



MECHANISM OF TUMOUR RESISTANCE IN SALMONELLA-IMMUNIZED MICE

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ABSTRACT

The experiments reported in this thesis investigated the tumour resistance induced by immunization of (BALB/cx C57BL/6)_{F₁} mice with a live vaccine of the intracellular bacterial parasite, Salmonella enteritidis 11RX (11RX). The aims were to define the cytotoxic effector cells, the mechanism by which they recognize tumour cells and to characterize the 11RX-sensitized T cells induced by 11RX infections which are involved in the generation of tumour resistance.

This study confirmed previous suggestions that activated macrophages are the principal cytotoxic cells present in the peritoneal cavity of 11RX-immunized mice. It was found that the lysis of tumour cells in vitro is dependent on contact between effector and target cells and that activated macrophages bind tumour cells more efficiently than do macrophages from normal mice. Unlabelled tumour cells could competitively and non-specifically inhibit the lysis of radioactively labelled tumour cells by peritoneal cells (PC) from 11RX-immunized mice.

Concanavalin A-activated splenic blast (blast) cells were compared to tumour cells for their ability to interact with activated macrophages. Blast cells were not lysed by PC from 11RX-immunized mice and did not inhibit the lysis of tumour cells in vitro. However, blast cells did bind to monolayers of activated macrophages and to a lesser degree to normal macrophages. The extent of blast cell binding to the two macrophage types was equal to that observed with tumour cells. Unlike the adherence of tumour cells to activated macrophages, the bond between blast cells and activated macrophages was labile and dissociated with incubation in vitro suggesting that the two types of interactions were qualitatively different.

Spleen cells from llRX-immunized mice could transfer to naive recipients the ability to recall resistance to intraperitoneal (ip) tumour challenge upon ip injection of llRX antigen extract. When the ip route was used to transfer spleen cells, high levels of ip tumour resistance could be recalled three weeks later.

Tumouricidal activity could be induced in inflammatory macrophages by culturing them in vitro with lymphoid cells from llRX-immunized mice and llRX antigen. The lymphokines, Interleukin 2 (IL2) and Macrophage Activation Factor (MAF) are released by llRX-sensitized lymphoid cells when they are stimulated in vitro with llRX antigen. Lymphoid cell populations differ in their ability to release these lymphokines and, furthermore, the ability to release MAF did not correlate directly with the release of IL2. Treatment of lymphoid cells with anti-Thy 1.2 antibody and complement abolished their ability to release the two lymphokines.

From these observations the following conclusions were drawn. The tumour resistance induced in mice by llRX immunization is effected mainly by activated macrophages and is mediated by llRX-sensitized T cells which are long-lived. These sensitized T cells can activate tumouricidal macrophages by releasing MAF when stimulated with bacterial antigens. The sensitized T cells which release MAF may be different from those which release IL2.

Lysis of tumour cells by activated macrophages requires the binding of target cells by effector cells which is mediated by surface structures common to tumour cells. The binding structures present on normal dividing blast cells appear to be different from those present on tumour cells. This may explain why blast cells are not killed by llRX-activated macrophages.

This thesis contains no material previously submitted by me for a degree in any University and to the best of my knowledge and belief it contains no material previously published or written by any other person except where due reference is made in the text.

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ABBREVIATIONS USED IN THIS THESIS

^{125}I -UdR	$[\text{}^{125}\text{I}]\text{-5-iodo-2'-deoxyuridine}$
^{125}I -EAT	^{125}I -UdR-labelled EAT cells
^{125}I -P815	^{125}I -UdR-labelled P815 cells
^{125}I -blast	^{125}I -UdR-labelled Con A-activated blast cells
^3H -TdR	<u>[methyl-^3H]</u> -thymidine
^3H -P815	<u>[methyl-^3H]</u> -thymidine-labelled P815 cells
^{51}Cr	$\text{Na}_2^{51}\text{CrO}_4$
^{51}Cr -EAT	$\text{Na}_2^{51}\text{CrO}_4$ -labelled EAT cells
^{51}Cr -EL4	$\text{Na}_2^{51}\text{CrO}_4$ -labelled EL4 cells
^{51}Cr -MPC11	$\text{Na}_2^{51}\text{CrO}_4$ -labelled MPC11 cells
^{51}Cr -P815	$\text{Na}_2^{51}\text{CrO}_4$ -labelled P815 cells
^{51}Cr -blast	$\text{Na}_2^{51}\text{CrO}_4$ -labelled Con A-activated blast cells
*EAT/Ag	suspension of 10^6 ^{125}I -EAT cells/ml in medium containing $50\ \mu\text{g}$ 11RX antigen/ml
11RX	<u>Salmonella enteritidis</u> 11RX
Ab	antibody
Ag	antigen
CB6F ₁ mice	(BALB/c x C57BL/6)F ₁ hybrid mice
CCS	conditioned culture supernatants
CPM	counts per minute
CS	Con A-conditioned spleen cell supernatant containing IL2 activity
Con A	Concanavalin A
Con A blast cell	Con A-activated splenic blast cells
DMEM	Dulbecco's modified Eagle's minimal essential medium

EAT	Ehrlich ascites tumour
EMEM	Eagle's minimal essential medium
FCS	foetal calf serum
IL2	interleukin 2 (formerly T cell growth factor)
LNC	lymph node cells
LPS	lipopolysaccharide
MAF	macrophage activation factor
MIF	macrophage migration inhibition factor
MMP	methyl- α -D-mannopyranoside
NK cells	natural killer cells
PC	peritoneal cells from normal or immunized mice
PL	peritoneal lymphocytes
PMN	polymorphonuclear
SD	standard deviation of the mean
SMP	standard MAF preparation
TSA	tumour-specific antigen(s)
§	section
ip	intraperitoneal(ly)
iv	intravenous(ly)
lyc	lymphocytes
ppi PC	PC from mice injected ip with proteose-peptone broth
s.e.m	standard error of the mean
μ Ci	microcuries
μ g	micrograms
μ m	microns

TO MY MOTHER AND FATHER



CHAPTER ONE

TUMOUR RESISTANCE INDUCED BY INFECTION WITH
INTRACELLULAR BACTERIAL PARASITES1.1 The role of immune responses in controlling the growth of tumours

The notion that the immune system may have a role in preventing neoplasia is not a new one. Such ideas were advanced early this century by Ehrlich and by his contemporaries (reviewed by Green and Shevach, 1974). In more recent times, Burnet was one of the many workers who developed and popularized this concept (Burnet, 1970). He proposed that somatic mutations were a frequent event in vivo and that these led both to malignancy and to the expression of new surface antigens. He suggested that a mechanism, which he called immune surveillance, detected and destroyed many of these cells before they gave rise to tumours. Hence, it would follow that when tumours appear, the process of immune surveillance has failed.

This hypothesis and other similar ones provided the stimulus for extensive studies directed at analysing the immunogenic and antigenic nature of tumours, and the role immune responses may play in controlling tumour growth. The study presented in this thesis is not concerned with ability of tumours to induce immune responses in susceptible hosts. Nevertheless, it seems appropriate to very briefly summarize the evidence that immune responses can be mounted to tumours and the effectiveness of such responses in controlling tumour growth.

Many tumours induced experimentally in animals bear tumour specific antigens (TSA) not found on normal cells. Such TSA can induce both humoral and cell-mediated immune responses which can be protective for the host (reviewed by Lamon, 1974; Herberman, 1977; Ting and

Herberman, 1977). However, many TSA are not immunogenic and tumours vary greatly in their immunogenicity (reviewed by Baldwin, 1973; Herberman, 1977). Furthermore, despite the demonstration that TSA may be immunogenic, tumours may continue to grow in the face of a demonstrable immune response to the tumour (reviewed by Baldwin, 1973; Hellström and Hellstrom, 1974).

Several mechanisms have been proposed to explain this paradox. These include the presence of serum "blocking factors" (reviewed by Hellström and Hellström, 1974; Baldwin, 1973; Hellström, Hellström and Nepom, 1977) and the induction of specific and non-specific suppressor cells which may limit the magnitude of immune responses to TSA (reviewed by Noar, 1977, and by Kripke, 1981). Finally, tumours might avoid destruction by the immune system by secreting pharmacological substances which suppress host cytotoxic effector cells or prevent them from accumulating at the site of tumour growth (reviewed by North, Spitalny and Kirstein, 1978 and Sundharadas, Chung and Cantrow, 1978).

It has been suggested that many human tumours may also be immunogenic (i.e. possess TSA) because cells derived from them can be killed in vitro by lymphocytes from the same patient (reviewed by Hellström and Hellström, 1974) and because immunoglobulin which can bind to tumour cells has been detected in the sera of cancer patients (reviewed by Lewis et al., 1978). However, the specificity of these reactions has been questioned. Numerous workers have failed to demonstrate that the cytotoxicity against tumour cells which can be mediated by patients lymphoid cells is specific, and, it appears that in many instances the cytotoxic activity was due to natural killer (NK) cells which kill cells from tumour cell lines in a non-specific manner (Takasugi, Mickey and Terasaki, 1972; Skurzak et al., 1973; Klein, E., 1980). Furthermore, immunotherapy of tumours which is based on the these observations of immune reactivity of patients to their own tumours has failed to alter the course of cancer in humans (Alexander, 1981). One of

the reasons for this is that human tumours may lack appropriate TSA or, if they do possess them they are not immunogenic. In general, it has been found that heteroantisera which were thought to identify TSA on human tumour cells have, upon further analysis, been shown to detect quantitative rather than qualitative differences between normal and cancer cells (Old, 1981). Although some human tumours such as malignant melanoma may have TSA, some of which are restricted to autologous tumours and others shared amongst allogeneic tumours of the same origin (Old, 1981), it appears that these antigens may not be immunogenic. This is because over 70% of all melanomas tested bear a common TSA, yet most of the patients bearing these tumours do not have detectable antibodies in their serum (Old, 1981).

A large number of observations by different workers have raised doubts regarding the role of the immune system in preventing the growth of spontaneously arising tumours, both in animals and humans. For example, most spontaneously arising tumours in animals are poorly, if at all, immunogenic (Prehn, 1976; Hewitt, 1978). Also, according to the theory of immune surveillance, immunosuppression and immune deficiency should increase tumour incidence. Whilst immunosuppression can make experimental animals more susceptible to chemical and viral oncogenesis (reviewed by Stutman, 1975), the data available for humans (e.g. chronically immunosuppressed organ transplant patients) shows that it is mainly the incidence of lympho-reticular tumours which is increased (Penn, 1978, 1981). Thus, at best, immune surveillance may apply to a restricted variety of tumours. Alternatively, other mechanisms such as chronic antigenic stimulation (from the graft or infections), defective immunoregulation, infection with Epstein-Barr virus or a combination of all three, may be involved in stimulating the growth of lymphoid tumours (Penn, 1981).

There are no conclusive data that induction of immune responses to tumours has a role in the prevention or treatment of cancer (Alexander,

1981). Indeed, it has been postulated that weak immune responses to TSA may in fact stimulate the growth of tumours (Prehn, 1976). However, there are several approaches, which do not require that tumours bear (potentially) immunogenic TSA which may still prove to be useful in developing effective immunotherapeutic strategies (Weiss, 1981). In particular, one approach includes the stimulation of non-specific resistance to tumours by agents such as intracellular parasites and other agents that activate the reticuloendothelial system (RES) (Weiss, 1981).

1.2 The induction of non-specific tumour resistance by infection with microbial pathogens

Infection of humans and animals with microbial pathogens and other agents which can activate the RES can stimulate host resistance to tumour growth. Epidemiological observations indicate that the incidence of tumours is lower in patients suffering from active tuberculosis or osteomyelitis and those who have suffered from typhoid, paratyphoid, scarlet fever or diphtheria (reviewed by Nauts, Fowler and Bogatko, 1953). The first evidence that bacterial infections could mediate the destruction of established tumours was provided by W.B. Coley late last century (reviewed by Nauts et al., 1953). He observed that an inoperable sarcoma regressed in a patient who had suffered two acute streptococcal infections. He subsequently found that he could induce permanent regression of tumours in some patients by deliberately infecting them with Streptococci or injecting them with a mixture of toxins from β -haemolytic streptococci or Serratia marcescens. In 1969 Nauts presented further evidence of a correlation between bacterial infections and the increased incidence of spontaneous tumour regression in cancer patients.

These observations have been confirmed in many experimental models. The growth of syngeneic transplantable tumours can be reduced or totally suppressed in animals infected with Mycobacterium bovis strain

Bacillus Calmette-Guerin (BCG) (Old, Clark and Benacerraf, 1959; Halpern et al., 1959; Biozzi et al., 1959), Toxoplasma gondii or Besnoitia jellisoni (Hibbs, Lambert and Remington, 1971) and the avirulent Salmonella enteritidis 11RX (11RX) (Hardy and Kotlarski, 1971).

Similar effects have been noted with killed Corynebacterium parvum (Halpern et al., 1966; Woodruff and Boak, 1966), zymosan (Bradner, Clarke and Stock, 1958), the lipopolysaccharide (LPS) from gram negative bacteria (Hager et al., 1969; Tripodi Hollenbeck and Pollack, 1970), and the methanol extraction residue of BCG (Weiss, Bonhag and Leslie, 1966). Furthermore, it has been found that infection of mice with intracellular parasites can delay the appearance of "spontaneous" (Hibbs, Lambert and Remington, 1971) and carcinogen-induced (Ashman, Cook and Kotlarski, 1979) tumours in mice.

Because the induction of tumour resistance by infection with intracellular bacterial parasites (IBP) such as BCG, Listeria, 11RX, is a non-specific by-product of the specific immune response to such parasites, it seems appropriate at this point to discuss the characteristics of the immunity induced by IBP. Unless otherwise stated, the characteristics of immune responses to IBP apply also to intracellular protozoan parasites such as Toxoplasma and Besnoitia.

1.3 Resistance to intracellular bacterial parasites

1.3.1 The carrier state

BCG, Listeria, Salmonella and Brucella are classified as facultative, IBP because, like the protozoan parasites Toxoplasma and Besnoitia, they can survive and multiply within normal macrophages. These organisms eventually kill the infected cells, presumably as a result of the uncontrolled multiplication of the parasites (Collins, 1974). IBP establish a persisting infection or a "carrier state" when they infect normal, unimmunized mice. During this phase live bacteria are harboured in various tissues. The carrier state is generally

established in tissues rich in macrophages, although, the route of infection and dose of bacteria used for challenge are factors that affect their distribution. For example, in mice infected intravenously (iv) with avirulent llRX organisms, the carrier state is confined to the liver and spleen, whilst after intraperitoneal (ip) infection, llRX organisms are not restricted to these two organs. They are also found in the peritoneal cavity (Ashley, 1976; Ashley and Kotlarski, 1982a). When lethal doses of llRX or small doses of the highly virulent Salmonella typhimurium C5 are injected into mice, Salmonella can also be found in the blood (Davies and Kotlarski, 1974).

The duration of the carrier state induced by different IBP is characteristic for each parasite. Listeria (Mackness, 1962; North, 1973a, b) and llRX (Davies and Kotlarski, 1974; Ashley and Kotlarski, 1982a) organisms take 10 days and 40 days respectively to be eliminated from the spleens of normal mice, whilst Brucella abortus can be found in high numbers in the spleen more than 60 days after infection (Riglar and Cheers, 1980). BCG infections can persist for at least 120 days (Collins, 1974) and sometimes even life-long infections are established (Gray and Affleck, 1958).

1.3.2 Requirement for activated macrophages to control IBP infections

Phagocytic cells such as macrophages are an important component in resistance to infection. Bacteria such as staphylococci and streptococci when opsonized with specific antibody are rapidly phagocytosed and destroyed by normal macrophages. However, macrophages with enhanced bactericidal properties are required to effectively control and eliminate IBP infections. Lurie first showed that macrophages from BCG vaccinated rabbits were far more efficient in controlling the growth of tubercule bacilli than those from normal rabbits (Lurie, 1942). Since then, it has been demonstrated by many workers that resistance to IBP requires the generation of macrophages with enhanced phagocytic and bactericidal

capacity (reviewed by North, 1974; Collins, 1974). Mackaness (1962) coined the term "activated" macrophages to describe such cells. The ability to stimulate host production of activated macrophages is peculiar to live IBP; most killed vaccines, whilst capable of stimulating humoral responses, do not induce effective immunity (Holland and Pickett, 1956, Holland and Pickett, 1958; Ushiba et al., 1959 and Collins 1969a, b).

Immunity to IBP can be divided into two phases. The first phase of resistance is non-specific in its expression because during this phase animals can control a challenge of either the homologous IBP or a heterologous one. This phase can be detected only during the bacterial carrier state and is associated with increased activity of the host RES. During this phase activated macrophages can be recovered from infected animals. One of the earliest observations which helped to characterize the non-specific nature of this phase of immunity was made by Pullinger (1936) who showed that guinea pigs infected with Mycobacteria were resistant to Brucella infection. In 1957 Nyka showed that the converse was also true. It has also been shown that infection of mice with Mycobacteria makes them resistant to infection with Salmonella (Howard et al., 1959) and Listeria (Blanden, Lefford and Mackaness, 1969; Mackaness, 1964). Mackaness (1964) showed that activated macrophages were involved in cross-resistance to IBP since macrophages from Brucella-infected animals killed Listeria in vitro. Similarly, cross resistance in Salmonella and Listeria infected mice is associated with macrophages that can inactivate either parasite in vitro (Blanden, Mackaness and Collins, 1966; Blanden, 1968).

Once the immunizing bacteria have been eliminated, the RES activity returns to normal levels, activated macrophages can no longer be recovered and animals do not show non-specific resistance to IBP. During the second phase of immunity, resistance is specific for the immunizing bacteria indicating the "classic" immunological nature of resistance to IBP infections. The immunized animals clear a challenge inoculum of the

vaccine strain of organisms much more rapidly than normal mice, but only after a short time lag of 1-3 days during which the challenge organisms multiply (Mackanness, 1964; Collins, 1968; Coppel and Youmans, 1969). Once recalled by challenge with the immunizing strain, immunity is again non-specific in its expression. Unlike the induction of immunity to IBP, the second phase of resistance in immunized mice can be "recalled" by dead bacteria or protein extracts prepared from them (Halliburton and Hinsdill, 1972). Resistance to Toxoplasma and Besnoitia also requires activated macrophages and animals infected with these intracellular parasites are highly resistant to challenge with a variety of phylogenetically distinct parasites (reviewed by Sharma and Remington, 1981). Because Toxoplasma establishes life-long infections (Remington, Melton and Jacobs, 1961) cross-resistance to bacteria can be detected even 7-8 months after primary infection. However, mice immunized 5 months earlier with Toxoplasma are not resistant to Trypanosoma cruzi unless given a "booster" injection of Toxoplasma. This demonstrates that immunity to Toxoplasma also shows two phases (Williams, Sawyer and Remington, 1976).

The increased tumour resistance induced by infection with various intracellular parasites has also been ascribed to activated macrophages, because macrophages with the ability to kill tumour cells in vitro have been isolated from mice infected with Toxoplasma, Besnoitia, Listeria (Hibbs, Lambert and Remington, 1972; Krahenbuhl and Remington, 1974) or BCG (Evans and Alexander, 1972; Hibbs, 1973; Cleveland, Meltzer and Zbar, 1974). Furthermore, the peritoneal cells (PC) from mice infected with 11RX are also cytotoxic in vitro (Ashley and Hardy, 1973). Activated macrophages can also be harvested from animals immunized with non-viable agents such as killed C. parvum (Olivotto and Bomford, 1974; Ghaffar et al., 1974), LPS and double stranded RNA (Alexander and Evans, 1971; Glaser et al., 1976) or, with the living metazoan parasite Nippostrongylus brasiliensis (Keller and Jones, 1971).

More recently, there has been a great deal of interest shown in the lymphoid cells which exhibit "natural cytotoxicity" i.e. the ability to lyse tumour cells in vitro without prior immunization or manipulation of the effector cell donors. These cells are known as natural killer (NK) cells and are particularly relevant to this discussion because infection of animals with IBP (discussed in section (§) 3.1) or viruses (reviewed by Welsh, 1981) can enhance the activity of NK cells. The characteristics of NK cells and their interaction with tumour cells are discussed in detail in §1.12.

1.3.3 Sensitized thymus-derived lymphocytes induced during IBP infections mediate macrophage activation

Although activated macrophages are responsible for the destruction of IBP, immunity is mediated by sensitized T lymphocytes induced during IBP infections. In the presence of specific antigen, the sensitized lymphocytes bring about the macrophage activation that is required for the elimination of the IBP organisms.

Cell transfer experiments showed conclusively that lymphocytes were responsible for immunity to IBP. In 1969, Mackaness found that normal recipients infused with splenic lymphoid cells from Listeria-infected mice were protected against a lethal challenge with Listeria. Macrophage precursors in the spleen cell suspensions were not responsible for the transfer of resistance because the protective activity of spleen cells was destroyed when the lymphoid cells were pretreated in vitro with anti-lymphocyte globulin (Mackaness and Hill, 1969). This was confirmed in cell transfer experiments using rats. Thoracic duct lymph was used as the source of sensitized lymphocytes to reduce contamination by macrophages (McGregor, Koster and Mackaness, 1971; Koster and McGregor, 1971; Koster, McGregor and Mackaness, 1971). Similar results have been obtained from studies of immunity to Mycobacteria (Lefford, McGregor and Mackaness, 1973) and Salmonella

(Davies, 1975).

It is now well established that the sensitized lymphocytes which mediate resistance to IBP are T cells. This conclusion was reached following the demonstration that T cell-deficient mice are more susceptible to infection with Mycobacteria (Takeya et al., 1967; North, 1973a, 1974) and Salmonella (Collins, 1974; Davies and Kotlarski, 1976), and that Listeria-sensitized lymphoid cells treated with anti-Thy 1 and complement were unable to transfer resistance to normal mice (Lane and Unanue, 1972; North, 1973b).

The protective capacity of the sensitized donor lymphocytes requires the presence of recipient, bone-marrow derived macrophages. This proves that as in actively immunized animals, macrophages are necessary for the destruction of IBP. Tripathy and Mackaness (1969) found that recipients pretreated with the anti-mitotic drug vinblastine (VBL), cyclophosphamide or X-irradiation, treatments which destroy macrophage precursors in the bone-marrow, cannot be protected against Listeria challenge with splenic lymphoid cells from Listeria-immunized donors.

1.3.4 The life span and migratory characteristics of the lymphocytes generated during IBP infections

At the time this study was commenced, some work had been carried out characterizing the lymphocytes generated during Listeria and Mycobacteria infections in terms of their migratory behaviour within the body, their life-span and their sensitivity to VBL. It was found that there are major differences between the sensitized lymphocytes induced during infection with Listeria and infection with Mycobacteria.

During IBP infections there is a lymphoproliferative response (North, 1972; North, Mackaness and Elliott, 1972) characterised by the production of sensitized dividing lymphocytes (North, 1973b) and release of these cells into the circulation (McGregor, Koster and Mackaness,

1971; Lefford, McGregor and Mackaness, 1973). The presence of large numbers of sensitized lymphocytes in spleen and thoracic duct lymph is transient during Listeria infections whilst large numbers are maintained for long periods of time in BCG-infected rodents. It appears that this is because Listeria infections, unlike those with BCG, do not induce large numbers of long-lived sensitized lymphocytes.

In Listeria immunized mice, large numbers of rapidly dividing lymphocytes which can adoptively immunize naive recipients (protective lymphocytes) are present in the spleen only during the carrier state. From days 6 to 12 after infection, when the infecting organisms are being eliminated (North, 1973b), there is a 100-fold decrease in the number of "protective lymphocytes" present in the spleen of these animals, suggesting that most of the protective lymphocytes are short-lived and are produced only in the presence of live Listeria. This conclusion is supported by the results of cell transfer experiments which show that the lymphocytes, taken when their protective capacity is maximal, have very short recirculating and protective life spans (McGregor et al., 1971; North, Berche and Newborg, 1981).

The protective lymphocytes induced by BCG infections in rats are long-lived (Lefford et al., 1973) and viable Mycobacteria are not required for the maintenance of large number of these cells. For example, over a period of 41 weeks the protective capacity of TDL of BCG infected rats declined only 2-3 fold, although the number of bacteria that could be recovered from the liver and spleen of these animals was reduced approximately 400,000 fold (Lefford and McGregor, 1974).

The notion that the lymphocytes generated during BCG infection are long-lived is supported by the finding that TDL taken 8-28 days after immunization have a long functional life span in recipients (Lefford et al., 1973; Lefford and McGregor, 1978). In addition, they can recirculate from blood to lymph, and are non-dividing because they are resistant to VBL (Lefford et al., 1973). It has been established that

the latter two properties are characteristic of long-lived, small lymphocytes (Everrett and Tyler, 1967; Gowans and Knight, 1964).

It is possible that the differences in the protective lymphocytes induced during Listeria and Mycobacteria infections are quantitative rather than qualitative. This is because during the course of both infections, the sensitized lymphocytes that are induced mature from VBL sensitive (dividing) to VBL resistant (resting) lymphocytes (McGregor and Logie, 1973; Lefford et al., 1973; North and Deissler, 1975). However, for reasons which are not understood, infection with Mycobacteria results in the induction of far more VBL resistant lymphocytes than infection with Listeria. The protective capacity of VBL resistant lymphocytes taken from rats 28 days after infection with BCG is as high as that of lymphocytes taken earlier after immunization (Lefford et al., 1973) when they are totally sensitive to VBL. It is not known whether the small numbers of non-dividing protective lymphocytes which are present in the spleens of Listeria-immunized mice after the carrier state has been eliminated are long-lived, but evidence provided by Jungi and Jungi (1981) which is discussed in § 7.9.4 suggests that they may have a long half-life.

1.3.5 Newly formed protective lymphoblasts accumulate at sites of inflammation

Further characterization of the lymphocytes induced following Listeria infection has been made by studying their ability to accumulate at sites of inflammation. Using adoptive immunization experiments, it has been established that cells from sterile peritoneal exudates induced in Listeria infected rats and mice are more protective than TDL, spleen cells and resident peritoneal cells from similar animals, when these are compared on a cell-per-cell basis (North and Spitalny, 1974; Koster et al., 1971). These results suggest that newly-formed Listeria-immune lymphocytes can enter sites of inflammation via the bloodstream (Koster

et al., 1971). Autoradiographic analysis of cells obtained from various tissues of rats which have been injected iv with radioactively labelled immunoblasts or small resting lymphocytes obtained from the thoracic duct of Listeria infected rats, has revealed that only immunoblasts can enter sites of inflammation such as the stimulated peritoneal cavity (Koster and McGregor, 1971; McGregor and Logie, 1974). Small, labelled lymphocytes which are found in sterile peritoneal exudates are derived from immunoblasts which enter the exudate and revert to non-dividing cells (Koster and McGregor, 1971; McGregor and Logie, 1974). Consistent with the notion that only dividing lymphocytes can enter sites of inflammation, subsequent experiments showed that treatment of Listeria-infected donors with VBL prior to induction of a peritoneal exudate, prevents the accumulation of protective lymphocytes in the peritoneal cavity (McGregor and Logie, 1973, 1974; North and Spitalny, 1974). Experiments by North and Spitalny (1974) showed that the number of sensitized lymphocytes which accumulate in peritoneal exudates of Listeria infected animals is dependent on the rate that they are produced at the site of infection.

The physiological conditions at the site of inflammation which are responsible for the entry of Listeria-specific sensitized lymphoblasts are short-lived. It has been established that the sensitized lymphocytes will enter peritoneal exudates only during the first 24 h after induction (North and Spitalny, 1974; McGregor and Logie, 1974). However, because the protective capacity of exudates continues to increase until a peak on day 3, it has been concluded that the protective lymphocytes continue to divide in the peritoneal cavity. This is supported by the demonstration that administration of VBL to infected donors 24-72 h after exudate induction greatly decreases the protective capacity of exudate cells in transfer experiments. Cells capable of mediating immunity to Listeria became completely insensitive to the action of the antimitotic drug only 96 h after induction of the exudate (North and Spitalny, 1974).

It seems relevant to note that the protective capacity of the peritoneal exudate lymphocytes decays rapidly after the third day of induction. In other words, the rapid decay of protective capacity of peritoneal exudate cells correlates with the loss of dividing cells with protective activity. Little is known of the fate of those mediator cells which enter the peritoneal cavity and revert to non-dividing cell forms. It is unlikely that they can move out of the peritoneal cavity because ip transfer of protective exudate cells from Listeria-immune mice does not protect normal mice against iv Listeria challenge (North and Spitalny, 1974).

1.4 The characteristics of tumour resistance induced by IBP

The tumour resistance induced as a result of macrophage activation is a "local" phenomenon because IBP and other macrophage activating agents have their greatest therapeutic effect when they are given at the site of tumour challenge. It has been demonstrated that the growth of tumours in experimental animals is suppressed if tumour cells are injected with BCG, C. parvum or Listeria monocytogenes as a mixture at the same site, but not if they are injected separately at similar but distant anatomical sites (Zbar, Bernstein and Rapp, 1971; Baldwin and Pimm, 1971; Bartlett, Zbar and Rapp, 1972; Likhite and Halpern, 1973; Youdim, Moser and Stutman, 1974; Bast et al., 1975a, b).

A similar conclusion can be reached when the resistance of IBP-immunized mice to ip tumour challenge is analysed. It was found that the duration and the degree of tumour resistance is dependent on several factors including the routes of immunization and tumour challenge, the distribution of the parasites and their persistence in various tissues of the infected animals. The greatest protection against ip tumour challenge is observed in animals which have been previously or simultaneously immunized with IBP ip rather than iv (Halpern et al., 1966; Smith and Scott, 1972; Pimm and Baldwin, 1975; Hibbs, 1975a; Ashley

et al., 1976; Ashley and Kotlarski, 1982a). Some protection to ip tumour challenge is obtained after iv immunization with llRX but the resistance induced is generally weaker and of shorter duration than that induced by ip immunization (Ashley et al., 1976; 1982a).

The much greater resistance to ip tumour challenge that is induced by ip immunization of mice with BCG or llRX correlates with the establishment of a bacterial carrier state at this site. For example, Hibbs (1975a) showed that viable Mycobacteria could be recovered from the "peritoneal" lymph nodes of mice injected ip with BCG, but not from mice injected with BCG iv or intramuscularly. He showed that this correlated with the ability of the ip immunized mice to reject an ip challenge with syngeneic tumour cells and the presence of tumourcidal macrophages in the peritoneal cavity. Similarly, llRX establishes a carrier state in the peritoneal cavity after ip but not iv immunization (Ashley, 1976; Ashley and Kotlarski, 1982a).

Non-specific tumour resistance requires the persistence of the immunizing IBP, which is characteristic of the first phase of the immune response to IBP (discussed in § 1.3.1). The time during which cytotoxic macrophages can be recovered from immunized mice is determined by the duration of the carrier state. Krahenbuhl and Remington (1974) found that cytotoxic peritoneal macrophages could be recovered from Listeria-infected mice for only 12 days, which is approximately the length of time the organisms can be recovered from the spleen. In contrast, Toxoplasma and Besnotia establish life-long infections in mice and cytotoxic peritoneal macrophages can be recovered from such mice for over 6 months.

A detailed analysis of the tumour resistance induced by ip injection of llRX has revealed a more complex relationship than the one outlined above. It has been found that mice are still highly resistant to ip challenge with Ehrlich Ascites Tumour (EAT) cells 40 days after ip immunization with llRX, even though they do not contain any viable llRX

organisms in the peritoneal cavity and their PC are no longer cytotoxic for tumour cells in vitro (Ashley, 1976; Ashley and Kotlarksi, 1982a).

In addition, Ashley and colleagues have reported further parallels between non-specific tumour resistance and the cross-resistance to heterologous intracellular parasites which is induced by immunization with IBP. They showed that long after ip tumour resistance has waned and cytotoxic PC are no longer found in llRX-immunized mice, both activities can be "recalled" by injecting mice with a protein extract from llRX organisms (Ashley, Kotlarksi and Hardy, 1974; Ashley and Kotlarksi, 1982b). This effect is mediated by llRX-sensitized T cells because splenic T cells from llRX-immunized donors can transfer the ability to recall tumour resistance to normal mice (Ashley, Kotlarski and Hardy, 1977).

It has been suggested that the reason why IBP induce the most effective resistance to tumour growth when they are injected at the same site as that used for tumour challenge is that the IBP may also be acting as adjuvants. They may be potentiating the response to tumour antigens in the same way that BCG can potentiate the delayed hypersensitivity response to sheep erythrocytes and numerous other antigens (Miller, Mackaness and Lagrange, 1973; Mackaness, Lagrange and Ishibashi, 1974). It has, in fact, been reported that mixtures of tumour cells and BCG or C. parvum organisms can potentiate the specific immune response to immunogenic tumours when they are inoculated subcutaneously into experimental animals. This aspect is discussed in §3.1. Nevertheless, as stated earlier, the tumour resistance induced by intracellular parasites need not involve any specific immune response to tumour antigens, but can be a non-specific consequence of the immune response to bacterial antigens.

Zbar et al. (1970) demonstrated that the intradermal growth of a guinea pig hepatoma can be inhibited by eliciting a delayed-type hypersensitivity reaction to mycobacterial antigens at the site of tumour

growth. Ashley and his colleagues demonstrated that the in vivo destruction of ^{131}I (or ^{125}I)-UdR-labelled EAT cells injected ip into 11RX-immunized mice is so rapid, that it is extremely unlikely that the generation of an immune response to tumour is responsible for the removal of the tumour. By washing out the contents of the peritoneal cavity of 11RX-immunized mice at various times after ip challenge with 10^6 ^{131}I (^{125}I)-EAT cells, it was established that, within 12-24 h of challenge, less than 1% of the injected radioactivity was cell-associated. In some experiments less than 0.3% of the radioactivity could be recovered. During this time 60-90% of the counts injected ip into normal mice could be recovered and were cell-associated (Ashley, 1976; Ashley and Kotlarski, 1982a). This is consistent with the finding that PC from similar 11RX-immunized mice can lyse ^{51}Cr -EAT cells in vitro in 10-20 h (Ashley and Hardy, 1973; Ashley and Kotlarski, 1982a).

1.5 Biochemical markers for activated macrophages

As mentioned previously, the term "activated macrophages" describes a population of macrophages which had a greatly enhanced ability to kill IBP and which were subsequently shown to kill tumour cells in vitro (Hibbs, 1973). A great deal of time, thought and effort has gone into attempts to characterize the differences between normal and activated macrophages. It was found that they are better endowed than normal macrophages in many aspects including their size and ability to spread on glass, their phagocytic and digestive capacity, their content of acid hydrolases and their respiratory and mitotic rates (reviewed by Edelson, 1981).

There are biochemical differences between activated and normal macrophages, but these changes do not necessarily mean that a macrophage population is activated in the functional sense. For example, Nogueira, Gordon and Cohn (1977) showed that, although both thioglycollate-induced and activated macrophages can secrete more plasminogen activator, only

the activated macrophages are capable of killing Trypanosoma cruzi in vitro. Other characteristics that are shared by activated and inflammatory macrophages include an increased respiratory burst during phagocytosis, the ability to transport and metabolize glucose-1-¹⁴C to ¹⁴CO₂ more rapidly than normal macrophages, and lower levels of plasma membrane-associated ecto-5'-nucleotidase than those detected in plasma membranes of normal cells (Karnovsky and Lazdins, 1978; Soberman and Karnovsky, 1981).

It is possible that monoclonal antibody technology may help to define antigenic markers exclusive to activated macrophages. Recently Lohmann-Matthes and her colleagues described three monoclonal antibodies which can selectively reduce the in vitro tumouricidal activity of activated macrophages in different assay systems. Whether these antigens are unique to activated macrophages has yet to be elucidated (Sun and Lohmann-Matthes, 1982).

Whilst no biochemical changes have yet been found that are exclusive to the activated macrophages, it has been proposed that differences in the activities of various enzymes in different macrophage populations, may represent the presence of macrophages at different stages of the activation pathway or at different stages of differentiation (Cohn, 1978; Karnovsky and Lazdins, 1978; Edelson, 1981; Sorg and Neuman, 1981). For example, the levels of plasma membrane alkaline phosphodiesterase I are higher than normal in inflammatory, non-tumouricidal macrophages elicited by thioglycollate and are reduced in tumouricidal macrophages activated by C. parvum or pyran copolymer (Morahan, Edelson and Cohn, 1980; cited in Edelson, 1981). The changes in the expression of biochemical markers can vary between inflammatory macrophages elicited by different agents. In contrast to thioglycollate induced macrophages, proteose-peptone and glycogen induced inflammatory macrophages have virtually the same amount of alkaline phosphodiesterase I as do normal resident macrophages; the decrease in

5'-nucleotidase is greatest in macrophages elicited by thioglycollate, less in those elicited by glycogen and even less in those elicited by proteose peptone. Similarly, there is a greater increase in plasma membrane-associated leucine aminopeptidase in tumouricidal macrophages activated by pyran copolymer than in those activated by C. parvum (Morahan et al., 1980, cited in Edelson, 1981).

At first sight it would appear that the ability of tumouricidal macrophage populations to secrete H_2O_2 or a serine protease-associated cytotoxic factor and to strongly bind tumour cells in vitro may be characteristics unique to activated macrophages. However, as discussed in §7.5 macrophage populations may express one or more of these attributes without being cytotoxic in vitro. This suggests that for activation to be complete, several independently controlled changes must occur in the same cells, i.e. part of the difficulty in defining the biochemical attributes characteristic of activated macrophages may be due to the fact that populations of cells, rather than individual cells within a population are being analysed. Because the populations always contain a mixture of cells, it is impossible to determine which particular set of biochemical markers characterise activated macrophages, as distinct from the markers which can be attributed to suspensions of cells which may contain mixtures of a varying proportion of activated, inflammatory and normal macrophages.

1.6 The mechanism of macrophage activation in vitro.

1.6.1 Role of soluble factors (MAF)

In §1.3.3 evidence is presented that the process of macrophage activation during the immune response to IBP requires the interaction of sensitized T cells with antigen. Dissection of the macrophage activation process has been facilitated by the use of in vitro cell culture techniques and the availability of mice with genetic defects in their ability to generate tumouricidal, activated macrophages during infections

with IBP. Such approaches have shown that macrophage activation can be mediated by soluble factors that are released from sensitized T cells in an immunologically specific manner (Farr, Wechter, Kiely and Unanue, 1979a).

Non-cytotoxic, normal or inflammatory peritoneal macrophages can develop tumouricidal activity when cultured in vitro with lymphoid cells from IBP infected mice and specific antigen. Cytotoxicity does not develop when lymphoid cells from unimmunized mice are used, if antigen is omitted from the cultures or if antigen from an unrelated IBP is used (Ruco and Meltzer, 1977; Farr et al., 1979a).

The cell-free supernatants from cell cultures which generate tumouricidal macrophages contain a soluble factor designated Macrophage Activation Factor (MAF) which can activate purified populations of non-cytotoxic macrophages (Ruco and Meltzer, 1977; Farr et al., 1979a). MAF activity can also be detected in the culture supernatants of normal spleen cells stimulated with the T cell mitogen, Concanavalin A (Con A)(Fidler, Darnell and Budmen, 1976).

1.6.2 Macrophages must internalize MAF to be activated

Little has been done to clarify the mechanism of macrophage activation by MAF. However, there is some evidence that, in order to activate macrophages, MAF needs to bind to receptors on these cells. Once binding has occurred, MAF appears to be taken up by macrophages. If macrophages are treated with proteases, fucosidases or incubated with fucose they are less responsive to MAF than control macrophages. This suggests that the MAF receptors on macrophages are glycoprotein containing fucose (Remold, 1973; Fox, Gregory and Feldmann, 1974). The requirement for binding to ensure uptake of MAF into macrophages can be bypassed by using phosphatidyl choline liposomes containing MAF. Depending on the design of the experiment, insertion of MAF into macrophages can occur by phagocytosis or by fusion of the liposomes with

cell membranes. The results obtained confirmed the conclusion that, to be effective, MAF has to be taken up by macrophages. Liposome-encapsulated MAF was shown to be 20,000 times more active in inducing tumouricidal activity in normal macrophages than free MAF. Treatments which blocked or removed the macrophage surface-receptor for MAF, and therefore inhibited the activity of free MAF, did not inhibit the activity of liposome-encapsulated MAF (Poste et al., 1979; Sone, Poste and Fidler, 1981).

1.6.3 Different macrophage subpopulations vary in sensitivity to MAF

Not all macrophage populations are equally responsive to MAF. For example, suspensions of inflammatory macrophages, which are known to be biochemically different from normal ones, are more readily activated by MAF than are resident macrophages (Ruco and Meltzer, 1978a; Hibbs et al., 1977; Farr et al., 1979a). It has, in fact, been suggested that responsiveness to MAF is associated with a particular stage of macrophage differentiation; the variation in responsiveness to MAF of different macrophage populations may, therefore, simply reflect variations in the numbers of MAF-responsive macrophages present within each population. Thus, Ruco and Meltzer (1978a) accounted for the difference in responsiveness to MAF of induced and resident macrophage populations by suggesting that both populations contain MAF-responsive cells, but that induced exudates contain more of them.

MAF responsive macrophages appear to be recruited from bone marrow and the presence of a large number of macrophages rich in peroxidase in induced exudates suggests that MAF-responsive macrophages are immature (Ruco and Meltzer, 1978a). However, the possibility that their presence is purely coincidental has not been excluded because Lee, Wong and McIntyre (1981) have found that the increased MAF-responsiveness of subsets of glycogen-induced macrophage suspensions did not correlate with their peroxidase activity. Furthermore, Hibbs et al. (1977) reported

that resident peritoneal macrophages are easier to activate with MAF after being cultured in vitro for 72 h.

1.6.4 The role of lipopolysaccharides in macrophage activation by MAF

Although the mechanism of activation of macrophages by MAF is not known, it is believed to be a complicated, multistep process. At least one of the steps in the sequence leading to the activated state requires a signal that is delivered by, or can be mimicked by bacterial lipopolysaccharide (LPS) (Weinberg, Chapman and Hibbs, 1978; Ruco and Meltzer, 1978b, c; Hibbs et al, 1977). The action of LPS may only be required late in the activation process and macrophages must be altered or "partially activated" to be able to respond.

Meltzer and his co-workers found that some mice, such as those of the C3H/HeJ strain which are resistant to the effects of LPS (Watson and Riblet, 1974), do not develop cytotoxic macrophages after infection with BCG (Ruco and Meltzer, 1978d). In contrast, the closely related LPS-sensitive C3H/HeN strain, can develop these cells. Cross-breeding studies showed that the mutation which prevents C3H/HeJ mice from generating tumouricidal, activated macrophages is the same as, or is very closely linked to, the LPS-responsiveness gene (Ruco, Meltzer and Rosenstreich, 1978). Functional analyses suggested that the defect stems from the inability of macrophages from C3H/HeJ mice to respond to activation signals because the spleen cells of C3H/HeJ and C3H/HeN mice were equal in their ability to release MAF (Ruco and Meltzer, 1978b). Furthermore, it was found that C3H/HeJ macrophages were only partially defective in their ability to respond to the "LPS-like" signals present in MAF preparations because the macrophages from BCG-infected mice can be activated to the cytotoxic state if cultured with very high ($\mu\text{g/ml}$) quantities of LPS or very high concentrations of MAF. Macrophages from uninfected control C3H/HeJ mice, however, are not tumouricidal after exposure to this level of endotoxin or MAF (Ruco and Meltzer, 1978b, c).

This is consistent with similar findings by Russell et al. (1977). They reported that macrophages isolated from progressing Moloney virus-induced sarcomas which are not cytotoxic in vitro can be induced to become cytotoxic by the addition of ng/ml concentrations of LPS to the cultures. Activation of thioglycollate-induced macrophages, however, requires at least 1000 times more LPS.

1.6.5 Macrophage activation proceeds as a series of steps

Meltzer and his colleagues have proposed that the activation of responsive macrophages by MAF occurs in at least two sequential steps, the first being an induction or priming step which is followed by an expression or triggering step (Ruco and Meltzer, 1978c; Meltzer, 1981a). The MAF signal that is required in the triggering step of macrophage activation can be mimicked by LPS and other agents, including Con A (Ruco and Meltzer, 1978b). It appears that during the priming phase MAF-treated macrophages become responsive to the "LPS-like" signal present in MAF preparations. This model is based on the results of experiments in which macrophages were treated sequentially with a limiting amount of MAF and ng/ml concentrations of LPS.

The effect of LPS is rapid and can be detected after 15 min. Its activity is synergistic with MAF (Ruco and Meltzer, 1978c), and can be abrogated by the antibiotic polymyxin B which binds the lipid A portion of LPS (Ruco and Meltzer, 1978b). These experiments established that the sequence of exposure of macrophages to MAF and LPS is important. Macrophages exposed to limiting amounts of MAF followed by ng/ml amounts of LPS are tumouricidal in vitro. Macrophages do not develop tumouricidal activity if the sequence is reversed (Ruco and Meltzer, 1978c).

The activation of tumouricidal macrophages by MAF in the absence of added LPS can be also resolved into two steps (Meltzer, 1981a). Results similar to those discussed above were obtained when macrophages

were treated sequentially with MAF at two different concentrations. A 5 h incubation with low levels of MAF or a 1 h incubation with high levels of MAF does not induce tumouricidal activity in macrophages. However, exposure of macrophages to low concentrations of MAF for 4 h, followed by an additional 1 h incubation with high concentrations of MAF will result in as much cytotoxic activity as an 8 h exposure to high concentrations of MAF. Again, the sequence of treatment was also important: macrophages treated for 1 h with a high concentration of MAF followed by a low concentration of MAF for 4 h were not tumouricidal (Meltzer, 1981a).

The second activation signal that is present in supernatants containing MAF is unlikely to be LPS since polymyxin B has no inhibitory effect. Furthermore, the effect is lost after heating supernatants containing MAF at 60°C for 30 minutes, a treatment which has no effect on other biological effects of LPS (Meltzer, 1981a).

Whilst the data described above suggest that two functionally distinct signals are present in supernatants containing MAF, fractionation of such supernatants on Sephadex G100 has not resolved the two signals into two molecular species (Leonard, Ruco and Meltzer, 1978). It is possible that a sequence of changes are induced in macrophages through continued stimulation by one molecular species. Meltzer (1981a) hypothesises that the changes which occur in inflammatory macrophages following binding of MAF may include qualitative and/or quantitative alterations of surface receptors for MAF, development of new intracellular effector compartments, or of coupling systems to transduce receptor signals to effector compartments so that macrophages can respond to other differentiation signals from the same molecule.

By studying the genetic defect which prevents P/J mice from acquiring tumouricidal, activated macrophages during BCG infections, Boraschi and Meltzer have found evidence which indicates that the priming and triggering signals may be provided by different molecules (reviewed

by Meltzer, 1981b). The defect in P/J mice is genetically and functionally distinct from that carried by C3H/HeJ mice and it appears to be due to an inability of P/J spleen cells to release priming signals. Lymphokines prepared from the spleen cells of P/J mice, unlike those prepared from C3H/HeN and C3H/HeJ mice, are very poor activators of induced macrophages even when used at high concentrations and in the presence of LPS. They are, however, as effective as those from C3H/HeN mice at delivering the triggering signal to macrophages from BCG-infected C3H/HeJ mice (reviewed by Meltzer, 1981b).

Recent work by Nakamura and Meltzer (1981) suggests that the interaction of MAF-treated macrophages with target cells may be the final signal needed for the induction of tumouricidal activity. Maximum cytotoxicity occurs when target cells are added to macrophages while they are being treated with MAF. Their presence is not required during the first two hours incubation with MAF, but is essential during the subsequent 6 hours if maximal tumouricidal activity is to be induced.

1.6.6 Modulation of the activated state

Hibbs et al. (1977) proposed that macrophages can travel "backwards and forwards" along the differentiation pathway from normal macrophages to tumouricidal activated macrophages. This was based on a number of their in vitro observations. For example, they observed that tumouricidal activity is labile and is lost during overnight culture in vitro or by the addition of components of various adult sera, but they also found that cytotoxic activity can be restored by adding low concentrations of LPS or MAF. Consistent with their proposal, Russell et al. (1977) showed that macrophages recovered from regressing Moloney virus-induced sarcomas lost cytotoxicity when cultured in vitro for 24 to 72 h, but their activity could be restored by adding ng/ml quantities of LPS to the culture medium.

It is possible that inhibition of in vitro tumouricidal activity

of activated macrophages by various adult sera may be due to the presence of cholesterol. Chapman and Hibbs (1977) have resolved the inhibitory activity of adult sera into two molecular weight ranges, (equivalent to the molecular weights of IgM and IgG). One of these inhibitors behaved as a low density lipoprotein fraction which is known to be rich in cholesterol (Jackson, Morrisett and Gotton, 1976). These workers also demonstrated that the inhibitory activity of adult serum components could be reversed by exposure to low concentrations of LPS or MAF. The former finding has been confirmed by Ruco and Meltzer (1978b).

Although the mechanism which regulates expression of cytotoxic activity of activated macrophages has not been defined, Hibbs and Chapman (1977) propose that lipids could modulate the fluidity and/or receptor density of the cell membrane of activated macrophages. This could result in changes in binding capacity of the macrophages, interference with the transduction of recognition signals to the macrophages and/or inability to deliver a lethal hit to the target cells.

Ruco and Meltzer (1977) have provided contradictory data. In their in vitro system the time during which induced macrophages are responsive to the various stimuli in the activation sequence is brief, and that once cytotoxicity is lost, it cannot be regained. Furthermore, they found that activated macrophages which have lost their tumouricidal activity due to prolonged culture in vitro cannot be stimulated by LPS or MAF to be cytotoxic even though the cells are still viable (Ruco and Meltzer, 1978c).

1.6.7 Characterization of MAF

Conditioned culture supernatants of the type which contain MAF can also contain other activities such as Migration Inhibitory Factor (MIF) (discussed in §6.1), Interleukin 1 (IL1) (Finke, Sharma and Scott, 1981), thymocyte mitogenic protein (Farr, Kiely and Unanue, 1979b) and Interleukin 2 (IL2) (Finke et al., 1981; Prowse, 1981), Interferon

(Neuman and Sorg, 1977) and colony stimulating factor (Parker and Metcalf, 1974). The relevance of these various activities in macrophage activation or in the production of MAF is not known because, in general, supernatants from in vitro immune reactions have only been characterized functionally. There are only a few reports in the literature of the successful separation of the active molecular species from those effecting other activities.

MAF is a protein because its activity is destroyed by proteases (Leonard et al., 1978; Kniep et al., 1981). It is unlikely that it is glycosylated because it does not bind to Con A-Sepharose (Leonard et al., 1978) and its activity is not inhibited by procedures which prevent glycosylation of proteins or remove the carbohydrate moiety from them. Furthermore, "deglycosylated" MAF behaves chromatographically like untreated MAF (Kniep et al., 1981).

Estimates of the molecular weight of MAF differ. For example, Kniep et al. (1981) and Leonard, Ruco and Meltzer (1978) give figures of 3.0×10^4 M_r and 5.5×10^4 M_r respectively. These differences may not be real, however, since in both reports MAF eluted in a broad peak of activity and estimates of M_r varied greatly from experiment to experiment. Non specific binding of MAF to Sephadex may contribute to the variation in M_r estimates (Kniep et al., 1981).

Physiochemical fractionation of culture supernatants shows that MAF is clearly distinct from some other factors. MAF and MIF have been separated by Kniep et al. (1981) by isoelectric focusing in the presence of 8M urea and by size separation on Ultrogel AcA44.

MAF can be distinguished from IL2 because MAF has isoelectric points of pH 7.4 and pH 8.4 in the presence of urea (Kniep et al., 1981) whilst IL2 has isoelectric points of pH 4.3 and 4.9 (Mochizaki, Watson and Gillis, 1980).

1.7 In vitro assays for the anti-tumour activity of macrophages

The ability of macrophages to kill tumour cells can be measured in a variety of different in vitro assays. These are reviewed by Bean et al. (1976) and by Nelson, Hopper and Nelson (1978). Some of these are described below and include assays which measure the release of radioisotopes from prelabelled tumour cells and assays which measure the inhibition of cell growth.

(a) Assays which rely on membrane rupture

The cytoplasmic proteins of target cells are labelled by incubating the cells in vitro with $\text{Na}_2^{51}\text{CrO}_4$. Damage to the cell membrane of target cells results in leakage of ^{51}Cr from the tumour cells. The assays are generally terminated at 18-20 h after adding labelled target cells to the effector cell population. As a general rule, it is not feasible to use longer assay times with this technique because the spontaneous release of label from target cells is unacceptably high after 20 h (Bean et al., 1976; Brunner, Engers and Cerottini, 1976). This technique may not detect target cell damage due to cytostasis. Other radiolabels that have been used in this type of assay include ^{99}Tc and ^3H or ^{14}C proline (Bean et al. 1976).

(b) Assays which rely on nuclear disintegration

Cell death can be monitored by measuring the breakdown of nuclear DNA. Target cells are prelabelled with tritiated thymidine (^3H -TdR) or [^{125}I]-5-iodo-2'-deoxyuridine (^{125}I -UdR), washed and cultured with effector cells. The spontaneous release of nuclear label from targets is usually very slow, thus cytotoxicity assays can be conducted for up to 72 h (Holm and Perlmann, 1971; Bean et al., 1976). This allows detection of cytotoxic mechanisms which may not be detected using ^{51}Cr release assays. Use of ^{125}I -UdR has certain advantages over ^3H -TdR. ^{125}I -UdR released from dead cells is not reutilized as readily by

dividing cells as $^3\text{H-TdR}$ (Bean et al., 1976). Being a gamma emitter, ^{125}I can be counted directly without the processing of samples which is required for liquid scintillation counting of β emitters such as ^3H . The advantage of using $^3\text{H-TdR}$ is that it is far less toxic for target cells than $^{125}\text{I-UdR}$ (reviewed by Nelson, Hopper and Nelson, 1978).

(c) Assays which measure the inhibition of cell division

Cytostatic effects of macrophages can be detected by determining whether target cells at the end of the culture period will incorporate $^{125}\text{I-UdR}$ or $^3\text{H-TdR}$. Inhibition of uptake of either label can be due to target cell death or an arrest in DNA synthesis in otherwise healthy, viable cells (Bean et al., 1976). There is a draw back of this technique. Since macrophages and dead cells can release thymidine which can be reutilized (Opitz et al., 1978), "cytostasis" may simply be due to competitive inhibition of uptake of radiolabelled nucleoside in the absence of any effect on target cell division.

Alternatively, cell multiplication or cytostasis can be measured by counting the numbers of target cells present in cultures at the end of each assay.

(d) Assays which measure the detachment of adherent target cells

Antitumour effects of activated macrophages can be detected by measuring the detachment of adherent target cells in a microcytotoxicity assay. Macrophages and tumour cells are cultured together for up to 72 h. The culture vessels are then washed, stained and the remaining attached tumour cells counted by microscopy. Alternatively, radiolabelled adherent target cells can be used and the residual attached radioactivity can be measured (O'Toole and Clark, 1976; Oldham and Herberman, 1976).

1.8 Nature of the cells killed by macrophages

The in vitro cytotoxic activity of activated macrophages is selective for tumour cells and is not directed towards any known antigenic determinant on tumour cells. Hibbs, Lambert and Remington (1972b) found that activated macrophages obtained from mice infected with intracellular parasites were cytotoxic for both syngeneic and allogeneic tumour cell lines but not for highly immunogenic allogeneic fibroblasts. Furthermore, although the non-tumourigenic BALB/3T3 fibroblasts were not killed by syngeneic or allogeneic activated macrophages, two tumourigenic cell lines derived from them were lysed (Hibbs, 1973). These data have been confirmed many times by a large number of different workers (reviewed by Weinberg and Hibbs, 1978, and Nelson, Hopper and Nelson, 1978). However, some normal cells may not be totally resistant to the cytotoxic effects of activated macrophages (reviewed by Keller, 1981). Lohmann-Matthes, Kolb and Meerpohl (1978) reported that normal cells could be killed by macrophages activated in vitro with MAF. However, they showed that they were less susceptible than tumour cells of the same histological origin. In addition, they found that not all of the normal cells examined were susceptible and that higher macrophage to target cell ratios were required to kill susceptible normal cells than tumour cells.

The cytotoxic (and cytostatic) activity of normal inflammatory macrophages has not been examined as thoroughly. Keller (1976a) has shown that, although proteose-peptone induced rat peritoneal macrophages could inhibit the proliferation of "every rapidly proliferating cell line irrespective of transformation, species derivation, cell type, or growth characteristics," there was "no evident correlation between susceptibility to cytostasis and degree of transformation." In addition, normal fibroblasts were not killed by these macrophages and the sensitivity of transformed cells varied but did not correlate with their degree of malignancy. In general, where killing was detected, the levels obtained were low compared to those reported for activated macrophages.

In a later report, Keller (1978) showed that cells taken from early passages of normal mouse epidermal cells were nearly as susceptible to the low level of cytotoxic activity of normal macrophages as some of the more susceptible tumour cell lines.

1.9 Mechanism of tumour cytotoxicity by activated macrophages

1.9.1 Cytotoxicity requires binding of macrophages to tumour cells

It became apparent from experiments conducted a decade ago that the mechanism by which activated macrophages kill tumour cells requires that the effector and target cells are in close proximity. Cleveland, Meltzer and Zbar (1974) found that if adherent tumour cells were cultured in Petri dishes that contained a small central area of BCG-activated macrophages, 16-32 h later the tumour cells which initially adhered in this area were pyknotic and fragmented. In contrast, the tumour cells distal to the macrophage monolayer were healthy and had grown to confluence. Similar findings have been reported by Hibbs and his colleagues (reviewed by Weinberg and Hibbs, 1978). These studies and those of Holterman, Klein and Casale (1973) and Kaplan, Morahan and Regelson (1974) provided good evidence that cytotoxicity was not effected by a long-lived, soluble cytotoxic factor.

Visual examination revealed that there are physical interactions between tumour cells and activated macrophages during the cytotoxic reaction in which there is contact between target and effector cells. Hibbs (1974) reported that activated macrophages could extend pseudopods towards tumour cells and transfer lysosomal granules into them whereas normal ones did not do so. Bucana et al. (1976) confirmed these results and ultrastructural studies showed that although there were areas of close association between tumour cell and activated macrophage surface membranes, they did not fuse. The interaction between activated macrophages and tumour cells is highly dynamic. Microcinematographic

analysis revealed that BCG-activated macrophages clustered around, on and under neoplastic cells. Contact of individual macrophages with tumour cells lasted about 2 h. After this time they proceeded to make contact with another tumour cell. There were contacts made between activated macrophages and non-neoplastic cells, and normal macrophages with neoplastic and normal cells but these were less frequent and clustering of macrophages around the target cells was not seen (Meltzer, Tucker and Bruer, 1975a).

Recently it has been demonstrated that the interactions described above are represented by a strong selective binding of tumour cells to activated macrophages, and that activated macrophages can bind tumour cells more efficiently than resident or inflammatory macrophages. Piessens (1978) reported that oil-induced guinea pig macrophages treated in vitro with MAF bound twice as many syngeneic line 10 hepatoma cells as did untreated control macrophages. The increased binding was selective for tumour cells because there was no difference in the number of splenic lymphocytes or erythrocytes that were bound by MAF-treated macrophages. This finding has been confirmed and extended by Marino and Adams (1980a, b). They established that monolayers of BCG-activated macrophages can bind non-adherent syngeneic and allogeneic tumour-cells to a 4-15 fold greater extent than monolayers of resident or thioglycollate-induced macrophages. Activation did not result in an increase in the small amount of binding of splenic lymphocytes observed with resident and thioglycollate induced macrophages.

Binding of tumour cells to BCG-activated macrophages was temperature and time dependent, with maximal binding being reached after 60 minutes at 37°; it was dependent on the density of macrophages and tumour cells in the tissue culture wells. The binding sites on the macrophages could be saturated with large numbers of target cells (Marino and Adams, 1980a). Activated macrophages have to be metabolically active to bind tumour cells because heat-killed macrophages and those treated

with the glycolysis inhibitor, iodoacetate, did not bind tumour cells. In contrast, this is not required of tumour cells (Marino and Adams, 1980a).

1.9.2 The relationship of binding to cytolysis

Not surprisingly, there is a close correlation between the ability of activated macrophages to bind tumour cells and their ability to lyse them. Marino and Adams (1980b) have found that treatments which increase or decrease in vitro binding of target cells to activated macrophages have a corresponding effect on the in vitro cytolysis of tumour cells. In contrast, they showed that the small numbers of normal cells which do bind to activated macrophages are not lysed by them. Nor are those tumour cells which bind to non-cytotoxic, thioglycollate-induced macrophages.

Marino and Adams (1980b) demonstrated that binding of tumour cells to activated macrophages occurs via determinants which are not present on lymphocytes because the addition (and binding) of unlabelled tumour cells, but not lymphocytes, inhibited the lysis of subsequently added labelled tumour cells. They also provided direct evidence that tumour cells had to be bound to macrophages to be killed by them. Tumour cells which were not bound by activated macrophages after 1 h of co-culture were not lysed if they were removed at this time and then cultured with thioglycollate-induced macrophages. On the other hand, tumour cells which had bound during the 1 h of co-culture were lysed (Marino and Adams, 1980b).

Although these data suggest that the binding of tumour cells to activated macrophages is a prerequisite for cytolysis, subsequent studies by Adams and his colleagues have established that it is not sufficient to ensure that lysis occurs. Lysis also requires the release of a cytotoxic factor. These data are discussed in §7.5.

1.10 The nature of the tumour-cell receptors on cytotoxic macrophages

The finding that the in vitro tumouricidal activity of macrophages requires binding of tumour cells to effector cells, strongly suggests that macrophages recognize determinants on the tumour cell surface. The receptors on macrophages that mediate the recognition/binding of tumour cells are still only poorly characterized.

1.10.1 Receptors on specifically-cytotoxic macrophages

Reports of specifically cytotoxic macrophages have appeared in the literature for some time (reviewed by Weinberg and Hibbs, 1978 and Nelson, Hopper and Nelson, 1978). In general, it is likely that reports of the ability of macrophages to exhibit antigen-specific cytotoxicity against individual types of tumour cells is due to the acquisition by macrophages of specific, cytophilic immunoglobulin or immunoglobulin-like molecules.

Specific macrophage cytotoxicity mediated by a T cell-derived factor was demonstrated by Evans et al. (1972) using macrophages from DBA/2 mice immunized with heavily irradiated syngeneic lymphoma cells. T cells from immunized mice released a factor which "armed" normal macrophages, but not spleen cells, by conferring cytotoxic activity on them in a specific manner (Evans et al. 1972; Lohmann-Matthes et al. 1973). This factor was termed Specific Macrophage Arming Factor (SMAF). SMAF appeared to have two separate binding sites: an immunologically specific one for the tumour cells used for immunization and a "non-specific" one which binds to macrophages from syngeneic and allogeneic mice and to macrophages from rats. SMAF was not conventional immunoglobulin because it was produced by T cells and had two different molecular weights: one species had a molecular weight of 300,000 whilst the other was 50,000-60,000 which is less than intact immunoglobulin (Evans et al., 1972)

A role for specific antibody in macrophage cytotoxicity was first

established by Granger and Weisser (1966). They showed that heating macrophages from mice immunized against the allogeneic SaI tumour to 56° released a factor which had immunoglobulin-like properties: it could agglutinate the cells of the immunizing cell line and could fix complement. These observations were supported by those of Hoy and Nelson (1969). They showed that the serum of C57BL/6 mice which had been allo-immunized against the SaI tumour or A/J strain skin grafts had antibodies which could bind to normal macrophages and facilitate the adherence of SaI tumour cells or A/J lymphocytes to the macrophages. This phenomenon in which antibody mediates the cytotoxic activity of effector cells is called Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). In order to lyse tumour cells in the presence of antibody, macrophages have to be physiologically altered. It has been found that resident macrophages are not active in ADCC whilst those from mice injected with thioglycollate broth, C. parvum or BCG are (Bast et al., 1979, Yamazaki et al., 1976; Pasternack, Johnson and Shin, 1978a; Koren, Anderson and Adams 1981; Koren, Meltzer and Adams, 1981; Nathan et al., 1980; Lohmann-Matthes, Domzig and Taskov (1979); Domzig and Lohmann-Matthes, 1979).

In addition to macrophages, a variety of other cell types from normal animals can kill antibody coated target cells in vitro, whereas neither the cells nor the antibody are cytotoxic on their own. Therefore, it would seem appropriate at this point to digress and briefly discuss the characteristics of ADCC.

1.10.2 Antibody-dependent cell-mediated cytotoxicity

The first demonstration of ADCC was by Möller in 1965 who showed that lymph node cells (LNC) from allogeneic or semi-syngeneic normal mice could kill an MCA-induced mouse sarcoma in vitro, but only in the presence of rabbit-antimouse antibody. These findings have been

confirmed and extended by many independent workers (reviewed by Pearson, 1978).

The interaction of effector cells and target cells occurs via the Fc region of immunoglobulin. This is because ADCC effector cells have Fc receptors (Perlmann, Perlmann & Wigzell, 1972), and ADCC can be inhibited by Ab-Ag complexes, heat aggregated IgG (van Boxel et al., 1973; Scornick et al., 1974) or Fc reactive material such as staphylococcal protein A and rheumatoid factor (Austin and Daniels, 1976). Furthermore, ADCC does not occur if Fab or F(ab)'₂ fragments or mercaptoethanol-treated antibodies are used (Möller and Svehag, 1972; Perlmann, 1972; MacLennan, 1972; van Boxel et al., 1974).

Although the Fc region of immunoglobulin is required for ADCC, complement is not required because ADCC can occur (1) using heat inactivated antiserum, (2) in the presence of the complement inhibitor carrageenan (Pollack and Nelson, 1973), (3) at dilutions of antiserum which are too low to cause complement mediated lysis of target cells (Perlmann, Perlmann and Wigzell, 1972; Pollack and Nelson, 1973) and (4) with sera and cells from mice deficient in their ability to synthesize the complement components C'4 to C'6 (van Boxel et al., 1974).

All subclasses of IgG can mediate ADCC, but they may vary in their efficiency to do so (reviewed by Pearson, 1978). There is some evidence to suggest that IgM may also participate in ADCC (Dennert and Lennox, 1973; Lamon et al., 1975; Blair, Lane and Marr, 1976). Recent experiments, using monoclonal IgM, suggest IgM may require the presence of small amounts of IgG in order to participate in ADCC because IgM can synergise with suboptimal amounts of IgG in mediating ADCC (Shen et al., 1981). This suggests that, in earlier experiments, the activity of IgM may have been due to contaminating IgG in the antibody preparation.

As mentioned previously, several different cell types can effect ADCC. These include macrophages and polymorphonuclear (PMN) cells (Temple et al., 1973; Gale and Zighelboim, 1974) as well as a poorly

defined effector cell known as the Killer (K) cell. K cells have neither T cell or B cell markers and have dense, ovoid, eccentric nuclei. They have complement receptors and, not surprisingly Fc receptors for IgG (Greenberg et al., 1975). Although they possess Fc receptors, they are not phagocytic (Allison, 1972; Greenberg et al., 1973a). However, they may be related to macrophages because they are weakly adherent and have the same size and density as macrophages (Greenberg, Shen and Roitt, 1973b). This notion is consistent with the observation that promonocytes from bone marrow cultures can lyse antibody-coated tumour cells (Lohmann-Matthes, Domzig and Taskov, 1979; Domzig and Lohmann-Matthes, 1979).

It appears that the different effector cells may be restricted in the target cell-type they can lyse. Sanderson (1975) demonstrated that sensitized chicken erythrocytes are lysed by adherent and non-adherent effector cells, whereas only the non-adherent cells were active against two cell lines. Similar findings were reported by Nelson et al., (1976), using human peripheral blood leukocytes as effector cells.

1.10.3 Receptors on non-specifically cytotoxic, activated macrophages

The tumour receptors on activated macrophages are even less well characterized than those on specifically cytotoxic macrophages. They appear to be protein in nature because treatment of macrophages with trypsin (Marino and Adams, 1980a) or with inhibitors of protein synthesis such as pactamycin, puromycin or actinomycin D (Sharma and Piessens, 1978a) inhibit binding of tumour cells. This is consistent with the finding that these drugs abrogate the cytotoxic activity of MAF-activated macrophages (Sharma and Piessens, 1978a). Furthermore, the activity or expression of these receptors is controlled by cytoskeletal structures of the macrophage because cytochalasin A and B, VBL and colchicine treatment of macrophages will inhibit the augmented binding of tumour cells to MAF-activated macrophages (Sharma and Piessens, 1978b).

1.11 The role of soluble cytotoxic factors secreted by macrophages.

Despite the failure to demonstrate the involvement of soluble cytotoxic factors in the cytotoxic mechanism of activated macrophages (discussed in §1.9.1), other workers have reported the presence of cytotoxic and cytostatic activities in culture supernatants conditioned by activated macrophages in the presence or absence of tumour cells (reviewed by Weinberg and Hibbs, 1978). Some of these activities may not be involved in the killing of tumour cells by activated macrophages. For example, the lytic activity of some conditioned supernatants is not selective for tumour cells (Pincus, 1967; Piper and McIvor, 1975; Reed and Lucas, 1975; Kramer and Granger, 1972) and Calderon and his co-workers have shown that the cytostatic activity of their macrophage-conditioned supernatants was not selective for tumour cells (Calderon and Unanue, 1975; Calderon, Williams and Unanue, 1974). In addition, there are a number of examples where the selectivity, or otherwise, of the factors was not established (Heise and Weiser, 1969; Melsom et al., 1974). Notwithstanding these considerations, the incomplete characterization of the mechanism of killing of tumour cells has revealed that certain molecules such as thymidine, arginase, serine proteases, tumour necrosis factor and hydrogen peroxide which are secreted by macrophages may be cytotoxic for tumour cells. Whether or not any of these molecules are involved in the cytotoxic mechanism appears to be dependent on several factors; these include the source of the macrophages, the type of target cell used, the composition of the culture medium, the type of assay used to detect killing and/or cytostasis and the ability of effectors and targets to contact each other. These considerations are emphasised in the discussion presented in the next section.

1.11.1 Effect of thymidine

Macrophages from inflammatory exudates will inhibit the

proliferation of tumour cells in vitro, as assessed by either inhibition of the uptake of $^3\text{H-TdR}$, direct cell counting, or measurement of nuclear DNA content (Keller, 1974; Keller, 1976; Keller et al., 1976; Remington, Stadecker et al., 1977).

It is likely that thymidine, released from the macrophages, can cause the cytostasis observed because excess thymidine can block the division of cultured cells (Stadecker et al., 1977). Macrophages produce and release thymidine by de novo synthesis (Stadecker et al., 1977) and degradation of nuclear material from target cells killed by macrophages provides another source of thymidine for secretion (Opitz et al., 1975)

Whether cytostasis is observed probably depends on the tumour targets used. Although the division of tumour cells is more sensitive to blocking than that of mitogen-stimulated lymphoid cells, variation in sensitivity has been reported between different tumour cell lines. For example, concentrations of thymidine as low as 10^{-6} M have an effect on division of EL-4 thymoma cells, whereas normal lymphoid blast cells require 3,000 times this concentration. On the other hand, P815 mastocytoma cells require 250 times more thymidine than EL-4 cells to achieve a 50% inhibition of $^3\text{H-TdR}$ uptake (Stadecker et al., 1977).

1.11.2 Effect of arginase

In 1975 Currie and Basham showed that "conditioned" medium from cultures of macrophages apparently contained a cytotoxic soluble factor which was selective for tumour cells. Follow-up studies established that the cytotoxic factor released by cytolytic macrophages was arginase. It functioned by depleting culture medium of arginine because cytotoxicity could be inhibited by the addition of excess arginine (Currie, 1978). It was established that the selective toxicity of arginine depletion reflects an inability of tumour cells to survive periods of arginine starvation. Currie and Basham (1978) showed that, to survive, tumour

cells do not require higher concentrations of arginine than normal cells.

These workers did not define the lesion produced by limiting levels of arginine, but suggested two alternatives. Because arginine is a precursor for ornithine which is involved in the urea cycle, leading to the production of polyamines, growth inhibition could occur through a reduction in polyamine levels. They did not favour this alternative because the addition of ornithine to culture medium did not inhibit the cytotoxicity caused by arginine depletion (Currie, 1978). The more likely alternative is that lack of arginine affects protein synthesis. Weisfeld and Rouse (1977) have shown that depletion of arginine does not inhibit initiation of DNA synthesis by cells, whereas RNA and protein synthesis is depressed (Weinberg and Beker, 1970). Furthermore, Currie and Basham (1978) showed that addition of citrulline to culture medium ensured normal proliferation of tumour cells, probably because arginine was produced from citrulline via argino-succinate. Arginine starvation also leads to chromosomal abnormalities (Freed and Schatz, 1969). The selective toxicity of arginine depletion reflects an inability of tumour cells to survive periods of arginine starvation rather than a need for increased arginine levels. Normal cells can survive such deprivation (Currie and Basham, 1978).

The importance of arginine deprivation as the mechanism of tumour growth inhibition by macrophages may depend on the tumour cell line used and the source of the macrophages. Whilst Currie and Basham (1978) showed that paired normal and neoplastic cell lines differed in their susceptibility to arginine deprivation, they also showed that different tumour cell lines vary in their susceptibility to the toxic effects of arginine starvation. The hamster and rat tumour cell lines used in their experiments were more susceptible than tumour cell lines obtained from mice and humans. Furthermore, Fishman (1980) concluded that macrophages from different species differ in their ability to secrete arginase because he found that inflammatory macrophages from mice contain more

arginase than those from rats. He also established that low density macrophages are richer in arginase than those with a higher buoyant density.

Reports from Nelson and his colleagues indicate that, even when using the same tumour cell line, the relative importance of arginase to the cytotoxic mechanism may be determined by the effector cell population and the assay system employed. They found that using a 48 h ^{125}I -UdR release assay, the addition of 200 ug/ml arginine to the culture medium did not inhibit the release of ^{125}I -UdR from prelabelled C-4 methylcholanthrene-induced fibrosarcoma cells by PC from 11RX-immunized mice, and produced only a small reduction when PC from C-4-tumour-bearing mice were used as effector cells (Hopper, Harrison and Nelson, 1979). However, Farram and Nelson (1980) reported that the addition of arginine almost completely inhibited the killing of C-4 tumour cells in a microcytotoxicity assay. In this assay, detection of cytotoxicity is dependent on the inhibition of target cell proliferation and their detachment from the culture vessel.

1.11.3 Effect of serine proteases

The involvement of serine proteases in the tumouricidal and microbicidal functions of activated macrophages has been shown in a variety of systems (Hibbs et al., 1977; Buchmüller and Mael, 1980; McLeod and Remington, 1980; Adams, 1980; Adams et al., 1980). Serine protease inhibitors can enhance the killing of phagocytosed Leishmania (Buchmüller and Mael, 1980) or inhibit the killing of Toxoplasma (McLeod and Remington, 1980) and tumour cells (Hibbs et al., 1977; Adams, 1980; Adams et al., 1980). Protease inhibitors may affect the activation of macrophages (Hibbs et al., 1977; Buchmüller and Mael, 1980) and can inhibit their tumouricidal activity (Hibbs et al., 1977; Adams, 1980; Adams et al., 1980; Piessens and Sharma, 1980). A cytotoxic factor in serum-free medium "conditioned" by BCG activated macrophages cultured in

the absence of tumour cells has the properties of a serine protease (Adams et al., 1980).

Thioglycollate-induced macrophages do not spontaneously secrete the cytotoxic factor, however they will do so when stimulated by LPS, a procedure which induces cytotoxic activity in these cells (Adams et al., 1980). Adams et al. (1980) showed that the protease responsible for killing of tumour cells may be a unique one, synthesised and secreted only by cells capable of killing tumour targets. They did this by examining the protease activity of fractions of culture supernatants obtained from cytotoxic and non-cytotoxic macrophages. The activated macrophages they used were those obtained from BCG infected animals and thioglycollate-induced ones which were stimulated by addition of LPS; non-stimulated thioglycollate-induced macrophages were the other cells used. They detected 5 peaks of protease activity in fractions prepared from the culture supernatant of non-cytotoxic macrophages; an additional peak of protease activity was present in culture supernatants conditioned by BCG-activated macrophages and thioglycollate-induced macrophages stimulated with LPS. The cytotoxic activity co-chromatographed with this new peak of protease activity and could be inhibited by the addition of serine protease inhibitors.

This cytotoxic protease was selective in its action. It killed a wide variety of tumour cells but had no effect on non-malignant cells such as primary explant mouse embryo fibroblasts, normal lymphocytes and a nontransformed human cell line (Adams et al., 1980). It is secreted when BCG-activated macrophages are cultured by themselves, but its release is augmented when tumour target cells (but not lymphocytes) are added (Johnson, Whisnat and Adams, 1981).

Because anti-proteases are present in body fluids such as plasma, it is obvious that the cytotoxic activity of this serine protease will be rapidly inactivated when secreted by tumouricidal macrophages. If the protease is to effect the lysis of tumour cells, it needs to be protected

from the extracellular environment. Adams et al. (1980) have proposed this may occur when activated macrophages bind tumour cells because the gap between the two cell membranes is less than 200 Angstroms wide. The diffusion into the gap of serum anti-proteases would be restricted because they are large molecules ranging in molecular weight from 55,000 M_r to 670,000 M_r , therefore serine proteases secreted into the intercellular space would be able to damage the tumour cell membrane. Such a model is consistent with their finding that two protease inhibitors of 6,300 and 184 M_r respectively, can inhibit the lysis of tumour cells by BCG-activated macrophages when the assay is conducted in serum-supplemented tissue culture medium (Adams, 1980), and that, in the absence of serum, the target cells are lysed even if binding of activated macrophages to tumour cells is inhibited by an intervening cell impermeable membrane. As predicted, lysis in serum-free medium was inhibited by protease inhibitors (Adams and Marino, 1981).

1.11.4 Tumour necrosis factor

The sera from BCG-immunized mice injected with LPS can cause the necrosis of established tumours when injected into tumour-bearing recipients. Furthermore, such sera can be cytotoxic for some, but not all, tumour cell lines in vitro (reviewed by Ruff and Gifford, 1981). This activity is called Tumour Necrosis Factor (TNF) and is released by activated macrophages stimulated with LPS (Ruff and Gifford, 1981). TNF is distinct from the serine protease described in the previous section since its activity is unaffected by protease inhibitors (Ruff and Gifford, 1981).

1.11.5 Oxygen Metabolites

It has been known for some time that when neutrophils phagocytose particles the Hexose Monophosphate (HMP) shunt is activated and oxygen metabolites including superoxide anion (O_2^-), hydroxyl radicals ($\cdot OH$)

and hydrogen peroxide (H_2O_2) are generated and secreted. The possibility that some or all the above species are involved in tumour cell killing by macrophages is implied by two findings. Firstly, by-stander tumour cells are killed during phagocytosis of opsonized zymosan particles by polymorphonuclear (PMN) leukocytes by a mechanism involving the release of oxygen metabolites from PMN cells (Clark and Klebanoff, 1975; Edelson and Cohn, 1973), and secondly, that during phagocytosis macrophages, like neutrophils, undergo an oxidative burst and HMP shunt activation and release oxygen metabolites (reviewed by Johnston, 1978). Furthermore during phagocytosis or stimulation with surfactants such as phorbol myristate acetate (PMA), activated and elicited macrophages have a higher level of HMP shunt activation, as detected by O_2^- release, than resident ones (Johnston, Godzik and Cohn, 1978).

More compelling evidence that oxygen metabolites may be involved in tumour cytolysis by activated macrophages was presented by Nathan et al., 1979a, b). They reported that BCG-activated macrophages stimulated by PMA kill tumour cells a rapid manner i.e. within 4-6 h. The cytotoxic metabolite appears to be H_2O_2 because the abolition of H_2O_2 secretion by depleting the cultures of oxygen or glucose inhibited cytolysis as did the presence of scavengers of H_2O_2 such as catalase or thioglycollate medium (Nathan et al., 1979b) where as scavengers of O_2^- and $.OH$ had no effect. Furthermore, thioglycollate-induced macrophages did not release H_2O_2 or lyse tumour cells when stimulated with PMA which correlates with the known lack of cytotoxic activity of these cells (Nathan et al., 1979a). Consistent with the notion that activated macrophages kill tumour cells by secreting H_2O_2 , Piessens Hallowell and Churchill (1981) found that catalase inhibited the lysis of syngeneic hepatoma cells by MAF-activated guinea pig macrophages.

Nevertheless, there is evidence which suggests that H_2O_2 does

not play a role in tumour cell killing. Doubts have been raised as a result of attempts to correlate the ability of macrophages to secrete this metabolite with their ability to kill tumour cells. While it could be argued that failure to detect oxygen metabolites is due to their highly reactive nature which ensures that they do not persist for long once they are secreted, their release from macrophages has been demonstrated by a number of workers who have usually found a lack of correlation between release and cytotoxic activity of the macrophages. Cohen, Taffet and Adams (1982) found that macrophages from mouse strains in which BCG infection does not induce in vitro tumouricidal macrophages were still capable of releasing H_2O_2 on exposure to PMA. In addition, whilst the cytotoxic activity of activated macrophages is maintained during overnight culture by the presence of LPS, their ability to release H_2O_2 is not. In addition, the validity of concluding that H_2O_2 is a cytotoxic effector molecule can be questioned on the basis of experimental design. In many investigations the experimental conditions used appear highly contrived and unphysiological. For example, apart from the use of antibody coated target cells (Nathan and Cohn, 1980) in many experiments the release of H_2O_2 is stimulated by pharmacological agents, in particular PMA (Nathan et al., 1979a, b). Yet the activation of the HMP shunt and the stimulation of H_2O_2 release by activated macrophages are not generalized phenomena associated with membrane interactions. For example, LPS and digitonin are inactive in this regard (Nathan et al., 1979a), and tumour cells do not induce the HMP shunt or O_2^- production in C. parvum activated macrophages (Bryant and Hill, 1982). Furthermore these workers found that scavengers of H_2O_2 did not inhibit tumour killing (Bryant and Hill, 1982).

Piessens et al. (1981) have also expressed difficulty in interpreting their finding that catalase inhibits the cytotoxic activity of MAF-activated guinea-pig macrophages. They found that the amount of enzyme needed to cause this effect was 3×10^8 times higher than that

necessary to inactivate the amount of H_2O_2 generated by effector cells during the assay. They suggest therefore, that catalase may be acting on some other substrate for which it has a lower affinity than H_2O_2 . In addition, it appears that in the absence of stimulants such as PMA or Ab, MAF-activated macrophages may not be able to release enough H_2O_2 to kill tumour cells. For example, in order for xanthine oxidase coated particles to lyse tumour cells they need to produce H_2O_2 at a rate 50,000 times faster than MAF-activated macrophages.

Notwithstanding these misgivings, H_2O_2 may have a role in the cytotoxic mechanism if, as proposed for serine proteases, high concentrations of it can be generated in the intercellular space between tumour cell-macrophage conjugates. It follows that high concentrations of catalase would be needed to inhibit cytotoxicity because, being a large molecule ($M_r = 232,000$), it would not diffuse efficiently into this space. There is some in vitro evidence that H_2O_2 and serine proteases can interact in the lysis of target cells. Non-cytotoxic amounts of serine proteases and H_2O_2 act synergistically in lysing tumour cells in vitro (Adams et al., 1981). Furthermore, pretreatment of serine protease-resistant tumour cells with non-lytic amounts of H_2O_2 renders them sensitive to serine proteases. Therefore, since activated macrophages secrete H_2O_2 at a low level in the absence of any added stimulus (Nathan and Klebanoff, 1982) this may be sufficient to predispose certain target cells to lysis by serine proteases.

Finally, as implied above, there is good evidence that H_2O_2 released by activated macrophages is an important cytotoxic molecule in ADCC by activated macrophages (Nathan and Cohn, 1980). They found that Ab coated tumour cells were as effective as PMA in inducing the release of H_2O_2 from activated macrophages. Cytotoxicity was inhibited by depletion of glucose and oxygen from the culture which inhibits H_2O_2 release, and was similarly reduced in the presence of the H_2O_2 scavenger, thioglycollate broth.

1.12 Natural killer cells

As mentioned previously in §1.3.2, there is a class of cytotoxic lymphoid cells in normal mice known by the generic title of NK cells that can lyse some, but not all, tumour cells in vitro in the absence of added antibody and without prior immunization of the host. Amongst the first descriptions of natural cytotoxicity against tumour cells were those of Herberman and Kiessling and their respective co-workers. They demonstrated that spleen cells from normal mice were particularly active in lysing Moloney virus-induced leukaemia cells (Herberman et al., 1975a, b; Kiessling et al., 1975a, b). Since then, natural cytotoxicity has been reported in humans (Peter et al. 1975), primates (Clarke, Sturge and Falk, 1981), chickens and a wide variety of mammalian species (Leibold, Janotte and Peter, 1980). Although NK cells are found probably all species, the best characterized ones are those of mice. The characteristics of murine NK cells, their interactions with susceptible tumour cells and their possible role in vivo are discussed below.

1.12.1 Characteristics of NK cells

Initial analyses of murine effector cells showed that they were lymphocyte-like but could not be classified as conventional T cells, B cells or macrophages (reviewed by Kiessling and Wigzell, 1979 and Herberman et al., 1979). The effector cells possess high levels of the neutral glycolipid asialo-GM1 (Young et al., 1980), the Ly5 alloantigen (Cantor et al., 1979) and an allo-antigen designated NK1 which is selectively expressed on NK cells (Cantor et al., 1979).

More recently, however, it has become apparent that NK cells are a heterogenous population of effector cells which may differ in their origins and phenotypes. For example, NK activity is not restricted to cells of lymphoid origin because Lohmann-Matthes et al. (1979) demonstrated that promonocytes from bone marrow cultures could act as NK cells in vitro and Keller (1978) also reported that normal macrophages

may also exhibit natural cytotoxicity. Moreover "activated" NK cells i.e. cells whose activity has been augmented due to various IBP (see §3.1) and viral infections and other treatments which induce the release of interferon in vivo (reviewed by Welsh, 1981) appear to differ from "endogenous" NK cells found in unstimulated mice. They possess Fc receptors, express more Thy 1.2 antigens and are more adherent to plastic and nylon wool than normal NK cells (Herberman et al., 1978; Kiessling et al., 1980).

Several different groups of workers have shown that lymphoid cell suspensions consist of subpopulations of NK cells which differ in the target cells they can lyse (Kumar et al., 1979; Stutman, Paige and Figarella, 1978; Lust et al., 1981; Minato, Reid and Bloom, 1981). Various subpopulations can also be distinguished. The cytotoxic activity of some, but not all, subpopulations can be abolished by in vitro treatment of effector cells with anti-NK 1.2 and complement. Furthermore, lymphoid cells of mice which are homozygous for the recessive "beige" mutation show severely impaired in vitro NK cell activity for many but not all NK susceptible target cells (Lust et al., 1981). The "beige" mutation of mice is an autosomal recessive mutation which appears to result in a defect similar to the Chediak-Higashi syndrome in man; it was demonstrated that lymphoid cells from these mice could not kill several "classic" NK targets although they have functionally intact macrophages, B cells and T cells (Roder and Duwe, 1979). Furthermore, Minato et al. (1981) have established that NK cells can be divided into four subclasses on the basis of their expression of surface markers including Thy 1.2, Qa5, Lyt 2 and Ly 5, their target cell preference and the effects of interferon and IL2 on their in vitro activity. The notion that NK cells are heterogenous is further supported by analysis of cloned lymphoid cells with NK activity that have been maintained on long-term in vitro culture (Nabel et al., 1981; Dennert, Yogeeswaren and Yamagato, 1981; Brooks et al., 1982).

1.12.2 Evidence of a role for NK cells in vivo

Because NK cells are present in animals without prior exposure to tumours, it has been proposed that they may form a "first line of defence against nascent tumours" (Klein, E., 1980). Formal proof of this notion is not available, but there is some experimental evidence which suggests they may play an in vivo role in tumour resistance. For example, there is an inverse correlation between the susceptibility of tumour cells to lysis by NK cells in vitro and their tumourigenicity in vivo (Warner et al. 1977; Riesenfeld et al., 1980; Kiessling et al., 1975c). Furthermore, tumour cells from metastases can be more resistant to lysis in vitro by NK cells than cells from the primary tumour (Gorelik et al., 1979), a finding which implies that NK cells may exert a selective pressure during neoplastic disease in vivo.

A more direct approach has been to test the tumour resistance of normal mice made putatively NK cell-deficient by injection of agents, such as cyclophosphamide (Cy) or antibody to asialo-GM1, treatments which are known to reduce greatly the in vitro NK cell activity of lymphoid cells (Riccardi et al., 1980; Young et al., 1980). The rationale of this approach is that, if NK cells are important in the control of tumour cell growth, the treated mice would be less resistant to tumour challenge. Using this approach, Hanna and Fidler (1980) concluded that NK cells do have a role in vivo in controlling the growth of NK susceptible but not resistant tumours. They demonstrated that Cy-treated mice injected iv with in vitro cultured B16 melanoma cells, which are more susceptible to NK cells in vitro than uncultured ones, developed more pulmonary and extra-pulmonary metastases than untreated controls. Kawase et al. (1982) and Gorelik et al. (1982) arrived at a similar conclusion using mice treated with anti-asialo GM1 antibody. Consistent with these observations, Riccardi et al. (1981) and Gorelik et al. (1982) demonstrated that such mice had a reduced capacity to destroy iv injected radioactively-labelled NK susceptible tumour cells. Their conclusions

were supported by the finding that an infusion of a subset of normal spleen cells with the characteristics of NK cells restored the tumour resistance of treated animals (Hanna and Fidler, 1980; Riccardi et al., 1981; Gorelik et al., 1982). In general, it appears that manipulations or genetic defects that deplete mice of in vitro NK cell activity make them more susceptible to NK sensitive tumours but not resistant ones (Kawase et al., 1982; Talmadge et al., 1980).

The above studies can be criticized because the effects of Cy and anti-asialo GMI are transient and may affect cell types other than NK cells. However, similar results have been obtained using mice homozygous for the "beige" mutation. It has been established that beige mice are more susceptible than normal heterozygous bg/+ mice to the growth and metastasis of small inocula of tumour cells (Kärre et al., 1980; Talmadge et al., 1980) and, predictably, have a reduced ability to clear radioactively-labelled tumour cells (Kärre et al., 1980; Gorelik et al., 1982). Recently Warner and Dennert (1982) provided direct evidence of a role for NK cells in vivo. They demonstrated that injection of small numbers of cloned NK cells greatly enhanced the resistance of beige mice to artificial lung metastases induced by iv injection of tumour cells and to the induction of leukaemias by repeated low dose X-irradiation.

1.12.3 The mechanism of NK cell-mediated lysis

The mechanism by which NK cells lyse tumour cells is poorly understood but it involves the initial binding of target cells followed by the secretion of a cytotoxic factor(s) by the effector cells. However, binding is not sufficient for lysis to occur because some target cells which can bind to NK cells are not lysed (Collins, Patek and Cohn, 1981; Kunkel and Welsh, 1981).

Early studies involving competitive inhibition of NK cell lysis demonstrated that NK cells recognise surface structures common to all susceptible target cells irrespective of their origin and the known

differences in defined surface antigens (Herberman et al., 1975a; Kiessling et al., 1975a). These findings were consistent with a subsequent report by Roder and Kiessling (1978) that sensitive target cells, but not resistant ones, could bind to lymphocyte suspensions which had been enriched for NK cell activity. Furthermore, the degree of binding closely correlated with the sensitivity of the target cells to NK lysis, and with other variations in NK cell activity such as those observed with different mouse strains, or with suspensions obtained from different lymphoid organs taken from animals of various ages. Subsequently, Roder and his colleagues established that target cell binding is mediated by molecules of four molecular sizes ranging from 140,000 to 240,000 M_r which can be extracted from membranes of NK cell susceptible tumour cell lines. The molecules can inhibit the binding of susceptible tumour cells to NK cell enriched splenic lymphocytes. As predicted, these molecules are not present in the plasma membranes of tumour cells which do not bind to NK cells (Roder et al., 1979a; Roder, Ahrlund-Richter and Jondal, 1979b).

Little is known of the binding structures on susceptible cells. Contrary to an early hypothesis (Kiessling et al., 1975a), they are not necessarily murine leukaemia virus antigens because sensitivity to lysis does not correlate with the expression of serologically defined murine C viral proteins and glycoproteins (Becker, Fenyo and Klein, 1976), and some normal foetal and adult tissues can be lysed by NK cells (reviewed by Kiessling and Wigzell, 1981). Surface carbohydrates may form be part of the NK structures on susceptible target cells because simple sugars can inhibit NK cell lysis (Ades, Hinson and Decker, 1981). Alternatively, the carbohydrates may determine cell surface charge which may be a factor in NK cell-target cell interactions. For example, it has been found that the greater the amount of sialic acid on the target cell, and hence the greater its negative charge, the less sensitive they are to lysis by NK cells (Durdik et al., 1980; Yogeewaran et al., 1981).

As mentioned previously, not all tumour cells are lysed by NK cells and, as discussed above, some target cells are resistant to lysis because they do not bind to NK cells. However, binding of target cells to NK effectors, although necessary, does not automatically ensure that lysis of the cell will occur, i.e. it appears that the ability of target cells to bind is not directly related to their sensitivity to the cytotoxic mechanism. For example, Collins et al. (1981) have evidence to suggest that the sensitivity of some types of cells to the cytotoxic mechanism is restricted to an intermediate stage of transformation, whilst their ability to bind to NK cells can be a constant characteristic of cells during the entire process of tumourigenesis. They found that normal murine fibroblasts can bind to NK cells although they are not lysed. These become susceptible to lysis following in vitro transformation induced by MCA, when they are no longer dependent on anchorage for their growth and can form tumours in immuno-suppressed mice. When these tumours were passaged in immunocompetent mice, the tumours which eventually grew were resistant to NK cells but still possessed the recognition structures, as indicated by their ability to act as cold target inhibitors.

At least two mechanisms can be proposed to explain why target cells are resistant to lysis even though they have bound to NK cells. It is possible that resistant cells have a "membrane repair" mechanism(s) that is lacking in sensitive cells. Support for this view has been provided by Kunkel and Welsh (1981) and Collins et al. (1981) who demonstrated that NK cell-resistant fibroblasts and fibroblast cell lines which can bind to NK cells become sensitive to lysis when treated with metabolic inhibitors. This observation is similar to reports that metabolic inhibitors render some resistant tumour cells susceptible to lysis by antibody and complement (Schlager et al., 1977; Schlager and Ohanian, 1980).

Alternatively, resistant target cells may lack receptors for NK

cell cytotoxic factors (NKCF). Evidence for this possibility has been provided by Wright and Bonavida (1982, 1983). Their studies involved the use of a soluble factor (NKCF) which is selectively cytotoxic for NK cell-susceptible tumour cells. It is present in supernatants conditioned by murine spleen cells co-cultured with NK cell-susceptible targets but not resistant ones. This factor may not be the only cytotoxic factor involved in killing by NK cells because it is released by the subset of NK cells which is Thy 1.2⁻, asialo GM1⁺. Not surprisingly, NKCF is absorbed efficiently by NK cell-susceptible tumour cell lines but not resistant ones (Wright and Bonavida, 1982). The evidence they presented to support the proposal that tumour cells have receptors for NKCF which are distinct from NK-recognition structures is as follows. They demonstrated that several NKCF-resistant clones of the highly NK-susceptible YAC tumour cell line are poor targets for NK cells even though, like the parent cell line they bind to NK cells and induce the release of NKCF (Wright and Bonavida, 1983). They concluded that some tumour cells can bind to NK cells and activate the cytotoxic mechanism, even though they are resistant to the mechanism because they lack receptors for NKCF.

1.13 Aims of this study

This study was undertaken to examine some aspects of the mechanisms involved in the protection of mice infected with the IBP Salmonella enteritidis 11RX against challenge with transplantable tumours. Because tumour resistance appears to be a non-specific by-product of the immune response to 11RX, it seemed likely that such a study would provide some information regarding both anti-tumour effector mechanisms and also those involved in control of Salmonella infections. The data presented in this thesis are a continuation of previous studies.

1.14 Summary

The data discussed in this chapter establish that the immune response of animals to IBP induces non-specific resistance to other unrelated parasites and to the growth of transplantable tumours. IBP establish a persisting infection and activated macrophages are required for their elimination. Activated macrophages harvested from such animals are functionally and biochemically altered from normal macrophages and are cytotoxic for tumour cells in vitro. There is a suggestion that they may be cytotoxic to a lesser degree for some types of normal cells.

Macrophage activation is mediated by sensitized T cells during the immune response to IBP. During the early phase of the response, to some IBP, lymphocytes which confer protection are short-lived lymphoblasts. Some of these mature to long-lived, resting lymphocytes which are responsible for the recall of antibacterial resistance. Sensitized T cells activate macrophages by secreting soluble factors (MAF) when exposed to antigen. Activation by MAF proceeds as a complex sequence of steps in which LPS may participate.

Activated macrophages kill tumour cells by a contact dependent mechanism that involves the binding of tumour cells to the macrophages. Target cells damage is effected by cytotoxic factors secreted from activated macrophages. The nature of the tumour cell receptors on activated macrophages are poorly understood although specific antibody and SMAF released from T cells may mediate specific cytotoxicity. Macrophages are not the only cells type which are cytotoxic in the presence of specific antibody. In addition to activated macrophages, NK cells from normal animals can kill some tumour cells in a non-specific manner. Their activity is boosted by IBP and viral infections, and, like activated macrophages, they lyse sensitive targets by a contact dependent mechanism. This study was undertaken to investigate some aspects of the tumour resistance induced by llRX infections.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Mice

Inbred mice were used at 6-12 weeks of age. They were bred at the Medical School Animal House (The University of Adelaide) or at the Central Animal House (Waite Agricultural Research Institute, South Australia) from breeding stock originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA). (BALB/cJ x C57BL/6J) F_1 (CB6F $_1$) hybrid mice were used in most experiments. The other strains of mice used were DBA/2J, C57BL/6J, BALB/cJ and CBA/J. In addition to the inbred strains of mice mentioned above, Specific Pathogen Free mice of the LACA strain were used. These mice were bred as a closed colony.

2.2 Bacteria

Salmonella enteritidis 11RX (11RX) is a rough gram-negative organism which is avirulent for mice. Davies (1975) determined that the median lethal dose (LD $_{50}$) for CB6F $_1$ mice by the iv route is approximately 2×10^6 organisms.

11RX was maintained in a lyophilized form at 4° in sealed ampoules. Cultures were established from these by placing the contents of one ampoule into 10 ml of nutrient broth (Difco) and incubating at 37° overnight on a shaker. The culture was then plated on nutrient agar to obtain single colonies and to check that no contamination had occurred. A single colony of 11RX from this plate was inoculated into nutrient broth and cultured overnight as described above. This culture was used to inoculate nutrient agar slopes. Every week 11RX from a slope was streaked onto a nutrient agar plate and used as the source of the

organism for experiments. Each slope was used no more than 10 times and all the slopes were discarded and fresh ones prepared every three months.

2.3 Immunization of mice with llRX

CB6F₁ mice were immunized with 10^5 llRX organisms by the iv or ip route. For immunizing mice, an llRX colony was picked from the nutrient agar plate and inoculated into a 10 ml nutrient broth and incubated at 37°C overnight on a shaking platform. This culture was diluted 1 in 10 into a fresh broth and incubated as above for a further 3 h before being diluted to 5×10^5 organisms/ml in sterile physiological saline. This can be done quite accurately because it has been established that in 3 h cultures llRX organisms are growing logarithmically and are at a concentration of 2×10^9 organisms/ml. 10^5 organisms (0.2 ml) were injected iv using one of the lateral tail veins or ip. The actual number of organisms injected/mouse was determined by further diluting an aliquot of the suspension used to inject the mice and plating it out on nutrient agar to determine the viable count. The dose injected from experiment to experiment varied slightly but was always very close to 10^5 organisms/mouse. The small variations in the dose of llRX injected had no detectable effect on the experimental results. Unless otherwise indicated, all mice were used 6 to 8 days after ip immunization with llRX and will be referred to in this thesis as "llRX-immunized mice".

2.4 Preparation of llRX antigen extract

An llRX cytoplasmic protein antigen extract was prepared by a modification of the method of Ashley et al. (1974). llRX bacteria were disrupted using a French Press and the lysate was centrifuged at $100,000 \times g$ to remove cellular and nuclear debris. The cytoplasmic proteins were purified from the supernatant using phase separation in polyethylene glycol/dextran (Ashman and Kotlarski, unpublished results).

2.5 Lipopolysaccharide

LPS from the smooth Salmonella typhimurium C5 was obtained from Dr. R. B. Johnson (Department of Medicine, Royal Adelaide Hospital, South Australia). It was prepared by phenol extraction according to the method of Westphal, Lüderitz and Bister, (1952).

2.6 Anti-Thy1.2 antiserum

Anti-Thy1.2 antiserum was supplied by Dr. M. P. Ashley of this department. It was raised by immunizing AKR/J mice with thymus cells from CBA/J mice according to the method of Howard et al. (1971).

2.7 Media for preparation and culture of cell suspensions

All media and additives were prepared using deionized distilled water and analytical grade reagents. All solutions were sterilized before use by filtration through a membrane filter (Gelman Filtration Products, Michigan, USA) with 0.45 μm size pores.

2.8.1 Hanks' Balanced Salt Solution

Hanks' balanced salt solution (HBSS) was used for preparing and washing lymph node cell (LNC) and spleen cell suspensions, and tumour cell suspensions for routine in vivo tumour passage. It was prepared from tenfold concentrates of two stock solutions which were made following the recipe of Wellers et al. (1952). The HBSS was adjusted to pH7.4 by the addition of a sterile 1M solution of HEPES (Ultrol, Calbiochem-Behring Corp.) at pH 7.4 to give a final concentration of 20mM. For the preparation and washing of all other cell suspensions, tissue culture medium was used.

2.8.2 Tissue culture media

The culture media used in these experiments were Eagle's Minimal Essential Medium (EMEM) (GIBCO, New York, Cat# 410-1500) and Dulbecco's

modified EMEM (DMEM) (Flow Laboratories, Cat# 10-331-26). These were prepared from the powder base according to manufacturer's directions. They were supplemented with streptomycin (100 ug/ml) and penicillin (100 i.u./ml) (Glaxo, Australia Pty. Ltd.).

Either DMEM or EMEM buffered with 30mM HEPES (pH7.4) and containing 5% (v/v) heat-inactivated foetal calf serum (FCS) (Commonwealth Serum Laboratories or Flow Laboratories) were used for preparing and washing cells suspensions and will be referred to as "suspension media".

The medium used for in vitro cell culture (referred to as "culture medium") was always buffered with sodium bicarbonate (HCO_3^-), at a final concentration of 0.37% (w/v) for DMEM and 0.22% (w/v) for EMEM, and contained 10% FCS (v/v), unless otherwise stated in the text. Other additives which were sometimes added to these media were (a) 30mM HEPES, (pH7.4), (b) 1×10^{-4} M 2-mercaptoethanol (2ME, Sigma), and (c) 1 µg/ml Indomethacin (Merck). Their use is indicated in the text.

2.9 Composition of gaseous phases for buffering in vitro cell cultures

Cell cultures were at incubated 37° in one of two gas phases. For most purposes a humidified atmosphere of 10% CO₂ (Commonwealth Industrial Gases, South Australia) was used; however, for the culture of lymphocytes (e.g. preparation of Con A-activated blast cells, stimulation of lymphokine release and assays for Interleukin 2) a special gas mixture of 10% CO₂, 7% O₂ and 83% N₂ (prepared by C.I.G.) was used.

2.10 Trypan Blue for injection into mice

Trypan blue (Gurr, BDH Chemicals Pty. Ltd.) was prepared by dialysis against three changes of distilled water and then lyophilized. For injecting into animals it was redissolved in pyrogen free saline at 20 mg/ml and autoclaved. Mice received 4 mg of trypan blue by iv or ip route.

For use in vitro, lyophilized trypan blue was dissolved in tissue culture medium at 2 mg/ml and sterilized using a 0.45 μ m Millipore filter. The sterile solution was diluted in culture medium to the concentration required.

2.11 Cell counting

Cell suspensions were diluted into HBSS containing trypan blue at 1 mg/ml. The viable, unstained cells were counted using a haemocytometer. Cell numbers cited in the text refer to viable nucleated cells.

2.12 Differential Counting

Cell smears of PC were prepared using a Cytospin (Shandon Southern Pty. Ltd. England). They were fixed in methanol and stained with dilute Giemsa stain overnight. Three hundred cells in each smear were selected at random and examined to determine their cell type.

2.13 Tumour Cell Lines

Four tumour cell lines were used in these experiments. They were Ehrlich Ascites Tumour (EAT), the DBA/2 mastocytoma, P815, the C57BL/6 thymoma, EL4, and the BALB/c plasmocytoma, MPC-11. EAT was obtained in 1955 from the Roswell Park Memorial Institute (Buffalo, New York, U.S.A.) and was maintained by weekly serial ip passage of 10^7 EAT cells in specific pathogen free LACA mice. The LD_{50} by the ip route for $CB6F_1$ mice is approximately 200 cells (Hardy and Kotlarski, 1971).

The P815 mastocytoma was obtained from Dr K. Lafferty of the John Curtin School for Medical Research, Australian National University (Canberra, Australia). To grow the tumour to large numbers it was injected into DBA/2 mice and the tumour cells were then cryopreserved and stored at -80° . The P815 cells used in these experiments were all derived from this one batch of frozen cells. They were maintained by

in vitro culture in DMEM containing 10% FCS and HCO_3^- . Each culture was seeded at between 1×10^3 and 5×10^4 viable cells/ml and the cells were subcultured when they had grown to a density of $1-3 \times 10^6$ cells/ml.

The EL4 thymoma and the MPC-11 plasmacytoma were obtained from Dr A. Harris of the Walter and Eliza Hall Institute for Medical Research (Melbourne, Australia). They were also maintained by in vitro culture DMEM culture medium.

2.14 Immunization of mice with allogeneic tumour cells

BALB/c (H-2^d) mice were immunized against the alloantigens of C57BL/6 (H-2^b) mice by injecting them ip with 2×10^7 in vivo grown EL4 tumour cells. The mice were normally used 10 to 13 days later. Mice used after this time were boosted with another injection of 2×10^7 EL4 cells 2 days prior to the experiment.

2.15 Peritoneal Cells

PC were obtained by washing out the peritoneal cavities of mice with 2.5 ml of suspension medium containing heparin at 5 i.u./ml. Usually, the cells from four or five mice were pooled, washed once and resuspended in culture medium at 10^7 viable cells/ml.

2.16 Preparation of spleen and lymph node cell suspensions

The procedure was carried out aseptically. Mice were killed by cervical dislocation and their abdomen swabbed with 70% alcohol. Spleens were removed, cut into several pieces and gently dispersed one or two at a time in 5-10 ml of ice-cold HBSS using a ground glass homogenizer with a loose fitting pestle. To remove debris and cell clumps, the crude cell suspension was filtered through sterile cotton wool and immediately eluted with copious amounts of cold HBSS until the eluate was clear. The filtered suspension was centrifuged at $400 \times g$ for 5 min. If the spleen

cells were to be injected back into mice, the cell pellet was resuspended in HBSS, otherwise cold suspension medium (EMEM) was used. The concentration of viable leukocytes in the suspension was determined by the trypan blue exclusion method. The suspension was then diluted to the desired concentration.

A similar method was used to prepare lymph node cell suspensions.

2.17 Splenic lymphocytes

Splenic lymphocytes were separated from erythrocytes by centrifugation on a discontinuous Percoll density gradient consisting of a 2 ml layer of 50% Percoll and a 2 ml layer of 60% Percoll. The cells at the 50%/60% interface were washed three times in suspension medium and then used as targets in cell binding assays.

2.18 Blast cells

2.18.1 For use in in vitro cytotoxicity assays, cold target inhibition assays and cell binding assays

Spleen cell suspensions were prepared from normal CB6F₁ mice. Blast cells were prepared by taking 40 ml of suspension containing 4×10^7 viable spleen cells and incubating it with 40 ug Con A (Pharmacia) for three days at 37° using 10% CO₂, 7% O₂ and 83% N₂ as the gas phase. The medium used was EMEM supplemented with 1×10^{-4} M 2ME. After incubation, the cells were harvested and dead cells and erythrocytes were removed by centrifugation on a discontinuous Percoll gradient consisting of a layer of 30% Percoll and a layer of 50% Percoll. The blast cells at the 30%/50% Percoll interface were recovered and washed three times with medium before being resuspended to the desired concentration.

2.18.2 For use in IL2 assay

Spleen cell suspensions at 1×10^6 /ml were prepared from normal

CB6F₁ mice or SPF LACA mice. The medium used throughout was EMEM with additives as described above, including Con A as described above. The cell suspension was dispensed in 1 ml volumes into 16 mm wells of Linbro tissue culture trays (Linbro, Cat# 76-033-05) and incubated for 3 days at 37° in a gaseous phase of 10% CO₂, 7% O₂ and 83% N₂. Prior to use in the IL2 assay, the blast cells were resuspended using a Pasteur pipette, pooled, washed once with cold suspension medium, counted and made up to 4 x 10⁵ viable cells/ml.

2.19 Adherence of PC to Plastic

PC were adjusted to 1 x 10⁷/ml. Five mls of cell suspension were added to 100 x 20 mm plastic tissue culture dishes (Costar, Cambridge, MA, USA) and incubated for at 37° for the length of time stated in the text. The non-adherent cells were harvested by agitating the dishes and removing the culture fluid, as described in the text. The plates were then washed by squirting another 5 mls of warm medium onto the monolayers and the non-adherent cells obtained by this washing procedure were added to those originally harvested. The pooled non-adherent cells were centrifuged and resuspended in culture medium to the desired cell concentration.

Samples of adherent cells which were used in in vitro cytotoxicity assays (Chapter 3) were prepared in the following way. Multiwell tissue culture trays (Linbro 76-003-05) containing 10⁶ PC in 0.2 ml of medium/well were incubated for 3 h at 37°. The non-adherent cells each well were removed by gently sucking the medium up and down several times with a Pasteur pipette and then removing as much of the culture medium as possible. Fresh culture medium was then added to each well and the washing procedure repeated and the non-adherent cells and culture medium removed as before. Finally, target cells were added to the monolayers in 0.2 ml of medium.

2.20 Preparation of PC which do not adhere to nylon wool

Nylon wool (Fenwall-Leukopac) was soaked overnight in diluted acid according to the method of Greaves, Janossy and Curtis (1976). It was rinsed thoroughly with copious amounts of deionized, distilled water. The rinsing was continued until the electrical conductivity of the washings was the same as that of deionized, distilled water and the nylon wool was then dried. Columns were prepared by packing 600 mg amounts of the nylon wool into the barrel of 10 ml glass syringes. They were sterilized by autoclaving. Prior to use each column was equilibrated with DMEM culture medium overnight at 37° in a CO₂ incubator. PC were applied to each column in 2 ml of DMEM culture medium containing 5×10^7 cells and the columns were incubated for 1 h at 37°. The non-adherent cells were eluted slowly using 10 ml of warm medium. These cells were washed and resuspended to the desired concentration.

2.21 Preparation of PC which do not adhere to glass wool

Fine glass wool was soaked overnight in concentrated sulphuric acid, rinsed with copious amounts of deionized distilled water as described above and then dried. The columns were constructed by packing 1.4 g of treated glass wool into the lower 8 ml of a 10 ml syringe and were sterilized by autoclaving. Before use, the columns were rinsed with 20 ml of culture medium (DMEM) and incubated for 1-2 h at 37° in a CO₂ incubator.

2×10^8 PC in 2 ml of medium was applied to the columns which were then incubated for 1 h at 37° in a CO₂ incubator. The non-adherent cells were eluted by applying sufficient warm, suspension medium to the column to ensure that the eluate was clear.

2.22 Fractionation of PC on Percoll density gradients

The material used to fractionate PC on the basis of buoyant density was Percoll (Pharmacia). Percoll was diluted to 90%

concentration (v/v) in 10 x HBSS containing 20 mM HEPES. The 90% Percoll suspension was then diluted to the required strength with single strength HBSS. Percoll density gradients were constructed in 15 mm x 100 mm siliconized glass culture tubes.

2.22.2 Discontinuous density gradients

Discontinuous density gradients were constructed by layering with a Pasteur pipette 2 ml volumes of Percoll suspension at different concentrations starting with the densest one. The concentrations used are stated in the text. 5×10^7 to 1×10^8 PC in 2 ml of HBSS were carefully layered onto the gradient and centrifuged at $400 \times g$ for 30 min at 4° using a swing-out rotor. To prevent swirling and mixing of the layers, 10-15 sec were taken to accelerate the rotor to the correct speed and it was allowed to decelerate without braking.

The cells sedimenting at the interfaces of the different layers were recovered from the gradient with a Pasteur pipette. They were washed 3 x with 5-10 ml of suspension medium and the number of viable cells determined before suspending to the required concentration.

2.22.3 Continuous density gradients

To ensure proper delivery of the various concentrations of Percoll, each culture tube was clamped upright in a retort stand and a long fine hollow needle was lowered to the bottom of each tube and clamped vertically in place. Gradients were prepared by layering, with the aid of a peristaltic pump, 1 ml volumes of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% and 70% Percoll into each tube starting with the least concentrated one. The discontinuous gradients were stored at 4° for 3 days to ensure that they had become essentially continuous and linear. This procedure was established by collecting 0.25 ml fractions of the prepared gradients on days 1, 2 and 3 after preparation and measuring the refractive index of each fraction (data not shown).

Two x 10^8 PC in 2 mls were layered onto each gradient which was then centrifuged in a swing-out head at $400 \times g$ for 30 min at 4° as described above. The gradient was fractionated by lowering a fine hollow needle to the bottom of the centrifuge tube and pumping out 1 ml fractions starting with the most dense one. The fractions were washed three times, adjusted to 10^7 viable cells/ml and tested in a standard in vitro cytotoxicity assay.

2.23 Removing polymorphonuclear cells from cell suspensions

Contaminating polymorphonuclear cells were removed from cell suspensions by centrifuging the cells on a Sepalymph gradient (Teva, Israel) at $400 \times g$ for 20 min at room temperature. Cells at the interface were harvested with a Pasteur pipette, washed and resuspended to 10^7 viable cells/ml.

2.24 Radiolabelling of Cells

Cells were labelled with one of the following radioisotopes (Amersham International plc, Buckinghamshire, England):

- (a) $\text{Na}_2^{51}\text{CrO}_4$ (^{51}Cr) specific activity of 250-500 $\mu\text{Ci}/\text{ug}$,
- (b) 5-[^{125}I]-Iodo-2'-deoxyuridine (^{125}I -UdR) specific activity of 5Ci/mg,
- (c) [methyl- ^3H]-Thymidine (^3H -TdR) specific activity of 25 Ci/m mol.

2.24.1 Labelling cells with ^{125}I

EAT cells were labelled with ^{125}I -UdR by either of two methods.

(i) in vivo

EAT cells growing in the peritoneal cavities of mice were labelled with ^{125}I -UdR in vivo using a technique similar to that of Hofer, Prensky and Hughes (1969) and Porteous and Munro (1972). LACA or CB6F₁

mice were injected with 10^7 EAT cells ip. One day later each mouse was injected with a total of 1.5 μCi of ^{125}I -UdR, given as 4 separate injections over a period of 24 h. The labelled tumour cells were harvested 2 days later by rinsing the peritoneal cavity with 5 ml cold suspension medium. The cells were washed by layering 1.5 ml of FCS under the cell suspension and centrifuging at $250 \times g$ for 3 min. They were resuspended in fresh medium, counted and diluted to the desired concentration.

(ii) in vitro

Two days after injecting mice ip with 2×10^7 EAT cells, the EAT cells were harvested by peritoneal lavage, washed once and resuspended to a concentration of 5×10^6 cells/ml in DMEM containing 10% FCS and HCO_3^- . Fifty to 75 nCi of ^{125}I -UdR/ 10^7 cells was added to the cell suspension which was then incubated at 37°C in 10% CO_2 in air for 2 h, with occasional swirling. The cells were washed as above.

In some in vitro cytotoxicity assays P815 tumour cells and Con A-activated blast cells labelled with ^{125}I -UdR were used as targets. They were labelled by incubating the cells at 5×10^6 /ml with 2 μCi ^{125}I -UdR for 1 h at 37° using the same culture medium as above. The cells were washed once and incubated in culture medium at 37° for a further hour. The cells were then centrifuged through a layer of FCS to remove any radioactivity which was not cell-associated.

2.24.2 Labelling cells with ^{51}Cr

One ml suspensions of tumour cells, Con A-activated blast cells or lymphocytes at $5\text{--}10 \times 10^6$ cells/ml in DMEM supplemented with 10% FCS and 0.37% HCO_3^- were incubated with 50 or 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ for 1 h at 37° with occasional swirling. The bulk of the unincorporated radiolabel was removed by adding 10 ml of cold suspension medium and centrifuging the cells at $400 \times g$. The cell pellet was resuspended in 5 ml of fresh suspension medium and washed by centrifugation through a

layer of FCS as described above. The cells were resuspended in 2 ml of culture medium (DMEM), the concentration of viable cells was determined and adjusted to the desired value.

2.24.3 Labelling cells with ^3H -TdR

P815 tumour cells for use as targets for in vitro cytotoxicity assays were labelled with ^3H -TdR by culturing P815 cells at a concentration of 5×10^5 cells/ml in culture medium (DMEM) with $1.25 \mu\text{Ci/ml}$ of ^3H -TdR for 6-8 h. The cells were washed in the same way as ^{51}Cr -labelled target cells.

2.25 The measurement of anti-tumour activity in vivo

The in vivo anti-tumour activity of mice was tested by two methods. One measures the clearance of ^{125}I -EAT cells from the peritoneal cavity whilst the other method measures the whole body retention by radioactivity of mice injected ip with ^{125}I -EAT cells.

2.25.1 Clearance of ^{125}I -EAT cells from the peritoneal cavity

Mice were injected ip with 10^6 ^{125}I -EAT cells and the amount of cell-associated radioactivity which could be recovered from the peritoneal cavities of these mice was measured at various times after challenge. The technique used for harvesting cells from each peritoneal cavity and determining the radioactivity present in each cell pellet is described in detail by Ashley and Kotlarski (1982a). Each mouse was killed by cervical dislocation and its peritoneal cavity washed out with 5 ml of heparinized HBSS. 4 ml of the fluid was withdrawn and 3 ml placed into a 6 ml plastic serology tube (Medical Plastics Pty. Ltd. South Australia). If necessary, an aliquot of the remaining 1 ml was used to determine the number of PC recovered. 1 ml of FCS was layered under each of the 3 ml PC suspensions, using a Pasteur pipette. The tubes were centrifuged at $400 \times g$ for 10 min, all of the supernatants

including most of the FCS were then removed and the radioactivity in the cell pellets was measured. The radioactivity of a sample of the injected ^{125}I -EAT cells was also measured at the same time so that the recovery of radioactivity from each mouse could be calculated as a percentage of the injected radioactivity.

2.25.2 Whole body retention of ^{125}I

Mice were injected with 10^6 ^{125}I -EAT cells ip. The rate of in vivo destruction of the tumour cells was determined by measuring the whole body retention of ^{125}I by individual mice injected ip with 10^6 ^{125}I -EAT cells. The technique used was based on that of Hofer, Prenskey and Hughes (1969). The uptake of free ^{125}I by the thyroid was suppressed by supplementing the drinking water of the mice with 1 mg/ml sodium iodide (BDH Chemicals Ptd. Ltd.) at least 24 h before challenge with ^{125}I -EAT cells. NaI was maintained in their drinking water of the experimental mice during the 5-6 days their whole-body radioactivity was monitored. External contamination of the mice with ^{125}I was minimized by changing their sawdust daily, immediately before their radioactivity was to be measured.

To measure the radioactivity of individual mice, each mouse was confined to the lower portion of a plastic tube (10 cm x 3.7 cm) and placed in the well of sodium iodide-crystal scintillation detector (Nuclear Chicago). This was done immediately after the mice were challenged with ^{125}I -EAT cells, on day 1 and 2 and then on days 4-6. The retention of ^{125}I by each mouse was expressed as a percentage of the radioactivity detected in that mouse immediately after challenge. Because ^{125}I has a half-life of 60 days the counts from the mice over the six day period were not corrected for the natural decay of the isotope. The whole-body retention for a group of mice was expressed as the geometric mean of the ^{125}I retained by individual mice multiplied or divided by 1 standard deviation (SD).

2.26 In vitro cytotoxicity assay

The culture medium used was DMEM supplemented with 10% FCS, HCO_3^- and HEPES. The anti-tumour activity of 11RX-immunized mice was measured in vitro by the ability of their PC to lyse ^{51}Cr -labelled tumour cells. The assay is described in detail by Ashley and Kotlarski (1982a). In the standard in vitro cytotoxicity assay 1.5 ml of various PC suspension at 10^7 cells/ml were mixed with an equal volume of ^{51}Cr -target cells at 10^5 cells/ml. Eight replicate 0.2 ml samples of each mixture were dispensed in multiwell tissue culture trays (Linbro 76-003-05) so that each well contained 10^6 PC and 10^4 ^{51}Cr -labelled target cells. In some experiments the numbers of PC and target cells were varied as stated in the text. Control wells, included to determine spontaneous release of ^{51}Cr , contained only 10^4 ^{51}Cr -target cells in medium. In addition, 0.8 ml of the remainder of each cell suspension was dispensed into individual plastic serology tubes. The radioactivity in this aliquot of each cell mixture was measured and used to determine the total number of counts added to each of the wells. Four replicate wells were assayed at 10 h and at 20 h for ^{51}Cr release into the medium by sampling the top 0.1 ml of medium from each well with an plunger-type pipette. To estimate the total releasable amount of ^{51}Cr , 0.5 ml samples of the ^{51}Cr -target cell suspension were dispensed into three glass tubes and lysed with a few drops of chloroform. After vigorously vortexing the three tubes and allowing them to stand for 1 h at room temperature, the tubes were centrifuged at $1000 \times g$ to pellet the chloroform and cellular debris. The top 0.25 ml of supernatant was removed from each tube and transferred to another series of three tubes. The radioactivity in both the supernatant and the pellet samples was measured and the total releasable amount of label was calculated using the following formula:

$$\% \text{ Total release} = 100 \times \frac{2 \times \text{CPM in supernatant}}{\text{CPM in supernatant} + \text{CPM in pellet}}$$

For most target cells the total releasable ^{51}Cr was always close to 85% of the ^{51}Cr incorporated. When ^{125}I or ^3H labelled target cells were used, the total releasable counts were taken to be 100%. Spontaneous release of ^{51}Cr was approximately 1-1.5%/hour when EAT cells and P815 cells were used as targets. The results were expressed as percentage cytolysis which was defined operationally by the equation:

$$\% \text{ cytolysis} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous release}}{\text{total releasable } ^{51}\text{Cr} - \text{spontaneous release}} \times 100\%$$

When ^{51}Cr -EL4 and ^{51}Cr -MPC11 cells were used as targets, the assays were terminated at 5 h and 10 h respectively due to the unacceptably high spontaneous release of ^{51}Cr at later times.

2.27 Cold Target Inhibition of in vitro cytotoxicity

The medium used was DMEM supplemented with 10% FCS, HCO_3^- , and HEPES. Eight replicate, two-fold serial dilutions of unlabelled target cells in a volume of 0.1 ml were made in multiwell tissue culture trays using an 8 channel automatic pipette (Titertek Cat # 77-859-00). The concentration of the unlabelled cells ranged from 3.2×10^5 to 1×10^4 cells/well. A cell suspension containing 10^6 PC and 10^4 ^{51}Cr -labelled target cells in a volume of 0.1 ml was added to all the wells of one quadruplicate set of titrations. To control for the effect that medium depletion by extra target cells may have on the spontaneous release of ^{51}Cr from labelled target cells, a 0.1 ml volume containing only 10^4 ^{51}Cr -labelled target cells was added to each well of the other set of titrations. The trays were centrifuged at $400 \times g$ for 5 min before being incubated and processed in the usual way to determine cytotoxic activity. For calculating the % cytolysis in the presence of each concentration of target cells, the equation in §2.26 was used. However, the spontaneous release was taken to be the amount of label released from ^{51}Cr -target cells incubated in the presence of the same number of cold target cells as that used to inhibit cytotoxicity by PC.

2.28 Killing of Tumour Cells Across Cell Impermeable Membranes

Membrane filters (Sartorius-Membranfilter, GMBH, Göttingen, West Germany) with 0.22 μm or 0.45 μm pores were cut into five millimeter diameter discs with a paper punch. Following soaking overnight in deionized distilled water, the discs were rinsed and autoclaved in fresh deionized distilled water. Prior to use, the membrane discs were rinsed with and soaked in sterile culture medium. These membranes were used to separate effector and target cells in the wells of the tissue culture trays described above. The cells that were to be placed below the membrane were dispensed in quadruplicate in 0.2 ml volumes into the wells. The trays were centrifuged at 400 x g for 5 min and then a membrane was placed into each well using sterile, fine forceps. Care was taken not to trap any air bubbles or damage any of the membranes. The tray was centrifuged again to ensure the membranes were positioned exactly onto the cell layer below them and the supernatant in each well was removed with a Pasteur pipette and the second cell population was added in 0.2 ml. After centrifugation to layer the second cells population onto the membrane discs, the trays were treated as in a conventional in vitro cytotoxicity assay.

2.29 Cell-binding assay

PC at concentrations stated in the text were dispensed in quadruplicate in 0.1 ml volumes into multiwell tissue culture trays. After centrifuging at 400 x g for 5 min at 4°, the trays were incubated for 1 h at 37° to allow the macrophages to adhere. Non-adherent cells were resuspended by vortexing the trays on a Tray Shaker (Titertek CAT # 77-472-00) for 1 min at maximum speed. All of the supernatants were removed and those within a group were pooled. The number of cells in each pooled supernatant was determined. This allowed for calculation of the number of adherent cells remaining in the wells. ⁵¹Cr-labelled cells were added to the wells in 0.1 ml volumes and contact with the

monolayers was facilitated by centrifuging the trays at 20 x g for 10 min at 4°.

After incubating for 30 min at 37°, the unbound targets were resuspended by vortexing as above. The culture fluid containing loose cells was removed, and 0.1 ml of fresh medium at 37° was added and the trays vortexed again for another 30 seconds. This procedure was repeated another two times. After the final wash the medium in each well was replaced with 0.1 ml of a 0.4% v/v solution of Triton X100 (Ajax Chemicals Australia) in HBSS. (This level of Triton X100 had been shown in preliminary experiments to release 100% of the ⁵¹Cr from labelled cells). After incubating the trays for several hours at 37° in a humidified atmosphere, 0.05 ml samples from each well were taken and the amount of ⁵¹Cr present determined. The binding of cells to the monolayers was expressed as:

$$\% \text{ binding} = \frac{\text{counts bound to monolayer} - \text{counts bound to plastic alone} \times 100}{\text{counts added to monolayer}}$$

2.30 Sugars used in an attempt to inhibit PC cytotoxicity

The following sugars were added to the medium for in vitro cytotoxicity and cell binding assays. Except where indicated they were purchased from BDH Chemicals Ltd. Analytical grade: galactose, lactose, mannose, L (+) rhamnose, Methyl- -D-mannopyranoside (MMP) (Pharmacia), melibiose (A grade Calbiochem.). Laboratory grade: D (-) arabinose, N-acetyl-galactosamine (B grade Calbiochem.) N-acetyl-glucosamine (B grade Calbiochem.), meso-inositol. They were dissolved in culture medium at 200mM and the solutions were sterilized by filtration.

2.31 Testing lymphoid cells for the ability to release lymphokines

2.31.1 Induction of lymphokine release

Two ml of lymphoid cell suspensions at the concentrations stated in the text were cultured in duplicate in 16 mm diameter wells of Linbro

tissue culture trays. In most experiments the tissue culture medium used was serum-free EMEM supplemented with 2ME and indomethacin.

The trays were incubated for 20 h at 37° in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂. Following incubation, the top 1.5 ml of supernatant from each culture were collected aseptically, without disturbing the cell layer. The supernatants were stored at -20° until they were assayed for lymphokine activity.

2.31.2 Assays for lymphokines in culture supernatants

The two lymphokine activities measured in culture supernatants Interleukin 2 (IL2; formerly T Cell Growth Factor) and Macrophage Activation Factor.

2.31.3 Interleukin 2 assays

The presence of IL2 in culture supernatants was determined by the method of Lafferty et al. (1980a) and is based on the ability of IL2 to maintain the division of T cell blasts. Duplicate sets of six two-fold serial dilutions of culture supernatants were prepared in 0.05 ml of fresh EMEM, supplemented with 10% FCS, 0.22% HCO₃⁻ and 1 x 10⁻⁴ M 2ME, in the wells of flat bottomed multiwell trays. The supernatants were titrated for 6 wells. The positive control included in all assays was an IL2 standard of known activity (see §2.32.2). This was titrated in duplicate as above for 12 wells starting at a dilution of 1 in 100. The negative controls were 24 wells of medium without any IL2.

2 x 10⁴ Con A-activated blast. cells in 0.05 ml of medium were added to all the wells and the trays incubated at 37° for 20 h in an atmosphere of 10% CO₂, 7% O₂ and 83% N₂. After this time the cells were pulsed with 1 µCi ³H-TdR for 4-6 h added in a volume of 0.05 ml/well. To recover the cells present in each well, a Cell Harvester (Titertek Cat# 78-100-00) was used. This machine harvests 1 row of 12 wells at a time. It deposits the cells onto discs punched from

glass fibre mats (Titertek Cat# 78-105-05) and washes them with distilled water. The mats were dried and individual discs were removed and placed into plastic mini-scintillation vials (Filtrona Plastics, Australia). One ml of scintillation fluid (Aqueous Counting Scintillant II, Amersham, Arlington Heights, IL, USA) was added to each vial and the vials were capped, placed into carriers and their radioactivity determined in a liquid scintillation counter (Beckman Instruments Inc., USA, model LS 7500) which has a counting efficiency of greater than 50%.

A dilution of any culture supernatant used in the assay was considered to have IL2 activity if the counts incorporated by the blast cells cultured in it were more than 3 SD above the mean number of counts incorporated by blast cells cultured in medium alone.

2.31.4 Macrophage Activation Factor

The culture medium used in the assay was DMEM supplemented with 10% FCS and HCO_3^- . Culture supernatants were tested for MAF activity by determining whether proteose-peptone-induced macrophages treated with the supernatants were cytotoxic for P815 cells in vitro.

Unless otherwise stated in the text, the following procedure was used. The culture supernatants were titrated in duplicate as described in the assay for IL2 except that the volume used was 0.1 ml. A standard preparation of MAF was titrated for 12 wells, and fresh medium was added to a further 12 wells. 5×10^5 PC from CB6F_1 mice injected 2 days previously with 2 ml 10% proteose-peptone broth ip, were added to the wells in a volume of 0.1 ml. The trays were incubated for 8 h at 37° in a humidified atmosphere of 10% CO_2 in air before removing the non-adherent cells and washing the monolayers.

This was done by aspirating the culture fluid from all the wells and replacing it with 0.1 ml of warm suspension medium (DMEM or EMEM). The trays were vortexed vigorously for 45 seconds with a tray shaker, the fluid was removed and replaced with another 0.1 ml of suspension medium

and the washing procedure repeated. After removing the washing fluid, 1×10^4 ^{51}Cr -P815 or ^3H -P815 cells in 0.1 ml of medium were added to each of the wells. The trays were then re-incubated at 37° as before. If ^{51}Cr -P815 cells were used as target cells, the assay was terminated at 20 h. When ^3H -P815 cells were used as targets, the assay was terminated at 48 h. Cytotoxicity was determined either by measuring the amount of ^3H or ^{51}Cr that was released in the supernatant or by measuring the amount of ^3H that could be harvested from the wells using a cell harvester. For measuring ^3H in cell-free supernatants, 0.1 ml samples of the supernatant were placed into plastic mini-scintillation vials and 2 ml of ACSII scintillation fluid was added to each vial.

A dilution of any culture supernatant was considered to have MAF activity if, (1) macrophages pretreated with it caused the release of ^{51}Cr or ^3H from labelled P815 cells which was greater than 3 SD above the mean amount of label released by P815 cells cultured with untreated macrophages or (2) if the amount of ^3H that could be harvested from the wells was less than 3 SD below the mean amount of ^3H that could be harvested from wells where ^3H -P815 cells were cultured with macrophages preincubated in medium only.

2.32 Preparation of lymphokine standards

2.32.1 Macrophage Activation Factor

The MAF standards used in the above assays were cell-free supernatants from cultures of PC from 11RX-immunized mice which were incubated with 11RX antigen. DMEM supplemented with FCS and HCO_3^- was the culture medium used. A mixture of PC at $10^6/\text{ml}$ and 11RX antigen at $20 \mu\text{g}/\text{ml}$ were dispensed tissue culture flasks; the volumes used were 10 ml for the small flasks (Falcon 3013) and 40 ml for the large flasks (Falcon 3024). The flasks were flushed with a gas mixture consisting of 10% CO_2 , 7% O_2 and 83% N_2 , sealed and incubated upright for 20 h at 37° .

The culture fluid was centrifuged at $400 \times g$ for 10 min to sediment the cells and sterilized by filtration through a Millex GS filter (Millipore Corporation, Bedford, MA, USA) with $0.22 \mu\text{m}$ pores. One ml aliquots of the filtrate were dispensed into sterile plastic containers (Mallingkrodt, Australia) and stored at -20° . Each aliquot was used only once.

Three standard MAF preparations (SMP) were used during the course of this study. SMP1 was prepared using the PC from day 40 ip 11RX-immunized CB6F mice injected 3 days prior to cell harvest with 3 ml of 10% proteose-peptone broth. Uninduced PC from day 16 ip 11RX immunized CB6F₁ mice was used in making SMP2. SMP3 was made using uninduced PC from CBA mice injected ip 12 days earlier with 10^6 11RX per mouse.

2.32.2 Preparation of Interleukin 2 standard

Spleen cell suspensions were prepared from outbred LACA mice at a concentration of 5 ml/spleen. The medium used was serum-free EMEM containing 2ME and 5 $\mu\text{g/ml}$ of Con A. Fifteen ml volumes of the cell suspension were dispensed into a series of 75 cm^2 plastic tissue culture flasks (Falcon 3024). The flasks were gassed with a mixture of 10% CO_2 , 7% O_2 and 83% N_2 , sealed and the spleen cells allowed to interact with Con A and adhere to the plastic by incubating the flasks horizontally for 2 h at 37° . At this time excess, unbound Con A was removed by the following procedure which was designed to avoid detaching the adherent cell monolayer. The supernatant was decanted from each flask and, whilst holding the flask with the cell monolayer uppermost, 15 ml of warm HBSS was poured onto the contralateral surface of each flask. All the flasks were rolled slowly so that the HBSS washed over the cell monolayer once and the supernatant were discarded and the washing procedure repeated twice. After washing, 30 ml of fresh 2ME-supplemented serum-free EMEM were added to the flask and all the

flasks were flushed with the gas mixture described above and incubated for a further 17 h at 37°.

After this time all the culture supernatants were collected and any loose cells removed from it by centrifugation at 400 x g for 10 min. The supernatants were concentrated 10-fold in the cold by ultrafiltration through type YM Diaflo ultrafilters (Amicon, Lexington, MA, USA). The concentrate was filter sterilized using disposable Millex GS filters with 0.22 μ m size pores, dispensed into small aliquots and stored frozen. Lafferty et al. (1980a) have reported that IL2 prepared by this method is contaminated with less than 0.18 μ g/ml of Con A.

CHAPTER THREE

CHARACTERIZATION OF THE EFFECTOR CELLS RESPONSIBLE FOR TUMOUR
RESISTANCE IN SALMONELLA ENTERITIDIS 11RX IMMUNIZED MICE3.1 Introduction

Resistance to tumour challenge can be demonstrated in mice after either ip or iv injection of live 11RX (Hardy and Kotlarski, 1971; Ashley et al., 1974; Tindle et al., 1976; Ashley et al., 1976; Ashley and Kotlarski, 1982a, 1982b). Cytotoxic PC can be recovered from these mice but the nature of the effector cells present in the PC suspensions has not been clearly defined. Cytological examination of smears of cytotoxic PC on the sixth day of an ip 11RX infection reveals several cell types (see Table 3.6 and 3.8) many of which have been reported to be cytotoxic in vitro by various workers using a number of different model systems (see Chapter 1).

The tumour resistance induced by BCG and C. parvum has been ascribed to activated macrophages (Hibbs, 1974; Cleveland, Meltzer and Zbar, 1974; Olivotto and Bomford, 1974; Scott, 1974; Ghaffar et al., 1974). Macrophage monolayers from BCG or Toxoplasma infected mice prevent the in vitro growth of tumour cells, lyse tumour cells in vitro as assessed by radio-isotope release but have no effect on the growth of non-transformed cells lines (Hibbs, 1973, 1974b; Hibbs, Lambert and Remington, 1972b; Meltzer, Tucker, Sanford and Leonard, 1975b). Normal macrophages lack the cytotoxic and cytostatic capacity of their activated counterparts.

There is evidence to suggest that the tumour resistance induced by BCG may also involve NK cells. Recently several groups have shown that NK cell activity is increased by infection with BCG (Wolfe, Tracey and

Henney, 1976; Tracey, et al., 1977; Wolfe, Tracey and Henney, 1977) or injection of C. parvum (Ojo, et al., 1978), and that the effect of ip injection of BCG on NK cells, like the effect on macrophages is local in nature. This increase in NK cell activity is transient, occurring early after infection, peaking at days 4-6 and declining steadily to background levels by day 22. In vitro killing of tumour cells, as determined by ⁵¹Cr release from appropriate targets, was rapid, reaching a plateau at 4 h (Tracey, et al., 1977), unlike the in vitro killing obtained when macrophages are the effector cells. The lysis of tumour cells by macrophages is a much slower process. ⁵¹Cr release assays which measure the damage caused by macrophages to target cell cytoplasmic membranes are conducted over 18-20 h (Lohmann-Matthes, Schipper and Fisher, 1972; Marino and Adams, 1980a) whilst assays which monitor nuclear disintegration take 48-72 h (Meltzer et al., 1975).

CTL generation can also be modulated by infection with IBP. Thoracic duct lymphocytes from rats infected with live Listeria monocytogenes but not killed Listeria can kill a wide variety of allogeneic and syngeneic cell lines provided that they are restimulated in vitro for 2-3 days with Listeria antigens (Woan and McGregor, 1981; Woan, McGregor and Forsum, 1981). Little or no cytotoxic activity is detected in TDL before incubation with antigen and none is obtained if they are cultured in the absence of antigen. It was subsequently established that the cytotoxic cells were T cells (Woan, McGregor and Goldsenieder, 1981).

Whilst pretreatment with BCG or C. parvum alone induces non-specific immunity to tumour cells, due to activation of cytotoxic macrophages and NK cells, these agents can also play a role in the generation or enhancement of a specific immune response to tumour antigens. Injection of killed C. parvum (Likhite and Halpern, 1974) or viable BCG (Zbar et al., 1972; Bartlett, Zbar and Rapp, 1972) into established tumours can cause their complete regression. These hosts are

also resistant to subsequent challenge with the homologous but not any antigenically distinct tumour. Animals which have been injected with vaccines containing a mixture of viable or irradiated tumour cells and BCG or C. parvum will control the growth of a secondary tumour challenge at a distant site (Scott, 1975; Hawrylko, 1975a, b; Tuttle and North, 1976a; Baldwin and Pimm, 1971; Zbar et al., 1970) and will show delayed hypersensitivity to the tumour (Hawrylko, 1975b).

The specific immunity detected some time after injection of BCG and tumour cells is due to T cells. Resistance can be adoptively transferred to normal recipients using spleen cells and lymph node cells from animals treated with the vaccines (Hawrylko, 1975b; Scott, 1975; Tuttle and North, 1976a; Dye, North and Mills, 1981) and these cells are also specifically cytotoxic in vitro (Parr et al., 1977; Mills, North and Dye, 1981). Treatment of the recipient mice with 800R of gamma irradiation did not abrogate the systemic transfer of tumour resistance, with cells from immunized mice, leading in the conclusion that the transferred lymphocytes were the cytotoxic effector cells in vivo (Tuttle and North, 1976b).

The effect of C. parvum and BCG appears to be local in nature. Specific tumour immunity is not generated if either of these agents are given at a anatomical site distant from the tumour cells (Baldwin and Pimm, 1971; Scott, 1975; Hawrylko, 1975b). There are two possible explanations for this. The first one proposed by Parr, Wheller and Alexander (1977) is that that BCG infection selectively recruits T cells to the site of inflammation resulting in the sensitization of increased numbers of tumour-specific T cells. However, there is an alternate explanation. Activated macrophages induced by infection with BCG may be more efficient than normal ones at presenting tumour antigens to specific T cells. This notion is consistent with the recent report by Britz et al. (1982) that activated macrophages may be instrumental in presenting haptens in a manner which ensures that contact sensitivity is

induced i.e. a form of cell mediated immunity.

The evidence which suggests that macrophages are involved in llRX-induced tumour resistance is discussed in detail in §1.4. In summary, it has been established that resistance to tumours in llRX-immunized mice has characteristics analogous to resistance to IBP. Tumour resistance wanes after the elimination of the bacterial carrier state but can be recalled in long-term llRX-immunized mice by ip injection of llRX antigens. Furthermore, the ability to recall tumour resistance can be transferred to normal mice with splenic T cells from immunized donors and its expression requires the presence of radiation-sensitive host cells.

Several in vivo and in vitro techniques are available for analysing the cytotoxic cells generated during llRX infections. Agents such as carrageenan, silica or trypan blue which are taken up by macrophages and interfere with their function have been used by different workers to determine whether macrophages are involved in tumour resistance.

Carrageenan is a long-chain, high molecular weight sulphated polygalactose which is readily phagocytosed by macrophages. It is resistant to digestion by lysosomal enzymes and induces osmotic swelling, rupturing of lysosomes and cell death (Catanzaro, Schwartz and Graham, 1971). Carrageenan also inhibits complement activity (Borsos, Rapp and Crisler, 1965; Davies, 1965) and affects blood coagulation (Schwartz and Kellarmeyer, 1969; Anderson and Duncan, 1965). Carrageenan injected ip causes a reduction in the number of peritoneal cells and in the percentage of macrophages that can be harvested from the peritoneal cavity. The loss of macrophages is partially compensated for by an increase in the proportion of other cell types, notably lymphocytes (Nelson and Nelson, 1978a). Delayed hypersensitivity and specific sinecommitant tumour immunity are depressed by carrageenan treatment (Nelson and Nelson, 1978a, b) as are the anti-tumour effects induced by

C. parvum and BCG (Keller, 1977).

Silica, like carrageenan, is taken up by macrophages into phagocytic vacuoles (Allison, Hartington and Birbeck, 1966). It has been reported to be toxic for these cells although the evidence is somewhat conflicting. Silica is widely disseminated through the body after ip or iv injection and widespread macrophage destruction has been seen in the tissues where it localizes (Pearsall and Weisser, 1968). The silica preparations which have been used vary greatly in particle size and this may be a cause of the variation in the reported effects (van Loveren, Snoek and Otter, 1977). Van Loveren et al., (1977) found that repeated ip injection of mice with silica of 0.012 μm particle size resulted in the disappearance of both macrophages and lymphocytes from the peritoneal cavity, although the reduction in numbers of lymphocytes was not as great. In other reports, a single ip injection of silica into rodents (5 μm average particle size) caused an increase in total peritoneal cell and macrophage numbers, although some of the cytopathic effects of silica on macrophages were seen (Keller, 1976b; Du Buy, 1975). It has been suggested that the toxicity of silica is due to its affinity for the membranes of secondary lysosomes causing them to become permeable. The observation that polyvinyl-pyridine-N-oxide, which also binds to silica, renders silica non-toxic to macrophages supports this view (Allison, et al., 1966). Silica pretreatment of mice has reduced tumour resistance induced by BCG and C. parvum (Keller, 1977), early specific concomitant tumour immunity (Nelson and Nelson, 1978b) and allograft immunity (Pearsall and Weiser, 1968).

Trypan blue is an azo dye which inhibits lysosomal enzyme activity (Beck, Lloyd and Griffiths, 1967), is rapidly taken up by macrophages but not by lymphocytes, neutrophils, eosinophils, and basophils (Padewar, 1973) and readily stains secondary lysosomes of macrophages (Hibbs, 1974). Activated, macrophages from from BCG or T. gondii infected mice are not cytotoxic after pretreatment with trypan blue in vitro or in vivo

(Hibbs, 1974). Similarly, mice infected with BCG or I. gondii are no longer resistant to challenge with tumour cells after treatment with trypan blue (Hibbs, 1975b). Trypan blue reduced the tumour resistance of pyran copolymer treated mice and the in vitro cytotoxic activity of their macrophages (Morahan and Kaplan, 1976; Kaplan and Morahan, 1976)

To gain more conclusive evidence that activated macrophages were responsible for the cytotoxic activity detected in the peritoneal cavity of llRX-immunized mice, the effect of trypan blue on in vitro cytotoxicity and in vivo tumour resistance was investigated using short-term and long-term ip-immunized mice and long-term iv-immunized mice which had been injected with recall antigen. Trypan blue was used as an anti-macrophage agent rather than silica or carrageenan because it was readily available and because at the time this work was commenced there were no reports that trypan blue had any effects on other cell types. It had also not been established that trypan blue is anti-complementary and that it exerts an effect(s) on the blood clotting system.

In addition, the effector cells of llRX-immunized mice which were cytotoxic for tumour cells in vitro were further characterized by physical means i.e. on the basis of adherence to plastic or nylon wool and on the basis of buoyant density in Percoll. In addition to being tested against EAT target cells, in some experiments cell fractions were tested against a tumour cell line which is resistant to lysis by NK cells but not activated macrophages and one that is susceptible to both.

3.2. The effect of trypan blue on the clearance of ^{125}I -EAT from mice immunized with *S. enteritidis* llRX

Mice immunized with llRX are resistant to challenge with EAT cells and other tumour cell lines (Tindle et al., 1976; Ashley and Kotlarski, 1982a). Measurement of the fate of ^{125}I -iododeoxyuridine labelled tumour cells injected ip into mice provides a short-term in vivo assay of

tumour resistance. This is done by determining the amount of ^{125}I that can be recovered from the peritoneal cavity of immunized mice at various times after tumour challenge (Ashley, 1976; Ashley and Kotlarski, 1982a). The extent of ^{125}I clearance correlates well with the degree of resistance shown by mice immunized with 11RX by different routes or at different times (Ashley, 1976; Ashley and Kotlarski, 1982a).

Mice were immunized with 11RX by either the iv or the ip route at various times before ip challenge with ^{125}I -EAT, as indicated in the text below. Trypan blue was injected iv or ip into some of the mice in each group 2 to 3 h prior to challenge with ^{125}I -EAT. Their ability to clear ^{125}I -EAT from the peritoneal cavity was compared to that of untreated, 11RX-immunized mice. All the mice were sacrificed and the amount of cell-associated ^{125}I which remained in each peritoneal cavity was measured at times which had been established as being suitable in preceding experiments (Ashley, 1976; Ashley and Kotlarski, 1982a). These experiments showed that short-term ip-immunized mice took 12 h to clear ^{125}I -EAT to below the limit of detection, whilst long-term ip-immunized mice and recalled, long-term iv-immunized mice took about 24 h to do the same. Consequently, depending on the group of mice, ^{125}I -retention was measured 8, 16 or 22 h after challenge. Fig 3.1 shows that trypan blue had little, if any, effect on the clearance of ^{125}I from normal, unimmunized mice injected with ^{125}I -EAT and that the clearance of ^{125}I from normal mice injected ip with heat-killed ^{125}I -EAT was also unaffected by trypan blue.

3.2.1 Short-term ip immunized mice

Six days after ip immunization with 10^5 11RX, mice were capable of rapidly clearing an ip challenge of 10^6 ^{125}I -EAT cells. Injection of trypan blue into these mice prior to tumour challenge greatly reduced their ability to clear ^{125}I (Fig 3.2A).

FIGURE 3.1

The effect of trypan blue administration on the clearance of live
and heat-killed ^{125}I -EAT cells from normal mice

Mice were injected ip with 10^6 live (closed symbols) or heat-killed (open symbols) ^{125}I -EAT cells. These mice are the controls for the experiments shown in Fig 3.2 and the data indicate the amount of label retained by normal mice (\bullet \circ) or normal mice injected with trypan blue iv (\blacksquare \square) or ip (\blacktriangle \triangle) 2-3 h prior to challenge with ^{125}I -EAT cells. ^{125}I retention was measured 6 h (a), 16 h (b) or 22 h (c) after challenge with labelled tumour cells.

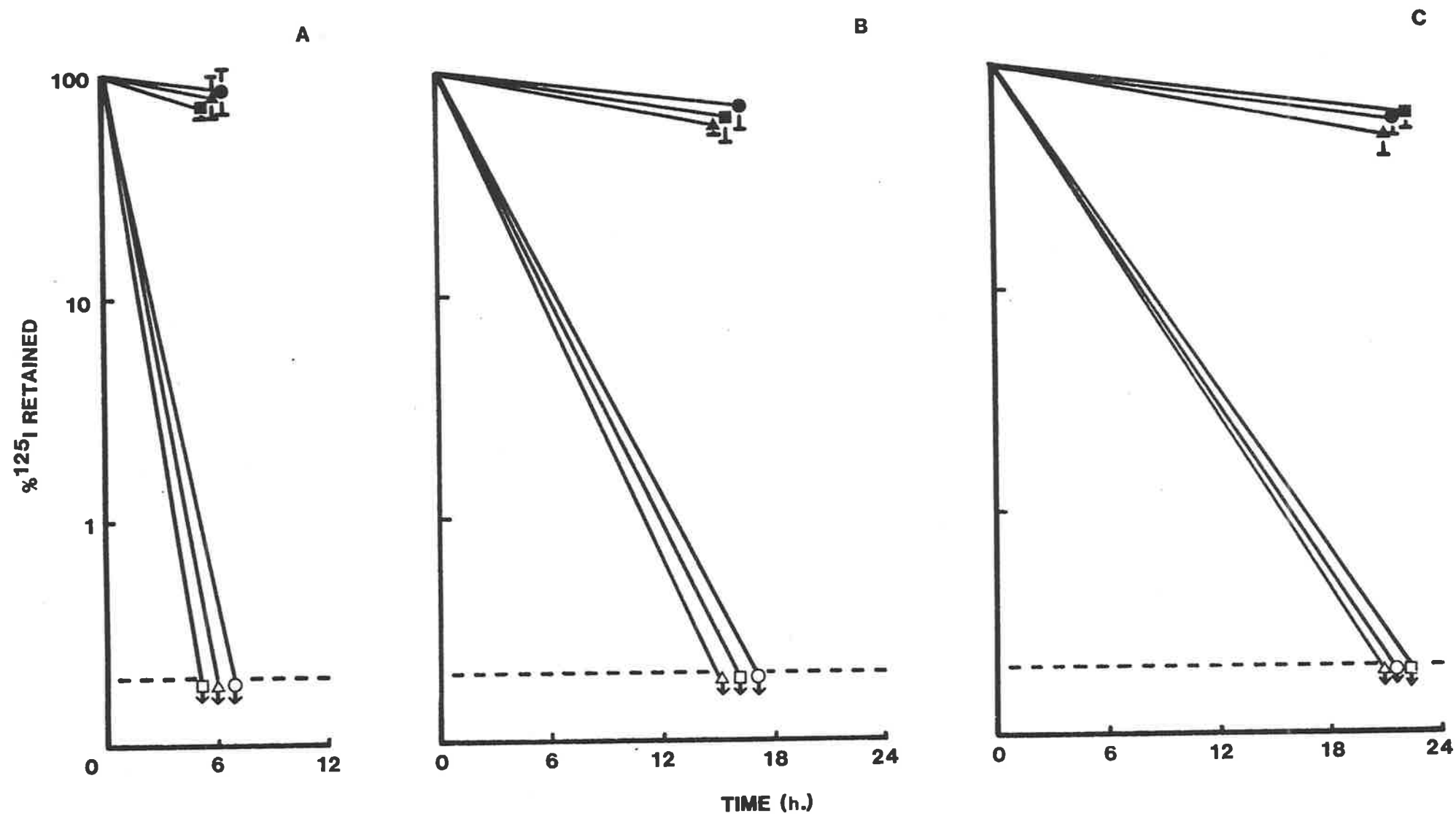
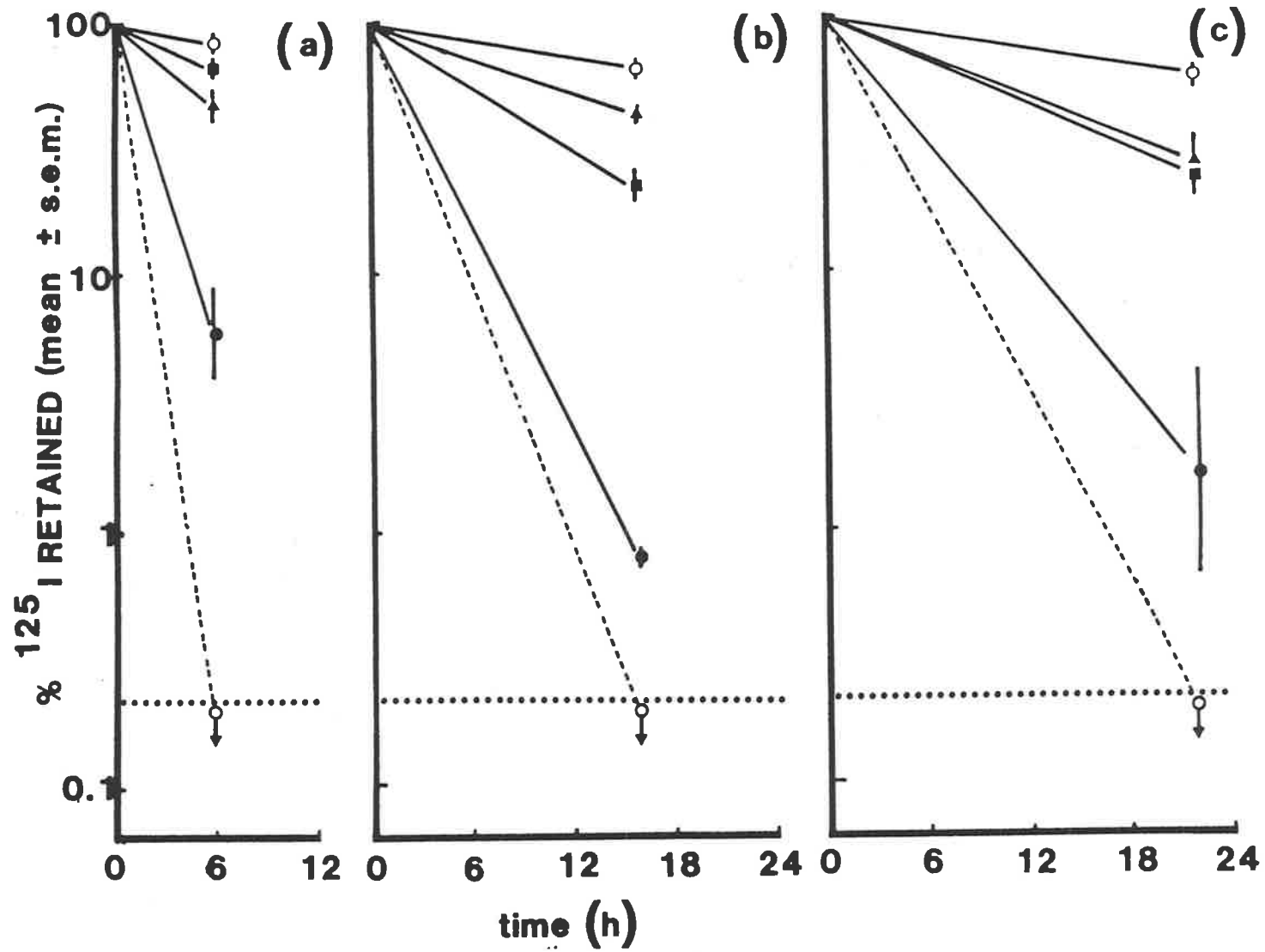


FIGURE 3.2

Effect of trypan blue on the clearance of ^{125}I -EAT cells
from the peritoneal cavity of 11RX-immunized mice

- (a) Clearance from short term ip immunized mice
- (b) Clearance from mice immunized iv 40 days previously and injected with recall Ag.
- (c) Clearance from mice immunized ip 40 days previously.
- (○) normal mice, no trypan blue (a, c) or long term iv immunized mice not injected with 11RX Ag (b).
- (■) immunized mice, trypan blue iv.
- (▲) immunized mice, trypan blue ip.
- (●) immunized mice, no trypan blue.
- (...) the limit of detection.
- (↓) below the limit of detection.

The maximum rate of clearance is indicated by retention of ^{125}I in normal mice injected ip with heat-killed ^{125}I -EAT cells (-----○).



3.2.2 Long-term iv immunized mice recalled with llRX antigen ip

Mice were injected with 10^5 llRX iv and 40 days later some were injected with 10 ug llRX antigen ip. Two days later they were challenged with 10^6 ^{125}I -EAT. Administration of trypan blue to "recalled" mice prior to challenge led to greater retention of cell-associated ^{125}I than that observed in "recalled" mice not given trypan blue (Fig 3.2B).

3.2.3 Long-term ip immunized mice.

Ashley and colleagues (Ashley, 1976; Ashley and Kotlarski, 1982a) showed that the PC from mice immunized ip with llRX 40 days previously are not active in vitro against ^{51}Cr -EAT. However, these mice are still resistant to EAT and begin to clear ^{125}I about 4 h after being challenged ip with ^{125}I -EAT (Ashley and Kotlarski, 1982a). To determine whether macrophages were involved in mediating tumour resistance at this time, mice were injected ip with llRX and 40 days later they were injected with trypan blue 3 h prior to challenge with ^{125}I -EAT. The results in Fig 3.2C shows that administration of the dye reduced the clearance of tumour cells.

From the results presented in this section it is clear that trypan blue does not produce its effect by simply inhibiting the breakdown of DNA from killed tumour cells because normal and immunized mice treated with trypan blue could still clear heat-killed ^{125}I -EAT cells from the peritoneal cavity. It follows therefore that the reduction in the ip clearance of ^{125}I -EAT cells by llRX-immunized mice injected ip or iv with trypan blue is consistent with the notion that activated macrophages are the effector cells responsible for tumour resistance in vivo.

3.3. The cytolytic activity of PC from trypan blue-treated llRX-immunized mice

The results of the above experiments showed that the tumour resistance in llRX-immunized mice could be inhibited by an

anti-macrophage agent, thus providing further circumstantial evidence that macrophages are involved in the in vivo effects on tumour cells in this model. If the cytotoxic effector cells which are active in vivo are also active in vitro then the in vitro cytolytic ability of PC of 11RX-immunized mice should be impaired by treatment with trypan blue. The cytotoxic activity of suspensions of PC from 11RX-immunized mice injected with trypan blue at various times before harvesting the PC was tested in a standard in vitro cytotoxicity assay against ^{51}Cr -EAT cells, i.e. by co-culturing 10^6 PC with 10^4 ^{51}Cr -EAT cells for 20 h in multiwell tissue culture trays.

3.3.1 Short-term 11RX immunized mice

Mice immunized ip with 11RX 6 days previously were used in a series of in vitro experiments. PC suspensions from these mice were prepared at various times after ip or iv injection of trypan blue and used in a standard in vitro cytotoxicity assay. The times used are indicated in Table 3.1. The series consisted of the same experiment carried out on 9 separate occasions using three different preparations of trypan blue. On six occasions differential counts were made on smears of the PC suspension obtained. Total cell recoveries were determined on seven separate occasions. The results of two of these experiments are shown in Table 3.1. All the data obtained indicated that 4 to 12 h after ip injection of trypan blue the cytolytic activity of the PC was greatly reduced. The PC had recovered some or most of their activity 18-24 h after ip injection of trypan blue.

In some experiments, the peritoneal washout fluid from which the cells had been removed was collected and the amount of trypan blue present was measured with a spectrophotometer by absorption at 650 nm. Most of the trypan blue was cleared from the peritoneal cavity within 24 h of injection. For example, only 0.2 mg and 0.02 mg of trypan blue remained in the peritoneal cavity of mice at 6 h and 24 h after injection

TABLE 3.1

The in vitro cytotoxic activity of PC from 11RX-immunized mice
injected with trypan blue at various times prior to harvesting PC[#]

Expt	Route of trypan blue injection*		Time of trypan blue injection (hours before PC collection)			
			No injection	6	12	24
1	ip	Number of PC/mouse	37×10^6	44×10^6	30×10^6	33×10^6
		Differential counts				
		macrophages	80	76%	75%	66%
		lymphocytes	10	0%	7%	18%
		polymorphonuclear cells	10	24%	18%	16%
	% Cytolysis [@]	37.3 ± 1.8	16.6 ± 2.7	15.2 ± 1.5	36.8 ± 2.7	
2	iv	Number of PC/mouse	26×10^6	24×10^6	29×10^6	ND [§]
		Differential counts				
		macrophages	69%	62%	65%	ND
		lymphocytes	10%	7%	11%	ND
		polymorphonuclear cells	21%	31%	24%	ND
	% Cytolysis	55.2 ± 1.1	34.6 ± 0.4	8.3 ± 5.5	ND	

(§) Not done

(*) 4 mg/mouse

(@) Measured at 20 h, mean \pm s.e.m

(#) Mice were immunized ip with 10^5 11RX 6 days before harvesting PC.

of 4 mg of the batch of trypan blue used in experiment 1, Table 3.1. Presumably this explains why the PC had recovered some, if not all, of their cytolytic activity at this time. Whether in some experiments the lack of complete recovery of cytolytic activity of the PC at 24 h after trypan blue injection correlated with less efficient clearance of trypan blue was not established.

The PC from short-term ip-immunized mice injected iv with trypan blue also showed a decrease in cytolytic activity. However, this reduction in activity was clearly evident only 12 h after injection of trypan blue. The time taken for the cytotoxic activity of the PC to recover after iv administration of trypan blue was not investigated.

3.3.2 Recalled, long-term iv-immunized mice

The cytotoxic activity of PC from recalled 40 day iv-immunized mice injected with trypan blue ip was determined. Mice were immunized with 11RX iv, 40 days later they were injected ip with 10 µg of 11RX antigen and two days later their PC were harvested and used in a standard in vitro cytotoxicity assay. Some of these mice were injected with trypan blue at various times before recovering the PC. Table 3.2 shows that 6-12 h after trypan blue injection the PC lacked cytotoxic activity but had recovered it 24 hours after the injection of trypan blue. The effect of trypan blue injection on the composition of the PC suspensions is not known because no smears were made of the PC.

3.4 The in vitro activity of cytotoxic lymphocytes from allo-immunized mice injected with trypan blue

Experiments were carried out to determine whether administration of trypan blue in vivo could inhibit the cytotoxic activity of cells other than activated macrophages. This was done by measuring the effect of trypan blue on the cytotoxic activity of peritoneal lymphocytes (PL). Cytotoxic PL were induced by immunizing BALB/c mice ip with the C57Bl/6

TABLE 3.2

The in vitro cytotoxic activity of PC from recalled,
11RX-immunized mice injected with trypan blue[#]

Time of trypan blue injection*	PC/mouse x 10 ⁻⁶	% Cytolysis (mean \pm s.e.m)	
		10h	20h
No trypan blue	12.5	11.4 \pm 1.2	25.4 \pm 2.7
6	25.0	-2.9 \pm 0.6	7.0 \pm 2.0
12	28.0	-4.0 \pm 0.3	2.6 \pm 1.8
24	23.0	2.0 \pm 0.4	39.8 \pm 5.0

(#) Mice were immunized 40 days previously with 10⁵ 11RX iv and injected ip with 10 μ g 11RX antigen 2 days prior to harvesting the PC. The target cells used were ⁵¹Cr-EAT cells. The spontaneous release of ⁵¹Cr was 17.2% at 10h and 21.7% at 20h.

(*) Hours before PC collection

thymoma EL4. Some of these mice were injected ip with trypan blue and 12 h later their PC were harvested. Suspensions of PL were prepared by depleting adherent cells using a glass wool column. To remove contaminating polymorphonuclear (PMN) cells, the eluted cells were centrifuged on a Sepalymph density gradient. They were tested against ^{51}Cr -EL4 cells at an effector to target cells ratio of 100:1. The results in Table 3.3 show that injection of trypan blue did not reduce the activity of the PL in an in vitro cytotoxicity assay against ^{51}Cr -EL4 whereas the activity of PC from 11RX-immunized mice against both ^{51}Cr -EL4 and ^{51}Cr -EAT was reduced using the same batch of trypan blue.

3.5 The cytotoxic activity of PC from short-term immunized mice after pretreatment in vitro with trypan blue

Experiments were conducted in which PC from 11RX-immunized mice were pretreated with trypan blue prior to being used in an in vitro cytotoxicity assay. Other workers in the field (Hibbs, 1974; Kaplan and Morahan, 1976) pretreated activated macrophages with trypan blue at a non-toxic concentration of 0.4 mg/ml for 24 h prior to the addition of tumour cells. This protocol could not be used because PC from 11RX-immunized mice rapidly lost cytotoxic activity in a ^{51}Cr release assay when preincubated at 37° for any length of time prior to the addition of target cells (for example, see Table 3.6 and Table 3.7). Accordingly, the incubation time was reduced to 1 h. PC at 10^7 /ml in culture medium were incubated with trypan blue at concentrations ranging from 1.0 mg/ml to 0.03 mg/ml. After incubation, the PC were washed, made up to 10^7 /ml and tested in an in vitro cytotoxicity assay against ^{51}Cr -EAT. The data presented in Fig 3.3 show that pretreatment of PC with the two lowest concentrations of trypan blue enhanced cytotoxicity but, as the concentration of dye was increased, the cytolytic activity of the PC was reduced in a dose dependent manner. The cytolytic activity of

TABLE 3.3

The in vitro cytotoxic activity of cells from allo-immunized and 11RX-immunized mice injected ip with trypan blue

Effector cells	Mice injected ip with trypan blue [#]	% Cytolysis (mean \pm s.e.m.)	
		⁵¹ Cr-EL4 [♠] 4 h	⁵¹ Cr-EAT [Ⓜ] 20 h
BALB/c anti-EL4 PL [¶]	No	76.4 \pm 3.7	ND*
	Yes	81.4 \pm 3.7	ND
PC from ip 11RX-immunized mice [§]	No	29.2 \pm 2.1	26.3 \pm 2.0
	Yes	4.3 \pm 1.3	5.3 \pm 0.5

(#) 4 mg ip.

(*) not done.

(¶) Cytotoxic PL were harvested from BALB/c mice immunized 10 days previously with 2×10^7 EL4 cells ip.

(§) PC were harvested from mice immunized with 10^5 11RX ip 6 days previously.

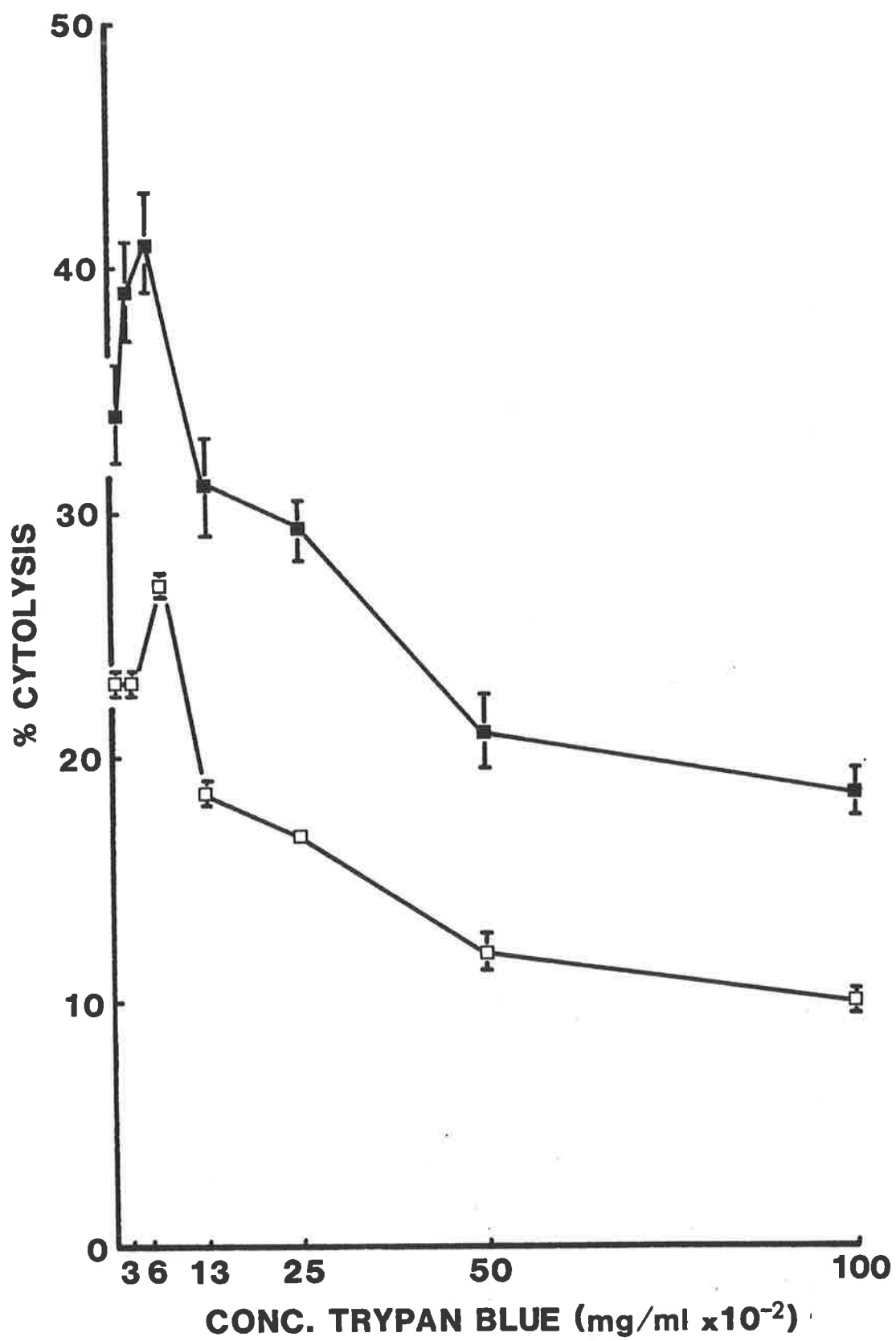
(♠) The spontaneous release of ⁵¹Cr from ⁵¹Cr-EL4 cells was 27.4%.

(Ⓜ) The spontaneous release of ⁵¹Cr from ⁵¹Cr-EAT cells was 24.0%.

FIGURE 3.3

The cytolytic activity of PC treated in vitro with trypan blue

PC at 10^7 /ml from mice immunized 6 days earlier with 10^5 11RX ip were incubated with trypan blue at various concentrations for 1h at 37° . They were washed and then tested for cytotoxic activity against ^{51}Cr -EAT cells in a standard in vitro cytotoxicity assay. Cytolysis was measured at 10 h (\square) and 20 h (\blacksquare).



the PC was not completely abolished using this protocol. There was a 55% reduction in the cytolysis observed at 10 h and 40% reduction of the cytolysis observed at 20 h when the PC were pretreated with 1 mg/ml of trypan blue. Based on these and earlier observations reported above, it is possible that trypan blue takes longer than 1 h to exert its maximum effect on cytolytic cells.

3.6 The in vitro cytotoxic activity of PC in the presence of trypan blue

In an attempt to increase the effectiveness of the trypan blue treatment, without the problem of associated loss of cytotoxicity due to preincubation at 37°, it was decided to add trypan blue to the culture medium used for the in vitro cytotoxicity assays. As a preliminary experiment the toxicity of the dye for ^{51}Cr -EAT cells and ^{51}Cr -EL4 cells was assessed by its effect on the spontaneous release of the label over 20 h. 10^4 labelled tumour cells were dispensed into multiwell tissue culture trays in 0.2 ml of medium containing different concentrations of trypan blue. The release of label from the EAT cells was measured at 10 h and 20 h whilst that from the EL-4 cells was measured at 4.5 h. The data in Table 3.4 show that trypan blue at the concentrations used (1 mg/ml to 0.03 mg/ml) was not toxic for either tumour and caused a small but reproducible reduction in the background release of ^{51}Cr .

Experiments were carried out to see whether the presence of trypan blue during the cytotoxicity assay reduced the in vitro cytotoxicity of PC from 11RX-immunized mice. Various doses of trypan blue were added to the culture medium containing cytotoxic PC and ^{51}Cr -EAT at a ratio of 100:1. These mixtures were dispensed into multiwell trays and the in vitro cytotoxicity assay was carried out as usual, using 10h and 20h sampling. Results in Fig 3.4 show that trypan blue inhibited the 10 h cytolysis of ^{51}Cr -EAT in a dose dependent manner and that the reduction

Table 3.4

The effect of trypan blue on the spontaneous release
of label from ^{51}Cr -EAT and ^{51}Cr -EL4 cells*

Trypan blue (mg/ml)	% ^{51}Cr release (mean \pm s.e.m)		
	EAT		EL4
	10 h	20 h	4.5 h
0	11.8 \pm 0.6	18.3 \pm 0.8	51.1 \pm 2.9
0.13	9.4 \pm 0.4	17.7 \pm 0.3	44.3 \pm 0.7
0.25	8.7 \pm 0.2	15.0 \pm 0.2	41.0 \pm 1.6
0.5	8.8 \pm 0.4	15.5 \pm 0.4	41.3 \pm 2.6
1.0	7.0 \pm 0.5	15.1 \pm 0.3	44.9 \pm 2.5

(*) ^{51}Cr -EAT cells and ^{51}Cr -EL4 cells were suspended to 5×10^4 cells/ml in medium containing trypan blue. 0.2 ml aliquots of the cell suspensions were dispensed into multiwell tissue culture trays and ^{51}Cr release was measured at the times indicated.

was more complete than with pre-incubation of the effector cells with trypan blue (Fig 3.4 cf 3.3). This result was consistent with the cytotoxic cells being macrophages. At 20h, however, the presence of trypan blue actually increased cytolysis over and above that obtained with PC in the absence of trypan blue. The mechanism of this effect was not investigated, although the results of a subsequent experiment shown in Fig 3.5 indicated that enhanced cytolysis occurred only in the presence of PC from mice immunized with 11RX. ^{51}Cr -EAT cells were not killed in the presence of trypan blue and PC from normal mice but trypan blue enhanced the lysis of tumour cells by PC from immunized mice, even though in this experiment, the PC showed low cytotoxic activity in the absence of trypan blue.

During the course of this work reports appeared in the literature that trypan blue could affect the activity of cytotoxic lymphocytes as well as macrophages if it is present throughout the duration of an in vitro cytotoxicity assay (Scornick, Ruiz and Hoffman, 1979). This was confirmed in the following experiment using cytotoxic BALB/c anti-EL4 PL (Table 3.5). Cytotoxic PL from alloimmunized mice or PC from 11RX-immunized mice were added to ^{51}Cr -EL4 cells at a ratio of 100:1. Trypan blue at the final concentrations indicated in Table 3.5 was added to the cell mixtures and the cell suspensions were dispensed into multiwell trays. ^{51}Cr release was measured 4h later. The addition of trypan blue to in vitro cytotoxic assays inhibited the lysis of ^{51}Cr -EL4 by both the PC from 11RX-immunized mice and the by PL from alloimmunized mice. It was obvious that the use of trypan blue in vitro was not a valid method for determining the nature of the cells active in in vitro cytotoxicity assays and an alternate approach had to be used.

3.7 Characterization of cytotoxic peritoneal cells

Characterization of the cells which are responsible for the in vitro cytotoxic activity of PC suspensions of 11RX-immunized mice was

FIGURE 3.4

The in vitro cytolytic activity of PC in the
presence of trypan blue

Mice were immunized with 10^5 11RX ip and their PC tested against ^{51}Cr -EAT cells in a standard in vitro cytotoxicity assay 6 days later. Trypan blue was added to the culture medium and was present through out the duration of the assay. Cytolysis was measured at 10 h (\square) and 20 h (\blacksquare).

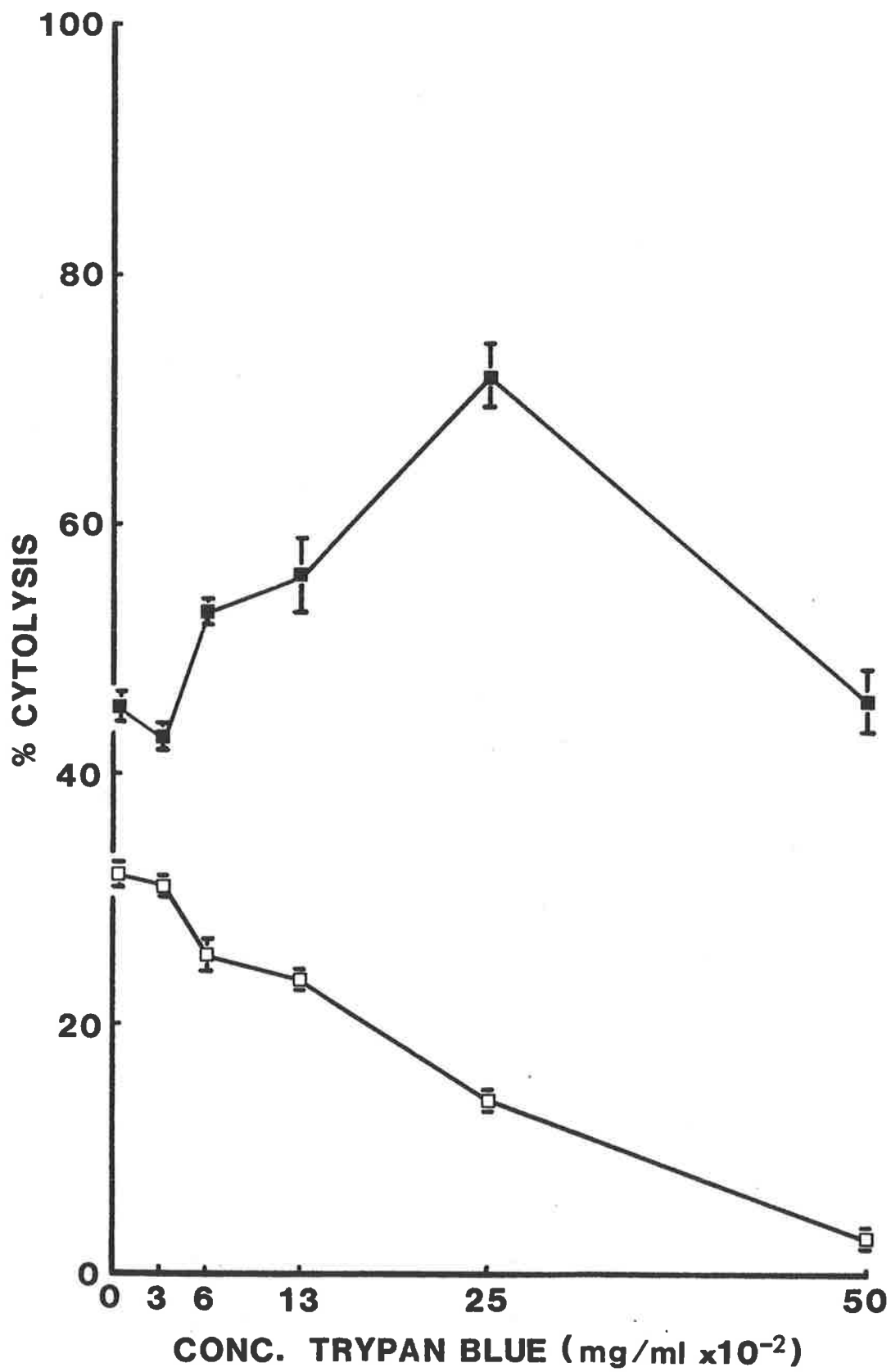


FIGURE 3.5

The in vitro cytolytic activity of PC in the
presence of trypan blue

PC from normal mice (□) or mice immunized 7 days previously with 10^5 11RX ip (■) were tested against ^{51}Cr -EAT cells in a standard in vitro cytotoxicity assay in the presence of trypan blue. The experiment was carried out in the same way as the experiment designed to obtain the data presented in Fig 3.4.

Cytolysis was measured only at 20 h.

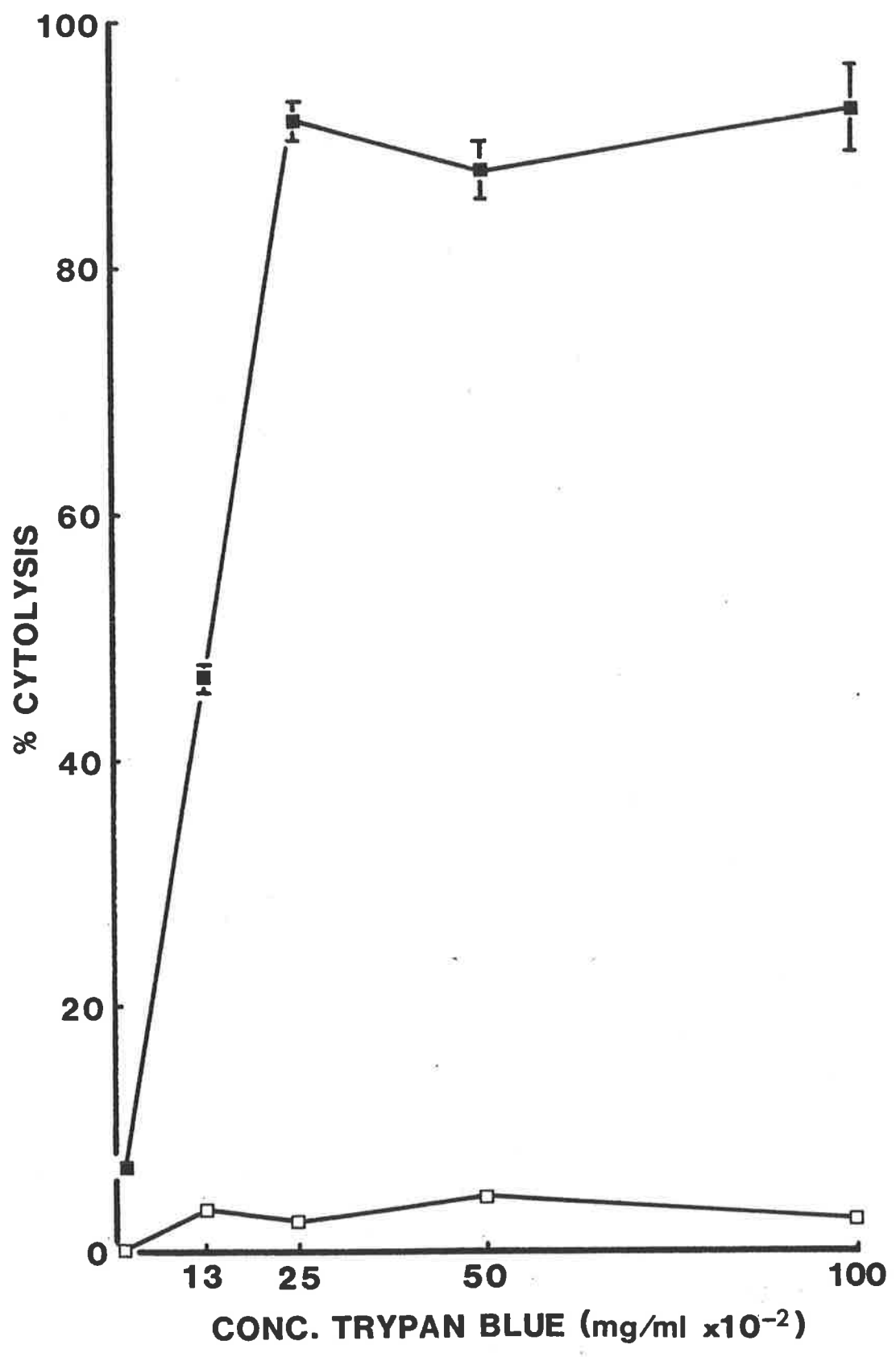


TABLE 3.5

Effect of the presence of trypan blue on the
in vitro cytotoxicity activity of PL and PC

% Cytolysis of ^{51}Cr -EL4 at 4 h (mean \pm s.e.m.)		
Trypan blue (mg/ml)	BALB/c anti- EL4 PL ^(¶)	PC from 11RX- immunized mice ^(§)
0	55.1 \pm 2.2	41.8 \pm 1.3
0.125	49.4 \pm 1.5	36.0 \pm 1.6
0.25	19.8 \pm 1.6	14.4 \pm 1.0
0.5	7.5 \pm 1.5	10.2 \pm 1.1

(¶) PL were prepared from BALB/c mice immunized with 2×10^7 EL4 cells ip 29 days prior to cell harvest. The mice were given a second injection of the same dose of cells 2 days before the experiment.

(§) PC were harvested from mice immunized with 10^5 11RX ip 6 days previously.

attempted by fractionating the PC on the basis of adherence to plastic and nylon wool and on the basis of their buoyant density before assaying the various cell fractions obtained for cytotoxic activity.

3.7.1 Adherence to Plastic

PC from short-term ip-immunized mice were fractionated into populations which adhered to plastic and those which did not. Adherent cell monolayers from 10^6 PC were prepared in multiwell trays by allowing the cells to adhere for 3 h at 37° and washing away the non-adherent cells as described in §2.19. Non-adherent cells were obtained by incubating PC at 10^7 /ml in plastic tissue culture dishes for the same period of time to allow the adherent cells to attach. The loose cells were harvested by agitating the dishes to resuspend these cells and then collecting the supernatants. The dishes were washed by squirting them with a jet of warm suspension medium and repeating the above procedure. All the non-adherent cells were pooled and the cytotoxic activity of these and the adherent cells was assayed. In the case of the non-adherent cells, the usual effector to target ratio of 100:1 was used as well as a ratio of 17:1 because only 17% of the PC were non-adherent. The results obtained are shown in Table 3.6. The non-adherent PC were inactive when the 17:1 ratio was used, but were as effective as the unfractionated PC kept on ice when the usual 100:1 ratio was used. Adherent cell monolayers prepared from 10^6 PC were as effective as unfractionated PC preincubated at 37° . These results indicated that the majority of the cytotoxic activity detected using 10^6 unfractionated PC resided in the adherent cells. Consistent with this conclusion was the observation that addition of 17×10^4 non-adherent cells to the monolayers caused only a slight increase in cytotoxicity. Differential counts (Table 3.6) showed that the number of macrophage-like cells found in the non-adherent fraction was 40% of that found in the unfractionated PC population. Thus, the cytotoxic activity

TABLE 3.6

Fractionation of PC from 11RX-immunized mice
on the basis of adherence to plastic

Type	Cells tested [¶] Number/Well	% Cytolysis (mean \pm s.e.m.) of ⁵¹ Cr-EAT at 20 h
PC [§] kept on ice for 3 h	10 ⁶	70.1 \pm 2.2
Unfractionated PC, preincubated at 37° for 3 h prior to assay [§]	10 ⁶	50.5 \pm 0.9
Adherent PC	Monolayer from 10 ⁶ PC	57.5 \pm 1.7
Non-adherent PC*	17 x 10 ⁴	15.9 \pm 0.6
Non-adherent PC	10 ⁶	71.2 \pm 2.9
Non-adherent PC + Adherent PC	17 x 10 ⁴ + monolayer from 10 ⁶	63.1 \pm 1.9

(¶) From mice immunized 6 days previously with 10⁵ 11RX ip.

(§) Differential counts: macrophages 45%, lymphocytes 28%, PMN 27% and mast cells 1 cell in 500 PC counted.

(*) Differential counts: macrophage-like cells, 17%, lymphocytes 48%, PMN 32%, mast cells 4%.

of the "non-adherent" cells may have been due to residual macrophages detached from the plastic surface during washing of the dishes or due to NK cells or both.

The presence of poorly adherent "macrophage-like" cells was confirmed by using a different procedure to remove adherent PC. The PC were allowed to adhere to plastic dishes for 1 h and the non-adherent cells were resuspended by rocking the dishes very slowly 4 or 5 times. The culture medium was removed and 5 ml of fresh warm medium was gently added to the dishes and the rocking procedure was repeated. The non-adherent cells were dispensed into fresh dishes and allowed to adhere for another 1 h. The cells which did not adhere after this time were collected as described above. Using this method, only 8% of the PC were non-adherent, but cytotoxicity assays using these non-adherent cells confirmed the results reported above (data not shown). Smears of these cells indicated that there was a great reduction in the proportion of macrophages from 51% in the starting population to 8% in the non-adherent population. PMN cells were reduced from 30% to 9% whilst lymphocytes were increased in proportion from 20% to 82%.

3.7.2 Adherence to nylon wool

To test for the presence of NK cells in the non-adherent cell population and to deplete the PC of macrophages more effectively, nylon wool rather than plastic was used as a substrate for adherence. Nylon wool has been shown to deplete macrophages to extremely low levels (Jerrels et al., 1980) and to enrich for NK cells if they are present in cell suspensions subjected to this treatment (Roder and Kiessling, 1978).

5×10^7 PC from mice immunized ip with 11RX 6 days previously were applied to nylon wool columns. After, incubation for 1h at 37°, the non-adherent cells were eluted with warm suspension medium. The proportion of cells which could be eluted was always approximately 10% of those applied. The eluted cells and unfractionated PC were tested in an

in vitro cytotoxicity assay against three different tumours, namely P815, MPC11 and EAT. P815 was chosen because it is resistant to killing by NK cells but is susceptible to lysis by macrophages, whilst MPC11 was chosen because it is susceptible to killing by both cell types (Roder et al., 1979c). When tested at an effector to target cell ratio of 100:1, the unfractionated PC lysed all three tumour targets (Table 3.7). At this ratio, the eluted cells killed MPC11 to the same extent as unfractionated PC although they showed a markedly reduced cytotoxic activity against both P815 and EAT tumour cells.

3.8 Bouyant density fractionation of PC on discontinuous gradients

In an attempt to separate different cell populations from one another, PC were fractionated on the basis of their bouyant density. The medium used was Percoll which is a suspension of colloidal silica particles (mean diameter = 21-22 nm) which has been made non-toxic by coating with polyvinylpyrrolidone (Pharmacia, 1980). It was decided to use discontinuous density gradients because they were technically simpler to prepare than continuous ones. This was done by layering 2 ml volumes of Percoll suspensions diluted to different strengths in HBSS. The concentrations of Percoll used were 30%, 50% and 70% respectively. 5×10^7 to 1×10^8 PC in 2ml of HBSS were gently layered onto the gradient using a Pasteur pipette and then centrifuged at $400 \times g$ for 30 min. Cells sedimenting at the 0-30% Percoll interface (fraction A), the 30-50% interface (fraction B), the 50-70% interface (fraction C) and those penetrating the 70% Percoll layer (fraction D) were recovered from the gradient using a Pasteur pipette. The cells were washed 3 x in medium, counted, and if sufficient cells were present in the fractions they were tested for cytotoxic activity in the usual manner at an effector to target cell ratio of 100:1. Cell smears were also made of the fractions obtained and differential counts performed to determine the cellular composition of each fraction.

TABLE 3.7

Cytotoxic activity of PC eluted from
nylon wool columns

Effector cells	% Cytolysis* (mean \pm s.e.m.)		
	$^{51}\text{Cr-MPC11}$	$^{51}\text{Cr-P815}$	$^{51}\text{Cr-EAT}$
	10 h	20 h	20 h
Normal PC	1.1 \pm 3.6	-0.4 \pm 1.7	-2.3 \pm 0.4
Unfractionated PC §	36.8 \pm 4.1	53.9 \pm 2.6	32.9 \pm 1.6
Unfractionated PC § preincubated 1 h at 37 $^{\circ}$	25.4 \pm 2.3	59.0 \pm 2.5	20.6 \pm 1.8
Non-adherent cells §	28.2 \pm 4.0	14.8 \pm 4.1	4.8 \pm 0.4

(*) Effector to target cell ratio used was 100:1.

(\S) From mice immunized 6 days previously with 10^5 11RX ip.

3.8.1 PC from recalled, iv immunized mice

Six mice which had been immunized iv with 11RX 54 days previously were injected with 10 µg of 11RX Ag and 2 days later their PC were harvested. When fractionated on a discontinuous Percoll density gradient, cell populations were recovered which were enriched in cytotoxic activity. Table 3.8 shows that the cells from fraction B were more cytotoxic than those from fraction C or from unfractionated PC suspensions. The cells from fraction C were as cytotoxic as unfractionated PC. Since very few cells were recovered in fractions A and D it was not possible to measure the cytotoxic activity of the least dense and most dense PC. Whilst the cellular composition of fraction B was identical to that of unfractionated PC, a greater proportion of the cells in fraction B were macrophages when compared to those in fraction C. Fraction C was enriched for PMN cells and lymphocytes. Although this method did not fractionate the cell types into pure populations the results suggestive that the cytotoxic cells are macrophages.

3.8.2 PC from short-term ip immunized mice

2×10^8 PC from mice immunized 6 days previously with 10^5 11RX ip were fractionated on a discontinuous Percoll gradient and the fractions tested in an in vitro cytotoxicity assay using ^{51}Cr -EAT as the target. The cells in fraction C were enriched for cytotoxic activity (Table 3.9) whilst the activity of the cells from fraction B was similar to that of the starting population. As in the previous experiment, not enough cells were present in fractions A and D to be tested in an in vitro cytotoxicity assay. Differential counts of smears made of PC fractions showed that enrichment of cytotoxic activity correlated with an increase in PMN cells and a decrease in macrophages in the cell suspension. This result implied that PMN cells may also be cytotoxic in this system. However, this possibility could not be distinguished from the alternate explanation that at day 6 after an ip infection with 11RX the high

Table 3.8

The cytotoxic activity and composition of fractions of PC from 11RX iv-immunized mice injected with 11RX antigen, obtained using discontinuous Percoll gradients[ⓐ]

(ⓐ) Mice were immunized with 10^5 11RX iv, 54 days later they were injected with 11RX antigen ip and their PC harvested 2 days later. The cells in fraction A are those which do not penetrate the gradient, fraction B consists of cells banding at the 30%-50% Percoll interface, fraction C contains cells which band at the 50%-70% interface whilst those cells penetrating the 70% Percoll layer are in fraction D.

(*) Not determined. Cytotoxic activity was not determined because very low numbers of cells were recovered.

		CELL FRACTION				
		Unfractionated	A	B	C	D
Cytolysis	10h	37.1 ± 0.5	ND*	51.4 ± 1.1	29.8 ± 1.4	ND*
	20h	67.2 ± 2.5	ND	80.3 ± 1.6	66.7 ± 2.0	ND
Number of cells recovered (x 10 ⁻⁶)		79.8 loaded	1.0	52.0	25.0	2.2
<u>Differential Counts (%)</u>						
	macrophages	74	60	72	48	15
	PMN cells	5	0	2	9	10
	lymphocytes	21	40	26	43	63
	mast cells					12

Table 3.9

The cytotoxic activity and composition of fractions of PC from ip immunized mice obtained using discontinuous Percoll gradients[@]

	CELL FRACTION		
	Unfractionated	B	C
Cytolysis* 10h	23.8 \pm 0.2	28.3 \pm 0.5	56.2 \pm 0.3
20h	31.8 \pm 0.4	37.0 \pm 1.0	63.6 \pm 1.3
Number of cells recovered (x 10 ⁻⁶)	207 loaded	155	188
<u>Differential Counts(%)</u>			
macrophages	66	86	40
PMN cells	23	9	45
lymphocytes	11	5	15

(@) Mice were immunized with 10⁵ 11RX ip 6 days previously. The cells in fraction B consists of cells banding at the 30%-50% Percoll interface, whilst those in fraction C band at the 50%-70% interface.

(*) Mean \pm s.e.m.

density macrophages were more cytotoxic than those of lower density.

A major limitation of discontinuous density gradients used in the above experiment is that the cells in each fraction span a very broad density range. Technical information supplied by Pharmacia showed that fraction B would contain cells with a buoyant density in the range of 1.04-1.06 g/ml and the cells in fraction C would have a density from 1.06-1.08 g/ml.

3.9 Buoyant density fractionation of PC using continuous gradients

To obtain better cell separation, a continuous Percoll gradient was constructed as described in Materials and Methods. Briefly, a discontinuous gradient was constructed using 1 ml Percoll steps of the following concentrations: 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% and 70%. The gradient was then stored for 3 days at 4° to allow diffusion of the steps to occur. Fig 3.6 shows the refractive indices of 0.25 ml fractions of the gradient after this time. It is clear that the individual steps had diffused and that the gradient had become essentially linear and continuous.

2×10^8 PC from mice immunized ip with 11RX 6 days previously were fractionated on such Percoll gradients and the cells which were recovered were assayed for in vitro cytotoxic activity against ^{51}Cr -EAT. As can be seen from Fig 3.7, the PC could be fractionated on the basis of buoyant density to yield subpopulations which, when tested at an effector to target cell ratio of 100:1, were found to be depleted of cytotoxic activity (fractions 1, 2, 3) or enriched for cytotoxic activity (fractions 6, 7, 8); three fractions (4, 5 and 9) had the same activity as the starting PC population. Differential counts done on smears made of the various fractions (Fig 3.7) showed that in fractions 1, 2 and 3 there was a depletion of macrophages and an enrichment for PMN cells and lymphocytes. The fractions with enriched cytotoxic activity contained a greater proportion of macrophages than the starting

FIGURE 3.6

Generation of a continuous linear Percoll gradient

The graph shows the refractive indices of 0.25 ml fractions of a gradient formed by allowing a discontinuous gradient with 1 ml steps to diffuse for 3 days at 4°.

- (□) refractive index of individual 0.25 ml fractions
- (─) refractive index of initial 1 ml Percoll steps used to prepare the discontinuous density gradient.

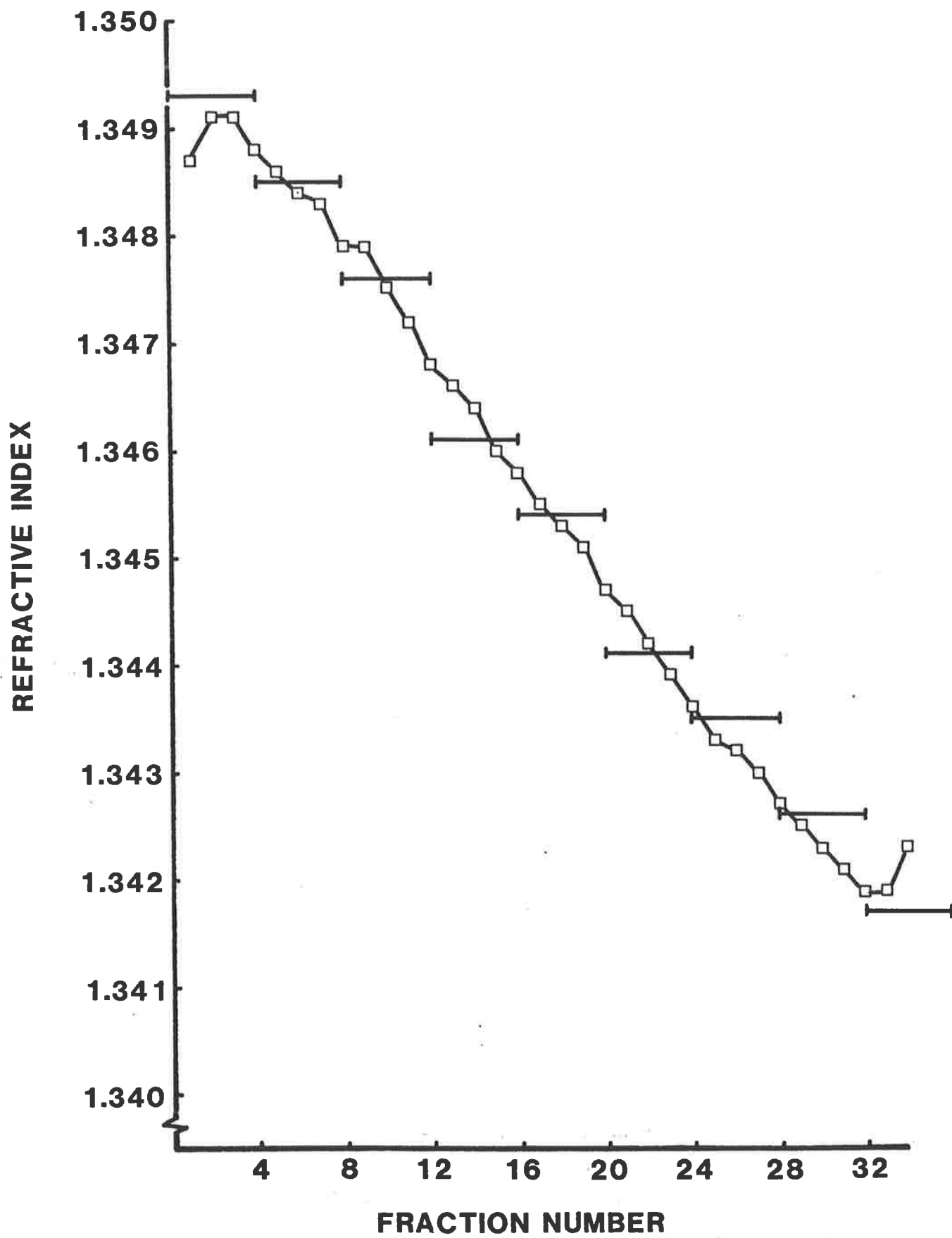
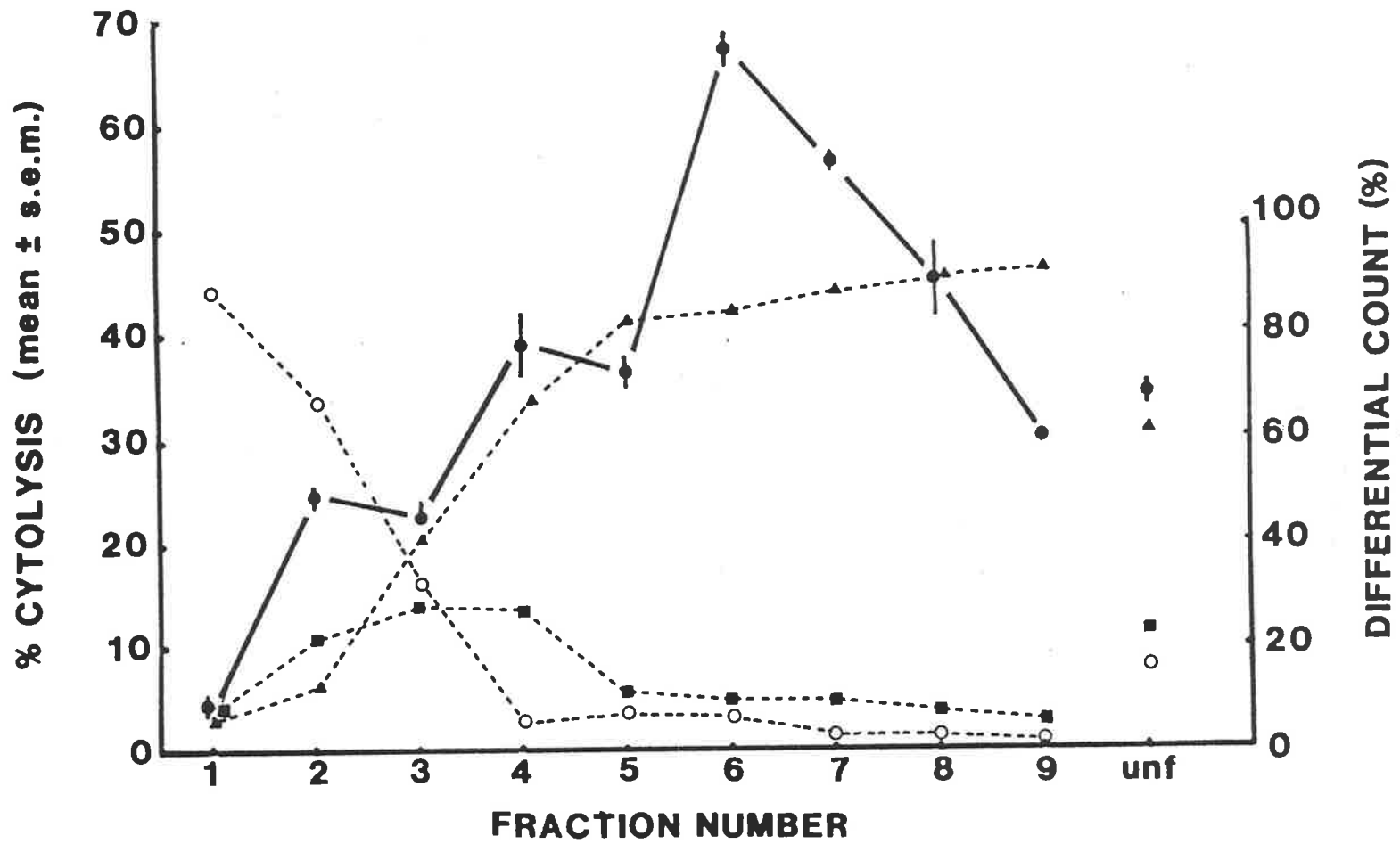


FIGURE 3.7

The cytotoxic activity of PC from 11RX-immunized mice
fractionated on continuous Percoll gradients

Cytolytic activity at 10 h (●) and differential counts of fractions of PC obtained using Percoll density gradients and unfractionated PC (unf). Fraction 1 was the densest one and 9 the least dense. Differential counts indicate lymphocytes (○), polymorphonuclear cells (■) and macrophages (▲).

PC were obtained from mice immunized ip 6 days previously with 11RX.



population. This result strongly supported the notion that most of the cytotoxic effector cells detected in vitro were macrophages.

3.10 Summary and conclusions

In an attempt to characterize the effector cells responsible for tumour resistance in Salmonella enteritidis 11RX-immunized mice the anti-macrophage agent trypan blue was used in both in vivo and in vitro experiments. Resistance was measured in vivo by the clearance of ^{125}I from the peritoneal cavity of mice injected ip with ^{125}I -EAT cells. The in vitro correlate was measured by lysis of ^{51}Cr -labelled tumour cells by PC from 11RX-immunized mice. Pretreatment of resistant mice with trypan blue greatly reduced both ^{125}I clearance and the non-specific in vitro cytolytic activity of their PC. The treatment of cytotoxic PC with trypan blue prior to use in an in vitro cytotoxicity assay also inhibited tumour cell killing as did the continued presence of the anti-macrophage agent during the assay. In the latter case the effect of trypan blue was not restricted to macrophages alone; alloantigen-specific, cytotoxic peritoneal lymphocytes were affected as well. Trypan blue treatment of alloimmunized animals did not, however, inhibit the in vitro cytotoxic activity of peritoneal lymphocytes. These data support the notion that macrophages are responsible for most of the cytotoxic activity detected by in vivo and in vitro assays.

Fractionation of cytotoxic PC on the basis of adherence to plastic or nylon wool and buoyant density, coupled with the use of appropriate cell targets, confirmed the above conclusions. However, other cells such as natural killer cells were also present in the PC suspensions and made some contribution to in vitro cytotoxicity when the appropriate cell targets were used.

CHAPTER FOUR

THE INTERACTION OF ACTIVATED MACROPHAGES WITH
NORMAL AND NEOPLASTIC CELLS4.1 Introduction

It is clearly established that susceptibility to macrophage mediated lysis is a property of transformed and neoplastic cells. Hibbs (1973) and Cleveland et al., (1974) showed that tumour cells did not grow when cultured with activated macrophages whilst the density to which normal cells grew in their presence was reduced only slightly (Cleveland et al., 1974; Keller, 1974). As discussed in §1.11, cytotoxic factors such as hydrogen peroxide, the enzymes arginase and serine proteases and the nucleotide thymidine, which are all secreted by activated macrophages, have been implicated in the mechanism(s) of tumour cell killing.

There is evidence to suggest that the difference in susceptibility to macrophage-mediated killing which distinguishes normal cells from neoplastic cells may be due to the altered characteristics of neoplastic cells which render them susceptible to the above mentioned factors, rather than due to an ability of macrophages to distinguish between the normal and neoplastic states. Arginine depletion inhibits the growth of tumour cells and normal cells (Currie, 1978; Weisfeld and Rous, 1977) but only normal cells can survive extended periods of arginine deprivation. A similar difference exists in the effect of excess thymidine on the two cell types. Cell division is inhibited by excess thymidine (Xeros, 1962), an effect which is lethal to tumour cells but not normal cells (Lee, Giovanella and Stehlin, 1977). Since activated macrophages release thymidine and arginase constitutively (Stadecker et al., 1977; Currie,

1978), it follows that within a tumour mass growing in vivo or tumour cells co-cultured with macrophages in vitro, tumour cell death by such mechanisms would not necessarily require the direct interaction of macrophages with the tumour cells. Tumour cell death could occur simply by the continued maintenance of conditions such as high thymidine or arginase levels in the local microenvironment.

Other evidence, however, strongly suggests that activated macrophages are capable of recognizing surface components of tumour cells. Microscopic examination of activated macrophage-tumour cell cultures show that effector and target cells are in close physical association (Hibbs et al., 1972b; Meltzer, Tucker and Bruer, 1975a; Bucana et al., 1976; Keller, 1974), whilst such associations are rarely seen when normal macrophages are used or when activated macrophages are cultured with normal cells (Meltzer et al., 1975; Keller, 1974; Keller et al., 1976).

During the course of this study two groups have shown that activated macrophages can bind tumour cells but not normal splenic lymphocytes in vitro (Piessens, 1978; Marino and Adams, 1980a). Their data suggest that tumour cells possess surface structures that are not present on normal cells. It is possible that the results reflect the fact the tumour cells were a dividing cell form whereas suspensions of lymphocytes contain mainly non-dividing cells. In other words, it may be that activated macrophages can discriminate between dividing and non-dividing cells, rather than normal and neoplastic cells, and that only tumour cells are killed following binding to the macrophages.

That macrophages can influence the function of normal cells has been well documented, in particular by Keller (Keller, 1974; Keller, 1976a; Keller, 1978; Keller, 1981). Proteose-peptone induced rat macrophages can inhibit the proliferation of normal cell lines as well as those which are transformed or tumourigenic (Keller, 1974; Keller, 1976; Keller, 1978). Target cells are arrested in the G1 phase of the cell

cycle (Keller et al., 1976). Other workers have shown that this effect on normal cells does not necessarily require direct contact with macrophages since culture medium conditioned by macrophages alone can inhibit the division of normal cells (Calderon and Unanue, 1975; Calderon, Williams and Unanue, 1974; Nelson, 1973; Waldmann and Gottlieb, 1973). Consistent with this is the observation of Keller (1974) that the proliferation of some tumour cells and normal cells was inhibited when they were co-cultured with macrophages in vitro even though there was little evidence for the formation of aggregates between the two cells types. However, Cabilly and Gallily (1981) reported that cytostasis of primary explant fibroblasts by thioglycollate induced murine macrophages occurred only if the target cells were artificially bound to macrophages by Con A or antibody.

Evidence that non-tumourigenic dividing cells may interact with cytotoxic activated macrophages was provided by Keller (1979) who showed that foetal and neonatal rat liver cells were capable of competitively inhibiting the in vitro killing of tumour cells by C. parvum activated macrophages. Adult liver cells did not have this property and liver cells were less active if they were taken from animals which were older than neonates. This was interpreted to mean that macrophages probably recognize determinants that are common to both tumour cells and foetal cells but which are absent on adult cells. An alternate explanation is that activated macrophages interact with the dividing cells in the foetal liver and that the lack of competitive activity by young and adult liver cells was due to the lack of dividing cells in the more mature organs.

The experiments presented in this chapter were designed to test the hypothesis that normal, dividing, adult cells and tumour cells could interact with activated macrophages in a similar way. The tumour cells used were the P815 tumour cell line which was maintained by in vitro culture and EAT cells which were passaged in vivo. The normal dividing cells were Con A-activated splenic blast (Con A blast) cells. These two

cell types were compared for their susceptibility to lysis by PC from 11RX-immunized mice and their ability to competitively inhibit the lysis of ^{51}Cr -P815 and ^{51}Cr -EAT cells. They were also compared to normal splenic lymphocytes for their ability to bind to macrophages from 11RX-immunized mice. Finally, an attempt was made to determine whether the interaction of P815 cells and activated macrophages was mediated by carbohydrates. Unless otherwise stated in the text, the PC were obtained from mice 6-8 days after ip injection of 10^5 11RX.

It was found that, whilst blast cells and P815 tumour cells were bound more effectively than non-dividing lymphocytes, blast cells were resistant to lysis by tumouricidal PC, thereby supporting the notion that activated macrophages could interact with normal, dividing cells without making them susceptible to lysis by the mechanisms responsible for killing tumour cells. However, in contrast to some of the previously reported data, blast cells did not inhibit cytolysis of tumour cells. Experiments designed to resolve this paradox suggested that only tumour cells could interact with activated macrophages for extended periods of time. During the course of this work reports appeared in the literature in which the interaction of activated macrophages with tumour cells, LPS-activated splenic blast cells (Marino, Whisnat and Adams, 1981) and Con A blast cells (Hamilton and Fishman, 1981) was compared. Their results will be discussed in Chapter 7 in relation to those presented below.

Before investigating the ability of activated macrophages to bind dividing normal and neoplastic cells it was necessary to determine whether the killing of P815 and EAT cells by 11RX-activated macrophages was a contact-dependent phenomenon. This was investigated in experiments where the conditions were varied in an attempt to reduce or increase the probability of contact between effector and target cells. The parameters used were variations in the effector to target cell ratio, or the surface area of the culture vessels used, separation of the effector and target

cells using a cell impermeable membrane and addition of Ab directed to alloantigens on target and effector cells, in an attempt to facilitate contact.

4.2 The effect of varying the effector to target cells ratio on in vitro cytotoxicity

If tumour killing is dependent upon establishing contact between effector and target cells, then it follows that as the number of PC used in an assay is reduced it would take longer to achieve a given level of killing of a standard number of tumour cells. Increasing the number of tumour cells used would increase the probability of an effector cell coming into contact with a target cell so that a standard number of PC could kill more tumour cells at any one time. In vitro cytotoxicity assays were performed in which the number of PC or the number of tumour cells were varied. The cytolysis observed when either parameter of the assay was changed is compared to the cytolysis in a standard in vitro cytotoxicity assay i.e. in an in vitro assay where 10^6 PC are co-cultured with 10^4 ^{51}Cr -labelled tumour cells. The results of these experiments are shown in Fig 4.1 and support the concept that killing of tumour cells is dependent on contact with PC. As can be seen in Fig 4.1A, the extent and the rate of cytolysis decreased as the number of PC used in the assay was reduced while keeping the number of tumour cells constant at 10^4 /well. The cytolysis attained at 4 h with a given effector to target cells ratio was not reached until 10 h when half the number of PC were used. Similarly, the level of cytotoxicity detected at 10 h was not reached until 20 h when the number of PC added was halved.

The results in Fig 4.1B show that a decreasing proportion of the target cells were lysed when the number of P815 cells added to a constant number of PC (10^6) increased. However, it is apparent that the total number of target cells killed increased when larger numbers of them were used in the assay (Fig 4.1C). These results showed that the rate of

FIGURE 4.1

The kinetics of lysis of ^{51}Cr -P815 cells by PC from 11RX-immunized mice
at different effector to target cell ratios

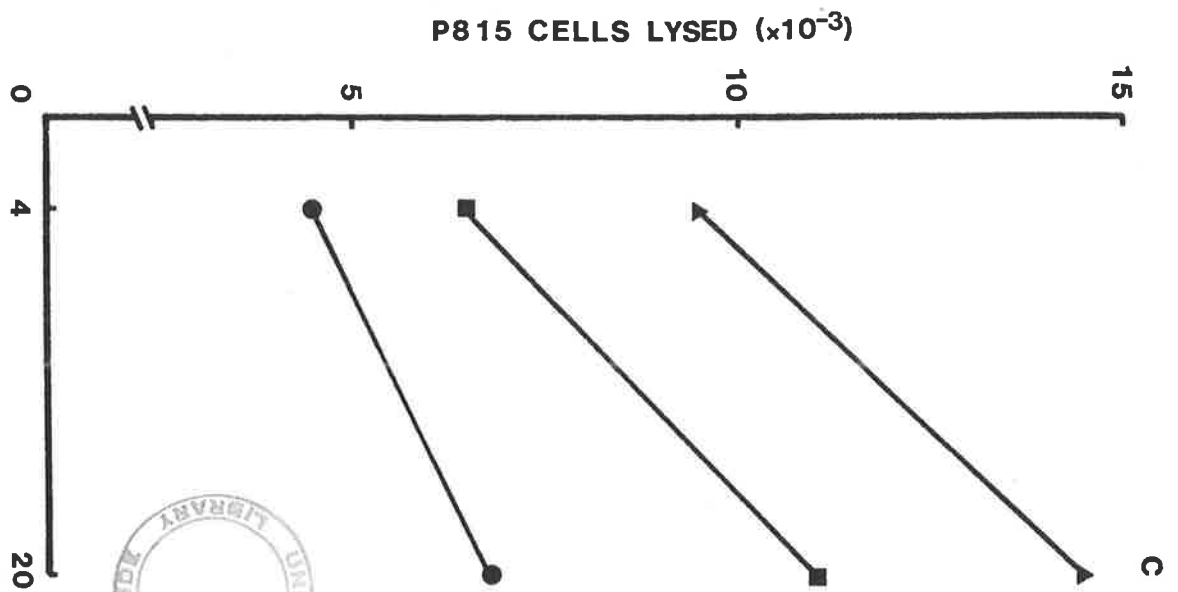
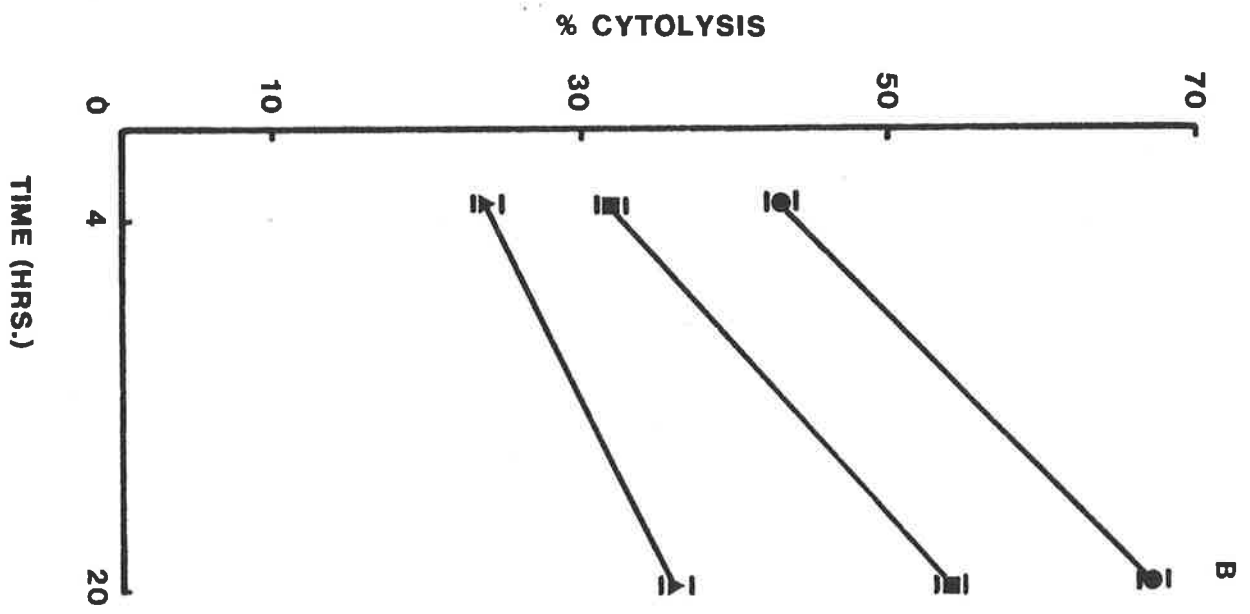
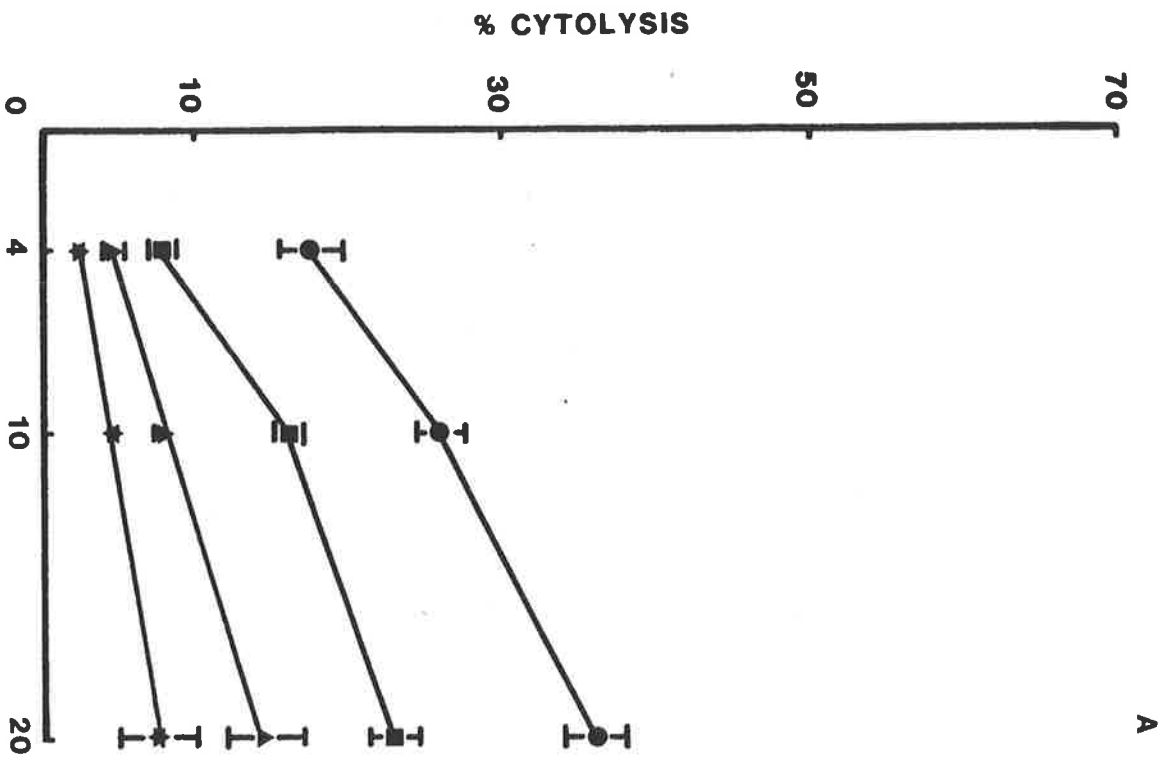
A: The effect of varying PC numbers.

PC were harvested from day 7 11RX-immunized mice and the following numbers were used as effector cells in an in vitro cytotoxicity assay with 10^4 ^{51}Cr -P815 cells: 10×10^5 (●), 5×10^5 (■), 2.5×10^5 (▲), 1.3×10^5 (★). Cytolysis was measured at 4 h, 10 h and 20 h.

B: The effect of varying the numbers of ^{51}Cr -P815 cells.

PC were harvested from 6 day 11RX-immunized mice and 10^6 PC/well were tested for in vitro cytotoxic activity against the following numbers of ^{51}Cr -P815 cells: 1×10^4 (●), 2×10^4 (■), and 4×10^4 (▲). Cytolysis was measured at 4 h and 20 h.

C: The data from panel B expressed as the number of P815 cells killed.



cytolysis was dependent on the number of PC and tumour cells used in the assay.

4.3 The role of soluble factors in the lysis of tumour cells by PC from 11RX-immunized mice

while supporting the concept that cell contacts are required for tumour cell killing, the results of the above experiments do not eliminate the possibility that killing occurs because macrophages release soluble cytotoxic factors or because they deplete the culture medium of nutrients. In the first experiment (Fig 4.1A) it could be argued that reducing the number of cytotoxic PC reduced the concentration of cytotoxic factors in the medium during the course of the assay or reduced depletion of essential nutrients. In the second experiment (Figs 4.1B and 4.1C), the same result would be obtained if the amount of cytotoxic material in the medium was not sufficient to lyse all of the extra target cells added to the wells. Experiments which were designed to distinguish between these alternatives are presented below.

4.3.1 The effect of low cell density on in vitro cytolysis

An approach used to investigate the role of soluble cytotoxic factors or medium depletion in macrophage-mediated cytolysis was to carry out in vitro cytotoxicity assays in tissue culture vessels with different surface areas. It was reasoned that an increase in the surface area would reduce the chances of tumouricidal PC and ^{51}Cr -EAT making contact with each other. The volume of tissue culture medium and the number of PC and ^{51}Cr -EAT cells was kept constant so that the concentration of "soluble cytotoxic factors" that may have been produced by the PC was unchanged. Mixtures of PC from 11RX-immunized mice and ^{51}Cr -EAT cells at a ratio of 100:1 were dispensed into tissue culture vessels at confluent and less than confluent cell density. 10^6 PC and 10^4 ^{51}Cr -EAT in 0.3 ml were dispensed into 4 replicate wells with a diameter

of 6 mm or 17 mm and incubated at 37° for 20 h before harvesting 0.1 ml aliquots of the supernatant from each well. These were used to determine the amount of ^{51}Cr release which had occurred during the assay period. The results in Table 4.1 show that the tumour killing in the 17 mm wells was greatly reduced when compared to that occurring in the smaller wells. Similarly, when 5×10^6 PC and 5×10^4 ^{51}Cr -EAT in 3 ml were allowed to interact in 50 mm tissue culture dishes, the cytolysis which occurred was less than that when the same number of cells in 3 ml were incubated in 17 mm wells (Table 4.1). Both results suggest that cell contact is important in tumour cell killing.

4.3.2 Separation of tumour cells and PC by cell impermeable membranes

The above data are against a "soluble factor" being responsible for killing in this system. This view is supported by experiments using cell impermeable membranes to separate cytotoxic PC from target cells. Sartorius filters of five millimeter diameter with 0.45 μm or 0.22 μm pores were used to separate effector and target cells in the wells of multiwell tissue culture trays as described in §2.28. The results in Table 4.2 show that no significant tumour killing was obtained when cytotoxic PC were separated from ^{51}Cr -EAT by 0.45 μm Sartorius membranes. Addition of unlabelled EAT to the PC at a ratio of 1:100 did not result in any increase in killing of ^{51}Cr -EAT cells on the other side of the Sartorius membranes. This implied that a long-lived cytotoxic factor was not constitutively produced and was not induced when the effector cells came in contact with EAT. A similar result is seen in Table 4.5 where ^{51}Cr -P815 cells were used as target cells.

4.4 The effect of antibody on the cytolysis of ^{51}Cr -P815 cells by cytotoxic PC

Although it is generally accepted that activated macrophages can kill tumour cells in the absence of Ab specific for the tumour cells,

TABLE 4.1

The in vitro cytotoxic activity of PC in culture vessels
of various surface areas*

Type of culture vessels	Number of cells		Volume in vessel (ml)	% Cytolysis [§] (mean \pm s.e.m.)
	PC	⁵¹ Cr-EAT		
6mm wells	10 ⁶	10 ⁴	0.3	53.0 \pm 1.6
17mm wells	10 ⁶	10 ⁴	0.3	13.7 \pm 1.8
17mm wells	5x10 ⁶	5x10 ⁴	3.0	32.6 \pm 0.4
50mm dishes	5x10 ⁶	5x10 ⁴	3.0	13.4 \pm 1.0

(*) PC were obtained from mice immunized ip with 11RX 6 days previously.

(§) Measured at 20 h.

TABLE 4.2

Lack of tumour killing across cell-impermeable membranes*

Cells above membrane	Cells below membrane	% Cytolysis [#] (mean \pm s.e.m.)
PC + ⁵¹ Cr-EAT	none	37.8 \pm 0.6
none	PC + ⁵¹ Cr-EAT	43.8 \pm 2.4
PC	⁵¹ Cr-EAT	7.9 \pm 1.4
⁵¹ Cr-EAT	PC	8.7 \pm 0.9
PC + ⁵¹ Cr-EAT	EAT	45.0 \pm 1.8
PC + EAT	⁵¹ Cr-EAT	8.6 \pm 2.9
EAT	PC + ⁵¹ Cr-EAT	38.5 \pm 1.6
⁵¹ Cr-EAT	PC + EAT	7.1 \pm 1.5
PC + ⁵¹ Cr-EAT	no membrane	46.2 \pm 1.8.

(*) PC were obtained from mice immunized with 10^5 11RX 6 days previously. 10^6 PC and 10^4 ⁵¹Cr-EAT cells above or below or separated by Sartorius membranes with 0.45 μ m pores. 10^4 unlabelled EAT cells were included in some cultures.

(#) Cytolysis was measured at 20 h. The spontaneous release of ⁵¹Cr-EAT cells in medium alone was $20.3 \pm 0.6\%$ and was unaffected by the presence of the membranes.

there are reports in the literature indicating that in some in vitro systems this is not the case. Bast et al. (1979) showed that PC taken from mice after injection with C. parvum lysed murine ovarian adenocarcinoma cells in vitro in a 4 h assay only in the presence of anti-tumour Ab. Similarly, Yamazaki et al. (1976) reported that BCG- or LPS-activated macrophages lysed tumour cells in vitro only when tumour-specific Ab was present. In both cases the presence of these Abs did not render normal macrophages cytotoxic. Therefore, experiments were performed to determine whether Ab could enhance the cytolytic activity of PC from 11RX-immunized mice.

The antiserum used was prepared by injecting CBA mice with 2×10^7 P815 cells ip every week for 8 weeks. A pool of serum was prepared from blood obtained from these CBA mice. The serum was diluted 1/10 in HBSS, sterilized by filtration and stored frozen in 1 ml aliquots. It was used in the following experiments at a final dilution of 2×10^{-3} . The antiserum contained Abs directed to the MHC determinants of DBA/2, which are also present on the cells of BALB/c and CB6F₁ mice. Accordingly, Abs in this antiserum should increase binding between appropriate effector and target cells either by crosslinking P815 cells and CB6F₁ PC via the MHC antigens or by promoting the adherence of Ab coated tumour cells to the Fc receptors present on PC.

The experiments reported in §4.2 were repeated to determine the effect of Abs on the kinetics of cytolysis when the number of PC and ⁵¹Cr-P815 cells were varied. The effect of antiserum on in vitro cytotoxic activity when the number of PC were reduced is shown in Fig 4.2A. At the standard effector to target ratio of 100:1, the presence of Abs increased cytolysis at 4 h and 10 h but appeared to have no effect on the cytotoxicity detected at 20 h. A similar effect was seen at an effector to target cell ratio of 50:1. When the effector to target cell ratio was further reduced to 25:1 (data not shown) and 12.5:1, cytotoxicity at all 3 time points was higher in the cell mixtures

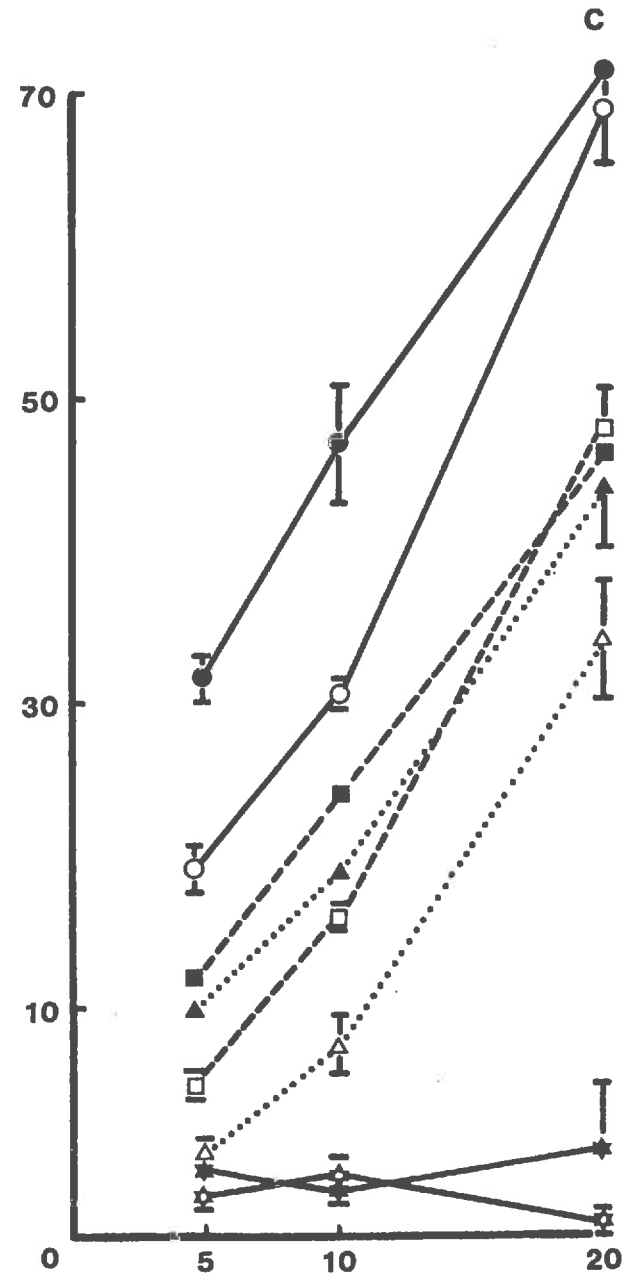
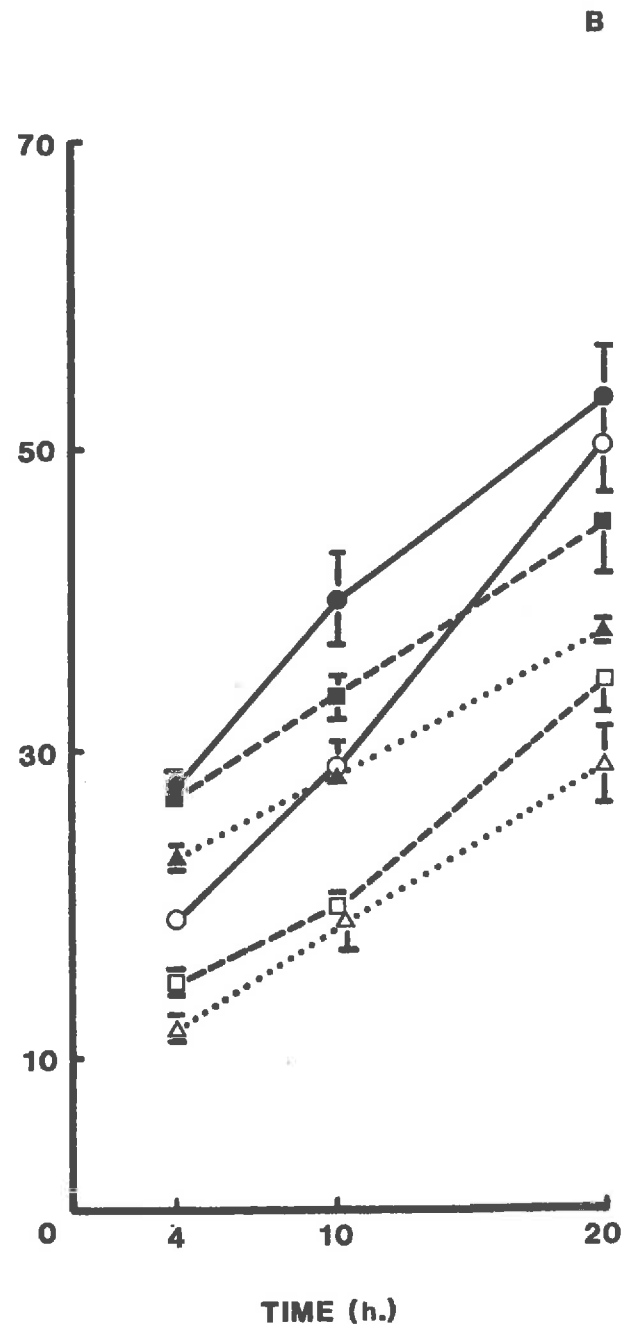
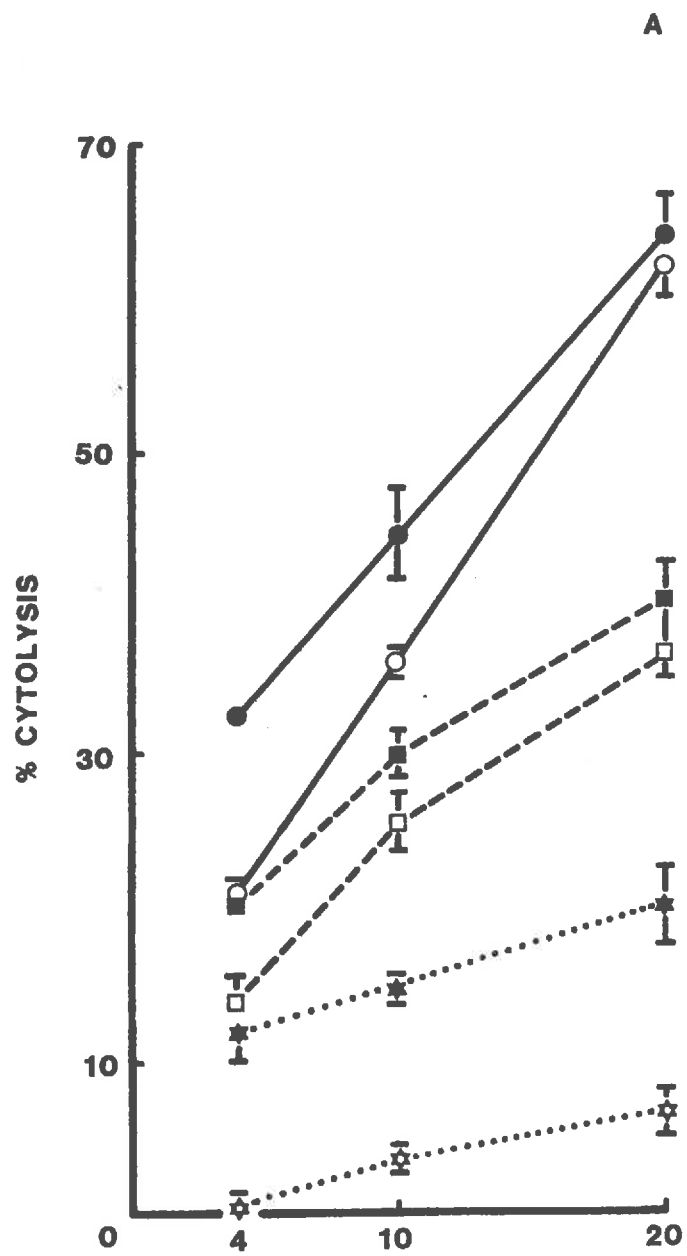
FIGURE 4.2

The effect of antibody on the kinetics of cytolysis of ^{51}Cr -P815 cells by PC from 11RX-immunized mice

PC were harvested from mice immunized with 11RX ip 7 days previously. They were tested against ^{51}Cr -P815 cells in a standard in vitro cytotoxicity assay. Their cytotoxic activity was compared to the in vitro cytotoxic activity of PC at different effector to target cells ratios, and to the in vitro cytotoxic activity of PC taken at different times after 11RX immunization.

A 2×10^{-3} dilution of CBA-anti-H-2^d antiserum was omitted from (open symbols) or added to (closed symbols) the culture medium. ^{51}Cr release was measured at 4-5 h, 10 h and 20 h after commencement of co-culture.

- A: The cytotoxic activity of different numbers of PC from day 7 11RX-immunized mice. 1×10^4 ^{51}Cr -P815 cells/well were co-cultured with the following numbers of PC: 10×10^5 (○ ●), 5×10^5 (□ ■), and 1.3×10^5 (☆ ★).
- B: The cytotoxic activity of PC from day 7 11RX-immunized mice cultured with different numbers of ^{51}Cr -P815 cells. 1×10^6 PC/well were cultured with the following numbers of ^{51}Cr -P815 cells: 1×10^4 (○ ●), 2×10^4 (□ ■), 4×10^4 (△ ▲).
- C: The cytotoxic activity of PC from normal mice (☆ ★) and those taken from mice 7 days (○ ●), 10 days (□ ■) and 13 days (△ ▲) after 11RX immunization.



containing antiserum. The greatest effect of antiserum was observed at the lowest effector to target cell ratio used, it caused a 3 fold increase in cytolysis at 20 h. The results in Fig 4.2B shows that antiserum enhanced the cytotoxic activity of PC when higher than usual numbers of target cells were used. Cytolysis was increased at all time points tested.

Because Abs could increase the level of cytolysis at 20 h when the experimental conditions used resulted in low in vitro cytotoxic activity of PC, experiments were carried out to determine whether antiserum could augment the cytotoxic activity of PC taken from immunized mice at times after they are maximally cytolytic i.e. after day 7 (Ashley and Kotlarski, 1982a). A standard in vitro cytotoxicity assay in the presence or absence of antiserum was carried out using PC from unimmunized mice or mice immunized ip with 11RX 7, 10 and 13 days previously. The data in Fig 4.2C show that PC from normal mice were not cytotoxic for ^{51}Cr -P815 cells in the presence of antiserum, and that antiserum did not increase the cytotoxic activity of day 10 and 13 PC to that of day 7 PC. In all cases, Ab caused a marked increase in cytolysis early in the assay (4 h and 10 h) but a difference at 20 h was seen only with 13 day PC, and this effect was only marginal at best.

In summary, antiserum can augment the level of cytolysis in a 20 h ^{51}Cr release assay when the experimental conditions are arranged such that the cytotoxic activity of the PC is low i.e. when high numbers of tumour cells or low numbers of effector cells are used or when the PC are taken from mice later than 7 days after 11RX immunization. As mentioned previously, this may occur because Ab enhances the contact between activated macrophages and tumour cells. Cell-binding experiments were conducted to determine whether this did occur in the experiments described above. The results from this experiment are presented in §4.8.3 and indicate that Ab does enhance binding.

4.5 Con-A activated splenic blast cells as targets for tumouricidal PC

As stated previously, it is generally accepted that only tumour cells are killed by activated macrophages although some investigators have detected killing of normal cells. Con A blast cells are one of the cell types which have reported to be susceptible to the cytolysis by activated macrophages (Lohmann-Matthes, Kolb, and Meerpohl, 1978). Therefore, it seemed worth re-examining the killing of blast cells by activated macrophages in the context of establishing whether susceptibility to lysis is a characteristic of tumour cells or all dividing cells.

Con A blast cells from CB6F₁ mice and P815 tumour cells were compared as targets in in vitro cytotoxicity assays using PC from short term ip immunized mice. The Con A blast cells were prepared by incubating spleen cells at 1×10^6 /ml with 1 μ g/ml of Con A in DMEM culture medium at 37° in 10% CO₂, 7% O₂ and 83% N₂ for 3 days. They were depleted of the few remaining erythrocytes, dead cells and small lymphocytes by centrifugation on a discontinuous density gradient consisting of a layer of 30% Percoll and one of 50% Percoll. The Con A blast cells, which banded at the interface of these two layers were harvested and washed as described in §2.22.2. Several experiments were conducted in which ⁵¹Cr-labelled Con A blast (⁵¹Cr-blast) cells were used as targets for PC in a standard in vitro cytotoxicity assay. The results of these experiments are summarized below.

4.5.1 Summary of experiments using ⁵¹Cr-blast cells as target cells

It was found that ⁵¹Cr-blast cells were not suitable targets because they were "fragile" in vitro. For example, the spontaneous release of label from ⁵¹Cr-blast cells in culture medium was much higher than that from ⁵¹Cr-P815 cells, namely as high as 60% at 20 h (data not shown). Interpretation of data was further complicated because it appeared that PC were providing the necessary conditions for

maintaining the viability of ^{51}Cr -blast cells in culture. When ^{51}Cr -blast cells were cultured with PC from normal and 11RX-immunized mice, the amount of label released over 20 h was 2-3 fold less than if the target cells were cultured in medium alone (data not shown). Possible indication of cytolysis of Con A blast cells by PC from 11RX-immunized mice was obtained. It was consistently found that at 20 h more label was released from ^{51}Cr -blast cells when they were cultured with 11RX-activated PC than with normal PC, although there was little if any difference at 4 h and 10 h (data not shown). The amount of label released was always much less than that obtained when ^{51}Cr -blast cells were cultured in medium alone, giving a negative value for % cytolysis when the usual calculation was made. When the calculation was made using the amount of ^{51}Cr released by blast cells in the presence of normal PC as spontaneous release, the % cytolysis of ^{51}Cr -blast cells in the presence of 11RX-activated PC was much less than the value obtained with ^{51}Cr -P815 cells and was in the range of 10-15% (data not shown). This appeared to confirm the findings of other workers which show that, although some non-tumourigenic cells can be killed by activated macrophages, they are much poorer targets than tumour cells (Lohmann-Matthes et al., 1978; Hamilton and Fishman, 1981)

4.5.2 Criticism of previous studies using Con A blast cells as target cells

A major criticism of the above experiment and those reported by others (Lohmann-Matthes et al., 1978; Hamilton and Fishman, 1981) where in vitro cytotoxicity assays are carried out using Con A blast cells as targets is that no steps were taken to ensure that the Con A blast cells would continue to divide during the period of the assays. It is now well established that Con A blast cells require the presence of the lymphokine IL2 to maintain cellular division for even relatively short periods of in vitro culture. In its absence, Con A blast cells cease to

proliferate, revert to the appearance of small resting lymphocytes and eventually die (Smith, 1980; Lafferty et al., 1980b). That the Con A blast cells did revert was confirmed in one of the experiments reported above. This was done by visually, using an inverted microscope. It is possible, therefore, that Con A blast cells were poor targets for activated macrophages because in co-culture with PC they reverted to non-dividing cells which lacked the structures required for interaction with activated macrophages. Alternatively, it could also be argued that Con A blast cells are totally resistant to lysis by activated macrophages but appear to be lysed by tumouricidal PC because, in the absence of IL2, they become unhealthy and "fragile". Accordingly, to distinguish between these two possibilities the above experiment were repeated in the presence of IL2. The source of IL2 was a Con A-stimulated spleen cell culture supernatant (CS) which had been concentrated 10-fold by ultrafiltration (Lafferty et al., 1980a) as described in §2.32. No attempt was made to purify IL2. In the experiments reported below, IL2 was present in the tissue culture medium as a 10^{-2} dilution of CS, a concentration which maintained proliferation of Con A blast cells for at least 24 h.

4.5.3 ^{125}I -blast cells are not lysed by PC from 11RX-immunized mice

Before proceeding with the studies outlined above, a preliminary experiment was conducted to determine whether ^{125}I -UdR- and ^3H -TdR labelled P815 cells were suitable as target cells in a standard in vitro cytotoxicity assay i.e. to establish whether these nuclear labels are released with the same kinetics as ^{51}Cr in the model system used in the present studies. The reason for doing this was that it seemed likely that the spontaneous release of radioactivity from ^{125}I -labelled Con A blast cells would be much lower than the release obtained from ^{51}Cr -labelled ones, making them more suitable as targets. The results of this experiment are given in Chapter 6, (Table 6.10) and indicate that

^{125}I -UdR and ^3H -TdR could be used to label P815 cells for use in a 20 h in vitro cytotoxicity assay because, in the presence of PC from short-term 11RX-immunized mice, radioactivity is released from ^{125}I -P815 and ^3H -P815 cells with similar kinetics to ^{51}Cr ^{51}Cr release from ^{51}Cr -P815 cells.

Consequently, Con A blast cells from CB6F_1 mice were labelled with ^{125}I -UdR by the method described in §2.24.1 (ii) and were used as targets in the presence of IL2 in a standard in vitro cytotoxicity assay. The effector cells were PC from short-term immunised mice, and the assays were set up in duplicate because sampling was done at 10 h and 20 h. As predicted, the results indicate that ^{125}I is a more suitable label for Con A blast cells than ^{51}Cr . The spontaneous release of ^{125}I was approximately 1%/hour. This was not markedly affected by the addition of normal PC. Furthermore, no evidence of lysis of the Con A blast cells by PC from 11RX-immunized mice was obtained. When compared to the spontaneous release of ^{125}I from Con A blast cells cultured in medium alone, the cytotoxicity was calculated to be 2.2% and 3.2% at 10 and 20 hours in the presence of tumouricidal PC and 3.3% and 2.1% at 10 and 20 hours in the presence of normal PC. These results clearly establish that Con A blast cells are not killed by activated macrophages when IL2 is present in the culture medium and support the notion that the cytolysis of Con A blast cells by activated macrophages detected by others may have been due to the fragile nature of the target cells which were labelled with an inappropriate marker (Lohmann-Matthes et al., 1978) or were cultured in the absence of IL2 (Lohmann-Matthes et al., 1978; Hamilton and Fishman, 1981).

4.6 The inhibition of cytolysis by unlabelled tumour cells

Because the killing of tumour cells by activated macrophages is non-specific and requires contact, it could be argued that the interaction between the two cell types is mediated by surface structures

common to all tumour cells. If this were the case, then addition of unlabelled tumour cells should reduce the number of labelled tumour cells that are killed by activated macrophages, due to competitive binding to effector cells. This was shown in cold-target inhibition experiments which were performed in the following way. Two-fold serial dilutions of the unlabelled cells used for inhibition were prepared in quadruplicate in multiwell tissue culture trays in a volume of 0.1 ml. To each of these wells a 0.1 ml volume of a cell suspension containing PC at 1×10^7 cells/ml and ^{51}Cr -tumour cells at 1×10^5 /ml was added. Since the additional cells used for inhibition could contribute significantly to the depletion of nutrients and possibly affect the spontaneous release of ^{51}Cr simply due to metabolic effects, parallel cultures were set up in which the labelled tumour cells without PC were incubated with the additional cells at the same concentrations as those used to inhibit cytolysis. The amount of ^{51}Cr released from tumour cells in the presence of the unlabelled cells alone was the "spontaneous release" value used for calculating the cytolysis of tumour cells by PC in the presence of the cells used for inhibition. Cytolysis was measured at 20 h and the results from 3 separate experiments are shown in Fig 4.3.

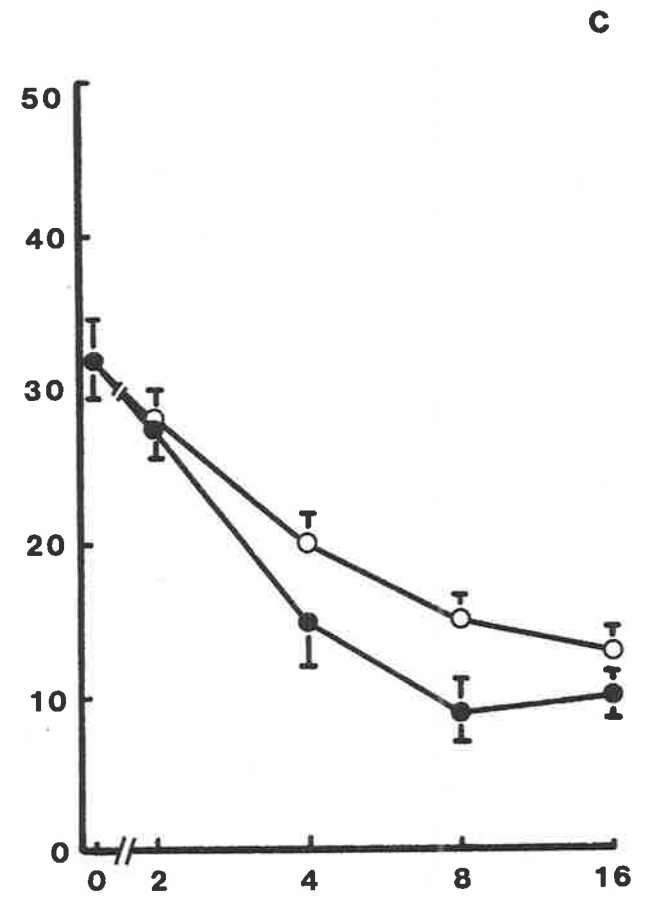
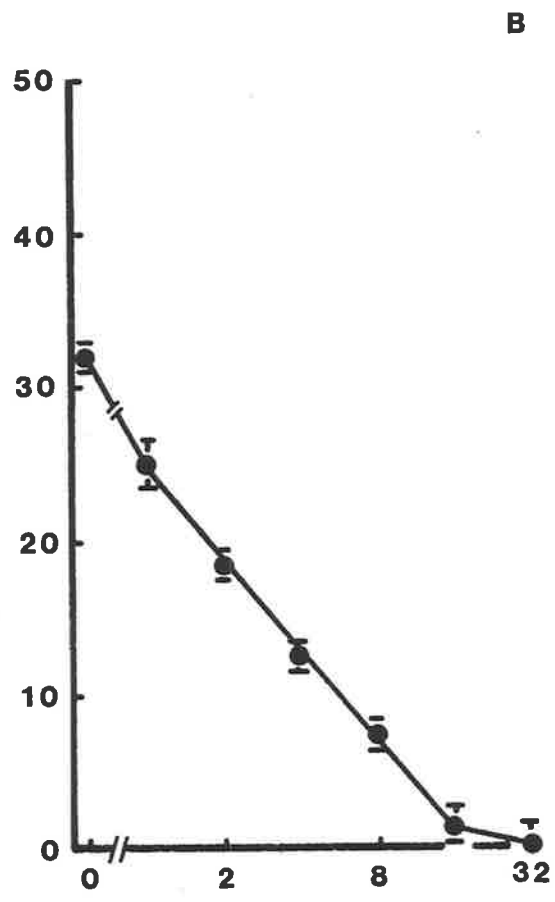
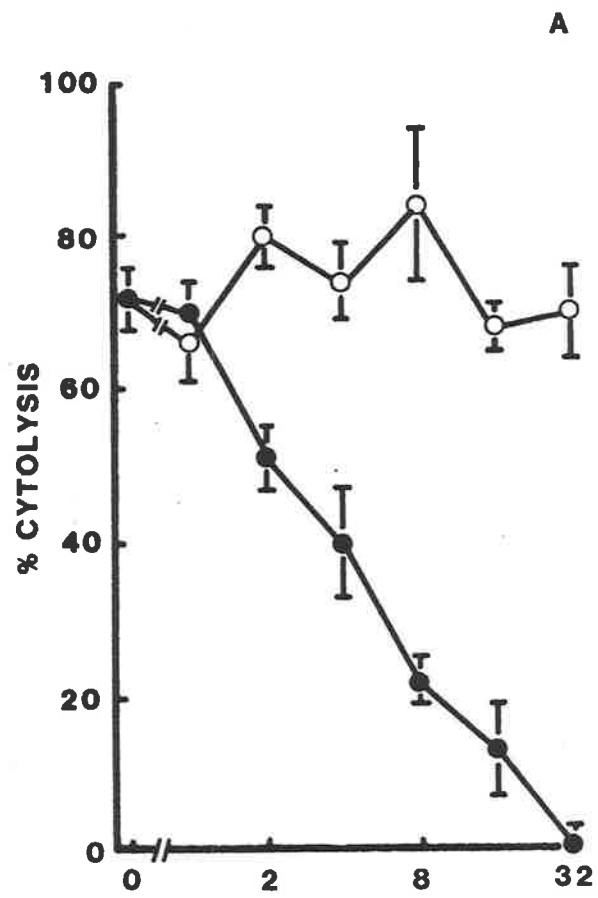
The effect of adding unlabelled P815 cells or normal PC on the lysis of ^{51}Cr -P815 cells is shown in Fig 4.3A whilst the effect of unlabelled EAT cells on the lysis of ^{51}Cr -EAT targets was investigated in a second experiment and the data are shown in Fig 4.3B. The in vitro killing of both types of tumour cells was inhibited by unlabelled tumour cells in a competitive manner. Addition of as few as two unlabelled tumour cells for every ^{51}Cr -labelled tumour cell resulted in a marked reduction of killing of both tumours. Normal peritoneal cells present at a level as high as 32 times that of the labelled targets did not significantly inhibit the killing of ^{51}Cr -P815 suggesting that the inhibition observed was not simply due to steric hindrance by unlabelled target cells.

FIGURE 4.3

Cold target inhibition of in vitro PC-mediated cytotoxicity
by unlabelled homologous and heterologous tumour cells

- A: Inhibition of PC-mediated lysis of ^{51}Cr -P815 cells using unlabelled P815 cells (●) or normal PC (○).
- B: Inhibition of PC-mediated lysis of ^{51}Cr -EAT cells using unlabelled EAT cells.
- C: Inhibition of PC-mediated lysis of ^{51}Cr -P815 cells using unlabelled P815 cells (○) or EAT cells (●).

PC from 11RX-immunized mice were co-cultured with 10^4 ^{51}Cr -P815 or ^{51}Cr -EAT cells in the presence of various numbers of target cells. In panels A and B the PC were from day 7 and day 6 11RX-immunized mice and were tested at an effector to target cell ratio of 100:1, whilst in panel C they were from day 8 11RX-immunized mice and were tested at an effector to target cell ratio of 50:1. Cytotoxicity was measured at 20 h.



As predicted, the inhibition by cold tumour cells was non-specific because unlabelled EAT or unlabelled P815 cells could inhibit lysis of ^{51}Cr -P815 cells equally well (Fig 4.3C). The experimental design used to demonstrate lack of specificity differed slightly from that used for other inhibition experiments. The effector to target cell ratio used was 50:1 and a smaller range of unlabelled tumour cells used. Similarly, killing of ^{51}Cr -EAT cells by PC from 11RX-immunized mice could be inhibited equally well by unlabelled P815 cells or unlabelled EAT cells (Kotlarski and Andrus, personal communication).

4.7 Con A activated-blast cells as cold target inhibitors of cytolysis

Although it was found that Con A blast cells were not lysed by activated macrophages, the experiments in §4.5 did not eliminate the possibility that Con A blast cells could be binding to macrophages via determinants similar to those present on tumour cells, but were not lysed because they are resistant to the cytotoxic mechanism(s) which kill tumour cells. If this were true, Con A blast cells should inhibit the lysis of tumour cells by activated macrophages. To test this possibility experiments were carried out to compare Con A blast cells with unlabelled P815 cells for their ability to competitively inhibit the lysis of ^{51}Cr -P815 and ^{51}Cr -EAT cells by PC in vitro. The experimental design used was the same as that described for previous inhibition experiments with the exception that CS was added to the culture medium as a source of IL2 when Con A blast cells were used as inhibitor cells.

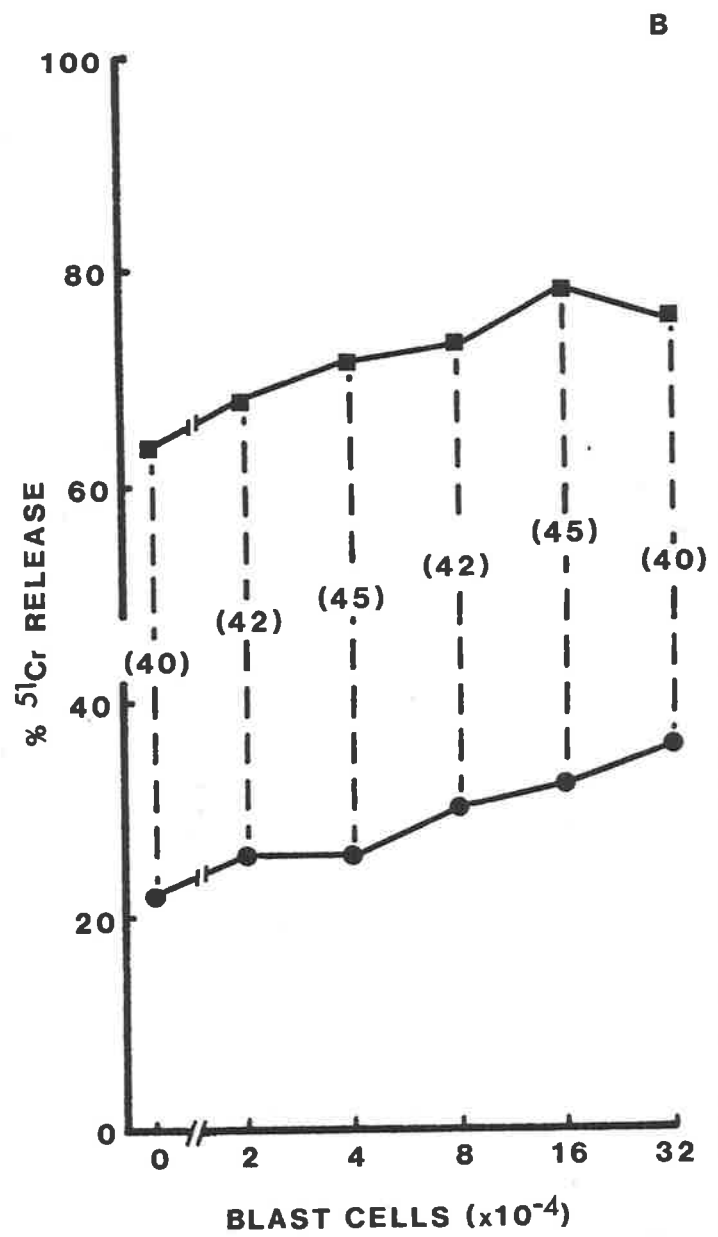
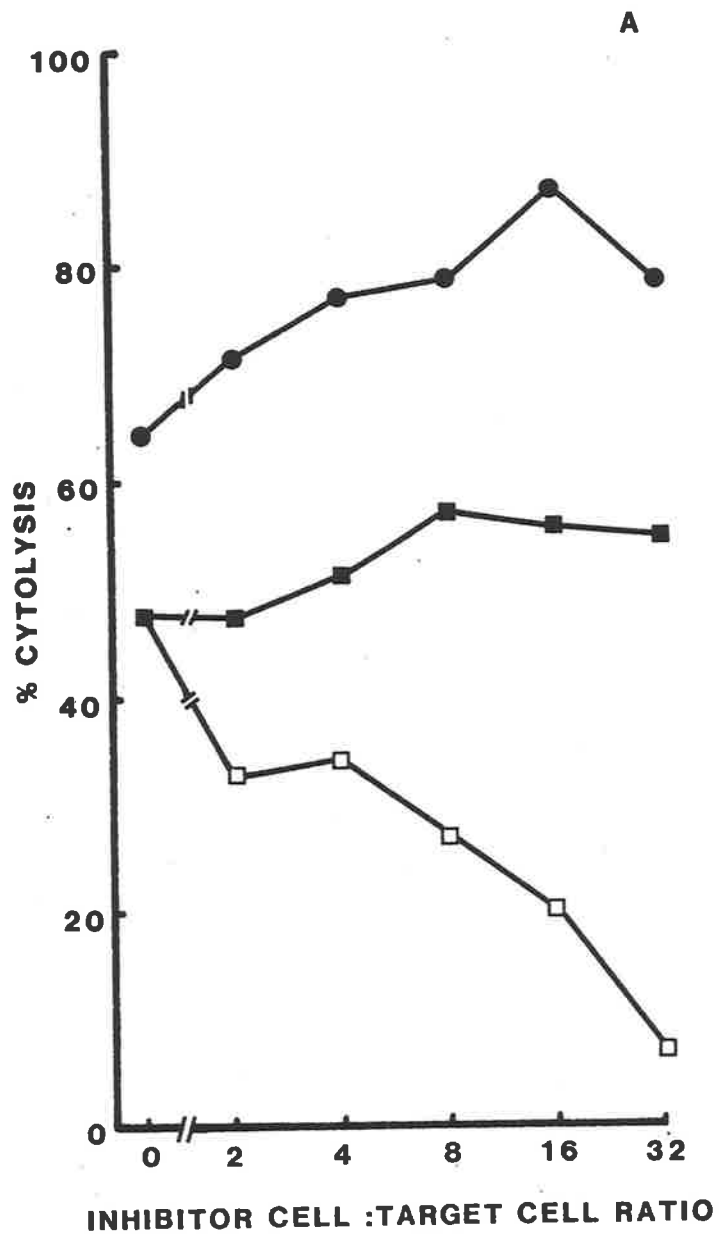
4.7.1 Con A blast cells do not inhibit the lysis of ^{51}Cr -P815 cells

The results in Fig 4.4A confirmed those of previous experiments showing that unlabelled P815 cells competitively inhibit the lysis of ^{51}Cr -P815. In the absence of IL2, Con A blast cells did not inhibit cytolysis and, in fact, caused some increase in cytotoxicity when 8×10^4 or more Con A blast cells were added/well. When CS was added

FIGURE 4.4

Comparison of the ability of Con A-activated blast cells and tumour cells to competitively
inhibit the PC-mediated lysis of ^{51}Cr -P815 cells

- A: Cytolysis of ^{51}Cr -P815 cells in the presence of unlabelled P815 cells (\square), blast cells (\blacksquare), or blast cells in the presence of IL2 (\bullet).
- B: The amount of ^{51}Cr released from ^{51}Cr -P815 cells cultured in the presence of IL2 and different numbers of blast cells with (\blacksquare), or without (\bullet) 10^6 PC.



to the tissue culture medium, the in vitro cytotoxic activity of the PC was markedly increased. This suggested that either a lymphokine activity such as MAF was also present in CS (data not shown) or that the blast cells were cytotoxic for tumour cells, especially when their proliferation is maintained by IL2. Even in the presence of IL2 Con A blast cells did not inhibit the lysis of P815 target cells. Cytolysis again increased in a dose dependent fashion. In Fig 4.4B the data are presented as the total % ^{51}Cr released by ^{51}Cr -P815 cells when cultured with Con A blast cells only or with a mixture of Con A blast cells and PC. The two curves are parallel and at all Con A blast cell concentrations used, the PC from short term-immunized mice caused the release of an additional 40%-45% of the incorporated label from ^{51}Cr -P815 cells.

4.7.2 The lysis of ^{51}Cr -P815 cells by Con A blast cells is due to cytotoxic cells

To determine whether the increased cytolysis of ^{51}Cr -P815 cells in the presence of Con A blast cells was due to medium depletion or cytotoxic cells, ^{51}Cr -P815 cells and Con A blast cells in the absence of IL2 were cultured together or separated by a 0.22 μm Sartorius membrane. When 3.2×10^5 Con A blast cells and 10^4 ^{51}Cr -P815 cells were co-cultured on top of the membranes the release of label was $39.0 \pm 1.2\%$ at 20 h. This was reduced to $28.0 \pm 0.8\%$ when the two cell populations were separated by the membranes. The release of label from ^{51}Cr -P815 cells cultured alone on top of the membrane was $21.4 \pm 0.3\%$. Since the increase in ^{51}Cr background release was not as great when the Con A blast cells and ^{51}Cr P815 cells were separated by a membrane, it was concluded that the release of ^{51}Cr was mainly due to the presence of cytotoxic cells in the Con A blast cell population. This is consistent with findings reported frequently by others (Bevan and Cohn, 1975). Collectively, these results imply that under these experimental conditions the cytotoxic activities of the PC and Con A blast cells were

expressed independently and were additive. These observations tend to suggest the possibility that the increased cytotoxicity observed in the presence of IL2 may be due to cytotoxic blast cells.

4.7.3 Con A blast cells do not inhibit lysis of ^{51}Cr -EAT cells

The previous experiment was repeated using ^{51}Cr -EAT cells as well as ^{51}Cr -P815 cells as targets. EAT cells were used as targets in an attempt to avoid the complication observed in the experiments above where P815 cells were lysed by the Con A blast cells. The reasoning behind this approach was that since EAT cells can grow in any strain of mouse, they probably lack detectable surface MHC antigens necessary for allograft rejection. Thus it seemed likely that they would not be lysed by the Con A blast cells. The data presented in Table 4.3 show that, as predicted, ^{51}Cr -EAT cells were not lysed by the Con A blast cells whilst ^{51}Cr -P815 cells were. Even in the presence of IL2, Con A blast cells did not inhibit the lysis of EAT cells.

4.8. The ability of activated macrophages from 11RX-immunized mice to bind tumour cells

The lack of cold target inhibition by Con A blast cells suggested that Con A blast cells did not interact with activated macrophages. Alternatively, it was possible that inhibition did not occur because, although the Con A blast cells were bound by activated macrophages, they did not compete for the cytolytic mechanism. To distinguish between these two possibilities experiments were carried out to assay the ability of activated macrophages to bind to various cell types.

The cell binding experiments were carried out as follows. Macrophage monolayers from PC obtained from 11RX-immunized mice were prepared in quadruplicate in multiwell tissue culture trays as described in §2.29. There was experiment-to-experiment variation in the density of the adherent cell monolayers formed with a given number of PC.

TABLE 4.3

The effect of blast cells on the cytolysis of ^{51}Cr -P815 cells
and ^{51}Cr -EAT cells in the presence of IL2^(a)

Blast cells per well ($\times 10^{-4}$)	^{51}Cr -EAT cells		^{51}Cr -P815 cells	
	% ^{51}Cr release in absence of PC ^(#)	% cytolysis ^(c) (mean \pm s.e.m)	% ^{51}Cr release in absence of PC ^(#)	% cytolysis ^(c) (mean \pm s.e.m)
0	27.2 \pm 1.1	31.3 \pm 2.4	49.2 \pm 2.3	91.2 \pm 2.4
2	27.4 \pm 0.4	ND	58.8 \pm 1.6	ND*
8	28.3 \pm 0.7	30.0 \pm 0.5	61.4 \pm 1.1	82.7 \pm 5.2
32	27.9 \pm 1.1	27.6 \pm 0.6	69.0 \pm 1.0	87.1 \pm 7.9

(a) Mixtures of 10^4 ^{51}Cr -P815 cells or ^{51}Cr -EAT cells and various numbers of blast cells were cultured in the presence of IL2 with or without 10^6 PC from mice immunized with 11RX 8 days previously.

(#) The background ^{51}Cr release from labelled tumour cells when cultured only with blast cells.

(c) The cytolysis of labelled tumour cells by PC in the presence of blast cells.

(*) Not determined.

^{51}Cr -labelled target cells were added to the wells and sedimented onto the adherent cell monolayers by gently centrifuging the trays at $20 \times g$ for 10 min. Following incubation for 30 min at 37° , most of the unbound tumour cells were removed by vortexing the tray on a tray shaker and aspirating the culture fluid from the wells. To ensure virtually complete removal of unbound cells, the monolayers were washed by adding fresh warm medium and vortexing the trays again before removing the fluid. This procedure was repeated twice. After washing, a 0.4% solution of Triton-X100 in HBSS was added to the wells to lyse the bound target cells. The amount of radioactivity released into the medium was measured and the binding of the target cells was expressed as a percentage of the counts added to the wells at the beginning of the experiment. The first set of experiments reported below were designed to characterize the stoichiometry of ^{51}Cr -P815 tumour cell binding to activated macrophages.

4.8.1 The effect of varying the concentration of P815 cells on binding to activated macrophage monolayers

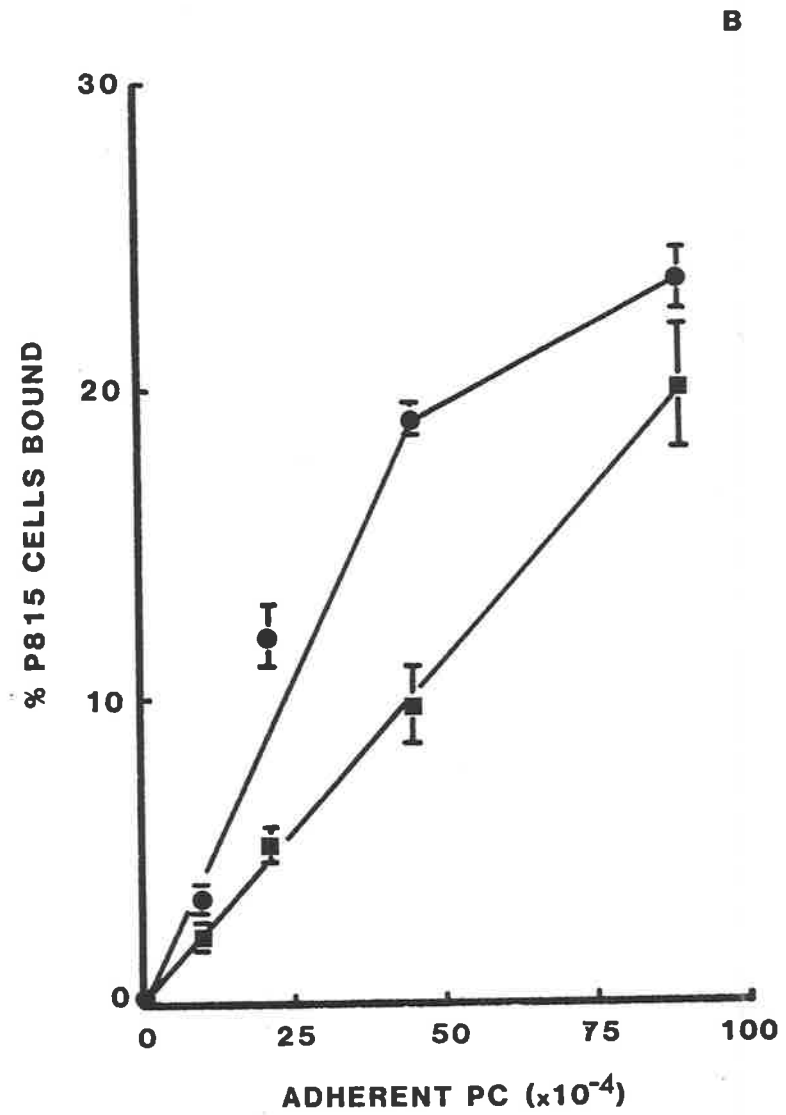
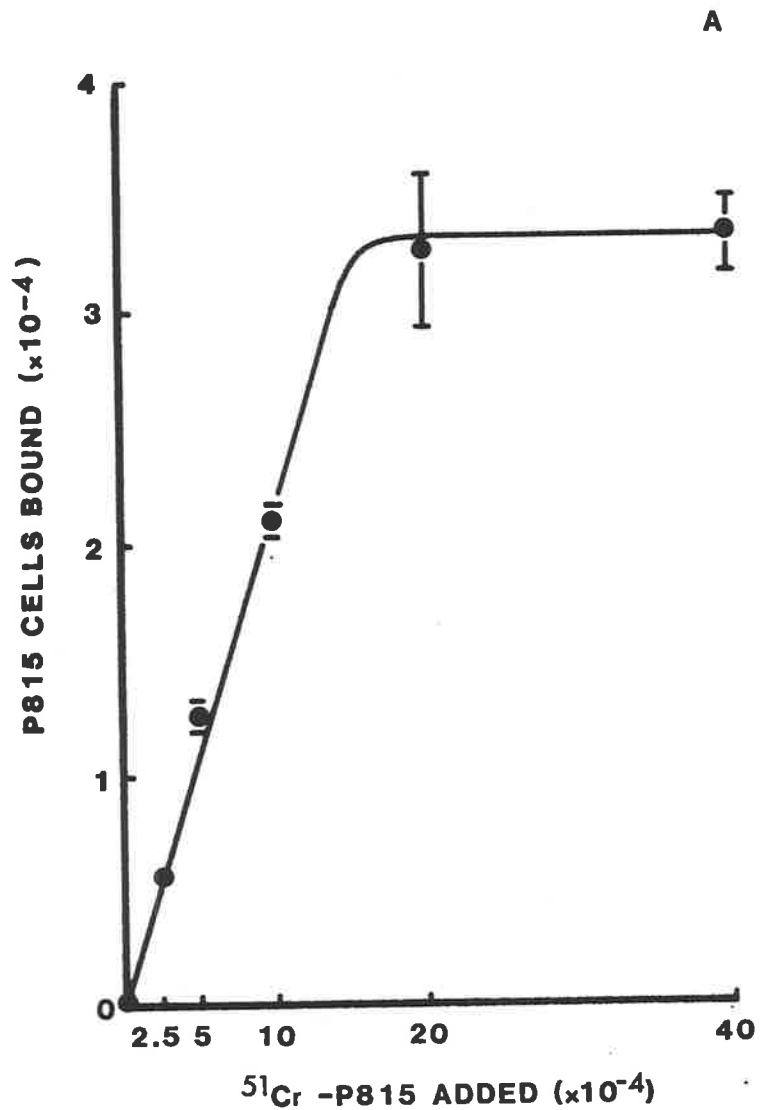
The experiments were done using monolayers of activated macrophages which were prepared using 10^6 PC/well, and adding various concentrations of ^{51}Cr -P815 cells to them. A typical results is shown in Fig 4.5A. The number of cells bound increased linearly with the number of P815 added up to a concentration of 2×10^5 P815 cells/well at which point a plateau was reached. Visual examination showed that 2×10^5 P815 cells formed a complete monolayer in 6 mm diameter wells, providing an obvious explanation for the plateau effect observed. The percentage binding was constant (approximately 20% in this experiment) and maximal in the linear part of the curve i.e. at ^{51}Cr -P815 concentrations below 2×10^5 cells/well.

FIGURE 4.5

The stoichiometry of binding of ^{51}Cr -P815 cells to activated macrophages from 11RX-immunized mice

- A: The effect of varying the numbers of ^{51}Cr -P815 cells on their binding to 6.4×10^5 adherent PC.
- B: The effect of varying the numbers of adherent PC on the binding of 5×10^4 (■) and 2×10^5 (●) ^{51}Cr -P815 cells.

The macrophage monolayers were prepared with PC from day 6 11RX-immunized mice and the results are expressed as the mean \pm s.e.m of the numbers of ^{51}Cr -P815 cells bound in 4 replicate wells.



4.8.2 The effect of varying the number of adherent cells on the binding of P815 cells

The effect of varying the number of macrophages on the binding of a constant number of ^{51}Cr -P815 cells was also examined in a series of experiments. One of these is presented below. Monolayers of activated macrophages were prepared from two-fold serial dilutions of PC, starting with a concentration of 10^6 cells/well and ^{51}Cr -P815 cells were added at a concentration of 2×10^5 or 5×10^4 /well. The binding assay were carried out as described above. The data in Fig 4.5B show that the percentage of tumour cells bound increased in a linear fashion as the number of macrophages were increased and that the slope of the binding curve was steeper when the higher number of target cells was added to the wells. However, when using the higher number of ^{51}Cr -P815 cells, the slope of the binding curve decreased when the density of the adherent cell monolayer was increased beyond a certain concentration. This suggests that at this concentration of adherent cells the number of binding sites on the macrophages for tumour cells were almost sufficient to bind the maximal number of ^{51}Cr -P815 cells from a dose of 2×10^5 ^{51}Cr -P815 cells. Regardless of the number of ^{51}Cr -P815 cells used, a similar proportion was bound by the densest macrophage monolayer.

The results from these two experiments showed that the adherent PC from short term immunized mice are capable of binding tumour cells and that, within certain limits, the binding is directly proportional to the number of P815 cells and adherent PC used.

4.8.3 The effect of antibody on the binding of P815 cells to activated macrophages

The experiments in §4.4 showed that the presence of alloantibodies enhanced the cytolysis of ^{51}Cr -P815 cells, especially at low effector to target cell ratios. To establish whether the mechanism involved an increase in the binding of ^{51}Cr -P815 cells to activated macrophages,

1×10^4 ^{51}Cr -P815 cells were allowed to bind to activated macrophage monolayers of different densities in the presence or absence of Abs specific for the tumour cells. The results in Table 4.4 show that Ab increased the binding of tumour cells and that it was more pronounced as the concentration of adherent PC decreased. Five and a half times more target cells were bound by the least dense monolayer when Ab was present; at the highest concentration of adherent cells the increase was only 2-fold.

4.8.4 Binding of P815 cells to normal macrophages and to macrophages obtained at different times after immunization

To determine whether the ability to bind ^{51}Cr -P815 cells correlated directly with the cytotoxic activity of PC, binding experiments were carried out using PC suspensions of differing cytotoxic activity. The results from 2 experiments are presented below.

Experiment 1: Confluent macrophage monolayers were prepared from mice immunized seven days previously with 11RX i.p. and from age and sex matched unimmunized control mice. Since normal macrophages spread less than activated ones, the monolayers were prepared from twice as many PC/well as those prepared from 11RX-immunized mice. 2×10^5 ^{51}Cr -P815 cells were added to the monolayers and the binding assay was carried out as described above. Monolayers from immunized mice bound P815 to a much greater extent than those from unimmunized mice. 8.0×10^4 P815 cells were bound by 6.9×10^5 activated macrophages whilst 2.1×10^4 P815 cells were bound by 1.7×10^6 normal macrophages. This is a 9.5-fold difference in the tumour binding capacity of the two cells forming the monolayers.

Experiment 2: Fig 4.6 shows the binding of 2×10^5 ^{51}Cr -P815 cells to macrophage monolayers prepared from PC of normal mice and those from PC obtained at 3 different times after 11RX immunization. The macrophages from all four groups of mice were tested at 3 different cell

TABLE 4.4

The effect of antibody on the binding of ^{51}Cr -P815 cells
to monolayers of 11RX-activated macrophages[@]

Numbers of PC used to form monolayer ($\times 10^{-5}$)	% ^{51}Cr -P815 cells bound (mean \pm s.e.m)		
	without Ab	with Ab	Ratio*
1.3	1.2 \pm 0.2	6.6 \pm 0.6	5.5
2.5	3.6 \pm 0.2	13.1 \pm 1.2	3.6
5.0	8.3 \pm 1.2	21.9 \pm 1.2	2.6
10.0	21.0 \pm 1.4	39.5 \pm 1.1	1.9

(@) 10^4 ^{51}Cr -P815 cells were allowed to bind to macrophage monolayers prepared using various numbers of PC obtained from mice immunized ip with 11RX 7 days previously. CBA anti-H-2^d antiserum was present in some wells at a final dilution of 2×10^{-3} .

(*) The ratio of counts bound to monolayers of a given density in the presence and absence of Ab.

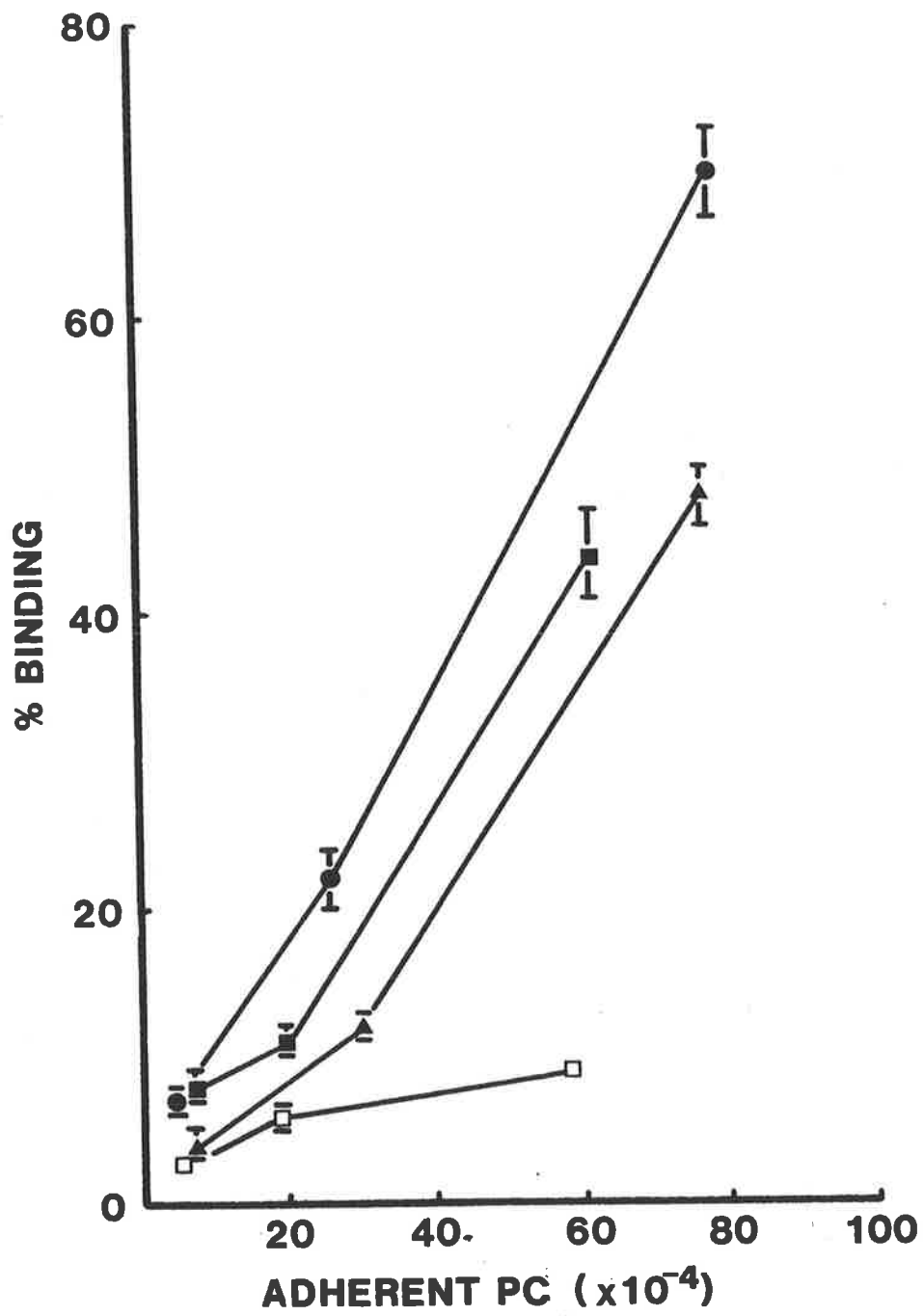
FIGURE 4.6

The binding of ^{51}Cr -P815 cells to macrophages
at different times after 11RX-immunization

Macrophage monolayers were prepared with PC harvested from normal mice (\square), and mice immunized with 11RX ip 6 days (\bullet), 11 days (\blacktriangle) and 40 days (\blacksquare) previously. Approximately 58%, 78%, 76% and 61% (respectively) of the PC adhered to the wells. 2×10^5 ^{51}Cr -P815 cells/well were used to compare the binding ability of the various monolayers.

The cytolytic activity of the various PC populations against ^{51}Cr -P815 cells at 10 h in a standard in vitro cytotoxicity assay was:

normal PC	$-1.4 \pm 1.1\%$
day 6 after 11RX	$47.2 \pm 0.9\%$
day 11 after 11RX	$22.6 \pm 2.1\%$
day 40 after 11RX	$7.1 \pm 1.4\%$



densities by preparing adherent cell monolayers from 1×10^5 , 3.3×10^5 and 10^6 PC/well. Although the in vitro cytotoxic activity of PC declined by day 40 to near that of normal PC (please see legend to Fig 4.6 for data), the ability of the macrophage monolayers to bind ^{51}Cr -P815 cells was not reduced to the same extent.

Thus, whilst binding appears to be necessary for tumour lysis, there is no direct correlation between the cytotoxic activity of macrophages and their ability to bind tumour cells. The necessity for tumour cells to be closely associated with cytotoxic PC for lysis to occur is consistent with the observation in the above experiments that ^{51}Cr -P815 cells bind to activated macrophages and that normal macrophages, which are not cytotoxic, bind them to a much lesser extent. Such observations are in keeping with those reported by Marino and Adams (1980).

4.9 The lysis of tumour cells bound to adherent cytotoxic PC

It is possible that the proportion of the tumour cells which bind to monolayers and are lysed may reflect the proportion of cytotoxic macrophages in that population. Assuming that the ratio of target cells bound by cytotoxic macrophages and non-cytotoxic macrophages remained the same regardless of the adherent cell density, then, over a range of macrophage densities the lysis of the bound tumour cells would be expected to remain constant. Similarly, in a situation where the concentration of the target cells was varied and that of the effector cells kept constant, the proportion of the bound tumour cells which would be lysed should also remain unchanged. It follows that the decrease in cytolysis normally detected when the numbers of PC used for in vitro cytotoxicity assays are reduced would not occur if, at the beginning of the assay, target cells were allowed to bind to macrophages and any unbound cells were then removed before any killing has occurred. To test this prediction the following experiments were carried out.

4.9.1 Experimental design

In the first experiment macrophage monolayers at two different cell densities were used and the number of ^{51}Cr -P815 cells added to them was varied, whilst in the second experiment the concentration of adherent PC was varied and the concentration of ^{51}Cr -P815 cells was held constant. The experimental design which is described below was used for both experiments. Macrophage monolayers of different densities were prepared in the usual way and various known numbers of ^{51}Cr -P815 cells were allowed to bind. After removing the unbound tumour cells, 0.2 ml of fresh tissue culture medium was added to the wells and the trays were incubated at 37° for 20 h. Subsequent to removing a 0.1 ml samples from each well to measure ^{51}Cr release, 0.1 ml of 0.4% Triton X-100 was added to each well to solubilize the intact cells and release all the ^{51}Cr so that the radioactivity bound to each macrophage monolayer at the beginning of the experiment could be determined.

To calculate the % cytolysis of the bound tumour cells, it was necessary to estimate the spontaneous release of ^{51}Cr obtained if the tumour cells bound to activated macrophages had been cultured in medium alone. To do this, ^{51}Cr -P815 cells were cultured at concentrations ranging from 800 cells to 2.5×10^4 cells/well in medium alone. It was found that the spontaneous release at 20 h increased as fewer tumour cells were present in the wells and ranged from 23.5% at the highest concentration to 32% at the lowest concentration of ^{51}Cr -P815 cells. The spontaneous release of ^{51}Cr for each of the doses of ^{51}Cr -P815 cells bound to macrophage monolayers was estimated from the results of this experiment (data not shown).

4.9.2 The effect of varying the number of ^{51}Cr -P815 cells

Fig 4.7 shows the lysis of bound tumour cells when the number of ^{51}Cr -P815 cells added to the wells was varied from 6×10^3 to 1×10^5 . The percentage of tumour cells binding to macrophages was

FIGURE 4.7

The lysis of ^{51}Cr -P815 cells bound to monolayers of activated macrophages

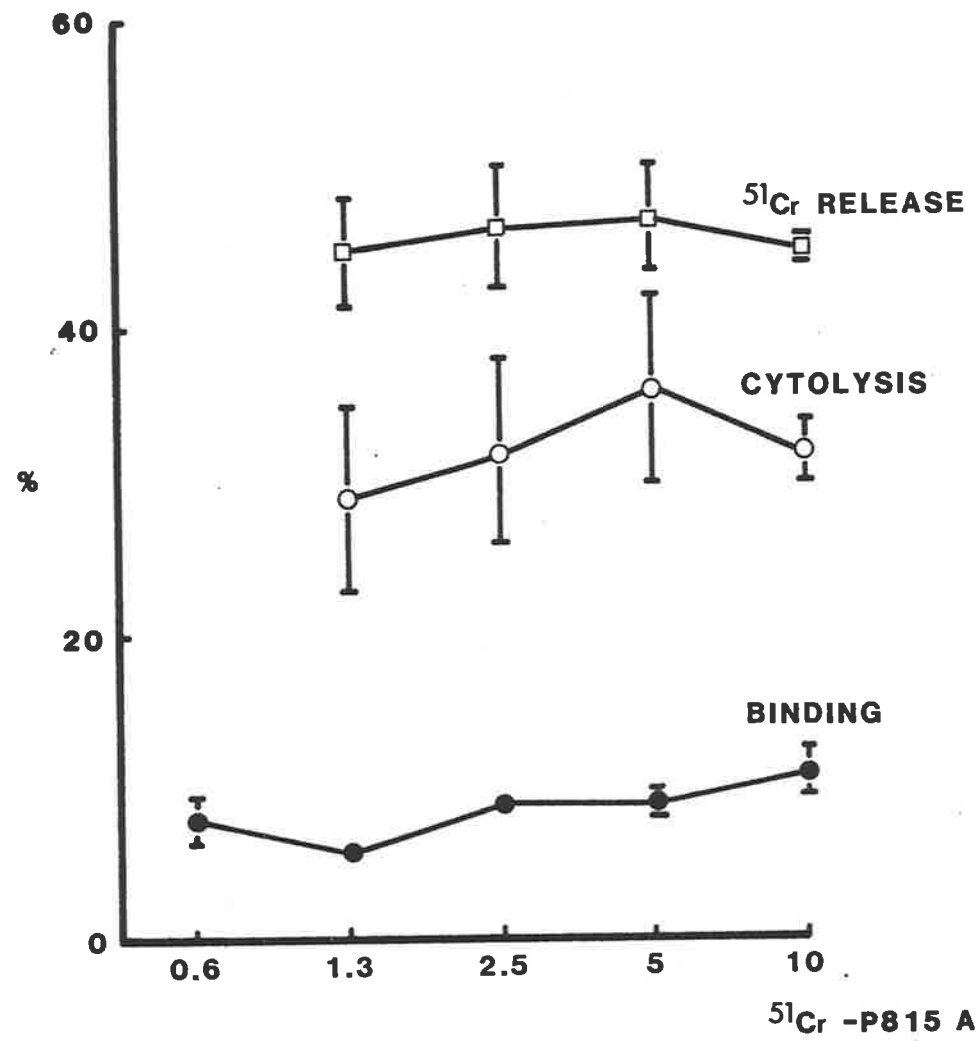
I: The effect of varying tumour cell concentration

Various numbers of ^{51}Cr -P815 cells were allowed to bind to monolayers of of 2.2×10^5 or 7.2×10^5 adherent PC from mice immunized 8 days previously with 10^5 11RX ip. After 30 min at 37° , unbound ^{51}Cr -P815 cells were removed, fresh medium was added to the wells and the trays were incubated for 20 h at 37° .

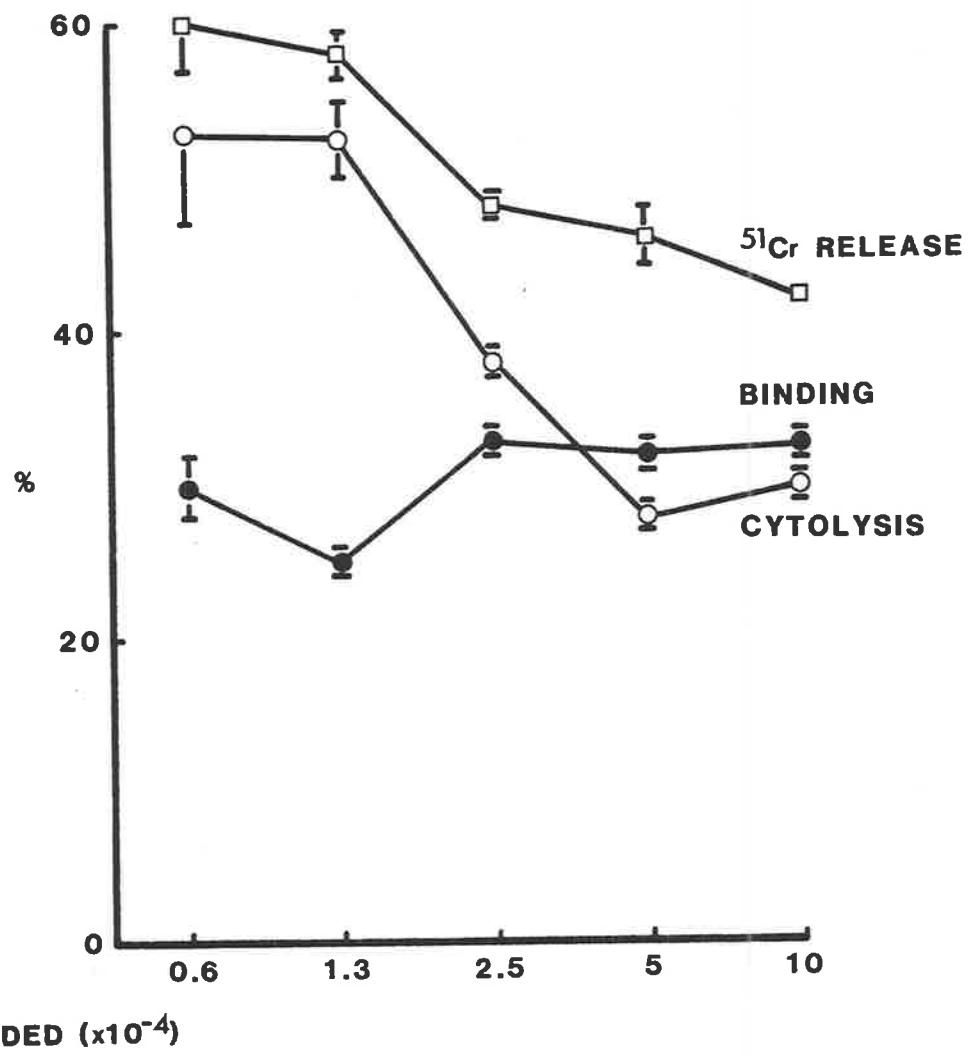
The % target cells bound, the % ^{51}Cr release from them and % cytolysis were determined as described in the text. The results are expressed as the mean \pm s.e.m from four replicate wells.

ADHERENT PC PER WELL:

2.2×10^3



7.2×10^5



directly related to the density of macrophages and was constant over the whole range of doses of target cells used. The % cytolysis of the tumour cells bound to 2.2×10^5 adherent cells was also constant at approximately 32%. However, when monolayers of the higher macrophage density (7.2×10^5 /well) were used there was a difference in the % cytolysis which occurred. The cytotoxicity was higher with small numbers of bound ^{51}Cr -P815 cells. As the number of bound ^{51}Cr -P815 cells increased, the % cytolysis decreased to the level obtained with the less dense macrophage monolayers.

4.9.3 The effect of varying the number of macrophages

The relationship between tumour cell binding and lysis when the density of the macrophage monolayers is varied over a 4-fold range is shown in Fig 4.8. As in previous experiments, the proportion of cells bound was related to the number of adherent cells in the wells. Over a 4-fold range of effector cells the cytolysis of target cells bound when 1×10^4 ^{51}Cr -P815 cells were added to the monolayers was constant. However, when 1×10^5 ^{51}Cr -P815 cells were allowed to bind to the adherent cell monolayers, the % ^{51}Cr release from and consequently the % cytolysis of the bound tumour cells varied. Cytotoxicity increased with the density of the macrophage monolayers, up to a concentration of 3.7×10^5 adherent PC and then declined.

It is clear that the prediction made above appears to hold only for a limited range of adherent cell and target cell doses. The appropriate conditions were obtained when various numbers of ^{51}Cr -P815 cells are added to macrophage monolayers of low density, or, when the density of the adherent cells is varied and small numbers of ^{51}Cr -P815 cells are allowed to bind. The causes for these limitations were not investigated.

FIGURE 4.8

The lysis of ^{51}Cr -P815 cells bound to monolayers of activated macrophages

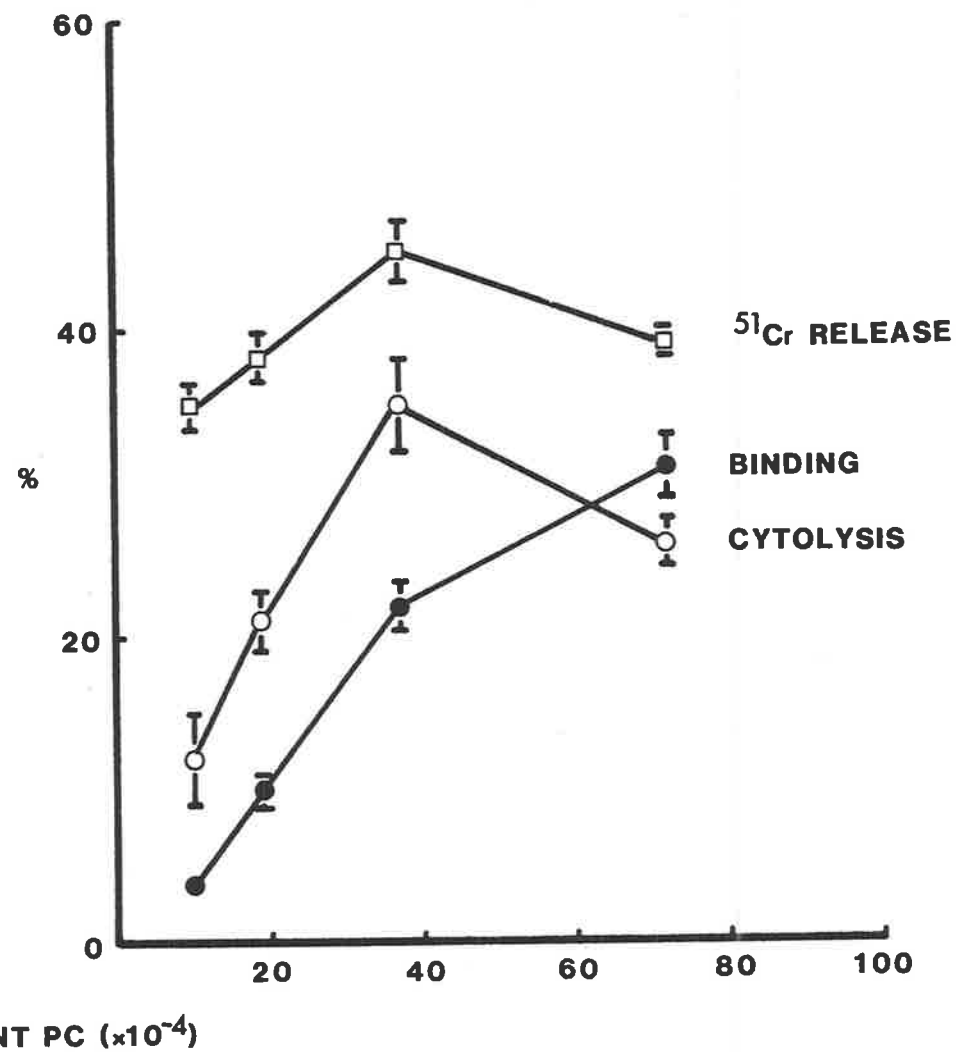
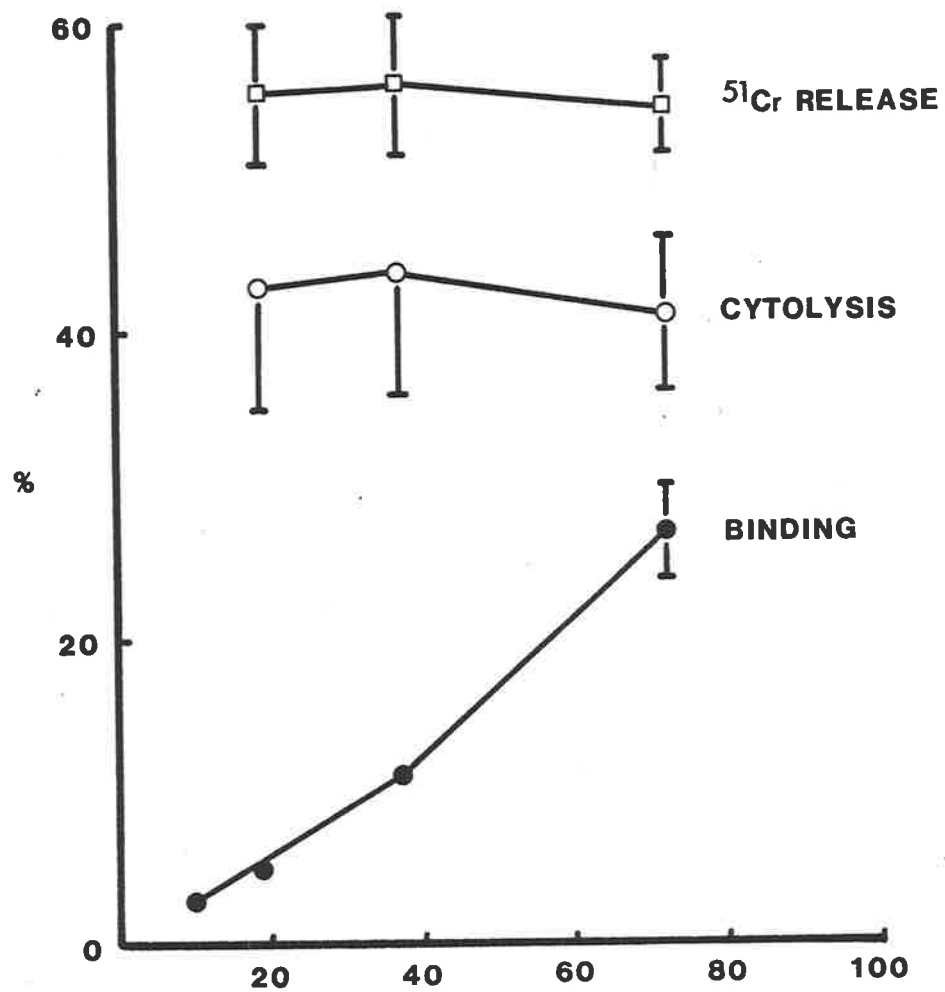
II: The effect of varying the numbers of adherent PC

The PC were obtained from mice immunized 8 days previously with 10^5 llRX ip. Monolayers of adherent PC were prepared at various cell densities and 1×10^4 or 1×10^5 ^{51}Cr -P815 cells were added and allowed to bind to them for 30 min. After this time unbound ^{51}Cr -P815 cells were removed, fresh medium added to the wells and the trays incubated for 20 h at 37° .

The % target cells bound, the % ^{51}Cr release from them and % cytolysis were determined as described in the text. The results are expressed as the mean \pm s.e.m from four replicate wells.

NUMBER OF ^{51}Cr -P815 CELLS ADDED PER WELL 1×10^4

1×10^5



4.10 The binding of normal cells to activated macrophage monolayers

To determine whether the binding of cells by activated macrophages monolayers was restricted to tumour cells alone, the adherence experiments were repeated using Con A blast cells and normal splenic lymphocytes. The Con A blast cell suspensions were prepared as described previously in §2.18.1. Splenic lymphocytes were obtained from cell suspensions prepared from spleens of normal CB6F₁ mice. They were purified on Percoll gradients consisting of a layer of 50% Percoll and one of 60% Percoll as described in §2.17 and used in the binding assay after labelling with ⁵¹Cr in the usual way.

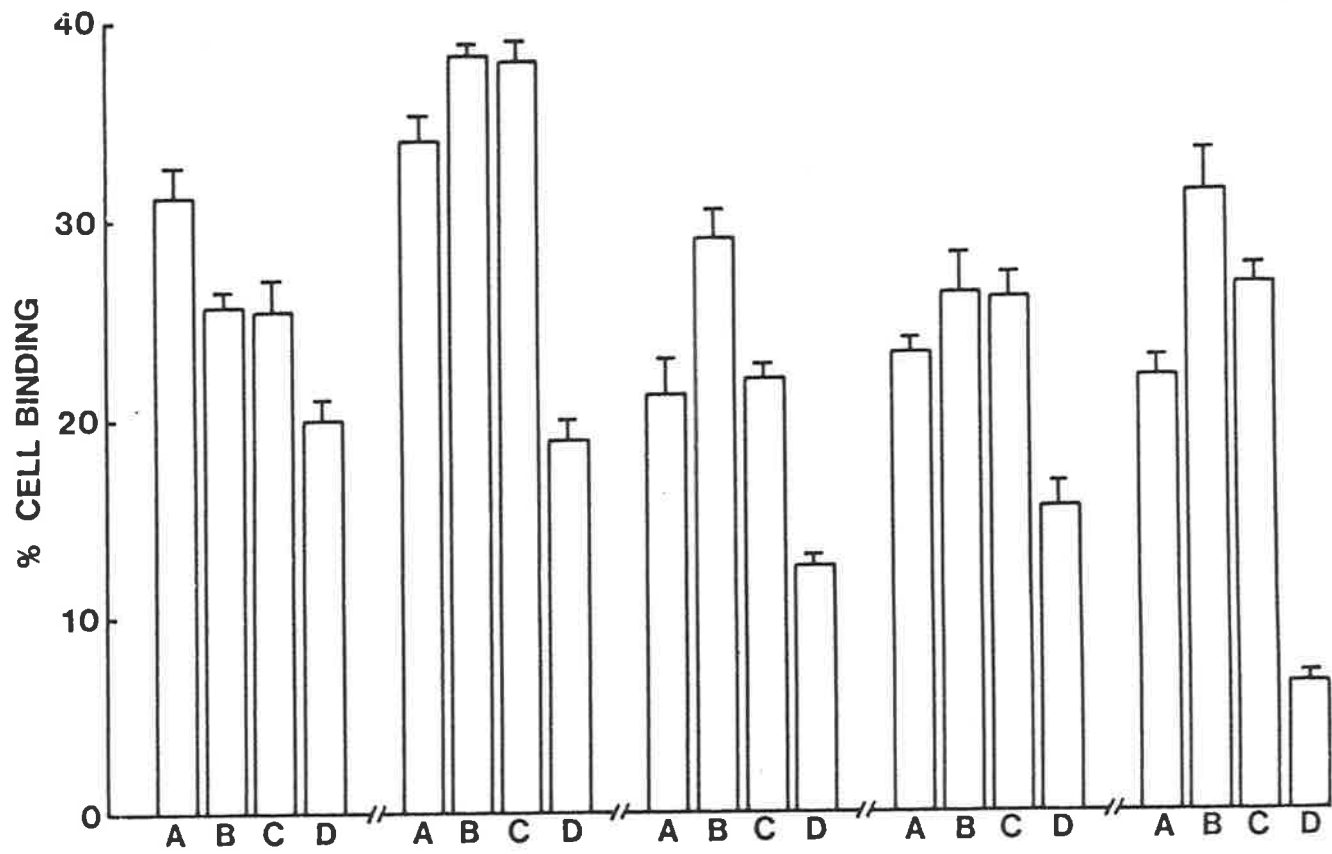
When comparing the binding of the various cells types, a constant number of 1×10^5 cells/well, forming less than a complete monolayer, was added to the wells because the cell types used varied in size and different numbers of them would have been required to form complete monolayers of targets. To make the results obtained for the binding of the different targets comparable, "non-limiting" numbers of macrophages was used i.e. monolayers prepared from 10^6 PC. IL2 was not included in the culture medium unless otherwise stated. This is because the assays were of short duration and it was assumed that the Con A blast cells would not have reverted to non-dividing cell forms during this time.

The results from five separate experiments comparing the binding of tumour cells, Con A blast cells and splenic lymphocytes (lyc) to activated macrophages are shown in Fig 4.9. It can be seen, that the ability of activated macrophages to bind Con A blast cells was equal to or better than their ability to bind P815 tumour cells. Residual Con A played little or no part in the interaction between Con A blast cells and activated macrophages because the presence of 100 mM methyl- α -D-mannopyranoside (MMP), an inhibitor of Con A, had little or no effect on the binding of the Con A blast cells. The concentration of MMP used could abolish any effect that added Con A had on the binding of cells to the adherent cell monolayers. This was determined in a

FIGURE 4.9

A comparison of the binding of tumour cells and dividing and non-dividing lymphocytes to activated macrophages

The results of five separate experiments measuring the binding of: (A) ^{51}Cr -P815 cells, (B) ^{51}Cr -blasts, (C) ^{51}Cr -blasts in the presence of 100 mM MMP and (D) ^{51}Cr -lyc, to monolayers of 11RX-activated macrophages. The monolayers in each well were prepared from 10^6 PC and the targets were used at 1×10^5 cells/well. The results are expressed as the mean \pm s.e.m. of the % of target cells bound in four replicate wells.



preliminary experiment using activated macrophage monolayers and splenic lymphocytes exposed to 5 µg/ml of Con A for 30 min, a pretreatment which increased binding by a factor of 4. In the presence of 100 mM MMP, the binding of the pretreated spleen cells was reduced to that of untreated lymphocytes (data not shown).

Splenic lymphocytes from normal mice were also bound by 11RX-activated macrophage monolayers but with less efficiency than P815 tumour cells and Con A blast cells (Fig 4.9). The average values of the results of six experiments showed that 11RX-activated macrophage monolayers bound nearly twice as many P815 cells as normal lymphocytes.

This difference is not as great as that reported by Marino and Adams (1980a) and contradicts the evidence of Marino, Whisnat and Adams (1981) who reported that BCG-activated macrophages did not bind LPS-activated splenic Con A blast cells to any great extent. To ensure that the data reported here were not obtained simply because the 11RX-activated macrophage monolayers were not washed with enough force to remove loosely bound target cells, the binding experiments were repeated using the washing technique of Marino and colleagues. The monolayers were washed 4x with a plunger type pipette. Even using this method for removing unbound cells, the large differences in the ability of activated macrophages to bind malignant and normal cells which were reported by Marino and Adams (1980a) and Marino, Whisnat and Adams (1981) could not be confirmed. In one experiment (data not shown), P815 cells, Con A blast cells and splenic lymphocytes were bound equally well by activated macrophage monolayers of different densities whilst on the other occasion lymphocytes were bound even more efficiently than the tumour cells, especially when low numbers of adherent PC were used.

Data in §4.8.4 showed that larger numbers of ⁵¹Cr-P815 tumour cells bound to macrophages from 11RX-immunized mice than to macrophages from normal mice. If the efficient binding of tumour cells and Con A blast cells to 11RX-activated macrophages was due to the same mechanism,

then Con A blast cells should also bind less efficiently to normal macrophages. This prediction was tested in an experiment described below.

4.11 The binding of Con A-activated blast cells to normal macrophages

The same pools of PC as those used for the first experiment discussed in §4.8.4 were used for this experiment. 1×10^5 Con A blast cells were added to each well which contained a monolayer of either 6.9×10^5 activated macrophages or 1.7×10^6 normal macrophages. The results indicated that on a per cell basis, activated macrophages were 7-fold more efficient than normal macrophages at binding Con A blast cells because the % Con A blast cells bound was 45% and 21% respectively.

4.12 Attempts to reconcile the differences between the results obtained from binding and cold target inhibition experiments

The two findings that Con A blast cells did not inhibit the killing of ^{51}Cr -P815 cells and ^{51}Cr -EAT cells but could bind to activated macrophages to the same degree as tumour cells appear to be contradictory. There are several explanations which could reconcile the two sets of data. Firstly, the most trivial explanation is that the presence of the tumour cells created unfavourable culture conditions which resulted in the death of the Con A blast cells so that they could no longer act as cold target inhibitors. Secondly, since there is some evidence that the binding of tumour cells to activated macrophages can augment the release of at least one cytotoxic factor (Johnson, Whisnand and Adams, 1981), the binding of Con A blast cells could also do the same. If Con A blast cells are resistant to the cytotoxic mechanism because such factors cannot attach to the Con A blast cell surface, the concentration of cytotoxic factors in the medium could increase, resulting in bystander lysis of unbound tumour cells. Finally, Con A blast cells could be inefficient cold target inhibitors of cytolysis if their binding to activated macrophages was only temporary. Detachment of

Con A blast cells a short time after binding would leave the macrophages free to interact with tumour cells. Experiments were carried out to test these possibilities.

4.12.1 The effect of unlabelled P815 cells on the viability of ^{51}Cr -blast cells co-cultured with PC from 11RX-immunized mice

To determine whether the presence of P815 cells resulted in the death of Con A blast cells when co-cultured with PC, the following experiment was conducted. 10^6 PC from normal or immunized mice and 10^4 ^{51}Cr -blast cells were cultured without added IL2 for 10 h in the absence of unlabelled P815 cells or with concentrations of P815 cells in the in the range of $2-32 \times 10^4$ cells/well. The results obtained indicate that it was unlikely that many Con A blast cells died when they were cultured with P815 cells in the presence of activated macrophages. There was little lysis of the ^{51}Cr -blasts in the presence of PC from immunized mice, and lysis was not affected by adding up to 3.2×10^5 unlabelled P815 cells (data not shown). In addition, it is worth noting that it is unlikely that the release of ^{51}Cr from ^{51}Cr -blast cells was due to the mechanism(s) involved in tumour cell lysis, because, the low level of cytolysis was not reduced by adding P815 cells.

4.12.2 Con A blast cells do not induce the release of cytotoxic factors from PC

The following experiment was performed to determine whether Con A blast cells induced the release of a cytotoxic factor from 11RX-activated PC. Mixtures of 11RX-activated PC and Con A blast cells were separated from ^{51}Cr -P815 with Sartorius membrane filters with $0.22 \mu\text{m}$ pores in an experiment similar to that presented in Table 4.2. No evidence was obtained that addition of Con A blast cells to activated macrophages induced the release of soluble factors which were cytotoxic to tumour cells, (Table 4.5). Separation of cytotoxic PC from ^{51}Cr -P815 cells

TABLE 4.5

The effect of blast cells on the lysis of ^{51}Cr -P815 cells*

Cells below membrane	Cells above membrane	% Background release (mean \pm s.e.m)	% Cytolysis (mean \pm s.e.m)
PC + ^{51}Cr -P815	(no membrane)		55.8 \pm 2.3
PC + ^{51}Cr -P815	None		55.6 \pm 4.7
PC	^{51}Cr -P815		17.5 \pm 2.1
PC + Blasts	^{51}Cr -P815		24.7 \pm 2.0
None	^{51}Cr -P815	21.3 \pm 0.4	-
^{51}Cr -P815	None	29.5 \pm 3.7	-
Blasts	^{51}Cr -P815	23.1 \pm 1.1	-

(*) Cells were used at the following concentrations: PC at 10^6 /well, ^{51}Cr -P815 at 10^4 /well and blast cells at 7×10^5 /well. The final volume of culture medium/well was 0.2 ml. ^{51}Cr release was measured at 20 h.

produced a large reduction in the release of ^{51}Cr from labelled tumour cells. Addition of 7×10^5 Con A blast cells to 10^6 PC did not cause a marked increase in the killing of the P815 cells on the other side of the membrane.

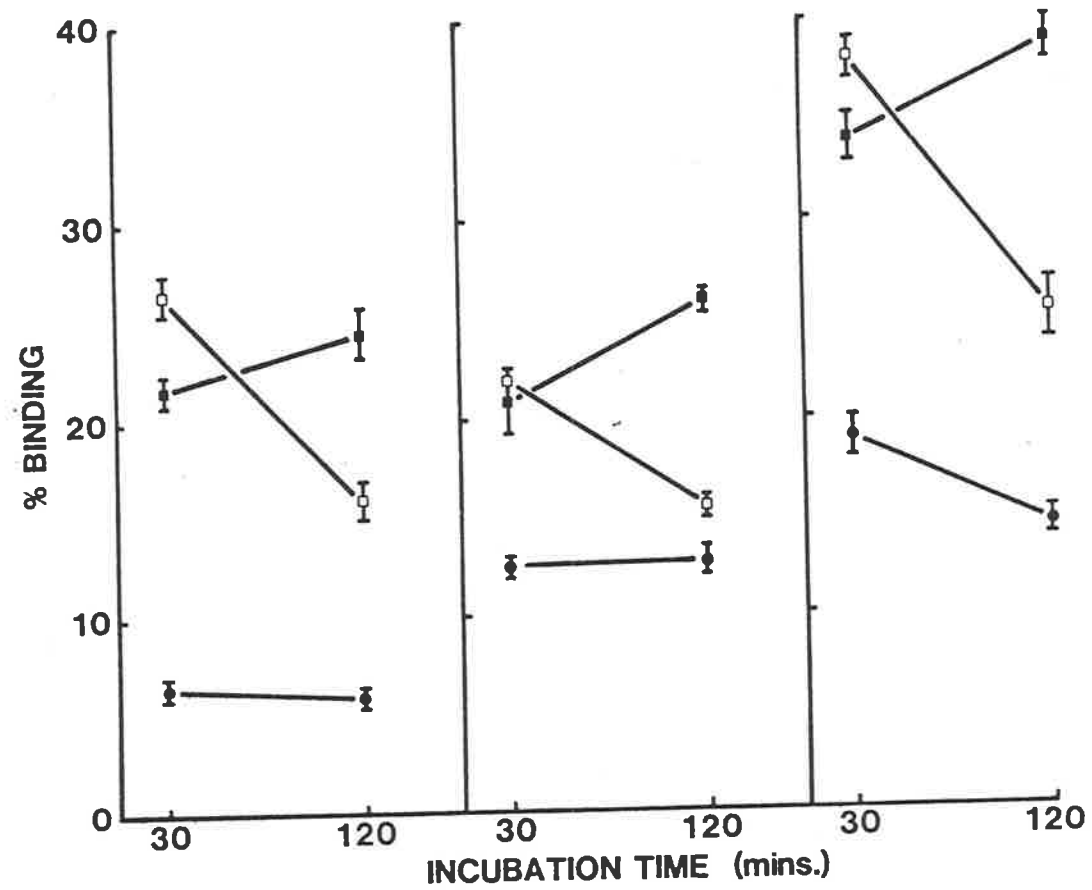
4.12.3 The binding of Con A blast cells to macrophage monolayers is short-lived

Some evidence was found to support the suggestion that the binding of Con A blast cells to activated macrophages was only temporary. Activated macrophage monolayers were prepared from 10^6 PC in two separate trays and 10^5 ^{51}Cr -labelled cells of various types were allowed to bind for 30 min or 120 min. The % binding of ^{51}Cr -labelled cells at these two times is compared in Fig 4.10. The results indicate that the number of Con A blast cells bound by activated macrophage monolayers decreased whilst the number of P815 cells bound was unchanged or increased and the number of normal lymphocytes bound was unchanged. It seemed possible that the detachment of Con A blast cells from activated macrophages reflected their reversion to resting lymphocytes during the 120 min incubation in vitro, because the experiment was carried out in the absence of IL2. For this reason, the above experiment was repeated using culture medium containing IL2. In addition, 100 mM MMP was also added to some of the wells to ensure that the binding observed was not due to the action of any residual Con A in the CS preparation which was used as the source of IL2. As stated earlier, the CS was prepared using the method of Lafferty et al. (1980a) which has been designed to ensure that the amount of Con A present in the CS is negligible. It was therefore not surprising to find that the addition of MMP had no effect on the results obtained. The results of this experiment (data not shown) confirmed the previous findings. The addition of CS did not maintain the binding of the Con A blast cells to the adherent cell monolayers, suggesting that reversion to a non-dividing

FIGURE 4.10

The effect of prolonged incubation on the binding of target cells

^{51}Cr -P815 cells (■), ^{51}Cr -blasts in the presence of MMP (□) and ^{51}Cr -lyc (●) were allowed to bind to monolayers of 11RX-activated macrophages for 30 min or 120 min. The monolayers in each well were prepared from 10^6 PC and the targets were used at 1×10^5 cells/well. The results are expressed as the mean \pm s.e.m. of the % of target cells bound in four replicate wells.



state was not the mechanism involved in their detachment.

4.13 The effect of simple sugars on the cytolysis of P815 cells by PC

The nature of the structures on the surface of tumour cells that are recognized by activated macrophages is unknown. There is some evidence suggesting that the interaction may involve carbohydrate moieties on the tumour cell or macrophage. Weir, Grahame, and Ögmundsdottir (1979) reported that certain sugars could inhibit the binding of macrophages to monolayers of tumour cells and embryo fibroblasts. Monosaccharides and oligosaccharides can inhibit NK cell activity (Ades, Hinson and Decker, 1981), spontaneous cytotoxicity by cultured human peripheral blood monocytes (Muchmore, Decker, and Blaese, 1981) and the cytotoxic action of invertebrate cells on mammalian target cells (Decker, Elmholt and Muchmore, 1981). Similarly, cytotoxic T cells may also interact with their targets via sugar-specific receptors (Black *et al.*, 1981). If activated macrophages recognize carbohydrates on tumour cells, then the addition of appropriate sugars should inhibit the in vitro cytolysis of targets. This possibility was tested in the following experiment. Mixtures of cytotoxic PC and ^{51}Cr -P815 cells at a ratio of 100:1 were prepared in the usual manner in medium containing one of the following sugars: galactose, lactose, mannose, rhamnose, MMP, arabinose, -melibiose, N-acetyl-glucosamine, and N-acetyl-galactosamine. Two separate experiments were performed with slightly different conditions employed on each occasion. In the first experiment the concentration of the sugars was 5 mM and cytolysis was measured at 10 h. However, because no inhibition was obtained, in the second experiment a higher the concentration of the sugars was increased to 20 mM and cytotoxicity was measured at 4 h rather than 10 h. This was done to reduce the possibility that the sugars were being metabolized to inactive forms during the in vitro cytotoxicity assay. The results in Table 4.6 show that none of the sugars used caused a marked decrease in

TABLE 4.6

The effect of simple sugars on the in vitro cytotoxic activity
of PC from 11RX-immunized mice[@]

EXPERIMENT 1 (5 mM sugar) EXPERIMENT 2 (20 mM sugar)

Sugar Added	% Cytolysis (mean \pm s.e.m)	% Cytolysis (mean \pm s.e.m)
	10 h	4 h
NONE	51.9 \pm 1.9	19.0 \pm 1.7
galactose	52.2 \pm 3.6	17.8 \pm 0.4
lactose	46.0 \pm 2.8	21.6 \pm 0.9
D (+) mannose	43.2 \pm 3.8	18.3 \pm 0.4
L (+) rhamnose	51.9 \pm 2.0	18.3 \pm 0.8
MMP	45.8 \pm 2.8	14.8 \pm 0.3
D (-) arabinose	53.8 \pm 3.6	16.1 \pm 1.8
melibiose	49.8 \pm 2.5	14.9 \pm 0.9
N-acetyl-galactosamine	46.6 \pm 1.8	22.5 \pm 2.0
N-acetyl-glucosamine	46.4 \pm 3.3	19.3 \pm 1.1
meso-inositol	ND*	21.2 \pm 1.1

(@) Various sugars were tested for their ability to inhibit the lysis of ⁵¹Cr-P815 cells by PC in a standard in vitro cytotoxicity assay. The PC were obtained from mice immunized 7 days previously with 10⁵ 11RX ip.

(*) Not determined.

cytotoxicity in either experiment, indicating that the interactions between activated macrophages and P815 cells do not involve any of these sugars.

4.14 Summary and conclusions

The lysis of P815 tumour cells and EAT cells by activated macrophages is dependent on contact with the effector cells. The requirement for contact is consistent with the increased ability of macrophages from 11RX-immunized mice to bind tumour cells in vitro and the finding that the enhanced cytolysis of P815 cells by PC from CB6F₁ 11RX-immunized mice in the presence of CBA anti-H-2^d antiserum correlated with enhanced binding of target cells to the activated macrophages. The binding of tumour cells to macrophages was necessary but not sufficient to ensure cytolysis as evidenced by the finding that, late after 11RX immunization, macrophages could still bind P815 cells as effectively as those taken from mice 6-8 days after immunization but their in vitro cytotoxic activity was much lower. The ability of activated macrophages bind tumour cells can be competitively and non-specifically inhibited by unlabelled tumour cells, implying that the surface structures that activated macrophages recognize are present on all tumour cells.

Experiments were carried out to determine whether these structures were exclusive to tumour cells, or were also present on dividing, normal cells such as Con A blast cells but not on non-cycling cells such as splenic lymphocytes. P815 tumour cells and Con A blast cells were bound by monolayers of activated macrophages equally well and more effectively than splenic lymphocytes in a 30 min cell binding assay. Normal macrophages bound fewer P815 cells and Con A blast cells than macrophages obtained from 11RX-immunized mice. This suggests that activated PC have an increased number of the receptors required for the binding of tumour cells and Con A blast cells and that the surface structures which

interact with macrophage receptors are present on both dividing and non-dividing cells. Attempts to demonstrate that the structures are carbohydrates were not successful.

Although there are similarities in the binding of P815 tumour cells and Con A blast cells to macrophage monolayers, it appears that the interaction of Con A blast cells with PC from 11RX-immunized mice is qualitatively and quantitatively different to that of tumour cells. ^{125}I -Con A blast cells are totally resistant to lysis by tumouricidal PC in a 20 h in vitro cytotoxicity assay. This is consistent with the finding that, unlike tumour cells, Con A blast cells did not competitively inhibit the lysis of ^{51}Cr -P815 cells and ^{51}Cr -EAT cells by activated PC. The lack of cold target inhibition by Con A blast cells could not be explained by the release of cytotoxic factors resulting from the interaction of Con A blast cells with activated macrophages. However, the finding that with time, Con A blast cells, but not tumour cells, dissociate from monolayers of activated macrophages suggests that binding of the two cell types may occur through different receptors. Therefore, the detachment of initially bound Con A blast cells would leave the macrophages free to interact with tumour cells.

In summary, these data suggest that Con A blast cells are resistant to lysis by activated macrophages for one or more of the following reasons: (A) they may bind to a different subpopulation of activated macrophages, or to the same population via different receptors and as a consequence they do not activate the cytotoxic mechanism, (B) if they do interact with the same subpopulation of macrophages through the same receptors as tumour cells, the interaction may not be of sufficient duration for the macrophages to deliver their "lethal hit", (C) even if they do activate the cytotoxic mechanism when they bind to macrophages, they may be resistant to the cytotoxic mechanism which lyses tumour cells.

CHAPTER FIVE

THE LIFE SPAN OF LYMPHOCYTES MEDIATING THE RECALL
OF TUMOUR RESISTANCE IN LONG-TERM 11RX-IMMUNIZED MICE5.1 Introduction

Tumour resistance in mice infected with 11RX is a non specific manifestation of the specific immune response to the intracellular bacterial parasite. As such, it decays as the immunizing bacteria are eliminated from the liver and spleen (Ashley et al., 1974; Ashley and Kotlarski, 1982b) in much the same way as resistance to heterologous intracellular bacteria does (Mackaness, 1964; Collins, 1968; Coppel and Youmans, 1969). By 40-50 days after iv infection or 150 days after ip infection, 11RX-immunized mice can no longer control an ip challenge with 10^6 EAT cells (Ashley et al., 1974; Ashley and Kotlarski, 1982a). However, very high levels of ip tumour resistance can be "recalled" in long-term immunized mice by injecting them ip with 10 μ g of a protein extract from 11RX organisms (Ashley et al., 1974; Ashley and Kotlarski, 1982b). This phenomenon is analogous to the recall of bacterial immunity with bacterial antigenic extracts (Halliburton and Hinsdill, 1972) which requires the presence or injection of specific antigen but, once induced, is non-specific in its expression. Sufficient levels of tumour resistance to cause an increase in survival time following ip challenge with 10^6 EAT tumour cells can be recalled with 11RX antigen as much as 250 days after immunization. Injection of 11RX antigen up to 370 days after immunization results in detectable clearance of a challenge with 125 I-EAT cells at a faster rate than occurs in normal controls.

The ability to recall tumour resistance can be transferred to

naive mice with splenic lymphocytes from long-term llRX-immunized mice provided llRX antigen is injected shortly before or at the time of tumour challenge (Ashley et al., 1977). The lymphocytes which mediate the recall of ip tumour resistance are T cells because pre-treatment of donor spleen cells with anti-Thy 1.2 and complement in vitro greatly reduces the ability of recipients to clear a subsequent challenge of ^{125}I -EAT cells with the result that the mice succumb to tumour growth and die as quickly as normal, untreated mice.

The observation that protective levels of tumour resistance against a highly lethal tumour challenge can be recalled in mice immunized with llRX a considerable time earlier imply that sensitized T cells are maintained in llRX-immunized mice for a very long time after the clearance of organisms. Two possible mechanisms can be envisaged by which this can occur. Firstly, long-lived, sensitized lymphocytes are generated in high numbers during the infection with llRX and are present in the animals for a long period of time. Alternatively, non-viable bacterial antigens persisting in immunized mice after clearance of the infection, maintain a population of short-lived, sensitized lymphocytes.

One piece of evidence suggesting that llRX antigens may indeed persist comes from the observation that whilst PC from day 40 ip-immunized mice are poorly cytotoxic in vitro, mice at this time are still highly resistant to ip tumour challenge (Ashley, 1976; Ashley and Kotlarski, 1982a). A possible explanation for the 4-6 h lag before such mice begin to kill ^{125}I -EAT cells is that the injection of tumour cells may be causing the release of llRX antigens sequestered in PC, thereby recalling resistance. However this is only circumstantial evidence and long-term persistence of these antigens, or lack of persistence has yet to be formally demonstrated. It is clear that a role for persisting antigens in maintaining a pool of llRX-sensitized T cells is purely speculative.

In this chapter are presented the results of experiments designed

to measure the functional life span of sensitized T cells present in long-term immunized mice. The experimental strategy involved transferring sensitized lymphocytes to normal mice and at various time thereafter injecting them with 10 µg of 11RX antigen and challenging them with 10^6 ^{125}I -EAT cells. The 11RX antigen and the labelled tumour cells were injected as a mixture, which will be referred to as *EAT/Ag in this chapter. Tumour killing was assessed by measuring the whole body retention of ^{125}I in each mouse over a six day period. To suppress the uptake of free ^{125}I by the thyroid of the mice, non-radioactive sodium iodide was added to their drinking water at least 24 h before challenge and the mice maintained on it for the duration of the experiment. Previous studies by Ashley and his colleagues have established that radioactivity is cleared much more rapidly in mice which are resistant to the tumour challenge than in normal, unimmunized mice and that the rate of clearance of the label correlates with the level of resistance of mice at various times after immunization (Ashley, 1976; Ashley et al., 1977; Ashley and Kotlarski, 1982a, b). This short-term in vivo assay for tumour resistance is highly sensitive and will detect low levels of tumour resistance which do not result in prolongation of survival (Ashley, 1976; Ashley et al., 1977; Ashley and Kotlarski, 1982a).

The spleen cells of iv immunized mice were used as a source of 11RX-sensitized lymphocytes because it had already been established that they were capable of transferring the ability to recall tumour resistance to normal mice (Ashley et al., 1977). So as to minimize the possibility of transferring viable organisms and actively immunizing the recipients, mice were used as spleen cell donors no earlier than 40 days after immunization. By this time their spleens contained very few if any viable 11RX organisms (Ashley, 1976; Ashley and Kotlarski, 1982a).

Even before carrying out these experiments, it was clear that the route chosen (iv or ip) for transferring the spleen cells could impose constraints on the interpretation of experimental results. To measure

the functional half-life of sensitized lymphocytes after ip transfer the decay in the tumour resistance that could be recalled needs to reflect the death of mediator lymphocytes. Ideally, therefore, they should not migrate from that anatomical compartment for the duration of the experiment, (up to 4 weeks). This is not likely to be so, because lymphocytes have been shown to migrate and recirculate (Gowans and Knight, 1964). This would mean that any decrease in the level of tumour resistance that could be recalled may in fact, simply be a measure of the rate at which sensitized lymphocytes leave the peritoneal cavity and distribute through out the body. There is very little information in the literature regarding the fate of lymphoid cells transferred by this route. Catanzaro et al. (1978) showed that the majority of ^{51}Cr -labelled peripheral blood and peritoneal lymphocytes injected ip could still be found at this site 24 h later; the authors thereby suggested that cells lost the ability to migrate once they entered the peritoneal cavity. Unfortunately no data were provided on the distribution of the cells at later times. Earlier work by Gillette and Lance (1972), showed that ^{51}Cr -labelled, pooled lymph node lymphocytes could migrate from the peritoneum to the liver, spleen and lymph nodes in 48 h; however the percentage of cells remaining in the peritoneum at this time was not shown.

At the time these experiments were commenced it was hypothesised that the recall of tumour resistance in long-term iv-immunized mice was mediated by recirculating lymphocytes capable of migrating to the peritoneal cavity. Therefore the spleen cells would have to be transferred iv, if one hoped to mimic what was occurring in the donor. By using this route, however, a large number of spleen cells would need to be transferred since 80% of the splenic lymphocytes that can be detected 24 h after iv transfusion into recipient mice are distributed between the liver, spleen, lymph nodes and the Peyer's patches (Zatz and Lance, 1970). In addition to these considerations, it is possible that only

lymphoblasts may be able to enter the peritoneal cavity so that by using the iv route one may be measuring the activity of only one of a number of subpopulations of lymphocytes capable of activating macrophages to kill tumour cells in vivo, (for a detailed discussion of this point please see §5.5).

5.2 Dependence of in vivo cytotoxicity on the number of spleen cells transferred

In the experiments demonstrating that tumour resistance could be transferred with spleen cells from 11RX-immunized mice and was mediated by T cells, only a single dose of spleen cells was used (Ashley et al., 1977). Because it was intended to estimate the functional half-life of sensitized cells it was necessary to determine whether the measurement of whole-body radioactivity was sufficiently sensitive to detect the effect of two-fold differences in the number of spleen cells transferred.

Fig 5.1A shows the ^{125}I retention by groups of mice which had been challenged ip with a mixture of *EAT/Ag, and graded doses of spleen cells from day 94 iv-immunized mice. Clearance of ^{125}I greater than that shown by control mice, was detected when as few as 1×10^7 spleen cells were injected along with the tumour challenge. Groups of mice which received increasing doses of spleen cells could be distinguished by their clearance of ^{125}I , which was dose dependent. Radioactivity was cleared most rapidly by mice injected with heat-killed ^{125}I -EAT cells. Within 24 h approximately 90% of the label was excreted. The remainder was cleared much more slowly with approximately 2-3% of the radioactivity persisting in the mice at day 6. The measurement of in vivo tumour death by this technique is limited by the retention of this significant amount of radioactivity which is not associated with the cells which can be washed from the peritoneal cavity (Ashley, 1976; Ashley and Kotlarski, 1982a). This established that the technique was sufficiently sensitive to detect 2-fold differences in the number of spleen cells transferred.

FIGURE 5.1

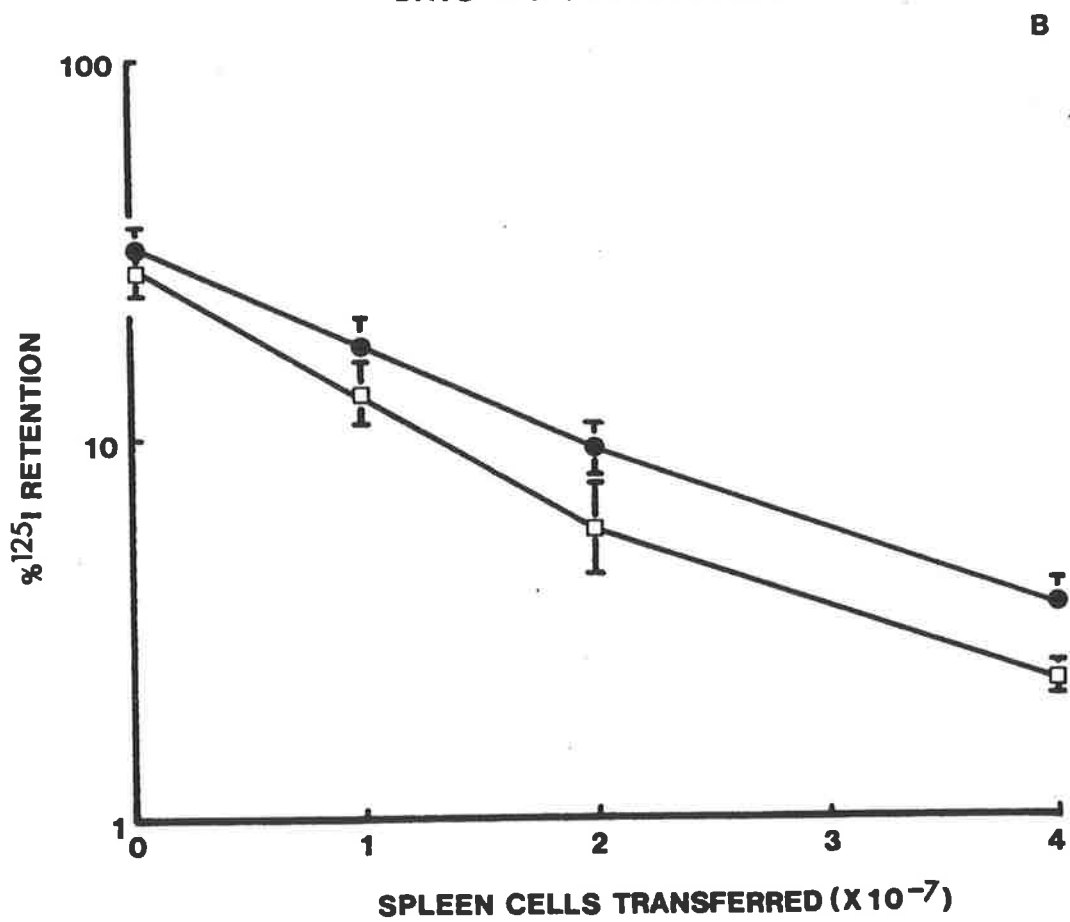
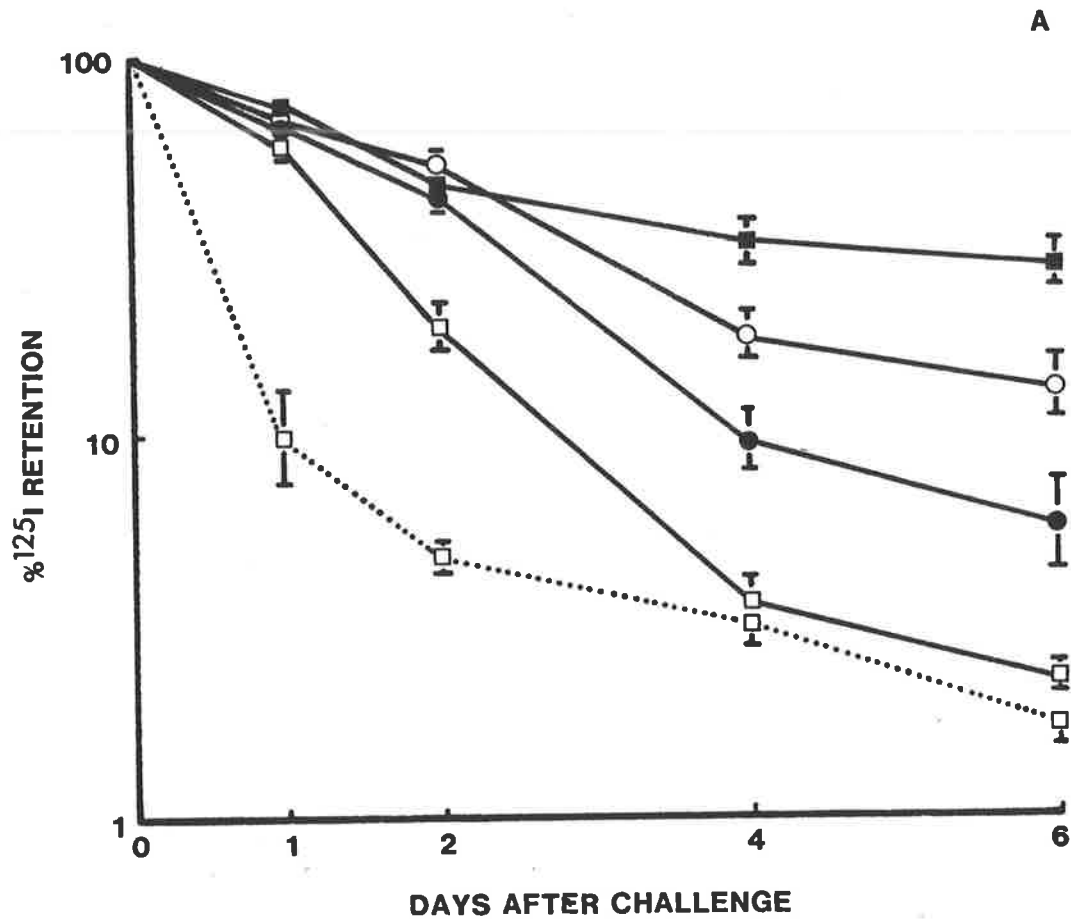
The relationship of ip tumour resistance to
the dose of spleen cells transferred

Resistance to ip tumour challenge was measured as retention of ^{125}I by mice injected ip with cell suspensions containing a mixture of 10^6 ^{125}I -EAT cells, 10 μg of 11RX antigen and various numbers of spleen cells from donors which were immunized with 10^5 11RX iv, 94 days previously. The dose of spleen cells injected per mouse was: 4×10^7 (□), 2×10^7 (●), 1×10^7 (○) or no spleen cells (■). A group of normal mice was injected with 1×10^6 heat-killed ^{125}I -EAT cells (...□...).

A: ^{125}I retention in recipients following challenge.

B: Retention of ^{125}I by recipients as a function of spleen cell dose on day 4 (●) and day 6 (□) after challenge.

Each line shows the results from a group of 5 mice and each point shows the geometric mean (x/\pm s.e.m) of the ^{125}I retained by individual mice at different times after transfer.



The data show that percentage of ^{125}I retained on days 4 and 6 after challenge is related to the dose of cells transferred (Fig 5.1B). The logarithm of % ^{125}I retained by mice given a limiting dose of spleen cells at the time of challenge with *EAT/Ag was directly proportional and linearly related to the number of spleen cells transferred.

However, one of the limitations imposed by the retention of residual ^{125}I is that once a particular dose of spleen cells is reached (in this experiment 4×10^7 per mouse), any increase in cell dose which could only serve to increase the resistance of recipients will not be reflected as an increase in the amount of label that is cleared at days 4-6. This is because recipients injected ip with 4×10^7 spleen cells will retain as much ^{125}I 4-6 days after challenge as normal mice injected with heat-killed ^{125}I -EAT cells. In this chapter such a dose of spleen cells is considered to be a "non-limiting" dose.

The speed in which tumour cell destruction is detected after challenge with ^{125}I -EAT cells also appears to be dependent on the dose of spleen cells transferred. Thus, mice which had received 4×10^7 spleen cells had cleared ^{125}I faster than control mice by the first day after challenge. In mice injected with 1×10^7 or 2×10^7 spleen cells enhanced clearance of ^{125}I was not detected until day 2.

In summary, these results confirm and extend those of Ashley *et al.* (1977). They show that the ability to recall tumour resistance can be transferred with spleen cells from long-term iv llRX-immunized mice, that the level of cytotoxicity is dependent on the dose of cells transferred, and that the assay is capable of detecting 2-fold differences in the number of spleen cells transferred.

5.3 Systemic transfer of the ability to recall tumour resistance

The above experiments showed that tumour resistance could be transferred locally. A study aimed at establishing whether systemic transfer of tumour resistance is possible is discussed in the following

section. Experiments were carried out to determine whether the recall of tumour resistance in long-term iv llRX-immunized mice is due to migrating lymphocytes. Preliminary experiments indicated that very little, if any, ip tumour resistance could be recalled when mice were challenged ip with *EAT/Ag immediately after iv injection of spleen cells from llRX-immunized mice (data not shown). Low, but significant levels of tumour resistance could be recalled if three days were allowed to elapse before the recipients were challenged with *EAT/Ag. This implied that the failure recall tumour resistance until day 3 after iv injection of spleen cells into recipients was because lymphocytes were unable to migrate to the peritoneal cavity immediately after transfer. Therefore, in subsequent experiments, 3 days were allowed to elapse between the time of iv cell transfer and tumour challenge. A direct comparison of the tumour resistance that could be recalled on days 0 and 3 after ip or iv transfer of spleen cells from immunized mice is shown in the experiment depicted in Fig 5.2 and is part of a larger experiment discussed in §5.5.2. Spleen cells from long-term iv-immunized mice were injected iv or ip into normal recipients. Immediately after transfer some mice were challenged ip with *EAT/Ag and their retention of ^{125}I was monitored for the next six days. It can be seen that by day 2 after challenge the ability of mice which were injected with spleen cells by the iv route to clear a challenge of ^{125}I -EAT cells was much less than that of mice which were injected ip and was indistinguishable from that of mice which received no spleen cells (Fig. 5.2A). The rest of the mice were challenged with *EAT/Ag on day 3 after transfer. The data confirmed earlier findings that mice which received spleen cells iv 3 days before challenge killed ^{125}I -EAT cells more efficiently than those challenged immediately after cell transfer, but again this rate of killing was much lower than that which could be transferred by the ip route (Fig 5.2B). This result supports the the notion that spleen cells cannot migrate efficiently immediately after cell transfer and is consistent with

FIGURE 5.2

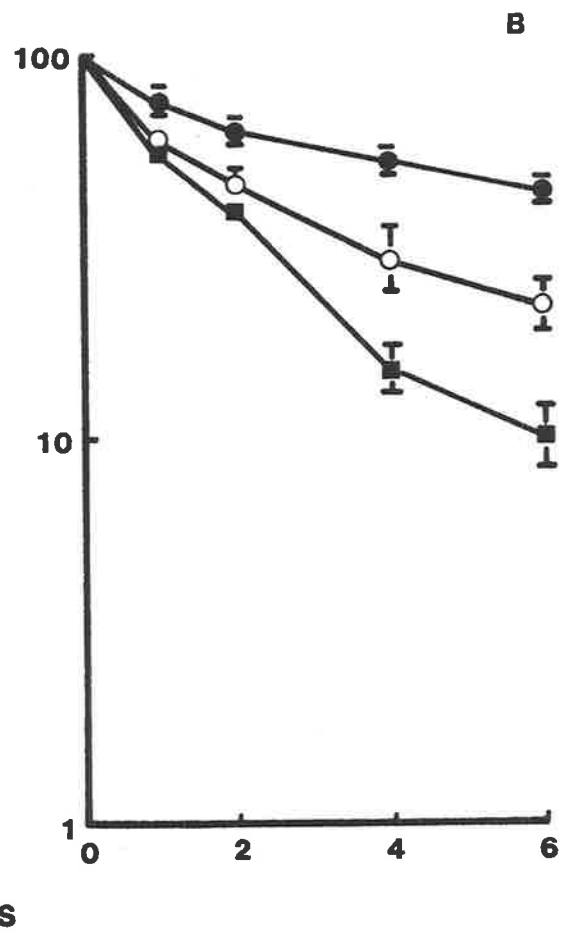
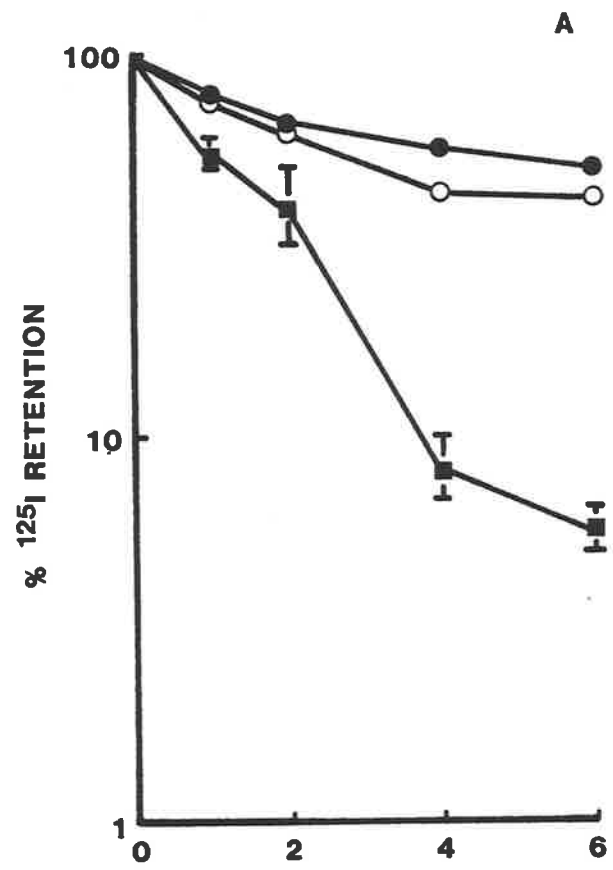
Comparison of ^{125}I retention in mice challenged with ^{125}I -EAT cells and 11RX antigen
following ip or iv transfer of spleen cells from immunized mice

A: Retention of ^{125}I in recipients challenged immediately after cell transfer. Mice were injected with 2.2×10^7 spleen cells iv and, shortly after, challenged with 10^6 ^{125}I -EAT cells and 10 μg 11RX antigen (■). Other recipients were injected ip with a mixture of 2.2×10^7 spleen cells, 10^6 ^{125}I -EAT cells and 10 μg 11RX antigen (○). Control animals received only ^{125}I -EAT cells and 11RX antigen (●).

B: Retention of ^{125}I in recipients challenged 3 days after cell transfer. Mice were injected with cells ip (■) or iv (□) or with nothing (●) at the same time as those in A. Three days later they were challenged ip with a mixture of 10^6 ^{125}I -EAT cells and 10 μg 11RX antigen.

Donor mice were immunized iv 56 days previously with 10^5 11RX.

Each line shows the results from a group of 5 mice and each point shows the geometric mean (x/\pm s.e.m) of the ^{125}I retained by individual mice at different times after transfer.



findings by Freitas (1976) (cited in Parrott and Wilkinson, 1981) that ^{51}Cr -labelled lymphocytes injected iv into mice take up to 6 h to leave the capillary bed of the lungs and 24-72 h to transit the spleen and liver before appearing in the lymph nodes.

5.4 The functional life span of 11RX-sensitized lymphocytes after systemic transfer

The following experiments established the length of time iv-transferred lymphocytes could be detected in recipient mice. Groups of normal CB6F_1 mice were injected iv with graded doses of spleen cells from long-term immunized mice or only the media used for suspending cells. At day 3 and intervals thereafter groups of 5 recipients were challenged with *EAT/Ag. Fig 5.3 shows that mice which had received iv injections of 5×10^7 or 2.5×10^7 spleen cells from immunized mice 3 days previously could kill ^{125}I -EAT cells as judged by ^{125}I retention in these animals. It is also apparent that the level of ip tumour resistance that was recalled depended on the dose of spleen cells transferred. Three days after spleen cell transfer mice which had received 1.3×10^7 spleen cells iv retained ^{125}I -EAT to the same extent as mice given no spleen cells from immunized donors. All the recipients which were challenged 11 or 20 days after transfer cleared ^{125}I at the same rate as control mice. Thus on day 11 and 20 after transfer mice which had received even the highest dose of 5×10^7 spleen cells iv behaved the same as control mice in their ability to clear ^{125}I -EAT cells. This implies that during the 8 day interval which was allowed to elapse between the first and second time of challenge, the activity of iv transferred spleen cells had decayed 4-fold. From these results the functional half-life of spleen cells was estimated to be 4 days at the most, indicating that the lymphocytes which transferred the the ability to to recall tumour resistance acted systemically for only a short time.

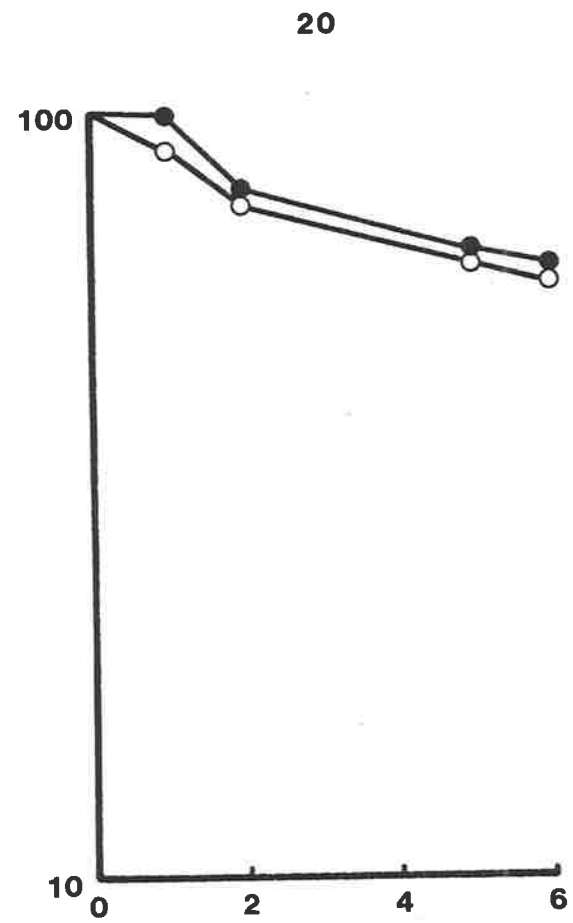
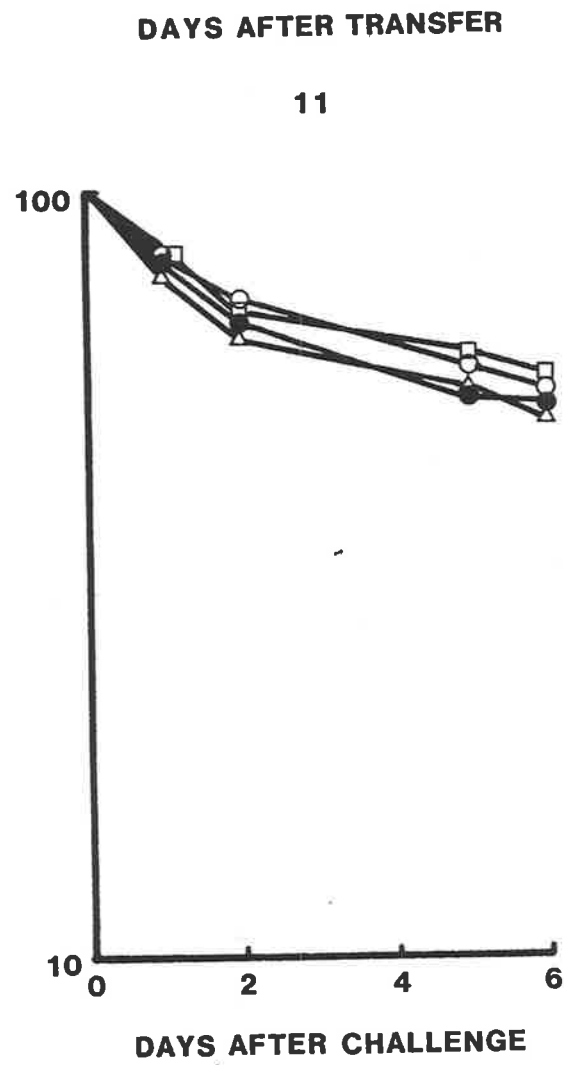
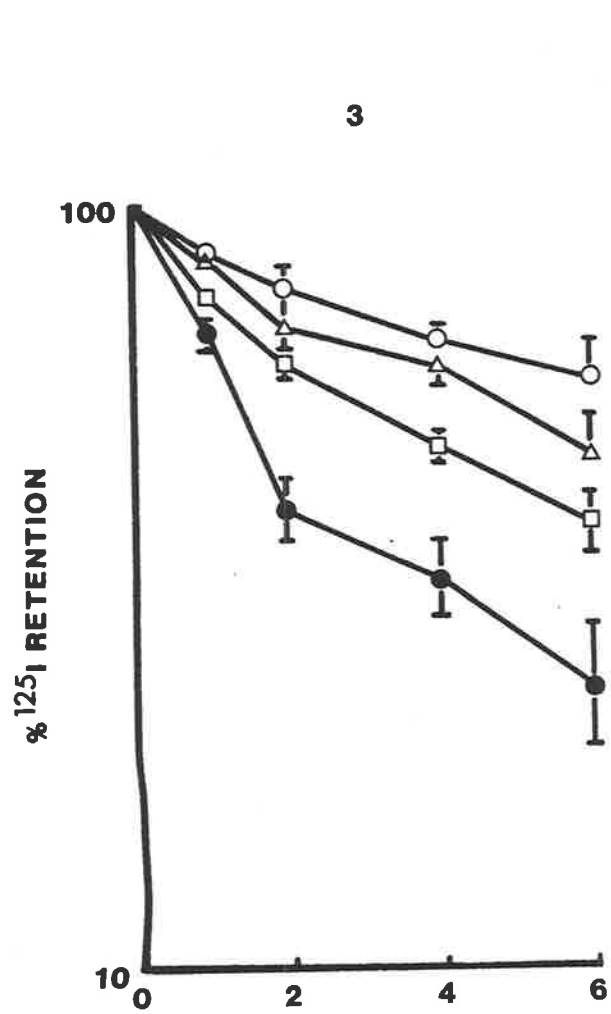
FIGURE 5.3

The decay of tumour resistance after iv transfer of spleen cells from 11RX-immunized mice

Normal mice were injected iv with one of the following doses of spleen cells from mice immunized iv with 10^5 11RX 41 days previously: 5×10^7 spleen cells (●), 2.5×10^7 spleen cells (□), 1.3×10^7 spleen cells (Δ), cell suspension medium only (○).

Recipients were challenged with 10^6 ^{125}I -EAT cells and 10 μg 11RX antigen ip on days 3, 11 or 20 after spleen cell transfer.

Each line shows the results from a group of 5 mice and each point shows the geometric mean ($\bar{x} \div \text{s.e.m}$) of the ^{125}I retained by individual mice at different times after transfer.



This experiment was repeated to determine the life span of the lymphocytes after iv transfer more closely. A single spleen cell dose of 2.2×10^7 cells per mouse was used. This experiment is discussed in §5.5.2 and the rate of decay is compared to the decay of activity obtained after the same number of cells were transferred ip.

5.5. The functional life span of sensitized lymphocytes after local transfer

The results of the previous experiment are consistent with the notion that the lymphocytes which transfer the ability to recall tumour immunity have a short functional life span. However, the finding that a higher level of tumour resistance could be detected in mice injected with spleen cells from immunized donors at the same (ip) site as the tumour challenge suggested that only a small proportion of the lymphocytes may find their way to the peritoneal cavity after iv transfer. The previous experiments were not designed to distinguish between the effect of a "dilution" of the lymphocytes after iv transfer due to circulation and distribution throughout the body and the possibility that the experimental design was selecting for a particular subpopulation of sensitized lymphocytes which entered the peritoneal cavity some time after cell transfer.

As discussed earlier in §1.3.5 the work of Koster et al. (1971), Koster and McGregor (1971) and North and Spitalny (1974) showed that the ability of lymphocytes to enter an inflamed peritoneal cavity was restricted to large dividing lymphocytes and any small lymphocytes found in the exudate were probably derived from them. Small long-lived lymphocytes which were transfused iv into normal recipients did not find their way into inflammatory exudates. Since the cells which transfer immunity to Listeria most efficiently are large, dividing, short-lived lymphocytes it seemed possible that a similar population was being detected after systemic transfer of sensitized spleen cells. Therefore,

the low level of tumour resistance which was recalled after iv transfer of spleen cells from long-term llRX-immunized mice may have been mediated by large lymphocytes entering the peritoneal cavity in response to the inflammation caused by the ip injection of llRX antigen and tumour cells. Unless T cells generated by immunization with llRX are different from those induced by Listeria, any long-lived, small lymphocytes capable of recalling tumour immunity should not have been able to enter the peritoneal cavity and cause the macrophage activation required for the destruction of ^{125}I -EAT cells. In an attempt to determine whether long-lived small lymphocytes were present in the spleen cell suspensions of long-term immunized mice, the life span of the lymphocytes mediating recall of tumour resistance was measured after transfer by the ip route. The results from several of these experiments are presented below.

5.5.1 Experiment 1: Decay of spleen cell activity after ip transfer

Groups of mice were injected ip with 1×10^7 or 4×10^7 spleen cells from long-term iv-immunized mice. Some recipients were challenged with *EAT/Ag immediately after transfer whilst other groups were challenged with *EAT/Ag at weekly intervals there after. The clearance of ^{125}I by the recipients is shown in Fig 5.4. The data indicate that the level of tumour resistance that could be recalled did not decrease markedly over the course of the experiment (21 days). This contrasted greatly with results obtained when spleen cells had been transferred iv. Some evidence for the decay of activity could be inferred by comparing the ^{125}I retention of the different groups on the second day after challenge. By day 21 mice which received 1×10^7 spleen cells took at least 2 days to start clearing ^{125}I faster than normal mice whilst mice challenged earlier than that were already doing so by day 2.

If all the sensitized spleen cells in the inocula had a functional life of 4 days (as estimated by the experiments in §5.4) it would follow that by day 21 (approximately 5 half lives) recipients should have

FIGURE 5.4

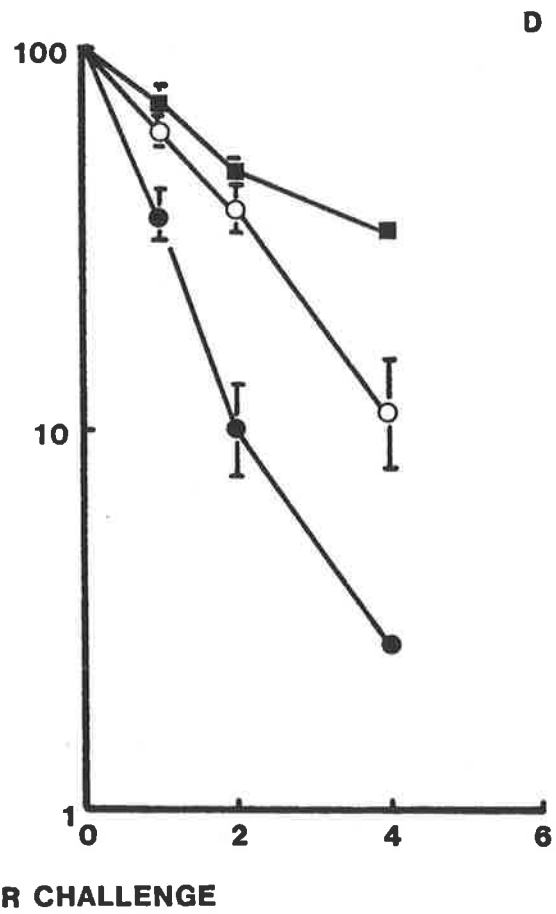
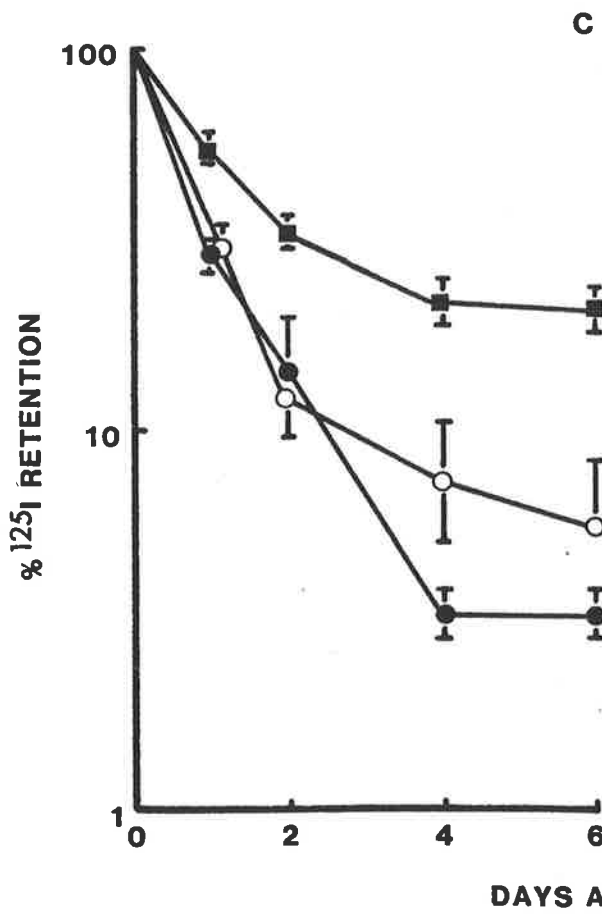
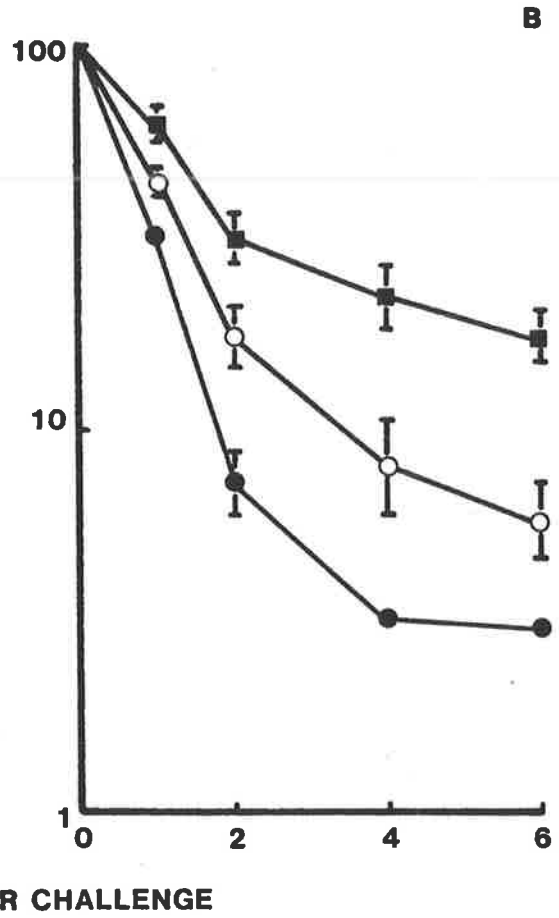
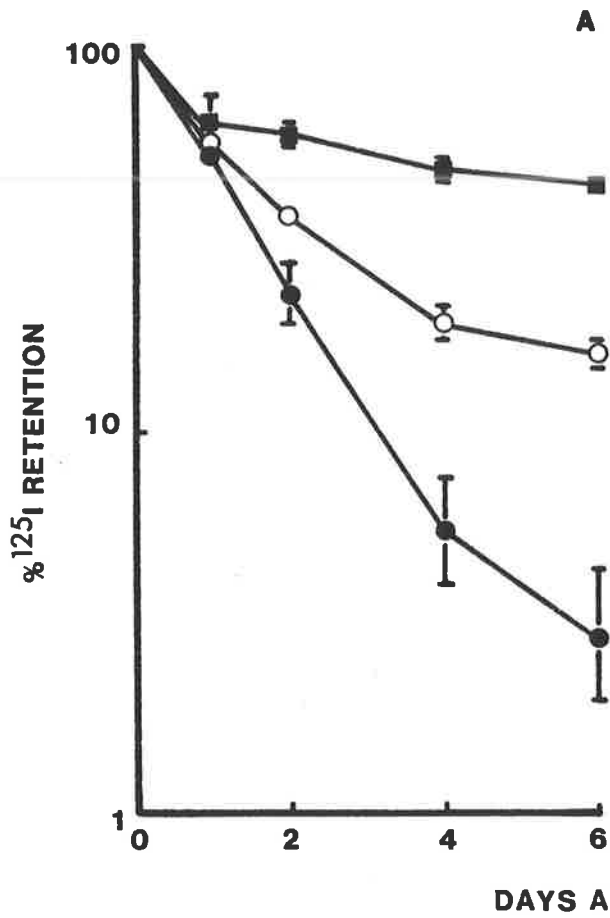
The decay of tumour resistance in mice injected ip
with spleen cells from immunized mice

Recipients were injected ip with 4×10^7 (●) or 1×10^7 (○) spleen cells, or cell suspension medium only (■). Groups of 5 recipients were challenged with a mixture of 10^6 ^{125}I -EAT cells and 10 ug 11RX antigen at the following times:

- A: at the time of transfer,
- B: 7 days after transfer,
- C: 14 days after transfer,
- D: 21 days after transfer,

Donors were immunized with 10^5 11RX iv 60 days previously

Each line shows the results from a group of 5 mice and each point shows the geometric mean (\times/\div s.e.m) of the ^{125}I retained by individual mice at different times after transfer.



shown very little activity, since the resistance detected could have been mediated by no more than the equivalent of 1×10^6 spleen cells given at the time of tumour challenge. This is a spleen cells dose which was found to be about 10-fold less than that required to transfer detectable ip tumour resistance when *EAT/Ag was injected at the same time as the spleen cells (data not shown).

5.5.2 Experiment 2: Direct comparison of the decay of lymphocyte function after iv and ip transfer

The evidence presented indicates that the protection against ip tumour challenge persists for a much longer period in recipients when spleen cells are transferred locally rather than systemically. Since the iv transfer experiments and Experiment 1 above were not carried out at the same time and different batches of donors at different times after immunization were used for these experiments, it could be argued that the the different rates of decay could be a reflection of the specific activity of the different spleen cell suspensions used for transfer. This is because if any of the spleen cell suspension had been particularly active, then a "non-limiting" cell dose would have been given, and 21 days may have been too short a time to detect any decay. To overcome this objection the decay of tumour resistance after ip or iv transfer was compared directly.

A large number of normal mice were injected ip or iv with 2.2×10^7 spleen cells prepared from the same batch of long-term immunized mice. At daily intervals for the first six days and then on the 12th day after transfer, recipients were chosen randomly and challenged with *EAT/Ag. The ^{125}I retention of mice challenged at various times after cell transfer is shown in Fig. 5.5. As expected, greater clearance of radioactivity occurred in mice injected with spleen cells ip than in mice injected iv. The level of protection that could be recalled after iv transfer increased to a maximum on days 3-4 and then

FIGURE 5.5

Comparison of the rate of decay of tumour resistance after iv or ip
transfer of spleen cells from 11RX-immunized mice

Spleen cell suspensions were prepared from mice immunized 56 days previously with 10^5 11RX iv. Recipients were injected with nothing (●) or 2.2×10^7 spleen cells iv (○) or ip (□). Different groups were challenged ip with 10^6 ^{125}I -EAT and 10 µg 11RX antigen at the following times:

A: at the time of transfer,

E: 4 days after transfer,*

B: 1 day after transfer,

F: 5 days after transfer,*

C: 2 days after transfer,

G: 6 days after transfer,*

D: 3 days after transfer,

H: 12 days after transfer,*

Each line shows the results from a group of 5 mice and each point shows the geometric mean ($\bar{x} \div \text{s.e.m}$) of the ^{125}I retained by individual mice at different times after transfer.

* please see the next page for panels E, F, G and H.

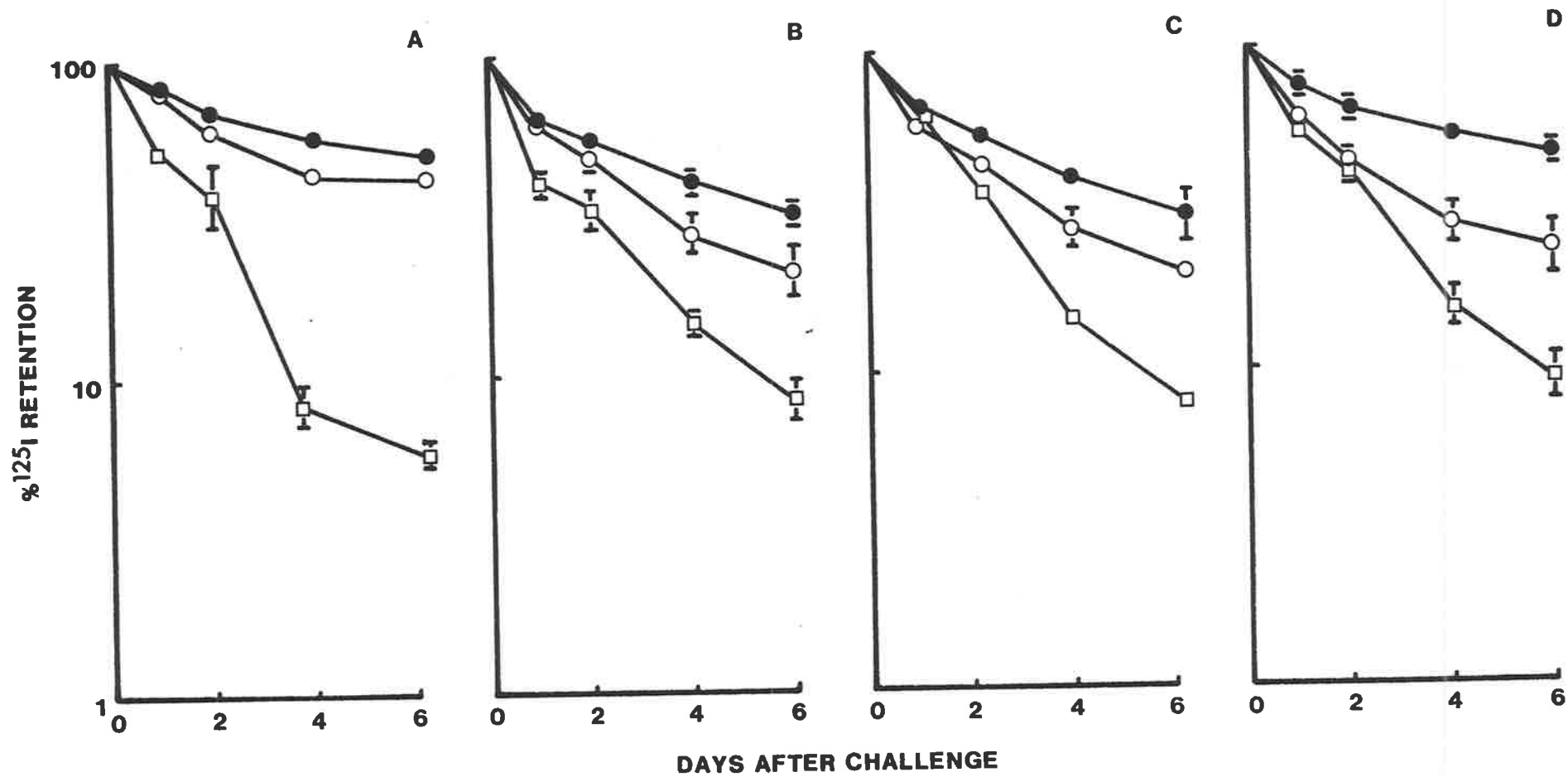


FIGURE 5.5 (CONTINUED)

The clearance of ^{125}I from spleen cell recipients challenged ip with 10^6 ^{125}I -EAT and 10 μg 11RX antigen at the following times after transfer:

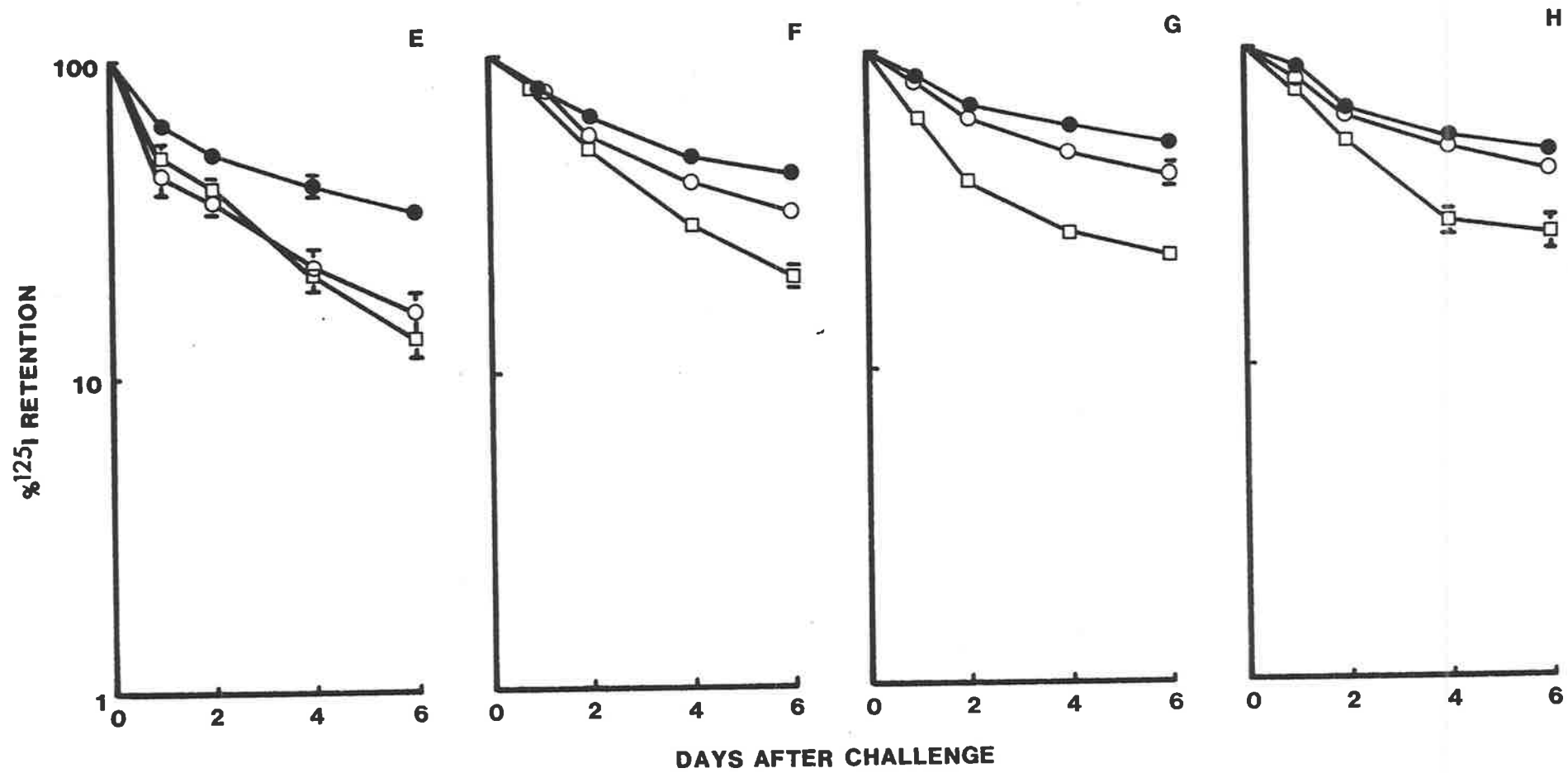
E: 4 days after transfer,*

F: 5 days after transfer,*

G: 6 days after transfer,*

H: 12 days after transfer,*

Each line shows the results from a group of 5 mice and each point shows the geometric mean ($\bar{x} \div \text{s.e.m}$) of the ^{125}I retained by individual mice at different times after transfer.



rapidly declined to normal levels. The protection afforded by ip transfer did not increase at any time but decayed steadily over the first 4 days and then remained relatively constant until day 12. The clearance curves of sets of recipients challenged on day 4 were coincident. If the functional half-life of the cells had been the same after iv and ip transfer, the level of tumour resistance that could be recalled would have decayed at the same rate so that by day 12 after transfer both groups would have cleared ^{125}I at the same rate as control mice. Since this was not the case, it follows that at least some of the lymphocytes in the spleens of long-term iv-immunized mice had a long life span. In addition, this experiment suggested there may be an initial decay in the activity of the spleen cells shortly after ip transfer. This could be due to the death of short-lived lymphocytes present as a subpopulation in the spleen cells transferred or due to the migration of a subset of lymphocytes out of the peritoneal cavity.

5.5.3 Experiment 3: Comparison of the decay of spleen cell activity in recipients and donors

It is possible that the estimates for the functional half-life of 11RX-sensitized T cells which mediate the recall of tumour resistance are inaccurate because of the unnatural site of transfer. The rate of decay was, therefore, compared to that occurring in the donors. A large batch of CB6F_1 mice were immunized iv with 11RX and some of them were used as spleen cells donors at various intervals between 67 and 93 days after immunization. In addition to this, on day 67 a large number of normal mice were injected ip with 4×10^7 spleen cells from some of the immunized donors. Over the next 27 days groups of these "day 67" recipients were taken at random and challenged with *EAT/Ag. At these same times some of the remaining donors were taken, spleen cell suspensions at various concentrations were prepared from them, mixed with *EAT/Ag and injected ip into normal mice. The clearance of ^{125}I in the

"day 67" recipients at various times after transfer was compared to that of "day 74", "day 78", and "day 94" recipients.

The results in Fig. 5.6 show that the degree of tumour resistance that could be transferred with a standard number of spleen cells decreased over a 24 day period. The degree of tumour resistance transferred with doses of 2×10^7 and 4×10^7 spleen cells from donors at day 70 was very similar. By day 6 after tumour challenge recipients retained only 4% of the counts injected into them, indicating that these doses of cells were "non-limiting". On day 74 after immunization, a dose of 1×10^7 spleen cells transferred less activity than the two higher cell concentrations, suggesting that this dose was becoming limiting. A dose of 2×10^7 spleen cells did not become limiting until 94 days after immunization.

The level of tumour resistance that could be recalled in "day 67" recipients over 26 days remained very high. For most of the experiment their activity paralleled that of mice which had received 4×10^7 spleen cells at the time of challenge, from mice immunized 70-78 days previously. By day 26 after transfer, the level of tumour resistance that could be recalled in "day 67" recipients had dropped to that transferred with 2×10^7 spleen cells from day 93 donor mice. These results suggest that the activity of llRX-sensitized lymphocytes decays at the same relatively slow rate or at only a slightly faster one in recipients than in donors. The three experiments presented above clearly show that ip tumour resistance can be recalled for a long time after local, but not systemic, transfer of spleen cells. These data correlate with the observation discussed earlier that tumour resistance can be recalled in llRX-immunized mice for a long time after immunization.

5.6 Summary and conclusions

The experiments presented in this chapter confirm and extend the results of Ashley et al. (1977) that the ability to recall ip tumour

FIGURE 5.6

The rate of decay of T cell activity in recipients
and 11RX-immunized spleen cells donors

A comparison was made of the tumour resistance that could be recalled in spleen cell recipients, and the resistance that could be transferred with spleen cells from donors at different times after immunization with 11RX.

A large group of mice were immunized with 10^5 11RX iv and 67 days later normal recipients were injected ip with 4×10^7 spleen cells (●) prepared from some of the donors. These recipients were challenged at various times after transfer with ^{125}I -EAT cells and $10 \mu\text{g}$ 11RX antigen. At different intervals from days 70 to 90 after immunization, other normal mice were injected ip with a mixture of 10^6 ^{125}I -EAT cells, $10 \mu\text{g}$ 11RX antigen and the following numbers of spleen cells from some of the remaining donors:

- 4×10^7 spleen cells (○),
- 2×10^7 spleen cells (▲),
- 1×10^7 spleen cells (□),
- no spleen cells (■).

Each line shows the results from a group of 5 mice and each point shows the geometric mean (x/\div s.e.m) of the ^{125}I retained by individual mice at different times after transfer.

DAYS AFTER IMMUNIZATION 70

DAYS AFTER TRANSFER 3

74

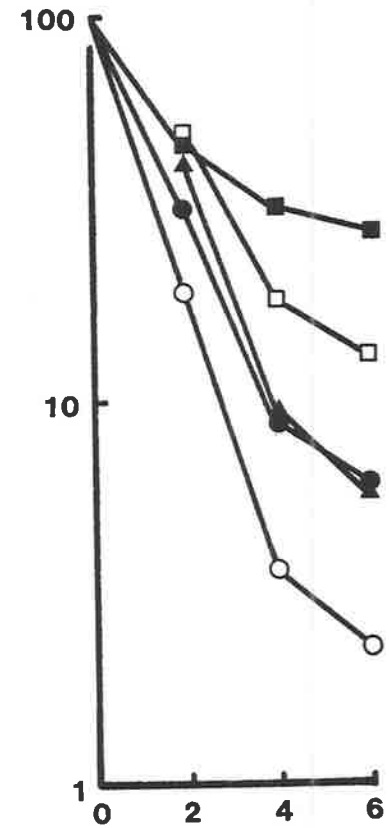
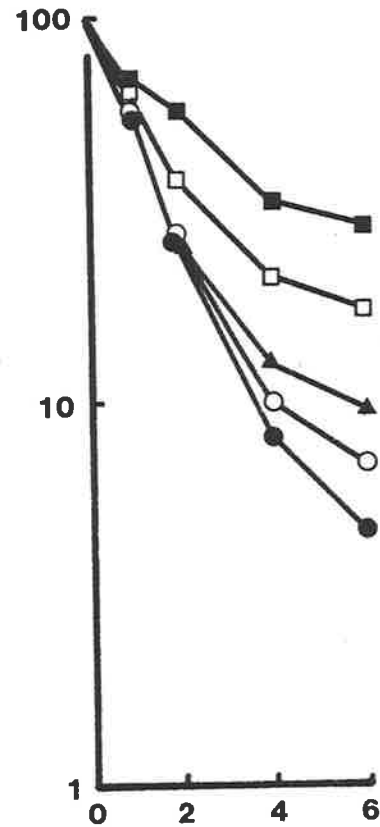
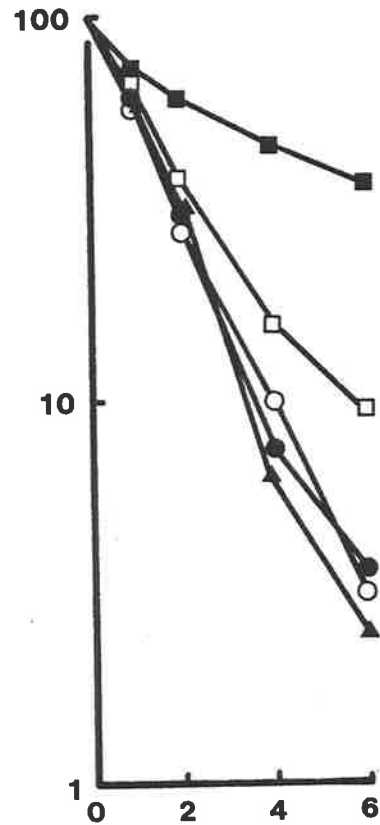
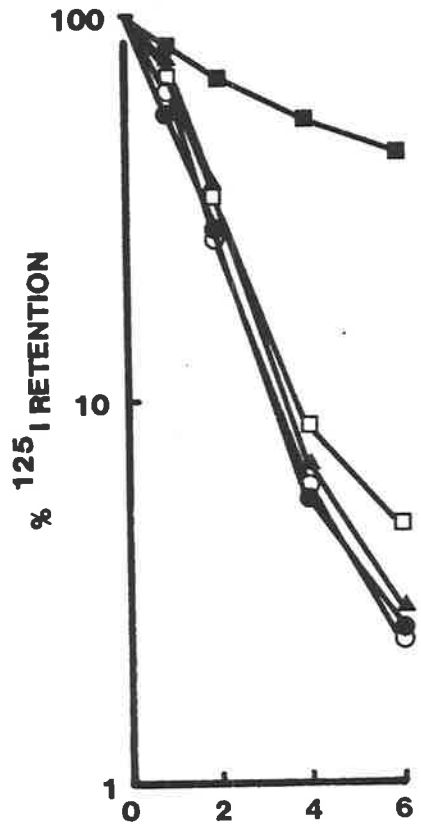
7

78

11

94

27



DAYS AFTER CHALLENGE

resistance can be transferred to naive recipients with spleen cells from long-term llRX-immunized mice. It was found that the systemic transfer of tumour resistance was weak and transient with the donor lymphocytes appearing to have a functional half-life of 4 days. The ip tumour resistance that could be recalled after local transfer of spleen cells was stronger and more enduring and it appeared that the activity of the llRX-sensitized T cells in the recipients decayed at the same rate as in the spleens of donor mice.

Because Ashley et al. (1977) established that the ability of recipients to recall tumour resistance was abolished if the spleen cells were pretreated with anti Thy 1.2 and complement, it is concluded that the recall of ip tumour resistance in long-term llRX-immunized mice is mediated by sensitized T cells with a long functional life span. It is possible that short-lived T cells may also have a role in recalling tumour resistance.

CHAPTER SIX

LYMPHOKINE RELEASE DURING LLRX INFECTION

6.1 Introduction

Following the initial demonstration that humoral immune responses required collaboration between thymus derived and bone marrow derived lymphocytes (Claman, Chaperon, and Triplett, 1966; Miller and Mitchell, 1968; Mitchison, 1971), cellular interactions have been shown to be involved in the control of all immune responses. Such interactions can be mediated, in vitro at least, by soluble factors released by lymphocytes (lymphokines) or macrophages (monokines). These molecules are produced by regulatory cells of the immune system and exert either a stimulatory or suppressive effect effect on cells of the same or different type.

During the immune response to intracellular bacterial parasites there is a proliferative response in the lymphoid organs of the infected animal which reflects, in part, the expansion of T and B lymphocyte clones specific for microbial antigens. Animals develop delayed hypersensitivity to the parasite antigens which is associated with cellular immunity (reviewed by Turk, 1975) and their macrophages have a greatly enhanced capacity to kill the infecting organisms as well as unrelated intracellular parasites and tumour cells (discussed in §1.3.2).

The possibility that soluble factors may be involved in the manifestation of CMI was first raised in the early 1960's as a result of in vitro analysis of the delayed hypersensitivity reaction. David, Lawrence and Thomas, (1964a) showed that, in the presence of specific antigen, small numbers of PC from guinea pigs showing DTH to hapten-protein conjugates inhibited the in vitro migration of macrophages

obtained from normal animals. Fractionation of the PC from immunized guinea pigs showed that the inhibition of macrophage migration was mediated by sensitized lymphocytes. Subsequently, the cell-free supernatants from such cultures were also found to inhibit the migration of normal macrophages in vitro (Bloom and Bennett, 1966; David, 1966). This activity in the culture supernatants was termed Migration Inhibition Factor (MIF) and has since been described in murine, rabbit and human systems (reviewed by Bloom, 1971; David and David, 1972; Rocklin et al., 1980).

The phenomenon of migration inhibition in vitro correlates well with the presence of DTH in the intact animal (David et al., 1964; Bloom and Bennett, 1966; Borel and David 1970). The PC from guinea pigs showing DTH to an antigen, but not those which only possess circulating antibody or are tolerant to the antigen, will be inhibited in their migration in vitro when cultured with the specific antigen (reviewed by Bloom, 1971). MIF has also been detected in the plasma and sera of BCG-immunized mice which have been injected iv with living BCG cells or Old Tuberculin (Salvin, Youngner and Lederer, 1973). Furthermore, the amount of MIF present in the sera of mice previously immunized with different BCG-cell-wall-in-oil vaccines and injected iv with Old Tuberculin correlates with their degree of resistance to BCG (Salvin et al., 1975).

In addition to MIF, a macrophage chemotactic factor (ChF) is present in the culture supernatants from antigen stimulated lymphoid cells (Ward, Remold and David, 1969; 1970). ChF has been described in systems using sensitized lymphoid cells from guinea pigs, rats and man (reviewed by Rocklin, Bendtzen and Greinender, 1980). Physicochemical analysis of active culture supernatants revealed that guinea pig MIF and ChF activities are on separate molecules (David and David, 1972; Rocklin et al., 1980). It was proposed that MIF and ChF may both play a role in vivo in the DTH reaction i.e. by attracting and ensuring the

accumulation of macrophages at the site where a DTH reaction is elicited. This is supported by the finding that injection of MIF-containing supernatants into the skin of normal guinea pigs results in the development of an inflammatory reaction which resembles DTH (reviewed by Bloom, 1971; and David and David, 1972) and can mimic in vivo phenomena associated with the hypersensitive state e.g. the "macrophage disappearance" reaction (reviewed by Rocklin et al., 1980).

Further evidence that lymphokines are involved in cellular immunity was provided by the finding that MIF-containing culture supernatants also have marked morphological, biochemical and functional effects on macrophages (Nathan, Karnovsky and David, 1971; Nathan, Remold and David, 1973) so that they resemble in vivo activated macrophages. After treatment with the culture supernatants, macrophages resemble in vivo activated ones in their bacteriostatic (Fowles et al., 1973) and tumouricidal activity (Piessens, Churchill and David, 1975). Whenever the same culture supernatants or sera have been assayed for MIF and MAF activity, they have both been present (Piessens, Churchill and David, 1975; Chapman and Hibbs, 1977). For this reason, it was accepted that MAF and MIF were different manifestations of the same biologically active molecule (Nathan et al., 1973; David, 1975). However, this has now been disproved by Kniep et al. (1981) who has succeeded in separating the two activities into two different molecular species and by Erickson et al. (1982) who have reported that a T cell hybridoma that releases MAF into the supernatant does not release MIF. The mechanism of macrophage activation as it is understood at present is discussed in detail in §1.6. It is generally accepted that activation of PC by MAF represents the in vitro manifestation of the mechanism which operates in vivo in animals infected with IBP and leads to the production of activated macrophages capable of killing the IBP.

Recent evidence indicates that other lymphokine activities may be present in culture supernatants of lymphoid cells obtained from animals

immunized with IBP. For example, IL2 is released by the PC from mice immunized with live Listeria (Finke, Sharma and Scott, 1981) and from the lymph node cells of mice infected with the metazoan parasite Nematospiroides dubius (Prowse, 1981) provided that lymphoid cells are cultured in the presence of appropriate antigen. Consistent with this is the observation of Kaufmann and Hann (1982) that addition of Listeria antigen could induce release of IL2 from cloned Listeria specific T cells which can confer immunity to normal mice.

The results of the present studies reported in the subsequent sections of this chapter support these recent observations. At the time studies were commenced, very few data had been published on the ability of lymphoid cells from animals infected with IBP to release MAF (Ruco and Meltzer, 1977) and none on their ability to release IL2 in vitro. Since then it has been established that (1) both MAF and IL2 are released when normal spleen cells are cultured in vitro with the presence of Con A (Fidler, Darnell and Budman, 1976; Watson et al., 1979) and (2) T effector cells, namely those capable of lysing target cells bearing the appropriate alloantigen, release IL2 when exposed in vitro to either Con A or specific antigen (Andrus and Lafferty, 1981). It therefore seemed worthwhile to establish whether lymphoid cells from llRX-immunized mice could release IL2 and/or MAF when they were cultured in vitro with llRX antigens. The lymphoid cells used were obtained from various anatomical sites of from mice at different times after infection with llRX. This was done to establish whether the release of IL2 could provide a more convenient and technically simpler assay for effector T cells responsible for CMI to IBP than assays for release of MAF and/or MIF. The results of these studies are presented in the following sections.

6.2 The release of Interleukin 2 by llRX sensitized lymphoid cells

Lymphoid cells from llRX-immunized mice were tested for their

ability to release IL2, by culturing them in 16 mm wells in 2 ml of serum-free EMEM containing 1×10^{-4} M 2-mercaptoethanol, 20 $\mu\text{g/ml}$ of 11RX antigen and 1.25 $\mu\text{g/ml}$ of Indomethacin in a gas atmosphere of 10% CO_2 , 7% O_2 , or 87% N_2 . The antigen concentration to be used was established in preliminary experiments which indicated that 20 $\mu\text{g/ml}$ of antigen induced more IL2 release than 2 or 20 $\mu\text{g/ml}$ (data not shown). Indomethacin was included in the cultures because a report by Lafferty et al. (1980) suggested that conditioned supernatants contain a factor which inhibits the expression of IL2 activity. Lafferty et al., (1980b) established that IL2 could not be detected in conditioned media unless they were concentrated 10-fold by ultrafiltration through Amicon PM10 membranes. The inhibitory material is apparently absorbed by Amicon PM-10 membranes (Lafferty et al., 1980b). Because the manufacturer states that prostaglandins are absorbed by such membranes it was considered possible that the inhibitor could be prostaglandins. For this reason Indomethacin, a known inhibitor of prostaglandins synthesis (Shen and Winter, 1977), was included in cultures of sensitized cells. An additional reason for adding Indomethacin to cultures has been provided quite recently. Walker et al. (1983) have shown that prostaglandins also inhibit IL2 production.

The presence of IL2 in conditioned supernatants was detected in a blast cell proliferation maintenance assay developed by Lafferty et al., (1980a). It relies on the ability of IL2 to maintain the division of 3 day Con A-activated blast cells of mice in culture overnight and is described in detail in §2.31.3. Briefly, conditioned media were screened for IL2 activity by preparing duplicate, 2-fold, serial dilutions of the test supernatants in 0.05 ml of EMEM supplemented with 10% FCS and 1×10^{-4} M 2-mercaptoethanol in multiwell tissue culture trays. 2×10^4 blast cells in 0.05 ml of medium were added to all the wells containing supernatant dilutions and the trays were incubated at 37° for 20 h. After this time, blast cell proliferation was measured by pulsing

each well with 1 μ Ci of ^3H -TdR for 4 h before harvesting the cells. Duplicate titrations of a standard lymphokine preparation of high IL2 activity were included in each assay as positive controls. The standard IL2 preparation was obtained in the same way as the IL2-rich CS used in the experiments in §4.5.2 and was used in the assay at a starting dilution of 1:100. "Reversion controls" were included to determine the number of counts per minute (cpm) that were incorporated by blast cells which had reverted to the non-dividing state. These consisted of 12-24 wells in which blast cells were incubated in fresh medium without lymphokine for the duration of the assay.

Spleen cells from long-term llRX-immunized mice were used in the initial experiments. These cells were used because it had been established that they could transfer the ability to recall tumour resistance to normal mice (Ashley et al., 1977) as well as resistance to challenge with llRX (Davies, 1975). In addition, PC were also tested because it had been reported that during Listeria infection, sensitized lymphocytes migrate to the peritoneal cavity (Jungi, 1981) and because experiments by Attridge, Kotlarski and Bushell (unpublished observations) showed that PC are rich in llRX-sensitized lymphocytes. The presence of sensitized lymphocytes in PC suspensions was established by demonstrating that PC can transfer to naive mice the the ability to recall tumour resistance and to manifest a local delayed hypersensitivity reaction to llRX antigens. Suspensions of spleen cells and PC from normal, unimmunized mice and mice immunized with llRX iv 54 days previously were used at a concentration of 5×10^6 for spleen cells and 2×10^6 and 4×10^6 for PC from immunized and normal mice respectively. These were cultured in a volume of 2 ml in 16 mm wells of Linbro tissue culture trays in the presence or absence of llRX antigen. Supernatants of the cultures were harvested at 20 h. The results in Table 6.1 established that sensitized lymphocytes from llRX-immunized mice release IL2 when cultured with llRX antigen in vitro and that, 54 days after immunization,

Table 6.1

Lymphoid cells from immunized but not normal mice release
IL2 when cultured with antigen[@]

Antigen added	I. Spleen*		I. PC*		N. Spleen*	N. PC*
	-	+	-	+	+	+
Dilution of CCS	³ H incorporated (CPM)					
4	1048	<u>5394</u> [§]	1601	<u>9319</u> [§]	1413	1788
8	1118	<u>4624</u>	1670	<u>5805</u>	1406	1886
16	1533	2131	2393	<u>4254</u>	1632	1502
32	1579	2310	1650	<u>2820</u>	1703	2198
64	ND [#]	ND	ND	2072	ND	ND

(@) Mice were immunized 54 days previously with 10^5 11RX iv.

(§) Underscored numbers are greater than 3 SD above the mean number of counts incorporated by blasts cultured in medium alone which was 2,600 CPM.

(*) I. Spleen = Spleen from immunized mice.

I. PC = PC from immunized mice.

N. Spleen = Spleen from normal mice.

N. PC = PC from normal mice.

(#) Not determined.

the peritoneal cavity contains a greater proportion of these cells than the spleen. The data also show that the presence of 11RX antigen in culture supernatants does not mimic IL2 because no activity was detected in supernatants of spleen cells and PC from normal mice cultured in the presence of 11RX antigen.

To determine the relationship between cell dose and IL2 activity of conditioned culture supernatants (CCS), the following experiment was performed. Mice immunized 14 days previously with 10^5 11RX iv were injected with 3 ml of 10% proteose peptone broth and their PC harvested 2 days later. The number of cells used in culture containing 11RX antigen was 4, 2, and 1×10^6 proteose peptone induced (ppi) PC/well. 4×10^6 ppi PC were also cultured in the absence of antigen. The results in Fig 6.1 show that the IL2 activity of CCS was linearly related to the number of lymphoid cells cultured because the amount by which the culture supernatants could be diluted before they lost IL2 activity doubled as the number of PC in the culture was doubled. As in the previous experiment, CCS in which ppi PC had been incubated were not capable of maintaining the division of Con A activated blast cells if 11RX antigen was omitted from the medium used for culturing the ppi PC.

6.3 Requirement for T cells in the release of IL2

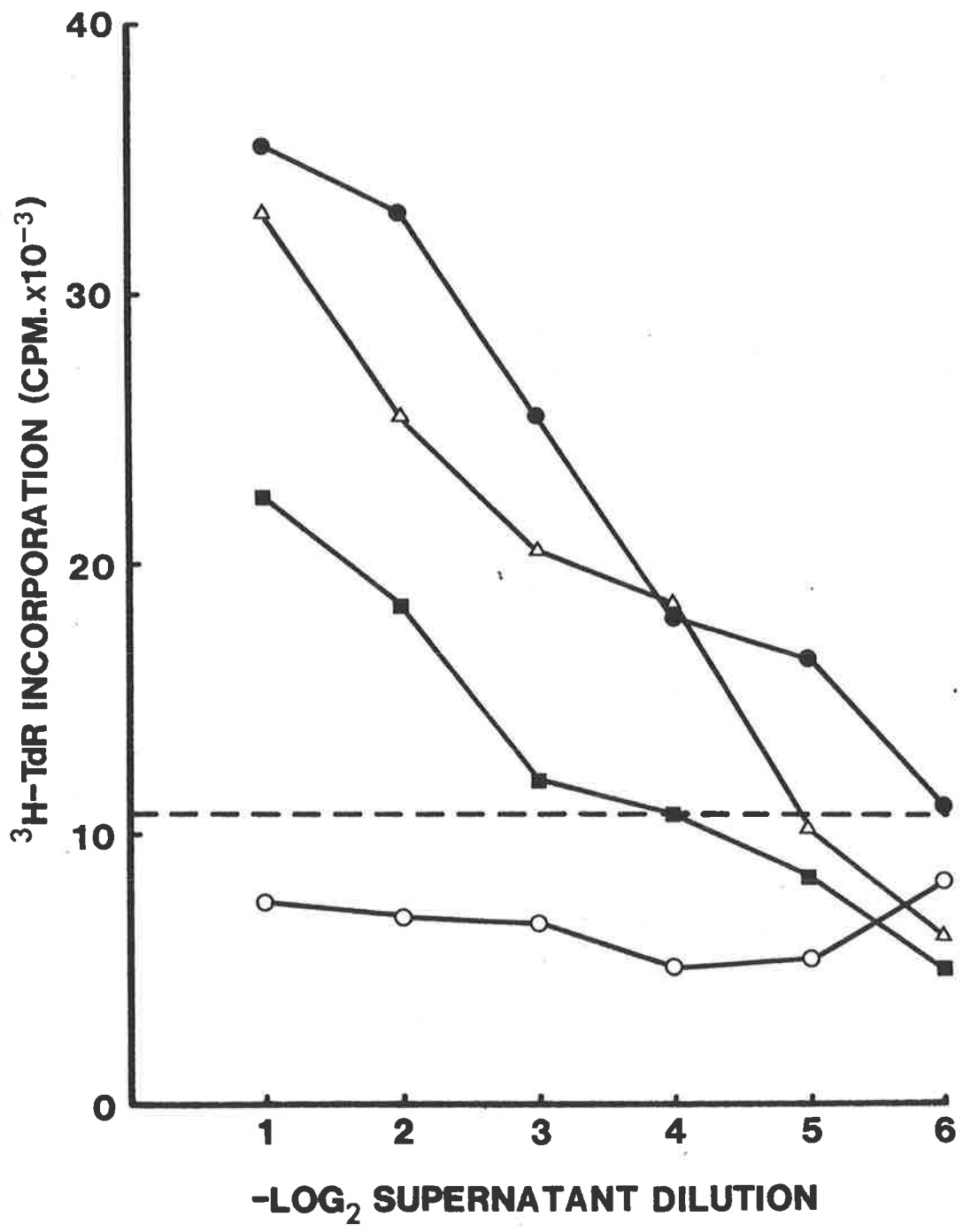
To establish that T cells are required for the release of IL2 by PC from 11RX-immunized mice the following experiment was performed. PC from day 29 ip immunized mice were incubated at 1×10^7 /ml in a 1:10 dilution of anti-Thy 1.2 antiserum at room temperature for 30 min. The cells were then centrifuged and resuspended to the same volume in EMEM containing 10% Guinea Pig Complement (GPC). After incubation at room temperature for 30 min, these cells were washed, resuspended and cultured at a density of 10^6 cells/ml in the presence or absence of 20 μ g of 11RX antigen/ml. Cultures containing 10^6 untreated PC and ones containing 10^6 /ml of PC which had been treated only with complement

FIGURE 6.1

The release of IL2 by ppi PC from 11RX-immunized mice

Mice were immunized iv with 1×10^5 11RX. On day 14 they were injected ip with 3ml of 10% proteose peptone broth. Their PC were harvested 2 days later and cultured for 20h in the presence or absence of 20 $\mu\text{g/ml}$ 11RX antigen. Cell culture supernatants (CCS) were taken and assayed for IL2 activity by culturing 2×10^4 blast cells for 20 h in dilutions of the various CCS. The CCS assayed were from cultures containing 1×10^6 (■), 2×10^6 (Δ), or 4×10^6 (●) ppi PC and 20 $\mu\text{g/ml}$ of 11RX antigen, or 4×10^6 ppi PC (○) incubated in the absence of antigen.

(-----) 3 SD above the mean number of CPM incorporated by blast cells incubated in fresh medium without IL2.



were also included. CCS were harvested after 20 h of culture and were tested for IL2 activity in the usual way. The CCS obtained from cultures of untreated PC or those treated with complement only, had a titre greater than 32 (data not shown). This was reduced to less than 4 when PC were treated with anti-Thy 1.2 and complement. The end point of an IL2 titration is taken as the reciprocal of the greatest dilution of CCS which induced incorporation of ^3H which was 3 SD above the incorporation obtained by with blast cells cultured without IL2.

6.4 The anatomical distribution of lymphocytes releasing IL-2 at various times after llRX immunization

Because results of the experiment reported in §6.2 indicated that lymphoid cell suspensions may vary in their ability to release IL2, the ability of lymphoid cells from different anatomical sites taken at different times after immunization were tested for their ability to release IL2 on exposure to antigen in vitro. The cells were obtained from the spleen, the mesenteric lymph nodes and the peritoneal cavity of immunized mice. In most of the experiments described below, spleen cells and LNC were cultured at a concentration of 5×10^6 per well in 2 ml of medium whilst the PC were usually cultured at 4×10^6 cells per well unless otherwise indicated. The CCS were harvested at 20 h and their IL2 activity assayed in the usual way. The end point was calculated in the usual way.

The results from one such experiment are shown in Table 6.2. They confirm the finding that lymphoid cell populations do not release IL2 in the absence of llRX antigen. Differences in ability to release IL2 were detected when antigen was added to the cultures. For example, no detectable IL2 activity was present in the CCS of antigen stimulated LNC cultures, whilst all three different PC suspensions were found to release IL2. In contrast, however, spleen cells from llRX-immunized mice released only small amounts of IL2 and then only late after immunization.

TABLE 6.2

The ability of various lymphoid cell populations to release
IL2 in vitro after llRX immunization

Immunization (time, route)	IL2 Titre					
	spleen		mLNC [§]		PC	
	+ [Ⓔ]	- [¶]	+	-	+	-
14 days, iv [@]	< 2	< 2	< 2	< 2	16	< 2
95 days, iv [#]	8	< 2	< 2	< 2	32	< 2
14 days, ip [*]	2	< 2	< 2	< 2	> 64	< 2

(@) Cells were taken from mice which had been immunized iv with llRX 14 days previously.
Spleen cells and LNC were cultured at 5×10^6 /well.
PC were cultured at 4×10^6 /well.

(#) Cells were taken from mice which had been immunized iv with llRX 95 days previously.
Spleen cells and LNC were cultured at 5×10^6 /well.
PC were cultured at 2×10^6 /well.

(*) Cells were taken from mice which had been immunized ip with llRX 14 days previously.
Spleen cells and LNC were cultured at 5×10^6 /well.
PC were cultured at 4×10^6 /well.

(§) Mesenteric lymph node cells.

(Ⓔ) Lymphoid cells cultured in the presence of antigen.

(¶) Lymphoid cells cultured in the absence of antigen.

The inability of LNC and spleen cells to release IL2 14 days after immunization with llRX may not necessarily be due to lack of llRX-sensitized lymphocytes. It is possible that these organs may not contain the appropriate antigen presenting cells, or that they contain cells which suppress the release of IL2. These possibilities were tested in the experiments described below.

6.5 Is the inactivity of LNC and spleen cells due to the lack of antigen presenting cells?

Prowse (1981) established that LNC from N. dubius infected mice would not release IL2 unless normal peritoneal cells were added to the cultures, presumably to act as antigen presenting cells. This implies that in LNC suspensions shortage of antigen presenting cells may limit the release of IL2. Therefore, experiments were conducted to determine whether LNC from llRX-infected mice could release IL2 when stimulated with llRX antigen in the presence of additional antigen presenting cells.

6.5.1 Ability of normal PC to present antigen

First, it was established that PC from normal mice can present antigen, as determined by release of IL2 from suitably sensitized lymphocytes. The cells used were obtained from PC of mice which had been immunized 67 days previously with 10^5 llRX iv. An aliquot of this PC suspension was depleted of macrophages by allowing them to adhere to plastic dishes for 1 h at 37° . Approximately 4% of the cells were recovered in the non-adherent fraction. 4×10^5 non-adherent cells were cultured in 2 ml of medium with 20 $\mu\text{g/ml}$ antigen in the presence or absence of 2×10^6 PC from normal mice. Control cultures which contained antigen and 2×10^6 normal PC or 4×10^5 non-adherent PC from immunized mice were included as controls. The supernatants were harvested in the usual way 20 h after incubation and the IL2 activity of these is shown in Fig 6.2. Whilst the unfractionated PC from immunized

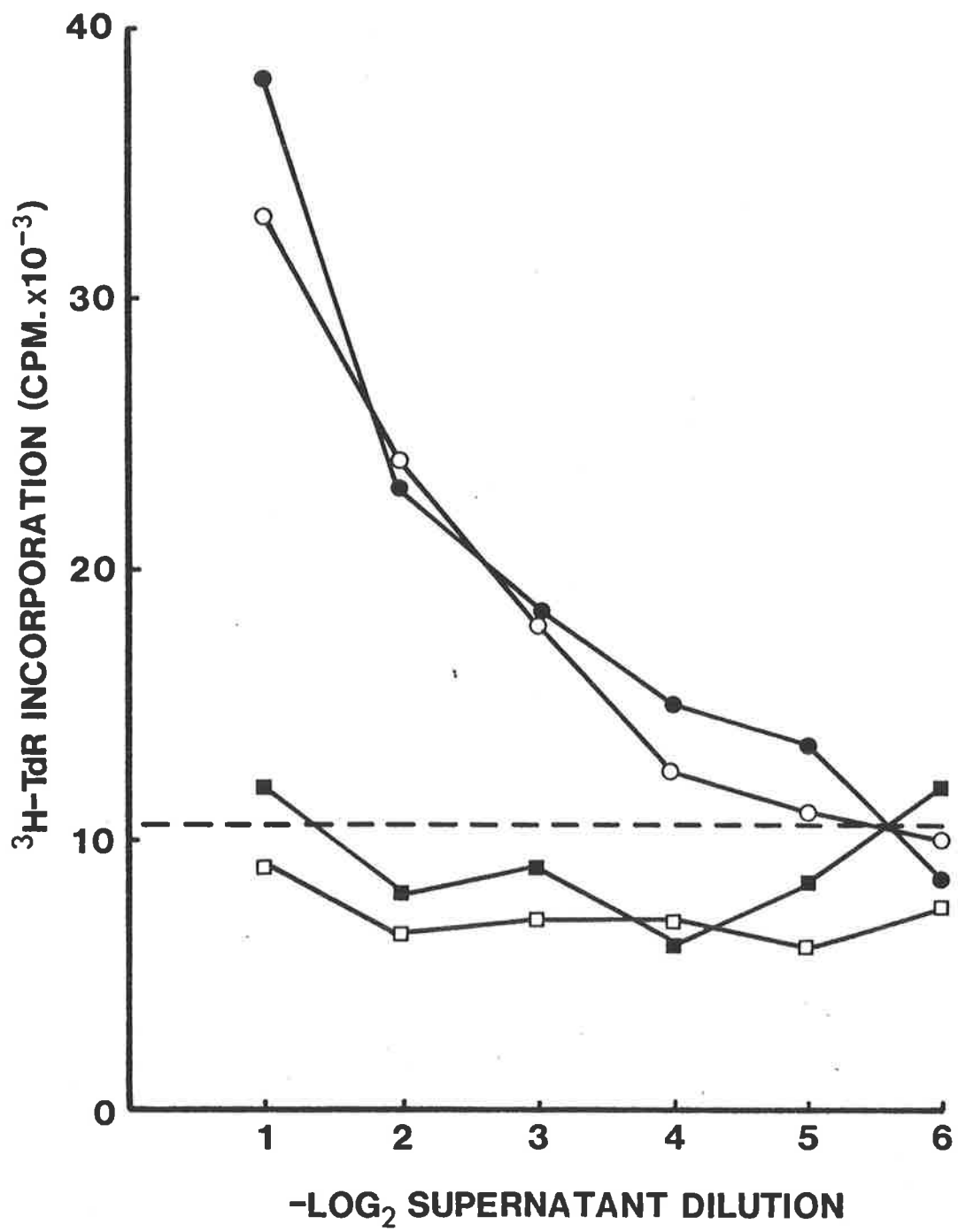
FIGURE 6.2

The ability of PC from normal mice to act
as antigen presenting cells

IL2 activity was assayed in the CCS of 20 h cultures in which the following numbers of cells from day 67 iv immunized mice were incubated with 11RX antigen:

- (●) 2×10^6 unfractionated PC from 11RX immunized mice.
- (■) adherent cells from 2×10^6 normal PC.
- (□) 4×10^5 non-adherent PC from 11RX-immunized mice.
- (○) 4×10^5 non-adherent PC from 11RX-immunized mice co-cultured with 2×10^6 PC from normal mice.

(-----) 3 SD above the mean number of CPM incorporated by blast cells incubated in fresh medium without IL2.



mice released IL2, neither the adherent PC from 11RX-immunized mice nor the non-adherent cells from such PC released the lymphokine when cultured with 11RX antigen. As expected, a mixture of these latter two populations released IL2, but only in the presence of 11RX antigen. Thus 2×10^6 normal PC could present antigen and induce the release of IL2 from sensitized lymphocytes.

6.5.2 Effect of adding normal PC on the ability of lymphoid cell suspensions to release IL2

(i) Lymph node cells

To ensure that lack of antigen presenting cells was not preventing release of IL2 from LNC, these cells were cultured with 11RX antigen in the presence of normal PC. Cell suspensions were prepared from the mesenteric lymph nodes and peritoneal cavities of mice immunized with 10^5 11RX ip 33 days previously. 5×10^6 LNC were cultured with 11RX antigen in the presence or absence of 2.2×10^6 PC from normal mice. As controls, duplicate cultures of 4×10^6 PC from the immunized mice and 11RX antigen were included. The results in Fig 6.3 show that PC from the the immunized mice released high levels of IL2 but LNC from the same animals did not release IL2 even when cultured in the presence of PC from normal mice.

(ii) Spleen cell suspensions

A similar experiment was carried out to determine whether spleen cells from short-term 11RX-immunized mice would release IL2 when cultured with 11RX antigen and normal PC. The results in Fig 6.4 show that the spleen cells from mice immunized iv 15 days previously did not release IL2 when incubated with 11RX antigen in vitro although their PC did. Addition of normal PC to the spleen cells cultured with 11RX antigen did not result in the release of IL2 in the culture medium.

These results suggest that the mesenteric lymph nodes and spleens of mice immunized with 11RX on day -33 or -15 respectively, did not

FIGURE 6.3

The inability of LNC to release IL2 in the presence
of normal peritoneal cells and 11RX antigen

IL2 activity was assayed in the CCS of cultures in which 5×10^6 LNC of mice immunized 33 days previously with 10^5 11RX ip were incubated for 20 h with 11RX antigen in the presence (■), or absence (□) of 2.2×10^6 PC from normal mice. In other cultures 4×10^6 PC from the immunized mice were cultured in the presence (●) or absence (○) of 11RX antigen.

(-----) 3 SD above the mean number of CPM incorporated by blast cells incubated in fresh medium without IL2.

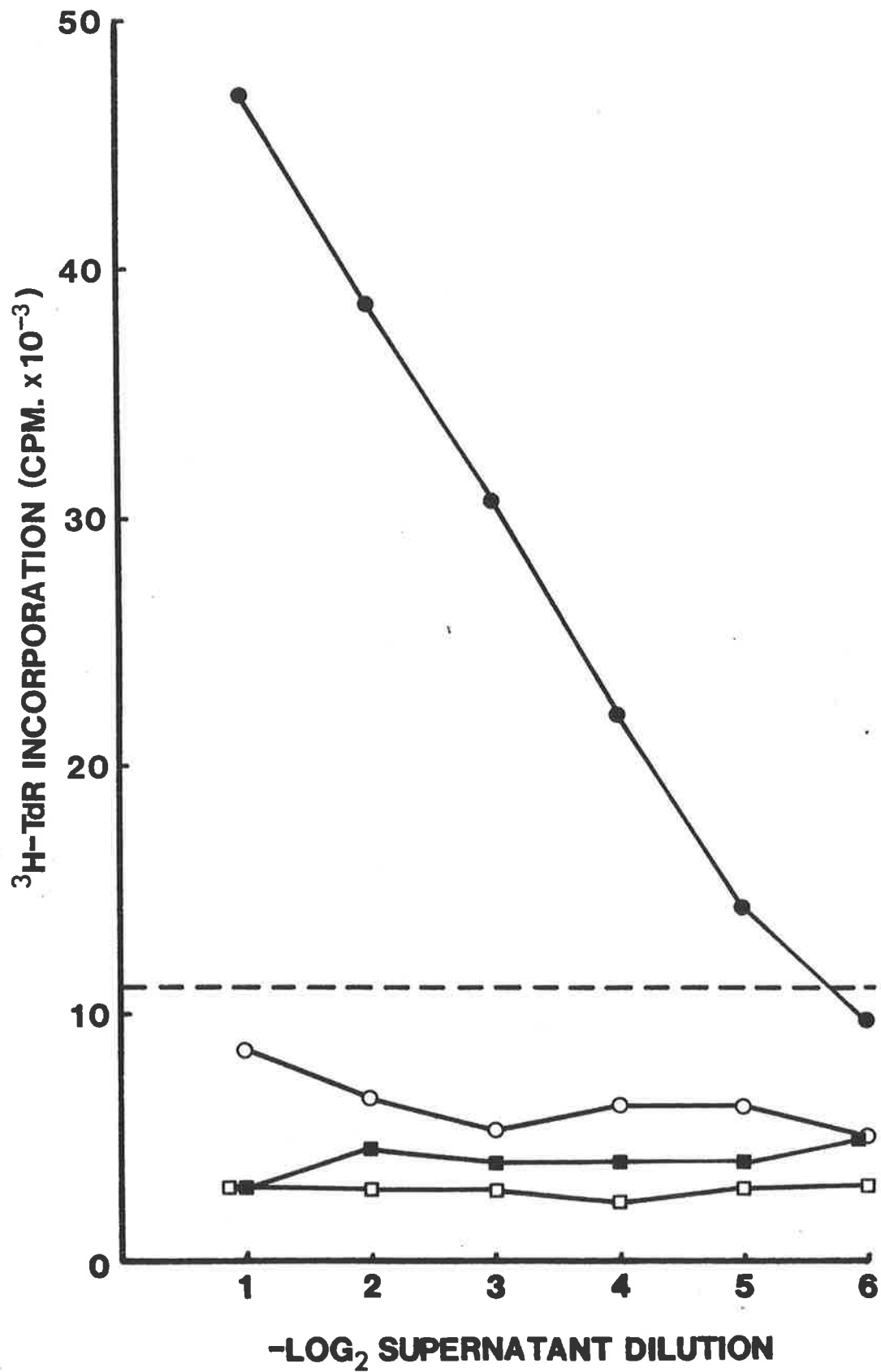
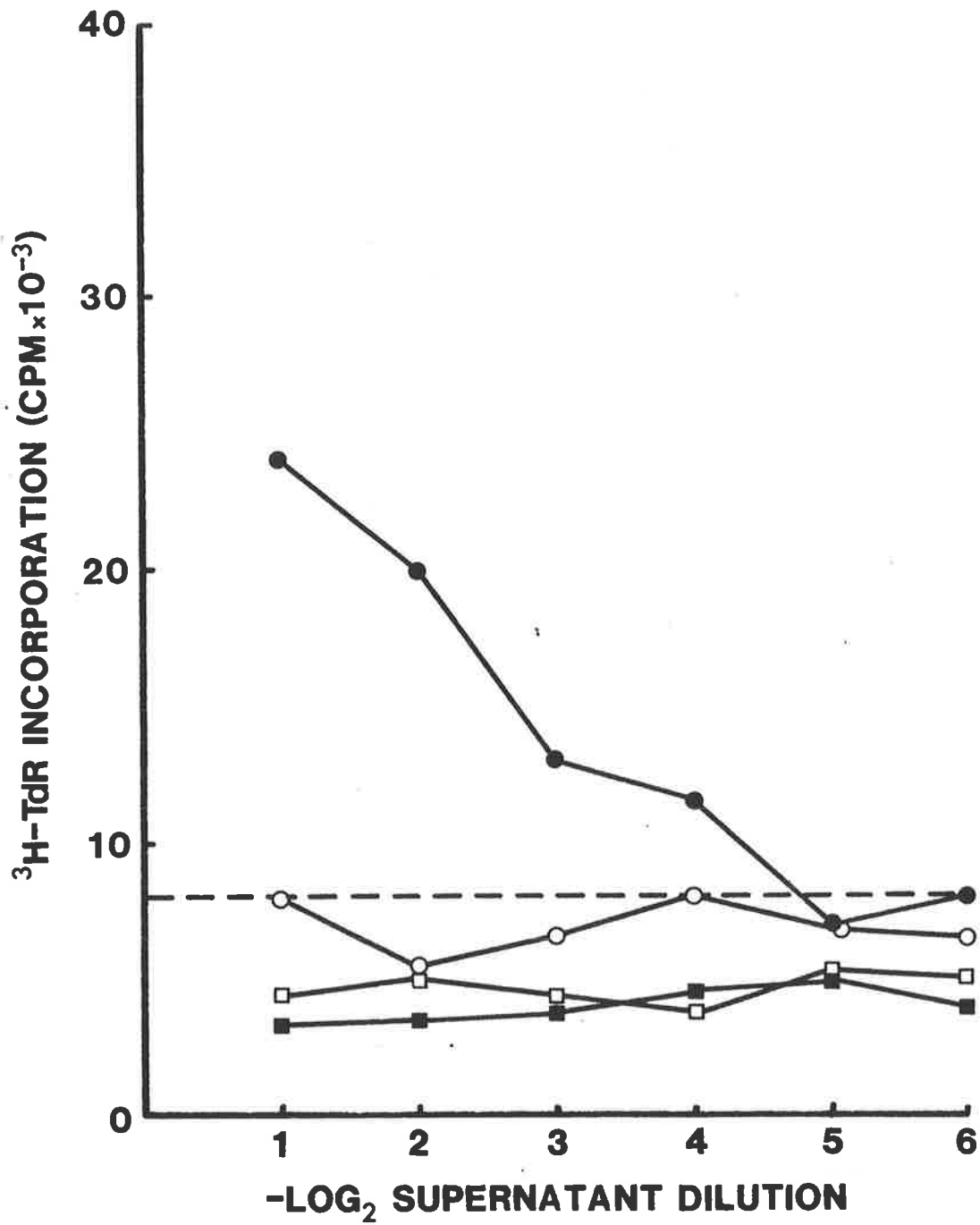


FIGURE 6.4

The inability of spleen cells early after 11RX infection to release
IL2 in the presence of normal peritoneal cells and 11RX antigen

Mice were immunized 15 days previously with 10^5 11RX iv. IL2 activity was measured in the CCS of 2 ml cultures where 5×10^6 spleen cells were incubated for 20 h with 20 $\mu\text{g/ml}$ of 11RX antigen in the presence (■), or absence (□) of 4×10^6 PC from normal mice. Control cultures contained 4×10^6 PC from the immunized mice with (●) or without (○) 11RX antigen.

(-----) 3 SD above the mean number of CPM incorporated by
blast cells incubated in fresh medium.



contain cells capable of releasing IL2. Alternatively, if they were present, release of IL2 may have been prevented by suppressor cells. The presence of suppressor cells which inhibit the ability of T and B cells to antigens in vitro, has been reported in the lymphoid organs of animals infected with intracellular parasites (Scott, 1972a, b; Wing and Remington, 1977; Kongshavn et al., 1977; Baird and Kaplan, 1977; Eardley and Jayawardena, 1977; Youdim and Sharman, 1976; Watson et al., 1975; Riglars and Cheers, 1980). The experiments presented in the following section address this possibility.

6.6 Is the lack of IL2 secretion due to suppression

The experimental design was as follows. Spleen cells or LNC from 11RX-immunized mice were co-cultured with PC from 11RX-immunized mice which could release IL2 in the presence of antigen. It was assumed that, if suppressor cells were present in the spleen or LNC suspensions, their presence would inhibit or reduce the release of IL2 from PC.

The results from four separate experiments are shown in Fig 6.5. In the first experiment (Fig 6.5A), 2×10^6 PC from mice immunized iv 66 days previously were cultured with 11RX antigen in the absence or presence of 5×10^6 spleen cells or LNC from mice immunized with 11RX iv 19 days earlier. This resulted in a 2-4 fold reduction in the IL2 titre of the CCS. Such an effect was not observed, however, when the experiment was repeated using the PC from day 61 iv immunized mice and the LNC or spleen cells from mice which had been immunized ip 14 days previously. The titration curves for all three supernatants were very similar (Fig 6.5B).

In the experiment shown in Fig 6.5C 5×10^6 spleen cells and 4×10^6 PC from mice immunized iv 15 days previously were cultured together or separately in the presence of 11RX antigen. In this experiment spleen cells did release IL2, but only at a low level. The addition of spleen cells to the PC cultures did not reduce the IL2

FIGURE 6.5

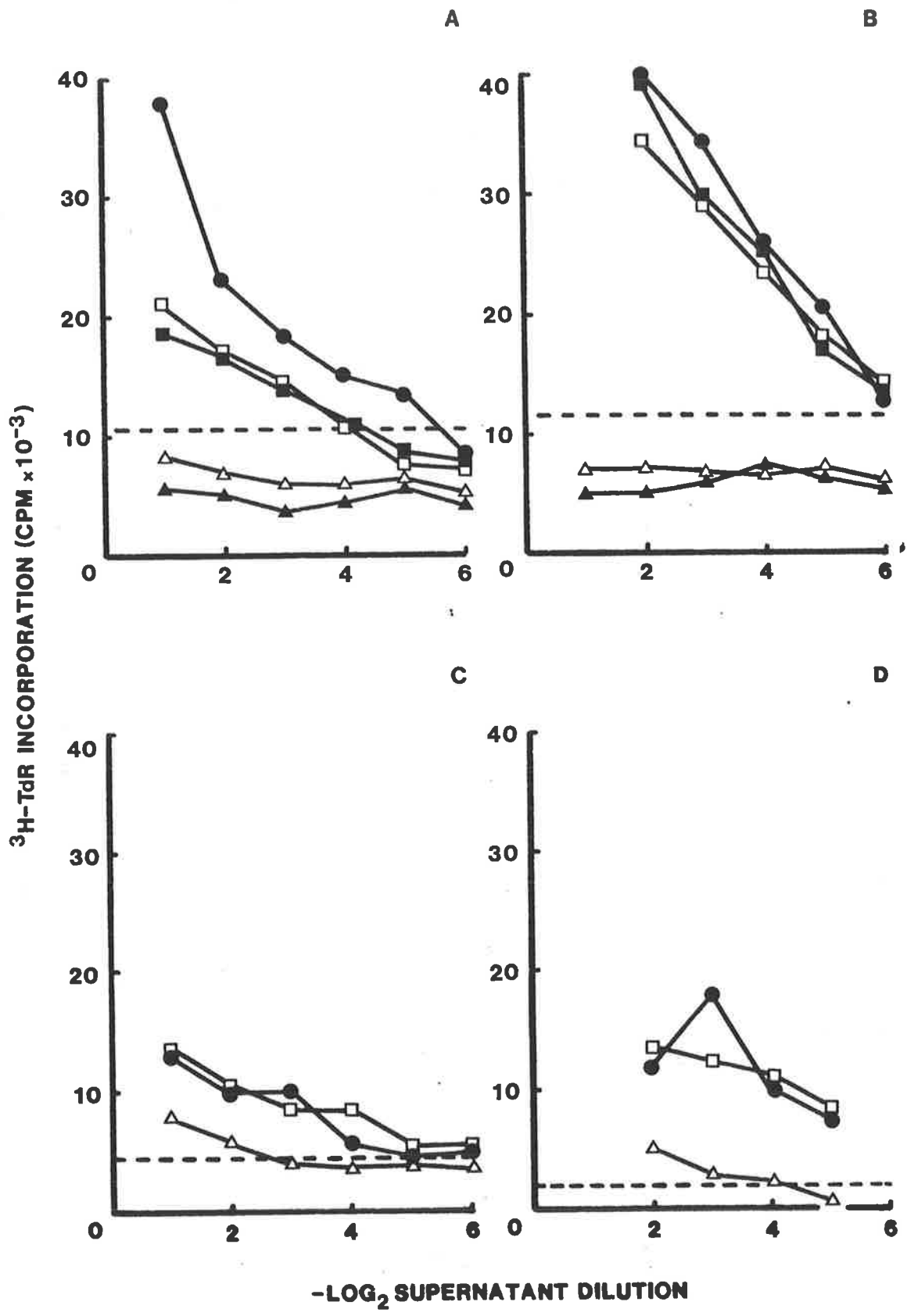
The lack of suppressive cells in the spleen
and LNC of 11RX-immunized mice

The results of 4 separate experiments investigating the possible presence of cells in spleens or lymph nodes of immunized mice which prevent the release of IL2 in vitro. IL2 activity was assayed in the supernatants of cultures in which the following cells were incubated for 20 h with 11RX antigen:

- (●) PC in the absence of other cell types.
- (□) PC with spleen cells.
- (■) PC with LNC.
- (△) LNC in the absence of other cell types.
- (▲) Spleen cells in the absence of other cell types.

The numbers of cells used in each of the experiments were as follows:

- A: 2×10^6 PC from Day 66 iv immunized mice.
 5×10^6 spleen cells or LNC from mice immunized iv 15 days previously.
- B: As for A except that PC were from day 61 iv immunized mice and the spleen cells and LNC were from mice immunized ip with 11RX 14 days previously.
- C: 4×10^6 PC and 5×10^6 spleen cells were obtained from day 15 iv immunized mice.
- D: As for A except that PC and spleen cells were both obtained from day 29 ip immunized mice.



activity of the CCS. A similar result was obtained using 2×10^6 PC and 5×10^6 spleen cells from day 29 ip immunized mice (Fig 6.5D). These experiments provided no evidence for the presence of suppressive cells in the spleens or the mesenteric lymph nodes of iv and ip immunized mice.

6.7 Preliminary attempts to detect MAF release

The initial experiments which were designed to obtain evidence that T cells from llRX-immunized mice could release MAF were carried out using PC from long-term llRX-immunized mice. These cells were used because they were not cytotoxic for tumour cells in vitro (Ashley, 1976; Ashley and Kotlarski, 1982a) and because the experiments reported in the previous sections of this chapter established that such lymphoid cells could release IL2 when stimulated with llRX antigen. The experimental approach used in the initial studies to detect MAF was the two-pronged one reported by Farr et al. (1979a). This involved attempts to activate the PC from long-term llRX-immunized mice by culturing the cells in the presence llRX antigen and to detect lymphokines in the conditioned media of such cultures. In addition, lymphocyte-enriched suspensions of the PC were prepared and their ability to activate macrophages of normal PC was determined by culturing a mixture of these two cell types in the presence and absence of llRX antigen. The experimental details and the results obtained are presented in the following two sections.

6.7.1 Activation of PC from long-term llRX-immunized mice

Normal mice and mice which had been immunized with llRX ip or iv 35-40 days previously were injected ip with 3 ml of 10% proteose peptone broth and their ppi PC were harvested 3 days later. In most experiments, the number of mice used was 4/group. A lymphocyte enriched (non-adherent) cell suspension was obtained from an aliquot of the ppi PC suspension of llRX-immunized mice by depleting the PC of adherent cells

using the method described in §2.19. Duplicate cultures of ppi PC from normal and 11RX-immunized mice were prepared by dispensing 10^6 PC with or without 20 ug/ml of 11RX antigen in 1 ml of medium (DMEM) containing HCO_3^- and FCS into 16 mm wells of Linbro tissue culture trays. In addition, duplicate wells containing a mixture of 10^6 normal ppi PC and 10^5 non-adherent ppi PC from 11RX-immunized mice and 20 μg of 11RX antigen in 1 ml were also prepared. Control wells containing 10^6 non-adherent ppi PC and 20 μg 11RX antigen were also included. This was done because of the observation reported in §3.7 that non-adherent PC prepared by the same method as that used in this series of experiments contained some macrophage-like cells. Following incubation of the cultures at 37° for 24 h in a gas atmosphere of 10% CO_2 , 7% O_2 and 83% N_2 , the CCS were harvested using aseptic technique, and stored frozen until they were used in the experiments described in the following section (6.7.2). Adherent monolayers of all the cultures were prepared by removing non-adherent cells using the washing technique described in §2.19. 10^5 ^{51}Cr -P815 cells were added to each washed monolayer in a 1 ml volume of culture medium. These cultures were incubated for a further 20 h using the same culture conditions as described above. After this time the top 0.5 ml of the culture medium in each well was harvested and the amount of ^{51}Cr present in each sample was measured to determine whether tumour cell killing had occurred.

The results of an experiment of this type are presented in Table 6.3. The data indicate that macrophages can be activated in vitro by a process that requires the presence of both antigen and sensitized lymphocytes. This was inferred from the finding that macrophages from the ppi PC suspensions of long-term 11RX-immunized mice were cytotoxic for ^{51}Cr -P815 cells if they were incubated with antigen whereas they were not cytotoxic if incubated in medium only (cultures 5 and 6, cf 2 and 3) and because macrophages from normal mice became cytotoxic only when normal ppi PC were cultured with 11RX antigen and non-adherent PC

TABLE 6.3

Activation of PC by 11RX antigen^(†)

Culture number	Source of PC ^(§)	11RX antigen	Non-adherent PC ^(#)	Cytolysis (%) ^(@)
1	normal	-	-	-1.5
2	D40 i.p.	-	-	-2.5
3	D40 i.v.	-	-	-1.1
4	normal	+	-	0.4
5	D40 i.p.	+	-	20.5
6	D40 i.v.	+	-	15.9
7	normal	+	D40 i.p.	25.5
8	normal	+	D40 i.v.	29.5
9	-	+	D40 i.p.	-0.3
10	-	+	D40 i.v.	0.5

(†) 10^6 PC from long-term 11RX-immunized mice or normal mice were incubated for 24 h in 16 mm wells in the presence or absence of 20 µg/ml of 11RX antigen. The adherent cells were then tested for cytotoxic activity.

(§) All mice were injected ip with 3 ml of 10% proteose peptone broth 3 days prior to harvesting PC.

(#) 10^6 non-adherent ppi PC from 11RX-immunized mice were added to some cultures as indicated.

(@) The cytolysis of 10^5 ^{51}Cr -P815 cells measured at 20 h, mean of two replicate cultures.

from 11RX-immunized mice (cultures 7 and 8, cf 1 and 4). It is unlikely that the cytotoxic activity of the macrophages in cultures 7 and 8 was due to the contamination of the non-adherent PC with macrophages from 11RX-immunized mice because the few adherent cells which were present after the lymphocyte enriched (non-adherent) PC had been incubated for 20 h with 11RX antigen did not lyse ^{51}Cr -P815 cells (cultures 9 and 10). These data confirm the results of Farr et al. (1979) and are consistent with the notion that during 11RX infections macrophage activation is mediated by sensitized lymphocytes, probably involving the release of MAF.

6.7.2 Activation of macrophages by conditioned culture media

To determine whether sensitized lymphocytes of 11RX-immunized mice released MAF, the CCS obtained from the experiment discussed above were tested for MAF activity. Two-fold dilutions of the CCS were made in fresh culture medium (DMEM). PC were obtained from normal mice injected 3 days previously with 3 ml of 10% proteose peptone broth. A series of duplicate cultures containing 10^6 PC and 1 ml of a particular dilution of one of the various CCS were prepared in 16 mm wells and incubated at 37° for 24 h. After removing the non-adherent cells and supernatants by using the washing technique described previously, 10^5 ^{125}I -P815 cells were added to each macrophage monolayer. The trays were incubated for 40 h and the amount of ^{125}I released into the medium was measured at this time. The incubation period was extended to 40 h because it seemed possible that the sensitivity of the assay would be increased by doing this. ^{125}I -UdR was used to label P815 instead of ^{51}Cr this isotope gave a lower spontaneous release in long-term assays (see §6.16). The results of this experiment are shown in Table 6.4. They establish that only the CCS from cultures 5, 6, 7, and 8 (as described in Table 6.3) were capable of activating ppi macrophages from normal mice to kill tumour cells. The cultures from which these CCS were obtained were the

Table 6.4

Macrophage activation by culture supernatants

Culture number providing CCS [@]	Concentration of CCS (%)			
	50	25	12.5	6.3
	% Cytotoxicity [§]			
1	4.2	N.D.*	N.D.	N.D.
2	2.8	N.D.	N.D.	N.D.
3	1.3	N.D.	N.D.	N.D.
4	1.2	0.8	1.4	5.2
5	19.7	11.7	0.6	0
6	16.5	10.3	1.6	0.5
7	17.2	4.4	-3.1	-1.4
8	12.3	2.0	-1.3	-1.6
9	0.6	0.3	1.0	-0.1
10	0	-0.1	0.1	-0.6

(@) Supernatants from cultures described in Table 6.3.

(§) Measured at 40 h. The spontaneous release of ¹²⁵I was 19.4% whilst release of label in the presence of untreated macrophages was 15.3%.

(*) Not determined.

only ones which had cytotoxic activity when assayed after 20 h culture with 11RX antigen (Table 6.3). In other words, the results indicate that the activation of peritoneal macrophages which was detected in the previous experiment was probably mediated by soluble factors. The data also indicate that MAF was not released from lymphoid cells in the absence of antigen and that 11RX antigen did not cause the release of MAF from lymphoid cells of unimmunized mice (CCS 1, 2, 3 and 4). The fact that induced macrophages from normal mice cultured in supernatants 9 and 10 were not cytotoxic indicates that sensitized lymphocytes could release MAF only in the presence of 11RX antigen and adherent cells. It appears that the number of contaminating adherent cells present in the lymphocyte-enriched PC population was too low to induce the release of detectable levels of MAF in the presence of 11RX antigen. The finding that CCS 4, 9, and 10 did not activate macrophages indicates that the 11RX antigen in the culture medium did not mimic the effects of MAF.

6.8 Development of an assay for MAF in culture supernatants

Because of the intention to assay a large number of samples, it was decided to scale down the MAF assay using multiwell tissue culture trays. The following protocol was adopted and is described in detail in §2.31.4. CCS were titrated using two-fold serial dilutions in a volume of 0.1 ml of culture medium (DMEM supplemented with HCO_3^- and FCS) and 5×10^5 ppi PC were added to the wells in 0.1 ml of the same medium giving a final volume of medium of 0.2 ml per well. In at least 4 wells ppi PC were incubated in fresh medium alone. Unless otherwise stated, the ppi PC used in all subsequent experiments were obtained from normal CB6F₁ mice 48 h after ip injection of 2 ml of 10% proteose peptone broth. The trays were incubated for 24 h at 37° in a humidified atmosphere containing 10% CO₂ in air. Prior to the addition of labelled target cells, the medium was removed from all wells and replaced with 0.1 ml of warm suspension medium (EMEM or DMEM). The non-adherent

PC were removed by resuspending them using a tray shaker and aspirating the medium from each well. All the adherent monolayers of cells were washed by repeating this procedure. Ten thousand ^{51}Cr -P815 cells were then added to each well in a volume of 0.2 ml of culture medium (DMEM) and the trays incubated again using the conditions discussed above. 20 h later 0.1 ml of supernatant was obtained from each well and the amount of ^{51}Cr present in each sample was measured. A dilution of CCS was considered to have detectable MAF activity if the cytotoxic activity of ppi macrophages incubated in it was greater than 3 standard deviations above the mean cytotoxic activity of macrophages from ppi PC incubated in fresh medium alone. In the following experiments three preparations of MAF were used as standards. They were prepared as described in §2.32.1 and will be referred to as SMP1, SMP2 and SMP3.

To determine whether the amount of cytolysis detected was dependent on the number of PC used, an experiment was carried out according to the above protocol using dilutions of SMP 1 as a source of lymphokine and 1.3×10^5 , 2.5×10^5 or 5×10^5 ppi PC per well. The ppi PC were obtained from normal mice injected with 3 ml of proteose peptone broth 3 days previously. Fig 6.6 shows that the greatest amount of cytotoxicity occurred when 5×10^5 ppi PC were added to the wells. Accordingly, this number of PC was added to the wells in all subsequent assays for MAF (unless otherwise indicated). This number of ppi PC formed virtually a complete monolayer of adherent cells.

6.9 T cells are required for the release of MAF

To confirm that T cells are also required for the release of MAF by PC from 11RX-immunized mice, aliquots of the culture supernatants from the experiment in §6.3 in which anti-Thy 1.2 and complement treated PC were cultured with 11RX antigen were assayed for MAF activity. Cytotoxicity was tested in an 20 h assay using ^{51}Cr -P815 cells as targets. The results in Table 6.5 confirm that the release of MAF

FIGURE 6.6

The cytotoxic activity of macrophages from ppi PC
treated in vitro with two-fold dilutions of SMP1

1.3×10^5 (■), 2.5×10^5 (Δ), and 5×10^5 (●), ppi PC were treated for 24h with SMP1 and the macrophages present in each cell suspension were then tested for their cytotoxic activity against 10^4 ^{51}Cr -P815 cells in a 20h assay. The spontaneous release of ^{51}Cr was 17.9%.

Vertical bars represent 1 SD from the mean.

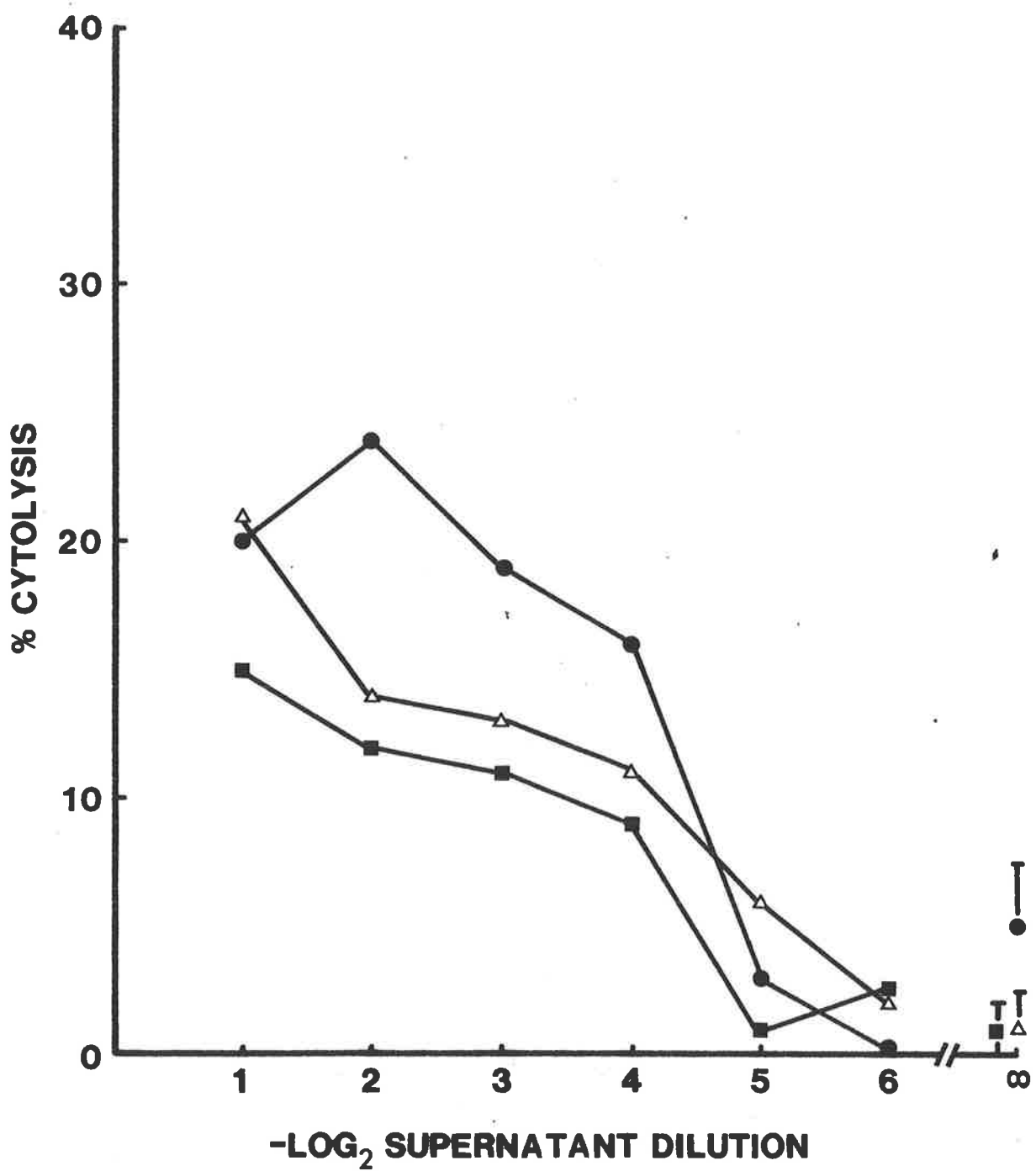


TABLE 6.5

Effect of anti-Thy 1.2 and complement on the release of MAF
by lymphoid cells from 11RX-immunized mice

Treatment of PC used for preparation of CCS [#]	% Cytolysis* (20 h)
Medium only	33
GPC only	37
Anti-Thy 1.2 and C'	6

(#) The CCS were prepared by incubating the PC from mice immunized 29 days previously with 10^5 11RX ip, pretreated as described above, at a concentration of 10^6 /ml with $20 \mu\text{g/ml}$ of 11RX antigen for 20 h. The CCS were removed and stored at -20° until assayed.

(*) MAF activity was determined by incubating 5×10^5 ppi PC for 24 h with a 1/2 dilution of CCS from the cultures described above. The adherent cells from such cultures were tested for cytotoxic activity against 10^4 ^{51}Cr -P815 cells in a 20 h assay.

requires the presence of T cells because media conditioned by anti-Thy 1.2 and complement-treated 11RX-sensitized PC did not activate macrophages from ppi PC. Treatment of PC with GPC alone had no effect. In addition, the result further indicates that MAF activity in culture supernatants was not simply due the presence of 11RX antigen.

6.10 The cytostatic activity of lymphokine-treated macrophages

From microscopic examination of the cultures in the multiwell tissue culture trays at the conclusion of each MAF assay, it soon became evident that during the 20 h period when tumour targets were incubated with macrophage monolayers, the number of tumour cells present in various wells increased, decreased or remained the same. In other words, treatment of ppi PC with CCS produced macrophages which were cytotoxic, cytostatic or allowed the multiplication of tumour target cells. It also became apparent that ^{51}Cr release detected only with those macrophage monolayers which were cytotoxic for tumour cells. The possibility that cytostatic as well as cytotoxic effects could be measured in the same MAF assay were tested in the following experiment.

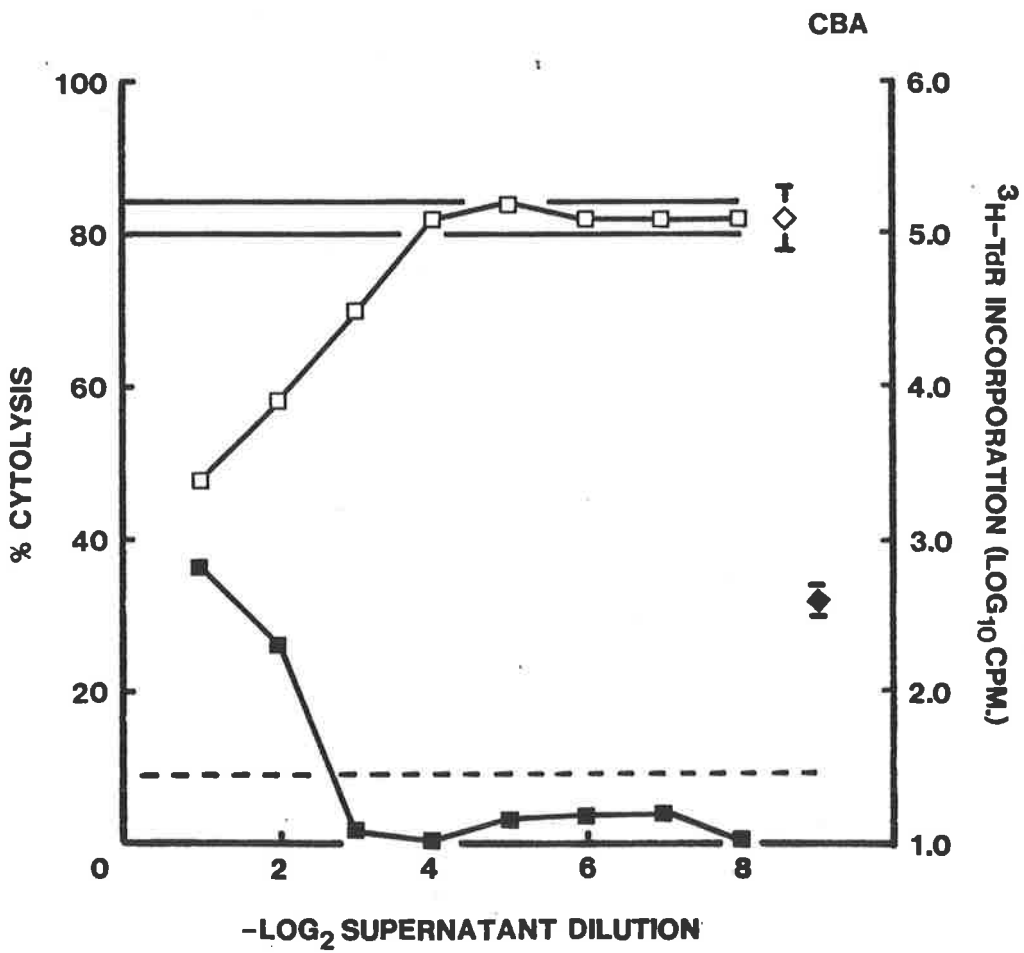
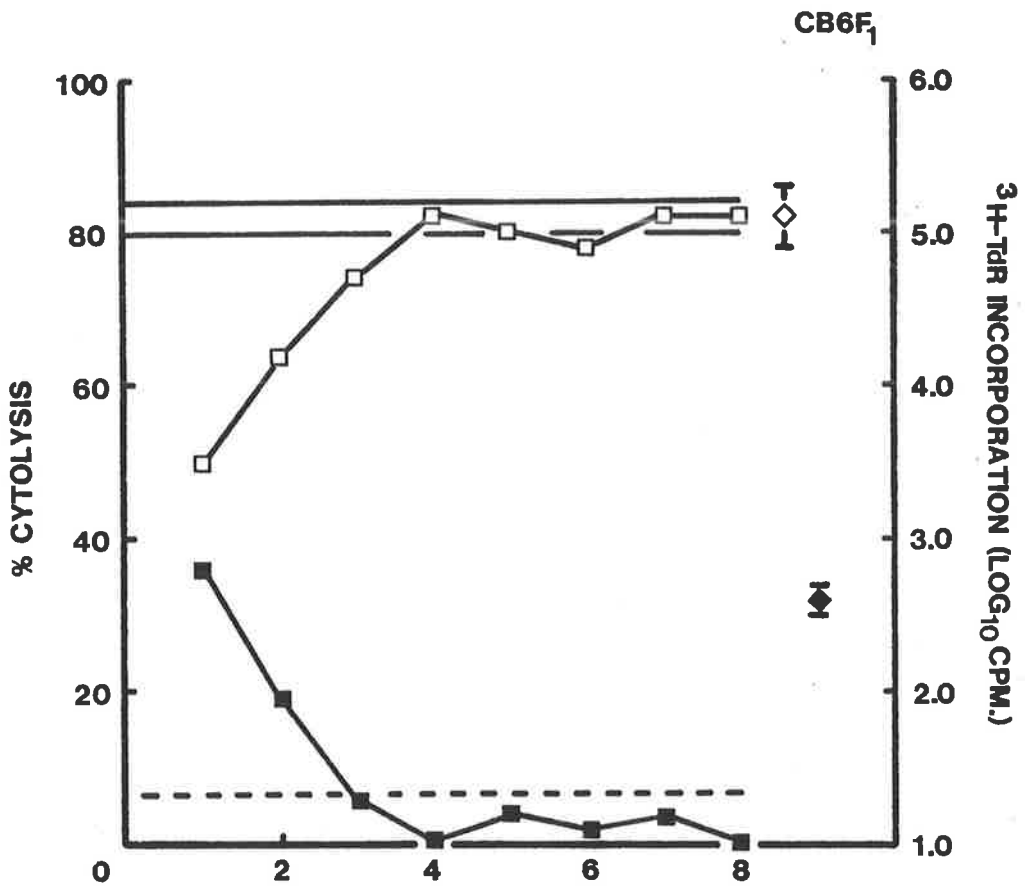
A MAF assay using ppi PC of CB6F₁ and CBA/J mice and various dilutions of SMP1 was performed using the standard protocol. At the conclusion of the assay, 1 μCi of $^3\text{H-TdR}$ (in 0.025 ml) was added to each of the wells. After the trays had been incubated at 37° for 4 h in 10% CO₂ in air, the contents of each well were harvested on to glass fibre discs using a cell harvester and the amount of $^3\text{H-TdR}$ incorporated measured by liquid scintillation counting. The results obtained confirmed those obtained by visual examination of the wells prior to cell harvesting (Fig 6.7). Large clusters of tumour cells were seen in wells which were subsequently found to incorporate a great deal of the $^3\text{H-TdR}$ added. Not surprisingly, very little $^3\text{H-TdR}$ was incorporated in the wells where cytolysis had occurred and very few tumour cells were seen. As the cytolysis decreased, $^3\text{H-TdR}$ incorporation

FIGURE 6.7

The cytolytic and cytostatic activity of macrophages
from ppi PC treated with lymphokine

PC were harvested from normal CB6F₁ and CBA mice which had been injected ip 2 days previously with 2ml of 10% proteose peptone broth. A standard MAF assay was carried out using these cells and the standard SMP1. At the completion of the assay 1 μ Ci of ³H-TdR was added to the wells. After a further 4 h incubation the cells were harvested and the incorporation of ³H measured by liquid scintillation counting.

- (■) Cytolysis of ⁵¹Cr-P815 cells
- (□) CPM incorporated by cells pulsed for 4h with ³H-TdR.
- (◇) Incorporation of ³H-TdR by ⁵¹Cr-P815 cells cultured in medium alone, (spontaneous release control wells).
- (◆) Background level of radioactivity detected by liquid scintillation counting of unpulsed ⁵¹Cr-P815 cells.
- (-----) The level of cytolysis which is 3 SD above the mean cytolysis shown by macrophages from untreated induced PC.
- (————) 3 SD above and below the mean number of CPM incorporated by ⁵¹Cr-P815 cells which had been pulsed for 4h with ³H-TdR after being cultured for 20 h with macrophages from untreated induced PC.



increased until it reached a maximum in the wells containing macrophages pretreated with a 1:16 or higher dilution of SMP1. It was also evident that cytostatic effects could be detected. Wells containing macrophages of ppi PC pretreated with a 1:8 dilution of SMP1 had fewer tumour cells than those where higher dilutions of SMP1 had been used. These wells showed little, if any, cytotoxicity towards ^{51}Cr -P815 cells but induced a great deal of cytostasis. Similar results were obtained using both CBA and CB6F₁ PC indicating that the lymphokine activities detected were not mouse-strain specific. Although the results indicate that "limiting" dilutions of MAF could induce cytostatic rather than cytolytic effects on ppi PC, they did not suggest that assaying for cytostasis would greatly increase the sensitivity of the assay for lymphokine activity. However, the experiments in the following section (6.11.1) suggest that, on certain occasions, the cytostasis assay may be markedly more sensitive.

6.11 Variability of the MAF assay

The results in the previous sections clearly established that MAF could be detected using a 20 h ^{51}Cr release assay. However, results obtained using SMP1 as the source of lymphokine(s) indicated that the assay for MAF was subject to a great deal of variation.

6.11.1 Lack of reproducibility of MAF assays using SMP1

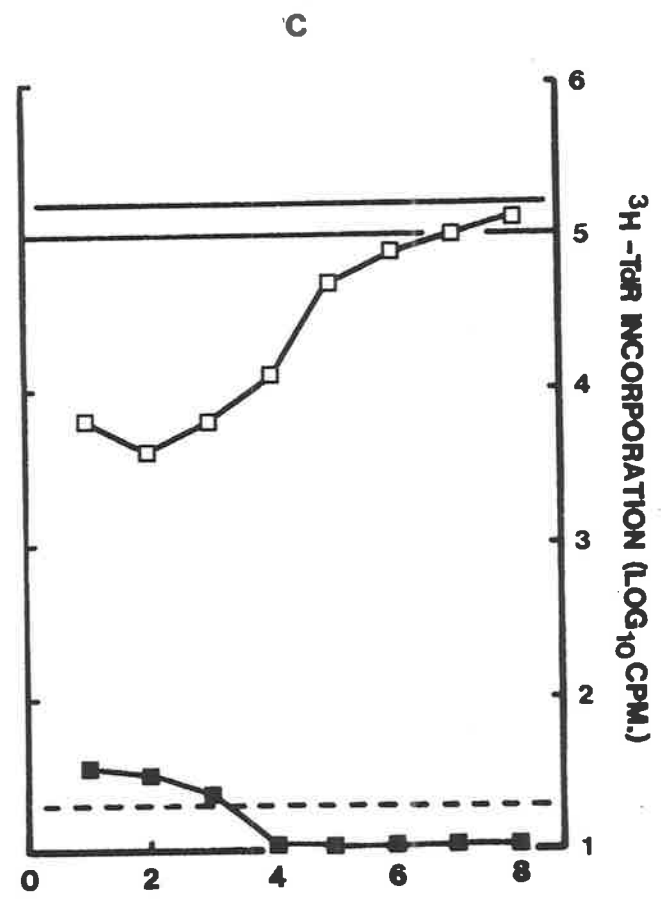
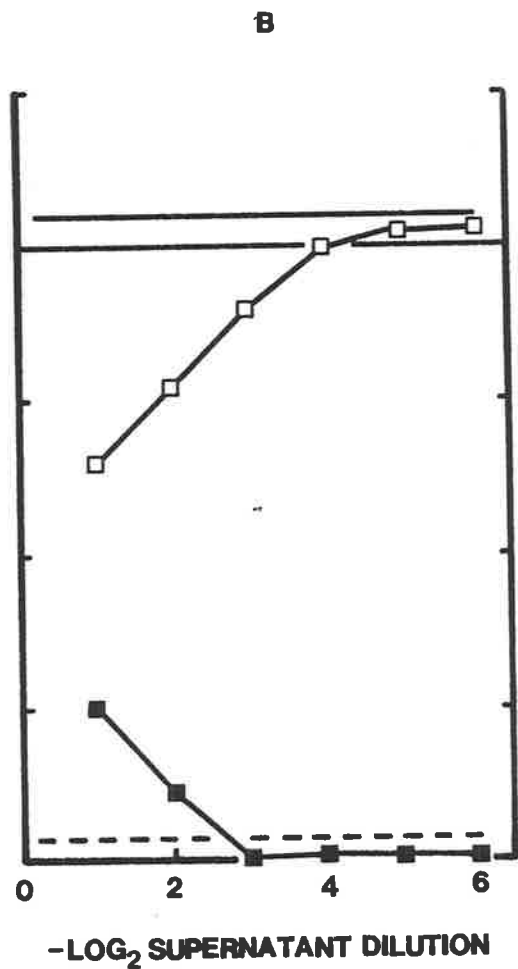
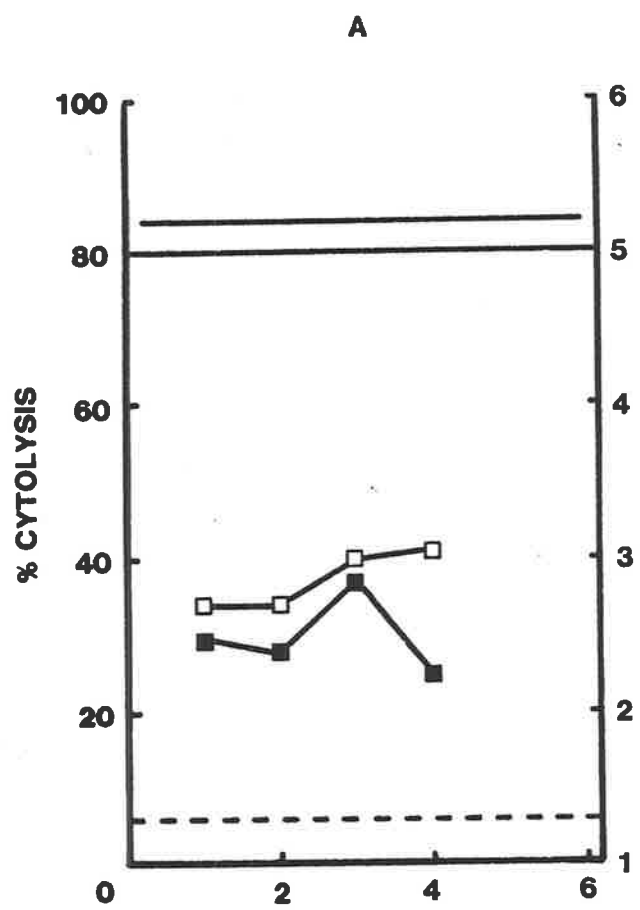
The cytotoxic and cytostatic activity of macrophage monolayers prepared from ppi PC treated with SMP1 in three different experiments is shown in Fig 6.8. It is evident that the cytolytic activity of monolayers treated with a particular concentration of lymphokine varied markedly from experiment to experiment as did the amount by which the preparation could be diluted before it lost activity. Although the cytostatic activity of the monolayers was high in all three experiments, the potency of the lymphokine preparation appeared to vary even more when this assay was used. On all three occasions no visually detectable

FIGURE 6.8

Variation in the cytolytic and cytostatic activity of macrophages
from lymphokine treated ppi PC in 20 h assays

The results of three separate experiments designed to examine the in vitro cytolytic and cytostatic activity of macrophages from lymphokine(s)-treated ppi PC against ^{51}Cr -P815 cells.

- (■) Cytolytic activity against 10^4 ^{51}Cr -P815 cells by macrophages from 5×10^5 ppi PC which were pretreated for 24 h with dilutions of SMP1.
- (□) The incorporation of ^3H -TdR by ^{51}Cr -P815 cells at 20-24 h of incubation. 1 μCi of ^3H -TdR was added to each well at 20 h to measure tumour cell proliferation
- (-----) 3 SD above the mean cytolytic activity of macrophages from untreated, induced PC.
- (————) 3 SD above and below the mean number of counts incorporated by ^{51}Cr -P815 cells when pulsed with ^3H -TdR after being cultured for 20h in the presence of macrophages from untreated PC.



tumour cell growth occurred in the wells showing low levels of ^3H -TdR incorporation. For this reason it seemed highly unlikely that the variation in inhibition of ^3H -TdR incorporation was simply due to competition by cold thymidine secreted by lymphokine-treated macrophages. Since freshly thawed, identical aliquots of the same lymphokine preparation were used on all three occasions, the results from these experiments suggested that some other parameter necessary for the full expression of cytolytic activity was varied from experiment to experiment. The following section presents experiments carried out in an attempt to define these parameters.

6.12 Effect of varying time of incubation with ppi PC with conditioned culture supernatants

The standard protocol used for the MAF assays was altered by incubating ppi PC with dilutions of SMP1 for 5, 14 or 26 h instead of the usual 24 h. The ppi PC were obtained from CB6F_1 and CBA mice. The cytotoxic and cytostatic activities of the macrophage monolayer obtained after SMP1 treatment were determined as described in §6.11.1. Significant, but very low levels of cytotoxicity were detected only when ppi PC were pretreated with SMP1 for 26 h (Fig 6.9). Somewhat surprisingly, ppi PC showed cytostatic activity for ^{51}Cr -P815 cells even if they were pre-incubated in the absence of MAF, provided the pre-incubation period was no longer than 5 h. Approximately 50% inhibition was obtained in the presence of these cells. Pre-incubation in SMP1 enhanced the cytostatic activity of these cells and induced cytostatic activity in cells preincubated for 14 h and 26 h, which had no cytostatic activity if incubated in medium alone. The potency of SMP1 appeared to decrease with time, becoming less effective at the higher dilutions used. These data led to the conclusion that SMP1 contained a factor(s) capable of maintaining the cytostatic activity which is normally lost during prolonged incubation in vitro. The finding that

FIGURE 6.9

The effect of varying the incubation time with lymphokine(s) on the cytotoxic and cytostatic activity of macrophages from ppi PC

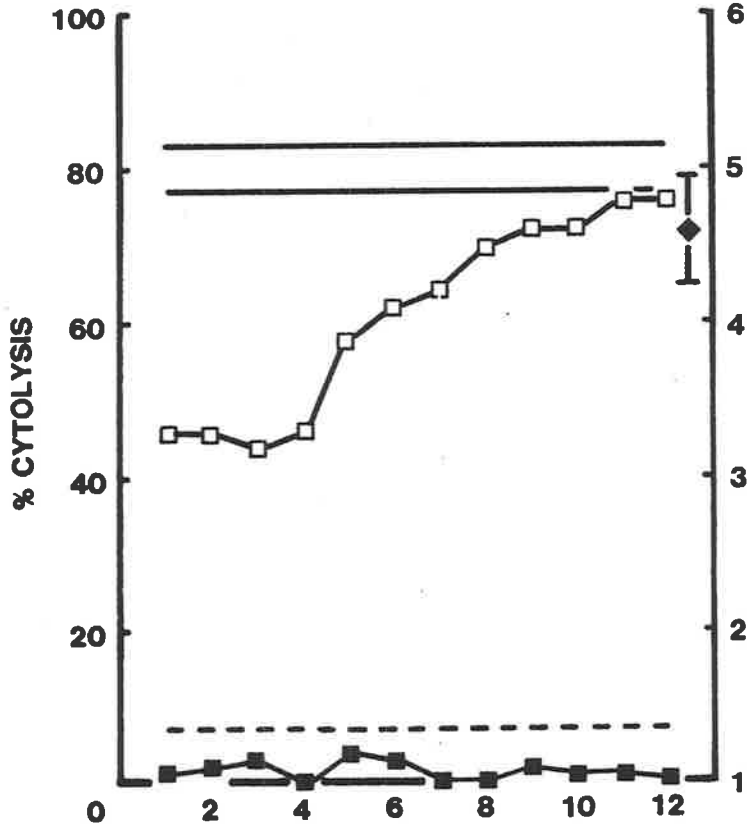
CB6F₁ and CBA mice were injected with 2 ml of 10% proteose peptone broth and the PC harvested 2 days later. 5×10^5 PC were pretreated for 5, 14 or 26 h with dilutions of SMP1.

- (■) Cytolysis at 20 h of 10^4 ^{51}Cr -P815 cells by macrophages from lymphokine-treated PC.
- (□) CPM of ^3H -TdR incorporated from 20-24 h by P815 cells incubated with macrophages from lymphokine(s)-treated ppi PC.
- (◆) Mean \pm 3SD of CPM incorporated by ^{51}Cr -P815 cells when pulsed with ^3H -TdR after being cultured for 20h in the presence of macrophages from untreated PC.

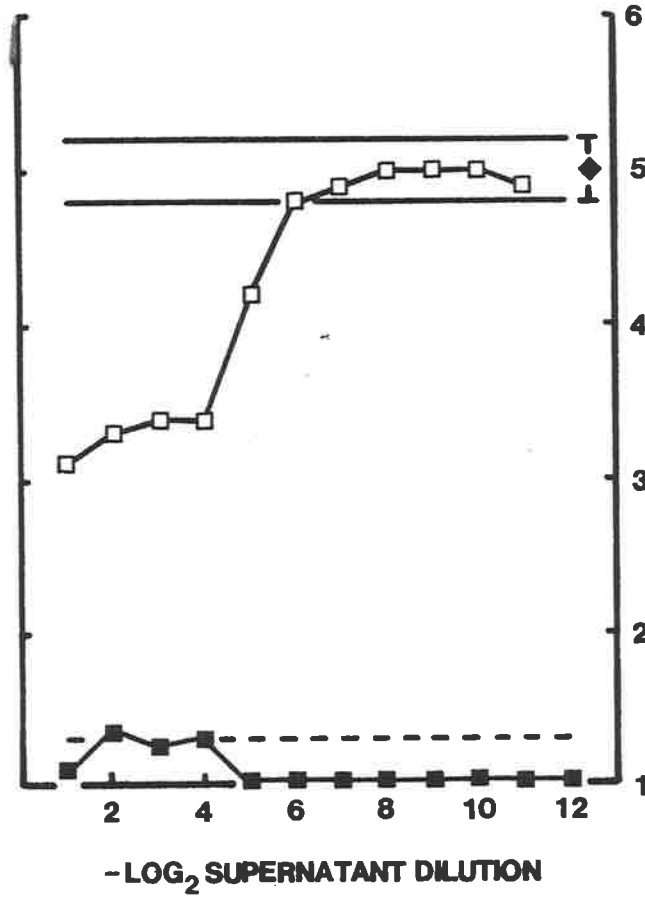
- (-----) 3SD above the mean cytolytic activity of macrophages from untreated induced PC.
- (————) 3 SD above and below the mean CPM of ^3H -TdR incorporated from 20-24 h by ^{51}Cr -P815 cells cultured in medium alone.

HOURS INCUBATED WITH LK

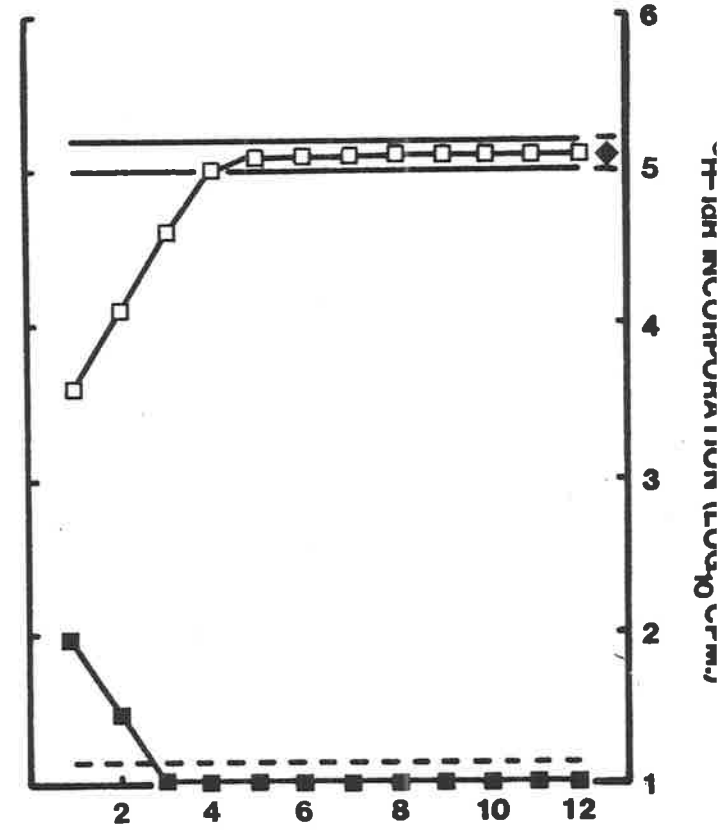
5 HRS.



14 HRS.



26 HRS.



ppi PC have cyostatic activity is consistent with those reported by Keller (1976) and Keller et al. (1976), using ppi PC of rats.

6.13 Effect of adding LPS and prolonging incubation time prior to harvesting

Several reports have shown that MAF can activate macrophages to kill tumour cells in a ^{51}Cr release assay only if LPS is also present during the activation step (Taramelli, Holden and Varesio, 1981; Taramelli and Varesio, 1981; Taffet, Pace and Russell, 1981; Marino and Adams, 1982). It is proposed that LPS delivers a signal to macrophages that is required for the expression of cytotoxic activity by MAF treated macrophages (Ruco and Meltzer, 1978c; Marino and Adams, 1982) FCS can provide a source of LPS in tissue culture media but the LPS content of FCS varies from batch to batch (Wienberg, Chapman and Hibbs, 1978). It was possible, therefore, that the variable nature of the MAF assay may have been due to different levels of LPS contamination of the different batches of FCS used during these experiments. Another source of variation may be the length of time required for tumour cells to be killed by in vitro activated macrophages. If cytotoxicity is not complete by 20 h, variations from experiment to experiment may occur. These possibilities were investigated in the following experiments.

A comparison was made of the ability of the SMP2 preparation of MAF to activate macrophages in the presence or absence of small amounts of Salmonella typhimurium C5 LPS. LPS was used at a concentration of 50 ng/ml, a level which has been reported to induce cytolytic activity in macrophages which are at a stage of non-cytolytic activation but which does not induce cytotoxicity in normal induced macrophages (Russell, Doe and McIntosh, 1977). The experiment was set up in duplicate to allow 20 and 48 h measurements to be made.

Induced PC were cultured with a 1:4 dilution of SMP1 or 50 ng/ml of LPS or both in a final volume of 0.2 ml. Some of the cultures were

incubated for 24 h at 37° before removing the non-adherent cells and supernatants and adding radioactively labelled P815 cells. In other wells, 10^4 labelled P815 cells were included with the PC during culturing with lymphokine or LPS or both. ^{51}Cr -P815 cells were used as targets in the 20 h assays and ^3H -P815 cells were used in the 48 h assays. The P815 cells were labelled with ^3H -TdR by suspending them at a concentration of $2.5 \times 10^5/\text{ml}$ and incubating them overnight with $5 \mu\text{Ci}/\text{ml}$ of ^3H -TdR. At the end of the 48 h assay period, tumour cell death was assessed by harvesting the contents of the multiwell trays onto glass fibre discs using a cell harvester and measuring the amount of ^3H -TdR that could be recovered. It was assumed that the harvesting sampled all the labelled cells and that any decrease in radioactivity, compared to control values, was due to the death of labelled target cells and breakdown of DNA.

The results in Table 6.6 show that very little, if any, cytotoxicity was detected in a 20 h ^{51}Cr release assay when ^{51}Cr -P815 cells were cultured with PC in the presence of lymphokine(s) or LPS. The addition of C5 LPS did not augment the activity of the lymphokine preparation. However, a high degree of cytostasis was evident when ^{51}Cr -P815 cells were incubated for 24 h with ppi PC in the presence of lymphokine(s), LPS or both (Table 6.7). Whilst ppi PC inhibited ^3H -TdR incorporation in the absence of lymphokine or LPS, the addition of these agents to the cultures decreased ^3H -TdR incorporation a further 100-fold. Addition of lymphokine or LPS to tumour cells alone had only a marginal effect on their ^3H -TdR incorporation.

Adherent cells from ppi PC which had been preincubated for 24 h with SMP2, LPS or a combination of both also showed poor cytotoxicity against P815 in a 20 h ^{51}Cr release assay (Table 6.6). The cytostatic activity of these PC was not measured. In both cases, however, cytotoxicity was detected 48 h after the addition of ^3H -P815 cells. This was true regardless of whether ^3H -P815 cells were added to the

Table 6.6

The effect of LPS on the cytotoxicity induced by
lymphokine in 20 h and 48 h assays

Additives		Cytotoxic activity of			
		ppi PC ^①		treated macrophages [#]	
MAF	LPS	% Cytolysis	% ³ H recovered	% Cytolysis	% ³ H recovered
		20 h [⊘]	48 h*	20 h [⊘]	48 h*
-	-	0.6 ± 2.4	65.8 ± 5.2	-6.4 ± 4.4	81.8 ± 7.4
+	-	3.2 ± 2.0	32.6 ± 3.4	8.8 ± 5.6	58.0 ± 1.9
-	+	5.8 ± 2.4	36.6 ± 1.7	8.3 ± 1.5	74.0 ± 2.0
+	+	0.9 ± 3.2	33.6 ± 1.7	8.2 ± 2.0	58.3 ± 0.3

(@) Immediately after harvesting the ppi PC, 5×10^5 PC were cultured with 10^4 labelled P815 cells and the additives indicated in this table.

(#) 5×10^5 ppi PC were cultured for 24 h with medium or with SMP1 or LPS or both. The medium and non-adherent cells were removed and replaced with 10^4 labelled P815 cells in 0.2 ml of medium.

(⊘) Measured at 20 h using ⁵¹Cr-P815 cells.

(*) Measured at 48 h using ³H-P815 cells and expressed as a percentage of counts recovered from wells where target cells were cultured in the absence of effector cells.

(¶) See Table 6.7 for inhibition of ³H-TdR incorporation by ⁵¹Cr-P815 cells cultured with ppi PC and the additives described.

TABLE 6.7

$^3\text{H-TdR}$ incorporated by $^{51}\text{Cr-P815}$ cells cultured with ppi PC§

$^{51}\text{Cr-P815}$ cells with			$^3\text{H-TdR}$ incorporation*
PC@	MAF#	LPS $\text{\textcircled{d}}$	(CPM)
-	-	-	113600 \pm 5600
+	-	-	15200 \pm 300
+	+	-	150 \pm 10
+	-	+	150 \pm 10
+	+	+	200 \pm 6
-	+	-	88700 \pm 6000
-	-	+	97000 \pm 2200
-	+	+	ND $\text{\textcircled{f}}$

(§) From the experiment shown in Table 6.6.

(@) $^{51}\text{Cr-P815}$ cells were cultured for 20 h with ppi PC.

(#) MAF (SMP1) was used at a final dilution of 1/4.

($\text{\textcircled{d}}$) LPS used at a final concentration of 50 ng/ml.

(*) Background counts = 40 CPM.

($\text{\textcircled{f}}$) Not determined.

induced PC together with the lymphokine preparation or LPS or whether the induced PC were preincubated with these agents for 24 h prior to adding tumour cells. Again, no additive or synergistic effect of lymphokine and LPS could be detected. This result led to the conclusion that long-term assays are more sensitive and detect cytotoxicity not evident at 20 h.

6.14 Cytotoxic activity of lymphokine-treated macrophages is consistently detected using long-term assays

In preliminary experiments designed to characterize the cytotoxic activity of macrophages from lymphokine-treated ppi PC in long-term assays it was found that 8 h preincubation induced more cytotoxic activity than 24 h (data not shown). Therefore, in subsequent assays for MAF, ppi PC were pretreated with conditioned culture supernatants for 8 h prior to the removal of non-adherent cells and the addition of ^3H -P815 cells. The protocol for the long-term assays was essentially the same as that for the 20 h ^{51}Cr release assay. The main alteration from the experiment described above is that the P815 cells were labelled at 5×10^5 cells/ml with lower amounts of ^3H -TdR and for a shorter period of time (1.25 $\mu\text{Ci/ml}$ for only 6-8 h).

The activity of SMP2 in 4 separate assays is shown in Fig 6.10. The amount of radioactivity recovered when ^3H -P815 cells were cultured with macrophages from untreated, ppi PC was generally somewhat higher than that seen when target cells were cultured in medium alone. There was variation from experiment to experiment in the amount of radioactivity recovered from the wells in which ^3H -P815 cells were cultured in the absence of macrophages. However, it was never less than 60% and in some experiments it was as high as 90%. The reasons for this variation were not investigated.

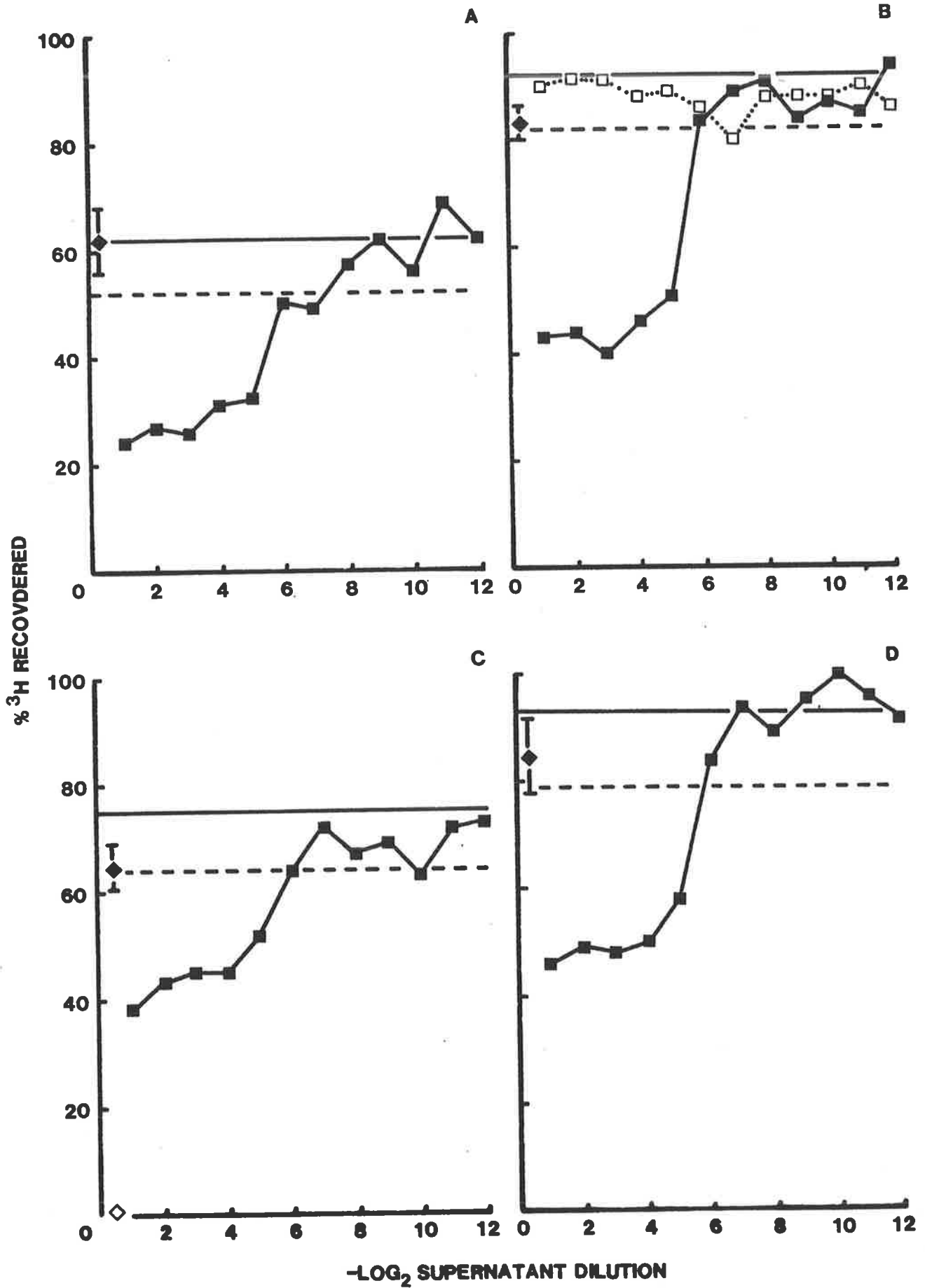
In one experiment, the cytotoxic activity of the non-adherent cells from lymphokine pretreated ppi PC was tested. This was done in the following way. The washing procedure was modified slightly from that

FIGURE 6.10

The results of 4 replicate experiments showing the cytotoxic activity of macrophages from lymphokine pre-treated induced PC in a long-term assay

CB6F₁ mice were injected with 2 ml of 10% proteose peptone broth and the PC harvested 2 days later. 5×10^5 ppi PC were pre-treated for 8 h with dilutions of SMP2 and the macrophage monolayers prepared from them were then cultured with 1×10^4 ³H-P815 cells from 44 to 48 h. At the end of this time the cellular contents of the wells were harvested and the percentage of ³H-TdR recovered determined.

- (■) % CPM recovered when ³H-P815 cells were cultured with macrophages from SMP2 pre-treated ppi PC.
- (□) % CPM recovered when ³H-P815 cells were cultured with the non-adherent cells from SMP2 pre-treated ppi PC.
- (◆) Mean \pm 3 SD of the % CPM recovered when ³H-P815 cells were cultured in medium only.
- (-----) 3 SD below the % CPM recovered when ³H-P815 cells were cultured with macrophages from untreated ppi PC.
- (————) mean % CPM recovered when ³H-P815 cells were cultured with macrophages from untreated ppi PC.



used in other experiments. The top 0.1 ml of medium was removed from all the wells and the tray vortexed to resuspend the non-adherent cells. The medium from the wells in which ppi PC were treated with SMP2 was transferred to empty wells. The macrophage monolayers were washed as usual and ^3H -P815 cells then added to all the wells including the ones containing the non-adherent cells from lymphokine-treated ppi PC. Predictably, cytotoxicity was not seen in the wells in which the target cells were cultured with the non-adherent cells recovered from lymphokine treated induced PC (Fig 6.10B). In the 4 assays the MAF titre of SMP2 was between 1:32 and 1:64. These experiments established that this protocol for a MAF assay was more reproducible and more sensitive than that using ^{51}Cr -P815 cells in an 20 h ^{51}Cr release assay.

6.15 Some cell populations appear to have cytotoxic activity when long-term assays are sampled with cell harvester

In the experiments reported in Table 6.6 and other experiments not presented here, ppi PC were found to be cytotoxic for ^3H -P815 cells when tested without prior incubation in medium, lymphokines or LPS. There are several possible reasons for this. It could have been due to overlabelling of the target cells making them more fragile than normal. This would be consistent with results shown in Fig 6.10 where macrophages from ppi PC cultures in the absence of lymphokine were not cytotoxic for P815 cells labelled with lower concentrations of ^3H -TdR. However Keller (1976) reported that rat ppi PC are cytotoxic for tumour cells in vitro and, therefore, it could be argued that the lymphokine(s) detected in this assay was not MAF (which induces cytotoxic activity in normal macrophages), but rather a lymphokine(s) which maintained in vitro cytotoxic activity. Alternatively, it was possible that the ppi PC were not cytotoxic but "sticky" allowing some of the ^3H -P815 cells to adhere strongly enough to prevent them from being recovered using the cell harvester. Experiments, designed to distinguish between the latter two

possibilities were carried out and the results are presented below.

Experiment 1 A standard in vitro cytotoxicity assay was carried out in which ^3H -P815 cells were co-cultured with normal PC or PC from short-term 11RX-immunized mice. At 20 h the medium in all the wells was sampled for ^3H release and the contents of the wells were then harvested onto glass fibre discs. The radioactivity recovered in the supernatants and on the discs is shown in Table 6.8. The results clearly show that all the radioactivity added to the wells when ^3H -P815 cells were cultured with PC from 11RX-immunized mice or medium alone could be recovered by sampling the supernatant and using the cell harvester. However, when cultured with normal PC, the sum of the counts in the supernatant and that on the discs was clearly less than the total added. Because very few counts were recovered in the supernatants, it seemed reasonable to conclude that the failure to recover all the radioactivity was because normal macrophages were "sticky" for ^3H -P815 cells.

Experiment 2 A similar experiment was carried out using ppi PC preincubated for 8 h with or without lymphokine(s). In this case the sum of the counts in the supernatant and those harvested onto the discs approximated closely those added to the wells at the beginning of the experiment, and thus it appears that when ppi PC are preincubated for 8 h prior to the addition of target cells, the macrophages are no longer "sticky". Because of these findings, this experiment was repeated and cytotoxicity was assessed by measuring the amount of label released into the medium.

Experiments 3-5 The results of three different experiments in which normal and ppi PC were used are shown in Table 6.9. It is clear from the results of experiment 3 that normal PC and ppi PC were not cytotoxic for ^3H -P815 cells in a long-term assay if tested immediately

TABLE 6.8

Comparison of ^3H released into supernatant and
that recovered with cell harvester

Expt	^3H -P815 incubated with	S/N [#] CPM	Disc CPM	Total* CPM	CPM added to wells [£]
1 [@]	medium	169	4087	4256	4360
	Normal PC	226	2573	2799	4232
	11RX PC	3307	888	4195	4184
2 [§]	medium	450	3136	3586	3500
	ppi macrop [¶]	554	2997	3551	3500
	MAF ppi macrop [£]	2302	1307	3609	3500

(@) 10^4 ^3H -P815 cells were cultured for 20 h with medium or 10^6 PC from normal mice or mice immunized with 11RX ip 6 days previously.

(§) 10^4 ^3H -P815 cells were cultured with medium or macrophage monolayers from ppi PC preincubated 8 h previously with a 1/10 dilution of SMP2.

(#) Supernatant (2 x counts in 0.1 ml of supernatant).

(*) Sum of CPM in the supernatant and on the disc.

(£) CPM present in an aliquot of 10^4 ^3H -P815 cells added to the wells.

(¶) Macrophage monolayers from ppi PC preincubated for 8 h with medium in the absence of lymphokines(s).

(£) Macrophage monolayer from ppi PC cultured for 8 h with a 1/10 dilution of SMP2.

after being harvested from mice. In experiment 4, ppi PC were treated with SMP2 for 8 h or 24 h and the macrophage monolayers obtained from these were tested for in vitro cytotoxic activity using ^{51}Cr -P815 cells in a 20 h assay and ^3H -P815 cells in a 48 h assay. As in previous experiments, macrophages from lymphokine-pretreated PC were poorly cytotoxic at 20 h. It should be noted however that macrophages from ppi PC incubated in medium only for 24 h were more cytotoxic at 20 h than those treated with MAF. The reason for this is not known and this effect was not observed in any other experiment of this type. When the assay period was extended to 46 h, treated but not untreated macrophages were markedly cytotoxic for ^3H -P815 cells. The results indicated that MAF induces maximal cytotoxicity in macrophages by 8 h and that it decreases if the induction period is extended to 24 h. These kinetics for the development of macrophage tumouricidal activity are similar to those reported by Ruco and Meltzer (1977). Experiment 5 shows that MAF induces cytotoxicity in macrophages from normal PC but that they are less responsive to the effects of lymphokines than are the macrophages from ppi PC. This is consistent with previous reports in the literature that responsiveness to MAF is associated with a particular stage of macrophage differentiation (Ruco and Meltzer, 1978; Hibbs etc, 1977). Furthermore the degree of activation of macrophages from normal PC, unlike those of ppi PC is variable from experiment to experiment (data not shown).

The experiments discussed in this section have established that cytotoxicity can be measured in a long-term assay by either sampling the supernatants for ^3H release or by harvesting the cells and measuring cell-associated radioactivity. However, caution must be exercised if using the latter method because ^3H -P815 cells appear to "stick" to PC from normal mice if co-cultured with them immediately after being harvested from mice. This gives the impression that they are cytotoxic. This problem does not arise if PC are preincubated in medium for 8 h prior to adding target cells and cell-associated ^3H can be used to

TABLE 6.9

The in vitro cytotoxic activity of macrophages from lymphokine
-treated ppi PC as measured by a long-term ^3H release assay

Expt	Type of PC used	PC per well ($\times 10^{-5}$)	Treat- ment of PC [§]	Time [¶] (h)	Cytolysis		
					^{51}Cr -P815 20 h	20 h	^3H -P815 46 h
3 [£]	N [@]		-	0	-0.6 \pm 3.4	3.1 \pm 0.9	-1.2 \pm 2.1
	ppi [#]		-		-2.3 \pm 3.2	2.4 \pm 0.3	-1.1 \pm 2.0
4 [£]	ppi	5	-	8	0.2 \pm 3.2		1.5 \pm 1.0
	ppi	5	+	8	6.9 \pm 6.2		43.2 \pm 2.0
	ppi	5	-	24	13.2 \pm 4.3		0.5 \pm 1.4
	ppi	5	+	24	7.9 \pm 4.0		21.0 \pm 1.5
5 [£]	N	5	-	5			0.0 \pm 0.5
	N	2	+	5			0.6 \pm 1.1
	N	5	+	5			21.6 \pm 8.1
	ppi	5	-	5			-0.7 \pm 0.9
	ppi	2	+	5			34.3 \pm 1.3
	ppi	5	+	5			50.3 \pm 1.1

(@) PC from normal mice.

(#) Proteose peptone induced PC.

(£) PC were tested for cytotoxic activity immediately after harvesting from mice.

(£) PC were pretreated with MAF prior to removal of medium and non-adherent cells and the addition of 10^4 labelled P815 cells.

(§) PC were preincubated with medium (-) or with a 1/10 dilution of MAF (+). SMP2 and SMP3 were used in experiments 3 and 4 respectively.

(¶) Length of time PC preincubated with MAF or medium before addition of target cells.

determine cytotoxicity. Furthermore, the use of the cell harvester is a more convenient technique to use, especially when large numbers of CCS are assayed for MAF activity.

6.16 The effect of delaying the addition of target cells on the cytotoxic activity of lymphokine-activated macrophages

The finding that macrophages activated in vitro take longer to kill P815 cells than do macrophages activated by in vivo infection with 11RX implies that the cytotoxic mechanism(s) used by the two types of macrophages is different. However, these data are consistent with an alternate hypothesis. It is possible that the same mechanism(s) is used by both types of macrophages but that with in vitro activation further changes, subsequent to MAF treatment, need to occur to the macrophages before they become cytotoxic. If this were the case, then similar amounts of cytotoxicity should be detected when tumour cells are added immediately after pre-incubation with MAF or only 24 h prior to sampling. This possibility was tested in the following experiment in which the cytotoxic activity of in vitro and in vivo activated macrophages were compared. Mice were injected ip with 10% proteose peptone broth or with 10^5 11RX iv and their PC harvested on days 2 and 6 respectively. The PC from 11RX-immunized mice were tested immediately after harvesting in a standard in vitro cytotoxicity assay against P815 cells labelled with ^{51}Cr , ^3H , or ^{125}I . Three replicate sets of cultures were prepared and cytolysis was measured at 14.5, 20 and 23.5 h. The ppi PC were incubated at 2×10^5 cells/well for 8 h with medium only or with a 1/2 dilution of SMP2. The macrophage monolayers were washed and 10^4 ^3H -P815 cells were added to them either immediately or 16 h later. The cultures to which ^3H -P815 cells were added immediately after MAF treatment were set up in duplicate and sampled for ^3H release at 20 h or 44 h whilst in the cultures where the addition of the target cells was delayed, ^3H release was measured at

Table 6.10

Comparison of the cytotoxic activity of in vitro
and in vivo activated macrophages

Effector cells added to culture	Time P815 cells added to cultures (h)	Isotope used	% cytotoxicity (h)			
			14.5	20	23.5	44
11RX PC [@]	0	³ H	65.6	78.1	78.6	ND*
		¹²⁵ I	65.1	66.5	66.7	ND
		⁵¹ Cr	80.0	74.5	91.2	ND
MAF-treated macrophages ^β	0 [#]	³ H	ND	18.9	ND	63.5
	16 [§]		ND	1.2		ND

(@) PC from mice injected ip with 10⁵ 11RX 6 days previously.

(β) Macrophage monolayers from ppi PC pretreated for 8 h with a 1/2 dilution of SMP2.

(#) ³H-P815 cells were added to the cultures immediately after pretreatment of ppi PC with SMP2.

(§) ³H-P815 cells were added to the cultures 16 h after pretreatment of ppi PC with SMP2.

(*) Not determined.

20 h. The results in Table 6.10 do not support the hypothesis that macrophages develop cytotoxic activity some time after the MAF treatment. It is clear from these results that PC (macrophages) from 11RX-immunized mice can induce the release of all three radioisotopes within 14.5 h of co-culture of effector and target cells. In contrast, it takes 44 h for a similar amount of ^3H to be released from ^3H -P815 cells co-cultured with in vitro activated macrophages. The data are consistent with the conclusion that the reason why incubation for 44 h apparently results in greater tumour cell killing is due to a lag between the time of delivery of the cytotoxic effect and the release of label into the supernatant (cf line 4 and line 5 of results in Table 6.10). In addition, it is worthwhile noting that the cytotoxic activity of MAF treated macrophages is less if the addition of target cells is delayed by 16 h (compare cytolysis at 20 h for lines 4 and 5). This is consistent with the observation by Ruco and Meltzer (1977) that MAF-treated macrophages lose activity when MAF is removed from the culture.

6.17 The relationship between cytostasis and cytotoxicity

Although killing of ^3H -P815 cells by in vitro activated macrophages appears to be quite slow, the experiments in §6.10, 6.11 and 6.12 established that by 20 h after co-culture with lymphokine-treated macrophages, the proliferation of P815 cells was inhibited. It seemed possible that one of the differences in the cytotoxic mechanism of in vitro and in vivo activated macrophages is that cytostasis is induced earlier by in vivo activated macrophages than those activated in vitro. A number of experiments were performed to investigate this possibility.

Replicate sets of cultures were prepared in which unlabelled P815 cells were incubated with PC from 11RX-immunized mice or with macrophage monolayers from normal and ppi PC pretreated with MAF or medium. An additional replicate set of cultures was prepared using ^3H -P815 cells to allow measurement of the cytotoxic activity of the various effector

cells. At various times during the incubation, a set of the cultures containing unlabelled P815 cells was pulsed for 2 h with 1 μ Ci/well of ^3H -TdR. In some experiments the incorporation of ^3H was measured by harvesting the contents of each well on to glass fibre discs, then any adherent labelled cells remaining in each well were solubilized by replacing the fluid in each well with 0.1 ml of 0.5% SDS. The trays were incubated overnight in a humidified incubator and the contents of each well were removed with an automatic pipette and the radioactivity in each sample determined in the usual way. The amount of ^3H incorporated was calculated to be the sum of the counts per disc and in the SDS lysate. In experiments only the cell harvester was used was used for sampling.

The results from three such experiments are presented in Table 6.11. In experiment 1, only the cell harvester was used for sampling. In experiments 2 and 3, SDS solubilization was used as well. It was difficult to get reproducible results using this experimental protocol. However, in general there was no apparent difference in the time of induction of cytostasis in P815 cells by in vitro and in vivo activated macrophages. Furthermore, the degree of cytostasis induced did not correlate with the rate or amount of tumour killing. Experiment 1 shows that incubation with MAF induced similar levels of cytostatic activity at about the same rate in both normal and ppi macrophages although the % cytotoxic activity was clearly different. However, cell titration experiments showed that macrophages from MAF-treated normal PC were less cytostatic than from MAF-treated ppi PC (data not shown). This is consistent with the differences seen in their cytotoxic activity. The rate of killing was the same for both types of macrophages.

In experiments 2 and 3 there was a four-fold increase in the killing of ^3H -P815 cells by MAF-treated macrophages between 20 and 40 h of incubation whilst the increase was only 1.4-2-fold with PC from 11RX-immunized mice. In experiment 2 in vitro activated macrophages induced cytostasis much earlier (within the first 2 h) than PC from

TABLE 6.11

The induction of cytostasis by cytotoxic PC

- (@) PC from mice immunized with 11RX ip. Numbers in parentheses show days after immunization. PC were tested for cytotoxic activity immediately after harvesting from mice.
- (#) ppi PC and normal PC were pretreated with a 1/5 dilution of MAF (+) or with medium alone (-) for the hours shown in parentheses. SMP2 was used in experiment 1 and SMP3 in experiments 2 and 3. The cytotoxic activity of the adherent cell monolayers were then tested.
- (§) PC added per well.
- (£) Expressed as a percentage of the counts incorporated by P815 cells cultured in medium alone (six replicates per sample).
- (*) Mean \pm s.e.m from quadruplicate determinations.
- (¶) The increase in cytotoxicity from 20 h-40 h, calculated as cytotoxicity at 40 h divided by cytotoxicity at 20 h.
- (£) Not determined.

Expt	Cell type used	PC per well (x 10 ⁻⁵)	MAF#	% ³ H incorporation [‡] time (h)				% Cytolysis* 20 h 40 h		Ratio [¶]
				0-2	3-5	6-8	24-26			
1	ppi PC	2	- (15)	79	69	47	92	3.3 ± 0.6	0.7 ± 0.9	1.9
			+ (15)	12	14	5	4	17.1 ± 1.6	32.9 ± 1.0	
	normal PC	2	- (15)	66	84	84	93	0.4 ± 0.5	-4.8 ± 0.8	1.6
		+ (15)	22	19	12	4	6.2 ± 1.4	10.1 ± 3.7		
	11RX PC [®] (7)	2	-	23	10	2	1	12.8 ± 1.6	25.7 ± 3.6	2.0
2	ppi PC	2	- (8)	116	75	103	95	12.0 ± 1.0	48.1 ± 3.1	4.0
			+ (8)	17	15	18	3			
	11RX PC (6)	2	-	82	51	28	12	23.3 ± 1.3	42.7 ± 6.4	2.0
		10	-	ND [‡]	ND	ND	ND	43.6 ± 2.1	64.7 ± 5.9	1.5
3	ppi PC	2	- (8)	93	97		100	7.2 ± 2.4	30.4 ± 2.0	4.2
			+ (8)	48	46		16			
	11RX PC (7)	2	-	63	40		21	14.9 ± 0.7	20.7 ± 0.8	1.4
		10	-	ND	ND		ND	43.1 ± 3.3	68.7 ± 1.4	1.6

11RX-immunized mice, however, in experiment 3 both effector cell populations induced cytostasis within the first 2.5 h.

Because of the experimental design used in these three studies, it is not possible to exclude the possibility that the inhibition of $^3\text{H-TdR}$ incorporation by P815 cells, at the early stages of co-culture with macrophages, is due to competitive inhibition by thymidine secreted from effector cells. However, the rapid inhibition of $^3\text{H-TdR}$ observed in these experiments is consistent with a recent report by Hamilton *et al.*, (1982) that cytostasis of tumour cells, as determined by measuring the DNA content of the cell, occurs as early as 2-3 h after commencement of co-culture with activated macrophages.

In summary, no reasons could be provided for the variable nature of the MAF assay. It seemed that some external factor(s) may have determined whether tumour cells would be killed in a slow or rapid manner and these were not being kept constant from one experiment to the next. These results confirmed that cytotoxicity by MAF pre-treated macrophages was greater in a 48 h rather than a 20 h assay.

6.18 The relationship between IL2 and MAF release by lymphoid cells at different times after immunization with 11RX

The ability of lymphoid cells to release IL2 and MAF was examined in the following time course experiment. Groups of CB6F_1 mice were injected with 1×10^5 11RX by the iv or the ip route. At weekly intervals thereafter until week 5, 4 mice were taken from each group and PC, spleen cell and mesenteric LNC suspensions were prepared from them. The suspensions were cultured separately at 2×10^6 cells per well in 2 ml of medium for 20 h in the absence or presence of 11RX antigen. In addition to this, LNC were also cultured in the presence of 2×10^6 PC from normal mice. The culture supernatants were harvested and stored at -20° until they were tested for MAF and IL2 activity. The IL2 activity

was determined using the usual assay, and long-term (48 h) assays were carried out to quantitate MAF, using the cell harvester for sampling. Standard preparations of lymphokines were also included in each assay.

The results obtained showed that there were differences in the characteristics of IL2 and MAF release by the lymphoid cells of 11RX immunized mice. Regardless of the route used for immunization, both PC and spleen cells were active in releasing MAF whilst only PC appeared to be active in releasing IL2. The ability to release MAF was, in part, related to the distribution of the infecting organisms i.e. spleen cells were more active than PC after iv infection, whilst the converse was true if the 11RX organisms were injected by the ip route. Since 11RX organisms are not found in the peritoneal cavity after iv infection, it appears that the lymphocytes that release MAF and IL2 are capable of migrating from the spleen to the peritoneal cavity. Spleen cells lost their ability to release MAF by week 5 after immunization whereas PC could still release MAF. PC were also highly active in their ability to release IL2, whereas spleen cells had little or no ability to do so.

Fig 6.11 shows that as early as one week after ip infection with 11RX, both PC and spleen cells could release MAF when cultured with 11RX antigen. The activity of the spleen cells plateaued at week 2 and remained at that level until week 4. At week 5 it declined to the limit of detection (a titre of 4). The MAF activity of supernatants obtained from PC continued to rise until week 3 of the infection and remained at a high level until week 5. In comparison, only the PC released IL2 in high amounts when cultured with antigen in vitro. The ability of PC to release IL2 increased until week 3 and remained high thereafter. Lymphoid cells from normal mice cultured with 11RX antigen released neither IL2 or MAF into the culture medium, and medium containing antigen which had been incubated for 20 h in the absence of cells was similarly inactive in both assays (data not shown).

The release of IL2 and MAF by the lymphoid cells of mice immunized

FIGURE 6.11

The release of IL2 and MAF by the lymphoid cells of mice
at weekly intervals after iv immunization with 11RX

Mice were immunized with 10^5 11RX iv and at various times thereafter groups of mice were taken and lymphoid cell suspensions were prepared from them and cultured at 2×10^6 cells/ml in the presence or absence of 20 $\mu\text{g/ml}$ 11RX antigen. The culture supernatants were tested for IL2 and MAF activity.

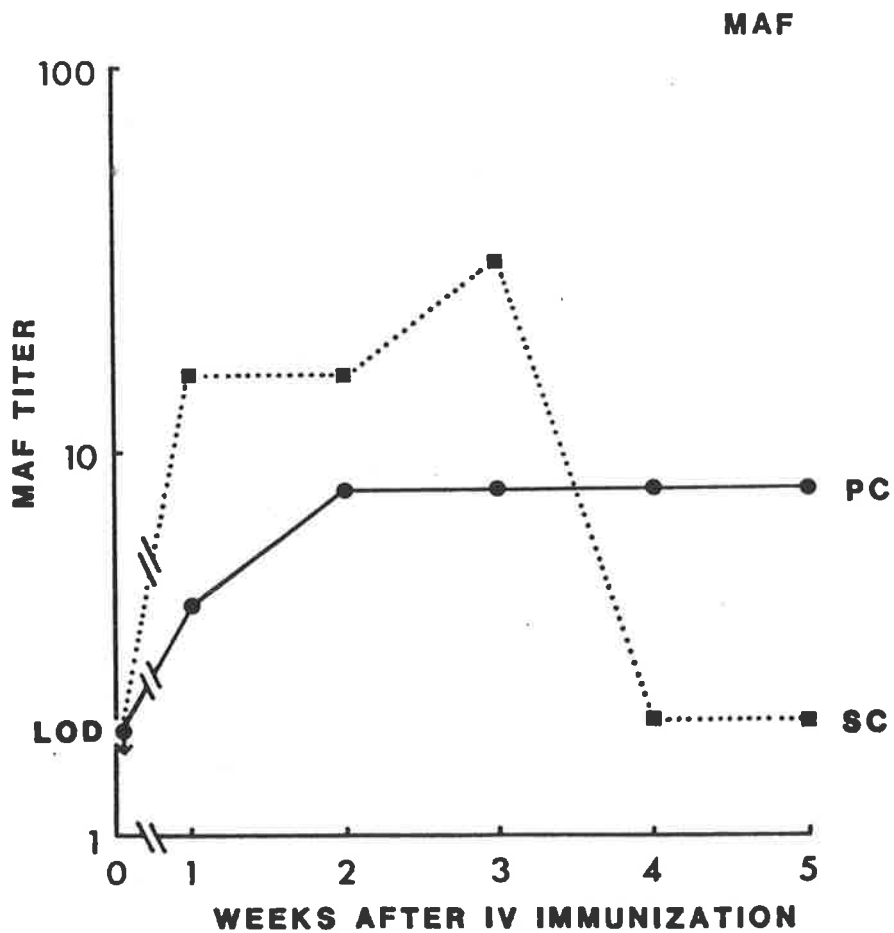
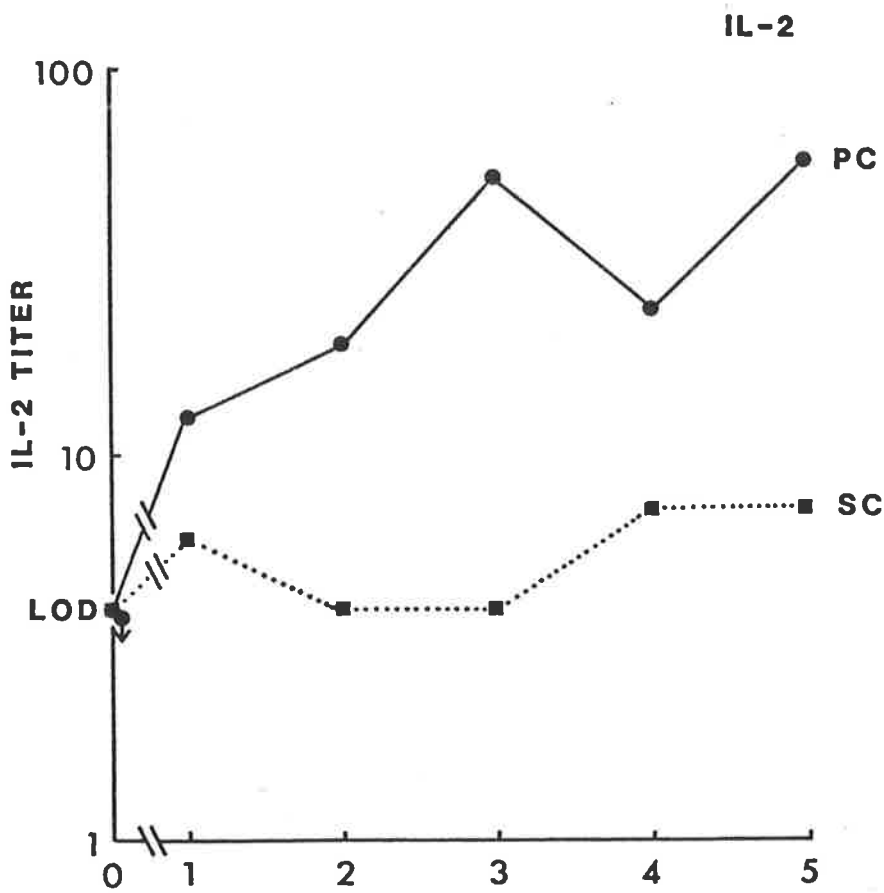
Lymphokine activities of supernatants from cultures containing:

(●) PC + 11RX antigen

(■) Spleen cells + 11RX antigen

LOD Limit of detection.

(↓) Below the limit of detection, i.e. no IL2 or MAF activity detected in supernatants at a 1/4 or 1/2 dilution, respectively.



with 11RX iv is shown in Fig 6.12. Spleen cells released somewhat higher levels of MAF than those of ip immunized mice, and maximum levels were already released 1 week after infection. Similar levels were released at weeks 2 and 3 and after this time MAF activity declined to baseline levels. The ability to release MAF developed more slowly in PC from iv immunized mice. Peak activity was released from week 2 onwards, although the titres were lower than the peak titres released from PC of ip immunized mice. As in the previous experiment, using cells from ip immunized animals, PC from iv immunized animals released high amounts of IL2 when exposed to 11RX antigen in vitro. Activity was detected as early as 1 week after infection and progressively increased up to week 5. Spleen cells from these animals released little or no IL2.

The ability of mesenteric LNC suspensions from ip and iv immunized mice to release lymphokines in the presence of 11RX antigen was tested 2 and 4 weeks after immunization. No MAF or IL2 activity was detected in any of the CCS, even when normal PC were added to the cultures of LNC (data not shown).

From the results presented in this section, it is likely that MAF release is a better in vitro indicator than IL2 release of the presence of effector T cells involved in CMI to 11RX. This is because cell suspensions prepared from spleen, which early after immunization contain activated macrophages to eliminate the infecting organisms, release MAF but not IL2 when cultured with 11RX antigen in vitro. Consistent with this view is the finding by Attridge and Kotlarski (unpublished observations) that the PC of mice immunized with a killed 11RX vaccine release IL2 in vitro in the absence of CMI in the intact animal.

6.19 Summary and conclusions

The experiments presented in this chapter establish that macrophages present in inflammatory exudates of normal mice can be activated in vitro to kill P815 cells when cultured with 11RX-sensitized

FIGURE 6.12

The release of IL2 and MAF from the lymphoid cells of mice
at weekly intervals after ip immunization with 11RX

Mice were immunized with 10^5 11RX ip and at various times thereafter groups of mice were taken and lymphoid cell suspensions were prepared from them and cultured at 2×10^6 cells/ml in the presence or absence of 20 $\mu\text{g/ml}$ 11RX antigen. The culture supernatants were tested for IL2 and MAF activity.

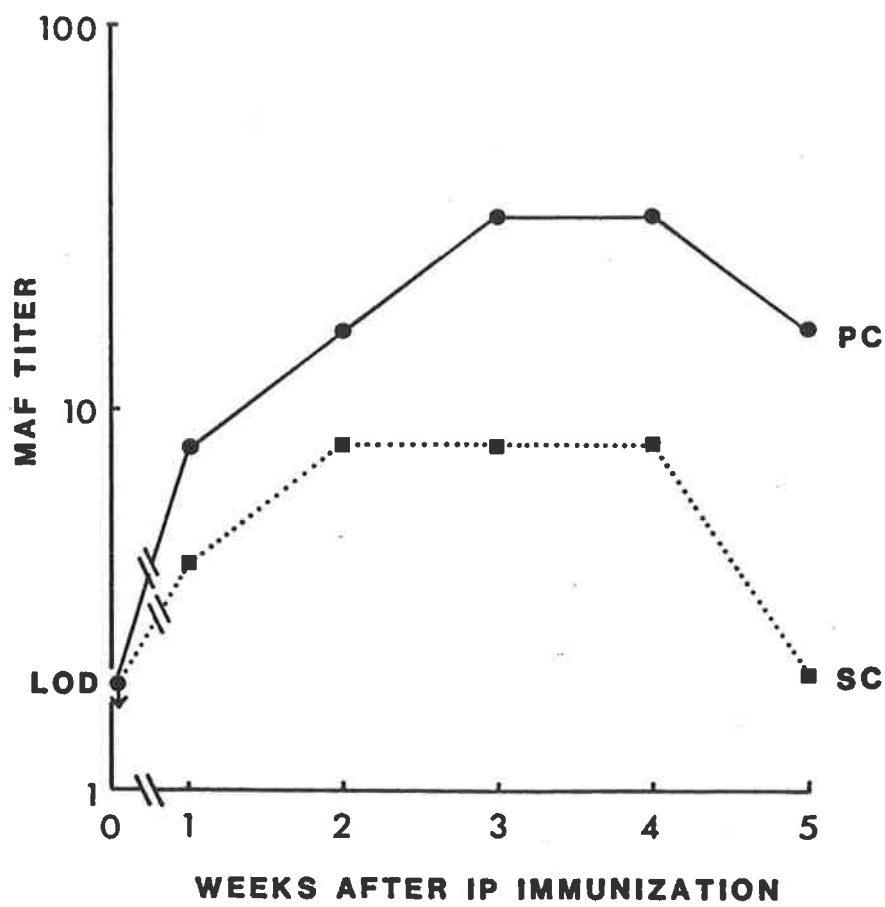
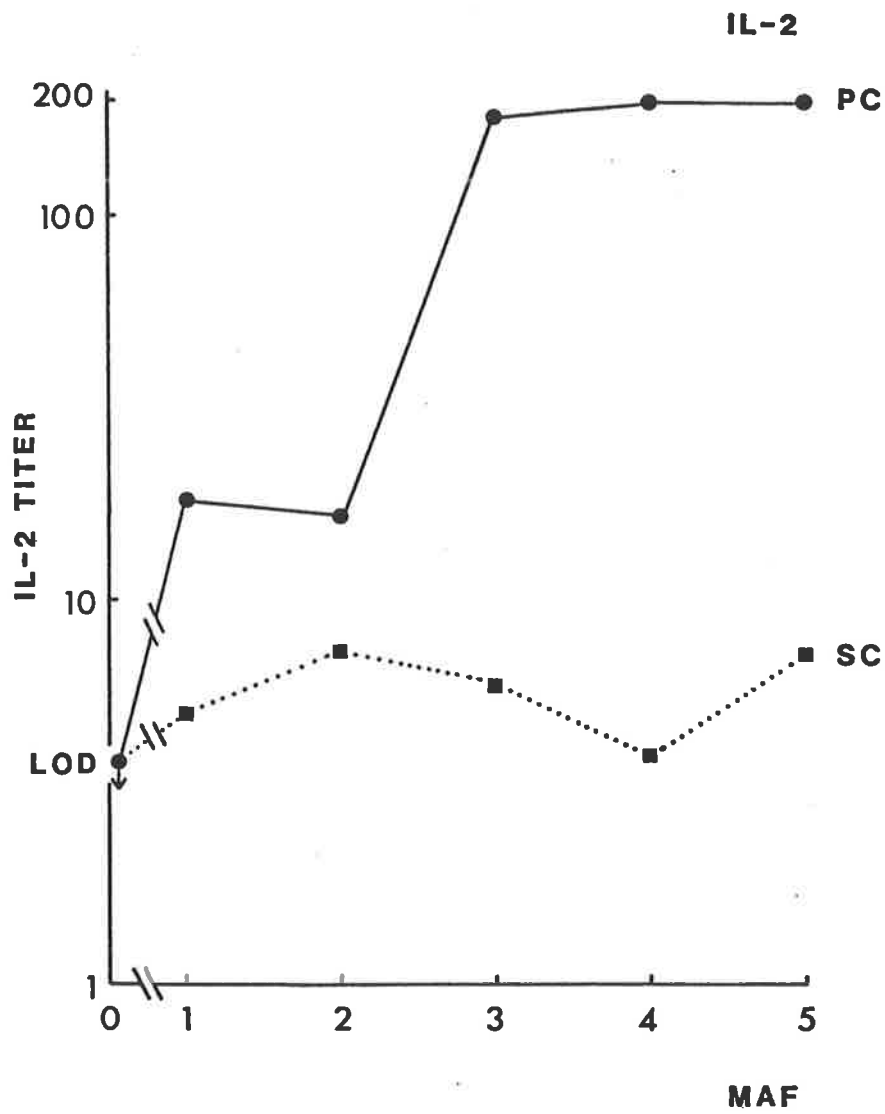
Lymphokine activities of supernatants from cultures containing:

(●) PC + 11RX antigen

(■) Spleen cells + 11RX antigen

LOD Limit of detection.

(↓) Below the limit of detection, i.e. no IL2 or MAF activity detected in supernatants at a 1/4 or 1/2 dilution, respectively.



lymphocytes and llRX antigen. Activation is mediated by a soluble factor(s). Cell-free supernatants from antigen-stimulated lymphoid cell cultures can also induce cytotoxic activity in normal and inflammatory macrophages. The production of MAF by sensitized lymphoid cells requires T cells, llRX antigen and adherent cells which apparently act as antigen presenting cells.

Cytotoxicity can be detected after 20 h culture of ^{51}Cr -P815 cells with MAF-treated macrophages. However, the amount of cytotoxic activity at this time is variable. Although cytotoxic activity could be detected by culturing ^{51}Cr - or ^3H -P815 cells with MAF-treated macrophages for 20 h, the degree of cytotoxicity obtained was variable and often quite low. Cytotoxic activity was consistently detected when the assay time was extended to 40-48 h and ^3H -P815 cells were used as targets. In comparison, the cytotoxic activity of PC (macrophages) activated in vivo by ip infection with llRX was consistently expressed within 20 h of incubation, irrespective of the radioactive label used to tag the target cells. These results suggest that these macrophages kill target cells (or, more precisely, induce the release of the radioactive labels used) by a mechanism which is different from that of in vitro activated macrophages.

Attempts to define the parameters responsible for the apparent variability in the cytotoxic activity of MAF-treated macrophages were unsuccessful, but it does seem certain that a relatively long-time of contact between these cells and tumour targets is required before release of radioactive label into culture supernatants occurs. Although in vitro activated macrophages induce little or no release of radioactivity from labelled tumour target cells during the first 20 h of co-culture, cytostasis was apparently induced by this time indicating that macrophages had an effect on tumour cells long before significant release of radioactivity could be detected. However, there did not appear to be any direct relationship between the onset of cytostasis as determined by

inhibition of ^3H -TdR incorporation by tumour cells and the rate at which prelabelled tumour cells released radioactivity when co-cultured with in vivo and in vitro activated macrophages.

IL2 is also released by lymphoid cells from 11RX-immunized mice and, like MAF, requires the presence of T cells, 11RX antigen and adherent cells for its release.

Not all lymphoid cells release IL2 or MAF. Mesenteric LNC did not release the two lymphokines, whilst spleen cells taken from mice during the 11RX carrier state release only small amounts of IL2. The lack of IL2 release by LNC and spleen cells appeared to be due to a lack of lymphokine releasing cells rather than a lack of antigen presenting cells or the presence of suppressive cells. This conclusion was reached because the addition of normal PC to LNC and spleen cell cultures did not induce the release of IL2 in the presence of antigen and because spleen cells and LNC did not inhibit the release of IL2 by PC from 11RX-immunized mice.

Direct comparison of MAF and IL2 release by lymphoid cells at various times after 11RX immunization revealed that the pattern of release was not the same for both lymphokines. This suggests that the two lymphokines are released by different subsets of T cells or, if released by the same cells, their release is not regulated by the same control mechanism(s).

CHAPTER SEVEN

THE MECHANISM OF TUMOUR RESISTANCE IN 11RX-IMMUNIZED MICE

7.1 Introduction

Mice infected with the intracellular bacterial parasite Salmonella enteritidis 11RX are highly resistant to the growth of transplantable tumours. The induction of high levels of non-specific tumour resistance is a feature common to many if not all infections with intracellular parasites (discussed in §1.2 and 1.4). The purpose of this study was to better define the mechanism of this resistance and the data presented in this thesis deal with two different aspects. One is the nature of the cytotoxic effector cells and the means by which they recognise tumour cells, and the other is the characteristics of the sensitized lymphocytes which are generated during 11RX infections and which ultimately control and mediate the expression of tumour resistance.

7.2 The nature of the effector cells responsible for tumour resistance in 11RX immunized mice.

As discussed in detail in §1.3.2 and §3.1, previous studies of the tumour resistance generated by infections with intracellular parasites other than 11RX have clearly shown that activated macrophages from infected animals can kill tumour cells in vitro. Although evidence for a role of macrophages in vivo is not conclusive it is consistent with the observation that tumour resistance is abrogated by treating infected animals with anti-macrophage agents such as silica, carrageenan and trypan blue. In view of this it was not surprising that the data of Ashley and colleagues (discussed in Chapter 1 and §3.1) suggested that activated macrophages were also responsible for the tumour resistance

generated during llRX infections. However, their studies did not exclude the possibility that other cell types may be involved.

This study has clearly established that the resistance of llRX-immunized mice to the growth of transplantable tumours is effected principally by activated macrophages, with NK cells possibly having a minor role. This conclusion is based on the results of experiments which have used a variety of techniques to identify the cytotoxic cell(s). These include the use of the anti-macrophage agent trypan blue to abrogate the ability of llRX-immunized mice to destroy of ^{125}I -EAT cells in vivo, and to inhibit the in vitro cytotoxic activity of their PC. The second approach was to fractionate PC on the basis of adherence to plastic or nylon wool, and on the basis of their buoyant density, before assaying the various fractions for in vitro cytotoxic activity.

7.2.1 The effect of trypan blue on cytotoxicity

It was found that trypan blue inhibited the clearance of ^{125}I -EAT cells from the peritoneal cavity of llRX-immunized mice and greatly reduced the in vitro cytotoxic activity of their PC. These results confirm the results of others that trypan blue can inhibit the tumour resistance induced by intracellular parasites and other macrophage-activating agents (Hibbs, 1974; Hibbs, 1975b; Kreider, Bartlett and De Freitas, 1978; Morahan and Kaplan, 1976).

Consistent with reports that appeared in the literature during the course of this study, it was found that trypan blue cannot be used in vitro to determine whether the cytotoxic activity of a cell population is due to macrophages. The presence of trypan blue in the tissue culture medium inhibited equally well the in vitro cytotoxic activity of PC from llRX-immunized mice and PL from allo-immunized mice. This result confirms those of Scornick et al. (1980) and is consistent with reports that trypan blue can inhibit antibody responses by affecting lymphocyte function (Strauss et al., 1977), and that it can inhibit the complement

mediated phagocytosis by PMN leukocytes (Gukian et al., 1978).

Trypan blue appears to have a more restricted activity in vivo and the data available suggest that it affects macrophages selectively. For instance, the in vivo and in vitro activities of lymphoid cells from trypan blue treated mice can be at least partially restored by depleting them of adherent cells (Kripke et al., 1977). Chronic treatment of mice with trypan blue delays, but does not abolish, the rejection of highly immunogenic, ultra-violet radiation-induced fibrosarcomas (UVT) by syngeneic mice (Kripke, et al., 1977; Kreider, Bartlett and DeFreitas, 1978). Since cytotoxic T cells can be obtained from UVT-bearing mice (Urban et al., 1982), the delay in rejection may be due to the inactivation of macrophages with rejection being mediated by cytotoxic lymphocytes which are insensitive to trypan blue. Consistent with this interpretation is the finding that trypan blue treatment does not abolish tumour resistance of mice immunized using schedules which induce specific cytotoxic T cells (Kripke et al., 1977; Wu and Kearney, 1980; Kreider et al., 1978; Urban, et al., 1982; Benjamini et al., 1977; Parr et al., 1977). The observation that PL from trypan blue-treated allo-immunized mice, but not PC from llRX-immunized mice were cytotoxic for ^{51}Cr -EL4 in vitro provides further evidence that trypan blue does not inhibit the activity of CTL but does inhibit the cytotoxic activity of activated macrophages when injected ip into mice.

Studies of the inhibition of antitumour activity by trypan blue indicate that activated macrophages play a role in the tumour resistance of llRX-immunized mice not only early after immunization, when llRX organisms are present in the peritoneal cavity, but also after the carrier state has been eliminated e.g. (1) in mice 40 days after ip immunization with llRX, and (2) in iv immunized mice injected with llRX antigen, a treatment which recalls tumour resistance after the primary phase of tumour resistance has waned.

The reduction of the in vitro cytotoxic activity of PC from

short-term 11RX-immunized mice and recalled mice by injection of trypan blue correlates with abrogation of tumour resistance and suggests that activated macrophages are the effectors cells active both in vivo and in vitro. However, alternate explanations are possible. In some of the experiments ip injection of trypan blue into 11RX-immunized mice increased the total number of PC recovered from the mice and altered their cellular composition. Because of this it was possible that the reduction in the in vitro cytolytic activity of the PC was due to the cytotoxic cells being "diluted out". This is considered to be unlikely because diluting the PC from 11RX-immunized mice with PC from normal mice in a ratio of 1:3 (normal to immunized) caused only a small reduction in cytolytic activity, i.e. from 45% to 36% (Sawyer, 1980). In most of the experiments described in §3.3.1 the "dilution factor" was of the same order, yet the reduction in cytolytic activity was much more profound.

It was found on some occasions that ip injection of trypan blue 6-12 h prior to harvesting PC did not completely inhibit the in vitro cytotoxic activity of PC. This observation requires some comment. Because the experimental approaches used in the present study were not designed to provide an explanation for this finding, it is not possible to distinguish between a number of alternative possibilities presented below. Some macrophages may be cytotoxic even though they have ingested the dye. Alternatively, it is possible that the residual cytotoxic activity may be due to macrophages that are part of the cellular influx which occurs during the first 10 days of an ip 11RX infection (Ashley, 1976; Ashley and Kotlarski, 1982a), entering the peritoneum at a time when there is insufficient trypan blue remaining to inactivate them. Furthermore, in addition to macrophages, PC suspensions may contain cytotoxic effector cells which are not inhibited by trypan blue or, finally it is possible that the dye or a contaminant such as endotoxin, may have induced cytotoxic activity in the PC. Support for this alternative was obtained in some experiments when PC from normal mice

injected with trypan blue 24 h earlier exhibited significant levels of cytotoxic activity in vitro (data not shown).

7.2.2 Fractionation of cytotoxic PC on Percoll density gradients

The use of density gradients to fractionate PC suspensions prior to assaying their in vitro cytotoxic activity provided further evidence that activated macrophages were the effector cells mainly responsible for tumour resistance in 11RX-immunized mice. This was most convincingly demonstrated when the PC from short-term 11RX ip immunized mice were fractionated on continuous density gradients. Fractions which contained a lower proportion of macrophages than the starting population had reduced cytotoxic activity, whilst fractions which had the same proportion of macrophages as the starting population or were enriched in the proportion of macrophages they contained were at least as cytotoxic for ^{51}Cr -EAT cells as unfractionated PC. Since all the fractions were tested at an effector to target cell ratio of 100:1, it is clear that, on a cell for cell basis, their in vitro cytotoxic activity was dependent on the proportion of macrophages present in the fractions. This was confirmed using PC suspensions from long-term 11RX iv immunized mice which had been injected ip with 11RX antigen 2 days prior to harvesting the PC. The PC were fractionated on a discontinuous Percoll gradient and the cytotoxic activity of the fractions compared to that of unfractionated PC. The results obtained conflict with those of Hopper, Harrison and Nelson (1979) who found that the PC of CBA mice re-infected ip with live 11RX several weeks after iv immunization had similar cytotoxic activity regardless of their sedimentation velocity (size) or the proportion of macrophages present in the fractions assayed. It is possible that the results obtained may reflect differences between the in vitro cytotoxicity assays used by Hopper et al. (1979) and the standard assay used in the experiments reported in this thesis. Their assays were carried out over 48 h using ^{125}I -UdR labelled fibrosarcoma

cells which were not killed in a 20 h ^{51}Cr release assay. Cytotoxicity was not detected prior to 22 h of culture with macrophages, indicating that the actual cytotoxic mechanism is probably different from that causing the rapid lysis of ^{51}Cr -EAT or ^{51}Cr -P815 cells by PC from 11RX-immunized CB6F_1 mice. It is, therefore, possible that cells with different cytotoxic potential might have been detected. It is worth noting that kinetics of tumour killing similar to those reported by Hopper et al. (1979) were obtained during this study when PC from CB6F_1 mice immunized with 11RX ip 2 weeks previously were used (data not shown).

The results in Fig. 3.7 shows that the macrophages from different fractions are not equally cytotoxic. Although, fractions 5 to 9 each contained at least 80% macrophages, the higher density fractions showed the greatest enrichment of in vitro cytotoxic activity. It is not possible to comment on the specific cytotoxic activity of the different macrophage subpopulations because the fractions were tested only at the standard effector to target cell ratio of 100:1. The finding that PC subpopulations with similar proportions of macrophages vary in their cytotoxicity is consistent with a report by Lee and Berry (1980) that C. parvum-activated macrophages are heterogenous in their function. They found that, although antigen presenting function for in vitro immune responses and the cytostatic activity of PC are both associated with macrophages, the highest antigen presenting activity was associated with small to medium sized macrophages whilst the largest macrophages were the most effective inhibitors of tumour cell proliferation in vitro.

It was not established whether the in vitro cytotoxic activity of the higher density fractions (1-4) was due to the small numbers of macrophages present or was due to the activity of the other cell types found in the fractions. However, the results of experiments discussed below in which PC were fractionated on the basis of adherence to plastic or nylon wool suggests that non-macrophage-like cells can be cytotoxic in vitro. Similarly, Hopper et al. (1979) reported that the cytotoxic

activity of the cell fractions containing small PC was reduced when the non-adherent cells were removed which also suggests that non-macrophage like cells from 11RX-immunized mice can be cytotoxic in vitro.

7.2.3 Fractionation of cytotoxic PC into adherent and non-adherent subpopulations

Fractionation of cytotoxic PC into plastic-adherent and non-adherent cell populations supported the view that most of the in vitro cytotoxic activity was due to macrophages. Monolayers of adherent PC were as cytotoxic for ^{51}Cr -EAT cells as unfractionated PC. The observation that non-adherent PC were poorly cytotoxic for ^{51}Cr -EAT cells unless they were concentrated and used at the standard effector to target cell ratio of 100:1 showed that non-adherent cells made only a minor contribution to the in vitro cytotoxic activity of PC from short-term 11RX-immunized mice. Even when great care was taken to exhaustively deplete the PC of plastic-adherent cells and to ensure that the non-adherent cells were not contaminated with macrophages, the non-adherent cell population was still cytotoxic in vitro at an effector to target cell ratio of 100:1. This showed that the in vitro cytotoxic activity of non-adherent PC was not necessarily due to contaminating, weakly adherent macrophages but to other cell types, possibly NK cells whose activity is also boosted by agents such as BCG or C. parvum (Wolfe et al., 1977; Ojo et al., 1978).

The presence of NK cells was confirmed by filtering PC through nylon wool columns and comparing suspensions of the non-adherent cells with suspensions of unfractionated PC for their cytotoxic activity against P815 tumour cells which are NK resistant and MPC11 tumour cells which are NK susceptible (Roder et al., 1979c; Wiltrot, Brunda and Holden, 1982). It was established that EAT cells, like P815 cells, are poor targets for NK cell-mediated lysis. Because nylon wool non-adherent PC from unimmunized mice were not assayed, it was not established whether

the NK cells detected in the PC suspensions from 11RX-immunized mice were present prior to 11RX infection and/or whether 11RX infection increased their number or activity.

It is possible that non-adherent PC prepared by removing those cells adherent to plastic and those adherent to nylon wool contain different cell types because non-adherent PC eluted from nylon wool columns were less cytotoxic for ^{51}Cr -EAT cells than those obtained by using plastic dishes to remove adherent cells. Nylon wool may have filtered out some of the cytotoxic cells present in the cytotoxic PC suspensions simply on the basis of size as may occur with glass bead columns (Shortman et al., 1971). Alternatively, it is possible that there is a population of cytotoxic cells which may bind to nylon wool and not plastic. Such weakly adherent cells may be related to the weakly adherent cells reported by Steinman et al., (1979) or they may be "activated" NK cells which differ in several ways from "endogenous" NK cells found in normal, untreated mice. Kiessling et al. (1980) found that activated NK cells are more adherent to nylon wool than endogenous NK cells, and can kill some tumour target cells that are resistant to lysis by endogenous NK cells, e.g. P815 tumour cells (Tracey et al., 1977; Welsh and Zinkernagel, 1977).

7.2.4 The relative contribution of activated macrophages and NK cells to tumour resistance induced by 11RX infections

The experiments reported in this thesis did not assess the relative contributions of activated macrophages and NK cells to the in vivo control of tumour growth in 11RX-immunized mice. Circumstantial evidence that NK cells may have an in vivo role in tumour resistance of normal mice has already been discussed in some detail in §1.12.2. The contribution that activated macrophages and NK cells could make to the control of tumour growth in 11RX-immunized mice may depend on the target cells used. Wiltrout et al. (1982) suggested that some NK

cell-susceptible tumour-cell lines are actually resistant to in vitro cytolysis by activated macrophages and vice versa. One would predict, therefore, that the destruction of P815 and EAT cells would be effected principally by activated macrophages.

If 11RX infections do augment NK cell activity, then they may contribute to tumour cell destruction only if the mice are challenged early after immunization. This is because the augmentation of NK cell activity by BCG infection is transient; it peaks on day 6 and has returned to normal levels by day 22 (Tracey et al., 1977). It seems likely that 11RX would induce a similar, rather transient increase in NK cell activity.

The relative contribution of activated macrophages and NK cells to tumour resistance could be tested by immunizing NK cell deficient beige mice (Roder and Duwe, 1979) and their normal littermates with 11RX and comparing the rate of growth of a challenge dose of tumour cells in these mice. Similarly, the effect of injecting monoclonal antibodies specific for cytotoxic macrophages (Sun and Lohmann-Mathes., 1982) or antisera directed to NK cell markers such as Ly5 or asialo-GM₁ (Kasai et al., 1979, Kasai et al., 1980; Young et al., 1980) on tumour resistance in 11RX-immunized mice is another experimental approach which could help to resolve this question.

7.3 The requirement for cell contact in the lysis of tumour cells by PC

The cytotoxic activity of PC from 11RX-immunized mice is effected by a mechanism which requires that the activated macrophages are in contact with, or in close proximity to, the tumour target cells. This is because cytolysis was reduced if the in vitro cytotoxicity assays were carried out under conditions which reduced the ability of the effector and target cells populations to come into contact with each other. It was found that the rate of tumour cell-killing was reduced if the number of PC used in the in vitro cytotoxicity assay was less than 10^6 /well or

if the usual number of PC and ^{51}Cr -EAT cells were cultured in vessels with a larger surface. Moreover, cytolysis was almost totally inhibited when the effector and target cells were separated by a cell impermeable membrane.

These observations argue against the involvement of long-lived soluble cytotoxic factors mediating the in vitro cytotoxic activity of llRX-activated macrophages and are consistent with those previously reported in studies using macrophages activated by other agents (Cleveland et al., 1974; Hibbs, 1974; Hibbs, Lambert, Remington, 1972b; Holtermann, Klein and Casale, 1973; Kaplan, Morahan and Regelson, 1974). If soluble cytotoxic factors are involved in the cytotoxic mechanism of llRX-activated macrophages then, for one or more of the following reasons, they have a short range of action: (1) they can not be generated in high enough concentrations to be effective over relatively large distances, (2) the factors are inactivated too rapidly for an effective concentration to be built up over a distance equivalent to the thickness of a Sartorius membrane, (3) the factors bind to Sartorius membranes, or (4) when unlabelled EAT cells are added to the PC in an attempt to induce the cytotoxic factor(s), the factor(s) is absorbed by the tumour cells. Whilst none of the possibilities were excluded by the experiments reported, Adams and Marino (1981) have shown that the lysis of tumour cells by proteases secreted from BCG-activated macrophage is not inhibited by similar membranes although these proteases are inactivated by serum (Adams, 1980). Furthermore Reidarson et al. (1982a, b) reported that tumour cell-inducible macrophage cytotoxins cannot be detected in culture supernatants if the tumour cells are not removed when the cytotoxins are being released because they adsorb to the tumour cells. Thus, the second and fourth proposals appear to provide the more likely explanation for the inability to demonstrate the release of cytotoxic factors.

7.4 Effect of antibody on the cytolytic activity of PC

The observation that cell-cell contact is necessary for cytotoxicity may explain some reports that PC from mice injected ip with C. parvum (Bast et al., 1979) or macrophages from mice injected ip with LPS or BCG (Yamazaki et al., 1976) are not cytotoxic for ^{51}Cr -tumour cells in vitro unless anti-tumour antibody is present during the assay. It is possible that the experimental conditions did not ensure effective target cell-effector cell interactions unless Ab was present to facilitate close contact. For example, Yamazaki et al. (1976) conducted their in vitro cytotoxicity assays at an effector cell density which is 5-fold less than that in the standard assay used in the present study. Furthermore, their assays were conducted for only 8 h rather than 20 h. In other words, it is possible that Ab may have increased the rate of cytolysis by mediating or enhancing the contact between tumour cell and macrophages which is necessary for the lysis of tumour cells to occur. This notion would be consistent with the observation that CBA-anti-H-2^d antiserum increased the lysis of ^{51}Cr -P815 cells by PC from 11RX-immunized mice and increased the binding of the target cells to macrophage monolayers prepared from such PC (§4.8.3).

However the enhancement of macrophage-target cell contact may not be the only mechanism by which Ab augments cytolysis because in the experiments of Bast et al. (1979), the PC density was no less than that in the standard in vitro cytotoxicity assays used in the experiments reported in this thesis. In contrast to the results presented in this thesis, they detected no killing of tumour cells at 4 h.

Several explanations are possible. (1) Because unfractionated PC were used in this study and that of Bast et al. (1979), it is possible that the increase in or induction of cytolytic activity of PC by antibody was due to the involvements of additional effector cells such as granulocytes, neutrophils and K cells which are active in ADCC (reviewed by Pearson 1978).

(2) The tumour cells used in the experiments by Yamazaki et al. (1976) and Bast et al. (1979) may be resistant to the cytotoxic serine proteases released by activated macrophages. Antibody may have been required for cytolysis in order to induce the release of H_2O_2 by macrophages (Nathan et al., 1980) to which the tumour cells may be susceptible and/or the H_2O_2 released may have interacted with serine proteases to lyse the tumour cells.

(3) Alternatively, it is possible that the macrophages used by Yamazaki et al. (1976) and Bast et al. (1979) in their cytotoxicity assays were only "partly" activated and antibody was providing a triggering signal similar to that delivered by LPS (Ruco and Meltzer, 1978b). Lang, Domzig and Lohmann-Matthes (1980) have recently reported that macrophages treated with concentrations of MAF too low to induce non-specific in vitro cytotoxic activity, can lyse tumour cells coated with quantities of antibody insufficient to mediate ADCC by untreated macrophages.

Lang et al. (1980) suggested that "partly" activated macrophages possessed the cytotoxic machinery for lysing tumour cells but not the receptors for binding them and antibody induced cytotoxicity by circumventing the need for such target cell-binding structures. However, recent data reported by Marino and Adams (1982) indicate that this suggestion may not be correct. They have found that MAF induces the expression of receptors which augment tumour cell-binding by macrophages and that a second signal delivered by ng/ml concentrations of LPS is necessary for the induction of the cytotoxic mechanism. Because several different agents apparently can deliver the second signal (Ruco and Meltzer, 1978b; Schreiber, Altman and Katz, 1982) it is possible that antibody may be doing the same.

It is unlikely that in this study antibody increased PC cytotoxicity by delivering a "trigger" to partly activated macrophages in the PC suspension. This is because antibody did not restore the

cytotoxic activity of PC taken 13 days after 11RX immunization. These PC suspensions contain macrophages with enhanced tumour binding activity but are poorly cytotoxic using the standard cytotoxicity assay and thus appear to be only partly activated.

7.5 Relationship of the ability of activated macrophages to bind and lyse tumour cells

The requirement for contact or close association between cytotoxic PC and tumour cells for cytolysis to occur is consistent with observations of Marino and Adams (1980b) which were published during the course of this study. They found that strong physical binding of tumour cells to BCG-activated macrophages needs to occur before they are lysed. The results presented in this thesis confirm their findings that tumour cells bind strongly to activated macrophages. They will be discussed in detail in §7.7.2. In concordance with more recent studies from Adams' laboratory it was established that the ability of activated macrophages to bind tumour cells can be dissociated from their ability to kill tumour target cells. It was found that, although the in vitro cytotoxic activity of PC from 11RX-immunized mice peaks on day 6 and decreases thereafter so that by day 40 it has decreased to almost the same activity as that shown by PC from normal mice (Ashley, 1976; Ashley and Kotlarski, 1982a), the ability of their macrophages to bind tumour cells remained high during the whole of this period. This result confirms a similar observation by Adams and Marino (1981) using macrophages of BCG-immunized mice and is consistent with two other studies which establish that the ability of macrophages to bind tumour cells and their ability to secrete cytotoxic serine proteases are controlled independently.

In one series of experiments Adams, Marino and Meltzer (1981) used macrophages from three different strains of mice, namely the C3H/HeJ, A/J and C3H/HeN strains. The reason for using these mice is that while C3H/HeJ and A/J mice have a genetic defect which affects their ability to

respond to activation signals and ensures that BCG infection does not induce tumouricidal macrophages, C3H/HeN mice are normal and BCG infection of these mice induces macrophages which kill tumour cells in vitro. They injected all three mouse strains with BCG and then compared the ability of their macrophages to bind tumour cells and to secrete a cytotoxic serine protease in vitro. They found that the macrophages of C3H/HeN mice could bind tumour cells and release the cytotoxic factor, whereas those of A/J mice secreted normal amounts of cytotoxic factor but had a markedly reduced capacity to bind tumour cells. Macrophages of C3H/HeJ mice bound tumour cells well but secreted only small amounts of cytotoxic factor. It follows, therefore, that the binding of tumour cells to "activated" macrophages may not necessarily result in killing of these cells.

Confirmation of this has been provided in another study by Marino and Adams (1981). They demonstrated that, although the ability of BCG-activated macrophages to bind tumour cells in vitro showed only a slight diminution with time, their cytotoxic activity and ability to release the cytotoxic factor had decayed markedly during a culture period of 18 h. Both of these activities could be restored by the addition of LPS to the cultured macrophages. Furthermore, addition of LPS at a concentration of 20 ng/ml maintained the two activities during the 18 h culture period. The binding capacity of the macrophages was not altered in the presence of LPS.

7.6 The susceptibility of Con A blast cells to lysis by activated macrophages

There are conflicting reports in the literature regarding the susceptibility of normal cells to the cytotoxic effects of activated macrophages (reviewed by Keller, 1981). Con A blast cells are amongst the normal cells reported by others to be susceptible to cytolysis by activated macrophages, although the amount of lysis is not as great as

that obtained with tumour cells (Lohmann-Mathes et al., 1978; Hamilton and Fishman, 1981). Their data however, are difficult to interpret since the authors did not take into consideration that Con A blast cells require IL2 to survive relatively short periods of in vitro culture. Therefore the "cytotoxicity" that they reported may simply have been due to the unhealthy state of the blast cells.

Early experiments conducted during the present study were also done without adding IL2 to the culture medium. They could be interpreted to indicate that Con A blast cells labelled with ^{51}Cr , could be lysed by tumouricidal PC, as judged by the release of ^{51}Cr into the culture medium. However, the assay system was considered to be an unsatisfactory one because of the large amount of spontaneous release of ^{51}Cr that occurred when the blast cells were cultured in medium alone. Normal PC appeared to provide better conditions for the survival of blast cells in vitro, because less ^{51}Cr was released when they were added to the culture medium. PC from 11RX-immunized mice were not as effective in reducing ^{51}Cr release, although less label was released in their presence than in medium alone. The difference in the amount of ^{51}Cr released in the presence of normal and 11RX-activated PC may reflect cytotoxic activity directed against the Con A blast cells, but it is difficult to be confident that this interpretation is correct. It seems equally likely that blast cells are damaged and release ^{51}Cr because the activated macrophages produce changes in the culture medium, which are unfavourable to blast cells since they are metabolically much more active than normal PC. This view is supported by the finding that the release of label from ^{51}Cr -blast cells cultured with PC from 11RX-immunized mice was not detected until 20 h whilst it could be detected as early as 4 h when tumour cells were used, and the demonstration that unlabelled P815 cells could not competitively inhibit the release of label from ^{51}Cr -blast cells.

The present findings that ^{125}I -blast cells are not lysed by

tumouricidal PC when IL2 is present in the tissue culture medium, also support the latter interpretation and clearly establish that Con A blast cells are resistant to the cytolytic effects of l1RX-activated macrophages. The data indicate that when normal dividing cells are used as targets, the culture conditions must be optimized for the normal functioning of the target cells and that the radioisotope used for labelling them should be chosen to ensure as low a rate of spontaneous release as possible. $\text{Na}_2^{51}\text{CrO}_4$ appears unsuitable for labelling blast cells if they are to be used in long-term assays. Even in the presence of IL2, the spontaneous release of label from ^{51}Cr -blast cells at 20 h was as high as 50% (data not shown). ^{125}I -UdR is a better label to use because, over the same period of time, the spontaneous release of ^{125}I from labelled blast cells in the presence of IL2 was only 20%.

7.7 The recognition of tumour cells by activated macrophages

One of the most interesting questions related to the biology of macrophage-target cell interactions is:- why do tumour cells and normal dividing cells differ so greatly in their susceptibility to lysis by activated macrophages? The notion that simple metabolic differences between the two cells types render tumour cells more susceptible to the cytotoxic effects of macrophage-secreted products such as thymidine and arginase has been discussed previously in §1.11 and §4.1. However, there is a great deal of evidence that activated macrophages can "recognise" tumour cells much better than normal cells (discussed in §1.9). The most pertinent studies which have provided very good evidence that activated macrophages have receptors that preferentially bind tumour cells have been presented by Piessens (1978) and Adams and his colleagues (Marino and Adams, 1980a). These appeared in the literature during the course of the studies reported in this thesis and will be discussed in the context of data presented in the present study.

7.7.1 The results of cold target inhibition studies

(a) Activated macrophages recognize structures which are present on all types of tumour cells

Collectively, the results of cold target inhibition experiments reported in this thesis and those of Hamilton and Fishman (1981), and the binding experiments of Marino and Adams (1980 a, b) provide very good evidence that activated macrophages are non-specifically tumouricidal because they recognize determinants present on the surface of all tumour cells. The present findings that unlabelled P815 cells and EAT cells could competitively inhibit cytolysis of ^{51}Cr -P815 confirm the observations by Hamilton and Fishman (1981) that unlabelled tumour cells can competitively inhibit the lysis of antigenically unrelated tumour cells by LPS-activated macrophages. They are also consistent with reports that activated macrophages could bind to all tumour cells tested and that tumour cells (Marino and Adams, 1980a, b) and cell membranes prepared from them (Marino, Whisnat and Adams, 1981) can inhibit the binding of unrelated tumour cells to monolayers of BCG-activated macrophages.

(b) Activated macrophages do not interact with dividing T cells in the same way as they do with tumour cells

It was proposed at the beginning of this study that surface determinants common to all dividing cells, both normal and malignant, mediate the apparently non-specific recognition of tumour cells by activated macrophages. It was also suggested that only tumour cells are susceptible to the lytic mechanism. This notion was investigated using two experimental approaches. One was to study the binding of tumour cells, Con A blast cells and normal splenic lymphocytes to monolayers of 11RX-activated and normal macrophages and the results obtained will be discussed in §7.7.2. This study was complemented by comparing the

ability of unlabelled tumour cells and unlabelled Con A blast cells to competitively inhibit the lysis of ^{51}Cr -P815 cells and ^{51}Cr -EAT cells by PC from 11RX-immunized mice. The results of cold target inhibition experiments clearly show that Con A blast cells in the presence of IL2 do not inhibit the lysis of ^{51}Cr -P815 or ^{51}Cr -EAT cells by PC from 11RX-immunized mice. In other words, the results do not support the hypothesis that the tumouricidal activity of activated macrophages involves the recognition of surface structures common to all dividing cell forms.

These findings are not consistent with those of other workers who published their findings during the course of the present study. In 1979 Keller reported that foetal liver cells (but not adult liver cells) could competitively inhibit the lysis of tumour cells by C. parvum activated macrophages. More recently a finding by Hamilton and Fishman (1981) provided further evidence for the existence of surface structures common to all dividing cell forms. They reported that non-malignant dividing cells such as thymocytes, Con A-activated spleen cells, human diploid fibroblasts and normal rat kidney cells, but not resting cells such as splenic and lymph node lymphocytes, could competitively inhibit the lysis of tumour cells by LPS-activated macrophages.

(c) Possible reasons why the findings of cold target inhibition experiments differ from those of Hamilton and Fishman

No attempt was made to resolve experimentally the differences between the present results and those obtained by Hamilton and Fishman (1981). However, some of the experiments reported in this thesis, which were directed towards clarifying the other aspects of the present study, do provide some evidence that they may simply reflect differences in the assay system. The differences which may be most important include (1) the use of ^3H -TdR-labelled rather than ^{51}Cr -labelled target cells and (2) the use of macrophages activated with LPS during the cytotoxicity

assays rather than in vivo activated ones. These and other possible reasons for not being able to confirm the results of Hamilton and Fishman (1981) are discussed below in some detail.

i Reutilization of label

$^3\text{H-TdR}$ released from dead target cells, can be reutilized by other dividing cells in culture whereas this does not occur with ^{51}Cr (Bean et al., 1976). It is possible that in the experiments of Hamilton and Fishman (1981) the dividing, unlabelled tumour cells which were used for cold target inhibition did reutilize at least some of the $^3\text{H-TdR}$ which was released from pre-labelled target cells. This would result in "cold target inhibition" which was apparent rather than real. Their finding that mitomycin C treated unlabelled tumour cells had less inhibitory activity than untreated ones is consistent with this interpretation. However, an alternate explanation is that mitomycin C treatment inhibits the expression of the binding structures required for interaction with activated macrophages, an explanation that would be favoured by Hamilton and Fishman (1981). Unfortunately, their experiments were not designed to distinguish between these possibilities. The finding in the present study that normal macrophages actually enhanced the survival of blast cells in vitro and that small numbers of activated macrophages could stimulate blast cell proliferation (data not shown) shows that reutilization of $^3\text{H-TdR}$ needs to be formally excluded before the inhibitory effects of blast cells and thymocytes can unequivocally be attributed to competition with labelled tumour target cells for binding sites on activated macrophages.

ii The use of "normal" cell lines

The use of cell lines as "normal" cells for cold target inhibition studies needs to be treated with caution. It is well known that normal cells undergo numerous changes (for example, changes in chromosome

numbers and morphology) when adapting to in vitro growth and that prolonged cultivation of cells in vitro can result in cells acquiring a malignant phenotype (reviewed by Sanford, 1965; Handleman et al., 1977). For instance, the BALB/3T3 cell line which, according to various criteria, is non-transformed (Aaronson and Todaro, 1968), can give rise to tumours (Boone, 1975). Furthermore, Tucker et al. (1981) have shown that during spontaneous transformation in vitro, murine fibroblasts may become increasingly tumourigenic before they become susceptible to killing by activated macrophages. Whether activated macrophages can bind such tumourigenic, "non-susceptible" cell lines is not known. In Hamilton and Fishman's experiments, thymocytes and human diploid fibroblasts appeared to be the least inhibitory of all the dividing cells examined. This suggests that, if binding structures capable of interacting with activated macrophages are present on all dividing cells, they may increase in number upon transformation, or that there are additional factors involved in the recognition of transformed cells by activated macrophages.

iii Possible differences in the way various tumour cells interact with macrophages

It can be argued that comparisons between the data of Hamilton and Fishman (1981) and those presented in this thesis cannot be made because the tumour cells used were not the same. This is because different types of tumour cells may not bind to macrophages with the same efficiency and/or may do so via different receptors or they may associate with subpopulations of macrophages. For example Hamilton and Fishman's data indicate some specificity of inhibition whereas this has not been observed by other workers. Hamilton and Fishman showed that macrophage mediated lysis of Abelson virus-transformed normal rat kidney cells is inhibited more efficiently by homologous target cells than by G1 lymphoma cells. However, EL4 thymoma cells and membranes prepared from them can

inhibit the binding of P815 tumour cells to monolayers of BCG-activated macrophages, results which suggest lack of tumour specific inhibition (Marino and Adams, 1980 a,b; Marino, Whisnat and Adams, 1981). The present study provided evidence consistent with latter observations. When P815 cells and EAT cells were used in inhibition studies, no tumour specific inhibition was detected, and thus, it is unlikely that Con A blast cells did not inhibit the lysis of EAT and P815 target cells because the tumour cells are non-lymphoid in origin.

iv Competition may not involve tumour-binding receptors on macrophages

It is possible that in the experiments of Hamilton and Fishman (1981, 1982a) G1 lymphoma cells and Con A blast cells were not competing for receptors used by activated macrophages to bind tumour cells. The receptors involved may have been those described by Lipsky and Rosenthal (1973) which are found on oil-induced macrophages and can bind thymocytes in an antigen-independent fashion. Hamilton and Fishman (1981, 1982a) used oil induced macrophages in their studies and activated them with LPS during the in vitro cytotoxicity assays. Their finding that Con A blast cells could competitively inhibit the cytolysis of G1 lymphoma cells whereas non-dividing lymphocytes did not do so is consistent with the observation that lymph node lymphocytes do not bind to oil-induced macrophages to the same extent as thymocytes (Lipsky and Rosenthal, 1973). It was not established that LPS had induced the changes in the macrophages which result in the augmented binding of tumour cells. The cells were induced macrophages which had been pretreated in vitro for 24 h with LPS, but no data were provided to indicate that the degree of tumour cell binding obtained was higher than in untreated macrophages (Hamilton and Fishman, 1982a). It is possible that binding was not augmented because attachment of LPS-treated macrophages to culture dishes and target cells could be disrupted by vigorous washing (Hamilton and Fishman, 1982a). This is not a characteristic of the binding of tumour

cells to 11RX or to BCG-activated macrophages (present studies; Marino and Adams, 1980a).

7.7.2 The results of cell binding experiments

(a) Activated macrophages bind tumour cells and dividing and non-dividing lymphocytes with similar efficiency

As mentioned previously, the results of the cell binding experiments reported in this thesis confirm the results of Marino and Adams (1980a, b) that tumour cells can bind to activated macrophage monolayers more efficiently than monolayers of resident macrophages. It was also found that 11RX-activated macrophage monolayers could bind tumour cells and Con A blast cells equally well, whilst normal spleen lymphocytes were bound with half the efficiency.

These results do not agree with those from Adams' group. They obtained much greater differences in the binding of tumour cells and dividing and non dividing lymphocytes to BCG-activated macrophage monolayers (Marino, Whisnat and Adams, 1981; Marino and Adams, 1980a). The reasons for the discrepancies are not immediately apparent although they are probably associated with differences in the techniques used for these studies. For example, although spleen cells were used as the source of lymphocytes in both studies, Marino and Adams (1980a) used non-adherent spleen cells obtained by filtration through nylon wool, whilst the selection in the present study was on the basis of buoyant density. Secondly, the washing procedure used in the present study is probably gentler. It is, however, unlikely that this did not remove cells which were more loosely bound than tumour cells, because similar results were obtained when a washing technique similar to that reported by Marino and Adams (1980a) was used. The demonstration of differences in the binding of blast cells and P815 cells to 11RX-activated macrophages monolayers and macrophages from resident PC supports the view

that unbound cells could be removed by the washing technique used. Finally, the agents used to activate macrophages were different. This may have resulted in the activation of macrophages with different properties. For example, the macrophages used in most of these experiments were taken at a time when Salmonella organisms were present in the peritoneal cavity (Ashley and Kotlarski, 1982a). It is possible, therefore that LPS may be associated with these cells and may have played a role in binding. However, the finding that peritoneal macrophages from long-term llRX iv immunized mice injected with llRX antigen 2 days prior to harvesting PC bound all three target cell types equally well, suggests that LPS played no significant role in binding.

A major difference between the cell binding assay used by Adams' group and the one used in these experiments is the way cell contacts were achieved. In the present study, the target cells were sedimented onto the macrophage monolayers by gentle centrifugation 4°, to ensure that contact between macrophages and target cells occurred rapidly and almost synchronously. This was followed by incubation at 37° for 30 min. Marino and Adams (1980a) conducted all procedures at 37° and established contact between targets and macrophages by rocking the cultures and allowing the target cells to sediment on to them at unit gravity. Because in most of the cell binding experiments reported in this thesis the trays were incubated at 37° for only 30 min, it is likely that the temperature of the culture medium was at 37° for much less than 30 min. Therefore if the binding of blast cells to activated macrophages was weak or unstable at 37° the procedure used in the present study may have ensured that such weak interactions were detected.

(b) The binding of Con A blast cells to activated macrophages is transient

The notion that binding of blast cells by macrophages is unstable at higher temperatures is supported by the finding that, even in the

presence of IL2, fewer blast cells were bound to the 11RX-activated macrophages after 120 min than 30 min. This is consistent with earlier reports that the antigen-independent binding of thymocytes to macrophages is transient (Lipsky and Rosenthal, 1975; Lopez, Vatter and Talmage, 1977). In most experiments, the number of ^{51}Cr -P815 cells that were bound increased moderately over this time. Since the binding of blast cells to 11RX-activated macrophages is much less stable than the binding of tumour cells, it is not so surprising that blast cells cannot inhibit the lysis of P815 cells even if the same receptors were used to bind blast cells and tumour cells to activated macrophages. The findings of Balk, Walker and Mescher (1981) provide a precedent for this possibility. They established that the binding of target cells to CTL is an equilibrium process and that splenic lymphocytes, which are much poorer targets than tumour cells (Bevan and Cohn, 1975) will bind to CTL, but with lower affinity than the binding of tumour cells. Spleen cell-CTL conjugates readily dissociate with time; approximately 50% are dissociated in 2 h, whereas the binding of tumour cells to CTL is stable for several hours at least (Balk and Mescher, 1981). Although only tumour cells can displace tumour cells conjugated to CTL, both spleen cells and tumour cells of the appropriate haplotype can displace lymphocytes bound to CTL (Balk and Mescher, 1981).

In summary, the most likely explanation for the observation that LPS-activated blast cells bind to monolayers of BCG-activated macrophages much less effectively than tumour cells seems to be that binding is temperature dependent i.e., that more stable conjugates may be formed at temperatures lower than 37°.

(c) Does binding of tumour cells and blast cells to activated macrophages require the same receptor?

These experiments did not establish whether activated macrophages bound tumour cells and Con A blast cells via the same receptor(s). The

data available suggest that the binding of normal dividing T cells and tumour cells to macrophages may be related because there are similarities between the antigen-independent binding of thymocytes to induced macrophages and the apparently non-specific binding of tumour cells to activated macrophages. In both examples binding requires that the macrophages but not the target cells are viable, it requires the presence of cations and is inhibited by inhibitors of glycolysis such as iodoacetate (Lipsky and Rosenthal, 1973; Marino and Adams, 1980a). The finding that 11RX-activated macrophage monolayers can bind both tumour cells and blast cells more efficiently than normal macrophages also suggests that the same macrophage receptor binds both types of cells. This notion is consistent with the recent observation by Hamilton and Fishman (1982b) that murine Con A blasts can inhibit the binding of lymphoma cells to normal macrophages and macrophages activated in vitro with MAF and LPS. However, the observations in the present study are also compatible with the existence of two distinct types of receptors which are present in increased numbers on activated macrophages. Furthermore, as the Con A blast cells used in these experiments are cytotoxic in vitro, the present experiments do not exclude the possibility that their binding to activated macrophages is mediated by receptors on the blast cells and that the macrophage is the "passive partner" in the interaction. It is possible that this could be resolved by determining whether binding still occurs using heat killed macrophages or heat killed blast cells or whether Con A blast cells can inhibit the binding of ^{51}Cr -P815 cells to monolayers of 11RX-activated macrophages.

7.7.3 The role of carbohydrates in the recognition of tumour cells by activated macrophages

The nature of macrophage receptors for tumour cells is unknown although several lines of evidence suggest that they may recognise carbohydrate structures on the target cells (Weir et al., 1979; Muchmore

et al., 1981). The finding that a Con A-resistant mutant of Chinese Hamster Ovary cells (CR-7) with alterations in its surface glycoproteins (Wright, Jamieson and Ceri, 1979) is not killed by C. parvum or pyran activated macrophages whilst the parent cell line is lysed, suggests that target cell carbohydrates may be important in the interaction of tumour cells with activated macrophages (K.C. Lee and I. Sugawara, personal communication). This is further supported by the recent report by Brunda et al. (1983) that monosaccharides can inhibit the lysis of tumour cells by in vitro activated macrophages. In the experiments reported in this thesis the lysis of P815 cells by PC from 11RX-immunized mice could not be inhibited with simple sugars. Possible reasons for the lack of success are: (1) the surface sugars on P815 cells recognized by activated macrophages are not the ones used in the experiments, (2) the sugars are rapidly pinocytosed and metabolized by the PC and/or the tumour cells: for example, the half-life of ^{125}I -mannose-BSA on the surface of alveolar macrophages is less than 5 minutes (Stahl et al., 1980), and (3) activated macrophages recognize oligosaccharides; thus, even if any of the sugars used the experiments were part of the oligosaccharide chain, their ability to compete for the receptor would be low.

7.8 Characterization of lymphocytes induced by immunization with 11RX

Resistance to infection with intracellular parasites as well as the increased resistance of infected hosts to the growth of transplantable tumours is due to a cellular immune response to parasite antigens. Evidence for this and for the importance of T cells in this phenomenon have been discussed previously in §1.3.3 and §5.1. Antigen specific T cells mediate at least two aspects of 11RX-induced tumour resistance. Firstly, they activate macrophages which are important for eliminating the infectious organisms and for destroying tumour cells and secondly, they provide a pool of sensitized lymphocytes which are

responsible for the rapid recall of the non-specific resistance to intracellular parasites and to tumour cells when the host is reexposed to the immunizing parasite or injected with antigenic extracts of the parasites (Ashley et al., 1977). At the time this study was commenced, little was known of the nature of T cells which mediate the recall of tumour resistance in Salmonella-immunized mice. In this study the T cells induced during llRX infections were characterized by determining their functional life span and their ability to release the lymphokines IL2 and MAF in vitro.

7.9 The functional life-span of lymphocytes mediating the recall of tumour resistance in llRX immunized mice.

A pool of sensitized T cells is maintained in llRX-immunized mice for a long time because tumour resistance can be recalled in these mice for up to a year after the primary infection with llRX has been cleared (Ashley et al., 1974; Ashley and Kotlarski, 1982b). Several mechanisms can be proposed which may ensure the maintenance of sensitized T cells. For example, the T cells induced by llRX infection may be intrinsically long-lived and survive in the immunized host long after the infecting organisms have been cleared. Alternatively, depots of non-viable llRX antigens maintain a pool of short-lived lymphocytes. Evidence is available which indicates that immunological memory in rodents immunized with Mycobacteria (Lefford and McGregor, 1974; Lefford et al., 1973) and Listeria (Jungi and Jungi, 1981) is due to long lived lymphocytes. It would, therefore, not be surprising to find that the sensitized T cells present in llRX-immunized mice are also long-lived.

The results of cell transfer experiments reported in this thesis indicate that spleen cells from long-term llRX-immunized mice contain a major population of long-lived and a minor one of short-lived sensitized T cells. Both can mediate the recall of tumour resistance. Their relative contribution to the recall of ip tumour resistance in spleen

cell recipients appears to be determined by the route used for cell transfer.

7.9.1 Short-lived lymphocytes are detected after iv transfer of spleen cells

When spleen cells from 11RX-immunized mice were transferred iv, there was a dramatic decrease in the level of resistance to ip tumour challenge that could be recalled in recipients as the time between transfer and recall increased. The functional half-life of the mediator T cells was calculated to be 4 days. This is similar to the short duration of specific, systemic tumour immunity in mice adoptively immunized with lymph node cells from donors vaccinated with irradiated tumour cells and C. parvum (Tuttle and North, 1976b), and the short-lived resistance to Listeria in recipients of spleen cells taken from donor mice during an active Listeria infection (North et al., 1981). These findings are consistent with the conclusion, drawn from the results of studies with T cells of Listeria-immunized mice, that only short-lived lymphocytes can enter the stimulated peritoneal cavity (North and Spitalny, 1974; Koster, McGregor and Mackaness, 1971; Koster and McGregor, 1971; McGregor and Logie, 1974). In other words, when the iv transfer route is used, the cells which mediate recall of ip tumour resistance have characteristics which are similar to those of effector cells present in the spleen during a Listeria infection. They are large, dividing lymphocytes with a short recirculating and functional life span (McGregor, Koster and Mackaness, 1970, 1971; North, 1973b; McGregor and Logie, 1973; North and Deissler, 1975; North, Berche and Newborg, 1981). The short time interval during which in vivo tumour resistance can be recalled is consistent with the observations of Sprent and Miller (1976a) that alloantigen-activated thoracic duct T cells transferred iv to syngeneic recipients take about 10 days to leave the recirculating lymphocyte pool. From their data it can be estimated that it takes 4-5

days for the number of these cells present in the thoracic duct lymph to be reduced by one-half.

7.9.2 Long-lived lymphocytes are detected after ip transfer of spleen cells

In contrast to the rapid decay of T cell activity after iv transfer of spleen cells, it was found that if the spleen cells of immunized mice were injected ip, tumour resistance could be recalled for a much longer time after cell transfer. It was concluded that long-lived llRX-sensitized T cells with a functional half-life of at least 21 days were also present in spleen cells of long-term llRX-immunized mice. The possibility that the increased functional half-life of T cells after ip transfer was only an apparent one, simply due to the injection of recipients with non-limiting numbers of short-lived effector cells was eliminated by the direct comparison of the rates of decay of T cell function in recipients injected iv or ip with small numbers of spleen cells prepared from the same donors. The results again showed that the decay was slower after ip transfer. It is also unlikely that the "environment" of the peritoneal cavity increased the longevity of llRX-sensitized T cells because the rate of decay of T cell activity in the donors and recipients was similar. These results confirm and extend the results of Lefford et al. (1973), Lefford and McGregor (1974) and, Jungi and Jungi (1981) that immunological memory to intracellular parasite infections is mediated by long-lived sensitized lymphocytes. In addition, they confirm and extend the findings of Catanzaro et al. (1978) that lymphocytes migrate poorly from the peritoneal cavity.

It could be argued that the persistence of T cell activity after ip transfer of spleen cells was due to the transfer of viable llRX organisms which actively immunized the recipients or maintained the production of short-lived T cells. Several reasons make this unlikely: (1) Donor mice were used no earlier than 40 days after llRX immunization;

at this time very few, if any, viable llRX organisms are present in the spleen (Ashley, 1976; Ashley and Kotlarski, 1982a). (2) If sufficient llRX organisms were present in the spleen cell inoculum to immunize the recipients, then one should be able to recall resistance for quite long periods of time in mice injected iv with spleen cells; this is clearly not the case. (3) Infections of normal mice with small numbers of llRX did not cause detectable macrophage activation (data not shown). Furthermore Attridge and Kotlarski (unpublished observations) found that mice injected with a mixture of ^{125}I -EAT cells and spleen cells from day 23 iv immunized donors without added llRX antigen, cleared the label at the same rate as mice injected with just a mixture consisting of ^{125}I -EAT cells and normal spleen cells. This indicated that, even though the spleens from these immunized mice contained about 10^3 viable llRX organisms (Ashley, 1976; Ashley and Kotlarski, 1982a), the spleen cell suspensions used for injection did not contain sufficient organisms to provide a "recall" stimulus. (4) If very small numbers of llRX organisms were transferred with the spleen cell suspension, it is unlikely that they would have "sensitized" the recipients to show recall activity because no tumour resistance can be recalled in mice immunized 14 days previously with 100 or 10 llRX organisms iv (I. Kotlarski, personal communication).

7.9.3 Relative contribution of long-lived and short-lived lymphocytes in recall of tumour resistance

Although the experiments reported in this thesis were not designed to measure the relative proportions of long-lived and short-lived llRX-sensitized T cells in donor spleen cell suspensions, it appears that in the spleen of long-term llRX-immunized mice long-lived cells greatly out-number short-lived lymphocytes. It could be reasoned that, if the short-lived lymphocytes made a significant contribution to the total in vivo activity of the spleen cell suspension, then, after ip transfer

of spleen cells, a bimodal decay would be expected in the ip tumour resistance that could be recalled. That is, there would be an initial rapid drop reflecting the decay of the short-lived lymphocytes, followed by the much slower rate of decay due to the remaining long-lived lymphocytes. Since this effect was seen in only one of three experiments, it is likely that there are very few short-lived lymphocytes in the spleens of long-term llRX-immunized mice.

The presence of small numbers of short-lived (presumably dividing) lymphocytes in donor spleen cell suspensions suggests that there may be small numbers of bacteria in the spleens of long-term llRX-immunized mice. Although it is impossible to exclude their presence, previous studies (Ashley, 1976; Ashley and Kotlarski, 1982a) show that there could not be any more than 5 viable llRX organisms per spleen. It is also possible that there may be small depots of non-viable llRX antigens persisting in the spleens at this time and these may be responsible for maintaining a low rate of division of llRX antigen specific T cells.

Alternatively, it may be incorrect to conclude that short-lived, dividing llRX-sensitized T cells were present in the donors prior to harvesting cells. It is possible that all of the llRX sensitized lymphocytes in the spleen cell suspension were non-dividing but that some began to proliferate as a consequence of iv transfer into normal recipients. This suggestion is based on the finding by Sprent and Miller (1976a) that activated lymphoblasts and small thoracic duct lymphocytes from normal mice underwent some proliferation after injection into syngeneic recipients in the absence of any apparent antigenic stimulus.

7.9.4 The possible role of long-lived non-recirculating peritoneal lymphocytes in recalling tumour resistance

Because the ability to recall tumour resistance is transferred poorly by the iv route, and the majority of llRX-sensitized splenic lymphocytes are long-lived cells which cannot enter the peritoneal

cavity, it seems unlikely that the recall of ip tumour resistance in long-term llRX-immunized donors is mediated by cells present in the spleen of the animals. The recall of tumour resistance in long-term llRX iv immunized mice is probably mediated mainly by sensitized, long-lived, non-recirculating peritoneal lymphocytes which are the progeny of some of the short-lived lymphocytes that enter the peritoneal cavity during the early stages of the iv infection. This view is based on Jungi's findings that during the early phase of Listeria infections, specific lymphoblasts extravasate and settle in tissues such as the peritoneal cavity and the testes and some of them become tissue-positioned, long-lived memory T cells (Jungi and Jungi, 1981), that mediate the recall of resistance to Listeria. Several experimental procedures show that during Listeria infections many specific lymphocytes leave the recirculating lymphocyte pool and become immobilized in body tissues. Jungi (1981a) found that chronic thoracic duct drainage, which depletes rats of recirculating lymphocytes, did not abolish resistance of immunized rats to intratesticular challenge with Listeria. Consistent with this were the results of parabiosis experiments in which normal rats whose circulation was joined to that of Listeria-immunized partners at different times after infection were tested for their resistance to a Listeria challenge. Normal rats, joined to immunized rats early, but not late after infection, were protected against Listeria challenge, but the normal partners were never as resistant as the infected ones (Jungi, 1980a, b, 1981a). This was true even if parabiosis was established before infection and was maintained for 21-24 days thereafter (Jungi, 1980a, b; 1981). Further evidence for the existence of tissue positioned, long-lived memory lymphocytes in Listeria immunized rats is discussed below.

Several lines of evidence suggest that such a mechanism may also operate during llRX infections. It was found that resident PC taken from mice shortly after iv infection with llRX released IL2 and MAF when

stimulated in vitro with llRX antigen. Because iv immunization of CB6F₁ mice with llRX sets up an infection in the liver and spleen but not the peritoneal cavity (Ashley, 1976; Ashley and Kotlarski, 1982a), this indicates that sensitised T cells migrate from the spleen to the unstimulated peritoneal cavity of immunized mice. This is consistent with the observations of Jungi (1981b) that lymphocytes with in vitro reactivity to Listeria antigens can accumulate in the unstimulated peritoneal cavity of rats immunized subcutaneously with Listeria. More direct evidence for the presence of sensitized lymphocytes in the peritoneal cavity following iv immunization with llRX has been provided by Attridge and Kotlarski (personal communication). They have shown that PC as well as spleen cells from iv llRX-immunized donors can transfer the ability to recall tumour resistance to normal mice. This is consistent with the finding of Jungi and Jungi, (1981) that the resident peritoneal lymphocytes from Listeria-immunized rats can adoptively immunize normal recipients. Furthermore, since the Listeria specific PL are long-lived (Jungi and Jungi, 1981) it is likely that the llRX-specific peritoneal lymphocytes, like those found in the spleen of long-term iv immunized mice, are long-lived.

7.10 The release of lymphokines by lymphoid cells from llRX immunized mice.

The action of lymphokines released by antigen-stimulated lymphocytes in the control and expression of immune responses is well established. IL2 and MAF are included amongst the lymphokines which are important in the immune response to intracellular parasites. IL2 is released by helper T cells and provides the second signal required for the differentiation and continued proliferation of effector T cells (Lafferty et al., 1980; Wagner et al., 1980; Smith, 1980). As discussed previously (§1.6.1 and §6.1) MAF is released when sensitized T cells are incubated with antigen.

This study confirms and extends the finding of others that IL2 and MAF are released in vitro when lymphocytes from IBP-immunized animals are stimulated with antigen. Characterization of the different lymphoid cell populations for their ability to release both lymphokines at different times after llRX infection was made. Spleen cells and PC from llRX-immunized mice release IL2 and MAF when cultured for 20 h with soluble llRX antigen in vitro. This is consistent with recent reports that PC from Listeria-immunized mice will release IL2 in vitro (Finke et al., 1981; Kaufmann et al., 1982) and that the spleen cells from BCG immunized mice (Ruco and Meltzer, 1977), and the PC of Listeria-immune animals release MAF (Farr et al., 1979).

7.10.1 Differences between macrophage activation in vitro by lymphokines and activation in vivo by llRX infection

The ability to induce cytotoxic activity in normal and ppi macrophages by treating them in vitro with T cell-derived lymphokines supports the view that macrophage activation is mediated by T cells. It was evident, however, that lymphokine treatment does not activate macrophages to the same degree as in vivo llRX infection. Differences in the degree of activation were indicated by the fact that lymphokine-treated macrophages take much longer to kill P815 cells (or, more precisely, to bring about release of radioactive label) than PC from llRX-immunized mice. These differences may be related to a difference in the metabolic activity of in vitro and in vivo activated macrophages. Not too surprisingly, PC from llRX-immunized mice are much more metabolically active than lymphokine treated macrophages prepared from ppi PC. As an indication of this, it was found that 30 mM HEPES had to be added to the DMEM culture medium, containing 0.37% HCO_3^- , when in vivo activated PC are cultured to ensure that the pH is maintained at or near pH 7 (Ashley, 1976; Ashley and Kotlarski, 1982a). Addition of HEPES is not required when MAF-activated cells are cultured.

Attempts to increase the degree of macrophage activation by adding LPS to the lymphokine preparations used failed to induce cytotoxic activity that could be detected in a 20 h ^{51}Cr release assay. This is in contrast to the reports of other workers who claim that LPS does induce activation in MAF treated macrophages (Taramelli et al., 1980; Taramelli and Varesio, 1981; Pace et al., 1981; Marino and Adams, 1982). In experiments conducted in this laboratory, PC induced with a variety of sterile irritants and incubated with various preparations of lymphokines and endotoxins also failed to kill ^{51}Cr -P815 cells in a 20 h assay (Attridge and Kotlarski, unpublished data).

In most experiments, little or no cytotoxicity could be detected at 20 h, but target cell division was always found to be inhibited at this time when ever this was assayed. Such inhibition may be an earlier indicator of target cell damage than ^3H release. Although not investigated in this study, this notion is consistent with findings by Pasternack, Johnson and Shin, (1978a) that the proliferation of lymphosarcoma cells can be inhibited by starch-induced macrophages and alloantibody in the absence of ^{51}Cr release. Yet, when these tumour cells were separated from the macrophages and cultured in fresh medium, they failed to resume cell division even though they are metabolically active. The inhibited tumour cells died in the following 24-48 h (Pasternack et al., 1978b).

An inability of lymphokine-treated but not in vivo activated macrophages to secrete high amounts of proteases and nucleases may be a reason why cytotoxicity is not detected in short-term assays using lymphokine-activated macrophages. This is consistent with data reported by Roberts and Vasil (1982). In an experimental system similar to the one employed in this study, they found that ^3H -L929 cells co-cultured for 24 h with lymphokine-treated macrophages were dead, according to visual evidence, yet ^3H was not released. However, by adding DNA'ase to cultures at this time, large amount of label were released into the

medium.

7.10.2 Spleen cells and LNC release little or no IL2

The anatomical distribution of lymphocytes that release IL2 and MAF differ during the course of llRX infections. LNC released neither lymphokine whereas PC from iv and ip llRX-immunized mice release both MAF and IL2. The finding that spleen cells from such animals can secrete MAF but release little or no IL2 established that inability to release IL2 in the presence of llRX antigen does not necessarily indicate an absence of llRX-reactive T cells in the lymphoid cell population being examined.

The inability of murine spleen cells to release IL2 in vitro after iv llRX immunization may be related to the observation that spleen cells from rodents immunized with C. parvum, Mycobacteria and Brucella are unable to respond to antigens and mitogens such as Con A (Scott, 1972a, b; Wing and Remington, 1977; Kongshavn et al., 1977; Baird and Kaplan, 1977; Eardley and Jayawardena, 1977; Youdim and Sharman, 1976; Watson et al., 1975; Riglars and Cheers, 1980). Because IL2 is necessary as a second signal for the activation of effector T cells (Lafferty et al., 1980b; Wagner et al., 1980; Smith, 1980) the results from this study suggest that the hyporesponsiveness of spleen cells from IBP infected animals to T cell mitogens may be due to an inability of their T cells to release IL2. It would therefore be interesting to determine whether Con A can induce release of IL2 from the spleens of llRX-immunized mice. In the studies cited above it was found that activated macrophages in the spleens of IBP-infected animals can suppress T cell activation. Prostaglandins released by macrophages may mediate such suppression. For example, prostaglandins released from activated macrophages can inhibit T cell activation by Con A in spleen cells from C. parvum infected rats (Gemsa, 1981).

At first sight, it would appear that IL2 secretion by spleen cells of llRX-immunized mice may be suppressed by activated macrophages,

possibly via the secretions of prostaglandins. Although this explanation cannot be totally excluded by the experiments presented in this thesis, several reasons (discussed below) make this explanation seem unlikely. In other words, the more likely explanation is that spleen cells and LNC obtained from mice shortly after 11RX immunization are unable to release detectable amounts of IL2 is due to a paucity of 11RX-specific IL2 releasing cells in these tissues.

Notwithstanding the fact that indomethacin does not inhibit all suppressive mechanisms by activated macrophages (reviewed by Hoffeld, Metzger and Oppenheim, 1981), the drug which is an inhibitor of prostaglandin synthesis (Shen and Winter, 1977) was included in all cultures in which lymphoid cells were stimulated with 11RX antigen. Secondly, in contrast to the studies reported by Gemsa (1981) using rats immunized ip with C. parvum, Cahill and Hopper (1982) have shown that during a primary ip infection of CBA mice with 11RX, there is no increase in the spontaneous secretion of prostaglandins by macrophages. Although they found that 11RX-activated macrophages released more prostaglandins in vitro when stimulated with opsonized SRBC or C3-coated zymosan particles, they did not study the effects of 11RX antigen on prostaglandin release. Thirdly, the results of cell-mixing experiments do not support the view that suppressor cells present in the spleens or mesenteric lymph nodes of 11RX-immunized mice or a lack of antigen presenting cells prevent the release of IL2. These results, however, do not exclude the possibility that IL2-releasing peritoneal lymphocytes may be insensitive to the suppressive mechanism operating in the spleen or alternatively that IL2 release from spleen cells requires antigen presenting cells of a type not found in PC.

7.10.3 The possible action of suppressor cells in controlling the in vitro release of IL2 and MAF by PC early after 11RX infection

The fact that PC can be induced to release IL2 at a time when

large numbers of activated macrophages are present could also be used as evidence that activated macrophages do not suppress release of IL2 under conditions used in this study. However, it is possible that partial suppression of IL2 release may occur early after immunization because PC release far more IL2 if harvested on or after week 3 of infection than during the first two weeks. This may be related to the previous findings by Ashley and colleagues that by week 3 very few infectious organisms can be recovered from the peritoneal cavity and that the PC have lost most of their in vitro cytotoxic activity (Ashley, 1976; Ashley and Kotlarski, 1982a). In other words although there is no direct evidence for suppressor cells in the spleen cell suspensions of 11RX infected mice, it is possible that such cells are present in the peritoneal cavity during the first two weeks after ip infection, a time when PC contain activated macrophages (Ashley, 1976; Ashley and Kotlarski, 1982a). This would be consistent with the finding that macrophages from C. parvum treated mice and MAF-treated macrophages can inhibit the mitogen-stimulated release of MAF from normal lymphocytes (Varesio, Holden and Taramelli, 1981; Taramelli, Holden and Varesio, 1981). Cell fractionation studies using PC obtained shortly after immunization should resolve this possibility.

During this part of the discussion it has been tacitly assumed that the amount of LK released by lymphoid cells reflects the number of 11RX-sensitized T cells present in the lymphoid cell suspensions. However, it is possible that differences between cell populations at any one time during the infection or in the same cell population at different times may also reflect differences in the activity of antigen presenting cells in the cell suspensions. For this reason it would be desirable to compare the numbers of lymphokine releasing cells by more direct means e.g. using a constant source of antigen presenting cells such as irradiated normal spleen cells, normal PC or various cell lines and culturing these in the presence of 11RX antigen with various numbers of nylon wool-purified T cells.

7.10.4 The relationship between the lymphocytes which release MAF in vitro and those which confer protection in vivo

No direct or indirect evidence has been presented that the T cells which release MAF in vitro are the same as the lymphocytes which transfer the ability to recall tumour resistance. However, it is tempting to speculate that this may be the case. Recently Attridge and Kotlarski (personal communication) have found that spleen cells and peritoneal cells taken shortly after iv immunization with 11RX are very effective in transferring the ability to recall tumour resistance to normal mice. These observations provide circumstantial evidence for the notion that the release of MAF by lymphoid cells may be a good indicator of the presence of effector T cells in lymphoid cell suspensions. Certainly, it is true that the appearance of MAF-releasing cells after iv immunization with 11RX appears to be similar to the production of protective effector T cells in the spleens of Listeria-immunized mice (North, 1973; Jungi, 1980a; North and Deissler, 1975). Both appear related to the actual bacterial load in that organ. Secondly, the presence of larger numbers of MAF-releasing lymphocytes in PC than in the spleen following the loss of the carrier state in iv-immunized mice is consistent with Jungi's reports after the clearance of Listeria from rats, the unstimulated peritoneal cavity contains high numbers of protective sensitized lymphocytes even though they are no longer present in the draining LNC or thoracic duct (Jungi, 1980a, b; Jungi, 1981a, b; Jungi and Jungi, 1981).

The anatomical dissociation of IL2 and MAF production during the immune response to 11RX suggests that the two lymphokines may be released by different lymphocyte subpopulations. There are reports in the literature that are consistent with this conclusion. Kaufmann and colleagues found that the T cells from Listeria-immunized mice which release IL2 in vitro when stimulated with killed Listeria are $Lyt\ 1^+ 2^- 3^-$ whilst those which transfer protection to naive recipients i.e. cause macrophage activation in vivo are

Lyt 1⁺ 2⁺ 3⁺ (Kaufmann et al., 1982; Kaufmann, Simon and Hahn, 1979; Hahn and Kaufmann, 1982). Recently Kelso et al. (1982) found that some clones of cytotoxic lymphocytes which produced MAF when stimulated with antigen in vitro did not release IL2. However, this conflicts with the recent finding by Kaufmann et al. (1982) that several biological activities may be mediated by a single T lymphocyte population. They established that cloned T cell lines from Listeria infected mice can mediate several functions including the release of IL2 in vitro and the transfer of protection to normal mice.

7.11 Summary, conclusions and future directions

This study examined some of the aspects related to the mechanism of tumour resistance of CB6F₁ mice immunized with the IBP Salmonella enteritidis 11RX. It has confirmed that activated macrophages are responsible for most of the tumour killing observed in vitro and that they are probably induced during 11RX infections by soluble factors released from antigen stimulated T cells.

Cytotoxicity requires contact between activated macrophages and tumour cells and there is no evidence that long-lived soluble cytotoxic factors play a role in cytotoxicity in vitro. The finding that unlabelled tumour cells could inhibit the cytolysis of unrelated, labelled tumour cells suggests that the interaction between effector and target cells is mediated via surface determinants common to most, if not all tumour cells. Activated macrophages appear to have an increased number of receptors for these determinants. Although cell binding is required for cytotoxicity, tumour killing is not an automatic consequence of this interaction. These findings suggest that the abilities of macrophages to bind and kill tumour cells are controlled independently.

Con A blast cells and splenic lymphocytes also bind to activated macrophages. However, their interaction is qualitatively different from that of tumour cells. Con A blast cells are resistant to lysis by PC

from 11RX-immunized mice and do not inhibit the cytolysis of tumour cells by cytotoxic PC. A possible explanation for this was provided by the demonstration that the binding of blast cells to activated macrophages is transient, whereas that of P815 cells is much more stable. From these results, there is no evidence to suggest that the non-specific recognition of tumours by activated macrophages involves determinants common to all dividing cells.

Characterization of the sensitized lymphocytes induced by 11RX infections revealed that both long-lived and short-lived lymphocytes can mediate the ability to recall tumour resistance after the immunizing 11RX organisms are eliminated.

This study also demonstrated that soluble factors released by antigen stimulated 11RX-sensitized T cells, can induce cytotoxic activity in normal and inflammatory macrophages in vitro. However, the activation process in vitro does not completely reflect activation in vivo because lymphokine-treated macrophages take longer to kill tumour cells in vitro than those from 11RX-immunized mice. The release of IL2 and MAF by lymphoid cell populations of 11RX-immunized mice was studied in some detail. Their ability to release IL2 did not correlate with their ability to release MAF. It was concluded that different subpopulations of T cells may be required for the release of the two lymphokines; alternatively it is possible that there are different mechanisms which control the release of MAF and IL2 from the same population of T cells.

This study could be extended to further characterize the surface structures on tumour cells and the macrophage receptors which mediate binding. Such investigations may be facilitated by studying the interaction of activated macrophages with tumour cell lines which are resistant to their cytotoxic effects e.g., the Con A resistant clone of Chinese Hamster Ovary cells mentioned in §7.7.3 and the DNA virus transformed hamster cell line (SV40 HGl) which is tumourigenic in allogeneic animals (Cook, Hibbs and Lewis, 1980).

The characterisation of the sensitized lymphoid cells induced following immunization with 11RX is also by no means complete. Further studies are required to determine whether the tentative conclusion that the effector cells present in 11RX-immunized mice may have characteristics similar or identical to those induced following immunization with Listeria monocytogenes is a valid one. Studies using peritoneal and spleen cell suspensions obtained from animals shortly after immunization with 11RX may provide information which is particularly useful for this purpose. In addition, the reasons for the dissociation of IL2 and MAF release from sensitized lymphoid cells warrants further investigation as does the role of different types of antigen presenting cells in inducing cellular versus humoral immune responses to IBP.

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