The effect of bacterial infection or bacterial extracts on development of immunity to tumours in mice

Pâtricia Jane Sawyer B.Sc.Hons.(Adel.)

Department of Microbiology and Immunology

University of Adelaide

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CONTENTS

Abstract

Immunization of mice with viable, rough, avirulent *Salmonella enteriditis* 11RX increases their resistance to challenge with Ehrlich ascites tumour.

The model used in this project was the recall of tumour resistance in long-term 11RX immunized mice. Recall is elicited with a protein-rich extract of 11RX which is, in itself, not protective in normal mice.

The aim of this study was to use previously developed assays to characterize and fractionate 11RX extracts in order to obtain pure material. Another objective was the investigation of the effects of other strains of bacteria or extracts in the same experimental system.

The main results of the project were:

(i) The 11RX extracts consist of many different proteins, and contain only a small proportion of other bacterial components. The proteins have not been isolated in pure form.

(ii) It has been shown to be unlikely that bacterial endotoxin, in itself, is responsible for the recall phenomenon.

(iii) 11RX extracts were fractionated using filters of different sizes. Similar doses of filtered material can mimic the recall activity of the original extracts. This activity is not unique to one particular molecular weight division.

(iv) The 11RX extracts refered to in this abstract are made from the cell sap of the bacteria. Mixtures made from the outer or inner cell membrane of 11RX can mimic some aspects of the recall effect, when administered to long-term immunized mice.

(v) Cell sap extracts prepared from other bacteria in the Enterobacteriaceae group can elicit recall in long-term 11RX immunized mice. A similar extract made from Salmonella typhimurium M206 can recall mice immunized with M206.

(vi) An attempt was made to induce resistance in CBA mice to challenge with the syngeneic tumour RI leukaemia by immunization with 11RX. It was largely unsuccessful.

These findings are discussed in relation to present knowledge concerning the anti-tumour effects of bacterial vaccines in experimental animal models. The application of these studies to human cancer immunoprophylaxis and immunotherapy is also discussed.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

P.J. Sawyer

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Abbreviations

B cell or B lymphocyte	bone marrow-derived lymphocyte
T cell or T lymphocyte	thymus-derived lymphocyte
PPD	purified protein derivative of tuberculin
BCG	*Mycobacterium bovis* strain bacillus Calmette-Guérin

INTRODUCTION

1. Historical Aspects

Observations that human malignant growths sometimes undergo regression following acute bacterial infection were made many years ago. The earliest work of this type was carried out by Busch in 1866 (cited by Shear and Perrault, 1944). He reported in detail two cases of patients with sarcomas where the tumours shrunk markedly after spontaneous attacks of erysipelas. He then attempted to induce the infection in a woman with multiple sarcomas. His first attempts failed but he persisted and achieved some success. Following the appearance of the infection the large tumour shrank about 1 cm per day and the smaller tumours completely regressed. Subsequently, however, the large tumour grew rapidly again. Busch's work is all the more interesting since it was carried out before the advent of modern bacteriology.

Coley is often quoted as the first clinician to exploit the early observations on a large scale. Starting in 1891, he attempted to produce streptococcal infections in patients with inoperable tumours. When it became apparent that the infection could be fatal, he inoculated the patients with cultures of bacteria sterilized by heating or with sterile culture filtrates. However, neither of these treatments had any effect on tumour growth. In 1892 he learnt of the work of Roger which suggested that the toxins of Serratia marcescens could augment the virulence of other organisms (cited by Nauts, Fowler, and Bogatko, 1953). Coley mixed these toxins with those of the Streptococcus and filtered the mixture - the first preparation of "Coley Mixed Toxins". Fifteen different types of extract were used to treat human tumours over the

next 60 years, usually intra-tumour (i.t.) injection was used. Out of a total of 1200 cases listed by Nauts and colleagues, there were 300 in which complete regressions were elicited by toxin therapy.

Research work was also carried out in laboratories. Using an E. coli toxin extract Andervont (1936) elicited temporary regressions of murine tumours. These included a spontaneous mammary carcinoma and a primary sarcoma induced by a hydrocarbon carcinogen. He found that the toxin was only effective against fully established tumours. Some of the striking regressions he observed were associated with a haemorrhagic reaction and resulting damage to the tumour vasculature. Shwartzman (1936) and Gratia and Linz (1931) were among other pioneers who used toxins from gram-negative bacteria to induce necrosis in animal tumours.

Lack of continued interest in Coley's toxins was probably due to the deleterious side effects of the treatment and the development of radiotherapy and chemotherapy techniques for the treatment of cancer.

Other olinicians also made observations on the association between tumour regression and infectious disease in man. Shear claimed that 75% of the spontaneous remissions in childhood leukaemia observed in an American hospital had occurred after acute infection (Shear, 1950). Jacobsen (1934) cited data which shows a low incidence of tumours in people with tuberculosis, osteomyelitis, typhoid, para-typhoid, scarlatina, and diphtheria. He emphasised the importance of an active reticuloendothelial system (RES) in coping with neoplasia and Nauts et al., (1953) concluded that the increased incidence of cancer was connected with the advent of modern asepsis.

In the 1940's when Medewar demonstrated the immunological basis of tissue rejection, Gross discovered that some animal tumours expressed tumour specific transplantation antigens (Gross, 1943). It therefore seemed likely that immunological mechanisms played a role in host defence against cancer and interest was revived in the possible exploitation of bacteria or their extracts for cancer immunotherapy.

Early workers in this field used crude ill-defined preparations of bacterial "cultures", "toxins", or "filtrates". The gram-positive Streptococcus used by Coley in his mixtures was known to elaborate an exotoxin, but, in the light of further information, it is unlikely that it was responsible for the tumour necrosis in man or animals induced by toxin extracts. Shear emphasised that the active material was a protein-free product of gram-negative bacteria (Shear and Perrault, 1944). This is consistent with Coley's observation that it was necessary to include a Serratia marcescens extract in his effective toxin preparations. Probably the most important component of the toxins used for tumour therapy is endotoxin (or lipopolysaccharide, refered to in the following pages as ET). This is a major component of the cell walls of most gram-negative bacteria. It can elicit numerous biological and pharmacological effects in animals and man.

2. Anti-tumour effects of endotoxins

In order to elucidate the ways in which toxin therapy operates, it is relevant to look at the data describing the effects of purified ETs. Before proceeding further, it is important to note that most workers agree that ET exerts no direct toxic effect on tumour cells.

Complexes of lipid and polysaccharide from various micro-organisms of widely differing taxonomy have been reported to be active against solid tumours (reviewed by Whistler, Bushway, Singh et al., 1976). The results indicated that differences in the type or dose of extract, strain of mouse or rat, size and type of tumour and routes of administration may give rise to divergent effects (Whistler et al., 1976).

In 1943 Shear and Turner isolated Serratia marcescens ET. When injected i.p. into mice bearing subcutaneous (S.C.) implants of 8 day old Sarcoma 37 this material induced extensive tumour necrosis. Complete destruction of the tumour did not occur and the surviving tumour cells were responsible for the death of the animals. Due to its toxicity, clinical tests were not performed with this ET.

Numerous workers have used the non strain specific mouse tumours Ehrlich ascites tumour (EAT), Sarcoma 180 or TA3-Ha to study tumour prophylaxis or necrosis elicited by various ETs (Sugiura, 1953; Hager, Bailey, Hampers et al., 1969; Mizuno, Yoshioka, Akamatu et al., 1968; Yang and Nowotny, 1974; Nigam, 1975). Preparations used included Shear's ET, and ETs and glycolipids extracted from various gram-negative bacteria.

In the case of EAT, evidence for protective or therapeutic effects of ET is conflicting when one compares the data of these workers. Taking this into account, ETs can probably

protect mice against challenge with EAT (intraperitoneal (i.p.)/i.p. or s.c./i.p.), and can be of therapeutic value when given systemically after a solid tumour implant. Only Sugiura (1953) claims to show that ET was therapeutic for EAT in the ascitic form as distinct from the solid form and this result remains in doubt. Indeed, Parr, Wheeler, and Alexander (1973) have shown that ET is not therapeutic for other ascitic murine tumours. A large solid tumour mass is the optimum target for ET's therapeutic actions, with alteration of vasculature playing an important role in tumour regression. However, Hager et al., (1969) showed that a disturbance to the blood supply was not related to the prophylactic effects of ET against ascitic EAT.

Hager et al., (1969) tested the anti-tumour effects of ET using a number of different mouse strain and tumour combinations and found the strain of mouse used was important, even with EAT. C57BL/6J and C57L/J mice were completely protected against i.p. growth of EAT if ET was given i.p. 10 days prior to challenge. They observed little effect in any system if ET was given after the tumour or injected together with the tumour challenge.

Yang and Nowotny (1974) demonstrated that as little as 1 µg of ET rendered mice resistant to challenge with TA3-Ha given in the same site.

ET can inhibit the growth of syngeneic as well as non-specific animal tumours. As mentioned before, the work of Andervont indicates that ET was also effective against primary murine tumours. Tripodi, Hollenbeck, and Pollack (1970) found that admixture of ETs with Sa1 spindle cell sarcoma prior to challenge of mice resulted in tumour growth inhibition if low

doses of tumour cells were used. Parr et al., (1973) described the mouse lymphoma L5178Y and the fibrosarcoma FS6 as being susceptible to ET treatment. The most marked effects were observed when ET was injected into s.c. or intra-dermal (i.d.) tumours, or when it was given systemically to mice bearing solid tumours. In contrast to these reports, Hager et al., (1969) found that the dose of ET which protected mice against EAT challenge did not protect them against the growth of a polyoma, a myeloid leukaemia or a lymphosarcoma.

Ribi, Granger, Milner, et al., (1975) studied the effects of ET on the growth of transplantable, syngeneic guinea pig line 10 tumours. At the time of i.t. injection of ET the i.d. tumours were 6-12mm in diameter and had metastasized to the regional lymph nodes. At a dose of 300 µg ET had no effect on tumour growth. However, when administered in combination with oil and a special lipid component from mycobacterial cell walls ET produced cure rates of up to 90% (see also section 5.2(ii)).

Whistler et al., (1976) review the numerous other experimental animal tumour systems in which ETs have been demonstrated to be efficacious in slowing tumour growth.

2.1 The active component of the endotoxins

The portion of the ET complex which is responsible for its tumour necrotic and growth limiting properties has been the subject of disagreement between various workers. The ET preparations used contain lipid, polysaccharide and in some cases protein impurities. As early as the 1940's there were indications that the effective portion could be lipid. Shear and Turner (1943) found that trypsin digestion did not affect the potency of their material. Nitrogen content could be lowered drastically with no effects. The active material consisted of

sugar and phospholipid in which no protein could be detected. The most recent work indicates that Lipid A is the critical component. This is not surprising, as Westphal, Lüderitz and coworkers have shown that Lipid A, when suitably solubilized, will mimic many of the biological activities of its parent molecule (Lüderitz, 1970).

Papers contributing to the idea that Lipid A is the active component are those of Kasai, Aoki, Watanabe et al., (1961), Mihich, Westphal, Lüderitz et al., (1961), Nowotny, Golub, and Key (1971), Nigam (1975) and Chang and Nowotny (1976). However, the work of Parr et al., (1973) provides the most definitive study. They found that 250 µg of solubilized Lipid A induced tumour necrosis and regression when injected into mice bearing a syngeneic lymphoma. They obtained similar results using 100 µg of glycolipids extracted from S.minnesota R595. For both preparations the effective anti-tumour dose was approx. 10 times higher than that of smooth ETs. This correlates exactly with the differences in LD_{50} of the materials in mice, the lipids being the less toxic (Galanos, Rietschel, Lüderitz et al., 1971). Hence it may be that Lipid A or glycolipids are as effective as smooth ETs in inducing tumour necrosis, when this "therapeutic index" is taken into account.

Another important observation was made by Alexander and Evans (1971). They demonstrated that when either ET or Lipid A was present in the culture medium, a monolayer of mouse peritoneal macrophages inhibited the growth of lymphoma cells. On a weight basis there was little difference in the effectiveness of the two preparations.

The above results, and those in the other papers noted, leave little doubt that Lipid A can mimic the anti-tumour effects of ET.

2.2 Mechanism of the anti-tumour effects of endotoxins

For the reasons discussed above, in the 1940's immunologists began to take an interest in the experiments of Coley, Andervont, Shear and other workers in the field of toxin therapy. However, it was not until early this decade that a more academic interest in the mode of action of ET led to an intense period of experimentation.

The evidence available in the literature shows that both non-immunological and immunological factors are involved in the anti-tumour activity of ET. The following sections consider some of the information relevant to this problem.

2.2.1 Role of the Shwartzman reaction in the ET induced necrosis of established tumours

The Shwartzman phenomenon has been demonstrated with guinea pigs and rabbits but not with mice. Injection of a few µgs of ET s.c. induces a mild skin inflammation. If 24 hours later a similar injection is given intravenously (i.v.) the skin site becomes haemorrhagic within 2 hours. The venules become blocked by leukocyte platelet thrombi and polymorphs. At the end of 4 hours the skin is the site of deep purple lesions. This reaction develops so rapidly, unlike immunological responses, that it has long been the butt of immunologists' jokes.

In 1936 Andervont described similar necrotic reactions in solid mouse tumours which occurred 2-4 hours after i.v. injection of ET. Solid tumours are largely dependent on vascular

supply and local haemorrhage regularly leads to tumour cell damage. On the other hand, growth of ascitic tumours leads to the damage of the oemental and visceral blood vessels and red blood cells in the ascitic fluid are a usual terminal occurrence.

Are the two phenomena related? The solid tumour vasculature without prior exposure to ET responded to ET like the blood vessels in the skin of previously sensitized animals.

Parr et al., (1973) found that when tumour bearing mice were treated with heparin prior to the injection of ET the degree of tumour necrosis was reduced in comparison with the appropriate controls. This suggests that clotting in the venules does play a part in the initiation of solid tumour regression. Since heparin can inhibit the Shwartzman reaction (Good and Thomas, 1953), we can conclude that ET induced tumour regression can, in part, be attributed to a Shwartzman like phenomenon.

In contrast, there is evidence which indicates that a Shwartzman reaction is not an important factor in the ET induced suppression of tumour growth. Tripodi et al., (1970) prepared an alkali-treated ET extract. Unlike native ET, this extract did not inhibit murine sarcoma growth when injected s.c. together with tumour cells although it could elicit local tissue necrosis in rabbits.

2.2.2 Role of host immune response

Parr et al., (1973) presented evidence which showed that immunogenic murine tumours were more responsive to ET therapy than those with little or no intrinsic immunogenicity. These workers also found that although haemorrhagic necrosis still occurred in L5178Y lymphomas in ET treated mice which had been

irradiated with 400 rads, there were no tumour regressions. Administration of anti-lymphocyte serum or cortisone had similar effects. Although these treatments may induce other pathological changes, it is probable that immunosuppression is the cause for the lack of effect of ET on tumour growth in these mice.

Parr et al., (1973) showed that the effect of x-irradiation was less marked when mice bearing the FS6 fibrosarcoma were treated with ET. This tumour contains 40% macrophages, which are known to be radioresistant when fixed in the tissues. This suggests that tumour associated macrophages have a role to play in ET induced tumour regression. The observation is compatible with Evan's (1973) conclusion that there is a relationship between the high macrophage content of rat tumours and low metastatic rates.

From the evidence one could hypothesise that the initial vascular damage to a solid tumour elicited by ET is followed by the infiltration of host cells or mediators into the tumour and by macrophage activation. The importance of these factors may vary with the type of tumour studied and with the route of injection of ET (i.e., whether it is given i.t. or systemically).

2.2.3 Natural hypersensitivity to endotoxin

Many workers point out that the effects of ET may well depend on the host being presensitized by ET producing bacteria in the normal gut flora (Nelson, 1969; Burnet, 1971). Skin reactions to ET are intermediate between Arthus-type reactions and delayed-type hypersensitivity (DTH). There is a similarity between the reaction of a sensitized animal to the

antigen given i.v. and that produced by ET in a normal animal (Stetson, 1961).

Schaedler and Dubos (1964) showed that pathogen-free or germ-free mice were highly resistant to the toxic effects of ET. On acquiring intestinal flora from other mice the animals became susceptible. Kim and Watson (1966) did, however, show that germ-free piglets were just as sensitive to the toxic effects of ET as conventional animals. They suggested that hypersensitivity may be involved in the fever response to ET, but that the toxic effects were the manifestation of a primary response.

It seems likely that some of the actions of ET are due to the host being presensitized and that this is why minute amounts of ET can be extremely potent and the reactions are accelerated.

2.2.4 Role of activated macrophages

Data from in vivo and in vitro experiments indicates that the activated macrophage is probably the principle effector cell involved in the ET induced inhibition of tumour growth.

Hager et al., (1969) showed that protection against i.p. challenge with EAT could be transferred to normal recipients with peritoneal macrophages from ET treated mice, but not with spleen cells or serum. Berendt and Saluk (1976) harvested peritoneal cells from mice 3 days after i.p. administration of ET. These cells, or the cell-free peritoneal fluid, transferred resistance to normal mice to challenge with TA3-Ha tumour. These workers showed that the fluid did not contain residual ET, but it was not established whether or not the soluble factor accounted for the activity of the cells. Currie

and Basham (1975) had shown that after 24 hours of culture in the presence of ET rat macrophages released a soluble factor which lysed tumour cells but not normal cells. In contrast, other workers have found that contact between tumour cells and ET activated macrophages is required if cytostatic or cytolytic effects are to be observed (Alexander and Evans, 1971; Bruley-Rosset, Florentin, Khalil et al., 1976).

Alexander and Evans (1971) obtained some other pertinent results. Adherence-purified peritoneal macrophages taken from ET treated mice inhibited the growth of lymphoma or sarcoma cells in vitro. Similar effects were demonstrable if macrophages from normal mice were incubated with tumour cells and ET in vitro. Only after 2 or 3 days of culture was a cytotoxic effect observed. The macrophages used could be taken from mice which were syngeneic or allogeneic with respect to the tumour cells.

The results of the in vitro experiments discussed above show that ET can activate macrophages directly via non-immunological mechanisms. In this context, it is interesting that nude mice, which are genetically T cell deficient, possess activated peritoneal macrophages (Cheers and Waller, 1975; Meltzer, 1976; Emmerling, Finger, and Hof, 1977). It is probable that the increased gut flora of these mice will attack the immune-deficient wall of the intestine and the resulting damage will allow bacterial products, such as ET, to get through and activate macrophages. Indeed, Meltzer (1976) showed that germ-free nude mice did not possess activated peritoneal macrophages.

However, there is data which shows that ET can activate macrophages indirectly via immunological mechanisms. Wilton,

Rosentreich, and Oppenheim (1975) studied guinea pig macrophage activation by ET *in vitro*, using ^{14}C-glucosamine uptake as their criterion of activation (Hammond and Dvorak, 1972). They found that activation only occurred when B cells were added to pure cultures of macrophages. In addition, cell-free supernatants from ET stimulated B cells caused quiescent macrophages to take up glucosamine. In light of this report, it is interesting that Yoshida, Sonozaki, and Cohen (1973) reported that guinea pig B cells stimulated by ET produced migration inhibition factor (MIF). MIF was the first described of the lymphokine mediators of cellular immunity, originally thought to be elaborated when sensitized T cells were stimulated with specific antigen. Since then, Bloom, Stoner, Gaffney *et al.*, (1975) have demonstrated that T cell depleted guinea pig spleen cells sensitized to tuberculin could produce MIF upon stimulation.

A report of Carswell, Old, Kassel *et al.*, (1975) provides suggestive evidence that immunological factors can be involved in the interactions of ET with macrophages. Serum from mice injected i.v. with *Mycobacterium bovis* strain BCG or *Corynebacterium parvum* and injected i.v. with ET 14-21 days later contained a factor which induced necrosis in solid mouse tumours if injected i.v. into tumour-bearing hosts. Both treatments were essential if tumour necrosis factor (TNF) was to be produced. At the time of large scale necrosis haemorrhage was not evident, and TNF did not affect the growth of normal cells in culture but it had a cytostatic effect on tumour cells. It seemed likely that TNF was a product of activated macrophages, as when it was abundant in the blood there was widespread pykonosis and disruption of the macrophages in the hyperplastic spleens of the mice.

2.3 Conclusions

In conclusion, to what extent the anti-tumour action of ET is immunological in nature is uncertain. ET can activate mouse and rat macrophages by direct contact, although work with guinea pig macrophages suggests that B cells are involved in this process. Parr and colleagues did show that immunosuppressive measures abrogated the therapeutic effect of ET for some murine tumours, and in addition that immunogenic tumours were the most susceptible to ET treatment.

The clinical application of ET for tumour immunotherapy is likely to remain a memory from the days of Coley because the material is very toxic. Its therapeutic action thrives on large tumour masses, and not on small numbers of cells. Although it can induce regressions of large solid tumours, any metastases would probably not be eliminated.

Bacteria used as immunotherapeutic agents in man may contain ET. However, the standard gram-negative type of ET has not been described in two of the main agents, BCG and C.parvum, although their cell walls do contain lipids and polysaccharides.

3. Inhibition of tumour growth by intracellular parasites

Preamble

Hibbs (1975) reviews the "immunopotentiators" (a term coined by Sir Peter Medawar) which have been shown in experimental animals to increase non-specifically the resistance of the host to infection with micro-organisms and to tumour growth.

Chronic infection with intracellular parasites and other organisms such as BCG, Salmonella enteriditis, Listeria monocytogenes, LDH elevating virus, M-P virus, haemolytic anaemia virus, protozoa, and metazoa will all stimulate host resistance non-specifically.

Other agents which can activate the RES and act as adjuvants have similar properties to the above mentioned live organisms. Among these are ET, methanol extraction residue of BCG, and killed bacteria such as C.parvum, B.pertussis, and M.butyricum.

Resistance is termed non-specific because there does not have to be an antigenic cross reaction between the stimulating agent and the challenge micro-organism or tumour. For example, mice infected with L.monocytogenes or BCG are resistant to challenge with the unrelated bacterium S.typhimurium (Mackaness, 1964; Senterfitt and Shands, 1970). Kierszenbaum (1975) showed that i.v. injection of C.parvum protected mice against infection with the protozoan T.cruzi.

3.1 Activated macrophages

Intracellular parasites can, by definition, multiply within the phagocytic cells of a host, and may kill these cells. Killing of these parasites has been shown to require

the participation of "activated" macrophages. These cells are capable of killing not only the homologous infecting organism but also a wide range of unrelated intracellular pathogens and tumour cells.

The mechanism of activation of macrophages with heightened microbiocidal properties was elucidated by Mackaness and colleagues (reviewed by Collins, 1974). The intracellular parasites in a host provide a source of antigen which sensitizes thymus-derived lymphocytes. Activated macrophages are induced by the interactions of these cells with macrophages. Non-specific cellular resistance declines with the disappearance of the antigenic stimulus from the host but can be rapidly recalled by reintroduction of the sensitizing antigen.

There is evidence that such a mechanism is involved in the induction of immunity to Listeria, Salmonella, and Mycobacteria (North, 1975; Davies and Kotlarski, 1976b; Lefford, 1975). In the case of Listeria, there is no correlation between antibody titres and the immunity induced to these bacteria (North, 1975). However, the induction of resistance to S.typhimurium in mice requires specific antibody as well as a cell-mediated immune response (Davies and Kotlarski, 1976a). In the case of immunity to Listeria, the sensitized T cells are short-lived, but there is evidence that mice immunized with BCG or Salmonella remain immune hundreds of days after the initial sensitization, suggesting that the sensitized lymphocytes are long-lived (reviewed by Davies, 1975).

In vitro studies have shown that sensitized T lymphocytes release a plethora of biologically active substances called lymphokines, some of which are involved in the induction of macrophages with increased bactericidal or bacteriostatic

capacity. For example, Patterson and Youmans [1970] immunized mice with M.tuberculosis H37Ra and then incubated the splenic lymphocytes with H37Ra in vitro. When incubated with supernatants from these cultures normal mouse macrophages could inhibit the intracellular growth of viable H37Ra. Fowles, Fajardo, Leibowitch et al., [1973] stimulated guinea pig lymphocytes with the mitogen Concanavalin A in vitro. The partially purified culture supernatants were incubated with normal guinea pig macrophages. Such macrophages suppressed the growth of L.monocytogenes. Similar effects were observed by other workers who used supernatants from mixed leukocyte cultures or from Toxoplasma stimulated sensitized spleen cells (Godal, Rees, and Lamvik, 1971; Krahenbuhl and Remington, 1971).

As in the case of immunity to the intra-cellular parasites, there is data which shows that T cells and lymphokines play an indirect role in tumour destruction. Evans and Alexander [1972] incubated lymphocytes from BCG-immunized mice with PPD in vitro. The supernatants from these cultures "armed" normal macrophages, so that they became "activated" when incubated with PPD i.e., they became non-specifically cytotoxic for various tumour cell lines. The lymphokine in the culture supernatant was designated specific macrophage arming factor (SMAF) and was derived from sensitized T cells. Similar experiments were done using tumour cells for immunization instead of BCG. The "armed" macrophages were able to kill only the tumour cells used for sensitization, whereas the "activated" cells showed no such specificity (Evans and Alexander, 1972). Lohmann-Matthes, Zeigler et al., [1973] described a lymphokine called "macrophage cytotoxicity factor"

which is similar to SMAF. Piessens, Churchill, and David (1975) described another lymphokine which does not require the presence of the sensitizing antigen to induce activated macrophages.

In vivo, local persistence of sensitizing antigen ensures the continued presence of a population of tumouricidal macrophages. Thus Meltzer, Tucker, Sanford et al., (1975) could recover tumouricidal macrophages for more than 42 days after i.p. injection of mice with Phipps strain BCG. However, peritoneal macrophages from mice immunized i.p. with Glaxo strain BCG 10-14 days previously were cytotoxic for tumour cells in vitro only if PPD was added to the cultures (Parr, 1974; Evans and Alexander, 1972). These results presumably reflect the fact that the Phipps strain of BCG induces chronic infection, whereas the Glaxo strain persists for a relatively short time in mice. This is supported by the observation that mice infected with BCG by the i.p. route but not by the i.v. or intramuscular (i.m.) route could limit the growth of an i.p. tumour challenge, and possessed peritoneal macrophages which were cytotoxic for tumour cells in vitro (Hibbs, 1975). Furthermore, if PPD is injected i.p. into mice which have been immunized i.d. with BCG, the peritoneal macrophages become non-specifically cytotoxic for tumour cells in vitro (Ruco and Meltzer, 1977).

Further evidence for the role of macrophages in tumour cell destruction comes from in vivo experiments. Macrophages have been shown to be in close association with disintegrating tumour cells after the i.t. injection of BCG into guinea pigs (Snodgrass and Hanna, 1973). It is known that silica blocks macrophage activity and it has been demonstrated that

administration of silica to rats will abrogate the tumour regression otherwise induced by the injection of methanol extraction residue of BCG (Allison, Harington, and Birbeck, 1966; Hopper, Pimm, and Baldwin, 1976).

The experiments of Hibbs and colleagues suggest that the lysozomal enzymes of activated macrophages are responsible for tumour cell destruction. Trypan blue is not toxic for mouse peritoneal macrophages, but it is taken up by the macrophages and stored in secondary lysozomes. It inhibits the activity of lysozomal hydrolases (reviewed by Hibbs, 1976). The treatment of mice with non-toxic doses of trypan blue abrogates BCG or Toxoplasma induced non-specific resistance to tumour challenge (Hibbs, 1976). In vitro, the tumouricidal activity of activated macrophages is inhibited if the cells are exposed to non-toxic levels of trypan blue (Hibbs, 1976). The transfer of the contents of the lysozomes from activated macrophages into tumour cells probably involves fusion of the tumour cell and macrophage membranes. Indeed, hydrocortisone is known to stabilize membranes, and the cytotoxic effects of activated macrophages are inhibited if they are incubated with hydrocortisone in vitro (Hibbs, 1976). However, there is some data which suggests that contact is not required (section 2.2.4).

Although activated macrophages can lyse or inhibit the growth of diverse cells such as tumours, bacteria, and protozoa, they do not affect cells of normal tissues (Hibbs, Lambert, and Remington, 1972a; Hibbs, 1973; Currie and Basham, 1975; Piessens et al., 1975). Tumour cells appear to be recognised not because of any distinct antigenic composition, but because they have an altered membrane structure which endows them with the ability to grow uninhibited by contact

in vitro (Hibbs, 1973). This has been termed "nonimmunologic recognition" by various workers, but the concept may require re-evaluation in the light of evidence indicating that some micro-organisms share antigens with tumour cells (section 9).

3.2 The relationship between DTH and cell-mediated immunity

DTH can be elicited with microbial antigens in animals infected with intracellular parasites. There is usually a close temporal relationship between cutaneous DTH and anti-microbial immunity, and both can be adoptively transferred with sensitized T lymphocytes, not serum. Mackaness (1969) suggested that DTH and cell-mediated immunity (CMI) to microbial infection are different manifestations of the same immunological phenomenon.

Some workers have tried to resolve this question by eliciting cell-mediated responses in animal hosts by different procedures, and the results obtained are conflicting. For example, Dodd (1970) showed that guinea pigs undergoing systemic DTH to bovine gamma globulin were resistant to challenge with *Listeria*, but Forbes (1966) did not obtain this result in mice using ovalbumin as antigen. Goihman-Yahr, Raffel, and Ferraresi (1969) harvested peritoneal macrophages from guinea pigs mounting a systemic DTH response to BCG and these cells suppressed the growth of *Listeria in vitro*. This was not the case if the animals were undergoing systemic DTH to contact skin allergens or to allogeneic cells. However, Blanden (1969) showed that induction of a graft-versus-host reaction in F1 mice by the injection of parental cells resulted in the peritoneal macrophages of these mice acquiring heightened microbiocidal properties. Furthermore, the relationship between specific DTH and acquired antituberculous resistance has

been a subject of controversy for some years (reviewed by Collins, 1974). In this case, it is relevant to note that just because it is not possible to demonstrate cutaneous DTH in an infected animal does not imply that the host does not possess sensitized lymphocytes (Collins, 1974).

Considering anti-tumour resistance induced by the intracellular parasites, there are numerous examples in which tumour growth has been shown to be limited at the site of DTH responses to microbial antigens. Addition of PPD or other mycobacterial antigens to a tumour challenge is an effective way of preventing tumour growth in previously vaccinated animals (Parr, 1974; Zbar, Wepsic, Borsos et al., 1970). Indeed, it has also been shown that tumour growth is suppressed at the sites of DTH reactions to non-mycobacterial hapten-protein conjugates, or to an antigenically different tumour (Zbar, Bernstein, and Rapp, 1971; Zbar et al., 1970). In addition, treatments which abrogated the development of DTH to PPD also abolished the therapeutic effect of BCG for guinea pig skin tumours. Examples were the administration of anti-lymphocyte serum or of large numbers of BCG by the i.v. route (Hanna, Snodgrass, Zbar et al., 1973; Zbar et al., 1971).

In the previous section it was noted that activated macrophages are the effector cells of the anti-tumour resistance. In this context it is relevant to note that in the mouse, the time course for the presence of tumouricidal peritoneal macrophages after an i.p. injection of PPD into sensitized hosts closely approximates the time course of the cutaneous DTH response to PPD (Ruco and Meltzer, 1977). In addition, it was emphasised in section 3.1 that lymphokines play a role in the induction of macrophages with increased bactericidal or tumouricidal capacity. MIF is a lymphokine known to be

elaborated when animals mount DTH responses and it has been demonstrated that i.t. administration of purified MIF extracts or MIF containing sera will induce tumour regression in guinea pigs and mice (Bernstein, Thor, Zbar et al., 1971; Salvin, Youngner, Nishio et al., 1975).

Although CMI and DTH are known to be associated with sensitized T cells, the associated lymphokines, such as MIF, can be produced by both T and B cells (section 2.2.4). The data of Salvin, Sonnenfeld, and Nishio (1977) suggested that sensitized T cells were necessary if B cells were to release MIF when animals were re-exposed to the sensitizing antigen.

In conclusion, the relationship between DTH and CMI is still largely unresolved. Although the evidence in this section indicates that DTH to mycobacterial antigens and anti-tumour resistance induced by Mycobacteria are intimately related, this may not always be the case (section 4.2.3, 5.1).

3.3 Conclusions

Some of the important points provided by the evidence in the literature are:-

(a) in many instances macrophage activation is induced in an immunologically specific manner.

(b) the efferent limb of the bactericidal or tumouricidal action of activated macrophages is non-specific.

(c) sensitized T cells and their lymphokine products play a major role in macrophage activation. However, macrophages have also been demonstrated to be activated directly by immunopotentiators (sections 4.2.3 and 7). In addition, there is evidence which suggests that B cells produce lymphokines.

Pathways for tumour cell destruction not involving activated macrophages have also been described. Non-specific cytolysis by mediators or lymphoid cells has been demonstrated *in vitro* in some experimental systems. Ruddle and Waksman (1968) and Meltzer and Bartlett (1972) observed that PPD-stimulated tuberculin sensitive lymphoid cells or their culture supernatants were non-specifically cytotoxic for fibroblast and tumour cell monolayers. Granger and Williams (1971) described a soluble product of specific antigen or mitogen stimulated lymphocytes called "lymphotoxin", which was cytotoxic for a broad spectrum of cultured cells. Youdim (1977) also described a lymphotoxin-like mediator which was involved in *L.monocytogenes* induced cell-mediated cytolysis of mouse B16 melanoma cells *in vitro*. An *in vivo* role for lymphotoxin has not been demonstrated.

There are several pathways by which tumour cells can be destroyed in an immunologically specific manner. Among these are T cell mediated cytolysis, antibody-dependent cell mediated cytolysis, B cell mediated cytolysis, and specific macrophage mediated cytolysis. Discussion of these mechanisms is outside the scope of this introduction, as they involve specific recognition between the effector cells and the targets. However, this does not mean that there is not an element of specificity for the tumour in the inhibition of tumour growth by the intracellular parasites. Bacteria and tumours have been reported to share antigens (section 9), and in addition specific tumour immunity does sometimes develop as a consequence of the immunopotentiator acting as an adjuvant in the host response to tumour antigens (e.g., sections 4.2.1, 4.2.3, and 7).

4. Anti-tumour effects of Mycobacteria

Preamble

The original BCG strain was isolated by Calmette and Guérin at the Pasteur Institute. Attenuation of the original strain needed 13 years and 231 passages before the ultimate attenuated one was produced. BCG has been used for 500,000,000 vaccinations against tuberculosis since 1921 with few casualties (Bast, Zbar, Borsos et al., 1974).

BCG is, like other intracellular parasites, a stimulator of the RES. BCG can also act as an adjuvant; both cellular and humoral responses to heterologous antigens are heightened in BCG infected animals. Lewis and Loomis (1924) showed that tuberculous guinea pigs injected with SRBC developed higher haemolysin titres to SRBC than did normal animals. Dienes and Schoenheit (1927) demonstrated that guinea pigs inoculated in a tuberculous area with egg-white, or pollen produced high antibody titres and developed DTH to these antigens. In this case the effects of the tuberculous infection were expressed systemically. Removal of the site of antigen inoculation and the draining lymph node several hours after antigen injection did not alter the DTH or antibody response in the test animals.

Incorporated in an oily emulsion, killed Mycobacteria can sensitize an animal to tuberculin and can heighten immune responses, particularly DTH, to protein antigens incorporated in the adjuvant mixture (Freund and McDermott, 1942). The materials in Freund's adjuvant induce a macrophage-containing granuloma at the injection site, and the oil droplets aid in the dissemination of antigen to the lymph nodes where it will persist for a long time.

The anti-tumour potential of BCG was first demonstrated when tumour growth was suppressed in mice and rats which had been infected with BCG prior to tumour challenge (Halpern, Biozzi, Stiffel et al., 1959; Biozzi, Stiffel, Halpern et al., 1959; Old, Benacerraf, Clarke et al., 1961). In the classic paper of Old and associates, which reported results with a large number of mouse tumours, BCG was protective in 45% of the lines tested, did not affect another 45% and facilitated tumour growth in 10%.

Since the early 1960's studies with experimental animal tumour models have become increasingly oriented toward immunotherapy, rather than immunoprophylaxis. As clinicians are commonly confronted with patients who already have tumours, this development is hardly surprising. However, it is realised that the therapy of tumour masses which have metastasized in a host presents great problems. Data discussed in the following sections shows that BCG can cope with only a limited tumour burden, and that it has little ability to limit metastasis.

Of necessity, clinicians have to base their approach to human cancer immunoprophylaxis or immunotherapy largely on the empirical results from animal models. For this reason, the following sections are concerned firstly with some of the findings from studies with experimental animal tumours, and secondly with clinical trials.

4.1 Immunoprophylaxis with Mycobacteria

Some of the experimental systems in which treatment with BCG, complete Freund's adjuvant (CFA), or BCG cell walls has been shown to be of positive value in reducing tumour incidence,

delaying tumour appearance, or prolonging the survival of the animals are shown in Table 1. BCG heightens resistance to non-strain specific transplantable tumours, syngeneic transplantable tumours and primary spontaneous tumours. BCG can also protect the host against the cancer inducing agents such as radiation, chemicals and viruses.

4.1.1 Inhibition of carcinogenesis by Mycobacteria

Most workers administer Mycobacteria to animals before or after giving the carcinogen or oncogen, prior to the appearance of visible tumours. In some animal models the outcome has altered when the timing of prophylaxis was varied. For example, Ankerst and Jonsson (1972) worked with a model in which mice developed palpable tumours 12 weeks after being infected with adenovirus type 12 at birth. A single dose of BCG given at 3-4 weeks of age decreased tumour incidence. If BCG was administered at 9 weeks, no protection was observed.

Different workers have observed either a temporary or a permanent decrease in the incidence of spontaneous leukaemia in AKR mice after treatment with BCG or CFA (reviewed by Bast et al., 1974). These conflicting observations are probably the result of different schedules of BCG or CFA administration. Variables such as type of virus, species, sex, age of animals used and timing of CFA treatment are undoubtedly responsible for reports which claim inhibition of viral oncogenesis by CFA on the one hand, and enhancement on the other (Hibbs, Lambert, and Remington, 1972b). Surprisingly, few studies have examined the effect of administering BCG to animals at more than one dose level.

Table 1. Experimental animal tumour models used to study immunoprophylaxis with Mycobacteria

Animal	Tumour		Carcinogenic or Oncogenic agent	Route of administration		Refs.
	Primary	Transplant		BCG	tumour or agent	
mouse			3-methyl cholanthrene	i.v. 86 days after MCA	i.m.	1
			strontium^{90}	s.c. at tumour appearance	i.p.	2
hamster			benzo(a) pyrene in Fe_2O_3	i.v. BCG cell walls	intratracheal	3
			Polyoma virus	i.p. 7 days of age	i.p. 21 days of age	4
mouse			Friend disease virus	i.v. 21 days before virus	i.v.	5
			Friend disease virus	i.p. CFA 77 days s.c. before virus	i.p.	6
			Moloney sarcoma virus	interscapsular 28 days before MSV	i.m. + BCG	7
		Fibrosarcoma (syngeneic)		i.v. 7 days before tumour	s.c.	1
		mammary tumour (syngeneic)		i.v. 7 days before tumour	s.c.	1

Table 1 ctd.....

	mammary tumour			i.v.53 days of age	spontaneous	1
	AKR leukaemia			i.v.63 days of age	spontaneous	1
	AKR leukaemia			i.v.70 days of age	spontaneous	4
	AKR leukaemia			i.p.CFA time s.c.not stated	spontaneous	6
	mammary tumour			i.p.CFA time s.c.not stated	spontaneous	6
		Sarcoma 180 (non-specific)		i.p.CFA 70 days s.c.before tumour	i.p.	6
		Leukaemia L1210 (syngeneic)		i.p.CFA 63 days s.c.before tumour	i.p.	6
		B16 melanoma (syngeneic)		various routes 10 days before tumour	various routes	8
		Lewis lung tumour (syngeneic)		i.v. 14 days before tumour	s.c.	9
		lymphoma or fibrosarcoma (syngeneic)		i.p. 14 days before tumour	+ PPD route not stated	10

References

1. Old, Benacerraf, Clarke et al., (1961).
2. Nilsson, Revesz, and Stjernsward (1965).
3. Zwilling, Springer, and Kaufman (1977).
6. Hibbs, Lambert, and Remington (1972b).
7. Schwartz, Zbar, Gibson et al., (1971).
8. Proctor, Auclair, and Lewis (1976).

Table 1 ctd.....

4. Lemonde (1973).
5. Larson, Ushijima, Florey et al., (1971).
9. Mathé (1976).
10. Parr (1974).

Heightened cellular immune responses in animals treated with Mycobacteria have correlated with the inhibition of carcinogenesis. For example, CFA treated mice which resisted viral oncogenesis and spontaneous tumour development were known to have activated macrophages, which were present even 10 weeks after CFA administration (Hibbs et al., 1972b). Schinitzky and coworkers applied the carcinogen dimethylbenz (a) anthracene to the skin of mice and then injected the mice with BCG i.v. prior to promoting tumour development with croton oil. Mice which were free of tumours after BCG treatment had a greater cutaneous DTH response to PPD than did tumour bearing mice (cited by Bast et al., 1974).

Inhibition of carcinogenesis by BCG has been attributed to the stimulation of a natural immune surveillance system against tumours. This concept was developed by Burnet, who proposed that T lymphocytes had evolved in order to eliminate potentially malignant cells which were constantly arising in the host by somatic mutation (Burnet, 1970). Although the outlines of this theory have broadened since this time, it is still a controversial subject for which there is a lack of clearcut evidence. In view of the current information, analysed recently by Allison (1977), it is unreasonable to either be uncritical of the surveillance hypothesis or to reject it totally. A discussion of the literature relating to immune surveillance is outside the scope of this introduction.

BCG can affect host responses other than the immune response. For example, BCG may limit viral oncogenesis by stimulating interferon release (Bast et al., 1974). The levels of enzymes which are responsible for the metabolism of chemical carcinogens may change when BCG is administered to an

animal (Bast et al., 1974). In addition, BCG treatment can act in concert with other biological manipulations to inhibit tumour development. For instance, fasting and fighting, or fasting and pregnancy, together with BCG administration, were more effective than any one treatment in prolonging survival and reducing the incidence of spontaneous leukaemia in AKR mice (Lemonde, 1966). Fasting, fighting, and pregnancy all induce an increase in the levels of corticosterone steriods. In summary, immunological, hormonal, and metabolic factors can all influence carcinogenesis.

4.1.2 "Primed site" protocols with live BCG and irradiated tumour cells

Although it is well known that BCG can act as an adjuvant (section 4.0) the conditions for inducing specific tumour immunity with BCG have not been well examined. Acquisition of specific systemic tumour immunity would be of great advantage to a host in which the tumour has metastasized and may not be accessible to local treatment with BCG. Mackaness and colleagues investigated this problem in an immunoprophylactic model utilizing P815 mastocytoma which is syngeneic for DBA/2 strain mice. They chose P815 because it is normally poorly immunogenic, and BCG treatment was likely to be more advantageous to the host than in the case of highly immunogenic tumours.

Hawrylko and Mackaness (1973a) showed that growth of a P815 challenge in mouse footpads was only temporarily inhibited if the tumour was given admixed with BCG. Moreover, the tumour-free survivors were not all immune to rechallenge with P815. More efficacious was a "primed site" or "split adjuvant"

protocol, in which BCG was injected into one site followed by injection of irradiated P815 into the same site and P815 challenge in a distant site. The tumour challenge did not require admixed BCG for the protocol to be effective and the survivors possessed specific tumour immunity (Hawrylko and Mackaness, 1973a).

The maximum degree of tumour specific immunity was demonstrable when irradiated P815 cells were injected into the BCG sites at the time of peak DTH response to BCG antigens (Hawrylko and Mackaness, 1973b). The immunity generated by the irradiated P815 against the primary challenge was short lived (10 days) in comparison to that stimulated by the challenge itself. Most mice which were protected against the first tumour challenge were still immune 100 days later (Hawrylko and Mackaness, 1973a). There is evidence that irradiation of tumour cells lowers their immunogenicity (Alexander, Delorme, Hamilton et al., 1968). The most likely explanation for these results is not that tumour-associated antigens have been altered, but that the irradiated cells do not provide the persisting antigenic stimulus of replicating cells (Herberman, 1977). For similar reasons, viable micro-organisms or dead organisms in an oil emulsion are superior immunogens to dead organisms given in saline (Collins and Mackaness, 1970).

Although the "primed site" protocol may represent successful tumour immunoprophylaxis in some models, its application is limited. Ghose, Guclu, Tai et al., (1976) experimented with the highly lethal transplantable lymphomas EL4 in C57BL/6J strain mice and L2 in AKR strain mice. Using the Hawrylko and Mackaness protocol, they obtained some protection

against the growth of EL4 although the tumour challenge had to be mixed with BCG. The long term survival of these mice was still a poor 10%. With the L2 these workers were uniformly unsuccessful with any protocol; even at the site of an ongoing DTH response to BCG the growth of L2 tumours was not inhibited. The L2 thymoma is non-immunogenic, and Ghose et al., (1976) could not generate any CMI to L2 by a number of different manipulations.

4.1.3 Clinical studies

Only two studies have provided evidence that administration of BCG early in life is effective in preventing childhood leukaemia and other human cancers.

Davignon, Lemonde, Robillard et al., (1971) carried out such a trial in Quebec. An analysis of data relating to deaths from acute childhood leukaemia occurring among children immunized with BCG at birth revealed that leukaemia mortality in this group was half as common as in children given no BCG ($p = 0.001$). Crispen and Rosenthal (1976) also obtained results which indicated the efficacy of BCG vaccination in preventing cancer. Their study was retrospective and looked at the death rates from leukaemia and other cancers of those vaccinated as newborns at Cook County Hospital, Chicago. Those among the vaccinated group who left Chicago were included in the study which spanned the period 1957-1969. The controls were drawn from the Chicago population, and adjusted for deaths for the study period. There was a reduction in cancer deaths of 74% in the vaccinees as compared to the controls ($p = 0.001$). Conformation of the validity of the conclusions made in the studies of Davignon and Crispen was

obtained. It was shown that there were no differences between the vaccinees and controls with respect to the number of non-cancer deaths (i.e., road accidents or trauma).

However, other data exist which show no such difference with respect to deaths from cancer between vaccinated and non-vaccinated individuals. Comstock, Martinez, and Livesay (1975) reported the results of a Puerto Rican trial in which children from 1-18 years of age (50% were in the 7-12 year group) were vaccinated from 1949-1951. The annual rates of cancer deaths were 10.3 per 100,000 for the vaccinees and 7.2 per 100,000 for the controls. The vaccinees were, however, protected from death due to tuberculosis.

Comstock, Livesay, and Webster (1971) undertook another controlled immunization trial in America on people of a higher mean age than in the above study and obtained similar results. However, Crispen and Rosenthal (1976) questioned the efficacy of the vaccination procedure used in the latter trial. The 1972 Medical Research Council trial in Britain, using teenagers, provided no useful data because the vaccinee group was too small for valid statistical analysis. The indications were, however, that BCG was beneficial. A major limitation of these latter studies is that a large proportion, or all, of the participants were older than 5 and had survived the period of highest incidence for childhood leukaemia.

In conclusion, BCG immunoprophylaxis in humans is a relatively uncomplicated and less hazardous procedure than is the treatment of cancer patients with BCG (section 6 (ii) b). However, further investigations need to be carried out before a conclusion can be made on the efficacy of BCG vaccination in

preventing neoplasia in humans. As the above evidence shows, at the present time the results of clinical trials are conflicting.

4.2 Immunotherapy with Mycobacteria

4.2.1 The guinea pig model

This model was developed by workers at the National Cancer Institute, Maryland, U.S. It utilizes strain 2 guinea pigs and syngeneic line 10 tumour cells. From a recent paper it appears that this tumour is immunogenic (Zbar, Ribi, Kelly et al., 1976a), although Bast et al., (1974) had claimed that it was not immunogenic.

The guinea pigs receive a single i.d. injection of 10^6 tumour cells. Seven days later, a tumour nodule 1cm in diameter is present with metastases in the first draining lymph node. Control animals, or those with the tumour nodule excised at this stage, die from metastases. If the regional lymph node is also excised the guinea pigs are protected. Intratumour injection of 10^7 BCG cures about 60% of the animals, with regression of i.d. nodules and elimination of metastases (Bast et al., 1974).

An important difference between BCG cured guinea pigs and surgery cured guinea pigs is that the former animals possess specific tumour immunity (Bast et al., 1974).

(i) Importance of tumour burden

Intratumour BCG treatment is not curative if the guinea pigs bear advanced i.d. line 10 tumours (Bast et al., 1974).

Hanna, Peters, and Fidler (1976a) attempted to design a model in which they could demonstrate that

the specific tumour immunity generated by i.t. BCG treatment of skin tumours was of some therapeutic value if the guinea pigs also bore disseminated tumours. Their rationale was that the guinea pig model was of little relevance to the clinical situation, in which patients with solid tumours will often succumb because of distant metastases which are present at the time of local treatment.

Hanna et al., (1976a) injected 10^2-10^6 line 10 tumour cells i.v. into guinea pigs bearing 6 day old line 10 skin tumours (1cm diam.), and one day later BCG was injected into the local tumours. The skin tumours did not regress if the animals had received 10^3-10^6 tumour cells i.v., indicating that a relatively small distant tumour burden interfered with local immunotherapy. If, however, the skin tumours and draining lymph node were excised 42 days after BCG treatment, only 10% of the guinea pigs which had received 10^3 tumour cells i.v. died. 10^3 tumour cells injected i.v. were fatal for 85% of normal guinea pigs. Clearly, as long as the appropriate surgical measures were taken to reduce the tumour burden, the specific tumour immunity generated by local BCG treatment could deal with a small load of distant metastases.

A further imperfection of the guinea pig model is that there is a very short latent period between the i.d. inoculation of tumour cells and the growth of a large tumour nodule. This is a characteristic of most transplantable experimental animal tumours,

and is not like the clinical situation in which it probably takes several years for a human tumour to grow into a 1cm diameter nodule. Jessup, Riggs, and Hanna (1977) inoculated small numbers of line 10 cells into guinea pigs by the i.d. route and allowed the tumours to develop to 1cm in diameter. After the excision of dermal tumours and draining lymph nodes in animals with 22, 29, or 41 day old grafts, 20%, 60%, and 86% of the guinea pigs were able to reject a second tumour challenge, respectively. If instead of excision, BCG was injected i.t., the majority of guinea pigs with 22 or 29 day old tumours were cured. However, guinea pigs with 41 day old tumours had many nodal metastases and only the dermal nodules regressed after BCG treatment. The data indicated that gross tumour burden was a more important factor than specific tumour immunity in determining the effects of local BCG therapy.

(ii) Route of BCG administration

Clinicians prefer to avoid the use of the i.t. route of injection because of adverse side effects (section 6 (ii) b). However, few other modes of administration will cure guinea pigs with line 10 skin tumours. Routes of injection of BCG which were not effective included i.v., i.p., intranodal, foot-pad, and i.d. distant from the tumour site (Zbar et al., 1976a). Scarification with BCG, at a different site from an excised tumour, but in the same region of lymphocytic drainage, is a method of administration frequently employed in human cancer

immunotherapy. This approach was not curative in the guinea pig model (Hanna, Peters, Gutterman et al., 1976b).

Zbar et al., (1976a) found that if BCG was given i.d. adjacent to line 10 skin tumours the guinea pigs were cured, providing that the dermal tumour nodule was excised 7 days after BCG treatment.

These results clearly demonstrate the requirement for close contact between BCG and tumour cells if therapy is to be effective.

(iii) Location of tumour

Deposits of line 10 tumour in the peritoneal cavity, muscle, lymph nodes, and lung are resistant to local BCG immunotherapy (Zbar et al., 1976a).

(iv) Response to mycobacterial antigens

Guinea pigs bearing i.d. line 10 tumours must mount a cellular response of the delayed hypersensitivity type toward BCG antigens if local BCG treatment is to be effective (section 3.2).

Zbar et al., (1976a) proposed that the response to BCG antigens at the site of established line 10 tumours results in the activation of macrophages which can then kill the tumour cells. Direct evidence for macrophage involvement is lacking and mainly relies on histological, ultrastructural and cinemicrographic analyses (e.g., Bast et al., 1974; section 3.1; Bucana, Hoyer, Hobbs et al., 1976).

The results of in vitro studies have been confusing. Fidler, Budmen, and Hanna (1976) harvested peritoneal macrophages from guinea pigs whose line

10 tumours had regressed following BCG treatment. These cells were cytotoxic for line 10 cells *in vitro* at effector to target ratios of 100:1. In contrast, ratios of 10,000:1 were needed when lymphocytes from these animals were used. The lymphocytes were pooled from spleen and lymph nodes. The relevance of these results to the *in vivo* situation was rendered doubtful by the observation that macrophages from guinea pigs dying of progressive tumour growth were cytotoxic for tumour cells *in vitro* at 100:1 ratios. Serum from tumour-bearing animals did not abrogate this cytotoxicity, so it was unlikely that humoral factors blocked this interaction *in vivo* (section 6 (i)). Whether or not a suppressor cell was involved was not determined.

Fidler, Kataoka, and Hanna (1976) proposed that *in vitro* data relevant to the immune status of tumour bearing or BCG tumour cured guinea pigs may have been found if cells from the lymph nodes draining the tumour site had been used in the above study. They demonstrated that lymphocytes and macrophages from the regional lymph nodes of cured animals were cytotoxic for line 10 cells *in vitro* at 100:1 ratios. The *in vivo* role of these lymphocytes is uncertain. If they are line 10 sensitized lymphocytes, responsible for the specific tumour immunity of the cured animals, it might have been expected that they could have been found in other lymphoid organs, but this was not the case (c.f. above study). Unfortunately, *in vitro* assays with

regional lymph node cells from tumour bearing guinea pigs could not be done because of the extent of line 10 metastases in the nodes.

Thus, at present, there is no *in vitro* cell mediated cytotoxicity assay which reflects the immune status of donor guinea pigs. However, other evidence is consistent with the involvement of BCG sensitized lymphocytes and activated macrophages in the BCG mediated immunotherapy of line 10 guinea pig tumours.

(v) Response to tumour antigens

Complete tumour regression in the guinea pig model is likely to require the development of specific tumour immunity. Zbar *et al.*, (1976a) studied a number of tumours which were able to grow i.d. in guinea pigs and found that the tumours which did not regress after BCG treatment were not detectably immunogenic, with one exception. Their data also showed a correlation between tumour immunogenicity and the number of tumour cells that could be destroyed during BCG treatment. For example, for a non-immunogenic tumour, 10^5 tumour cells did not grow if mixed with BCG and implanted i.d. into guinea pigs. This figure was $> 10^6$ for immunogenic tumours. Other workers have obtained similar results with transplantable rat and mouse tumours (sections 4.2.3 and 4.2.4).

How the specific anti-tumour response is augmented by BCG is not fully understood. Rapp, Kleinschuster, Lueker *et al.*, (1976) proposed that the

destruction of line 10 tumour cells by BCG activated macrophages promotes the development of specific tumour immunity. These workers could not correlate the acquisition of specific tumour immunity with DTH to tumour antigens, but their colleagues could transfer specific tumour immunity to normal guinea pigs with lymphocytes from animals immunized with a mixture of BCG and line 10 cells (Smith, Harmel, Hanna et al., 1977). In addition, guinea pigs bearing 1cm diam. line 10 skin tumours were cured if they were injected with such lymphocytes by the intracardiac route (Smith et al., 1977).

(vi) Conclusions

The guinea pig model is one of the few in the literature in which animals bearing established tumours are used for the study of immunotherapy. Many animal models are not truly immunotherapeutic as they involve the grafting onto animals of tumour cell inocula admixed with BCG. In addition, it is relevant to the clinical situation to investigate the effects of other forms of treatment such as surgery in an animal model.

Many of the requirements for successful BCG treatment of i.d. line 10 tumours are the same as those for dermal malignant melanoma in humans (section 4.2.5 (i)). Furthermore, new approaches elucidated in this model, such as administration of BCG prior to surgery, may be useful in the treatment of humans with early stage cancers (this section (i), (ii) and section 4.2.5 (i)). An important result

of experiments with the guinea pig model is that injection of BCG into i.d. tumours will not cure the animals if there are extensive regional or visceral metastases (i).

It would be useful if the guinea pig model were extended to include a number of other tumours, preferably which grow in sites other than skin and which vary in histology and etiology to line 10. "A second basis on which to support the general applicability of experimental models is to avoid reliance on a single tumour line or primary tumour system" (Bartlett, Kreider, and Purnell, 1976).

4.2.2 Mouse fibrosarcomas

These models were initially described by Bartlett, Zbar, and Rapp (1972) and are useful for the comparisons they provide with the guinea pig model. These workers showed that 3 transplantable syngeneic fibrosarcomas, of widely differing immunogenicities, did not grow in BCG immunized mice when given as an i.d. challenge admixed with BCG. Subsequently, using tumour 1038, it was found that prior immunization was not required for tumour growth inhibition. The 1038 tumour is described as "moderately antigenic" (Bartlett et al., 1972) and it is maintained by serial passage in immunodepressed mice.

In an apparent contrast to the guinea pig model, mice which had been challenged with tumour alone developed tumour specific immunity, but this was abrogated by BCG. Other experiments showed that this was not always the case. Bartlett et al., (1972) injected BCG into established i.d. 1038 tumours, then excised the tumours later and demonstrated that tumour

specific immunity had developed. As Bartlett and coworkers proposed, it is likely that challenging mice with a mixture of 1038 cells and BCG resulted in the killing of 1038 in an "innocent bystander" reaction because of the vigorous host response to BCG. Consequently, the 1038 tumour did not have time to grow and sensitize the host to tumour antigens.

Chung, Zbar, and Rapp (1973) established that short-lived BCG sensitized T cells and DTH to BCG antigens play an important part in the BCG mediated inhibition of 1038 tumour growth as is the case in the guinea pig model (section 3.2).

4.2.3 Rat tumours

Baldwin and Pimm (1971) showed that rat sarcoma cells would not grow if implanted s.c. into rats in admixture with BCG. No suppression was obtained if BCG was given separately either near or contralaterally to the tumour inoculum. The s.c. growth of a non-immunogenic tumour, AAF57, was inhibited by contact with BCG (Hopper, Pimm, and Baldwin, 1975). However, larger numbers of immunogenic tumour cells were susceptible to BCG treatment. For example, when 200 µg of BCG was used, 10^3-10^4 cells was the relevant figure for AAF57, but 5×10^5 cells did not grow in the case of the strongly immunogenic sarcoma Mc7 (Hopper et al., 1975; Baldwin and Pimm, 1971). Tumour specific immunity developed after BCG induced the growth inhibition of immunogenic tumours (Baldwin and Pimm, 1971).

BCG suppressed the growth of transplanted rat tumours in athymic mice (Pimm and Baldwin, 1975b). It appears that neither DTH to BCG antigens, or the development of tumour specific immunity are needed for BCG to inhibit tumour growth in this model. These results were confirmed by Moore who found that immunosuppression by thymectomy and whole-body

irradiation only partially abrogated BCG contact induced inhibition of rat osteosarcoma growth (cited by Pimm and Baldwin 1975a).

In the model of Baldwin and colleagues the effector cells are activated macrophages (section 3.1). In view of the above results, the implication is that BCG can activate macrophages by a T cell independent mechanism. Indeed, the work of other groups indicates that BCG can activate macrophages by direct contact. Bruley-Rosset et al., (1976) showed that normal mouse macrophages became tumouricidal after incubation in vitro with BCG for 4 days. Florentin, Huchet, Bruley-Rosset et al., (1976) injected mice with BCG by the i.v. route. They demonstrated that peritoneal macrophages from these animals were cytotoxic for tumour cells in vitro. Maximum cytolysis was demonstrable with cells taken 3 days and 14 days post infection, and at 7 days there was little cytolysis. It is likely that the early activation of macrophages by BCG was by a direct mechanism, whereas in time a T cell response to BCG antigens developed. North (1969) demonstrated that 14 days were required for an optimal immune response to BCG to develop in a murine host. In conclusion, the evidence in the literature suggests that BCG can activate macrophages by direct contact and also by immunological mechanisms which require sensitized T lymphocytes.

Hopper and Pimm (1976) demonstrated that enrichment of rat tumour cell inocula with syngeneic normal macrophages facilitated the contact suppression induced by BCG. When the macrophage content of tumour D23 was increased from 10% to 50%, the number of tumour cells which did not grow when inoculated s.c. with BCG could be increased 20 fold. Tumour growth was

not affected when macrophages alone and not BCG were added to tumour deposits. Similar data relating to the proportion of tumour associated macrophages and the success of ET mediated therapy were obtained by Parr et al., (1973) (section 2.2.2).

One of the more important aspects of the work of Baldwin and coworkers has been their study of BCG treatment of tumour deposits in the lung. Baldwin and Pimm (1973b) reported that spontaneous pulmonary metastases, appearing after the surgical removal of rat tumours growing s.c., were less frequent following the i.v. injection of BCG. This effect is probably due to the fact that i.v. injected Mycobacteria home to the lungs and activate alveolar macrophages which arrest the pulmonary deposits (Hopper and Pimm, 1976; Lefford, 1971). However, Baldwin and Pimm (1973a) observed that the growth of artificial pulmonary metastases resulting from the i.v. injection of weakly immunogenic tumour cells was greatly enhanced if BCG was added to the challenge inoculum.

Hopper, Pimm, and Baldwin (1976) suggested that contact suppression of tumour growth by BCG could be applicable to the treatment of immunosuppressed patients, as long as their macrophages were functional. However, their relative lack of success with BCG therapy of non-immunogenic rat tumours suggests that some form of host immune response is needed if tumour growth is to be suppressed. Injection of BCG into established s.c. sarcomas (0.5-2.3cm diam) is relatively ineffective in suppressing tumour growth, even in rats presensitized to BCG (Baldwin and Pimm, 1971). This contrasts with the results obtained in the guinea pig model. Nevertheless, the work with tumour deposits in the lung is an example of the successful BCG therapy of visceral metastases in the rat.

4.2.4 Leukaemias and lymphomas

Mathé and colleagues established the use of a combination of irradiated tumour cells and BCG for leukaemia therapy, and determined the maximum number of tumour cells that could be eliminated with immunotherapy. They used a syngenic transplantable murine leukaemia L1210 which is weakly immunogenic, and about half the mice given 100 tumour cells will survive. Mathé, Pouillart, and Lapeyraque (1969) demonstrated that a large percentage of mice given 10^5 L1210 cells s.c. were protected if they were given irradiated cells s.c. and multiple i.v. BCG injections beginning 24 hours after tumour challenge. This effect was not observed if more than 10^5 L1210 cells were used. Irradiated cells administered alone represented efficacious therapy if less than 10^5 cells were given as a challenge. BCG alone provided no protection, even if the mice were given 5 injections of the material. Mathé (1976) reported that treatment with BCG and irradiated leukaemic cells cured 60% of mice grafted with 10^3 Rauscher leukaemia cells, but as yet has not reported on the application of his protocol to the treatment of a range of leukaemias of differing immunogenicity. Unfortunately, an initial promising study of Mathé and colleagues in which irradiated leukaemia cells and BCG were used for the therapy of leukaemia in humans has not been confirmed by similar subsequent work (section 4.2.5 (ii)).

Parr (1972) investigated immunotherapeutic regimes which utilized irradiated tumour cells and BCG using four syngeneic transplantable murine lymphomas. Injection of 10 cells i.p. of any one of the tumours killed all of the recipients. L5178Y lymphoma is highly immunogenic and does not metastasize, but the opposite applies to lymphomas L5178Y-M, TLC5, and TLX9.

Parr gave tumour cells i.p. or s.c. and then 24 hours later, or 4 hours in the case of TLC5, administered irradiated cells i.p. or s.c. followed by BCG i.p. For a given tumour whether or not protection was observed depended on the route of tumour challenge. For example, immunotherapy was effective if mice had been given 10^3 L5178Y cells i.p., but not if 10^5 cells were given s.c., although the livers of these mice were less infiltrated with tumour than in the case of control mice. This result contrasts with the spectacular regressions obtained by Mathé using immunotherapy to treat s.c. implants of L1210. Parr's protocol was ineffective against i.p. or s.c. grafts of L5178Y-M or TLX9 or i.p. grafts of TLC5. The lack of efficacy of treatment correlated well with the lack of immunogenicity of the tumour and its ability to metastasize.

Some workers have investigated the effects of interspersed chemotherapy and immunotherapy for the treatment of animal leukaemias, as chemotherapy is invariably used in the treatment of human leukaemia (section 4.2.5 (ii), (iii)). For example, Mathé, Halle-Pannenko, and Bourut (1974) found that if BCG was given before cyclophosphamide to mice grafted with L1210 cells tumour growth was accelerated, but if it was given after it increased the efficacy of small doses of the drug. Florentin (1976) suggested that BCG stimulated lymphocytes were more susceptible to the immunosuppressive action of cyclophosphamide than resting cells. Pearson, Pearson, Gibson et al., (1972) used as a model the murine leukaemia LSTRA, grafting of 10^4 tumour cells s.c. usually results in death of the mice on days 12-15. These workers administered 1, 3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) to mice 7 days after injecting 10^4 LSTRA cells s.c. At this stage the disease was

systemic. Drug treatment cured 12% of the mice, but additional treatment with BCG resulted in long-term survival of 68%. Various other studies show that if BCG therapy is combined with drug treatment the resultant effect on the tumour bearing host can be either deleterious or beneficial (reviewed by Gutterman, Mavligit, and Hersh, 1976a).

As is apparent from the above discussion, there is little data to show that BCG (with or without irradiated tumour cell vaccines) can be immunotherapeutic in animals bearing advanced leukaemias. Usually the treatment is given soon after s.c. tumour challenge which means that most animal models have little relevance to the clinical situation.

4.2.5 Clinical studies

(i) Melanoma

Dramatic regressions of i.d. metastases were observed in preliminary studies when patients with disseminated malignant melanoma were treated with i.t. BCG (Morton, Eilber, Malmgren *et al.*, 1970). Bast *et al.*, (1974) computed the results of this study and subsequent ones carried out by 12 other groups and reported that BCG injected lesions regressed in 58% of cases, and non-injected nodules in 14%. The 5 year survival of patients with disseminated malignant melanoma is less than 3%, and 20% have recurrences of melanoma limited to the skin. This poor prognosis has stimulated studies on i.t. immunotherapy with BCG.

Similar factors influence the success rate of BCG therapy of melanoma in humans and of line 10 skin tumours in guinea pigs (section 4.2.1). The

site and size of the melanoma nodules are important, s.c. and visceral tumours being more resistant to BCG treatment than i.d. tumours (reviewed by Rosenberg and Rapp, 1976). Although some groups have reported regressions of non-injected nodules, these have usually been in the region of BCG drainage, and not in contralateral sites. Indeed, Siegler observed that in $^{4}/_{11}$ patients given i.t. BCG new nodules appeared while those injected were regressing (cited by Rosenberg and Rapp, 1976).

Morton, Eilber, Holmes et al., (1976) observed patients with malignant melanoma for 1-8 years after they had received i.t. BCG but no surgery. Thirty-two percent remained tumour free, but all of these people had had intracutaneous tumours, none of those with s.c. or visceral tumours remained disease free. However, Gutterman, Mavligit, Burgess et al., (1976b) report unpublished results which indicate that the response rates of melanoma patients with liver metastases may be improved following the i.v. administration of BCG.

Morton et al., (1976) studied the effects of BCG therapy as an adjunct to surgery in patients in whom the melanoma had spread to the regional lymph nodes (Stage II) and in those in whom it had spread to various distant sites (Stage III). BCG was administered systemically after surgery and in some cases irradiated melanoma cells were also given. Eighty-five percent of Stage II patients who have had radical surgery alone will usually succumb to

recurrence within 2 years. With immunotherapy this percentage was lowered and the 2 year survival rate was improved. Forty percent of the Stage III patients treated with surgery and immunotherapy remained disease free for 2 years, and the remainder survived longer. The efficacy of the therapy with tumour cells could not be evaluated because the number of patients was too small. The results of 3 other trials with Stage II patients have confirmed the usefulness of the BCG therapy (reviewed by Gutterman et al., 1976a).

Results obtained in the guinea pig model indicated that treatment with BCG prior to surgery cured the majority of animals (section 4.2.1 (i), (ii)). Workers at the National Cancer Institute have begun clinical studies which utilize this knowledge, and the preliminary results are promising. Four out of four patients with Stage I melanoma who were given i.t. BCG before surgery had complete regression of the primary tumour (Zbar et al., 1976a).

(ii) Acute lymphoblastic leukaemia

Mathé (1969) had a group of 30 patients with acute lymphoblastic leukaemia (ALL) who had been given chemotherapy and had been in remission for at least 2 years. Ten patients were given no further treatment and all relapsed within 130 days. The other 20 patients received weekly treatments with BCG by scarification or were injected with killed allogeneic ALL cells or were given both treatments. All of these patients remained in remission 3-13

years after the initiation of immunotherapy (Mathé, de Vassal, Delgado et al., 1976). It was not possible to determine which of the immunotherapeutic protocols was the most successful as the number of patients was too small.

Since this time, similar studies by other workers have not confirmed Mathé's results. Two controlled trials, one in the U.S.A. and one in Britain, investigated the use of BCG treatment in children with ALL who were in remission. No encouraging results were obtained (reviewed by Powles, 1976). In these trials the BCG used was not the Pasteur strain used by Mathe and was presented differently. In addition, ALL cells were not used. Two further studies in which Pasteur BCG was used also yielded no positive results (Powles, 1976). Leventhal, LePourhiet, Halterman et al., (1973) compared the effects of two different modes of immunotherapy in ALL patients in remission. They observed no difference in the duration of remission of patients who were given Pasteur BCG and ALL cells in a Mathé protocol and those given cells and a chemotherapeutic drug. In any case, both groups relapsed within a relatively short time.

At the moment there is not sufficient evidence suggesting that BCG immunotherapy is efficacious in the treatment of ALL. In view of the fact that good results have been achieved with intensive combination chemotherapy, the use of BCG does not appear justified at present.

(iii) Acute myelogenous leukaemia

Powles and colleagues treated acute myelogenous leukaemia (AML) patients who were in remission with various forms of chemotherapy and immunotherapy. In their first study, patients were given either chemotherapy alone or chemotherapy plus immunotherapy. The immunotherapy consisted of BCG (Glaxo) and irradiated allogeneic leukaemia cells given percutaneously at separate sites weekly. Patients receiving both treatments survived longer (median 510 days) than patients maintained on chemotherapy alone (median 270 days, $p = 0.03$). This difference was mainly due to a markedly increased survival time of the chemo-immunotherapy patients after they relapsed (Russell, Chapuis, and Powles, 1976). Little difference in remission length or survival was observed if unirradiated leukaemia cells were given instead of irradiated cells (Russell et al., 1976).

It was not clear from this trial whether immunotherapy augmented the patient's response to his tumour, or whether it permitted the patients to tolerate higher doses of drugs because of bone marrow stimulation. The lack of advantage of using live tumour cells suggested that augmentation of specific anti-leukaemic immunity was not involved. Other groups have found that immunotherapy with BCG alone will prolong the survival of patients with AML (reviewed by Oliver, 1977). An example is the study of Gutterman et al., (1976b). Patients in AML remission received no further therapy, chemotherapy, or live BCG

by scarification. Patients maintained on BCG had a prolonged disease-free interval compared to the other groups.

The results of these trials indicate that immunotherapy can prolong the survival of patients with AML.

(iv) Lung cancer

McKneally, Maver, and Kausel (1977) have shown that a single dose of live BCG (Tice strain) delivered into the pleural space after surgery prolonged the post-operative disease free interval and survival among patients with lung cancer (Stage I). This was not the case if Stage II or III patients were treated. The median duration of observation in this study was 640 days. The BCG treated patients were also given isonicotinic acid hydrazide (INH) as were the control patients.

In another trial, Roscoe, Pearce, Ludgate et al., (1977) treated patients with lung cancer with surgery and then BCG (Glaxo strain) was administered for a number of weeks either i.d. or percutaneously. After 33 months there was no significant difference in the median survival times of the BCG treated groups and the control group. This disappointing result presumably reflects the need for contact between BCG (or BCG stimulated cells) and the tumour cells, as exemplified in some animal models (e.g., sections 4.2.1, 4.2.3). Another factor contributing to the inefficacy of immunotherapy was probably the fact that a high proportion of patients had advanced

disease i.e., they were not Stage I patients. In each group, 37% of patients not only had squamous tumour but also had tumour in the mediastinal nodes.

(v) Colorectal cancer and breast cancer

A poor prognosis is associated with cancer of the colon or rectum (stage Duke's C), in which tumour has metastasized to the regional lymph nodes. Patients are usually treated with surgery, but preliminary evidence indicates that administration of BCG by scarification after surgery results in prolongation of the disease-free interval and survival (Gutterman et al., 1976a).

In a controlled trial, 23/44 patients with disseminated breast cancer who were treated with chemotherapy alone died, whereas only 5/45 given BCG by scarification in addition to chemotherapy died ($p = 0.005$). The period of observation in this study was 15 months (Gutterman et al., 1976b).

Zbar et al., (1976a) suggested that patients with colon carcinoma (stage Duke's C) or primary breast cancer would be likely to benefit from the BCG followed by surgery approach mentioned in the section on Melanoma.

(vi) BCG immunotherapy and immunocompetence

a. DTH responses to microbial antigens or DNCB

In most animal models it is necessary for the animals to be able to mount a DTH response to mycobacterial antigens if BCG treatment is to result in tumour regression or suppression (e.g., sections 4.2.1, 4.2.2). In humans, the ability to respond to

some antigens with positive skin tests can be correlated with successful immunotherapy.

Melanoma patients in whom lesions regressed after i.t. BCG treatment were PPD positive before therapy or mounted a response after BCG treatment. On the other hand, non-responders to therapy tended to remain anergic (Morton et al., 1970; reviewed by Bast et al., 1974). In addition, Morton et al., (1970) and Pinsky, Hirshaut, and Oettgen (1972) found that most melanoma patients who responded to i.t. BCG treatment could develop DTH to dinitrochlorobenzene (DNCB). Hersh, Gutterman, Mavligit et al., (1976) showed that melanoma patients with a good prognosis after BCG treatment could mount a vigorous DTH response to dermatophytin prior to therapy.

In conclusion, the available evidence suggests that cancer patients who respond to BCG treatment have an intact immune system. ". . . nearly all responses in patients treated in this way (with BCG) have occurred where crudely tested delayed hypersensitivity mechanisms have been intact." (Br. Med. Jnl. editorial "BCG in Cancer" 19 June 1976).

b. Responses to tumour antigens

Studies with experimental animal tumours have shown that BCG can augment the host immune response to tumour antigens (sections 4.1.2, 4.2.1, 4.2.2, 4.2.3, 5.1, 5.2 (iii)).

Perhaps in the expectation that BCG will have a similar adjuvant effect in humans, as well as its

non-specific effect, BCG therapy has sometimes been combined with the administration of irradiated tumour cells or other forms of tumour antigen. However, there is little evidence to suggest that human tumour-associated antigens are immunogenic (Herberman, 1977). The evidence from animal studies indicates that the frequency of immunogenicity among naturally occurring tumours is low (Baldwin, 1976). It may be incorrect to assume that the in vitro cell-mediated or humoral responses demonstrated against autologous human tumour cells reflect in vivo anti-tumour responses. For example, lymphocytes from many normal human donors are cytotoxic for different tumour cell lines (reviewed by Pross and Baines, 1977).

With the assay techniques presently available, the use of specific active immunotherapy in man "is somewhat analagous to giving insulin for diabetes without being able to monitor the level of the blood sugar" (Gutterman, 1977). It remains to be established whether the use of tumour cell vaccines and BCG represents more effective immunotherapy than BCG treatment alone.

5. Anti-tumour effects of killed Mycobacteria and mycobacterial extracts.

Preamble

Living BCG rather than dead Mycobacteria or their extracts have been prefered for immunopotentiation because living micro-organisms are more effective in inducing cell-mediated anti-microbial immunity. Vaccination with live BCG for the prevention of tuberculosis in man has been without complications. However, it is doubtful that such treatment also prevents neoplasia (section 4.1.3). It may be that repeated vaccinations are required, as suggested by Crispen and Rosenthal (1976), in which case it would be desirable to use non-viable vaccines. Repeated inoculation of live BCG may result in immunoparalysis rather than immunostimulation and has occasionally been associated with severe hypersensitivity reactions and death (section 6 (ii) b). There is always a chance that BCG infection will become progressive, particularly if the hosts are immunosuppressed cancer patients. Furthermore, different commercial batches of live vaccines may contain different proportions of live: dead bacilli and may vary in their state of attenuation (Mackaness, Auclair, and Lagrange, 1973; Crowle, 1972).

The use of non-viable mycobacterial vaccines for the immunoprevention or immunotherapy of cancer would eliminate some of these problems, although anaphylactic reactions could still occur after a long course of injections. Evidence from studies in animals and man has indicated that non-living mycobacterial vaccines can induce an effective level of resistance to tuberculosis (reviewed by Crowle, 1972). "Almost every mycobacterial product so far tested has some antituberculous activity . . ."

(Collins, 1974). The following sections look at studies in which the anti-tumour effects of non-viable mycobacterial preparations were investigated in animals and man.

5.1 Immunoprophylaxis with extracts of BCG

Weiss and his colleagues have carried out extensive investigations using a preparation called methanol extraction residue (MER) of BCG (reviewed by Weiss, 1976). MER is the portion which remains after exhaustive extraction of phenol-killed, acetone washed BCG with methanol at 56°C. The material is administered to animals as a fine suspension in saline without the addition of oil, and it can persist in the host for months. Like live BCG, it is an immunopotentiator and is sometimes more effective than BCG. Other advantages are its stability, low toxicity, and that it induces only a low degree of tuberculin hypersensitivity when compared with live or phenol-killed BCG.

Early studies with solid syngeneic mouse tumours which involved the use of MER as a prophylactic agent in direct comparison with live or dead BCG showed that the effects of MER were highly dose dependent (Weiss, Bonhag, and Leslie, 1966). MER slowed or prevented tumour growth when given i.p. before s.c. implantation of a Balb/c uterine sarcoma, a C3H hepatoma, an R111 mammary carcinoma, or a C3H osteogenic sarcoma. These were either old established tumours, or those in the first or second transplant generation. Facilitation of tumour growth was, however, observed in some cases and some of the prophylactic effects were abolished by doubling or halving the dose of MER. Sometimes live BCG was effective in cases in which MER or dead BCG were not and vica versa.

Haran-Ghera and Weiss (1973) studied the action of MER on the development of leukaemia in C57BL/6 mice infected with radiation leukaemia virus. MER lengthened the latent period and reduced tumour incidence if given in multiple administrations after some tumour cells had already arisen. Given earlier, MER stimulated tumour growth. The possibility of some of the effects of MER being due to heightened immune response against the virus were not eliminated.

Lavrin, Rosenberg, Connor et al., (1973) showed that the timing of MER administration was critical in determining whether or not tumour development was inhibited in mice treated with the carcinogen MCA.

Minden, Wainberg, and Weiss (1974) evaluated the prophylactic effects of MER against the growth of line 10 skin tumours in guinea pigs. They also used BCG-SS which is a supernatant of a 200,000 x g centrifugation of a sonically disrupted heat-killed BCG culture. Intradermal injection of 0.5 - 1.0 mg of MER or of 25-50 µg of BCG-SS was followed at various times by the inoculation of line 10 cells at a separate i.d. site. Either treatment caused initial tumour nodules which formed to regress and the combined results were 11/23 cures. All control guinea pigs grew tumours and died. Some of the MER treated animals were given a tumour challenge some months later and were still resistant. Of the animals which survived the initial tumour challenge $^{8}/_{10}$ showed a high degree of cutaneous DTH to BCG-SS, but only $^{4}/_{10}$ did so in response to PPD. This presumably shows that PPD may not be truly representative of some important mycobacterial antigens.

It is interesting that the above prophylactic effects of BCG extracts were confined to particular breeding colonies of strain 2 guinea pigs (Zbar, Minden, McClatchy et al., 1976b). Workers in Israel and Colorado could obtain the results just described but those in Maryland could not. It is the latter group who claim that injection of live BCG prior to challenge with line 10 tumour cells does not result in inhibition of tumour growth (Zbar et al., 1971). Reports of shared antigens between line 10 and BCG have come from the Colorado group (section 9) and this may have some bearing on the conflicting results. In any case, this example serves to emphasise the desirability of autochthonous tumour models.

In summary, immunoprophylactic anti-tumour activity is not an exclusive property of living Mycobacteria. In section 4.1 (and Table 1) it was pointed out that killed Mycobacteria in the form of CFA and BCG cell walls in oil were effective prophylactic agents. However, persistence in the host is not restricted to living bacilli or their derivatives in oily vehicles. The BCG extracts MER and BCG-SS have prophylactic effects if administered to animals as suspensions in saline. Nevertheless, it is difficult to choose the appropriate dose of MER and it is also possible for tumour growth to be facilitated if the host is pretreated with MER.

5.2 Immunotherapy with killed Mycobacteria and mycobacterial extracts.

(i) Killed Mycobacteria

The ability of killed Mycobacteria to act as immunotherapeutic agents is dependent on whether or not they are presented in such a way that they persist and induce CMI in the host.

Important variables are the species of animal used, dose and route of administration of the vaccine, and whether or not an oil vehicle is used. The following studies provide some examples.

Intradermal implants of line 10 tumour cells will not grow if inoculated together with heat-killed BCG into guinea pigs sensitized to BCG (Zbar et al., 1971). However, only living BCG suppressed tumour growth in unsensitized animals, at least when similar doses of killed and live organisms were used (Zbar et al., 1971). These results were consistent with the observation that killed BCG did not "promote" a delayed-type hypersensitivity response in guinea pigs (Zbar et al., 1971). Recently, Yarkoni, Rapp, and Zbar (1977) demonstrated that heat-killed M.smegmatis or M.tuberculosis induced regressions of established line 10 tumours if incorporated into mineral oil prior to i.t. injection. Presumably, addition of the oil meant that the organisms provided a persisting antigenic stimulus at the tumour site.

It is important to note that the vaccines containing mineral oil used by Ribi, Zbar, Rapp and coworkers which are refered to in these sections contain a final concentration of oil of only 1%. The lesions produced by i.t. injection of such mixtures in guinea pigs heal completely (Yarkoni et al., 1977). In contrast CFA contains 50% oil and induces severe and lasting local reactions.

Chung et al., (1973) observed that heat-killed BCG suppressed the i.d. growth of 1038 mouse fibrosarcomas if given together with the tumour challenge. However, if equal numbers of organisms were compared, live BCG was more effective. Cutaneous DTH to PPD in mice given killed BCG was comparable to

that in mice given live BCG. In contrast, Blanden reported that heat-killed BCG administered i.v. did not sensitize mice for a DTH response to PPD (cited by Chung et al., 1973). The reason for this apparent difference in results is probably that killed organisms sensitize mice adequately for a dermal DTH response when given i.d. but not i.v. (Crowle, 1975).

Baldwin, Cook, Hopper et al., (1974) showed that s.c. growth of rat sarcomas or a hepatoma was suppressed if tumour cells were inoculated in admixture with radiation-sterilized BCG. In addition, pulmonary growth of i.v. injected sarcoma cells was limited if the cells were administered together with the killed vaccine. On a weight basis, live BCG was more effective than killed BCG against s.c. tumour grafts. Pimm and Baldwin (1975a) found that pleural sarcoma growth was suppressed if the tumour cells were inoculated together with the killed vaccine. The route-dependency of these anti-tumour effects was not investigated, but it is likely that killed BCG, like live BCG, will only suppress the growth of these rat tumours if injected at the site of tumour challenge (section 4.2.3).

(ii) BCG cell walls in oil

In the guinea pig, BCG cell walls without mineral oil droplets produce little skin inflammation and lack anti-tumour effects (Zbar et al., 1976a). However, i.t. injection of BCG cell walls emulsified in mineral oil will cure guinea pigs with i.d. deposits of line 10 tumour (Zbar, Ribi, and Rapp, 1973). Baldwin and Pimm (1973a) have also shown that such vaccines are effective in limiting the pulmonary growth of rat sarcomas.

Association of BCG cell walls with metabolizable oils such as Vitamin A, pristane, or peanut oil results in relatively

ineffective vaccines (Zbar et al., 1976a). The component of the cell walls which facilitates their attachment to mineral oil droplets is termed P3 and is a type of lipid called trehalose mycolate (Ribi et al., 1975). The P3 molecule administered alone is non-toxic, non-antigenic, and is unable to sensitize guinea pigs to mycobacterial antigens (Ribi et al., 1975). However, in combination with mineral oil and complex bacterial glycoproteins P3 can act as an adjuvant or immunotherapeutic agent in guinea pigs. The host response to such vaccines is characterized by granuloma formation with the accumulation of large numbers of macrophages (Kelly, 1977).

The necessary properties of the antigenic component in the mineral oil vaccines have yet to be determined. Granger, Yamamoto, and Ribi (1976) found that DTH responses of guinea pigs to BSA were facilitated if the sensitizing injections consisted of BSA admixed with P3 and mineral oil. Such vaccines, containing BSA or PPD, will protect mice against challenge with tubercule bacilli (Ribi, Toubiana, Strain et al., 1978). However, Zbar et al., (1976a) found that injection of mixtures of P3, oil and BSA into established guinea pig tumours did not bring about tumour regression. It may be that mycobacterial protein antigens are an absolute requirement in such a vaccine because successful immunotherapy in this model may depend on a cross reaction between BCG and tumour antigens (section 9).

During their search for non-viable preparations of defined chemical nature Ribi et al., (1975) found that i.t. injection of mixtures of P3, Salmonella glycolipids and oil cured guinea pigs with line 10 skin tumours. Subsequently, Kelly,

Granger, Ribi et al., (1976) showed that ET from Coxiella burneti could substitute for the Salmonella ET in the vaccines. The rickettsial ET is several thousand times less toxic and considerably less pyrogenic than Salmonella ET, and so this work may lead to a revival of interest in ET as an anti-tumour agent. In addition, Ribi's group is at present trying to determine the essential structural requirements which endow P3 with its properties (e.g., Ribi et al., 1978).

This section would not be complete without mention of the use of BCG cell wall vaccines in the treatment of "Cancer Eye". Hereford cows develop a naturally occurring, metastatic, squamous cell carcinoma of the eye. Kleinschuster, Rapp, Lueker et al., (1977), injected BCG cell walls incorporated into mineral oil into the lesions of such animals at a dose of 390ug for $1cm^3$ of tumour. Regression or arrest of the disease was observed in 71% of the treated animals whereas in control animals, or in animals given cell walls in aqueous phase, the disease was progressive with lymph node metastases.

As Kleinschuster and coworkers point out, this model is more analogous to human cancer than the majority of animal models because the carcinoma is autochthonous and slow to develop. The cows are long-lived and techniques and dosages of agents used may be relevant to the clinical situation. Unfortunately, long-term studies have not yet been done, nor has the efficacy of immunotherapy been compared with surgical treatment. It is not known if lymph node metastases are eliminated after i.t. injection of BCG cell walls as is the case in the guinea pig model.

(iii) MER

In the guinea pig model i.t. injection of MER leads to tumour regression and the development of specific tumour immunity. However, this result was only observed when strain 2 guinea pigs from certain breeding colonies were used (Wainberg, Margolese, and Weiss, 1977; see also section 5.1).

Weiss (1976) reported that MER treatment of murine leukaemias was efficacious if irradiated tumour cells were administered with MER. In studies with mouse mammary carcinomas he found that it was necessary to combine MER treatment with radiation therapy or chemotherapy.

Treves, Cohen, Feldman et al., (1976) demonstrated that the incidence of spontaneous metastases following surgical removal of s.c. implants of the mouse Lewislung tumour was decreased if the mice were given MER. The survival of the mice was also prolonged with MER treatment.

Hopper et al., (1975) found that MER was as effective as live BCG in suppressing the s.c. growth of rat tumours when given in admixture with the tumour cells. The treatment was more effective against strongly immunogenic rather than weakly immunogenic tumours.

5.2.1 Clinical studies

(i) Killed BCG

Shipyard workers who use asbestos can develop diffuse mesothelioma of the pleura and peritoneum. The duration of survival from first symptoms to death is short (13-21 months) and of the many forms of treatment used none are curative (Elmes, 1973). Pimm and Baldwin (1975a) reported that Elmes administered radiation-killed BCG to a small number of patients

with pleural mesothelioma. There were no side effects other than a limited fever and there was a reduction in the rate of accumulation of pleural fluid.

(ii) BCG cell walls in oil

Yamamura, Azuma, Taniyama et al., (1976) administered BCG cell walls in oil (final oil concentration 1%) i.d., intralesionally, or intrapleurally to patients with lung cancer (Stage IV). The control group were maintained on chemotherapy, the other group received both chemotherapy and immunotherapy. Fifty percent of the control patients survived for the average time of 4 months, but 50% of patients given immunotherapy survived for 14 months or longer. Preliminary results suggest that oil-attached BCG cell walls may be useful for the immunotherapy of melanoma, leukaemia, and Hodgkin's disease (Yamamura et al., 1976). Yamamura and colleagues did not report that there were any severe side effects resulting from use of the cell wall vaccine.

(iii) MER

The immune status of patients with various forms of neoplasia has been evaluated after treatment with MER by skin testing with microbial antigens or DNCB and in vitro lymphocyte blastogenic responses to mitogens. Phase I and II trials of this type have shown that i.d. or s.c. administration of MER can be immunosuppressive, immunostimulatory, or without effect (reviewed by Mikulski and Muggia, 1977).

Necheles, Rausen, Kung et al., (1976) showed that MER was of use in the treatment of childhood neuroblastoma. The duration of remission was lengthened in children treated with chemotherapy and MER, so that 65% of the children were in remission for 15 months. The median remission period for children given only chemotherapy was 9 months.

MER was injected into all the lesions of 8 patients with metastatic cutaneous melanoma (Krown, Pinsky, Hirshaut et al., 1976). Four of these people had previously responded to i.t. BCG but had relapsed. Complete regression of all lesions was observed in 1 patient, partial regression in 6 patients, and in 1 patient all the lesions progressed. This was not a controlled trial, but the results indicate that MER may be of benefit in melanoma therapy.

The most promising results have been obtained with the MER treatment of adult patients with AML (Cuttner et al., cited by Mikulski and Muggia, 1977). Eight patients were given only chemotherapeutic drugs, and another 7 were given i.d. injections of MER as well as chemotherapy. The latter group were in remission for the average time of 15½ months, compared to 3½ months for the former group ($p = 0.005$). The median survival time of patients receiving MER and drugs was also increased compared to the control group ($p = 0.004$).

MER is well tolerated when given by the i.d. route in humans, causing sterile abscesses and local pain, but few cases of fever (Weiss 1976; Mikulski and Muggia, 1977). In the study of Krown and associates i.t. administration of MER caused fever, chills and hypotension, but the symptoms were severe in only $^1/_8$ patients. Mikulski comments that there have been some reports of severe anaphylactic reactions to MER, particularly in patients previously treated with BCG. He advocates the use of smaller doses of MER because of a preliminary report which suggests that minute amounts of MER can "evoke" DTH. However, this terminology implies that small amounts of MER can be used to elicit DTH in sensitized hosts, but not to induce DTH.

(iv) Elicitation of DTH reactions at tumour sites in skin cancer patients

Klein and his colleagues observed that cutaneous and mucosal cancers in man regressed if the skin overlying the lesions was painted with contact allergens such as DNCB when the patient had been presensitized to the allergen. The concentrations used were insufficient to elicit DTH reactions in normal skin (reviewed by Klein, Papermaster, Case et al., 1975).

Patients with multiple skin tumours who could not be sensitized to these chemicals often reacted to microbial antigens, presumably because they had been sensitized to micro-organisms before the tumours developed. Some of the antigens used included PPD, mumps antigen, streptokinase-streptodornase, candida extracts, histoplasmin, coccidioidin, blastomycin and pertussis extracts (Klein, Holtermann, Milgrom et al., 1976). At first Klein administered these microbial antigens by i.t. injection. It was later found that topical application of the antigens in semi-solid creams was also satisfactory.

Many patients over 50 years old responded to PPD injection and administration of BCG to the non-responders resulted in positive responses to PPD and other antigens (Klein et al., 1976). Twenty-four patients with extensive skin tumours were given local immunotherapy with microbial antigens or DNCB and 5 years after treatment 95% of these patients were free of tumour (Klein, Holtermann, Case et al., 1974). Sixty-six patients were observed for shorter periods of time after immunotherapy and the results were also encouraging. Most of these patients had been treated with chemotherapy and surgery prior to Klein's study. Local immunotherapy was less effective

against deep-seated lesions and widely disseminated cancers (Klein et al., 1974).

One of the most interesting of Klein's findings was that DTH reactions are of more marked intensity in skin with pre-malignant lesions than in normal skin. In addition, some growths which are not large enough to be clinically detectable become clearly discernable at the sites of DTH reactions and may be eliminated (Klein, 1969) Patients with multiple skin cancer syndromes develop tumours throughout their lives after the initial manifestation of the disease. Standard treatments (i.e. surgery, radiation) have no effect on tumour incidence in these patients. However, with Klein's local immunotherapy protocol the incidence of tumours in 7 patients with xeroderma pigmentosum was reduced so that few surgical interventions were needed during observation periods ranging from 3-7 years (Klein and Holtermann, 1972). Normally, the xeroderma patients would require as many as 100 surgical procedures per year. This result indicates the usefulness of the skin challenge technique as a prophylactic measure.

Klein and coworkers have shown that passive transfer of DTH to otherwise anergic skin cancer patients is of immunotherapeutic value. Klein (1969) took peripheral leukocytes from patients sensitized to various chemicals and injected them s.c. into 8 patients who had been shown to be non-responders to these chemicals. Within 8-24 hours after administration of the cells a DTH reaction to the sensitizing agent could be demonstrated in the recipients and the challenge response was shown to result in the regression of cutaneous tumours.

In addition, Klein and colleagues have treated a few patients with lymphokine extracts. The preparations used were supernatant fluids from cultures of mitogen stimulated lymphocytes, or from continuous cultures of a human lymphoblast cell line (Holtermann, Papermaster, Rosner et al., 1975; Papermaster, Holtermann, Rosner et al., 1974). It was demonstrated in animals or with in vitro experiments that the supernatants from the continuous culture line contained skin reactive factor, lymphotoxin and monocyte activating factor (Papermaster et al., 1974). The patients treated included those with multiple superficial basal cell carcinomas, metastatic mammary carcinoma, reticulum cell sarcoma and multiple squamous cell carcinomas. Administration of the lymphokine extracts resulted in partial or complete regression of cutaneous lesions in $^{8}/_{13}$ patients (Klein et al., 1976).

5.3 Conclusions

At the time of writing the only reports relating to the characterization of mycobacterial extracts with anti-tumour activity were concerned with P3 (section 5.2 (ii)). MER and BCG-SS (section 5.1), which do not require admixture with oil to induce their anti-tumour effects, have not been fractionated.

Further guidelines for the preparation of mycobacterial extracts with anti-tumour activity may come from experiments on the induction of DTH with defined antigens. For example, although most water soluble antigens cannot sensitize hosts for DTH unless incorporated in oil, they can if substituted with certain molecules. Coon and Hunter (1973) demonstrated that BSA-lauric acid conjugates sensitized guinea pigs for a DTH response to native BSA. Migliore-Samour, Floc'h, Maral et al.,

(1977) showed that if water-soluble peptidoglycan fragments extracted from M.tuberculosis var. hominis strain Test were conjugated to palmitic acid and injected into guinea pigs together with ovalbumin the animals mounted DTH responses to ovalbumin. As well as lipid substitution, methylation and iodoacetylation of proteins are known empirically to enhance DTH responses to these proteins (Crowle, 1975).

Substituted protein antigens probably induce DTH in hosts because, like antigens-in-oil, they localize in the T dependent areas of lymphoid organs (Crowle, 1975; Coon and Hunter, 1973). However, it is incorrect to assume that any conjugate of lipid and protein can induce DTH. For example, although BCG cell walls contain 61% lipid (Kotani, Kitaura, Hirano et al., 1959) emulsification in mineral oil is required for BCG cell walls to stimulate a similar degree of CMI to that induced by live BCG (e.g., Zbar et al., 1976a; Larson, Ribi, Wicht et al., 1963; Ribi, Anacker, Barclay et al., 1971). The ratio of lipid to protein and the type of lipid (i.e., saturated or unsaturated) are important in determining the adjuvant activity of mycobacterial peptidoglycolipids (results of Hiu and coworkers, cited by Mathé, 1976).

In conclusion, the evidence discussed in the previous sections shows that killed Mycobacteria and mycobacterial extracts have anti-tumour activity in animals and in man. Extrapolating from studies investigating DTH induction, it is possible that anti-tumour effects will not be confined to the crude extracts currently in use, but that the active components can be isolated and if necessary be chemically modified so that they provide the appropriate antigenic stimulus. In view of the fact

that the administration of live BCG to humans is a potentially hazardous procedure, it appears justified to propose that more emphasis should be placed on the development of mycobacterial extracts as agents of cancer immunotherapy or immunoprophylaxis.

6. Adverse effects resulting from treatment with Mycobacteria or derivatives

(i) Animal models

If adjuvants are given prior to the grafting of tumour cells they may trap lymphocytes which could otherwise respond to tumour antigens by virtue of the vigorous inflammatory response they induce. An example is provided by a report of Meltzer and Leonard (1973). Normally, line 1 tumour cells will regress if implanted i.d. into strain 2 guinea pigs and this process is associated with the development of specific tumour immunity. However, tumour rejection was impaired in animals which had been immunized 28 days previously with CFA. This was so whether or not lymph nodes draining the tumour challenge site had been exposed to CFA, and so was not due to morphological damage to the lymph nodes. It is likely that the response to CFA resulted in lymphocytes being unable to participate in a primary cellular response to tumour antigens because if CFA was administered to animals immune to line 1 a subsequent tumour challenge was rejected.

These workers also found that line 10 tumour cells grew progressively if inoculated i.d. together with BCG into guinea pigs pretreated with CFA, although a DTH response to mycobacterial antigens developed at the tumour site. This provided a demonstration that specific tumour immunity, presumably abrogated by the CFA treatment, is important in the BCG-mediated immunotherapy of line 10 grafts (section 4.2.1 (v)). If the time period between CFA administration and tumour challenge is lengthened, tumour growth is suppressed if the tumour cells are given in admixture with BCG or PPD (Zbar et al., 1970, 1971).

When BCG is given to animals together with tumour cells the development of specific tumour immunity is often facilitated (e.g., sections 4.2.1, 4.2.3). However, in some cases the host response to BCG causes the too rapid destruction of tumour cells and this process is prevented (section 4.2.2). This could be disadvantageous to the host if the tumour had metastasized prior to BCG treatment. The adverse effects refered to in this and the above paragraphs are dependent on the tumour under consideration being immunogenic.

Animal studies have also shown that the administration of BCG of high virulence or in large doses can result in disorganization of lymphoid tissues and thus alteration of their functions (Mackaness et al., 1973).

The variable effects of BCG treatment on tumour growth are related to the dose of BCG used, and the route and timing of BCG administration relative to tumour challenge. Chee and Bodurtha (1974) found that in mice pre-immunized with a high dose of BCG, B16 melanoma cells grew faster than normal, whereas if a low dose was used the tumours grew at a reduced rate. Pimm and Baldwin (1975a) reported that the pleural growth of rat tumours was facilitated if BCG was inoculated i.v. or s.c., but was suppressed if BCG was given intrapleurally. Wepsic, Harris, Sander et al., (1976) observed enhancement of tumour growth in rats immunized with BCG cell walls in oil 14 days prior to i.m. challenge with Morris hepatoma cells. However, tumour incidence and size were similar in control rats and in rats given the BCG vaccine 12 days after tumour challenge. Bansal and Sjögren (1973) showed that administration of BCG to rats 2 weeks prior to, or at the time of, grafting

of a polyoma tumour results in inhibition of tumour growth, but if BCG was given when the tumour was palpable tumour growth was accelerated.

Some workers have proposed that the enhanced tumour growth sometimes observed after the administration of BCG to animals is due to the increased production of serum factors which interfere with cell-mediated immune responses to tumour antigens. For example, using their rat polyoma tumour model, Bansal and Sjögren (1973) correlated the inhibition of tumour growth by BCG with augmented *in vitro* tumour-specific lymphocyte-mediated cytotoxicity. Promotion of tumour growth after BCG treatment correlated with the accelerated appearance in the serum of a factor which blocked this cytolysis. Ankerst and Jonsson (1972) found that one particular schedule of BCG treatment was ineffective in preventing oncogenesis in mice by adenovirus type 12. In these mice blocking factors appeared in the serum earlier than was the case with control mice or mice in which administration of BCG reduced tumour incidence. Tumour specific antibody, tumour antigen and specific immune complexes have been implicated as blocking factors (reviewed by Baldwin and Robins, 1976).

BCG can also induce production of suppressor cells in a host, but whether these cells are involved in the BCG mediated enhancement of tumour growth has not been established. Geffard and Orbach-Arbouys (1976) demonstrated that spleen cells from mice injected i.v. with high doses of BCG could actively suppress cell-mediated responses such as graft-versus-host reactions or mixed lymphocyte reactions. They could demonstrate these effects with purified T cells. Florentin *et al.*, (1976)

observed that BCG given i.v., rather than s.c., to mice resulted in a marked depression of the response of spleen cells to Con A *in vitro* which was consistent with the induction of suppressor macrophages. These workers could not correlate these results with any *in vivo* experiments.

Inappropriately administered tumour antigens can interfere with BCG-mediated tumour immunotherapy. Baldwin and Robins (1976) could suppress the s.c. growth of rat sarcomas by contralateral inoculation of tumour cells admixed with BCG. However, if these animals were also given i.p. injections of irradiated tumour cells or solubilized tumour antigen, tumour rejection was impaired. Hawrylko (1975) showed that tumour immunity developing in response to the injection of irradiated murine P815 tumour cells into BCG infected s.c. sites was abrogated by the i.v. injection of irradiated tumour cells. This effect could not be demonstrated in splenectomized mice suggesting that the formation of antibody was involved in the abrogation of tumour immunity.

Thus, BCG treatment can result in stimulation of tumour growth and abrogation of the development of specific tumour immunity. These effects could be due to lymphocyte trapping, rapid destruction of tumour cells at BCG sites, alteration of lymphoid organ architecture, increased production of serum blocking factors or the induction of suppressor cells. In addition, the adjuvant effect of BCG on host response to tumour antigens can be rendered ineffective in conditions of tumour antigen excess.

It is by no means well established that all incidences of BCG-induced tumour growth facilitation can be ascribed to one

of the above mentioned mechanisms -- some of which have yet to be demonstrated as operative *in vivo*. Moreover, although within one experimental model certain conditions may favour acceleration of tumour growth, in another model similar conditions may lead to suppression of tumour growth. For example, Baldwin and Pimm (1973a) observed that the number of lung nodules was greatly increased if rats were given BCG together with weakly immunogenic tumour cells by the i.v. route. In contrast, Proctor *et al.*, (1976) found that the number of lung nodules was either unchanged or decreased if mice were given BCG i.v. on the same day as they were challenged i.v. with weakly immunogenic B16 melanoma cells.

(ii) Clinical studies

a. Facilitation of tumour growth

There is some evidence suggesting that BCG therapy can result in accelerated tumour growth in humans. Schwarzenberg, Simmler, and Pico (1976) observed a "probable" growth enhancement of solid tumour growth in 3/10 patients given i.v. BCG. There were indications that administration of BCG induced immunosuppression. While there was no change in the immune responsiveness of anergic patients after this treatment, patients able to mount allergic responses prior to receiving BCG were unresponsive after treatment. Mastrangelo mentioned that tumours grew more rapidly than normal in 5 melanoma patients following i.t. BCG, and Levy reported a single melanoma patient in whom tumour growth was accelerated after i.t. BCG (cited by Rosenberg and Rapp, 1976).

b. Side effects

Intralesional injection of live BCG causes severe side effects. The complications usually noted are chills, fever, and malaise. Three fatalities have been reported which were due to anaphylactic reactions after a long course of injections (Bast et al., 1974; Schwarzenberg et al., 1976). Liver malfunction, including hepatitis, has been observed in patients who were given i.t. BCG (Bast et al., 1974).

Although BCG is found in the blood soon after i.t. injection, progressive infection is rare. Nevertheless, Sparks, Albert, and Breeding (1977) advocate the simultaneous administration of INH to melanoma patients receiving i.t. BCG to prevent the BCG replicating. They found that rats bearing s.c. implants of a metastatic tumour survivied longer if given INH i.p. and BCG i.t. than if given only BCG. In addition, Chung et al., (1973) have shown that administration of INH to mice does not affect the BCG-induced suppression of i.d. fibrosarcoma growth.

It could be predicted from the results with experimental animal tumours that patients with acute leukaemia or with visceral metastases would benefit from i.v. BCG therapy. Few trials have been carried out because of the possibility of severe complications. However, Whittaker, Lilleyman, Jacobs et al., (1973) reported only temporary fever when BCG was given i.v. at monthly intervals to 2 patients with AML. Schwarzenberg et al., (1976) observed chills, malaise, and temporary fever when BCG was administered i.v. to patients with solid tumours. Hepatic granulomas were demonstrable in some patients, suggesting that liver dysfunction may have been

observed in the long term. Mathé (1976a) cautions that i.v. BCG therapy is potentially hazardous as the tendency of the bacilli to clump could lead to pulmonary complications.

McKneally et al., (1977) reported that two lung cancer patients became seriously ill, and one died after being given intrapleural BCG. The dose of BCG used was considerably higher than that recommended for intrapleural use.

Severe complications have not been observed in people to whom BCG has been administered by scarification, i.d., or percutaneously. There have been no fatalities in trials which have included several hundred patients (Bast et al., 1974).

Large doses of BCG administered orally are well tolerated in man, but there is little evidence to suggest that oral BCG induces tumour regression. Administration of BCG orally to patients with malignant melanoma produced beneficial effects in a few patients (Falk, Mann, and Langer, 1973). Klein, Holtermann, Papermaster et al., (1973) found that oral administration of BCG was a highly effective way of inducing cutaneous responsiveness to PPD in skin cancer patients. Mild analgesics ameliorated the side effects of chills, fever, and nausea.

In short, the side effects of BCG immunotherapy depend on the dose, route of administration, number of previous immunizations, and the strain of BCG used. Although the i.t. route of injection probably represents the most effective form of therapy against solid tumours (e.g., section 4.2.5 (i)), it is associated with the worst side effects.

7. Anti-tumour effects of C.parvum

Corynebacterium parvum is a gram-positive bacterium which has only low pathogenicity in man and does not produce endotoxins or exotoxins. Live or dead, C.parvum is a potent stimulator of the RES and can also act as an adjuvant. The anti-tumour effects of C.parvum have been investigated with killed organisms. The following discussion does not distinguish between C.parvum and the related organism C.granulosum.

Halpern, Biozzi, Stiffel et al., (1966) were among the first to show that administration of C.parvum could protect mice against the growth of subsequently transplanted tumours. Woodruff and Boak (1966) were the first to demonstrate an immunotherapeutic role for C.parvum. Systemic administration of C.parvum to mice delayed the growth of mammary adenocarcinomas grafted s.c. 8-12 days earlier.

Most of the experiments done with C.parvum have examined tumour immunotherapy rather than immunoprophylaxis. The following discussion relates some of the findings of such studies.

If C.parvum is added to a challenge inoculum of rodent tumour cells prior to s.c. grafting, inhibition of tumour growth will occur (Likhite, 1975; Pimm and Baldwin, 1977; Likhite, 1976). In the mouse, injection of C.parvum into established tumours suppresses tumour growth, and often complete regressions will be observed (Scott, 1974b; Milas, Hunter, Bašić et al., 1975; Likhite and Halpern, 1974; Woodruff and Warner, 1977). However, s.c. administration of C.parvum at a site distant from primary murine tumours rarely slows tumour growth (Scott, 1974b; Likhite and Halpern, 1974;

Milas et al., 1975). In rodents, i.v. or i.p. administration of C.parvum after, or together with, an i.v. inoculum of tumour cells prolongs survival of the animals and reduces the number and size of lung nodules (Milas, Hunter, and Withers, 1974; Bomford and Olivotto, 1974; Pimm and Baldwin, 1977). Simultaneous intrapleural administration of C.parvum and tumour cells prevents tumour growth in rats (Pimm and Baldwin, 1977). In rodents, efficacious local immunotherapy with C.parvum has been observed with strongly and weakly immunogenic tumours.

In the mouse, systemic administration of C.parvum has been reported to have little effect on s.c. tumour growth in comparison with local immunotherapy (Scott, 1974a; Woodruff and Warner, 1977; Likhite and Halpern, 1974). In view of these reports, it is surprising that Suit, Sedlacek, Wagner et al., (1976) and Milas, Hunter, Bašić et al., (1974) demonstrated that i.v. C.parvum treatment was more effective than i.t. treatment in producing regressions of s.c. murine fibrosarcomas. The latter tumours are strongly immunogenic, whereas those studied by Scott and others were weakly immunogenic. In addition, variables such as content of tumour associated macrophages, extent of vascularity, and site of tumour graft would be expected to affect the outcome of systemic immunotherapy. Data is not available which relates to the first two points although Suit et al., (1976) found that i.p. or i.d. sarcoma deposits were more susceptible than i.m. tumours to therapy with i.v. or i.p. injected C.parvum.

Similar correlations may not hold in different species. For example, in the rat, Pimm and Baldwin (1977) reported that

the s.c. growth of a strongly or weakly immunogenic tumour was enhanced when C.parvum was administered i.v. at the same time as tumour challenge.

In contrast to the experiments with solid tumours, there are few reports which show that C.parvum inhibits the growth of murine leukaemias. For example, i.p. injection of C.parvum 24 hours after the i.p. injection of leukaemia cells into AKR mice had little effect on survival (Lamensans, Stiffel, Mollier et al., 1968). Multiple injections of C.parvum given i.v. soon after the s.c. grafting of L1210 leukaemia cells into syngeneic hosts failed to affect tumour growth (Mathé, Pouillart, and Lapeyraque, 1969). However, Pearson et al., (1972) found that administration of both the drug BNCU and C.parvum to mice bearing disseminated LSTRA leukaemia was very effective therapy in terms of the number of long-term survivors (see also section 4.2.4).

In the mouse, the in vivo anti-tumour effects of C.parvum are associated with the relative distribution of C.parvum in the various organs. The localization pattern of s.c., i.t., and i.v. injected ^{125}I or FITC labelled C.parvum was studied by Dimitrov, Greenberg, and Denny (1977) and Scott and Milas (1977).

These workers found that i.v. injected C.parvum persisted in the spleen and liver, but was cleared more rapidly from the lungs. After s.c. administration, most of the bacteria were restricted to the site of injection, with high concentrations in the draining lymph nodes and none in the contralateral nodes. Intralesionally injected C.parvum was similarly distributed, but the systemic spread of the bacteria was more rapid

after i.t. than s.c. injection, presumably because of the superior vascularization of the tumour nodule in comparison with normal tissue.

In summary, the results of numerous experiments show that the anti-tumour effects of C.parvum are likely to be best exploited if the route of administration of C.parvum is such that close association between tumour cells and cells stimulated by C.parvum is obtained.

Responses to C.parvum and tumour antigens during immunotherapy

As in the case of BCG, the properties of C.parvum which have been shown to be fundamental to its anti-tumour effects are its capacity to activate macrophages and to act as an adjuvant in the host response to tumour antigens.

Activation of macrophages

The anti-tumour effects of C.parvum administered by the i.v. route also occur in irradiated or T cell depleted mice and so do not depend upon a heightened response to tumour antigens (Woodruff, Ghaffar, and Whitehead, 1976; Woodruff and Warner, 1977).

It has been shown that activated macrophages are induced in T cell depleted mice after i.v. injection of C.parvum, so it is most likely these cells which are responsible for the above effects (Bomford and Christie, 1975). The growth inhibitory effect of systemically administered C.parvum against s.c. grafted tumours is abrogated if the host is treated with gold salts, a specific macrophage toxin (McBride, Tuach, and Marmion, 1975). Surprisingly, however, silica did not abolish the suppressive effect of i.v. C.parvum against lung metastases of the mouse Lewis lung tumour (Jones and Castro, 1977).

Peritoneal and pulmonary macrophages from C.parvum treated mice have been shown to inhibit DNA synthesis and growth of syngeneic sarcoma and leukaemia cells in vitro, even if taken from T cell depleted mice (Olivotto and Bomford, 1974; Ghaffar, Cullen, and Woodruff, 1975).

Ghaffar et al., (1975) and Christie and Bomford (1975) did not obtain activated macrophages after incubation of normal mouse macrophages with C.parvum in vitro. However, oil-induced normal macrophages were activated with this treatment (Christie and Bomford, 1975). These workers suggested that in vivo C.parvum could mimic the first stage of the oil-induction process, by producing a factor chemotactic for macrophages.

However, C.parvum can also activate macrophages by a T cell dependent pathway. Normal mouse macrophages were shown to be activated after incubation, in vitro, with C.parvum and spleen cells from C.parvum immunized mice (Christie and Bomford, 1975). Treatment of these spleen cells with anti-θserum and complement abolished activation. Incubation of normal macrophages with supernatants from cultures of sensitized spleen cells and C.parvum was sufficient to induce macrophage activation. This data indicates that a T cell produced lymphokine is involved in the activation process (section 3.1).

It seems likely that C.parvum, like live BCG, can activate macrophages directly or by T-dependent mechanisms. An alternative explanation, suggested by Bomford and Christie (1975), is that the mice used in some studies are naturally sensitized to C.parvum and, even if T-depleted, will possess some sensitized T cells and so can mount an immunological response to C.parvum antigens.

C.parvum as an adjuvant to tumour antigens

It was noted in the section on immunotherapy with BCG that i.t. injection of BCG, or challenge of an animal with a mixture of tumour cells and BCG, would often result in the surviving animals having acquired specific tumour immunity (e.g., sections 4.2.1 and 4.2.3).

Similar therapeutic regimes utilizing C.parvum in mice also induce specific tumour immunity (Scott, 1974b; Likhite and Halpern 1974; Gupta, Morahan, and Kaplan, 1978). It has been suggested that regression of the primary tumour induced by i.t. C.parvum therapy depends upon potentiation of the host response to tumour antigens because i.t. C.parvum therapy is ineffective in T cell depleted mice (Scott, 1974b). However, this requirement for T cells could be interpreted as indicating that a T cell mediated response to C.parvum is required (previous section).

In addition, the mode of action of C.parvum in i.t. therapy appears to vary with the tumour and mouse strain used. For example, Kaplan and Morahan (1976) observed that i.t. injection of mouse sarcomas with C.parvum resulted in regression of the primary tumour even in T cell depleted hosts.

Thus, in some cases of i.t. treatment with C.parvum, although the host may respond to tumour antigens such a response is not required for regression of the primary tumour.

In the mouse, there is one example in which successful i.t. C.parvum therapy was definitely associated with a T cell response to C.parvum antigens. Injection of C.parvum into established Meth A tumours in the footpads of Balb/c mice was

followed by tumour regression if the hosts had been previously sensitized to C.parvum. This effect was only demonstrable 5-15 days after sensitization, which corresponded to the period of active CMI to C.parvum antigens (Tuttle and North, 1975).

In the rat, suppression of the growth of immunogenic tumour cells following the s.c. injection of C.parvum and tumour cells resulted in the generation of specific tumour immunity (Pimm and Baldwin, 1977). However, the inhibition of tumour growth was probably not due to an adjuvant effect of C.parvum for tumour antigens. It was found that whole-body irradiation of the hosts had no effect whereas depletion of macrophages abrogated tumour resistance (Pimm and Baldwin, 1977). These workers obtained similar results using BCG in rats (section 4.2.3).

Mice injected s.c. with a mixture of C.parvum and irradiated P815 mastocytoma cells developed specific tumour immunity which did not occur using irradiated cells or C.parvum alone. These mixtures suppressed the growth of P815 cells growing in footpads when injected into contralateral footpads 3 days after tumour challenge (Scott, 1975). Bomford (1975) reported similar findings with a murine fibrosarcoma.

Reports of the effect of systemic C.parvum treatment on the development of specific tumour immunity in the mouse are conflicting (e.g., Scott 1974a; Milas, Hunter, Bašić et al., 1974; Purnell et al., 1976).

In short, C.parvum given locally or systemically can augment the host response to tumour-associated antigens, but whether or not this response is essential for the inhibition of primary tumour growth is debatable. Variations in the

species or strain of animal used, tumour immunogenicities, doses of C.parvum injected, and experimental protocols can give rise to different results.

Enhancement of tumour growth by C.parvum

C.parvum can also enhance tumour growth, and the chances of this occurring are increased if high doses of C.parvum are used (Bomford, 1977). Prior s.c. injection of C.parvum into mice enhanced the growth of s.c. implants of mouse tumours and the effect was more marked if the C.parvum was injected i.v.(Bomford, 1977). Woodruff and Warner (1977) showed that tumours which usually regressed after transplantation into normal mice failed to do so if the mice were treated systemically with C.parvum.

The prophylactic or therapeutic effects of irradiated tumour cells can also be reduced or abolished by systemic injection of C.parvum or by injection of irradiated cells mixed with large doses of C.parvum (Smith and Scott, 1972; Scott, 1975; Bomford, 1975).

Two mechanisms have been proposed to explain the immunosuppressive effects of C.parvum, but whether these are involved in enhancement of tumour growth has not been established.

Allwood and Asherson (1972) showed that prior i.v. injection of C.parvum into mice inhibited DTH to contact-sensitizing chemicals because small lymphocytes were prevented from localizing at the site of application of the chemical. In C.parvum treated mice the localization of these cells at the skin site was depressed by 66% in comparison with control mice. This dramatic suppressive effect was not observed if other adjuvants such as CFA and B.pertussis were used. Lymphocyte trapping in

mice given i.v. C.parvum would prevent the host responding to a subsequent tumour challenge (reviewed by Bomford, 1977).

C.parvum also induces cells which inhibit the division of lymphocytes responding to tumour antigen. Kirchner, Glaser, and Herberman (1975) reported that i.p. injection of C.parvum into mice resulted in the appearance of splenic macrophages which inhibit the in vitro cytolysis of tumour cells by spleen cells sensitized to tumour antigen.

The data of Bomford (1977) suggested that lymphocyte trapping rather than active suppression was likely to be responsible for the promotion of tumour growth by C.parvum in murine hosts. In either case, immunogenic tumours should be the most susceptible to the promotion of tumour growth by C.parvum. By definition, non-immunogenic tumours cannot sensitize host small lymphocytes.

The above studies have led to cautionary comments on the use of C.parvum for tumour immunotherapy in man. One example is the following: "Corynebacteria, which are not better tolerated than BCG, should not be administered without sophisticated monitoring of the immune functions of the patients, because they inhibit T cells" (Mathé, 1976a). However, there is little evidence to suggest that C.parvum promotes tumour growth or is immunosuppressive in cancer patients (Oettgen, Pinsky, and Delmonte, 1976; Bomford, 1977).

7.1 Comparisons of the anti-tumour effects of BCG and C.parvum in animal models

(i) Ribi, Milner, Granger et al., (1976) found that oil-attached BCG cell walls were 30x more effective on a weight basis than C.parvum in inducing regressions of established i.d. line 10 tumours in guinea pigs.

(ii) Likhite (1976) used a syngeneic transplantable rat tumour, 13762 mammary adenocarcinoma, to compare the anti-tumour activity of 10^6 live BCG with that of 1mg of C.parvum. He found that tumour growth was inhibited if cells were mixed with C.parvum prior to s.c. or i.d. implantation. If BCG was used the growth of i.d. grafts was suppressed, but the growth of s.c. implants was facilitated. Tumour growth was inhibited when C.parvum was given at a separate site (i.p. or i.d.) after the i.d. injection of tumour cells. In these circumstances BCG enhanced tumour growth.

In contrast to Likhite, Kreider, Bartlett, and Purnell (1976) found that s.c. injection of 13762 cells mixed with BCG (10^6) resulted in tumour rejection. They did not compare the efficacy of BCG and C.parvum.

(iii) Scott and Bomford (1976) compared the potentiation of tumour specific immunity induced in mice by BCG and C.parvum. The tumours used were the P815 mastocytoma (Scott, 1974a and b) and the M4 fibrosarcoma (Bomford, 1977).

Mice were immunized with a s.c. injection of a mixture of irradiated P815 cells and C.parvum. When BCG was used as an adjuvant it was given using the "primed site" protocol of Mackaness and coworkers (section 4.1.2). Various times after immunization the mice were challenged with P815 cells and it was found that the immunity induced by C.parvum was longer lasting than that induced by BCG. Equivalent weights of BCG and C.parvum were compared in these experiments.

In the case of the M4 tumour, M4 cells were injected s.c. into the hind footpads of mice and 2 days later mixtures of adjuvant and irradiated tumour cells were injected into the

contralateral footpad. For a given tumour burden, higher doses of BCG were needed to inhibit tumour growth to the same extent as C.parvum. It was found that only C.parvum suppressed the growth of large inocula of tumour cells.

Conclusions

More appropriate comparative experiments will be done when the active moieties of BCG and C.parvum have been isolated. However, as the whole organisms are at present used in clinical studies the experiments described above do provide some useful information—although it is apparent that each experimental tumour model may give different answers. It is also important to note that different strains of BCG have been shown to vary in their immunological properties [Mackaness, Auclair, and Lagrange, 1973].

7.2 Clinical studies with C.parvum

Israël and Halpern (1972) reported the results of a controlled trial in which C.parvum was administered to patients with various types of advanced metastatic visceral cancer. One half of the patients (70) received regular treatment with drugs, the second group also received the drugs but were given C.parvum s.c. once a week. Chemotherapy was stopped when platelet or leukocyte counts were low, but otherwise therapy was continued for the life of the patient. The group given C.parvum survived 5 months longer than the control patients ($p < 0.001$). This result held for the largest subgroup of patients who had squamous cell carcinoma of the lung ($p = 0.07$). Patients who were PPD positive before therapy had a better prognosis. Higher doses of drugs could be administered to patients given C.parvum.

Israël and colleagues also found that patients with oat cell carcinoma of the lung, sarcomas, metastatic breast cancer or malignant melanoma survived longer if they were given C.parvum s.c. in addition to chemotherapy (cited by Oettgen et al., 1976). In this case, the trials began after excision of the primary tumour and regional lymph nodes. The period of observation was 1 year. In addition, Israël, Edelstein, Depierre et al., (1975) administered C.parvum by the i.v. route to patients with various disseminated cancers. Out of a total of 20 patients, the lesions in 8 patients regressed to less than 50% of their initial size after 4-16 weeks of therapy. Furthermore, the lesions in 11 patients with malignant melanoma completely regressed following i.t. injection of C.parvum (Israël, 1974-cited by Oettgen et al., 1976).

C.parvum has not been implicated as the cause of any fatalities in man. Intravenous injection of C.parvum results in more side effects (e.g., fever, chills, increased blood pressure, headache, etc.) than s.c. administration (reviewed by Oettgen et al., 1976).

After considering the evidence available in the literature, Oettgen et al., (1976) concluded that the general immunocompetence of cancer patients treated with C.parvum formed no consistent pattern. Their criteria included DTH responses to microbial antigens and DNCB, lymphocyte counts, and the responsiveness of lymphocytes to mitogens in vitro.

8. Anti-tumour effects of C.parvum extracts

McBride, Dawes, and Tuach (1976) prepared acidic polysaccharide extracts from C.parvum by acid or alkaline hydrolysis of whole organisms. The anti-tumour activity of extracts derived from 0.7mg of C.parvum was compared with that of 0.7mg of C.parvum. The acid extract was as effective as C.parvum in preventing the development of lung tumour nodules if given i.v. to mice 1 day before the i.v. injection of fibrosarcoma cells. The extracts could limit the growth of i.p. or s.c. grafts of fibrosarcoma if given in admixture with tumour cells. However, the extracts had little immunotherapeutic effect against 3 day old tumours, unlike whole C.parvum. These workers found that the extracts did not induce a significant antibody response or splenomegaly if administered to mice, and they concluded that whole C.parvum elicited much greater anti-tumour effects.

Millman, Scott, and Halbherr (1977) used the murine P815 mastocytoma model to investigate the anti-tumour effects of C.parvum extracts. Established footpad tumours were injected with the preparations. Although whole C.parvum inhibited tumour growth, RNA and cell wall extracts had little anti-tumour activity. Riveros-Moreno, Bomford, and Scott (1978) also found that cell walls had little anti-tumour activity against the murine M4 fibrosarcoma. However, they did demonstrate that cell walls elicited DTH in C.parvum sensitized mice, and that tumour growth was inhibited if cell walls were injected i.t. into sensitized mice. Nevertheless, no complete regressions occurred, which contrasted with the 100% complete regressions observed if similar amounts of whole C.parvum were

injected i.t. into unsensitized mice. The lack of activity of cell walls could not be explained on the basis of any difference in localization and persistence in mice in comparison with whole C.parvum. It seemed likely that cell walls were not the components responsible for the anti-tumour effects of whole C.parvum.

Indeed, Millman et al., (1977) have shown that injection of a putative cytoplasmic C.parvum extract into P815 footpad tumours resulted in suppressed tumour growth in both the injected footpads and in uninjected contralateral footpads. The growth inhibition was more marked than that elicited with 5x the amount (in terms of total nitrogen) of whole cells. In addition, whole C.parvum only limited the growth of those footpad tumours into which it was injected.

The above results are promising as these workers appear to have achieved an enrichment of anti-tumour activity after fractionation of C.parvum. The designation "cytoplasmic" for Millman's extract has not been verified as it has not been characterized. Thus it has yet to be determined if both cell wall and non-cell wall components are needed for extracts of C.parvum to have anti-tumour activity, as was proposed by Riveros-Moreno and coworkers.

9. Antigenic cross reactions between bacteria and tumours

It is possible that the prophylactic or therapeutic effects of BCG against some tumours may be specific in expression in view of reports that BCG and tumour cells share antigens. There have been reports of antigenic cross reactions between BCG and the line 10 guinea pig tumour, Rous sarcoma cells, a mouse melanoma, and a human melanoma (Bucana and Hanna, 1974; Faraci, Barone, and Schour, 1975). On the other hand, no such cross reaction was demonstrated between BCG and line 1 guinea pig tumour and a mouse plasma cell tumour (Bucana et al., 1974).

Minden, Sharpton, and McClatchy (1976) showed that the line 10 tumour also shares antigens with Listeria monocytogenes and probably with Brucella abortus and Salmonella typhimurium. This is interesting in view of the data of Minden, McClatchy, and Farr (1972) which indicated that BCG shared antigens with L.monocytogenes and other heterologous bacteria. Minden et al., (1976) did not observe any cross reactions between line 1 tumour cells and the bacteria. These workers have shown that it is unlikely that the antigens involved on the line 10 tumour are normal tissue antigens. However, whether or not these antigens are identical with tumour specific transplantation antigens or whether they constitute determinants relevant to the anti-tumour effects of the bacteria remains to be established.

Similar considerations may apply in the case of C.parvum and tumour cells. James and coworkers investigated changes in immunoglobulin levels following the administration of C.parvum to mice. They adopted this approach in order to determine

whether or not these immunoglobulins would bind to tumour cells, which would reveal whether or not C.parvum and tumour cells shared antigens. They showed that giving C.parvum, or other adjuvants, to normal or tumour-bearing mice resulted in a marked increase in the production of immunoglobulins of most classes (James, Willmott, Milne et al., 1977). These immunoglobulins bound to syngeneic tumour cells, embryo cells, adult kidney cells and adult spleen cells in vitro. Tumour cells incubated in these sera did not exhibit reduced growth following implantation in mice, so there is no evidence that these serological changes are relevant to the anti-tumour effects of C.parvum. It may be that C.parvum shares antigens with both normal and tumour cells, or that administration of C.parvum results in increased synthesis of auto-antibodies or of pre-existing anti-tumour antibodies.

If the antigenic similarities in nature are as broad as some of the preceeding evidence suggests some basic immunological precepts may need redefinition.

10. Conclusions

Administration of various "immunopotentiators" to animal hosts produces a number of effects on tumours including:

(a) suppression or regression of tumour growth,

(b) enhancement of tumour growth,

(c) potentiation of specific tumour immunity.

Which of these effects is observed depends upon the model system considered. Some important factors in determining the outcome of treatment with these agents are as follows:-

(a) anatomical location, type, size and immunogenicity of the tumour;

(b) dose, route, and timing of BCG or C.parvum administration;

(c) the species of animal;

(d) host complement of T lymphocytes. In some cases, but not all, there is an absolute requirement for T cells if suppression of tumour growth is to be observed;

(e) in some models it is possible that successful immunotherapy relies upon an antigenic cross reaction between the immunopotentiator and the tumour.

Looking back over the data in the literature it is evident that few advanced experimental animal tumours will regress completely if the host is treated with BCG or C.parvum. Evidence relating to tumour immunoprophylaxis in animals is impressive, but there is little evidence to suggest that this approach will work in humans.

Animal models which resemble human cancer in its many forms are few and far between, simply because it is not easy

for the scientist to obtain and work with spontaneous, slowly metastasizing, autochthonous tumours in small laboratory animals. Nevertheless, the models available have allowed fairly thorough elucidation of the mechanisms of action of the immunopotentiators. It is impressive that BCG and C.parvum have shown some clinical efficacy in view of the difficulties of extrapolating immunotherapeutic protocols from the laboratory to the clinic.

The treatment of cancer patients with BCG and C.parvum results in alleviation of the disease process rather than elimination of all tumour cells. Immunotherapy has prolonged the survival time of some cancer patients. However, in some cases in which BCG or C.parvum administration has been combined with chemotherapy, it is not clear if these agents counteract the drug-induced myelosuppression or if they are acting via immunotherapy (e.g., Oettgen et al., 1976).

For ethical reasons, until recently immunotherapists have been treating patients with advanced cancer only when the other available modes of treatment have failed. In these people, the tumour burden has been reduced using surgery, radiation, and chemotherapy. Studies by Rapp and coworkers in guinea pigs indicate that a better approach could be to employ immunotherapy in patients with early stage cancers, and then to use other treatments. "Certain clinical situations seem unsuitable for immunotherapy. These would include patients who come to the physician with metastatic disease present in the lungs, liver, brain, or bone" (Zbar et al., 1976a).

It is difficult to propose ways in which BCG and C.parvum could be presented to the cancer patient and induce a

more effective anti-tumour response, either specific or non-specific in expression, than is the case with protocols currently employed. A problem central to tumour immunology is the apparent lack of immunogenicity of tumour cells in their human hosts. Moreover, the words "tumour immunology" embrace diverse immunological situations. "Tolerance of spontaneous or syngeneic tumours provides another setting which again is very heterogeneous, ranging from highly vascularized rapidly growing sarcomas through lymphomas to slowly growing epitheliomas. Different tumours shed different amounts of tumour-specific antigens with varying degrees of immunogenicity" [J.H. Humphrey, 1976, British Medical Bulletin 32 pp. 182]. Although the problem of the immunogenicity of human tumour cells is of great importance to the field of tumour immunotherapy it is outside the terms of reference of this introduction.

BCG and C.parvum have now been used for some years in the treatment of cancer patients. In the next few years much progress will undoubtedly be made in the study of host-tumour cell relationships. During this period scientists should continue to characterize components of BCG and C.parvum. However, "it should be emphasized that indiscriminate use of BCG by physicians in the treatment of neoplasia may often result in a disservice to the patient" [Laucius, Bodurtha, Mastrangelo et al., 1974]. Another pertinent comment is that of Currie [1976]: ". . .irrational forays into cancer wards armed with hope and a few ampoules of BCG are likely to leave the frontiers of knowledge singularly unmoved."

MATERIALS AND METHODS

Mouse strains

The inbred strains of mice used for the experiments in this project were:-

(i) (BALB/cJ ♀ x C57BL/6J ♂) F1 hybrids (CB6F1), bred in the University of Adelaide Medical School Animal House. Breeding pairs were obtained from Jackson Laboratories, Maine, U.S.A.

(ii) CBA/J strain mice, maintained as above.

Male and female mice, 8-12 weeks of age were used in experiments.

In addition, LACA (specific pathogen free) strain mice, from a closed colony, were used in some cases for EAT passage and for the *in vivo* radio-isotope labelling of EAT cells. These mice were obtained from the University of Adelaide Central Animal House.

Bacterial strains

(i) *Salmonella enteriditis* 11RX

This rough strain is avirulent for mice and was described by Ushiba, Saito, Akiyama *et al.*, (1959). The median lethal dose, (LD_{50}), determined after i.v. challenge of CB6F1 mice, is approx. 2×10^6 organisms (Davies, 1975).

(ii) *Salmonella typhimurium* C5, *Salmonella typhimurium* M206, *Salmonella enteriditis* Se 795, *Escherichia coli* BV.

These strains were kindly provided by Dr. R. Davies. The original references describing these organisms have been cited in Davies (1975).

(iii) Salmonella haarlem 09, Salmonella greenside 050

These strains were kindly provided by Dr. S.H. Neoh.

(iv) Escherichia coli K12 P400

This strain was kindly provided by P.A.Manning.

Maintenance and use of bacteria

The bacteria were kept as lyophilised cultures in sealed ampoules, and used to set up nutrient agar slopes (Blood Agar Base, Difco) every 3 months. The slopes were stored at 4°C.

Bacteria were cultured at 37°C overnight on nutrient agar plates to obtain single colonies. Liquid cultures were obtained by inoculating colonies into nutrient broth (double strength Bacto Nutrient Broth, Difco and NaCl 5g/l) and incubating overnight at 37°C on a shaker. Logarithmic (log) phase cultures were obtained by subculture of overnight cultures ($^1/_{10}$ dilution) into fresh broth which was incubated for 2½ - 3 hours at 37°C with shaking.

When a number of litres of bacteria were grown for use in the preparation of antigen extracts, the content of each culture flask was checked by plating samples onto nutrient agar plates.

Mice were immunized with log phase cultures of bacteria which had been diluted to the appropriate concentration in saline (0.9% w/v). The numbers of organisms which had been injected were checked in retrospect by viable counting. The numbers given in the tables and figures in the text (e.g. 10^5 11RX i.v.; 1.2×10^7 M206 i.v.) are the estimated doses. In the tables in Results "10^5 11RX i.v. day-X" refers to the fact that the mice were given 11RX X days prior to the experiment.

Determination of numbers of bacteria in the peritoneal cavity, liver and spleen

Mice were killed by cervical dislocation, swabbed with alcohol and the abdominal skin was reflected.

(i) The peritoneal cavity was washed out by injecting 1 ml of sterile saline through a 19 gauge needle, massaging the abdomen and withdrawing the fluid. The fluid was kept on ice until it was plated.

(ii) The spleen and liver were exised and rinsed with sterile saline. Each organ was put in a sterile glass bottle to which was added 3-10 ml of sterile saline. Samples were kept on ice and later homogenised with an electrically-driven Ultra-Turrax homogeniser. The probe of this device was washed successively with alcohol, and 2 lots of saline between samples.

(iii) Duplicate or triplicate 0.1 or 0.2 ml aliquots of each sample (or an appropriate dilution) were plated onto nutrient agar plates which were incubated at 37°C overnight.

Media used for cell suspensions

Media were made with deionised distilled water and analytical grade chemicals and were sterilized by filtration through Millipore membrane filters (0.45μm pore size).

(i) Hanks' balanced salt solution (HBSS) was routinely used for the preparation of tumour cell suspensions for injection into mice. HBSS was made by diluting stock solutions, prepared as described by Weller, Enders, Robbins et al., (1952). HBSS was adjusted to pH 7.4 by the addition of sterile $NaHCO_3$ solution

(4.4% w/v) or sterile HEPES. HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethane sulphonic acid) was added as a solution of pH 7.4, containing 1M HEPES buffered with NaOH.

(ii) For in vitro cytotoxicity assays, the media used were made from Dulbecco's modified Eagle's Minimal Essential Medium (DMEM). DMEM was made up from a powder (Grand Island Biological Company, N.Y., U.S.A.) and penicillin (100 units/ml) and streptomycin sulphate (100 ug/ml) were added.

(a) For preparing and washing cell suspensions the medium was adjusted to pH 7.4 with NaOH solution (2.5N) and HEPES buffer (final concentration 30mM). Heat-inactivated foetal calf serum (FCS) was added to a final concentration of 5% (v/v). FCS was prepared from blood obtained from the South Australian Meat Corporation Abbatoirs, and was stored at -15°C.

(b) The culture medium was prepared by adjusting the pH of the basic DMEM and antibiotics to pH 7.4 with NaOH (2.5N). Buffering was provided by HEPES (final 30mM) and $NaHCO_3$ (final 0.37% w/v). The medium was filtered, and prior to use the pH was readjusted by flushing with 5% CO_2 in air. Filtered FCS was then added to a final concentration of 10% (v/v).

(iii) Tumour cell suspensions for routine mouse passage were prepared in sterile glass bottles. All other cell suspensions were prepared in sterile siliconised Pyrex glass tubes with screw caps. Cells were kept on ice until used. Tubes were flushed with 5% CO_2 in air if they contained cells suspended in the culture medium.

Tumours

(i) EAT was obtained in 1955 from the Roswell Park Memorial Institute, N.Y., U.S.A. The tumour, which has no mouse strain specificity, was maintained in CB6F1 or LACA mice by serial i.p. passage using 10^7 cells. In CB6F1 mice, the LD_{50} by the i.p. route is approx. 200 EAT cells (Hardy and Kotlarski, 1971).

To prepare tumour cell suspensions, mice which had been injected i.p. with 10^7 EAT cells 7 to 10 days previously were killed and the ascitic fluid was aspirated. The fluid was mixed with medium and then centrifuged (250 x g for 1.5'). The cells were washed 3 times and resuspended in medium. The final cell suspension from one mouse usually contained approx. 10^9 EAT cells, of >95% viability.

(ii) RI (radiation-induced) leukaemia was obtained in 1976 from Dr. D.I.Connell, Chester Beatty Research Institute, England. RI is syngeneic for CBA mice. In the Institute the tumour is maintained in CBA/Lac mice, and in our laboratories in CBA/J mice. Serial passage involved weekly i.p. injections of 10^5 RI cells. Death is abrupt with only moderate ascites development-metastasis is probably a major factor (Smith and Scott, 1972). Inoculation of 10 RI cells i.p. will cause death (Smith and Scott, 1972; and see text).

The method for preparing RI cell suspensions was essentially the same as for EAT except that medium containing heparin (10 I.U./ml) was used to

wash out the peritoneal cavity. In addition, some of the cells formed clumps during the washing procedure, and these were removed by sieving suspensions through fine wire mesh. For experiments, cells were usually taken from passage mice at 6 to 9 days, at which time 2-4 x 10^8 RI cells could be harvested, generally of 70-$>$95% viability.

Peritoneal Exudate Cells (PEC)

The term PEC refers to peritoneal cells which were harvested from mice which had not been treated with stimulants such as oil or thioglycollate.

The peritoneal cavity was washed out as described previously, except that 2.5 ml of cell suspension medium containing heparin was used instead of 1ml of saline.

The washout fluids from 4 or more mice were pooled and centrifuged (400 x g for 7'). The cells were taken up in suspension medium, counted, washed once and then resuspended to the desired concentration in culture medium.

Cell counting

(i) Tumour cell or PEC suspensions were diluted into saline containing trypan blue (0.1% w/v). Viable (unstained) cells were counted using a haemocytometer. Cell numbers in the text refer to viable nucleated cells.

(ii) In some experiments, PEC suspensions were counted using a model F Coulter particle counter (Coulter Electronics Inc., Florida, U.S.A.).

Aliquots of PEC suspensions were diluted into saline or phosphate buffered saline (pH 7.2) from

which particles had been removed by filtration through a Millipore Filter (0.45μm pore size). Prior to counting, a solution of Saponin (Coulter Electronics Inc.) was added to samples to a final concentration of 0.01% (w/v). Saponin lysed any red blood cells in the samples.

Coulter counting did not differentiate between viable and dead cells. Invariably, PEC suspensions were 100% viable.

(iii) It was sometimes of interest to estimate the total number of PEC per mouse, and this was approximated as follows:-

$$\frac{\text{total PEC harvested} \times \left(\text{volume of medium used for washout} + 0.2^{*}\right)}{\text{volume of washout fluid aspirated}}$$

* 0.2ml was the volume of fluid assumed to be in the cavity prior to the injection of medium.

Radioisotope labelling of EAT cells

(i) ^{51}Cr was obtained as a sterile saline solution of sodium chromate (specific activity = 100 to 300 mCi/mg chromium) from the Radiochemical Centre, Amersham, England.

For in vitro experiments 100μCi of ^{51}Cr was added to 1ml of suspension medium which contained 5×10^6 EAT cells. For in vivo experiments the medium contained 5×10^7 EAT cells. The mixtures were incubated at 37°C for 60', and were shaken occasionally. The labelled cells were washed 5 times, were enumerated using trypan blue, and were resuspended to the desired concentration in the

appropriate medium. The γ radioactivity of the suspensions was determined by placing samples in a well-type scintillation counter [Auto-Gamma Spectrometer, Packard Instrument Co., Illinois, U.S.A.]. The ^{51}Cr-EAT suspensions were generally 85-95% viable.

(ii) 5-(^{125}I)-iodo-2'-deoxyuridine (^{125}IUdR = 10 or 110 mCi/mg) was obtained as a sterile aqueous solution from the Radiochemical Centre and was diluted into HBSS or DMEM prior to use.

Each mouse was injected i.p. with 10^7 EAT cells followed the next day by 4 i.p. injections of ^{125}IUdR at intervals of 3 hours. The total amount of ^{125}IUdR injected was 3μCi. ^{125}IUdR-EAT cells were harvested 2 days later by rinsing the peritoneal cavity with suspension medium. The fluid was centrifuged and the cells were washed once and resuspended in HBSS or DMEM.

(iii) In one experiment, specified in the text, ^{131}IUdR (10 mCi/mg) was used instead of ^{125}IUdR. In this case each mouse was injected with a total of 40μCi of ^{131}IUdR.

In vivo assays for recall of resistance to EAT

(i) Whole-body retention of ^{125}IUdR

Normal mice and long-term 11RX immunized mice were injected i.p. with 0.2mls of medium or saline containing various μg quantities of 11RX extracts. Control mice were those which received medium or saline, or were uninjected.

One day before tumour challenge, the mice were given unlabelled sodium iodide (0.1% w/v) in their drinking water. This was done to suppress the retention of radioactivity due to the uptake of labelled iodide by the thyroid.

One, two, or three days (as specified) after the injection of extracts, the mice were challenged i.p. with 10^6 ^{125}I-EAT cells. This dose represented approx. 5,000 LD_{50} doses. At various times thereafter, the whole-body radioactivity of the individual mice was monitored. Each mouse was put into a plastic tube (10cm x 3.7cm) and placed in the well (4.5cm diam. x 5.7cm deep) of a sodium iodide crystal scintillation detector (Nuclear Chicago, Searle and Co., Illinois, U.S.A.).

The amount of radioactivity injected was determined by measuring the radioactivity of the mice soon after challenge. The ^{125}I retention measured on the following days was calculated as a percentage of the injected radioactivity, after correcting for isotope decay.

In some experiments killed labelled EAT cells were injected into normal mice. These cells had been incubated at 56°C for 30'.

In a few cases, resistance or susceptibility to tumour growth was also assessed by recording the survival times of the mice injected with labelled EAT.

(a) In the experiment in which ^{131}I-EAT cells were used, the radioactivity of the mice was measured using the counting chamber (10.8cm diam. x 20.3cm long) of an ARMAC scintillation detector (Packard Co.). Individual mice were monitored, and the radioactivity of groups of mice was also determined by placing the mice in a jar (8cm diam. x 14cm long).

(ii) Clearance of radioisotope-labelled EAT cells from the peritoneal cavity

Mice which were treated as mentioned previously (i) were challenged i.p. with 10^6 EAT cells labelled with ^{51}Cr or ^{125}I. Sixteen to 24 hours later (as specified in the text) each mouse was killed and the peritoneal cavity was rinsed out with 5ml of saline. The radioactivity of a standard aliquot of the wash-out fluid was measured in the Auto-Gamma counter. The radioactivity of a sample of the injected cell suspension was also determined so that the recovery of radioactivity from each mouse could be calculated as a % of the injected radioactivity.

It had been determined by Ashley (1976) that the ^{125}I or ^{51}Cr recovered in such washout fluids as described above was cell-associated.

The in vitro cytotoxicity assay for recall of resistance to EAT

Mice were treated in the same manner as described for the in vivo assays except that on the day when they would otherwise have been challenged with EAT, their PEC were harvested.

Suspensions of test PEC and ^{51}Cr-EAT cells were mixed and dispersed into Microtiter plastic tissue culture trays (flat-

bottomed, Cooke Instruments) so that each well contained 10^4 ^{51}Cr-EAT cells and 10^6 PEC in 0.2ml of culture medium. Each assay included control wells containing ^{51}Cr-EAT only. The trays were covered with loose fitting lids and incubated at 37°C in a CO_2 incubator (100% humidity, 5% CO_2 in air).

At various times thereafter, quadruplicate wells were assayed for ^{51}Cr release into the medium by removing 0.1ml of medium from each well using an automatic pipette with disposable tips. Samples were taken without disturbing the cells, which were settled at the bottom of the wells.

An estimation of the amount of ^{51}Cr release equal to 100% tumour cell lysis was obtained by lysing duplicate samples of ^{51}Cr-EAT cells with chloroform. Approx. 80% of the total incorporated radioactivity was released using this treatment. Ashley (1976) had determined that this treatment killed 100% of the tumour cells using trypan blue staining. The ^{51}Cr chloroform release was taken to represent the total releasable ^{51}Cr.

Results from this assay were calculated as follows:-

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

(i) Note: The in vivo and in vitro assays mentioned above were described in more detail by Ashley et al., (1974) and Ashley (1976). In this project CB6F1 mice were used in the experiments described in Results, sections 3-6. Either CB6F1 mice or CBA mice (as specified) were used for the experiments in Results, section 7.

Test for DTH

A micrometer-operated syringe, fitted with a 30 or 26 gauge needle, was used to inject various μg quantities of 11RX extracts in 0.01-0.02ml of saline into the right hind footpads of mice. At various times after injection, usually 4, 24, 48, 72 and 96 hours, the thickness of both hind feet was measured in triplicate to the nearest 0.05mm with dial gauge callipers (Kröplin, Germany or Mercer, England). The degree of swelling of the right hind footpad was estimated by subtracting the thickness of the left foot from the thickness of the right foot. The results in the text are expressed as % increase in footpad thickness.

Preparation of 11RX extracts

The protein-rich extract used by Ashley, Kotlarski and Hardy (1974) was termed 11RX antigen. This term is also used to describe preparations made since this time, including those in which modified extraction procedures were used. Methods used for making 11RX antigen are described in (i), (ii), and (iii) below. All preparations termed 11RX antigen were derived from 78,000-100,000 x g supernatants of disrupted 11RX.

In the interests of clarity, a diagram showing the essential details of extraction of the various 11RX antigen preparations is included at the beginning of the text.

Other extracts used in this study were derived from 78,000 x g pellet material and the extraction techniques are described in (iv) below.

(i) 11RX antigen prepared from bacteria disrupted with ultrasound

The method was previously described by Ashley (1976). These extracts were available at the beginning of this project.

Washed 11RX from an overnight culture were suspended to approx. 30mg/ml and disrupted with ultrasound at 4°C (until the OD_{650nm} was halved) in 10mM Tris-HCl pH 7.8 containing 5mM $MgCl_2$, 5mM 2-Mercaptoethanol and nucleases (10µg/ml DNase and 10µg/ml RNase; Calbiochem.). The suspension was then centrifuged, first at low speed to remove whole bacteria and large debris (8,000 x g for 20') and then at high speed to remove ET and other cell envelope components (100,000 x g for 2 hours at 4°C). The supernatant was dialysed for 16 hours at 37°C against Tris buffered $MgCl_2$ (containing sodium azide, 0.02% w/v) in the presence of more nucleases (a further 10µg/ml of each). This was followed by extensive dialysis against distilled water and then deionised distilled water at 4°C. This period was generally 6 days. The final day's dialysis was done against deionised distilled water which did not contain azide. The remaining non-dialysable material (Batch I, II, or III) was lyophilised and stored dry at 4°C.

A similar preparation made during this project was termed Ultrasound supernatant + pellet (U.S. SN + pellet). The yield was 1.5% (w/w) of the 11RX.

(ii) 11RX antigen prepared from bacteria disrupted in the French Pressure cell.

Batch L was prepared essentially as above (i) except for the following variations:-

Washed 11RX from an overnight culture were suspended in approx. one hundredth the original culture volume of buffer. The buffer used was the same except that it was 50mM in Tris not 10mM as used previously. The cells were broken at 4°C in a French Pressure cell (Aminco, Silver Spring, Maryland,U.S.A.)

at 12,000 p.s.i. The high speed centrifugation was 78,000 x g for 2 hours.

Another preparation made in the same way was termed French Press supernatant + pellet (F.P. SN + pellet). The yield was 12.5% (w/w) of the 11RX.

(iii) Other 11RX antigen preparations

(a) Soluble or particulate antigens

During the 37°C incubation step mentioned above in (i), a flocculent precipitate formed, resulting in the presence of both soluble and particulate material in the dialysis sac and thus in the antigen preparations.

In some cases the soluble and particulate matter was separated by centrifugation (78,000 x g for 1 hour) prior to the further dialysis steps. The pellet was washed and resuspended in buffer. Supernatant and pellet material was then extensively dialysed and lyophilised as described previously. Such preparations are termed F.P. SN, U.S. SN, or F.P. pellet in the text.

(b) 11RX antigen prepared using phase separation

Nucleases were not used at any stage during these extractions.

Washed 11RX from overnight or log phase cultures were suspended in 50mM Tris-HCl pH 8.5 (without 2-Me or $MgCl_2$) and broken in the French pressure cell as described previously. The high-speed spin supernatant was incubated for 2 hours at 37°C in order to obtain soluble and particulate material. The particulate material was discarded. The supernatant was then

treated essentially as described by Clark, Peden, and Symons (1974) with some modifications.

To every 28ml of supernatant, a solution containing the following ingredients was added slowly:- 53ml of 50mM Tris-HCl pH 8.5 containing 100mM NH_4Cl and 27g of NaCl, and 9.3ml of 20% (w/w) Dextran 500 (Pharmacia) in water and 26.0ml of 30% (w/w) polyethylene glycol 6000 (PEG; Union Carbide) in water. This procedure was carried out at 4°C with stirring, and the mixture was stirred for a total of 2 hours. The suspension was then centrifuged at 3,000 x g for 10' and the top PEG phase was then recovered. The bottom dextran phase was discarded. NaCl was removed by dialysing the PEG phase overnight at 4°C against 5 changes of Tris-HCl buffer pH 7.8 (different molarities were used, from 10mM to 100mM). The contents of the dialysis sac were centrifuged (8,000 x g for 20') in order to remove a precipitate which had formed.

PEG was removed by virtue of the fact that it did not bind to DEAE-cellulose (DE-52; Whatman) whereas the protein antigen did under the conditions used. 50-100ml of PEG phase was applied to columns of 80-100cc in volume and the unbound material was eluted overnight using the appropriate buffer.

Protein antigen material formed a dark band near the top of the column and was eluted with buffer containing 0.5M NaCl. In some cases the eluate contained a small amount of insoluble material, and this was removed by centrifugation. The eluate was then dialysed

against buffer to remove NaCl and was stored at -15°C at protein concentrations of 1-8mg per ml. In the text, these extracts are refered to as DE-52: PSE (DE-52: Phase separated extract).

The yield of DE-52: PSE from log phase cultures was an average 3% (protein/w) of the 11RX. For overnight cultures this figure was 4%. Dry weights were not done for DE-52: PSE. These figures are the means from 4 different batches of DE-52: PSE from log phase cultures, or 3 batches from overnight cultures.

(iv) Cell envelope components

(a) Triton X-100 extraction

Washed bacteria (11RX or P400) from log phase cultures were disrupted in the French Pressure cell in 10mM HEPES pH 7.4. Large debris was removed by low speed centrifugation (8,000 x g for 20') and the cell envelope pellet was collected by high speed centrifugation (78,000 x g for 1 hour).

Cell envelopes were washed in 10mM HEPES pH 7.4 containing 2mM $MgCl_2$. Extraction was then carried out according to Schnaitman (1971a). The envelopes were resuspended to a protein concentration of approx. 10mg/ml in 10mM HEPES buffer pH 7.4 containing 2% (v/v) Triton X-100, and then incubated for 20' at room temperature. The mixture was then centrifuged (78,000 x g for 1 hour, 4°C) and the extraction was repeated on the pellet. The pellet was washed twice with distilled water and then resuspended in distilled water and stored in aliquots at -15°C. Pellet material is refered to in the text as Triton-insoluble wall (TIW).

The supernatant material containing Triton X-100 was freed of the detergent by incubation overnight at -15°C with 2 volumes of cold 95% ethanol. The ethanol precipitate was collected by centrifugation (10,000 x g for 20') and washed twice with 70% ethanol. The precipitate was resuspended in distilled water and stored in aliquots at -15°C. This material is refered to in the text as Triton-soluble envelope (TSE).

(b) Sodium dodecyl sulphate extraction

This procedure was essentially that of Braun and Rehn (1969). Washed 11RX from a log phase culture were suspended in distilled water and disrupted in the French Pressure cell. After breakage 0.1M EDTA pH 7.4 was added to the mixture to a final concentration of 0.01M. Both low speed and high speed pellets (see (a)) were collected, combined, washed in distilled water and resuspended in distilled water.

The cell pellets from 45 litres of culture (22g dry weight of bacteria) were suspended in a volume of 90mls and this suspension was added dropwise with stirring to 600mls of boiling 4% sodium dodecyl sulphate (SDS). The mixture was stirred for another 2 hours while it cooled and then kept overnight at room termperature. Insoluble material was then collected by centrifugation (78,000 x g for 20', room temperature), washed 3 times with distilled water and then taken up in distilled water and stored at -15°C. This material is refered to in the text as SDS cell wall extract.

(c) 11RX ET

11RX ET was made using the phenol/water method of Westphal, Lüderitz, and Bister (1952) or the phenol/chloroform/petroleum ether technique of Galanos, Lüderitz, and Westphal (1969).

Salmonella typhimurium C5 ET was kindly provided by Dr. B. Reynolds.

(v) Note: Prior to injection into mice, all solutions containing particulate matter were exposed briefly to ultrasound.

Lipid extraction

To each 1ml of sample 5ml of MeOH was added and this mixture was then incubated for 10' at room temperature. 5ml of $CHCl_3$ was then added, and there was a further 10' incubation. The mixture was then centrifuged (3,000 x g for 10', 4°C) and the supernatant was kept. The extraction was repeated on the pellet (using 2ml of MeOH and 5ml of $CHCl_3$) and the supernatants were pooled.

To the supernatants, 4ml of 0.1M KCl was added with mixing, then the mixture was left to stand at 4°C for 15' after which it was cleared using hot tap water. The top (MeOH) phase was discarded. The lower phase was then washed with 5ml MeOH and 4ml 0.1M KCl using the above procedure.

The lower phase was left to evaporate in a Pyrex test tube in a water bath (45°C) in a fume-cupboard. The phosphorus content of the residue was then estimated as later described.

Analysis of 11RX extracts

(i) Protein estimation

Three methods were employed for estimating the protein content of extracts.

(a) The assay method used for most of the samples in this project was that of Schacterle and Pollack (1973) which is a rapid version of the Lowry method. It is refered to in the text as the Lowry method.

(b) Before and after protease treatment of Batch L antigen, (described in the text) a micro Biuret method was used for protein estimation. The reagents for this method are as follows:-

1. 3% (w/v) sodium hydroxide
2. 17.3g sodium citrate
   10.0g sodium carbonate
   1.73g cupric sulphate

These were dissolved separately in distilled water, with heating, added together, and made up to 100ml in a volumetric flask. The solution was kept in a dark bottle.

The Biuret reagent was prepared by mixing 4ml of 1 with 0.2ml of 2. To each 0.5ml of sample, 0.5ml of the reagent was added with mixing, and the mixture was left to incubate for 15' at room temperature. Optical density (O.D.) was then read at 330nm against an appropriate blank.

In both assays (a) and (b), bovine serum albumin (BSA) was used as the standard.

(c) O.D. measurements were used to estimate the protein content of PEG phase material, as PEG interfered with the

other assays. O.D. was also used to determine the protein content of DE-52; PSEs. mg/ml of protein was approximated using the equation 1.45 A_{280nm} - 0.74 A_{260nm} for a 1cm light path (Williams and Chase, 1971).

(ii) Nucleic Acid estimation

The amounts of nucleic acid in extracts were not quantified. Samples containing only soluble material were considered to be grossly contaminated with nucleic acid if their A_{280nm}/A_{260nm} ratios were 0.5 or less. If this ratio approached 2.0 (i.e., 1.5-1.8), and there was no O.D. reading at 320-340nm, then the material was considered to be largely protein (Williams and Chase, 1971). The nucleic acid content of particulate extracts was not determined.

(iii) Carbohydrate estimation

The carbohydrate in extracts was estimated using the phenol-sulphuric acid method. To each 0.5ml of sample, 0.5ml of 5% phenol was added with mixing. To this mixture 2.5ml of conc. H_2SO_4 was added. Glucose was used as the standard, and the O.D. of samples was read at 490nm.

(iv) 2-Keto-3-deoxyoctonate (KDO) estimation

Samples (0.2ml) were first added to 0.25ml 0.2M H_2SO_4 and boiled for 8' to release any ET bound KDO. After cooling, KDO was estimated using the thiobarbituric acid method of Weissbach and Hurwitz (1959). 11RX ET was the standard. In the text the values % ET (w/w) were calculated from KDO measurements assuming that any KDO in extracts was derived from whole ET molecules, not fragments.

(v) Phosphorus estimation

Organic phosphate in extracts was ashed using the method of Ames and Dubin (1960). To each 0.10ml of sample (various different amounts were used), in a Pyrex test tube, 3 drops of 10% Mg $(NO_3)_2.6H_2O$ in 95% ethanol were added. The material was ashed by shaking the tube over a Bunsen flame until the fumes disappeared. Usually, samples were ashed twice to ensure complete conversion to inorganic phosphate. After cooling, 0.3ml of 0.5N HCl was added to each tube, the tube was then capped and heated in a boiling water bath for 15' to hydrolyse to phosphate any pyro-phosphate formed during ashing.

The phosphorus content of the ashed and hydrolysed samples was then estimated using the method described by Fiske and Subbarow (1925). KH_2PO_4 was used as a standard, and ATP was used as a further check. Both these materials were put through the ashing procedure along with test samples. It was assumed that 1µm of PO_4^{-3} contained 30.9µg of P and the results in the text are expressed as % P (w/w).

Protease assay

The method used to determine if 11RX extracts contained proteases was essentially that described by Rinderknecht, Geokos, Silverman, et al., (1968).

The substrate, Remazo brilliant blue covalently linked to Hide powder, was kindly provided by R. Douglas-Broers. 10mg of this powder was weighed into each of a number of test tubes. The test samples in 50mM Tris-HCl pH 7.8 were then added (volume 3ml). The tubes were incubated at 37°C for 60' with

occasional shaking. The reaction was stopped by placing the tubes in ice. The tubes were then centrifuged (3,000 x g for 10') and the amount of blue dye released was determined by reading the OD of the supernatants at 595nm. For an assay mix volume of 3ml, ΔOD_{595} (sample-blank) = 2.9 was taken to represent 1 arbitrary unit of protease activity. Pronase was used as a positive control.

Fractionation

The gels used for column chromatography were DE-52 and Sephadex G200 (Pharmacia). Gels were handled as recommended by the manufacturers, i.e., in terms of swelling procedures, flow rates, and sample volumes. The chromatography is described in the text.

For ultrafiltration, an Amicon PA-10 pressure ultrafiltration cell was used (Amicon Corp., Mass., U.S.A.) together with either PM-10 (10,000 daltons molecular weight exclusion) or PM-30 (30,000 daltons molecular weight exclusion) membranes.

Polyacrylamide gel electrophoresis

(i) Non-denaturing; without SDS

The method used was the disc gel system using Tris-Tricine buffers described by MacGregor, Schnaitman, Normansell et al., (1974).

Gels were loaded with 50µl of sample at a protein concentration of 1mg/ml. Gels were stained for protein with Coomassie brilliant blue (Swank and Munkres, 1971) and densitometer tracings were obtained with a Quick Scan Jnr. gel scanner (Helena Labs. Corp., Texas, U.S.A.)

(ii) Denaturing; with SDS

In this case both sample and running buffers contained SDS.

Slab gels

Samples for slab gel electrophoresis were prepared, electrophoresed, and stained for protein according to Lugtenberg, Meijers, Peters et al., (1975).

Sample preparations were heated for 5' at 100°C immediately prior to loading onto the gels. Each gel was loaded with 12.5µl of sample containing protein at a concentration of 1mg/ml.

The stained, dried slab gels were photographed.

RESULTS

1. Preamble - The anti-tumour effects of Salmonella enteriditis 11RX

Hardy and Kotlarski (1971) demonstrated that immunization of mice with viable, rough, avirulent 11RX protected the mice from subsequent challenge with EAT. Mice could eliminate an i.p. challenge of EAT most effectively if they had been recently infected with 11RX by the i.p. route. Resistance induced by i.v. immunization was less effective (Ashley *et al.*, 1974). Mice which had been immunized i.v. 50 days previously with 11RX exhibited no resistance to i.p. tumour challenge, but for approx. 20% of mice immunized by the i.p. route resistance lasted for 90-100 days (Ashley *et al.*, 1974).

Ashley *et al.*, (1974) showed that the tumour resistance of long-term i.p. or i.v. immunized mice could be recalled by the i.p. injection of a protein-rich extract of 11RX at the time of, or a few days prior to, tumour challenge. The extract, termed 11RX antigen, did not protect normal mice against challenge with EAT. 11RX antigen elicited a DTH response in 11RX immunized mice (Davies, 1975).

If normal mice were injected i.p. with a mixture of spleen cells from long-term immunized mice and 11RX antigen, they became resistant to challenge with EAT. Resistance was shown to be associated with T lymphocytes in the donor spleens and radiation-sensitive cells in the recipient (Ashley, Kotlarski, and Hardy, 1977). Glass adherent cells which were cytotoxic for EAT cells *in vitro* could be harvested from the peritoneal cavities of recently infected or recalled mice (Ashley, 1976).

Tumour specific immunity is not involved in this model, and it is unlikely that there is an antigenic cross reaction between 11RX and EAT (Ashley, 1976; Tindle, Neoh, Ashley et al., 1976).

The aims of the project which is the subject of this thesis were to further analyse and purify 11RX antigen preparations, and also to look into whether or not the recall phenomenon was unique to either 11RX or EAT.

2. Characterization of 11RX antigen preparations

The 11RX antigen preparations used by Ashley and co-workers were made by disrupting overnight cultures of washed, live 11RX, removing large debris by low speed centrifugation, and then taking the supernatant of a 100,000 x g centrifugation. Nuclease treatment and dialysis were used to obtain the protein-rich 11RX antigen preparations. When this project was begun, it was known that such extracts contained approx. 95% protein and very little carbohydrate or nucleic acid.

Other workers classify 34,000-150,000 x g supernatants from disrupted micro-organisms as cytoplasmic extracts. For example, such extracts have been prepared from S.typhimurium, M.tuberculosis, Histoplasma capsulatum, and Mycoplasma arginini (Ames, 1974; Kanai and Youmans, 1960; Domer, 1976; Alexander and Kenny, 1977).

However, it has been known for some years that most methods of cell disruption lead to some solubilization of cell membrane components via mechanical breakdown, or because lytic enzymes act on them, despite the use of low temperatures (Rogers and Perkins, 1968). Thus it was possible that 11RX antigen preparations contained all of the constituents of intact cells, albeit in different proportions.

Certain bacterial components were not likely to be the active moiety (ies) of 11RX antigen. These were ET, glycolipid, polysaccharide, and RNA. These materials have anti-tumour activity when administered to animal hosts which have not been actively immunized with bacteria. In addition, these extracts usually elicit immediate-type hypersensitivity

reactions. Recall of tumour resistance and DTH responses are elicited with protein or polypeptide antigens in immunized hosts (Parr, 1974; Collins, 1974). At the time of writing there was only one report which claimed that pure lipid (Lipid A from Salmonella ET) elicited a DTH reaction (Marks, Jackson, and Cooper, 1975). The criterion used by these workers was inhibition of macrophage migration in vitro. At the time of writing, these results have not been confirmed using in vivo experiments.

Thus, in terms of the majority of data available, it was considered likely at the commencement of this project that protein was responsible for the recall and DTH responses elicited by 11RX antigens.

The following sections describe the composition and some properties of 11RX antigen preparations.

2.1 Composition of 11RX antigen preparations

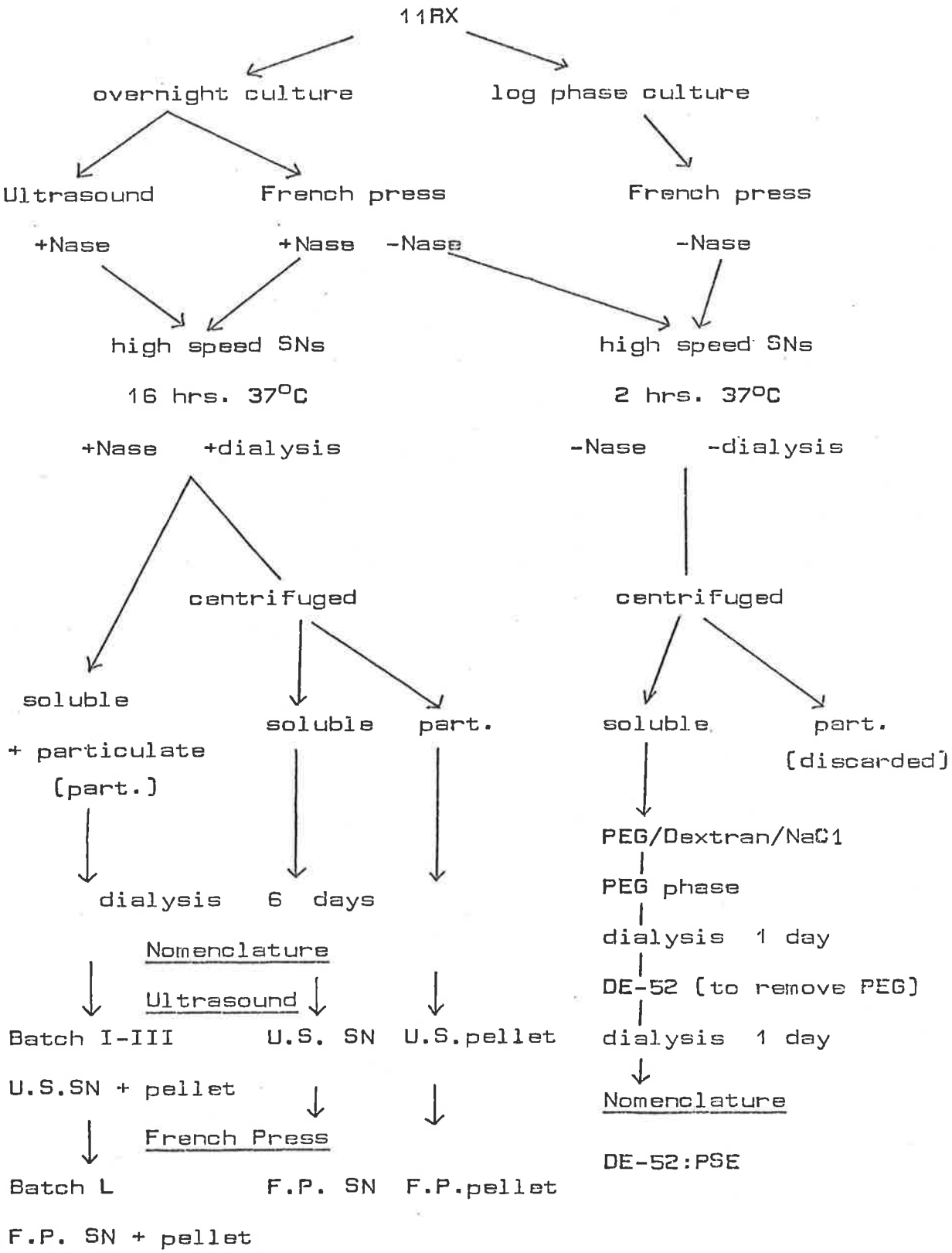
A number of different batches of 11RX antigen were used in this project. They were all found to be similar to the preparation used by Ashley et al., (1974) in their anti-tumour effects and in their ability to elicit DTH reactions in 11RX immunized mice. All the batches were derived from 78,000-100,000 x g supernatants of disrupted 11RX, but there were some differences in extraction procedures. The preparative techniques are described in detail in Materials and Methods, given in outline below, and shown diagramatically in Fig. 1.

A noticeable property of the preparation used by Ashley et al., (1974) was that it contained soluble and particulate material. The latter component formed when high speed

Fig. 1

Flow diagram of the preparation of

11RX antigens


11RX
overnight culture
log phase culture
Ultrasound
French press
French press
+Nase
+Nase
-Nase
-Nase
high speed SNs
high speed SNs
16 hrs. 37°C
2 hrs. 37°C
+Nase
+dialysis
-Nase
-dialysis
centrifuged
centrifuged
soluble
+ particulate
(part.)
soluble
part.
soluble
part.
(discarded)
dialysis
6 days
PEG/Dextran/NaCl
PEG phase
dialysis 1 day
DE-52 (to remove PEG)
dialysis 1 day
Nomenclature
Ultrasound
Batch I-III
U.S. SN
U.S.pellet
U.S.SN + pellet
Nomenclature
DE-52:PSE
French Press
Batch L
F.P. SN
F.P.pellet
F.P. SN + pellet

supernatants were incubated at 37°C. It was of interest to determine if the soluble and particulate matter was of different composition, and whether or not both contained recall antigen(s). The two components were separated by centrifugation, either during extraction, or after lyophilization. Soluble antigen extracts are refered to as SN preparations:- U.S. SN or F.P. SN depending on whether or not ultrasound or the French Pressure cell was used to disrupt the 11RX. Particulate antigen extracts are termed pellet preparations e.g., F.P. pellet.

In some cases, extracts were not treated with nucleases at any stage of preparation. Instead, phase separation was employed to remove nucleic acids. These antigens are termed DE-52: PSEs (DE-52: phase separated extracts) and were derived from soluble material only. All the other 11RX antigens originated from overnight cultures of 11RX, but in the case of some DE-52: PSEs log phase cultures were used. The composition of DE-52: PSEs is considered in a separate section to that describing other 11RX antigens (section 2.1.6).

It was of interest to determine the differences in composition, e.g., protein, nucleic acid, and carbohydrate content, of high speed supernatants and 11RX antigen preparations. The main differences between the former and the latter were the dialysis and 37°C incubation steps. It was pertinent to determine the necessity for 16 hours of 37°C incubation, nuclease treatment, and dialysis so this is why data on high speed SNs is given in the text.

2.1.1 Protein content of high speed SNs and 11RX antigen preparations

Protein content of extracts was estimated using the Lowry method. This technique was in general prefered to O.D. measurements because samples often contained particulate matter. In addition, high speed SNs contained a lot of nucleic acid or nucleic acid fragments, and this meant that the use of O.D. for protein estimation would be subject to inaccuracies (Herbert, Phipps, and Strange, 1971).

A comparison of protein content to dry weight for high speed SNs was not done in this project. Maaløe and Kjeldgaard (1966) reported that protein, estimated using the Lowry method, accounted for 70-85% of the dry weight of Salmonella under a wide range of growth conditions. As cell envelope protein constitutes a relatively small proportion of cell dry weight (5-10%), this means that the bulk of a high speed SN will consist of protein. The remaining material will be DNA (3% of cell dry weight) and RNA (10-30% of cell dry weight, depending on growth conditions).

Quantitative comparisons of the protein content of high speed SNs and the resultant 11RX antigens showed that a high proportion of protein was lost during the dialysis procedure. The figure was 36% (±5% S.Error, average of 5 extracts). This was the case with either F.P. or U.S. derived SNs, SNs incubated for 2 hours or 16 hours at 37°C, and nuclease treated or non-nuclease treated SNs. It seemed likely from this result that the large amount of dialysable protein was present because endogenous proteases had degraded native proteins

during the 37°C incubation. This possibility was later explored [section 2.1.5].

11RX antigen preparations [e.g., Batch II, Batch L, F.P. SN] contained 90-100% protein as estimated using the Lowry method in comparison to the dry weight of the preparations. The variations in protein content between batches did not represent absolute differences, but were due to the limitations of the assay method. The percentages of constituents other than protein were estimated using the appropriate specific assays.

a. Proportions of soluble and particulate protein in 11RX antigens

In the case of SN + pellet antigens [e.g., Batch I-III, Batch L, F.P. SN + pellet], the proportion of particulate protein was highest in those extracts which were made using 11RX disrupted in the French Press.

Two different 11RX antigen preparations made using the French Press contained 34% and 44% [Batch L] of total protein in particulate form. Two extracts made using ultrasound contained 12% and 14% [Batch II] of total protein in particulate form.

b. Polyacrylamide gel electrophoresis [PAGE] of 11RX antigens

(i) Non-denaturing; without SDS

Samples of Batch II and F.P. SN were electrophoresed under non-denaturing conditions and densitometer scans of gels stained for protein are shown in Fig. 2. Use of this technique revealed that 11RX antigens contained a large number of different proteins which were presumably resolved on the basis of both size and charge differences. [e.g., positions of BSA and hemoglobin].

Fig. 2

Disc PAGE, without SDS, of 11RX antigens

1. Batch II

2. F.P. SN.

3. protein standards *

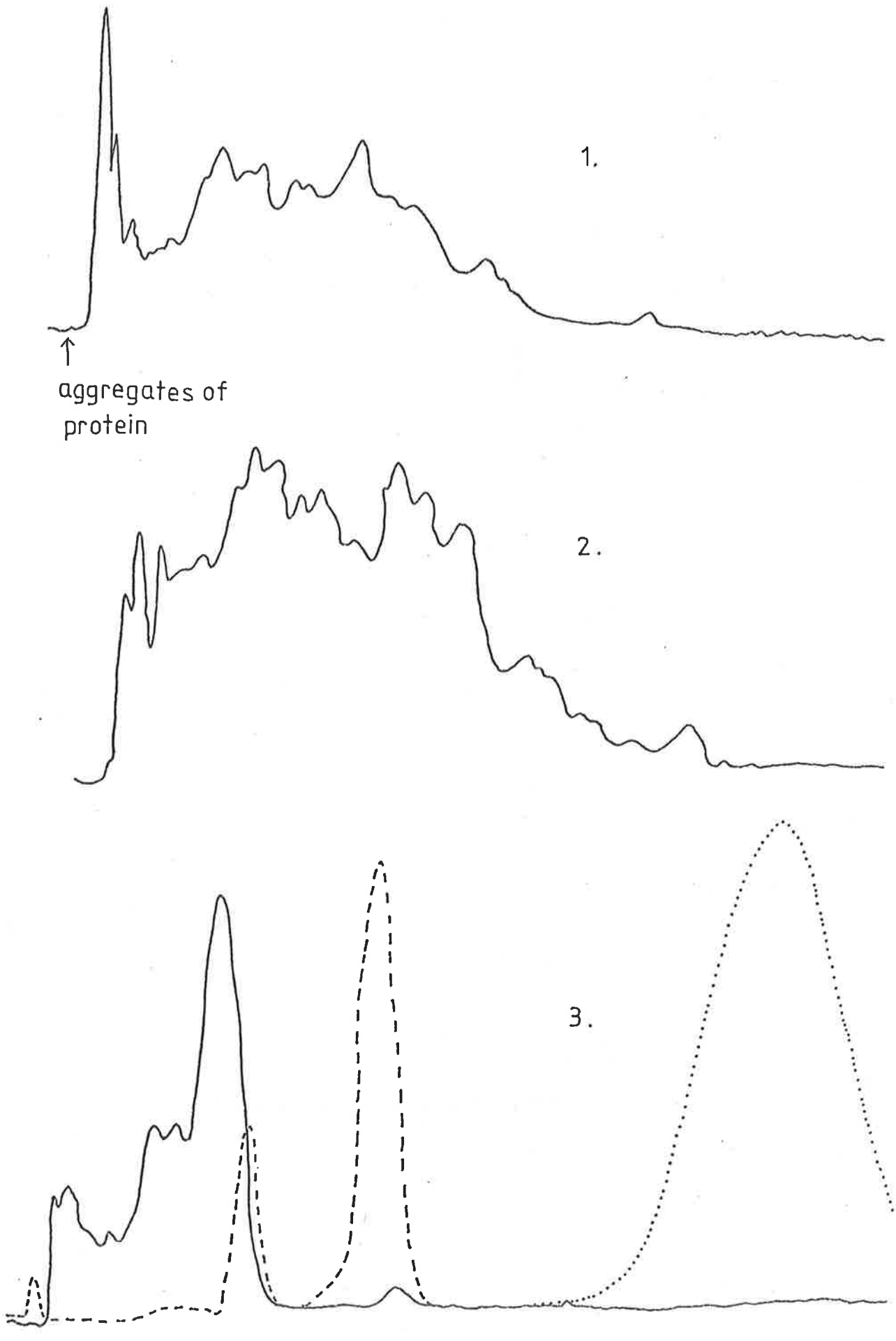
Direction of migration: left to right

* Transferrin (molecular weight 77,000 daltons)

BSA (molecular weight of monomer 69,000 daltons)

Hemoglobin (molecular weight 63,000 daltons)

The protein standards were electrophoresed on separate disc gels concurrently with 1. and 2.


1.
aggregates of protein
2.
3.

The protein profiles of Batch II and F.P. SN did not correspond exactly, showing that there were differences in protein composition between these two extracts. The particulate protein in Batch II antigen was not resolved using this technique.

(ii) Denaturing; with SDS

The boiling of proteins in SDS and 2-Mercapto-ethanol extensively disrupts hydrogen, hydrophobic, and disulphide linkages. Most insoluble proteins are solubilized and dissociated into their individual polypeptide chains. All polypeptides behave as anions as a result of complex formation with SDS, and so when electrophoresed in the presence of SDS will be resolved largely according to molecular weight differences (Shapiro, Viñuela, and Maizel, 1967). SDS PAGE was used in this project so that the protein profiles of soluble and particulate 11RX antigens could be compared.

To be able to directly compare different proteins with PAGE it is preferable to run the samples next to each other. This was done using a long thin slab of polyacrylamide, on which up to 22 samples were run, giving patterns of perfect correspondence.

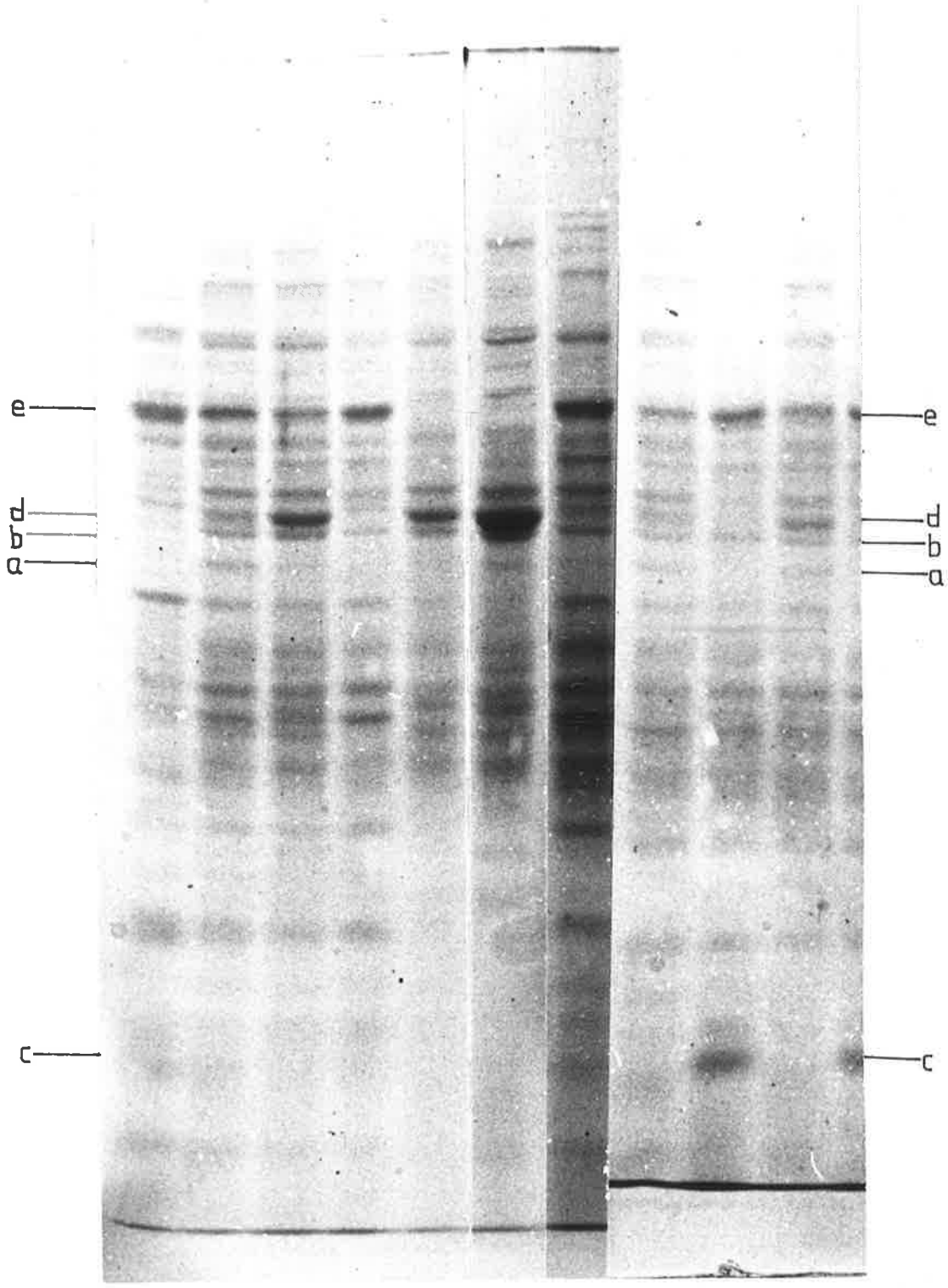
When various batches of 11RX antigen were electrophoresed using this method, it was apparent that their protein compositions were similar (Fig. 3). However, differences in the proportions of individual protein bands were observed. For example, in the case of three SN + pellet antigens prepared using ultrasound, Batch I No. 1), Batch II (No. 2 & 8), and U.S. SN + pellet (No.9);

Fig. 3

Slab PAGE, with SDS, of 11RX antigens

1. Batch I
2. Batch II
3. Batch L
4. Batch L SN
5. Batch L pellet
6. F.P. pellet
7. F.P. SN
8. Batch II
9. U.S. SN + pellet
10. F.P. SN + pellet

Direction of migration: top to bottom.


e
d
b
a
c
e
d
b
a
c
1. 2. 3. 4. 5. 6. 7. 8. 9. 10.

Batch I is missing band a and b, U.S. SN + pellet is missing band a, but Batch II has both bands a and b. U.S. SN + pellet has a prominent band c which is not evident in either of the other two extracts. Of the two SN + pellet antigens made using the French Press, Batch L (No. 3) and F.P. SN + pellet (No. 10), most bands appeared to be shared. When comparing ultrasound and French Press extracts, Batch L and F.P. SN + pellet are similar in composition to Batch II rather than to Batch I or U.S. SN + pellet. However, Batch L contains a lot more band d protein than Batch II. In short, different batches of antigens did not have the same protein composition.

In addition, soluble and particulate antigens had different protein compositions. Batch L SN (No. 4) and F.P. SN (No. 7) can be compared with Batch L pellet (No. 5) and F.P. pellet (No. 6) respectively. Clearly, band d is prominent in the pellet antigens, but band e is absent. On the other hand the SN antigens contain little band d, but a lot of band e. Similar comparisons were not made for U.S. preparations. This result indicated that the particulate protein mixture did not consist only of aggregates of the soluble proteins. If this had been the case, the protein profiles of soluble and particulate antigens would have been the same. The origin of the particulate protein was later investigated (section 2.1.7).

2.1.2 Nucleic acid content of high speed SNs and 11RX antigen preparations

As is evident from Fig. 1, during the extraction of most of the 11RX antigens used in this project, nucleases were added (see also Materials and Methods). The exception was in the case of phase separated extracts.

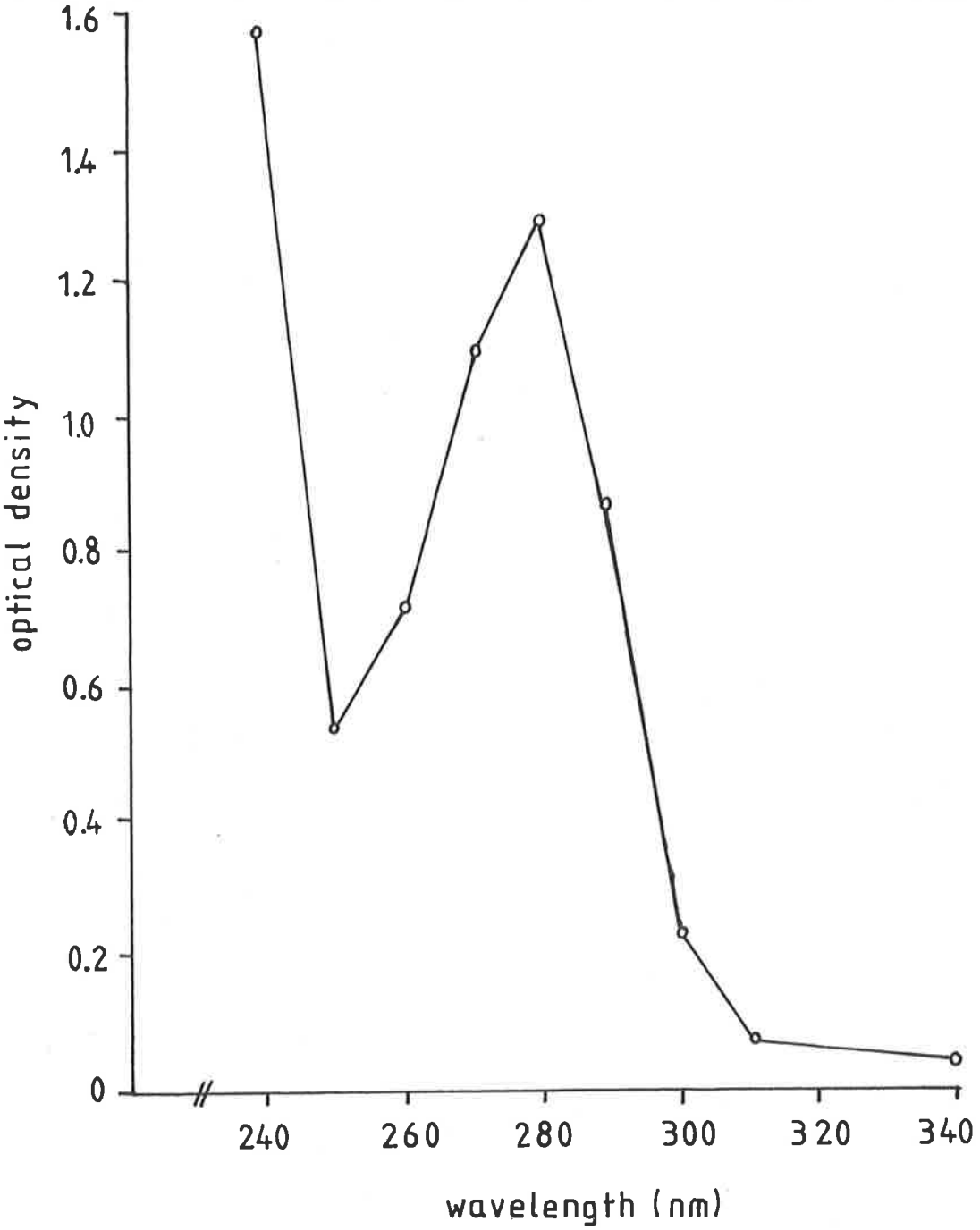
As in the case of protein determinations, the nucleic acid content of extracts containing particulate matter could not be estimated using O.D. measurements. High speed SNs contained a lot of nucleic acid derived material as was demonstrated by their A_{280nm}/A_{260nm} ratios of 0.5. For soluble 11RX antigen preparations this ratio approached 2.0, which is characteristic of proteins. A 240-340nm scan of an F.P. SN preparation (ratio 1.8) is shown in Fig. 4. Nuclease treatment combined with dialysis was obviously an effective way of obtaining nucleic acid-free preparations.

The particulate material in 11RX antigens was not assayed for its DNA and RNA content; it was found that most of the nucleic acid fragments produced during cell rupture were quite small, since dialysis of high speed SNs which had not been nuclease treated led to the loss of 260nm absorbing material. The A_{280nm}/A_{260nm} ratios of two such dialysed SNs were 0.93 and 1.2. However, as ratios of 1.5-1.8 were demonstrable for the soluble 11RX antigens used routinely (i.e. nuclease treated or phase separated), some form of treatment other than dialysis was thought to be desirable to ensure that all nucleic acid derived material was removed.

According to data presented by Kwapinski (1972) there is the following relationship between 280/260 ratio and % nucleic acid in a sample:-

Fig. 4

U.V. optical density scan of a solution of F.P. SN,11RX antigen


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
optical density
240
260
280
300
320
340
wavelength (nm)

Ratio	0.92	%	4.3
	1.2		1.5
	1.5		0.5
	1.8		0

2.1.3 Carbohydrate and ET content of high speed SNs and 11RX antigen preparations

The phenol-sulphuric acid method was used for estimating the carbohydrate content of extracts. This method detects ribose, deoxyribose, and different types of hexose, and so approximates to a universal reagent (Herbert et al., 1971). This technique was used because one aim of this project was to determine the chemical nature of the moiety(ies) of 11RX antigen involved in recall of tumour resistance; i.e., whether it was carbohydrate or protein. Thus, it was appropriate to use a method which was not selective in the type of sugar it could detect.

2-keto-3-deoxyoctonate (KDO) is a component unique to the ETs of gram-negative bacteria. The content of ET in extracts was estimated by assaying for KDO, using 11RX ET as the standard.

As in the case of protein (section 2.1.1) the results indicated that a large amount of carbohydrate and ET was present in high speed SNs which was not present in 11RX antigen preparations. For example, 4 different high speed SNs contained 0.810, 0.120, 0.084, and 0.720 mg/ml carbohydrate. After dialysis, these figures were 0.370, 0.026, 0.010, and 0.120 mg/ml respectively. Three high speed SNs contained 0.380, 0.270, and >0.800 mg/ml ET. After dialysis these figures were <0.33, <0.053, and 0.100 mg/ml respectively. The

same specifications applied to these extracts as to those in which protein content was compared (section 2.1.1). These results showed that a high proportion of carbohydrate and ET in high speed SNs was dialysable. This was confirmed when these extracts were filtered using a 10,000 dalton molecular weight exclusion membrane.

More carbohydrate and ET (approx. 80%) in high speed SNs was of small molecular weight than protein (36%). As the high speed SNs consisted mainly of protein in any case, this meant that 11RX antigen preparations were enriched in protein. Clearly, dialysis was an essential step in the extraction procedure. The proportions of carbohydrate and ET in some 11RX antigens are shown in Table 2. The figures are expressed as a percentage of the dry weight of the sample, except for Nos. 12 and 13 where they were calculated in terms of protein estimated using the Lowry method. The $<$ figures represent the limits of detection of the assays. If the ET remaining in 11RX antigens is in the form of low molecular weight fragments, then the % ET figures are over-estimates.

The data showed that carbohydrate and ET content of preparations varied from batch to batch irrespective of the method used to rupture the organisms. No significant differences in the carbohydrate and ET content of the soluble (i.e. F.P. SN, U.S. SN) compared to particulate (i.e. F.P. pellet) antigen preparations were observed.

The structure and origin of the ET and carbohydrate fragments in crude 11RX extracts or in 11RX antigen preparations was not determined. The KDO detected in the samples may have been associated with KDO-Lipid A complexes, or KDO

Table 2

Carbohydrate and ET content

of 11RX antigen preparations.

Preparation	[a]% carbohydrate	[a]% ET
1. Batch I	1.4	[b]N.D.
2. Batch II	< 1.3	N.D.
3. Batch III	4	< 0.3
4. Batch L	2	N.D.
5. U.S. SN	4	< 4
6. U.S. SN + pellet	3	< 3
7. F.P. SN	2.1	0.3
8. F.P. pellet	0.5	< 4
9. F.P. SN + pellet	1.7	< 3
10. F.P. SN	0.2	0.5
11. F.P. SN	0.1	0.9
12. F.P. pellet	0.1	0.2
13. F.P. pellet	N.D.	< 0.4

[a] details given in text

[b] Not done

alone rather than with any sugar residues. The presence or absence of Lipid A was not ascertained. There are two possibilities for the origin of the fragments. They could have been split off the fully synthesised ET found in the cell wall, or may be precursor molecules detached from the cytoplasmic membrane or ribosomes.

An alternative method for measuring ET content of extracts is the haemagglutination inhibition technique which indicated that 11RX antigen preparations contain only low levels of ET, comparable to those detected using assays for KDO (I. Kotlarski, personal communication).

2.1.4 Phosphorus content of 11RX antigens

It is generally recognised that the lipid constituents of gram-negative bacteria are localised in the inner and outer membranes of the cell envelope (Salton, 1967). Thus one method which was used to determine if 11RX antigen preparations contained any membrane fragments was to estimate lipid associated phosphorus. This was done by extracting samples with $CHCl_3$-MeOH in order to obtain any lipid free of other material, and then assaying this for its total phosphorus content.

In some cases, the total phosphorus content of samples was estimated without prior $CHCl_3$-MeOH extraction. Any phosphorus detected in these samples could be associated with nucleic acid, lipid, or both.

Samples of 11RX ET, 11RX envelope and 11RX Triton-soluble envelope were assayed for phosphorus content as positive controls because they were known to contain lipid. The lipid from one sample each of ET and envelope was extracted prior

to assay. This was probably not necessary as Osborn, Gander, Parisi et al., (1972) showed that the envelope from S.typhimurium contained only 1% of the total cellular DNA or RNA, even if nucleases were not used in the extraction. The values of % P obtained for 11RX envelope fractions (shown in Table 3) were comparable to those obtained for E.coli and S.typhimurium by other workers (Koplow and Goldfine, 1974; Osborn et al., 1972; Schnaitman, 1970).

When compared with the above preparations, 11RX antigens contained very little phosphorus (Table 3; $<$ represents limit of detection of the assay). Similar values were obtained for unextracted and $CHCl_3$-MeOH extracted samples. Any phosphorus in 11RX antigens was probably associated with lipid, not nucleic acid (section 2.2.2). For $CHCl_3$-MeOH extracted samples, phospholipid content can be roughly calculated assuming a molecular weight of 700 for phospholipid, and assuming 1 mole of phosphorus per mole of phospholipid. Using this approximation, 11RX antigens contained $<$ 4.6% phospholipid by weight.

2.1.5 Protease activity in 11RX extracts

As noted in section 2.1.1, high speed SNs contained a large amount of dialysable protein. It was important to determine if this protein was an artifact produced by proteolytic degredation of native proteins which had occurred during extraction. The presence of proteases in 11RX antigens could only result in protein degredation over a period of time, and complications during fractionation studies.

The assay for protease activity involved measuring the amount of dye released from a dye-protein substrate after

Table 3

Phosphorus content of 11RX extracts.

Extract	% Phosphorus by weight
1. 11RX ET	3.0[L] - 4.0
2. [a]11RX envelope	>1.6, 1.6, >0.6[L]
3. [a]11RX Triton-soluble envelope	1 - 2%
4. F.P. SN	<0.15%[L]
5. F.P. SN + pellet	<0.15%[L]
6. F.P. pellet	<0.15
7. Batch L	<0.2

[a] see also section 5.2.

[L] determined on material extracted with chlorform-methanol.

2. 3 different extracts.

incubation at 37°C for 1 hour. Pronase was used as a positive control.

There was no demonstrable protease activity in 1500 µgs of Batch II or F.P. SN 11RX antigens (Table 4; < represents limit of detection of the assay). High speed SNs and cell envelopes were extracted from log phase and overnight cultures of 11RX, and samples were assayed for protease content immediately after extraction. Nucleases were not added during cell disruption. No significant protease activity could be detected in these extracts (Table 4).

The latter result was surprising, as other workers have reported that crude cell-free E.coli extracts contain soluble proteases which hydrolyse casein at 37°C (Chaloupka and Liebster, 1959; Kowit, Nang-Choy, Champe et al., 1976). The Rinderknecht method used in this project is said to be more sensitive than the casein digestion technique as each of 5 proteases assayed by Rinderknecht et al., (1968) could be detected at a concentration of 200 ng per ml. However, it is evident from Table 4 that pronase could not have been detected if ng quantities had been used in this project. The sensitivity of the assay varies markedly depending upon the number of dye molecules per 1000 amino acid residues of the protein (Rinderknecht et al., 1968). Hence had a different batch of substrate been used the limit of detection could have been lowered. However, the data does permit the conclusion that if any proteases were present in 11RX extracts they were there in minute amounts.

Whether or not endogenous proteins were being degraded in 11RX extracts was not determined. Kowit et al., (1976)

Table 4

Estimations of the protease content of 11RX extracts and nucleases.

Sample	*amount in µg	Units of protease activity
1. Pronase	12.5	0.2
	1.1	0.07
Batch II	1500	< 0.03
F.P. SN	1500	< 0.03
2. Pronase	12.0	0.46
a. Log phase 11RX		
9,000 × g SN	540	< 0.03
78,000 × g SN	370	
cell envelope	100	
b. overnight 11RX		
9,000 × g SN	670	< 0.03
78,000 × g SN	530	
cell envelope	200	
3. Pronase	12.0	0.37
RNase	10	< 0.03
	1570	
DNase	10	< 0.03
	360	0.35

* by weight, except for 2.a. and b. which are protein based on measurements using the Lowry method.

estimated that the level of proteolytic activity in E.coli extracts versus casein was 3 to 10 fold higher than autolytic activity. It has been shown that approx. 2-5% of endogenous proteins in E.coli cell-free extracts were degraded per hour at 37°C (Kemshead and Hipkiss, 1974; Chaloupka and Liebster, 1959). The latter workers showed that the reaction was linear for 8 hours of incubation. In view of this report, if the protein lost during dialysis of 11RX high speed SNs represented 37°C degredation products, one would have expected to find more dialysable protein in extracts incubated for 16 hours at 37°C compared with those incubated for 2 hours at 37°C. This was not the case (section 2.1.1). In addition, it was later found that this protein was present in 11RX high speed SNs which were kept at 4°C (section 4.4). This being the case, it was unlikely that this protein could all be accounted for as proteolytic degredation products. Possibly, the protein is an artifact produced by shear forces during cell disruption. It is unlikely that it is produced by proteolytic degredation which occurs during cell culture. Even in the case of cells in the stationary phase, the in vivo rate of protein degredation in E.coli is only 0.2% per hour (Chaloupka and Liebster, 1959). At present, the origin of the dialysable protein remains undetermined.

Although protease activity could not be found in 11RX extracts, it was in fact detected in a sample of the batch of DNase which was used for most of the extractions described in this text (Table 4). The RNase used was found to be protease free. It was thought advisable to discontinue the use of DNase, so phase separation was chosen to remove DNA and RNA from high speed SNs. It is discussed in the following section.

2.1.6 Phase separation of high speed SNs

Liquid polymer phase partition is a technique devised by Albertsson for the separation of proteins from nucleic acids. It has been used during the purification of enzymes and nucleic acids (Albertsson, 1970). In this project, a polyethylene glycol (PEG)-Dextran 500-NaCl system was utilized. It has been employed by numerous workers as an initial step in the purification of enzymes from micro-organisms (e.g., Okazaki and Kornberg, 1964; see also Materials and Methods).

Both log phase and overnight cultures of 11RX were disrupted in the French Pressure cell and used to make phase separated antigens. It was of interest to determine if antigens made from log phase cultures were different to those prepared from overnight cultures. It has been reported, at least in the case of certain cell envelope proteins, that the protein composition of exponentially growing cells is different to that of stationary phase cells (Schnaitman, 1974a; Braun, 1975).

Prior to phase separation, 11RX high speed SN was incubated for 2 hours at 37°C, and the particulate material was removed by centrifugation. The shortened incubation period allowed sufficient time for any aggregates to form (section 2.1.7), and should have minimized the extent of any proteolysis by endogenous proteases if this was taking place. After the soluble material had been partitioned the PEG phase had an A_{280nm}/A_{260nm} ratio typical of proteins, showing that nucleic acid or nucleic acid fragments had separated into the dextran phase. In addition, most of the ET derived material (as detected using the assay for KDO) was in the dextran phase.

The rest of the steps involved in the preparation of DE-52: Phase separated extracts (DE-52: PSEs) are described in Materials and Methods and shown schematically in Fig. 1.

Using SDS slab PAGE, the protein composition of DE-52: PSEs was compared with that of 11RX antigens which had been prepared using nucleases. Looking at Fig. 5 it can be seen that all antigen preparations have similar protein compositions, although there are some differences in the relative amounts of different protein bands. For example, clearly DE-52: PSE from a log phase culture (No. 5) has a large amount of band f, although F.P. SN (No. 4) has relatively little. However these variations probably represent only the differences between individual antigen batches, as noted previously (section 2.1.1 b (ii)). For instance, DE-52: PSEs Nos. 6 and 3 (overnight and log phase, respectively) have little, if any, band f in contrast to Nos. 2 and 5 (both from log phase cultures). This result was confirmed when gels of Nos. 6 and 5 were rerun (Nos. 8 and 9, respectively).

In conclusion, DE-52: PSEs from log phase or overnight cultures did not differ grossly in protein composition to 11RX antigens prepared using nuclease treatment. In addition, a more dramatic difference between the SDS PAGE protein profiles of 11RX antigens made with nucleases and DE-52: PSEs would have been expected if significantly more proteolytic degredation had occurred in the former case.

The dry weights of DE-52: PSEs were not determined. PEG interfered with protein estimation by the Lowry method, so the protein content of PEG phases and DE-52: PSEs was determined from O.D. measurements. For DE-52: PSEs protein content

Fig. 5

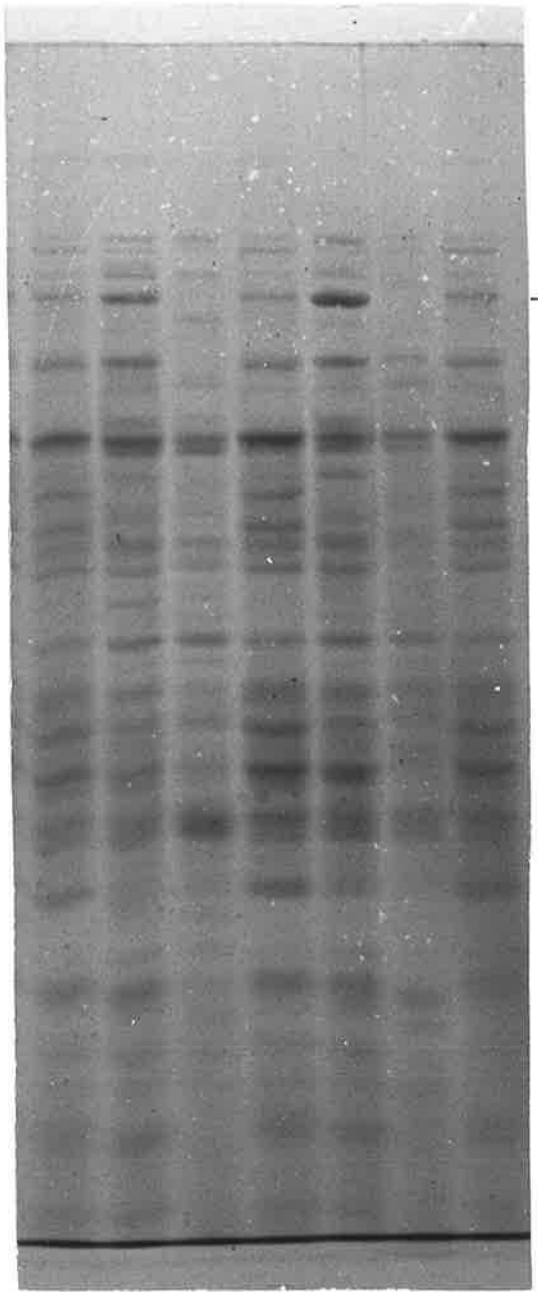
Slab PAGE, with SDS, of 11RX antigens

1. F.P. SN
2. DE-52:PSE (from a log phase culture)
3. DE-52:PSE (from a log phase culture)
4. F.P. SN
5. DE-52:PSE (from a log phase culture)
6. DE-52:PSE (from an overnight culture)
7. F.P. SN
8. No. 6 repeat electrophoresis
9. No. 5 " "

2., 3., 5., and 6., different batches of DE-52:PSE

1., 4., and 7., same batch of F.P. SN

Direction of migration: top to bottom



1. 2. 3. 4. 5. 6. 7.

8. 9.

estimated using O.D. was approx. 70% of that determined using the Lowry method (average of 3 extracts). Four batches of DE-52: PSE contained from 1-3% carbohydrate and < 3% ET; where the %'s are expressed as a proportion of protein content (measured using O.D.).

The ability of DE-52: PSEs to recall tumour resistance was later assessed (section 4.2).

2.1.7 Cytoplasmic membrane proteins in high speed SNs

One obvious explanation for the appearance of particulate material in high speed SNs incubated at 37°C was that it represented the reaggregation of membrane fragments which had been broken off the cell envelope during cell rupture. A report in the literature indicated that this was probable. MacGregor and Schnaitman (1973) noticed that particulate material formed when a 200,000 x g SN of disrupted E.coli was incubated for 2 hours at 32°C. They prepared Triton X-100 soluble material from the particulate matter and also from the cell envelope. Using SDS PAGE they showed that the protein composition of the two samples was identical. Schnaitman (1971a) had shown that Triton X-100 solubilized cytoplasmic membrane proteins, not cell wall proteins, and so this was good evidence that the high speed SN particulate matter contained cytoplasmic membrane proteins (see also section 5.2).

The same procedure was carried out in this project with 11RX high speed SN particulate material and 11RX envelopes. Using slab SDS-PAGE, the Triton X-100 solubilized particulate material was shown to be very similar in protein composition to Triton X-100 solubilized cell envelope (Fig. 6). This indicated that the particulate material did contain some cytoplasmic membrane proteins.

Fig. 6

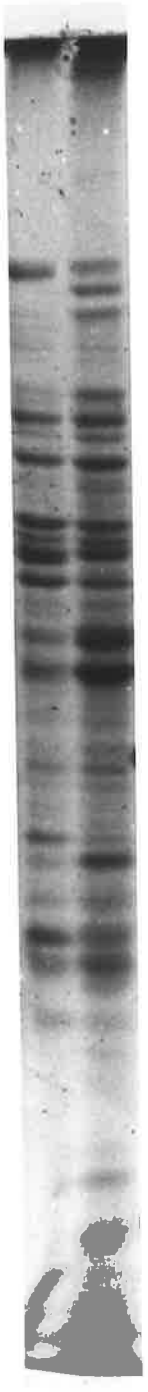
Slab PAGE, with SDS, of 11RX extracts

1. Triton-solubilized particulate material from a high speed SN.

2. Triton-soluble envelope.

Direction of migration: top to bottom

[Log phase 11RX cultures were used to prepare both 1. and 2. The bacteria were disrupted in the French press. In the case of 1. the particulate material was collected after the incubation of high speed SN material at 37°C for 2 hours. Nucleases were not used].



1. 2.

In this study, and in that of MacGregor, the Triton X-100 insoluble portion of particulate material was not characterised. MacGregor commented that the proteins of the Triton X-100 insoluble fraction were not identical with either cytoplasmic membrane or outer membrane proteins. She concluded that this fraction contained cytoplasmic and ribosomal proteins, some of which were probably denatured.

Whether or not cytoplasmic membrane lipid was present in high speed SN particulate material in addition to protein was not investigated by MacGregor. The low phosphorus content of 11RX antigens containing particulate matter indicated that there was little phospholipid present (section 2.1.4). This was a surprising result, as Riviere and Azoulay (1971) commented that distinctive membrane bilayers (as observed in the e.m.) were a characteristic of the particulate material as it formed in E.coli high speed SNs. It may be that the routine dialysis of 11RX high speed SNs to obtain 11RX antigen extracts resulted in the loss of phospholipid, as the average molecular weight of phospholipid is 700 (Osborn et al., 1972). Whether this was the case was not determined.

Schnaitman (technical notes for laboratories: E.coli cell envelope fractionation, 1975) comments that after removal of particulate material from E.coli high speed SNs (after a 2 hr. incubation), the remaining cytoplasmic fraction should contain less than 5% contamination by cytoplasmic membrane. Therefore, soluble SN type, 11RX antigens probably still contain cytoplasmic membrane material.

MacGregor also demonstrated that the cytoplasmic membrane protein in E.coli high speed SNs was not newly formed precursor

material, but was broken off existing membrane. The question then arose as to whether or not high speed SNs or 11RX antigens contained similarly fragmented cell wall material. This is considered below.

2.1.8 Outer membrane proteins in 11RX antigens

11RX antigens contained detectable amounts of ET (or ET derivatives, section 2.1.3), and, as mentioned above, the particulate material contained cytoplasmic membrane proteins. It therefore seemed likely that they contained similarly fragmented cell envelope components such as cell wall proteins. Two reports in the literature suggested a method for determining if 11RX antigens contained certain outer membrane proteins.

Ames (1974) found that 3 outer membrane proteins of S.typhimurium LT2 could be detected on SDS polyacrylamide gels if 100°C heat was used to solubilize cell envelopes in SDS. These proteins were not identifiable on gels if 37°C was used for solubilization. No such temperature-dependent effect was observed if the cytoplasmic fraction of the cell was used. Koplow et al., (1974) also reported differences in electrophoretic behaviour of E.coli CR34 outer membrane proteins solubilized at 70°C or 100°C. Again this effect was not noted if the E.coli cytoplasmic fraction was similarly treated.

In this project, samples of 11RX Triton-insoluble wall (containing outer membrane proteins, see section 5.2) and Batch II, 11RX antigen were heated in SDS at 37°C for 2 hours and some samples were given an additional incubation at 100°C for 5'. The samples were electrophoresed on disc gels in the presence of SDS using the pH 7.2 buffer system of Maizel.

References to sample preparation and the gel system are not given in Materials and Methods, but can be found in Schnaitman (1974b). After staining for protein, O.D. scans of the gels revealed that the banding pattern obtained was dependent on the temperature of solubilization in the case of 11RX Triton-insoluble wall, but not Batch II (Fig. 6a). No temperature dependent effect was noted if 11RX Triton-soluble envelope (see previous section) was electrophoresed (Fig. not shown, c.f. Koplow et al., 1974). The protein profiles of 11RX Triton-insoluble wall shown in Fig. 6a are not comparable in some respects to those depicted for S.typhimurium Triton-insoluble wall using the same gel system (Schnaitman, 1974a). This is because these particular gels were overloaded with protein. When whole 11RX cell envelopes were electrophoresed using the Bragg-Hou system the protein composition observed was similar to that of S.typhimurium envelopes (Fig. not shown; Schnaitman, 1974b and a). These results were confirmed when an 11RX Triton-insoluble wall extract was electrophoresed using the Lugtenberg system (section 5.2).

Thus, Batch II, 11RX antigen does not contain heat-modifiable outer membrane proteins. Whether 11RX antigen preparations contained Braun's lipoprotein, murein, or non-heat modifiable outer membrane proteins was not established. The possibility is considered in the discussion section.

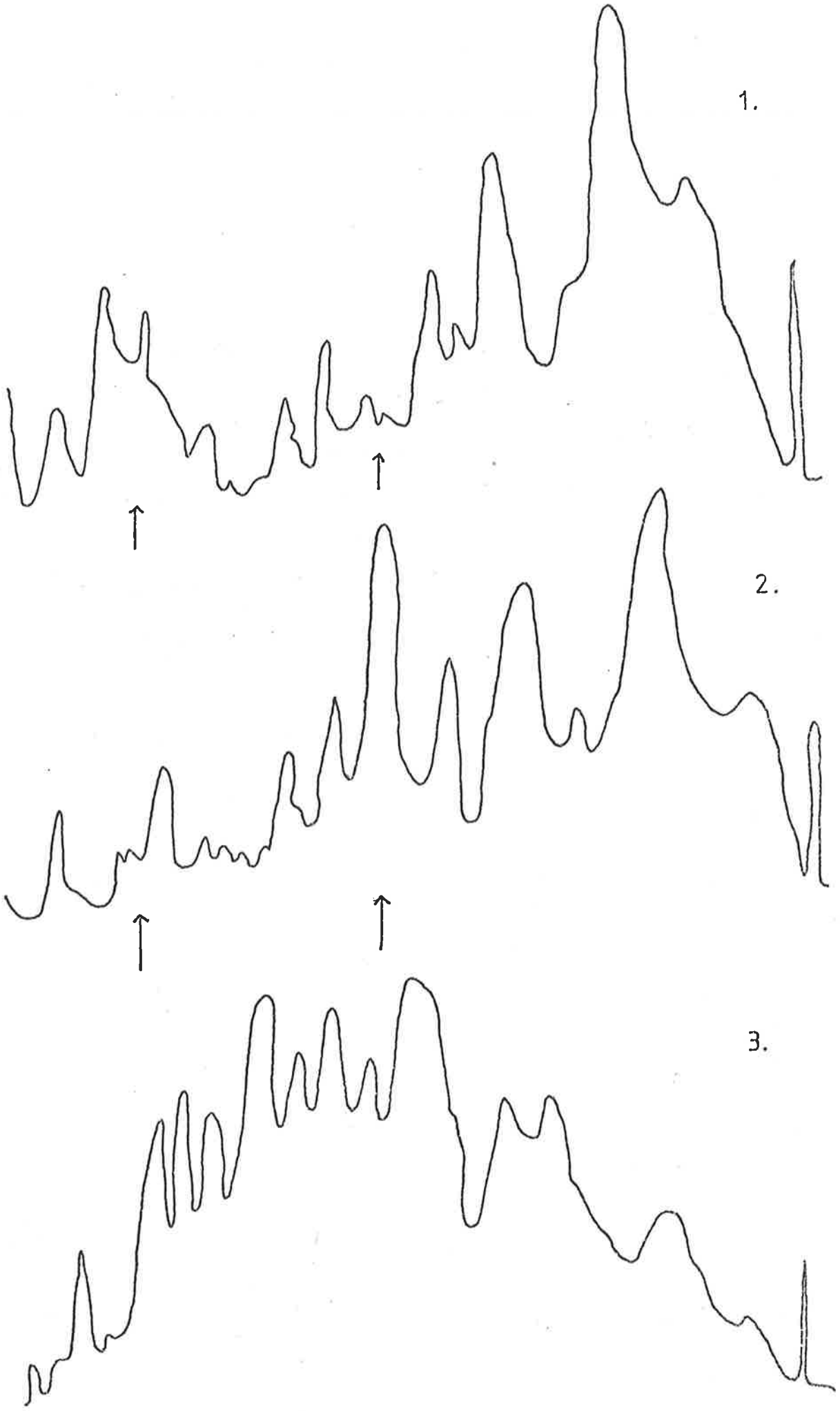
Fig. 6a

Disc PAGE, with SDS, of 11RX extracts

1. Triton-insoluble wall: heated at 37°C only.
2. Triton-insoluble wall: heated at 37°C + 100°C.
3. Batch II, 11RX antigen: either 37°C only
   or 37°C + 100°C.

Direction of migration: left to right.

The arrows show the points of difference between 1. and 2.


1.
2.
3.

3. Properties of 11RX antigens

The data relating to the composition of 11RX antigens did not throw any light on the chemical nature of the component responsible for the recall of tumour resistance. In an attempt to resolve this problem, 11RX antigens were subjected to 100°C heat, strong alkali, 2-Mercaptoethanol, proteases, or periodate treatment. Recall activity of untreated and treated preparations was compared, in the expectation that if recall antigen(s) was protein, certain of the above treatments should inactivate it, whereas others would leave it unaffected. Similar considerations applied if recall antigen(s) was lipid or carbohydrate.

The results are discussed in the following sections.

3.1 Effect of boiling

Most proteins are denatured by heat, in particular the activity of most enzymes is heat labile. Notable exceptions are some bacterial cell wall proteins (Sabet and Schnaitman, 1971; Braun, 1975). Carbohydrates and lipids are also heat resistant.

Batch II antigen was boiled for 1 hour at a concentration of 50 µg per ml in saline (Expt. 1). In addition, Batch L was boiled for 30' at 1mg per ml in 50mM Tris-$CaCl_2$ pH 8.4, and was diluted after cooling to 50 µg per ml in saline (Expt. 2). In the solution of Batch L aggregates formed after boiling, so it was exposed briefly to ultrasound before dilution. Recall activity of boiled and untreated antigens was assessed using the whole-body ^{125}IUdR retention assay. The data, shown in Table 5, indicated that boiling did not affect the ability of the antigens to recall tumour resistance in long-term 11RX immunized mice.

Table 5

Effect of boiling on the recall activity of 11RX antigen preparations.

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in whole mouse on day 3 (Expt. 1) or day 2 (Expt. 2) (arith. mean ± S.E.)			
	Normal		[c] Immunized	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
saline 0.2ml	40.4±5.9	54.4±3.6	25.2±4.6	51.3±3.5
[d] 11RX antigen 10 µg	26.8±4.6	42.0±6.6	5.3±1.0	8.7±2.0
boiled 11RX antigen 10µg	22.4±2.9	37.5±3.9	8.1±1.1	7.6±0.9
None, heat-killed EAT i.p. on day 0	2.9±0.3	10.6±0.7		

[a] 10^6 ^{125}I-EAT i.p. day 0.

[b] mean of individual mouse counts 4-6 mice per group.

[c] Expt. 1 10^5 11RX i.v. day-123.
Expt. 2 as in Table 8 ii.

[d] Expt. 1 Batch II.
Expt. 2 Batch L.

The results suggested that if recall antigen is a protein it certainly is an unusual one. It seemed probable from this experiment that the active moiety of recall antigen is cell wall protein, carbohydrate, or lipid.

3.2 Effect of alkali

The effects of alkali on some bacterial components are well documented. Alkaline hydrolysis of ET releases ester-linked, but not amide linked, fatty acids from Lipid A (Wu and Heath, 1973). Alkali treatment also results in deacylation of the polysaccharide portion of ET. Ester linked fatty acids were cleaved from E.coli lipoprotein during alkali treatment, and 50% of the α-helical structure of the native protein molecule was irreversibly lost (Braun, 1975). Antigenicity (due to the polypeptide portion) was unaffected, but mitogenicity (due to the lipid portion)was abolished (Braun, 1975). In short, if recall antigen was a lipid, or an O-Acyl carbohydrate, its activity should not be retained after alkali treatment.

In the case of proteins, alkali may destroy the native conformation, and this can be reversible depending on the protein involved. The tertiary structure of a protein can be lost while order is preserved in the secondary structure, and thus antigenicity may be retained. Changes in pH notably affect the solubility of proteins (refs. from Schachman, 1963).

To 5 mg of Batch II antigen in 0.9ml of saline was added 0.1ml of 1 M NaOH. The solution was left at 56^{o}C for 1 hour and then neutralized with 0.1 M HCl. The particulate material in this extract was solubilized by this procedure. Prior to injection into mice, the solution was diluted to 50 µg per ml in saline.

Alkali treatment of Batch II slightly lowered the recall effect elicited in long-term immunized mice, as assessed using the *in vitro* cytotoxicity assay. The cytolysis demonstrable with PEC from mice given alkali-treated Batch II was 80% of that obtained using PEC from mice to which the same amount of untreated Batch II had been administered.

The preservation of recall activity in alkali-treated Batch II was inconsistent with lipid or O-Acyl carbohydrate being the active moiety of recall antigen.

3.3 Effect of 2-Mercaptoethanol

Batch L was dissolved in 0.5M 2-Mercaptoethanol (2-Me) and the solution was left to stand for ½ hour at room temperature. 1M iodo-acetamide was then added to neutralize the reaction, and the mixture was dialysed against distilled water overnight. For injection into mice the mixture was diluted into saline assuming no alteration in protein concentration. A solution of dialysed 2-Me and iodo-acetamide was similarly diluted and injected to provide the appropriate control. The data,in Table 6,showed that this treatment did not affect the ability of Batch L to recall tumour resistance in 11RX immunized mice. This implied that if the active moiety is protein, its function does not depend on a conformation held together by S-S bonds.

3.4 Effect of proteolytic enzymes

Batch L was treated with proteases in 10mM $CaCl_2$ adjusted to pH 8.4 with 50mM Tris buffer. Batch L was incubated with 5% by weight of trypsin and 5% by weight of pronase for 6 hours at 37°C. Another 5% by weight of pronase was added at this time and the mixture was incubated for a further 14 hours.

Table 6

Effect of 2-Mercaptoethanol

on the recall activity of 11RX antigen, Batch L

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in whole mouse on day 2 (arith. mean ± S.E.)	
	[d] Immunized	[c] p
Buffer 0.2ml	16.9 ± 2.0	—
Batch L 5 µg	8.5 ± 1.0	0.005
[e] 2-Me treated Batch L	7.3 ± 1.0	0.005

[a] 10^6 ^{125}I-EAT i.p. day 0.

[b] mean of individual mouse counts 5 or 6 mice per group.

[c] Probability value for comparison of % retained with that of buffer group by Student's t-test.

[d] 6 x 10^9 11RX orally day-24.

[e] details given in text.

The reaction was stopped by adding EDTA to a final concentration of 20mM and boiling the mixtures for 10'. Control mixtures of buffer and buffer containing trypsin and phonase were treated similarly. It was observed that the particulate material in Batch L was solubilized after protease treatment.

Before and after protease treatment, the Biuret assay was used to estimate the protein content of samples. Unlike the Lowry method, the Biuret assay does not detect amino acids, but relies on the presence of peptide bonds for the measurement of protein concentration. This means that if proteases degrade a protein sample a reduction in Biuret measurable protein should be observed. This was found to be the case when either Batch L or BSA was treated with proteases (Table 7). According to the assay most of the protein (97%) in Batch L was degraded by proteases. If EDTA was added at the start of the incubation period instead of $CaCl_2$ neither Batch L or BSA was degraded (Table 7).

The 3 different mixtures of protease treated Batch L shown in Table 7 were assessed for their ability to recall tumour resistance using the whole-body ^{125}IUdR retention assay. The mixtures were diluted with saline for injection into mice assuming no reduction in the protein content of samples. Control solutions were diluted similarly. The data, shown in Table 8, indicated that the protease treatment reduced the effectiveness of Batch L by approx. 2-3 fold, but did not abolish recall activity completely.

These results were confirmed by an additional experiment (I. Kotlarski, unpublished observations). Batch II antigen was treated with thermolysin and then the mixture was

Table 7

Protease treatment of

11RX antigen, Batch L

		[a] Biuret µg protein/ml		
Sample	pH	not treated	treated	% redn.
BSA	[c]4	500	572	—
BSA	8	448	188	58
Batch L	[c]4	160	200	—
1. Batch L	8	240	[b]< 6	>97
BSA	8	500	100	80
BSA	8	688	108	84
2. Batch L	8	216	< 6	>97
3. Batch L	8	230	< 6	>97

[a] corrected for buffer and protease O.D. contribution.

[b] limit of detection.

[c] EDTA added at the start of the incubation period.

1., 2., and 3. as used in Table 8.

Table 8

Effect of protease treatment on the recall activity of 11RX antigen, Batch L

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in whole mouse on day 1 (i) or day 2 (ii) (arith. mean ± S.E.)	
	normal	[d] immunized
i buffer 0.2ml	34.4 ± 5.1	41.6 ± 1.5
buffer + proteases 0.2ml	39.6 ± 3.8	41.0 ± 2.8
Batch L 5 µg	23.8 ± 2.2	8.6 ± 0.9
1. treated Batch L[c]	41.6 ± 1.9	24.0 ± 3.6
ii buffer 0.2ml	54.4 ± 3.6	51.3 ± 3.5
buffer + proteases 0.2ml	60.4 ± 8.2	48.2 ± 1.8
Batch L 5 µg	42.0 ± 6.6	8.7 ± 2.0
2. treated Batch L[c]	53.6 ± 4.6	22.6 ± 4.5
3. treated Batch L[c]	46.9 ± 7.7	24.8 ± 5.5
none, heat-killed EAT i.p. day 0	10.6 ± 0.7	

[a] 10^6 ^{125}I-EAT i.p. day 0.

[b] mean of individual mouse counts 4 or 5 mice per group.

[c] details given in text.

[d] i 10^5 11RX i.v. day-94.
ii 10^5 11RX i.v. day-60.

dialysed. Although 10mg per ml of protein, as measured using the Lowry method, had been added to the thermolysin, only 0.7mg per ml could be detected after dialysis using the same method (93% reduction). The mixture was diluted with saline for injection into pre-immunized mice assuming no loss of protein. The thermolysin treated Batch II was as effective as untreated Batch II in recalling tumour resistance.

The preceding data indicated that there were a number of possibilities regarding the nature of active moiety of recall antigen, including the following:-

(i) it is not protein, or

(ii) it is protein, but it is -

(a) protease resistant. Some native proteins are resistant to proteolysis, especially those with α helical regions such as E.coli lipoprotein (White, Handler, and Smith, 1968; Braun, 1975).

(b) susceptible to proteolysis, but sufficient of it remained undegraded and was able to elicit recall of tumour resistance. Very small quantities (i.e., 0.3 µg by weight) of 11RX antigen can elicit recall (section 4.1).

(c) protected from enzymic attack by a carbohydrate or lipid moiety.

3.5 Effect of periodate

Periodic acid and its salts cleave carbon-carbon bonds between adjacent dihydroxy positions in sugar moieties of carbohydrates. Treatment of Batch L with sodium metaperiodate was carried out according to Sabet and Schnaitman (1971). To end the reaction, glucose was added to the reaction mixture.

The control solution was that in which glucose was added to Batch L and periodate at the start of the incubation period. For injection into mice the mixtures were diluted with saline. The control buffer, which contained glucose and periodate, was diluted similarly.

The results, shown in Table 9, indicated that periodate treatment had failed to affect the recall activity of Batch L, suggesting that carbohydrate was not the active moiety of recall antigen. It was later found that 0.5 mg of Batch L did not consume any periodate after a 1 hour incubation period, although using a similar amount of glucose it was demonstrated that 2.7 moles of periodate were consumed per mole of glucose after 1 hour. The method used to assess periodate consumption was that described by Keleti and Lederer (1974).

3.6 Conclusions

These experiments did not resolve the chemical nature of recall antigen. The evidence in section 3.2 indicated that the active moiety is not lipid, and that in section 3.5 was suggestive, but not conclusive, evidence that it is not carbohydrate.

This leaves the possibility that the active moiety is protein, but the data in section 3.1 and 3.4 showed that if this is the case, the protein is likely to be atypical, or it is associated with non-functional lipid or carbohydrate groups. This information would be consistent with the active moiety being a cell wall protein rather than a cytoplasmic protein.

However, it is possible that there is more than one recall antigen (section 4.4, 5.2, 5.3). Although one

Table 9

Effect of periodate treatment on the recall activity of 11RX antigen, Batch L

[a] Treatment i.p. on day-2:-	[b] % ^{125}I retained in whole mouse on day 2 (arith. mean ± S.E.)
	[c] Immunized
[d] control buffer 0.2ml	36.3 ± 9.5
[d] control, Batch L 5 µg	5.8 ± 0.5
[d] Batch L, periodate treated	9.6 ± 2.9

[a] 10^6 ^{125}I-EAT i.p. on day 0.

[b] mean of individual mouse counts, 4-5 mice per group.

[c] as used in expt. ii of Table 8.

[d] details given in text.

moiety[ies] retained activity after some treatments, the activity of the other[s] may have been destroyed. A more informative answer on the chemical nature of recall antigen[s] requires preparation of more defined extracts.

4. Attempts at fractionation of 11RX antigen preparations

4.1 Recall dose response

In order to look for enrichment of activity when fractionating 11RX antigen preparations, it would be desirable to know the dose characteristics of the recall response. During this project, the *in vitro* cytotoxicity assay was used to investigate the recall dose response using 11RX antigen Batch II. Before the results of these experiments are described, the reasons for choosing this particular assay method are detailed.

One assay which could have been used is the footpad assay. Mice mount a definitive cutaneous DTH response to 11RX antigen only a few days after immunization with live 11RX (Davies, 1975). In contrast, mice are used for *in vivo* or *in vitro* recall of tumour resistance experiments 40-50 days after i.v. immunization. If used prior to this time, the mice possess some cellular immunity without having been injected with recall antigen. It is frequently practically impossible, and indeed undesirable, to keep large numbers of infected mice for a number of weeks. Use of the footpad assay for screening antigen fractions would eliminate this problem. However, as discussed in the introduction, the relationship between different cell mediated immune responses is not well defined. This is so in the case of recall of resistance to EAT and the DTH response elicited in the footpads of sensitized mice. Although the footpad assay was used in some cases to assess the activity of 11RX extracts, it is not known whether the antigen(s) involved in recall and DTH elicitation are the same.

There were 3 assay techniques to choose from which are used to assess the recall of tumour resistance. In the case of the whole-body retention assay, the differences observed between recalled and non-recalled mice are limited by events subsequent to tumour cell killing (Ashley, 1976). Cytotoxic events can be followed with heightened sensitivity if the contents of the peritoneal cavity are washed out after challenge with labelled tumour. Alternatively, an assay of similar sensitivity to the latter is the in vitro cytotoxicity assay in which the tumouricidal capacity of PEC from recalled mice is assessed. In addition, if the in vitro assay is used, any changes in PEC number per mouse following the injection of 11RX extracts can be easily determined.

Thus, in terms of sensitivity, the in vitro cytotoxicity assay was a good choice for dose response experiments. In addition, compared with the other recall assays it represented a more rapid method for assessing the recall potential of large numbers of fractions of 11RX antigen preparations. However, it did have some limitations which are detailed in the next few paragraphs.

Experiments done during this project showed that the in vitro cytotoxicity assay did not give reproducible results from day to day in terms of the maximum percentage cytolysis. Using PEC from similarly treated mice, and the same PEC: target cell ratios, the maximum cytolysis at 20 hours varied from 80% to 10% from day to day. These results were confirmed by other workers in the same laboratory.

One of the reasons for this variation was the fact that PEC suspensions used were pools of PEC from individual mice.

The proportion of cytotoxic PEC in such a pool would vary from day to day depending on how many of the PEC were harvested from mice which had "missed" an i.p. injection of recall antigen (i.e. received it in the gut rather than i.p.). The following experiment demonstrated this point. In one *in vitro* assay, 45% cytolysis was obtained with PEC from 4 immunized mice which had been injected with 11RX antigen Batch II. The cytolysis was lowered to 36% if the same PEC were mixed in a 3:1 ratio with non-cytolytic PEC from a normal mouse which had received Batch II.

In the case of the whole-body retention assay, in which individual mice were counted, a long-term immunized mouse which had "missed" an injection of 11RX antigen was easily identified during counting on the day after tumour challenge.

Another problem with the *in vitro* assay was that it was not possible to assess what small differences in % cytolysis meant in terms of tumour cell killing *in vivo*. In addition, the 100:1 PEC: EAT ratio was used in all experiments, although the total number of PEC harvested from each group of mice was variable (Fig. 8).

The results of 3 *in vitro* dose response experiments done with Batch II antigen are shown in Fig. 7a, 7b, and 7c. In each experiment long-term immunized mice, and normal mice in the case of Fig. 7a and b, were injected with 0.1-100 µg of Batch II per mouse. Fourty to 48 hours later their PEC were harvested and the ability of these cells to lyse ^{51}Cr-EAT target cells was assessed.

The long-term immunized mice used for the experiment of Fig. 7a had been orally immunized 102 days previously. The

Fig. 7 (continued on next page)

Recall dose response with Batch II, 11RX antigen using the in vitro cytotoxicity assay

PEC from normal mice (----)

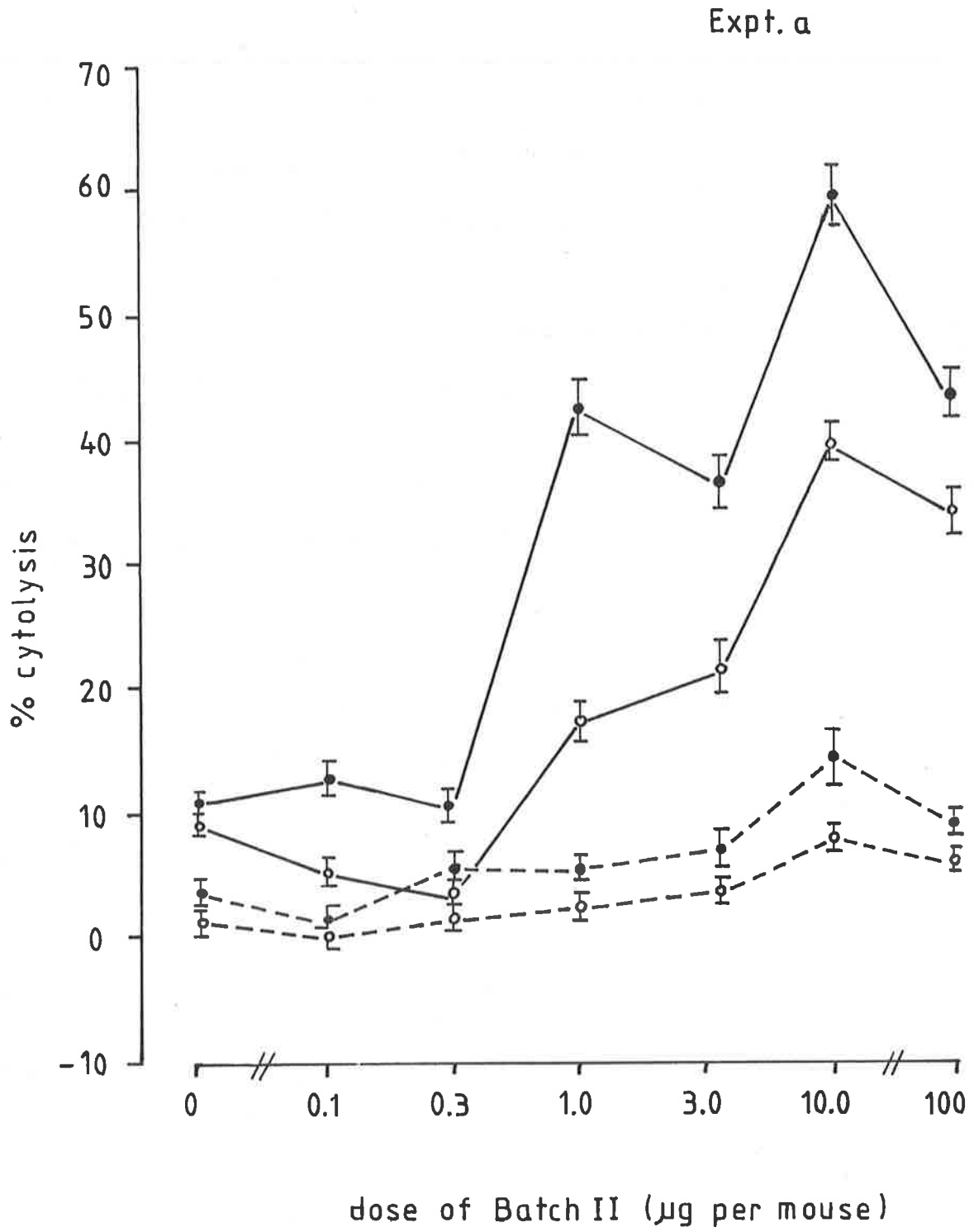
PEC from long-term immunized mice (——)

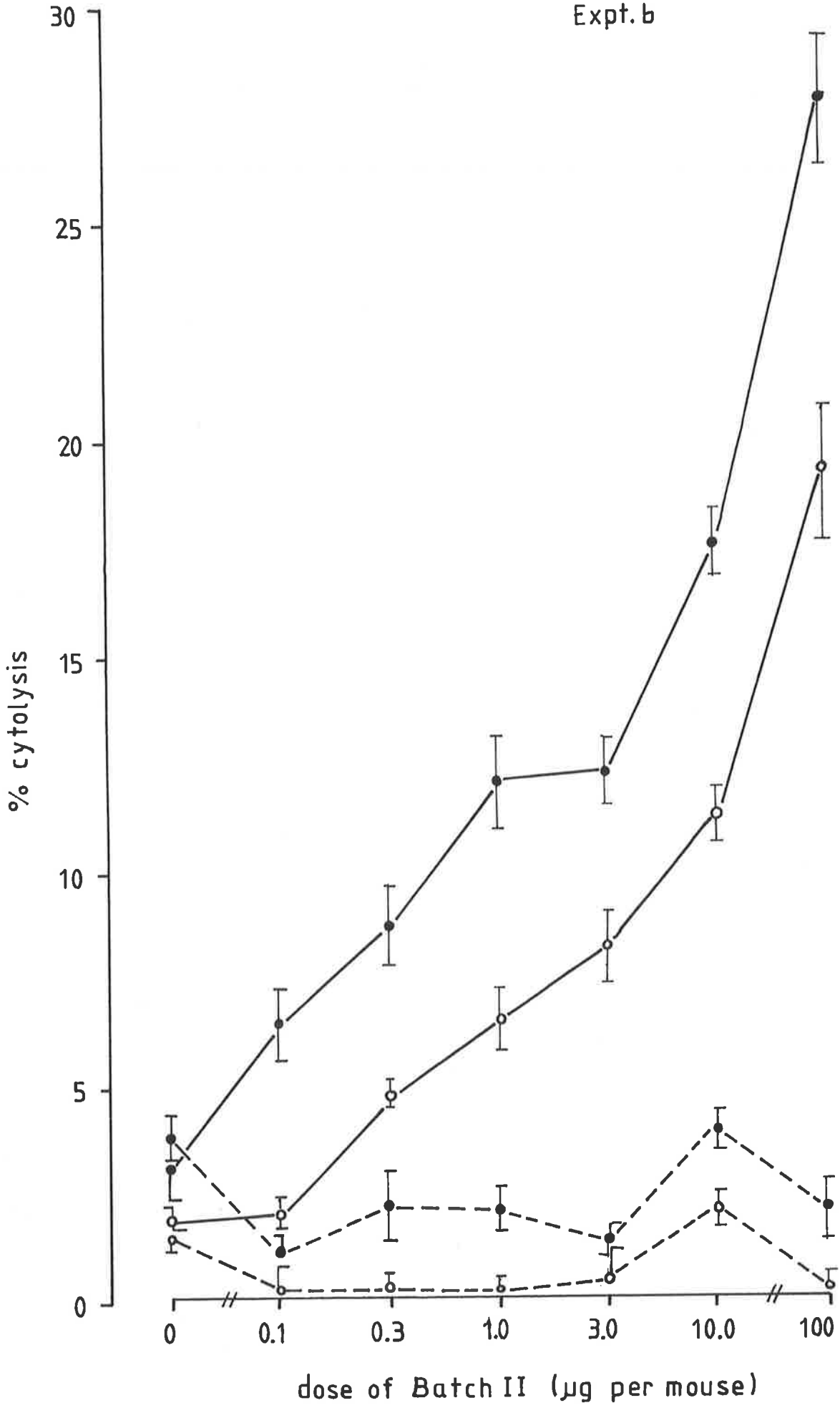
PEC were pooled from groups of 4 mice given 0.2ml saline or various doses of Batch II i.p. 2 days prior to assay.

Each point represents the mean cytolysis from quadruplicate wells. The vertical bars represent ± 1 S.Error.

Expt. a : % cytolysis measured after a 5 hr. (o) or a 10 hr. (●) incubation.
Immunized mice had received 7×10^9 11RX orally 102 days previously.

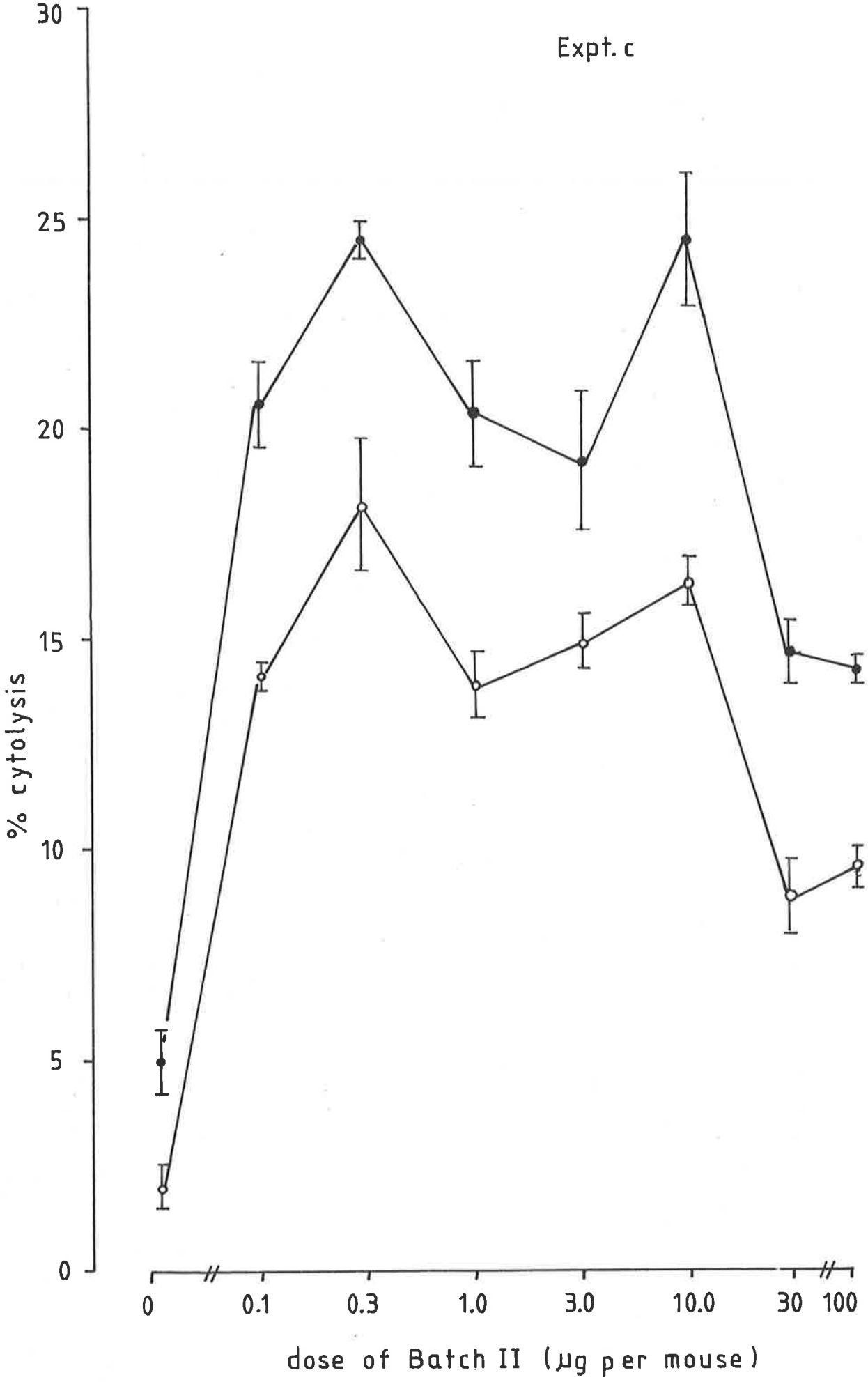
Expt. b : % cytolysis measured after a 8½ hr. (o) or a 20 hr. (●) incubation.
Immunized mice had received 10^5 11RX i.v. 48 days previously.


Expt. a
70
60
50
40
30
20
10
0
-10
% cytolysis
0
0.1
0.3
1.0
3.0
10.0
100
dose of Batch II (µg per mouse)


Expt. b
30
25
20
15
10
5
0
% cytolysis
0
0.1
0.3
1.0
3.0
10.0
100
dose of Batch II (µg per mouse)

.... Fig. 7 ctd.

Expt. c : % cytolysis measured after a 8½ hr. (-o-)
or a 20 hr. (-●-) incubation.
The mice had received 10^5 11RX
i.p. 74 days previously.


Expt. c
30
25
20
15
10
5
0
% cytolysis
0
0.1
0.3
1.0
3.0
10.0
30
100
dose of Batch II (µg per mouse)

cytolysis at 10 hours obtained with PEC from these mice which had been injected with 1, 10, or 100 μg of Batch II was 43%, 60% and 45% respectively. Less than 15% cytolysis was observed with PEC from mice given 0.1 or 0.3 μg of Batch II. In Fig. 7b, the mice used had been immunized with 11RX i.v. 48 days previously. The 20 hour cytolysis figures obtained using 1, 10 or 100 μg of Batch II were 12%, 17% and 28% respectively. Less than 10% cytolysis was demonstrable with PEC from mice given 0.1 or 0.3 μg of Batch II. In both experiments PEC from normal mice given Batch II were not significantly cytotoxic.

The results of these experiments were similar to those reported by Ashley et al., (1974). These workers tested the recall activity of 1 μg, 10 μg and 100 μg of Batch II using the whole-body retention assay with ^{131}I-EAT. In mice immunized i.p. 135 days previously with 11RX these doses of antigen were similarly effective in recalling resistance to EAT (c.f. Fig. 7a). In the case of mice immunized i.v. 50 days prior to antigen injection, 100 μg was more effective than 1 or 10 μg (c.f. Fig. 7b). All doses had very little effect on the clearance of labelled tumour in normal mice.

In the experiment shown in Fig. 7c, 0.1 and 0.3 μg of Batch II elicited a surprisingly high recall response, but in the range 1-100 μg, 10 μg is the optimal dose. The mice used in this experiment had been injected with 11RX i.p. 74 days previously.

Overall, the results of these experiments suggested that the response of long-term immunized mice to different doses of Batch II varied with the time and route of immunization.

For each group of mice from the experiments of Fig. 7a, b, and c a total PEC per mouse figure was estimated as described in Materials and Methods. For expt. a the Coulter particle counter was used for cell counting, but in expts. b and c a haemocytometer was used. The percentage increase in PEC number of the mice given Batch II was determined relative to the groups given saline, for both the normal and pre-immunized mice. These figures are shown in Fig. 8.

Total PEC numbers were determined because there was some indication that an influx of PEC induced in pre-immunized mice after the injection of 11RX antigen could be associated with the recall of tumour resistance. Ashley (1976) had found that a high degree of cytolysis was demonstrable if PEC were harvested from pre-immunized mice 1-2 days after the injection of 11RX antigen, but not at 0, 4, or 6 days. This was associated with an influx of PEC on days 1-2.

The data shown in Figs. 7 and 8 show that there was no correlation between the fluctuations in PEC numbers and the tumouricidal capacity of PEC taken from mice given different doses of 11RX antigen. In expts. a, b, and c PEC number was highest in pre-immunized mice which had been given 0.1 μg of Batch II. In contrast, the cytolytic capacity was greatest when the mice had been injected with 10 μg in Expt. a, 100 μg in Expt. b, and 0.3 or 10 μg in Expt. c. The maximum number of PEC which could be harvested from normal mice was from those mice which had received the highest dose of antigen. These results showed that an increase in PEC number induced by the injection of recall antigen did not imply an increase in cytotoxic PEC. However, it may have been more informative

Fig. 8

% Increase in total PEC number per mouse* of mice given various doses of Batch II, 11RX antigen relative to mice given saline.

PEC from normal mice (----)

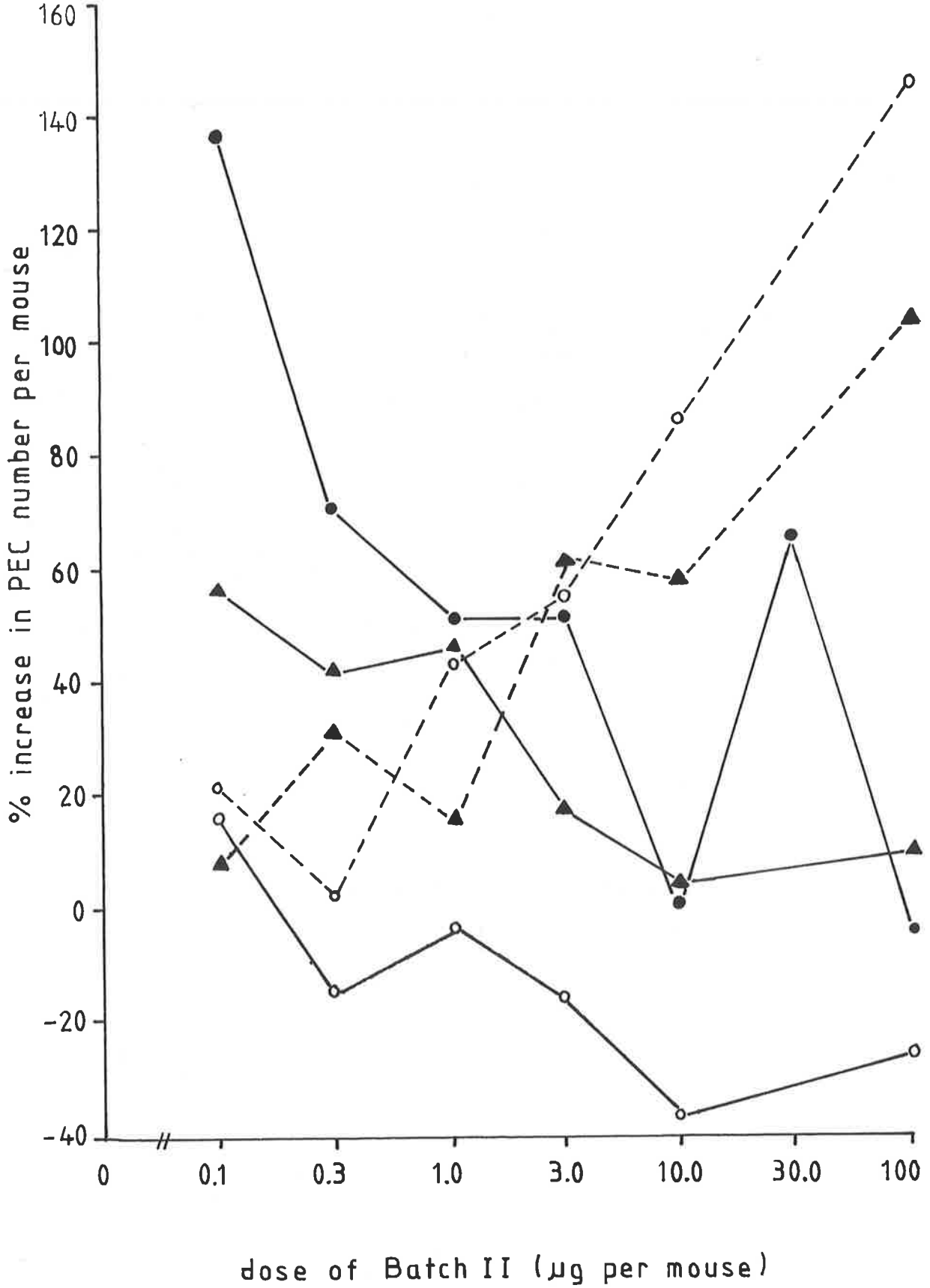
PEC from long-term immunized mice (——)

o Expt. 7a

▲ Expt. 7b as in Fig. 7

● Expt. 7c

*PEC were pooled from groups of 4 mice. The total PEC number per mouse for each group of mice was therefore determined as an average.


160
140
120
100
80
60
40
20
0
-20
-40
% increase in PEC number per mouse
0
0.1
0.3
1.0
3.0
10.0
30.0
100
dose of Batch II (µg per mouse)

to do differential counts on PEC suspensions. Ashley (1976) showed that the cytotoxic PEC in 11RX immunized mice injected with 11RX antigen were glass adherent and hence probably macrophages.

In conclusion, the results obtained using the in vitro cytotoxicity assay indicated that when screening fractions of 11RX antigen for recall potential it would be desirable to test a range of doses of both the original preparation and each fraction. This would limit the number of fractions which could be assayed at any one time. An alternative would be to use a dose of fraction equivalent in weight to a dose of the 11RX antigen preparation which elicited a suboptimal recall response. If there is any enrichment of recall activity in the fraction, it should induce PEC of greater cytotoxic capacity than the same dose of the starting material.

Note:-

Recently, Dr. L. Ashman determined the dose response characteristics of DTH elicitation with 11RX antigen using the footpad assay. She found that at 48 hours after antigen injection, the maximum increase in footpad thickness was elicited by 10 µg or 100 µg of Batch II (approx. 45% increase). Using 1 µg the increase was 22%, and with 0.1 µg it was 12%. These results show that the footpad assay is of comparable sensitivity to the in vitro cytotoxicity assay.

4.2 Recall of tumour resistance with soluble and particulate 11RX antigens

It has already been pointed out that some 11RX antigen preparations contained both soluble and particulate material (i.e., Batch I-III, Batch L, F.P. SN + pellet, U.S. SN +

pellet), while others consisted of only soluble (F.P. SN, U.S. SN, DE-52; PSE) or particulate material (F.P. pellet). All of these extracts could recall tumour resistance in long-term immunized mice. As the separation of soluble and particulate material was a form of fractionation, the data relating to the recall activity of some of these extracts is presented in this section.

Batch III had been stored dry at 4°C for some months before the commencement of this project. It was suspended in saline and separated into soluble and particulate material by centrifugation (150,000 x g, 1 hour). The pellet was washed once and then resuspended in saline. Protein content of the supernatant and pellet was estimated using the Lowry method. The recall activity of 1-100 µg of Batch III (based on dry weight), supernatant, or pellet (based on protein) was assessed using the whole-body retention assay. This is the only experiment described in the text in which ^{131}I-EAT was used instead of ^{125}I-EAT. On the day of tumour challenge ^{131}I was monitored by counting the mice in groups, and this figure was divided by the number of mice to give an average individual mouse count. Thereafter, each mouse was counted, and the ^{131}I retention figures shown in Table 10 were calculated by dividing this count by the day 0 individual count. The data indicated that both supernatant and pellet material could recall tumour resistance as effectively as Batch III, at least over the dose range tested.

These results were confirmed when freshly prepared 11RX antigen extracts were used. The particulate material was separated from the soluble material prior to the extensive

Table 10

Recall activity of soluble and particulate material from 11RX antigen, Batch III

[a] Treatment i.p. on day-2 :-	[b] % ^{131}I retained in whole mouse on day 4 (arith. mean ± S.E.)
	[c] Immunized
saline 0.1ml	20.8 ± 2.2
Batch III 1µg	2.7 ± 0.2
10µg	3.1 ± 0.4
100µg	2.1 ± 0.2
[b] pellet 1µg	2.7 ± 0.6
10µg	2.4 ± 0.3
100µg	2.7 ± 0.2
[b] supernatant 1µg	2.5 ± 0.3
10µg	2.7 ± 0.4
100µg	4.0 ± 0.4

[a] 10^6 ^{131}I-EAT i.p. on day 0.

[b] details given in text.

[c] 10^5 11RX i.v. day-63, 5-7 mice per group.

dialysis step (see Materials and Methods). Six days after lyophilization, 10 µg by weight of F.P. SN + pellet, F.P. SN, or F.P. pellet was injected i.p. into normal and long-term immunized mice. Recall activity was assessed using the whole-body retention assay. The day 3 retention figures, which are shown in Table 11, correlated with the results of the previous experiment. In addition, the extracts did not affect the clearance of ^{125}I-EAT in normal mice, and treated pre-immunized mice survived longer than the control mice. The differences between the proportion of mice surviving in the various treated immunized mice were not significant. This was shown in another experiment, in which the dose of extracts used was 100 µg. Freshly made U.S. SN + pellet and U.S. SN preparations had comparable recall activity to F.P. extracts when assayed at a dose of 10 µg (Table 11). U.S. pellet material was not tested.

The ability of DE-52: PSE to recall tumour resistance was assessed using the in vitro cytotoxicity assay. Pre-immunized mice were injected i.p. with 3 µg of DE-52: PSE and PEC from these mice were harvested 2 days later. The % cytolysis obtained using these cells was comparable to that demonstrated using cells from mice given 3.2 µg of Batch II (Table 12). The relative effectiveness of DE-52: PSEs and Batch II on a weight basis was not determined.

In view of the fact that both supernatant and pellet preparations had recall activity, supernatant extracts were chosen for further attempts at fractionation. The rationale was that separation of the proteins in the water-insoluble pellet material was likely to be more complicated technically.

Table 11

Recall activity of soluble and particulate 11RX antigen preparations

[a] Treatment i.p. on day-2 :-	[b]% ^{125}I retained in whole mouse on day 3 (arith. mean ± S.E.)	
	Normal	[d] Immunized
None	35.7 ± 1.8 [e] ($^0/_5$)	37.0 ± 6.9 [e] ($^0/_5$)
[c] Batch II	33.7 ± 2.1 ($^0/_5$)	3.8 ± 0.4 ($^5/_5$)
French press preps.		
F.P. SN + pellet	29.1 ± 5.7 ($^0/_5$)	3.8 ± 0.6 ($^4/_4$)
F.P. SN	30.9 ± 2.8 ($^0/_4$)	4.6 ± 0.6 ($^3/_5$)
F.P. pellet	23.6 ± 5.0 ($^0/_5$)	3.8 ± 0.4 ($^5/_5$)
Ultrasound preps.		
U.S. SN + pellet	35.6 ± 2.0 ($^0/_5$)	4.4 ± 1.2 ($^4/_5$)
U.S. SN	24.8 ± 4.9 ($^0/_5$)	5.8 ± 1.2 ($^3/_5$)
None: heat-killed EAT i.p. on day 0	4.2 ± 0.5 ($^4/_4$)	

[a] 10^6 ^{125}I-EAT i.p. on day 0.

[b] mean of individual mouse counts.

[c] doses of extracts; 10 µg per mouse.

[d] 10^4 11RX i.v. day-92.

[e] proportion of mice alive on day 30.

Table 12

Recall activity of 11RX antigen, DE-52: PSE

-assessed using the in vitro cytotoxicity assay

[b] PEC from mice given the following i.p. on day-2 :-	[a] % cytolysis at 10 hrs. (arith. mean ± S.E.)	
	[b] Normal	[b] Immunized
saline 0.2ml	3.4 ± 0.5	11.8 ± 1.0
Batch II 3.2 µg	6.7 ± 1.0	36.3 ± 1.2
[c] DE-52:PSE 3.0 µg	4.9 ± 0.1	26.0 ± 2.2

[a] mean from quadruplicate wells.

[b] 4 mice per group, mice as used in expt. of Fig. 7a.

[c] from a log phase culture.

F.P. SNs were used because these were obtained in greater yields than U.S. SNs. In addition, DE-52: PSEs were also used. The results discussed in a previous section (2.1.6), and above, showed that DE-52: PSEs were similar in composition and possessed the recall antigen specificity(ies) of 11RX antigen preparations made using nucleases to remove DNA and RNA.

The initial aim of the fractionation experiments was to determine if all fractions or only a few possessed recall activity. Doses of fractions administered per mouse were 3-10 µg, which were in the optimal range for a recall response (section 4.1). It became apparent that most of the fractions tested had recall activity at this dose level, suggesting that there may be more than 1 recall antigen.

4.3 DE-52 chromatography of F.P. SN

Cowie, Cohen, Bolton et al., (1959) prepared a crude cell-free extract from E.coli by disrupting cells in the French pressure cell and removing large debris. This material was loaded onto a DEAE-cellulose column equilibrated in phosphate buffer pH 7 or Tris succinate buffer pH 7.6. It was eluted with a linear salt gradient from 0.0M-0.8M NaCl. Many protein peaks were resolved, as estimated using the Lowry method to assay each fraction. The activity of 3 different cytoplasmic enzymes was shown to be associated with 3 distinct protein peaks. These results indicated that 11RX antigen preparations would probably bind to an anion exchanger, so this chromatographic technique was used to determine if any fractionation of recall antigen(s) could be achieved.

Twenty mg of an F.P. SN 11RX antigen preparation was applied to a DE-52 column (1 x 67 cm) equilibrated with 20mM Tris-HCl pH 7.7. The column was washed with 2 column volumes of the same buffer and then the bound protein was eluted with a continuous salt gradient from 0.0M-0.5M NaCl. This technique resolved 5 protein peaks (Fig. 9).

Fractions no. 9-13, 44-48, 49-54, 55-62, and 63-79 were pooled, and each pool was dialysed against buffer to remove salt. Ten µg of each pool was injected i.p. into long-term immunized mice and normal mice, and there were 4-6 mice per group. The doses were based on protein estimated using the Lowry method. F.P. SN and Batch II were used as positive controls and were injected at a dose level of 10 µg by weight. Two days later the mice were challenged with ^{125}I-EAT and label retention was followed by whole-body counting the mice. The results are shown in Table 13.

Neither the 11RX antigen preparations nor the fractions affected tumour retention in normal mice. On the basis of day 1 retention figures, Batch II was most effective at recalling tumour resistance (14% retained c.f. 85% in buffer group). F.P. SN, and pools 55-62 and 63-79 facilitated tumour clearance to a lesser degree than Batch II (30-37% retained), but were more effective than pools 9-13, 44-48, and 49-54 (56-65% retained). By day 4 all immunized mice which had been injected with 11RX antigen preparations or fractions thereof had retained much less tumour compared with the control group. However, 60 days after tumour challenge the only surviving mice were in those groups which had been injected with Batch II, F.P. SN, or pool 63-79. The latter extracts

Fig. 9

DE-52 chromatography of

F.P. SN, 11RX antigen

details given in text

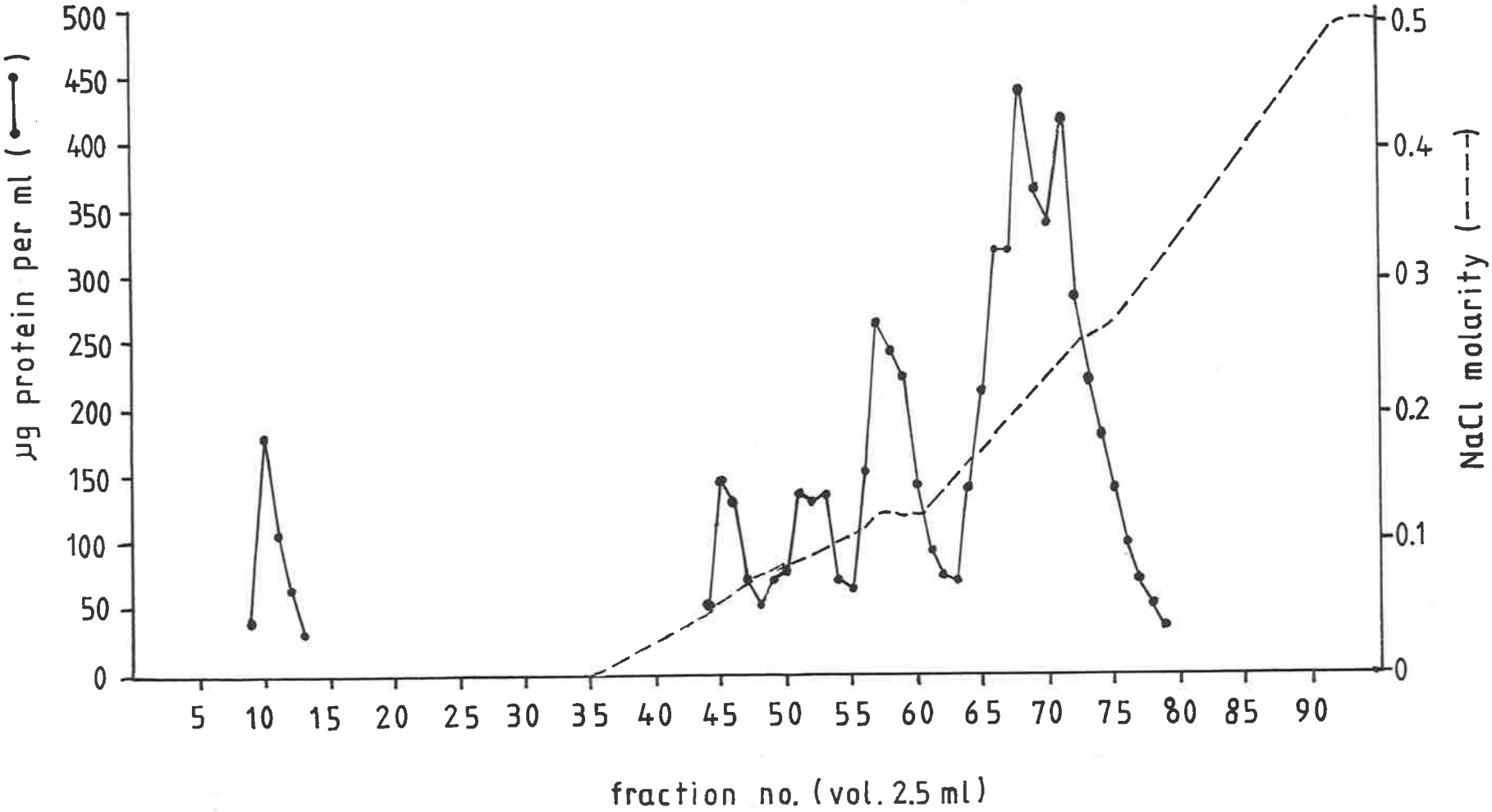

µg protein per ml (——)
500
450
400
350
300
250
200
150
100
50
0
0.5
0.4
0.3
0.2
0.1
0
NaCl molarity (– – –)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
fraction no. (vol. 2.5 ml)

Table 13 (continued on next page)

Recall activity of DE-52 fractions of 11RX antigen, F.P. SN -assessed using the ^{125}I whole-body retention assay

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in whole mouse on day 1 (arith. mean ± S.E.)	
	Normal	[c] Immunized
buffer 0.2ml	73.8 ± 2.2	84.5 ± 2.3
[d] Batch II	72.5 ± 3.8	14.2 ± 2.8
F.P. SN	78.0 ± 6.3	30.8 ± 5.4
Fraction No.:		
9-13	78.8 ± 5.3	59.8 ± 6.0
44-48	83.3 ± 3.0	56.4 ± 8.7
49-54	81.2 ± 2.9	65.3 ± 5.7
55-62	75.5 ± 1.6	36.8 ± 5.9
63-79	74.7 ± 2.8	28.8 ± 3.8
None: heat-killed EAT i.p. day 0	10.7 ± 1.2	

[a] 10^6 ^{125}I-EAT i.p. on day 0.

[b] mean of individual mouse counts, 4-6 mice per group.

[c] 10^5 11RX i.v. day-102.

[d] details of extract doses given in text.

..... Table 13 ctd.

Treatment i.p. on day-2	% ^{125}I retained on day 4	
	Normal	Immunized
buffer	25.0 ± 2.8 [e] $(^0/_6)$	24.5 ± 2.7 [e] $(^0/_4)$
Batch II	24.8 ± 3.6 $(^0/_6)$	2.7 ± 0.3 $(^3/_5)$
F.P. SN	34.2 ± 1.8 $(^0/_5)$	2.8 ± 0.4 $(^1/_5)$
Fraction No.:		
9-13	26.8 ± 3.7 $(^0/_6)$	8.2 ± 2.0 $(^0/_5)$
44-48	32.0 ± 0.9 $(^0/_6)$	8.9 ± 3.1 $(^0/_5)$
49-54	27.7 ± 2.4 $(^0/_6)$	7.8 ± 3.5 $(^0/_5)$
55-62	33.8 ± 1.4 $(^0/_6)$	3.2 ± 0.6 $(^0/_5)$
63-79	28.0 ± 3.2 $(^0/_6)$	2.8 ± 0.2 $(^3/_5)$
None: heat-killed EAT i.p. on day 0	2.4 ± 0.5 $(^5/_5)$	

[e] proportion of mice alive on day 60.

were also the only effective recall antigens if mean time to death up to day 60 was used as the criterion of resistance to tumour growth, viz., buffer (25.8 ± 1.1 days), 9-13 (26.8 ± 0.4), 44-48 (34.2 ± 4.6), 49-54 (29.5 ± 1.7), 55-62 (26.6 ± 0.9), 63-79 (49, 44), F.P. SN (44.3 ± 1.0), Batch II (42, 44). The ± refers to S.Error.

The recall activity of the DE-52 fractions was also assessed using the in vitro cytotoxicity assay. The pools were injected at a dose of 5 ug of protein per mouse, Batch II and F.P. SN at 10 µg by weight. The results are shown in Table 14. Only pool 9-13 did not induce cytotoxic PEC.

The combined results of the experiments indicated that at a dose level of 5 or 10 µg pools 44-48, 49-54, 55-62, and 63-79 had recall activity. However, using survival as the criterion pool 63-79 was the only fraction which was as effective, or more effective, than the starting material. Pool 63-79 contained 57% of the total protein eluted from the DE-52 column, and when it was electrophoresed (PAGE, without SDS) it was found that its protein composition was similar to that of F.P. SN, indicating that little fractionation had been achieved. The other pools were not studied using PAGE.

It was decided that fractionation on the basis of size, rather than charge, differences should be a better method for separating the proteins in 11RX antigen preparations, so molecular sieve chromatography and ultrafiltration were subsequently employed.

The above results also suggested that it would be preferable to use more than 1 assay technique to establish that a fraction has recall activity. In particular, it appeared

Table 14

Recall activity of DE-52 fractions of 11RX antigen, F.P. SN -assessed by the in vitro cytotoxicity assay

[a] PEC from mice given the following i.p. on day-2 :-	[b] % cytolysis at 20 hrs. (arith. mean ± S.E.)
	[a] Immunized
saline 0.2ml	5.7 ± 0.7
[c] Batch L F.P. SN	14.9 ± 1.0
	14.8 ± 1.3
Fraction No.:	
9-13	7.2 ± 1.0
44-48	12.1 ± 0.3
49-54	15.4 ± 1.6
55-62	18.4 ± 1.6
63-79	14.8 ± 1.4

[a] 10^5 11RX i.v. day-86. 3-4 mice per group

[b] mean from quadruplicate wells

[c] details of extract doses given in text

that day 1 ^{125}I-EAT whole-body retention or survival were more sensitive indexes of recall than the in vitro cytotoxicity assay or day 4 ^{125}I-EAT retention. It was not practically possible to use all assays for all fractions, so the assays later used in the filtration experiments were the in vitro cytotoxicity assay or the day 1 ^{125}I-EAT whole-body or peritoneal cavity retention assays. The data presented in the following section shows that the results obtained using one of these 3 assays agreed with the findings of the 2 remaining assays.

4.4 Size fractionation of F.P. SN, DE-52: PSE, and high speed SN

Fifteen mg of an F.P. SN preparation in 2ml was applied to a Sephadex G-200 column (2.5 x 40cm) previously equilibrated in 20mM Tris-HCl pH 7.5 and elution was carried out with the same buffer. The protein profile of the fractions so obtained is shown in Fig. 10, and is typical of other G-200 fractionations which were done with F.P. SNs.

The results indicated little resolution, presumably because of the large number of proteins in F.P. SNs as was evident if samples were electrophoresed in non-denaturing or denaturing conditions (section 2.1.1 b). PAGE without SDS gave better resolution of the proteins in F.P. SN than G-200 chromatography, probably because in the former case separation is achieved on the basis of both size and charge differences (Hedrick and Smith, 1968).

Which G-200 fractions contained recall antigen(s) was not ascertained. Pressure ultrafiltration represented a more

Fig. 10

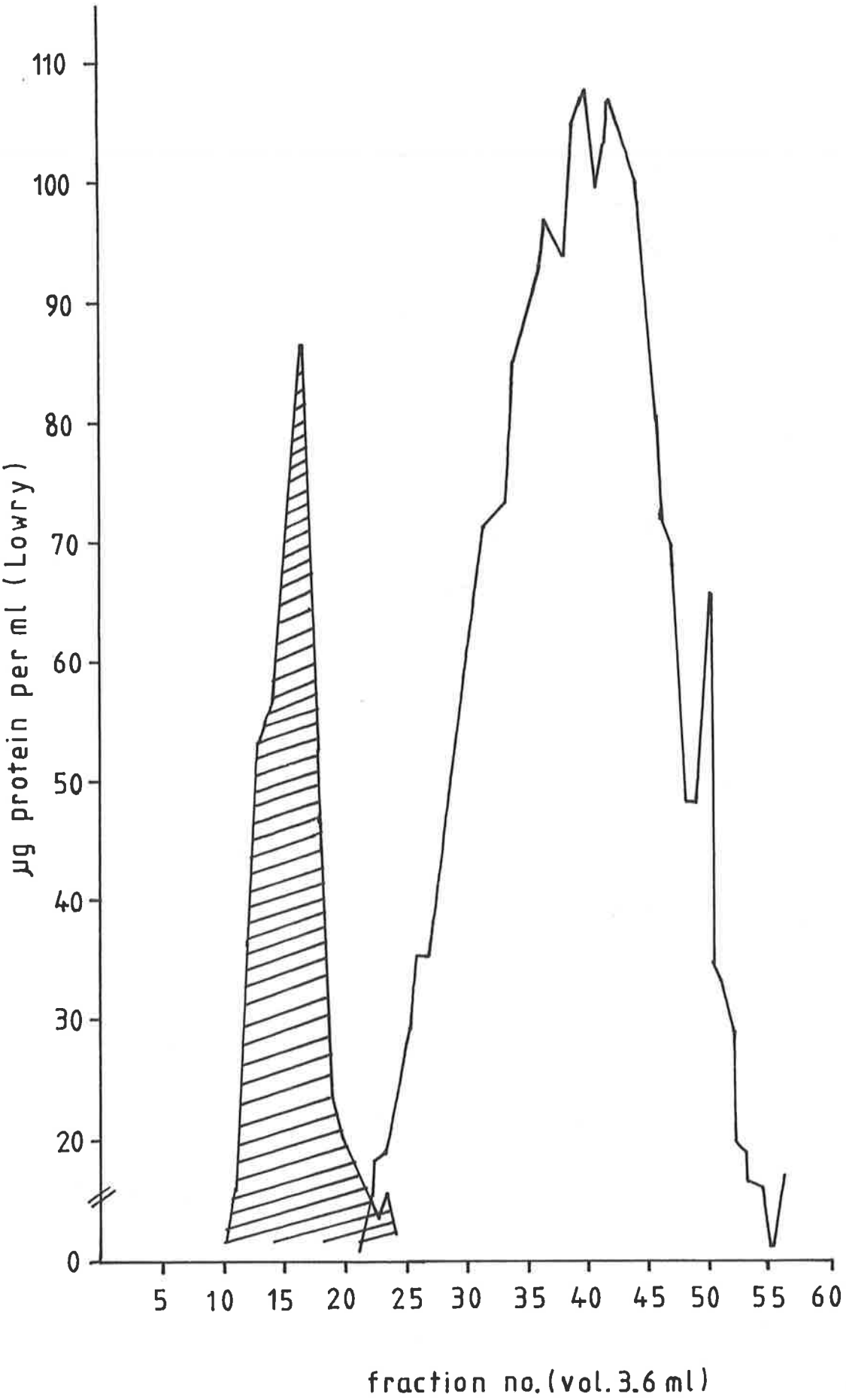
Sephadex G-200 chromatography

of F.P. SN, 11RX antigen

details given in text

shaded area = position of elution of blue dextran

(determined by O.D. 650_{nm})


110
100
90
80
70
60
50
40
30
20
0
µg protein per ml (Lowry)
5
10
15
20
25
30
35
40
45
50
55
60
fraction no. (vol. 3.6 ml)

rapid method for determining the molecular weight (m.wt.) with which recall activity was associated. Two batches of F.P. SN were filtered using a membrane which excluded material of less than 10,000 daltons (10K) m.wt. Seventy or 90% of the protein put into the pressure cell was recovered in the concentrate and filtrate. Five or 10% of the recovered protein was in the filtrate. Thus, although F.P. SNs were dialysed for 6 days prior to lyophilization they did still contain some low m.wt. protein.

Whether or not the F.P. SN 10K filtrate material could recall tumour resistance was assessed using the whole-body retention assay. Four μg was the dose injected per mouse, based on protein estimated with the Lowry method. In addition, 10 μg, or 100 μg by weight of the original F.P. SN were also administered to mice. There were 4 normal mice per group and 3 long-term immunized mice per group. The data shown in Table 15, day 1 ^{125}I retention, indicated that the filtrate facilitated tumour clearance in immunized mice to a degree comparable to that obtained using 10 μg or 100 μg of F.P. SN.

The association of F.P. SN recall activity with low m.wt. material again raised the question of how free these antigen preparations were of proteolytic degredation products (sections 2.1.1, 2.1.5). It was possible that fractionation of F.P. SN preparations was a pointless exercise if some fractions retained recall activity because they consisted of degredation products of other fractions. Consequently, DE-52: PSEs were used for the next experiments investigating the m.wt. with which recall activity was associated. In this way,

Table 15

Recall activity of 10K m.wt. filtrate of 11RX antigen, F.P. SN

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in whole mouse on day 1 (arith. mean ± S.E.)	
	Normal	[c] Immunized
saline 0.2ml	50.4 ± 3.4	33.9,34.2,32.6
F.P. SN, 10 µg	N.D.	19.0,13.6,13.7
100 µg	40.0 ± 0.8	13.7,14.9,15.2
[d] <10K filtrate, 4 µg	42.2 ± 2.1	8.8,12.0, 6.7
None: heat-killed EAT i.p. on day 0	6.2 ± 0.4	

[a] 10^6 ^{125}I-EAT i.p. on day 0

[b] mean of individual mouse counts, in the case of normal mice. Individual mouse counts shown for long-term immunized mice.

[c] 10^5 11RX i.v. day-104

[d] details given in text

the probability of degredation products confusing the results was at least reduced, even if not eliminated (section 2.1.6).

In the case of two DE-52: PSEs (from log phase cultures) filtered using a 10K m.wt. exclusion membrane, 1 or 5% of the protein in the extracts could be recovered in the filtrate. Like F.P. SN preparations, DE-52: PSEs had been dialysed during extraction. The Lowry method was used to measure protein during filtration of DE-52: PSEs.

One of the above DE-52: PSEs was also filtered through a 30K m.wt. exclusion membrane. After filtration with the 10K membrane, the concentrate was washed with buffer 4X and then the same procedure was carried out using the 30K membrane. The recovery of protein after filtration of DE-52: PSE through 10K and 30K membranes accounted for only 40% of the starting material, and 97% of this protein was of $>$30K m.wt. Some of the loss of material could probably be accounted for by that in the buffer washes after filtrations— the protein content of these was not determined. It was also possible that some of the protein absorbed to the membranes.

The filtrates were tested for recall activity using the <u>in vitro</u> cytotoxicity assay. Doses of 3-4 µg of protein per mouse were used in comparison with 4 µg of DE-52: PSE protein, and in one case 10 µg by weight of Batch II,11RX antigen preparation. Different batches of DE-52: PSEs were used in the experiments shown in Table 16. The results showed that material of $<$10K m.wt. from DE-52: PSEs had recall activity. In the case of Expt. 2, this material induced PEC which were considerably less cytotoxic for EAT than those obtained from mice treated with Batch II (21% and 56% cytolysis, respectively). Unfiltered DE-52: PSE was not assayed

Table 16

Recall activity of material of different m.wts. from 11RX antigen, DE-52: PSE

[a] PEC from mice given the following i.p. on day-2 :-	[b] % cytolysis at 20 hrs. (arith. mean ± S.E.)		
	Normal	[c] Immunized	
	Expt.2	Expt.1	Expt.2
buffer 0.2ml	0.4 ± 0.3	9.3 ± 2.7	8.3 ± 0.4
Batch II 10 µg	[f] 15.3 ± 1.5		55.7 ± 0.6
[d] DE-52:PSE 4 µg		22.5 ± 2.1	
[d] <10K filtrate 4 µg		23.0 ± 2.2	
3 µg	4.1 ± 0.9		20.5 ± 0.9
<30K filtrate 4 µg		34.2 ± 0.8	
>30K concentr 4 µg		24.2 ± 2.7	

[a] 4 mice per group

[b] mean from quadruplicate wells

[c] Expt.1 10^5 11RX i.v. day-104

Expt.2 10^5 11RX i.v. day-85

[d] details of DE-52:PSEs and filtration given in text.

[f] Footnote: Batch II appears to have "recalled" the normal mice. This will also be evident in other sections of the text. Rather than being a property of the 11RX antigen preparations used, this reflects the immune status of the normal mice, which was influenced by the condition of the animal house. Similar phenomena have been mentioned in the literature (e.g., Evans and Alexander, 1972).

in this experiment. It had been shown previously to be comparable to Batch II in recall activity when used at the 3 µg dose level (section 4.2). Thus from this experiment it appeared that the 10K filtrate was not enriched in recall antigen. However, in Expt. 1, DE-52: PSE, 10K filtrate, and 30K concentrate had comparable recall activity, at least at the dose tested. The 30K filtrate was more active by comparison (34% as c.f. 23-24% cytolysis).

As it had been found that low m.wt. material from DE-52: PSEs was able to recall tumour resistance, the question again arose of whether this was native or degraded material. It seemed desirable to determine the size distribution of recall antigen activity using 11RX extracts which had been handled as little as possible. For this reason, filtration experiments were done with freshly prepared high speed SNs. In two separate experiments, freshly grown 11RX from an overnight culture, or previously frozen 11RX from a log phase culture were disrupted using the French pressure cell. The 78,000 x g supernatants were obtained and kept at 4°C. Nucleases were not used, and the extracts were not dialysed. Particulate material was not removed—it did not form because high ionic strength buffer (0.1M Phosphate pH 7.2 or 7.7) was used for extraction and the filtrations were done at 4°C. High speed SNs were prepared, filtered, and injected into mice for assay of recall activity within one day. This procedure should have considerably minimized the chances of any of the proteins in the extracts being degraded by endogenous proteases.

The high speed SNs were filtered through 10K and 30K m.wt. exclusion membranes using the washing procedure

described previously. Protein was measured in the SNs, filtrates, and concentrates using the Lowry method. In the case of the experiment utilizing a high speed SN from an overnight culture of 11RX, the fact that the membranes did not exclude proteins of $>$10K or $>$ 30K m.wt. was verified using a solution of hemoglobin (m.wt. 63,000 daltons). A small amount, in terms of colour, was observed to be excluded by the 30K membrane. Presumably this was free heme. Forty % (c.f. DE-52: PSE filtrations) of high speed SN protein was recovered in filtrates and concentrates after filtration through both 10K and 30K membranes. For the high speed SN made from an overnight culture, 69%, 16% and 15% of the protein recovered was present in the 10K filtrate, 30K filtrate, and 30K concentrate, respectively. All of the carbohydrate (as estimated using the phenol-sulphuric acid method) was accounted for in the 10K filtrate. For the high speed SN from a log phase culture the above figures were 34%, 2%, and 64%, respectively.

These results provided confirmation that high speed SNs from 11RX not treated with nucleases contained a large proportion of low m.wt. protein (section 2.1.1). However, it appears from the above result that high speed SNs from overnight cultures contained more $<$10K m.wt. protein than dialysable protein (section 2.1.1). This may be so because ultrafiltration has a different selectivity to dialysis (Sober, Hartley, Carroll et al., 1965). In addition, the high speed SN from an overnight culture contained more $<$10K m.wt. protein than did the SN from the log phase culture. How representative were the above mentioned size distributions of proteins in high speed SNs was not established.

The filtrates and concentrates were assayed for recall activity using the peritoneal cavity retention assay. In

expt. 1 fractions from the high speed SN prepared from an overnight culture of 11RX were used at a dose level of 5 µg of protein per mouse. In expt. 2 fractions from the high speed SN prepared from a log phase culture were used at a dose level of 10 µg of protein per mouse. The day 1 ^{125}I retention data is shown in Table 17.

In both expts. 1 and 2, pre-immunized mice which had been injected with high speed SN, 10K filtrate, 30K filtrate, or 30K concentrate retained much less ^{125}I than the buffer injected controls. The % retained by the former groups of mice was comparable with that observed in normal mice given killed tumour cells. The high speed SNs and some of the filtrates contained the nucleic acid, carbohydrate, and ET fragments which are normally removed during the preparation of 11RX antigens (sections 2.1.2, 2.1.3). However, these extracts did not facilitate tumour clearance in normal mice to the same extent as they did in pre-immunized mice (Table 17). Non-specific anti-tumour effects can be ascribed to native ET molecules, which are not present in high speed SNs as they are found in the 78,000 x g pellet (section 5.1).

In conclusion, the results of the filtration experiments with DE-52: PSEs and high speed SNs suggested that more than one constituent of a particular size is able to recall tumour resistance in long-term immunized mice. Material of $<$10,000 daltons, between 10,000 and 30,000 daltons, and $>$30,000 daltons m.wt. originating from overnight or log phase cultures was shown to have recall activity. The conditions of the experiments with high speed SNs were such that little more could have been done in order to prevent any proteolytic

Table 17

Recall activity of material of different m.wts. from 11RX high speed SNs

[a] Treatment i.p. on day-2 :-	[b] % ^{125}I retained in peritoneal cavity of mouse on day 1 (arith. mean ± S.E.)			
	Normal		[c] Immunized	
	Expt.1	Expt.2	Expt.1	Expt.2
None	42.0 ± 4.5	N.D.	29.6 ± 4.3	N.D.
buffer 0.2ml	40.8 ± 3.3	64.8 ± 0.6	16.3,23.1,27.4	39.6 ± 6.2
[d] hi.sp. SN	20.0 ± 6.8	31.9 ± 9.9	[e] <1	10.6 ± 0.9
[d] <10K filt.	40.0,47.0,34.0	43.6 ± 6.1	<1	10.0 ± 0.3
<30K filt.	29.8 ± 1.7	32.5 ± 2.9	<1	11.1 ± 1.0
>30K concenr	9.5,6.0,17.8	46.3 ± 3.3	<1	12.9 ± 2.6
None, heat -killed EAT i.p. on day 0	<1	9.1 ± 0.1		

[a] 10^6 ^{125}I-EAT i.p. on day 0

[b] mean of individual mouse peritoneal cavity counts. 4-5 mice per group, except where individual %'s shown.

[c] Expt.1 10^5 11RX i.v. day-47.
Expt.2 10^5 11RX i.v. day-89.

[d] details of high speed SNs and filtrates in text.

[e] limit of detection

degredation occurring during extraction. Protease inhibitors were not used because it was desirable to keep the amount of extraneous material in the extracts to a minimum. As significant degredation probably does not occur during culture (ref. given in section 2.1.5), it seemed likely that the filtrates and concentrates were not cross-contaminated. Nevertheless, the origin of the large amount of low m.wt. protein in 11RX high speed SNs was not determined.

5. Anti-tumour effects of 11RX cell envelope components

Preamble

Therapy and prophylaxis of experimental animal tumours with ET and mycobacterial cell walls was discussed in the Introduction. These agents commonly induce anti-tumour effects in normal animals, in contrast to 11RX antigen preparations. High speed SN was chosen as the source of 11RX recall antigen in order to avoid ET contamination and thus any non-specific anti-tumour effects (I. Kotlarski-personal communication). However, 11RX cell envelope represented a source of some protein-rich components which were potential recall antigens. The next sections deal with the effects of 11RX ET and other cell envelope components on the cellular responses of normal and 11RX immunized mice. The assays used were those employed for the study of the recall and DTH phenomena.

5.1 11RX endotoxin

In initial studies, while looking for recall antigens, Ashley et al., (1974) obtained an extract termed 11RX ultrasonicate which was a low speed SN from ultrasonicated 11RX. Intraperitoneal injection of 100 µg of this material into normal mice 1 day prior to challenge with 10^6 EAT i.p. prolonged the survival of the mice from approx. 20 to 50 days. The extract was highly protective for long-term 11RX immunized mice challenged with EAT, prolonging their survival from approx. 30 to 90+ days. These workers postulated that the anti-tumour effect induced in normal mice by 11RX ultrasonicate was due to the presence of 11RX ET in the extract.

This was confirmed in this project when the anti-tumour effects of purified ET from 11RX were investigated. ET was

prepared by either the phenol-water or the Galanos techniques.

Two or 10 µg of 11RX ET facilitated the clearance of EAT in normal or pre-immunized mice, as assessed using either the ^{125}I-EAT whole-body retention assay or the ^{51}Cr-EAT peritoneal cavity retention assay (Table 18 a and b). ET from the smooth strain S.typhimurium C5 had a similar anti-tumour effect to that of rough ET from 11RX (Table 18b). Batch L 11RX antigen did not affect tumour retention if administered to normal mice showing that the ETs did not elicit a recall effect because the normal mice had become infected (Table 18b).

Although the anti-tumour effects of 11RX ET were demonstrable in vivo, i.p. administration of 11RX ET to normal or 11RX immunized mice did not render the peritoneal cells highly cytotoxic for EAT in vitro. The results of experiments which utilized the in vitro cytotoxicity assay are shown in Table 19. The normal mice used in Expts. 1, 3 and 4 must have been naturally infected, as administration of 11RX antigen preparations to these mice induced PEC cytotoxic for EAT (see footnote Table 16, section 4.4). When PEC were taken from mice given 10 µg of 11RX ET or C5 ET 2 or 3 days previously the cytolysis observed was either not significantly different to the control value (PEC from saline treated mice) or was 2-5 fold less in magnitude in comparison to the cytolysis demonstrated with PEC from mice given 11RX antigen preparations.

Thus, as judged on the basis of results obtained from either in vivo or in vitro assays, the anti-tumour effects

Table 18

The anti-tumour effects of 11RX ET -assessed with in vivo ^{125}I and ^{51}Cr retention assays

a.

* Treatment i.p. on day-1 :-	[c] % ^{125}I retained in whole mouse on day 3	
	Normal	[e] Immunized
saline 0.2ml	41	22
11RX ET 2 µg	19	5
None: heat-killed EAT i.p. on day 0	4	

b.

* Treatment i.p. on day-2:-	[d] % ^{51}Cr retained in peritoneal cavity of mouse at 15 hrs. (arith. mean ± S.E.)	
	Normal	[e] Immunized
saline 0.2ml	29.4 ± 4.2	26.7 ± 2.4
Batch L 10 µg	35.3 ± 1.3	6.2 ± 1.2
11RX ET 10 µg	12.2 ± 1.0	10.1 ± 1.6
C5 ET 10 µg	9.2 ± 1.1	5.9 ± 1.6

* 10^6 ^{125}I[a] or ^{51}Cr[b]-EAT i.p. on day 0

[c] average figure obtained from counting mice in groups in Armac counter. 5 mice per group.

[d] mean of individual mouse peritoneal cavity counts 5 mice per group.

[e] 10^5 11RX i.v. day-50[a]
10^5 11RX i.v. day-78[b]

Table 19 (continued on next page)

The anti-tumour effects of 11RX ET

-assessed by the in vitro cytotoxicity assay

[a] PEC from mice given the following i.p. on day-2 :-	[b] % cytolysis (arith. mean ± S.E.)			
	Normal		[d] Immunized	
	Expt.1	Expt.2	Expt.1	Expt.2
saline 0.2ml	0.4 ± 0.3	-0.5 ± 0.4	8.3 ± 0.4	0.10 ± 0.4
[c] 11RX antigen	15.3 ± 1.5	0.2 ± 0.4	55.7 ± 0.6	10.9 ± 0.8
11RX ET 10 µg	3.8 ± 0.9	-0.7 ± 0.8	3.2 ± 0.9	3.2 ± 0.6

[a] 4-5 mice per group

[b] Expt.1 20 hrs. Expt.2 8 hrs.
mean from quadruplicate wells

[c] Expt.1 Batch II 10 µg
Expt.2 Batch L 10 µg

[d] Expt.1 as used in Table 16 Expt.2
Expt.2 10^5 11RX i.v. day-64

..... Table 19 ctd.

[a] PEC from mice given the following i.p.	% cytolysis at 20 hrs.	
	[b] Normal	
	Expt.3	Expt. 4
saline 0.2ml	6.0 ± 1.5	2.4 ± 0.4
Batch L 10 µg	29.4 ± 2.5	12.7 ± 0.9
11RX ET 10 µg	13.0 ± 1.5	4.2 ± 0.3
[d] C5 ET 10 µg	10.8 ± 1.0	2.9 ± 0.1
[c] 11RX carrier mice		30.8 ± 1.6
[c] recalled 11RX mice		27.2 ± 1.3

[a] 4-5 mice per group
Expt. 3 treatment i.p. day-2
Expt. 4 treatment i.p. day-3

[b] see footnote Table 16

[c] included in Expt.4 as positive controls
10^5 11RX i.p. day-7 or
10^5 11RX i.v. day-90 and 10 µg Batch L i.p. day-3

[d] ET from S.typhimurium C5

of isolated 11RX ET were distinguishable from those of 11RX antigen preparations.

A further difference between the biological properties of 11RX ET and 11RX antigen preparations was evident when the reaction which followed the injection of 11RX ET into mouse footpads was observed. 1 µg or 10 µg of 11RX ET induced a swelling in the footpads of normal mice which became apparent at 4 hours and was still evident 96 hours later (Table 20). The degree of swelling was similar over the 24 hour-96 hour period. Batch L did induce some swelling in normal mice, but this was maximal 4 hours after injection and was negligible by 24 hours (Table 20). Both Batch L and 11RX ET induced an increase in thickness of the footpads of 11RX carrier mice, but in the case of ET the feet were still swollen at 96 hours, again indicating that the reactions induced by 11RX ET were not related to true DTH (Table 20, Nelson and Boyden, 1964).

In summary, when given i.p. to normal or pre-immunized mice, 11RX ET facilitated the clearance of an i.p. challenge of EAT. The <u>in vitro</u> cytolytic effect against EAT target cells demonstrable with PEC from mice given 11RX ET was small and variable compared with that obtained with PEC from recalled mice. Injection of 11RX ET, but not 11RX antigen, into normal mouse footpads resulted in a swelling which was still prominent 4 days later.

These findings indicate that 11RX ET, in itself, cannot be the moiety of 11RX antigen preparations which is responsible for recall or DTH responses elicited in 11RX immunized mice. However, the possibility that the active moiety[ies] of 11RX antigen is composed of an ET component associated

Table 20

A comparison of the footpad swelling in normal and 11RX carrier mice following the injection of 11RX ET or 11RX antigen, Batch L

[a] Treatment in L.H.f.p.[c] at 0 hrs. :-	% Increase in footpad thickness (arith. mean ± S.E.)			
	4 hrs.	24 hrs.	48 hrs.	96 hrs.
Normal				
1 Batch L[d]	10.9 ± 1.7	0.5 ± 0.4	2.6 ± 1.1	N.D.
1 11RX ET 10 µg	7.1 ± 1.9	58.4 ±11.1	54.9 ± 7.7	34.8 ± 3.8
2 11RX ET 1 µg	29.5 ± 3.3	11.2 ± 1.0	10.9 ± 2.5	14.5 ± 2.7
[b] Immunized				
1 Batch L[d]	11.5 ± 0.4	28.4 ± 6.9	14.9 ± 4.3	2.7 ± 1.1
1 11RX ET 10 µg	0.7 ± 0.6	67.4 ±12.1	73.2 ± 4.5	42.5 ± 5.1
2 11RX ET 1 µg	16.0 ± 2.8	45.1 ± 6.3	30.9 ± 4.7	13.0 ± 2.6

[a] 5 mice per group in Expt.1
10 mice per group in Expt.2

[b] 10^5 11RX i.v. day-4 (1)
10^5 11RX i.v. day-11 (2)

[c] f.p. = footpad

[d] dose 10 µg

with protein, or that such a molecule has recall potential, has not been excluded by the data presented in this text. Obviously such an ET component would not possess the properties of native ET.

5.2 11RX envelope, Triton-insoluble wall, and Triton-soluble envelope

Preamble

As the nomenclature used to describe the components of the cell envelopes from gram-negative bacteria may vary, that used in this text is given below.

The envelopes consist of 3 layers. The outermost is termed outer membrane and contains ET, phospholipid and protein. Braun's lipoprotein probably fixes the outer membrane to the middle layer of murein (peptidoglycan). The murein and outer membrane are collectively termed the cell wall. Innermost is the cytoplasmic membrane, which consists of protein and phospholipid and is separated from the cell wall by the periplasmic space. The mode of attachment of the murein to the cytoplasmic membrane is uncertain (Schnaitman, 1971a and b; Braun, 1975).

Schnaitman (1971a) established that the cell wall proteins of E.coli O111 are insoluble in Triton X-100 at room temperature, whereas cytoplasmic membrane proteins are solubilized. In addition, morphological evidence from electron micrographs indicates that the entire cytoplasmic membrane is soluble in Triton X-100 (Schnaitman, 1971a). Schnaitman (1971b) also found that a large proportion of the cell wall phospholipid and ET from E.coli O111 is solubilized by Triton X-100. Hence, at least in the case of E.coli O111, Triton-

insoluble wall (TIW) consists of cell wall proteins, murein, cell wall phospholipid and ET. Triton-soluble envelope (TSE) contains cytoplasmic membrane, and some cell wall phospholipid and ET.

Schnaitman (1974a) also found that the cell wall proteins, but not the cytoplasmic membrane proteins, of Salmonella typhimurium and several Shigella are insoluble in Triton X-100. The Triton extraction procedure was employed in this project to investigate the protein composition of 11RX antigen particulate material (see section 2.1.7). In addition, it was of interest to determine if 11RX TIW or 11RX TSE had recall activity, and the results are described below.

Results

Samples of 11RX envelope components were subjected to PAGE in the presence of SDS. Fig. 11 shows the protein profiles of gels of 11RX TIW and 11RX TSE. As the cell envelope of E.coli K12 has been extensively characterized, E.coli K12 strain P400 envelopes were also extracted with Triton for the purposes of comparison. The protein profiles of P400 TIW and P400 TSE are also shown in Fig. 11. P400 TIW (No. 1) has the 4 major proteins a, b, c, d in the 40K region which have been shown to be characteristic of E.coli K12 outer membrane (Lugtenberg et al., 1975). 11RX TIW (No. 5) appears to have only 3 proteins in this region, as is the case for S.typhimurium G30 (Lugtenberg, Bronstein, van Selm et al., 1977).

The ET and phosphorus content by weight of 11RX envelope, 11RX TIW, and 11RX TSE are shown in Table 21. 11RX TIW is much richer in ET than 11RX TSE. This contrasts with the data in Schnaitman's (1971b) paper which indicated that Triton

Fig. 11

Slab PAGE, with SDS, of

11RX and E.coli P400

envelope extracts

1. P400 TIW
2. P400 TSE: 1st extraction only
3. 11RX TSE: 2nd extraction only
4. P400 envelope
5. 11RX TIW
6. 11RX TIW
7. 11RX envelope
8. 11RX TSE: 1st extraction only

Direction of migration: top to bottom

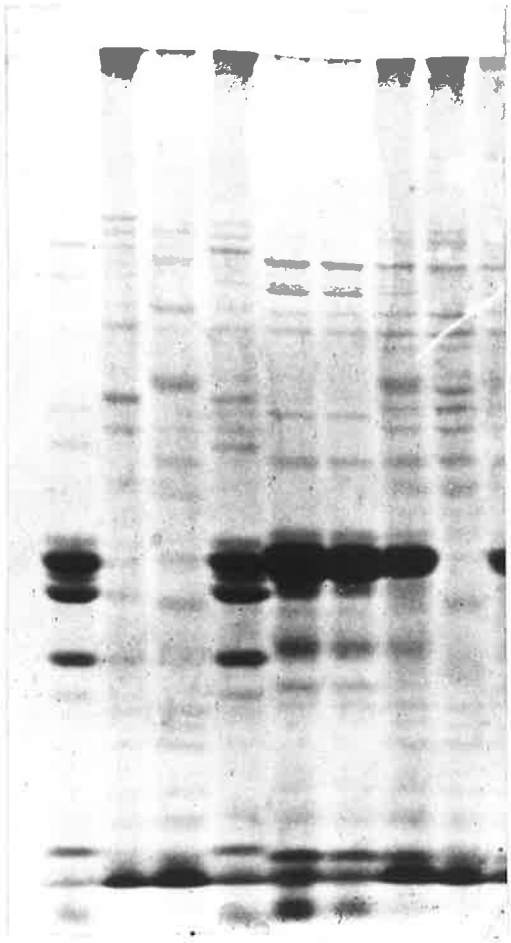

ca.40K
1. 2. 3. 4. 5. 6. 7. 8.

Table 21

Phosphorus and ET content

of 11RX cell envelope extracts

Extract	[a] % P	[a] % ET
11RX envelope	[b] 1.6	>12.8
11RX TIW	1.2	>14.1
11RX TSE:-		
1st. extraction	1.0	1.2
2nd. extraction	1.3	4.1

[a] by weight. Total P. Not lipid associated.

[b] see also Table 3, done on different extracts.

solubilized a large proportion of the ET in E.coli 0111 (J-5) envelopes. This may be due to the fact that 11RX ET is rough, whereas J-5 was synthesizing smooth ET under the conditions used by Schnaitman. Whether Triton solubilized 11RX cell wall phospholipid was not determined.

The 11RX envelope extracts were tested for their ability to enhance the clearance of an i.p. challenge of EAT in normal and 11RX immunized mice. Ten µg of each extract (based on dry weight) was injected i.p. 2 days prior to challenge with ^{125}I-EAT. Lowered whole-body ^{125}I retention on day 2 relative to the saline treated control mice was taken as an indication of protection from tumour growth.

The results, shown in Table 22, indicated that 11RX TSE had a similar effect to Batch L because it "recalled" long-term immunized mice, and had relatively little effect on EAT clearance in normal mice. On the other hand, 11RX envelope and 11RX TIW facilitated labelled tumour clearance in both normal and immunized mice, presumably because they contained a lot of ET (Table 21, see also section 5.1).

However, 11RX TIW was shown to have "recall" activity in experiments which utilized the in vitro cytotoxicity assay. Intraperitoneal injection of 10 µg of 11RX TIW (by weight) into immunized mice, but not normal mice, induced PEC cytotoxic for EAT (Table 23). The observed cytolysis was comparable to that obtained with PEC from mice recalled with Batch L, and was 4 fold higher than that demonstrated using PEC From immunized mice given 10 µg of 11RX ET (Table 23). Thus, the data showed that 11RX TIW could mimic the recall phenomenon in vitro, presumably because it contained material with properties similar to those of 11RX antigen.

Table 22

Anti-tumour effects of 11RX envelope extracts - assessed the whole-body retention assay

[a] Treatment i.p. on day-2:-	[b] % ^{125}I retained in whole mouse on day 2 (arith. mean ± S.E.)	
	Normal	[e] Immunized
saline 0.2ml	58.5 ± 4.4	50.8 ± 5.2
[c] Batch L	41.9 ± 4.9	11.0 ± 1.7
11RX envelope	7.5 ± 0.7	13.5 ± 2.8
11RX TIW	11.4 ± 1.3	14.8 ± 2.7
[d] 11RX TSE	32.8 ± 5.7	8.2 ± 1.4
None, heat-killed EAT i.p. on day 0	9.3 ± 1.2	

[a] 10^6 ^{125}I-EAT i.p. on day 0

[b] mean of individual mouse counts. 5 mice per group.

[c] dose of extracts = 10 µg

[d] 1st. Triton extraction

[e] 10^5 11RX i.v. on day-60

Table 23

Anti-tumour effects of 11RX envelope extracts - assessed by the in vitro cytotoxicity assay

[a] PEC from mice given the following i.p. on day-2:-	[b] % cytolysis at 8 hrs. (arith. mean ± S.E.)	
	[e] Normal	[e] Immunized
saline 0.2ml	-0.5 ± 0.4	0.1 ± 0.4
[c] Batch L	0.2 ± 0.4	10.9 ± 0.8
11RX TIW	0.7 ± 0.3	14.2 ± 0.8
[d] 11RX TSE	4.8 ± 0.6	23.0 ± 1.2
11RX ET	-0.7 ± 0.8	3.2 ± 0.6

[a] PEC pooled from groups of 4 mice

[b] mean from quadruplicate wells

[c] [d] as in Table 22

[e] as in Expt.2, Table 19

The cytolysis obtained with PEC from long-term immunized mice given 10 µg of 11RX TSE (by weight) was much higher than that obtained using PEC from similarly treated normal animals (Table 23). Thus, the specificity evident in the *in vivo* anti-tumour effects of 11RX TSE was also apparent *in vitro*. However, the cytolysis demonstrable with PEC from 11RX TSE treated normal mice was greater than that observed using Batch L induced normal PEC, presumably because the *in vitro* "recall" effect of 11RX TSE was also of greater magnitude than that of Batch L.

The 11RX envelope extracts were injected into the footpads of normal and 11RX immunized mice and the changes in footpad thickness were monitored. The results are shown in Table 24. 11RX envelope, 11RX TIW, and 11RX TSE induced more swelling in the feet of 11RX carrier mice than in the feet of normal mice. However, 11RX TIW and 11RX envelope, but not 11RX TSE or Batch L, induced swelling in the normal mouse footpads which was apparent 24 hours after injection. However, this non-specific response was small in comparison to that induced by isolated 11RX ET (see section 5.1) although 11RX TIW and 11RX envelope contain ET. The findings were similar when different batches of 11RX TIW and 11RX envelope were injected at a dose of 10 µg by weight in direct comparison to 5 µg of 11RX ET (data not shown). Nevertheless, the footpad reactions elicited by 11RX TIW or 11RX envelope in normal or immunized mice resembled Arthus reactions not DTH.

Although 11RX TSE did mimic the specificity of Batch L, the reaction it elicited in the footpads of immunized mice was not similar to DTH as the swelling was maximal at 4 hours

Table 24

Changes in footpad thickness following the intrafootpad injection of 11RX envelope extracts

Treatment in L.H.f.p. at 0 hrs.:-	[a] % Increase in footpad thickness (arith. mean ± S.E.)			
	4 hrs.	24 hrs.	48 hrs.	72 hrs.
Normal				
saline 0.02ml	3.1 ± 1.5	0.1 ± 0.1	0.2 ± 0.2	1.0 ± 0.7
[c] Batch L	4.1 ± 1.5	0.2 ± 0.2	0.2 ± 0.1	2.1 ± 1.2
11RX envelope	29.0 ± 3.2	5.2 ± 1.7	2.1 ± 0.7	2.2 ± 0.7
11RX TIW	28.5 ± 0.9	11.3 ± 2.8	3.1 ± 1.6	2.9 ± 1.1
[d] 11RX TSE	21.9 ± 1.1	1.4 ± 0.7	2.2 ± 1.1	2.0 ± 0.6
[b] Immunized				
saline 0.02ml	4.2 ± 1.1	0.9 ± 0.6	0.4 ± 0.2	N.D.
Batch L	13.5 ± 3.0	9.3 ± 2.8	13.5 ± 2.2	11.6 ± 2.8
11RX envelope	37.7 ± 4.3	29.5 ± 5.5	27.6 ± 3.5	14.6 ± 2.0
11RX TIW	36.4 ± 1.9	38.0 ± 6.2	22.5 ± 4.1	12.3 ± 4.2
11RX TSE	23.8 ± 3.0	12.2 ± 2.0	10.0 ± 1.2	8.3 ± 1.6

[a] 5 mice per group

[b] 10^5 11RX i.v. on day-7

[c] as in Table 22

[d]

after injection (Table 24). In summary, neither 11RX envelope, 11RX TIW, or 11RX TSE induced a footpad response which was of similar characteristics to that elicited by 11RX antigen preparations.

In conclusion, the in vitro experiments with 11RX TIW indicated that 11RX cell wall contains recall antigen(s). In other assays the anti-tumour effects of 11RX TIW could not be distinguished from the non-specific effects of ET. 11RX TSE contains recall antigen(s) as judged with in vivo or in vitro tests for recall of tumour resistance. 11RX TSE was not fully characterized, but the evidence in the literature indicates that it probably contains cytoplasmic membrane and cell wall components (see beginning of section). Whether the recall activity of 11RX TSE is due to cytoplasmic membrane or cell wall material was not determined.

5.3 SDS cell wall extract

To investigate the recall potential of cell wall components, preparations free of ET are essential. With this in mind, log phase cultures of 11RX were disrupted in the French pressure cell and the low speed and high speed pellet material was collected by centrifugation and boiled in SDS. Under these conditions the cell wall murein-lipoprotein complex remains insoluble, but other cell components are solubilized (Braun, 1975). Two batches of insoluble SDS cell wall extract of 11RX contained 2.9 or 4% ET by weight and 0.7 or 0.6% Phosphorus by weight.

The two SDS cell wall extracts were tested for recall activity using the whole-body retention assay. The dose of extracts used was 10 µg by weight. In one experiment mouse survival was also followed. The results, shown in

Table 25, indicated that the SDS extracts "recalled" tumour resistance in long-term immunized mice, but had little effect on the retention of ^{125}I-EAT in normal mice. The anti-tumour effect was similar to that of 11RX antigen Batch L.

One of the SDS extracts was injected into the footpads of 11RX carrier mice and normal mice. As was the case if the mice were given Batch L, the swelling apparent over the 24-48 hour period occurred only in the infected mice and was largely diminished by 96 hours (Table 26). Thus it appeared that 11RX SDS cell wall extracts could mimic the recall effect and, in addition, elicit a DTH response in immunized mice.

The composition of 11RX SDS cell wall extract was not determined. Braun and colleagues have shown that E.coli, S.typhimurium, S.minnesota and S.usumbura all contain complexes of murein with covalently bound lipoprotein (Braun and Rehn, 1969; Braun, Rehn, and Wolff, 1970). In most Enterobacteria the murein layer is also closely associated with non-covalently bound major outer membrane proteins of approx. 40,000 daltons m.wt. (Lugtenberg et al., 1977). These proteins will usually remain cross-linked with murein at 60°C in SDS, but will be dissociated at 80°C-100°C (Lugtenberg et al., 1977). However, in some cases, the 100°C SDS insoluble extracts are contaminated with these outer membrane proteins (Braun, 1975). Hence, the 11RX SDS cell wall extracts may have contained some outer membrane proteins as well as murein-lipoprotein.

Table 25

Anti-tumour effects of

11RX SDS cell wall extracts

[a] Treatment i.p. on day-2:-	[b] % ^{125}I retained in whole mouse on day 2 (arith. mean ± S.E.)	
	Normal	[d] Immunized
Expt.1		
saline 0.2ml	51.8 ± 4.8 [c] ($^0/_4$)	49.6 ± 4.8 [c] ($^0/_5$)
Batch L 10 µg	39.5 ± 6.6 ($^0/_5$)	11.7 ± 1.1 ($^5/_5$)
[e] SDS cell wall extract 10 µg	36.7 + 5.7 ($^0/_5$)	8.9 + 0.5 ($^5/_5$)
Expt.2		
saline 0.2ml	61.3, 38.5, 42.4	35.4 ± 5.9
[e] SDS cell wall extract 10 µg	32.3 ± 3.4	11.2 ± 2.7
[f] SDS cell wall extract 10 µg	43.3 ± 6.3	13.3 ± 7.6

[a] 10^6 ^{125}I-EAT i.p. on day 0

[b] mean of individual mouse counts.
4-5 mice per group, except where retention of 3 mice is recorded individually in Expt.2

[c] proportion of mice alive on day 40

[d] 10^5 11RX i.v. on day-55 (Expt.1)
10^5 11RX i.v. on day-92 (Expt.2)

[e] 4% ET, 0.6% P

[f] 2.9% ET, 0.7% P

Table 26

Characteristics of the footpad reaction following the injection of 11RX SDS cell wall extract

Treatment in L.H.F.p. at 0 hrs.:-	[a] % Increase in footpad thickness (arith. mean ± S.E.)			
	4 hrs.	24 hrs.	48 hrs.	96 hrs.
[b] Normal				
[c] Batch L SDS cell wall extract	10.9 ± 1.7	0.5 ± 0.4	2.6 ± 1.1	N.D.
	22.3 ± 3.3	0.2 ± 2.2	0.1 ± 0.1	N.D.
[b] Immunized				
Batch L SDS cell wall extract	11.5 ± 0.4	28.4 ± 6.9	14.9 ± 4.3	2.7 ± 1.1
	7.9 ± 2.7	48.9 ± 13.6	23.2 ± 6.4	4.9 ± 1.7

[a] 5 mice per group

[b] as in Table 20(1), section 5.1

[c] dose of extracts = 10 µg

5.4 Conclusions

There were at least 5 sources of recall antigen(s): soluble 11RX antigens, particulate 11RX antigens, 11RX TIW, 11RX TSE and 11RX SDS cell wall extracts. Data was presented which indicated that high speed SNs or soluble 11RX antigens (DE-52: PSEs) contained more than one recall antigen (section 4.4). Furthermore, although most of the above extracts probably share components, it is likely that some are different in composition to others, e.g., 11RX TSE and 11RX SDS cell wall extract. This confirms the possibility that there is more than one recall antigen.

6. Recall antigen(s) in Enterobacteria other than 11RX

Preamble

Intraperitoneal immunization with Salmonella strains other than 11RX will increase the resistance of mice to subsequent i.p. challenge with EAT (S.H. Neoh, unpublished results). The criterion taken as an indication of resistance to tumour growth was as follows: LACA strain mice were injected i.p. with 10^5 bacteria and 7 days later were challenged i.p. with 10^6 EAT. The percentage of mice alive on day 28 after challenge, when all the control mice were dead, provided an index of protection. Forty-five out of 86 Salmonella strains screened in this survey protected 50% or more of the mice against tumour challenge. 11RX protected 80% of the mice. The properties of protective strains are currently being examined. It appears that the number of bacteria recovered from the tissues on day 7 is not related to the induction of resistance.

It was of interest to determine if various bacteria other than 11RX contained recall antigen(s), and to include in the strains used one found to be non-protective in the above study. Davies (1975) had established that extracts from Enterobacteria, made in the same way as Batch II, 11RX antigen, elicited DTH reactions in 11RX immunized mice. These extracts were made from S.typhimurium C5, S.adelaide, S.minnesota 218S, S.enteriditis Se 795, E.coli BV, Citrobacter, and V.cholera 569B. Batch II and these preparations were similar in protein composition, as judged by PAGE without SDS (S.H. Neoh, unpublished results).

In the strain survey mentioned above, it was found that S.adelaide was protective and Citrobacter and 218S were non-protective. BV and 569B were not tested, and 795 and C5 are lethal for LACA mice at a dose of 10^5 organisms. Thus, data was available which indicated that the ability of an enterobacterial strain to protect mice against EAT growth was not related to the possession of antigens able to elicit DTH responses in 11RX immunized mice. This finding is similar to a report of Collins and Mackaness (1968), in the field of anti-microbial rather than anti-tumour immunity. These workers found that although S.pullorum was a rich source of antigens for eliciting DTH responses in mice infected with various Salmonellae, immunization with live S.pullorum was relatively ineffective in protecting mice against challenge with the virulent organism S.enteriditis SM^R.

Results

Antigen preparations were made from the following bacteria in the manner used for F.P. SN, 11RX antigen preparations: S.typhimurium M206, S.typhimurium C5, S.enteriditis 795, S.greenside 050, S.haarlem 09, and E.coli BV. In the strain survey, the protection index for S.greenside 050 was 10%, and for S.haarlem 75%. The extracts were stored frozen at concentrations of 1-2mg/ml of protein, based on measurements obtained using the Lowry method. Their dry weight was not determined. The carbohydrate to protein ratio of the extracts, expressed as a percentage, ranged from 1.9-3.2%.

Fig. 12 shows protein profiles of SDS PAGE of these extracts in comparison to an F.P. SN, 11RX antigen preparation. Although it is evident that the amounts of certain protein

Fig. 12

Slab PAGE, with SDS, of

P.aeruginosa OEP and

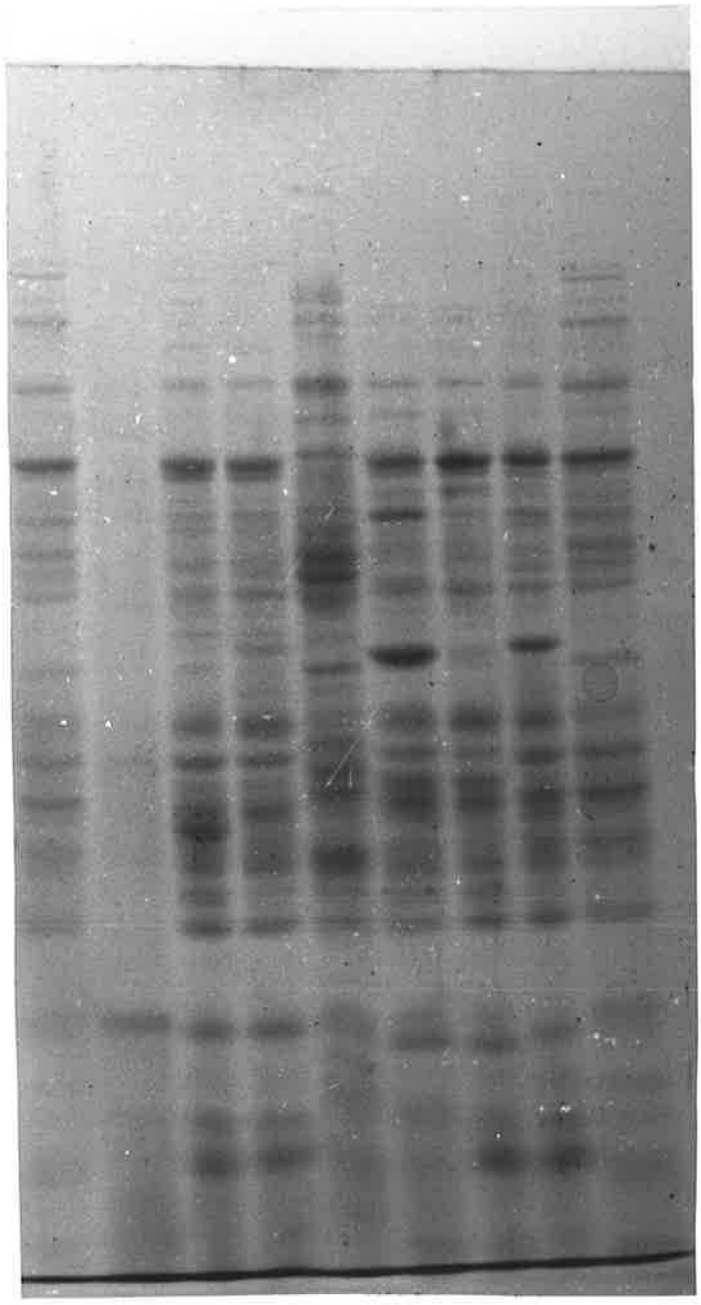
F.P. SN extracts from selected

Enterobacteria

1. 11RX F.P. SN
2. P.aeruginosa OEP
3. S.typhimurium M206 F.P. SN
4. S.typhimurium C5 F.P. SN
5. E.coli BV F.P. SN
6. S.greenside 050 F.P. SN
7. S.haarlem 09 F.P. SN
8. S.enteriditis Se 795 F.P. SN
9. 11RX F.P. SN

1., 9., same batch of 11RX F.P. SN

Direction of migration: top to bottom



1. 2. 3. 4. 5. 6. 7. 8. 9.

bands are variable between extracts, they are very similar in protein composition. This is the case for the preparations from S.haarlem (No. 7) and S.greenside (No. 6).

OEP is also shown in Fig. 12 (No. 2). This extract is "original endotoxin protein" from the ET of Pseudomonas aeruginosa (Homma, 1968). It was kindly provided by Dr. J.Y. Homma. OEP appears to be a pure protein as judged by PAGE. However, Hoshi, Kanzawa, Kuretani et al., (1972) reported that OEP contained 85% protein and 4.1% sugars, the latter as assessed by gas chromatography. In this project, the OEP sample used contained 8.4% carbohydrate, where the carbohydrate is expressed as a % of dry weight.

Hoshi et al., (1972, 1973) showed that administration of doses of the order of 0.10-0.80 µg/mouse of OEP to mice resulted in inhibition of the growth of i.p. inoculated EAT or Sarcoma 180 cells. It is likely that OEP elicited a recall effect in the mice used by these workers since Pseudomonas could be recovered from their intestinal tracts (J.Y. Homma to I. Kotlarski, personal communication).

The recall activity of the enterobacterial extracts was assessed using the day 1 ^{125}I-EAT whole-body retention assay. The dose of extracts administered was 10 µg of protein per mouse, except in the case of OEP which was given at a dose of 10 µg by weight. Either 11RX antigen Batch L or F.P. SN was used as a positive control, at a dose of 10 µg by weight. The mice used were long-term 11RX immunized mice, and normal mice. The results, shown in Table 27, are the combined data from 2 or 3 experiments for each extract, except in the case of the S.greenside preparation. In the latter instance, the extract

Table 27

Recall activity of OEP and F.P. SN type extracts from selected Enterobacteria

[a] Treatment i.p. on day-2:-	[b] % ^{125}I retained in whole mouse on day 1 (arith. mean ± S.E.)	
	Normal	[c] Immunized
none	46.4 ± 2.6	N.D.
saline 0.2ml	55.8 ± 3.4	43.8 ± 2.6
Batch L	40.1 ± 7.6	10.1 ± 1.4
11RX F.P. SN	51.7 ± 2.9	18.1 ± 2.6
P.aeruginosa OEP	48.4 ± 4.1	10.3 ± 1.2
F.P. SNs from:-		
S.typhimurium M206	47.1 ± 4.2	22.8 ± 2.8
S.typhimurium C5	46.1 ± 4.8	19.0 ± 4.1
S.enteriditis Se 795	42.4 ± 2.7	16.7 ± 2.3
S.greenside 050	40.0 ± 1.7	11.6 ± 3.1
S.haarlem 09	50.8 ± 3.2	15.6 ± 1.9
E.coli BV		
None: heat-killed		
EAT i.p. on day 0	15.2 ± 3.2	

[a] doses given in text; 10^6 ^{125}I-EAT i.p. on day 0

[b] mean of individual mouse counts
5-6 mice per group
combined results of 2 or 3 experiments

[c] mice used had received either:-
10^5 11RX i.v. on day-59, -87, or -103

was re-tested using the ^{51}Cr peritoneal cavity retention assay, and similar results were obtained (data not shown.) The data indicates that all the preparations could recall tumour resistance in immunized mice, but had no effect on tumour clearance in normal mice.

Thus, recall antigen(s) is not unique to 11RX. In addition, it is evident that S.greenside contains recall antigen(s), although immunization with live S.greenside did not protect mice against EAT challenge, as determined in the strain survey experiments. The extracts were not tested for their ability to elicit DTH reactions in 11RX immunized mice. Of particular interest was the finding that OEP had recall activity. Although not completely pure, it is considerably less heterogeneous than the other preparations (11RX antigen, 11RX TSE etc.) used during this project. It is not of high speed SN origin but is from the cell wall of P.aeruginosa. According to Homma (1968) it is not the protein component of Boivin-type antigens, as these can be separated from OEP during the extraction process.

6.1 Immunization and recall with S.typhimurium M206

S.typhimurium M206 is an attenuated smooth strain which can be used to immunize mice against the virulent strain S.typhimurium C5 (Jenkin, Rowley, and Auzins, 1964). In later studies it was found that M206 was much less effective than 11RX in inducing resistance to challenge with the virulent strain S.enteriditis Se 795 (Rowley, Auzins, and Jenkin, 1968). Ashley (1976) showed that i.p. immunization with 10^5 M206 was relatively ineffective compared to 11RX immunization in protecting mice against i.p. challenge with EAT. M206 was

not screened in the strain survey mentioned in the previous section.

The assays for recall used in this project involve keeping mice immunized i.v. with 11RX for 40-50 days so that their cellular immunity declines sufficiently for a recall effect to be clearly demonstrable. The above reports indicated that M206 induces a less "active" immunity than 11RX, and consequently that mice given M206 i.v. could probably be recalled only a short period after immunization. In order to determine if this was the case CB6F1 mice were immunized i.v. with 1.2×10^7 M206 organisms (LD_{50} approx. $8-9 \times 10^7$ organisms). Twenty-one days later no M206 could be recovered from the liver, spleen, or peritoneal cavity of these mice. Thus, in comparison to 11RX, i.v. injected M206 was rapidly cleared from the tissues of the CB6F1 mice (11RX data in Ashley, 1976).

Whether it was possible to recall tumour resistance in M206 immunized mice was assessed using the whole-body retention assay and the in vitro cytotoxicity assay. Ten µg (based on protein) of the M206 SN antigen extract was injected i.p. into normal mice or mice given 1.2×10^7 M206 i.v. 20 or 23 days previously. The results are shown in Table 28. The data indicated that it was possible to demonstrate an anti-tumour effect in the immunized mice given the extract. Hence, the M206 organisms must have induced sufficient sensitization for a recall effect to be elicited. Possibly, M206 treated mice could be recalled earlier than 20 days after immunization. This was not investigated.

Table 28

Recall of resistance to EAT

in S.typhimurium M206 immunized mice

a. whole-body retention assay

* Treatment i.p. on day -2:-	[c] % ^{125}I retained in whole mouse on day 2 (arith. mean ± S.E.)	
	Normal	[e] Immunized
saline 0.2ml	25.6 ± 3.3	25.6 ± 2.8
M206 F.P. SN 10 µg	14.9 ± 3.7	5.5 ± 1.3
None: heat-killed EAT i.p. on day 0	6.6 ± 0.5	

b. in vitro cytotoxicity assay

PEC from mice given the following i.p. on day-2:-	[d] % cytolysis at 20 hrs. (arith. mean ± S.E.)	
	Normal	[e] Immunized
saline 0.2ml	-3.4 ± 3.5	-0.9 ± 2.1
M206 F.P. SN 10 µg	-0.6 ± 2.1	16.9 ± 1.2

* 10^6 ^{125}I-EAT i.p. on day 0

[c] mean of individual mouse counts

[d] mean from quadruplicate wells. Combined results from 2 expts.

[e] 1.2×10^7 S.typhimurium M206 i.v.
on day-20 (a)
on day-23 (b)
4-7 mice per group in both a. and b.

7. RI leukaemia

An additional aim of this project was to investigate whether immunization of mice with 11RX protected them against challenge with tumours other EAT. The tumour chosen for study was RI leukaemia, which is a tumour syngeneic for CBA mice. It had already been established that i.v. injection of C.parvum into CBA T_6 T_6 mice induced peritoneal and lung macrophages which inhibited the DNA synthesis of RI cells in vitro (Olivotto and Bomford, 1974). Smith and Scott (1972) found that i.v. or i.p. C.parvum pretreatment prolonged the survival of mice which were challenged i.p. with 10 or 10^2 RI cells.

In Table 29 the results of LD_{50} experiments with i.p. injected RI cells are shown. Inoculation of 10 cells caused death 16-18 days later. However, there was some variation in the results, probably due to clumps of 4-5 cells in the cell suspensions which were not removed by a sieving procedure. Subsequently, glass wool filtration was found to be an excellent way of obtaining single cell suspensions. The doses given in Table 29 refer to viable cells, but the viability of suspensions varied from 70->95%.

The LD_{50} dose of 11RX given i.p. to CBA mice was determined to be between 5×10^7 and 10^8 bacteria per mouse. By the i.v. route it was between 2.9×10^6 and 1.2×10^6 organisms. The fate of 11RX in the liver, spleen and peritoneal cavity was followed in CBA mice injected with 9×10^4, 9×10^5, and 9×10^6 11RX i.p. The data, shown in Figs. 13a, b, and c, indicated that by day 11 the numbers of bacteria in the organs or the peritoneal cavity were 3-4 logs less than

Table 29

LD_{50} experiments with RI leukaemia in normal CBA mice

No. of RI cells given i.p.:-	[a] M.T.D. ± S.E.		
10	16.0 ± 0.8[b] ($^{1}/_{6}$)	17.7 ± 3.2[b] ($^{2}/_{5}$)	N.D.
10^2	17.8 ± 2.3 ($^{0}/_{6}$)	21.3 ± 1.7 ($^{0}/_{6}$)	17.2 ± 2.0[b] ($^{0}/_{5}$)
10^3	18.5 ± 2.6 ($^{1}/_{5}$)	14.7 ± 2.2 ($^{0}/_{6}$)	15.0 ± 0.6 ($^{0}/_{5}$)
10^4	16.5 ± 1.6 ($^{1}/_{5}$)	18.8 ± 2.3 ($^{0}/_{6}$)	14.0 ± 2.7 ($^{0}/_{5}$)

[a] mean time to death of mice which died up to day 35 after challenge

[b] proportion of mice alive on day 35
Each column represents the results of a different set of experiments.

Fig. 13 [continued on next page]

Recovery of 11RX organisms from CBA mice after infection by the i.p. route

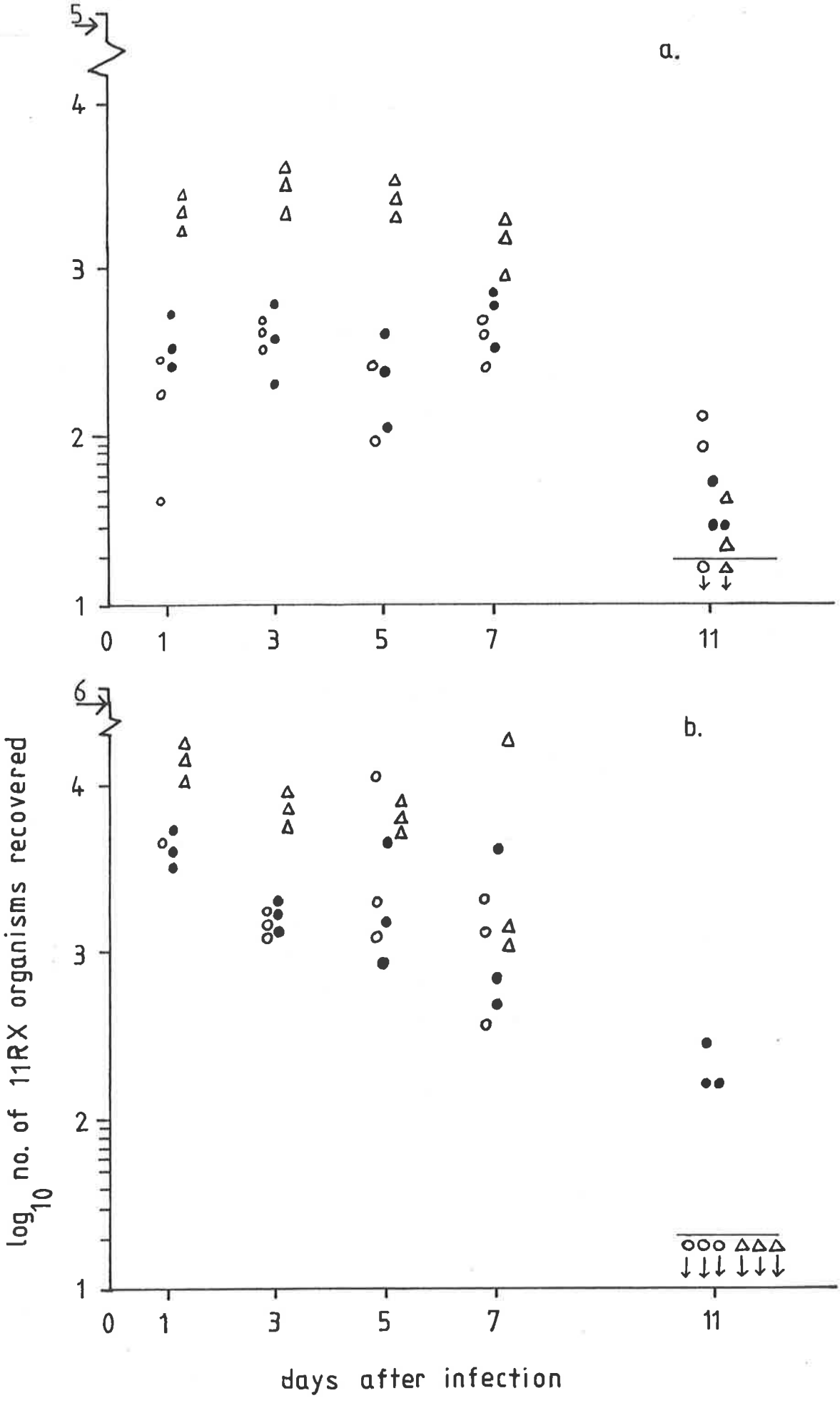
- ● spleen
- o liver
- Δ peritoneal cavity

Each point represents the number of organisms recovered from one mouse.

The horizontal bar represents the limit of detection.

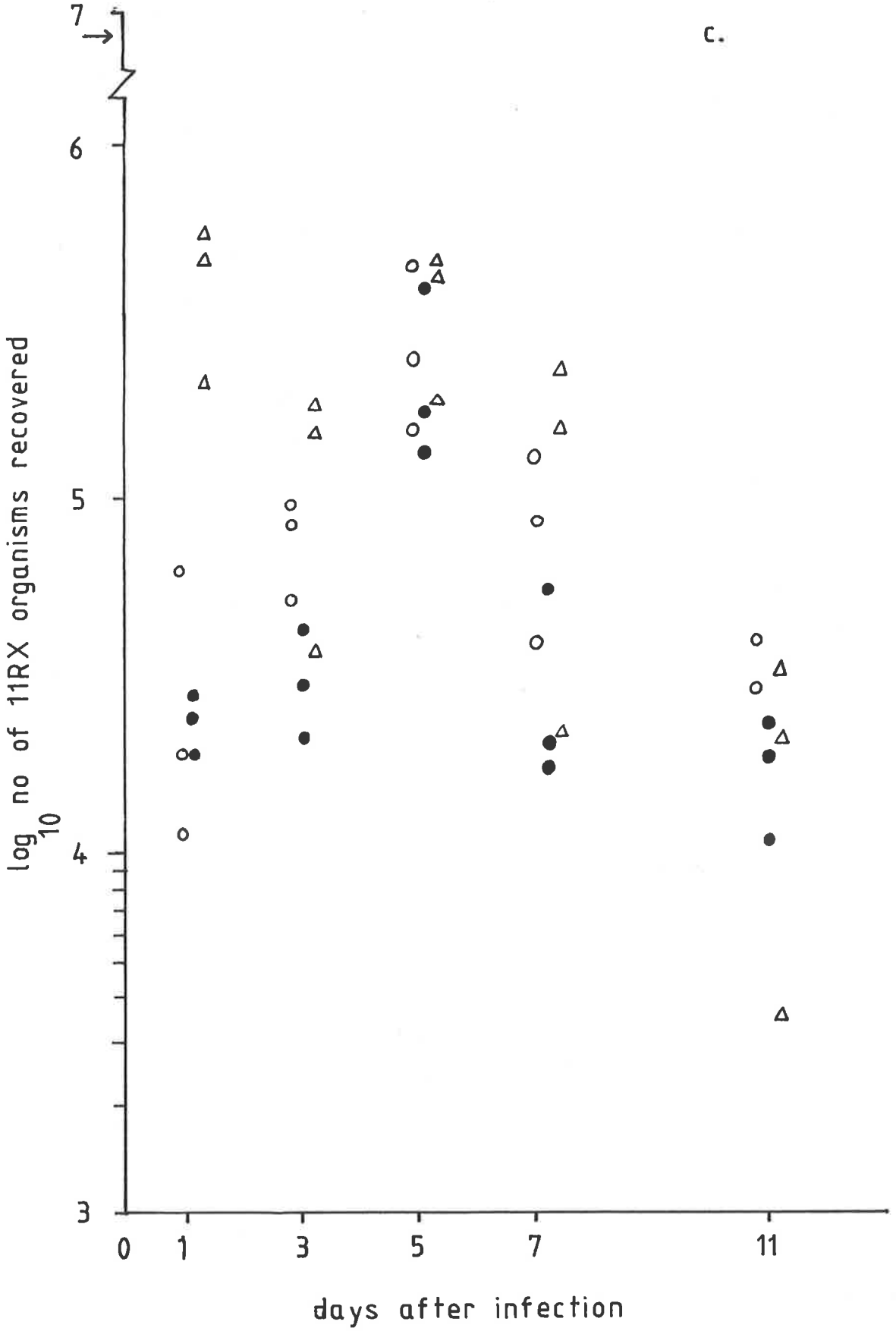
a. 9×10^4 11RX i.p. on day 0

b. 9×10^5 11RX i.p. on day 0


a.
b.
5
4
3
2
1
6
4
3
2
1
0
1
3
5
7
11
log10 no. of 11RX organisms recovered
days after infection

..... Fig. 13 ctd.

c. 9×10^6 11RX i.p. on day 0


c.
7
6
5
4
3
log10 no of 11RX organisms recovered
0
1
3
5
7
11
days after infection

the inoculum dose. No 11RX organisms were recovered from these mice 20 and 26 days after infection. This rapid elimination of 11RX contrasted with the persistence of a carrier state in CB6F1 mice given 10^5 11RX i.p. or i.v. (Ashley, 1976); and may be related to the resistance of the CBA mice to infection with virulent Salmonellae (Plant and Glynn, 1976).

Four days after i.v. or i.p. infection of CBA mice with 11RX, DTH reactions could be elicited in the footpads with Batch L, 11RX antigen (Table 30). This indicated that the mice had been sensitized to 11RX. The duration of sensitization was not determined.

In view of the lack of persistence of 11RX organisms in CBA mice, it seemed possible that an effective level of non-specific resistance was not generated in these animals by 11RX. Therefore, before experiments with RI leukaemia were commenced, the anti-tumour effects induced by 11RX immunization of CBA mice were assessed using EAT. Table 31 shows the results of an in vitro cytotoxicity assay experiment. A 100:1 PEC:EAT ratio was used (10^4 EAT/well), as this was found to be the optimum ratio when ratios from 20:1 to 200:1 were tested, varying either PEC or EAT numbers. The cytolytic capacity of the PEC was high 4 days after infection with 5×10^6 11RX i.p., but had declined markedly by day 13. In view of the data shown in Fig. 13 this suggested that cytotoxic cells were present only when reasonably high numbers of live 11RX could be recovered from the tissues.

The suitability of 5×10^6 11RX i.p. as an immunizing schedule was checked in further experiments. The presence of

Table 30

DTH response elicited in 11RX infected CBA mice with 11RX antigen

[a] Group of mice:-	% Increase in footpad thickness (arith. mean ± S.E.)			
	4 hr.	24 hr.	48 hr.	[c] 72-96 hr.
[b] 11RX i.p.	5.4 ± 1.0	10.4 ± 3.0	18.9 ± 3.2	7.3 ± 0.6
11RX i.v.	12.6 ± 2.4	20.2 ± 3.3	19.2 ± 4.1	11.0 ± 2.4
Normal	6.9 ± 1.0	2.6 ± 1.6	[d] 0	[d] 0

[a] 5 or 6 mice per group
10 µg Batch L in L.H.f.p. at 0 hrs.

[b] 5×10^6 11RX i.p.
or 10^5 11RX i.v. on day-4

[c] i.p. mice 96 hrs. Other groups 72 hrs.

[d] no increase in foot thickness

Table 31

PEC cytotoxic for EAT

in 11RX infected CBA mice

[a] PEC from CBA mice given the following:-	[b] % cytolysis at 20 hrs. (arith. mean ± S.E.)
Nothing	-2.8 ± 1.3
5×10^6 11RX i.p. on:-	
day-4	33.4 ± 3.8
day-8	18.0 ± 1.0
day-13	4.5 ± 0.7

[a] PEC pooled from groups of 4 mice

[b] mean from quadruplicate wells

cytotoxic PEC and resistance to EAT challenge were both monitored 4 days after infection with 11RX. The results are shown in Table 32. Although injection of 5×10^7 11RX i.p. induced cytotoxic PEC, it resulted in accelerated tumour growth because the challenged mice died earlier than the controls. 2.5×10^7 11RX i.p. prolonged the survival of mice challenged with EAT and induced cytotoxic PEC. The best results, in terms of cytotoxic PEC and resistance to EAT challenge, were observed if 10^7 or 5×10^6 11RX were given i.p. Thus, 5×10^6 11RX i.p. was chosen as an immunizing dose in RI protection experiments.

RI was not a good target cell for the in vitro cytotoxicity assay. Although 6 hours after ^{51}Cr labelling of RI cells the spontaneous release was of the order of 10 to 20%, by 20 hours it was 40-70%. The lower figure was obtained if double the standard FCS concentration was used. For this reason, experiments with RI were confined to survival studies.

The results, shown in Table 33, indicated that if CBA mice were injected with 5×10^6 11RX i.p. 4 days prior to challenge with 10^6 EAT the majority were protected against tumour growth (c.f. Table 32). This was not the case if the challenge was 10^2 or 10^3 RI cells i.p. Injection of 5×10^6 11RX i.p. 6 days prior to challenge with 10 or 10^2 RI cells i.p. did not prolong the survival time of the mice relative to that of the control mice. Injection of 10^5 11RX i.v. 4 or 6 days prior to injection of 10^2 RI cells was also without effect as an immunization procedure.

In conclusion, 4 days after i.p. infection with 5×10^6 11RX organisms CBA mice were sensitized to 11RX antigen and

Table 32

Anti-tumour effects of 11RX immunization in CBA mice - assessed using EAT

[a] Treatment prior to taking PEC or to EAT challenge:-	[b] % cytolysis at 20 hrs. (arith. mean ± S.E.)	[c] M.T.D. ± S.E.
Normal CB6F1 mice	7.1 ± 0.9	17.6 ± 0.4 [d] ($^{0}/_{5}$)
CB6F1: 10^5 11RX i.p. on day-4	25.7 ± 0.7	— ($^{5}/_{5}$)
Normal CBA	3.5 ± 0.4	16.3 ± 0.6 ($^{0}/_{6}$)
[e] Infected CBA:-		
5×10^6 11RX i.p.	20.5 ± 1.4	day 31 ($^{4}/_{5}$)
10^7 11RX i.p.	27.6 ± 2.0	day 35 ($^{2}/_{3}$)
2.5×10^7 11RX i.p.	16.0 ± 1.3	34.6 ± 2.7 ($^{0}/_{5}$)
5×10^7 11RX i.p.	14.9 ± 2.6	7.2 ± 3.2 ($^{0}/_{6}$)

[a] PEC pooled from groups of 4 mice

[b] mean from quadruplicate wells

[c] M.T.D. up to day 49 of mice which died following challenge with 10^6 EAT i.p. on day 0

[d] Proportion of mice alive on day 49

[e] all mice infected on day-4

Table 33

Resistance of 11RX infected CBA mice to challenge with RI leukaemia

Group of CBA mice:-	[a] M.T.D. ± S.E. following i.p. challenge with:			
	10^6 EAT	10 RI	10^2 RI	10^3 RI
Normal	17.0 ± 2.5 [b] ($^1/_6$)	15.0 ± 0 ($^0/_5$)	16.0 ± 1.2 ($^0/_4$)	15.0 ± 0.6 ($^0/_5$)
Infected:- [d] 11RX i.p.	day 43 ($^2/_3$)		18.3 ± 2.1 ($^0/_5$)	21.8 ± 0.8 ($^1/_5$)
[d] 11RX i.v.			[c] 15.2 ± 3.6	
[e] 11RX i.p.		[c] 17.6 + 0.6	[c] 16.6 ± 1.6	
[e] 11RX i.v.			[c] 13.2 ± 1.9	

[a] M.T.D. up to day 45 of mice which died following tumour challenge on day 0

[b] The figures in brackets represent the proportion of mice alive on day 45

[c] 5 mice per group; none alive on day 45

[d] on day-4: 5×10^6 11RX i.p. or 10^5 11RX i.v.

[e] on day-6: as above

their PEC were cytotoxic for EAT cells *in vitro*. However, some of the similarly immunized CBA mice succumbed to challenge with 10^6 EAT cells, whereas all CB6F1 mice immunized with 10^5 11RX i.p. survive a challenge of 10^6 EAT given i.p. 4 days later (Table 32; Ashley, 1976). Hence, as the 11RX immunization schedule used did not protect all CBA mice against EAT, it is not surprising that these mice were not resistant to challenge with approx. $10\text{-}10^2$ LD_{50} doses of the highly lethal RI leukaemia.

DISCUSSION

Recall of tumour resistance with 11RX antigen--parallels in the literature

Inhibition of tumour growth has followed the injection of Mycobacteria, C.parvum or their derivatives into the tumour sites of animals or humans sensitized to these organisms. The eliciting injection of bacterial antigen is either given i.t., in admixture with tumour cells, or prior to tumour challenge (Introduction, section 3.2, Table 1, sections 4.1.2, 5.2 (i), 5.2.1 (iv), 7, 8). Recently, Buckspan, Hojvat, and Skamene (1977) found that tumour growth in mice was also suppressed at the sites of DTH reactions to L.monocytogenes.

The model system used in this project can be added to the above list. The anti-tumour effects of 11RX antigen preparations in 11RX immunized mice resemble those of PPD in BCG immunized hosts (Evans and Alexander, 1972; Parr, 1974; Zbar et al., 1970; Ruco and Meltzer, 1977). Although in this study the recall of i.p. resistance to EAT was used as an assay system, the recall activity of 11RX antigen is not limited to the i.p. site. It is also demonstrable at s.c. sites in mice immunized with 11RX by the i.v. or i.p. routes (Ashley, Neoh, Kotlarski et al., 1976). 11RX antigens probably have to persist at the tumour site if effective tumour resistance is to be generated by 11RX antigen or viable 11RX (Ashley et al., 1974; 1976). A requirement for "contact" between the bacterial vaccine and the tumour cells for tumour growth inhibition is evident in other experimental animal tumour models and in the clinical situation (e.g., Introduction, sections 4.2.1 (ii), 4.2.3, 4.2.5 (i), (iv), 5.2.1 (iv), 7).

The available evidence indicates that activated macrophages with non-specific bactericidal and tumouricidal properties are the effector cells of the resistance induced by viable 11RX or elicited by 11RX antigen [Rowley et al., 1968; Davies, 1975; Ashley and Hardy, 1973; Ashley, 1976]. These findings are consistent with those of other workers investigating the anti-tumour effects of intracellular parasites or their derivatives in animals [Introduction, sections 3.1, 3.2, 4.1.1, 4.2.1 (iv), 7]. Furthermore, the involvement of sensitized T lymphocytes in the recall effect suggests that a T cell produced lymphokine(s) may be involved in the macrophage activation process [Ashley et al., 1977]. This information is also in agreement with the results of other groups [Introduction, sections 3.1, 3.2, 4.2.2, 7]. Workers in this laboratory are at present studying the mechanism of the recall effect.

Fractionation and characterization of 11RX antigen preparations.

Motive

The main reason for attempting to purify 11RX antigen preparations was to determine the chemical nature of recall antigen. The essential structural requirements of recall antigens are not known. In most cases crude bacterial extracts [i.e., cytoplasmic fraction, cell walls, whole cells] are used to evoke anti-tumour responses in immunized hosts [e.g., Introduction, sections 4.1.2, 5.2 (i), 7, 8, Results]. An exception to this is PPD [Introduction, sections 3.1, 3.2, 5.2.1 (iv)]. However, although PPD is partially pure, the component associated with the expression of PPD's anti-tumour

effects in sensitized hosts has not been characterized. PPD contains at least 3 distinct proteins, and also a lipid moiety which endows it with mitogenicity for B lymphocytes (results of Seibert, 1949 - cited by Sultzer and Nilsson, 1972; Melchers, Braun, and Galanos, 1975).

It may be incorrect to assume that recall antigen is protein in nature by analogy with studies on DTH elicitation in immunized hosts (refered to briefly in Results, section 2). Firstly, although it is true that DTH is usually elicited with proteins, there are some exceptions. For example, DTH has been demonstrated in animals against dextran and non-protein antigens from Mycobacteria (Crowle, 1975; refs. cited by Wong, Pickett, and Froman, 1970). Secondly, it has not been established if the antigens responsible for recall of tumour resistance or DTH elicitation are one and the same.

Findings

The moiety responsible for the recall activity of 11RX antigen preparations was not isolated in pure form. It appeared that there was more than one component of 11RX antigens or high speed SNs with recall activity (Results, sections 4.2, 4.3, 4.4). If lower doses of fractions had been administered to mice in the assays of recall activity it may have been found that one fraction was more active than the others on a weight basis (i.e., dose response experiments in Results, section 4.1).

The presence of low m.wt. protein in 11RX high speed SNs (Results, sections 2.1.1, 4.4) contrasts with at least one report in the literature: "... an E.coli 150,000 x g supernatant fraction appears to contain little or no protein which

elutes from a Sephadex G-100 column with an apparent molecular weight of less than 25,000-30,000" (unpublished data cited in the discussion of Kemshead and Hipkiss, 1976). Although these workers did not present data directly relating to this point, their work concerning proteolytic activity in E.coli cell-free extracts is consistent with their comment.

The data obtained during this project did not confirm the possibility that this difference in results is due to the presence of proteases in 11RX high speed SNs or antigens (Results, section 2.1.5). High speed SNs used to prepare phase separated 11RX antigens were incubated for 2 hours at 37°C as compared to 16 hours for nuclease treated extracts, but the SDS PAGE protein profiles of the resultant antigens were very similar (Results, section 2.1.6). There was, however, a large amount of dialysable or low m.wt. protein in high speed SNs as estimated using the Lowry assay. This was not present in high levels in the resultant antigen preparations, either phase separated or nuclease treated, which had been stored dry or frozen for some time (Results, section 4.4).

The following explanation could account for the above observations. Possibly, low m.wt. protein (or a large proportion of it) in 11RX high speed SNs is only apparently present due to interference with protein estimation by the Lowry method which is caused by a low m.wt. non-protein component. Such a component(s) may be an ET or carbohydrate derivative (Results, section 2.1.3). Herbert et al., (1971) comment that glucose and other substances can give rise to false positives of the protein content of microbial cell

extracts if the Lowry method is employed. Perhaps a similar phenomenon accounts for the observed inaccuracy of the Lowry method (2 fold too high), as compared to polyacrylamide gel scanning, for determining the protein content of outer membrane samples from wild type and mutant E.coli K12 strains (van Alphen et al., 1977 Biochimica et Biophysica Acta 466: pp. 261-2).

The Lowry method was used during this project for the reasons stated in Results, section 2.1.1. The protein content of <10K m.wt. filtrates from 11RX high speed SNs was not determined using other techniques. If the above mentioned explanation is correct then the doses, in terms of protein, of <10K m.wt. filtrates administered to mice in the assays of recall activity were much less than 5 or 10 µg (Results, section 4.4). This may indicate that the <10K m.wt. filtrate is enriched in recall antigen activity compared to other m.wt. fractions, if recall antigen(s) is protein in nature.

The above comments do not necessarily imply that no proteolytic degradation occurred during the extraction of 11RX high speed SNs. It seems likely that degradation did occur in those cases in which the DNase was used for preparation of 11RX antigens (Results, section 2.1.5). The question of the size distribution of proteins in 11RX high speed SNs could be resolved by filtration or gel chromatography of fresh extracts obtained from 11RX grown in the presence of radioactively-labelled amino acids. Appropriate measures would need to be taken to limit the aggregation of these extracts and to minimize the possibility of proteolytic degradation. In the light of the results quoted in an earlier

paragraph, it is relevant that Kemshead and Hipkiss (1974, 1976) followed the protein content of their E.coli extracts using labelled amino acids rather than the Lowry method (see top of pp. 188). Finally, it was found that high speed SNs from S.typhimurium C5 or S.typhimurium M206 organisms, extracted in the presence of nucleases, also contained a large amount of dialysable protein as determined using the Lowry method (results not shown). Data relevant to this point was not obtained for the extracts from other strains of Enterobacteria refered to in Results, section 6.

Characterization of 11RX antigen preparations revealed that they were largely protein in nature (Results, sections 2.1.1-2.1.4). However, the chemical nature of recall antigen(s) was not established (Results, sections 3.1-3.4). The retention of recall activity by 11RX antigens following some of the treatments described in section 3 may be accounted for on the basis of the incompleteness of the treatments rather than on the basis of the resistance of recall antigen(s) to a particular denaturing procedure. For example, many proteins retain 20% of their native ordered structures in the denatured state (Tanford, 1968) and this may be sufficient for them to retain some biological activity. Proteins may retain antigenicity after severe 2-Me treatment because non-covalent bonds will hold the polypeptide chains together although disulphide bonds have been disrupted. Polysaccharides could be unaffected by a 1 hour treatment with periodate even though monosaccharides are oxidised (Keleti and Lederer, 1974). However, as the data indicates that there is more than one component in 11RX antigen preparations with recall activity the results of such experiments can only be subject

to ambiguous interpretations if the components are different in nature and/or have different properties.

The results discussed in section 6 show that recall antigens are present in the high speed SN fraction from Enterobacteria other than 11RX. In addition, OEP, an extract from the cell walls of P.aeruginosa, has recall activity. Which moiety of OEP is responsible for this activity was not determined. As well as protein and carbohydrate OEP probably contains a large proportion of phospholipid as it contains 1.1% phosphorus [Hoshi et al., 1972, presumably by weight % P, although not stated; see also Results, section 2.1.4].

Cell envelope components in 11RX antigens

It is incorrect to refer to 11RX high speed SNs as cytoplasmic extracts. Particulate material from high speed SNs was found to contain cytoplasmic membrane proteins, some of which may be present in the remaining soluble material (Results, section 2.1.7). This presumably applies to soluble and particulate 11RX antigen preparations, although this was not established. Some workers are not aware of the work of MacGregor and Schnaitman (1973) in this area (i.e., those refered to in Results, section 2; Kemshead and Hipkiss, 1974; Chaloupka and Liebster, 1959; Kowit et al., 1976). Presumably this is because they do not observe aggregates forming in their high speed SN fractions because of the buffer systems and temperatures which they employ to carry out the extractions of their particular micro-organisms (MacGregor and Schnaitman, 1973). In the case of 11RX high speed SNs extracted in low ionic strength buffers (i.e., 10mM Tris-HCl), and depending on the protein concentration, aggregation was observed to varying degrees at 37°C, room temperature, and 4°C.

Other cell envelope components (ET, phospholipid and heat-modifiable outer membrane proteins) were found to be present in relatively low levels, if at all, in 11RX preparations (Results, sections 2.1.3, 2.1.4, 2.1.8 respectively).

Whether 11RX antigens contained Braun's lipoprotein, murein, or non heat-modifiable outer membrane proteins was not determined. A report in the literature relates to Braun's lipoprotein being found in E.coli cytoplasm. Seven % of the total cellular lipoprotein was found in the cytoplasmic

fraction of lysed E.coli spheroplasts (Braun, 1975). Braun comments that this is due to contamination from cell envelopes. Braun's figure allows an approximate calculation which indicates that lipoprotein could constitute 0.3% of the cytoplasmic fraction by weight. The latter figure was arrived at assuming 9×10^5 molecules of lipoprotein per cell (free in outer membrane and bound to murein), m.wt. of lipoprotein 7,500 daltons, dry weight of E.coli cell 0.32×10^{-12}g, cytoplasmic fraction 80% of cell dry weight. Extrapolating to 11RX antigen preparations, they could contain a maximum of 0.03 µg of lipoprotein per 10 µg of dry weight, assuming that 11RX lipoprotein is similar to E.coli lipoprotein (refs. in Results, section 5.3, and see also the following section in this Discussion).

If the contamination of 11RX antigen preparations or 11RX high speed SNs with certain cell wall constituents is as low as the above calculation suggests, it will be detectable using sensitive immunological techniques such as crossed immunoelectrophoresis (e.g., Alexander and Kenny, 1977; Owen and Salton, 1977).

The recall activity of cell envelope components

Experiments with Triton X-100 and SDS extracts from the cell envelopes of 11RX organisms indicated that the cell wall contains recall antigen(s) (Results, sections 5.2, 5.3). In addition, it was found that particulate 11RX antigen preparations and Triton solubilized cell envelope material contain recall antigen(s) (Results, sections 4.2, 5.2). Whether the recall activity of the latter extracts could be ascribed to their cytoplasmic membrane or non-cytoplasmic membrane

constituents was not determined. Possibly, a relatively pure extract of 11RX cytoplasmic membrane could be prepared using the method of Osborn et al., (1972) and tested for recall activity.

11RX cell walls represent the obvious choice for further fractionation studies of recall antigen preparations. Two of the reasons for originally choosing soluble 11RX antigen preparations for these studies were (i) detergent extraction of particulate extracts, whether from high speed SNs or cell envelopes, may have resulted in denaturation of recall antigen(s) and (ii) their antitumour effects were not complicated by the non-specific effects of ET. However, the cell walls of enterobacterial strains have now been extensively characterized and are of simpler composition than high speed SN fractions. Some cell wall components have been isolated free of ET and have retained biological or antigenic activity. Moreover, recently Dr. L. Ashman isolated the equivalent of Braun's lipoprotein from 11RX cell envelopes and demonstrated that the lipoprotein can recall resistance to EAT challenge in long-term 11RX immunized mice. The lipoprotein does not affect the clearance of an i.p. challenge of EAT if given to normal mice.

The outer membrane proteins of approx. 40,000 daltons m.wt. may be potential recall antigens. Diedrich, Summers, and Schnaitman (1977) obtained highly pure extracts of outer membrane proteins 1 and 2 from E.coli strains using mild Triton and SDS extraction. These extracts retained antigenic activity after purification. A number of workers have reported that purified outer membrane proteins from E.coli do

not contain any bound ET or phospholipid (Schnaitman, 1974b; Manning, 1977). However, it is clear that the biological activity of some outer membrane proteins is destroyed by Triton or SDS treatment, or at least nullified in the presence of these detergents (e.g., Pugsley and Reeves, 1976). Nevertheless, it is probable that some proteins could be isolated in native form from 11RX outer membranes and tested for recall activity.

The recall effect and DTH

Although it is debatable whether DTH and the different forms of CMI are the same phenomenon (Introduction, section 3.2), the inhibition of tumour growth elicited by 11RX antigen in 11RX immunized mice presumably occurs at the site of a DTH reaction.

The results of this project suggest that components from different parts of 11RX organisms have recall activity (Results, sections 4.2, 5.2, 5.3). This finding is similar to those of other workers investigating the multiple specificities represented in the development of DTH to micro-organisms. Thus, culture filtrates or cytoplasmic material from Salmonella, Mycobacteria, and Histoplasma can elicit DTH reactions in appropriately immunized hosts (Collins and Mackaness, 1968; Janicki, Wright, and Good, 1976; Kanai, Youmans, and Youmans, 1960; Domer, 1976). The same applies to cell wall or ribosomal material from Mycobacteria and Histoplasma (Kanai, Youmans, and Youmans, 1960; Domer, 1976).

Anti-tumour effects of 11RX ET

PEC from mice given 11RX ET were not highly cytotoxic for EAT cells *in vitro* (Results, section 5.1). This is an apparent contrast with other reports which describe the *in vitro* anti-tumour effects of macrophages from ET treated mice (Alexander and Evans, 1971; Bruley-Rosset *et al.*, 1976). The latter workers found that peritoneal macrophages from mice given 200 µg of *S.typhi* ET either s.c. or i.v. were cytotoxic for L1210 cells *in vitro*. Their work cannot be compared with this study, as the dose and routes of administration of ET are different. Alexander showed that macrophages from mice given 10 µg of *Shigella* ET 7 days and 1 day previously were cytostatic for tumour cells *in vitro*. 11RX ET induced PEC may have inhibited the *in vitro* growth of EAT, but this effect would not have been detected using the ^{51}Cr release assay. However, other data indicate that the explanation may be more involved. Highly cytotoxic PEC *can* be harvested from 11RX ET treated mice at certain times after ET administration (L. Ashman, unpublished results).

It is also pertinent to note that isolated macrophages were not used in this project in the *in vitro* cytotoxicity assay. The observed effects may have been complicated because the biological activity of peritoneal lymphocytes was in some way altered by ET administration (e.g., Shands, Peavy, Gormus *et al.*, 1974). In addition, it is known that some of the *in vitro* mitogenic effects and *in vivo* anti-tumour effects of ET vary with the extraction procedure used for preparing the ET, and with the bacterial strain used as a source of ET (Skidmore, Morrison, Chiller *et al.*, 1975; Ng, Butler, Chen *et al.*, 1976).

The mechanism of the *in vivo* anti-tumour effects of 11RX ET has not been elucidated. Lipid A is probably the active moiety, as alkali treatment of 11RX ET destroys its *in vivo* activity against EAT (L. Ashman, unpublished results). The latter result is in agreement with other reports in the literature (Introduction, section 2.1).

The claim that Lipid A is involved in the cell-mediated immune response of rats to *Salmonella* is controversial (Marks *et al.*, 1975—refered to in Results, section 2). In contrast to the results of these workers, there are reports which indicate that ETs inhibit the *in vitro* migration of macrophages from normal (i.e., not actively immunized) animals (reviewed by Nelson, 1969). Furthermore, at least in normal mice, ETs from *Salmonella* induce an Arthus-type of skin response (Collins and Mackaness, 1968; Results, section 5.1).

RI leukaemia

The schedules of 11RX immunization used in this project did not protect CBA mice against i.p. challenge with 10^2 RI cells (Results, section 7). 11RX organisms do not establish a carrier state in CBA mice (Results, section 7). 11RX may protect CBA mice against RI challenge, and provide a more effective level of resistance against EAT, if given in high doses or as a repeated course of injections. However, infection of CBA mice with micro-organisms other than *Salmonella* may induce superior non-specific resistance, as CBA mice are relatively resistant to infection with *Salmonella* (Plant and Glynn, 1976).

Looking closely at the study of Smith and Scott (1972) it is evident that i.v. or i.p. administration of 1.4mg of

C.parvum to CBA mice 7 days before i.p. challenge with 10 RI cells prolonged the survival of the mice. The majority of control mice had died by day 16, but 40% of the treated mice were alive on day 28. If the challenge was 10^2 RI cells all the control mice died by day 16, the i.v. treated mice by day 18, and the i.p. treated mice by day 21. If the mice were challenged with 10^2 RI-var. cells both treated and control groups died between days 12-15. RI-var. was a form of the RI which had been passaged through mice for 18 months, and was much less immunogenic than the RI which these workers obtained from frozen stocks.

Scott and Warner (1976) gave CBA T_6 T_6 mice 14 weekly injections of 35 ug of C.parvum either i.v. or s.c. Ten days after the last injection the mice were given 10^2 RI cells i.p. It is not clear from the reference given by these workers whether the RI used was from a mouse passaged stock, or a tissue culture line. The control mice died between days 13-14, the s.c. treated mice between days 15-16, and the i.v. treated mice between days 17-18. These differences were statistically significant. Eight days after the last C.parvum injection the mice mounted DTH responses to C.parvum, possessed antibodies against C.parvum in their serum, and had enlarged livers and spleens.

The above references demonstrate that i.p., i.v., or s.c. administration of C.parvum to mice does not protect them against i.p. growth of 10^2 RI cells. Although treated mice survived a few days longer, this could not be termed protection, and this effect was not observed if a mouse passaged form of RI was used.

The data from the C.parvum studies suggests that RI grows rapidly enough from inocula of 10^2 cells to overcome any non-specific anti-tumour effects generated by treatment with intracellular parasites. For example, Smith and Scott (1972) found that although i.p. pretreatment with C.parvum did not protect CBA mice against i.p. challenge with 10^2 RI cells, the same immunization schedule afforded CBA mice strong protection against i.p. growth of the syngeneic T3 fibrosarcoma. A similar explanation was offered by Hibbs, Lambert, and Remington (1971) when they found that chronic infection with intracellular protozoa protected mice against i.p. challenge with Sarcoma 180, but not against i.p. challenge with leukaemia L1210.

It has been shown that prior immunization of mice with live 11RX induces resistance to transplantable tumours other than EAT. Pretreatment i.p. with 11RX protects LACA mice against i.p. challenge with the non-strain specific tumours Krebs ascites tumour and Sarcoma 180, and also Balb/c mice against PC6 plasmocytoma (Tindle et al., 1976). Intravenous infection of mice with 11RX results in suppression of the growth of s.c. inocula of the above tumours, B16 melanoma, amelanotic B16, and a mammary carcinoma. The growth of the latter 3 tumours was followed in semi-syngeneic hosts (Ashley et al., 1976).

Workers are investigating the effects of 11RX immunization and administration of recall antigen preparations on the growth of other murine tumours in their syngeneic hosts.

In conclusion, further work rests with the characterization of microbial recall antigens and investigation of their

anti-tumour effects. Presumably, relatively few studies in progress are concerned with recall effects because of the demonstrated superiority of live organisms in inducing non-specific immunity. However, if recall antigens are given in repeated doses to the appropriately immunized hosts the resulting anti-tumour effects can be similar to those induced by chronic infection with intracellular parasites (e.g., Ruco and Meltzer, 1977). Moreover, Edmund Klein's work with skin cancer patients provides a demonstration of the immunotherapeutic and immunoprophylactic benefits which result from the use of recall antigens in humans (Introduction, section 5.2.1 (iv)). Nevertheless, the administration of repeated doses of recall antigens to sensitized hosts is a potentially hazardous procedure, as is the administration of many doses of live organisms (Introduction, section 5).

It is quite probable that the purification to homogeneity of extracts from BCG, C.parvum, 11RX etc. will result in preparations which have little or no anti-tumour activity in hosts which have not been actively immunized with live organisms (e.g., Introduction, section 8; comment made by D.W. Weiss in 1976 relating to MER fractionation - in general discussion of paper Ann. N.Y. Acad. Sci. 276: pp. 548). Possibly such pure preparations could be modified chemically or by the addition of oils or other substances so that they are able to induce non-specific resistance when administered to normal hosts (Introduction, sections 5.2 (ii), 5.3). However, one compromise could be to use unmodified pure extracts as therapeutic or prophylactic anti-tumour agents in hosts presensitized to the parent or a related organism.

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