INFECTION AND IMMUNITY, Nov. 1977, p. 393-399

Vol. 18, No. 2
Printed in U.S.A.

Mycoplasma-Dependent Activation of Normal Lymphocytes: Mitogenic Potential of Mycoplasmas for Mouse Lymphocytes

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Received for publication 26 April 1977

Nonviable preparations of a wide variety of glucose-utilizing mycoplasma species, including *Acholeplasma laidlawii* and *Spiroplasma citri*, were found to be mitogenic for mouse lymphocytes. Particularly strong reactions were obtained with *Mycoplasma synoviae*, *M. gallisepticum*, *M. pneumoniae*, *S. citri*, and a strain of *M. fermentans* that was previously isolated from a leukemic patient. Nonviable preparations of arginine-utilizing mycoplasmas inhibited the uptake of [^{3}H]thymidine by lymphocytes, but this effect could be reversed by heat treatment or arginine supplementation, and a stimulatory effect was then observed. Viable *M. arthritidis* was also found to have a mitogenic effect, as detected by an increased uptake of [^{3}H]thymidine by normal lymphocytes and by autoradiographic techniques in which an increase in the numbers of transformed cells was seen. These observations provide the potential for enhanced immunological responsiveness or lymphokine-mediated inflammation in mycoplasma-infected hosts.

Mycoplasmas have been shown to alter lymphocyte functions in a number of ways. In the first paper of this series, we reported that viable *Mycoplasma arthritidis* and *M. hominis*, arginine-utilizing species, induced normal mouse lymphocytes to become cytotoxic for allogeneic and syngeneic mouse target cells (2), thus providing a possible mechanism for in vivo inflammation. We also demonstrated that the cytotoxic reaction was not due to arginine depletion of the medium (3). Paradoxically, both nonviable and viable preparations of these organisms have been shown to suppress the mitogenic response of lymphocytes to phytohemagglutinin both in vitro and in vivo and to suppress antibody production in vitro and in vivo (4, 5, 7, 13, 15, 21). Other studies suggest that glucose-utilizing mycoplasmas can activate lymphocytes. Thus, Ginsburg and Nicolet (14) reported that viable *M. pulmonis* was mitogenic for normal rat lymphocytes, and Cole et al. (10) found that nonviable preparations of this species were mitogenic for mouse lymphocytes. More recently, Biberfeld and Gronowicz (6) showed that nonviable *M. pneumoniae* stimulated the uptake of [^{3}H]-thymidine by both normal mouse and guinea pig lymphocytes. Another example of mycoplasma-mediated activation of lymphocytes is apparent by our observations that many mycoplasma species induce interferon in sheep and human lymphocyte cultures (11).

The present study was undertaken to further define the mitogenic potential of mycoplasmas for normal lymphocytes and to resolve the paradoxical observations on the inhibitory and stimulatory effects of *M. arthritidis* on lymphocyte functions.

MATERIALS AND METHODS

Mycoplasma strains and culture procedures. *M. arginini* M732 (sheep), *M. canis* M725 (dog), *M. gallisepticum* M722 (turkey), *M. hyorhinis* M718 (swine), *M. hominis* M711 (man), and *M. pneumoniae* M710 (man) were obtained from the National Institutes of Health, courtesy of M. F. Barile. The sources of the following mycoplasmas are as follows: *M. fermentans* "G" (man), D. G. ff Edward; *M. fermentans* K10 (man), M. G. Gabridge; *M. hyosynoviae* 25591 (swine), R. F. Ross; *M. pulmonis* JB (mouse), D. Taylor-Robinson; *M. synoviae* 25204 (chicken), American Type Culture Collection; *Spiroplasma citri* Maroc 6 (plant), J. G. Tully. The following mycoplasmas were supplied by the authors: *M. arthritidis* 158p10p9 (man, passaged in rodents); *M. arthritidis* 14124p10 iv (rat); *M. felis* CO (cat); *Mycoplasma* sp. G-S1 (bush baby); *Acholeplasma laidlawii* U2 (fetal lamb kidney cells).

M. synoviae was cultured according to Aldridge (1) and *S. citri* was grown according to Saglio et al. (20). *M. pneumoniae* antigen, kindly supplied by L. Washburn, was grown as a monolayer on glass by the procedure of Somerson et al. (23). All other mycoplasmas were grown in modified Hayflick medium and were assayed for colony-forming units as described in the first paper of this series (2). Media for *M. pulmonis* were supplemented with a final concentration of 1%

(vol/vol) of a sterile 2-mg/ml solution of diphosphopyridine nucleotide (DNP-102; Sigma Chemical Co., St. Louis, Mo.). Broth cultures containing viable organisms were checked for identity by the growth inhibition test (8), using FAO/WHO reference antisera or specific antisera prepared in our laboratories. Mycoplasma antigens were prepared by sedimenting log-phase broth cultures at 27,000 × *g* for 30 min, washing them three times in sterile phosphate-buffered saline, suspending them in sterile deionized water, and subjecting them to sonic treatment for 2-min intervals in an ice water bath until free of cultivable organisms. All antigens were stored at −70°C until use. Protein determinations were performed according to Lowry et al. (16).

Complement-fixing and metabolic inhibiting antibodies in the sera of donor mice were detected as previously described (10). Positive control sera were run with each set of assays.

Preparation of lymphocyte suspensions. Spleen and node lymphocytes from individual Swiss Webster mice were prepared as described in the first paper of this series (2) and suspended in complete RPMI medium supplemented with 5% (vol/vol) heat-inactivated human serum (AB+) and 100 U of penicillin G per ml to give a final concentration of 1.5×10^6 cells per ml. Suspensions containing less than 30% viable cells were discarded. Lymphocytes from CBA mice were similarly prepared except that only spleens were used and that the final lymphocyte suspension of 5×10^6 cells per ml was prepared in RPMI medium containing L-glutamine and a final concentration of 2 mM 2-mercaptoethanol. Lymphocyte suspensions from CBA mice were prepared either from individual mice or from pools consisting of two to three mice each.

Measurement of lymphocyte transformation. The mitogenic properties of a variety of nonviable mycoplasma antigens for three separate lymphocyte suspensions taken from individual Swiss Webster mice (female, 7 to 8 weeks old; Simonsen Laboratories, Gilroy, Calif.) were tested according to the macrotest procedure detailed previously (10). In this test, nonviable antigens were added in 0.2-ml amounts to triplicate tubes containing 2 ml of the lymphocyte suspension. Final antigen concentrations consisted of 1, 5, and 15 μg of protein per ml. Negative and positive controls consisted of lymphocytes treated with medium alone or with 0.2 ml of a 1:50 dilution of phytohemagglutinin (HA15; Burroughs Wellcome & Co. [U.S.A.] Inc., Tuckahoe, N.Y.), respectively. The lymphocyte cultures were incubated for a total of 72 h, using a 20-h pulse with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; Amersham/Searle, Arlington Heights, Ill.) before harvest. Lymphocytes were harvested as previously described (10), and trichloroacetic acid precipitates were dissolved in NCS tissue solubilizer and counted in 10 ml of a Liquafluor (New England Nuclear Corp., Boston, Mass.)-toluene mixture (42:938 ml) with a Chicago 720 series scintillation counter. Counting efficiencies were approximately 30%.

The transformation of CBA mouse (CBA/J; Jackson Laboratories, Bar Harbor, Me.) spleen lymphocytes was studied with the microtiter system detailed in the second paper of this series (3). In the present experiments, RPMI 1640 medium contained 2 mM 2-mercaptoethanol in place of human serum (24). Nonviable antigens in 0.025-ml amounts were added to 0.2 ml of lymphocyte suspension (5×10^6 cells per ml) to give final concentrations of 1, 5, and 15 μg of protein antigen per ml. Negative and positive control wells received 0.025 ml of medium alone or 0.025 ml of a 1:50 dilution of phytohemagglutinin or 0.025 ml of a 250-μg/ml solution of lipopolysaccharide (*Escherichia coli* O55, B5; Difco Laboratories, Detroit, Mich.), respectively. Lymphocyte cultures were incubated for 72 h using an 18-h pulse with 0.125 μCi of [^{3}H]thymidine (6.7 Ci/mmol) per well. Harvesting procedures were described in the second paper of this series (3). One unlabeled well of each antigen/lymphocyte mixture was cultured for 72 h in appropriate mycoplasma medium to ensure absence of viable organisms.

The mitogenic potential of viable mycoplasmas was also tested by the microtiter procedure. Mycoplasma suspensions containing 10^4, 10^5, or 10^6 colony-forming units per 0.025 ml were added to each well. Negative and positive controls for lymphocyte transformation were as above. After 42 h of incubation, the wells were supplemented with 0.025 ml of medium alone or medium containing 100 or 250 μg of gentamicin. After a further 8 h of incubation, one well of each group was cultured to test for viable mycoplasmas, and the remaining wells were pulsed with 0.125 μCi of [^{3}H]-thymidine for an additional 18 h.

In all experiments the uptake of [^{3}H]thymidine was expressed as counts per minute minus background radiation. Blastogenic indexes were calculated as the ratio of counts per minute in lymphocyte cultures containing antigen or mitogen to counts per minute in lymphocyte cultures alone.

Autoradiography. Pulsed lymphocyte cultures in the presence and absence of mitogens or mycoplasma preparations were transferred to acid-cleaned slides and dried at 37°C. After fixation in ice-cold acetone for 5 min and washing and drying, the slides were coated with Kodak NTB2 nuclear tract emulsion and exposed for 7 days at 4°C. The slides were developed in D-19 developer (Eastman Kodak Co., Rochester, N.Y.), fixed according to standard procedure, and stained by the Giemsa technique.

RESULTS

Mitogenic properties of nonviable mycoplasmas. Both the macrotest and the microtest procedures for lymphocyte transformation showed that a wide range of mycoplasma antigens enhanced the uptake of [^{3}H]thymidine when added to normal Swiss or CBA mouse lymphocytes. Since there was a close correlation between the results obtained with these two procedures, the data from the microtest only are shown (Table 1). None of the lymphocyte cultures contained viable mycoplasmas at the time of harvest. The lymphocytes from all animals responded to phytohemagglutinin and lipopolysaccharide, indicating the presence of functional T and B lymphocytes. None of the mycoplasma media used exhibited either an in-

TABLE 1. *Mitogenic effects of nonviable mycoplasmas for normal CBA mouse lymphocytes, microtest procedure*

Mycoplasma antigen[a]	Mean cpm[b] (and blastogenic index[c]) in lymphocytes exposed to:							
	Control (no mitogen)	Phytohemagglutinin	Lipopolysaccharide	Protein antigen				
				1 μg/ml		5 μg/ml		15 μg/ml	
M. pulmonis JB (G+)	297	5,693 (21.9)	4,671 (17.8)	840	(2.8)	740	(2.6)	1,268	(4.4)
M. canis (G+)	1,100	10,754 (10.2)	11,061 (8.3)	2,730	(2.3)	4,875	(3.4)	3,625	(2.5)
M. felis (G+)	536	7,353 (13.7)	4,224 (7.7)	1,023	(1.9)	1,030	(1.9)	1,118	(2.1)
Mycoplasma sp. GS1 (G+)	536	7,353 (13.7)	4,224 (7.7)	1,507	(2.8)	1,849	(3.4)	2,260	(4.3)
M. gallisepticum (G+)	297	5,693 (21.9)	4,671 (17.8)	2,455	(8.7)	1,919	(6.7)	73	(0.3)
M. synoviae (G+)	297	5,693 (21.9)	4,671 (17.8)	921	(3.2)	1,905	(6.7)	2,260	(8.0)
M. fermentans G (G+)	536	7,353 (13.7)	4,224 (7.7)	1,202	(2.3)	2,229	(4.2)	1,645	(3.1)
M. fermentans K10 (G+)	297	5,693 (21.9)	4,671 (17.8)	1,473	(5.2)	2,481	(8.8)	2,879	(10.1)
M. hyorhinis (G+)	1,100	10,754 (10.2)	11,061 (8.3)	5,162	(3.8)	7,165	(4.8)	2,727	(2.0)
M. pneumoniae (G+)	1,100	10,754 (10.2)	11,061 (8.3)	2,086	(2.7)	3,296	(2.4)	4,819	(5.1)
M. hyosynoviae (A+)	978	9,062 (9.4)	5,452 (5.6)	971	(1.0)	177	(0.2)	122	(0.1)
M. hominis (A+)	536	7,353 (13.7)	4,224 (7.7)	354	(0.6)	95	(0.2)	235	(0.4)
M. arginini (A+)	536	7,353 (13.7)	4,224 (7.7)	102	(0.2)	91	(0.2)	66	(0.1)
A. laidlawii (G+)	978	9,062 (9.4)	5,452 (5.6)	2,509	(2.6)	4,560	(4.7)	3,908	(4.0)
S. citri (G+)	536	7,353 (13.7)	4,224 (7.7)	4,668	(8.7)	4,628	(8.6)	1,437	(2.7)
Mycoplasma medium[d]	517	8,301 (16.6)	7,291 (14.8)	464	(0.9)	433	(0.8)	517	(1.0)

[a] G+, Glucose-utilizing species; A+, arginine-utilizing species.

[b] Each value represents the mean of three determinations per mouse, using three individual mice. Several antigens were tested with each lymphocyte suspension.

[c] Mean blastogenic index calculated by averaging the indexes obtained from individual mice. The index is the ratio of counts per minute in lymphocytes exposed to mitogen to the counts per minute in lymphocyte cultures containing no mitogen.

[d] Medium consisted of Hayflick broth, *M. synoviae* broth, and *S. citri* broth. Mean results are shown. The indexes obtained with individual media ranged from 0.8 to 1.1, irrespective of protein concentration.

crease or a decrease in uptake of [^{3}H]thymidine at any of the concentrations tested.

All of the glucose-utilizing mycoplasma species caused enhanced uptakes of [^{3}H]thymidine, although only weak reactions were obtained with *M. felis* (mean blastogenic indexes of 1.9, 1.9, and 2.1 with 1, 5, and 15 μg of protein antigen per ml, respectively). Stronger reactions were obtained with *M. gallisepticum*, *M. synoviae*, and *S. citri* (mean blastogenic indexes of 6.7, 6.7, and 8.6, respectively, with 5 μg of protein per ml). *M. fermentans* K10 consistently exhibited a greater mitogenic potential than did the G strain. Using the macrotest procedure, *M. gallisepticum* and *M. synoviae* exhibited mean indexes of 24.2 and 12.4, respectively, with 5 μg of antigen per ml (data not shown). Although the degree of transformation was largely dose dependent, in some cases, i.e., *M. gallisepticum* and *S. citri*, there was evidence that 15 μg of antigen per ml was inhibitory for lymphocyte activity.

The arginine-utilizing mycoplasmas (*M. hyosynoviae*, *M. hominis*, and *M. arginini*) suppressed uptake of [^{3}H]thymidine by lymphocytes at antigen concentrations higher than 1 μg/ml (Table 1). We and others have shown that *M. arthritidis* exerts a similar effect (3, 9). Since suppression of lymphocyte activity is generally considered to be due to arginine depletion of the medium, experiments were next undertaken to determine whether the observed inhibition could be reversed by heat treatment of the mycoplasma antigens or by supplementation of the medium with excess arginine. The data obtained with *M. arthritidis* 158p10p9 are summarized in Table 2. Whereas arginine supplementation only partially reversed the inhibitory effect of *M. arthritidis* antigen, heat treatment of the antigen resulted in a marked increase in uptake of [^{3}H]thymidine as compared with that seen in lymphocyte cultures alone. Thus, at 5 μg of antigen per ml in regular RPMI medium, untreated antigen exhibited a mean index of 0.4, whereas heated antigen resulted in indexes of 3.9 and 4.0. Similar experiments were conducted with other arginine-utilizing mycoplasmas. With *M. hyosynoviae*, *M. hominis*, and *M. arginini*, untreated antigens at 5 μg/ml suppressed uptake of [^{3}H]thymidine (indexes of 0.5, 0.2, and 0.2 respectively), whereas antigens heated for 2 h at 56°C increased uptake of [^{3}H]thymidine (indexes of 2.5, 3.3, and 4.7, respectively).

Although these results indicate that both arginine- and glucose-utilizing mycoplasmas are mitogenic for normal mouse lymphocytes, additional experiments were undertaken to confirm that the donor mice were not presensitized against the stronger mitogenic species. Thus, mice were bled before lymphocyte harvest, and the individual sera were tested for the presence of complement-fixing and metabolic-inhibiting

TABLE 2. *Effect of heat and arginine supplementation on the mitogenicity of nonviable M. arthritidis 158p10p9 antigen for normal mouse lymphocytes*

Material tested	Mean cpm[a] (and blastogenic index[b]) in lymphocyte cultures exposed to:					
	Control (no mitogen)	Phytohemagglutinin	Lipopolysaccharide	Protein antigen		
				1 μg/ml	5 μg/ml	15 μg/ml
Regular medium						
Control	395	8,699 (21.9)	3,688 (9.5)			
Untreated antigen				323 (0.9)	142 (0.4)	164 (0.5)
Heated, 56°C, 30 min				840 (2.3)	1,257 (3.9)	1,337 (3.8)
Heated, 56°C, 2 h				905 (2.3)	1,529 (4.0)	1,382 (4.1)
Medium + arginine (0.5%)						
Control	174	1,865 (8.8)	1,323 (7.7)			
Untreated antigen				249 (1.5)	194 (1.9)	82 (0.7)
Heated, 56°C, 30 min				366 (2.3)	635 (3.7)	1,035 (5.5)
Heated, 56°C, 2 h				429 (2.2)	811 (4.5)	1,164 (6.3)

[a] Each value represents the mean of three determinations per lymphocyte pool and three separate lymphocyte pools consisting of two spleens per pool.

[b] Mean blastogenic index obtained by averaging the indexes from individual lymphocyte pools.

antibodies against *M. synoviae*, *M. gallisepticum*, *M. fermentans* K10, *S. citri*, and *M. arthritidis*. All of the lymphocyte suspensions exhibited mitogenic reactions when treated with antigens of the latter species, but no evidence of antimycoplasma antibodies were found in the sera of these donor mice.

Mitogenic properties of viable mycoplasmas. In the first paper of this series (2), we reported that viable *M. arthritidis* and *M. hominis* induced normal CBA lymphocytes to become cytotoxic for allogeneic and syngeneic target cells. Since this reaction might be mediated by a lymphokine or by a cytotoxic lymphocyte population produced in association with lymphocyte transformation, the following experiments were conducted to determine whether viable *M. arthritidis* exhibited mitogenic properties for normal lymphocytes.

Lymphocytes from CBA mice were infected with 10^4 to 10^6 colony-forming units of viable organisms per well for 42 h and then treated for 8 h with either regular medium or medium containing gentamicin before an 18-h pulse with [^{3}H]thymidine. Mycoplasmas were recovered in large numbers (not quantitated) from the infected lymphocytes at the time of [^{3}H]thymidine addition and at the time of harvest. In contrast, infected lymphocytes treated with gentamicin were free of cultivable organisms at both times. The mitogenic potential of viable *M. arthritidis* is recorded in Table 3. In the case of *M. arthritidis* 14124p10 iv, all doses of organisms resulted in an enhanced uptake of [^{3}H]thymidine (mean indexes ranging from 7.5 to 12.3) with no differences apparent between lymphocyte cultures receiving gentamicin and those that did not. Similar results were obtained with *M. arthritidis* 158p10p9, although high concentrations of organisms (10^6 colony-forming units per well) suppressed uptake of [^{3}H]thymidine even in the presence of gentamicin.

To further control for the possibility that enhanced uptake of [^{3}H]thymidine was due to the presence of replicating but "noncultivable" organisms, autoradiography was performed on representative lymphocyte cultures from the previous experiment. The results are summarized in Table 4. Transformed lymphocytes appeared as cells filled with black grains, whereas the presence of viable organisms was indicated by an enhancement of background grains and by occasional increased grains at lymphocyte surfaces. The results confirmed that viable *M. arthritidis* strains 14124p10 iv and 158p10p9 were, in fact, mitogenic for normal CBA lymphocytes. A close correlation was seen between the numbers of transformed lymphocytes, as detected by autoradiography, and the degree of uptake of [^{3}H]thymidine, as measured by scintillation counting (Table 3). Again, high concentrations of *M. arthritidis* 158p10p9 failed to induce lymphocyte transformation.

DISCUSSION

The studies reported in this communication have established that a wide variety of arginine- and glucose-utilizing mycoplasmas, including *A. laidlawii* and *S. citri*, are mitogenic for mouse lymphocytes. With the exception of *M. pulmonis*, none of the species tested has been isolated from normal mice, and no evidence of humoral antibodies against the stronger mitogenic species was found in the sera of donor mice. These observations indicate the absence of a presensitized lymphocyte subpopulation.

TABLE 3. *Mitogenic properties of viable M. arthritidis for normal CBA mouse lymphocytes*

M. arthritidis strain	Gentamicin (μg/well)	Mean cpm (and blastogenic index[a]) in lymphocyte cultures exposed to:					
		Control (no mitogen)	Phytohemagglutinin	Lipopolysaccharide	Mycoplasma		
					5×10^4 CFU[b]/ml	5×10^5 CFU/ml	5×10^6 (CFU/ml)
14124p10 iv[c]	None	608	9733 (17.7)[a]	5623 (10.4)	6725 (12.3)	5780 (9.8)	4265 (7.5)
	100	460	7097 (16.5)	4986 (9.3)	4960 (11.0)	4371 (10.1)	4756 (9.9)
	250	225	5285 (23.6)	2366 (10.7)	2203 (9.8)	2150 (9.5)	2203 (9.9)
158p10p9[d]	None	907	9389 (10.8)	4647 (5.3)	2418 (2.8)	641 (0.8)	90 (0.1)
	100	666	7340 (11.0)	3743 (5.6)	3876 (5.8)	3678 (5.5)	52 (0.1)
	250	448	5541 (12.4)	2668 (5.9)	1923 (4.2)	2582 (5.7)	56 (0.1)

[a] Mean blastogenic index obtained by averaging the indexes with individual lymphocyte pools.
[b] CFU, Colony-forming units.
[c] Mean values from two lymphocyte pools.
[d] Mean values from three lymphocyte pools.

TABLE 4. *Autoradiographic determination of mitogenic effects of viable M. arthritidis for normal CBA lymphocytes*

M. arthritidis strain	Gentamicin (μg/well)	Transformed lymphocytes (%)[a] in lymphocyte cultures exposed to:					
		Control (no mitogen)	Phytohemagglutinin	Lipopolysaccharide	Mycoplasma		
					5×10^4 CFU[b]/ml	5×10^5 CFU/ml	5×10^6 CFU/ml
14124p10 iv[c]	None	1.5	40.5	12.5	28.5	23	21
	100	0.5	33	10	13.5	21.5	15
	250	0	30.5	8.5	17	13.5	12
158p10p9[d]	None	1.6	28	16	11	0	0
	100	0.3	35.7	12.7	20.3	24.3	0
	250	1.0	31	13.3	9.3	16	0

[a] Each recorded result represents the mean of three separate counts per autoradiographic slide, using two slides per lymphocyte suspension and two or three separate lymphocyte suspensions.
[b] CFU, Colony-forming units.
[c] Mean values from two lymphocyte pools.
[d] Mean values from three lymphocyte pools.

Although some species, i.e., *M. synoviae*, *M. gallisepticum*, *M. fermentans* K10, *M. pneumoniae*, and *S. citri*, induced strong mitogenic reactions, another, i.e., *M. felis*, consistently exhibited weak reactions. The strong mitogenic reaction induced by *M. fermentans* K10 is of interest in view of the fact that this organism was originally isolated from a leukemic patient and that it produces a leukemoid disease of mice after intravenous injection (19). The laboratory-maintained G strain of *M. fermentans* appeared to be much less mitogenic.

This study has also resolved some of the paradoxical observations in regard to the interaction of arginine-utilizing mycoplasmas with mammalian lymphocytes. It has been known for some time that the inhibitory effect of these organisms on lymphocyte responsiveness to phytohemagglutinin in vitro is due to exhaustion of the arginine content of media by arginine deiminase activity (4). Similar observations have been made with nonviable disrupted mycoplasmas (21). We have now shown that heat treatment of these disrupted mycoplasma preparations abrogates their inhibitory effect toward mouse lymphocytes, thereby allowing a heat-stable mitogenic factor to be expressed. This latter observation has now been confirmed with human and rabbit lymphocytes (unpublished results). Additional studies with both quantitative uptake of [^{3}H]thymidine and autoradiographic techniques demonstrated that viable *M. arthritidis* strains 14124p10 iv and 158p10p9 were also mitogenic for mouse lymphocytes. However, the number of organisms used in these studies was found to be critical, since, in some cases, i.e., with strain 158p10p9, the highest inoculum (10^6 colony-forming units per well) caused an inhibition of lymphocyte metabolism. Callewaert et al. (7) and Barile and Leventhal (4) also reported inhibition of lymphocyte functions in the presence of viable arginine-utilizing mycoplasmas. These observations might best be explained by excessive replication of the organisms, thus leading to arginine depletion of the medium or other toxic effects before the [^{3}H]-thymidine pulse. In this regard, it is of particular relevance that in the second paper of this series

(3), we showed that *M. arthritidis* 158p10p9 did, in fact, replicate in the presence of lymphocytes and that it depleted arginine from the medium to a much greater extent than did strain 14124p10 iv.

The nature of the mitogenic component present in mycoplasmas is not known. Biberfeld and Gronowicz (6) showed that the mitogenic property of *M. pneumoniae* was heat stable. In contrast, Ginsburg and Nicolet (14) found that heat treatment abolished the transforming activity of viable *M. pulmonis*. Additional studies in our laboratories (unpublished results) have shown that heat treatment at 56°C for 2 h or at 121°C for 15 min does not affect the mitogenic properties of nonviable antigens of *M. synoviae*, *M. fermentans*, *M. gallisepticum*, *M. pulmonis*, *M. arthritidis*, or *S. citri*. These observations, together with those of Biberfeld and Gronowicz (6), who demonstrated that *M. pneumoniae* was a B-cell mitogen, might suggest that endotoxin could be the active component. Smith et al. (22) have detected lipopolysaccharides in aqueous phenol extracts of *Thermoplasma*, *Acholeplasma*, and *Anaeroplasma* species. However, no activity was detected in *S. citri*, *M. gallisepticum*, *M. arthritidis*, or *M. hyorhinis* species that we have shown to be mitogenic. Furthermore, collaborative studies with G. Nelson and S. Marcus (Department of Microbiology, University of Utah), using the *Limulus* lysate gelation test, have shown that, although a few of the antigens used in the present study contained low levels of endotoxin (< 0.1 μg per 5 μg of antigen protein), there was no correlation between degree of blastogenesis and presence or absence of endotoxin (unpublished results).

The finding that arginine- as well as glucose-utilizing mycoplasmas are mitogenic for normal lymphocytes provides a potential explanation for the mycoplasma-induced lymphocytotoxicity reactions reported in the first paper of this series (2). Thus, numerous studies have shown that nonspecific mitogens induce normal lymphocytes to produce a variety of lymphokines and to develop cytotoxic properties. Since cytotoxic effector activity precedes blastogenesis and is not dependent on deoxyribonucleic acid replication (17, 18), its development might be expected to be less susceptible to arginine depletion produced as a result of replicating organisms. It should be noted that previously we demonstrated a lymphocytotoxicity reaction to mouse fibroblasts as early as 18 h postinfection with a wide range of mycoplasma inocula (3).

The role played by lymphocyte activation in the inflammatory lesions caused by mycoplasmas remains to be defined. However, lymphokines are receiving increasing attention as mediators of inflammation, and lymphokine-containing preparations have recently been shown to induce synovial hyperplasia after intra-articular injection into rabbits (25). The apparent ability of mycoplasmas to activate normal lymphocytes clearly provides the potential for an early inflammatory response before the development of a sensitized lymphocyte population. The reaction of persisting mycoplasma antigen (9, 12) with sensitized lymphocytes would ensure continued inflammation.

In conclusion, we have now shown that mycoplasmas induce lymphocytes to produce interferon (11), to become cytotoxic for target cells (2, 3), and to undergo nonspecific transformation. The relationship between these phenomena and the lymphocyte subpopulations responsible are now under study.

ACKNOWLEDGMENTS

We thank L. Srisukonth, P. Thorpe, and P. Caskey for skilled technical assistance. We are indebted to G. Nelson and S. Marcus (Department of Microbiology, University of Utah College of Medicine) for performing the *Limulus* lysate gelation tests for the quantitation of lipopolysaccharides.

This investigation was supported by Public Health Service (PHS) grant AM02255 from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMD) and by a grant from the Kroc Foundation. K. E. Aldridge was supported by PHS fellowship 5 F22 AM02361 from the NIAMD.

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