THE DEVELOPMENT IN MICE OF LOCAL INTESTINAL IMMUNITY TO ENTEROBACTERICEAE

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TO MY FAMILY:

FOR THEIR LOVE, UNDERSTANDING AND SUPPORT.

TO MY TEACHERS:

WHO GAVE ME THE LIGHT AND SHOWED ME THE WAY.

CONTENTS

LIST OF ABBREVIATIONS USED IN TEXT

885	*Salmonella typhimurium* 885
BCG	Bacillus Calmette-Guérin
C5	*Salmonella typhimurium* C5
C5 OMP antigen	Salmonella C5 outer membrane protein antigen
CMI	cell-mediated immunity
CPA	cyclophosphamide
DTH	delayed-type hypersensitivity
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G.I. tract	gastrointestinal tract
GALT	gut-associated lymphoid tissue
HA unit	haemagglutination unit
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
LPS	lipopolysaccharide
MØ	macrophage(s)
MHC restriction	major histocompatibility complex restriction
P.P.	Peyer's patch(es)
s.c.	subcutaneous(ly)
SPF mouse	specific pathogen free mouse
SRBC	sheep red blood cells
TNF	tumour necrotising factor

SUMMARY

The development of immunity to Salmonella at the local mucous surface of the small intestine has been investigated. After oral administration an avirulent Salmonella typhimurium/Escherichia. coli hybrid 885 infected the Peyer's patches and grew for 3-4 days before declining in numbers. The primary infected mice were subsequently protected from challenge by a virulent Salmonella strain. Passive transfer of high titre specific antiserum failed to protect. Administration of cyclophosphamide inhibited the antibacterial activity of the spleen but not that of the Peyer's patches. It was deduced that Peyer's patches have already been primed against common enterobacterial antigens in the natural state due to their proximity to the bacterial flora of the gut.

The decreasing number of bacteria in the Peyer's patches 4 - 5 days following primary oral infection coincided with increasing numbers of macrophages in the Peyer's patches of infected mice. The elimination of Salmonella 885 required activated macrophages whereas Escherichia coli 492 and Vibrio cholerae 569B were susceptible to normal macrophages.

Salmonella 885 were found to be extracellular in relation to the Peyer's patch cells. Nonetheless there was a loose association between the bacteria and some of the cells, the proportion of this

increased after infection from 6% to 50%. This bacteria/cell association was found to occur with a 1:1 ratio throughout the period of bacterial multiplication indicating that bacteria could move from one cell to another. Nonetheless the association with cells was sufficient to allow the bacteria to move through the Peyer's patches with the cell traffic and to be transported towards the incoming macrophages which could then kill the bacteria. Macrophages could be recovered from Peyer's patches at varying times after oral infection and when these cells were used in vitro to measure bactericidal effects, it was found that at the time when large numbers of macrophages entered the Peyer's patches, these cells were already activated and possessed superior antibacterial powers.

The traffic of cells through these lymphoid follicles is seen as a key element in both the dissemination of T lymphocytes able to express delayed type hypersensitivity and in the carriage of organisms towards activated macrophages.

STATEMENT

The material in this thesis has not been previously submitted for a degree in any university, and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

Vichai Marneerushapisal

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CHAPTER I

HOST DEFENCE TO INTESTINAL INFECTION

1.1 NONSPECIFIC INTESTINAL DEFENCE

The gastrointestinal tract of an animal is continually exposed to numerous microorganisms of various species. Carter and Collins (1974) showed that when mice were fed with 10^7 Salmonella enteritidis, only 0.25% of these organisms could be recovered after 6 hours. This efficient elimination of organisms from the gut is due to such mechanisms as: gastric acid production (Mossel and Oei, 1975), mucous secretions (Walker et al., 1982), intestinal motility (Kent et al., 1966), epithelial migration and sloughing (Moon, 1971), and microbial antagonism (Abrams and Bishop, 1966).

The stomach contents have an acid pH which is a formidable barrier. It kills microorganisms and prevents them from passing live into the intestine. Food in the stomach reduces the efficiency of the barrier. Similarly, starvation increases the stomach movements which results in a rapid passage of water or food through the opened pyloric sphincter and thus diminished killing due to gastric acid occurs (Mossel, 1976; Mossel and Oei, 1975).

The gastrointestinal (G.I.) tract is covered with epithelial cells which have a short life span being continually sloughed off and wiped away by intestinal movement. This also helps to control infection. It has been demonstrated that Vibrio cholerae adheres to epithelial cells (Jones and Freter, 1976). Without these cells, cholera cannot be retained in the gut and is flushed out by

peristalsis. Drugs, like opium, which inhibit peristalsis will markedly increase invasion by Salmonellae in the guinea pig (Takeuchi, 1967; Kent et al., 1966)

Goblet cells, observed in the small and particularly the large intestine, secrete mucus that coats the epithelial surface of the gut. It has been postulated that the mucus coat performs an important protective function by inhibiting attachment and penetration of microorganisms, antigens and toxins through the epithelium . Walker et al. (1982) have provided evidence that immune mechanisms are capable of stimulating mucus release from goblet cells. This mucus facilitates the clearance of immune complexes from the intestinal lumen. In addition, glycoproteins in mucus may possibly bind enterotoxins and thereby inhibit attachment of these to the villous membranes, where they initiate their activity.

The G.I. tract of animals becomes inhabited by a variety of normal flora just after birth. The ecology and effect of this normal flora has been studied in conventional and germ-free mice by Tannock and Savage (1974). They found that germ-free mice are more susceptible to typhoid than mice with conventional flora (Tannock and Savage, 1974; Abrams and Bishop, 1966; Collins and Carter, 1978). Such susceptibility results from the unimpeded growth of Salmonellae in the intestinal lumen. For example, Collins and Carter (1978) showed that the LD_{50} of Salmonella enteritidis in conventional mice is 10^6 but in germ-free mice is only 3-5 organisms. In addition, Ruitenberg and his colleagues (1971) reported that when germ-free mice were orally challenged with Salmonella panama the organism grew in the gut at nearly the same rate as in broth in vitro. It seemed that the presence of the

normal flora prevented or inhibited the growth of the invading microorganisms, which could otherwise grow freely. This indeed seems to be the case. Conventional mice are much more susceptible to Salmonella infection following oral streptomycin treatment (Miller and Bohnhoff, 1963; Meynell and Subbaiah, 1963). Meynell (1963) and Bohnhoff et al. (1964) demonstrated that normal flora is accompanied by the presence of volatile fatty acids which are toxic to Salmonellae. Other mechanisms, such as maintenance of pH and oxidation-reduction potential by normal inhabitants may contribute. Savage (1972) demonstrated that a normal flora stimulates intestinal motility. Limitation due to the competition for nutrients, space and receptor sites between microorganisms is clearly a significant factor. A striking example of this was provided by Davidson and Hirsh (1975) who showed that the colonization of the intestine of pigs with a non-enteropathogenic Escherichia coli strain with adhesive properties (K88 positive) prevented disease when these pigs were challenged with an enteropathogenic E. coli possessing the same antigens.

1.2 THE IMMUNITY OF THE GUT

Studies by Burrows et al. (1947) showed that protection against cholera correlates with antibody (known as coproantibody) found in the stools of infected hosts. Freter (1956) demonstrated that circulating antibody was ineffective in preventing neonatal infection by Vibrio cholerae, but that good protection could be obtained when the antibody was located in the intestinal tract. This became clearer when Heremans and his colleagues (1959)

discovered a new immunoglobulin (Ig) class, namely, IgA which Tomasi and Bienenstock (1968) showed predominates in external secretions. This secretory IgA (sIgA) differs from its serum counterpart in being an 11S dimer of approximate mol. wt. 390,000 daltons with a complex structure consisting of two subunits of 7S monomer, with a linking J chain and a molecule of secretory piece. This special molecular structure makes sIgA markedly resistant to the proteolytic enzymes present in the intestines. It bathes the entire gut lumen and can block the adherence of bacteria to mucosal surfaces (Williams and Gibbons, 1972; Fubara and Freter, 1973; Bellamy et al., 1975), thereby preventing colonization. The ability of secretory antibodies to form complexes with bacterial toxin (e.g. cholera toxin) and thus prevent their binding to intestinal microvillus membranes is well documented (Clancy and Bienenstock, 1976).

Studies of naturally occurring virus infection and immunization regimens, using in particular live poliomyelitis (Sabin) vaccine, have established an important virus neutralizing activity for sIgA (Ogra and Karzon, 1969; Ogra et al., 1968). Walker and Isselbacher (1974) showed that secretory antibodies interfere with the uptake of luminal dietary protein antigens by forming complexes on the intestinal surface which prevent the migration of antigen to the cellular membrane and stop pinocytosis. This phenomenon of antigen exclusion may help protect the host from food allergy or immunological diseases of the gut.

Secretory IgA does not activate the classical pathway of complement. It may be capable of activating the alternative pathway (Robertson et al., 1976) although this has been disputed (Colten and Bienenstock, 1973) as has its ability to opsonise antigens for

phagocytosis (Reynolds and Thompson, 1973; Steele, Chaicumpa and Rowley, 1975).

1.3 RESPONSE OF THE GUT TO ADMINISTERED ANTIGEN

Once the significance of secretory IgA at the mucous surface was realised, it was important to determine how to induce the maximum response in this class of immunoglobulin. Pierce and Reynolds (1975) using cholera toxoid as antigen found that the best result was obtained by immunizing animals subcutaneously and following with an oral booster. Their studies in dogs were subsequently repeated in rats with the same immunization schedules of parenteral immunization and oral boosting. The results agreed with the previous one. However, Bloom and Rowley (1979) and Horsfall and Rowley (1979) working with mice found that live Vibrio cholerae given orally several times followed by a parenteral booster, was the most effective stimulus to intestinal antibody responses. Their studies also emphasized an immune memory of the mucosal immune system, as they found that after an antigenic booster, the antibody response (measured by the number of plaque-forming antibody-producing cells) appeared more quickly, was higher and more prolonged than in a primary response (Bloom and Rowley, 1979). Similar conclusions have been reached by other investigators (Mestecky et al., 1978). In contrast André et al. (1973) failed to demonstrate any memory response in the gut.

There are interesting questions remaining to be answered: why oral immunization induces immunoglobulin mainly of the IgA class, and why mucosal immune response and systemic tolerance occur after oral antigen administration. Some evidence suggests that T

lymphocytes regulate the mucosal immune system. There is an interesting finding by Ahmed, La Brooy and Rowley (1983) following studies in mice by feeding with Vibrio cholerae. They found IgA T helper cells and IgM T suppressor cells in the Peyer's patches of immune mice when they were transferred to syngeneic animals. Similar evidence has been provided by Elson et al. (1979) that when animals are immunized orally with antigen, IgA specific helper cells home to mesenteric lymph node but not to the spleen. Additionally, Richman et al. (1981) have identified a Peyer's patch T cell (TsX) capable of mediating suppression of an IgG anti-ovalbumin response after oral stimulation with antigen. This cell was shown to be capable of migrating from the Peyer's patches to the mesenteric lymph node and later to the spleen. Taken together, these results indicate that IgA-specific helper cells enhance the induction of IgA in the gut whereas systemic tolerance is mediated by IgG T suppressor cells which migrate from Peyer's patches and populate the spleen. Clearly further studies of isotype-specific T helper cells and isotype-specific (T cell) suppression are required.

1.4 CELL TRAFFIC IN THE GUT

After antigen challenge, lymphoblasts first migrate from the Peyer's patches to mesenteric lymph nodes, then pass via the thoracic duct through the general circulation and finally return to the lamina propria. During this migration the cells gradually change from lymphoblasts with Ig surface receptors into mature IgA secreting plasma cells (Guy-Grand et al., 1974).

The factors controlling this selective homing of the gut lymphoblasts remain undefined. It has been suggested that lymphoblasts bearing IgA are attracted towards the secretory component on the intestinal epithelium. But experimental evidence is sparse (Hopkins and Hall, 1975; McWilliams et al., 1975). Pierce and Gowans (1975) have proposed that antigen to which cells are primed amplifies their localization. Using cholera toxoid in rats, they observed that antigen-specific IgA-producing plasma cells could be found in the highest density in portions of the gut which had been challenged with antigen. Against this, Halstead and Hall (1972) demonstrated that syngeneic blasts transferred to antigen-free, neonatal rats migrate to the gut in the same way as they do to the gut of normal rats. Other work grafting foetal intestine to the kidney capsules of adult mice provided similar evidence, suggesting that in addition to antigen other factors determine the homing pattern of lymphocytes (Parrott and Ferguson, 1974). These conflicting results were clarified by the experiments of Husband and Gowans (1978) using Thiry-Villa intestinal loops in rats with cholera toxoid as the antigen. Their experiments illustrated that IgA precursors can migrate to the lamina propria independently of antigen, but that the presence of antigen markedly affects the number and distribution of the cells within the loop. Apparently there is antigen-induced local proliferation within the lamina propria itself, as well as recruitment of small recirculating lymphocytes.

One interesting finding has been reported by Elson, Heck and Strober (1979) who showed that IgA helper cells are abundant in the Peyer's patches, relative to the spleen. These suggest that IgA specific helper cells may be necessary not only for homing of

lymphoblasts but also for both the primary and secondary immune responses. The significance of IgA-specific T helper cells in this regard awaits clarification (Strober et al., 1978; Lum et al., 1980). There are other factors suggested to account for the high IgA/IgG ratio at secreting surfaces, for example, hormones (Weisz-Carrington et al., 1978) and blood flow (Ottaway and Parrott, 1979). However, no single factor has been shown to account for localization by itself; perhaps all may play a part.

Common mucosal immune system

As well as being the major immunoglobulin in the gut secretion, IgA also predominates in the external secretion from other organs, including salivary glands, bronchus and breast and possibly the urinary and genital tracts. Many similarities exist between the mucosal lymphoid tissue of these organs (Tenner-Rácz et al., 1979; Bienenstock and Befus, 1980). Lymphoblasts derived from these sites will selectively repopulate all mucosally associated lymphoid tissues in irradiated animals but with a selective preference for the organ of origin. Because of this connection between mucosal lymphoid tissues, Bienenstock used the term "common mucosal immune system" (Bienenstock, 1974; Bienenstock, McDermott and Befus, 1979).

This concept has important practical implications; for example, the newborn of many species (including human) receives a significant part of its intestinal antibody postpartum via the colostrum and milk (Brambell, 1970; Mach and Pahud, 1971). In humans, milk provides secretory IgA antibodies that are not internalized but function at the mucosal level in the intestine

(Hanson and Brandtzaeg, 1980). The most appropriate antibodies to protect the G.I. tract of the newborn would be directed against enteric organisms or other antigens normally acquired via the oral route. Thus the migration of the cells from a mother's G.I. tract to the mammary gland appears biologically relevant, since it would supply the newborn with antibodies directed against the antigens it is likely to meet in the immediate postpartum period. Similarly, with certain organisms such as moniliasis or gonococcus, migration between the pharynx and genital tract (if it occurs) could conceivably be beneficial, since these organisms affect both sites. However, these areas require further studies, i.e. the pattern of cell migration among these mucosal organs and whether the cells which migrate to remote sites still produce antibody without local antigen.

1.5 PEYER'S PATCHES - THE LYMPHOID ORGANS IN THE SMALL INTESTINE

Peyer's patches are gut associated lymphoid tissues (GALT) which were described in 1677 by Peyer. These subepithelial lymphoid organs occur throughout the small intestine. The number of Peyer's patches differ in animals, there being about 10-12 in a mouse. All Peyer's patches consist of a germinal centre, predominantly of B cells, with intervening corridors of thymus dependent cells (T cells). Lymphocytes enter from the general circulation through venules with specialized endothelia and leave via efferent lymphatic drainage to the mesenteric lymph nodes.

Peyer's patches are covered with specialized cuboidal microfold M cells (Owen and Jones, 1974). These cells lack a thick coated glycocalyx material at the cell surface and have only a thin

layer of cytoplasm. In addition, the surfaces of the Peyer's patch lymphoid follicles lack goblet cells and therefore have reduced surface mucus which allows the lymphoid cells to approach very closely to the gut lumen. Immediately beneath this specialized epithelium is a dome area, containing a mixture of B and T lymphocytes and macrophages. Unlike ordinary peripheral lymph nodes, Peyer's patches have no afferent lymphatics to deliver antigen. It has been shown that a range of antigen including latex particles (Bockman and Cooper, 1973), horseradish peroxidase (Owen, 1977) and bacteria (Carter and Collins, 1974) which can be seen in cell cytoplasm vesicles are transported into the dome area by M cells.

Peyer's patches consist mainly of lymphocytes and only very few macrophages (Faulk et al., 1971). The distribution of T and B cells in the Peyer's patches differs with the age of the animals. Before birth T cells predominate but after birth, following antigenic stimulation by ingested viral, bacterial and dietary antigens, B cells proliferate in the germinal centres. Germ-free animals do not develop a germinal centre unless antigen is administered orally (Pollard and Sharon, 1970). Similarly, foetal gut grafted under the kidney capsule does not develop germinal centres (Ferguson and Parrott, 1972).

A major function of the GALT is to supply cells committed to production of IgA in the lamina propria of the gut (Craig and Cebra, 1971; Guy-Grand, Griscelli and Vassalli, 1974; McWilliams et al., 1975). The importance of the Peyer's patches to the subsequent intestinal antibody response was demonstrated by the experiments of Cebra et al. (1976), who showed that when isolated loops of bowel were stimulated with antigen, those containing Peyer's patches gave

rise to an antibody response in both immunized and distal loops while challenge of loops lacking Peyer's patches resulted in only slight or no responses. Similar studies by Husband and Gowans (1978) reached the same conclusion. These experiments indicated that the Peyer's patches were the source of IgA producing cells, as previously suggested by homing studies of GALT by Craig and Cebra (1971).

1.6 MACROPHAGE ACTIVATION

The importance of macrophages as effectors of cell-mediated immunity to a variety of microorganisms is very clear. The organisms studied include many bacteria, protozoa and some viruses which are facultative intracellular parasites, e.g. Mycobacterium tuberculosis, Listeria monocytogenes, Brucellae and Salmonellae, Toxoplasma (Remington and Krahenbuhl, 1976) and ectromelia virus (Blanden and Mims, 1973).

The story goes back to 1893, when Metchnikoff suggested that phagocytic cells play a central role in host defence. However, the first direct evidence came from Luria (1942) who showed that macrophages from normal animals were unable to destroy Mycobacteria whereas macrophages from BCG vaccinated animals were able to do so. Suter (1953) confirmed the finding that macrophages from immunized animals had a greater bactericidal activity.

Studies of Mackaness using Listeria monocytogenes in mice provided much information. When a sublethal dose of virulent organisms was injected intravenously the organism multiplied in the spleen and liver for a few days and then the bacterial population declined markedly; thereafter such mice were resistant to challenge

with large numbers of virulent organisms and also developed a delayed inflammation reaction (DTH) when injected with Listeria antigen (Mackaness, 1971; Khoo and Mackaness, 1964). In addition peritoneal macrophages from resistant mice, when cultured in vitro, manifested resistance to infection with Listeria monocytogenes. This resistance both in vivo and in vitro decayed steadily. The bactericidal activities were demonstrated without immune sera. It seems antibody played a minor role, if any, in this resistance. In an experiment designed to illustrate this point, mice were passively immunized, or immunized actively with killed vaccine; although both procedures induced high humoral antibody titres, neither induced resistance. However, the transfer of immune lymphoid cells was effective. The cells responsible for this phenomenon were demonstrated to be T lymphocytes (North, 1973; Lane and Unanue, 1972; Simon and Sheagren, 1972).

Finally the expression of resistance is nonspecific. Several workers (Mackaness, 1962; Blanden, Mackaness and Collins, 1966) found that mice infected with Brucella abortus were also resistant to Listeria and similarly mice resistant to Salmonella were resistant to Listeria and vice versa.

Mackaness coined the term 'macrophage activation' to account for the increase in bactericidal capability that macrophages acquire during infection with facultative intracellular bacteria when the animal develops specific DTH.

Indeed activated macrophages from resistant infected mice possess a powerful microbicidal and tumoricidal activity. These observations posed interesting questions. How did the macrophages become activated? Where did they come from? It is now known that lymphokines (Dumonde et al., 1969) released from sensitized T

lymphocytes after reaction with specific antigens are able to activate macrophages (Godal et al., 1971; Simon and Sheagren, 1972; David and Remold, 1976). LPS has also been shown to be capable of activating macrophages (Cohn, 1978; Beller, Kiely and Unanue, 1980). When macrophages become activated, not only do they show powerful microbicidal and tumoricidal functions, but they also appear to have different physical features and biochemical characteristics. For example, such cells are bigger, they attach immediately to glass and spread more extensively. Cell metabolism is increased, e.g. increased glycolysis, hexose monophosphate shunt activity and increased enzyme levels - particularly hydrolytic enzymes (e.g. acid phosphatase, esterase) lysozyme and secretory enzymes (e.g. plasminogen activator) (Unkeless et al., 1974; Vassalli and Reich, 1977). None of these changes has proved a reliable indicator of macrophage activation by itself.

Thus there remains a lack of correlation between known biochemical changes, and the activated condition. This may be explained as follows:

1. Macrophages have functional heterogeneity (McIntyre, Rowley and Jenkin, 1967; Walker, 1976).

2. The number of cells which are activated when compared to the whole population may be small.

3. The microbicidal mechanisms are undoubtedly complex with many steps and operations at different levels, so identification is difficult. Nevertheless the oxygen-dependent pathway which generates the superoxide ion and hydrogen peroxide has been studied in considerable depth, and the best correlation has been shown between macrophage activation and hydrogen peroxide production

(Nathan et al., 1979a, b). It is worth noting that hydrogen peroxide is a potent antimicrobial and antitumour molecule (Stadecker et al., 1977; Kung et al., 1977).

CHAPTER 2

2.1 BACTERIAL DIARRHOEA

Diarrhoeal diseases remain a common problem in the world, leading to high infant mortality particularly in developing countries. Paediatric diarrhoea accounts for more fatalities than any other disease. The World Health Organization (WHO) investigated children in seven countries and demonstrated monthly diarrhoeal attack rates of up to 40% (van Zijl, 1966). In Guatemala, India and Indonesia careful prospective field studies have determined accurately the annual incidence of paediatric diarrhoea. They showed that there were one to two attacks per child per year, during the first three years of life. These attacks were fatal in 1-4% of the episodes, yielding a death rate from diarrhoea of 20-50 per 1000 children annually (van Zijl, 1966). From these results Rohde and Northrup (1976) calculated an estimate for the total number of episodes of diarrhoea in 1975 in children less than 5 years of age in Asia, Africa and Latin America. The result was approximately 500 million episodes of diarrhoea. Of these 1-4% would be expected to be fatal, resulting in the deaths of five to eighteen million children in 1975.

These high rates of mortality are in part due to the pre-existing disease and poor health conditions of the patients, who, because of malnutrition, are more likely to die from opportunistic infections. Neter (1975) reported that in patients with malignant diseases, bacteraemia (usually due to Salmonella typhimurium and Salmonella derby) often occurred. Children with malnutrition are more susceptible to diarrhoeal disease (Sutoto

et al., 1975). Clearly these patients are not able to mount an effective immune response to combat infection.

Diarrhoeal disease may be caused by bacteria, viruses, fungi and protozoa. This thesis will describe only the diarrhoeal diseases caused by gram-negative bacilli. Four pathogenic gram-negative bacilli are mainly responsible for diarrhoeal diseases: Vibrio cholerae, Shigella, Salmonella and E. coli. They are classified into 2 groups according to the ultimate cause of the diarrhoea: whether due to enterotoxigenic or invasive microorganisms.

The enterotoxigenic organisms, Vibrio cholerae and E. coli, cause diarrhoeal disease by liberating enterotoxins which activate cyclic AMP in the epithelial cells of the intestinal tract and result in increased fluid secretion (Field, 1971). Both species of bacteria need to adhere to the gut epithelium in order for the enterotoxin to have a direct effect on the epithelial cells. It has been demonstrated that E. coli possess special appendages called pili which enable them to stick to the gut epithelium (Moon, Nagy and Isaacson, 1977). Some other pathogens possess colonizing factors (Evans et al., 1977) and Vibrio cholerae adheres to the mucosal surface of the small bowel in the absence of obvious pili (Fubara and Freter, 1973; Schrank and Verwey, 1976). Microorganisms lacking adhesive factors will be flushed out by peristalsis. The enterotoxin interferes with cell metabolism; the epithelial cells secrete vast amounts of fluid but no signs of cell damage appear. The patients may suffer from severe dehydration, low blood pressure and coma, and may die.

The invasive group consists of Shigellae, Salmonellae and invasive E. coli. Only some biotypes of E. coli possess invasive

properties. All of them have to penetrate through the epithelial cells in order to cause diarrhoeal diseases. The invasion by Shigellae is accompanied by a marked inflammation of the gut and cellular damage. Blood and white cells are usually found in the stools. Shigellae are non-motile and do not produce H_2S which make them different from Salmonellae. The pathogenesis of Shigellae and Salmonellae are different and will be described separately.

All known species of the genus Shigella are pathogenic for man. Shigellae appear to be the most virulent in terms of the small numbers of organisms that are required to initiate diarrhoea (Levin et al., 1973; DuPont et al., 1972) and small amount of exotoxin to cause death (van Heyningen, 1968). As few as 10 organisms of Shigella dysenteriae taken orally are enough to cause diarrhoea when compared to Vibrio cholerae which requires one million organisms (Hornick, 1978). Apart from the protein exotoxin of diphtheria toxin and the botulinus toxin, Shigella enterotoxin is the most powerful toxin known. However, the mechanism of its action is unknown. In order to cause disease Shigella infection requires that the organisms must penetrate the epithelial surface (Formal et al., 1976). The mechanism of penetration remains undefined, as does the ensuing pathogenesis of the disease.

Salmonella is an exceedingly large genus, comprising approximately 1400 serotypes, most of which are widely distributed in nature as enterobacteria of animals. Some of these strains are responsible for gastroenteritis whereas the others cause bacteraemic enteric fever. Salmonella typhi was the first member to be described and is the causative organism of typhoid fever.

2.2 TYPHOID FEVER

Typhoid fever, or a disease very similar to it, was known long before Hippocrates. It has a typically variable onset and exhibits a classical type of pyrexia with a "step-ladder" rise. Fever is maintained for the first 2-3 weeks and then subsides. Typhoid fever has variable symptoms, signs, duration and severity. Without knowing the etiology of typhoid fever Pierre Bretonneau and Pierre Louise (1829) attempted to clarify the picture of typhoid fever from pathogenic and post-mortem appearance to separate true typhoid from all other gastrointestinal tract infections which were often confused with one another. They described the inflammation of the Peyer's patches, their enlargement and ulceration. Not until 1880 was the causative agent, Salmonella typhi, discovered by Carl Joseph Ebeth.

Typhoid fever is a significant public health problem in many areas of the world. Attack rates in some countries are very high; in some endemic areas of Egypt rates as high as 2.8% of the population have been reported (Messih, 1961).

Contaminated food and water are the principal sources of infection. Infection may be reduced by improving the water supply, sanitation and personal hygiene. Immunologists have tried to develop an effective vaccine for 50 years, without great success until very recently when a live attenuated vaccine (Ty 21a) has been successfully tried in Egypt. Chemotherapy offers an alternative approach to the control of typhoid fever. The antibiotic chloramphenicol was first used in 1948 and since then has changed the course of the disease and improved the prognosis of patients. It cut short the course of disease from 30 to 3.5 days.

Unfortunately, such therapy gives rise to drug resistant strains. Indeed a typhoid epidemic in Mexico in 1972 was caused by a strain resistant to a number of antibiotics, including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Weissman et al., 1973; Rodriguez-Leiva, 1979; and Davies, 1979).

In developing countries vaccination would seem to offer the best method for control of the disease. In order to have a chance of improving vaccination procedures it will be necessary to understand the immune interaction of both host and bacteria at the cellular level.

2.3 PATHOGENESIS OF TYPHOID

Typhoid fever which occurs in humans can be i) very severe, ii) self-limiting or iii) subclinical leading to a carrier state. In the last case the organism will be shed in stools for months or years. A typhoid-like disease that occurs in mice and which is caused by either Salmonella typhimurium or Salmonella enteritidis has similar signs and effects. These two bacterial strains are usually used in mice as a model to study the pathogenesis of human typhoid, its immunological aspects and the host-parasite relationships.

Animals usually contract Salmonella infection via the oro-faecal route. After ingestion the bacteria pass along the gastrointestinal tract from the stomach to the small intestine. Initially Salmonellae infect certain specific areas such as the distal ileal mucosa and its Peyer's patches (Carter and Collins, 1974). The degree of Salmonella infection is dose dependent. If higher doses are used for challenge then many sites of the gut

become infected (Hohmann, 1979). Ozawa et al. (1973) suggested that these bacteria may be able to pass through or between the epithelial cells of the intestine without showing any tissue damage as in the case of Shigella invasion. However, it seems that the invasion of epithelial cells is not essential for pathogenesis, because only a small number of organisms were recovered from the gut epithelium, compared to a large number of Salmonellae in the adjacent Peyer's patches. In addition, bacteria in the columnar epithelial cells (Giannella et al., 1973; Takeuchi, 1967) are subsequently collected within the Peyer's patches via afferent capillaries (Carter and Collins, 1974). Only a small number of the bacteria in the intestine continue to multiply and disseminate to the mesenteric lymph node. Infection by avirulent Salmonella strains is halted in the mesenteric lymph node whereas the virulent strains multiply and spread through the thoracic lymph duct to the circulating blood and into various organs, particularly the spleen and the liver. Fever and diarrhoea are common symptoms of this disease and animals usually die of a systemic infection.

As Salmonellae do not produce enterotoxins like Vibrio cholerae or Shigellae, studies have been undertaken during the last decade to investigate how these infections cause death. Nothing conclusive has yet been established. Nevertheless reports by Giannella et al. (1975) and Gots, Formal and Giannella (1974) showed the Salmonella is capable of activating cyclic AMP in the gut and causing intestinal fluid secretion. The mechanism of Salmonella activation of adenylate cyclase is unclear but apparently differs from that of cholera toxin in that it is inhibited by indomethacin. Unfortunately, the above report is one of few which exist concerning

the possible pathogenesis of Salmonella infection. Clearly there is a need for further investigation in this particular area.

2.4 CHARACTERISTICS OF SALMONELLAE

Salmonellae are members of the Enterobacteriaceae:- non spore forming, straight rods, motile with peritrichous flagella. Salmonellae are easily cultivated on ordinary nutrient media and fail to ferment lactose and sucrose. With the exception of S. typhi and S. gallinarum, they all produce gas in glucose and hydrogen sulfide in triple sugar iron medium (TSI). Lack of urease has distinguished Salmonellae from Proteus spp.

Salmonellae are composed of a large group of organisms varying in antigenic structure. Kauffmann and White classified Salmonellae into different groups according to their O antigens and further differentiated into species and varieties on the basis of flagella antigens. The Kauffmann-White system now includes well over 500 varieties, many of which are listed as distinct species.

2.4.1 Antigenic structure of Salmonellae

At the surface of the cell body, there are somatic antigens (O antigens) associated with lipopolysaccharide (LPS) (in both motile and non-motile organisms) which is an integral part of the cell wall. These antigens are heat stable and are not destroyed by alcohol or dilute acid. These O antigens are the endotoxins of the Salmonellae bacilli and their antigenic specificity is determined by their polysaccharide components.

The O antigen (LPS) has long been known to be an important

virulence factor in Salmonellae. Rough (R) forms of bacteria that have lost the O specific side chains of the LPS are avirulent. Semirough forms, in which the length of these normally polymeric side chains is reduced, have reduced virulence in mice (Nakano and Saito, 1969; Valtonen, 1969). So it is logical to assume that O antigen is associated with the virulence of a bacterium and that antibody raised against O antigens should be protective.

A significant number of reports have investigated the protective activity of O antibodies and the effect of variation of the polysaccharide structure of O antigens; for example, in bacterial mutants or bacterial hybrids. Valtonen and Makela (1971) demonstrated that S. typhimurium with O antigens 4, 12 is more virulent than a strain expressing 9, 12. This was confirmed in a study by Lyman et al. (1971) who also found that strains carrying 9, 12 were less virulent than 4, 5, 12 carrying strains. It has been convincingly shown that antibody against any one of the O antigenic factors - 1, 4, 5 or 12 - serves to opsonize bacteria (Biozzi et al., 1963) and increases the rate of their clearance after intravenous injection, although this is not accompanied by significant protection against intravenous challenge. Furthermore, Auzins and Rowley (1969) investigated the importance of the O antigens of Salmonellae in resistant and susceptible mice. They found that the BALB/c strain is very susceptible to Salmonellae and that it lacks the ability to produce antibody against O-5 antigen as compared to the resistant Swiss White mice. It has been speculated that virulent bacterial species are more likely to occur among the higher chemotypes (i.e. species with larger LPS side-chains) because of their greater lipophilic character which might inhibit

phagocytosis (Lüderitz, Staub and Westphal, 1966). Others have demonstrated the role of O antigens in bacterial virulence:- O antigen enhanced bacterial resistance to bactericidal action of serum (Rowley, 1968) had anticomplementary activity (Morrison and Kline, 1977) and protected the bacterial membrane from enzymic activity (Rest, Cooney and Spitznagel, 1977).

The H antigens associated with the flagella are protein in nature and heat labile. Salmonellae usually possess flagella but the importance of these structures for virulence is not established. However, infected animals show a rapid increase in anti-H antibody although only for a short period. In addition, anti-H antibody does not possess bactericidal activity in vitro or opsonic activity in vivo (Hohmann, 1979). Tully and Gaines (1961) showed that the loss of H antigen from Salmonella typhi did not reduce the virulence of bacteria. Moreover, flagellae were not necessary for the colonization of the intestine (Tannock et al., 1975). Collins (1970) compared non-motile S. gallinarum, S. pullorum and motile strains of S. enteritidis for their ability to colonize the small intestine and found that both groups have equal ability. Subsequent studies confirmed the above finding, equal numbers of both motile and non-motile bacteria being recovered from the Peyer's patches of germ-free mice infected orally (Collins and Carter, 1978).

In contrast to the above results with Salmonella, Guentzel and Berry (1975), Jones and Freter (1976), Attridge and Rowley (1983) conducted similar studies in Vibrio cholerae. They all emphasized the importance of flagella as they found a close relationship between motility and virulence of Vibrio cholerae.

Nevertheless it was not the motility that was required *per se* but the production of a particular adhesin on the flagellum (Attridge and Rowley, 1983; Jones and Freter, 1976) which allowed *Vibrio cholerae* to adhere to and attack the cells by the action of cholera enterotoxin.

Typhoid bacilli and certain related organisms possess, in addition to endotoxin, a somatic antigen which is associated with virulence for animals. It has been designated the Vi or virulence antigen. It seems likely that other gram-negative organisms possess analogous virulence antigens, e.g. K antigens of *E. coli*. Vi antigen is present as an envelope antigen and prevents access of anti-O agglutinins to their specific somatic antigens, and it has anti-phagocytic ability (Bhatnagar, 1935). Tully *et al*. (1962) in experiments on chimpanzees indicated that *Salmonella typhosa* without Vi antigen required relatively more organisms to induce typhoid in contrast to organisms associated with Vi antigen. Gaines *et al*. (1961) supported their finding by showing Vi antigen enhanced the virulence of a non-Vi variant of *Salmonella typhosa*.

As to the significance of Vi antibody for protection Diena *et al*. (1974) revealed that immunisation of mice with a hybrid *Salmonella typhi-Salmonella typhimurium* strain which expressed Vi antigen, or with pure Vi antigen (Diena *et al*., 1975) conferred no protection. Also in human typhoid vaccine trials in Yugoslavia in 1962 using alcoholized Salmonella vaccine containing Vi antigen the same conclusion (Yugoslav Typhoid Commission, 1962) was reached.

2.4.2 Common enterobacterial antigen

In addition to the antigens that differentiate various

Enterobacteriaceae from each other, most of these organisms possess the core regions of the cell-wall lipopolysaccharide which are identical or extremely similar chemically (Lüderitz, Staub and Westphal, 1966). Gram-negative organisms share several cross-reactive antigens. One of these antigens known as "common enterobacterial antigen (CA)" was first described by Kunin (Kunin, Beard and Halmagyi, 1962). It can be extracted from the cells and detected by haemagglutination or haemolysis of antigen coated erythrocytes. It has been suggested that it might be possible to develop an immunizing agent from this common antigen that is capable of enhancing resistance to a wide range of enteric bacteria (Gorzynski, Ambrus and Neter, 1971).

2.5 NATURAL RESISTANCE TO SALMONELLA INFECTION

Natural resistance is the ability of a host to resist the usual pathological effects of an infectious organism in the absence of previously acquired specific immunity. One very good example is typhoid fever. It is a uniquely human disease. No other animals are infected with Salmonella typhi. Natural resistance may play a vital role in allowing an infected host to survive long enough to mount an effective immune response or possibly the genotype of a given host may determine whether it will be infected or resist the disease via other non-immune pathways. Despite the fact that the mechanisms of natural resistance are not well understood, genetic experiments using strains of inbred mice allow analysis of this phenomenon. Using such mice, natural resistance to a number of infectious organisms has been demonstrated to be under genetic control; there are many resistance genes which control resistance

to a number of organisms.

There are few genes in mice which express resistance to Salmonella infection. The Salmonella resistance gene Ity (Plant and Glynn, 1979) has been well studied and a small number of mouse strains have an abnormal allelic gene Lps^d (Rosenstreich, 1978) or xid (O'Brien et al., 1979) which confers susceptibility to Salmonella infection. Plant and Glynn (1979) made the first comprehensive study of resistance to Salmonella typhimurium in mice. They found that the susceptibility or resistance of inbred mouse strains is under the control of an autosomal gene in chromosome I, named Ity, to stand for immunity. The influence of this gene is profound. The susceptible mouse homozygous for Ity^s can be killed by as few as 10 Salmonella typhimurium while the resistant mouse (Ity^r) may survive when challenged by over 10^4 organisms. The control mechanism of the Ity gene is unknown but most workers agree that the Ity gene regulates the nonspecific ability of the host to control growth of Salmonellae in the spleen and liver. Hormaeche's finding supports their results by demonstrating that Salmonellae grow faster in the susceptible mouse than the resistant mouse strain (Hormaeche, 1979).

The Lps gene is located in chromosome 4. It has the ability to control the response of mice to lipopolysaccharide (LPS). There are two alleles n and d representing normal and defective responsiveness (Watson et al., 1978; Watson, Riblet and Taylor, 1977). Most of the inbred mouse strains are lipopolysaccharide responsive Lps^n, except for C3H/HeJ and C57Bl/10ScN strains which are known for endotoxin unresponsiveness (Lps^d). In vitro, cells of Lps^d mice - lymphocytes, macrophages and fibroblasts - respond poorly to endotoxin, especially the macrophages which fail to show

tumoricidal activity or any response to lymphokines such as MIF (Tagliabue et al., 1978).

CBA/N is the only one of the inbred mouse strains which has an x-linked recessive mutation gene which is defective in antibody production. This mouse is markedly deficient in B lymphocytes so it shows no response to the T cell independent antigen endotoxin (O'Brien et al., 1979). The inability to produce an effective antibody response is accompanied by susceptibility to Salmonella typhimurium, but the host can be rendered resistant by passive administration of anti-Salmonella sera.

2.6 THE ROLE OF ANTIBODY IN SALMONELLA INFECTION

It has been demonstrated that mice immunized with killed vaccines of Salmonella were protected from virulent strains, measured by an increased number of animals surviving challenge. Kenny and Herzberg (1968) studied living and heat-killed Salmonella vaccines, both of which produced bactericidal and haemagglutinating antibodies within 4 days. These workers claimed that killed vaccine could induce effective protection against both intraperitoneal and subcutaneous injection with S. typhimurium or S. enteritidis. Subsequent studies by Raettig (1976) found similar stimulation of nonspecific immunity when mice were immunized orally ten times with heat inactivated S. enteritidis. These mice were resistant to oral challenge with virulent S. typhimurium.

Jenkin and Benacerraf (1960) found that the amount of phagocytosis and rate of intracellular killing of the virulent organisms could be increased if the virulent organisms were first opsonized with specific antisera. Melendez et al. (1978) confirmed

their earlier finding. They demonstrated that the growth of S. enteritidis in vitro was inhibited by immune peritoneal exudate cells only in the presence of immune sera. Recently, Cooper, Johnson and Rowley (1983) reached the same conclusion, that the expression of bactericidal activity by peritoneal exudate cells required specific antibody.

In contrast to these results, Gorer and Schutze (1938) found that the resistance of mice to various strains of Salmonella bore no relationship to the level of antibody. Collins (1969a,b) revealed that animals immunized with killed vaccines of Salmonella produced high antibody titres. However, they could not control the growth of virulent strains in the spleen and liver. Further, he had observed a significant reduction in the size of the initial inoculum which attaches to the epithelial surface, allowing the host more time to develop immune responses and delay the dissemination of the organisms to the spleen and liver. Over the intervening years, many other workers investigated the resistance to intracellular bacterial parasites: antibody appears to be limited in value in protection against Mycobacterium, Brucella and Listeria and the other arm of immunity, the cellular system, seems to play the major role in resistance to these organisms.

2.7 CELL-MEDIATED IMMUNITY AGAINST INTRACELLULAR INFECTION.

Mechanisms of cell-mediated immunity (CMI) which afford protection against infection by facultative intracellular bacteria have been established. Some characteristics of this immune response are:

i) delayed-type hypersensitivity (DTH) to the causative agent normally accompanies the infection (Zinsser, 1921);

ii) immunization with live bacteria is required to induce the protection (Hobson, 1957; Mackaness, 1962; and Sulitzeanu, 1965);

iii) neither dead bacteria nor culture filtrate can induce DTH (Fleischner and Neyer, 1918) nor full protection (Hobson, 1957; Mackaness, 1962; Holland and Pickett, 1958); and

iv) adoptive transfer of protection cannot be fully achieved with specific antibody (Miki and Mackaness, 1964) and transfer of DTH requires lymphocyte transfer.

In the late 1960's Mackaness and his coworkers studied the development of immunity to the intracellular bacterium Listeria monocytogenes, and stated that the resistance of animals to infection depended upon the participation of two cell types (McGregor and Koster, 1971): specific T lymphocytes and mononuclear phagocytes (macrophages). T cells act as specific inducers and mononuclear phagocytes as nonspecific effector cells.

During the last 20 years a large number of workers have investigated the role of cell-mediated immunity directed against various intracellular parasites, e.g. Mycobacterium tuberculosis, Brucella abortus, Listeria monocytogenes, and some Salmonellae (Luria, 1942; Suter, 1953; Pomales-Lebron and Stinebring, 1957; Holland and Pickett, 1958; and Mackaness, 1964). Using Salmonella typhimurium, Hobson (1957) and Howard (1961) have shown that immune macrophages were able to inhibit and kill intracellular Salmonella which confirmed the findings of other workers (Saito et al., 1960).

Mackaness (1971) stated that the interaction between T-lymphocytes and specific antigen results in the liberation of macrophage activation factor (MAF) which increases the capacity of macrophages to kill bacteria and tumour cells. It should be emphasized that the expression of such antibacterial activity is nonspecific (Blanden, Mackaness and Collins, 1966; Mackaness, 1971). However, the resistance against the original infecting organism is always considerably greater than that against unrelated pathogens (Ruskin and Remington, 1968; Ruskin et al., 1969; and Frenkel and Caldwell, 1975).

Attempts have been made to activate normal macrophages in vitro by culturing them with specifically sensitized T lymphocytes plus antigen, or with the supernatant from antigenically or polyclonally stimulated lymphocytes. The bactericidal activity of such stimulated macrophages was examined by adding bacteria and performing viable counts at different intervals thereafter. Simon and Sheagren (1972) showed that the incubation of monolayers of normal macrophages with specific antigen and peritoneal lymphocytes obtained from animals with delayed-type hypersensitivity caused the macrophages to acquire greatly enhanced listericidal activity. Similar results were obtained by others (e.g. Jones and Youmans, 1973; Cole, 1975; Godal, Rees and Lamvik, 1971; Klun and Youmans, 1973; Buchmüller and Mauel, 1979). As a result of these studies we now know that "lymphokines" play an important role in macrophage activation as first clearly enunciated by Dumonde et al. (1969); Mackaness (1971).

Macrophage-mediated destruction of microbial pathogens in vivo is a local rather than a systemic event. At the sites of bacterial implantation the replicating bacteria lead to the expression of T cell-mediated inflammation (DTH). The macrophage

population in these infected areas is derived from bone-marrow and blood monocytes. Thus it is not surprising that emigrant macrophages harvested from inflammatory exudates are more susceptible to activation by lymphokines and to become microbicidal in vitro than are resident macrophages (Nogueira and Cohn, 1978).

The relative importance of blood-borne monocytes and resident macrophages in the expression of such immunity became clear when mice that were shielded except for their livers were irradiated - thus selectively destroying the capacity of liver macrophages to divide. Upon infection with Listeria monocytogenes, the shielded animals recruited typical macrophages into the liver and expressed immunity in that organ. Unshielded controls neither accumulated macrophages nor developed immunity (North, 1970). It was concluded that immigrant blood monocytes rather than local macrophages are responsible for the development of activated macrophages in infected tissues.

Antibacterial immunity and delayed-type hypersensitivity (DTH) appeared to be linked together. This is suggested by the close temporal relationship between the two phenomena (Collins and Mackaness, 1970; Mackaness and Blanden, 1967) and by the fact that both DTH and antibacterial immunity can be transferred by specific lymphocytes (Mackaness, 1971; Collins and Mackaness, 1968). Moreover expression of both DTH and protection depend on bone-marrow derived (Hahn, 1975; Lubaroff and Waksman, 1968), mononuclear phagocytes (Dienes and Mallory, 1932; Dvorak, 1974). In contrast, Youmans and Youmans (1969) were able to demonstrate that animals could be resistant to tuberculosis in the absence of detectable DTH, or could be rendered hypersensitive without any corresponding increase in resistance (Raffel, 1955; Rothschild, Friedenwald and

Bernstein, 1934).

One can therefore question if these two phenomena are discrete but coincidental events; for example it may be that delayed-type hypersensitivity (DTH) and antibacterial immunity are mediated by different subpopulations of T lymphocytes. Recently however, Kaufmann and Hahn (1982) succeeded in establishing cloned T cell lines which were specific for L. monocytogenes, and showed that a single cloned T-cell line was able to confer both DTH to Listeria antigen and protection against Listeria in vivo. This significant finding needs confirmation.

The cooperation between T lymphocytes and macrophages in the expression of cellular immunity works in two directions. As has been mentioned earlier T lymphocytes activate macrophages to become killer cells; in addition however, macrophages can regulate T lymphocyte proliferation and differentiation (Beller and Unanue, 1976, 1978). Further, this T cell and macrophage interaction is under MHC restriction; donors and recipients of T cells and macrophages have to share the same H-2I locus (Rosenthal and Shevach, 1973; Schwartz and Paul, 1976).

Zinkernagel (1974) provided evidence that transfer of immunity to Listeria by splenic T cells in mice is H-2 restricted. Additionally, the H-2I locus is necessary and sufficient for antibacterial protection (Zinkernagel et al., 1977).

There is no doubt that the best way to obtain protection against enteric organisms is to stimulate the local cellular immune system at the site of infection i.e. in the intestine. The existence of local CMI was shown by Galindo and Myrvik (1970). Using mycobacterial vaccines, Spencer et al. (1974) compared the development of local respiratory and systemic CMI in the guinea

pig. Animals immunized via the respiratory tract with either H37Ra (killed) or BCG (live) vaccines developed significantly greater local respiratory CMI than did animals immunized subcutaneously. Clearly local immunization is of great significance in protecting the initial site of infection; however, the mechanisms of cell-mediated immunity at the mucosal surface are still not well understood.

2.8 TYPHOID VACCINE

The development of vaccines to prevent typhoid fever has been aimed at agents with capacity to stimulate antibody production, which should be most relevant and most convenient to measure. For more than 50 years, both in animal experiments and human trials, this has been attempted, but Salmonella vaccines have been poorly protective compared to smallpox or poliomyelitis vaccines.

Vaccine trials for 12 years were sponsored by WHO and were aimed at raising antibody against any or all of the Salmonella typhi antigens (O, H and Vi) by treating the organism in different ways, for example alcoholized vaccine in which Vi antigen is well preserved, or acetone inactivated vaccine for preserving H antigen. The conclusion was that the increasing levels of both Vi and O antibodies did not correlate with the effectiveness of vaccines. Although H antibody did show some correlation with protection (Benenson, 1964), this finding conflicted with other studies (Tully and Gaines, 1961; Tully, Gaines and Tigertt, 1963) and so it was suggested that those vaccines which raised H antibody and were protective contained other labile protective antigens (Benenson, 1964). However, each of these vaccines afforded some protection,

particularly in children in the endemic area (Yugoslav Typhoid Commission, 1964; Hejfec et al., 1966; and Ashcroft et al., 1967). Ashcroft et al. (1967) suggested that, in such endemic areas, repeated ingestion of subinfective doses of typhoid bacilli may result in an immunized population and that vaccines then enhanced resistance by a booster effect. The protection would fail when heavily contaminated foods were ingested.

When all these results were considered together with the prevailing concept that typhoid carriers were immune to reinfection, it seemed that the optimal route of immunization against typhoid fever ought to be oral, leading to antigenic stimulation of antibody producing cells in lamina propria where the natural infection occurred. Two oral inactivated typhoid vaccines, Typhoral (Behring Co., Somerville, N.J.) and Taboral (Swiss Serum and Vaccine Institute, Bern, Switzerland) were put to the test. Again, antibodies against H, O, and Vi were increased to variable degrees among the volunteers. But resistance to S. typhi was less than with parenteral vaccine. The level of antibody did not correlate with protection. Moreover, there were frequent relapses of typhoid fever in patients with high O, H and Vi antibodies.

A mutant strain of Salmonella typhi which lacks the enzyme galactose epimerase - Ty2la - was isolated by Germanier and Fürer (1975). It was shown to be capable of synthesizing immunologically important cell wall lipopolysaccharide of the smooth type when galactose is supplied exogenously (as occurs in vivo). The bacterial uptake of galactose and its accumulation causes cell lysis. Therefore both the virulence and the protective capacity of such 'gal E' mutants depend on the activity of all of the enzymes responsible for metabolism of galactose and its distribution within

the bacterial cell.

As an initial test live oral vaccine Ty2la was tried in volunteers for protection against typhoid (Hornick et al., 1976) with encouraging results. This safe, live Ty2la vaccine has now been tried successfully on a large scale in Egypt (Wahdan et al., 1982).

Mice which survive Salmonella infection are resistant to re-infection. Similarly with mice which have been infected with sublethal doses of live Salmonella parenterally. These infected mice develop mechanisms which can eliminate a superinfecting dose of organisms from the liver and spleen (Mackaness, Blanden and Collins, 1966). This mechanism has the characteristics of the acquired cellular resistance which develops in tuberculosis, brucellosis and listeriosis. Infection or active immunization induces opsonic antibodies which enhance phagocytosis, but this alone will only protect a proportion of the animals against the lethal effects of the challenge (Jenkin and Rowley, 1963). However, immunization with live organisms also initiates the production of large numbers of activated macrophages by the host. Passive transfer studies using hyperimmune serum or sensitized cells leave no doubt that the latter are of primary importance in the control of intracellular infections, leading to eventual elimination of the bacteria from the tissues (Mackaness, 1971).

Both the livers and spleens of infected mice show an increase in weight and in ^{3}H thymidine uptake. Histological studies reveal that the infected foci or granulomas of these organs are filled with mononuclear phagocytes and macrophages.

Hohmann (1979) conducted studies in Salmonella immunity and found that when mice were infected with avirulent Salmonella which

persist in the Peyer's patches (the lymphoid organs of the small intestine), the organisms grow in the Peyer's patches for a few days and then decline in numbers. Afterwards these mice are resistant to the homologous strain and to a virulent strain of Salmonella typhimurium, C5. This is possibly due to the immunity occurring locally in these areas, as well as in the liver and the spleen. Recently, Moser et al. (1980) confirmed these results by demonstrating that Salmonella typhimurium G30 induced high intestinal IgA antibodies and gave good protection which was accompanied by DTH. These results indicated that live oral vaccines stimulated both the humoral and cellular immune systems.

2.9 AIMS

Because of the localisation of bacteria in the Peyer's patches we would like to know whether these lymphoid follicles are uniquely important to the development of immunity following oral immunisation. Some of the questions to be answered if possible are:

1. Does true local cellular immunity exist in the Peyer's patches of the small intestine?
2. Are there changes in cell population (lymphocytes and macrophages) during the development of immunity?
3. Is this local immunity associated with locally activated macrophages?

CHAPTER 3

MATERIALS AND METHODS

3.1 ANIMALS

Mice used were outbred, specific-pathogen-free LAC strain mice supplied by the Central Animal House of this University. They were originally obtained from the Medical Research Council Laboratory Animal Centre, Carshalton, Surrey, England. Both sexes of mice were used at 6-8 weeks of age; they were kept in the departmental Animal House and allowed to eat commercial mouse food and to drink tap water.

Rabbits were also obtained from the Central Animal House, age and sex were varied.

3.2 BACTERIAL STRAINS

Salmonella 885 is a Salmonella typhimurium-E. coli hybrid strain derived from a cross between Salmonella typhimurium SF 1142 x E. coli Hfr 492, which has the Salmonella biotype in addition to the expression of E. coli O-8 antigen. Salmonella 885^{SR} is an isolated streptomycin-resistant mutant.

Characteristics of Salmonella 885

Antigen	O-8 and H-i:1,2.
LD_{50} for mice	intravenous route = 10^6
	oral route = 10^{10}

Antibiotic susceptibility:-	Resistant to erythromycin, sulphafurazole, cloxacillin, and penicillin. Susceptible to chloramphenicol, streptomycin, tetracycline, ampicillin and neomycin.
Biochemical reaction:-	TSI media-acid butt, alkaline slant with H_2S. Ferment glucose with acid and gas, and do not ferment lactose and sucrose.

The antibiotic susceptibility test, biochemical reaction test and the pattern of growth and decline of organisms in the Peyer's patches were used to check the identity of Salmonella 885.

Salmonella typhimurium C5 is a smooth strain which is extremely virulent for mice - the LD_{50} is less than 10 organisms when challenged intravenously and 10^5 orally. $C5^{SR}$ is a streptomycin-resistant mutant.

E. coli F492, is a non-motile strain which expresses O-8 antigens. This organism and Salmonella 885 were obtained from Dr G. Schmidt, Max Planck Institute, Freiburg.

Vibrio cholerae 569B is a classical Inaba strain obtained from the Cholera Research Laboratories, Dacca.

All these organisms were kept as lyophilised cultures and stored at 4°. For experimental use, a new ampoule of lyophilised culture was opened every 3-4 weeks, subcultured in tryptic soy broth (Difco) and streaked onto a nutrient agar plate.

3.3 CULTURE MEDIA FOR BACTERIA.

3.3 a. Tryptic soy broth was used for the routine growth of bacteria. One colony of bacteria was transferred into a 100 ml broth, then cultured with shaking at 37° overnight (approximately 16-18 hours). A 1:100 dilution of this culture was counted in Helber Bacterial Counting Chamber. Bacteria were washed by centrifugation and resuspended in PBS at a concentration of 10^9/ml; if the organisms were to be administered orally, $NaHCO_3$ was added to the PBS to a final concentration of 3% (w/v).

3.3 b. The agar used was modified Luria agar - a differential and selective medium. Bacteria which do not ferment lactose, such as Salmonella, grow as red colonies on this medium. Addition of sodium sulphite provides an inhibitor for the normal flora of the intestine (e.g. micrococcus). However, when streptomycin-resistant organisms were used in experiments, streptomycin sulphate (at 100 µg/ml) was added instead of sodium sulphite in the Luria agar media. Agar plates were dried at 37° for 30-45 minutes and stored at 4° until required. These agar plates were used for all purposes, i.e. counting bacteria from the homogenized Peyer's patches, intestinal wall and gut content or in the bactericidal assay.

Luria Agar Formula

Difco tryptone	19 gm/L
Difco yeast extract	5 gm/L
NaCl	0.5 gm/L
Sodium sulphite	2.5 gm/L
Difco agar	15 gm/L.

Distilled water was added to 1000 ml. The pH was adjusted to 7.2 with 0.5 N NaOH or 0.5 N HCl. Each bottle containing 500 ml agar medium was then autoclaved at 15 lb/in^2 for 20 min. Just before use, 25 ml of 20% sterile lactose solution and 1 ml of 1% 2,3,5-triphenyl tetrazolium chloride (Calbiochem. San Diego, California) which had been sterilized by filtration were added to 500 ml of molten Luria agar media.

3.4 TISSUE CULTURE MEDIUM

RPMI-1640 was used primarily for all purposes. The powdered medium (Microbiological Associates Inc., Bethesda, U.S.A.) was dissolved in deionised, distilled water and buffered with sodium bicarbonate 0.84 gm/L and Hepes 4.76 gm/L (20 mM). The medium was adjusted to pH 7.2 and was sterilized by filtration through a Millipore membrane filter (0.45 µm pore size). Usually 5-10% of heat inactivated foetal calf serum (Flow Laboratories) was added to the medium before use. When antibiotics were required both streptomycin sulphate (100 µg/ml) and penicillin G (100 units/ml) were added.

3.5 ANTIGENS

3.4 a. Lipopolysaccharide (LPS) preparation

LPS was prepared by the technique described by Westphal, Lüderitz and Bister (1952). An overnight culture of Salmonella typhimurium 885 was washed and resuspended in saline to a concentration of 20 mg/ml. The bacterial suspension was heated to

65° and then mixed with an equal volume of pre-heated 90% phenol solution with constant stirring, then maintained at this temperature for 20 min. After cooling to room temperature the mixture was spun at 700x*g* for 20 min at 4° in an MSE centrifuge (Measuring and Scientific Equipment Ltd. England). The aqueous layer was removed and kept at 4°. The phenol layer was then re-extracted with an equal volume of water. The two aqueous layers were pooled and dialysed against distilled water at 4° overnight. The contents of the dialysis bag were mixed with 5 volumes of ethanol and 0.5 gm of sodium acetate and left for 4 h at 4°. The resultant LPS precipitate was collected by centrifuging at 700x*g* for 30 min. After solubilization in distilled water and dialysis against distilled water overnight, the LPS solution was centrifuged at 1,000x*g* for 10 min. The supernatant was subjected to further centrifugation at 100,000x*g* for 1 hour in a Spinco Ultracentrifuge (Beckman Instruments Inc., U.S.A.). The final pellet was dissolved in distilled water, dialysed against distilled water and lyophilized.

3.5 b Outer membrane protein antigens preparation

Outer membrane protein (OMP) antigens were prepared from *Salmonella enteritidis* 11RX and *Salmonella typhimurium* C5 by a technique previously described by Ashley, Kotlarski and Hardy (1974). An overnight culture of *Salmonella typhimurium* C5 was washed with distilled water twice and resuspended at a concentration of 30 mg/ml in magnesium chloride buffer (5 mM $MgCl_2$ in 10 mM tris-HCl, pH 7.8) containing 2-mercaptoethanol (5 mM) and nucleases [DNase and RNase, both at 10 µg/ml (Calbiochem.)]. The bacteria

were disrupted by ultrasonic vibration at 4°. The sonicate was centrifuged at 12,000xg for 15 min at 4° to remove large debris and then the supernatant was centrifuged again at 100,000 x g for 2 h at 4° to remove the lipopolysaccharide. The supernatant fluid was dialysed at 37° for 24 h against tris buffered magnesium chloride containing 0.01% sodium azide with DNase and RNase (10 µg/ml of each) to ensure that nucleic acid was completely digested. The antigen was then dialysed extensively against distilled water at 4°, lyophilised and stored at 4°.

3.6 COLLECTION OF SERUM

Blood was obtained from the retro-orbital plexus of mice or from the ear veins of rabbits. Blood was kept at 37° for 1 h and then at 4° for 3-5 h for maximum clot retraction. Serum was separated and sterilized by filtration and stored in small aliquots (0.5-1.0 ml) at -20°.

3.7 PREPARATION OF ANTISERA

Rabbit anti-885 antiserum

An overnight (sixteen hour) culture of Salmonella 885 was washed with PBS twice and resuspended in PBS at 10^9/ml. 0.5 ml of such a suspension was injected intravenously into 2-3 rabbits, twice a week for 3-4 weeks. Serum was collected 7 days after the last dose and tested for antibody titre. Once a high antibody titre was obtained, the rabbit was anaesthetised with pentobarbitone sodium and cardiac puncture was performed. Serum was separated, filtered

through 0.45 μm Millipore (Millipore Corporation, Bedford, Ma.) and stored in small aliquots without preservative at -20°.

Mouse anti-C5 antiserum

Mice were given 3-4 intravenous injections of 40 μg of alcohol-killed Salmonella typhimurium C5 vaccine at monthly intervals. After a further week, the mice were bled.

3.8 QUANTITATION OF ANTIBODY BY PASSIVE HAEMAGGLUTINATION

Sheep red blood cells (SRBC) were washed by centrifuging three times and resuspended to 2.5% v/v in saline containing 100 μg/ml of Salmonella 885 lipopolysaccharide. The SRBC were incubated with the antigen (LPS) for 60 min at 37° with rolling and then washed again three times. Cells were resuspended to 1% v/v in saline.

25 μl of two-fold dilutions of antisera were prepared in round bottom microtitre trays (Flow Labs) and equal volumes of sensitized SRBC were added. The trays were incubated at 37° for 1 h and a further 4° overnight. The last well demonstrating any agglutination was taken as the endpoint.

Two haemagglutination units were used for opsonization of bacteria in the bactericidal assay.

3.9 TECHNIQUE OF ORAL INFECTION

Mice were fed with 0.5 ml of bacterial suspension containing 5×10^8 Salmonella 885 in 3% $NaHCO_3$-PBS. This was administered

via a blunt 19 G needle which was gently introduced through the mouse oesophagus into the stomach.

3.10 RECOVERY AND ENUMERATION OF BACTERIA

Mice were killed by cervical dislocation and the small intestine separated from the surrounding mesentery and removed. Using a long blunt needle fitted to a plastic syringe 10-20 ml of cold normal saline was flushed through the intestinal lumen.

The outside of the intestine was washed again with saline. The Peyer's patches from each mouse were excised and pooled in 5 ml saline in a 20 ml wide-necked bottle. The rest of the intestine was placed in 10 ml saline if bacterial enumeration was required. The tissue was homogenised on ice with an Ultraturrax homogeniser (Janke and Kunkel, West Germany) at full speed for two 30 second periods. Duplicate 0.1 ml aliquots of appropriate dilutions of the homogenates were plated on Luria agar media.

3.11 PREPARATION OF SINGLE CELL SUSPENSION OF THE PEYER'S PATCHES

3.11 a. Mechanical preparation

The small intestine from each mouse was prepared and cleaned by the technique described in section 3.10. The Peyer's patches were excised and pooled together in 2 ml 10% FCS-RPMI 1640 medium. The patches were disrupted gently by three to four turns in a glass homogenizer. The cell suspension was passed through a 200 mesh sieve into a 10 ml conical centrifuge tube. The single cell suspension was washed once and resuspended in the same medium. The

preparation yielded approximately 10^7 cells per mouse. Viability of the cells tested by trypan blue was 85-90%. This preparation contained a high proportion of epithelial cells and was of course grossly contaminated by intestinal organisms.

3.11 b. "Scratching" technique

This method has the advantage of avoiding mechanical damage to cells and of providing a sterile cell suspension relatively free from epithelial cells.

The small intestine was removed from the mouse with aseptic precautions. After one end of the small intestine had been tied with cotton it was then filled with approximately 0.5 ml of 0.05% trypan blue and then the other end was tied. The closed intestine was washed with 200 ml sterile saline then spread in a small sterile plastic Petri dish (90 x 14 mm, Disposable Products Pty. Ltd., South Australia) containing 1 ml of 10% FCS-RPMI 1640 with antibiotics. Using a dissecting microscope each Peyer's patch was scratched with a size 19 hypodermic needle so as to pierce the serosal surface and allow the lymphoid contents to flow out. If the operation was sufficiently superficial the suspension of cells obtained contained no blue dye indicating that the intestinal epithelium had not been ruptured. With experience successful preparations could be obtained about three times out of four. The suspension was pipetted up and down several times with a small sterile pasteur pipette before filtering through 200 mesh sterile stainless steel sieve. The yield was usually approximately 5×10^6 cells/mouse, containing no epithelial cells, mostly mononuclear cells with a viability of more than 95%.

3.11 c. Collagenase digestion method

This technique was described originally by Cebra et al. (1977) for the preparation of mouse intestinal lamina propria cells using collagenase type I (clostridiopeptidase A, Sigma Chemical Co.). The details of the method and diagram are shown in Chapter 6.

3.12 DETERMINATION OF DIFFERENT CELLS FROM THE PEYER'S PATCHES

3.12 a. Determination of B cells by direct immunofluorescence

Single cell suspensions of the Peyer's patches from infected mice were prepared with a glass homogenizer. The cells were washed with 5% FCS-RPMI 1640 with antibiotics, counted and resuspended to a concentration of 2×10^8 cells per ml in medium supplemented with 0.1% sodium azide. A 0.1 ml aliquot of this suspension was centrifuged at 500xg for 5 min, and the cell pellet resuspended in 50 µl of rabbit anti-mouse µ chain antibody conjugated to fluorescein isothiocyanate (FITC) (which was kindly given by Dr Peter Ey and Stephen Gadd in our department) in the presence of 0.1% sodium azide to reduce pinocytosis. The cells were incubated in the dark for 45 min in an ice bath. After completing the incubation the cells were washed 3 times with 5% FCS-RPMI 1640 with antibiotics and the cell pellet was resuspended in 20 µl of the medium with 0.1% azide. A drop of cell suspension was placed on a slide, covered with a cover glass and sealed with finger nail polish. The number of cells with fluorescence was counted (out of a total of 200-300 cells) under the fluorescent microscope (Olympus BH-RFL-W).

3.12 b. Determination of T cells by direct immunofluorescence.

A similar procedure to that described above was used, except that the cells were incubated with fluoresceinated monoclonal anti-mouse Thy 1.2 (Miles-Yeda Ltd.) at 4° for 45 min and then treated as described in section 3.11 a.

3.12 c. Determination of macrophages

The experiments were performed in slide culture chambers, the design of which was kindly given by Dr D.M. Weir, University of Edinburgh, Medical School [Thomas, H.I.J. and Weir, D.M. (1972) Clin. Exp. Immunol. 12: 263]. The details of our modified version with smaller holes is shown in Chapter 6.

The suspension of Peyer's patch cells ($2x10^5$ in 100 µl RPMI 1640 with 10% FCS, 100 µg/ml streptomycin and 100 units/ml penicillin) was incubated with 10^7 latex particles in 20 µl culture medium in each well of the slide culture chamber. The macrophages were allowed to adhere to the glass slide for 2 h. After completing the incubation, the fluid was tipped away and each well washed with 200 µl culture medium five times. The adherent cells (macrophages) were stained with Wright's stain and examined at a magnification of 100 x using a light microscope. Adherent cells containing 3 or more latex particles were counted as macrophages (in a total area of 0.28 cm^2). Cell suspensions from a control normal mouse were included in the same slide culture chamber.

3.13 COLLECTION OF PERITONEAL EXUDATE CELLS

Mice were killed by cervical dislocation. The skin over the abdomen was flooded with 70% alcohol, then grasped with forceps and the loose skin cut with scissors. The skin was lifted up and away from the abdominal wall, taking care not to tear the abdominal wall. 2.5-3 ml of Hanks' balanced salt solution was introduced into the peritoneal cavity and massaged gently. The peritoneal fluid was withdrawn slowly and placed in a siliconized conical centrifuge tube in ice. The cells were washed by centrifuging 300-500x*g* for 5 min at 4°. The supernatant was discarded and the cells resuspended in 10% FCS-RPMI 1640 and kept in an ice bath.

3.14 ACTIVATION OF MACROPHAGES.

Mice were injected with 2×10^6 washed Salmonella 885 in 0.2 ml PBS intraperitoneally. Cells were harvested 7-9 days later for the experiments.

3.15 IN VITRO BACTERICIDAL ASSAYS.

3.15 a. Bactericidal assay by micro-culture plate (Flow Labs)

200 µl of 10% FCS-RPMI 1640 (with 20 mM Hepes and 0.84 gm/L $NaHCO_3$) containing 2×10^5 peritoneal cells was added to each well of sterile micro-culture trays (Flow Labs) and incubated at 37° in 5% CO_2 in air for 30-45 min. After incubation, the medium was removed from each well, and 10 µl of culture medium containing $2x10^2$ opsonized bacteria were added (bacteria were opsonized with

2HA units of specific antibody). The trays were centrifuged at 1500 x g for 10 min at 4° in a Coolspin centrifuge (PL 430, Model MSE, England), and then another 20 µl of culture medium was added to each well and the trays incubated at 37°, 5% CO_2 in air. Duplicate bacterial counts were taken at zero time (when the bacterial suspension was added) and at different intervals thereafter by adding an equal volume of 1% Triton-X100 and pipetting up and down a few times before transferring onto nutrient agar plates.

3.15 b. Bactericidal assay by micro-slide culture chamber.

An in vitro bactericidal micro-assay using the Weir micro-slide culture chamber followed a similar procedure. 20 µl of medium containing 4 x 10^4 macrophages (prepared using the collagenase method) were added to each well. After 2 h of incubation the supernatant fluid was decanted and the residual fluid was shaken out of the wells. 100-200 opsonized bacteria were added in 10 µl. Remaining steps were carried out as in 3.15 a. It should be noted here: 1) Care must be taken during the addition of macrophages to each micro-well to avoid air trapping. 2) The medium (10% FCS-RPMI 1640) was further supplemented with 10% fresh rabbit serum. 3) During isolation and enzymatic digestion of the Peyer's patches, antibiotics (200 units/ml of penicillin, 200 µg/ml streptomycin and 20 units/ml gentamycin) were added to the RPMI 1640 to avoid bacterial contamination by 885 organisms of the infected Peyer's patches.

3.16 WEIGHING THE ANIMAL AND ORGANS

Each mouse was weighed separately and then each of them was sacrificed by cervical dislocation. The small intestine was removed and washed through with cool saline solution. The Peyer's patches were excised and weighed with suitable precautions being taken to prevent drying during the process. The spleens were also weighed.

3.17 DELAYED TYPE HYPERSENSITIVITY

Salmonella enteritidis 11RX or Salmonella typhimurium C5 outer membrane antigen (Ashley, Kotlarski and Hardy, 1974) was injected into the hind footpad of sensitized mice. The left footpad was injected with 10 µl PBS and the right footpad with 10 µl (20 µg) protein antigen, using a micrometer operated glass syringe fitted with a 30 G needle. After 48 h and 72 h, the thickness of the footpads was measured with micrometer dial gauge calipers (Model 130, Mercer, England). A group of 5 tested mice was compared with control normal mice.

3.18 ASSAY FOR TUMOUR NECROTISING FACTOR (TNF)

The assay was based upon ^{3}H thymidine incorporation by the lymphoma cell line EL4. The cells were suspended at $4x10^{6}$/ml in Dulbecco's Minimal Essential Medium (DMEM) with 10% foetal calf serum and 10 mM of 2-mercaptoethanol. 50 µl aliquots of cell suspension ($2x10^{4}$) were incubated with equal volumes of serial two fold dilutions of TNF serum at 37°, 5% CO_2 in air for 24 h. The suspension was pulsed with 25 µl (1 µCi) of ^{3}H thymidine

(Radiochemical Centre, Amersham, Buckinghamshire, England) for 4-6 h. The ^{3}H thymidine incorporation was measured by sucking the mixture through a Harvester filter using a Titertek Cell Harvester (Flow Labs) and counting the radioactivity of the cells using a liquid scintillation beta counter (Beckman LS 7500 Liquid Scintillation System). Triplicates of each dilution of TNF serum, and controls (cells suspended without TNF serum), were included in these experiments.

3.19 QUANTITATION OF PROTEIN AND CARBOHYDRATE

Proteins were quantitated by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

Carbohydrate was determined by the anthrone reaction (Williams and Chase, 1968) using glucose as a standard.

3.20 HISTOLOGICAL PREPARATIONS

Organs and tissues (Peyer's patches, spleen and intestine) were fixed with Bouin's solution. Histological sections were made by dehydrating and embedding in paraffin. Sections were cut 5 μm thick and stained with haematoxylin-eosin. (The sections were kindly provided for us by the Pathology Department, courtesy of Dr Lomax-Smith to whom we are grateful.)

CHAPTER 4

I. THE PROTECTION OF MICE FROM *SALMONELLA TYPHIMURIUM* C5 BY FEEDING LIVE AVIRULENT SALMONELLA

II. EFFECT OF CYCLOPHOSPHAMIDE ON ANTIBACTERIAL ACTIVITY OF THE PEYER'S PATCHES AND SPLEEN.

4.1 INTRODUCTION

As stated in Chapter 2 the aim of this thesis is to investigate the immune events occurring at a local site namely the Peyer's patches of the small intestine where the initial reaction between cells and bacteria occurs. In this chapter we discuss the results of these investigations.

4.2 DEVELOPMENT OF ANTIBACTERIAL ACTIVITY OF THE PEYER'S PATCHES AGAINST SALMONELLA INFECTION.

Experiments have been designed using a *Salmonella-E. coli* hybrid strain (885). It is an avirulent strain of Salmonella which was derived by recombination between *S. typhimurium* 1142 and *E. coli* 492 and although it possesses the majority of the *S. typhimurium* genome and has the biotype of a Salmonella, it carries that portion of the *E. coli* genome specifying the O-8 lipopolysaccharide (Hohmann, Schmidt and Rowley, 1978).

After oral infection of mice with Salmonella 885, the organisms became established in the Peyer's patches of the small intestine, and the numbers peaked at 3 to 4 days and then declined

over 14 days (Fig. 4.1). The organisms could not be found in the spleen or liver at any time, and there were very few in the rest of the intestine. The rates of growth and decline of Salmonella 885 in the Peyer's patches showed a dose response, however, 5×10^8 bacteria was the optimal dose necessary to uniformly infect the Peyer's patches of all mice without killing them. Therefore this dose was used in each of our experiments unless otherwise indicated.

4.3 THE PROTECTION OF SALMONELLA 885 FED MICE AGAINST VIRULENT SALMONELLA TYPHIMURIUM C5.

As seen in Figure 4.1 the Peyer's patches of the Salmonella 885 fed mice begin to eliminate the organisms by day 4 to 5. It was of interest to determine whether this was due to a protective mechanism in the Peyer's patches which could protect mice from challenge with virulent Salmonella typhimurium C5.

Twenty-five mice were fed with Salmonella 885 5×10^8 bacteria on days 0, 3 and 20 and 25 control mice were caged in a separate room for the same period. A day after the last feeding, all mice, experimental and control, were challenged orally with 2×10^7 Salmonella typhimurium $C5^{SR}$. At varying times 5 mice from each group were sacrificed and the numbers of bacteria in the Peyer's patches and spleen were counted. The results are shown in Fig. 4.2. Non-immunised mice were unable to control the growth of bacteria in the Peyer's patches. The organisms grew continuously and spread to the spleen. All mice in the control group were sick or dying 3 days after challenge and all had died by day 5. However, the mice which had been orally immunised with Salmonella 885 controlled the growth and eventually eliminated the Salmonella

Figure 4.1

Number of Salmonella 885 recovered from the Peyer's patches of mice orally infected with 885 (5×10^8/mouse). No organisms were found in the spleen or liver, and relatively few organisms in the rest of the intestine.

Numbers shown at each point indicate the average values of five mice.

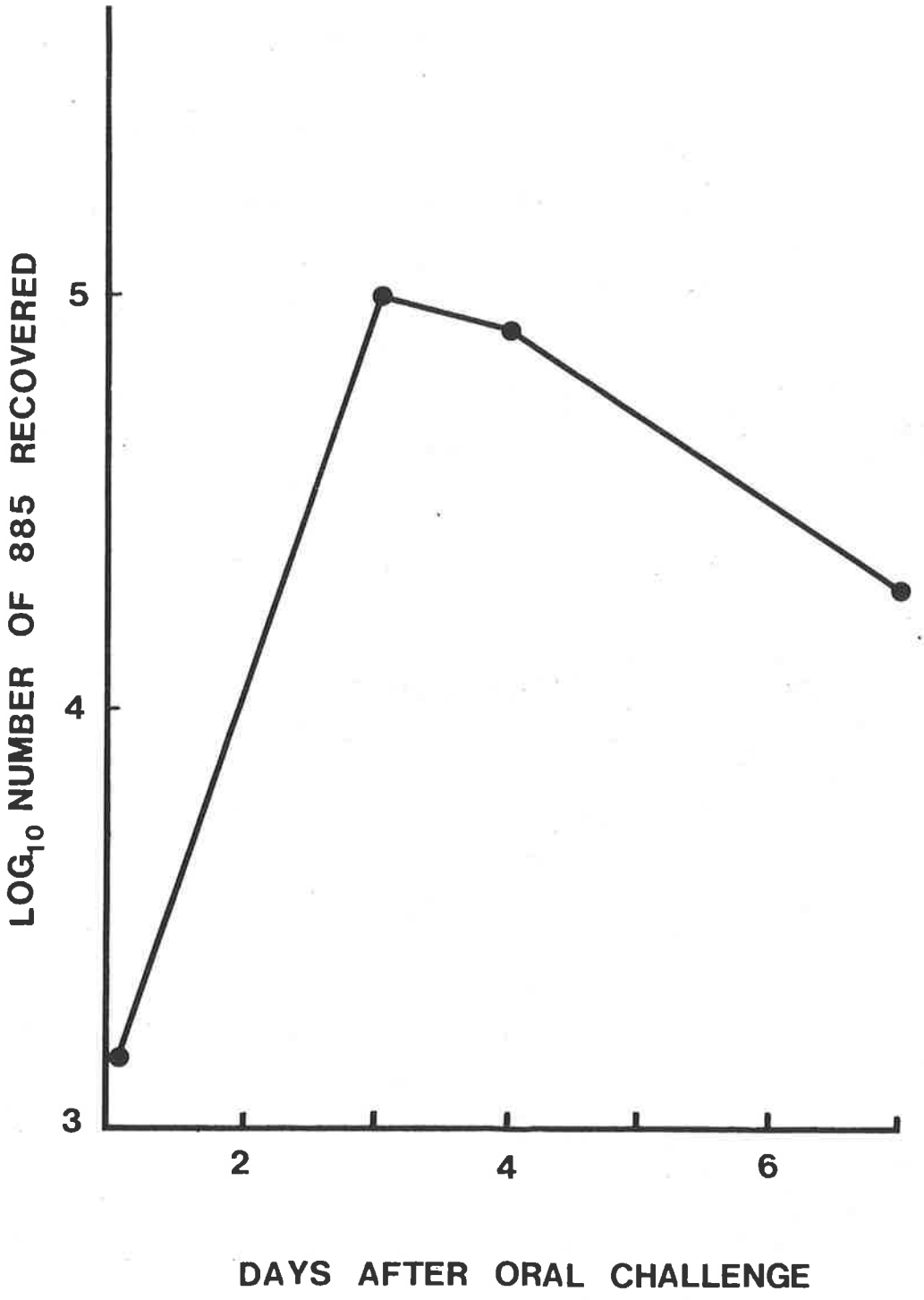
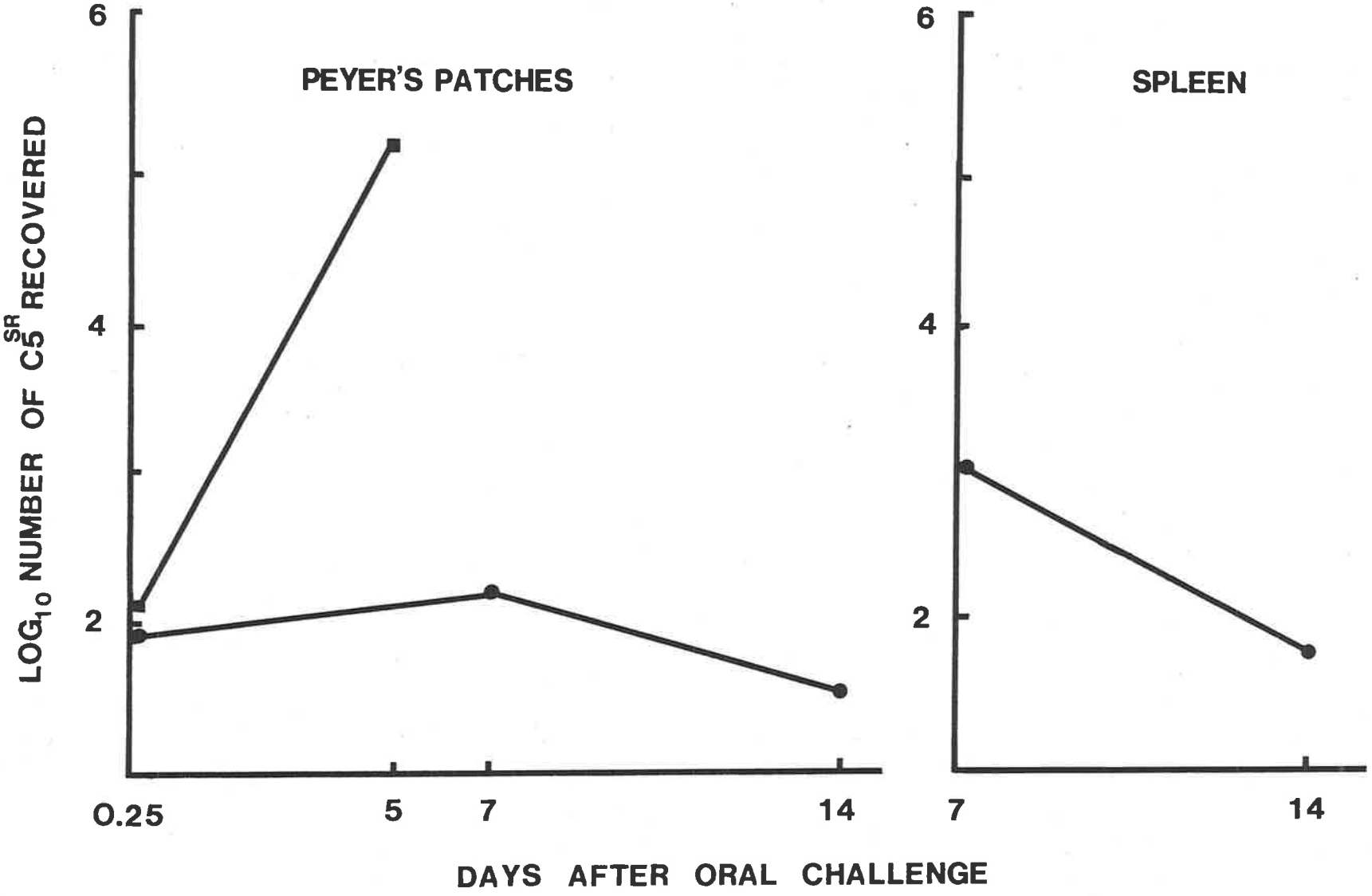

LOG10 NUMBER OF 885 RECOVERED
5
4
3
2
4
6
DAYS AFTER ORAL CHALLENGE

Figure 4.2

The number of Salmonella typhimurium $C5^{SR}$ recovered from the Peyer's patches and spleen of normal mice ■ and mice which had received 3 oral doses of live Salmonella 885 ● , 21 days prior to an oral rechallenge with 2×10^7 Salmonella $C5^{SR}$.


PEYER'S PATCHES
SPLEEN
6
4
2
6
4
2
LOG10 NUMBER OF C5SR RECOVERED
0.25
5
7
14
7
14
DAYS AFTER ORAL CHALLENGE

typhimurium $C5^{SR}$ challenge organisms. These organisms did spread to the spleen where they grew for a few days but again the spleen of the live Salmonella fed mice developed an antibacterial activity sufficient to eliminate them.

As reported by many authors, the resistance which develops rapidly after colonization with Salmonellae is not confined to homologous microorganisms (Collins, 1974; Blanden, Mackaness and Collins, 1966; Hohmann, 1979). Again our experiments are in accord with this finding.

4.4 EFFECT OF PASSIVE TRANSFER OF SPECIFIC ANTIBODY ON THE GROWTH OF VIRULENT SALMONELLA TYPHIMURIUM $C5^{SR}$ IN MICE

As previous results (Fig. 4.2) show, mice that have been fed with 885 organisms were resistant to virulent strains of Salmonella. During the eight day period before challenge the mice can be shown to have produced specific antibody and we wished to ascertain what role this antibody played in the protection by passively transferring antibody.

Eighty mice were divided into 4 groups and 2 groups were fed with Salmonella 885 (5×10^8 bacteria/mouse), the rest were kept in a separate room. On day 8, 20 normal mice and 20 Salmonella 885 fed mice were injected intravenously with 0.2 ml of anti-Salmonella C5 mouse serum with a haemagglutination titre of 1/2540 against C5 LPS coated SRBC and 15 min later all 4 groups of mice were challenged with $C5^{SR}$ (2×10^6 bacteria) intravenously. At different intervals 5 mice were sacrificed and bacteria were counted in the homogenised Peyer's patches and spleens.

The results (Figs. 4.3 and 4.4) showed that the passive intravenous transfer of high titre anti-Salmonella C5 mouse sera did not give absolute protection to the mice from subsequent challenge with Salmonella typhimurium $C5^{SR}$. Although the initial recovery of organisms from mice which received antibody was small, the bacteria continued to proliferate uninterrupted even in presence of antibody until some of the mice were dead. In addition, the passive transfer of antibody to mice delayed the spreading of the intravenously injected organisms from the spleen to Peyer's patches. Mice which had been fed with 885 organisms with or without transferred antibody looked normal and survived during the time of the experiment, by which time some of the remaining 5 normal control mice were dying.

4.5 CYCLOPHOSPHAMIDE AS AN IMMUNOSUPPRESSANT IN THIS SYSTEM.

Cyclophosphamide (CPA) (Bristol Laboratories, Crows Nest, N.S.W., Australia) is an alkylating agent which acts directly on nucleoprotein, causing cross-linking and distortion of the DNA. CPA has been used in the chemotherapy of cancer and as an immunosuppressive agent for the control of immune mechanisms involving antibody production and cell-mediated immunity, such as delayed-type hypersensitivity (DTH) and transplantation immunity (Berenbaum, 1962; Berenbaum, 1967; Turk, 1967).

CPA seems to have dual actions: it may suppress or it may enhance the immune response. These effects may be due to the fact that the population of cells involved in the immune response are heterogeneous and dynamic. The cells comprising this heterogeneous pool may be expected to differ in their susceptibilities as they differentiate and multiply. The timing of CPA administration, and

Figure 4.3

Effect of passively transferred anti-C5 antibody on recovery of Salmonella $C5^{SR}$ from the spleen following i.v. challenge of control mice and mice given 885 orally eight days previously.

Symbols: ● normal control mice; ○ normal mice with transferred antibody; ■ 885 fed mice; □ 885 fed mice with transferred antibody.

Numbers shown at each point indicate the average values of five mice.

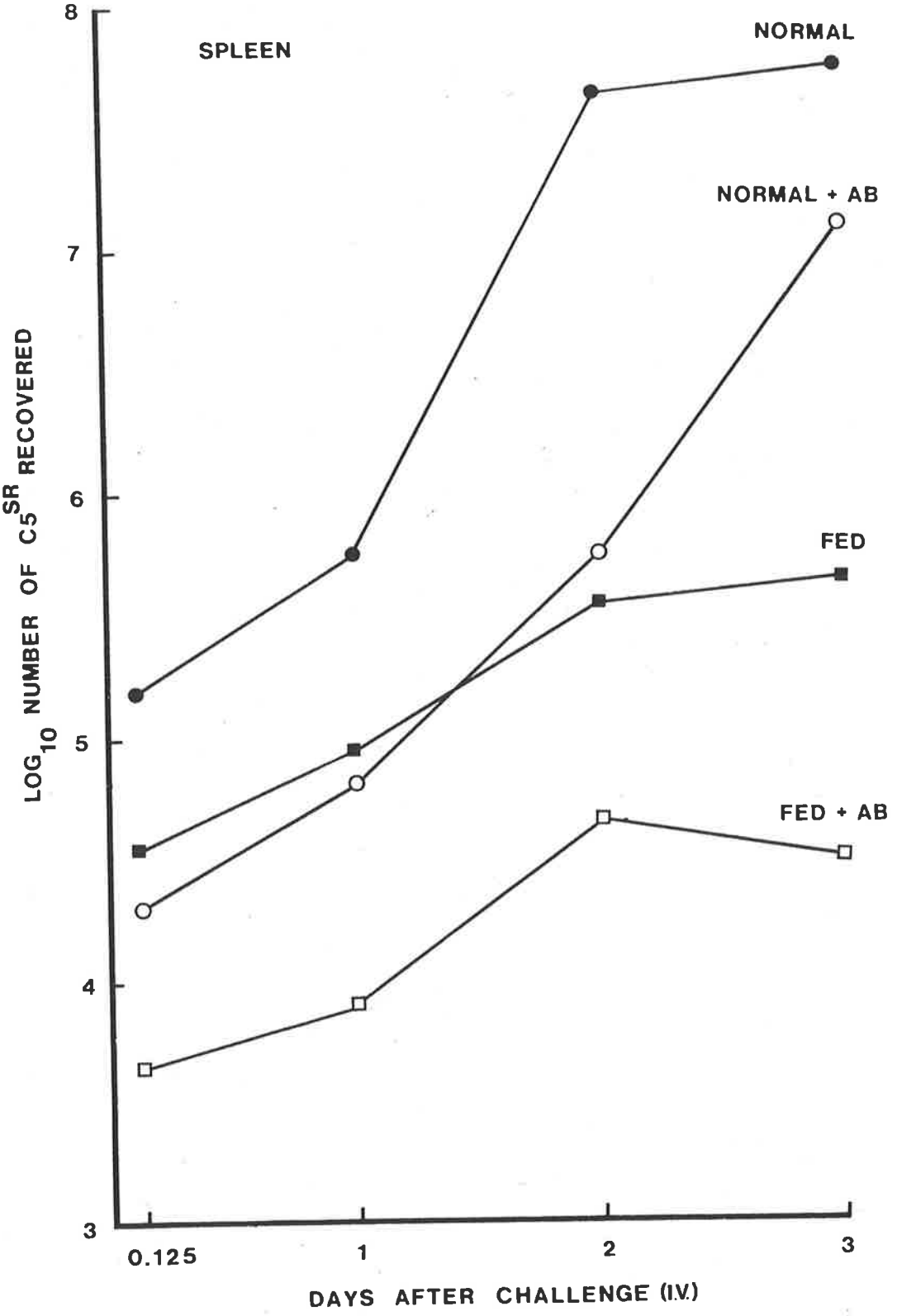
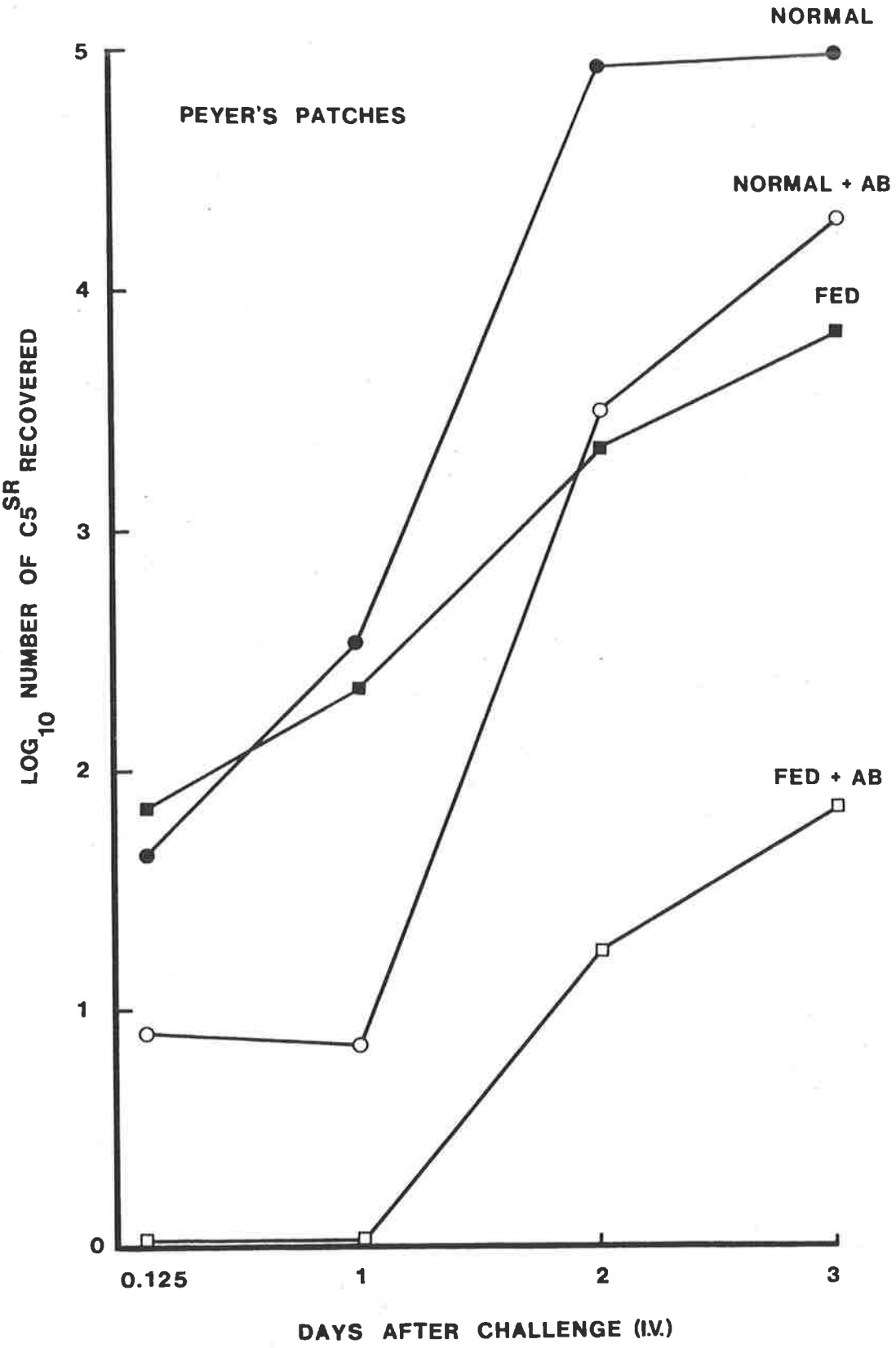

SPLEEN
NORMAL
NORMAL + AB
FED
FED + AB
8
7
6
5
4
3
LOG10 NUMBER OF C5SR RECOVERED
0.125
1
2
3
DAYS AFTER CHALLENGE (I.V.)

Figure 4.4

Effect of passively transferred anti-C5 antibody on recovery of Salmonella $C5^{SR}$ from the Peyer's patches following i.v. challenge of control mice and mice given 885 orally eight days previously.

Symbols: ● normal control mice; ○ normal mice with transferred antibody; ■ 885 fed mice; □ 885 fed mice with transferred antibody.

Numbers shown at each point indicate the average values of five mice.


NORMAL
PEYER'S PATCHES
NORMAL + AB
FED
FED + AB
5
4
3
2
1
0
0.125
1
2
3
LOG10 NUMBER OF C5SR RECOVERED
DAYS AFTER CHALLENGE (I.V.)

its dosage could be critical in determining its effect.

Since CPA causes nuclear damage it is particularly toxic to dividing cells or precursor cells which have a rapid turnover rate, such as lymphocytes.

We have already observed that some weakly virulent S. typhimurium strains, when introduced by suitable routes into mice, populate either the Peyer's patches or spleen. They grow in these sites for several days and are then eliminated, presumably because these lymphoid organs acquire the ability to kill bacteria in a manner characteristic of cellular immunity (Carter and Collins, 1974; Hohmann, Schmidt and Rowley, 1978). Tripathy and Mackaness (1969) showed that CPA suppressed the antibacterial activity of the spleen in controlling the growth of Listeria monocytogenes. They interpreted this to mean that CPA could prevent the sensitization and clonal expansion of T cells and in this way prevent the activation of macrophages. The development of antibacterial activity may take place in the spleen after intravenous injection of organisms or in the Peyer's patches after oral administration. It has already shown that CPA prevents this sequence of events in the spleen so that localized bacteria continue uninterrupted growth. The studies in this chapter aim to examine the effect of CPA on bacterial growth in the Peyer's patches.

4.6 EFFECT OF CYCLOPHOSPHAMIDE ON ANTIBACTERIAL ACTIVITY OF THE PEYER'S PATCHES.

4.6.1 Primary and secondary challenge

Some mice were given one infection (5×10^8 885 orally) and

given CPA (4 mg s.c.) on the same day. Other mice were given two oral infections (5×10^8 885 on day 0 followed by 5×10^8 885^{SR} on day 7) and given CPA on the day of the secondary challenge. CPA given at the time of primary or secondary infection had no apparent effect on the increase and decline in the numbers of bacteria in the Peyer's patches (Fig. 4.5). Other experiments failed to show any appreciable effect of CPA in doses ranging from 1 to 200 mg/kg given either orally or subcutaneously.

The weight changes which normally occur in this lymphoid tissue after infection was not markedly different in those mice given CPA (Fig. 4.6).

4.7 EFFECT OF CYCLOPHOSPHAMIDE ON ANTIBACTERIAL ACTIVITY OF THE SPLEEN.

4.7.1 Primary challenge:

Administration of 10^6 Salmonella 885 intravenously to mice resulted in the localization of about 4×10^4 organisms in the spleen. With normal mice, these organisms doubled in number by day 2, but then remained at this level. CPA given subcutaneously at the same time as the intravenously administered organisms allowed rapid and continued growth of the Salmonella 885 to occur (Fig. 4.7A).

Challenge of normal animals intravenously or orally was followed by an increase in spleen weight which was not altered by CPA as in the case of the Peyer's patches (Fig. 4.8).

Figure 4.5

Numbers of 885 or 885^{SR} organisms recovered from the Peyer's patches of mice after oral challenge.

A. Primary infection ●, 5×10^8 Salmonella 885 orally on day 0; ○, 5×10^8 Salmonella 885 orally plus 4 mg of CPA subcutaneously on day 0;

B. Secondary infection ■, 5×10^8 Salmonella 885 orally on day 0 and rechallenge with 5×10^8 Salmonella 885^{SR} on day 7; □, 5×10^8 Salmonella 885 orally on day 0 and rechallenge with 5×10^8 Salmonella 885^{SR} plus 4 mg of CPA subcutaneously on day 7.

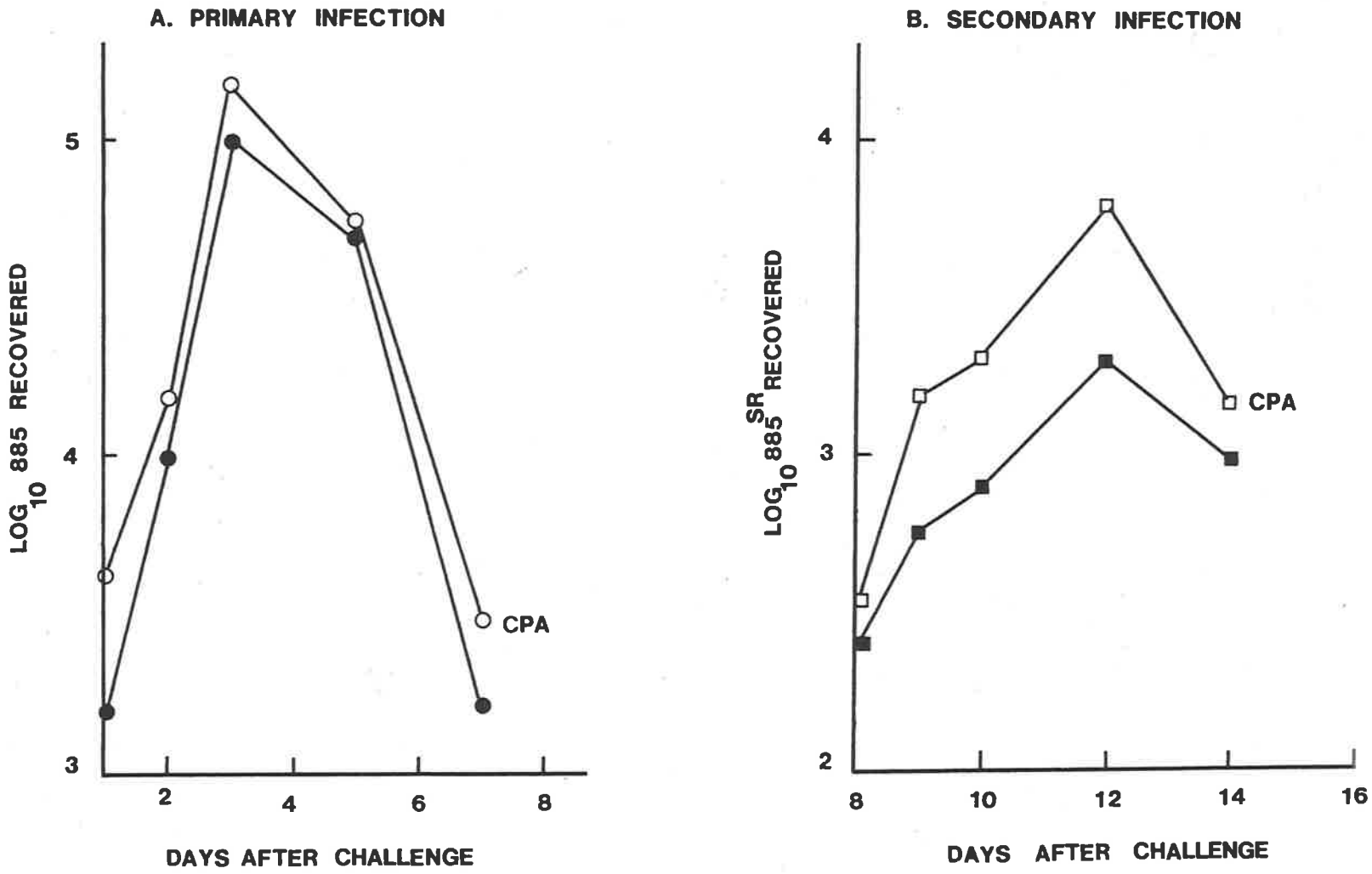

A. PRIMARY INFECTION
LOG10 885 RECOVERED
5
4
3
2
4
6
8
CPA
DAYS AFTER CHALLENGE
B. SECONDARY INFECTION
LOG10 885SR RECOVERED
4
3
2
8
10
12
14
16
CPA
DAYS AFTER CHALLENGE

Figure 4.6

Peyer's patch weight expressed as % of total body weight.

Symbols:

■ : mice fed 885 on day 0

● : mice fed 885 on day 0 with oral CPA on day 0.

Numbers in each point were the average values of 5 mice.

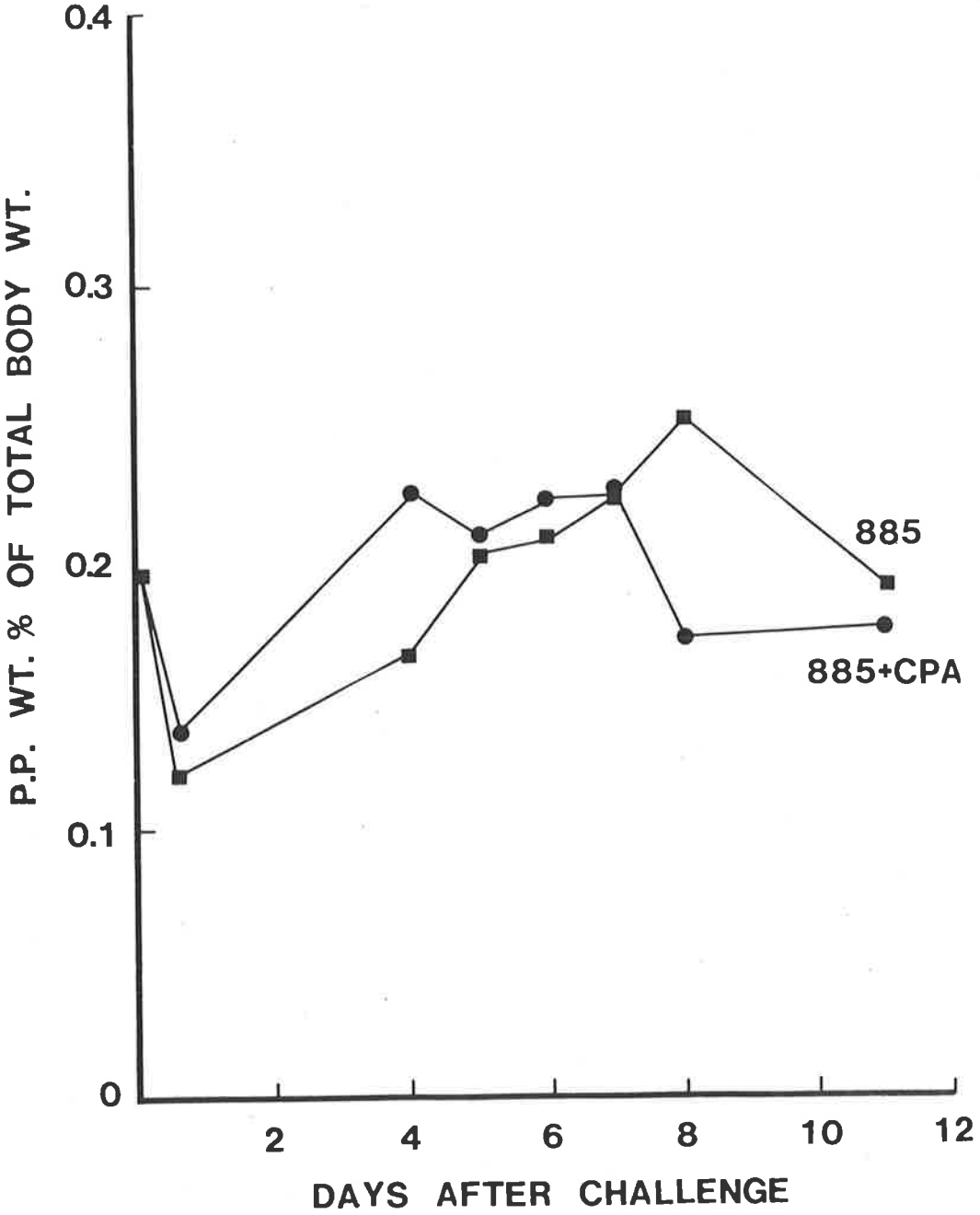

0.4
0.3
0.2
0.1
0
P.P. WT. % OF TOTAL BODY WT.
885
885+CPA
2
4
6
8
10
12
DAYS AFTER CHALLENGE

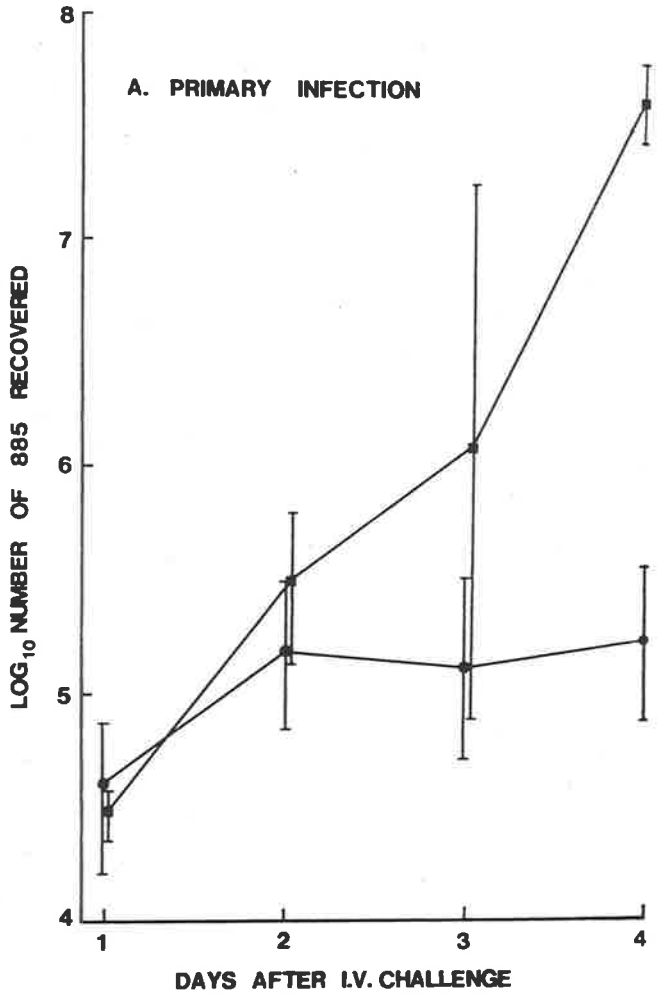
Figure 4.7

A. Primary infection

Numbers of organisms recovered from the spleens of mice after intravenous (i.v.) injection of 5×10^6 885 organisms. Symbols ●, normal mice injected on day 0; ■, normal mice injected with Salmonella 885 plus 4 mg of CPA subcutaneously on day 0. Bars shown at each point indicate the mean $\pm$ one standard deviation for the five mice studied.

B. Secondary infection

Recovery of organisms from the spleens of mice after secondary infection with 5×10^6 Salmonella 885^{SR} given intravenously. All mice received 5×10^8 Salmonella 885 orally on day 0 and were rechallenged with 5×10^6 Salmonella 885^{SR} intravenously on day 7. Another similarly treated group received CPA subcutaneously on day 7 in addition to the organisms. Symbols: ●, intravenous challenge with 5×10^6 Salmonella 885^{SR}; ■, intravenous challenge with 5×10^6 Salmonella 885^{SR} plus 4 mg of CPA subcutaneously. Bars shown at each point indicate the mean $\pm$ one standard deviation for the five mice studied.


A. PRIMARY INFECTION
LOG10 NUMBER OF 885 RECOVERED
8
7
6
5
4
1
2
3
4
DAYS AFTER I.V. CHALLENGE

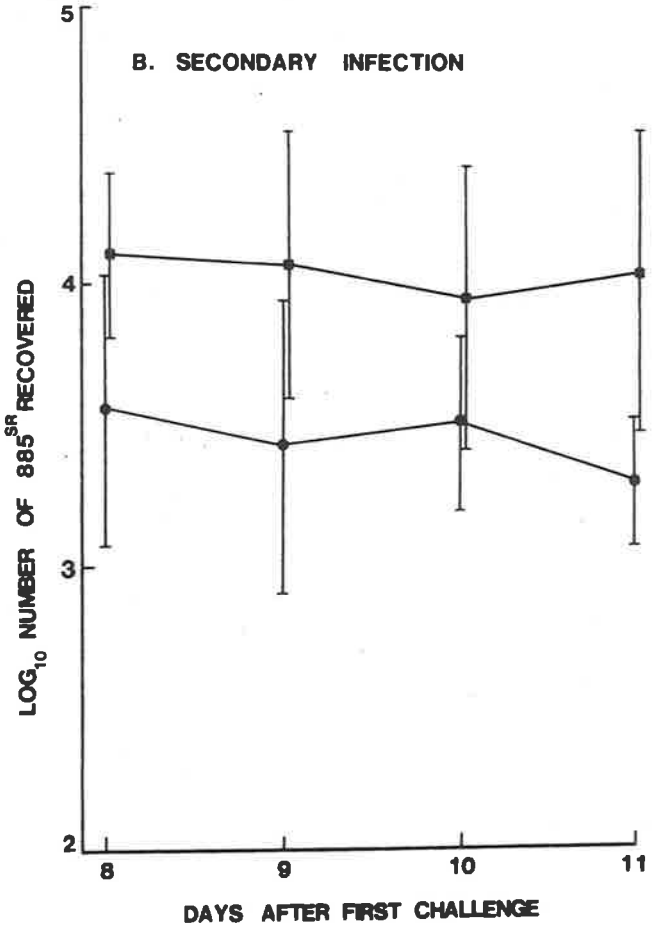

B. SECONDARY INFECTION
LOG10 NUMBER OF 885SR RECOVERED
5
4
3
2
8
9
10
11
DAYS AFTER FIRST CHALLENGE

Figure 4.8

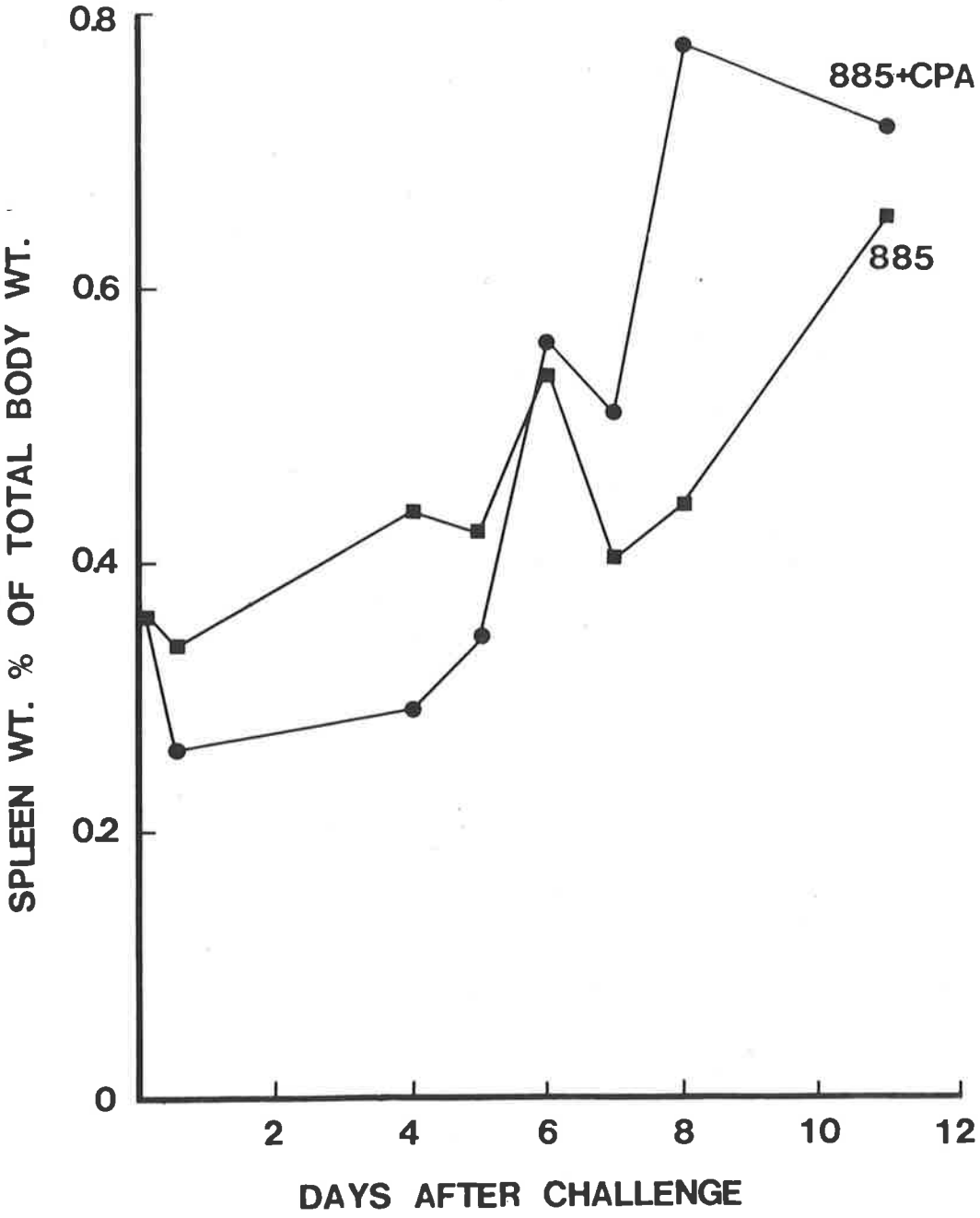
Spleen weight expressed as % of total body weight

Symbols:

■ : mice fed with Salmonella 885 on day 0

● : mice fed with Salmonella 885 and oral CPA on day 0

Numbers in each point were the average values of 5 mice.


0.8
0.6
0.4
0.2
0
SPLEEN WT. % OF TOTAL BODY WT.
885+CPA
885
2
4
6
8
10
12
DAYS AFTER CHALLENGE

4.7.2 Secondary challenge:

Fifty mice were fed 5 x 10^8 Salmonella 885. Seven days later all of these mice were challenged intravenously with 2 x 10^6 Salmonella 885^{SR} and 25 of them were given 4 mg of CPA subcutaneously. Figure 4.7B shows that the CPA did not alter the ability of these previously fed mice to control the growth of Salmonella 885^{SR} in the spleen.

On the other hand, the results were entirely different when this experiment was carried out so that the CPA was given at the same time as the initial oral dose of 5 x 10^8 Salmonella 885. Under these conditions, the mice given CPA at day 0 had diminished ability to control the splenic growth of an intravenous challenge of Salmonella 885^{SR} 7 days later (Fig. 4.9).

4.8 EFFECT OF CYCLOPHOSPHAMIDE ON ANTIBODY RESPONSE

As has been mentioned previously CPA suppresses the cell-mediated immunity and prevents antibacterial activity of the spleen. This drug may also affect antibacterial immunity by inhibiting antibody production.

Twenty mice were immunized with heat-killed Salmonella 885 (5 x 10^8/mouse) intravenously and ten of them were injected subcutaneously with 4 mg of CPA 2 hours later. Serum was obtained on day 3 and 7 and tested for haemagglutination of 885 LPS coated SRBC (Chapter 3).

As shown in Table 4.1 the administration of CPA totally suppressed the antibody production over this time period.

Figure 4.9

Effect of cyclophosphamide, given on the day of the initial feeding of organisms, on the splenic growth of a subsequent intravenous challenge.

Symbols: ●, 5 x 10^8 Salmonella 885 orally on day 0 and 5 x 10^6 Salmonella 885^{SR} intravenously on day 7. ■, 5 x 10^8 Salmonella 885 orally plus 4 mg of CPA subcutaneously on day 0, CPA again on day 4, and 5 x 10^6 Salmonella 885^{SR} intravenously on day 7.

Bars shown at each point indicate the mean $\pm$ one standard deviation for the five mice studied.

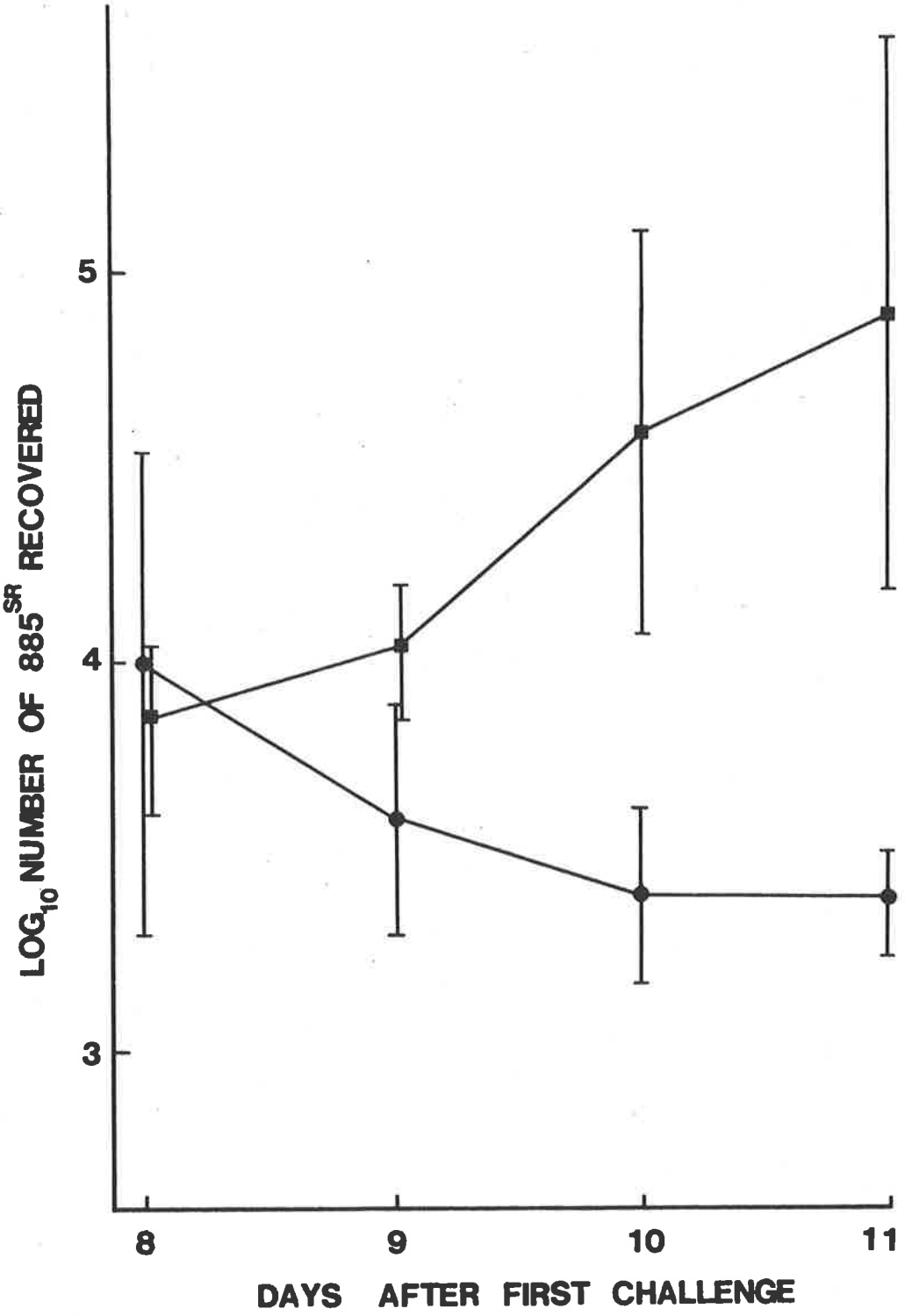

5
4
3
LOG10 NUMBER OF 885SR RECOVERED
8
9
10
11
DAYS AFTER FIRST CHALLENGE

Table 4.1

Effect of cyclophosphamide on antibody response following i.v. immunization of 5×10^8 heat-killed Salmonella 885.

Days after last dose	Haemagglutination titre	
	+ CPA	- CPA
Day 3	>1	1/32
Day 7	>1	1/128

4.9 CONCLUSIONS.

1. Local Peyer's patches immunity exists which increases following primary infection.

2. We confirmed that a live Salmonella vaccine can protect mice against virulent Salmonella typhimurium C5. This protection was not conferred on normal mice following passive transfer of specific, high titre antibody.

3. Giving CPA, at any time, did not affect the development of antibacterial activity in the Peyer's patches, whereas in the spleen, the antibacterial activity was abolished when CPA was given with the bacteria. However, there was no effect when CPA was given 7 days after the initial bacterial challenge. Initial priming of cells can be prevented by CPA e.g. in the spleen whereas in Peyer's patches CPA has no effect. We conclude from this that cells in the Peyer's patches are already primed. In support of this is the finding that after the spleen has been primed CPA has no effect.

4. CPA given together with bacterial antigen completely represses the antibody response.

CHAPTER 5

THE CELLULAR COMPOSITION OF PEYER'S PATCHES AND THE MIGRATION OF SALMONELLA 885 IN PEYER'S PATCHES IN ASSOCIATION WITH LYMPHOCYTE TRAFFIC

5.1 INTRODUCTION

In the previous chapter it was shown that approximately 4-5 days after feeding Salmonella 885 to mice the Peyer's patches developed an antibacterial activity to control the growth and elimination of bacteria which they did not possess originally. These primary infected mice were resistant to both the homologous strain and to the virulent strain of Salmonella. Since we have observed that the Peyer's patches of infected mice are bigger and more protuberant it is possible that the increased resistance could be due to a change in the numbers or the proportions of the various cells in the Peyer's patches. Alternatively it could be due to changes in the functions or activity in some cell population.

Our studies in this chapter are to investigate any quantitative or qualitative changes which may occur in the Peyer's patches after infection.

5.2 THE DISTRIBUTION OF MACROPHAGES AND LYMPHOCYTES IN THE PEYER'S PATCHES OF MICE CHALLENGED WITH SALMONELLA 885.

5.2.1 Enumeration of macrophages in the Peyer's patches.

We attempted to estimate the numbers of macrophages in the

Peyer's patches by incubating single cell suspensions of Peyer's patch cells on glass where the macrophages were allowed to adhere and phagocytose latex particles for 2 hours (5% CO_2, 37°). The cells with latex particles inside were counted under phase contrast. We used a Slide Culture Chamber, which was kindly given by Dr D.M. Weir, Edinburgh University, Scotland. It is a simple device consisting of a small rectangular silastane block with 10 holes in it which is placed between a haematological slide and a metal frame with matching holes. They are clamped together, so that each well is sealed and can hold 300 µl of liquid. When this chamber is unclamped the slide can be stained for morphological studies (Figure 6.2 in Chapter 6). Initially the single cell suspensions were prepared with a glass homogeniser. Very few adherent cells were found and most of these were not phagocytic. Better results were obtained with a modified preparation method.

A single cell suspension of the Peyer's patches of the fed mice in 10% FCS-RPMI 1640 were obtained by the scratching technique (Chapter 3). 100 µl of 2 x 10^5 of cell suspension was added to each well and incubated in 5% CO_2 at 37° for 2 hours. The fluid was tipped away and each well was washed gently with 5% FCS-RPMI 1640. 20 µl of medium containing 10^7 latex particles was added and incubated for a further 0.5 - 1 hour. The slides were then washed several times with the same medium and stained with Wright's stain. Cells containing 3 or more latex particles were counted inside the whole spot (approximately 0.28 cm^2). Controls were performed in a similar way on the same slide by using cell suspensions of the Peyer's patches from normal mice. The results of 2-3 mice of each experiment are shown in Table 5.1.

Table 5.1

The distribution of cells in the Peyer's patches of mice infected orally with Salmonella 885

Days after feeding 885	Peyer's patch cells[a]		
	Lymphocytes[b] (per 100 P.P. cells)		Macrophages[c] (per 5×10^6 P.P.cells)
	B cells	T cells	
0	42,59	22,28	2,400
1	46,55	34,34	2,500
2	47,54	27,30	3,500
3	41,50	25,33	5,000
4	39,48	24,30	32,200
5	28,30	19,27	66,300
6	59,63	30,30	61,200
7	40,45	26,28	69,300

a Isolated by the scratching technique

b From 2 duplicate slides 200-300 cells examined per slide

c Average of individual values from 2-3 mice (1-2 wells assayed for each mouse).

This Slide Culture Chamber provides a convenient method of quantitating macrophages. The results are reproducible and accurate. On the first few days after infection, approximately 3500 macrophages were found per 5 x 10^6 Peyer's patch cells and these started to increase by 10-25 times at day 4 and this number was maintained at least until day 7.

The morphology of Peyer's patch macrophages in the Slide Culture Chamber is shown in Fig. 5.1 (using opsonized bacteria instead of latex since this yielded a better photograph).

5.2.2 The number of B and T lymphocytes in the Peyer's patches

B and T lymphocytes were enumerated in mice infected with Salmonella 885 orally by the direct immunofluorescence technique. B lymphocytes were stained with fluorescein-conjugated rabbit anti-mouse μ chain antisera (Fig. 5.2) and T lymphocytes with fluorescein-conjugated mouse monoclonal anti-Thy 1.2 (Chapter 3). The proportions of B and T lymphocytes showed no significant changes throughout the 7 days following infection (Table 5.1). The B cell numbers at days 5 and 6 whilst deviating from the other counts are not regarded as significant. However, the total number of cells recovered from Peyer's patches of the infected mice a few days after bacterial challenge increased nearly 2-fold compared to the control mice.

5.3 THE ADHERENCE OF BACTERIA TO THE CELL SURFACE

The ability of various bacteria to adhere to the epithelial

Figure 5.1

The Peyer's patch macrophage isolated by collagenase technique from Salmonella infected mice orally on day 7. Macrophages showed adherence to hematological slide, and phagocytosed opsonized bacteria.

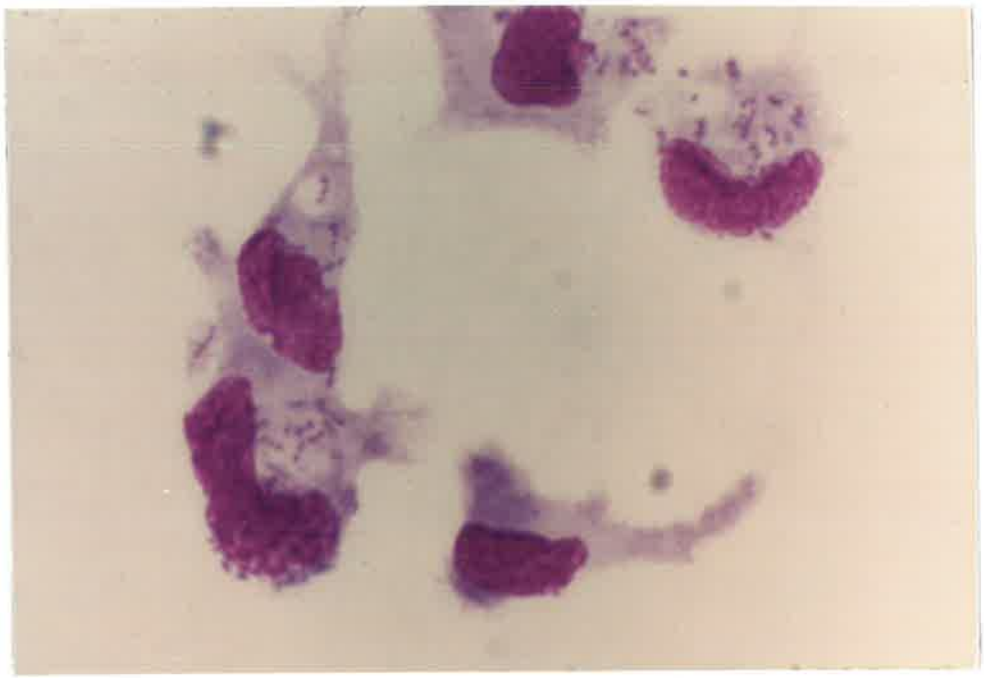
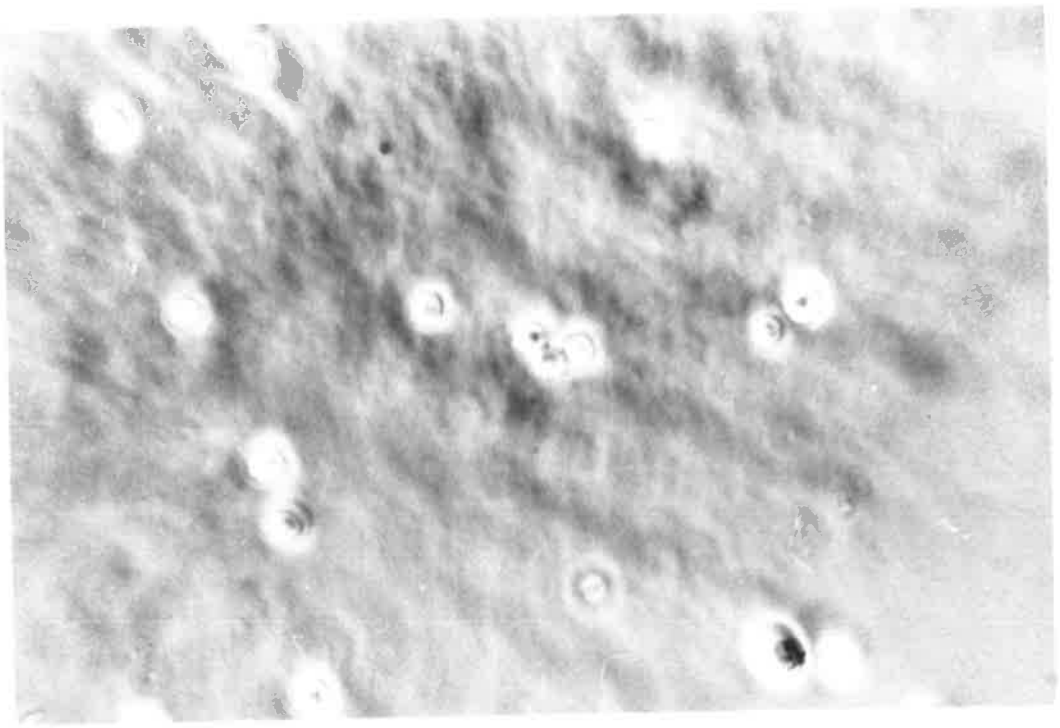
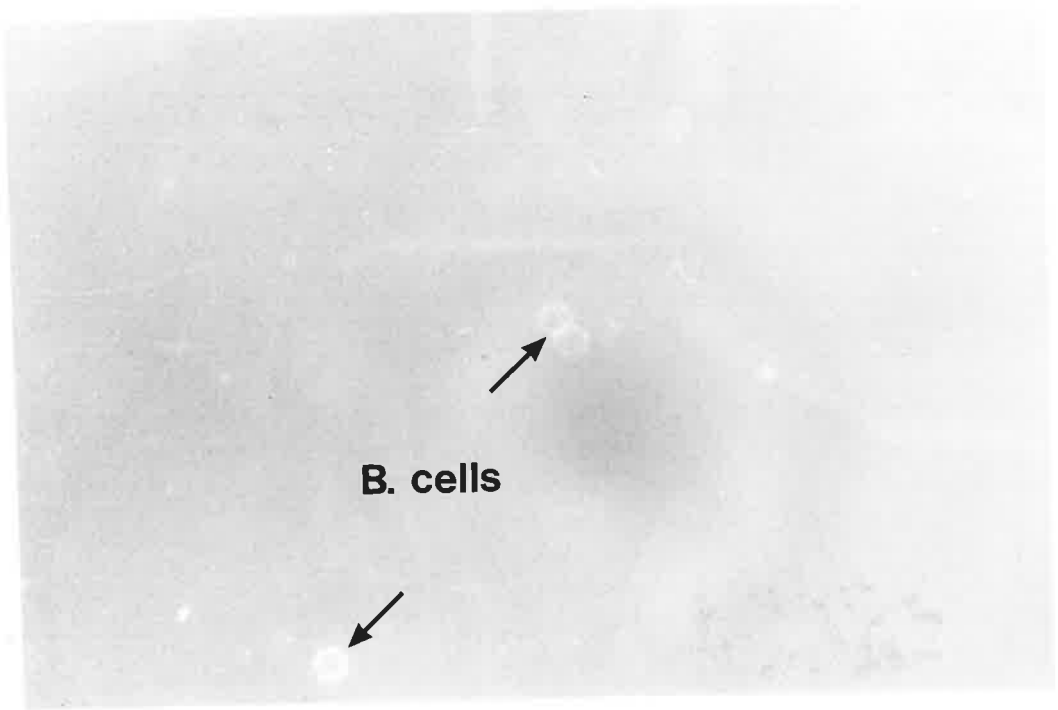


Figure 5.2

B lymphocytes from Peyer's patches of 4 day Salmonella 885 fed mice were detected by direct immunofluorescent technique. B lymphocytes showed fluorescence with FITC-conjugated rabbit anti-mouse μ chain.




B. cells

cells of the mucous surfaces of the respiratory, gastrointestinal and urogenital tract has provoked much interest over recent years. The bacteria are thought to possess certain molecular structures on their surfaces which enable them to bind to specific receptors of the host tissues. Further studies have shown that, in most cases, the ability of gram-negative bacteria to adhere to epithelial cells is associated with irregularly shaped and sized surface structures called fimbriae (Jones, 1977; Duguid, 1968), or pili (Brinton, 1965). Adherence of gram-positive bacteria is associated with hair-like projections (Beachey and Ofek, 1976). The binding afforded by these appendages has been widely investigated.

5.4 BACTERIA-CELL ASSOCIATION OF SALMONELLA FED MICE

It has been claimed that Salmonellae, like Mycobacterium, Brucella or Listeria, are intracellular parasites. In preliminary experiments single cell suspensions of the Peyer's patches from infected mice were centrifuged at 200x*g* and bacteria counted in the supernatant and in the cell pellet. Most of the bacteria were found in the supernatant fluid and few of them were associated with the cells at the bottom of the tube. This surprising finding led us to investigate the relationship between cells and bacteria in the Peyer's patches of the infected mice in a series of experiments.

Single cell suspensions in saline prepared from Peyer's patches of infected mice by glass grinder technique (Chapter 3) were centrifuged at 200x*g* for 5 min and aliquots of both the starting suspension and the supernatant were removed for counting. In the early stages of infection about 90% of the bacteria were free in the supernatant, but 8 days later this had changed, so that 50% of the

organisms were cell-associated (Table 5.2). The results suggested that this change was due to the immune responses occurring in the Peyer's patches and that 6-8 days after infection Ab-forming lymphocytes can link the organisms to the cells. This interpretation was supported by the finding that when 0.1% disodium ethylenediamine tetra-acetic acid (EDTA) was included in the washing fluid, all the bacteria were found to be free from the cells. In other words, we can presume that none of the recoverable bacteria came from within cells but were associated with the exterior of cells.

In Salmonella infection in our studies, only 10^3 organisms were found on day 1 and 10^5 on day 5. We now ask the question as to whether the 10^2 fold increase from day 1 to day 5 occurs via the multiplication at discrete foci on particular cells or whether there is a spreading of bacteria to other cells in the Peyer's patches. We have already seen in previous results that most of the bacteria in Peyer's patches are extracellular but that a varying proportion may be loosely cell-associated. In the following experiments attempts were made to assess this cell-association by allowing the cells to fall gently through a liquid column and to observe whether in their sedimentation they carried bacteria along with them.

5.4.1 "Falling test"

This experiment was designed to answer the above question by performing it under the best conditions to prevent dissociation of bacteria/cell aggregates.

A single cell suspension of the Peyer's patches of Salmonella

Table 5.2

Cell-associated bacteria in Peyer's patches after oral feeding with 885 organisms.

Day	% of bacteria in cell pellet after washing in:	
	Saline	Ethylenediamine-tetra-acetic acid 0.1%
2	6	1
4	28	N.D.*
6	29	0.3
8	46	0.1

Note: 0.1% EDTA does not affect the viability of Salmonella 885 or damage the cells in the time of the experiments.

* Not done.

885^{SR} fed mice was prepared by the scratching technique. One milliliter of this suspension (5×10^6 cells) was loaded gently onto 50 ml of cold 5% foetal calf serum/RPMI 1640 medium containing streptomycin in a 50 ml siliconized burette. The cells were allowed to settle by gravity at 4° for 2 hours. The cells and bacteria were counted in aliquots of the initial cell suspension and in the bottom 2 ml of the column at the end of the experiment. The results (Table 5.3) showed that all the bacteria were cell-associated on day 4, 5, 6 and 7 after infection. Unfortunately, we could not do the experiment with the cell suspension from the first 3 days of infected mice because there were not enough bacteria in the Peyer's patches. However, when bacteria were added in vitro to the Peyer's patch suspensions of normal mice, a similar association was found. It is important to note that in controls where the bacterial suspension was loaded onto the liquid column no sedimentation of bacteria had occurred at the end of two hours.

5.4.2 Fluctuation test

The Falling test revealed that bacteria and cells are associated but, since centrifugation of the suspensions in 0.1% EDTA was evidently capable of dissociating some of the bacteria from the cells, the bond between them may be weak. If this is so one might expect that the bacteria could move around within the Peyer's patches and that there would rarely be more than 1 bacteria associated with 1 cell since the cell:bacteria ratio is always greater than 100:1.

We attempted to derive data on this question by using a fluctuation test. This test was carried out by diluting the single

Table 5.3

The distribution of bacteria and cells during sedimentation of a single cell suspension taken from infected Peyer's patches six days after feeding 885^{SR}

Mouse no.	No. cells put on column	No. cells in bottom 2 ml*	% cells recovered	No. Bacteria put on column	No. Bacteria in bottom 2 ml	% Bacteria recovered
1	4.8×10^6	1.3×10^6	27	1,428	445	31
2	7.8×10^6	1.8×10^6	23	640	120	19
3	6.2×10^6	1.1×10^6	18	1,585	500	32
4	2.6×10^6	5×10^5	19	153	20	13

* 1 ml of the suspension of Peyer's patch cells was loaded onto a 50 ml column of 5% FCS-RPMI 1640 in the cold and allowed to settle for two hours. Two ml were then removed from the bottom of the column of liquid and both cell and bacterial counts were made. The same experiment has been done with 4, 5 and 7 day fed mice with similar results.

cell suspension of the Peyer's patches of Salmonella 885^{SR} fed mice until only few bacteria could be recovered from the last dilution. We then made a large number of spread plates at this dilution for bacterial counting. The expectation of this experiment was that if the bacteria were distributed uniformly there would be a similar number of colonies on every plate. On the other hand, if there was a discrete adherence of Salmonella 885 to certain cells the number of colonies on each plate would be widely variable. The results (Table 5.4) showed that every agar plate had a similar number of bacterial colonies. This indicated that bacteria were distributed uniformly among the cells. The bond that forms between them therefore must be weak, allowing the bacteria to move from one cell to the other. We observed bacteria attached to the Peyer's patch cells (mainly lymphocytes) under phase contrast.

5.5 EFFECT OF ADDITIONAL CHALLENGE

In order to determine the significance of any cell association of bacteria mice which had received one oral challenge with 5×10^8 Salmonella 885 were rechallenged orally with a similar dose of a streptomycin-resistant mutant (Salmonella 885^{SR}). Thus the growth of both variants could be assayed separately. When given separately both mutants pursue the same course of penetration and growth in Peyer's patches. Since the organisms occupy extracellular positions, it might be expected that the two inocula would mix and grow in the same ratio from then on. The results in Figure 5.3 did not prove this to be the case. Both the first and second challenge organisms were found to pursue an independent growth and decline pattern which clearly indicated that

Table 5.4

Fluctuation test

Number of Salmonella 885^{SR} on each plate		
Number of Peyer's patch cells [a] plated (in 0.1 ml)		
1×10^4	2×10^3	4×10^2
26	6,5,2	1,2
21	3,6,6	1,4
22	4,12	2,2
19	8,8,6	2,1
22	6,8,7	2,1
18	3,1,4	2,1
20	7,2,7	0,0
20	7,2,7	0,0
10	7,9	0,0
25	2,5,5	0,0

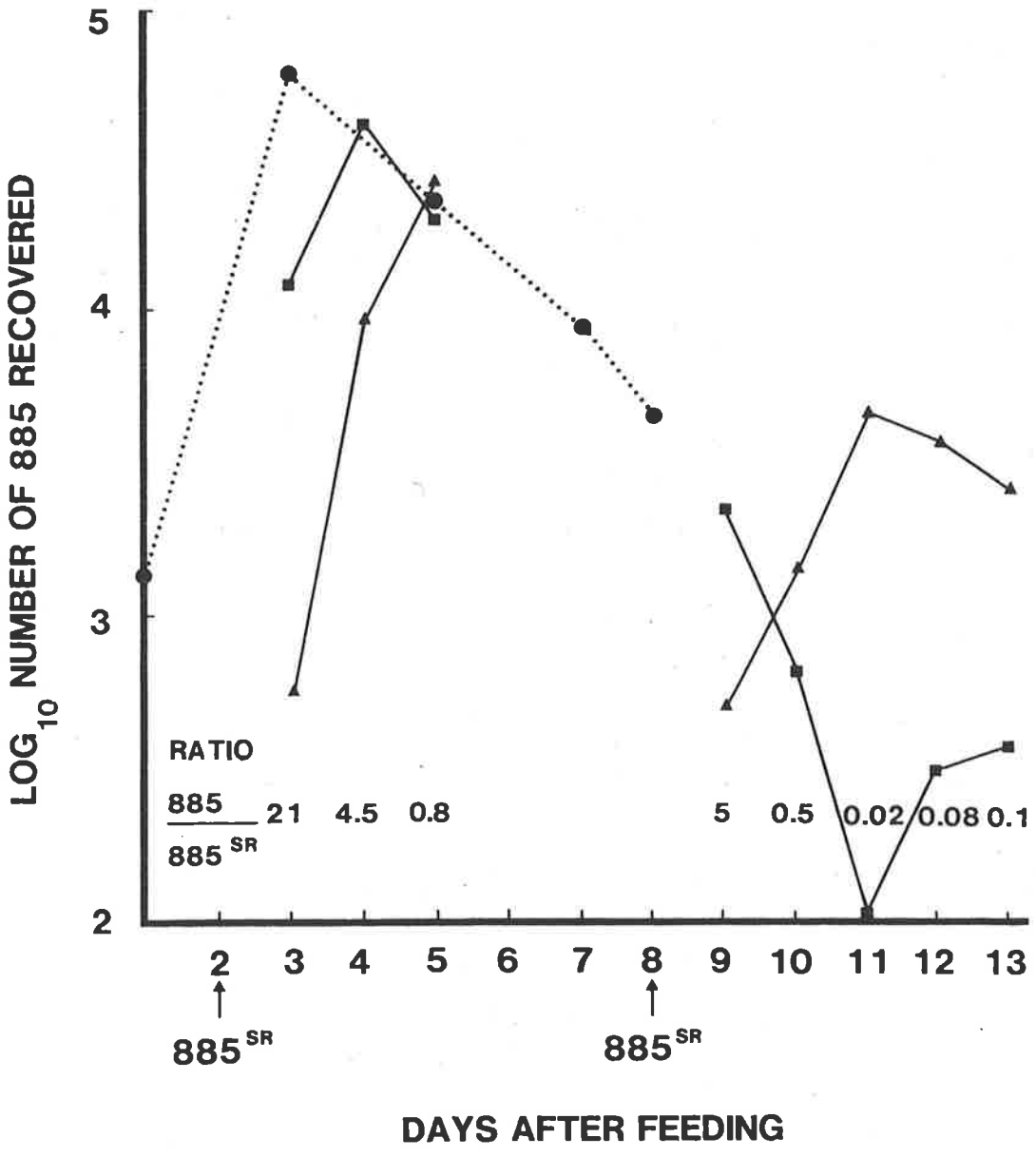
a Single cell suspension from Peyer's patches of 4 day Salmonella 885^{SR} fed mice was obtained by scratching technique. Peyer's patch cells were plated by the spreading plate technique (10^5 cells/ml).

Note: Similar results were also obtained with mice infected with Salmonella 885^{SR} orally on days 5, 6, 8.

Figure 5.3

The effect of superinfection with a marked strain of 885

Shows the simultaneous recovery of 885 and 885^{SR} from the Peyer's patches of mice. ● = Recovery of organisms from mice fed 885 on day 0, control series mean of 10 experiments; ■ = recovery of 885 from mice fed 885 on day 0 and 885^{SR} on days 2 or 8; ▲ = recovery of 885^{SR} from mice fed 885 on day 0 and 885^{SR} on days 2 or 8.


5
4
3
2
LOG10 NUMBER OF 885 RECOVERED
RATIO
885 / 885SR
21
4.5
0.8
5
0.5
0.02
0.08
0.1
2
3
4
5
6
7
8
9
10
11
12
13
885SR
885SR
DAYS AFTER FEEDING

these two extracellular populations did not mix. If the second inoculation was given at a time when the first batch of Salmonella 885 was being rapidly eliminated, the newly arrived Salmonella 885^{SR} pursued an independent course of growth and ultimate decline.

In other words, the two extracellular populations behaved as though they occupied separate compartments. This could mean that the cell-associated bacteria travel within the Peyer's patches in association with the lymphocyte traffic and that each inoculum must pursue this journey independently. Such movement seems to be a preliminary to elimination of the organisms.

5.6 CONCLUSIONS

Peyer's patches of Salmonella 885 fed mice appeared bigger than those of normal mice and the total number of cells doubled within a few days after infection. The per cent of T and B lymphocytes were not appreciably changed during the course of infection.

However, the number of phagocytic adherent cells (presumably Peyer's patch macrophages) showed an increase from approximately 2.5×10^3 per 5×10^6 cells on day 1 to as high as 7×10^4 on days 5-7.

Salmonella 885 are not intracellular during this local infection but are clearly associated with the outside of cells by loose bonds. Bacteria-cell association increased following the infection. Ninety-four per cent of bacteria were free on the second day after infection but only 52% remained so on day 8.

The "Falling test" and Fluctuation test give a clear picture of this bacteria-cell association. Only one cell in 5000 of the

day 1 Peyer's patch cells has a bacterium attached to its surface, but when the organisms multiply, the enlarging bacterial population is mobile and moves from cell surface to cell surface with which it has only a loose association and the ratio remains 1 bacterium/cell.

It seems probable that the association has considerable significance since the successive bacterial challenges were found to pursue separate growth curves. The two successive bacterial challenges did not mix and it seems possible that the initial challenge has moved along with the accompanying lymphocyte traffic from the area of penetration by the time the second challenge inoculum arrives. The second challenge inoculation may then associate with the trafficking lymphocytes and the process of growth and decline is repeated.

CHAPTER 6

THE ELIMINATION AND KILLING OF BACTERIA BY THE PEYER'S PATCH MACROPHAGES

6.1 INTRODUCTION

The increasing number of macrophages following oral administration of Salmonella 885 coincided with a decline of Salmonella 885 in the Peyer's patches. This inverse correlation between the number of macrophages and bacteria suggests a causal relationship due to the bactericidal action of the incoming macrophages (Fig 6.1).

Since it has been shown (Blanden, Mackaness and Collins, 1966) that activated macrophages are necessary for efficient killing of Salmonellae it seemed desirable to demonstrate the *in vitro* bactericidal activity of macrophages, isolated from the Peyer's patches, and to see if this activity changed with time after feeding.

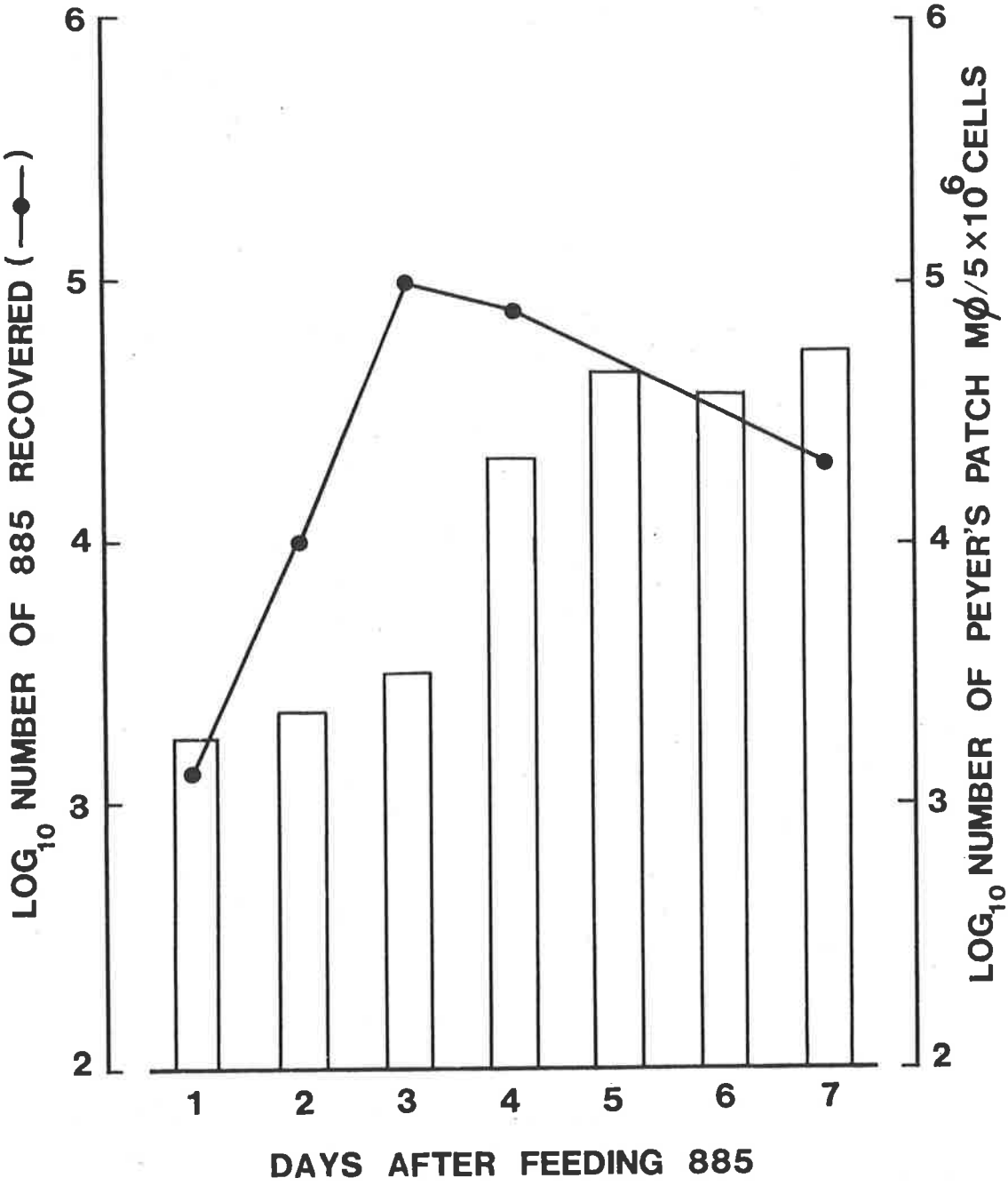
6.2 THE MECHANISMS OF PHAGOCYTOSIS AND BACTERICIDAL ACTIVITY

Macrophages have been proved to be the antibacterial effector cells and, after activation of the host's cellular immune system, are better equipped to ingest and kill Salmonellae and the other intracellular parasites (Hobson, 1957; Howard, 1961; Saito *et al.*, 1960; North, 1970; Jones and Byrne, 1980). When macrophages and organisms are in close contact, firm attachment occurs between the organism opsonized with antibody and complement to the surface of macrophage *via* Fc and C3 receptors). This attachment results in

Figure 6.1

Recovery of macrophages (isolated by the "scratching" technique) and Salmonella 885 from the Peyer's patches of mice after oral infection with Salmonella 885. Symbol: open bar = number of macrophages and (●) = number of Salmonella recovered.

The macrophages numbers were derived from the experiment in Table 5.1 and the bacterial numbers were derived from the experiment in Figure 4.1.


6
5
4
3
2
LOG10 NUMBER OF 885 RECOVERED (—●—)
LOG10 NUMBER OF PEYER'S PATCH Mϕ/5 X 10^6 CELLS
1
2
3
4
5
6
7
DAYS AFTER FEEDING 885

stimulation of the membrane of the phagocytic cells and triggers phagocytosis, fusion of the phagocytic vacuole with lysosomes, metabolic changes and finally destruction of intracellular organisms. The organisms may be killed by oxygen-dependent system and/or oxygen-independent system.

6.3 BACTERICIDAL ACTIVITY OF THE ISOLATED PEYER'S PATCH MACROPHAGES

There is no difficulty in measuring the bactericidal activity of large numbers of macrophages. This can be done in plastic tissue culture plates using approximately 4×10^5 macrophages/5mm diameter well and using such methods the interactions between peritoneal macrophages and bacteria have been well studied (Cooper, Johnson and Rowley, 1983). Unfortunately the number of macrophages in the Peyer's patches of normal mice is very small and may only total 10^4 or so in all the collected intestinal lymphoid tissue from one SPF mouse. Efforts were made to recover greater numbers of macrophages from the Peyer's patches and to miniaturise the technique of studying their reaction with bacteria. The first problem was satisfactorily solved using collagenase digestion as described in the accompanying scheme.

By this means it was possible to recover more than 10^5 macrophages from the Peyer's patches of mice which had been fed with 885, the numbers depending on the time since feeding. Note that this about doubles the number of recovered macrophages from that in Fig. 6.1.

Since satisfactory bactericidal experiments can be performed when 4×10^5 cells are spread on a surface 5 mm in diameter (i.e. surface area of flat-bottomed wells of a Flow Labs tissue culture

plate - 0.28 cm^2), it should be possible in theory to produce similar kinetics using 5 x 10^4 macrophages spread over 1/10 of the area. This was tried in two ways. (a) In capillary tubes using an interface with Ficoll-Hypaque as the area for macrophage/bacterial interaction, and (b) Using a miniature tissue culture chamber assembled on a glass microscope slide (micro slide culture chamber).

6.3.1 Method of isolating viable macrophages with collagenase.

We have tried different methods to isolate viable macrophages in large numbers. Best results were obtained when the Peyer's patches were digested with Clostridium histolyticum collagenase (Clostridiopeptidase A from Cl. histolyticum Type 1, Sigma Chemical Co., Mo., U.S.A.). This method provided sufficient macrophages in a healthy condition to perform in vitro experiments. The flow chart for Peyer's patch collagenase digestion and the macrophage isolation procedure is shown below.

Isolation of macrophages from the Peyer's patches with collagenase

Isolated Peyer's patches

↓

Incubate at 37° in 1 mM dithiothreitol (in 0.05% EDTA) for 15 min, wash twice with saline solution

↓

Cut into small pieces with scissors in 10% FCS-RPMI 1640 medium.

↓

Digest with collagenase (20 units/ml, clostridiopeptidase A) at 37° in a shaking water bath for 40-60 min.

↓

Pass through 200 mesh stainless steel sieve into 30 ml conical centrifuge tubes with 5 ml of foetal calf serum, centrifuge at 600 x g 10 min.

↓

Wash the cell pellet twice with 10% FCS-RPMI 1640.

↓

Resuspend in 10% FCS-RPMI 1640 at 2-4 x 10^6/ml

↓

Distribute 3ml into FCS pre-coated culture petri dish (50 x 13 mm).

↓

Incubate at 37°, 5% CO_2 for 2 hours.

↓

Tip off the liquid, wash twice with 10% FCS-RPMI 1640.

↓

Detach macrophages with 0.1% EDTA in PBS plus 5% FCS pH7.2.

↓

Pool macrophages into 30 ml conical centrifuge tube with 5 ml FCS, wash with 10% FCS-RPMI 1640: pure isolated macrophages are ready for use in bactericidal experiments. (Yield 300 µl of 3 x 10^6 cells/ml from 8 mice).

Note: Streptomycin 200 µg/ml, penicillin G 200 units/ml and gentamycin 20 units/ml were included in the medium except in the last step when pure isolated macrophages were resuspended in 10% FCS-RPMI 1640 with streptomycin 100 µg/ml before being used for the

bactericidal assay.

6.3.2 The bactericidal assay of isolated macrophages by the capillary tube method

The interaction of macrophages and opsonized Salmonella 885^{SR} was followed in graduated, siliconized capillary tubes (Clay-Adams, N.J., U.S.A). Each tube contained 2-4 x 10^4 macrophages + 1-2 x 10^2 bacteria in 10% FCS-RPMI 1640 supplemented with another 10% fresh rabbit serum in a total volume of 20 µl. Both ends of the tube were sealed with Parafilm (American Can Company, Greenwich, CT., U.S.A.) and incubated at 37°, 5% CO_2 in air either horizontally or vertically. Bacteria were counted at different time intervals by pipetting 20 µl of 1% Triton X100 (a non-ionic detergent) up and down in the tube several times in order to lyse the macrophages before blowing the contents onto the surface of streptomycin agar plates and spreading with a glass spreader. This concentration of Triton X100 did not affect the viability of 885^{SR} in the duration of our experiments.

At least 90% of the isolated macrophages were viable initially when stained with trypan blue but no killing activity was observed. It is possible that the macrophages were not able to spread and phagocytose on the Parafilm.

A modification designed to promote contact of bacteria with macrophages was then used. The idea was to add 20 µl of a macrophage-bacterial suspension (2 x 10^4 of macrophages + 200 bacteria) into 100 µl graduated, siliconized capillary tubes (Clay-Adams), one end of which was filled with 50 µl Ficoll-Hypaque in Dulbecco's medium (specific gravity 1.16) as a dense base before

sealing the tubes. The capillary tubes were centrifuged at 1500 rpm, 4° for 10 min and then incubated at 37° for different intervals before counting viable bacteria. The capillary tubes were cut with a diamond pen near the sealed end underneath the interface between Ficoll-Hypaque and the aqueous suspension and 20 µl of 1% Triton X100 was pushed through the capillary tubes onto the agar plates for the bacterial count. Slight bactericidal activity by the macrophages was observed but this was not consistent.

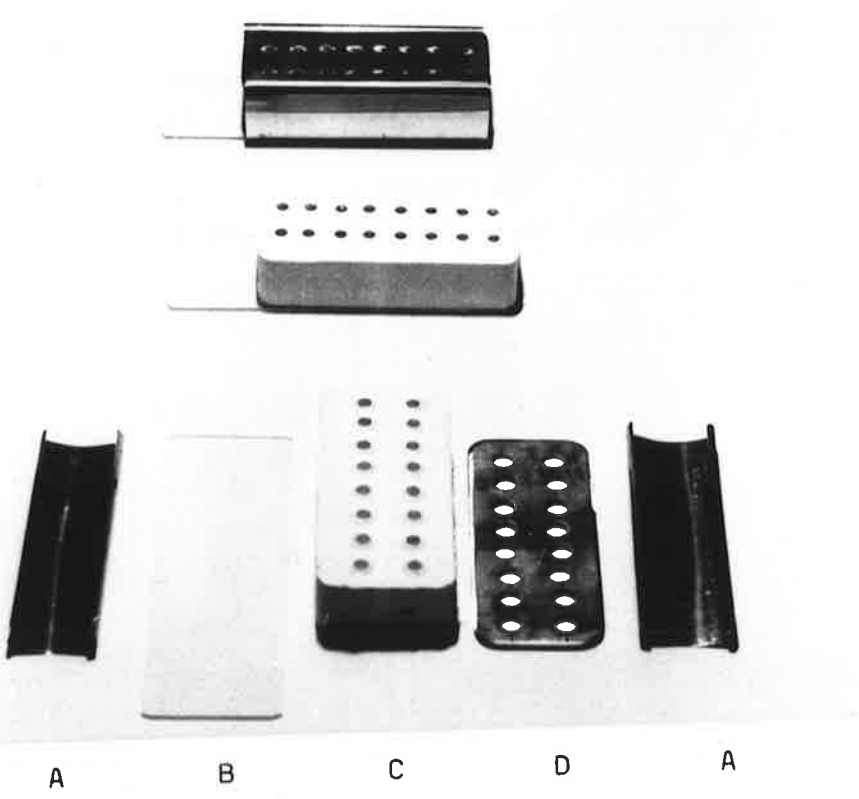
6.3.3 The bactericidal assay of the isolated macrophages by micro slide culture chamber.

Recently Cooper, Johnson and Rowley (1983) have demonstrated a good bactericidal activity of activated peritoneal macrophages with Salmonella typhimurium by allowing macrophages to adhere and spread on micro titration trays (Chapter 3) before adding opsonized bacteria. We decided to modify a slide culture chamber by reducing the size of each well so that it was 10 times smaller, therefore it would hold 30 µl of liquid (Fig. 6.2).

Twenty microlitres containing 4 x 10^4 of isolated Peyer's patch macrophages free of bacterial contamination (including 885 organisms) were added to each well, and the macrophages allowed to adhere and spread for 2 hours. The supernatant fluid was then tipped off and each well washed twice with culture medium. Bacteria were opsonized with a rabbit anti-885 serum diluted to 2 HA units. Into each well 200-250 opsonized Salmonella 885^{SR} were added in 10 µl of 10% FCS-RPMI 1640 with streptomycin (100 µg/ml) and supplemented with 10% fresh rabbit serum. Control wells without macrophages were included. In order to give maximum

Figure 6.2

The micro-slide culture chamber used in the bactericidal assay. The wells are approximately 0.028 cm^2 in area (cf. 0.28 cm^2 in the unmodified slide culture chamber).



A = metal clip

B = glass slide

C = silastane block

D = stainless steel cover

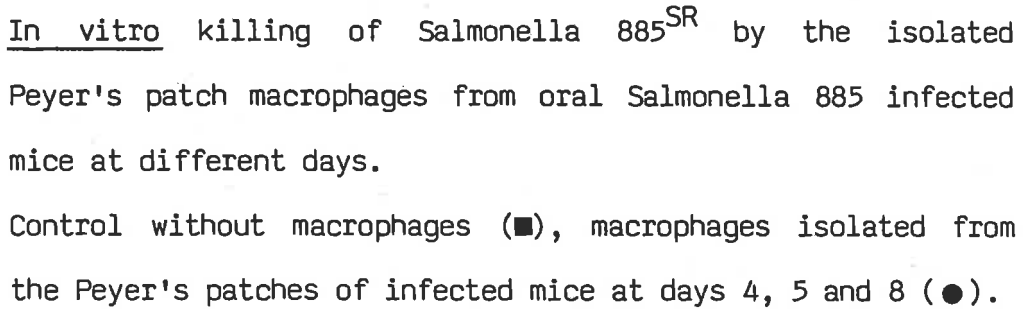
bacteria-macrophage contact micro slide culture chambers were centrifuged at 1500 rpm 4° for 10 min in an MSE Coolspin centrifuge, PL430 England. Bacteria were counted from duplicate wells before centrifugation at 0 time, and at different intervals thereafter by adding an equal volume of 1% Triton X100 and spreading on streptomycin agar plates.

As shown in Fig. 6.3, the macrophages isolated from the Peyer's patches of mice at different days after oral infection with Salmonella 885 showed an increase in bactericidal activity with a maximum being reached by day 8 at which time good killing occurred in the first 60 min of in vitro action. This increase in bactericidal activity of macrophages isolated from the Peyer's patches of mice after oral infection with Salmonella 885 was similar to that we observed with peritoneal macrophages of mice immunized i.p. with Salmonella 11RX (Davies, 1975).

6.4 PEYER'S PATCHES ARE THE SITES OF ANTIGEN SAMPLING

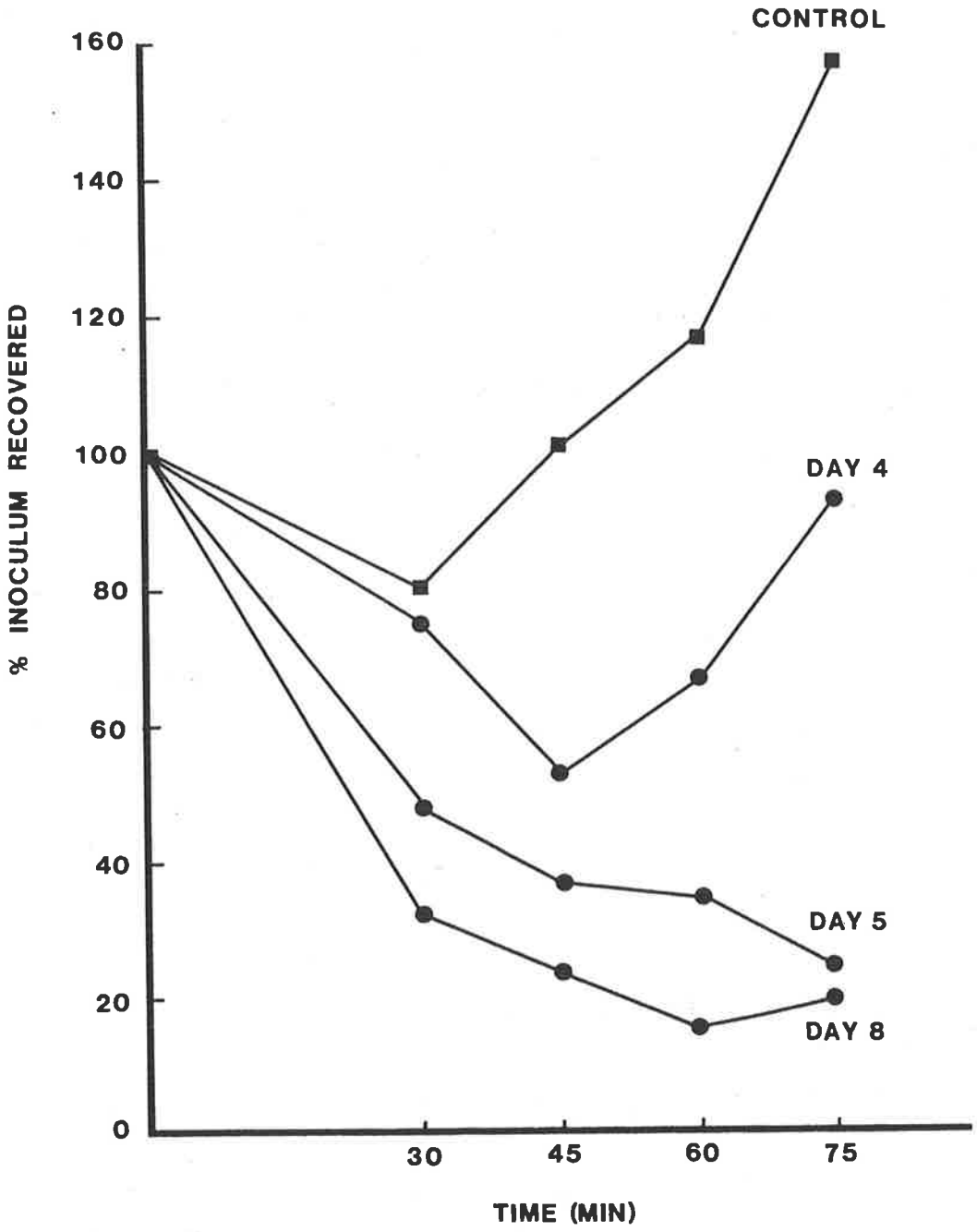
It has been demonstrated that specialized epithelial cells (M cells) which covered the dome area of the Peyer's patches could be seen transporting luminal material in the cell cytoplasm vesicles (Owen and Jones, 1974; Owen, 1977). A similar uptake of dead Mycobacterium, carbon particles and powdered erythrocytes confirmed that sampling was an active process (Owen and Jones, 1974). Peyer's patches can therefore be seen to be a site of antigen sampling from the intestinal lumen. We broadened our understanding of immunological response in the Peyer's patches by using three different bacteria to challenge mice orally namely Vibrio cholerae 569B, E. coli 492 and Salmonella 885, all capable of adhering to the

Figure 6.3



In vitro killing of Salmonella 885^{SR} by the isolated Peyer's patch macrophages from oral Salmonella 885 infected mice at different days.

Control without macrophages (■), macrophages isolated from the Peyer's patches of infected mice at days 4, 5 and 8 (●).


CONTROL
DAY 4
DAY 5
DAY 8
160
140
120
100
80
60
40
20
0
% INOCULUM RECOVERED
30
45
60
75
TIME (MIN)

intestine. Since the M cells were presumed to be sampling the bacteria adherent to the epithelial lining of the gut, we might expect that we would find these three different bacteria in the Peyer's patches in similar numbers if they were given to mice in equal numbers.

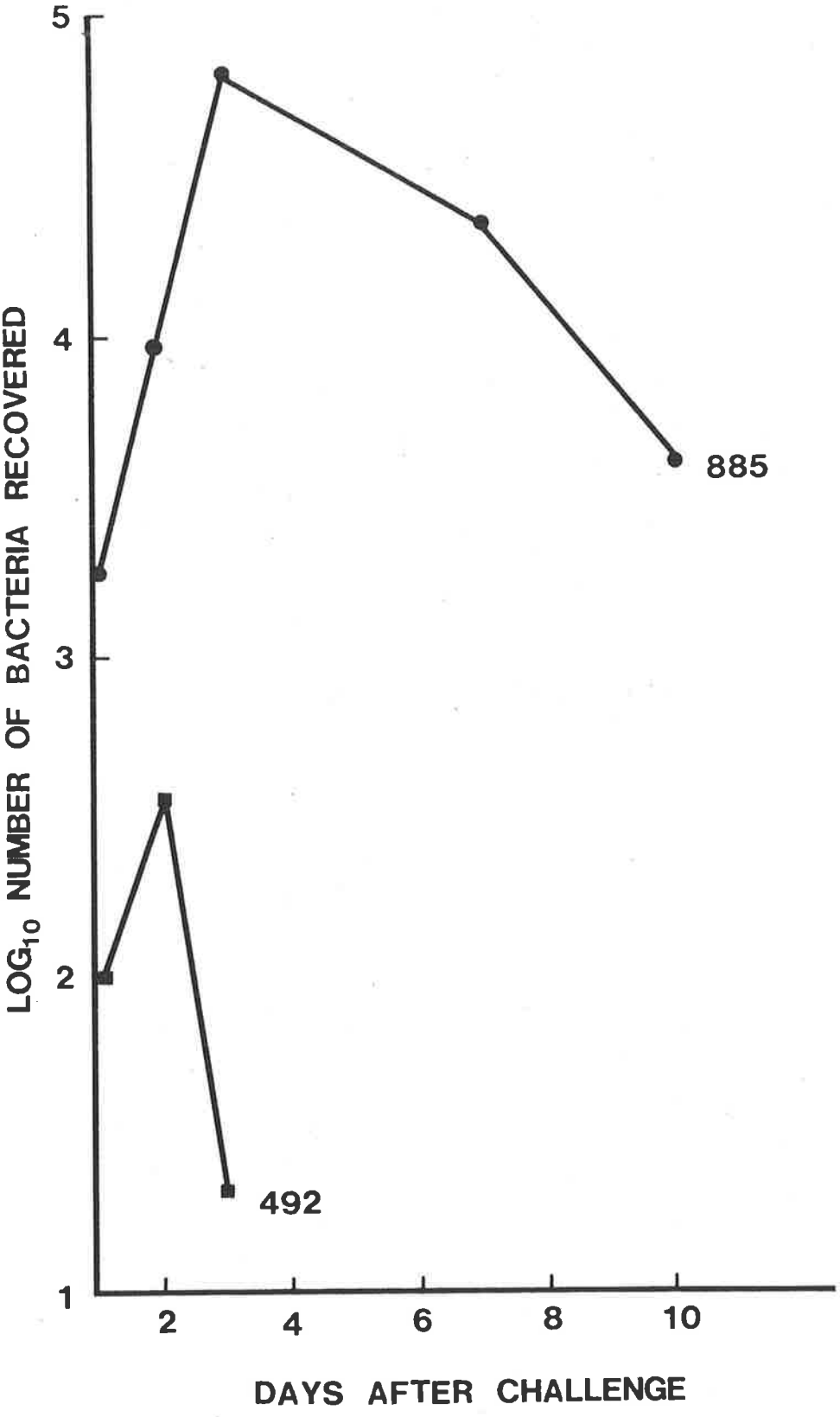
6.5 THE ELIMINATION OF THREE DIFFERENT BACTERIA BY THE PEYER'S PATCHES

Mice were divided into 3 groups of 35 mice which were challenged orally with 5×10^8 bacteria in 0.5 ml of PBS with 3% sodium bicarbonate. Each mouse received one kind of bacteria; either Vibrio cholerae 569B or E. coli 492 or Salmonella 885. On the following day five mice from each group were sacrificed and the small intestine was washed thoroughly. The bacteria in the Peyer's patches from each mouse were enumerated as described in Chapter 3.

The numbers of the three different bacteria recovered were markedly different. We never recovered any viable Vibrio cholerae 569B at any time. However, on day 1 approximately 100 E. coli 492 were recovered, i.e. about ten times less than Salmonella 885. By day 3 E. coli 492 were eliminated from the Peyer's patches while Salmonella 885 still continued increasing in number (Fig. 6.4). It seems probable that bacterial entry into Peyer's patches is due to pinocytosis by the covering M cells and this might be in proportion to the numbers of organisms attached to the lumenal surface of these cells. On this basis, since the three bacterial strains have capacities to adhere to gut epithelial cells we could expect to find similar numbers inside the Peyer's patches.

Figure 6.4

Recovery of bacteria from the Peyer's patches of orally infected mice at different days. Salmonella 885 (●) E. coli 492 (■) and Vibrio cholerae 569B (no organisms recovered). Each point shows the average from 5 mice.


5
4
3
2
1
LOG10 NUMBER OF BACTERIA RECOVERED
885
492
2
4
6
8
10
DAYS AFTER CHALLENGE

Since this is not so, an explanation could be that the penetrating organisms might be killed at vastly different rates. The fact that no Vibrio cholerae 569B were found in the Peyer's patches, could be due to a very high susceptibility of Vibrio cholerae 569B to the bactericidal action of macrophages and their rapid elimination.

Thus we proposed that the different rates of elimination of microorganisms in the Peyer's patches were due to the different degrees of susceptibility of each bacterial strain to the macrophages in that area. Therefore it was possible that Vibrio cholerae 569B is the most susceptible organism of the three and that Salmonella 885 is the most resistant to the bactericidal action of macrophages. This possibility was tested in the following in vitro experiment.

6.6 THE SUSCEPTIBILITY OF DIFFERENT BACTERIA TO THE BACTERICIDAL ACTION OF MACROPHAGES IN VITRO.

It was technically difficult to collect a large number of normal macrophages from the Peyer's patches. However these are easily obtained from the peritoneal cavity and these probably are a representative sample of all the animal's macrophages. We therefore performed the bactericidal assay with mouse peritoneal macrophages.

For these experiments, flat bottom 96 well titration trays (Flow Laboratories, Inc.) (Chapter 3) were used. The results shown in Table 6.1 solidly supported the hypothesis that the susceptibility of bacteria was Vibrio cholerae 569B > E. coli 492 > Salmonella 885 respectively. At 30 min 95% Vibrio cholerae 569B, 84% E. coli 492 and 20% Salmonella 885 were killed by macrophages. Bacterial controls without macrophages maintained their numbers for

Table 6.1

Comparison of killing rates by macrophages from normal mice in presence of 10% mouse serum, all bacteria preopsonised with 2HA-units specific antibody.

Time (min)	Number of bacteria recovered					
	Salmonella 885		E. coli 492		V. cholerae 569B	
	macrophage	-	macrophage	-	macrophage	-
0	231	237	256	256	476	506
30	185 (80)[a]	303 (127)	42 (16)	249 (97)	22 (5)	527 (104)
60	153 (66)	266 (112)	37 (14)	629 (245)	12 (2.5)	622 (122)

a The % of inoculum recovered is shown in parentheses.

30 min and then began to multiply.

6.7 CONCLUSIONS

Macrophages migrated into the Peyer's patches of the mice orally infected with Salmonella 885 probably under the influence of the cell-mediated immune response. The bactericidal activity of these cells could be demonstrated to defend host against invading microorganisms.

1. The number of macrophages in the Peyer's patches of mice orally infected with Salmonella 885 increased coinciding with the decline of Salmonella 885. These results suggested that bacteria were killed by macrophages which entered the Peyer's patches. Direct evidence from in vitro experiments showed that macrophages isolated from the Peyer's patches at this time were able to kill Salmonella 885. The best bactericidal activity was observed 8 days after mice had been infected with Salmonella 885 orally. These results agreed with the peak activity of mouse peritoneal macrophages activated with Salmonella.

2. As discussed earlier, results of others suggest that different kinds of bacteria, as well as other particles, are transported with similar efficiency into the Peyer's patches by the M cells. The very different levels of recovery of viable bacteria in mice fed with Salmonella 885, E. coli 492 and V. cholerae 569B may be explained by differences in susceptibility to killing by macrophages as indicated by our in vitro experiments.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

Acute bacterial diarrhoeal disease is a major cause of morbidity and mortality throughout the world. In developing countries, acute bacterial diarrhoea is recurrent and frequently disabling, and may be responsible for 50% of infant mortality (Gordon, 1971; Rohde and Northrup, 1976). While its impact is considerably less in the developed countries it remains a major cause of morbidity, hospitalization and loss of economic resources. In fact, acute diarrhoeal disease is second only to the common cold in causing absence from work (Dingle et al., 1964). Improvements in sanitation, personal hygiene and antibiotic treatment could greatly reduce the problems but a more attainable solution for typhoid would be to find an effective vaccine.

Vaccines which have been introduced since the last decade are still of questionable effectiveness. The development of immunity against Salmonella infection needs to be investigated, particularly at the cellular level to gain a better knowledge of the host-parasite relationship.

7.2 DEVELOPMENT OF IMMUNE RESISTANCE TO SALMONELLA INFECTION

Killed typhoid vaccines, which are able to produce high titres of specific antibody, have a limited success in protecting vaccinees except in endemic areas where the subjects experience repeated subinfective doses. The inability of these vaccines to

generate a cell-mediated immune response has been suggested as the reason for this failure (Nath et al., 1977). This suggestion is in agreement with the previous findings by several workers that resistance to Salmonellae depends mainly upon a cell-mediated immune mechanism (Ushiba et al., 1959; Howard, 1961; Blanden, Mackaness and Collins, 1966).

Salmonellae appear to be confined to the Peyer's patches of the small intestine after the ingestion of contaminated food or water. Virulent Salmonella strains are able to multiply and may spread through the circulation whereas avirulent strains are usually eliminated in a few days.

We have demonstrated the existence and development of immunity in the Peyer's patches after antigenic stimulation. Mice after a primary infection were resistant to a virulent strain, Salmonella typhimurium C5 (Fig. 4.2). MacDonald and Carter (1980) also found a similar result when they demonstrated that protective immunity to Listeria monocytogenes occurs in the Peyer's patches, lymph nodes and spleen. In addition, this protective immunity can be transferred to normal syngeneic mice with immune Peyer's patch cells, mesenteric lymph node cells or spleen cells. The development of immunity in the spleen and liver following oral administration was probably due to the cell traffic. The passive transfer of high titre specific antibody delayed the course of infection but failed to protect mice (Fig. 4.3 and Fig. 4.4). Therefore it appears that antibody plays a minor role in host defence to Salmonella. It seems clear that cell-mediated immunity is paramount in the protection of animals against Salmonella and the last section of this chapter will be devoted to discussing this.

7.3 ARE PEYER'S PATCHES ALREADY PRIMED?

It is worth noting that macrophages present in the Peyer's patches of conventional mice seem to be competent against many microorganisms. Cottier et al. (1975) could not detect any intact bacteria in Peyer's patches, despite these structures being a preferential site of entry for particles from the gut - for example, carbon particles introduced into the gastrointestinal tract could be located in the Peyer's patches.

The results presented in Chapter 4 are consistent with this observation. We found that the Peyer's patches of the small intestine of mice are already primed since CPA, an alkylating antimitotic agent, has no effect in controlling bacterial multiplication in the Peyer's patches. This is analogous to the situation with primed spleen which is capable of controlling bacterial growth in a manner which is not inhibited by CPA.

The control of multiplication and the elimination of intracellular bacteria such as Listeria from the spleen requires a state of activation of the macrophage, the ultimate effector cell. This activation is mediated by lymphokines which are released by sensitized T lymphocytes upon contact with antigen (Lane and Unanue, 1972; Simon and Sheagren, 1972; North, 1978). For its efficient development, this process requires the presence of a large number of sensitized T cells, which can only arise by clonal expansion of a relatively small number of pre-existing committed cells.

Tripathy and Mackaness (1969) were able to explain the dramatic effect of CPA on splenic control of bacterial multiplication in terms of prevention of the cell division necessary for the expansion of the appropriate T cell clones. Our results for

bacterial growth in the spleen confirm their earlier findings.

Peyer's patches, which are aggregates of lymphoid tissue with a structure basically similar to that of the spleen, were not affected by CPA treatment. A consistently greater number of Salmonella 885 were found in the Peyer's patches of CPA-treated animals 1 day after oral challenge, but the development of antibacterial activity after colonization of these tissues with Salmonella 885 was not changed by the antimitotic drug. It seems reasonable therefore, to suppose that clonal expansion of lymphocytes is not necessarily involved in the Peyer's patches in contrast to the situation with the spleen. Clearly, the intestinal lymphoid follicles differ from the spleen in the development of antibacterial activity and can be rendered resistant to the growth of bacteria in the absence of any such development in the spleen.

It seems feasible that the different observations made in the Peyer's patches and the spleen reflect differences in the degree of priming at the two sites. The Peyer's patches of conventional mice are likely to be constantly primed by antigens from many enteric organisms. Therefore, the different responses of the spleen and the intestinal lymphoid follicles to CPA could be due to differences in the degree of previous natural priming received. When the spleen was deliberately primed, the subsequent antibacterial responses were unaffected by CPA (Fig. 4.7B). Because deliberately primed spleens, like Peyer's patches, were able to control a subsequent challenge with the organisms even in the presence of CPA, it is possible that under these conditions there were enough sensitized T cells to provide macrophage-activating lymphokines in the absence of further T cell division. Deliberate priming of Peyer's patches shortened the period needed for an effective antibacterial capacity to develop

after rechallenge, but this development appeared to occur in the absence of massive cell multiplication. Our interpretations are supported by studies of Gadol et al. (1976) who evaluated the cell-mediated immune response in guinea pigs orally challenged with live or heat-killed Vibrio cholerae. They observed that cells from both immunized animals and unimmunized controls displayed macrophage-inhibitory factor (MIF) activity in vitro, even in the absence of Vibrio antigen. Levin et al. (1974) also reported a higher background of lymphokines (chemotactic activity) in control Peyer's patch cells than in control spleen cells. Taken collectively, these data indicate that the cells in the gut are in a constant state of activation and are further stimulated by oral vaccination.

7.4 MACROPHAGES AND INFECTION OF THE PEYER'S PATCHES

Most of the available evidence concerning immunological resistance to Salmonella infections is in favour of the idea that resistance requires both cellular and humoral immunity. The cellular immunity is mediated by T lymphocytes and macrophages and is only observed when mice are immunized with viable vaccines (Collins, 1973; 1974) and subsequently develop delayed-type hypersensitivity (Moser et al., 1980).

A few days after infection the Peyer's patches appear larger, and contain approximately twice the number of cells normally observed (Marneerushapisal and Rowley, unpublished data). However, immunofluorescent studies of the lymphocyte populations of Peyer's patches did not detect any great changes in the relative proportions

of B and T cells, during the course of infection (Table 5.1).

The antibacterial capacity of macrophages is greatly increased by the action of lymphokines released from sensitized T cells (Mackaness 1971; North, 1978). Recent work in our laboratory has investigated aspects of T cell involvement in cellular immunity against Salmonellae. M. Musa (personal communication) has been able to activate normal peritoneal macrophage populations by incubation with the supernatant from Con-A stimulated spleen cell cultures. In vitro these activated macrophages, unlike normal macrophages, are able to kill virulent Salmonella typhimurium C5.

When mice were infected with Salmonella orally, there was a dramatic increase in the number of macrophages in the Peyer's patches a few days after infection (Fig. 6.1). This is consistent with the observation by Hohmann (1979) that there is an increased number of cells staining with acid phosphatase (presumably macrophages) in the Peyer's patches approximately 7-10 days after Salmonella infection. A similar finding was made by Owen, Allen and Stevens (1981) during an electron-microscopic study of giardiasis, a self-limiting parasitic infection of the gut in mice. They also observed an accumulation of macrophages in the Peyer's patches, with the parasites visible inside the cells.

In our experiments the increase in the numbers of macrophages coincided with the decline in bacterial numbers, beginning on day 4 after infection (Fig. 6.1). This inverse correlation suggests that the elimination of Salmonellae from the Peyer's patches may be due to the migration of monocytes into these infected areas, and their subsequent differentiation into macrophages. Supportive evidence comes from earlier studies by other workers with Listeria

monocytogenes which showed that the degree of resistance in the liver and spleen was directly correlated with the number of macrophages in these organs (North, 1969; 1970). Moreover it has recently been demonstrated that the nonspecific resistance of Listeria-fed mice to Salmonella is due to the increased number of macrophages in the Peyer's patches of such mice (MacDonald, Bashore and Carter, 1982). Such observations suggest a role for macrophages in local intestinal immunity.

7.5 THE NATURE OF THE ASSOCIATION BETWEEN BACTERIA AND HOST CELLS

After feeding mice with 5×10^8 Salmonella 885 approximately 10^3 bacteria were recovered from Peyer's patches on day 1 and this increased to 10^5 organisms on day 4, a 100-fold difference (Fig. 4.1). This means that on the first day the bacteria adhered on average to only one in every 5,000 cells (total cells = 5×10^6 cells); during their multiplication over the next few days do the organisms remain bound to the original cells and form discrete foci or do they distribute themselves over the surfaces of neighbouring cells?

When cell suspensions from infected mice were allowed to settle by gravity in tissue culture medium, adherent bacteria settled with the cells whereas free bacteria did not settle in the period of the experiment (Falling test). We found that similar proportions of cells and bacteria had settled (Table 5.3), suggesting that all of the bacteria were cell-associated. Fluctuation tests then provided further information about the nature of this association. A large number of plates made at limiting dilutions contained similar numbers of colonies (Table 5.4); if

bacteria were associated with particular cells or in a discrete focus, we could expect a varying number of colonies to be recovered using this procedure. It therefore seems that the bacteria are evenly distributed throughout the population of cells within the Peyer's patch.

Finally, another question may be asked, is there any significance in this association? Superinfection experiments have some bearing on this. Mice were fed a streptomycin-sensitive strain of 885 organisms. Several days later the infected mice were fed again with a streptomycin-resistant mutant of 885 organisms (885^{SR}). Each variant could be enumerated separately. Our studies using EDTA suggested that the organisms are extracellular and able to move from one loose cell association to another (Table 5.2, 5.4); if the organisms were reversibly bound to the Peyer's patch cells, the two (antigenically identical) inocula would mix and would then multiply or decline together, maintaining the same ratio. That was not the case, the two populations displayed separate growth patterns, each passing through growth and decline phases independently of the other population. We suggest that the initial challenge moves, accompanying the lymphocyte traffic, and is therefore removed from the area of penetration by the time the second challenge inoculum arrives. If this is so it is likely that the lymphocytes traffic towards the incoming macrophages and the delay of several days in establishing local antibacterial activity is accounted for by the slow traffic of these two cell populations.

Similarly, James-Holmquest et al. (1974) have suggested that sperm with attached gonococci provide vehicular transport for their "hitch-hikers" through the cervical mucus. Other organisms may spread to different organs via the blood by attachment to

circulating cells. For example, the malarial parasite Plasmodium infects red blood cells and thus circulates throughout its host.

To survive, the host must get rid of pathogens, whereas for their part microorganisms have to evade phagocytosis and killing by the host defence system. Microorganisms may evade phagocytosis in a number of different ways. They may produce toxins which by killing the cell make phagocytosis impossible. They may fail to adhere to the phagocyte or if adherent, they may resist ingestion; in the latter situation survival is by no means certain however, since some of the phagocyte's bactericidal mechanisms operate extracellularly (Weiss et al., 1978; Blumenstock and Jann, 1981). We previously found that 885 organisms are extracellular and cell associated. Therefore one wonders whether the adherence of bacteria to the lymphocyte facilitates macrophage ingestion of bacteria or whether the binding allows the organism to avoid contact with macrophages. If so, the possible extracellular killing by macrophages needs to be examined. There is no evidence that lymphocytes are able to kill bacteria even though tumour cytotoxicity by lymphocytes has been reported (Hellström et al., 1971; Brunner and Cerottini, 1971).

The results in Chapter 5 showed that Salmonella 885 in the Peyer's patches were extracellular and associated with cells. It is interesting to speculate how these organisms adhere to the Peyer's patch cells (mainly lymphocytes). That some bacteria are able to associate with lymphocytes is already known. Mayer et al. (1978) showed that Bacillus melitensis binds to a determinant on B cells which is unlikely to be surface immunoglobulin. However, it can be clearly demonstrated that the Cowan I strain of Staphylococcus aureus (which produces protein A) binds to the Fc region of IgG, and this has proved useful in the identification of immunoglobulin

bearing cells (Ghetie, Nilsson and Sjöquist, 1974). It has been suggested that the binding of a mutant of E. coli to lymphocytes involves a lectin on the lymphocyte membrane and a carbohydrate on the bacterial cell wall (Mayer and Teodorescu, 1978). Our studies do not elucidate the mechanism by which Salmonella attach to cells. There is no evidence that Salmonella can produce protein A antigen, and We have not investigated the possibility of lectin-type binding which might be inhibitable by simple sugars.

7.6 REQUIREMENTS FOR INTRACELLULAR KILLING BY MACROPHAGES

Studies in our department have investigated the requirement for specific antibody in the killing of intracellular Salmonellae by murine macrophages. In 1963 Jenkin "persuaded" macrophages to engulf S. typhimurium by the ingenious approach of forming a phage-bacterium complex (using a phage to which the bacterium had been made resistant) and then adding antibody against the phage. Phagocytosis occurred and the bacteria were internalized but not killed; evidently antibodies specific for the bacteria themselves were required if intracellular killing was to occur.

Jones (1980) obtained similar results in studies with Toxoplasma gondii, as did Draper and D'Arcy Hart (1975) with Mycobacterium. Nevertheless, for the intracellular killing of Mycobacterium tuberculosis it was shown that, in addition to specific antibody, the macrophages had to be activated in order to destroy the bacteria. These studies indicate that there are different requirements for the effective phagocytosis and killing of different bacterial species, in terms of the need for specific antibody and the state of activation of the macrophages (Wright and

Malawista, 1972; Henson, 1971; Elsbach, 1977).

Recent studies in this laboratory by Cooper et al. (1983) have investigated the role of specific antibody in the killing of 2 different intracellular bacteria, Listeria monocytogenes and Salmonella typhimurium. They found that the killing of Salmonella typhimurium by normal or activated macrophages definitely required specific antibody, whereas no antibody was needed with Listeria monocytogenes. This latter bacterium was found to adhere to activated macrophages spontaneously - the binding did not occur via Fc receptors - and was killed in 10% foetal calf serum in RPMI 1640 medium in the absence of antibody.

The presence of serum seems to be sufficient to promote the intracellular killing of Listeria monocytogenes by activated macrophages. This suggestion is based on the work of Leijh et al. (1980) who found that extracellular stimulation by serum triggers the intracellular killing of Staphylococcus aureus by monocytes. No intracellular killing occurred in the absence of serum and the bactericidal activity was better with fresh serum than with heat-inactivated serum, suggesting the involvement of complement. Indeed a need for C3b on the target cells was suggested by the observation that, when the number of monocyte C3b receptors was decreased (by treatment with pronase or anti-monocyte serum) there was a decrease in intracellular killing.

7.7 BACTERICIDAL ACTIVITY OF THE PEYER'S PATCH MACROPHAGES

Our earlier results suggest that macrophages are effector cells in elimination of organisms (Fig. 6.1). If Peyer's patch macrophages are believed to be of paramount importance in defence

against infectious agents it would be desirable to directly demonstrate their bactericidal activity in vitro.

For in vitro bactericidal assays macrophages could be isolated in large numbers and in good condition by collagenase digestion. Haskill (1981) demonstrated that collagenase is a relatively mild proteolytic enzyme which effectively dissociates tumours and connective tissues. Collagenase does not destroy a variety of membrane markers (surface immunoglobulin; theta antigen; Fc receptors) on blood cells, ascites, inflammatory cells and tumour cells. We obtained a good yield with more than 90% viability by this method. We found that macrophages which were adherent and spread on glass or plastic were optimal for bactericidal studies.

The results in Chapter 6 are clear. Macrophages isolated from the Peyer's patches of mice fed with Salmonella 885 were able to kill pre-opsonized 885^{SR} organisms in vitro, if incubated in the presence of fresh rabbit serum. The number of macrophages recovered were similar on day 5 to 8 (Fig. 6.1), but the bactericidal activity of macrophages isolated on day 8 was the greatest (Fig. 6.3).

From this evidence we can draw the conclusion that the development of resistance in the Peyer's patches after primary infection is partly due to accumulation of macrophages which migrated into the infected areas and partly due to the state of activation of these cells (Fig. 6.1 and 6.3). The increase of bactericidal activity of the Peyer's patch macrophages is in agreement with the bactericidal activity of peritoneal macrophages when activated with Salmonella 885 intraperitoneally. The results also agree with other studies of the bactericidal capacities of

splenic, liver and peritoneal macrophages taken from mice infected with Salmonella (Davies, 1975; Zinkernagel, 1976). Also, MacDonald and Carter (1980) provided evidence that Listeria-fed mice are highly resistant to an oral challenge 7 days after primary immunization.

Macrophage activation is a local event and is most marked at the site of initiation of an immune reaction, as in granulomas (Lurie, 1942; Dannenberg, 1968; Dannenberg et al., 1968). Histologically, macrophages near the centre of granulomas appear to be the richest in lysosomal enzymes, and here bacteria show the most marked signs of destruction. Thus, any attempt to collect activated macrophages from sites other than those close to the cellular immune reaction will yield macrophages that are only marginally activated (Lurie, 1942; Dannenberg, 1968; and Dannenberg et al., 1968). By extrapolation one might expect that, following infection by the oral route, the macrophages from the Peyer's patches would display the greatest bactericidal activity compared to other areas.

It was recently reported by MacDonald, Bashore and Carter (1982) that there is nonspecific resistance in the Peyer's patches of mice fed with Listeria monocytogenes. Surprisingly, they could not demonstrate phagocytosis of bacteria by isolated Peyer's patch macrophages, although they found that macrophages from the liver and peritoneum did display bactericidal activity. One needs to emphasize that in our studies the Peyer's patch macrophages were isolated by very gentle methods; either by collagenase or by the scratching method, and in both cases the cells retained their ability to phagocytose and to spread on glass slides or plastic. Recently, Richman, Graeff and Strober (1981) have shown that Peyer's patch macrophage-enriched cells are capable of presenting antigen if

the patches are treated with collagenase prior to culture. In contrast, Challacombe et al. (1979) could not demonstrate antigen presention by Ia-positive adherent cells from the Peyer's patches. These studies suggest that the method of cell preparation might affect the outcome of the experiments.

7.8 MACROPHAGE HETEROGENEITY

There is mounting evidence that certain macrophage subpopulations perform certain functions; for example Ia-positive macrophages are necessary for antigen presention (Beller, Kiely and Unanue, 1980) and only certain subpopulations can perform antibody-dependent macrophage-mediated cytotoxicity (ADCC) (Sun and Lohmann-Matthes, 1982). Further, it has been suggested that the killing of tumour cells and Trichinella spiralis is mediated by different subpopulations of macrophages (Wing, Krahenbuhl and Remington, 1979). So we may ask whether there is a particular macrophage subpopulation which enters the Peyer's patches after Salmonella 885 infection and is responsible for elimination of the bacteria. The development of monoclonal antibodies to macrophage subpopulations should be helpful in clarifying this interesting question.

7.9 BACTERIAL COMPONENTS WHICH INFLUENCE SUSCEPTIBILITY TO PHAGOCYTOSIS.

We have studied the rate of elimination of three different gram-negative bacilli - Vibrio cholerae 569B, E. coli 492 and Salmonella 885 - which have capacities to adhere to epithelial

cells. If equal numbers of bacteria were orally administered to separate groups of mice then we might expect to find similar numbers of each strain in the Peyer's patches, because it seems that the M cells actively take up whatever organisms or antigens adhere to them and transport them into the Peyer's patches (Owen and Jones, 1974). The results shown in Fig. 6.4 did not support this expectation. Large numbers of Salmonella 885 were recovered from the Peyer's patches throughout the duration of the experiment (3 days) whereas E. coli 492 were recovered in smaller numbers for the first 2 days only. Vibrio cholerae 569B were not found at any time. Is it possible that Vibrio cholerae 569B do not enter the Peyer's patches? This seems unlikely because recently Ahmed, La Brooy and Rowley (1983) demonstrated the presence of IgA isotype-restricted T-helper cells and IgM isotype-restricted T-suppressor cells in the Peyer's patches of mice orally infected with Vibrio cholerae 569B. In their studies they transferred the Peyer's patch cells from mice orally primed with Vibrio cholerae 569B to syngeneic recipients and observed in the latter increased numbers of cells in the lamina propria which were synthesizing IgA antibodies directed against Vibrio antigens, but a decreased number producing anti-Vibrio antibodies of the IgM class. This study indicated that Vibrio cholerae antigens had entered the Peyer's patches of the small intestine and made contact with immunocompetent cells including macrophages. This in turn suggests that, in our experiments, the different rates of bacterial elimination reflect different susceptibilities of the bacteria to destruction by the Peyer's patch macrophages. Supportive evidence is provided by Collins and Carter (1978) who infected germ-free mice with three different Salmonella strains. They found that the rates of elimination of the strains

were related to their susceptibility to the bactericidal action of normal macrophages. In addition, the virulent strain of Salmonella was only killed by activated macrophages.

The susceptibility of microorganisms to the bactericidal activity of macrophages obviously depends on the genetic composition of the bacteria - those which are able to produce capsules are more resistant to phagocytosis and the bactericidal activity of phagocytic cells (Wood, 1960; Horwitz and Silverstein, 1980). Similarly, gram-negative bacteria with longer LPS chains are more resistant than those carrying shorter chains to the killing of macrophages (Stendahl and Edebo, 1972; Valtonen, 1977). Rest, Cooney and Spitznagel (1977) used well defined mutants of Salmonella typhimurium to demonstrate an increasing susceptibility to the bactericidal action of lysosomal extract with the progressive shortening of the lipopolysaccharide chains on the cell wall. These results have been supported by further studies by Modrzakowski and Spitznagel (1979) and Weiss et al. (1980) These workers studied the susceptibilities of bacteria to the bactericidal action of an extract of polymorphonuclear leukocyte granules which effects the splitting of bacterial phospholipid and thereby increases the permeability of the outer membrane. Since smooth strains with complete lipopolysaccharide side chains were resistant to the action of the granular extract it was postulated that the lipopolysaccharide sterically hindered binding of these proteins. Bacterial cell walls, therefore, seem to play a crucial role in bacterial resistance to phagocytes. Despite the fact that most of this work was performed using neutrophils and monocytes, macrophages possess similar oxygen-dependent and oxygen-independent bactericidal systems.

Confirmation of the importance of bacterial susceptibility was obtained in our studies involving the survival of several bacterial strains when incubated with peritoneal macrophages *in vitro* (Table 6.1). The rates of killing *in vitro* correlated with the rates of elimination *in vivo*, viz.: *Vibrio cholerae* 569B > *E. coli* 492 > *Salmonella* 885 (Table 6.1, Figure 6.4).

Thus it seems probable that bacteria and inert antigens are randomly sampled by the Peyer's patch dome cells in proportion to the extent of adhesion, and that the level of immunization induced will depend on the ability of the organisms or antigens to survive the digestive powers of the macrophages within the Peyer's patches.

7.10 CONCLUSIONS

In order to completely understand the importance of Peyer's patches in modulating or resisting enteric infections many things remain to be done. The present work could be extended in the future into the following areas.

1. Is there a certain subpopulation of macrophages which acts as the effector cell population in Peyer's patches?
2. The possibility of extracellular killing by macrophages of Salmonella 885 organisms which are adherent to lymphocytes.
3. The nature of the T cells which activate the Peyer's patch macrophages.
4. The nature of the adhesins which enable Salmonellae to adhere to intestinal cells.

5. Why does antibody promote ingestion by phagocytic cells yet prevent attachment and pinocytosis by M cells. Is this simply a reflection of the presence or absence of Fc receptors?

ADDENDUM

EFFECT OF SALMONELLA C5 OUTER MEMBRANE PROTEIN (OMP) ANTIGEN ON SALMONELLA FED MICE

During investigation of the cellular distribution in the Peyer's patches of mice orally infected with Salmonella (Chapter 5) we found that the time of appearance of macrophages was consistent with the concept that these cells infiltrate following the liberation of lymphokines from sensitized T cells (following their interaction with specific antigen). The elimination of Salmonella 885 from the Peyer's patches (Fig. 6.1, Chapter 6) is possibly due to the increased bactericidal activity of these infiltrated macrophages as shown in vitro.

This local accumulation of macrophages resembled a delayed type hypersensitivity focus and occurred at a time when the animals began to exhibit DTH to the bacterial antigens. It seemed possible that the protective significance of the monocyte influx could be assessed by examining the effect of directing the monocytes away from the Peyer's patches and towards other (artificially created) foci of delayed type hypersensitivity. If this diversion could be achieved, any change in the pattern of growth of Salmonella in the Peyer's patches would be likely to reflect the importance of a monocyte influx to local resistance.

A.1 EFFECT OF INJECTING BACTERIAL ANTIGENS IN THE COURSE OF THE INFECTION.

A.1.1. Enumeration of Salmonella 885 in the Peyer's patches after injection of C5 OMP antigen.

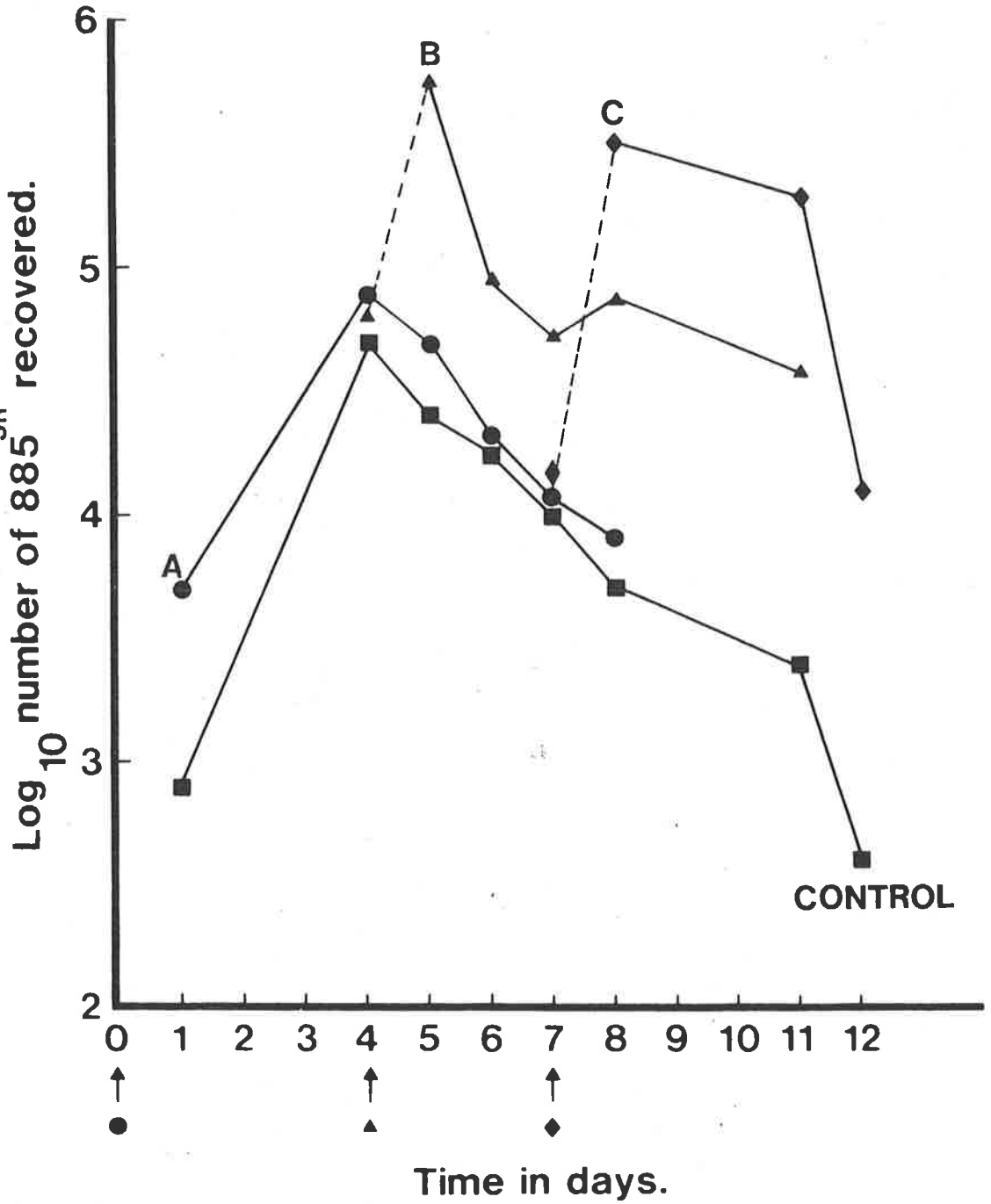
Previous studies had shown that the OMP of many different Salmonellae could produce DTH in mice infected with a particular serotype of Salmonella (Moser et al., 1980). We used a preparation from S. typhimurium C5, since this was available in quantity, but a similar preparation from 885 worked equally well. The plan was to inject crude outer membrane protein of Salmonellae (known to stimulate DTH in infected mice (Moser et al., 1980) into several sites subcutaneously and to count the numbers of organisms in the Peyer's patches of these mice by comparison with control mice, all mice having been orally infected with 885 some days earlier.

Doses of 80 μg of crude outer membrane protein of S. typhimurium C5 (in 40 μl PBS) were injected subcutaneously (into 4 sites - one hind foodpad, abdomen and both shoulders) into mice which had been orally infected with Salmonella 885 at different times. Within an hour the mice appeared sluggish and ill, with ruffled fur. When the numbers of bacteria in the Peyer's patches were counted 24 hours later their numbers had greatly increased by comparison with the uninjected control mice. The longer the period between oral infection and challenge with OMP, the greater the increase in the number of Salmonella in the Peyer's patches (Fig. A1). If the C5 OMP antigen was administered intraperitoneally or intravenously there were similar dramatic increases in the numbers of Salmonella in the Peyer's patches, but this effect was

Figure A1

Recovery of Salmonella 885^{SR} from the Peyer's patches of mice orally infected with Salmonella 885^{SR} (5×10^8) on day 0. Infected mice were challenged subcutaneously with 80 µg C5 OMP antigen on days 0, 4 or 7, (A,B,C respectively). Control mice were orally infected only.

Each point represents the average value from five mice.


6
5
4
3
2
B
C
A
CONTROL
Log10 number of 885SR recovered.
0 1 2 3 4 5 6 7 8 9 10 11 12
Time in days.

not observed using the oral route (Fig. A.2).

Initially we believed this to be due - as in the original hypothesis - to a deviation of macrophages from the infected Peyer's patches, but examining the numbers of macrophages in the Peyer's patches showed that these were similar to those in the matching control mice fed with Salmonella as shown in the experiments described below.

A.1.2. Effect of injecting C5 OMP antigen on numbers of macrophages in Peyer's patches.

A single cell suspension was prepared by "scratching" the Peyer's patches of mice fed with 885 organisms a few days earlier (Chapter 3). Cells (2×10^5) and latex particles (4×10^7) were incubated together for 2 h at 37° in a slide culture chamber (in a volume of 100 µl) to allow the macrophages to adhere to and phagocytose the particles. After incubation, the chambers were washed and slides were stained with Wright's stain. We found similar numbers of macrophages in the Peyer's patches of the infected mice challenged with C5 OMP antigen and the control (infected, unchallenged) mice. Thus, injecting C5 OMP antigen at different sites did not prevent the influx of macrophages into the Peyer's patches.

When activated peritoneal macrophages from mice given Salmonella 885 (2×10^6, i.p.) 7 days previously were incubated *in vitro* with C5 OMP antigens (1 µg and 2.5 µg) for 30 min. there were no signs of cell damage and the macrophages could perform bactericidal activity as well as control macrophages (Fig. A3).

Figure A2

Recovery of Salmonella 885^{SR} from the Peyer's patches of mice fed Salmonella 885^{SR} 3 days previously. Bacteria were enumerated 24 h after challenge with C5 OMP antigens by a variety of routes and doses, as indicated.

Each bar shows the average value from five mice.

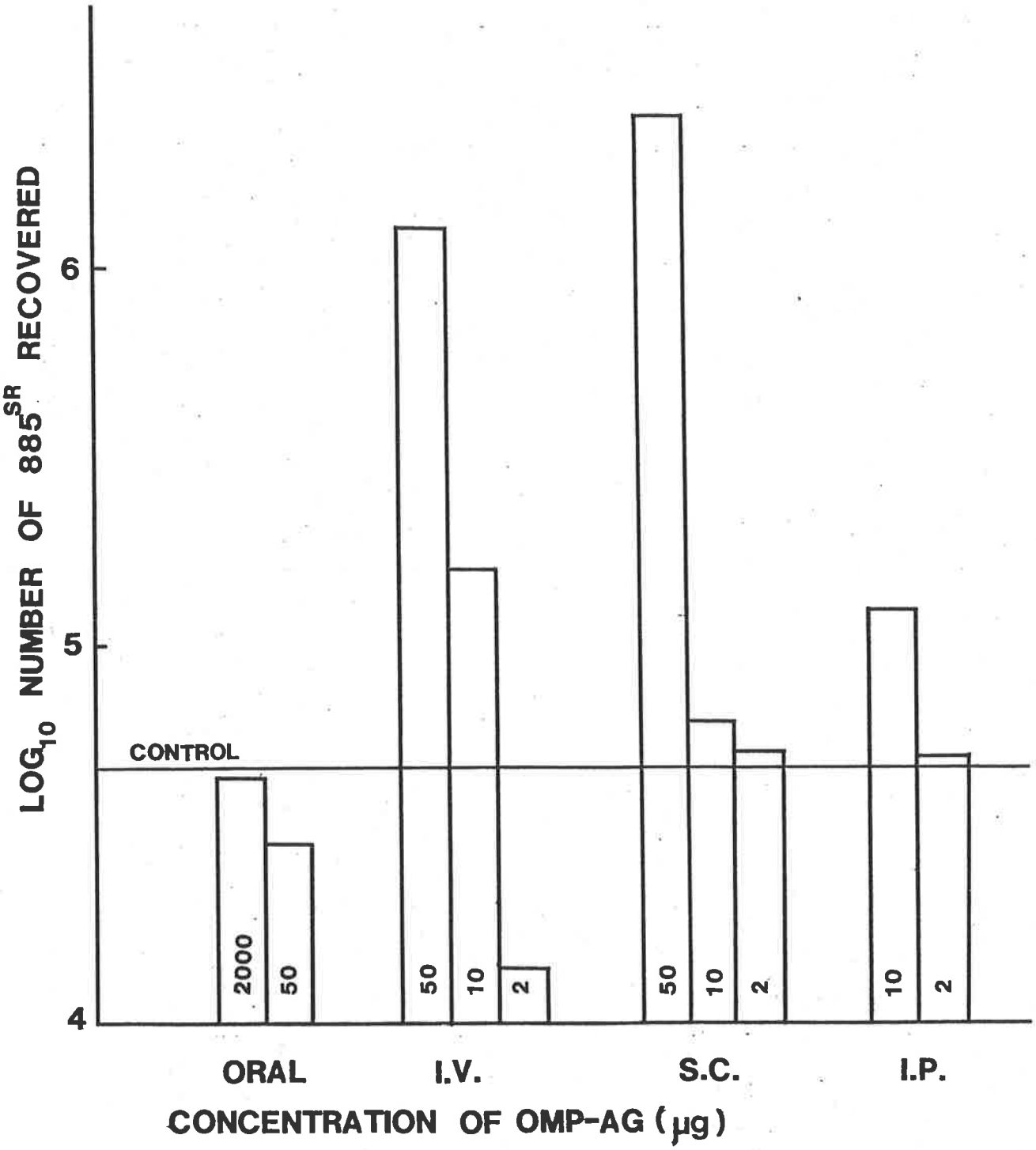

LOG10 NUMBER OF 885SR RECOVERED
6
5
4
CONTROL
2000
50
50
10
2
50
10
2
10
2
ORAL
I.V.
S.C.
I.P.
CONCENTRATION OF OMP-AG (μg)

Figure A.3

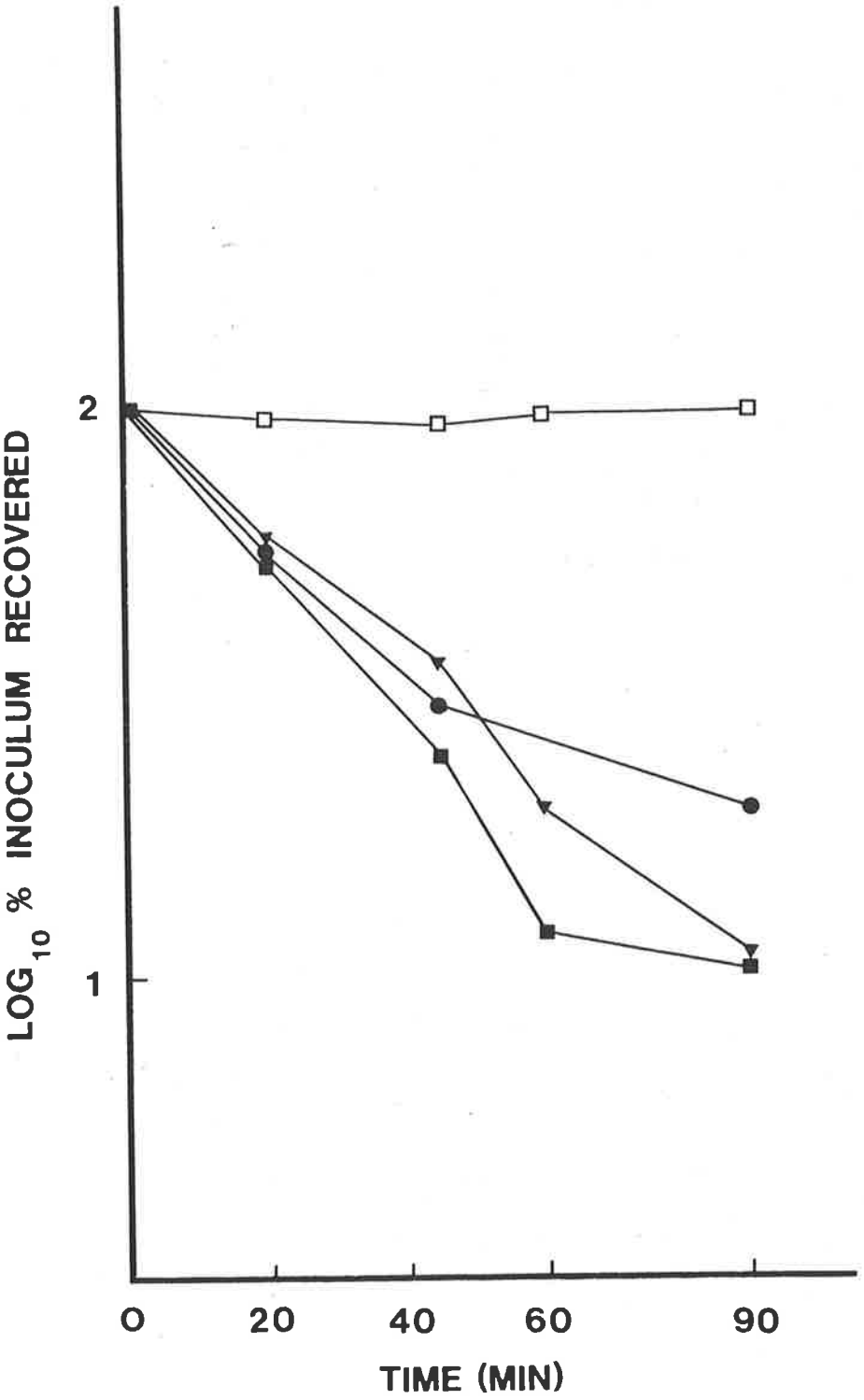
The bactericidal action of activated macrophages after incubation with C5 OMP antigen.

Bactericidal activity of activated peritoneal macrophages taken from mice given Salmonella 885 (2×10^6, i.p.) 7 days previously after incubation with 1 μg or 2.5 μg C5 OMP antigen for 30 min. The supernatant was decanted and opsonized Salmonella 885^{SR} were added. Duplicate viable bacterial counts were performed at different intervals.

Symbols: ● macrophages with no C5 OMP antigen;

▼ macrophages with 1 μg C5 OMP antigen;

■ macrophages with 2.5 μg C5 OMP antigen and □ bacteria alone.


LOG10 % INOCULUM RECOVERED
2
1
0
20
40
60
90
TIME (MIN)

A.2 TOXICITY OF SALMONELLA OMP FOR MICE

Following the injection of 50-80 μg of the C5 OMP antigen into Salmonella 885 infected mice, we found that at least 50% of the mice were dead by day 3. The question was whether the cause of death was due to direct toxicity of the antigen or hypersensitivity of the infected mice to the antigen. The results in Table A.1 revealed that mice fed with 885 were more sensitive to the antigen than normal mice. Most infected mice died 24 hours after C5 OMP antigen (50-200 μg) was injected. Injection of the C5 OMP antigen into normal mice was only lethal at doses of around 2000 μg. Mice fed 885 5 days previously were at least 40 times more sensitive to injected C5 OMP antigen.

A.3 THE REINFECTION OF THE PEYER'S PATCHES WITH SALMONELLA

It is not clear why the numbers of Salmonellae in the Peyer's patches dramatically increased after C5 OMP Ag challenge (Fig. A.1). Since macrophage numbers and activity appeared normal it was necessary to investigate whether the multiplication really occurred primarily in the Peyer's patches or whether this was a reflection of events elsewhere.

A.3.1 Effect of injecting C5 OMP antigen on the numbers of Salmonellae in the Peyer's patches, gut lumen and intestinal wall.

Ten mice were fed with Salmonella 885^{SR} and then divided into two groups. On day 6, one group was challenged with 80 μg of

Table A.1

Number of mice surviving after challenge with crude Salmonella C5 outer membrane antigen

	Mice fed 885 5 days earlier				Normal mice			
Days after challenge	C5 Ag (μg) 2000	200	50	10	C5 Ag (μg) 2000	200	50	10
Day 0	3*	3	3	3	3	3	3	3
Day 1	0	2	1	3	3	3	3	3
Day 2		0	0	3	0	3	3	3
Day 3				3		3	3	3
Day 4				3		3	3	3
Day 11				3		3	3	3

* There were 3 mice in each group.

C5 OMP antigen subcutaneously; 24 hours later the 5 mice from this group and the 5 control mice were sacrificed, and their gut contents collected and pooled. Peyer's patches were excised from the intestinal wall and pooled and the rest of the intestines also pooled separately. The pooled materials from each group were homogenized and duplicate viable counts made on streptomycin agar plates.

The results in Fig. A.4 showed that recovery of Salmonella 885 from the gut lumen, intestinal wall and the Peyer's patches increased 23,000, 1,500 and 33 times respectively by comparison with the controls. In the controls there were approximately 700 Salmonella 885^{SR} in the gut lumen, compared to 2×10^7 after injection with C5 OMP antigens. It therefore appears that the increased number of Salmonellae in the Peyer's patches (33 times) merely reflects the much greater number present in the gut lumen.

A.3.2 Reinfection of Peyer's patches by Salmonella from the gut lumen.

The possibility of overgrowth was tested by inhibiting the growth of 885^{SR} in the gut lumen by the non-absorbable antibiotic neomycin sulfate, to which Salmonella 885^{SR} is susceptible. Two groups of mice were compared: 20 mice fed with Salmonella six days earlier were used as controls; 20 similarly fed mice were given neomycin sulfate (10 mg in PBS orally at the time of antigen administration and again 4 hours later). Both groups were challenged with 25 µg of C5 OMP antigen subcutaneously. Viable counts were made from the Peyer's patches, intestinal wall and gut contents 2.50 hours, 5 hours (Fig. A.5) and 24 hours (Fig. A.6)

Figure A4

Recovery of Salmonella 885^{SR} from homogenised Peyer's patches, gut contents and small intestines of mice 24 hours after mice were challenged with C5 OMP antigen (s.c.). All mice had been fed with 885^{SR} (5×10^8) six days earlier and one group injected s.c. with 80 µg C5 OMP antigen. There were five mice in each group.

■- controls, no C5 OMP antigen injected

□- C5 OMP antigen injected s.c.

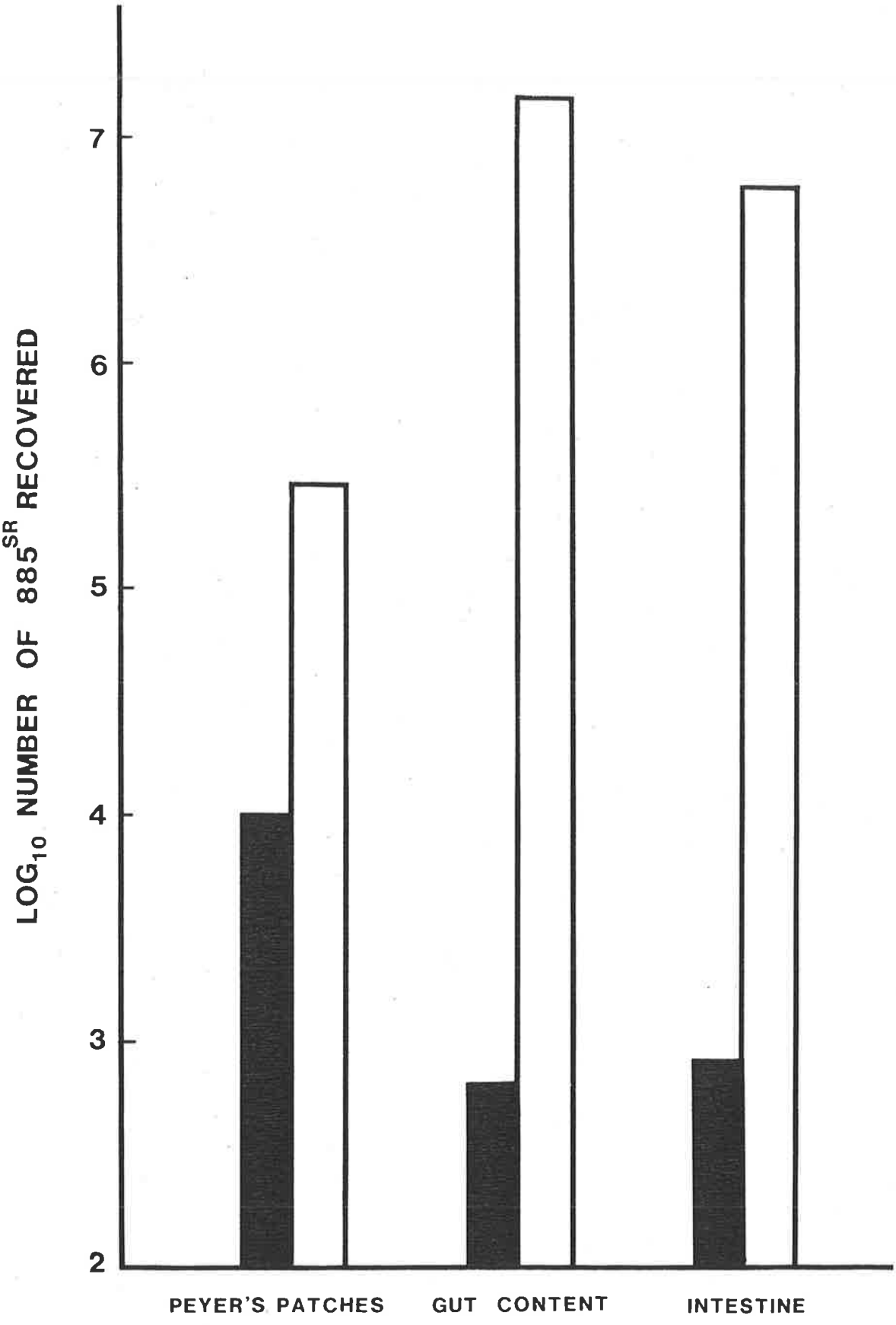

LOG10 NUMBER OF 885SR RECOVERED
7
6
5
4
3
2
PEYER'S PATCHES
GUT CONTENT
INTESTINE

Figure A5.

Recovery of Salmonella 885^{SR} from the Peyer's patches, gut contents and small intestines at 2.50 hours (solid bar) and 5 hours (open bar) after mice were challenged with 25 µg of C5 OMP antigen subcutaneously. All mice had been given Salmonella 885^{SR} (5×10^8) six days earlier and group T received 10 mg neomycin sulfate orally at the time of antigen administration and again 4 hours later.

Each bar represents the average number of organisms recovered from five mice.

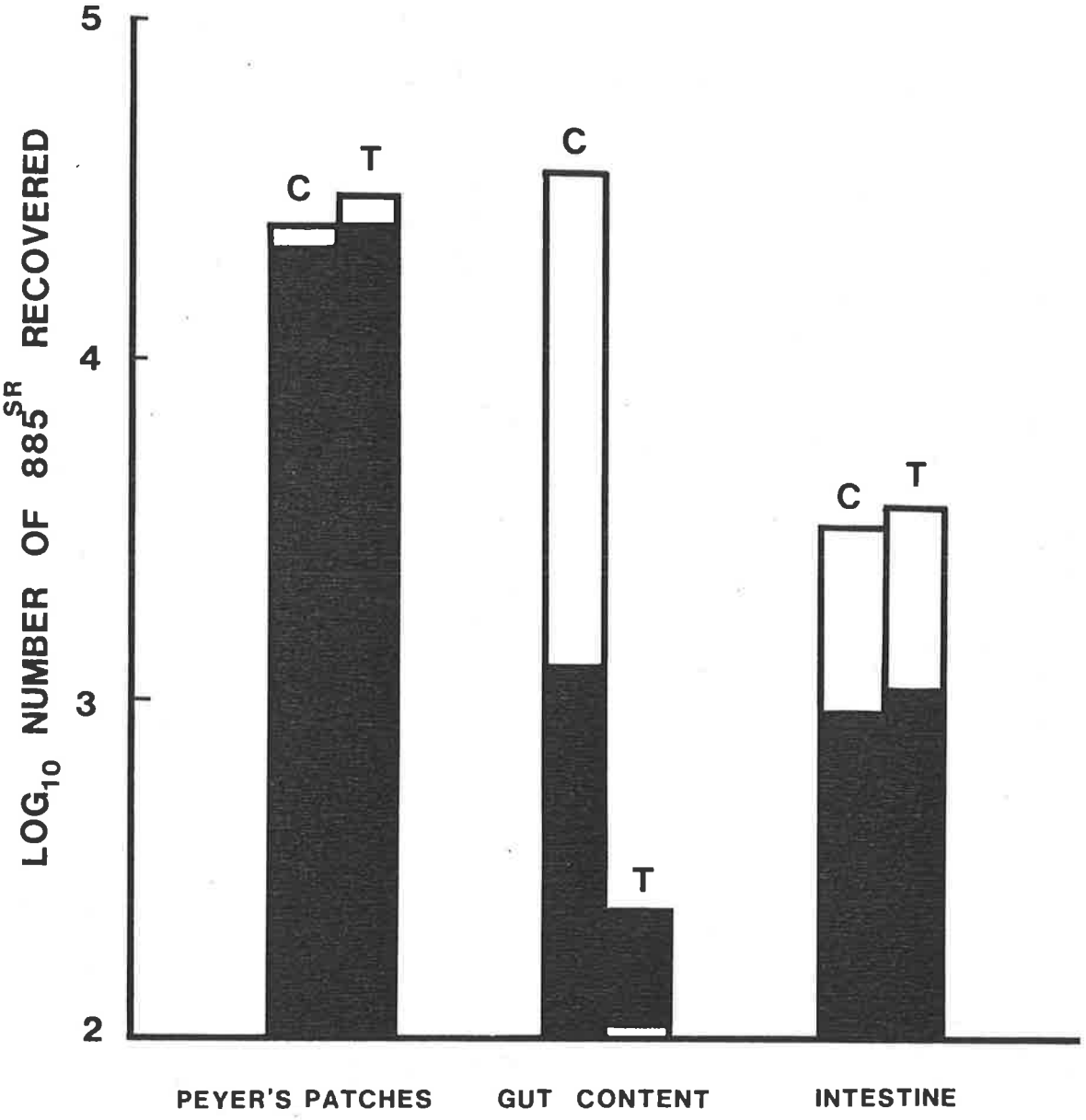
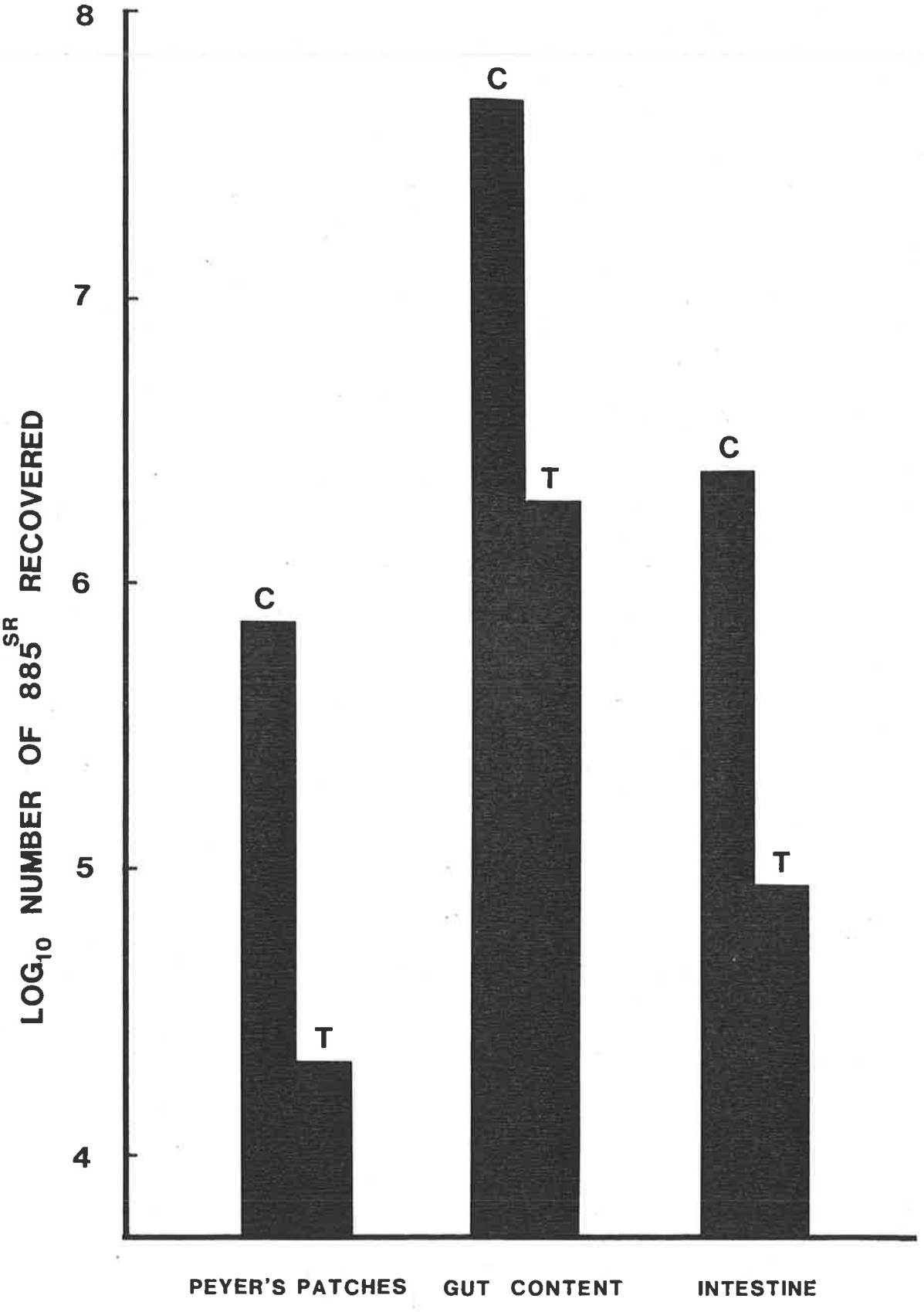

5
4
3
2
LOG10 NUMBER OF 885SR RECOVERED
C
T
C
T
C
T
PEYER'S PATCHES
GUT CONTENT
INTESTINE

Figure A6

Recovery of Salmonella 885^{SR} from the Peyer's patches, gut contents and small intestines 24 hours after mice were challenged with 25 µg of C5 OMP antigen subcutaneously. All mice had been given Salmonella 885^{SR} (5×10^8) six days earlier and group T received 10 mg neomycin sulfate orally at the time of antigen administration and again 4 hours later.

Each bar represents the average number of organisms recovered from five mice.


8
7
6
5
4
LOG10 NUMBER OF 885SR RECOVERED
C
T
C
T
C
T
PEYER'S PATCHES
GUT CONTENT
INTESTINE

later.

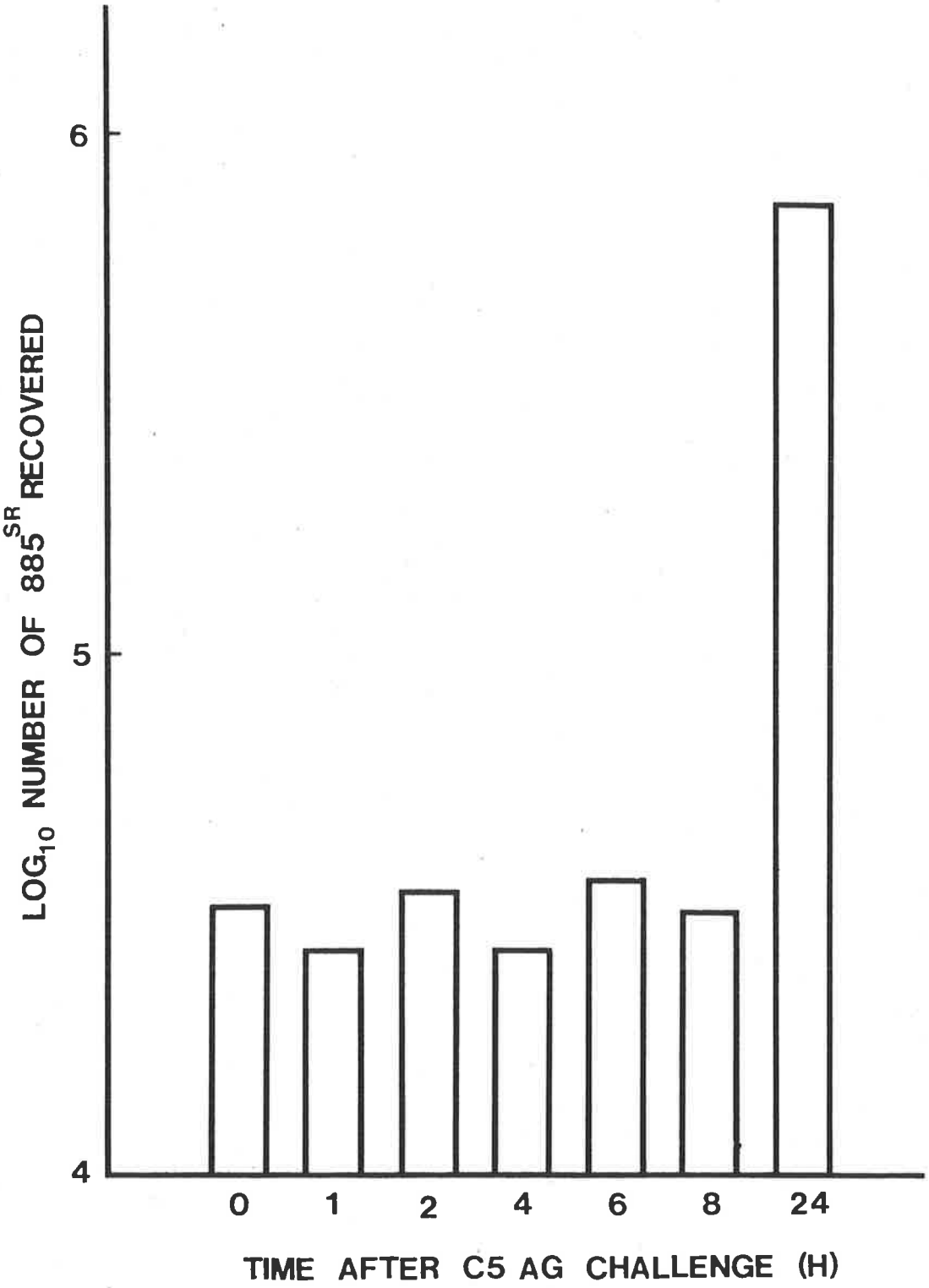
Neomycin sulfate did not kill Salmonella in the Peyer's patches or intestinal wall because this antibiotic is not absorbed from the intestine. It eliminated the free organisms in the intestinal lumen. Five hours after administering neomycin sulfate the number of Salmonella 885^{SR} in the Peyer's patches and intestinal wall had not changed and was similar in both groups. However neomycin had eliminated most of the organisms in the intestinal lumen. After 24 hours, the numbers of Salmonella 885^{SR} in the Peyer's patches, gut contents and small intestines of infected mice injected with C5 OMP antigen were increased as expected. However during this 24 hour period the Salmonella 885^{SR} in the gut lumen of infected mice challenged with OMP antigen and given neomycin sulfate was increased about 10,000-fold and because of this explosive Salmonella multiplication in the gut lumen the organisms reinfected the intestinal wall. We conclude that the increase of organisms was not due primarily to multiplication of the Salmonella in the intestinal wall itself but was due to reinfection from the greater numbers of organisms in the gut lumen. This is supported by the the fact that mice given neomycin from the beginning of the experiment did not show any change in numbers of organisms in the Peyer's patches even 24 h later.

The time course of bacterial growth in the Peyer's patches of C5 OMP-injected mice was followed more closely. The results in Fig. A.7. indicate that Salmonella 885^{SR} in the gut did not reinfect the Peyer's patches earlier than 8 hours after C5 OMP antigen challenge.

At this stage, whilst it was clear that the increased numbers of bacteria in the Peyer's patches arose from the overwhelming

Figure A7

Recovery of Salmonella 885^{SR} from the Peyer's patches of mice fed Salmonella 885^{SR} (5×10^8) 7 days earlier. These mice were challenged with 50 µg of C5 OMP antigen subcutaneously and the organisms in their Peyer's patches determined at the times indicated. Each bar represents the average number of organisms recovered from 5 mice.


6
5
4
LOG10 NUMBER OF 885SR RECOVERED
0
1
2
4
6
8
24
TIME AFTER C5 AG CHALLENGE (H)

numbers in the intestinal lumen, it is still puzzling to know why the mere injection of bacterial antigens subcutaneously should permit the rapid intraintestinal growth of organisms. Could this be due simply to prevention of peristalsis? To examine this possibility mice fed 885^{SR} eight days earlier were given 10 µg of methadone orally and the Salmonella enumerated 24 hours later in the Peyer's patches. This dose of methadone is able to inhibit peristalsis for at least eight hours as shown by giving trypan blue by mouth. Nevertheless this inhibition had no effect on the numbers of specific organisms in the Peyer's patches (Fig. A.8).

A.4 EFFECT OF INJECTING OTHER AGENTS INTO SALMONELLA 885^{SR} FED MICE.

Besides the outer membrane antigens which caused the multiplication of Salmonella 885^{SR} in the intestine, other non-specific substances such as zymosan, fumaropimaric acid, or carbon particles were tried. All three substances are capable of activating the complement cascade and of releasing permeability factors. Of these, carbon particles (5 mg intraperitoneally) produced the most marked effect whilst fumaropimaric acid (1 mg) showed little or no effect (Fig. A.9).

A.5 SERUM FACTORS LIBERATED IN MICE FED SALMONELLA AND INJECTED WITH OUTER MEMBRANE ANTIGEN.

It has been known for a long time that injection of colloidal material, including LPS, into mice which carry a variety of chronic infections such as BCG (Shear, 1944); Corynebacterium parvum (Shah,

Figure A8

Recovery of Salmonella 885^{SR} from the Peyer's patches, (solid bar) and intestinal wall (open bar) of mice fed Salmonella 885^{SR} (5×10^8) 8 days previously (control). Infected mice were either challenged with 50 µg of C5 OMP antigen subcutaneously or fed with 10 µg of methadone and viable counts were made from 5 mice 24 hours later.

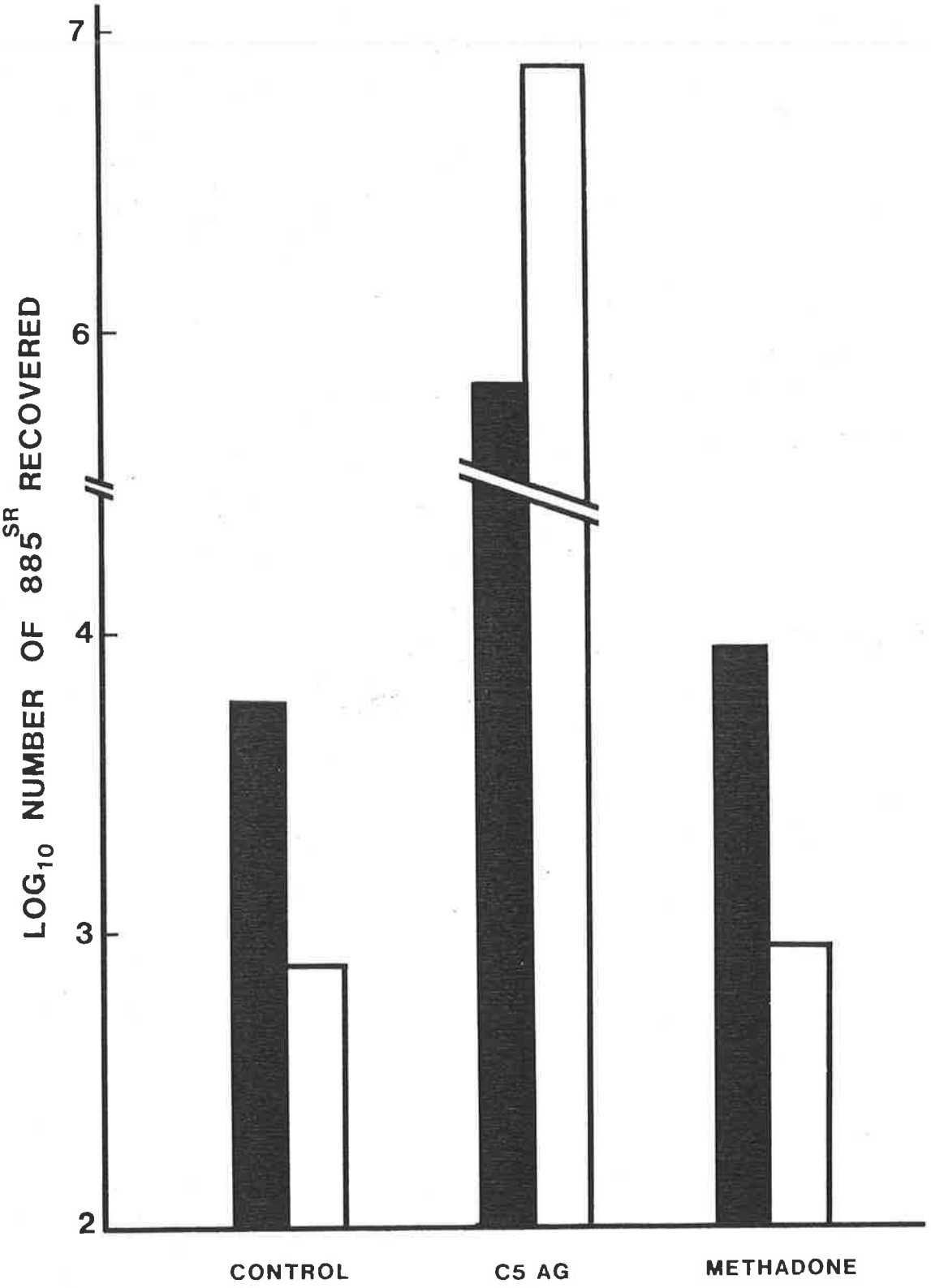
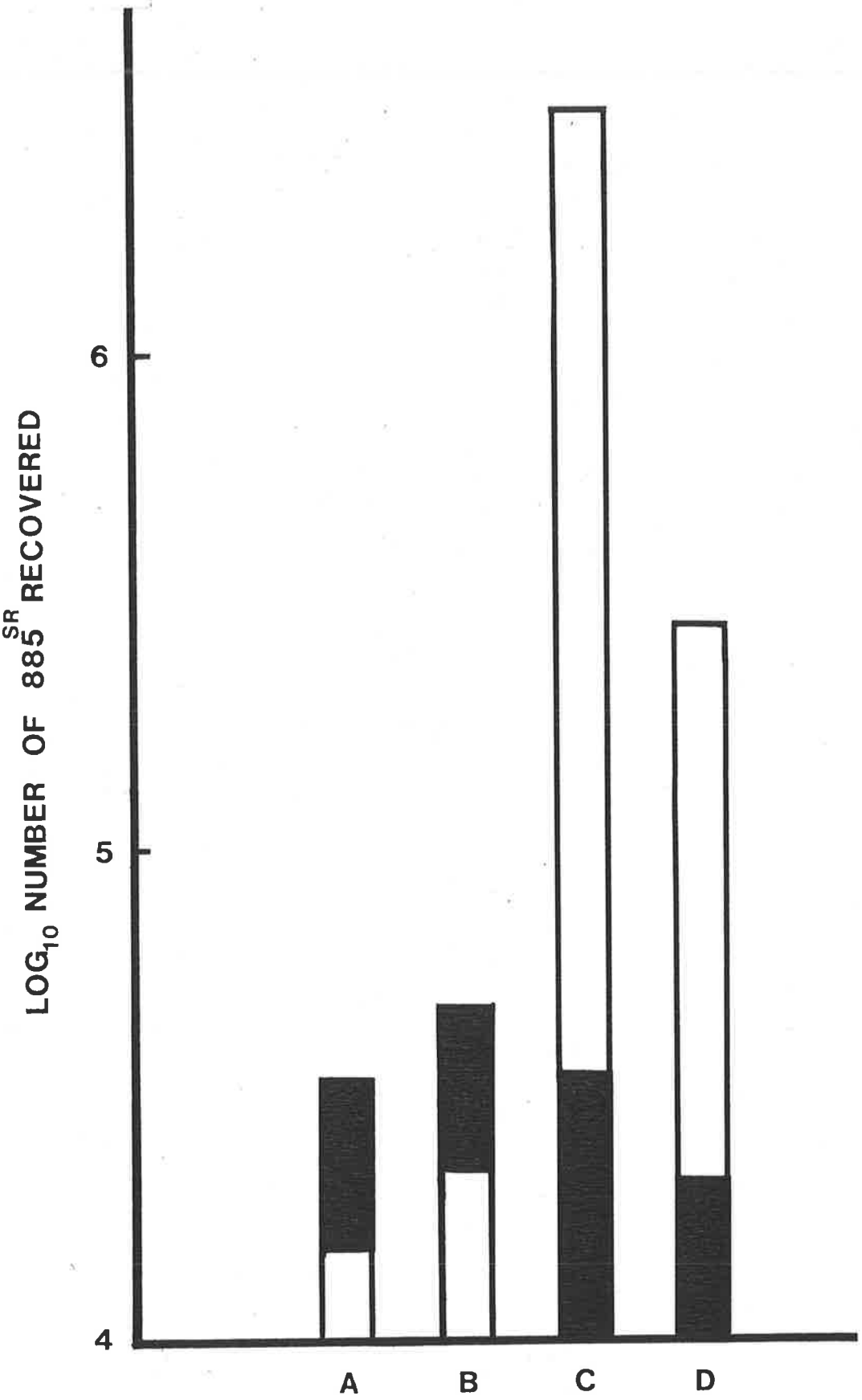

7
6
4
3
2
LOG10 NUMBER OF 885SR RECOVERED
CONTROL
C5 AG
METHADONE

Figure A9

Recovery of Salmonella 885^{SR} from the Peyer's patches of mice fed Salmonella 885 (5×10^8) 6 days previously. Bacteria were enumerated 4.30 hours (solid bar) and 24 hours (open bar) after challenge with fumaropimaric acid 1 mg (B), carbon particles 5 mg (C) or zymosan 500 µg (D) injected intraperitoneally. Controls were untreated and are shown in group A.

Each bar represents the average number of organisms recovered from five mice.


LOG10 NUMBER OF 885SR RECOVERED
6
5
4
A
B
C
D

et al., 1978); Schistosoma mansoni (Ferluga et al., 1979); Plasmodium vinckei (Clark and Clouston, 1980) may result in some dramatic effects. Such mice are much more sensitive to the toxic effects of LPS and rapidly exhibit fever, hypoglycemia and shock. There is also liberation into the serum of factors such as tumour necrotising factor (Carswell et al., 1975) which can kill many tumour cells both in vivo and in vitro. Within two hours of giving LPS to infected mice a variety of factors appear in the serum, such as lymphocyte activating factor, T cell replacing factor (Märnnel et al., 1980), lymphotoxin (Rosenau and Tsoukas, 1976), glucocorticoid antagonising factor (Shear, 1944) as well as factors involved in liver necrosis. There is evidence that these factors are released following the action of LPS or colloid on activated macrophages or reticuloendothelial cells (Hoffman et al., 1978). We realise now that injection of C5 OMP antigen (containing LPS) into mice orally infected with Salmonella 885 causes hypersensitivity and leads to an increase in intestinal permeability (see below). Based on these observations, we looked for an accompanying release of tumour necrotising factors into the serum to see whether there might be a relationship between this and the growth of bacteria in the gut.

A.6 TUMOUR NECROTISING FACTOR (TNF).

Tumour necrotising factor (TNF) can be found in the serum of animals sensitized to BCG (or certain other immunopotentiators) and challenged later with endotoxin. This factor causes the necrosis of some tumours when passively transferred to tumour-bearing animals. TNF has other unique characteristics such as lack of species specificity and the ability to discriminate between normal cells and

certain tumour cells *in vitro*. However, the mechanism by which these cells are killed is not clear yet and needs to be clarified.

Numerous transplanted tumours and transformed cells have been shown to have a high degree of sensitivity to TNF. These include: sarcomas S-180 (CD1-Swiss) and BP8 (C3H); leukemias EL4 (C57BL/6) and ASL1 (A strain), the mastocytoma P815 (DBA/2); and several transformed cell lines: L-929, Meth A cells and human melanoma cells (Helson *et al.*, 1975). In addition, Old (1976) reported that not all transformed cells are affected by TNF and he suggested that TNF presumably recognized a common feature of some transformed cells but not others (or normal cells).

Our studies agreed with the earlier findings of others. We found that mice fed with Salmonella 885 challenged with C5 OMP antigen after 1-2 hours contained TNF in serum, which exhibited cytotoxicity when cultured with leukemic EL4 for 24 hours. These experiments were kindly performed by Dr I. Kotlarski, with the results shown in Fig. A.10. The highest TNF activity was obtained when the serum was taken from infected mice 1-2 hours after challenge with C5 OMP antigen.

A.7 EFFECT OF PASSIVE TRANSFER OF TNF INTO MICE FED WITH SALMONELLA

It is possible that serum factors such as TNF which are liberated into serum may directly affect the numbers of Salmonella in the gut. The results shown in Fig. A.11 did not support this. Transferring TNF positive serum (0.3 ml) collected from infected mice after challenge with C5 OMP antigen did not alter the population of organisms in the intestinal contents.

Figure A10

The assay for TNF activity was performed as described in Section 3.18. The putative sources of TNF were serum pools prepared from mice fed 5 x 10^8 Salmonella 885 on day 8, and challenged subcutaneously with C5 OMP Ag 1 (■), 2 (△) or 4 (●) hours before bleeding. Three experiments gave similar results. (TNF assay kindly performed by Dr I. Kotlarski).

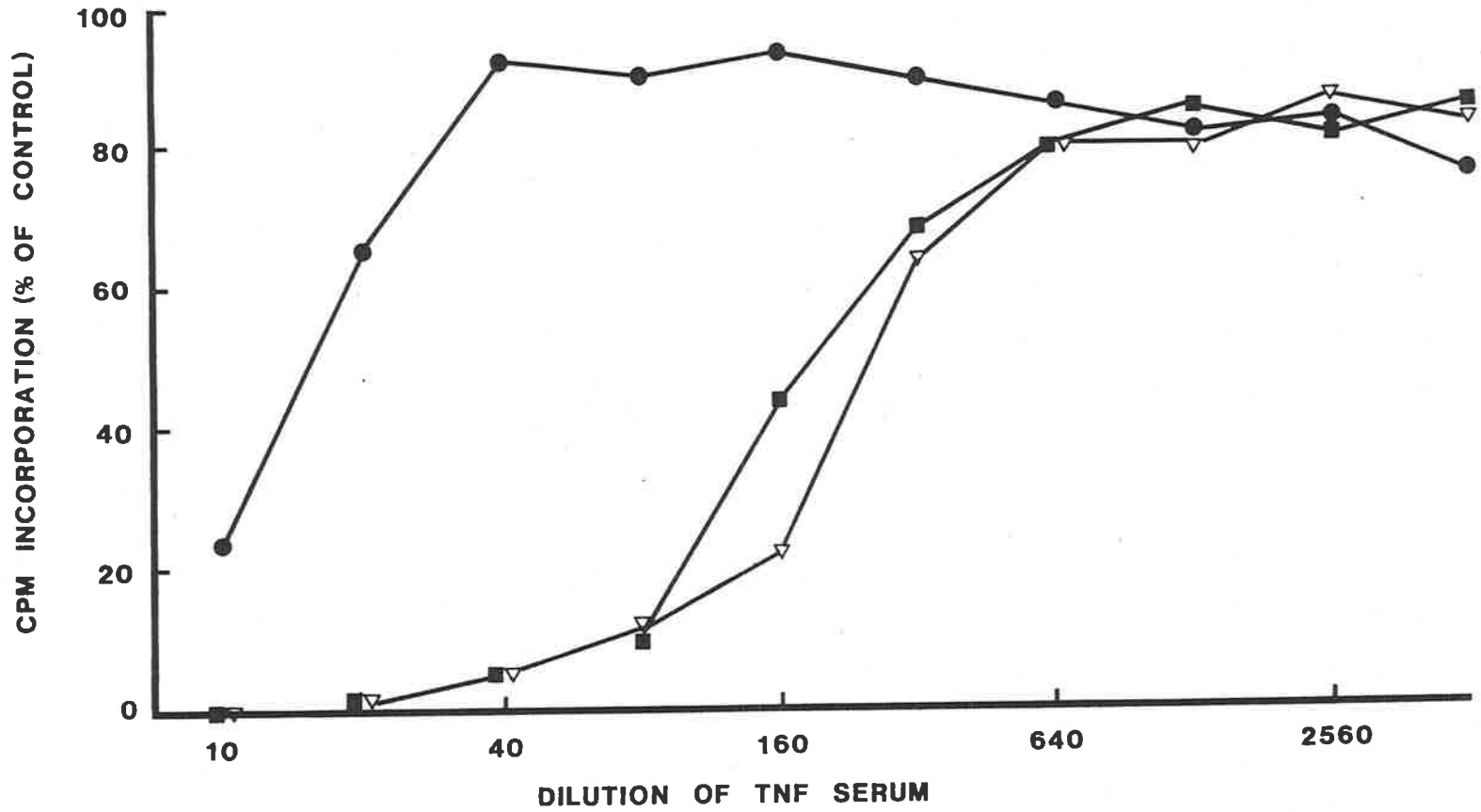

100
80
60
40
20
0
CPM INCORPORATION (% OF CONTROL)
10
40
160
640
2560
DILUTION OF TNF SERUM

Figure All

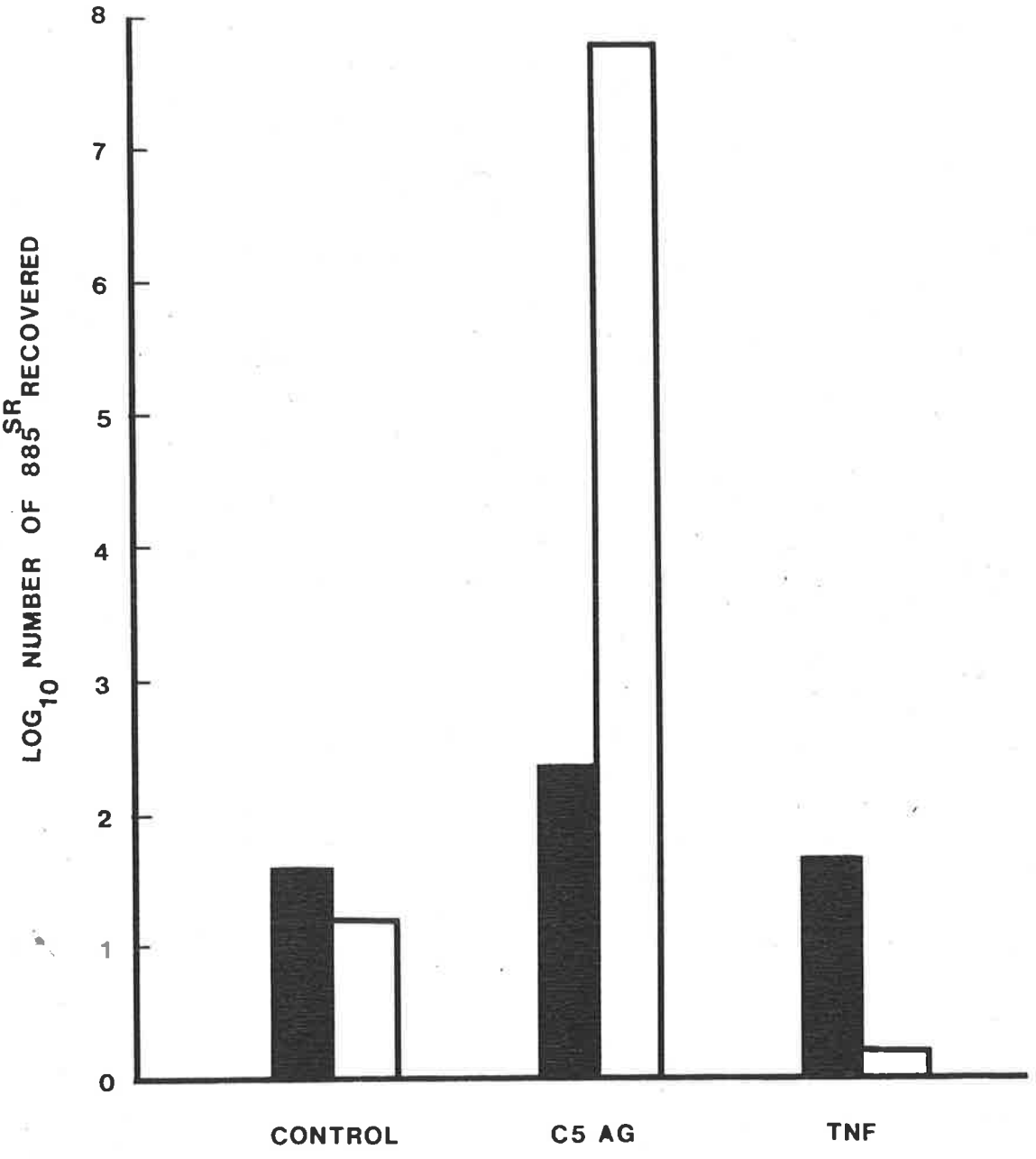
Recovery of Salmonella 885^{SR} from the intestinal contents of mice infected with 885^{SR} orally 7 days earlier.

One group was given 0.3 ml TNF serum intravenously, and the other group received 50 µg C5 OMP antigen subcutaneously and the rest served as controls. Mice were sacrificed 6 hours or 24 hours after the above treatments and numbers of specific organisms in the gut were counted.

Each bar represents the average number of organisms recovered from five mice.

■ 885^{SR} recovered at 6 hours after treatment.

□ 885^{SR} recovered at 24 hours after treatment.


LOG10 NUMBER OF 885SR RECOVERED
8
7
6
5
4
3
2
1
0
CONTROL
C5 AG
TNF

A.8 DISCUSSION

It is not known why injection of C5 OMP antigen into Salmonella orally infected mice should be followed by a massive multiplication of organisms in the gut lumen which later reinfect the intestinal tissues and the Peyer's patches. This huge growth of organisms in the gut is not due to diminished peristalsis nor is it obviously associated with TNF activity. Moreover this effect is not specific for the antigens of the infecting organism since other OMP, LPS, carbon particles or zymosan could also stimulate intestinal growth of organisms.

Obvious changes were observed in the small intestine of orally infected mice injected with C5 OMP antigen subcutaneously:- diarrhoea was found as expected; bile and mucus secretion were common and gas filled the intestinal lumen. Evans blue dye given intravenously 30 min before the C5 OMP antigen was found in the gut lumen, indicating some degree of increased permeability in the intestine. Haemorrhage into the Peyer's patches at the proximal end of the intestine was often found. The Peyer's patches were hard and firm when touched with the dissecting needle, and were accompanied by signs of intestinal inflammation and increased permeability. There were signs of liver damage (bile in the gut) which was confirmed by serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) determination in which the transaminase enzyme levels were elevated (data not shown). These results agree with earlier findings by others (Shands and Senterfitt, 1972). The results were possibly due to a localised Shwartzman type of reaction which can be produced by many colloids but to which the infected mice had been rendered more sensitive by

the growth of organisms in the lymphoid tissues of the gut.

Histological studies kindly carried out by Dr Lomax-Smith, of the Pathology Department of this University, revealed that after 1-2 hours of OMP antigen challenge there was necrosis and ulceration in the Peyer's patches. The mucosa of the small intestine was quite normal.

Salmonella infected mice are similar to BCG infected mice which show hypersensitivity to LPS (Suter et al., 1958). The injection of LPS caused profound physiological effects and overwhelming infection and death.

There is no doubt that the increasing number of Salmonella 885^{SR} in the Peyer's patches was due to several factors and because of the potent effect of LPS in many different systems it is hard to pinpoint a particular mechanism. However, it is worth noting that the gut lumen of mice fed with Salmonella challenged with OMP antigen obviously contained a large amount of bile which is a favourable nutrient for Salmonella organisms. That may enhance the growth of bacteria.

Another observation was that there was excessive secretion of mucus into the gut lumen. It has been shown that mucus provides protection for bacteria from the immunological defence system (Whitby and Rowley, 1959; Joo and Juhasz, 1968). It seems likely that the few bacteria in the gut lumen of mice fed with Salmonella begin to grow rapidly when provided with the exudate of fluid and bile which appears naturally in the intestine following injection of antigen and that this provides the initiative for the events followed in this chapter. We can presume that the previous feeding of Salmonellae (and no doubt other organisms) sensitizes the gut so that changes occur focally there following the s.c. injection of the

bacterial antigen. We must presume that the interaction does not depend on specific antigen/antibody reactions since very similar effects can be provoked using non-specific materials such as carbon or zymosan.

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