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Arthritis of Mice Induced by *Mycoplasma pulmonis*: Humoral Antibody and Lymphocyte Responses of CBA Mice

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Peak arthritis occurred 14 days after intravenous injection of *Mycoplasma pulmonis* and persisted in some mice at low levels for 84 days. A marked lymphocytosis occurred during the first week of infection with only a slight increase in polymorphonuclear leukocytes. Complement-fixing antibodies appeared in low titer 3 days after infection and moderate levels persisted for 84 days. The metabolic-inhibiting and mycoplasmacidal antibody responses were absent or minimal. *M. pulmonis* appeared to be mitogenic for mouse lymphocytes as evidenced by (i) increased uptake of [^{3}H]thymidine by normal lymphocytes exposed to various concentrations of nonviable *M. pulmonis* antigen, and (ii) a 13-fold increase in [^{3}H]thymidine uptake in lymphocytes taken from mice 3 days after infection with *M. pulmonis* in the absence of added antigen. Lymphocytes taken from infected mice transformed significantly more at all time periods than control lymphocytes when exposed to *M. pulmonis* antigen. This response was maximal at 3 days and minimal at 21 to 35 days after infection. Lymphocytes sensitized to *M. pulmonis* did not transform when exposed to *M. arthritidis* antigen or vice versa. *M. pulmonis* infection had no effect upon the mitogenic responses of lymphocytes to phytohemagglutinin or lipopolysaccharide. There was no statistically significant correlation between persistence of arthritis and degree of humoral antibody or lymphocyte responses. However, persisting arthritis was associated with a higher incidence of mycoplasma isolations.

Both *Mycoplasma pulmonis* (2, 23) and *Mycoplasma arthritidis* (12) induce a chronic arthritis of mice after intravenous injection of viable organisms. These models of chronic inflammation are of great interest since the etiological agents are quite different biochemically, antigenically, and in their interaction with mammalian cells.

Although complement-fixing antibodies are produced in rodents injected with *M. pulmonis* (9, 30), metabolic-inhibiting (MI) antibodies which might be more relevant to protection of the host have not been consistently detected (9, 22, 36, 38). Locally produced antibody, however, may play a role in protection against *M. pulmonis* respiratory disease (6).

Although a cell-mediated response to *M. pulmonis* is also believed to play a role in protection against the invading organisms (18, 38), the detection and development of cell-mediated responses have not been well documented. Similarly the effects of *M. pulmonis* infection on the responsiveness of T and B cells to mitogens remains unknown. It was recently reported that the migration of macrophages taken from mice infected with *M. pulmonis* was inhibited in the presence of a mouse synovial antigen yet was unaffected by *M. pulmonis* antigen (24). A complicating factor in these studies was that macrophages taken from infected mice migrated to a greater extent than did normal macrophages.

The present studies were undertaken to determine in more detail the humoral antibody responses of mice to *M. pulmonis* and to define the effect of *M. pulmonis* infection on the in vitro blastogenic responses of lymphocytes to homologous antigen. The lymphocyte responses to phytohemagglutinin (PHA) and bacterial lipopolysaccharide was also examined to determine T (26) and B (1) lymphocyte activity, respectively.

MATERIALS AND METHODS

Cultivation of mycoplasmas. *M. pulmonis* strain JB used in these studies was originally obtained from J. G. Tully. The growth medium used was a modification of that described by Hayflick et al. (7, 25). It consisted of Bacto PPLO agar or broth (Difco, Detroit, Mich.) supplemented to 15% (vol/vol) horse serum, 5% (vol/vol) fresh yeast extract, 1,000 U of

penicillin G per ml, 1% (wt/vol) glucose, and 1% (vol/vol) of a sterile 2-mg/ml solution of diphosphopyridine nucleotide (DPN-102, Sigma Chemical Co.). The medium was adjusted to pH 7.8. *M. arthritidis* strain 158 P10 P9 used in the cross-reactivity experiments was cultured as previously described (12, 21).

The preparation and concentration of mycoplasma suspensions used for injection and antigen production were as previously described (21), except that in the case of *M. pulmonis*, broth cultures were prepared by first inoculating stock cultures onto 200 ml of complete mycoplasma agar in a flat-bottomed flask. After 24 to 48 h of incubation at 37 C, 200 ml of complete mycoplasma broth was added, followed by an additional liter of broth 16 to 24 h later. This method increased the number of viable organisms obtained. The protein concentration of the antigen suspension was determined by the procedure of Lowry et al. (32).

Induction of arthritis and autopsy. Concentrated stock cultures of *M. pulmonis* frozen at −60 C were thawed and subjected to ultrasonic vibration for 4 s using a model S75 Sonifier equipped with a 0.32-cm microtip (Branson Instruments Inc., Danbury, Conn.). This procedure dispersed the clumps of organisms, thus producing more consistent and higher colony-forming units. Female CBA mice, 6 to 8 weeks old, were injected intravenously with 10^9 to 2×10^9 colony-forming units per 0.2 ml of the sonified suspension. Control mice were injected with 0.2 ml of complete mycoplasma broth. Four control mice and eight mice injected with *M. pulmonis* were sacrificed after 3, 7, 14, 21, 35, 56, and 84 days. Hematological, cultural, serological, and lymphocyte studies were performed on the same mice. Before the mice were sacrificed each animal was scored for arthritis by subjectively measuring joint swelling. Ankles, wrists, metatarsal joints, metacarpal joints, and digits were graded on a 0 to 4 scale and totaled. Then each animal was bled from the retro-orbital venous plexus using a heparinized 0.05-ml capillary tube. A viable mycoplasma count was performed on 0.025 ml of blood and a differential and total leukocyte count was performed on the remaining 0.025 ml. Animals were exsanguinated by cardiac puncture. Blood was allowed to clot and serum was harvested for serological tests.

Spleens and lymph nodes were removed aseptically and transferred to petri dishes containing 5 ml of RPMI 1640 medium (Microbiological Associates, Albany, Calif.) for the preparation of lymphocyte suspensions (vide infra).

Isolation of mycoplasma. Isolation of mycoplasma from wrist and ankle joints was attempted by removing hind and forepaws, exposing the tibiotarsal and radiocarpal joints, and by streaking the exposed surface on a PPLO agar plate. Mycoplasma isolations from spleens and nodes were also attempted by streaking 0.05 ml of the minced tissue suspensions on mycoplasma agar plates. After 7 days of incubation all cultures were observed for the presence of mycoplasma. Plates were scored from 0 to 4 depending on the number of colonies present (0, 0; 1 to 25, 1; 26 to 100, 2; 100 to 300, 3; greater than 300, 4).

Tissue culture media. The tissue culture medium employed throughout the experiments was RPMI 1640 with L-glutamine (Microbiological Associates) supplemented with 100 U of penicillin and streptomycin (Microbiological Associates) per ml and 2% (vol/vol) heat-inactivated (56 C for 30 min) human serum. Aliquots of the serum were stored at −20 C and thawed for each experiment.

Preparation of lymphocyte cultures. The technique used was a modification of that described by Colley (15). Spleens and nodes were placed in separate petri dishes containing 5 ml of tissue culture medium without serum. Tissues were teased gently with a scalpel blade. Suspensions were then filtered through a 60-mesh stainless-steel screen and centrifuged for 5 min at $200 \times g$. Three milliliters of prewarmed (37 C) 0.83% NH_4Cl, tris(hydroxymethyl)aminomethane-buffered to pH 7.2, was added to lyse erythrocytes (3). After gentle mixing, the suspensions were centrifuged at $200 \times g$ for 5 min. Cells were resuspended in 5 ml of fresh medium and recentrifuged at $200 \times g$. The number of viable cells was determined in a hemocytometer, using equal volumes of cells and trypan blue (prepared daily by mixing 4 parts 0.2% [wt/vol] aqueous trypan blue with 1 part 4.25% [wt/vol] aqueous NaCl [40]). The cells were resuspended to a final concentration of 1.5×10^6/ml of complete tissue culture medium. No culture with more than 22% dead cells was used.

Node and spleen suspensions of each mouse were distributed as follows. Two milliliters of the spleen lymphocyte suspension was dispensed into each of six plastic disposable tubes (16 by 125 mm; Falcon no. 3033). Twenty micrograms of lipopolysaccharide B (*Escherichia coli* O55;B5, Difco) in 0.2 ml was added to three of the tubes. The remaining spleen suspension was pooled with the lymph node suspension and dispensed into nine tubes. Pooled lymphocyte cultures contained from 10 to 30% node cells. Triplicate tubes of the pool were stimulated with 0.2 ml of *M. pulmonis* antigen containing 5 μg of protein or 0.2 ml of a 1:50 dilution of PHA reagent grade no. HA15 (Burroughs Wellcome, Tuckahoe, N.Y.). Three control tubes with no added mitogen were also included. Stimulants used for blastogenesis were pretitered for optimal transformation. All tubes were incubated at 37 C in 5% CO_2 and air for 72 h. Twenty-four hours prior to harvesting all cultures were pulsed with 1 μCi of tritiated thymidine (specific activity, 19 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.).

Measurement of blastogenesis. Cultures were harvested by a modification of the technique of Colley and DeWitt (16). Cultures were transferred to 12-ml glass conical centrifuge tubes and washed twice with cold phosphate-buffered saline, pH 7.2. Tubes were centrifuged at $200 \times g$ for 5 min after each wash. Cultures were then treated with 0.5 ml of 1 N NaOH and incubated for 10 min at 56 C, after which cells were treated twice with 2 ml of cold 5% trichloroacetic acid (centrifuged for 5 min at $1,250 \times g$ each time). After the second trichloroacetic acid treatment, precipitates were dissolved by adding 0.5 ml of NCS tissue solubilizer (Amersham/Searle) and agitating with a Vortex mixer. Ten milliliters of

scintillation fluid (42 ml of Liquifluor [New England Nuclear, Los Angeles, Calif.] per liter of scintillation grade toluene [Matheson Coleman and Bell, Los Angeles, Calif.]) was added to each tube. After thorough mixing the tube contents were transferred to scintillation vials (Packard no. 600175, Downers Grove, Ill.) and allowed to dark adapt for 5 h. Samples were counted in a Beckman LS-233 liquid scintillation counter with an automatic external standard (Beckman Instrument Inc., Fullerton, Calif.). Counts were converted to disintegrations per minute (dpm) by employing a standard curve relating percentage of efficiency to the external standard which allows for a quench correction. Most samples were counted at 35 to 40% efficiency. The dpm in the triplicate lymphocyte cultures were averaged. The results were expressed either as dpm or as a blastogenic index, i.e., the ratio of dpm in lymphocyte cultures with mitogen to the dpm in lymphocyte cultures without mitogen.

Cross-reactivity between M. pulmonis and M. arthritidis. Mice injected intravenously with *M. pulmonis* were sacrificed 5, 8, and 12 weeks later and were tested (as described previously) for the presence of a lymphocyte response to either *M. pulmonis* or *M. arthritidis* antigen. Five micrograms of antigen was added to each lymphocyte culture tube. Mice previously injected with 2×10^9 colony-forming units of *M. arthritidis* were sacrificed after 5 weeks and also tested for lymphocyte reaction to either *M arthritidis* or *M. pulmonis* antigen.

Humoral antibody tests. All mouse sera were heat inactivated at 56 C for 30 min prior to use. Antibody studies were carried out on individual mouse sera. Complement-fixing antibody was measured by the microtechnique of Casey (5) using the same antigen preparation employed for lymphocyte stimulation. The microtechnique of Taylor-Robinson et al. (39) was used to determine the presence of MI antibody in which inhibition of the metabolism of glucose was used as the indicator. Mycoplasmacidal antibody titers were determined as described by Cole and Ward (10).

Statistical analyses. The Welch t test was used for group comparisons since the variances in the groups could not be assumed to be equal. Statistical analyses were performed by M. Klauber, Division of Biostatistics, University of Utah College of Medicine.

RESULTS

Induction of disease and persistence of organisms. The numbers of infected and control mice used throughout this study are reported in Table 1. *M. pulmonis* strain JB induced maximum arthritis 14 days after injection. Arthritis scores ranged from 0 to 4. Low levels of arthritis persisted for the duration of the experiment. Mice harvested at 21 days were injected with a different pool of *M. pulmonis*: no arthritis developed in these mice and mycoplasmas were not recovered at sacrifice. At autopsy a purulent reaction was not seen in the joints at any time period.

Mycoplasmas were no longer cultivable from the blood after 3 days except from one mouse at 35 days. Mycoplasmas persisted in the spleens of two mice for 7 days and in the nodes of one mouse for 14 days. Survival of viable mycoplasmas was greatest in the joints and they were isolated in one case as late as 84 days after injection.

Effect of leukocyte counts. *M. pulmonis* induced a marked leukocytosis in mice which lasted 14 days after injection of the organisms (Fig. 1). Total leukocyte counts returned to normal by 21 days but exhibited an increase between 56 and 84 days as compared with control mice. Differential counts revealed that the leukocytosis was mainly lymphocytic in nature (Fig. 2). Only a slight increase in polymorphonuclear leukocytes was apparent. Maximum lymphocytosis occurred at 3 days after injection of the organisms. By 21 days lymphocyte counts had returned to control values. At 56 and 84 days after injection of the organisms lymphocyte counts were again elevated but polymorphonuclear leukocyte counts were normal.

Humoral antibody responses. The results of humoral antibody responses are summarized in Fig. 3. Complement-fixing antibodies were first detected in low titer 3 days after the injection of organisms and reached peak values at 7 to 14 days. Despite a slight decrease in antibody levels after 21 and 35 days, the titers remained high for the duration of the experiment. The highest individual titer observed was 1:1,280.

Mice responded poorly to *M. pulmonis* in the production of MI and cidal antibodies. Low levels of MI antibody were detected at 7, 14, and 21 days, and again at 56 days. No antibody was apparent 84 days after injection. Cidal antibody titers paralleled those observed in the MI test. Maximum titers detected were 1:80 for both the MI and cidal test. Difficulties were encountered in the cidal antibody test due to the tendency of *M. pulmonis* to grow in clumps. All suspensions required 5 s of sonification before use in the test. This property may explain previous failures to detect an antibody response, since clumps of organisms may resist the action of antibody.

None of the control mice developed a humoral antibody response to *M. pulmonis*.

Lymphocyte responses of control mice to M. pulmonis antigen. The results of lymphocyte responses are summarized in Table 2. Spleen-node lymphocyte cultures prepared from control mice and exposed to *M. pulmonis* antigen in vitro exhibited a consistently higher [^{3}H]thymidine uptake than lymphocytes not treated with *M. pulmonis* antigen. Statistical analysis of all results indicated a high level of

TABLE 1. *Isolation of mycoplasmas and development of arthritis*

Days after injection	No. of *M. pulmonis* mice	No. of control mice[a]	No. of infected mice from which mycoplasmas were cultured				Mice with arthritis (%)	Mean arthritis scores at sacrifice
			Blood	Spleen	Nodes	Joints		
3	7	4	4	4	2	3	0	0
7	6	4	0	2	0	4	33	0.4 (0–1)[b]
14	8	4	0	0	1	6	75	1.6 (0–4)
21	11	6	0	0	0	0	0	0
35	8	4	1	0	0	2	25	0.9 (0–4)
56	11	6	0	0	0	1	27	0.6 (0–3)
84	8	4	0[c]	0[c]	1[c]	0[c]	75	0.8 (0–1)

[a] None of the control mice developed arthritis. Blood, spleen, and nodes were all free of mycoplasmas.
[b] Range of arthritis scores.
[c] Only four *M. pulmonis* mice were cultured at this time period.

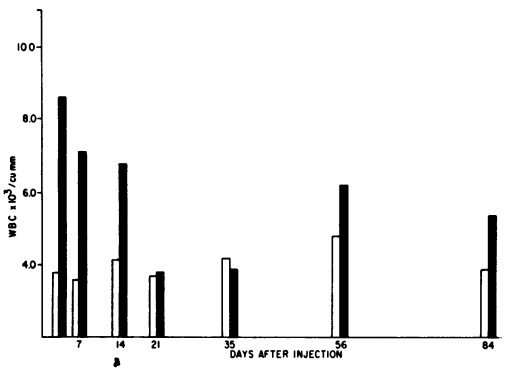

FIG. 1. *Total leukocyte (WBC) counts in control mice (open bars) and in mice injected with 10^9 colony-forming units of M. pulmonis (solid bars).*

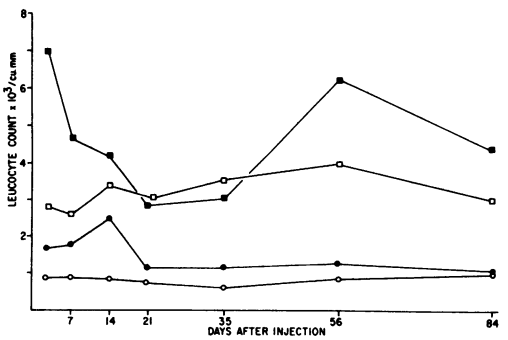

FIG. 2. *Changes in peripheral leukocyte types in mice injected with M. pulmonis. Polymorphonuclear leukocytes in control mice (○) and in infected mice (●). Lymphocytes in control mice (□) and in infected mice (■).*

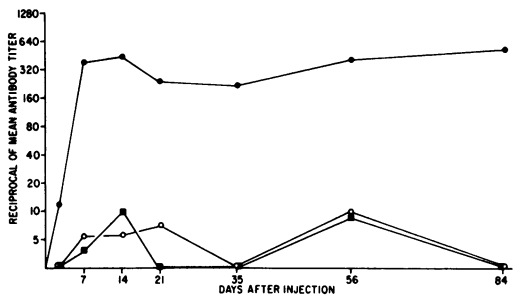

FIG. 3. *Humoral antibody responses of CBA mice to infection with M. pulmonis. Complement-fixing antibody (●), MI antibody (○), and mycoplasmacidal antibody (■).*

significance ($P = <0.05$). These results are of interest in view of the observations of Ginsburg and Nicolet, who reported that *M. pulmonis* was mitogenic for rat lymphocytes (20). A separate experiment was set up in which lymphocytes were harvested from four normal uninjected mice and exposed to various concentrations of *M. pulmonis* antigen. The results indicated that the mitogenic effects of *M. pulmonis* antigen were dose dependent (Table 3). Thus, 1 μg of *M. pulmonis* antigen resulted in a mean blastogenic index of 1.8, whereas 30 μg of antigen resulted in a mean index of 5.7. The effect observed was not due to sensitization against medium constituents since the donor mice used did not receive an injection of mycoplasma broth. The experiment was repeated using a new antigen prepared from a broth culture of *M. pulmonis*. The mean blastogenic indices from three mice were 8.0 with 40 μg of antigen,

TABLE 2. *Blastogenic responses of lymphocytes taken from control and infected mice to M. pulmonis antigen*

Days after injection	Control mice			*M. pulmonis*-infected mice			*P* values for indices[c]
	[3H]thymidine control (dpm)[a]	Antigen (dpm)	Index[b]	[3H]thymidine control (dpm)[a]	Antigen (dpm)	Index[b]	
3	2,046	3,306	1.7	26,555	303,090	18.6	<0.05
7	13,144	36,607	2.9	11,386	138,273	15.4	0.05
14	6,701	38,161	4.0	5,534	113,311	22.9	<0.005
21	5,680	11,454	1.6	5,735	26,787	5.4	<0.01
35	7,146	17,025	2.0	5,302	35,693	7.0	<0.005
56	8,984	14,097	1.4	9,150	57,522	7.7	<0.005
84	5,534	10,031	2.0	4,631	42,244	10.1	<0.05

[a] Background levels of [3H]thymidine uptake in the absence of antigen.
[b] Mean of individual indices. The indices consist of the ratios of [3H]thymidine uptake in lymphocyte cultures containing antigen as compared with [3H]thymidine uptake in cultures not containing antigen.
[c] *P* values calculated by comparing individual indices from control and infected mice.

TABLE 3. *Effect of M. pulmonis antigen on uptake of [3H]thymidine by normal mouse lymphocytes*

Mouse	Blastogenic indices[a] obtained with antigen at:			
	1 μg	5 μg	15 μg	30 μg
1	1.5	1.9	2.5	2.5
2	1.8	2.4	3.7	4.1
3	2.1	4.1	7.3	9.7
4	1.6	2.1	6.2	6.3
Mean	1.8	2.6	4.9	5.7

[a] Indices calculated from the ratio of disintegrations per minute in cultures containing antigen to those without antigen.

3.9 with 10 μg of antigen and 1.4 with 1 μg of antigen, respectively.

Lymphocyte responses of infected mice to M. pulmonis antigen. Lymphocytes taken from mice 3 days after injection with *M. pulmonis* showed a much greater uptake of [3H]-thymidine in the absence of added antigen (i.e., mean dpm of 26,555) as compared with lymphocytes from control mice (i.e., a mean dpm of 2,046; Table 2). These results were statistically significant ($P = <0.05$) at the 3-day time period. A similar observation was made in lymphocyte cultures prepared from spleens alone. At all other times no difference was detected between uptake of [3H]thymidine by control and sensitized lymphocytes.

The enhanced activity at 3 days could be due to the presence of viable mycoplasmas which (i) resulted in an immune interaction with early sensitized lymphocytes or (ii) directly incorporated [3H]thymidine. However, an examination of individual animals showed that there was no correlation between isolation of viable mycoplasmas from spleen and nodes on autopsy and degree of background [3H]thymidine incorporation. Another explanation is that *M. pulmonis* is mitogenic in vivo. Interestingly, the enhanced background values of [3H]thymidine uptake in infected mice at 3 days paralleled the marked lymphocytosis which occurred at this time (Fig. 2).

Lymphocytes taken from infected mice transformed significantly when exposed to *M. pulmonis* antigen. The effect was most pronounced at 3 days. Thus, despite elevated background levels of [3H]thymidine uptake at this time, the mean blastogenic index was 18.6. A comparison of dpm in sensitized (mean value: 303,090) as opposed to control lymphocytes (mean value: 3,306) exposed to *M. pulmonis* antigen revealed a ratio of 91.7.

Lymphocytes transformed at all other time periods, although the response at 21 and 35 days was somewhat less. Statistical analysis of individual indices indicated that the results were statistically significant at all times ($P = 0.05$ to <0.005).

Cross-reactivity between M. pulmonis and M. arthritidis. Both *M. pulmonis* and *M. arthritidis* infections of mice result in a similar chronic disease. Therefore, an attempt was made to determine whether the lymphocytes of mice injected with *M. arthritidis* would transform significantly when exposed to *M. pulmonis* antigen and vice versa. The results are summarized in Table 4.

Lymphocytes taken from mice injected with *M. pulmonis* exhibited enhanced uptake of [3H]thymidine when exposed to *M. pulmonis* antigen (mean index: 7.6) but did not transform in the presence of *M. arthritidis* antigen (mean index: 1.1). Control mice exhibited no signifi-

TABLE 4. *Specificity of antigen-mediated lymphocyte transformation*

Experimental group	Antigen tested	Blastogenic index obtained after the following days:			
		35	56	84	Mean[a]
M. pulmonis-injected mice	*M. pulmonis*	7.3 (4)[b]	5.4 (2)	9.6 (3)	7.6 (9)
	M. arthritidis	0.6 (4)	0.9 (2)	1.8 (3)	1.1 (9)
Control mice	*M. pulmonis*	1.1 (2)	1.1 (2)	1.8 (2)	1.3 (6)
	M. arthritidis	0.9 (2)	0.7 (2)	0.5 (2)	0.7 (6)
M. arthritidis-injected mice	*M. pulmonis*	2.5 (5)	NT[c]	NT	
	M. arthritidis	3.1 (5)	NT	NT	
Control mice	*M. pulmonis*	2.6 (4)	NT	NT	
	M. arthritidis	0.6 (4)	NT	NT	

[a] Mean index obtained by pooling results for all time periods.
[b] Number of mice.
[c] NT, Not tested.

cant transformation in response to either *M. pulmonis* or *M. arthritidis* antigens. Similarly lymphocytes from mice injected with *M. arthritidis* transformed in the presence of *M. arthritidis* antigen (mean index: 3.1) as compared with lymphocytes from control mice which did not transform (mean index: 0.6). *Mycoplasma pulmonis* antigen resulted in a similar level of [^{3}H]thymidine uptake in both control lymphocytes (mean index: 2.6) and lymphocytes sensitized to *M. arthritidis* (mean index: 2.5). The nonspecific transformation observed is consistent with the mitogenic property of *M. pulmonis* antigen.

These results suggest that there is no cross-reaction between *M. arthritidis* and *M. pulmonis*, and that the enhanced transformation of lymphocytes from sensitized animals in the presence of homologous antigen is specific.

Effect of M. pulmonis infection on the lymphocyte response to PHA and bacterial lipopolysaccharide. *M. pulmonis* infection had no significant effect on the response of lymphocytes to PHA or bacterial lipopolysaccharide (Table 5).

The lower indices (which were statistically significant at the 90% confidence level) obtained with PHA 3 days after injection with *M. pulmonis* coincided with higher background values of [^{3}H]thymidine uptake (Table 2). Comparison of dpm in control and sensitized lymphocytes exposed to PHA did not indicate any inhibitory effect.

Correlation between persisting arthritis humoral and cell-mediated antibody response and presence of viable organisms. At day 14 and later time periods mice were sorted into three groups exhibiting (i) no arthritis, (ii) arthritis scores of 1 to 2, and (iii) arthritis scores of >2. The comparisons undertaken are summarized in Table 6.

No correlation was apparent between degree of arthritis and humoral antibody responses. The somewhat higher mean blastogenic indices of arthritic mice were found not to be statistically significant (i.e., $P = 0.257$ for the comparison of groups i and ii and $P = 0.299$ for the comparison of groups i and iii). Combining the arthritic groups 2 and 3 resulted in a somewhat higher level of significance ($P = 0.123$) as compared to controls but this was still outside the 90% confidence levels.

A larger number of arthritic mice harbored mycoplasmas as compared with nonarthritic mice and, in addition, greater numbers of organisms were isolated. An examination of individual joints revealed that, by 14 days of infection, 73% of all joints were free of both arthritis and organisms, 6.3% of joints exhibited both mycoplasmas and arthritis, 15.3% of joints were arthritic yet harbored no mycoplasmas, and only 5% of mice harbored mycoplasmas in the absence of macroscopic arthritis.

DISCUSSION

Interest in members of the *Mycoplasmatales* as possible etiological agents of human rheumatoid arthritis was initiated by Sabin's observation that certain strains induced a chronic proliferative arthritis of mice (34, 35). Sabin's type C mycoplasma has now been identified as *M. pulmonis* (31). As recently reviewed, despite some reports describing isolation of mycoplasmas from rheumatoid arthritis tissues most investigators have failed to find such an association (14, 36). However, initiation of human rheumatoid arthritis by an unidentified infectious agent which may or may not persist remains an attractive hypothesis. The studies reported in

TABLE 5. *Effect of M. pulmonis infection in vivo on the mitogenic responses of lymphocytes to PHA and lipopolysaccharide*

Days after injection	Mean blastogenic indices[a]					
	PHA			Lipopolysaccharide		
	Control mice	*M. pulmonis* mice	*P* value[b]	Control mice	*M. pulmonis* mice	*P* value[b]
3	460.4	107.4	<0.1[c]	95.8	90.5	>0.5
7	167.5	244.9	>0.5	109.5	153.7	>0.5
14	264.6	306.9	>0.5	204.9	93.3	>0.5
21	208.6	188.6	>0.5	59.2	88.1	>0.5
35	217.2	166.0	>0.5	169.2	58.9	>0.1
56	119.6	137.3	>0.5	78.1	58.7	>0.5
84	162.0	225.5	>0.5	9.9	17.1	>0.1

[a] Ratio of disintegrations per minute in lymphocyte cultures exposed to mitogen as compared with disintegrations per minute in lymphocyte cultures without mitogen.
[b] *P* values calculated by comparing indices of individual mice from control and infected groups.
[c] See text.

TABLE 6. *Relationship between arthritis, immune response, and persistence of viable organisms*

No. of mice[a]	Arthritis score	Mean antibody titers			Mean blastogenic index	Mice harboring mycoplasmas (%)
		CF[b]	MI	Cidal		
22	0	343	6.7	3.3	5.1	13.6
8	1–2	443	3.6	<10	11.8	50
5	>2	347	<10	13.3	17.3	60

[a] Mice taken from day 14 and later groups only.
[b] CF, Complement fixing.

this paper examine the response of mice to *M. pulmonis* to eventually identify the arthritogenic pathway.

Most previous studies have failed to detect the presence of significant levels of MI antibody in either *M. pulmonis* respiratory disease or arthritis (36, 38, 22). Similarly we have failed to induce MI antibodies in Swiss Webster mice repeatedly injected with a variety of strains of *M. pulmonis* mixed with Freund adjuvant (9). In the present study using CBA mice, low activity in both the MI and cidal tests was detected. Whether these results reflect the strain of mouse used cannot be ascertained at this time. It should be noted that CBA mice do in fact appear to be more resistant to *M. pulmonis* (38). The serological heterogeneity within the species *M. pulmonis* (17, 22) could account for differences in immunogenicity. Studies to determine whether the weak antibody response of mice to *M. pulmonis* contributes to the chronicity of infection remain to be undertaken.

We have presented evidence for two mechanisms of transformation of lymphocytes by *M. pulmonis*, i.e., (i) a mitogenic effect toward lymphocytes and (ii) an immunologically specific transformation of sensitized lymphocytes. Our observations that normal unsensitized lymphocytes exposed to *M. pulmonis* antigen exhibit an increased uptake of [^{3}H]thymidine are consistent with those of Ginsburg and Nicolet (20) who reported that *M. pulmonis* was mitogenic for normal rat lymphocytes. An immune mechanism for this transformation, i.e., presence of specifically sensitized rat lymphocytes, was considered unlikely as the organisms exhibited a high transforming ability which was eliminated by heating at 60 C. In our study, none of the control mice had humoral antibodies against *M. pulmonis*, suggesting that the lymphocyte pool did not in fact contain a sensitized population.

M. pulmonis may also be mitogenic in vivo. Thus lymphocytes taken from mice 3 days after injection of *M. pulmonis* exhibited a 13-fold greater uptake of [^{3}H]thymidine in the absence of added antigen as compared with lymphocytes taken from control mice. Although there was no correlation between presence or absence of viable mycoplasmas and degree of transformation, a persisting nonviable antigen might have initiated the transformation of sensitized lymphocytes. Clearly *M. pulmonis* exerts a significant effect on the lymphocyte population of the animal. The massive lymphocytosis in the peripheral circulation at 3 days is evidence of this. Whether this latter observation represents an immune or nonspecific mitogenic response to

M. pulmonis remains to be defined.

There is evidence that *M. pulmonis* may affect the metabolism of other cell types. Thus, Harwick et al. (24), using the macrophage migration inhibition technique, observed that macrophages taken from mice infected with *M. pulmonis* migrated further in the absence of added antigen than did macrophages taken from normal mice. The increased migration of these macrophages may have counteracted any inhibitory effect of specific antigen and thus led to Harwick's failure to detect a cell-mediated response to *M. pulmonis*.

If *M. pulmonis* is in fact nonspecifically mitogenic in vivo, the systemic effects appear to be rapidly lost. Thus, lymphocytes taken from infected mice exhibit normal uptake of [^{3}H]thymidine by 7 days and the lymphocytosis in the peripheral circulation is declining at this time. Since *M. pulmonis* localizes and persists in joints, it is possible that the nonspecific mitogenic properties of these organisms enhance immune processes, thereby contributing to the inflammation. Despite the apparent mitogenic effect of *M. pulmonis*, lymphocytes taken from infected animals responded normally to PHA and lipopolysaccharide.

The present study has shown that lymphocytes taken from mice infected with *M. pulmonis* through 84 days transform significantly greater than normal lymphocytes when exposed to *M. pulmonis* antigen. Other experiments (unpublished) using Swiss Webster mice detected a mean blastogenic index of 20.7 in animals infected 120 days previously with *M. pulmonis*. Investigations are now being performed to determine whether this immunologically specific reaction is T cell dependent since antibody-producing B cells can undergo blastogenesis (19). No significant correlation was found between degree of blastogenesis and severity of arthritis, but a correlation was apparent between presence of mycoplasmas and arthritis. The role played by a cell-mediated immune response to persisting antigen in the arthritic process remains unknown at this time. In *M. pulmonis*-induced respiratory disease cell-mediated immunity appeared to be a significant factor in the development of the lesion since T cell-depleted animals exhibit much less severe pneumonic lesions (18). However, Taylor et al. (38) showed that, at least in the early stage of *M. pulmonis*-induced arthritis, a cell-mediated immunity appeared not to be responsible for the disease since prior thymectomy and X-irradiation, procedures which eliminate T cells, resulted in a more severe arthritis.

These observations suggest that a cell-mediated response plays a significant role in protection of the host against infection. The many failures in detecting a significant neutralizing antibody response in mice infected with *M. pulmonis* would support this view. It has also been shown that prior treatment with cyclophosphamide, which acts on B cells and rapidly dividing T cells (29), results in enhanced arthritis (38) and enhanced respiratory disease (36). The latter could be reversed by passive administration of sensitized spleen cells. Thus, in the early stages of the disease, treatments which impair host defenses and permit enhanced mycoplasmal multiplication appear to result in a more severe arthritis. Clearly, the role played by T and B cells in the maintenance of the disease needs additional study.

Other mechanisms of *M. pulmonis*-induced arthritis must also be considered. The ability of *M. pulmonis* to adsorb to mammalian cells (11, 27, 28, 33) provides the potential for cytotoxicity due to peroxide production (13) and altered antigenicity. The observations of Harwick et al. (24) are of interest since they showed that the migration of macrophages from arthritic mice was inhibited in the presence of normal synovium, thus suggesting the existence of an antigen common to parasite and host. In this regard we have previously presented evidence that *M. arthritidis* shares an antigenic component with rodent tissues (4).

We believe that a comparison of *M. pulmonis*-induced arthritis with that induced by *M. arthritidis* will provide valuable clues into the nature of chronic inflammation since these two agents are quite distinct. The present study has shown that there is no cross-immunity between the two organisms. Additional studies (Keyes and Cole, unpublished observations) have shown that mice previously infected with *M. pulmonis* are equally or more susceptible to *M. arthritidis*-induced arthritis as compared to untreated control mice. The two diseases are similar in their chronicity, in the persistence of organisms in the joints and in the poor neutralizing antibody responses of the host. Lymphocytes from infected animals transform at all time periods (8; B. C. Cole, L. Golightly-Rowland, and J. R. Ward, Ann. Rheum. Dis., in press) in the presence of homologous antigen, suggesting the presence of a cell-mediated immunity.

Identification of a common arthritogenic pathway for these two organisms would probably simplify an understanding of the disease process. However it also is quite likely that different mechanisms are operative. Understanding of these processes would shed light on

the etiological mechanisms of human rheumatoid arthritis and might point to possible multiple etiologies for disease.

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