REDUCTION IN THE SEVERITY OF *MYCOPLASMA PNEUMONIAE*-INDUCED PNEUMONIA IN HAMSTERS BY IMMUNOSUPPRESSIVE TREATMENT WITH ANTI-THYMOCYTE SERA

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PLATE XII

THE pathogenesis of pneumonia in man caused by *Mycoplasma pneumoniae* is poorly understood. However, the *M. pneumoniae*-infected Syrian hamster is a useful model, not only because this is one of the few small animals that can be successfully infected, but also because the histological characteristics of the pneumonia and the temporal development of antibody are similar to these features in man (Dajani, Clyde and Denny, 1965; Fernald, 1969).

Pneumonia in hamsters infected with *M. pneumoniae* consists of peribronchial and perivascular cuffing by lymphocytes, and this suggests that it is the result of a delayed hypersensitivity response in the lung. However, immunoglobulin is associated with many of the cells (Fernald, Clyde and Bienenstock, 1972), indicating that the response may not be a pure delayed hypersensitivity reaction. Nevertheless, other observations suggest that delayed hypersensitivity develops during *M. pneumoniae* infection. Thus, guinea-pigs and man develop skin hypersensitivity (Fernald, 1971; Mizutani *et al.*, 1971). Furthermore, lymphocytes from previously infected human subjects and guinea-pigs can be stimulated *in vitro* by *M. pneumoniae* antigens to undergo blast transformation (Leventhal *et al.*, 1969; Fernald, 1971; Biberfeld, 1972; Fernald, 1972), and the migration of macrophages can be inhibited by *M. pneumoniae* antigen (Arai *et al.*, 1971). Nevertheless, these *in-vitro* demonstrations of delayed hypersensitivity may not be relevant *in vivo*. However, with regard to the latter situation, Denny, Taylor-Robinson and Allison (1972) demonstrated that mice, immunosuppressed by thymectomy and X-irradiation and infected intranasally with *M. pulmonis*, developed lung lesions that were much less severe than those observed in infected but immunologically normal mice. Although the lung lesions in these mice and in *M. pneumoniae*-infected hamsters appear similar, this does not necessarily mean that thymus-dependent mechanisms are also important in the development of hamster lung lesions.

Because we are particularly interested in the hamster as a model for the human disease, the effect of thymus-dependent lymphocyte depletion on the development of *M. pneumoniae*-induced lung lesions in hamsters was investigated. Immunosuppression was effected by the use of anti-thymocyte serum. The present report records findings obtained with this model in two laboratories.

MATERIALS AND METHODS

Mycoplasma pneumoniae strains. Virulent *M. pneumoniae* strains P1-104166, B8-M129 and P5-427A, known to produce pneumonia in hamsters were used. The mycoplasmas

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were grown in thallium acetate-free mycoplasma medium for seven days, dispensed in 1-ml amounts and stored at -70°C . Each 1-ml portion contained 10^6 colour-changing units (*vide infra*).

Infection and immunosuppression of hamsters. Golden, Syrian hamsters weighing approximately 100 g were supplied by Coombehurst in England and by Engle's Hamstery in the USA. The hamsters were anaesthetised by intraperitoneal inoculation of sodium pentobarbitone (0.06 mg per g body weight), and infected by intranasal inoculation of 0.2 ml of the *M. pneumoniae* culture. As controls, other animals were given an equivalent intranasal inoculation of mycoplasma medium.

Hamsters were immunosuppressed by intraperitoneal inoculation of 0.002 ml per g body weight of pig anti-hamster thymocyte serum (ATS) supplied by Dr A. C. Allison (Clinical Research Centre) or rabbit anti-hamster thymocyte serum prepared by one of us (G.W.F.). The pig ATS was made by intravenous inoculation of 5×10^9 hamster thymocytes on two occasions 14 days apart (Levey and Medawar, 1966). The rabbit ATS was prepared by inoculating 10^8 thymocytes in complete Freund's adjuvant into the hind foot pads followed three weeks later by two intravenous injections, two days apart, of 10^8 thymocytes. The animals were bled 1 week later. The pig ATS was heat-inactivated at 56°C for 30 min. and absorbed with hamster erythrocytes before use; rabbit ATS was not heated or absorbed. The ATS was inoculated two days before infection with *M. pneumoniae* and daily thereafter until day 14 at which time the experiments were terminated. As controls, some hamsters were treated with normal pig or rabbit serum administered in the same way.

Mycoplasma medium. In both laboratories, liquid medium for isolation and growth of *M. pneumoniae* consisted of PPLO broth 70% (v/v), unheated horse serum 20% (v/v), yeast extract 10% (v/v) of a 25% (w/v) aqueous solution, glucose 0.1% (w/v), thallium acetate 0.05% (w/v), 1000 units of penicillin G per ml and phenol red 0.002% (w/v). The pH of the medium was adjusted to 7.8.

Mycoplasma isolation and identification. Two weeks after intranasal inoculation of *M. pneumoniae*, hamsters were killed by intraperitoneal injection of pentobarbitone and bleeding from the axillary vessels. Specimens from the pharynx were obtained by passing calcium-alginate-wool swabs deeply into the pharynx several times and then rinsing them in 1.8 ml of mycoplasma medium. This was regarded as a 10-fold dilution of the specimen. At the Clinical Research Centre, lungs were irrigated with 4 ml of medium to remove mycoplasmas, as described by Taylor-Robinson *et al.* (1972). At Chapel Hill, lungs were homogenised in mycoplasma medium to make a 10% (w/v) suspension, and in both laboratories specimens of brain, liver and spleen were homogenised in the same way. The numbers of mycoplasmas present in the blood and various other specimens were determined by making serial ten-fold dilutions of the samples in 1.8-ml volumes of medium contained in tightly capped vials. These were incubated at 37°C for at least 3 weeks, during which time they were observed for growth of mycoplasmas as indicated by a reduction in pH and consequent change in colour of the medium. The highest dilution then showing a change from pink to yellow was considered to contain one colour-changing unit (c.c.u.). Isolated organisms were identified as *M. pneumoniae* by the disk growth-inhibition technique (Clyde, 1964).

Histological procedures. Lungs were fixed in formol-saline. At the Clinical Research Centre, lungs were distended *in situ* with 4 ml of formol-saline administered through a cannula. After fixation, tissues were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. To compare the severity of the lung lesions of immunosuppressed and immunologically normal hamsters, the amount of cellular infiltration was graded from 0 to 3 and expressed as a lesion score (Fernald *et al.*, 1972). The degree of infiltration shown in fig. 1 represents a grading of 2.

RESULTS

Preparations of anti-hamster thymocyte serum (ATS), known to suppress cell-mediated functions, were used to investigate the importance of thymus-

dependent lymphocytes in the development of pneumonia in hamsters infected with *M. pneumoniae*.

Effect of immunosuppression on the recovery of M. pneumoniae

Sixty hamsters, half of them immunosuppressed, were examined 14 days after infection. In two out of four experiments mycoplasmas were isolated in significantly greater numbers ($P = <0.001$ and <0.01 ; Student's t-test) from the lungs of immunosuppressed animals than from the lungs of those that were immunologically normal (table I). However, there was no difference in the

TABLE I

The effect of treatment with anti-hamster thymocyte serum (ATS) on the recovery of Mycoplasma pneumoniae from the respiratory tract of hamsters 14 days after infection

Experiment		ATS prepared at	Site tested	<i>M. pneumoniae</i> isolations from		P value for numbers of organisms isolated
number	performed at			normal hamsters	immunosuppressed hamsters	
1	CRC	CRC	Pharynx Lungs	$3.5 \pm 0.4^*$ (8/8) [†] 4.0 ± 0.4 (4/8)	3.8 ± 0.3 (11/11) 6.3 ± 0.2 (9/11)	>0.1 <0.001
2	CRC	CRC	Pharynx Lungs	3.2 ± 0.3 (10/10) 4.9 ± 0.4 (10/10)	3.6 ± 0.3 (9/10) 6.1 ± 0.1 (9/10)	>0.1 0.01
3	Chapel Hill	CRC	Lungs	6.3 ± 0.3 (6/6)	6.8 ± 0.5 (5/5)	>0.1
4	Chapel Hill	Chapel Hill	Lungs	6.3 ± 0.3 (6/6)	7.0 ± 0.2 (4/4)	>0.1

CRC=Clinical Research Centre.

* Geometric mean $\pm$ standard deviation of number of organisms expressed as $\log_{10}$ colour-changing units per ml in experiments CRC at and as $\log_{10}$ colony-forming units per ml in experiments at Chapel Hill.

† Proportion of hamsters from which organisms were isolated.

number of organisms isolated from the throats of hamsters in either group and organisms were not isolated from blood, spleen, liver or brain of any of the hamsters.

Effect of immunosuppression on lung lesions

Pneumonia in immunologically normal hamsters infected with *M. pneumoniae* was characterised by peribronchiolar and perivascular cuffing by lymphocytes, as shown in fig. 1. The pneumonia in immunosuppressed hamsters was much less severe, as indicated by a reduction in the number of cells associated with the lesions (fig. 2). Furthermore, the lesion scores for immunosuppressed hamsters were significantly less ($P = <0.001$; Student's t-test) than those for immunologically normal animals (table II). In contrast to suppression of peribronchial infiltration, the extent of the bronchial exudate was not affected, although its character changed. Whereas immunologically normal hamsters

had a bronchial exudate of polymorphonuclear (PMN) leucocytes and large monocytes, the PMN leucocyte component was reduced in the exudate of animals treated with ATS; many of the ATS-treated animals appeared to have bronchial exudates consisting of monocytes only. Control normal and immunosuppressed hamsters given inoculations of medium only did not develop lung lesions.

TABLE II

The effect of treatment with anti-thymocyte serum (ATS) on the lung lesions of hamsters infected with Mycoplasma pneumoniae

Experiment number*	Lesion scores for		P value for lesion scores
	normal hamsters	immunosuppressed hamsters	
1	1.4±0.4 (4/4)†	0.4±0.3 (2/6)	0.05-0.1
2	1.7±0.3 (10/10)	0.3±0.2 (4/10)	<0.001
3	2.3±0.2 (6/6)	0.5±0.2 (3/5)	<0.001
4	2.3±0.2 (6/6)	0.4±0.2 (2/4)	<0.001

* The numbers refer to the same experiments as shown in Table I.

† Number of hamsters with pneumonia/number challenged.

DISCUSSION

We have already mentioned the observation that has led to the belief that delayed hypersensitivity contributes to the production of lesions in *M. pneumoniae* infections. Our present results indicate that a thymus-dependent function is concerned in the pathogenesis of pneumonia in the hamster, and are similar to those of Denny *et al.* (1972), who infected mice intranasally with *M. pulmonis* and immunosuppressed them by thymectomy and X-irradiation. In both studies, immunosuppression was associated with a reduction in the severity of lung lesions, and mycoplasmas multiplied slightly more in the respiratory tract of the immunosuppressed than of the control animals. However, isolation of *M. pneumoniae* organisms from non-respiratory sites of ATS-treated hamsters was not observed, whereas isolation of *M. pulmonis* from organs other than the lungs occurred in immunodeficient mice more frequently than in immunologically normal ones. The reason for this difference is not clear, but it may be that one or more of the following factors was responsible: (i) *M. pulmonis* organisms, present in undetectable numbers in extra-pulmonary sites, were able to multiply in the absence of cellular defence mechanisms; (ii) because the hamster is not the natural host for *M. pneumoniae* organisms, these mycoplasmas are unable to multiply in non-pulmonary sites; and (iii) the ATS was not sufficiently immunosuppressive to permit extra-pulmonary dissemination of the organisms in the hamster.

Although we have demonstrated the importance of thymus-dependent lymphocytes in the development of lung lesions in *M. pneumoniae*-infected hamsters, the role that these cells play in immunity is not known. Fernald *et al.*

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FIG. 1.—Vertical section of lung from an immunologically normal hamster 2 weeks after intranasal challenge with *M. pneumoniae*. There is peribronchiolar and perivascular cuffing by lymphocytes. $\times 45$.

FIG. 2.—Vertical section of lung from an immunosuppressed hamster 2 weeks after intranasal challenge with *M. pneumoniae*. There is minimal lymphocyte infiltration. $\times 45$.

(1972) have shown that the hamster-lung lesions comprise two distinct populations of lymphocytes, one of which contains immunoglobulin while the other does not. Hamsters develop lung lesions more rapidly on re-infection than on primary infection. Thus, the lesions are most extensive three days after infection and have resolved by day 14, a time when lesions are normally prominent. The predominant cell in these accelerated lesions is immunoglobulin-negative, and this suggests that cell-mediated, rather than antibody-mediated, mechanisms are major factors in immunity to *M. pneumoniae* infections. However, there is now some evidence to indicate that, in man, local secretory antibody may also be a factor in resistance to re-infection (Brunner *et al.*, 1973). Studies on infection of hamsters with *M. pneumoniae* after passive transfer of either serum or sensitised lymphocytes from immune hamsters may help to resolve the extent to which cell-mediated mechanisms play a part in resistance to *M. pneumoniae* infection.

SUMMARY

Intranasal inoculation of *Mycoplasma pneumoniae* into hamsters caused pneumonia characterised by peribronchial and perivascular cuffing by lymphocytes. Hamsters immunosuppressed by treatment with anti-thymocyte serum developed a much less severe pneumonia. The numbers of organisms isolated from the lungs of immunosuppressed animals were greater than those obtained from the lungs of immunologically normal animals but there was no evidence that extrapulmonary spread occurred. These results indicate the importance of cell-mediated mechanisms in the pathogenesis of this mycoplasma-induced pneumonia. The hamster model may help to establish the role of cell-mediated immunity in resistance to this type of infection.

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