HOST-BACTERIA RELATIONSHIPS AT THE SECRETORY SURFACES OF THE LUNG

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SUMMARY

The development of immunity in the mouse to intra-nasal infection with Klebsiella pneumoniae was investigated with regard to cellular and humoral aspects of immunity. Particular attention was paid to the possible role of local, humoral immunity in resistance to this organism.

Experiments were designed to investigate the role of antibody in the expression of cellular immunity due to the importance of the alveolar macrophage in the lung. Studies on the killing of Salmonella typhimurium and Listeria monocytogenes by normal and activated peritoneal macrophages revealed that antibody was required for the destruction of Salmonella but not Listeria by macrophages, irrespective of the degree of activation. The requirement of antibody for killing of Salmonella was related to the inability of this organism to bind to macrophages in the absence of antiserum. Listeria bound to macrophages in the total absence of serum. The binding of Listeria was dependent on the presence of Mg^{++} and Ca^{++} ions, while the antibody dependent binding of Salmonella was not. Thus the binding of Listeria was not due to the presence of cytophilic antibody. Acquired cellular immunity therefore does not change the requirement of antibody for macrophages in the destruction of certain organisms.

Humoral immunity was shown to protect mice to intra-nasal challenge with Klebsiella pneumoniae. Immunity could be produced by actively immunising mice intra-venously with vaccines or by passive

transfer of antiserum with the infecting dose of organisms. Both IgG and IgM derived from serum were shown to be effective in protection. Differences were observed between the protection mediated by these classes of antibody dependent on the nature of immunising vaccine used to raise the anti-serum. While antibody directed against capsular polysaccharide efficiently protected mice, a second non-capsular antigen that was heat-labile was also able to induce the production of protective antibodies. Antibodies to this antigen appeared in the IgG class and not the IgM class. The antigen was present in other strains of Klebsiella.

As humoral immunity was shown to be critical in resistance to this organism, and as cellular activation could not alter the requirement for antibody , it was decided to investigate the possible contribution of local humoral factors . Local immunisation of the respiratory tract produced immunity in the absence of high titres of serum antibody. Antibody could be detected in pulmonary secretions. Antibody in both IgA and IgG classes was detected and shown to be protective when passively transferred. While serum antibodies could promote the clearance of an avirulent organism from the lung following aerosol exposure, sIgA had no such effect. Fc receptors for IgG were detected on alveolar macrophages but no receptor for IgA was found. Protection mediated by IgA was probably not dependent on alveolar macrophage function. The possible contribution of polymorphonuclear leukocytes was investigated. IgA was not able to promote killing by these cells.

The protection mediated by IgA in the lung does not appear to rely on cellular events in the lower respiratory tract, the site of pathology in infection with Klebsiella. It is proposed that IgA functions solely in the upper respiratory tract in preventing temporary colonisation from occurring and inhibiting the spread of infection to the lower airways. A similar function for IgA and local immunity can be envisaged in human disease where colonisation of the upper respiratory tract by Klebsiella or other gram-negative bacteria appears to be the initiating event in nosocomially acquired infections.

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.

J. McA. Cooper

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ERRATA

P.	4	Line 5, occurring, not occuring
P.	10	Line 7, resemble, not ressemble
P.	36	Line 11, pneumonia, not pmeumonia
P.	67	Line 18, experiments, not experiment
P.	69	Line 4, assayed, not asayed
P.	98	Line 7, membrane, not meembrane
P.	105	Line 9, enhanced, not enhance
P.	111	Line 2, more than, not greater than
P.	111	Line 5, word missing is mins. (minutes).
P.	111	Line 15, resemble, not ressemble

Fig. 6.5. "Antiserum" on abscissa should read: "pulmonary secretions".

NOTE: The words "normal macrophage(s)" used throughout this text refer to resident macrophages, harvested from animals which had not been pre-treated before use.

CHAPTER 1

HOST DEFENSES TO PULMONARY INFECTION

In common with other external surfaces of the body, such as the skin and the gastro-intestinal tract, the respiratory tract is exposed continually to pathogenic and non-pathogenic organisms. Infection is fortunately rare and despite the large numbers of infectious organisms in inspired air, the lower airways remain quite sterile under normal conditions. The defenses involved rely on non-specific clearance of particles by mechanical means and on the contribution of humoral and cellular components of immunity.

These defenses do fail from time to time. The lungs and upper airways are frequent sites of primary infection with viruses and bacteria. Such infections may be due to exposure to overwhelming numbers of organisms, to exposure to pathogenic organisms to which the host is not immune or to the impairment of host defenses by environmental factors or by pre-existing disease. Bacterial pneumonia may also be secondary to pre-existing viral or bacterial infection of the airways. This thesis is concerned with the role of host defenses in protection against bacterial pneumonia. In particular, it is concerned with the relationships that may exist between the various components of humoral and cellular immunity as they occur in the respiratory tract. Attention is paid in detail to the factors determining resistance to gram-negative bacterial pneumonias that often occur in debilitated patients or as secondary infections.

1.1 NON-SPECIFIC PULMONARY DEFENSES

The anatomy of the airways is critical in determining the site at which inhaled particles deposit or sediment out of the inspired air. Particles of over 10 microns in size are removed within the nose by the filtering effect of the hairs and turbinates of the nasal cavity (Proctor and Wagner, 1967). Impaction of particles on the posterior wall of the nasopharynx removes particles of about 10 microns and it is only the smallest particles or droplets that reach the bronchi. With the high number of bronchial and bronchiolar divisions that occur, as the air flow decreases droplets sediment onto the walls of the airways. Brownian motion may result in the deposition of particles within the lung (Morrow, 1960). It is clear that most inhaled particles will deposit at some level within the respiratory tree, although particles of 0.1 to 0.5 microns in size tend to remain suspended in the air (Muir and Davies, 1967).

The importance of the site of deposition depends on the composition of the particle. In terms of micro-organisms, deposition may enhance their infective potential or diminish it dependent on the site. This was clearly demonstrated using aerosol infection with *Mycobacterium tuberculosis* (Wells, 1955). The size of particle generated by the aerosol and hence its site of deposition was related to infective potential. Droplets were generated over a wide range of sizes but the optimal size for production of infection was about 3 microns. Larger particles, even containing higher numbers of organisms, failed to be infective. It is clear that the most infective droplets were those that penetrated deep within the lung and that larger droplets were removed in the upper respiratory tract

where they were less infective.

Particles deposited within the upper respiratory tract are principally removed by muco-ciliary transport. Respiratory mucus is constantly moved towards the trachea and oropharynx by the action of the cilia of the pseudo-stratified epithelium of the respiratory tract. The removal of particles is extremely efficient in the upper respiratory tract. Particles deposited within the trachea are removed with a half-life of only 30 minutes (Yeates et al., 1975). This should be contrasted however with the much slower removal of fine aerosols that deposit in the lower respiratory tract. Bacterial aerosols have been reported to be removed at a rate of about 3-4% per hour (Green and Kass, 1964).

In addition to the mucus secreted into the upper airways, several other components are secreted that could be expected to play a role in the non-specific resistance of the lungs. Possible roles for lysozyme and lactoferrin have been suggested (Newhouse, Sanchis and Bienenstock, 1976a). Lysozyme is produced in high quantities by alveolar macrophages (Myrvik, Leake and Fariss, 1961) but its role in the lung has not been elucidated.

Complement components have also been reported to be present in bronchial secretions although generally only in low levels: C3, C4 and C6 have been detected (Reynolds and Newball, 1974) as have components of the alternative pathway (Newhouse, Sanchis and Bienenstock, 1976a). Again no direct evidence exists that these components exert any beneficial effect in the lung. In general, highly inflammatory reactions in the lung, as at other mucosal surfaces, appear to be damaging rather than beneficial. The low levels of complement components found, and the lack of IgM in bronchial secretions, suggest that their presence may not be

important *in vivo*.

Of the various proteins found in bronchial secretions, one of the most important, in general terms, is alpha-l-anti-trypsin. It is interesting to note that this protein is critical in preventing inflammatory and destructive changes mediated by other host enzymes in the respiratory tract. The deficiency of this protein, and the concomitant inflammatory reactions that do occur, result in the development of obstructive airways disease (Talamo, Allen and Kahan, 1968; Eriksson, 1965). These observations highlight the extreme danger of uncontrolled inflammatory reactions in the lung.

1.2 HUMORAL IMMUNITY IN THE LUNG

It is convenient to distinguish at this time between the upper and lower respiratory tracts. Kaltreider (1976) has defined the line of demarcation to be at the broncho-alveolar junction where the mucosal surface gives way to respiratory epithelium in the respiratory bronchioles and alveoli. This definition is particularly useful in discussing immunity in the lung. For most evidence that is available illustrates important immunological differences existing between these two divisions of the respiratory tree. The upper respiratory tract appears to be a classic, mucosal surface at which local immunity is expressed. The lower tract demonstrates evidence of cellular immune responses in an environment that more closely approximates that occuring systemically in the animal. Humoral immunity as expressed predominantly in the upper tract will be discussed first.

Secretions may be obtained from animals and humans by lavaging sections of the respiratory tract. Secretions from tracheal

washouts from rabbits have demonstrated that immunoglobulins of both IgA and IgG classes may be found in high concentration (Reynolds and Thompson, 1973a). It would appear, however, that the relative proportions of these two immunoglobulins vary according to the level of the respiratory tract. While IgA predominates in pharyngeal secretions, much higher amounts of IgG are found in secretions from the bronchus (Kaltreider and Chan, 1976). As already stated, IgM is found in only low concentrations in respiratory secretions (Kaltreider and Chan, 1976) or not at all (Reynolds and Thompson, 1973a; Hand, Cantey and Hughes, 1974). IgM is present in saliva, although at only one-sixth of the concentration of IgA (Kaltreider and Chan, 1976), and may therefore contribute to events in the pharynx.

The remaining immunoglobulin class of importance in the respiratory tract is, of course, IgE which is widely recognised for its importance in allergic reactions. As the antibody involved in such common ailments as allergic rhinitis and asthma, it is normally thought of as a producer of pathology within the lung. It appears to be secreted locally and is markedly increased in atopic individuals. It has been suggested that IgE may be important in protection against parasitic infection but its function in this is not clear (Kaltreider, 1976).

Both IgA and IgG may be produced locally by lymphoid tissue in approximation to the respiratory tract, although transudation of IgG from serum accounts for most of the IgG present in normal circumstances (Merrill, Naegel and Reynolds, 1980). Lymphoid tissue is found organised in bronchus associated follicules that closely ressemble the Peyer's patches of the intestine both histologically and immunologically (Bienenstock, Johnston and

Perey, 1973a,b) and in lymph nodes surrounding the bronchi. The similarity between the gut and bronchus associated lymphoid tissue has been taken as evidence in favour of a local system of immunity common to mucosal surfaces (Bienenstock et al., 1978).

1.3 LOCAL IMMUNITY

These general observations on the classes of immunoglobulins found in the respiratory tract and the finding of similarities between the arrangement of lymphoid tissue in the intestine and the bronchus are only recent observations in the history of the development of ideas concerning local immunity. From the earliest days of immunology, it was recognised that the responses of the host locally at the site of infection would alter the outcome of infection, and that these responses would not necessarily be those found systemically in the host. Besredka (1927), Calmette (1923) and Gay (1924, 1926) reviewed many experiments, dating back to Pasteur, using local immunisation to protect against local infection. The concept that local immunity might exist independently from systemic immunity dates from this time. Gay wrote in 1924: ' a general reaction as evidenced by serum antibodies is no indication of a superior local protection, for example, in the intestine.' Experiments, particularly within the intestinal and respiratory tracts, over the following twenty years confirmed the truth of this concept of local immunity.

Early observations on local immunity demonstrated that immunity could be developed by local immunisation in the absence of a systemic response. Bull and McKee demonstrated resistance to intra-nasal inoculation with pneumococci in rabbits that had been

locally immunised. No serum antibody could be demonstrated in these animals (Bull and McKee, 1927, 1928, 1929).

Burrows was one of the first to demonstrate that this form of immunity could be explained by the production of antibody in secretions at these surfaces. Studying cholera in guinea pigs, he observed that antibodies found in the faeces (copro-antibody) correlated well with resistance to the disease. By X-irradiating animals or by challenging animals late in an immune response, at a time when no copro-antibody could be recovered, it was possible to show that animals were not resistant despite the presence of high levels of antibodies in their serum (Burrows, Elliott and Havens, 1947; Burrows, Deupree and Moore, 1950).

At about this time, Fazekas de St. Groth reported similar correlations between antibody levels in nasal secretions and resistance to influenza in mice. He drew attention to the efficiency of local immunisation in producing this immunity and high levels of antibody (Fazekas de St. Groth and Donnelly, 1950a,b).

The systematic investigation of local immunity was made possible by the description of immunoglobulins belonging to a new class, IgA. This antibody was reported to be present in particularly high concentrations in those secretions that had been previously demonstrated to exhibit local immunity (Heremans, Heremans and Schultze, 1959).

The three basic characteristics of a local immune response have been stated as follows (Tomasi, 1970);

(a) secretory antibody may correlate with resistance better than serum antibody does,

(b) serum and local antibody levels vary independently,

(c) IgA is the predominant antibody found in secretions

expressing this immunity.

While these criteria clearly describe the characteristics of a local immune response, several important questions remain concerning the control of this immunity and the effector mechanisms whereby antibody, particularly IgA, produces immunity. Furthermore, it appears clear now that to the above criteria describing a single, individual local immune response, should be added the fact that immune responses occuring at mucosal surfaces seem to be related one to another and to form what has been called a ' common, mucosal immune system ' (Bienenstock et al., 1978).

1.4 CONTROL OF LOCAL IMMUNITY

The finding of high levels of IgA in mucosal secretions has been shown to be the result of local production of this immunoglobulin by plasma cells found in the lamina propria and other tissues in proximity to the mucosal surface. IgA produced in these circumstances is dimeric and contains an extra polypeptide, christened J chain (Koshland, 1975). These characteristics distinguish locally produced IgA from systemically produced, monomeric IgA.

Following secretion of this dimeric IgA from plasma cells, it passes through the epithelial cells of the mucosa and in doing so attaches a further polypeptide known as secretory component (Brandtzaeg, 1974). This complete molecule is described as secretory IgA (sIgA).

It became clear that the local predominance of sIgA at mucosal surfaces was due to the high number of plasma cells committed to its production at these sites. Thus the lymphoid tissue

present differed significantly from that found, for example, in the spleen where no such predominance of IgA secreting cells occurs. The differences existing between lymphoid tissue responsible for systemic responses and that responsible for local responses raised several important questions that remain only partly answered. Do these differences represent a true subdivision of the humoral immune system, with separate controls operating ? Alternatively, are the special qualities of local immune responses simply adaptive to the physiology of mucosal surfaces, in particular to the high antigen loads present at these surfaces ?

Bienenstock and Befus (1980) have reviewed the evidence in favour of the existence of a ' common mucosal immune system '. Much of this evidence deals with similarities that exist between the lymphoid tissue at these sites as has been mentioned. The most important evidence, however, concerns studies that demonstrate that not only are lymphocytes associated with these sites predestined to produce IgA but that these lymphocytes appear to home preferentially to mucosal sites in transfer experiments and appear to migrate between mucosal surfaces.

In 1964, Gowans and Knight reported that large lymphocytes could be isolated from the thoracic duct lymph of rats. When transfered to other rats, these cells could be seen to home preferentially to the gut wall and to appear there as plasma cells. It is now known that the thoracic duct is part of a normal migration route used by these cells of gut origin. Lymphocytes are sensitised in the Peyer's patches of the intestine to antigen of gut origin. From the Peyer's patches, the cells migrate via the lymphatics, the thoracic duct and the circulation to the intestinal wall where they differentiate into antibody producing cells (Craig and Cebra, 1971

). These cells are committed to IgA production predominantly. The localisation of these cells is not completely specific to the intestine. They do appear at other mucosal surfaces and in the spleen, but always appearing as IgA producing cells.

Similar large lymphocytes to those found in thoracic duct lymph, may be found in the bronchus-associated lymphoid tissue which appears to functionally and anatomically ressemble the Peyer's patches (Bienenstock, Johnston and Perey, 1973a,b). These large lymphocytes reappear preferentially in the bronchial wall and the gut after transfer (Rudzik et al., 1975).

These results emphasise that large lymphocytes stimulated by antigen at mucosal surfaces preferentially return to mucosal sites rather than to the spleen or other systemic lymphoid tissue. Studies on cells obtained from lymph nodes draining mucosae also demonstrate this propensity. These experiments also emphasise that lymphocytes may demonstrate organ specificity. Mesenteric lymph node cells localise within the intestinal wall while bronchial lymph node cells localise within the bronchial wall (McDermott and Bienenstock, 1979).

While these experiments are performed in situations where antigen may be expected to influence results greatly, similar localisation has also been reported in so-called antigen free situations (Halstead and Hall, 1972; Parrott and Ferguson, 1974). Hormonal, vascular and chemotactic factors have all been suggested as controlling mechanisms (McDermott, Clark and Bienenstock, 1980; Ottaway and Parrott, 1979; Parrott, 1979). It appears therefore that antigen alone may not explain the migration and homing of these lymphocytes predestined to IgA production. Antigen does play an important role in the localisation of specific antibody producing

cells however (Ogra and Karzon, 1969; Husband and Lascelles, 1974).

Pierce and Gowans (1975), and later Husband and Gowans (1978), investigated the localisation of cells producing specific antibody against cholera toxoid (ACC). Following local immunisation of the intestine, ACC could be detected in thoracic duct lymph. ACC from this lymph localised highly specifically within the intestine. If one of two Thiry-Vella loops was immunised, ACC localised in that loop rather than in the unimmunised loop. The high numbers of ACC present in the immunised loop was possibly due in part to local division of these cells after localisation. Husband and Gowans (1978) therefore presented this hypothesis: while antigen independent localisation of cells from thoracic duct lymph and other sources to mucosal sites may occur, antigen appears to play an important role in the localisation of specific antibody forming cells. Antigen driven localisation could therefore be expected to be important in situations where local immunity to a given antigen (micro-organism) is involved.

The mucosal surfaces of the body are continually exposed to exceedingly high levels of foreign antigens. In addition to controlling migration of lymphoblasts, antigen may also be responsible for determining the class of antibody these cells are committed to produce. Cebra and his colleagues have suggested an important role for environmental antigens in the control of the expression of genes for both the variable region and constant region of immunoglobulin (Gearhart and Cebra, 1979; Cebra et al., 1980). Clones of antibody producing cells may be produced from Peyer's patch cells. The class of antibody produced depends on the nature of the antigen involved. Cells producing antibody to bacterial antigens such as phosphorylcholine or inulin produce IgA. Such antigenic

determinants could be expected to occur within the intestine. A much lower percentage of cells producing antibody against non-bacterial antigens such as dinitro-phenol are IgA producers. This suggests that antigenic stimulation may control the expression of constant region genes, and that exposure to gut antigens in high levels produces the expression of genes for IgA, rather than IgM or IgG.

Cebra has proposed that mucosal lymphoid follicles impede the maturation of B lymphocytes to plasma cells. This agrees with the common observation that plasma cells cannot be recovered from Peyer's patches. The class of antibody produced by a plasma cell depends on the translocation of V_H genes next to C_H genes with excision of intervening C_H regions. Such excisions would be expected to follow the order of C_H regions on the chromosome: C_{μ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_{α} (Honjo and Kataoka, 1978). Cebra proposes that ' the more often a translocational event occurs in a B lymphocyte, the greater the overall probability that progeny would express the V_H/C_{α} gene ' (Cebra et al., 1980). If such translocation and excision steps were time or division dependent, then the proposed blocking of maturation of lymphocytes within the Peyer's patches and other mucosal lymphoid tissue could provide a suitable environment for this to occur.

Many of the special characterisitics of local immunity can be seen to result from the high antigen loads presented to mucosal surfaces. Antigen is responsible for localisation of antibody producing cells following local immunisation or local infection. Antigen also drives the genetic alterations required for the production of IgA at these surfaces. In contrast to these findings, antigen does not appear to account for the selective localisation of lymphoblasts to mucosal tissue. Furthermore, mucosal lymphoid

follicles have been proposed to block the maturation of lymphocytes. While no evidence exists that this occurs, evidence does exist that a special environment within these follicles predisposes to IgA committment of lymphocytes. These observations reinforce the functional studies that led to the development of the concept of a ' common mucosal immune system '. The control of these supposedly non-antigen dependent characteristics of the system are not understood clearly. The recent description of lymphocytes bearing Fc receptors for IgA (Lum, Benveniste and Blaese, 1980) and of IgA specific helper T-cells (Elson, Heck and Strober, 1979) may result in a better understanding of some of these problems.

While antigen independent controls do appear to operate within this system, it is clear that in experimental situations involving the production of local immunity, there is no better method than direct immunisation of the surface involved. This remains the central strategy in the development of vaccines to be used for prevention of disease at mucosal surfaces.

1.5 FUNCTION OF IgA

While investigations of the controlling mechanisms of local immunity, the biochemistry and genetics of the glycoproteins involved generate their own problems, the central importance of this system for the host is the efficiency with which it may resist disease at the body's surface. The mechanism by which locally produced immunoglobulins, particularly IgA, achieve this is far from clear.

In the realm of viral diseases, sIgA has been demonstrated to be able to neutralise viruses (Waldman and Ganguly, 1974) and

observations dealing with viral infection of the respiratory tract were some of the first to demonstrate the importance of locally produced antibody (Fazekas de St. Groth and Donnelly, 1950 a,b ; Amoss and Taylor, 1917). Against bacterial diseases, the role of sIgA is far less clear. Antibody of this class has been reported to neutralise toxins (Curlin and Carpenter, 1970; Kaur, McGhee and Burrows, 1972) but this action would not be expected to explain all the observations dealing with protection to bacterial diseases. Other anti-bacterial effects of IgA have been extremely difficult to demonstrate.

Complement fixation by sIgA has not been demonstrated. Myeloma IgA does appear to fix complement _via_ the alternative pathway (Klaus et al., 1979; Spiegelberg and Gotze, 1972). Bactericidal activity induced by sIgA has been described to occur in the presence of complement and lysozyme (Adinolfi et al., 1966; Hill and Porter, 1974) although Eddie, Schulkind and Robbins (1971) were unable to demonstrate this activity against _Salmonella typhimurium_. In contrast to these reports of the ability, allbeit limited, of IgA to promote complement dependent effects, IgA has been reported to inhibit complement dependent bactericidal activity of other classes of antibody (Griffiss, 1975; Griffiss and Bertram, 1977). It seems in general that complement fixing activity of sIgA is difficult to demonstrate and its function _in vivo_ in this respect must at the moment be considered to be minor.

Likewise, opsonic effects of sIgA have been poorly demonstrated. No significant opsonic activity has been reported using macrophages although Reynolds and Thompson (1973b) have reported a minor effect of IgA from respiratory secretions in promoting phagocytosis by alveolar macrophages. No Fc receptor for

IgA has been reported on macrophages of any source. Myeloma IgA has been reported to bind to polymorphonuclear leukocytes (Henson, Johnson and Spiegelberg, 1972; Lawrence, Weigle and Spiegelberg, 1975) although myeloma IgA has also been reported to block bactericidal activity of polymorphs in an Fc dependent fashion (Van Epps, Reed and Williams, 1978). By contrast with myeloma IgA, sIgA has been reported to lack opsonic activity for polymorphs (Wilson, 1972) although a complement dependent opsonisation effected by sIgA for these cells has been described (Kaplan, Dalmasso and Woodson, 1977). The possible relationship between IgA and polymorphs must be left unsolved, while no evidence exists of efficient opsonisation of particles for macrophages.

As already stated, Fc receptors for IgA have been described on lymphocytes but the implications of this are not clear (Strober et al., 1978; Lum et al., 1979).

Despite these conflicting and generally negative results, some comments on the role of IgA may be made. In investigating protection against cholera in the infant mouse model (Ujiiye et al., 1968; Neoh and Rowley, 1972; Chaicumpa and Rowley, 1972), Steele assessed the effectiveness of various classes of antibody (Steele, Chaicumpa and Rowley, 1974). Antibodies of IgG, IgM and sIgA classes were equally protective (by weight) in the gut, despite the far greater efficiency of IgG and IgM at promoting phagocytosis and complement fixation. The protection against cholera in the gut was not dependent on the presence of the Fc portion of the immunoglobulin molecule but could be produced by $F(ab')_2$ fragments. F(ab) fragments showed diminished activity. It was proposed that antibody acted by cross-linking the bacteria and interfering with colonisation (Steele, Chaicumpa and Rowley, 1975).

Colonisation of mucosal surfaces, whether by cholera or by invasive organisms, is often the first and critical step in development of disease. The attachment of bacteria shows specificity in terms of both the host and tissue involved (Gibbons, 1977; Savage, 1972). Presumably the interaction between bacteria and host epithelial cells is mediated by receptors that demonstrate some degree of specificity. Adherence and pathogenicity have been linked to the presence of particular surface structures of bacteria, for example piliation in gonococci (Swanson, Kraus and Gottschlich, 1971; Swanson, 1973). IgA has been shown to be able to interfere with this process of attachment and colonisation (Williams and Gibbons, 1972), although presumably it is no more efficient at this than other classes of antibody.

1.6 BRONCHO-ALVEOLAR CELLS

Humoral immune responses of a local nature appear to be important in the upper respiratory tract and the role of these defenses in resistance to bacterial infection will be considered in the following chapter. In addition to humoral defenses, cellular components of the immune system are present in the lower airways.

Cells may be obtained from the lung by brochial lavage in the same fashion that secretions may be sampled. The majority of cells found in broncho-alveolar washouts are alveolar macrophages. From 60-90% of cells have been identified as macrophages in a variety of studies (Reynolds, Kazmierowski and Newball, 1975; Ford and Kuhn, 1973; Kaltreider, Turner and Salmon, 1975). The remaining cells are predominantly lymphocytes, other cell types being rare in normal washouts. T-cells are more numerous than B-cells and the

ratio of the two cell types is similar to that found in serum (Daniele, Altose and Rowlands, 1975).

While these studies use cells obtained by pulmonary lavage, several functional studies have been performed using homogenised lung preparations. Lymphocytes from these preparations show differences in responses to those from spleen. In particular while demonstrating good mitogen-induced cytotoxicity, lung lymphocytes appear unable to mediate antibody dependent cellular cytotoxicity despite the presence of Fc receptors on a high percentage of cells (Hunninghake and Fauci, 1976a, b). It is clear that T-lymphocytes from the lung are immunocompetent and could be expected to play a role in cell-mediated responses.

In protection against bacterial infections, the central role of the alveolar macrophage has been emphasised by many studies. Green and Kass (1964a,b) followed the clearance of radio-labelled bacteria from the lung. Animals were exposed to bacterial aerosols and the lungs were removed from animals over the subsequent hours. Bacterial counts on lung homogenates showed rapid killing of the inoculum with only a slow removal of radio-activity. These experiments were interpreted as demonstrating rapid *in situ* killing by alveolar macrophages. Several similar studies also concluded that rapid *in situ* killing in the lung resulted from the action of resident macrophages (Laurenzi et al., 1964). Other cell types may be involved. While polymorphonuclear leukocytes are not found in normal bronch-alveolar washouts, the rapid influx of these cells into the respiratory tract has been accorded importance in the clearance of certain bacteria such as *K. pneumoniae* (Toews, Gross and Pierce, 1979; Rehm, Gross and Pierce, 1980).

Never-the-less, resident macrophages remain the front line defense against bacterial invasion of the alveoli. Alveolar macrophages are derived from circulating monocytes similarly to other tissue macrophages. Within the tissue of the lung, these cells undergo maturation and adaptation to aerobic environment. The cells then migrate into the alveoli (Bowden et al., 1969; Moore and Schoenberg, 1964). These macrophages may die in situ or be removed by muco-ciliary action in the bronchioles (Brain, 1970). A less important route of removal of these cells is via their active migration back into the interstitium (Tucker, Wyatt and Undery, 1973).

Macrophages obtained by lavage have been shown to possess considerable activity in vitro against bacteria (Reynolds and Thompson, 1973b). In general, however, alveolar macrophages have been found to be less efficient than peritoneal macrophages in bactericidal activity in vitro (Pavillard and Rowley, 1962; Pavillard, 1963; Degre, 1969). Alveolar macrophages from homogenised guinea pig lung are highly efficient in antibody dependent cellular cytotoxicity against chicken RBC. They are more active in this than blood monocytes (Hunninghake and Fauci, 1976b).

The ability of normal alveolar macrophages to destroy bacteria may be enhanced by immunisation of the animals resulting in the production of antibody (Jakab, 1976). It may also be enhanced by non-specific activation. While normal alveolar macrophages exhibit a limited capacity to destroy Listeria monocytogenes., activation of these cells by BCG immunisation results in greatly enhanced listericidal activity (Myrvik, 1972). Such a response is classic of cell-mediated responses involving the activation of macrophages by the products of lymphocytes.

1.7 MACROPHAGE ACTIVATION

Enhancement of the function of macrophages has been shown to be produced by two means. Specific antibody, acting primarily as an opsonin, will promote phagocytosis and appears to be essential for the phagocytosis of many organisms. Non-specific immunity may arise following infection with a variety of organisms, particularly intra-cellular parasites, that is characterised by increased microbicidal, and tumoricidal, function of macrophages.

That macrophages may vary in their ability to destroy micro-organisms was shown in studies involving resistance to Mycobacterium tuberculosis. Lurie (1942) demonstrated that macrophages from normal animals were unable to destroy mycobacteria whereas macrophages taken from immunised animals were able to do so. Suter (1953) confirmed the finding that macrophages from BCG immunised animals showed enhanced microbicidal activity.

Many studies followed showing that this immunity was non-specific and therefore did not ressemble humoral immune responses that were specific for the immunising organism (Boehme and Dubos, 1958). Animals recovering from infection with, for example, salmonella demonstrated resistance to infection not only with salmonella but also listeria. Mackaness coined the term 'activated macrophage' to describe cells obtained from animals recovering from infection with one of the intra-cellular parasites, such as brucella, listeria or salmonella (Mackaness, 1962, 1964). In a series of experiments with listeria, brucella and salmonella, he demonstrated that the resistance of animals to these infections lay in the enhanced microbicidal activity of their macrophages and

that antibody played an apparently minor role in this immunity (Mackaness, 1964; Blanden, Mackaness and Collins, 1966).

While serum from immune animals was shown to be ineffective at transferring this immunity, transfer of lymphoid cells was effective (Mackaness, 1969). The lymphoid cells responsible for this were demonstrated to be T-cells (North, 1973; Lane and Unanue, 1972). Simon and Sheagren (1972) reproduced these events in vitro. Guinea pigs were immunised with bovine gamma globulin (BGG) and lymphocytes obtained from their peritoneum or lymph nodes. These cells were then incubated in vitro with BGG and macrophages. The listericidal activity of these macrophages was then determined. Macrophages having been incubated with sensitised lymphocytes and the sensitising antigen showed enhanced listericidal activity. It was subsequently shown that the release of lymphokines from lymphocytes was responsible for these events. While specificity was required for the sensitising antigen, the subsequent activity of the macrophages was non-specific. Indeed activated macrophages were also shown to exhibit tumoricidal activity (Evans and Alexander, 1970).

These studies tended to emphasise the importance of cellular events while according antibody little if any role. This tendency is still in evidence (North, 1978). If the implication that activated macrophages no longer required antibody for phagocytosis were true, classic studies on the importance of opsonic antibody in immunity would need to be reassessed.

In addition to the functional characteristics discussed above, activated macrophages exhibit a variety of biochemical differences from normal macrophages. Many of these seem to reflect higher rates of metabolism. Cell protein is increased, as is rate of spreading and pinocytosis (Edelson, Zweibel and Cohn, 1975;

Rabinovitch and Stefano, 1973). Rates of phagocytosis are much higher, over 1,000 fold greater than resident macrophages (Bianco, Griffin and Silverstein, 1975). These factors alone would not seem to be responsible for the acquired capacity of these cells to destroy intra-cellular organisms, particularly as events following ingestion would appear to be critical. Activated macrophages show increased levels of lysosomal enzymes and some secretory enzymes such as plasminogen activator (Gordon, Unkeless and Cohn, 1974; Cohn and Benson, 1965). Never-the-less, many of these characteristics are shared by macrophages that are stimulated by a variety of substances such as thioglycollate, starch or casein. These cells often lack full microbicidal and tumoricidal activity as shown by lymphokine activated cells (Cohn, 1978). There have been several attempts to correlate activation with a particular biochemical quality, representing the pathway involved in killing. Attention has been paid to oxygen-dependent pathways; the production of superoxide ions, peroxidase and hydrogen peroxide have been studied. Best correlations would appear to be made between activation and H_2O_2 release (Nathan and Root, 1977; Nathan, Noguiera et al., 1979; Nathan, Brukner et al., 1979).

Whether or not activation may be truly correlated with H_2O_2 release, it must be born in mind that all such biochemical tests are of necessity performed on whole populations of macrophages. The percentage of activated macrophages in a given population may of course vary widely. Quantitative differences expressed in biochemical terms, while useful in comparing populations, should not replace functional definitions of acquired cellular immunity, that represent qualitative differences in the immunity of animal populations.

In general, activation appears to depend on the participation of lymphokines. Whether or not this is always true is not clear. A possible exception would appear to be the ability of endotoxin to produce activation. Doe and Henson (1978) have reported tumoricidal activity in macrophages, elicited *in vivo* with thioglycollate and subsequently activated *in vitro* with endotoxin. Peritoneal inflammation of this sort has been reported to result in exudates that are more susceptible to activation by lymphokines. The responsive cell appears to be the newly-arrived, peroxidase positive monocyte from the blood (Ruco and Meltzer, 1978). It is possible that similar activation of elicited macrophages is induced by endotoxin.

1.8 ROLE OF ANTIBODY IN PHAGOCYTOSIS.

The above studies have all emphasised the cellular nature of this immunity but the role of antibody should not be neglected. Antibody is generally accorded the role of opsonin in the process of phagocytosis, and it is clear that binding of a micro-organism to the surface of a macrophage *via* the interaction of antibody and the Fc receptor is very efficient at promoting phagocytosis. Acquired cellular immunity is essential, however, for good immunity to a variety of organisms that survive within normal macrophages. If the only role of antibody was to promote ingestion, then clearly the important events in immunity are those occuring post-ingestion and after antibody has played its part.

Such an assessment of the role of antibody may not be sufficiently accurate. Jenkin (1963) investigated the phagocytosis of *Escherichia coli* bearing phages. Antibody directed against the

phage was sufficient to promote ingestion, but killing of the bacteria did not proceed. Antibody directed against the bacteria, resulted in ingestion and killing. Similar observations have been made with Rickettsia mooseri. Normal human serum promotes the ingestion but not the killing of this micro-organism. When specific antibody is used as the opsonin, killing proceeds (Gambrill and Wisseman, 1973a, b).

The important post-ingestion event that appears to be antibody dependent is phago-lysosome fusion. This is illustrated in studies with Toxoplasma gondii. When normal macrophages are incubated with T. gondii and normal serum, the organisms are ingested and multiply within the cells (Anderson and Remington, 1974). In this situation, phago-lysosome fusion does not occur (Jones and Hirsch, 1972; Jones, Yeh and Hirsch, 1972). The use of specific antibody promotes fusion and death of the micro-organism occurs (Jones, Len and Hirsch, 1975; Anderson and Remington, 1974). Cell-mediated immunity to this organism may alter the course of infection in vivo and in vitro. Activation of macrophages, while not promoting killing of organisms within the phagosome, does result in the inhibition of growth of the micro-organism and cyst formation results (Remington, Krahenbuhl and Mendenhall, 1972; Frankel, 1967; Ruskin and Remington, 1969).

Studies of intra-cellular killing of Mycobacterium tuberculosis, while demonstrating the effect of antibody in promoting phago-lysosome fusion, show that macrophage activation is the critical factor in determining the fate of the organism. Normal macrophages phagocytose virulent M. tuberculosis but phago-lysosome fusion fails to occur (Armstrong and Hart, 1971; Hart et al., 1972). The organisms multiply freely within the phagosomes. Treatment of

the mycobacteria with immune rabbit serum results in phago-lysosome fusion but has no effect on the ability of the organisms to multiply within normal macrophages (Armstrong and Hart, 1975). Activation of macrophages by preparations of lymphocytes does inhibit the division of *M. tuberculosis* within macrophages *in vitro* (Klun and Youmans, 1973). Antibody alone is thus not able to materially alter the fate of ingested *M. tuberculosis*.

These studies show that antibody clearly has an important role to play not only in promoting attachment of bacteria and other parasites to macrophage membranes, but may also in events following ingestion.

1.9 ACTIVATION OF ALVEOLAR MACROPHAGES

While alveolar macrophages do not appear to be as bactericidally active *in vitro* as normal, peritoneal macrophages, several other observations suggest that they may exhibit some of the characteristics of activated cells. Alveolar macrophages have been found to contain higher levels of lysozyme and lysosomal enzymes (Pavillard, 1963; Myrvik, Leake and Fariss, 1961; Leake, Gonzales-Ojeda and Myrvik, 1964). They are also more active metabolically than peritoneal cells, with a greater oxygen consumption and hexose monophosphate shunt activity. Phagocytosis produces only a minor increase in shunt activity, whereas in peritoneal cells a great increase is noted during phagocytosis. This has been taken as evidence that alveolar macrophages are relatively mature compared with peritoneal macrophages (Myrvik, 1972). These changes appear to be similar to those described in activated macrophages.

Several other important differences have been noted between alveolar cells and those of peritoneal origin. Alveolar macrophages have been demonstrated to inhibit mitogen-induced lymphocyte proliferation (Holt, 1979). This capacity is not present in normal peritoneal populations but is present in activated peritoneal populations (Wing and Remington, 1977). This evidence was interpreted to mean that alveolar macrophages exert a controlling influence on T-cell mediated responses in the lung. Such an effect would be expected to diminish harmful hypersensitivity reactions.

These observations tentatively support a hypothesis that alveolar macrophages are activated in the normal situation, at least above normal levels seen in peritoneal cells. It is clear that the mechanism for stimulation or activation of macrophages exists in the lung. Immunocompetent T-cells are present in lung tissue and the constant exposure to antigen could be expected to result in some degree of stimulation, even in the absence of overt infection or disease.

Acquired cellular immunity may be expressed in the lung in a similar fashion to that observed elsewhere. Myrvik (1972) demonstrated enhanced listericidal activity in alveolar macrophages from animals immunised with BCG. *In vivo* acquired cellular immunity to listeria may be demonstrated but does appear qualitatively different from responses seen to systemic infection (Truitt and Mackaness, 1971). Mice were exposed to aerosols of *L. monocytogenes* and development of inflammatory infiltrates and disease were followed. Normal mice developed a low grade inflammatory response to aerosol infection but this was markedly less than the cellular response seen in the liver of normal animals infected systemically. Other mice were immunised systemically with *L. monocytogenes*. The

initial events in the lung following aerosol were similar in normal and immune mice in terms of the number of surviving listeria and the rate of their division. Subsequently, with the arrival of blood monocytes, immune mice mounted an effective defense against the listeria. It would appear that alveolar macrophages are not easily activated following systemic immunisation.

Similar separation of systemic and pulmonary cell-mediated immunity have been reported following vaccination with influenza virus (Waldman, Spencer and Johnson, 1972). It is clear from these studies using local immunisation, as opposed to systemic immunisation, that perfectly adequate cell-mediated responses can be mounted in the respiratory tract. Cell-mediated responses also occur following bacterial immunisations and infection (Reynolds, Thompson and Devlin, 1974; Cantey and Hand, 1974).

The lung is therefore able to call on a variety of immune responses to resist infectious disease. The following chapter will review evidence on the exact roles these defenses play in immunity to bacterial pneumonias.

CHAPTER 2

RESISTANCE TO BACTERIAL PNEUMONIA

The lung can be seen to possess a remarkable array of defenses against micro-organisms and other environmental hazards. Infection occurs in the respiratory tract under a variety of circumstances. This thesis is primarily concerned with events surrounding infection with bacteria, resulting in pneumonic disease. In particular, infection with Klebsiella pneumoniae is studied. K. pneumoniae is one of the most important of organisms producing secondary or nosocomial infection. It is an infection, therefore, that occurs in patients whose pulmonary, and general, defenses are weakened by pre-existing illness. The factors responsible for immunity to this organism were studied in the hope of delineating those defenses that would be most efficient at preventing infection.

2.1 PNEUMONIA DUE TO GRAM-NEGATIVE BACTERIA

Friedlander's original isolation of Bacillus Friedlanderi (now known as Klebsiella pneumoniae) from the lungs of victims of fatal pneumonia, led him to believe that this was the common causative organism of pneumonia. This belief was, of course, inaccurate. The majority of pneumonias are produced by gram-positive organisms such as pneumococci, Staphylococcus aureus or haemolytic streptococci. Of primary pneumonias (community acquired) only approximately 10% are caused by gram-negative organisms (Rose, Heckman and Unger, 1973; Dorff et al., 1973). By contrast with these infections, secondary or hospital acquired pneumonias tend to be caused by unusual organisms, particularly gram-negative bacteria.

In a series of 224 cases of hospital acquired pneumonia, gram-negative bacteria were isolated in 37% of cases. Those organisms normally responsible for primary pneumonias were isolated in only 20% of cases while no pathogen was isolated in 43% of cases (Graybill et al., 1973).

The most common organisms producing nosocomial pneumonia belong to three families; Enterobacteriaceae, Pseudomonaceae and Achromobacteraceae (Pierce and Sandford, 1974). The most common organisms are K. pneumoniae (Montgomerie, 1979) and P. aeruginosa (Graybill et al., 1973).

Gram-negative infections continue to produce high morbidity and mortality. Before the introduction of antibiotics, mortality for K. pneumoniae infection of the lung was reported to be as high as 97% in some series (Lampe, 1964). Even after the introduction of antibiotics mortality remains very high. Gram-negative pneumonias were reported to have a mortality of 51% (Graybill et al., 1973), whereas gram-positive infection (hospital acquired) had a mortality of 20%. The mortality of gram-negative infections was not altered by the introduction of gentamycin, indicating that even potent antibiotics could not cure these diseases.

These high rates of mortality are in part due to the presence of pre-existing disease in patients. Community acquired, primary klebsiella pneumonia is a disease of elderly men (Hyde and Hyde, 1943; Edmondson and Sanford, 1967). It is associated with alcoholism (Manfredi, Daly and Behnke, 1963), diabetes mellitus and chronic pulmonary disease (Holmes, 1956). Hospital acquired klebsiella pneumonia occurs most commonly post-operatively, following anti-microbial therapy, or respiratory assistance (Graybill et al., 1973). Clearly the patients contracting it are not

able to mount an effective defensive response to infection.

The most common route of infection appears to be by spread of the organisms from the pharynx to the lower respiratory tract. The mechanism of this is not clear but alternative means of infection such as following bacteraemia or direct inhalation of bacteria are difficult to demonstrate. Some reports exist of contamination of respiratory therapy equipment (Reinarz et al., 1965) but this appears to be rare. Pneumonia does follow colonisation of the upper respiratory tract. In a study of patients receiving anti-microbial therapy for primary pneumonia, colonisation of the respiratory tract by gram-negative organisms and S. aureus was observed. Superinfection occured in 13% of those colonised. Colonisation appeared to be the initial abnormality detected (Tillotson and Finland, 1969). Colonisation of the oro-pharynx was found not to occur in normal subjects exposed to a hospital environment, but to occur in patients exposed to the same enviroment. Colonisation correlated best with the severity of illness (Johansson, Pierce and Sanford, 1969).

It is clear that the development of infection with these organisms and the subsequent course of disease depends on the ability of the host to respond, rather than on antibiotic or other therapy. In particular, colonisation of the upper respiratory tract in seriously ill patients suggests that normal clearance mechanisms are not operative.

2.2 KLEBSIELLA PNEUMONIAE

K. pneumoniae is a capsulated, gram-negative organism. The biochemical characteristics of the genus Klebsiella have been

outlined in a Report (1963). This and the description of the species of Klebsiella given in Kauffmann (1966) are adhered to here. In particular, Klebsiella are characterised by a positive Voges-Proskauer reaction, and a negative methyl red reaction. They are distinguished from Enterobacter sp by being ornithine decarboxylase negative, lysine decarboxylase positive, and arginine dihydrolase negative.

K. pneumoniae may be serotyped according to capsular polysaccharide and less commonly by their O antigens. Over eighty capsule types of K. pneumoniae are reported (Heidelberger et al., 1978). Several other organisms possess capsules that are antigenically similar to these polysaccharides, in particular S. pneumoniae, for example K. pneumoniae type II polysaccharide cross-reacts with S. pneumoniae type II (Picoff, 1966).

In addition to their role in pulmonary infection, klebsiella produce infections of the urinary tract and wounds. No particular capsule type may be assigned to infections of these various sites (Blanchette and Rubin, 1980), although early studies on the virulence of klebsiella associated respiratory infection with capsular types 1-6. This does not appear to be true and many capsule types have been reported to occur in pulmonary infections and other infections (Richard, 1973).

Some studies have tended to implicate capsule production in virulence. Kerby (1950) demonstrated that non-capsulated klebsiella were removed at a faster rate from the blood-stream than were capsulated klebsiella. While a correlation does exist between resistance to phagocytosis and virulence, capsule size per se does not appear to be correlated with virulence (Hall and Humphries, 1958). Takahashi and his colleagues have reported that virulence

may vary amongst variants of one isolate of K. pneumoniae and that virulence of the variants may be correlated with excessive production of capsule (Takahashi, Yoshida and San Clemente, 1977). Similar findings are reported with K. ozaenea (Takahashi, Iwanii and Yoshida, 1978). Polysachharide production in pneumococci has been correlated with virulence (MacLeod and Krauss, 1950).

A correlation has been noted between piliation and lack of virulence in klebsiella species (Duguid, 1959). The significance of this is not clear, as in other bacterial species which infect mucosal surfaces piliation has been correlated with virulence (Swanson, 1973; Swanson, Kraus and Gottschlich, 1971).

The capsular polysaccharide of K. pneumoniae has several immunological effects. It has been reported to act as an adjuvant for both humoral responses (Nakashima, Kobayashi and Kato, 1971) and cellular responses (Lefevre and Agneray, 1977). It also promotes non-specific antibody production (Nakashima, Kojima and Kato, 1976). High doses of polysaccharide produce immunologic paralysis (Batshon, Baer and Shaffer, 1963; Nakashima, Kobayashi and Kato, 1971) in a similar fashion to pneumococcal polysaccharide. While promoting cell mediated hypersensivity reactions, capsular polysaccharide may also impair the intra-phagocytic bactericidal activity of peritoneal macrophages (Kato, Kato and Nakashima, 1976, 1979). While the importance of other observations is not clear, this last might be expected to have some influence on events in vivo.

2.3 RESISTANCE TO BACTERIAL PNEUMONIA

Following the description of Friedlander's bacillus and subsequently the pneumococcus as causes of pneumonia, much work was done on the development of resistance to these organisms and to the factors influencing natural susceptibility of animal species to them. Neufeld and Handel in 1910 (quoted in Robertson and Sia, 1927) showed that mice could be rendered immune to pneumoccocci by passive transfer of normal human serum. Bull and McKee (1921) showed that chicken serum could similarly promote resistance in mice.

The ability of serum factors to promote resistance was further demonstrated by <u>in vitro</u> studies. In general, these studies assayed the ability of serum to promote killing of pneumococci by blood leukocytes. Several animals, such as the cat and dog, were shown to be resistant to infection with pneumococci, virulent to other animals. The serum of naturally resistant animals was able to promote bactericidal activity in serum-leukocyte mixtures whereas the serum from susceptible animals was not (Robertson and Sia, 1924). Furthermore, this activity of serum was directed against strains of pneumococci that were avirulent for the animal tested, but not for virulent strains (Woo, 1926). Thus the serum factors responsible for bactericidal activity exhibited some specificity. Leukocytes taken from susceptible animals were able to kill pneumococci in the presence of serum from naturally resistant animals, thus demonstrating that no defect in the leukocytes was responsible for their susceptibility (Robertson and Sia, 1927).

These studies on serum from naturally resistant animals suggested the presence of opsonins specific for strains of

pneumococci. Acquired resistance was also studied. Robertson et al. (1928)showed that recovery from infection with pneumococcal infection of cats correlated with the appearance of pneumococcidal activity in the serum, as well as with increased agglutinative and mouse protective activity. This activity appeared at a time when bacteraemia ceased in these animals, and was also shown to be type specific for the organism.

K. pneumonia was reported to produce pneumonia in experimental situations similar to those for pneumococcal pneumonia (Lamar and Meltzer, 1912; Christie, Ehrich and Binger, 1928). While the pathology following intra-nasal inoculation of K. pneumoniae was similar to that with pneumococci most reports draw atention to the more destructive nature of klebsiella infection. Natural immunity may also be observed to K. pneumoniae. Chickens are able to resist both S. pneumoniae type II and the cross-reacting K. pneumoniae type II. Furthermore serum from naturally resistant chickens may transfer immunity to K. pneumoniae to susceptible mice (Picoff, 1966).

In contrast to these studies implicating opsonising antibody in resistance, particularly natural immunity, to bacterial pneumonia, Wood described phagocytosis occuring within alveoli apparently without the contribution of antibody. This phenomenon of 'surface phagocytosis' was thought to occur during consolidation due to the crowding of alveoli with phagocytic cells which were thereby brought into direct contact with bacteria. This was reported to occur with both pneumococcal and klebsiella infections (Wood and Irons, 1946; Wood, 1941). Such phagocytosis did not appear to result in destruction of the ingested bacteria nor did it alter the course of infection unlike type-specific antibody which resulted in

rapid destruction of bacteria (Wood, 1941).

2.4 LOCAL IMMUNITY TO PNEUMONIA

Bull and McKee (1927) showed that animals recovering from infection demonstrated immunity to re-inoculation, and that immunity also followed immunisation with killed vaccines of pneumococci. Later (Bull and McKee, 1928) they found that rabbits demonstrating any antibodies at all in their serum were fully resistant to intra-nasal challenge with pneumococci. In view of experiments at that time dealing with local immunity, they attempted to determine whether immunity might precede the finding of antibodies in the serum. They therefore performed several experiments aimed at demonstrating local immunity (Bull and McKee, 1929). Rabbits were immunised with vaccines of pneumococci intra-nasally, and their subsequent resistance determined. Serum levels of antibody were measured by complement fixation. Immunity could be detected in the absence of detectable serum antibody. They concluded that local immunity could develop against pneumococci.

Walsh and Cannon (1936) extended these studies. They demonstrated that intra-nasal immunisation produced specific protection against pneumococci in that non-specific irritants did not produce protection. They confirmed that immunity developed in the absence of detectable serum antibody. However, they clearly demonstrated that immunity following intra-nasal immunisation was not restricted to the respiratory tract, in that animals also became resistant to intra-venous challenge. They concluded that true local immunity was not produced by intra-nasal immunisation, but that systemic immunity occured, possibly due to the presence of extremely

low levels of serum antibody.

Unfortunately, few other studies using bacterial pulmonary infection to investigate local immunity exist. Local resistance to viral infection of the respiratory tract was thoroughly investigated and elegantly demonstrated by Fazekas de St. Groth and Donnelly (1950 a,b), but bacterial infections have been relatively neglected. More recently, local immunity to Franciscella tularensis has been described (Bellanti et al., 1967; Buescher and Bellanti, 1966) which tends to support the earlier studies of Bull and McKee. In the absence of the demonstration of antibodies in nasal secretions by these earlier workers at a time when serum antibody was undetectable, their results are not easily interpreted. The studies of Walsh and Cannon strongly suggest that systemic immunity is also involved but the possiblility remains that local immune mechanisms may be protective against bacterial pneumonia.

2.5 ACTIVE IMMUNITY TO BACTERIAL PNEUMONIA

The vast majority of more recent studies on the production of resistance to bacterial pneumonias has concentrated on systemic immunisation. In this regard the importance of capsular antigens rather than somatic antigens has been emphasised (Macleod et al., 1945; Heidelberger et al., 1947). Several studies exist demonstrating the efficiency of pneumococcal vaccines in protecting human populations against pneumococcal infection. Vaccines prepared by killing bacteria with heat were reported to be effective (Cecil and Austin, 1918; Cecil and Vaughan, 1919). Such vaccines appeared to be effective against given capsular serotypes. In view of the apparent importance of capsular antigens, and the incidence of local

abcess formation following the use of whole bacterial vaccines, purified capsular polysaccharide was used as a vaccine. Many early studies using this method failed to provide adequate controls or population sizes. Never-the-less, studies such as those of Smillie, Warnock and White (1938) where an epidemic of type 1 pneumococcal pneumonia in a mental hospital was halted by the use of specific capsular antigen suggested that immunisation could be effective. Studies by Macleod, Hodges, Heidleberger and Bernhard (1945) clearly demonstrated the efficiency of vaccination with capsular polysaccharide. Indeed, the use of vaccines decreased the incidence of pmeumonia not only in immunised subjects but also in unimmunised controls with whom the subjects mixed freely.

Similar studies on acquired resistance to gram-negative infections exist. Circulating antibodies to K. pneumoniae promote resistance to this organism (Chedid et al., 1969). In a clinical setting, vaccination against P. aeruginosa was shown to produce circulating antibodies and to protect patients at risk of infection (Polk, Borden and Aldrete, 1973).

2.6 NON-SPECIFIC IMMUNITY TO K. PNEUMONIAE

In addition to the possible role of antibodies in immunity to K. pneumoniae, non-specific immunity has also been described by Chedid and his colleagues. Immunity of mice infected intra-venously with K. pneumoniae could be enhanced by previous injection of apparently unrelated lipopolysaccharide (LPS) from salmonella (Chedid et al., 1968; Parant, 1968; Parant et al., 1967). This had been previously demonstrated with a variety of other organisms (Landy, Michael and Whitby, 1962; Rowley, 1955). The particular

mechanism of this immunity was not clear. Lethally irradiated mice could also be protected by this injection with LPS but in this case the effect was temporary and the mice could not be rendered immune (Galelli, Parant and Chedid, 1977). Bone marrow reconstitution in these mice did restore full immunity following injection with LPS. The ability of LPS to protect was inhibited by cyclophosphamide (Parant et al., 1976).

This capacity of LPS was not due entirely to its role as a polyclonal B-cell activator (Andersson, Sjoberg and Moller, 1972) as other B-cell activators did not exhibit this effect in vivo (Parant et al., 1976).

Neo-natal thymectomy did not result in any increase of susceptibility to infection (Parant et al., 1976). In this, klebsiella resembles listeria and salmonella to which thymectomised mice are also resistant despite the obvious importance of T-cells in acquired imunity to these infections (Campbell et al., 1974; Fauve and Hevin, 1974). However, the experiments dealing with reconstitution of irradiated animals tended to suggest that T-cells were not involved in non-specific immunity induced by LPS as no T-cell activity could be detected in reconstituted mice.

The mechanism of immunity remains unclear. Chedid, Parant and their colleagues have suggested that two cell types are involved. A cell that is radio-resistant appears to be responsible for an early inhibition of growth of the organism. However, a second, radio-sensitive cell is required for lasting expression of immunity. This cell is bone-marrow derived and the response appears to be T-independent. Many similarities exist between this model and other models of non-specific immunity to bacterial infection mediated by activated macrophages. LPS is thought to be able to

activate macrophages and it may be that the initial stage of this response (radio-resistant) is mediated by macrophages with the participation of already existent antibody. The second cell required for immunity is not easily identified. The response is inhibited by cyclophosphamide but is not mimicked by other B-cell activators. It seems possible on reviewing their data that this second phase is not a non-specific event. If non-specific reticulo-endothelial system activation were responsible for resistance in the first few days of infection, time would permit the establishment of an antibody response to the infection, and not in response to LPS. The known ability of cyclophosphamide to inhibit antibody responses (Marneerushapisal , and Rowley, personal communication) could evidently inhibit this response.

2.7 INTERACTIONS OF PULMONARY DEFENSES

Various studies can therefore be seen to implicate circulating (and local) antibody, and non-specific (possibly cell-mediated) responses in resistance to pulmonary pathogens. Other studies have emphasised the importance of particular aspects of pulmonary defenses; the non-specific, mechanical clearance mechanisms of the mucosal epithelium, the cells of the alveoli or antibody within bronchial secretions. The interaction of all these defenses and host-bacteria relationships within the lungs form the subject of the experiments reported in this thesis.

Previous studies have illustrated many of the important relationships that do exist within the lung. The recent studies of Reynolds and his colleagues have illustrated many important aspects of these relationships. Following intra-muscular or intra-nasal

immunisation of rabbits with an antigen of *P. aeruginosa*, antibodies can be recovered from the bronchial secretions. No IgM is detected but IgA and IgG immunoglobulins are routinely found in these secretions (Reynolds and Thompson, 1973a). Following intra-muscular immunisation antibody is found predominantly in the IgG class. Following local immunisation IgA antibody is also detected. The effect of these antibodies on phagocytosis of *P. aeruginosa* by alveolar macrophages was then studied (Reynolds and Thompson, 1973b). IgA was considerably less efficient at promoting phagocytosis than IgG, although intra-cellular killing proceded irrespective of the class of antibody. It should be reiterated that some opsonic effect of the IgA preparation was detected.

Studies on the Fc receptors of alveolar macrophages confirmed the presence of receptors for IgG but not for IgM. Receptors for complement components were also found but as complement is not normally found in high concentrations in bronchial secretions, complement presumably does not play an important role in the normal lung (Reynolds et al., 1975).

Cellular immune responses were also detected in this system by demonstrating migration inhibitory activity by respiratory lymphocytes. *In vitro* activation of alveolar macrophages did not alter phagocytic rates or intra-cellular killing of *P. aeruginosa* (Reynolds, 1974).

Such studies leave many questions unanswered. The ability of IgG antibody to opsonise bacteria for alveolar macrophages resembles findings in the peritoneum. However, the slight ability of IgA to promote phagocytosis does not agree with findings in the peritoneum where IgA has practically no effect (Steele, Chaicumpa and Rowley, 1974). Furthermore, the function of macrophages in an

environment containing high levels of IgA is unique to the respiratory system. Peritoneal and other tissue macrophages operate in environments in which IgG and IgM predominate. Several differences have been reported between the environment of the lower respiratory tract and serum, in particular the lack of IgM and complement components. The juxta-position of secretory immunoglobulins and expression of local immunity with the expression of cellular immunity is also noteworthy.

Studies on lower respiratory tract function considered in isolation from events in the upper respiratory tract avoid considerations of the possible interactions between these two areas. Bacterial infections of the lung make no such distinction. Colonisation of the upper respiratory tract by gram-negative bacteria in hospitalised patients precedes lower respiratory tract infection. Serum antibody to such bacteria has been reported to protect susceptible patients but might not be expected to influence events in the mucosal environment of the pharynx.

It is unfortunate that so few studies exist on the possible contribution of local immunity to bacterial pneumonias. This may be due in part to the demonstration of the importance of cells in the lower airways, however, such studies have generally relied on experimental, aerosol infection which is not the common route of infection, especially for gram-negative bacteria.

If cellular events are of paramount importance in the lower airways, then several points require further consideration. Reports of non-specific immunity to K. pneumoniae resemble findings with acquired cellular resistance to listeria, salmonella and other intra-cellular parasites. While klebsiella is not a facultative, intra-cellular organism, it appears that non-specific stimulation of

macrophages may result in improved resistance to that organism as it can for organisms such as E. coli. The importance of antibody following macrophage activation must therefore be considered carefully. If activation of macrophages can supplant the need for antibody, then cell-mediated responses as have been demonstrated in the lung may be sufficient for immunity despite the efficiency of antibody in the normal situation. There is no evidence that this occurs but while doubt remains concerning the role of antibody in resistance to intra-cellular organisms, amongst others, such questions remained unanswered. Activation certainly alters the macrophage membrane towards the expression of higher numbers of Fc receptors and might be expected to enhance minimal levels of phagocytosis, such as that said to be elicited by IgA with alveolar macrophages.

Certainly, alveolar macrophages differ metabolically and functionally from other macrophages and if any macrophage might be expected to possess Fc receptors for IgA, then these cells would be candidates. Early studies suggesting the existence of local immunity in the lung might thereby be explained.

While the pulmonary environment differs from that of other tissues as reflected in levels of serum components, it should be remembered that this environment is rapidly changed by inflammation as occurs during infection. Serum proteins such as complement and IgM might then enter the alveoli and bronchioles. The most startling demonstration of the effects of inflammation, however, are seen in the rapid influx of polymorphonuclear cells into the lung. While these cells cannot be obtained from washouts of normal lungs, they may rapidly appear following infection with organisms such as K. pneumoniae and have been reported to be the important cell in

control of this organism.

The possible importance of polymorphs further higlights the need for reappraisal of the possible contribution of IgA to immunity in the lung as these cells have been reported to possess receptors for myeloma IgA although no demonstration of bactericidal effect of sIgA and polymorphs has been made.

2.8 AIMS

The general aim of the studies reported in this thesis was to investigate the interactions of the various host defenses to pulmonary infection with _K. pneumoniae_. Studies were designed to investigate the following areas:

(a) Preliminary experiments were performed to study the requirement of antibody for the killing of bacteria by normal and activated macrophages. These experiments were performed using peritoneal macrophages and salmonella and listeria. These organisms were used rather than _K. pneumoniae_ for several important reasons. Most importantly, much information is available concerning the interaction between these bacteria and macrophages and of the importance of activation in their killing. The evidence in favour of a role of activation in resistance to klebsiella is far less satisfactory. Furthermore, considerable controversy has existed concerning the differing requirements of these two bacteria for antibody as an opsonin and it was hoped that some light could be shed on this problem.

(b) The importance of humoral immunity in resistance to _K. pneumoniae_ was studied. In relation to these studies, some of the properties pertaining to virulence of _K. pneumoniae_ in mice were

considered and in particular the importance of antigens that could be recognised by protective antibodies was investigated. Despite the early belief in the importance of capsular antigens, evidence was found of the existence of a non-capsular antigen against which antibody was highly protective.

(c) The possibility of local immunity to intra-nasally inoculated K. pneumoniae was investigated. The role of both active and passive immunity was studied and the ability of locally porduced antibody to promote protection was demonstrated.

(d) The interactions of various classes of antibody, IgG, IgM and IgA with pulmonary defenses were then examined. In particular their role in promoting aerosol clearance in addition to protection to intra-nasal challenge, their interaction with alveolar macrophages and with polymorphonuclear leucocytes were studied.

The results from these experiments will be discussed in terms of the relative importance of local and systemic humoral responses, and cellular responses in protection to gram-negative bacterial pneumonia. The importance of these factors in resistance to and recovery from disease and their relevance to an understanding of the predisposing factors involved in development of secondary or nosocomial infection will be discussed.

CHAPTER 3

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

Klebsiella pneumoniae Kpn1 was obtained from Dr. R.F. Berendt, Fort Detrick, Maryland (Berendt, Long and Walker, 1975). Its virulence was maintained by intra-nasal passage (section 3.9b). Its virulence for mice was determined regularly during the course of experiments.

Other strains of *K. pneumoniae* were obtained as follows. Strains 90 and 1153 from the Department of Microbiology, University of Queensland and strains 4, 21 and 25 from the Commonwealth Serum Laboratories, Parkville, Victoria. Other clinical isolates were also obtained from these sources and from the Institute of Medical and Veterinary Science, Adelaide, South Australia. A total of approximately 30 strains, including 90, 1153, 4, 21 and 25, were tested and found to be avirulent in the mouse. All strains were from specimens of sputum or urine from patients. Detailed clinical histories were not available.

Vibrio cholerae 569B is a classical Inaba strain obtained from the Cholera Research Laboratories, Dacca. It has since been kept in lyophilised cultures in this Department.

Strains of *Salmonella typhimurium* were obtained from departmental stocks. *S. typhimurium* C5 is a smooth, highly virulent strain in the mouse. *S. typhimurium* M206 is a smooth, avirulent

strain. Both are described in Furness and Rowley (1956). S. enteritidis 11RX is a rough strain of intermediate virulence for the mouse (Ushiba et al., 1959). S. typhimurium F885 and E. coli F492 had been recently obtained from Dr. G. Schmidt, Max Planck Institute, Freiburg. S. typhimurium F885 is a S. typhimurium-E. coli hybrid expressing the E. coli O8 antigen (Hohmann, Schmidt and Rowley, 1979). These strains and S. adelaide were obtained from departmental stocks, stored as lyophilised cultures at 4°.

3.2 CULTURE MEDIA FOR BACTERIA

Bacteria were grown on nutrient agar (Difco) or in liquid culture in double-strength nutrient broth (Difco). Cultures were grown with shaking at 37°. Cultures of klebsiella were grown in tryptic soy broth (Gibco). All cultures were used in log-phase. Cultures were maintained on nutrient agar slopes or plates stored at 4° from which colonies were selected for inoculation of broth as required. K. pneumoniae Kpnl was stored as bacterial suspension in 0.1% peptone in saline at -80°. Tryptic soy broth was inoculated with a freshly thawed aliquot of this suspension, grown for 16 hours at 37° with shaking and then sub-cultured to fresh tryptic soy broth and grown for 2-3 hours.

3.3 ANIMALS

Mice for general purposes were obtained from a closed colony maintained in specific pathogen free conditions at the Central Animal House of this University. They were originally obtained from the Laboratories Animals Centre, Surrey (LACA). Mice

were generally used at 6-8 weeks of age and mice of either sex were used.

Germ-free BALB/c mice were maintained in this department by Dr. D.J. Horsfall from breeding stock obtained from the Walter and Eliza Hall Institute, Melbourne.

After removal from the specific pathogen free or germ-free environments, mice were housed in a conventional animal house in this department.

Infant mice were obtained from a breeding colony of LACA mice maintained in this department. They were used at 4-6 days of age.

Guinea pigs and rabbits were obtained from the Central Animal House of this University.

3.4 COLLECTION OF SERUM

Blood was obtained from the retro-orbital plexus of mice or by cardiac puncture of guinea pigs (anaesthetised with ether) or rabbits (anaesthetised with pentobarbitone sodium). Serum was removed after clotting of the blood at 37^{0} for 1 hour and 16 hours at 4^{0}. Serum was stored in aliquots at -20^{0} and thawed for experimental use. No preservatives were added to serum.

3.5 COLLECTION OF INTESTINAL SECRETIONS

Mice were killed by cervical dislocation and their small intestines removed. These were rinsed in cold saline and the lumens were flushed with 1 ml of saline introduced through a blunt needle. The intestinal contents thus collected were homogenised (

Ultraturrax, Janke and Kunkel, West Germany) and centrifuged to remove debris (12000 x g, 20 minutes, 4^{0}). The supernatant was concentrated using Aquacide. All these manoeuvres were performed on ice or at 4^{0}. Secretions after concentrating were dialysed against phosphate buffered saline, pH 7.4, and stored at -20^{0}.

3.6 COLLECTION OF PULMONARY SECRETIONS

Mice were killed by cervical dislocation and their tracheas exposed. A blunt 19G. needle was introduced into the trachea and tied in place. 0.8 mls of Hanks' balanced salt solution (Ca^{++}, Mg^{++} free) was gently injected into the lungs and removed by syringing. This was repeated three times. Secretions were processed in the same fashion as intestinal secretions. After concentration and dialysis, they were stored at -20^{0} without the addition of preservative.

3.7 COLLECTION OF PULMONARY CELLS

Mice were killed by cervical dislocation and the lungs lavaged as desribed for the collection of secretions (section 3.6). For the collection of cells lungs were lavaged with Hanks' balanced salt solution (Ca^{++}, Mg^{++} free) containing 12mM Lignocaine, and the lavages were repeated six times to obtain maximal yields. The use of Lignocaine has been reported by Holt (1979) to increase yields of functionally active pulmonary cells.

3.8 COLLECTION OF PERITONEAL EXUDATE CELLS

Mice were killed by cervical dislocation and the skin over their peritoneum reflected. 2.5 mls of Hanks' balanced salt solution (Ca^{++}, Mg^{++} free) was introduced into the peritoneal cavity. After gently massaging the mouse's abdomen, the fluid was withdrawn. The fluid was centrifuged at approximately 400 x g for 2-4 minutes and the supernatant discarded. The cells were then resuspended in culture medium. Glass centrifuge tubes were siliconised and all solutions and cell preparations were kept on ice.

3.9 TECHNIQUES FOR IMMUNIZATION OR INFECTION

3.9a Intra-nasal inoculation

Mice were anaesthetised with pentobarbitone sodium (0.6 mg/g body weight given by intra-peritoneal injection; Sagatal, May and Baker). A blunt 19G needle and a disposable 1 ml syringe were used to inoculate 0.05 ml of bacterial suspension into the nose of mice. Live K. pneumoniae were suspended in tryptic soy broth and vaccines were suspended in saline for this inoculation.

3.9b Intra-nasal passage of K. pneumoniae

Mice were infected intra-nasally with 100-1000 Kpnl. After 2-3 days, when the mice were showing signs of illness, 3-5 mice were sacrificed and their lungs removed. The lungs were homogenised and new mice were immediately infected with 0.05 mls of this homogenate. This procedure was repeated 2-3 times and after the final passage,

samples of the homogenate were streaked on agar. After incubation, isolated colonies of Kpnl were picked from the plates and dispersed in 0.1% peptone saline. This suspension was diluted until barely turbid and 0.3 ml aliquots frozen at -80^{0} until required. Virulence of the passaged organism was maintained for 2-4 months under these conditions of storage but regular tests of virulence were performed.

For use, an aliquot of bacterial suspension was thawed and used to inoculate tryptic soy broth.

3.9c Oral immunisation

Mice were immunised with 0.5 ml of bacterial suspension in 25% saturated $NaHCO_3$. This was administered *via* a blunt 19G needle which was gently introduced through the mouse's oesophagus into the stomach.

3.9d Aerosol exposure

An aerosol exposure chamber was constructed exactly as specified by Ruppert et al., (1976). This chamber is designed to deliver an aerosol containing droplets of 1-5 microns in size. A bacterial suspension at 10^{11}/ml in saline was placed in a Hudson nebuliser. Compressed medical air (CIG, Adelaide) was passed through this nebuliser at 10-12 l/minute and a further 8-10 l/minute mixed with the resultant aerosol in a preliminary chamber. The aerosol was then passed into the exposure chamber, containing mice within mesh cages.

3.10 PREPARATION OF VACCINES

3.10a Heat-killed organisms

Log-phase cultures were centrifuged, washed in saline and resuspended to 5 x 10^9/ml. The bacteria were then steamed for 3 hours, washed by centrifuging three times and resuspended to a suitable concentration in saline.

3.10b Glutaraldehyde-killed organisms

Log-phase cultures were washed by centrifuging three times and resuspended to 10^9/ml in phosphate buffered saline, pH 7.4. Glutaraldehyde was added to a final concentration of 0.1%. The bacteria were stirred at room temperature for 15 minutes and then washed by centrifuging at least five times and then resuspended in saline.

3.10c Alcohol-killed organisms

Washed log-phase cultures of bacteria were resuspended in 70% v/v alcohol in distilled water and left at 4° for 24 hours. Bacteria were then washed three times and resuspended in saline.

3.10d Ultra-violet irradiation

Bacteria were suspended to 10^9/ml and irradiated with ultra-violet light for 30-40 minutes in a Petri dish containing a thin layer of bacterial suspension.

All vaccines were determined to be sterile by viable counting.

3.11 QUANTITATION OF ANTIBODY

3.11a Passive Haemagglutination

Sheep red blood cells (SRBC) were washed by centrifuging three times and resuspended to 2.5% v/v in saline containing 100 ug/ml of lipopolysaccharide or capsular polysaccharide. Lipopolysaccharide from V. cholerae was alkali-treated but other preparations were not. The cells were incubated with the antigen for 60 minutes at 37° with rolling and then washed again three times. Cells were resuspended to 1% v/v in saline.

Serial two-fold dilutions of antisera for testing were made in micro-titre trays (Flow Labs) and equal volumes of sensitised SRBC added. The trays were incubated at 37° for 1 hour and then at 4° for 16 hours. End-points were determined as the last well demonstrating a significant difference in settling pattern compared with control wells. 1 haemagglutinating unit (1 HAU) was defined as that amount of antibody just sufficient to produce detectable haemagglutination.

3.11b Haemolytic assay

A passive haemagglutination assay was set up as described above. After an initial incubation of 1 hour at 37°, the settling patterns were disturbed by shaking the trays and an equal volume of 5% fresh frozen guinea pig serum in saline added to the wells. The

end-point of haemolysis was determined after a further incubation at 37° for 1 hour.

3.11c Bacterial agglutination

Serial dilutions of antisera were prepared in saline and an equal volume of 5-10 x 10^{9} bacteria/ml was added. The tubes were incubated at 37° for 2 hours and then at 4° for 16 hours and the end-point of agglutination was determined by comparison with control wells. 1 bacterial agglutinating unit was likewise defined as that amount of antibody sufficient to produce detectable agglutination.

3.11d Quantitative precipitin test

Capsular polysaccharide of Kpn1 was serially diluted from 2000 to 10 ug/ml and an equal volume of antiserum added. The tubes were incubated at 37° for 1 hour and then at 4° for 2 days. The supernatants were removed and the precipitates dissolved in 0.1N NaOH and the OD_{280} determined. The passive haemagglutinating activity of the supernatants was determined. The amount of specific antibody was determined at equivalence point from a plot of precipitated protein <u>versus</u> polysaccharide concentration. Haemagglutinating activity at the equivalence point was reduced to less than 95%.

3.11e Enzyme linked immuno-absorbent assay

This assay is described in detail in Chapter 5 (Section 5.8)

3.12 IMMUNODIFFUSION

3.12a Single radial immunodiffusion (Mancini)

A modification of the technique of Mancini, Carbonara and Heremans (1965) was used. 1 mm layers of 1% w/v of agarose (Calbiochem) in 0.05M sodium barbital/HCl buffer, pH 8.2 were formed on glass slides, previously coated with 0.5% agarose that was baked onto the slides. Commercial goat anti-mouse heavy chain antisera were incorporated in the agarose layers (Meloy Laboratories) at concentrations determined to be suitable for detection of immunoglobulins in preliminary experiments. Dilutions of sera were added to wells cut in the agarose and dilutions of standard mouse serum (Meloy Laboratories) were included on every plate.

Plates were left at room temperature in a humid atmosphere for 2 days and then washed in saline, press dried between filter papers and this cycle was repeated 4 times. Plates were finally washed in distilled water and dried at 85°. Plates were stained with 1-2% Xylene Brilliant Cyanine G in methanol, water, acetic acid in the proportions 5:5:1. Plates were destained in this solvent.

Diameters of precipitin rings were determined and the concentration of standards plotted <u>versus</u> the square of the diameter. Concentration of samples were then determined from the standard graph.

3.12b Double radial immunodiffusion (Ouchterlony)

Similar layers of 1% agarose were prepared, but without the incorporation of antisera. Wells were cut in the form of rosettes

and serum and antisera (Meloy Laboratories) were added to wells in the desired pattern. Plates were incubated, washed and stained as for 3.12a.

3.13 PURIFICATION OF ANTIBODY

3.13a Protein-A Chromatography

The method of Ey, Prowse and Jenkin (1978) was used for the isolation of IgG from mouse serum and pulmonary secretions. The column was equilibrated with loading buffer, 0.1M phosphate, pH 8.2. The sample of serum (5 mls mouse serum or concentrated pulmonary secretions) was adjusted to pH 8.2 by the addition of 2 mls of loading buffer and sufficient 1M Tris-Hcl, pH 9. This sample was then loaded onto the column and the effluent collected and pooled. This was always checked to confirm that IgG was not present by Ouchterlony gel diffusion. The IgG bound to the column was eluted using 0.1M sodium citrate buffer, pH 3.3. The IgG was collected into equal volumes of 1M Tris-HCl pH 9 to neutralise the acid buffer. The IgG pool was dialysed immediately against phosphate buffered saline, pH 7.4, and then the IgG and the residual pool (after removal of IgG) were concentrated with Aquacide to the original volume of serum. The samples were then dialysed against saline.

3.13b. Sephadex G200 Chromatography

Separation of IgM from serum previously depleted of IgG by Protein-A chromatography was performed on Sephadex G200 using phosphate buffered saline at pH 7.4 for elution. Fractions

containing IgM were determined by Ouchterlony immunodiffusion. These were pooled, concentrated using Aquacide and dialysed against phosphate buffered saline. Concentrations of IgM were determined by Mancini immunodiffusion. IgA was not detected in these preparations by Ouchterlony immunodiffusion, and was not thought to contribute significantly to results obtained as determined by the activity of purified IgA in similar conditions.

3.13c Purification of Secretory IgA

Purified secretory IgA was obtained from intestinal washings of mice immunised orally with V. cholerae 569B. This preparation was kindly provided by Dr. D.J. Horsfall and Dr. L. Bloom. The secretions were purified by passage through Sephadex G200, Protein-A Sepharose 4B and an IgM immunoabsorbent column. No haemolytic activity was detected in this preparation against 569B LPS sensitised SRBC, but it retained good activity in the baby mouse protection test as described by Chaicumpa and Rowley (1972). IgA was the only immunoglobulin detected by immunodiffusion methods.

3.14 PREPARATION OF Fc FRAGMENTS OF RABBIT IMMUNOGLOBULIN

Immunoglobulins were prepared from rabbit serum by $(NH_4)_2SO_4$ precipitation, and the IgG isolated by chromatography on Protein-A Sepharose 4B. Fc fragments were prepared by digestion of the immunoglobulins by the method of Nisonoff et al., (1960). The digest was dialysed aginst phosphate buffered saline and fragments were separated from intact immunoglobulin by chromatography on a Sephadex G200 column. The fractions containing

the lower molecular weight fragments were pooled and concentrated to 200 ug/ml with Aquacide. The preparation was dialysed against saline.

3.15 CLEARANCE OF BACTERIA

To determine pulmonary clearance of bacteria, mice were exposed to aerosols of bacteria for 30-45 minutes. At the end of this time, several mice were sacrificed and their lungs removed and homogenised. Viable counts were determined on these homogenates. At intervals, further mice were sacrificed and counts performed on lung homogenates.

To determine peritoneal clearance of bacteria mice were intra-peritoneally injected with 10^6 live bacteria in 0.2 ml of saline. Mice were sacrificed at intervals and peritoneal lavage performed. The number of bacteria remaining in the peritoneum was determined by viable counting of the lavage fluid.

Bacteria were opsonised prior to aerosol or injection by the addition of antiserum to the final suspension of bacteria 15 minutes prior to infection. The bacteria were not centrifuged after opsonisation.

Rates of clearance were determined as the percentage of the initial inoculum cleared at each time point. The standard error of this percentage was determined by the method of Wilks (Green and Kass, 1964b).

3.16 DETERMINATION OF 50% LETHAL OR PROTECTIVE DOSES

To determine the 50% lethal dose (LD_{50}) of bacteria, mice were infected with 5-fold serial dilutions of bacteria. The 50% end-point was determined by the method of Reed and Muench (1938).

The 50% protective dose (PD_{50}) of antibody preparations was determined by opsonising a standard inoculum of bacteria (generally 10-20 LD_{50}'s of Kpnl for intra-nasal infection) with 2-4-fold dilutions of antibody. The PD_{50} was similarly determined by the method of Reed and Muench (1938).

3.17 ACTIVATION OF MACROPHAGES

Macrophages were activated _in vivo_ using one of two methods. 10^5 live _S. enteritidis_ 11RX were injected into the peritoneal cavities of mice and cells were harvested by peritoneal lavage six days later. Alternatively, 1 ug of _S. typhimurium_ C5 lipopolysaccharide was injected intra-peritoneally and the cells harvested after two days.

3.18 ASSAY OF HYDROGEN PEROXIDE PRODUCTION

H_2O_2 release from peritoneal exudate cells was determined by the method of Nathan and Root (1977) with the exception that whole populations of peritoneal exudate cells were used for the determination. Peritoneal exudate cells were obtained by lavage and centrifuged. They were resuspended to a concentration of approximately 10^6/ml in Dulbecco's phosphate buffered saline

with glucose. To 1 ml of cell suspension were added 1 ul of horse radish peroxidase 30 mg/ml and 1 ul of scopoletin 1 mg/ml. After incubation at 37^0 for 2 minutes, 1 ul of 0.3 mg/ml phorbol myristic acetate in DMSO was added. After a further incubation of 5 and 10 minutes, the fluorescence was measured (exciting wavelength 350 nm and emitted 460 nm) in a Ratio Fluorometer (Farrand Optical Co.). The loss of fluorescence was calculated over 5 minutes.

3.19 IN VITRO KILLING ASSAY

Peritoneal exudate cells were resuspended in RPMI 1640 (with 20 mM Hepes and 5.29g/l $NaHCO_3$) containing up to 30% foetal calf serum (inactivated by heating to 56^0 for 30 minutes). 200 uls of cell suspension was added to wells of micro-culture plates (Flow Labs, U.S.A.). 5×10^5 cells were added to each well to ensure the formation of a complete monolayer of cells. After incubation at 37^0 in 5% CO_2 in air for 2-3 hours, the medium was removed from the monolayers and fresh medium containing bacteria and antiserum was added to the wells. 30 uls of medium containing $2\text{-}5 \times 10^4$ bacteria were added to each well, this being a sufficient volume to cover the monolayer. Antiserum was generally added to a final concentration of 0.1%. Amounts of antibody contained in this concentration are cited with the results of experiments. The trays were incubated again at 37^0 and samples taken at intervals. For sampling, one tray was removed from the incubator and 30 uls of 1% Triton-X added to all the wells to disrupt the macrophages. Samples were then withdrawn from the wells and viable counts performed. Over the time period involved in the sampling process, this concentration of Triton-X did not affect the viability of the organisms used.

3.20 ADHERENCE OF BACTERIA TO MACROPHAGES

The ability of bacteria to adhere to macrophages was determined by the preparation of monolayers of macrophages on flying coverslips in Leighton tubes. Peritoneal exudate cells were obtained from normal mice and resuspended at 5 x 10^5 cells/ml in culture medium. The medium used depended on the experiment but was one of; RPMI 1640, RPMI 1640 with 10% heat-inactivated foetal calf serum, Dulbecco's balanced salt solution. 1 ml of cell suspension was added to each Leighton tube. After incubation at 37° in 5% CO_2 in air for 1 hour, the medium and non-adherent cells were removed and 1 ml of culture medium containing bacteria was added. Bacteria were added in excess (10^7/ml) to ensure good contact between macrophages and bacteria. After 30 minutes incubation at 37°, the monolayers were washed by pipetting fresh medium over the coverslips. The cells were stained with Wright's stain and the percentage of macrophages binding one or more bacteria was determined by light microscopy. Coverslips were prepared in triplicate and 100 cells were counted per coverslip.

3.21 ANTIGEN PREPARATIONS OF KLEBSIELLA

3.21a Ultrasonic disruption of bacteria

K. pneumoniae were grown in tryptic soy broth to late log phase, centrifuged and resuspended in saline. 1-5 x 10^{10} bacteria in 10 mls were ultrasonicated at 1.5-1.7 amperes (M.S.E. Ultrasonicator), four times for 20 seconds. Whole bacteria were then removed by centrifugation.

3.21b X-Press technique

A crude extract of bacteria was prepared by harvesting cells from a culture in tryptic soy broth, washed by centrifuging and resuspended in 0.01M HEPES, pH 7.4, to approximately 2 x 10^{10}/ml. A small quantity of DNase and RNase were added and the bacteria were cracked using an X-Press. $MgCl_2$ was added to 1mM after cracking and whole bacteria were removed by centrifugation.

3.21c Membrane proteins

A log-phase culture of K. pneumoniae was washed by centrifugation and resuspended in 0.75M sucrose in 10mM Tris-HCl, pH 7.8. The bacteria from a 1500 ml culture were suspended to a concentration of approximately 10^{10}/ml. An equal volume of lysozyme (400 ug/ml in 40mM EDTA, pH 7.8) was then added in the cold and stirred for 15 minutes. After this the bacteria were subjected to ultra-sonic disruption as above, but for three periods of 30 seconds. Whole bacteria were removed by centrifugation at 10000 x g and the membrane proteins were removed by centrifugation at 160000 x g for 2 hours. The pellet of membrane proteins was resuspended in 1 ml of 25% w/w sucrose in 10mM EDTA, pH 7.8. A gradient of 30-55% sucrose was prepared and the membrane preparation layered on this gradient. The gradient was centrifuged at 78000 x g for 18 hours. The centrifuge tubes were pierced at the bottom and 400 ul samples were collected. Polyacrylamide gel electrophoresis, in the presence of SDS, was performed on these fractions by Dr. P.A. Manning of this department (Achtman et al., 1978).

3.21d Polysaccharide and lipopolysaccharide

Polysaccharide was prepared from K. pneumoniae by the method of Batshon, Beer and Shaffer (1963). Lipopolysaccharide was produced from V. cholerae and S. typhimurium by the method of Westphal, Luderitz and Bister (1952).

3.22 PROTEIN AND CARBOHYDRATE DETERMINATION

Protein concentrations were determined by the Lowry modification of the Folin-Ciocalteu reaction (Lowry et al., 1951). Bovine serum albumin was used as a standard.

Carbohydrate concentrations were determined using the phenol-sulphuric acid method as detailed by Hodge and Holfreiter (1962). Dextrose was used as a standard.

CHAPTER 4

THE ROLE OF ANTIBODY IN THE RECOGNITION AND DESTRUCTION OF BACTERIA BY ACTIVATED MACROPHAGES

Amongst the various defenses of the host to bacterial pathogens, one of the most powerful is phagocytosis and killing of the parasite by cells of the reticulo-endothelial system. While these cells have been reported to possess some limited power of recognition of antigenic molecules (Gorzinsky, Macrae and Jennings, 1979), this appears to be restricted to the recognition of certain carbohydrate antigens as protein antigens are not recognised (Loor and Roelants, 1974). Several reports do exist of the ability of macrophages to bind particles without the aid of opsonins (Capo et al., 1979; Rabinovitch, 1967; Weir and Orgmundsdottir, 1977; Benoliel et al., 1980).

In general, however, macrophages require the presence of opsonins to facilitate the binding of foreign particles to their surfaces. Such binding is the preliminary to phagocytosis and is therefore essential in the destruction of a potential pathogen.

Two important classes of receptors on polymorphs and reticulo-endothelial cells have been demonstrated. These are the receptors for immunoglobulin and for components of complement. Macrophages, with which this chapter deals, have been demonstrated to possess receptors to the Fc portion of immunoglobulins of both IgG and IgM classes . Three separate Fc receptors for IgG have been described on mouse macrophages, one binding IgG_1 and IgG_{2b}, another IgG_{2a} and the third IgG_3 (Unkeless, 1977; Diamond and Scharff, 1980; Diamond and Yelton, 1981). All receptors appear to

be functionally distinct. The possible existence of Fc receptors for IgM on macrophages is still controversial, and its existence is often denied (Diamond and Yelton, 1981; Huber, 1980). Nevertheless, several reports do suggest that a receptor does exist for IgM (Lay and Nussenzweig, 1969; Walker, 1977). It may be that this receptor is cryptic as trypsin treatment appears to expose it (Haegert, 1979).

Immunoglobulins may also enhance the binding of micro-organisms via fixation of the third component of complement. Receptors for C3b and C3d have also been described (Ehlenberger and Nussenzweig, 1977). Complement activation by the alternative pathway may also promote phagocytosis via the receptor for C3 (Bar-Shavit, Raz and Goldman, 1979; Shurin and Strosser, 1978).

It is generally held that binding of particles via the Fc receptors results in internalisation, that is the phagocytosis of the particles. Whether or not such phagocytosis also follows binding by the complement receptors is a matter of some dispute (Dierich, 1980), although phagocytosis has been clearly described with activated macrophages (Edelson, Zweibel and Cohn, 1975).

The importance of opsonins in promoting phagocytosis has been shown with a wide variety of organisms (Robertson and Sia, 1927; Jenkin and Rowley, 1959; Jenkin and Benacerraf, 1960). Antibody plays a critical role in these studies in permitting phagocytosis to occur and it is clear that, without antibody, recognition and subsequent ingestion of the parasite will not occur under these conditions.

A second factor influencing the ability of the reticulo-endothelial system to deal with invading micro-organisms is its degree of activation. Lurie (1942) clearly demonstrated the

enhanced ability of rabbit macrophages to destroy Mycobacterium tuberculosis following exposure to the organism. Many subsequent studies, particularly those of Mackaness (1962, 1964, 1969) established the non-specific nature of this acquired immunity that was dependent on the development of a population of activated macrophages. This immunity was effective against a wide variety of so-called intra-cellular pathogens such as listeria, mycobacteria, brucella and salmonella. That this immunity was largely non-specific and that animals resistant to one of these organisms could also resist challenge by others argued against the importance of antibody in this process (Howard et al., 1959; Boehme and Dubos, 1958). Indeed many experiments with Listeria monocytogenes purported to demonstrate that antibody was not required for its phagocytosis and destruction by activated macrophages (Mackaness, 1969; McGregor, Koster and Mackaness, 1971). These findings were supported by the fact that passive transfer of antibody did not produce good protection, nor could killed vaccines that efficiently produced high levels of serum antibody protect animals against these organisms (Collins, 1969 a,b).

Nevertheless, some evidence was presented that, while animals could be rendered resistant to unrelated organisms, the homologous organism was handled more efficiently then heterologous ones. Mice immunised with L. monocytogenes could remove these organisms far more efficiently than M. tuberculosis and vice versa (Coppel and Youmans, 1969). Particularly in the case of salmonellae, it was found that the development of specific antibody could greatly enhance acquired cellular immunity (Davies, 1976). Furthermore, evidence had been presented that antibody was absolutely required for the phagocytosis of salmonellae (McIntyre, Rowley and Jenkin,

1967; Rowley, Auzins and Jenkin, 1968).

The general observations of the critical importance of antibody in promoting phagocytosis of many bacterial species, including salmonellae, appear to conflict with the overall findings of the ability of activated macrophages to express acquired imunity without the participation of antibody. Due to the particular biochemical and functional characteristics that suggest that alveolar macrophages may indeed be highly stimulated if not actually activated, it was important to understand whether or not activation of macrophages permitted phagocytosis to occur without the aid of antibody. The studies discussed above would suggest that at least some bacteria, such as L. monocytogenes, might be phagocytosed and destroyed in the absence of antibody although all such results must be viewed with caution. In some circumstances, sufficient cell-bound antibody may be eluted from macrophages to permit passive transfer of immunity (Rowley, Turner and Jenkin, 1964) so that, even in vitro in the absence of serum, sufficient antibody may be present to permit binding of micro-organisms to macrophages. Cytophilic antibody may be detected on macrophages even after four days of culture in medium, and may be capped by divalent anti-immunoglobulin reagents (Loor and Roelants, 1974).

Therefore, as a preliminary to considering the role of humoral and cellular components in pulmonary defense, it was decided to attempt to clarify the above conflict. The particular question investigated was whether activation altered any requirement for antibody by macrophages for the recognition or killing of bacteria. For these studies, Salmonella typhimurium and Listeria monocytogenes were chosen as organisms to which an animal may classically become resistant via acquired cellular immunity. The evidence presented

above suggests that they may vary in opsonic requirements.

4.1 DEVELOPMENT OF IN VITRO ASSAY

As the ubiquitous presence of antibodies leave all in vivo experiments open to doubt, these studies were performed in vitro. Furthermore, this permitted investigation of both the killing efficiency of cells and the initial interaction between bacteria and macrophages.

Many assays previously described for measuring the bactericidal capacity of macrophages fail to report any significant reduction in the total inoculum of bacteria. Several methods have been published that allow an initial period of contact between macrophages and bacteria, followed by the removal of extra-cellular organisms by mechanical means (Petersen et al., 1977; Benedsen et al., 1977; Spitalny, 1981). Others have suggested the use of temperature sensitive mutants that would not divide at 37^{0} (Hooke et al., 1978). Such methods seldom reproduce the rapid and efficient destruction of bacteria seen in vivo.

Other methods do attempt to determine the ability of macrophage cultures to destroy the total number of bacteria in culture without any such manipulation. In particular the methods of Degre (1969), Cohn and Morse (1959), Pavillard (1963) and Whitby and Rowley (1959) seem to reflect the true bactericidal activity of macrophages. Similar methods were therefore attempted.

The initial development of these system was carried out using Vibrio cholerae 569B. This organism is totally avirulent in the adult mouse and is rapidly destroyed in the mouse peritoneum.

Macrophages were obtained by peritoneal lavage,

centrifuged and resuspended in tissue culture medium (Dulbecco's medium or RPMI 1640) containing 10-20% heat-inactivated foetal calf serum (FCS). They were suspended at approximately 1-2 x 10^6 cells/ml. and bacteria were added at a final ratio of cells:bacteria of 10:1 - 100:1. Antiserum was added (0.1% mouse anti-569B). Siliconised tubed containing 1 ml. of cells and bacteria were incubated at 37^0, with rolling. Samples were taken and plated for viable counts over the next two hours. Despite several modifications to this procedure with regard to serum concentrations, cell and bacteria numbers and type of medium employed, poor killing results were obtained. Generally, a slight decrease in the growth rate of V. cholerae when compared with control wells was observed. Slightly improved results were obtained with RPMI 1640 rather than Dulbecco's medium.

It would appear that a major problem confronting in vitro assays is the ability of producing close contact between cells and bacteria. This problem was approached by the use of methods using macrophage monolayers rather than suspensions. Experiment were carried out using micro-culture trays. Mouse peritoneal exudate cells were allowed to adhere to the wells of these trays during an initial incubation of 3 hours at 37^0. Sufficient cells were added to ensure a confluent monolayer of adherent cells: this required at least 4-5 x 10^5 cells per well. The medium (RPMI 1640 and 10-30% FCS) and non-adherent cells were then removed by pipetting and a small volume of bacterial suspension was added. As a small volume of medium always remained in the wells following pipetting, 20-30 uls. of bacterial suspension were generally found to be sufficient to cover the well. Using this small volume, a macrophage to bacteria ratio of 10:1 could be used and efficient killing demonstrated.

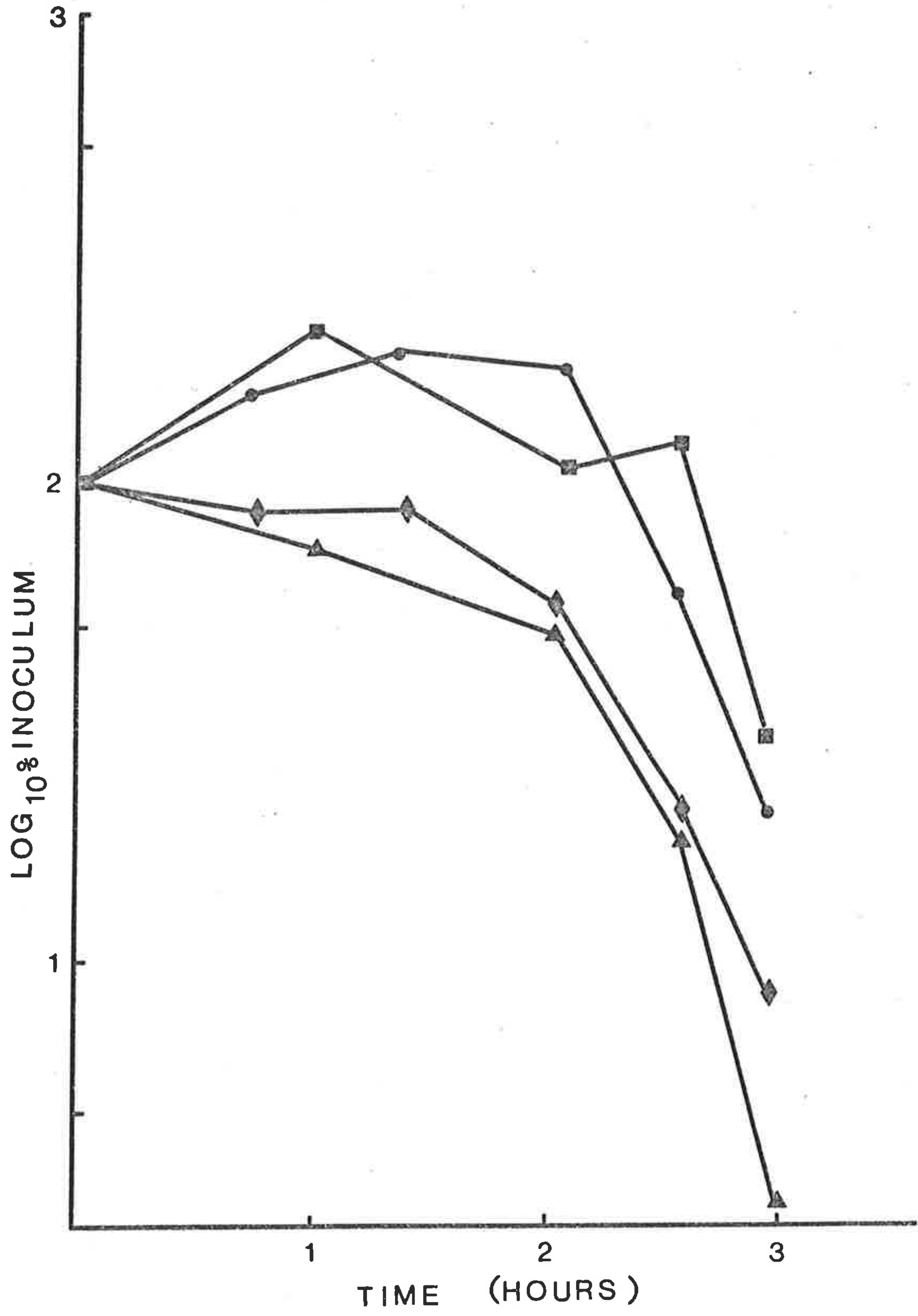
Generally, however, a slighly higher ratio was used : approximately 50:1.

Killing was determined by incubating the trays at 37^{0} and samples were obtained for viable counting during the ensuing hours. These were performed by disrupting the macrophages with Triton-X non-ionic detergent. The final concentration of Triton-X in the wells was 0.5%. This concentration did not cause a decrease in viability of any of the organisms tested during the course of sampling. Killing was therefore determined as the decrease in total number of bacteria in the wells during incubation. Control wells containing no macrophages were always included.

The medium used was RPMI 1640 (Grand Island Biochemical Co.) containing 20 mM HEPES . Incubations were carried out in 5% CO_2 in air. No antibiotics were found to be necessary and were not included. Maximal killing was observed in the presence of high concentrations of FCS. Up to 30% FCS was found to improve the performance of macrophages (Figure 4.1). The reason for this is not immediately obvious. It may be that FCS provides some opsonin or stimulating molecule for macrophages: perhaps even the presence of trace amounts of lipopolysaccharide might enhance the activity of these cells in vitro. It has been found more recently that lower quantities of heterologous serum can be used (FCS or rabbit serum) if it is fresh and not heat-inactivated (Musa and Marneerushapisal, personal communication). It has been suggested that this might represent the contribution of complement components, some of which are known to stimulate macrophages (Schorlemmer and Alllison, 1976). Homologous antiserum was also added in these experiments to permit killing to proceed. A concentration of 1/1000 was generally used as higher concentrations of mouse serum were found to be mildly

Figure 4.1

Effect of the concentration of foetal calf serum (FCS)on the killing of V. cholerae 569B by monolayers of normal peritoneal macrophages, in the presence of 2 HAU of antiserum. (●) 2% FCS, (■) 10% FCS, (♦) 20% FCS, (▲) 30% FCS.



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LOG 10 % INOCULUM
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2
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TIME (HOURS)

inhibitory. Such a concentration generally contained 1-2 haemagglutinating units (HAU), with the antisera that were available.

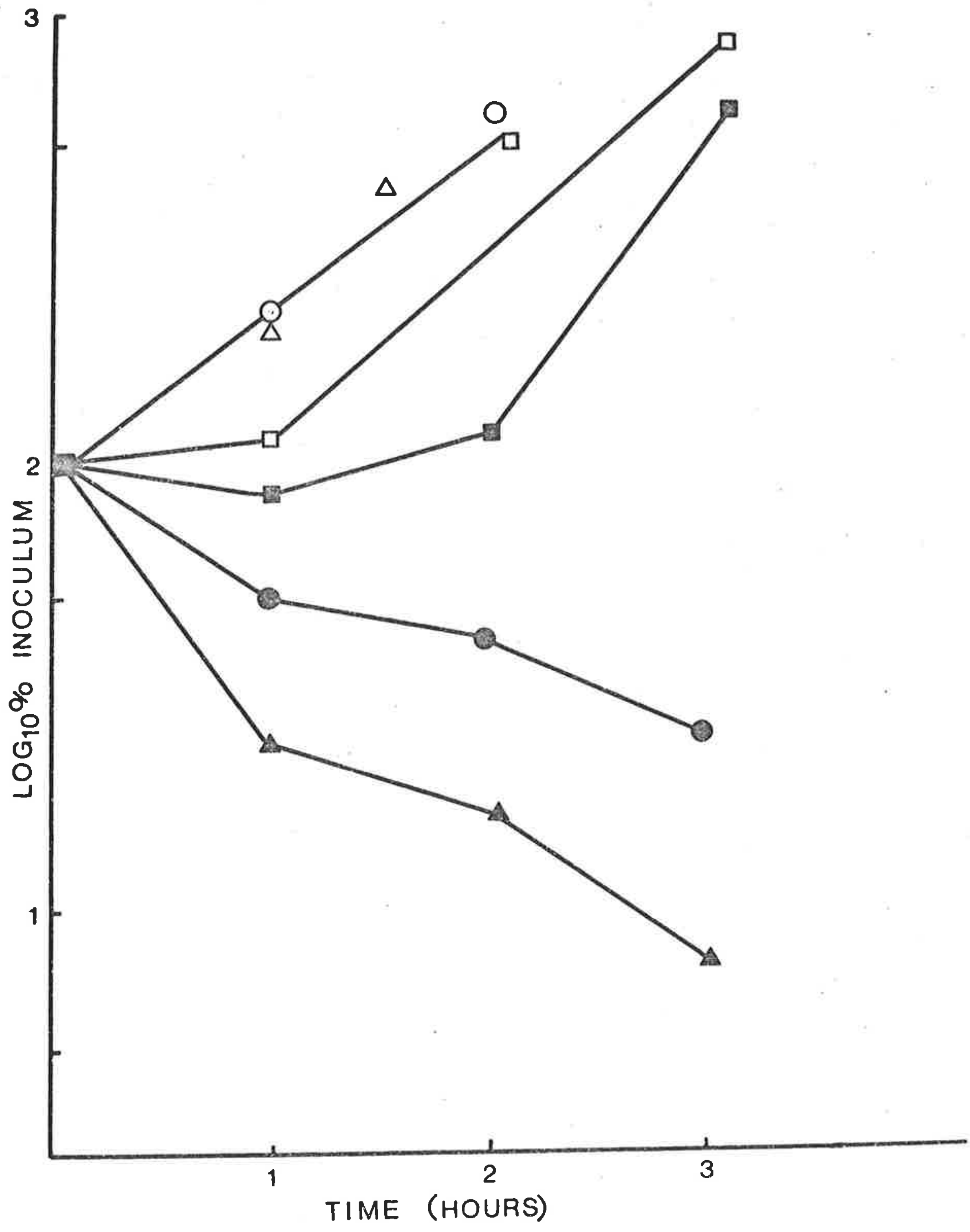
4.2 REQUIREMENT FOR ANTIBODY FOR THE DESTRUCTION OF SALMONELLAE

Normal macrophages were asayed for their ability to kill virulent Salmonella typhimurium C5 and it became clear that antibody was required for any killing to occur (Figure 4.2). This was found to be true in most experiments. However, some batches of FCS (Commonwealth Serum Laboratories, Victoria) were found to permit killing to occur in the absence of added antibody. Absorption of this serum with acetone-killed S. typhimurium removed this effect. Whether this was due to contamination of the FCS with trace amounts of antibody, presumably of adult bovine origin, or to the presence of other opsonins, effective at high concentrations of FCS, is not clear. With the exception of these batches of FCS, antibody was absolutely required for the killing of any salmonellae to occur. Antibody alone, in the absence of macrophages, did not produce significant killing of the inoculum. 0.5-1 HAU of mouse antiserum to S. typhimurium C5 was added to the cultures.

The virulence of bacterial species is generally determined by their ability to persist and multiply within the host (Collins, 1974). Hohmann, Schmidt and Rowley (1979), while studying oral infection of mice with salmonella sp., have demonstrated that virulent salmonellae are able to persist and multiply within the Peyer's patches of the intestine. Whether or not these events in the Peyer's patches represent interactions between bacteria and the macrophages known to exist within the patches (Lefevre, Hammer and Joel, 1979), it seems reasonable that this is the case.

Figure 4.2

Killing of S. typhimurium C5, by normal (○ , ●) and L.P.S.-activated macrophages (△ , ▲) from LACA strain mice in the absence (○ , △) or presence (● , ▲) of 2 H.A.U. of specific mouse antiserum. Control wells (□, ■).


LOG10% INOCULUM
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TIME (HOURS)

To determine if macrophages were capable of destroying avirulent salmonellae in the absence of antibody and if virulence could be correlated with requirements for antibody, strains of salmonellae of varying virulence to the mouse and an avirulent strain of *Escherichia coli*, as described by Hohmann et al. (1979) and summarised in Table 4.1, were cultured with normal peritoneal macrophages *in vitro* (Figures 4.3 and 4.4). A similar decrease in the number of bacteria was observed with all of these bacteria during the first hour of incubation. The avirulent strains (*S. typhimurium* M206 and *E. coli* F492) failed to increase in number during the following hours. The strains of intermediate virulence (*S. typhimurium* 885, *S. adelaide* and *S. enteritidis* 11RX) slowly increased while the highly virulent *S. typhimurium* C5 rapidly grew in the cultures. All strains required the presence of specific antiserum for any killing to occur. The virulence of these strains can therefore be correlated with their ability to grow in the presence of normal macrophages and not with varying requirements for antibody.

4.3 DESTRUCTION OF SALMONELLAE BY ACTIVATED MACROPHAGES

Two methods of *in vivo* activation of macrophages were used for these studies. Infection of mice with *S. enteritidis* 11RX *via* the peritoneum has been described to produce T-cell dependent activation of macrophages (La Posta, Ashley and Kotlarski, 1982). Peritoneal exudate cells were harvested six days after intra-peritoneal injection of 10^5 *S. enteritidis* (11RX-activated cells). Lipopolysaccharide (LPS) has been described in a variety of conditions to produce stimulation or activation of macrophages (Doe

Table 4.1

Characteristics of bacteria used in assays of phagocytosis (from Hohmann, 1980).

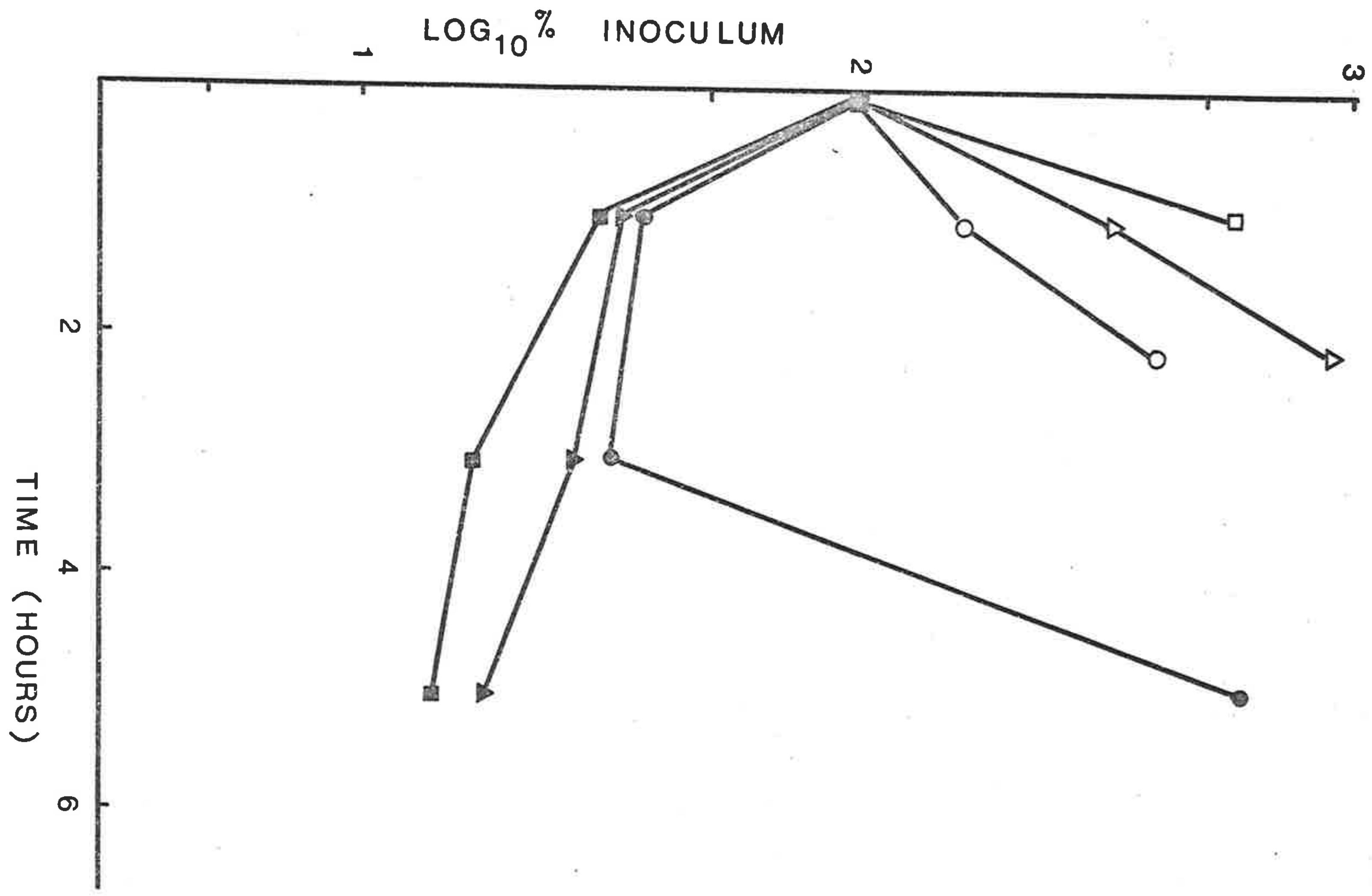
Bacterium	Oral LD_{50}	Multiplication*
S. typhimurium C5	10^{5}	12.5
S. typhimurium F885	10^{10}	10.0
S. enteritidis 11RX	10^{10}	11.6
S. adelaide	N.A.	N.A.
S. typhimurium M206	$>10^{11}$	0
E. coli F885	$>10^{11}$	0

* number of divisions/day in Peyer's patches

N.A. not available

Figure 4.3

Killing of bacteria by normal peritoneal macrophages in the presence of 2 H.A.U. of mouse antiserum *in vitro*. (●) *S. typhimurium* C5; (▲) *S. typhimurium* M206; (■) *E. coli* F492. Control growth in the absence of cells (○ , △ , □).



LOG10 % INOCULUM
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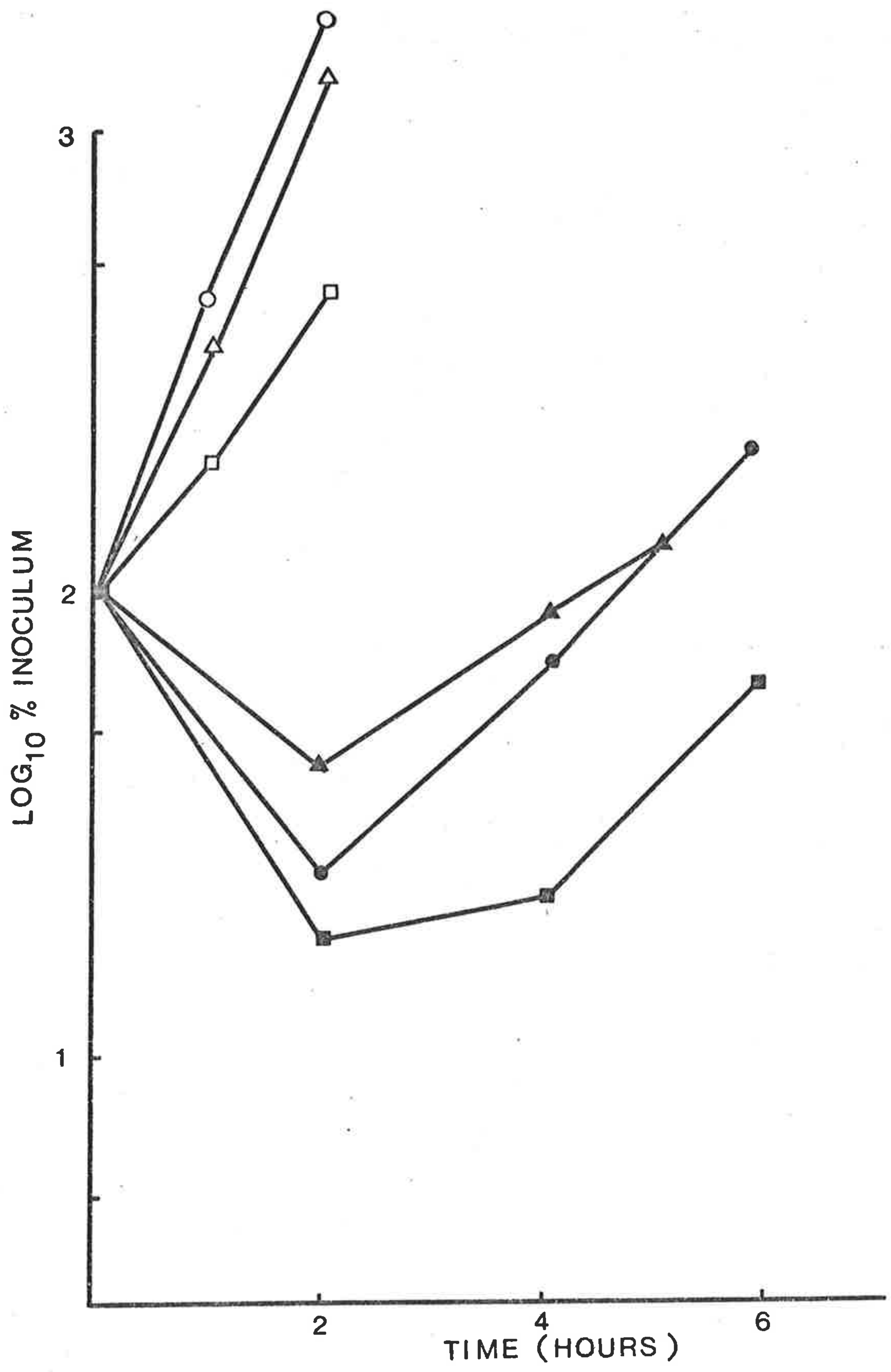
2
3
TIME (HOURS)
2
4
6

Figure 4.4

Killing of bacteria by normal peritoneal macrophages in the presence of 2 H.A.U. of mouse antiserum *in vitro*. (●) *S. enteritidis* 11RX; (▲) *S. adelaide*; (■) *S. typhimurium* F885. Control growth in the absence of cells (○, △, □).



LOG$_{10}$ % INOCULUM
3
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1
2
4
6
TIME (HOURS)

and Henson, 1978; Doe et al., 1978; Alexander and Evans, 1971; Currie and Basham, 1979). Mice were injected with 1 ug. of S. typhimurium C5 LPS and cells (LPS-activated)were harvested two days later. Doe and Henson (1978) used a two-stage procedure for activating macrophages with LPS and it is possible that initial eliciting of a peritoneal exudate with a stimulant such as thioglycolate is important for establishing full activation by this method. Cohn (1978) has suggested that LPS does not produce complete tumoricidal and microbicidal activation.

Both 11RX- and LPS-activated cells exhibited an enhanced capacity to destroy S. typhimurium C5 in the presence of antibody. Without antibody, activated macrophages were quite unable to kill these bacteria. Results from LPS-activated cells are shown in Figure 4.2. Similar effects were obtained with 11RX-activated cells. With respect to normal cells, these demonstrated no qualitative differences in their requirement for antibody. Whether or not quantitative differences existed was not determined. Reported changes in the density and avidity of Fc receptors on activated macrophages (Rhodes, 1975) suggest that activated cells might require lower levels of opsonising antibodies.

4.4 H_2O_2 PRODUCTION BY ACTIVATED MACROPHAGES

In addition to measuring the bactericidal capacity of these cells, their ability to release H_2O_2 was also determined. It has been suggested that this is the best biochemical correlate of activation (Nathan, Noguiera et al., 1979). Both LPS- and 11RX-activated cells were capable of releasing significant levels of H_2O_2 (Table 4.2). Macrophages from normal animals were unable

Table 4.2

Production of H_2O_2 by peritoneal exudate cells from specific pathogen free mice (S.P.F.) or conventional mice following in vivo activation by intra-peritoneal injection of S. enteritidis, 11RX, or 1 ug. of S. typhimurium L.P.S.

Mice	Activation	H_2O_2 Production*
S.P.F.	11RX	2.4$\pm$1.3
	L.P.S.	0.4$\pm$0.4
	nil	$<$ 0.1
Conventional	11RX	2.0$\pm$0.4
	L.P.S.	1.4$\pm$0.7
	nil	$<$ 0.1

*nM/10^6 cells/5 mins. $\pm$ 1 s.d.

to produce H_2O_2.

The mice used in all these experiments (LACA mice) were bred in specific pathogen free facilities (spf mice). During their use in experiments, they are housed under conventional conditions. We have previously reported that the immunoglobulin levels in the serum of these mice rapidly increased during their first exposure to conventional conditions (Horsfall, Cooper and Rowley, 1978). This appears to occur due to the increased but normal levels of antigen to which they are exposed. During the present studies, it was observed that lower numbers of cells could be harvested from the peritonea of spf mice compared with fully conventionalised animals (1-2 x 10^6 from spf mice compared with 5 x 10^6 from conventionalised mice). Furthermore the cells obtained from spf mice possessed very poor bactericidal activity. Most interesting, however, was the fact that LPS failed to elicit an active peritoneal exudate in these mice. Two days after LPS injection, the macrophages harvested from the peritoneum were still poorly bactericidal and released only low levels of H_2O_2 (Table 4.2). 11RX-activation was effective in these mice.

These observations perhaps reflect the two-step process of eliciting and activation used by Doe and Henson (1978) to produce active macrophages with LPS. Presumably the higher numbers of cells in the peritonea of normal, conventionalised mice are elicited in response to the same increased antigen loads responsible for increases in immunoglobulin levels. Cohn (1978) has suggested that macrophages similar to elicited cells are the important cells in the day to day functions of the reticulo-endothelial system. It seems reasonable that these higher antigen loads are reflected not only in the stimulation of the macrophage population but also in the

immunoglobulin levels, and thereby natural antibody levels, and that these effects act together to increase general resistance of the host.

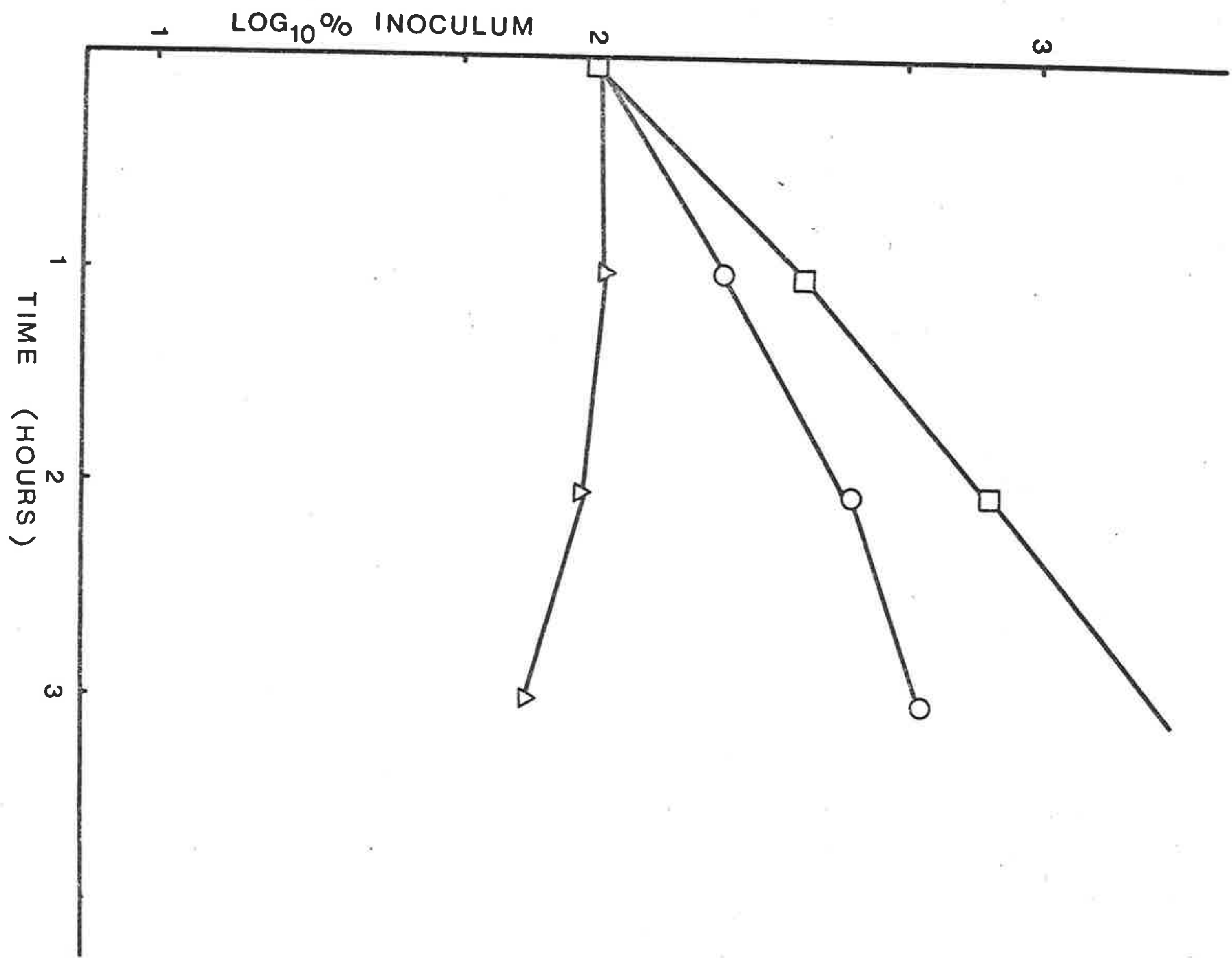
4.5 DESTRUCTION OF LISTERIA MONOCYTOGENES

The results with S. typhimurium endorsed the belief that both normal and activated macrophages required antibody for the destruction of this bacterium. It remained to be established if this was true for other, so-called intra-cellular bacteria. Similar experiments were therefore performed using L. monocytogenes. Initial experiments using normal and 11RX-activated cells failed to demonstrate any killing at all of L. monocytogenes, either in the presence or absence of antibody. It was found that killing could be demonstrated only if the bacteria and macrophages were mixed immediately after harvesting the cells and then added to micro-culture trays. That is, no initial incubation time for a macrophage monolayer to form could be allowed if killing was to be demonstrated. Instead bacteria and cells were mixed in numbers equivalent to those used in normal experiments and 30 uls. of this mixture was then added to the wells. In this circumstance, killing of L. monocytogenes could be demonstrated by 11RX-activated cells (Figure 4.5). Incubation of the peritoneal exudate cells for three hours prior to the addition of bacteria completely removed the ability of these cells to kill listeria (Figure 4.6). No killing could be observed with normal macrophages although some decrease in growth rate of the organism was noted. LPS-activated cells also failed to demonstrate killing.

The presence or absence of specific antiserum did not

Figure 4.5

Killing of *L. monocytogenes* by normal (○) and 11RX-activated (△) macrophages from LACA strain mice in the absence of antiserum *in vitro*. Bacteria were added at the beginning of *in vitro* cultivation. Control growth (□).



LOG_{10}% INOCULUM
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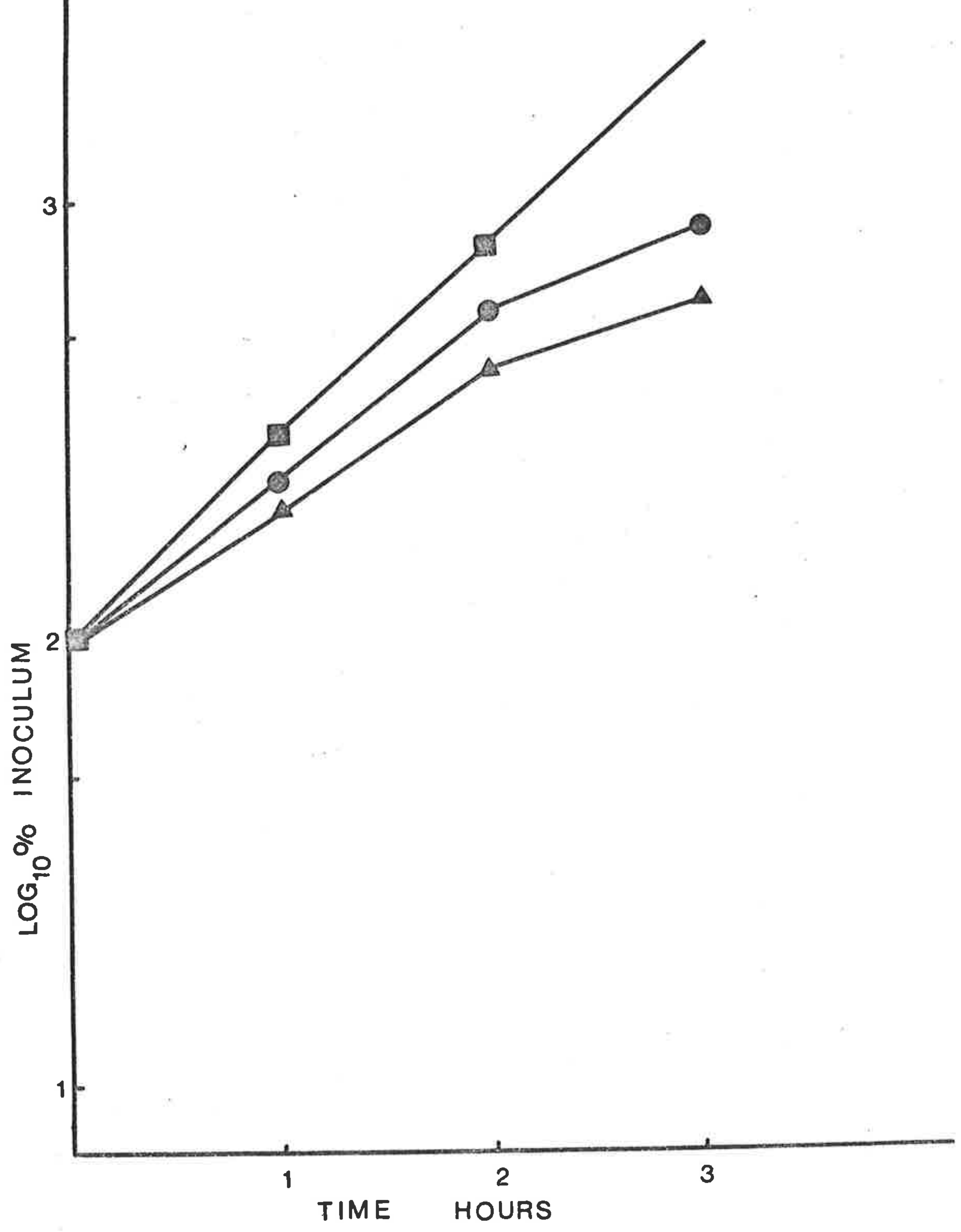
2
3
TIME (HOURS)
1
2
3

Figure 4.6

Killing of L. monocytogenes by normal (●) and 11RX-activated macrophages (▲) in the absence of antiserum in vitro. Macrophages were incubated for 3 hours before bacteria were added. Control growth (■).



3
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LOG10% INOCULUM
1
2
3
TIME HOURS

alter these findings. Approximately 1 bacterial agglutinating unit was added to the cultures. It was evident that no added antibody was required for the ingestion and killing of L. monocytogenes by activated macrophages.

Several important differences can therefore be seen between the killing of S. typhimurium and L. monocytogenes by normal and activated macrophages. Firstly antibody is obviously required for the destruction of salmonellae but does not appear to be needed in this system for the destruction of listeria. Secondly, normal macrophage populations contain a significant percentage of cells capable of killing virulent salmonellae. These cells cannot destroy listeria. Thirdly, enhanced killing of salmonellae by activated macrophages is not immediately lost on culture as is the activity against listeria. Fourthly, while LPS-activated cells are able to kill salmonellae better than normal cells, they are not able to kill listeria, and can therefore be considered as not fully activated despite an ability to produce significant levels of H_2O_2.

It is possible that some of these differences may be explained by the addition of endogenous LPS with salmonellae, particularly with regard to the apparent differences in degree of activation and the loss of activation on culture. Listericidal activity of cells cultured in the presence of LPS was not determined but might alter the interpretation of these findings.

Nevertheless, the primary aim of these experiments was fulfilled. The finding that antibody to salmonellae was required for their destruction but not for that of listeria helped explain the apparent conflict between studies implicating antibody in acquired immunity to salmonella and those supporting the generally, non-specific nature of cellular immunity particularly with regard to

listeria. However. the criticism mentioned earlier of the possible contribution of cell-bound antibody could still be applied to this system. Furthermore the presence of high levels of FCS in this assay could provide other opsonins that replaced a requirement for antibody. It was therefore decided to investigate further the initial interaction between macrophage and bacteria, with the hope of describing significant differences between the binding of salmonellae and listeria to macrophages.

4.6 BINDING OF BACTERIA TO MACROPHAGES

In order to follow the binding of bacteria to macrophages, monolayers of cells were prepared on flying coverslips in Leighton tubes. Approximately 1 x 10^6 cells in 1 ml. of tissue culture medium were added to the tubes and incubated at 37^0 for 1-2 hours. The coverslips were then washed by gently pipetting fresh medium over them and bacterial suspensions in medium were then added. A relative excess of bacteria was added (over 10^7/ml.) in order to ensure good contact between macrophages and bacteria. After a further incubation at 37^0, the monolayers were again washed and stained with Wright's stain. The coverslips were then examined and the percentage of macrophages with one or more bacteria attached was then determined. Coverslips were prepared in triplicate and 100 cells were counted on each coverslip.

When the cells were cultured in RPMI 1640 containing 10% FCS, S. typhimurium C5 was found to bind only in the presence of antibody (1 HAU). L. monocytogenes bound to macrophages in its absence and the presence of antibody (1 bacterial agglutinating unit) did not significantly increase this binding. Similar results

were obtained in RPMI 1640 in the absence of FCS and in Dulbecco's balanced salt solution (BSS). Clearly, FCS was not a source of opsonin for listeria (Table 4.5).

In a second experiment, L. monocytogenes and S. typhimurium C5 were mixed together and then added to the monolayers to determine if these organisms were binding to the same or different sub-populations of cells. S. typhimurium was incubated with specific antiserum prior to being added to the L. monocytogenes. No antiserum to listeria was incorporated. After incubation with these bacteria, the coverslips were washed and stained with Gram's stain in order to distinguish the two species. The two bacteria ,were seen to bind to the same cells in all circumstances and there was no evidence of inhibition of one species by the other.

4.7 INHIBITION OF BINDING WITH Fc FRAGMENTS

Of the known receptors on the macrophage surface, the Fc receptor is of particular importance in the binding of opsonised bacteria. The evidence presented above that the two organisms did not seem to compete with receptors on the macrophage surface could be quantitated better by inhibition of one or the other. Antibody mediated binding could be expected to occur via the Fc receptor, especially in the absence of serum-derived complement factors. Therefore, inhibition of binding via this receptor was attempted. Soluble complexes of tri-nitro-phenol (TNP) coupled to bovine serum albumin incubated in rabbit anti-TNP antiserum were prepared at varying ratios of antigen to antiserum. No inhibition of antibody-mediated binding of S. typhimurium could be produced when

these complexes were incubated with the cells before and during incubation with the bacteria. Nor could inhibition be produced using heat-aggregated rabbit IgG (1 mg/ml). The reasons for these failures are not obvious although the composition of soluble complexes that are efficient at binding to Fc receptors is critical and presumably the correct conditions were not found in this system (Harkiss and Brown, 1981).

An alternative approach was to prepare Fc fragments from rabbit IgG. Rabbit IgG is reported to be opsonic for mouse macrophages in an Fc dependent fashion (Steele, Chaicumpa and Rowley, 1975). Fc fragments were prepared from rabbit IgG by pepsin digestion and purified by chromatography on Sephadex G200. After concentration with aquacide and dialysis against saline to 200 ug/ml, these Fc fragments were incubated with the macrophage monolayers in RPMI 1640 at room temperature just prior to the addition of the bacteria and antiserum, and during a subsequent further 30 minute incubation. Fc fragments at 20 ug/ml produced significant inhibition of antibody-mediated binding of _S. typhimurium_. The binding of _L. monocytogenes_ was not inhibited by these fragments (Table 4.3).

4.8 EFFECT OF SUGARS ON THE BINDING OF LISTERIA TO MACROPHAGES

Orgmundsdottir and others (Orgmundsdottir and Weir, 1976; Orgmundsdottir, Weir and Marmion, 1978) have reported that binding of _Corynebacterium parvum_ to macrophages occurs in serum free medium by way of a lectin-like interaction that may be inhibited by a variety of sugars. This binding is also independent of antibody and it was possible that a similar mechanism could explain the

Table 4.3

Effect of Fc-fragments of rabbit IgG (1 mg./ml) on binding of bacteria to peritoneal macrophages.

Organism	Antiserum[1]	Fc	% cells[2]
L.monocytogenes	-	-	49$\pm$8
	-	+	41$\pm$6
S.typhimurium	+	-	44$\pm$4
	+	+	16$\pm$1

[1] 2 H.A.U. of LACA anti-C5 antiserum added with S. typhimurium.

[2] the percentage of macrophages binding bacteria $\pm$ 1 s.d.

attachmant of L. monocytogenes to macrophages, although the binding of listeria occurs in high concentrations of FCS which could be expected to inhibit the lectin-like binding of C. parvum.

Accordingly, a variety of sugars were assayed for their ability to inhibit the binding of L. monocytogenes (glucose, mannose, galactosamine, fructose, galactose, sorbitol, xylose, arabinose). Monolayers were prepared in serum free Dulbecco's BSS and sugars added to a final concentration of 20 mM 15 minutes before the addition of bacteria. No antiserum was added. No inhibition was evident with any of these sugars (examples of which are given in Table 4.4).

4.9 EFFECT OF Ca^{++} and Mg^{++} DEPLETION ON ATTACHMENT OF LISTERIA

Orgmundsdottir and Weir (1976) have also reported that attachment of C. parvum is dependent on the presence of divalent cations. Despite the lack of inhibition of attachment of listeria by Fc fragments, the possibility still remained that cell-bound antibody was present. Therefore attempts were continued to inhibit the attachment of listeria in some fashion that would clearly ditinguish it from antibody-mediated events.

Macrophages were cultured in Dulbecco's BSS, prepared without Ca^{++} or Mg^{++}. After washing of the monolayers, Dulbecco's BSS , without divalent cations and containing 0.1% EDTA , was added with bacteria. The attachment of S. typhimurium in the presence of antibody was not inhibited under these conditions. However, the binding of L. monocytogenes, in the absence of antiserum, was strongly inhibited (Table 4.5). L. monocytogenes did bind at normal levels in the presence of added antiserum under

Table 4.4

Effect of sugars on the binding of L. monocytogenes to macrophages monolayers in serum free medium.

Sugar (20mM)	% Macrophages binding listeria
nil	45 ± 15
glucose	65 ± 20
mannose	46 ± 9
galactose	31 ± 9

Table 4.5

Binding of bacteria to monolayers of peritoneal macrophages in Ca^{++}, Mg^{++} containing medium or in medium depleted of cations.

Organism	Medium	Antiserum[1]	% cells[1]
L.monocytogenes	Normal	-	51 ± 9
		+	59+13
	Ca^{++} Mg^{++}	-	15 ± 1
	depleted	+	48 ± 15
S.typhimurium	Normal	-	< 5
		+	67 ± 1
	Ca^{++} Mg^{++}	-	< 5
	depleted	+	60 ± 10

[1] 1 bacterial agglutinating unit of mouse anti-listeria antiserum, or 2 H.A.U. mouse anti-C5 antiserum added with respective bacteria.

[2] the percentage of macrophages binding bacteria.

these conditions.

This result clearly establishes that *L. monocytogenes* can bind to macrophages without the need for the presence of any antibody and that the existence of cell-bound antibody does not explain these findings. It appears that listeria uses a receptor on the macrophage membrane that is distinct from the Fc receptor and is not antibody-dependent.

4.10 CONCLUSIONS

While cell-mediated immunity has been demonstrated to be important in resistance to infection with salmonella, listeria and other intra-cellular organisms, these experiments demonstrate that important differences exist in the relative importance of antibody for the expression of this immunity to listeria and salmonella. While salmonellae will only bind to macrophages in the presence of opsonins, particularly antibody, listeria may attach to these cells *via* an unknown mechanism that does not require the presence of antibody. No evidence was found to support the concept that the process of activation alters the requirement of macrophages for antibody as an opsonin.

CHAPTER 5

HUMORAL IMMUNITY TO PULMONARY INFECTION WITH *KLEBSIELLA PNEUMONIAE*

The lungs are subjected to a continual barrage of infective particles from the environment. Non-specific defenses relying on filtering of the inspired air and removal of particles by muco-ciliary action are responsible in large measure for the protection of the airways from constant infection. Such defenses may fail in a variety of situations including many induced by environmental pollutants such as cigarette smoke.

In addition to these non-specific defenses, most arms of the immune system are represented in the lung and airways. Immunoglobulins are present at all levels of the respiratory tract and the development of humoral responses has been correlated with immunity to a variety of micro-organisms. In general, the ultimate method of disposal of pathogens reaching the lower respiratory tract appears to rest in the phagocytic activity of alveolar macrophages (Green and Kass, 1964 a,b). Antibodies appear to be important in this situation as opsonins for the promotion of phagocytosis by these cells (Robertson and Sia, 1927; Heidelberger et al., 1947). Particular attention was paid in these studies to the importance of antibody in promoting the phagocytosis of encapsulated organisms such as *Streptococcus pneumoniae* or *Klebsiella pneumoniae*.

The presence of macrophages in an environment of high antigen exposure such as the lung suggests the possibilty that stimulation or activation of these cells might occur. Alveolar macrophages do exhibit some characteristics of activated cells (as

discussed in Chapter 2). The ability of the lungs to resist potentially pathogenic micro-organisms could therefore also depend on the level of reticulo-endothelial activity expressed in the lung. In the light of findings with the importance of antibody in the expression of acquired cellular immunity to some organisms described in the preceding chapter, it is clear that both humoral and cellular components may be required for complete immunity.

While antibody has generally been found to be importanmt in mediating resistance to encapsulated organisms, non-specific immunity has been described to Klebsiella pneumoniae that appears to possess some of the characteristics of acquired cellular immunity. Parant and others (Parant et al., 1967; Parant et al., 1976) have reported that resistance to intra-venous challenge with K. pneumoniae may be enhanced by prior exposure of the animal to heterologous lipopolysaccharide. The particular pathways mediating this immunity are not clear. Experience with salmonellae, suggest that if this immunity is in part dependent on activation of the reticulo-endothelial system then this activation would not alter the assessment of the importance of antibody in resistance to this organism.

The aim of these studies was to investigate the development of immunity to Klebsiella pneumoniae in the mouse, and to investigate the various relationships that exist between humoral and cellular factors operating in the lungs. Immunoglobulins of IgA, IgG and IgE classes all exist in pulmonary tissue or secretions. IgA is present in highest quantities in the upper respiratory tract where it has been demonstrated to be protective against viral infection (Bellanti, Artenstein and Buescher, 1965; Smith et al., 1966). IgG would appear to be more plentiful in the lower airways (

Kaltreider and Chan, 1976). Immunoglobulins of this class could be expected to act as opsonins for alveolar macrophages (Reynolds and Thompson, 1973b). The possible role of IgE in protection to micro-organisms is not clear although its involvement in allergic reactions is all too clear (Kaltreider, 1976). Neither complement nor IgM are found in significant quantities in normal pulmonary secretions (Reynolds and Thompson, 1973a), although inflammation of the pulmonary tissue would be expected to permit the rapid exudation of serum factors,including IgM and complement, into these tissues.

While clear roles for serum derived IgM and locally produced IgG can be envisaged in destruction of bacteria in the lungs, little evidence exists that IgA may also provide protection. However the presence of high concentrations of IgA in the upper respiratory tract could surely influence resistance either in a positive way or in a negative fashion by inhibiting the action of other classes of immunoglobulin, as has been described in a complement dependent bactericidal system (Griffiss, 1975; Griffiss and Bertram, 1977). To investigate these interactions, the development of specific immunity to *Klebsiella pneumoniae* was studied. Antibody was found to be highly protective in the lung. Following the description of this immunity, the role of locally produced IgA antibody was then investigated and the interactions of immunoglobulin classes with alveolar macrophages studied. This chapter presents the evidence regarding humoral immunity to *K. pneumoniae*. In investigating the host-bacteria relationships in the lung, a new antigen of *K. pneumoniae* was described. Antibody against this non-capsular antigen is extremely protective.

5.1 MODEL OF MURINE KLEBSIELLA INFECTION

A model of pneumonia induced by klebsiella in a variety of laboratory animals has been described . Following intra-nasal infection of mice (Berendt, Long and Walker, 1975), or aerosol infection of rats (Berendt et al., 1977) with virulent K. pneumoniae, a destructive, purulent pneumonia develops. In mice, this is characterised by the development of polymorphonuclear infiltrates after the first 24 hours. These foci become quite extensive during the following 48 hours and infective foci, surrounded by capsular material, are also found in the lung. Infection of the liver, spleen and kidneys follows (Berendt, Long and Walker, 1975).

It was felt that this would provide a good model of the infection in humans. Accordingly, several strains of K. pneumoniae were obtained from clinical isolates. The majority of these (from the Commonwealth Serum Laboratories, Victoria; the Department of Bacteriology, University of Queensland; and the Institute of Medical and Veterinary Science, Adelaide) were from patients with pulmonary infections. Mice were anaesthetised with pentobarbitone sodium (0.06 mg/G, intra-peritoneally and bacterial suspensions were inoculated onto their nares, 0.05 mls. being inoculated. None of these strains were capable of producing fatal infections in mice. Several strains were passaged intra- peritoneally, and subsequently intra-nasally, but still failed to produce infection with less than 10^5-10^6 organisms, given intra-nasally.

The original strain described by Berendt, Long and Walker (1975) was kindly provided by Dr. Berendt, of Fort Detrick, Maryland. This organism, K. pneumoniae type 1, was highly virulent

to mice when given intra-nasally. The 50% lethal dose (LD_{50}) of this organism was less than 5 organisms. To maintain virulence in the organism during the course of experiments, intra-nasal passage was performed. Mice were infected intra-nasally (see section 5.2) and after 2-3 days bacteria were isolated from lung homogenates of infected animals by plating on nutrient agar. Colonies were taken from the plates and suspended in peptone saline. Aliquots of this suspension were stored at -80^{0} and thawed and inoculated into trypticase soy broth when needed.

All the organisms described as K. pneumoniae conformed with the biochemical reactions defined by the Subcommittee on the Taxonomy of the Enterobacteriaceae (1963) and Kauffmann (1966). In particular, all strains were; methyl red negative, Voges-Proskauer positive, lysine decarboxylase positive, arginie dihydrolase negative and ornithine decarboxylase negative.

5.2 INFECTION WITH KLEBSIELLA PNEUMONIAE TYPE 1

Mice were routinely anaesthetised and infected intra-nasally. Following infection with a lethal dose of organisms, the mice begin to appear ill after two days with ruffled fur and behaving lethargically. At this time, their lungs show gross evidence of consolidation, with a thick purulent exudate and areas of bullous destruction of lung parenchyma. Death occurs on the fourth to fifth day after infection.

The progress of the illness can be followed by performing viable counts on the lungs and other tissues of mice. Lungs, spleens and livers were removed from infected mice and homogenised. Viable counts were performed on these homogenates and on samples of blood

taken from the retro-orbital plexus (Figure 5.1). Viable organisms can be recovered from the lungs as soon as 6 hours following infection. No organisms are found in the blood or in other organs at this time. Infection of the lungs would therefore appear to occur by inhalation of the inoculum and not via direct invasion of the blood stream from the naso-pharynx. Infection thus appears to occur in the same fashion as in human patients where lower respiratory tract involvement follows colonisation of the naso-pharynx, presumably by inhalation of infected secretions (Tillotson and Finland, 1969).

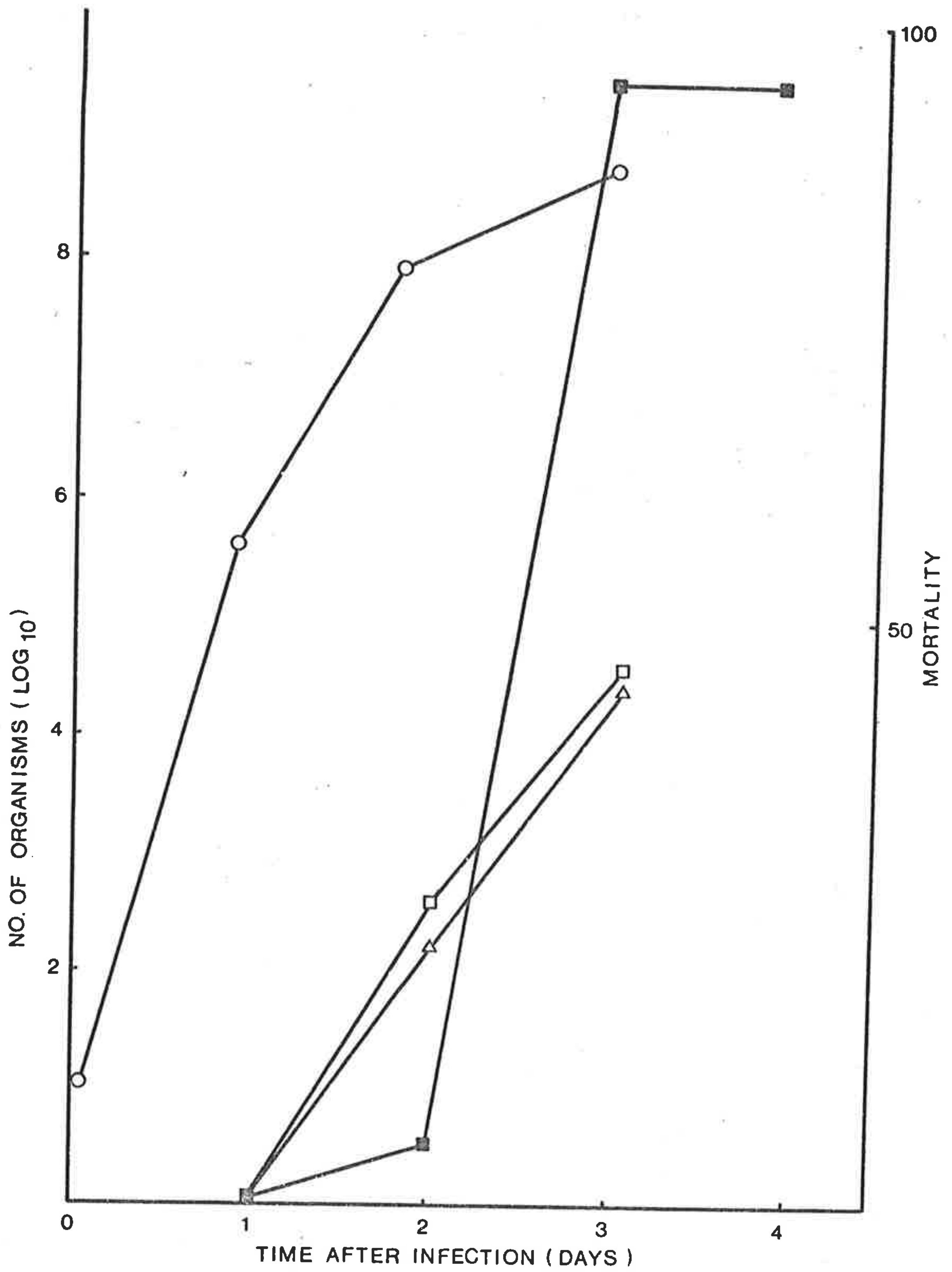
Septicaemia and infection of other organs occurs after two days and high numbers of organisms can subsequently be recovered from, the spleen and liver. At this time, large numbers of polymorphonuclear neutrophils may be found in bronchial washings of these mice. The total number of monocytic cells does not increase during the course of the infection (Figure 5.2). K. pneumoniae is poorly cleared from the lungs of granulocytopaenic mice and this has been sited as evidence that these cells are important in protection to this organism (Rehm, Gross and Pierce, 1980). Whether or not this massive influx of polymorphs is related to protection or recovery is not clear from these studies reporting a simple decrease in clearance rates.

5.3 PRELIMINARY PROTECTION EXPERIMENTS

By analogy with other pulmonary infections in which opsonising antibody has been reported to be critical in immunity , attempts were made to develop high levels of serum antibody in mice and to determine any subsequent protection. A vaccine was developed for active immunisation. In order to preserve all the antigens of

Figure 5.1

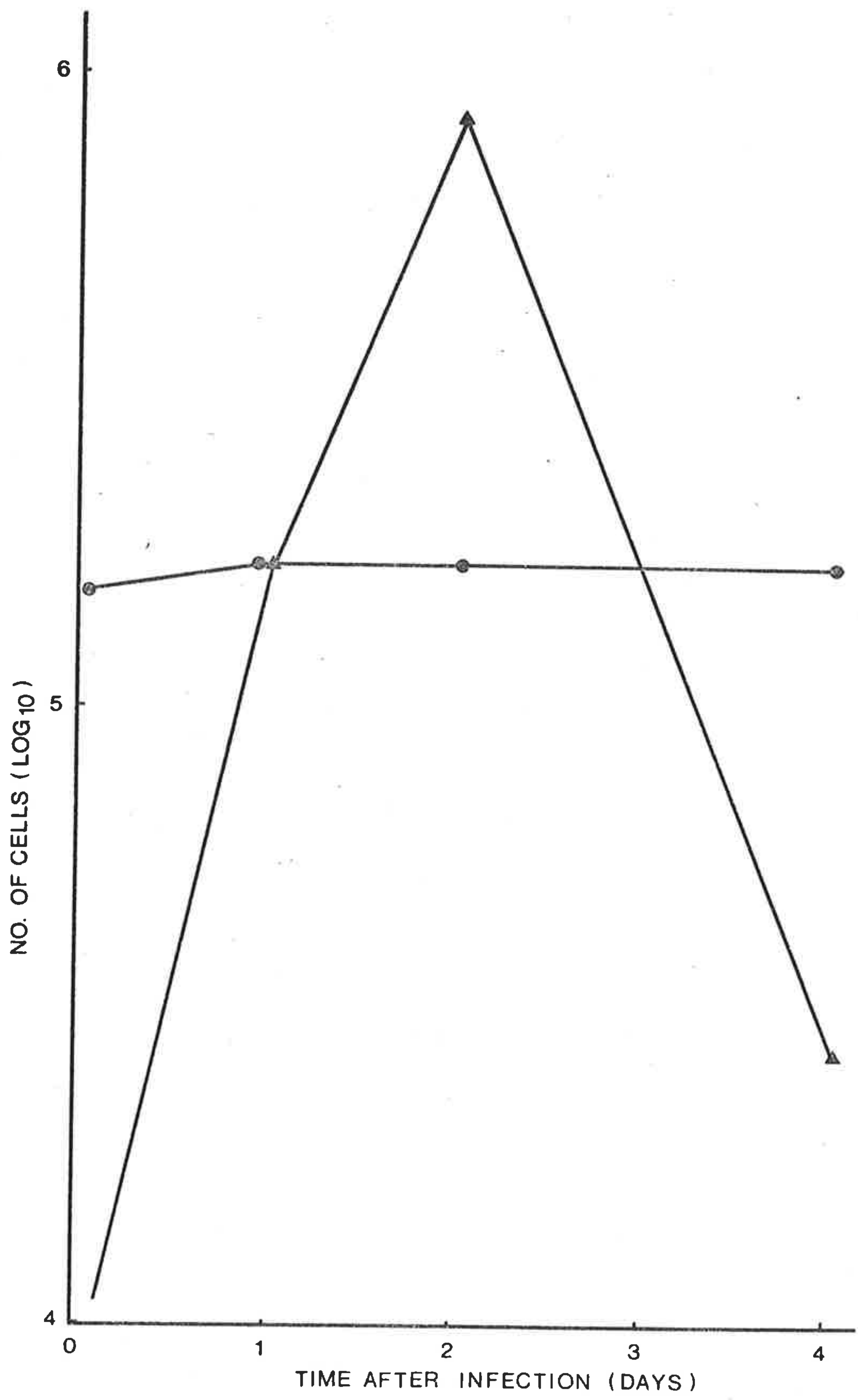
Growth of K. pneumoniae in the lungs (○), spleen (△) and blood (□) of mice infected with 12 organisms on day 0. The percentage mortality, of 20 mice, is also shown (■).



NO. OF ORGANISMS (LOG 10)
8
6
4
2
0
1
2
3
4
TIME AFTER INFECTION (DAYS)
100
50
MORTALITY

Figure 5.2

Recovery of cells from mouse lungs by bronchial lavage during infection with K. pneumoniae on day 0. Mononuclear cells (●), polymorphonuclear cells (▲).



6
5
4
NO. OF CELLS (LOG10)
0
1
2
3
4
TIME AFTER INFECTION (DAYS)

klebsiella, bacteria were exposed to ultra-violet irradiation to prepare the vaccine rather than more forceful means of killing the bacteria such as heat or alcohol. Mice were given three intra-venous injections of 10^8 ultra-violet irradiated organisms over two weeks. These animals were then challenged with Kpnl to determine the 50% lethal dose (LD_{50}). These mice were found to resist over 10^5 Kpnl given intra-nasally.

To determine if this protection was mediated by antibody, serum was obtained from mice after an extended course of injections (6 injections over a four week period). The antibody content of this serum was measured by haemagglutination of sheep red blood cells sensitised with capsular polysaccharide of Kpnl. Passive protection by this antiserum was assayed by incubating the bacteria with 2 haemagglutinating units (HAU) of antiserum for approximately 15 minutes, on ice. These bacteria were then inoculated into mice intra-nasally. The LD_{50} of these organisms was 2×10^3 organisms (determined by the method of Reed and Muench, 1938). This represented an increase of over 400-fold compared with unopsonised bacteria. Antiserum produced no loss of viability of the bacterial inoculum, the final concentration of antiserum being approximately 0.5%.

A standard protocol was designed to test the efficiency of various antibody preparations. An inoculum of between 20 and 50 organisms was found to give most consistent results in passive transfer experiments designed to determine the 50% protective dose (PD_{50}) of antisera. This inoculum was prepared from log-phase cultures the concentration of which was determined initially by counting in a Neubauer chamber. Viable counts were performed to confirm this estimate. The culture was then serially diluted in

trypticase soy broth to about 5-10 x 10^2/ml. Dilutions of antisera were then added and incubated on ice for a short period (10 to 20 minutes). Mice were then anaesthetised and infected intra-nasally.

The protective ability of serum was apparently linked to its antibody content determined by haemagglutination (Table 5.1). While the concentration of serum at the PD_{50} varies 80-fold, the haemagglutinating titre varies only 3-fold.

5.4 PROTECTION BY ANTIBODY POOLS FROM UV-SERUM

All sera assayed to this point were from animals immunised with ultra-violet irradiated organisms (UV-sera). As an initial step in the description of the protective ability of immunoglobulin classes, UV-serum was fractionated by chromatography on Protein A - Sepharose 4B. The IgG fraction was collected and was dialysed against phosphate buffered saline after acid-elution from the column, and then concentrated to the original volume of serum. The serum pool depleted of IgG (residual pool) was likewise concentrated. When these fractions were assayed for protection, the major proportion of the protective capacity was found to be in the IgG fraction. The PD_{50} of several IgG preparations was similar to that of whole serum when compared on the basis of haemagglutination. The residual pools were extremely poor at protection despite possessing similar haemagglutinating and haemolytic titres. Indeed, nearly 100 times the haemagglutinating titre of IgG was required to produce comparable protection (Figure 5.3). This was confirmed on several occasions.

The residual pools contained antibody with both haemagglutinating and haemolytic activity. Haemolytic activity was

Table 5.1

Correlation of haemagglutinating activity with protective capacity of UV-serum against intra-nasal challenge with Kpnl.

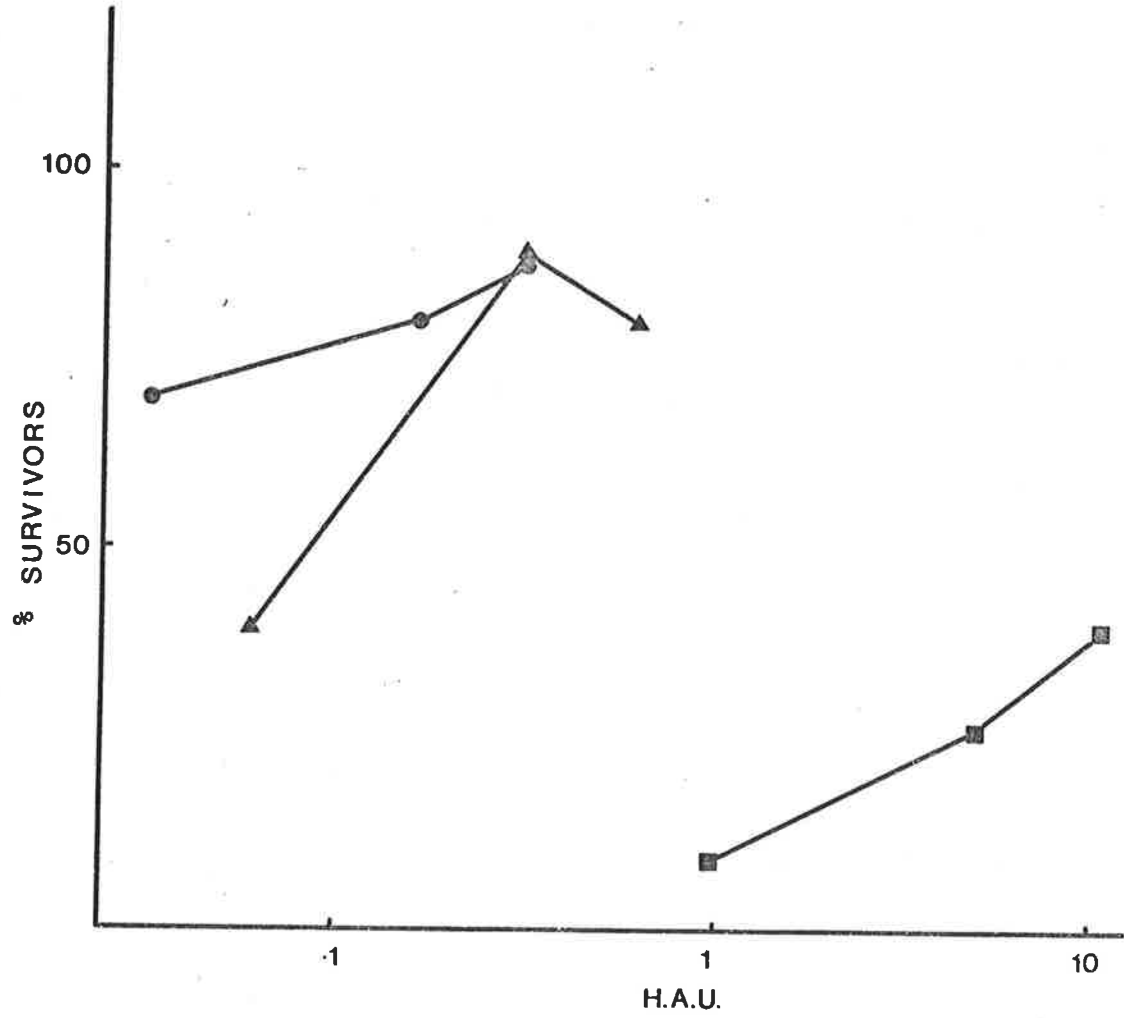
Serum	H.A.U.[1]	P.D.$_{50}$ as;	
		% serum[2]	H.A.U.[2]
A	512	6.0	1.5
B	4096	0.3	1.7
C	128	26	1.7

[1] H.A.U./ml. of serum

[2] amount of serum added to inoculum

Figure 5.3

Protection of mice to intra-nasal challenge with Kpnl by whole UV serum (●), IgG (▲), and the residual serum pool after removal of IgG (■). All control mice died. Percentage of experimental mice surviving are shown against the number of H.A.U. of antiserum given with the inoculum.


% SURVIVORS
100
50
·1
1
10
H.A.U.

detected by the incubation of serum with capsular polysaccharide sensitised red cells in the presence of fresh guinea pig serum, as a source of complement. Comparison of haemagglutinating and haemolytic titres confirmed that the principal class of antibody in the residual pool was IgM. This was confirmed by Ouchterlony gel diffusion, as was the purity of the IgG preparations.

Several alternative explanations appeared to be possible. If, as Green and Kass (1964a) have suggested, the alveolar macrophage is the important cell in immediate defense of the lower respiratory tract, then antibody could be expected to promote the destruction of micro-organisms by virtue of its opsonic capacities. IgM is known to be opsonic in the peritoneal cavity by two pathways. Lay and Nussenzweig (1969) have reported the presence of an Fc receptor for IgM on peritoneal macrophages. Secondly, IgM is highly efficient at fixing complement and can thereby also produce phagocytosis via complement receptors, especially for C3b, on macrophage membranes. It may be, however, that these pathways are not operative in the lung. Reynolds et al. (1975) failed to demonstrate Fc receptors for IgM on alveolar macrophages in humans . They have also reported that IgM-coated pseudomonas are not phagocytosed by alveolar macrophages (Reynolds and Thompson, 1973b). Furthermore complement components are found in very low concentrations in pulmonary secretions (Reynolds and Newball, 1974) and therefore might not be available for C3 mediated phagocytosis although C3b and C3d receptors have been reported on alveolar macrophages (Reynolds et al., 1975). As the residual pools of the UV serum are haemolytic in vitro, it seems likely that complement would be fixed in vivo if present.

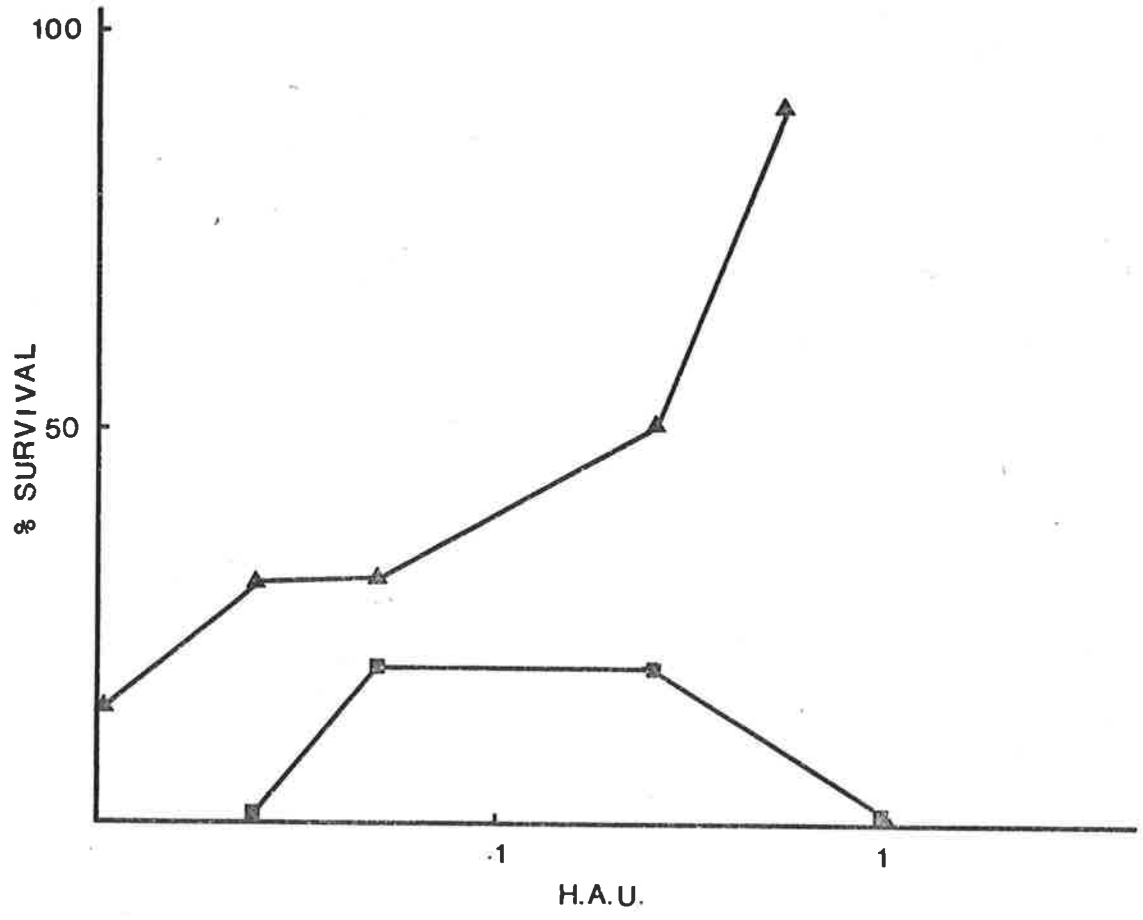
The results from the protection experiments tended to

support the in vitro evidence of these researchers. If these explanations were true, then differences should be observable between the protective activity of the residual pools in the peritoneum and in the lung. For in the peritoneum, IgM has been shown to be highly efficient at promoting destruction of bacteria (Steele, Chaicumpa and Rowley, 1974), whether this be by fixation of complement or by opsonisation. Kpnl is also highly virulent when given by intra-peritoneal injection, or indeed when given intra-venously to mice. The protective activity of the various preparations of UV-serum was determined using intra-peritoneal infection. No protection was produced in the peritoneum by the residual pool while IgG and whole serum was efficient at similar concentrations as used in the lung (Figure 5.4).

This result was unexpected due to the known activity of IgM in the peritoneum. The role of IgM in clearance of a non-virulent organism from the lung was then investigated to determine if the finding that IgM from UV serum failed to protect in the lung reflected a general inability of IgM to promote clearance of bacteria by lung defenses. The model of aerosol infection using Vibrio cholerae 569B will be described in detail in Chapter 6. This organism was chosen for study for several reasons. As a completely avirulent organism in the mouse, and one that is easily destroyed by both phagocytic cells and by the bactericidal activity of complement, it provides a convenient model for investigating the clearance of particles from the lower airways by the alveolar macrophages (Green and Kass, 1964a). Furthermore antisera against this organism was readily available for these studies, in particular purified secretory IgA was available and the production of IgA antibodies to Kpnl was difficult due to its virulence. This will be

Figure 5.4

Protection of mice to intra-peritoneal challenge with Kpnl by preparations of UV serum. (▲) IgG, (■) residual pool after removal of IgG. All control mice died. Percentage of experimental mice surviving are shown against the number HAU of antiserum given with the inoculum.



100
50
% SURVIVAL
·1
1
H.A.U.

further discussed in relation to local immunity. However, the aerosol model of infection was used in these studies to investigate if IgM per se was ineffective in the lungs at promoting bacterial removal. Antiserum to V. cholerae 569B was purified first using Protein A-Sepharose 4B and then Sephadex G200 chromatography. V. cholerae were incubated with 1.0 HAU of whole serum or IgM and spf mice were exposed to aerosols of these organisms. Lungs were removed from mice at intervals, homogenised and counts of viable bacteria in their lungs were performed. Mice received approximately 7×10^4 bacteria each following 40 minutes exposure. During three hours, 34% of unopsonised bacteria were removed, compared with 72% of serum opsonised and 62%, of IgM opsonised bacteria. These results were similar to those obtained with immune serum in other experiments (Table 6.1).

While this evidence was not decisive, it strongly suggested that IgM did promote clearance of non-virulent V. cholerae from the lung as efficiently as other classes of antibody. This suggested that the results obtained with Kpnl could not be explained on the basis of antibody class alone. It was possible that while anti-capsular haemagglutinating activity did appear to correlate with protection, antibodies against other antigens might also play a role and explain the apparent discrepancy between the protective activity of IgG and IgM when compared on the basis of anti-capsular activity.

It was decided at this time that other forms of vaccines should be used to raise antisera. From a practical point of view, ultra-violet irradiated bacteria were unstable when stored and had to be prepared freshly for use. Variations in sterility between preparations produced further complications due to the extreme

virulence of the organism, as only a few live organisms were required to produce lethal infection in mice by any route of administration used. In addition to these problems, the use of a variety of vaccines might bring to light the role of antibodies directed against other antigens.

5.5 PROTECTION BY ANTI-SERA TO HEAT LABILE AND HEAT STABLE ANTIGENS

Antisera were produced in mice by intra-venous injection of glutaraldehyde killed Kpnl (GK-serum) or heat killed Kpnl (HK-serum). Similar numbers of organisms were injected (10^8, six times over one month). Preliminary experiments were performed using HK-serum. In comparison with earlier, UV-sera this serum was far less protective on the basis of haemagglutination (Figures 5.3, 5.5). When IgG was removed from this serum by chromatography, considerable protective activity remained in the residual pool. The PD_{50} of these preparations was determined by the method of Reed and Muench (1938).

This protective activity was further quantified by determining the absolute amounts of antibody by quantitative precipitin reactions. Purified IgM was prepared by chromatography of the residual pool on Sephadex G200 and purity determined by Ouchterlony gel diffusion. No IgA immunoglobulin was detected in these fractions. Quantitative precipitin reactions using capsular polysaccharide, produced values of 5.9 ugm./HAU for IgG and 1.3 ugm./HAU for IgM. On the basis of amount of anti-capsular antibody in HK-serum, IgG appeared twice as protective as IgM (Table 5.2).

This evidence suggested that the differences in protective activity observed between IgG and the residual pool of UV-serum

Table 5.2

$P.D._{50}$ determinations on immunoglobulin fractions of HK-serum expressed in terms of H.A.U. and absolute amounts of antibody.

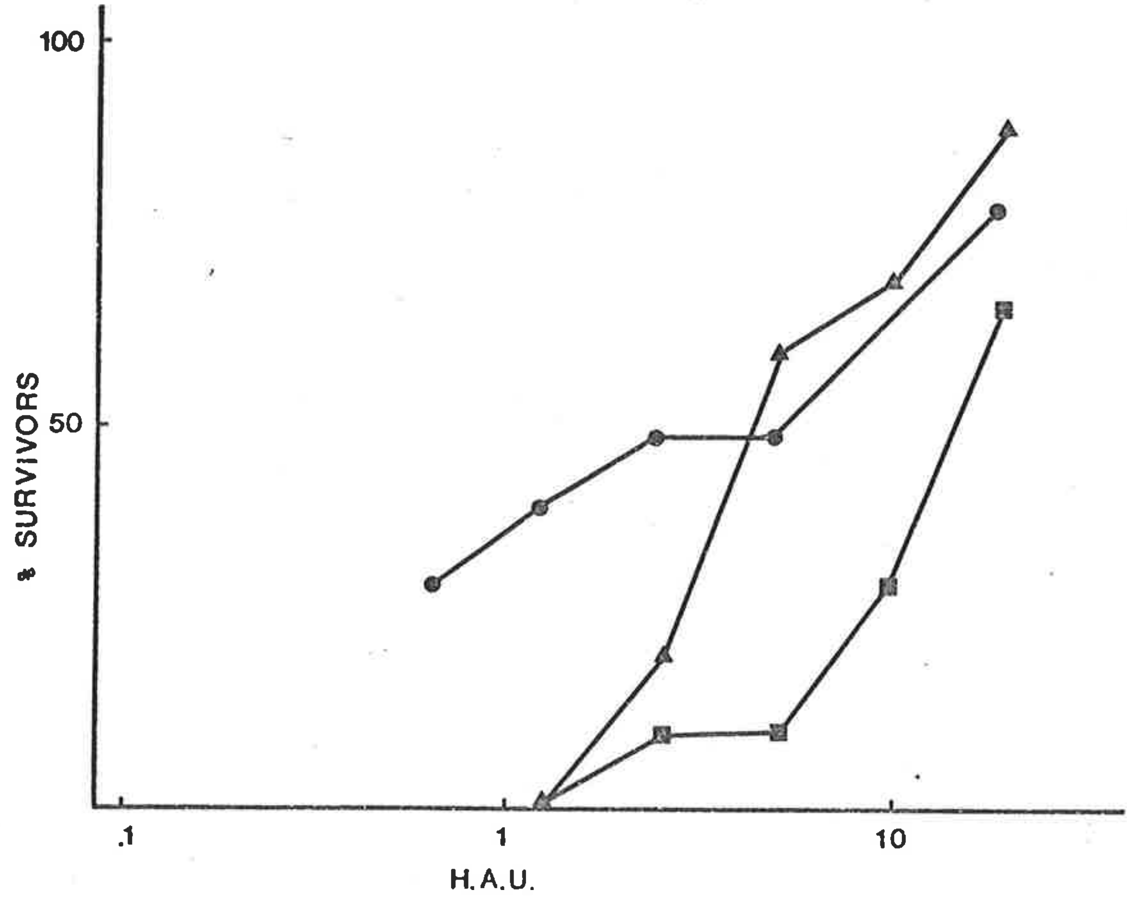
Preparation	ugms/H.A.U.[1]	$P.D._{50}$ as;	
		H.A.U.[2]	ugms[2]
IgG	5.9	0.57	3.4
residual pool	1.3	5.0	6.5

[1] ugms of specific antibody/ H.A.U. as determined by quantitative precipitin.

[2] $P.D._{50}$ expressed as H.A.U. and ugms. of specific antibody added to inoculum.

Figure 5.5

Protection of mice to intra-nasal challenge with Kpnl by whole HK serum (●), IgG (▲), and the residual serum pool after removal of IgG (■). All control mice died. Percentage of experimental mice surviving are shown against the number of H.A.U. of antiserum given with the inoculum.



100
50
% SURVIVORS
.1
1
10
H.A.U.

should not be explained by the class of antibody involved but rather by some characteristic of the serum.

GK- and HK-sera were compared. Both were capable of producing protection although HK-serum appeared slightly less efficient. To determine if different antigens were detected by these sera, they were exhaustively absorbed with heat-killed Kpnl. Absorption was repeated until no haemagglutinating activity remained in these sera. The sera were then used in protection experiments in concentrations equivalent to those of the parent sera . Absorption removed almost all the protective activity form HK-serum (Figure 5.6)but only a fraction of that of GK-serum (Figure 5.7). These results clearly established that antibodies present in GK-serum detected antigens that HK-serum did not. Presumably these were heat-labile antigens that were not related to the capsular polysaccharide, detected in haemagglutination assays.

If the presence of antibody against this antigen were to be invoked to explain the original observations with UV-serum, then this antibody could be expected to be predominantly IgG. The absorbed, GK-serum was therefore purified using Protein A. Fractions obtained by this purification were compared with fractions from unabsorbed GK-serum at equivalent concentrations of serum. All the protective activity of the absorbed serum was found to be in the IgG fraction (Figures 5.8 and 5.9). While it is clear that anti-capsular antibody is efficient at promoting protection, the second antigen can also be seen to play an important role in this. Given the high number of capsular types of klebsiella (over 80), the existence of a second antigen of importance in mediating _in vivo_ protection and possibly of less variability might well have important implications in resistance to _K. pneumoniae_. Given the

Figure 5.6

Protection of mice to intra-nasal challenge with Kpnl by whole HK serum (●)and HK serum absorbed with heat-killed Kpnl (▲). All control mice died. Percentage of experimental mice surviving are shown against the quantity of serum used in the inoculum. Haemagglutinating activity is shown in brackets.

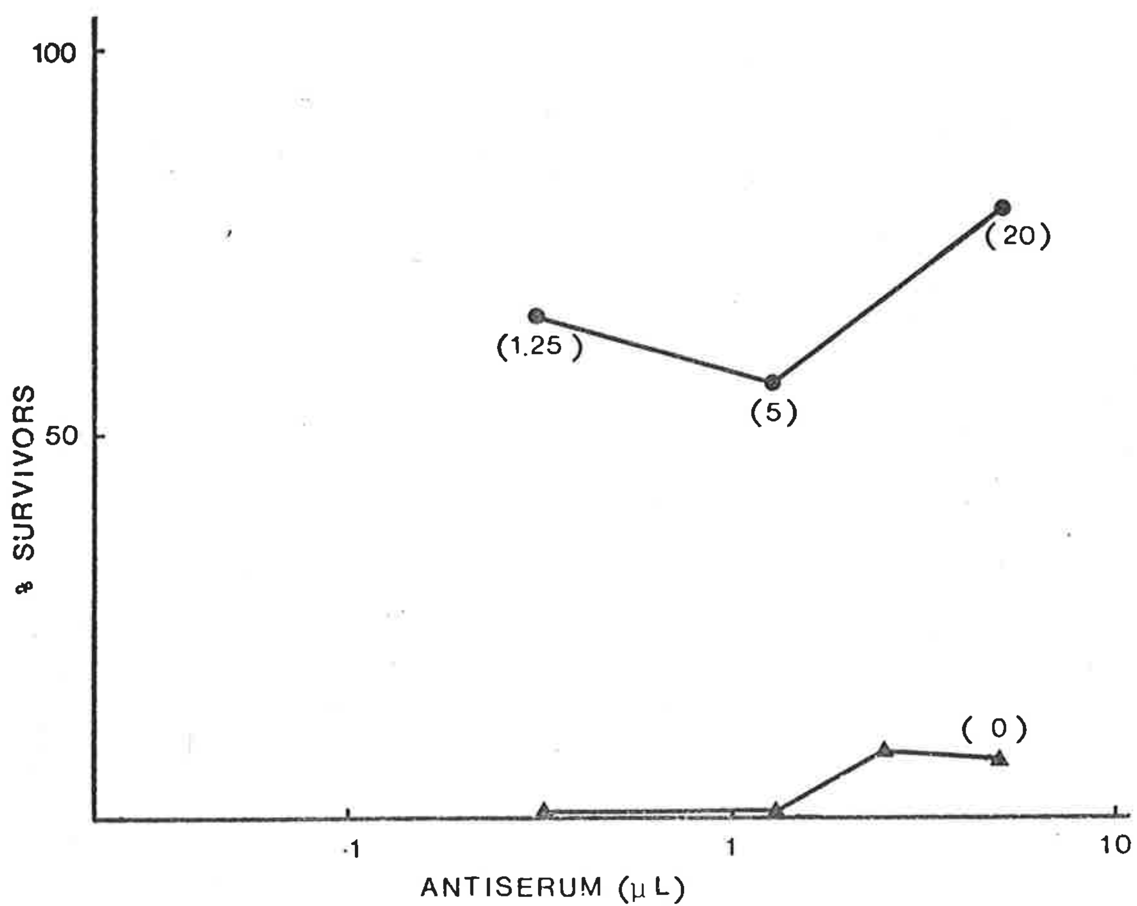
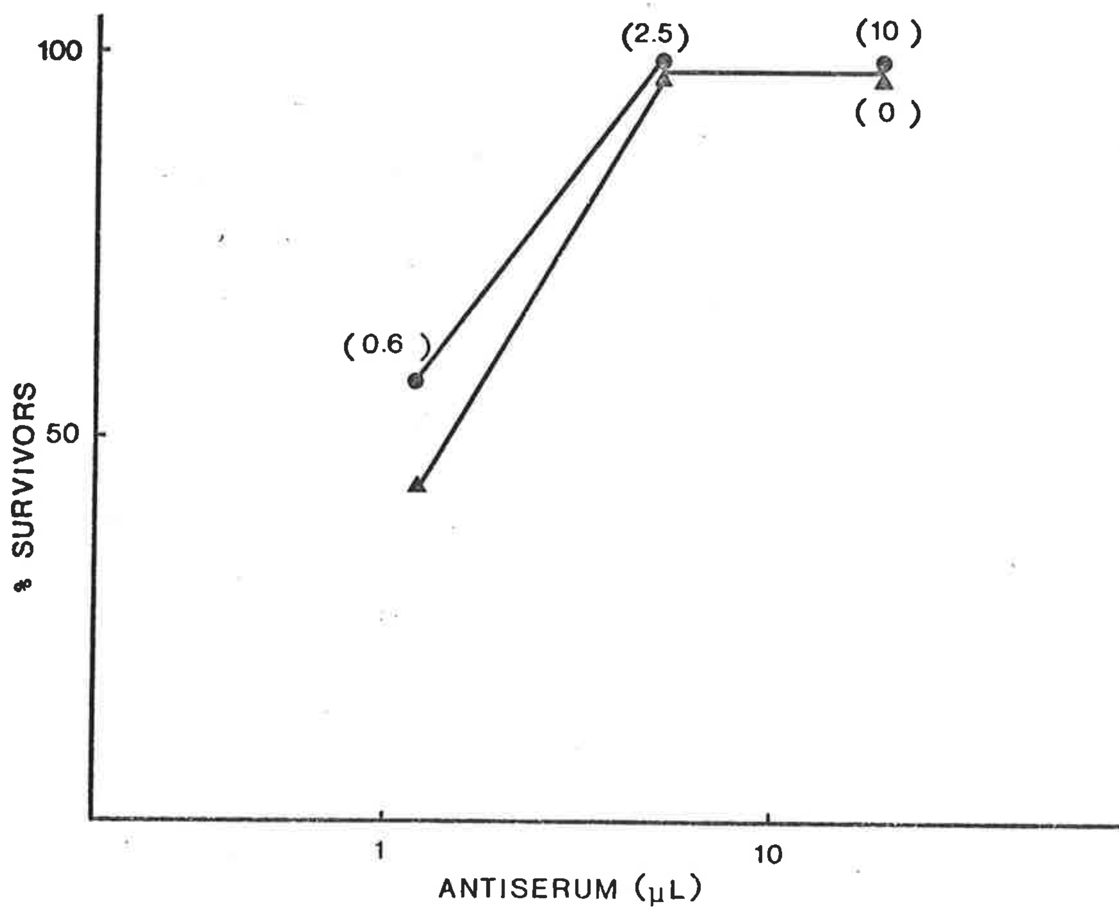

100
50
% SURVIVORS
(1.25)
(5)
(20)
(0)
·1
1
10
ANTISERUM (μL)

Figure 5.7

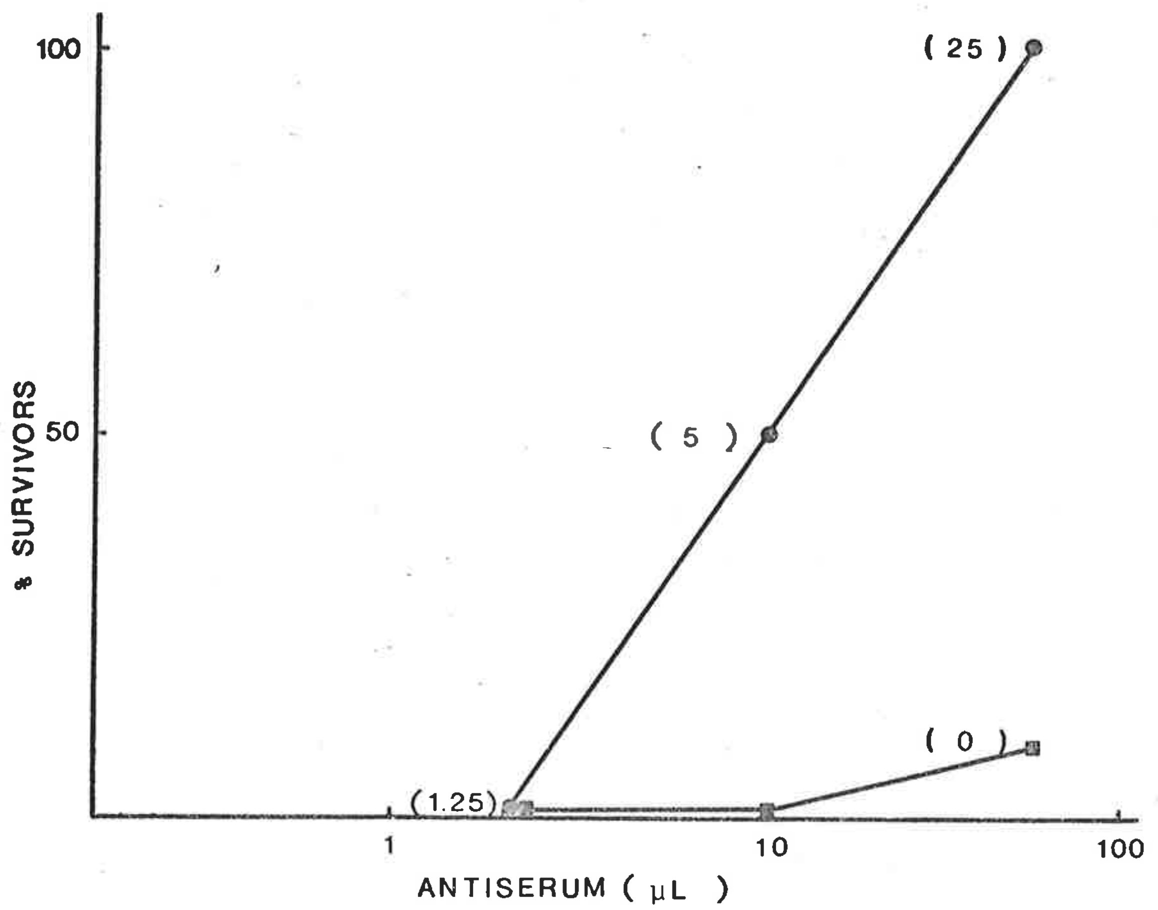
Protection of mice to intra-nasal challenge with Kpnl by whole GK serum (●)and GK serum absorbed with heat-killed Kpnl (▲). All control mice died. Percentage of experimental mice surviving are shown against the quantity of serum used in the inoculum. Haemagglutinating titres are shown in brackets.



100
50
% SURVIVORS
(2.5)
(10)
(0)
(0.6)
1
10
ANTISERUM (µL)

Figure 5.8

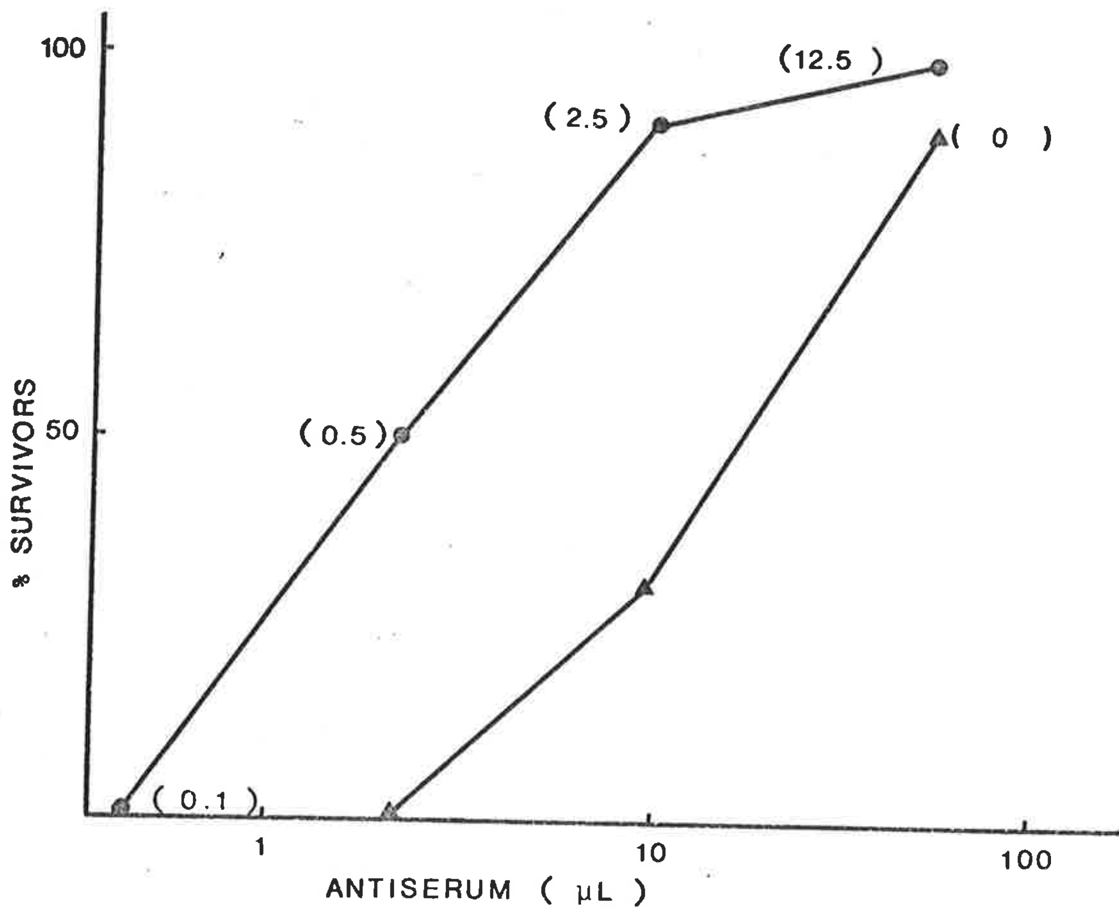
Protection of mice to intra-nasal challenge with Kpnl by the residual pool of GK serum (●)and the residual pool of GK serum absorbed with heat-killed Kpnl (■). All control mice died. Percentage of experimental mice surviving are shown against the quantity of serum used in the inoculum. Haemagglutinating titres are shown in brackets.



100
50
% SURVIVORS
(25)
(5)
(0)
(1.25)
1
10
100
ANTISERUM (µL)

Figure 5.9

Protection of mice to intra-nasal challenge with Kpnl by the IgG pool of GK serum (●)and the IgG pool of GK serum absorbed with heat-killed Kpnl (▲). All control mice died. Percentage of experimental mice surviving are shown against the quantity of serum used in the inoculum. Haemagglutinating titres are shown in brackets.


100
50
% SURVIVORS
(12.5)
(2.5)
(0)
(0.5)
(0.1)
1
10
100
ANTISERUM (μL)

copious production of capsular material by these organisms, the finding of a non-capsular antigen that may be recognised by antibody in vivo is of interest. To further investigate this antigen an assay was required to detect antibody directed against it.

5.6 PASSIVE HAEMAGGLUTINATION ASSAYS USING EXTRACTS OF Kpn1

Several techniques were attempted to develope a passive haemagglutination assay using extracts prepared from Kpn1 by ultra-sonication. Extracts were labelled with I^{125} using chloramine T. Percentage of extract binding to sheep red blood cells was then determined under a variety of conditions.

SRBC were incubated in the presence of extract and 0.05% glutaraldehyde for 1 hour at 37°. After washing by centrifugation, it was determined that only 0.6% of radio-activity bound to the cells. A similarly low percentage bound using chromic chloride coupling (Williams and Chase, 1976). The use of tanned SRBC and glutaraldehyde in the method of Imai et al., (1974) and Walker, Meinke and Weigle (1979), resulted in the binding of 9.4% of radio-activity.

None of these methods of coating SRBC resulted in a useful haemagglutination assay. It was therefore decided to utilise an ELISA to attempt to detect the antigen.

5.7 DEVELOPMENT OF ELISA DETECTING ANTIBODY TO KLEBSIELLA ANTIGENS

An ELISA was developed to permit detection of antibody against the heat-labile antigen in vitro in order to verify these results obtained in vivo and to attempt some characterisation of the

antigen. The system using alkaline phosphatase and p-nitro phenol phosphate described by Engvall and Perlmann,(1971) was used. Goat anti-mouse light-chain coupled to the enzyme was kindly provided by Mrs. P. Udomsantisuk of this department.

The antiserum was raised in goats by injection of purified mouse IgM. This antiserum was passed down a column of Sepharose 4B, to which was coupled mouse IgG_1 purified by Protein A chromatography (Ey, Prowse and Jenkin, 1978). Goat antibodies bound to this column were eluted using 1M propionic acid buffered to pH 2.4. The antibody eluted from this column reacted with all classes of mouse immunoglobulin by Oucterlony gel diffusion and subsequently in an ELISA. The antibody was coupled to enzyme using glutaraldehyde and the optimal concentration of coupled enzyme determined in an ELISA using micro-titre trays coated with $(NH_4)_2SO_4$ precipitated mouse immunoglobulin at varying dilutions.

An assay was developed to detect antibodies to Kpnl capsular polysaccharide (CPS-K). Micro-titre trays were directly coated with 100 ug./ml. CPS-K in bicarbonate buffer, pH 9.6. After an overnight incubation at 4°, the trays were washed and dilutions of antiserum for testing were added. Phosphate buffered saline containing 0.05% Tween-20 was used for washing the wells and for preparing the dilutions of antiserum. After a 6 hour incubation at room temperature, the wells were washed again and enzyme-coupled anti-mouse light-chain antiserum was added. A further overnight incubation at room temperature was generally used to permit maximal binding of the anti-mouse light chain antiserum, the wells washed and p-nitro phenol phosphate 1 mg./ml. in 10% diethanolamine added. The reaction was allowed to continue at room temperature until

suitable density of colour developed and this was then measured at OD_{405} in a micro-ELISA reader.

An assay was also developed to detect antibody to the heat-labile antigen. A log-phase of Kpnl was washed by centrifugation and resuspended to 2-5 x 10^9/ml. in 10 mM. Tris-HCl, pH 7.8. Aliquots of 15 mls. were ultra-sonicated at maximum intensity in an MSE Ultra-sonicator for four periods of 20 seconds. The bacteria were kept on ice during this entire procedure. Whole bacteria were removed by centrifugation and the protein concentration determined (Lowry et al., 1951)

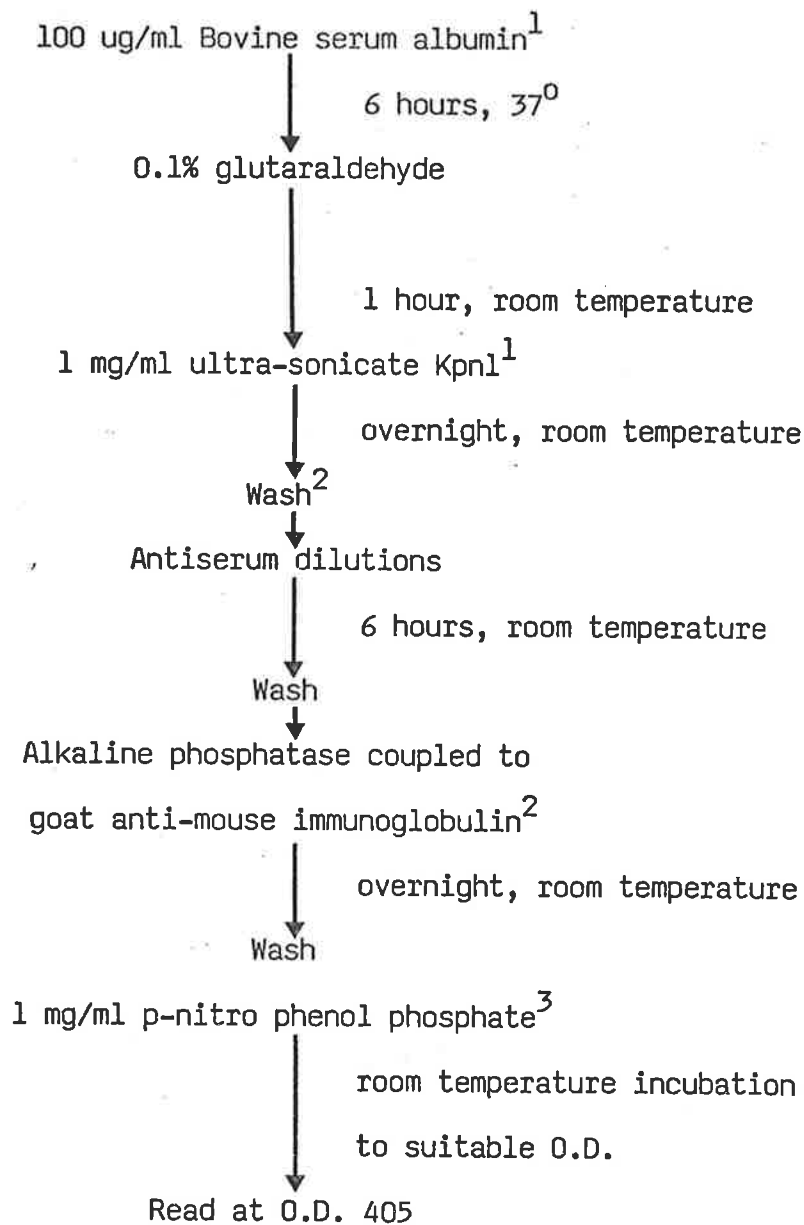
To determine optimal conditions for binding of these proteins to micro-titre trays, the ultra-sonicate was labelled with I^{125} using chloramine-T and incubated in wells under a variety of conditions. The wells were washed and cut from the trays. Bound radio-activity was then measured. Protein bound directly to the wells in carbonate buffer at pH 9.6, but this could be significantly increased by cross-linking the proteins to bovine serum albumin (BSA) already coating the wells. 1 mg./ml. of BSA was incubated in the wells for 1 hour at 37^0, 0.1% glutaraldehyde added followed by the ultra-sonicate, at mg./ml. After a further incubation at 37^0 for 6 hours, the wells were well washed and counted. This procedure bound over twice the amount of radio-actively labelled ultra-sonicate and produced greater sensitivity in the ELISA. The protocol used is given in Figure 5.10.

5.8 ACTIVITY OF ABSORBED SERA

GK- and HK-sera were then assayed for activity against both CPS-K and ultra-sonicate. It was confirmed that absorption with

Figure 5.10

Protocol of ELISA used to detect antibodies against crude extract of Kpnl



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[1] in phosphate buffered saline, pH 7.4

[2] PBS-0.05% Tween 20

[3] 10% diethanolamine

heat-killed bacteria had removed anti-polysaccharide activity from these sera. As this assay was approximately 100 times more sensitive then haemagglutination, this removed the doubt that trace amounts of anti-capsular antibody remained after absorption (Figures 5.11 and 5.12).

The same sera were assayed against ultra-sonicate. The results (Figures 5.13 and 5.14) confirmed that higher levels of antibody remained in absorbed GK-serum than in HK-serum. This supports the in vivo findings and also permits further attempts at characterisation of the antigens involved.

5.9 NATURE OF THE HEAT-LABILE ANTIGEN

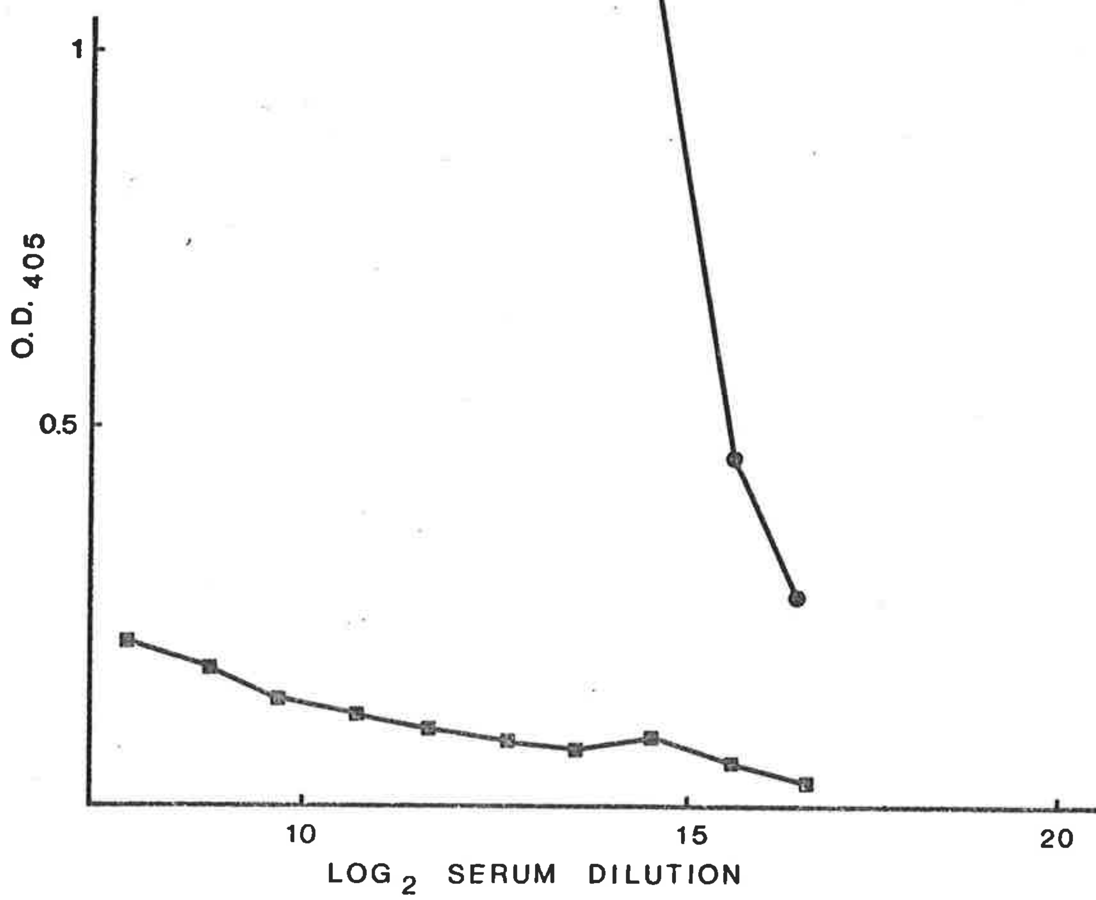
The results of the protection experiments and the ELISAs demonstrated the existence of a heat-labile, non-capsular antigen of Kpnl that was biologically important. The importance of this antigen would be greater if it was an antigen that was present in a variety of klebsiella and not simply Kpnl. On the other hand it might be related to the extreme virulence of this strain in mice and represent a virulence antigen.

Of the non-K antigens of klebsiella, the O antigens have received some attention for their importance in further classifying strains. However, they are classically heat-labile (Kauffmann, 1966) and are therefore not likely to be candidates for the heat-labile antigen.

Of possible surface structures of bacteria in general, pili or flagella may be important in virulence, however Kpnl would not appear to possess either of these structures. Klebsiella pneumoniae are non-motile and non-piliation is associated with

Figure 5.11

ELISA against CPS-K of HK serum before (●) and after (■) absorption with heat-killed Kpnl. Two-fold dilutions from 1/200.



O.D. 405
1
0.5
10
15
20
LOG 2 SERUM DILUTION

Figure 5.12

ELISA against CPS-K of GK serum before (●) and after (■) absorption with heat-killed Kpnl. Two-fold dilutions from 1/200.

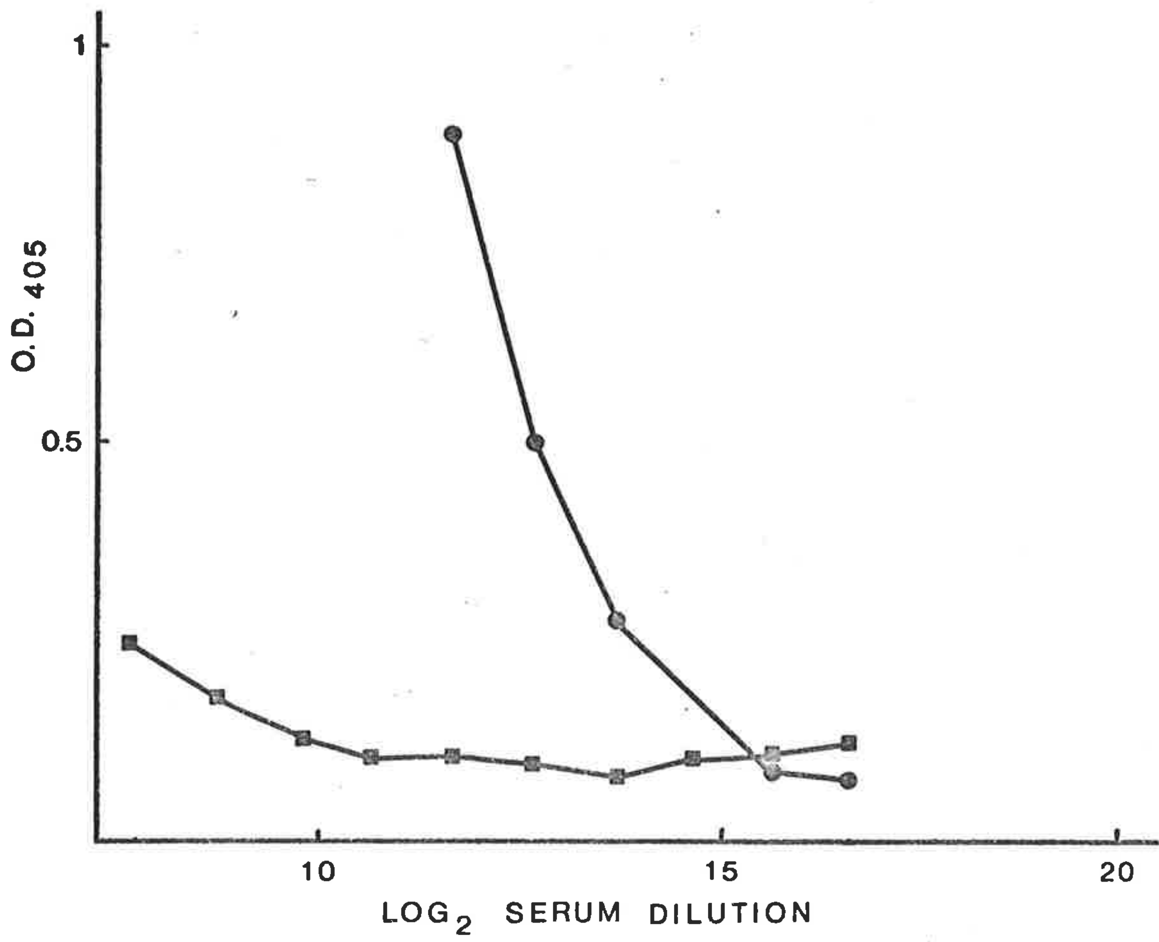
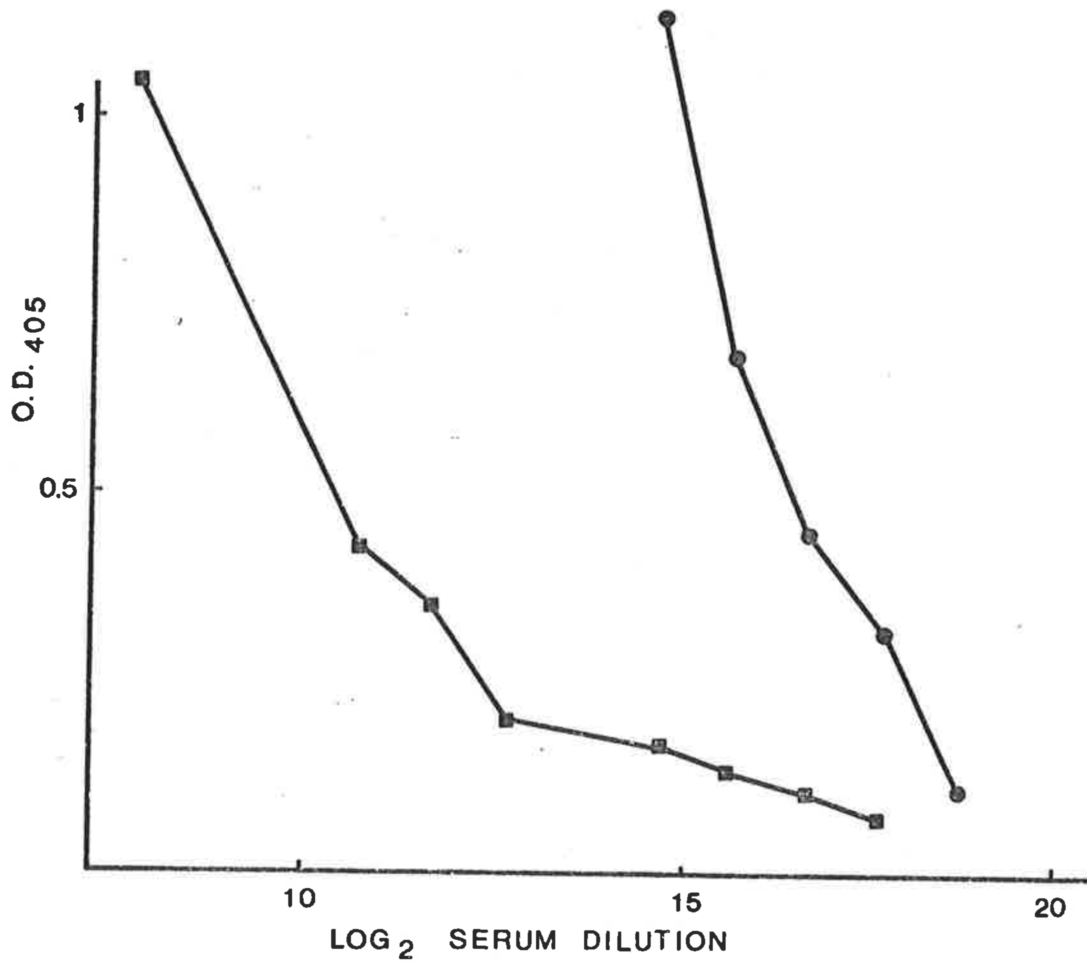

1
0.5
O.D. 405
10
15
20
LOG2 SERUM DILUTION

Figure 5.13

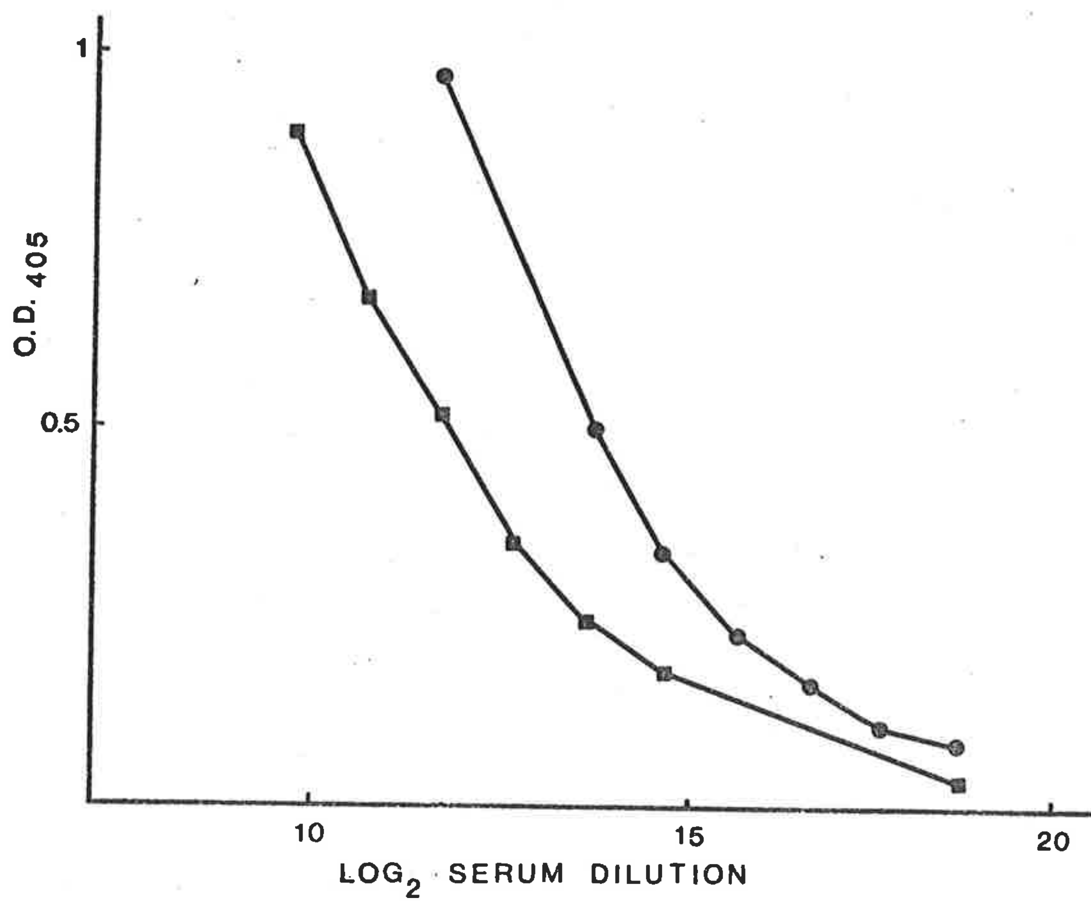
ELISA against ultra-sonicate of Kpnl of HK serum before (●) and, after (■) absorption with heat-killed Kpnl. Two-fold dilutions from 1/200.



O.D. 405
1
0.5
10
15
20
LOG 2 SERUM DILUTION

Figure 5.14

ELISA against ultra-sonicate of Kpnl of GK serum before (●) and after (■) absorption with heat-killed Kpnl. Two-fold dilutions from 1/200.



1
0.5
O.D. 405
10
15
20
LOG2 SERUM DILUTION

virulence of klebsiella, not piliation (Duguid, 1959). Pili could not be detected on Kpnl by direct red blood cell agglutination, pellicule formation on standing culture or by electron microscopy (performed by the Department of Biochemistry of this University). Electron microscopy did highlight the copious quantities of capsular material produced by this strain, underlining the surprising fact that apparently non-capsular antigens could bind antibody.

5.10 MEMBRANE PREPARATIONS OF KLEBSIELLA

To investigate whether this antigen could be detected in the outer membrane of Kpnl or in other strains of klebsiella, membrane preparations were made and run on polyacrylamide gels (PAG electrophoresis was kindly performed by Dr. P. Manning of this department). The membrane preparations of Kpnl were also separated on sucrose density gradients. Bacteria were ultra-sonicated following spheroplasting with lysozyme. This ultra-sonicate was pelletted at 160,000 x g and resuspended in 25% (w/w) sucrose in 10 mM EDTA pH 7.8. It was then layered on a 25-50% sucrose gradient and centrifuged at 78,000 x g for 18 hours. The centrifuge tubes were pierced and approximately 30 fractions were collected and run on SDS-PAGE. Fractions were also used to coat micro-titre trays and assayed for the presence of the heat-labile antigen by incubating the trays with a standard dilution of absorbed GK-serum and then proceeding with an ELISA as above. GK-serum absorbed with heat-killed Kpnl was used to avoid detecting capsular polysaccharide. Protein concentrations of the fractions were compared by reading their OD 280. The majority of protein and ELISA-detected activity was found in those densest fractions

corresponding with the outer membrane proteins on PAGE. ELISA-activity was closely associated with protein concentration, except in the least dense fractions (Figure 5.15) and therefore no clear interpretation can be made from these results. Outer membrane proteins were also present in all fractions as determined by PAGE and it may be that the heat-labile antigen is associated with these outer meembrane proteins.

PAGE on membrane preparations, kindly prepared by Dr. W. Chaicumpa, of other strains of klebsiella demonstrated similarity between them. Several of the strains lack one or other of the major outer membrane proteins (strains 21 and 90, Figure 5.16). The other proteins of Kpnl appear to be present in these strains and in Enterobacter aerogenes.

To determine if the heat-labile antigen was present in any of these strains two approaches were taken. An ELISA inhibition assay was developed. Wells were coated as normal with ultra-sonicate of Kpnl. A standard dilution of absorbed GK-serum was then added to the wells along with varying dilutions of ultra-sonicate from other strains. Inhibition of binding of the antiserum could then be quantitated by determining the total amount of protein of each ultra-sonicate required to produce a given percentage of inhibition. Unfortunately, results varied between preparations of these ultra-sonicates and the absolute values of protein given in Table 6 can only be taken as rough indication of the amount of antigen present.

The presence of this antigen in other strains could be verified by second absorptions of this antiserum with glutaraldehyde killed preparations of some of these strains. All strains of klebsiella tested produced some decrease in activity, however some

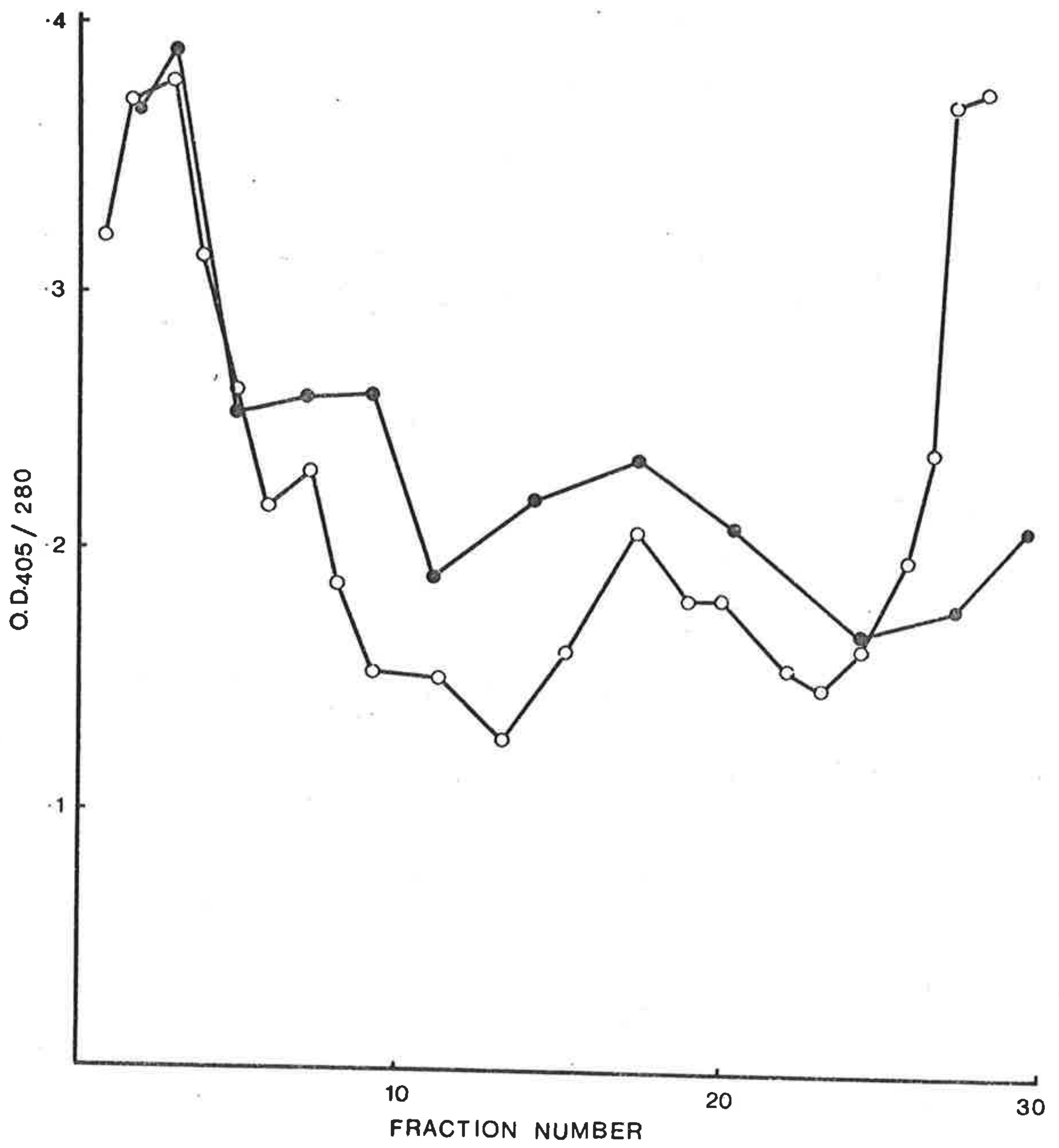
Table 5.3

Amounts of total protein from various strains of klebsiella producing 10% inhibition of binding of absorbed GK-serum to crude extract of Kpnl in an ELISA.

Strain	ugms.
Kpnl	1.3
90	1.6
1153	11.0
4	15.0
21	7.0
25	6.0
Bovine serum albumin	83

Figure 5.15

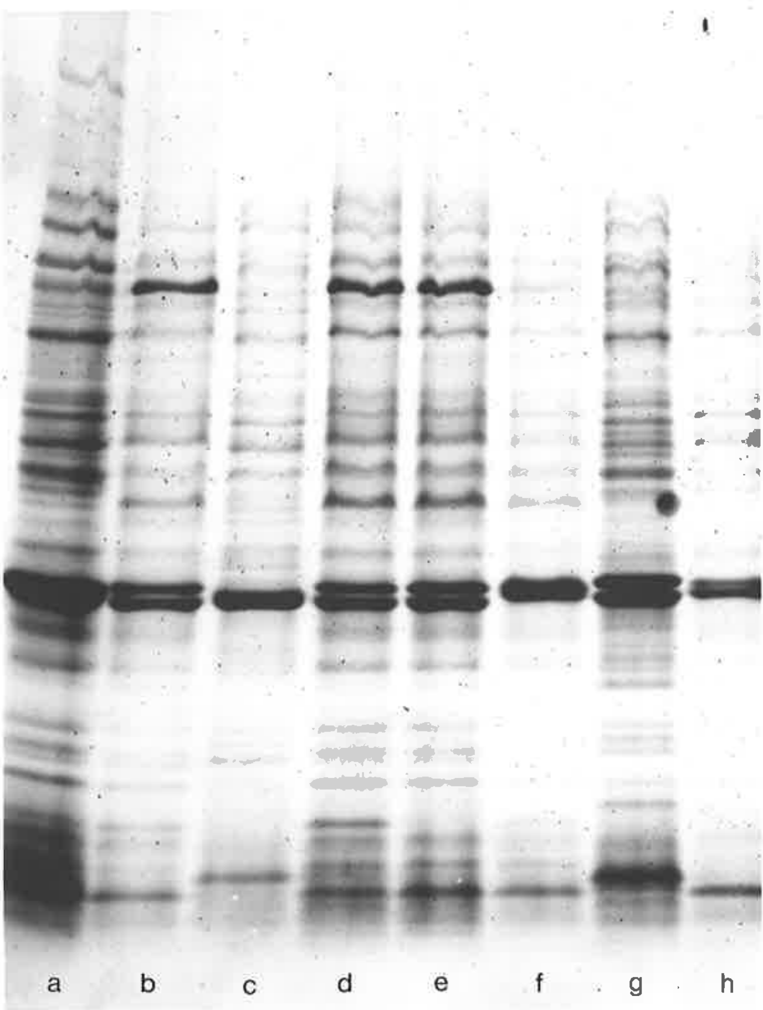
ELISA using fractions of sucrose density gradient of membrane proteins of Kpnl (●). Antiserum (GK serum absorbed with heat-killed Kpnl) added at constant concentration to all wells. Optical density of fractions at O.D. 280 also shown as measure of protein concentration (○).



·4
·3
·2
·1
O.D.405 / 280
10
20
30
FRACTION NUMBER

Figure 5.16

SDS-PAGE of strains of *K. pneumoniae* and *E. aerogenes*. From left to right; (a) E. aerogenes, (b) 4, (c) 21, (d) 23, (e) 25, (f) 90, (g) 1153, (h) Kpnl.


a b c d e f g h

strains, particularly 1153, produced a greater decrease in activity than even the homologous organism, Kpn1, when equal numbers of organisms were used for the absorption.

It therefore seems that while evidence from analysis of fractions from sucrose density gradients is compatible with the antigen being associated with the outer membrane proteins, comparison of the strains failed to permit identification of any protein that might be responsible. Nevertheless, the evidence does suggest that the antigen is widely represented amongst klebsiella strains.

5.11 CONCLUSIONS

Humoral immunity to K. pneumoniae is elicited by intra-venous vaccination with several different bacterial vaccines. Protection may be mediated by both IgG and IgM. Antibodies may be directed against either capsular polysaccharide or a second, heat-labile antigen, or antigens, that does not appear to be related to either the K or O antigens of K. pneumoniae but that does appear to be present in a number of other strains of klebsiella. It is clear therefore that immunity to this organism may be produced by systemic immunisation. The possible role of local immunisation in the lung will now be discussed.

CHAPTER 6

LOCAL IMMUNITY IN THE LUNG

Most recent studies dealing with immunity to bacterial infections of the lungs tend to emphasise the importance of lower respiratory tract events. In particular, the alveolar macrophage has been viewed as the most likely means of destruction of bacteria arriving in the alveoli following aerosol administration (Green and Kass, 1964a), although the possible contribution of polymorphonuclear leukocytes has also been studied (Rehm, Gross and Pierce, 1980). Evidence for this is primarily that bacteria are destroyed in situ and are not removed by the muco-ciliary system. The low levels of complement present in the secretions of the lung (Reynolds and Thompson, 1973a; Reynolds and Newball, 1974) endorse the belief that complement is not an important component of this defense. Problems of interpretation exist with all such studies. In particular, the contribution of serum factors, such as IgM and complement, during inflammatory reactions would radically change the environment of the alveoli, with respect to cell and humoral factors. Nevertheless, it is clear that the immediate response of pulmonary defenses must involve the alveolar macrophage. Presumably this cell is responsible for the day to day maintenance of sterility in the lower airways and for the destruction of avirulent micro-organisms present in inspired air.

Results presented so far are easily explained on the basis of the destruction of K. pneumoniae in the lung by cells, either macrophages or polymorphs. Antibody can be seen to promote resistance , when acquired either passively or actively. Such

findings are in agreement with earlier studies on the importance of immunisation on the removal of bacteria from the lung (Jakab, 1976). However, simple interpretations of this sort ignore events in the upper airways. While aerosol infection does occur frequently in clinical situations, the infective droplets are seldom small enough to permit the bacteria or other micro-organisms to be directly implanted in the alveoli. In many infections, colonisation of the nasopharynx or adjacent areas will precede pneumonia or other infection of the lung parenchyma. Events in the nasopharynx and nasal passages may therefore be critical in development of disease, well before alveolar macrophages or other components of alveolar defense are involved.

In this model of infection with K. pneumoniae intra-nasal inoculation is more effective at initiating infection in mice than aerosol exposure. It was found that the LD_{50} of Kpnl following intra-nasal administration is less than 5 organisms, while the LD_{50} by aerosol exposure is approximately 200 organisms. Clearly deposition in the lower respiratory tract does not promote infection as easily as deposition in the upper respiratory tract. Transient colonisation of the upper respiratory tract in this experimental model would therefore appear to be important in development of infection.

The mucosal surfaces of the upper respiratory tract differ immunologically from the bronchioles and alveoli. There is good evidence from studies characterising the lymphoid tissue of the upper airways (Bienenstock, Johnston and Perey, 1973a,b) and the secretions bathing these surfaces (Kaltreider and Chan, 1976; Hand, Cantey and Hughes, 1974) that they form part of the common mucosal immune system, as described by Bienenstock et al. (1978).

Secretory IgA predominates and local production of antibody of this class independently of serum antibody levels confirms that the upper respiratory tract should be considered as a site of expression of local immunity (Hand, Cantey and Hughes, 1974).

In striking contrast to the many studies on viral infection, little attention has been given to the possible role of local immunity to bacterial infection of the lung, despite early observations by Bull and McKee (1929) and Walsh and Cannon (1936) that local immunisation of the lung could produce protection to Streptococcus pneumoniae, in the absence of the development of serum antibody. Some recent evidence with pulmonary infection with Francisella tularensis support these findings (Bellanti et al., 1967; Buescher and Bellanti, 1966).

Anti-bacterial effects of IgA are notably difficult to demonstrate in vitro, but there is no doubt that in vivo protection to a variety of bacteria, particularly intestinal pathogens, may be mediated by antibodies of this class . In such situations IgA is physically separated from tissue based defenses that rely on phagocytosis or complement activation. IgA is generally considered to be poorly opsonic (Wilson, 1972; Reynolds, Kazmierowski and Newball, 1975; Steele, Chaicumpa and Rowley, 1974) and appears to interfere with complement activation of other classes of immunoglobulin in certain conditions (Griffiss, 1975; Griffiss and Bertram, 1977; Russell-Jones, Ey and Reynolds, 1980). It would appear unlikely to play a useful role in defense systems relying on either of these pathways, such as the lower respiratory tract. Yet if local immunisation does produce protection against a lower respiratory tract pathogen such as S. pneumoniae, this interpretation obviously requires alteration.

Several possible explanations exist. The action of IgA may be strictly localised to the nasal and pharyngeal membranes and exert protective effects at this level, possibly interacting with muco-ciliary transport. Alternatively, IgA may act in concert with alveolar cells to promote destruction of the bacteria. While Fc receptors for IgA have not been reported on macrophages, they are known to exist on lymphocytes (Strober et al., 1978; Lum, Benveniste and Blaese, 1980) and polymorphonuclear cells (Henson, Johnson and Spiegelberg, 1972; Lawrence, Wiegel and Spiegelberg, 1975). Certainly Fc receptor activity appears to differ between cell populations dependent on their maturity and degree of activation (Cline, 1975; van Furth, 1976). While Fc receptors for IgA might not exist on some macrophages, that does not exclude the possibility of their existence on alveolar cells.

IgA has been reported to be less opsonic than IgG for alveolar macrophages (Reynolds et al., 1973b) but several comments should be made concerning these studies. The experiments reported, while demonstrating a quantitative difference in phagocytic rate following opsonisation of particles with antibodies of these two classes, still demonstrate a positive effect of IgA in promoting phagocytosis. The antibody preparations used, however, do not appear to have been purified by removal of IgM by affinity chromatography and any interpretation is therefore suspect, as has been shown with other studies purporting to demonstrate an opsonic effect of IgA (Wernet et al., 1971). In experiments designed to demonstrate Fc receptors directly, IgA was not included in the study (Reynolds et al., 1975).

Studies described so far clearly demonstrate the important role of antibody in acquired resistance to <u>K. pneumoniae</u>. The

relationship of these antibodies with other pulmonary defenses is now to be investigated. In particular, the problems left unanswered by studies reported above with regard to local immunity to this pathogen, and the relationships between IgA and alveolar macrophages will be studied. Several important questions remain completely unresolved. Can local immunity be demonstrated in the lung against this pathogen ? Is IgA protective against bacterial infections in the lung ? Do alveolar macrophages possess receptors for IgA ?

The general strategy of these studies involved study of humoral responses in the lung, to the exclusion of the possibility of cellular responses. This was dictated by the experiments already described. Antibody given passively produced good protection against K. pneumoniae. Antibody appeared to be required for any destruction of K. pneumoniae to occur in the lung. Even if cellular responses did develop in the lung, as they are known to do to certain pathogens (Mackaness, 1971), they could not be expected to replace this requirement for antibody.

Prior to the development of a model of K. pneumoniae infection, several studies were performed using aerosol exposure of mice to Vibrio cholerae. The reasons for this were several fold and have already been partly discussed with regard to clearance experiments described using IgM (see Chapter 5). As V. cholerae is avirulent rapid clearance of this organism from the alveoli could be expected to occur, as does occur in the peritoneum (Table 6.3). Such clearance could therefore be expected to be produced by the normal defenses present in the alveoli, particularly the macrophages, in the absence of great inflammatory changes as occur with K. pneumoniae. Antisera were readily available against V. cholerae, in particular secretory IgA from mouse intestinal

secretions. The availability of these sera became critical due to difficulties encountered in obtaining secretions containing IgA antibody to klebsiella (Section 6.4).

6.1 CLEARANCE OF BACTERIAL AEROSOLS FROM THE LUNG

Many early reports (Green and Kass, 1964a,b; Laurenzi et al., 1964) drew attention to the extremely rapid destruction of bacterial aerosols _in situ_ in the lung and presumed that this was produced by the alveolar macrophages. Jakab (1976) reported that the clearance of such aerosols was greatly enhance by active or passive immunisation of the animals. Aerosols, providing the droplets are of a sufficiently small size, will be inspired directly into the alveoli or terminal bronchioles. The effectiveness of alveolar macrophages can therefore be determined _in vivo_ by following the clearance of harmless bacteria from the lungs.

For aerosol exposure of animals a chamber was built as outlined by Ruppert et al., (1976). This chamber was designed to produce a fine aerosol that would result in deposition of bacteria in alveoli. Although originally designed to expose mice arranged in six cages, great variability was observed in the doses of organisms received by mice in the various cages. Mice were therefore exposed in only one cage at a time with an air flow of 20 l/minute. Variability between doses received and the clearance of bacteria was found both from mouse to mouse, and from day to day. Some reasons for this variation were discovered, and will be discussed , but in general they remained a serious problem in obtaining significance of results.

For exposures, a log-phase culture of _V. cholerae_ 569B was

centrifuged and resuspended to 10^{11} organisms/ml. Air was passed through a Hudson nebuliser containing 17 mls of this suspension. Medical air from pressurised cylinders (CIG, Adelaide) was passed through the nebuliser at 10-12 l/ minute and an additional 8-10 l/ minute were added to this in the mixing chamber. Mice were exposed to aerosols for 30-40 minutes which resulted in an initial dose of the order of 5×10^4 organisms/ mouse as determined by viable count on homogenised lungs of mice sacrificed immediately after exposure (Time 0).

Initial experiments determined the rate of removal of *V. cholerae* from the lungs of mice by sacrificing mice at intervals, removing and homogenising their lungs and counting the number of viable bacteria. The results were interpreted by determining the percentage of the initial inoculum cleared;

$$\% \text{ Cleared} = \frac{\text{Initial count} - \text{Final count}}{\text{Initial count}} \times 100$$

The standard error of this ratio was calculated by the method of Wilks (Green and Kass, 1964b).

High variations in the ability of mice to clear aerosols was immediately obvious in the initial series of experiments. Similar observations have been made previously (Huber, La Force and Johanson, 1977). The daily variation observed was striking and it became clear that this related to the length of time mice spent in the conventional animal house of this department. It should be reiterated that these mice are bred and raised in a specific pathogen free (spf) animal house and are housed in a conventional animal house during the course of experiments. We have previously described rapid changes in the immunoglobulin levels in serum of mice during this process of conventionalisation (Horsfall, Cooper

and Rowley, 1979), and have observed in studies with macrophages that the peritoneal macrophages of these mice also undergo functional and adaptive changes. The extremely poor clearance ratios found in spf mice support the hypothesis that conventional levels of antigen are important in stimulating defenses in the animal to potential infection (Table 6.1). Germ free mice exhibited even poorer rates of clearance (11% cleared over 200 minutes).

To determine if these differences were due to the low levels of immunoglobulin in these animals or to low activity of their reticulo-endothelial system, bacteria were opsonised with specific antiserum before nebulising. Bacterial suspensions were incubated with 0.1 HAU of mouse anti-*V. cholerae* 569B antiserum for 15 minutes on ice and then nebulised in the normal fashion. The addition of antiserum enhanced the clearance of bacteria by spf mice suggesting that, in part, the poor clearance rates were due to lack of natural antibody. The clearance of bacteria by germ free mice was likewise increased, but only 34% was cleared in 150 minutes, still well below that observed in conventional mice and spf mice. This is evidence therefore of decreased reticulo-endothelial activity and it is clear that the changes of conventionalisation involve both cellular and humoral responses, that in the normal animal act in concert to promote resistance.

By contrast with these various mice in which antibody was able to promote increased clearance, 6 day old mice were quite unable to clear these organisms even when opsonised, suggesting that in these animals no effective reticulo-endothelial activity exists in the lung.

Table 6.1

Clearance of *V. cholerae* from the lungs of spf and conventionalised mice, and the effect of antiserum on this.

Mice:	SPF		SPF		Conventionalised	
Opsonin:	nil		Immune serum		nil	
Time[1]	Count[2]	% cleared	Count	% cleared	Count	% cleared
0	13.7±9.8	0	10.1±6.1	0	2.1±2.0	0
150	10.4±7.6	24.0±35.0	4.3±2.5	57.0±16.0	0.7±0.9	65.0±24.0
200	9.1±5.2	34.0±28.0	2.8±2.4	72.0±13.0	0.6±0.7	71.0±19.0

[1] minutes

[2] total viable count/lung x 10^{-4}

6.2 ROLE OF ANTIBODY IN PROMOTING CLEARANCE

The preliminary experiments described above clearly indicated that specific antibodies could enhance clearance of V. cholerae from the lung. This system therefore lent itself to an investigation of the effect of IgA in clearance of bacteria from the lower respiratory tract. Secretory IgA was purified from mouse intestinal secretions by Dr. L. Bloom, formerly of this department. These were raised in mice by feeding live V. cholerae on four occasions followed by an intra-venous injection of live organisms as described in Bloom and Rowley (1979). Small intestines from these mice were removed and secretions obtained from them by rinsing the lumens with 1 ml of saline. The pooled secretions were clarified by centrifugation, and the immunoglobulins purified by chromatography on Sephadex G200. The IgA pool was further purified by passage through an IgM immunoabsorbent column, to remove contaminating IgM. This preparation of IgA retained good protective activity in the baby mouse protection test (Chaicumpa and Rowley, 1972). The final preparation contained 6.3 ug./ml. of sIgA antibody as determined by this test, with a content of 0.05 ug./ml. of IgM as determined by complement dependent bactericidal assay. This demonstrates the extreme difficulty of removing trace amounts of other classes of immunoglobulin from preparations of IgA.

The rate of clearance of bacteria from the lungs of conventionalised mice is greater than that expected from muco-ciliary action alone, which could account for a decrease of 4-5% per hour (Green and Kass, 1964a). The experiments on conventionalisation suggest that natural antibody may account for this higher rate of removal in these mice. The effect of IgA on this

clearance was then determined. Bacteria were opsonised with an equivalent amount of IgA antibody, by weight, as was used for serum opsonisation. Comparison was made on the basis on the known concentration of IgA from baby mouse protection tests. IgA caused some delay in the clearance of V. cholerae, when compared with conventional mice both with and without added opsonins (Table 6.2). Clearance rates approached normal levels by 200 minutes. This suggests that while IgA was not effective in promoting killing, it was efficiently replaced by other opsonins and in fact did not interfere with the action of these.

The contention that IgA is able to inhibit effects mediated by other classes of antibody (Griffiss, 1975; Griffiss and Bertram, 1977) is critical to our understanding of the action of IgA. Similar experiments to those performed in the lung were performed in the peritoneum. Mice were injected intra-peritoneally with live V. cholerae 569B and mice were sacrificed at intervals and their peritonea lavaged. While immune serum actively promoted killing, sIgA did not (Table 6.3). However, it failed to retard the killing seen in conventional mice. It also failed to interfere with the clearance seen in actively immunised mice, which cleared 99.8% of the initial inoculum of unopsonised bacteria or bacteria opsonised with IgA during the first 60 minutes.

The reported blocking effect of IgA quoted above was described in vitro, in a complement dependent bactericidal system. Bactericidal activity of serum was therefore determined using guinea pig serum as a source of complement. The bactericidal titre of antiserum was determined (5×10^5 bactericidal units, equivalent to 1.7×10^4 50% protective doses). Bactericidal reaction was allowed to proceed in the presence of 2 bactericidal units of serum

Table 6.2

Effect of opsonins on the clearance of V. cholerae from the lungs of conventionalised mice.

Opsonin:	nil		Immune serum[1]		sIgA[1]	
Time[2]	Count[3]	% cleared	Count	% cleared	Count	% cleared
0	7.3±6.5	0	3.9±4.1	0	8.1±3.2	0
150	3.1±3.0	58.0±29.0	0.1±0.4	90.0±7.0	7.1±3.2	19.0±24.0
200	1.9±1.3	74.0±14.0	0.13±0.23	97.0±3.0	2.9±2.2	64.0±13.0

[1] 0.1 HAU of opsonin incubated with bacteria before nebulising

[2] minutes

[3] total viable count/lung x 10^{-4}

Table 6.3

Clearance of *V. cholerae* from the peritoneum of conventionalised mice.

Opsonin	nil		Immune serum[1]		sIgA[2]	
Time[3]	Count[4]	% cleared	Count	% cleared	Count	% cleared
0	2.7 ±0.2	0	2.2 ±0.2	0	2.7 ± 0.2	0
20	2.8 ±0.2	0	0.23±0.08	90.0±2.0	2.4 ±0.4	11.0±9.0
40	0.26±0.10	90.0±2.0	0.03±0.02	99.0±1.0	0.41±0.07	85.0±2.0

[1] 1/250 HAU

[2] 1/50 HAU

[3] minutes

[4] total viable count/lung x 10^{-4}

and excess sIgA (150-1250 fold excess determined by PD_{50}). Results, shown in Table 6.4, failed to demonstrate any blocking effect of IgA.

Thus in two critical tests of the in vivo and in vitro activity of IgA, no evidence was found that IgA could significantly block phagocytosis or complement fixation. The only delay demonstrated was quite reversible and could not be expected to have any biological significance.

6.3 Fc RECEPTORS ON ALVEOLAR MACROPHAGES

The evidence presented above suggested that IgA was not opsonic for macrophages present in the lung. This would be expected if these macrophages lacked Fc receptors for immunoglobulin of this class.

In order to determine this, cells were obtained from the lungs of mice by bronchial lavage. Mice were sacrificed and their tracheas exposed. These were intubated with a blunt needle and the lungs lavaged repeatedly with small volumes of Hanks' balanced salt solution (Ca^{++}, Mg^{++} free). This method is adapted from that of Ryning and Remington (1977). Approximately 5-10 x 10^5 mononuclear cells could be recovered from the lungs of each mouse. Peritoneal cells were also obtained by lavage for comparison. Monolayers of macrophages from both sources were prepared in micro-culture trays. Cells were centrifuged and resuspended in RPMI 1640, containing 20% FCS. They were incubated in the trays for 2 hours at 37^0 in 5% CO_2 in air. The monolayers were washed with fresh medium and 1% SRBC added. These SRBC were sensitised with V. cholerae 569B LPS. After a further incubation of 45 minutes, the

Table 6.4

Effect of sIgA on the bactericidal reaction of immune serum against *V. cholerae*.

V. cholerae[1] plus:	Viable count[2]
(a) diluent alone	7.4×10^3/ml
(b) serum[3]	3.4×10^3/ml
(c) serum[4]	< 40/ml
(d) sIgA[5]	6.4×10^3/ml
(e) serum[4] plus sIgA[5]	< 40/ml

[1] 10^4/ml plus 1/20 fresh guinea pig serum

[2] viable count after 60 minute incubation at 37°

[3] 1 bactericidal unit

[4] 2 bactericidal units

[5] equivalent to 100 times the haemagglutinating titre of 2 bactericidal units of serum

monolayers were washed again and inspected under a light microscope. Macrophages bearing more than greater than 5 SRBC attached were counted as positive.

Without the addition of antiserum, no rosettes were observed. LPS-coated SRBC were incubated with 0.1-1.0 HAU of antiserum for 15 prior to addition to the monolayers. Other cells were incubated with 1.0 HAU of sIgA. Rosettes were seen on over 80% of peritoneal cells using whole serum as opsonin, and approximately 20% of alveolar cells. No rosettes were observed in either population when sIgA was used as an opsonin. Fc receptors for IgG were specifically shown by the use of mouse anti-SRBC serum purified by Protein A chromatography (kindly provide by Dr. P. Ey of this department). Fc receptors for IgG were clearly demonstrated on both alveolar and peritoneal macrophages.

Alveolar macrophages therefore appeared to ressemble peritoneal macrophages in the possession of Fc receptors for IgG and not for sIgA. These results support the findings of Reynolds and Thompson (1973b) in vitro, and our own findings in vivo on the lack of activity of IgA in promoting clearance of bacterial aerosols from the lung. It seems clear that the alveolar macrophage, and other defenses present in the alveoli such as serum-derived complement, cannot be implicated in any local immunity mediated by IgA. Nor do these results lend any support to the concept that IgA might promote removal via the muco-ciliary system. Any possible role for IgA in protection would appear to lie in the upper airways or with components of lower airways defense not present in the normal animal, such as cellular infiltrates.

6.4 PRODUCTION OF INTESTINAL IgA ANTIBODY TO Kpnl

The evidence so far accumulated gave no indications of a role for local immunity in protection against bacteria as proposed by earlier studies (Walsh and Cannon, 1936). As previously stated, however, the normal route of infection by gram-negative organisms is not by aerosol but by colonisation of the nasopharynx. Furthermore, the experimental model of klebsiella infection used in this study uses intra-nasal infection, thereby also using the natural route. Any action of local immunity at this level could be demonstrated by this model.

Mouse intestinal secretions are an excellent source of secretory immunoglobulins as already described. Bloom and Rowley (1979) have clearly demonstrated that the feeding of live organisms to mice produces excellent local humoral responses in the intestine that may be significantly boosted by intra-venous injection of the same organisms. A similar protocol was therefore also attempted with Kpnl. Mice were fed 10^6 live Kpnl in 0.5 mls. of 25% saturated $NaHCO_3$. Approximately 40% of these mice died after the first immunisation and the remaining mice failed to demonstrate any resistance to a second dose, 30-40% again dying. The immunising dose was progressively decreased, 10^3 organisms permitting sufficient mice to survive four feedings. Mice were then boosted by intra-venous injection of 10^6 ultra-violet irradiated Kpnl. Serum and intestinal secretions were obtained one week after this injection.

Serum from these mice possessed a haemagglutinating titre of 256 against CPS-K. The intestinal secretions were partially purified as described above by passage through Sephadex G200.

Immunoabsorption columns were not used. No antibody activity was detected in the immunoglobulin pools, even after extensive concentration using aquacide. Nor was any protective activity found when these immunoglobulins were used to passively protect mice against an intra-nasal challenge with Kpnl.

6.5 LOCAL IMMUNISATION OF THE LUNG

As attempts to prepare antibody in the IgA class against Kpnl from intestinal secretions appeared to be quite unsuccessful, it was decided to approach the problem by locally immunising mice via the nose. Obviously, the advantages of live vaccination to promote optimal local responses would have to be surrendered. Even the use of antibiotics to protect mice against live organisms was found to be ineffective. Due to the importance of heat-labile antigens in this strain (see Chapter 5), a glutaraldehyde killed vaccine was decided upon. Mice were immunised with 10^8 glutaraldehyde killed Kpnl, given intra-nasally (i-n), intra-venously (i-v) or orally. Four vaccinations were made followed by the standard i-v booster. A week after this booster, mice were challenged i-n with Kpnl. This experiment was performed on two separate occasions. Mice were challenged in both experiments with doses of Kpnl from 1×10^1 to 1×10^5. Orally immunised mice exhibited no protection at all, that is, all succumbed to the lowest dose. So too did control mice that were unimmunised or had received only the final, i-v booster. Results from i-v and i-n immunised mice are presented in Table 6.5 .

Both i-v and i-n immunisation produce active protection in these mice. There is no correlation however between the resistance of these groups of mice and the haemagglutinating activity of their

Table 6.5

Effect of route of immunization on protection to intra-nasal challenge to <u>K. pneumoniae</u> and on passive haemagglutinating activity of serum.

Experiment	Immunization[1]	LD^{2}_{50}	H.A.U.[3]
A	i-n	991	64
	i-v	704	2048
B	i-n	398	32
	i-v	180	512

[1] route of immunization, intra-nasally (i-n) or intra-venously (i-v)

[2] 50% lethal dose of Kpn1 as number of organisms

[3] H.A.U./ml. of serum

sera. While the i-v immunised mice exhibited high levels of serum antibody (which varied proportionately with the LD_{50}), i-n immunised mice exhibited extremely low levels of circulating antibody. This finding suggested that local immunity had indeed been produced in the i-n immunised mice, as local immunisation had produced better protection than i-v immunisation in each experiment.

The demonstration that this was so rested on the finding of higher levels of local antibody in these animals.

6.6 ANTIBODY LEVELS IN SERUM AND PULMONARY SECRETIONS

As the resistance of these mice, particularly the i-n immunised group, did not appear to be related to serum antibody levels, pulmonary secretions were obtained from these mice. The same technique as used for obtaining pulmonary cells was used, with the difference that the cells were removed by centrifugation. The secretions were then concentrated with aquacide and dialysed against saline. The total protein concentrations of these preparations were determined by the method of Lowry et al., 1951. Very low levels of haemagglutinating activity were found in these secretions. An ELISA was performed as previously described. Activity against CPS-K and crude membrane extracts was assayed. Enzyme was coupled to both goat anti-mouse light chain and to goat anti-mouse alpha chain. This latter anti-serum was raised in goats by repeated injection of mouse IgA and purified by passage through columns of IgG subclasses and IgM coupled to Sepharose 4B. This antiserum recognised only IgA immunoglobulins, as determined by Ouchterlony gel diffusion and by ELISA against purified mouse immunoglobulins.

Extremely low levels of antibody were found in the serum of

i-n immunised mice when assayed by ELISA (Figure 6.1). However, some IgA antibody could be detected when goat anti-mouse alpha chain was used in this assay (Figure 6.2). By contrast, no IgA antibody was found in the serum of i-v immunised mice.

The total antibody levels in pulmonary secretions were then determined. These were considerably higher in i-n than in i-v immunised mice, as was the content of IgA antibody (Figures 6.3 and 6.4).

Immunoglobulins could be detected in the secretions of i-n immunised mice by single radial immunodiffusion. In addition to IgA, several sub-classes of IgG could be detected; IgG_1 and IgG_{2a} were detectable (Table 6.6). No IgM could be detected. Nor could significant levels of immunoglobulin be found in the pulmonary secretions of i-v immunised animals. Intra-nasal immunisation not only increases the levels of antibody in pulmonary secretions, particularly in the IgA class, but it would also seem to increase the total levels of immunoglobulin. The total increase in immunoglobulins detectable is due only in part to anti-klebsiella antibody. As these secretions possessed no haemagglutinating activity, the maximal possible concentration of IgG antibody present as determined by quantitative precipitin (see Chapter 5) cannot account for the total increase in this immunoglobulin class. Certainly, CPS-K from K. pneumoniae has been reported to act as an adjuvant under certain conditions (Nakashima, Kojima and Kato, 1976), and this may be the mechanism of production of a non-specific rise in immunoglobulins.

The finding that no IgM could be detected in these secretions is in agreement with other studies of pulmonary secretions that have reported either no detectable IgM or extremely low levels (Hand,

Table 6.6

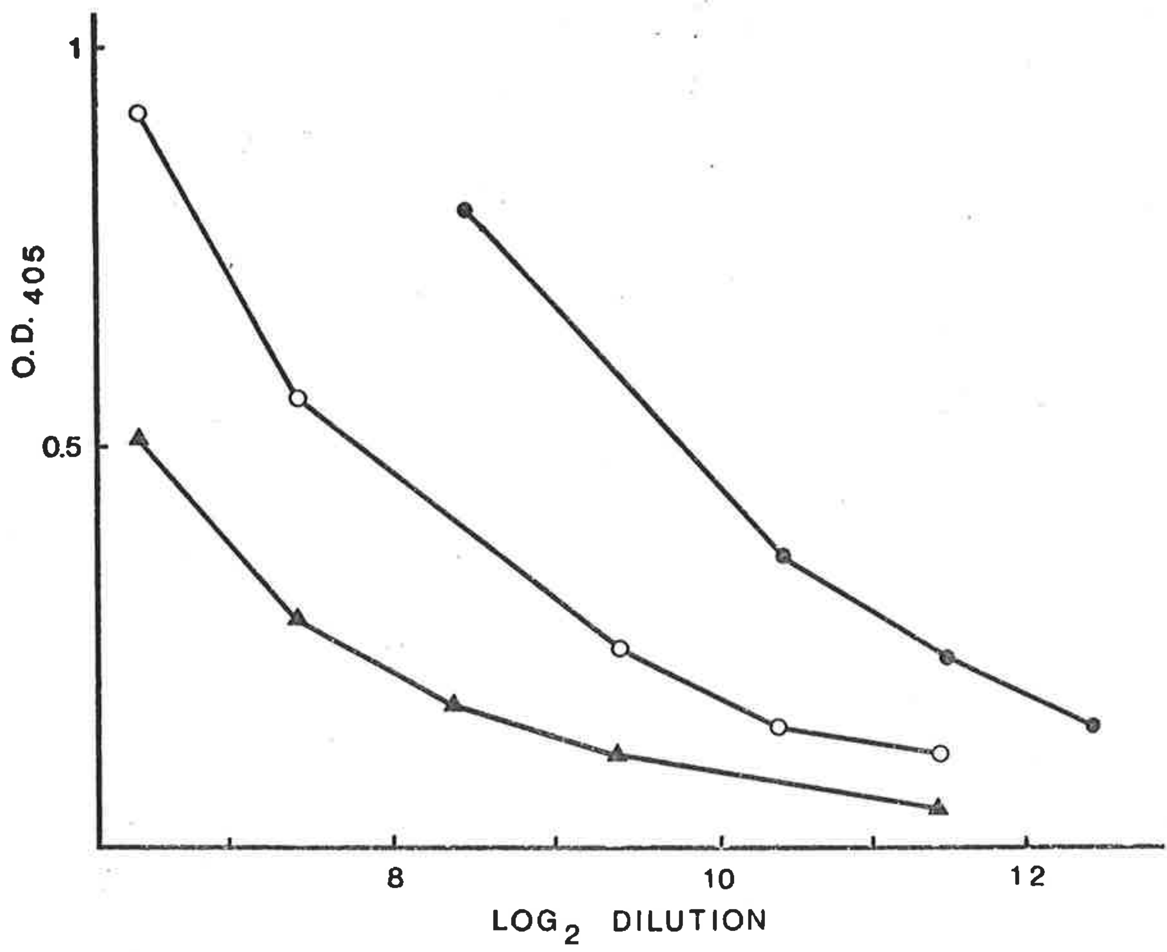
Content of immunoglobulin of lung secretions from control, intra-nasally (i-n) and intra-venously (i-v) immunised mice.

Immunoglobulin	Concentration*		
	control	i-n	i-v
IgA	<0.8	3.6	< 0.8
IgM	<0.4	< 0.2	< 0.4
IgG_1	<2.0	2.3	< 2.0
IgG_{2a}	<0.4	1.4	0.5

* concentration of immunoglobulin in mg/ 100 mgs of total protein in lung secretions of control and immunized mice.

Figure 6.1

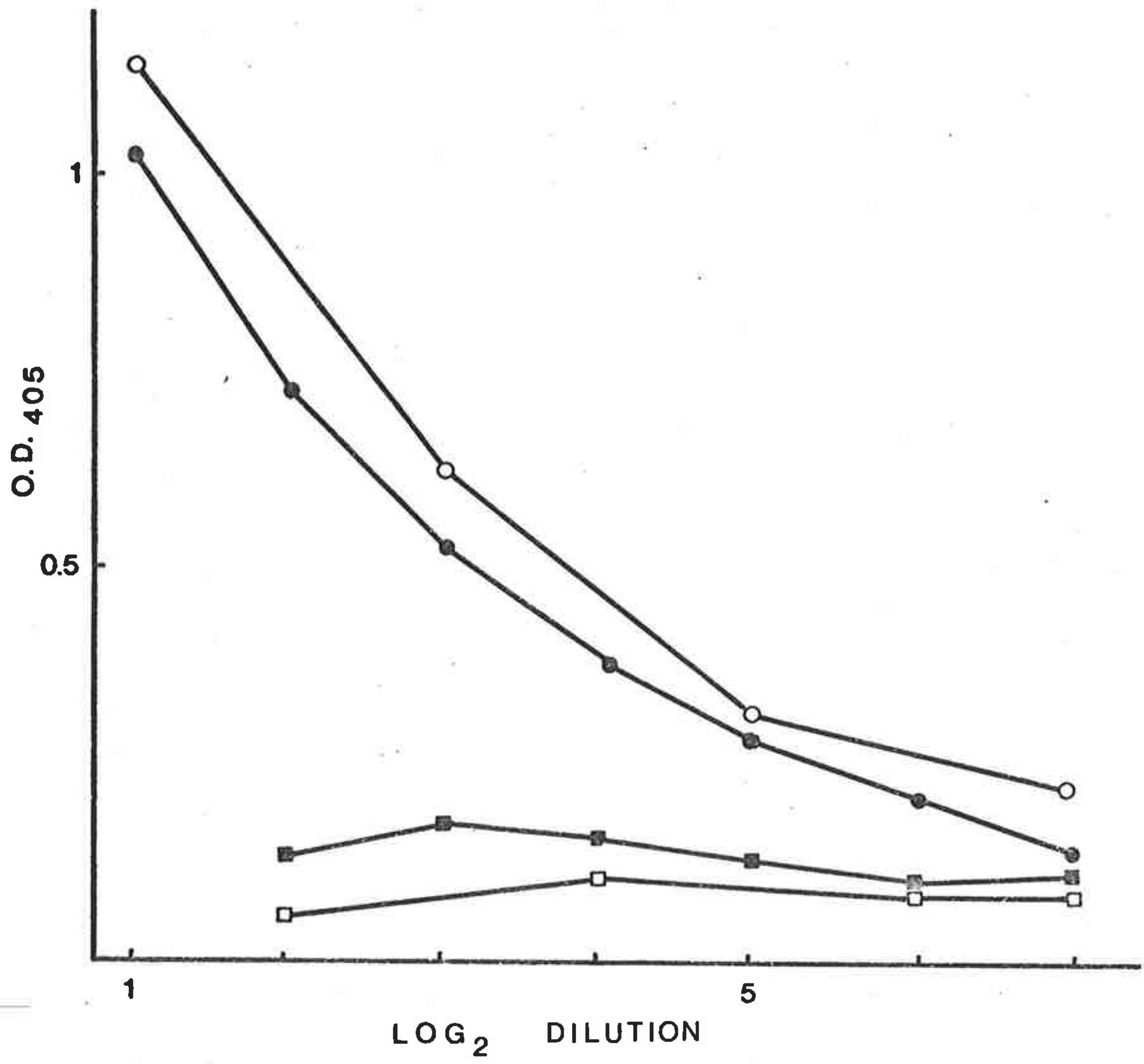
Antibody content of serum from control (▲)and i-n immunized mice (experiment A, ● ; experiment B, ○) detected by ELISA against CPS-K, and developed using goat anti-mouse Fab. Serum from i-v immunized mice recorded an $O.D._{405}$ of greater than 2.0 at dilutions of $1/2^{11}$.



1
0.5
O.D. 405
8
10
12
LOG 2 DILUTION

Figure 6.2

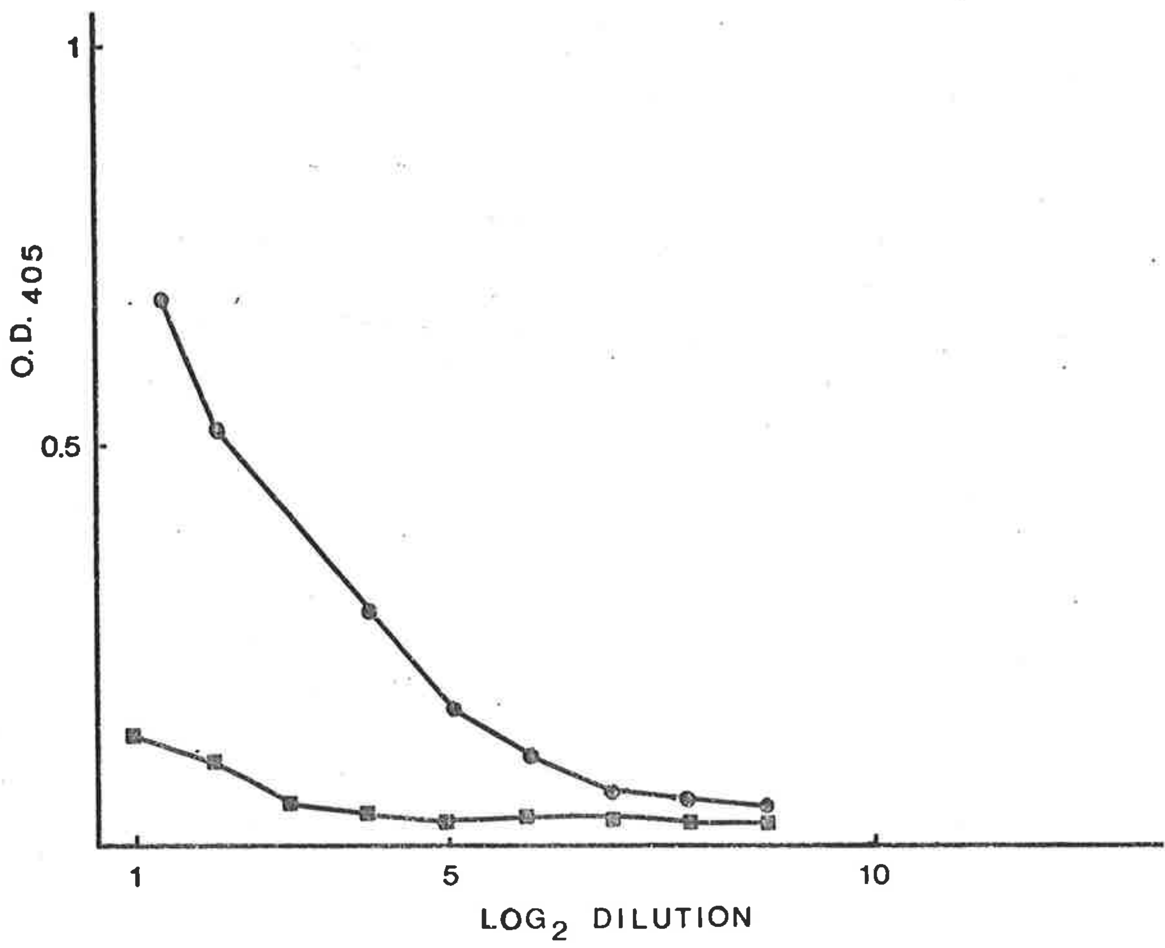
IgA antibody content of serum from i-v immunized (■ , □) i-n immunized mice (● , ○) detected by ELISA against CPS-K, and developed using goat anti-mouse alpha-chain, in two separate experiments; A (■ , ●) and B (□ , ○).



O.D. 405
1
0.5
1
5
LOG 2 DILUTION

Figure 6.3

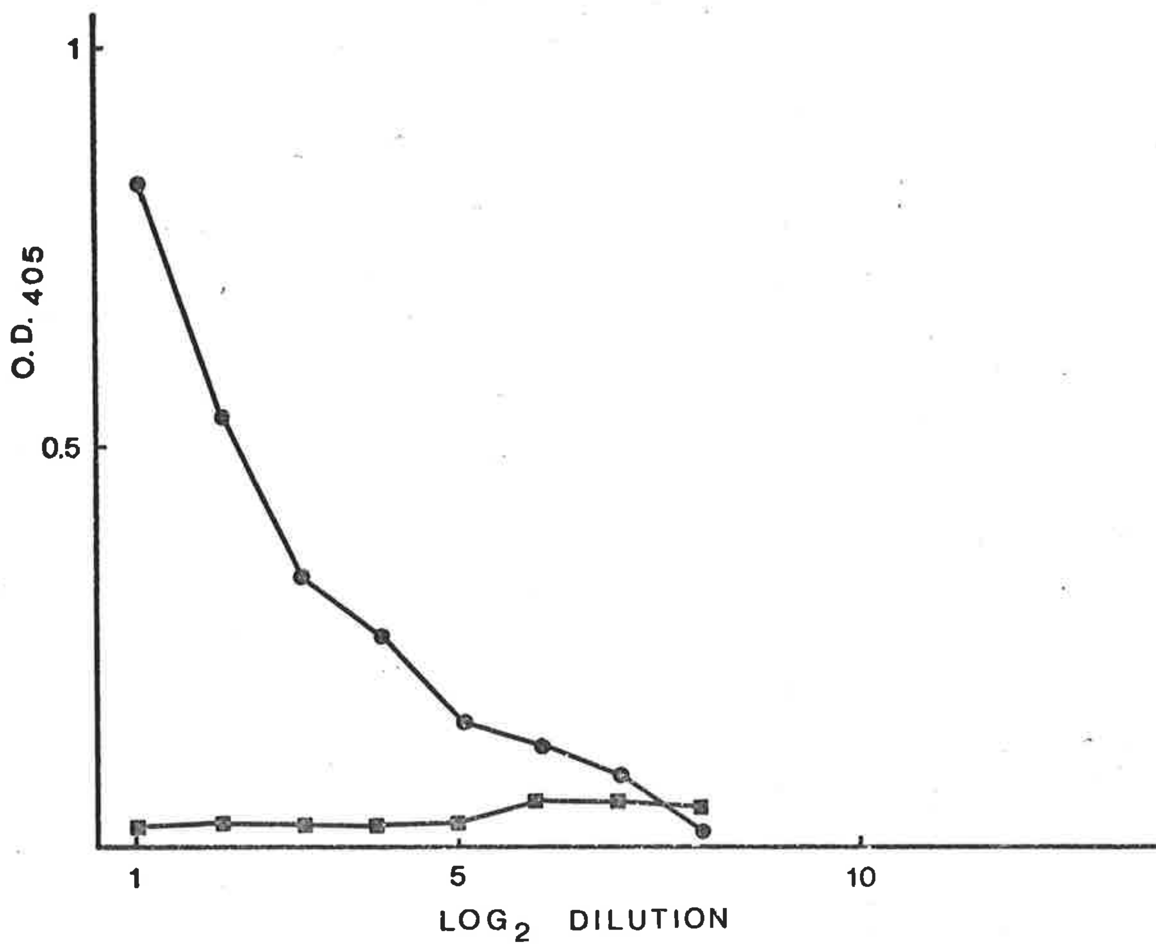
Antibody content of lung secretions from i-v immunized (■) and i-n immunized mice (●) detected by ELISA against crude extract of Kpnl, and developed using goat anti-mouse Fab.



1
0.5
O.D. 405
1
5
10
LOG2 DILUTION

Figure 6.4

IgA antibody content of lung secretions from i-v immunized (■) and i-n immunized mice (●) detected by ELISA against crude extract of Kpnl, and developed using goat anti-mouse alpha-chain.



1
0.5
O.D. 405
1
5
10
LOG 2 DILUTION

Cantey and Hughes, 1974; Reynolds and Thompson, 1973a). It suggests that the immunoglobulins detected are locally produced or selectively transported from serum and are not present due to contamination by serum.

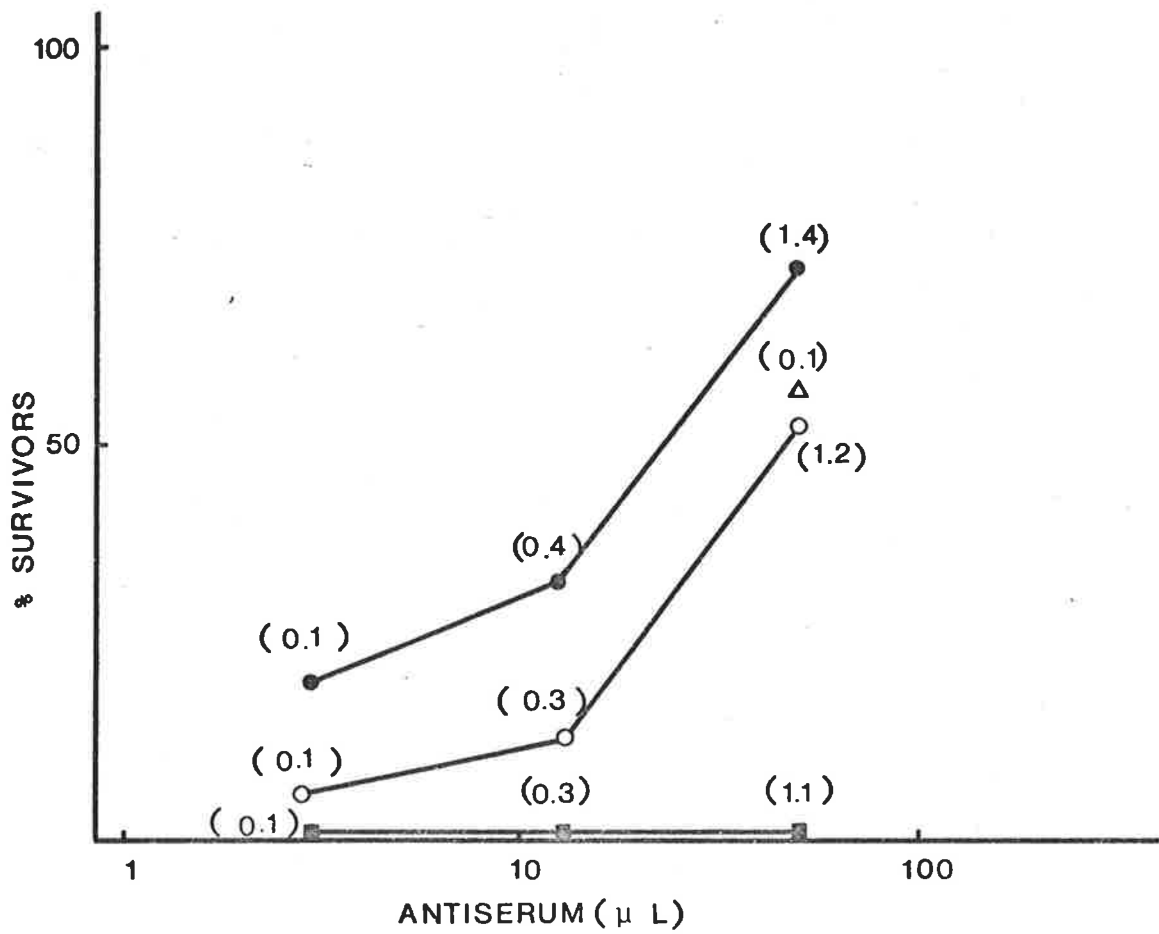
6.7. PASSIVE PROTECTION BY IgA

If the immunity produced in these mice was due to the presence of antibody in pulmonary secretions then these secretions might passively protect mice, similarly to antiserum as described. The extremely low levels of antibody in these secretions, on the basis of haemagglutinating activity, dictated that extremely high concentrations of the secretions should be used in the infecting dose of Kpnl. Secretions were obtained also from normal mice and concentrated to similar total protein levels. While secretions from normal mice failed to demonstrate any protection, those from I-N immunised mice produced good protection. 50% protection was produced at levels of approximately 1-2 HAU (Figure 6.5).

Immunoglobulins from these secretions were partially purified using Protein A chromatography. The IgG and residual pool were concentrated to the original volume of the secretions and dialysed against saline. The residual pool contained only IgA as determined by Ouchterlony gel diffusion. Neither pool had detectable haemagglutinating activity. Protective activity was found in both pools (Figure 6.5).

Figure 6.5

Protection to intra-nasal challenge with 20 Kpnl mediated by lung secretions from immunized mice (●), IgG prepared from these secretions (△) and secretions after removal of IgG (○). Normal secretions (■). Survival of mice shown plotted against quantity of secretions given with inoculum. Total protein concentrations shown in parentheses (mg/ml).



100
50
% SURVIVORS
(1.4)
(0.1)
(1.2)
(0.4)
(0.1)
(0.3)
(0.1)
(0.3)
(1.1)
(0.1)
1
10
100
ANTISERUM (μ L)

6.8 ROLE OF IgA IN THE LUNG

The above results have demonstrated that local immunisation of the lung may produce good protection to lower respiratory tract pathogens such as K. pneumoniae. The resistance produced is characterised by the local production of antibodies, particularly IgA but also IgG. Local immunisation produces circulating IgA antibody while systemic immunisation produces no antibody in this class. The protective effects of serum-derived IgM and IgG antibodies has already been described. It is easy to conceive of anti-bacterial immunity mediated by antibody of these two classes in the lung, whether one invokes phagocytosis or complement mediated bacteriolysis. The role of IgA is far less clear. Evidence from the clearance of bacterial aerosols and the finding that alveolar macrophages do not possess Fc receptors for IgA clearly does not indicate any role for these macrophages.

Rehm , Gross and Pierce (1980) have suggested a possible role for polymorphonuclear cells in resistance to K. pneumoniae. In general, the maximal polymorph response appears to occur after 2-3 days, at a time when no bacteria may be recovered from the lungs of infected mice. However, receptors for IgA have been described on polymorphs using human myeloma proteins (Henson, Johnson and Spiegelberg, 1972; Lawrence, Wiegel and Spiegelberg, 1975). In general, these seem to be receptors for monomeric IgA and not for sIgA and no in vivo correlates exist that would suggest an important biological role for these receptors. Nevertheless, such a mechanism could explain the results obtained with local immunisation against Kpn1.

To determine whether or not sIgA could be opsonic for

polymorphonuclear cells, the bactericidal activity of mouse polymorphs was determined in the presence of immune serum or sIgA. These experiments were kindly performed by Dr. J. Finlay-Jones of the Department of Clinical Microbiology, Flinders University, Adelaide. An exudate rich in polymorphs was elicited in the peritoneum of LACA mice by injection of 1 ml of brain heart infusion broth, 4 hours before harvesting the exudate cells by lavage. The resultant exudate contained approximately 70% polymorphs. After lavage, the cells were washed twice by centrifuging and resuspended to 5×10^6/ml, final concentration, in RPMI 1640 containing heat-inactivated mouse serum (up to 10%). V. cholerae were added to approximately 5×10^5/ml and immune serum or sIgA was added. The cell-bacteria mixture was prepared in test tubes that were then incubated with shaking at 37° and samples taken for viable counting during the incubation. Sampling was performed by disrupting the cells with Triton-X 0.1% in the presence of 10% foetal calf serum, a process that did not decrease the viability of the bacteria. V. cholerae was used in this experiment due to the availability of purified sIgA. No killing of bacteria occurred in the presence of sIgA and peritoneal exudate cells (Table 6.7).

These results do not support any role for IgA in opsonising bacteria for polymorphs in biological systems.

It has been stated previously that complement components are present at only low concentrations in bronchial secretions and would not appear to be of great importance to pulmonary resistance. Inflammation as occurs during infection could be expected to permit the transudation and exudation of serum proteins into the lung and complement might, in this circumstance, play a role. IgA has been reported to activate complement via the alternative pathway (Klaus

Table 6.7

Destruction of V. cholerae 569B by mouse polymorphonuclear leukocytes in vitro in the presence of heated, normal mouse serum (5-10%) and immune serum or sIgA, in excess (5 HAU).

Opsonin	Leukocyte associated killing* at:	
	60 minutes	120 minutes
nil	116	77
Immune serum	62	23
sIgA	102	86

* $\frac{\text{Viable count in presence of leukocytes}}{\text{Viable count in the absence of leukocytes}} \times 100\ \%$

et al., 1979),and might thereby be important in this situation, although it has always been difficult to demonstrate complement dependent bactericidal activity of IgA *in vitro*. This possible action of IgA would not appear to be important in resistance to Kpnl as this organism is resistant to the action of complement *in vitro* (Table 6.8).

While demonstrating the effectiveness of local immunity in protection against *K. pneumoniae* infection, these studies also show that this protection is not mediated by any bactericidal effect of locally produced sIgA in the lower respiratory tract. Presumably, therefore the site of action of sIgA is restricted to the upper respiratory tract.

Table 6.8

Efficiency of antiserum in promoting passive haemagglutination, passive haemolysis and complement dependent bactericidal reaction against V. cholerae 569B or Kpnl.

Antiserum, to	HAU[1]	HLU[2]	BU[3]
V. cholerae 569B	512	512	1.6×10^5
K. pneumoniae Kpnl	4096	8000	< 10

[1] haemagglutinating units/ml serum detected against SRBC coated with the homologous 569B LPS or Kpnl CPS-K.

[2] haemolytic units/ml serum using sensitised SRBC as for passive haemagglutination and 1/20 fresh guinea pig serum

[3] bactericidal units/ml using live homologous organisms in the presence of 1/20 fresh guinea pig serum

CHAPTER 7

DISCUSSION

No study of the role of immune responses in resistance to a particular infective agent can ignore questions of general importance in host-bacteria relationships. This study on acquired resistance to pulmonary infection with *Klebsiella pneumoniae* has demonstrated several general points of interest in addition to the implication of local and systemic immunity in this resistance.

7.1 ROLE OF ANTIGEN IN THE CONVENTIONAL ANIMAL

The importance of environmental antigens in maintaining normal levels of immunoglobulin in normal animals has been documented (Horsfall, Cooper and Rowley, 1978). Several results from this present study extend these observations and have further demonstrated that such effects may alter *in vivo* responses to micro-organisms in a significant fashion. Within the lung, alveolar macrophages appear to be responsible for the removal of organisms deposited directly in the alveoli by aerosol exposure. Phagocytosis and *in situ* destruction of these micro-organisms proceeds at varying rates within the normal animal dependent on the organism in question (Rehm, Gross and Pierce, 1980). Several organisms are quickly removed from the alveoli of animals. One such organism, reported above, is *Vibrio cholerae*. The rate at which this organism is removed is seen to depend on the antigenic load to which the animals have been exposed. Germ-free and specific pathogen free (s.p.f.) mice clear these organisms at an extremely slow rate when compared

with animals exposed to conventional levels of micro-organisms and antigen. S.p.f. mice show nearly normal rates of clearance of organisms when specific antibody is used to opsonise the bacteria. This indicates that the macrophages of such animals are active, and can be contrasted with responses in 6 day old mice in which even the addition of antiserum results in only poor clearance presumably indicating the immaturity of their reticulo-endothelial system. In adult s.p.f. mice, the poor clearance can be explained by the lack of opsonising antibody, presumably present as natural or cross-reacting antibody in the normal mouse. Such an explanation is consistent with evidence relating to immunoglobulin levels in conventionalising animals and the general importance of antibody in promoting phagocytosis.

Evidence from published studies (Horsfall, Cooper and Rowley, 1978) has shown that polyclonal increases in immunoglobulin levels occur in conventionalising animals (that is s.p.f. mice exposed to conditions in a conventional animal house). These increases may be simulated by exposure of s.p.f. mice to one strain of bacteria, in which case specific antibody accounts for a minor proportion of the resulting increase in immunoglobulin levels. The lung provides an experimental situation in vivo where this phenomenon may be studied and seen to be important. The mechanism by which increased antigen loads may enhance immunoglobulin levels is not clear. Several possibilities may be envisaged. Under some circumstances a disproportionately high response may be seen in the IgG_{2a} class. It may be that mitogens provided by bacterial colonisation of the intestine are responsible for this effect. Bacterial products such as lipopolysaccharide are reported to be polyclonal B-cell activators, and possibly some specific ones may

predispose to an increase in IgG_{2a} production.

While mitogens present amongst bacterial products can be used to explain these phenomena, the addition of foreign antigen may also be expected to alter the balance of immune responses in the host. For example, theories of network regulation of the immune system (Jerne, 1974) may be used to explain the development of the antibody repertoire by idiotype-anti-idiotype reactions even without the contribution of foreign antigens (Adam and Weiler, 1976). The general immunoglobulin levels controlled by such network interactions could be expected to change at a polyclonal level with the introduction of antigen. Such a change would not necessarily be specific and might simply represent the establishment of a new equilibrium within the immune system.

While the above discussion has emphasised humoral events, evidence has been presented in this thesis that cellular events may also be influenced by antigen loads. The alveolar macrophages do appear to possess normal ability to destroy V. cholerae. In other experiments, the efficiency of peritoneal macrophages from s.p.f. mice in bactericidal assays was shown to be impaired. This impaired activity was shown against Salmonella typhimurium and cannot be directly compared with events discussed above in the alveoli. V. cholerae is easily destroyed by mouse peritoneal macrophages while the degree of activation of these cells has been shown to alter importantly their bactericidal action towards S. typhimurium. This organism may more accurately reflect the degree of activity of macrophage populations. In addition to the impaired ability of peritoneal exudate cells from s.p.f. mice to destroy salmonellae, they responded to stimulation by lipopolysaccharide (LPS) less vigorously than cells from conventional mice, as measured by

H_2O_2 production. This would appear to be a further demonstration of the importance of antigen in maintaining normal immune responsiveness. Activation in vivo by intra-peritoneal injection of live S. enteritidis produced cells capable of enhanced release of H_2O_2 in both normal and s.p.f. mice. This method of activation involves T-cells while LPS stimulation does not appear to do so. The ability of cell populations to respond to LPS appears to vary according to their prior stimulation by antigen, or possibly other stimulants such as thioglycollate (Doe and Henson, 1978).

Normal levels of antigen induced by exposure to micro-organisms, but in the absence of disease resultant from these organisms, can therefore be seen to determine the background level of immune competence in the host. The exact mechanism of this is not clear, but effects both humoral and cellular events. This mechanism results in significant in vivo enhancement of responses to micro-organisms.

7.2 REQUIREMENT OF ANTIBODY BY ACTIVATED MACROPHAGES

Due to the importance attached to alveolar macrophages in defense of the lower respiratory tract, experiments outlined in Chapter 4 were designed to determine whether antibody's role as an opsonin remained important following activation of macrophages. Antibody has been thought to play little role in the phagocytosis of bacteria such as Listeria monocytogenes by activated macrophages (Mackaness, 1969). If the process of activation could alter the general requirement of antibody for the phagocytosis of a variety of organisms as previously shown (Robertson and Sia, 1927; Jenkin and Benacerraf, 1960; Jenkin and Rowley, 1959), then interpretation of

studies demonstrating the importance of opsonising antibody in protection to respiratory pathogens must be qualified.

Experiments performed in this study, demonstrated that antibody was absolutely required for the destruction of S. typhimurium by normal and activated macrophages in vitro. On the other hand, L. monocytogenes was destroyed in the absence of antibody, but only by activated cells. This difference in the requirements of the two organisms for opsonins confirms general findings that antibody to L. monocytogenes is not required for the expression of cell-mediated immunity to this organism (Mackaness, 1969; McGregor, Koster and Mackaness, 1971) whereas specific antibody greatly enhances acquired cellular immunity to S. typhimurium (Davies, 1976).

The ability of macrophages to kill L. monocytogenes in the absence of antibody was correlated with the ability of this organism to bind to macrophages under these conditions whereas S. typhimurium required the presence of antibody. The binding of L. monocytogenes to macrophages was dependent on Ca^{++} and Mg^{+} ions while antibody dependent binding was not. This finding answers the possible criticism of other in vitro studies that cell-bound antibody can explain demonstrations of binding of particles to macrophages in the apparent absence of antibody.

Binding of L. monocytogenes to macrophages differs from that previously reported for Corynebacterium parvum (Orgmundsdottir and Weir, 1976; Orgmundsdottir, Weir and Marmion, 1978) in that no inhibition could be obtained by sugars. Furthermore, binding of listeria occurs in the presence of 10-20% foetal calf serum and would appear therefore to be able to occur in vivo in the presence of high concentrations of protein.

These differences between the handling of salmonellae and listeria show the weakness of considering acquired immunity to the facultative intra-cellular organisms as though it were a generalised non-specific immunity. There is a tendency to consider host responses to these organisms to be similar. This is clearly not so as demonstrated above. Other evidence confirms this belief. Studies on the resistance of inbred mouse strains show that those resistant to salmonella may be susceptible to listeria and vice versa (Hormaeche, 1979; Cheers and McKenzie, 1978; Cheers et al., 1978).

Activation of macrophages does not appear to qualitatively alter opsonic requirements. The normal maturation of macrophages has been reported to result in altered expression of Fc-receptors on their membranes. Fc-receptor expression has been reported to increase during cell maturation (Cline, 1975; van Furth, 1976). Furthermore activation of macrophages produces an increase in number and affinity of these receptors (Rhodes, 1975). Such alterations in the expression of an important membrane receptor, possibly the most important in terms of the ability to promote phagocytosis, do not reflect more fundamental changes in the macrophage membrane that would completely abrogate the need for antibody. Certainly the amount of antibody required may be altered quantitatively but it would seem that the requirement for antibody depends on the inate ability of the organism to bind to macrophages and not on the character of the macrophage in question. Thus if antibody appears to be required for the ingestion of a given organism by normal macrophages, the same requirement can be expected during a cell-mediated response.

7.3 ROLE OF HUMORAL FACTORS IN RESISTANCE TO K. PNEUMONIAE

K. pneumoniae infection of mice was chosen as an animal model of lower repiratory tract infection. Acquired immunity to this organism was shown to be produced by systemic immunisation with various killed vaccines. Immunity could also be produced by passive transfer of antibody, in the form of antibody used to opsonise bacteria before exposure. This model therefore appeared suitable for the investigation of the interactions between various host defenses in the lung.

Human infection with K. pneumoniae, and other gram-negative organisms, generally follows colonisation of the upper respiratory tract of seriously ill patients. The infection is normally hospital-acquired. The animal model used in these studies involved intra-nasal inoculation of anaesthetised mice. The lethal dose of organism when given intra-nasally was considerably less than when given by aerosol. Thus upper respiratory tract deposition of this organism also appears to be important in the experimental situation.

Systemically immunised mice were strongly protected against infection and protection could be mediated by both IgG and IgM isolated from the serum of these mice. Protection could also be produced by intra-nasal immunisation of mice with killed K. pneumoniae. This was correlated with the production of antibody in the pulmonary secretions of these mice in the absence of high levels of serum antibodies. IgA and IgG from these secretions were protective in promoting passive immunity.

The role of immunoglobulin class in the lung was also investigated using aerosol clearance of V. cholerae. The use of this non-virulent organism proved to most helpful in these studies on the

efficiency of alveolar macrophages in clearing opsonised organism. Furthermore, the extreme difficulty of preparing IgA directed against K. pneumoniae in high quantities, excluded the possibility of performing these experiments with that organism. Difficulty was experienced as the most plentiful source of secretory antibody in the mouse is intestinal washings. To produce high levels of antibody in these secretions, immunisation with live bacteria is required. Due to the highly virulent nature of Kpn1 when given orally, this was not found to be practical. Intra-nasal imunisation of killed vaccine did result in the production of IgA but its preparation was extremely tedious and only low yields were obtained.

Experiments on aerosol clearance of V. cholerae, demonstrated that while IgG and IgM were efficient in promoting clearance from the lower respiratory tract, IgA was not. These experiments therefore differed importantly from those demonstrating protection to K. pneumoniae.

It is envisaged that the clearance of the non-virulent organism, V. cholerae, relies on the in situ killing by alveolar macrophages. The differing effects of IgG and IgA agree with the finding of Fc receptors for IgG but not for IgA on these cells. The presence of receptors for IgG on alveolar macrophages has been shown previously, although receptors for IgM were not demonstrated (Reynolds et al., 1974). The ability of IgM to promote clearance is not to be explained as easily. IgM receptors may be cryptic and may therefore be difficult to demonstrate on macrophages such as peritoneal cells (Haegert, 1979). IgM is extremely efficient at promoting phagocytosis in the peritoneum, whether this be by binding to an Fc receptor or by fixation of complement. Certainly, the ability of IgM to promote phagocytosis in the peritoneum is

paralleled by its activity in the lung. If complement is involved in the latter case, it may need to be derived by transudation from serum as it is not present in high amounts in pulmonary secretions from normal animals. Thus the exact mechanism whereby IgM promotes in vivo destruction of V. cholerae is unresolved. Cryptic Fc receptors on alveolar macrophages may exist, complement may be contributed from serum or low levels of complement present in normal secretions may be sufficient to promote phagocytosis.

Nevertheless, it is clear that IgA does not promote in situ killing of V. cholerae. This agrees with the finding that no Fc receptor for immunoglobulin of this class was detectable on alveolar macrophages and with the poor opsonising ability of IgA in vitro (Reynolds and Thompson, 1973b). The clear protective capacity of IgA in vivo against K. pneumoniae cannot be explained in terms of promotion of killing by alveolar macrophages. Several possible explanations existed.

A characteristic of pulmonary infection with K. pneumoniae is the massive influx of polymorphonuclear leukocytes into the alveoli. Polymorphs have been reported to possess receptors for IgA and possibly these cells could use IgA as an opsonin to promote bacterial killing. Experiments to investigate this were also carried out using V. cholerae due to the availability of purified sIgA antibody. These experiments failed to demonstrate any positive effect of IgA in polymorph bactericidal activity.

In addition, K. pneumoniae Kpnl is not susceptible to the action of complement in vitro and complement activation by the alternate pathway as has been reported for IgA (Klaus et al., 1979) would not appear to explain the protection seen in vivo.

The protection mediated by IgA cannot be explained by

events in the lower respiratory tract despite the fact that this is the site of major pathology in this infection. Rather events in the upper respiratory tract should be considered as the likely site of action. The importance of colonisation of the upper respiratory tract in development of human infection has been demonstrated in several studies. The higher virulence of Kpnl when given intra-nasally to mice compared to aerosol exposure has been mentioned suggesting that upper respiratory tract deposition of this organism is also important in the experimental situation. Attachment of bacteria to mucosal epithelium presumably influences the course of infection and in the susceptible host this initial step is critical. Normal host defenses in the upper respiratory tract appear to be impaired in these hosts.

IgA has been reported to interfere with the attachment of bacteria to epithelial cells (Williams and Gibbons, 1972) and such a mechanism could explain the experimental results. Bacteria unable to attach to the epithelium could presumably be removed by muco-ciliary action in the normal human or in the immune mouse. In the susceptible human, the predisposing factor may involve some aberration of this transport mechanism or some alteration of epithelial surfaces to permit colonisation. Equally some aberration of local immunity could be responsible for colonisation of susceptible hosts.

Local immunity to bacterial infection of the lung has therefore been demonstrated. The response reported appears to fulfill the criteria of classic local humoral immunity. Immunity may be produced by local immunisation, it does not appear to be closely related to serum antibody levels and locally produced IgA can be shown to be protective. Local immunity in this situation, however,

is not more effective then systemic immunisation, nor is IgA more effective than other classes of antibody. Serum antibodies may also contribute to immunity. Indeed, during inflammation of the respiratory tract during infection, the distinction between local and systemic events could be expected to become less important and both local and systemic antibody would contribute to immunity.

Furthermore, despite the demonstration that IgG and IgM can promote killing of bacteria deposited in the lower respiratory tract, the similarity of the efficiency of these classes and of IgA in promoting resistance to K. pneumoniae given by the intra-nasal route suggests that these antibodies may also be operative in the upper respiratory tract. IgM is of course not present at that site in the normal situation (Reynolds and Thompson, 1973a) but is introduced artificially in the experimental situation. IgG and IgM might share the mechanism used by IgA when they are introduced in this fashion into the upper respiratory tract. This would ressemble the situation seen in the intestine where all three classes of antibody are protective despite the normal predominance of IgA (Steele, Chaicumpa and Rowley, 1974). This point could be clarified by similar experiments to those of Steele, Chaicumpa and Rowley (1975) demonstrating that immunity within the intestine was not dependent on the Fc portion of the antibody molecule but was produced by $F(ab)_2$ fragments. If, in the lung, Fc activity was required for the action of IgG and IgM and not for IgA, different pathways of immunity could be proposed. It seems unlikely that the action of IgA is Fc dependent due to the demonstrated lack of importance of phagocytic or complement mediated events in the immunity produced by antibody of this class.

This demonstration of local immunity in the lung highlights

the importance of upper respiratory tract events in the development of lower respiratory tract disease. The immune mechanisms of the upper airways appear to act independently of those of the alveoli, IgA being unable to promote bactericidal reactions at this site.

7.4 ANTIGENS OF K. PNEUMONIAE

Amongst the antigens of klebsiella, capsular polysaccharide has received most attention. This is due to its importance in serotyping the organism and due to its many biological effects. Simply in terms of the copiousness of its production, it is the most easily observed antigen of this bacteria, evident in the luxuriant growth of klebsiella on nutrient agar. With respect to the immunology of infection, capsular antigens have also received great attention, partly due to observations of the efficiency of anti-capsular antibodies in promoting resistance and on the relative inefficiency of antibodies directed against somatic antigens (Macleod et al., 1946). The size of the capsule on klebsiella would suggest that the capsular antigen would be that most easily recognised by opsonising antibody.

Results presented agree with the finding that antibody directed against capsular polysaccharide is extremely protective. Both IgG and IgM antibodies directed against this antigen were raised using heat-killed vaccines of K. pneumoniae, and these antibodies promoted resistance to intra-nasal challenge with K. pneumoniae. Antisera raised against glutaraldehyde-killed klebsiella appeared more protective and protection could be produced by this antiserum after all anti-capsular activity had been removed by absorption with heat-killed klebsiella. Such absoption removed all

protective activity from antisera to heat-killed klebsiella.

These results suggested the presence of a heat-labile non-capsular antigen. Antibodies directed against this antigen were as protective as anti-capsular antibodies. An ELISA was developed to detect antibody against this antigen and an ELISA inhibition to detect the presence of antigen in membrane preparations of strains of K. pneumoniae. The antigen could be detected in several other strains, all of which were avirulent in the mouse but which were apparently virulent in humans as they were clinical isolates. The precise character of this antigen could not be elucidated. This strain of K. pneumoniae possessed neither pili nor flagella. The somatic antigens of the Enterobacteriaceae are classically heat-stable. The heat-labile antigen appeared to be common to several strains of klebsiella. Several antigens are shared by various members of the Enterobacteriaceae. The 'common enterobacterial antigen' does not appear to be important in this context as antibodies against it do not protect against klebsiella (McCabe and Greely, 1973). Antiserum to the Re antigen of strains of S. minnesota have been demonstrated to protect against heterologous organisms but these antisera were raised using heat-killed vaccines (McCabe, 1966). It has been suggested that such antigens may be important in resistance to klebsiella (Chedid et al., 1968) but the present results cannot be explained in this way.

The nature of the antigen must remain uncertain. However, it is clear that non-capsular antigens can play a significant role in vivo and this result stresses that attention should not be devoted to one antigen alone in consideration of host-parasite relationships even when good evidence exists of the importance of that antigen.

These results further emphasise the importance of labile antigens in virulence and in host immunity. Attempts to promote immunity to gram-negative organisms by employing vaccines composed of heat-killed organisms have often resulted in poor immunity, despite the retention of heat-stable antigens such as lipopolysaccharide. In particular, attempts to promote resistance to S. typhi using LPS as the immunogen have been unsuccessful despite the development of high levels of antibody to LPS (Tully, Gaine and Tiggert, 1965; Hornick et al., 1970). The importance of proteinaceous surface structures of several organisms, especially well demonstrated by the work of Swanson and his colleagues (Swanson, 1973; Swanson, Kraus and Gottschlich, 1971), has also supported the view that proteins easily destroyed by harsh chemical or mechanical treatments may be extremely important in host-bacteria relationships. The importance of non-LPS antigens has also been demonstrated with V. cholerae (Attridge, 1979). Thus, despite the interest associated with polysaccharide and lipopolysaccharide antigens, these molecules may not be the most important in determining the outcome of infection. By contrast the importance of polysaccharide antigens has been demonstrated with pneumococcal infection and in the present study with klebsiella.

7.5 CONCLUSIONS

Humoral responses are capable of producing immunity to pulmonary infection with K. pneumoniae. Protective antibodies are directed against at least two antigens; capsular polysaccharide and a heat-labile antigen.

Systemic and local humoral responses are protective. The

local immunity produced by intra-nasal immunisation is mediated by both IgG and IgA antibodies. Antibodies of these classes and of IgM class are protective against intra-nasal challenge to a similar degree.

Lower respiratory tract defenses, in particular destruction of bacteria by alveolar macrophages, are enhanced by IgG and IgM, but not by IgA.

IgA would appear to be protective only in the upper respiratory tract, possibly by interfering with colonisation or bacterial attachment to epithelial cells.

This demonstration of local immunity in the lung confirms earlier evidence of the possible importance of local responses in protection against bacterial infection of the lung (Bull and McKee, 1929; Buescher and Bellanti, 1966). Events in the upper respiratory tract may be seen to influence the subsequent development of disease in the lower respiratory tract. These results extend previous studies on the importance of local immunity in resistance to viral disease of the upper respiratory tract.

Cellular events have not been excluded but it has been shown that activation of macrophages by cell-mediated immunity does not remove the requirement for antibody as an opsonin, if the requirement exists. This requirement is dependent on the bacteria involved. As antibody has been shown to be important in resistance to K. pneumoniae, it is clear that cellular immunity could not be expected to replace this immunity but could of course supplement it.

The respiratory tract may therefore be expected to provide a fruitful model of local immunity for investigation of the precise mechanisms used by IgA, and other classes of antibody, to promote protection. Local immunity may play an important role in the initial

interactions of bacteria with the respiratory tract and determine the course of colonisation of the upper respiratory tract and hence the course of subsequent disease.

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