THE ROLE OF THYMUS-DEPENDENT IMMUNITY IN *MYCOPLASMA PULMONIS* INFECTIONS OF MICE

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PLATES XIX–XXI

THE relative roles of circulating antibodies, local secretory antibodies and cell-mediated immunity in mycoplasma infections are not known.

In man, *Mycoplasma pneumoniae* killed vaccine produces circulating antibodies, but these do not always protect against challenge by the living organism (Smith, Friedewald and Chanock, 1967). In hamsters, high levels of circulating antibody produced by parenteral vaccination with *M. pneumoniae* are also often poorly protective. On the other hand, hamsters previously infected with *M. pneumoniae* may have low levels of circulating antibody and yet be resistant to challenge (Fernald, 1969; Fernald and Clyde, 1970). Some type of local immunity would therefore appear to be more important than circulating antibody for resistance to infection. Searches for local antibody (IgA) in secretions from the respiratory tract of infected hamsters have been unsuccessful, although some has been detected in hyper-immunised animals (Fernald, 1970; Fernald and Clyde). Although it has not been found possible to demonstrate skin hypersensitivity in hamsters that have been immunised with *M. pneumoniae* vaccine, this does develop in immunised guinea-pigs, and lymphocytes both from these guinea-pigs and from previously infected persons can be stimulated *in vitro* by *M. pneumoniae* antigens (Leventhal *et al.*, 1969; Fernald, 1971).

These results suggest that cell-mediated immunity develops during *M. pneumoniae* infections, and the possibility that this type of immunity is more important for protection than circulating or local antibodies requires investigation. Cell-mediated immunity might also contribute to the cellular reaction of pulmonary mycoplasma infections. This speculation is based on the fact that the pulmonary lesions in *M. pneumoniae*-infected hamsters (Dajani, Clyde and Denny, 1965; Fernald, 1970) and in *M. pulmonis*-infected mice (Lutsky and Organick, 1966) show prominent peribronchiolar and perivascular cuffing by mononuclear cells, presumably lymphocytes. Another hint that cell-mediated immunity might play some part in protection against *M. pulmonis* infections comes from the observation that naturally infected, thymectomised mice and rats developed severe pulmonary lesions, with spread of mycoplasmas to the brain (Allison and Purcell, unpublished).

The present investigations were designed to analyse further the role of thymus-dependent immune reactions in protection against *M. pulmonis* infections in mice and in the development of pulmonary lesions.

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MATERIALS AND METHODS

Mice. Young adult CBA mice of either sex, weighing 25–45 g, were used in all experiments.

Ablation of thymus-dependent immunity. Mice were thymectomised when approximately 6–8 wk of age. Two weeks later they received 400 R of whole body X-irradiation. Some thymectomised and irradiated mice, as indicated in the text, were given 0·3-ml doses of rabbit anti-mouse-lymphocyte serum intraperitoneally, the day before infection, 2 days after infection and then weekly until the end of the experiment.

Mycoplasma pulmonis. Strain "Peter C, sub 8", obtained from Dr R. M. Lemcke as a broth culture, was inoculated into 100 ml of freshly prepared mycoplasma medium. After incubation at 37°C in a sealed bottle for 3 days, the medium was dispensed in 1-ml quantities into tightly capped vials and stored at –70°C. The infectivity titre of this stock culture was 10⁷ colour-changing units (CCU) per ml before freezing, and there was no fall in titre over the experimental period.

Medium. The medium (Chanock, Hayflick and Barile, 1962) consisted of 70 per cent. PPLO broth, 20 per cent. unheated horse serum, 10 per cent. of a 20 per cent. yeast extract, 0·1 per cent. dextrose, 0·002 per cent. phenol red, 1000 units of penicillin G per ml and 0·05 per cent. thallium acetate. After adjustment of pH to 7·8 it was used for isolating *M. pulmonis* and for infectivity titrations.

Infection procedure. About 2 mth after irradiation, mice were anaesthetised by intraperitoneal injection of sodium pentobarbitone (0·045 mg per g of body weight). An ampoule of *M. pulmonis* stock culture was thawed at 37°C and the mice were each given an inoculum of 0·0016 ml per g of body weight intranasally, by means of a tuberculin syringe and a 26-gauge needle; the volumes of culture given to the mice varied from 0·04 ml to 0·075 ml.

Collection of specimens. Mice were first anaesthetised by intraperitoneal injection of sodium pentobarbitone (0·06 mg per g of body weight). Blood samples were collected from the axillary vessels. Specimens from the pharynx were obtained by cutting through the skin and bones at the angles of the jaw and passing a cotton wool- or calcium alginate wool-tipped swab at least seven times deeply into the pharynx. The swab was rinsed thoroughly into 1–2 ml of mycoplasma medium, to give an arbitrary 1 in 10 dilution of the specimen. The lungs, *in situ*, were then washed with 1–2 ml of mycoplasma medium, this being injected and withdrawn three times through a cannula tied securely into the trachea (Taylor-Robinson *et al.*, 1972). The medium, after completion of the lavage, was regarded as a 1 in 10 dilution of the lung specimen. Portions of brain, liver and spleen were removed, weighed and thoroughly ground in a tissue grinder with sufficient mycoplasma medium to make a 10 per cent. suspension (w/v). Surgical instruments were always sterilised between the taking of samples from the different organs.

Mycoplasma isolation and identification. Isolation and titration of the amount of mycoplasma present in the blood and other specimens were carried out by making a series of ten-fold dilutions of the specimens in 1·8-ml amounts of mycoplasma medium contained in glass vials. These were tightly capped and incubated at 37°C for 10 days. They were observed at intervals for mycoplasma growth, which was indicated by a reduction in pH and consequent change in colour of the medium. The highest dilution causing a colour change from pink to yellow was considered to contain one CCU of infectivity. In some instances, to aid mycoplasma identification, liquid medium was subcultured to solid medium and colonies sought after incubation at 37°C in an atmosphere of 5 per cent. CO₂ in nitrogen (v/v).

Isolates were identified as *M. pulmonis* by either the disk growth-inhibition technique (Clyde, 1964) or the metabolism-inhibition method (Taylor-Robinson *et al.*, 1966).

Histopathology. After collection of the lavage specimens for mycoplasma isolation, the lungs were redistended with formol-saline through the tracheal cannula, removed from the chest and immersed in the same fixative. Specimens from other tissues were also fixed in formol-saline. After fixation, the tissues were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin.

Immunofluorescence. Grossly abnormal lungs from mice with *M. pulmonis* infections

were "snap-frozen" in acetone-carbon dioxide and stored at -70°C . Sections, approximately $7\text{ }\mu\text{m}$ thick, were cut at -25°C , fixed in acetone and stained with fluorescein-labelled anti-mouse-globulin rabbit antiserum. Sections of lungs, from the same mice, that were not fixed in acetone and lung sections from uninfected mice were stained as controls.

RESULTS

Effect of immunosuppression on M. pulmonis infections

Since thymectomy or treatment with anti-lymphocyte serum (ALS) render mice deficient mainly in cell-mediated immunity, these methods were used to study *M. pulmonis* infections in immunosuppressed and normal mice.

TABLE I

Effect of reduced thymus-dependent immunity on the growth of M. pulmonis in the respiratory tract of mice after intranasal inoculation

Experiment number	Immune status of the mice	Growth and average infectivity titre* of <i>M. pulmonis</i>	
		in the pharynx	in the lungs
1	Normal	6/6†(10 ^{4.8} CCU per ml)	6/6(10 ^{5.5} CCU per ml)
	Thymectomised and X-irradiated	6/6(10 ^{5.5} CCU per ml)	5/6(10 ^{6.6} CCU per ml)
2	Normal	13/22(10 ^{5.7} CCU per ml)	11/22(10 ^{6.3} CCU per ml)
	Thymectomised, X-irradiated and given anti-lymphocyte serum	15/15(10 ^{6.2} CCU per ml)	12/14(10 ^{6.1} CCU per ml)

* Expressed as colour-changing units (CCU) per ml for mice with positive cultures only.

† Proportion of mice from which *M. pulmonis* was isolated following intranasal inoculation.

Effect on respiratory-tract infection. The initial experiment was done with mice that had been only thymectomised and irradiated. Treated and normal mice were killed approximately 3 wk after intranasal inoculation of *M. pulmonis*, and examined for the amount of growth of the mycoplasma in the pharynx and lungs. As shown in table I, *M. pulmonis* was isolated from all the specimens taken from both the normal and thymectomised mice with the exception of the lung washing from one of the treated mice. Thus there was no significant difference in the rate of infection between the two groups. However, the average infectivity titres were about ten-fold higher, for both the pharyngeal and lung specimens, in the treated than in the control animals.

A second experiment was then done on mice that had been thymectomised, irradiated and also treated with ALS. Normal and immunosuppressed mice were inoculated on the same day and 4–6 animals in each group were killed at weekly intervals. The comparative results obtained for the normal

and the treated mice did not vary significantly over the 4-wk period of the experiment and so table I shows only the combined results for the whole period. Among 22 control mice, *M. pulmonis* was isolated from the pharynx of 13 and from the lungs of 11. In contrast, all 15 thymectomised mice gave positive pharyngeal cultures and 12 of 14 lung lavage specimens were positive. These differences between the two groups of mice were significant at the 1 per cent. level, by Fisher's exact test. The infectivity titres found in the specimens from the normal and treated mice were similar, in the case of both the pharynx and the lungs.

TABLE II

Effect of reduced thymus-dependent immunity on the spread of M. pulmonis from the lungs to other organs in mice following intranasal inoculation

Immune status of the mice	Number of mice used	Number of mice yielding isolations of <i>M. pulmonis</i> from the					Incidence of other organ infections in mice	
		pharynx or lungs	blood	brain	spleen	liver	given <i>M. pulmonis</i> intranasally	with confirmed respiratory infections
Normal	17	9	0	3	1	2	3/17 (18 per cent.)	3/9 (33 per cent.)
Thymectomised, irradiated and given anti-lymphocyte serum	15	15	1	8	4	1	10/15 (67 per cent.)	10/15 (67 per cent.)

The results from these two experiments suggest that reduction of thymus-dependent immunity in the mouse either makes it more sensitive to infection with *M. pulmonis* or allows *M. pulmonis* to grow more freely in the respiratory tract.

Effect on the spread of M. pulmonis to other organs. *M. pulmonis* has been isolated from the brains of naturally infected mice (Lemcke, 1961). Thymectomised young rats with naturally acquired infections develop severe pulmonary lesions, and *M. pulmonis* can be isolated from their brains (Allison and Purcell, unpublished observations). These findings prompted us to study how often spread of *M. pulmonis* occurred from the lungs to other organs after intranasal infection, in normal mice and in thymectomised, irradiated, ALS-treated mice. Table II shows the frequency of isolation of *M. pulmonis* from the respiratory tract, blood, brain, spleen and liver of these mice. Nine of 17 normal mice developed infections of the respiratory tract and from three of these mycoplasmas were also isolated from the brain; two of the three mice gave, in addition, positive liver cultures and the other a positive spleen culture. In contrast, all 15 thymectomised mice developed respiratory infections and in ten of them other organs were also infected; eight of these ten other infections involved the

brain. These results confirm that *M. pulmonis* can be isolated from non-respiratory organs, especially the brain, of normal, immunologically competent mice following respiratory infection, and demonstrate that dissemination occurs at least twice as frequently in mice with defective cell-mediated immunity.

Effect on histopathological changes in the lungs. Figure 1 illustrates the histology of *M. pulmonis* pneumonia in normal mice. There is extensive peribronchiolar and perivascular cuffing by lymphocytes and consolidation of large segments of the lung. The effect of thymectomy on the histological appearances is demonstrated in fig. 2; these particular sections were made from the mouse

TABLE III

Effect of reducing thymus-dependent immunity on the occurrence of perivascular and peribronchiolar round cell cuffing in the lungs of M. pulmonis-infected mice

Experiment number	Immune status of the mice	Proportion of mice developing pneumonia	Lesion score*
1	Normal	5/6	2·8
	Thymectomised and X-irradiated	3/6	0·7
2	Normal	5/22	1·8
	Thymectomised, X-irradiated and given anti-lymphocyte serum	5/15	0·6

* Average amount of perivascular and peribronchiolar infiltration (graded 0 to 4) in the lungs of mice with pneumonia. See text for details.

lung with the most extensive histopathological changes seen in this study. As compared with the normal, infected mouse, there is an over-all reduction in the amount of cuffing and consolidation. The distribution of round cells around the bronchioles and vessels is the same as in the normal mice but fewer cells are present. This reduction in the peribronchiolar and perivascular collections of lymphocytes after thymectomy was studied in more detail. Lung sections from mice killed in the first two experiments of this study were given code numbers; the mice all had pneumonia, as shown by the presence of any or all of the histological findings described above. The sections were then examined by two observers who graded the relative amounts of peribronchiolar and perivascular cuffing in each section as 0, 1, 2, 3 or 4. A "lesion score" for normal and thymectomised mice was then calculated by taking the total score for each group and dividing by the number of sections examined (table III). In both experiments the numbers of lymphocytes around bronchioles and blood vessels were greatly reduced in thymectomised mice; the lesion score for normal animals in the two experiments was 2·8 and 1·8, whilst the scores for thymectomised mice were 0·7 and 0·6.

The numbers of alveolar macrophages were also quite different in the normal

and thymectomised mice. No attempt was made to quantitate this finding, but the striking differences are illustrated in figs. 1c and 2c. In some normal, infected mice macrophages were present in such abundance that they were largely responsible for the appearance of consolidation. In thymectomised mice they were fewer in number. Large numbers of polymorphonuclear leucocytes were often seen in the alveoli and within the bronchioles of both normal and thymectomised mice (figs. 1c and 2c). There did not appear to be any obvious difference in the numbers of polymorphonuclear cells at these two sites in the two groups of animals, even allowing for the possibility that lung lavage may have removed some of the intrabronchiolar cells.

*Presence of cell-bound immunoglobulins in the lungs of mice with
M. pulmonis-pneumonia*

The lungs of mice that had developed severe pneumonia, including peri-bronchiolar and perivascular cuffing by lymphocytes, 2 to 3 wk after intranasal inoculation were examined for the presence of immunoglobulins by the immunofluorescence technique. Immunoglobulin was found within the lumen of bronchioles, appearing as multiple, fluorescent "globules", approximately 1–2 μ m in diameter, along the epithelial border (fig. 3a). Plasma cells were not seen in the cellular infiltrates around the blood vessels and bronchioles. Instead, there were varying numbers of large cells with irregular cytoplasmic processes, containing brightly fluorescent globules of the same size as those within the lumen of the bronchioles (fig. 3b). These cells, probably macrophages, were scanty in some areas of the lung and even where they were present in greatest numbers only a few of them contained the fluorescent immunoglobulin. Lung sections not fixed in acetone showed the same stained appearances as those that were fixed in acetone, indicating that the immunoglobulin was indeed "bound". Sections prepared from uninfected mouse lungs did not fluoresce. The failure to demonstrate plasma cells in the infected lungs is a further indication of the pre-eminent role of cell-mediated immunity.

DISCUSSION

In some viral and bacterial infections cell-mediated immunity is of greater importance than circulating antibody in defence of the host (Mackaness, 1970a and b; Allison, 1971a). Circulating antibodies are especially important in arbovirus and enterovirus infections, while in herpesvirus and poxvirus infections cell-mediated immune mechanisms seem to play the dominant role (Nathanson and Cole, 1970; Allison, 1971a). To explain these differences Allison (1971b) presented evidence supporting the view that viruses that stimulate cell-mediated immunity have antigens that infiltrate the plasma membrane of infected cells; this produces the self+x antigen system capable of sensitising lymphocytes. The role of cell-mediated immunity has been studied less well in bacterial infections, but Mackaness has demonstrated its major importance in infections caused by *Salmonella*, *Listeria* and *Brucella*, all facultative intracellular organisms.

M. PULMONIS INFECTIONS OF MICE

FIG. 1a.

FIG. 1b.

FIG. 1c.

FIG. 1.—Vertical sections of lung from an immunologically normal mouse 3 wk after intranasal inoculation of *M. pulmonis* showing: *a* (above). Extensive peribronchiolar and perivascular accumulations of round cells and large areas of consolidation. $\times 18$. *b* (below left). Peribronchiolar and perivascular cuffing by mononuclear cells, probably lymphocytes. $\times 100$. *c* (below right). Macrophages and polymorphonuclear leucocytes in an area of consolidation. $\times 1100$. Haematoxylin and eosin (HE).

M. PULMONIS INFECTIONS OF MICE

FIG. 2a.

FIG. 2b.

FIG. 2c.

FIG. 2.—Vertical sections of lung from an immunosuppressed mouse 3 wk after intranasal inoculation of *M. pulmonis* showing: *a* (above). Definite but minimal peribronchiolar and perivascular accumulations of round cells and only small areas of consolidation. $\times 18$. *b* (below left). Reduced amount of peribronchiolar and perivascular cuffing by mononuclear cells. The distribution of cells is the same as in immunologically normal mice. $\times 100$. *c* (below right). Many polymorphonuclear leucocytes but very few macrophages in an area of consolidation. $\times 1100$. HE.

M. PULMONIS INFECTIONS OF MICE

FIG. 3a.

FIG. 3b.

FIG. 3.—Sections of pneumonic lung, from mice infected with *M. pulmonis*, stained with fluorescein-labelled anti-mouse-globulin rabbit antiserum showing: *a* (above). Lumen of a bronchiole with multiple fluorescent globules situated along the epithelial border. $\times 1620$. *b* (below). Area of lung beneath the epithelial border. Large cells are seen which are assumed to be macrophages; one of these contains fluorescent globules, while the others show a diffuse fluorescence. $\times 1620$.

It is generally agreed that mycoplasmas can be found in polymorphonuclear leucocytes and macrophages (Zucker-Franklin, Davidson and Thomas, 1966; Jones and Hirsch, 1971) but whether they enter other cells is controversial (Organick, Siegesmund and Lutsky, 1966; Mazzali and Taylor-Robinson, 1971). However, there is no doubt that they do form a very close relationship with the plasma membrane of cells (Zucker-Franklin *et al.*; Manchee and Taylor-Robinson, 1969). Attachment to ciliated respiratory epithelial cells is probably the first step in the establishment of infection by *M. pneumoniae* (Sobeslavsky, Prescott and Chanock, 1968; Lipman and Clyde, 1969; Collier and Clyde, 1971). In view of this close host cell-mycoplasma relationship it is not surprising that cell-mediated immunity should play a role in mycoplasma infections. Previous experiments in animals and man have already indirectly implicated this mechanism (Fernald, 1972). We have now produced more direct evidence of its importance by showing that mice with defective cell-mediated immunity are more susceptible to infection by *M. pulmonis* and that there is greater dissemination of the infection in such mice. Moreover, although the polymorphonuclear leucocyte response in the immunosuppressed mice infected with *M. pulmonis* appeared to be normal, at least in the alveoli, there was a much reduced infiltration of lymphocytes around the bronchioles and blood vessels and many fewer alveolar macrophages, as compared with normal, infected mice. Since cuffing by lymphocytes is characteristic of cell-mediated immune reactions, and since this was diminished in the animals rendered deficient in cell-mediated immunity, it seems reasonable to assume that this aspect of immunity is responsible for the lesions normally referred to as "pneumonia". Whether pulmonary lesions are beneficial or harmful in *M. pulmonis* infections cannot be stated categorically from these studies, but the pneumonia may be the penalty the animal must pay to contain the infection. The presence of macrophages in the pulmonary lesions of normal mice and their decreased numbers in immunodeficient animals supports the concept that a cell-mediated immune reaction leads to macrophage infiltration.

The results of the fluorescent antibody studies are also consistent with a major role for cell-mediated immunity in *M. pulmonis* infections. If circulating antibody was of primary importance, plasma cells with immunoglobulin in their cytoplasm should have been found in the lungs of infected animals, and this was not so. We did, however, find bound immunoglobulin, in the form of "globules", both within the lumen of bronchioles and inside cells, probably macrophages, around bronchioles and blood vessels. The location of these globules at the epithelial cell border of bronchioles is very similar to that of *M. pulmonis* organisms in the lungs of infected mice (Organick and Lutsky, 1968). *M. pneumoniae* organisms have been found in a very similar position in the lungs of infected hamsters (Dajani *et al.*, 1965) and in infected organ cultures of both hamster (Collier, Clyde and Denny, 1969) and human embryo tracheas (Collier and Clyde). It is, therefore, likely that the observed immunoglobulin was bound to clusters of organisms. The presence of large amounts of immunoglobulin at the same site as antigen may explain the failure to demonstrate antibodies in respiratory secretions. We can only speculate on the role of the

immunoglobulin-containing cells, probably macrophages, located among the peribronchiolar and perivascular lymphocytes. This may indicate the way in which antigen or antigen-antibody complexes are removed from their primary site in the bronchiole and taken to a site where lymphocytes can be sensitised or recruited. Since a soluble toxin, other than peroxide, has not been demonstrated for either *M. pneumoniae* or *M. pulmonis*, it may be that this transport mechanism allows the organisms themselves to initiate the pathological changes in the lungs.

The present studies have not delineated conclusively the relative roles of circulating antibody and cell-mediated immunity in the pathogenesis of *M. pulmonis* infections in mice. It is possible, of course, that both types of immune reaction play a part. The immunofluorescence studies suggest that local secretory antibodies were formed within the bronchioles. Local antibodies were not detected by Smith *et al.* (1967) during *M. pneumoniae* infections in volunteers. On the other hand, Biberfeld and Sterner (1969, 1971) found them in the bronchial secretions following natural infection, although the possibility that these came from the blood has been pointed out (Fernald, 1970). Examination of the effects of administering serum antibody or sensitised lymphocytes from immune animals to infected, immunosuppressed animals may help to define their respective roles in the pathogenesis of infection.

In conclusion, we offer a tentative interpretation of our observations. *M. pulmonis* in mice multiplies close to the luminal plasma membrane of bronchiolar epithelial cells. Antibody is secreted and becomes attached to the organisms without affecting their infectivity. Polymorphonuclear leucocytes and macrophages migrate from the bronchiolar connective tissue and ingest the antibody-coated organisms. The leucocytes remain in the alveolar and bronchial exudates, but some of the macrophages with their ingested organisms return to the underlying connective tissue. At first, discrete organisms coated with immunoglobulin are visible within the macrophage cytoplasm, but later the organisms are digested and the cytoplasm shows diffuse fluorescence. Mycoplasma antigens, probably in macrophages, stimulate a cell-mediated immune response, and sensitised lymphocytes accumulate around infected bronchioles and adjacent blood vessels. Some lymphocytes transform to become large pyroninophile cells. Macrophage migration-inhibitory factor is liberated, and macrophages accumulate in the reaction sites. Thus develops the atypical pneumonia characteristic of mycoplasma infections. Another factor released from transforming lymphocytes "activates" macrophages (Mackaness), increasing their capacity to kill intracellular organisms, including mycoplasmas. Hence, little spread occurs from the original sites of infection in the lungs, although occasionally a few organisms may appear in other organs such as the spleen and brain, probably carried there in macrophages.

In thymectomised mice, the multiplication of organisms and the reactive polymorphonuclear leucocyte response probably both occur to about the same extent as in normal mice. However, there is a diminished cell-mediated immune reaction, so that the peribronchiolar and perivascular infiltrates are much less prominent than in normal mice. Furthermore, macrophages do not

become activated, and are less able to kill mycoplasmas. As in some virus infections (Allison, 1970), the opportunity for the mycoplasmas to spread to distant sites is therefore greatly increased.

SUMMARY

The pathogenesis of *Mycoplasma pulmonis* infection was studied in normal mice and in thymectomised, X-irradiated, anti-lymphocyte-treated mice. The immunosuppressed mice were more readily infected by the intranasal route than were normal mice, although the mycoplasmas multiplied to only a slightly greater extent in the lungs of the deficient animals. Spread of mycoplasmas from the lungs to other organs, such as the brain, occurred more frequently in the immunologically deficient mice. Cell-mediated immunity appeared to be important in the development of the pulmonary lesions. Thus, the lungs of normal mice showed marked peribronchiolar and perivascular cuffing by lymphocytes, but this was much less prominent in the immunosuppressed mice and macrophages were also fewer in number.

Immunofluorescence studies provided further evidence for the important role of cell-mediated immune mechanisms in *M. pulmonis* infections in mice. The way in which such mechanisms may operate is discussed.

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