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Immunopathology of Experimental *Mycoplasma pneumoniae* Disease

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Experimental *Mycoplasma pneumoniae* pneumonia in the Syrian hamster resembles natural disease of man and is characterized by peribronchial round cells as well as exudates in the air passages. The nature of these intralumenal exudates was analyzed to assess host-parasite interaction at the respiratory epithelial surface, where infection with the mycoplasma is concentrated. Cells recovered by tracheobronchial lavage revealed changing patterns during the course of experimental disease; free macrophages decreased, polymorphonuclear leukocytes increased, and rosettes of epithelial cells surrounded by leukocytes appeared. Studies on formation of the rosettes in vitro with *M. pneumoniae*, sera, and cells suggested that complement-dependent immune phenomena were involved. The cellular events were maximal by 2 weeks after inoculation of normal hamsters. When previously infected animals were challenged, the course of infection was shortened, peribronchial infiltrates appeared rapidly, and development of exudates was both exaggerated and accelerated. These findings suggest that leukocytes entering the pulmonary parenchyma and lumen participate in the immune response to *M. pneumoniae* and contribute to host protection.

In studies on the pathogenesis of *Mycoplasma pneumoniae* disease, the Syrian hamster has proven to be a useful experimental model (3). Intransal inoculation of this animal results in a sequence of pathologic (6) and immunologic (8) changes resembling those occurring in the natural human disease. Although the mycoplasma localizes superficially on the respiratory epithelium, the most typical lesion is peribronchial infiltration with lymphocytes and plasma cells. However, a variable degree of exudation occurs within the tracheobronchial tree. Since the most direct interaction between host and parasite is at the epithelial surface, analysis of events at this site during the course of experimental disease has been undertaken. This investigation was stimulated by evidence that protective immunity against *M. pneumoniae* may be mediated by local rather than humoral mechanisms (9).

This report concerns dynamic changes in the character of cells recoverable from the pulmonary passages during the course of experimental *M. pneumoniae* disease. The results reflect events occurring in the lung parenchyma and provide indications of local immune phenomena.

MATERIALS AND METHODS

Organisms. All experiments utilized a virulent *M. pneumoniae* strain, PI 104166, which has been characterized previously (9). A pool was prepared in Hayflick's broth medium (10) representing the sixth passage after isolation from a subject with pneumonia. Samples of log-phase culture, containing approximately 10^7 colony-forming units (CFU)/ml, were stored at −70 C until needed.

Animals. Young male hamsters weighing 100 ± 15 g were obtained from a local supplier and maintained in an isolated room. Uninoculated control animals of the same age included in all experiments were cultured for mycoplasmas and studied for evidence of pneumonia; no organisms or abnormal pulmonary histology were found. Procedures used for inoculation, quantitative cultures, and histological examination have been detailed elsewhere (6).

Serology. *M. pneumoniae* antibodies were measured in hamster sera by complement fixation by using reagents and a microtitration procedure described by Fernald (8).

Collection of pulmonary cells. Animals were anesthetized with sodium pentobarbital, and the tracheas were exposed immediately and cannulated with a blunt 19-gauge needle. Two milliliters of phosphate-buffered saline (PBS; 0.01 M, *p*H 7.2) were injected through the needle and immediately withdrawn, resulting in approximately 75% recovery of the original volume. It was anticipated that this procedure would yield a sample of "free" cells from the lower trachea, bronchi, and larger bronchial branches. A sample of the cell suspension was mixed with a drop of concentrated acetic acid, and total cells were counted in a hemocytometer chamber. The remaining cells were sedimented

at 500 × *g* for 10 min, PBS was decanted, and smears of the cellular material were prepared and air-dried for cytological examination.

Cytological methods. Replicate smears of material from each animal were fixed and stained by three methods to enable cell differentiation by both morphological and histochemical criteria. Techniques used were: (i) hematoxylin and eosin after fixation in 95% ethanol (15); (ii) methyl green and pyronin after fixation in absolute ethanol (14); and (iii) the acid phosphatase method of Barka and Anderson (1) after fixation in formalin (4%) with calcium chloride (1%) for 2 hr at 4 C. The staining reactions of various cell types were identified by preliminary study of hamster blood, bone marrow, and lung sections. Hamster leukocyte morphology differs from that of many other small animals, as described by Stewart, et al. (13). Experience with pulmonary cell suspensions revealed that small macrophages were difficult to differentiate from the larger lymphocytic cells, since both showed cytoplasmic pyronin staining. Accordingly, definitive differential cell counts were made by using the acid phosphatase-stained smears. Cells were counted as macrophages only if they revealed a strongly positive reaction; the other cell types encountered were either negative or very weakly positive for acid phosphatase by the method used. Differential counts, based on examination of at least 200 cells, were performed under code by the author. Changes in cell numbers were considered significant only if they differed from normal mean values by at least two standard deviations (see Table 1).

Procedures for in vitro studies of cell interactions. Normal respiratory epithelial cells were obtained by incubating excised hamster lungs, filled with 2 ml of 0.01% trypsin (Difco, 1:250) in Hanks solution (*p*H 8) through a tracheal cannula, at 37 C for 15 min. The fluid recovered was centrifuged for 15 min at 500 × *g*, and the cells were resuspended in 2 ml of PBS. Mycoplasmas were sedimented from log-phase culture by centrifugation at 15,000 × *g* for 30 min and then were resuspended in PBS. Equal parts of organism and epithelial cell suspensions were combined for 30 min at room temperature and then centrifuged at 500 × *g* and washed thrice with PBS to remove the bulk of unadsorbed organisms; the product contained about 10^5 cells and 10^5 to 10^6 CFU of mycoplasma per ml. Normal hamster leukocytes were prepared by gravity sedimentation, for 30 min., of heart blood collected in four parts of 3.8% sodium citrate solution and mixed with an equal volume of 3% Dextran-250 (Pharmacia) in normal saline. The leukocyte-rich plasma was aspirated and centrifuged lightly, and the cells were resuspended in PBS. This provided a final cell concentration of about 10^7/ml. Fresh normal hamster serum was obtained from heart blood clotted and separated at 4 C on the day of each experiment. Portions of the serum were inactivated by heating at 56 C for 30 min. The *M. pneumoniae* antiserum was a pool of sera taken from six hamsters 4 weeks after infection and was heated at 56 C for 30 min before use.

RESULTS

Initial experiments were designed to determine the quantity and character of cells recoverable by pulmonary lavage of normal hamsters, thus providing baseline data for interpretation of changes occurring during the course of experimental *M. pneumoniae* pneumonia. The significance of these changes was suggested by additional studies of organism-host cell interactions in vitro and by examinination of the response of previously infected hamsters to challenge inoculation.

Studies in normal hamsters. Pulmonary lavage was performed on a group of 10 normal hamsters, and the total and differential cell counts were determined for each animal. The data permitted calculation of mean values and an estimate of normal variations which could occur (Table 1). By using 2 ml of PBS as the lavage fluid, approximately 100,000 cells/ml could be recovered. The cell types were designated by morphological and histochemical criteria and included (in order of frequency) macrophages, lymphocytes of various sizes, polymorphonuclear leukocytes, and plasma cells. Variable numbers of single and clustered ciliated epithelial cells were found in most preparations but were not included in the cytological analyses. The reproducibility of the data was indicated by results from many additional normal animals serving as controls in other experiments to be described.

Cytologic changes during M. pneumoniae infection. A number of hamsters were inoculated intranasally with approximately 10^6 CFU of a virulent *M. pneumoniae* strain. At intervals, groups of six animals were anesthetized with sodium pentobarbital, and pulmonary lavage was performed to determine the number and types of cells present within the air passages. Heart blood was obtained for serological study, and then the lungs

TABLE 1. *Cytology of lower respiratory tract secretions in the normal hamster*

Cell type	No. of cells/ml[a]	
	Mean	Range (±σ)[b]
Macrophage	60	46–74
Small lymphocyte	22	9–35
Large lymphocyte	14	7–21
Polymorphonuclear	3	1–5
Plasma cell	2	0–4
Total	101	82–122

[a] From pulmonary lavage with 2 ml of phosphate-buffered saline. Values expressed × 10^3.

[b] Calculated from data on 10 normal animals.

TABLE 2. *Experimental Mycoplasma pneumoniae disease in the Syrian hamster*

Day post-inoculation	Infection[a]		Pneumonia[b]		Complement fixation[c]
	+/No.	Amt	+/No.	Degree	
1	6/6	5.3	0/6	0	0.7
3	6/6	7.0	1/6	1.0	0
5	6/6	7.6	1/6	1.0	0.3
7	6/6	7.9	4/6	1.9	0.8
9	6/6	6.7	4/6	2.5	0.8
15	6/6	5.9	3/6	1.7	2.5
21	6/6	6.3	5/6	1.6	3.2
28	5/6	4.3	5/6	1.6	2.7
42	0/6	0	0/6	0	3.2

[a] Number infected/number inoculated; amount, $\log_{10}$ colony-forming units per gram of lung (geometric mean).

[b] Number with pneumonia/number inoculated; degree; mean score for positive animals each rated at 1 to 4+.

[c] Geometric mean titer, $\log_2$.

were removed aseptically and divided for quantitative culture and histological examination. The data shown in Tables 2 and 3 are from an experiment representative of several which were performed.

The course of mycoplasma infection and the evolution of pulmonary pathologic changes indicated in Table 2 are characteristic of the model, as reported previously from this laboratory (3, 6, 9). All animals were infected, and maximum replication of the mycoplasma occurred by day 7. Evidence of pneumonitis was seen as early as 3 days but reached a peak 2 to 3 weeks after inoculation. By 6 weeks, both infection and pneumonia had cleared.

During the first 9 days after inoculation, the number of cells recoverable by pulmonary lavage (Table 3) was within or near the range established for normal animals. On day 15, the cell yield increased almost fourfold; the counts remained elevated through day 28 but returned to normal levels by day 42. Changes in total cell count thus corresponded with the development and clearing of pneumonia.

Examination of the nature of the cells showed several additional findings (Table 3). Macrophages, the most abundant cells in the normal hamsters, were sharply reduced in the first several days after inoculation and did not return to the normal range until day 28. Increases occurred in the numbers of lymphocytes at 3 to 5 days and again at 15 days, with the larger, more differentiated forms predominating. Polymorphonuclear leukocytes, which were rare in the normal hamster, accounted for much of the cell elevation on day 15.

An additional feature was noted in examination of smears of the endobronchial contents (Fig. 1). After the first week of infection, organized cellular formations were recovered with increasing frequency (Table 3). These consisted of epithelial cells surrounded by variable numbers of polymorphonuclear leukocytes. Based on the observations that the mycoplasma is intimately associated with the respiratory epithelial membrane (4, 5) and the appearance of rosettes corresponded to development of early serologic responses (8; Table 2), the possibility of immune phenomena was suggested. This was explored by analysis of the way in which presumed constituents of the rosettes interacted under controlled conditions in vitro.

Studies of cellular interactions in vitro. A series of substitution experiments was designed (Table 4) to evaluate the participation of epithelial cells,

TABLE 3. *Cellular composition of lower respiratory secretions during experimental Mycoplasma pneumoniae pneumonia*

Cell type	No. of cells at various times postinoculation[a]								
	1 day	3 days	5 days	7 days	9 days	15 days	21 days	28 days	42 days
Macrophage	18.2	6.1	18.0	17.5	9.2	29.5	32.6	56.4	45.6
Small lymphocyte	10.6	6.5	15.5	6.0	1.4	48.1	26.8	17.8	19.0
Large lymphocyte	38.2	54.5	89.4	32.0	12.6	117.3	55.9	74.5	19.3
Polymorphonuclear leukocyte	8.9	3.2	30.4	41.0	55.9	176.0	59.5	32.5	2.0
Plasma cell	5.3	3.5	4.4	1.0	0.6	7.6	3.9	1.8	3.1
Total	81.2	73.8	157.7	97.5	79.7	378.5	178.7	183.0	89
Rosette figures	0	0	0	0	2.3	2.0	0.8	0.5	0

[a] Mean number of cells per milliliter of phosphate-buffered saline used for trans-tracheal lavage (data from same animals shown in Table 2). Values are expressed $\times 10^3$.

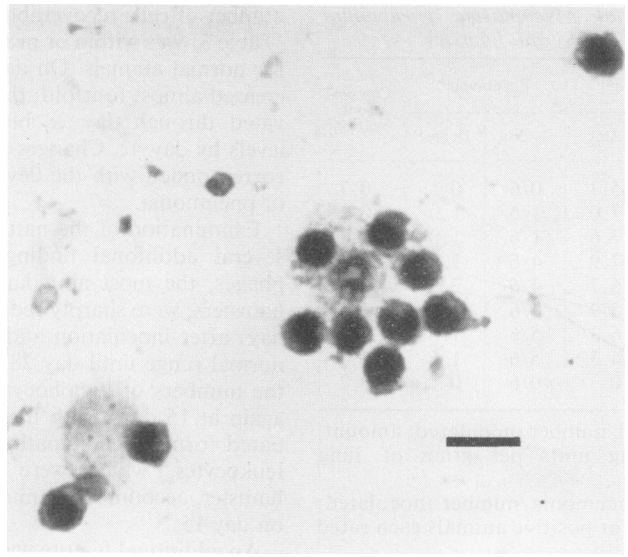

FIG. 1. *A cellular rosette, consisting of a central epithelial cell surrounded by polymorphonuclear leukocytes, from trans-tracheal lavage of a hamster inoculated 2 weeks previously with Mycoplasma pneumoniae (hematoxylin and eosin). Bar = 10 μm.*

TABLE 4. *Interaction of Mycoplasma pneumoniae in vitro with hamster sera, leukocytes, and respiratory epithelial cells*[a]

Components[b]					No. of rosettes produced (per ml)
Leukocytes	Mycoplasma	Epithelium	Serum		
			Immune[c]	Normal	
+	+	+	+	+ (Fresh)	$6 \times 10^3 - 13 \times 10^3$
+	+	+	+	+ (Heated)[c]	$1 \times 10^3 - 3 \times 10^3$
+	+	+	+	−	0
+	+	+	−	+ (Fresh)	0
+	+	+	−	+ (Heated)	0
+	+	+	−	−	0
+	+	−	+	+ (Fresh)	0
+	−	+	+	+ (Fresh)	0

[a] Incubated for 60 min at 4 C.
[b] Symbols: (+) 0.1 ml of component present; (−) component absent, 0.1 ml of phosphate-buffered saline present.
[c] Incubated for 30 min at 56 C.

M. pneumoniae, leukocytes, and *M. pneumoniae* antiserum and fresh serum factors in the formation of rosette figures. One-tenth-milliliter amounts of the components to be studied were combined and incubated in small glass tubes. Drops of the suspended material were then removed for rosette enumeration in a hemocytometer chamber and preparation of slides, which were stained with Wright's solution.

Rosette figures were produced in vitro (Fig. 2) which were identical to those occurring in vivo during experimental *M. pneumoniae* disease.

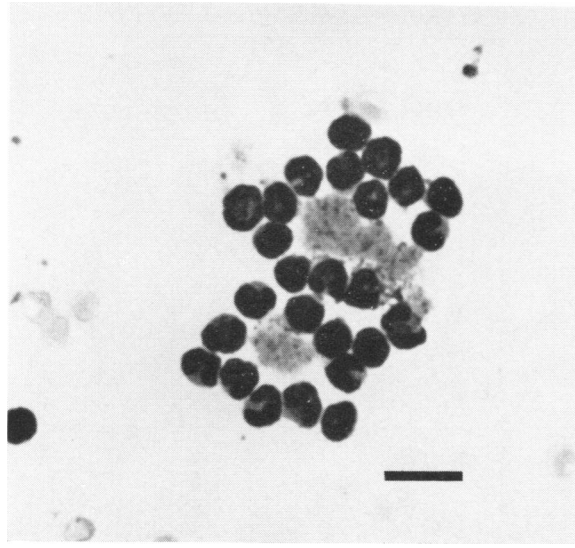

FIG. 2. *Rosette similar to that depicted in Fig. 1 produced in vitro by interaction of Mycoplasma pneumoniae, sera, and cells (Wright's stain). Bar = 10 μm.*

Serial studies altering incubation time and temperature revealed that the figures formed readily between 37 and 4 C but that they disappeared rapidly at 37 C. The results in Table 4 summarize three experiments conducted for 1 hr at 4 C.

Maximum rosette production required both antiserum and fresh normal serum; counts up to 130,000 rosettes/ml of suspension were obtained. Heating of the fresh serum consistently diminished the number of rosettes (below 30,000/ml). No true rosettes were seen if either immune serum, mycoplasmas, or epithelial cells were omitted from the reaction mixture. Occasional figures which were counted in control suspensions as rosettes proved to be leukocyte clumps when the stained smears were examined.

Studies of reinfected hamsters. To investigate the functional significance of the cytologic changes described above, additional experiments were performed by using hamsters reinfected with *M. pneumoniae*. A group of normal animals was inoculated with virulent organisms and maintained for 3 months to allow complete evolution of the experimental disease. They were then challenged with *M. pneumoniae* and sacrificed at intervals in groups of four to six for pulmonary lavage, quantitative lung culture, and pathological examination. Controls included normal animals (boarded for 3 months) inoculated with the challenge organisms and experienced animals inoculated with sterile broth medium.

The results are summarized in Table 5. It was possible to reinfect animals that previously had experienced *M. pneumoniae* disease; however, growth of the organism was limited, and both frequency and degree of infection were minimal by 2 weeks. Most of the animals developed peribronchial round cell infiltration within 3 days after challenge. There was an immediate and marked mobilization of phagocytes into the pulmonary passages, and rosette formations appeared after 3 days. These pathologic changes appeared earlier and were more marked than those occurring in animals infected for the first time (Tables 2, 3, and 5).

Two observations in control animals complicate interpretation of the experiment summarized in Table 5. The immune (previously infected) animals, though clear of mycoplasma infection, included two animals (of a group of six) with abnormal pulmonary histology consisting of a small focus of round cells near major bronchi in each animal. Although lymphoid collections are frequently seen in the lungs of rodents, this is unusual in the hamster (6, 7); accordingly, the changes were classified as a minimal degree of "pneumonia." The changes in mycoplasma-challenged immune animals at day 14 were identical and could represent a background upon which the more acute effects were superimposed. The inoculum used for initial infection and challenge of the animals was a broth culture of *M. pneumoniae*, and the possibility exists that some of the effects of challenge could represent sensitization to medium components, especially horse serum. Immune animals challenged with sterile broth revealed

TABLE 5. *Response of hamsters recovered from Mycoplasma pneumoniae infection to reinoculation*

Animal status[a]	Sacrifice day	Infection[b]		Pneumonia[c]		Total no. of cells lavaged ($\times 10^3$/ml)	No. of rosettes produced (per ml)
		+/No.	Amt	+/No.	Degree		
Immune (no challenge)	0	0/6		2/6	0.5	137.0	0
Immune (virulent organisms)	1	5/6	4.1	6/6	2.7	1,010.3	0
	2	5/6	3.3	6/6	2.8	605.7	0
	3	5/6	4.2	6/6	2.2	842.3	4×10^3
	7	6/6	4.6	6/6	1.3	307.7	0
	14	2/6	1.4	2/6	0.5	197.0	0
Immune (broth medium)	3	0/4		4/4	2.0	250.5	0
Normal (virulent organisms)	3	6/6	6.1	0/6	0	162.0	0
	7	6/6	6.4	1/6	0.5	314.0	0
	14	6/6	6.7	6/6	2.2	505.0	3.3×10^3
Normal (broth medium)	3	0/4		0/4	0	127.0	0
	7	0/4		0/4	0	141.0	0
	14	0/4		0/4	0	97.0	0

[a] Immune animals infected 3 months previously; challenge inoculum in parentheses.

[b] Number infected/number inoculated; amount, $\log_{10}$ colony-forming units per gram of lung (geometric mean).

[c] Number with pneumonia/number inoculated; degree, mean score for positive animals each rated at 1 to 4+.

occurrence of some peribronchial round cells and mildly elevated cell counts in the pulmonary passages; rosette figures were not produced. Thus, some reactivity to the medium was reflected cytologically; however, these changes were of less marked degree than those seen in animals challenged with the mycoplasma culture.

DISCUSSION

Evidence accumulated to date in various experimental model systems indicates that *M. pneumoniae* is an extracellular parasite (2, 4–6, 11). Although infection appears confined to the respiratory mucosa, there is intimate parasitism of host cell membranes achieved by a specialized attachment device of the organism (5). In this relationship, the mycoplasma is protected from the muco-ciliary clearance mechanism of the air passage but can damage epithelial cells and stimulate host immune responses. The lymphocytes and plasma cells which infiltrate the lamina propria probably mediate initial immune defenses. However, the interface between host and parasite is at the lumenal surface.

The studies reported describe and quantitate cellular events occurring in the tracheobronchial tree during the course of experimental *M. pneumoniae* disease and provide collation with the microbiology and several parameters of host response. "Free" macrophages, which were the predominant cells recovered by lavage of normal hamsters, were reduced in number throughout the period of maximal infection. This suggests that the process interferes with normal release of these phagocytes, possibly by their activation and fixation in the involved tissues. The appearance of increased lymphoid cells in the exudate corresponded to the peribronchial round cell infiltration and anteceded development of detectable serum antibody. Polymorphonuclear leukocytes were most numerous when the disease process reached its peak, including initiation of serologic responses, which suggests the occurrence of immune phagocytosis.

The leukocyte-epithelial cell configurations noted in the tracheobronchial exudates have no exact counterpart in other infectious diseases. The cellular formations resemble the rosettes of systemic lupus erythematosus, which occur when sera and leukocytes of certain patients are allowed to interact in vitro. Although the hamster rosette phenomenon could be reproduced with organisms and components from normal animals, further study is needed to define its exact nature and significance. The data suggest that rosette formation involves elements of complement activation,

leukocyte chemotaxis, immune adherance, and phagocytosis, accompanied by lysis of infected cells.

A relationship between immune events and the tracheobronchial exudates is suggested by the experiments involving reinfection of hamsters. The cellular responses evoked by second contact with *M. pneumoniae* were both accelerated and exaggerated relative to first infection. This included the early appearance of cellular rosette configurations, suggesting rapid mobilization of necessary opsonins. In association with these changes, pulmonary infection was not prevented, although its course was significantly shortened. Peribronchial round cell infiltration evolved more rapidly after challenge inoculation but qualitatively was similar to that seen in naive animals.

Atypical pneumonia in man is often accompanied by sputum production, and the material washed from the hamster lungs in these experiments may represent a crude counterpart. Frequent references in the clinical literature attest that purulent sputum often occurs in cold hemagglutinin-positive pneumonia, but detailed cytological studies have not been reported. Pilot studies of sputa from young adults with pneumonia suggest that cellular rosette formations like those reported for the hamster occur in natural *M. pneumoniae* disease but not in infections by several other etiologic agents (W. A. Clyde, Jr., *unpublished data*). If the frequency and specificity of this observation can be confirmed with additional data, a simple diagnostic technique is suggested. Specimens of this type could also be useful for assessing the interaction between *M. pneumoniae* and human respiratory cells (5, 11).

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