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Mitogenicity of *Mycoplasma fermentans* for Human Lymphocytes

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The in vitro stimulation response of human lymphocytes to *Mycoplasma fermentans* was examined. *M. fermentans* stimulated DNA synthesis in blood lymphocytes from all of 20 healthy subjects examined. Only one of these subjects had complement-fixing antibodies to *M. fermentans*. Lymphocytes from 21 of 22 adenoids and from 1 spleen were also stimulated to DNA synthesis by *M. fermentans*. The organism induced DNA synthesis in both B and T lymphocytes from adenoids and spleen and preferentially in T lymphocytes from blood. *M. fermentans* was shown to activate adenoid lymphocytes to non-antigen-specific antibody secretion demonstrable by a hemolytic plaque assay. It is concluded that *M. fermentans* can have a mitogenic effect on both B and T lymphocytes.

We have previously shown that *Mycoplasma pneumoniae*, an important pathogen of the human respiratory tract (16), can induce nonspecific, polyclonal antibody secretion in vitro in murine (5) and human lymphocytes (3). *M. pneumoniae* stimulated DNA synthesis in mouse spleen B cells (5) but not in human B lymphocytes (3). Human T cells, however, were stimulated by *M. pneumoniae* (3). In the present work we have examined the in vitro effect on lymphocytes of another human mycoplasma, *Mycoplasma fermentans*, and compared it with that of *M. pneumoniae*. No shared antigens between *M. fermentans* and *M. pneumoniae* have been demonstrated (22). *M. fermentans*, which has not been proven to be pathogenic, can be isolated from the urogenital tract in about 1% of healthy individuals (19) and may occasionally be present in arthritic joints (19). The results of the present study show that *M. fermentans* has a mitogenic effect on human lymphocytes, stimulating both B and T cells from adenoids and spleen and, preferentially, T cells from blood.

MATERIALS AND METHODS

Cell preparations. Adenoids were obtained from children undergoing adenoidectomy. A human spleen was obtained from a cadaveric kidney donor. The tissue was cut and homogenized through an iron net in RPMI 1640 medium (Biocult Laboratories, Paisley, Scotland). The cell suspension was passed through sterile gauze and then centrifuged in a Ficoll-Isopaque (Ficoll-Paque, Uppsala, Sweden) gradient (6). The spleen cells were also treated with carbonyl iron to remove phagocytic cells (see below). Blood lymphocytes from healthy adults were obtained by two alternative procedures. (i) Lymphocytes were purified from defibrinated blood by gelatin sedimentation (9). Phagocytic cells were removed with a magnet after treatment of the cell suspension with carbonyl iron (100 mg for 20 min), and erythrocytes were lysed by hypotonic treatment with 0.83% NH_4Cl solution. The cell population obtained usually consisted of more than 97% pure lymphocytes. (ii) Lymphocytes were purified from heparinized blood by centrifugation in a Ficoll-Isopaque gradient (6). This cell population usually contained 6 to 12% polymorphonuclear cells. In some instances this cell population was further purified by treatment with carbonyl iron as described above.

Culture conditions and assay for DNA synthesis. Cultures for determination of DNA synthesis were set up in triplicate or duplicate in tubes containing 10^6 lymphocytes, if not otherwise stated, in 1 ml of RPMI 1640 medium supplemented with a 10% AB serum pool (heated at 56°C for 30 min), L-glutamine penicillin, and streptomycin, and sometimes also 1% Trypticase soy broth (BBL). After 2 or 4 days of incubation, 0.1 μCi of [^{14}C]thymidine (The Radiochemical Centre, Amersham, England) was added to each tube. After further incubation overnight, the cells were harvested on membrane filters (Millipore Corp.). Radioactivity was measured in a liquid scintillation counter and expressed in counts per minute per culture. The mean for triplicate or duplicate tubes was calculated. A stimulation test was considered to be positive if the antigen induced a 2.5-fold or greater increase in thymidine uptake above the background value.

Cultures for determination of antibody synthesis were performed as described by Fauci and Pratt (11). Duplicate cultures of adenoid cells were set up in multiwell (1.5-cm-diameter wells) plastic plates (Linbro Scientific Inc.); 5 × 10^6 lymphocytes in 2 ml of RPMI 1640 medium supplemented as described above were placed in each well and incubated for 5 days. The AB serum pool used in these cultures and in parallel cultures for the determination of DNA synthesis had

been adsorbed three times with sheep erythrocytes (SRBC) (11).

Antigens and mitogens. Cultures were stimulated with *M. fermentans* antigen usually at a final dilution of 1/100, *M. pneumoniae* antigen usually at a final dilution of 1/100, or pokeweed mitogen (Grand Island Biological Co.) at a final dilution of 1/100 to 1/1,000. Dilutions of complete mycoplasma broth medium (see below) were added to control cultures. The *M. fermentans* antigen was prepared from organisms of strain PG18 (kindly obtained from E. A. Freundt, Aarhus, Denmark) grown in complete mycoplasma broth medium (7) composed of 7 parts mycoplasma broth (Difco), 2 parts unheated horse serum, 1 part 25% yeast extract, 1% glucose, 0.002% phenol red, 0.05 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; Calbiochem), and 1,000 U of penicillin per ml. The broth culture was centrifuged at 15,000 × *g* for 30 min, washed three times with phosphate-buffered saline, and finally resuspended in phosphate-buffered saline at a 100-fold concentration. The *M. pneumoniae* antigen was prepared as described previously (4) from organisms of strain FH (originally obtained from R. M. Chanock, Bethesda, Md.) grown in broth on a glass surface. Both antigens were ultrasonically treated and stored in small samples at −70°C. The antigens were heated at 56°C for 30 min before use. Various batches of antigen were used. The protein concentrations of undiluted antigen were between 3.5 and 4.5 mg/ml for *M. fermentans* antigens and between 1.2 and 1.6 mg/ml for *M. pneumoniae* antigens.

Assay for antibody-secreting cells. Cells were harvested, washed, counted, and assayed for direct plaque-forming cells (PFC) against SRBC by a modification of the Jerne-Nordin hemolysis-in-gel method, using an ultrathin-layer gel technique as described by Fauci and Pratt (11). PFC were counted after 3 h of incubation. The results are expressed as PFC per 10^6 viable lymphocytes.

Separation of B and T lymphocytes. B- and T-cell-enriched fractions were prepared by SRBC rosette formation followed by gradient centrifugation (20), as described in detail previously (3). The cells recovered from the sediment will be called T cells and those recovered from the fluid interphase will be called B cells, although these fractions were not completely purified. The frequency of T cells in the B- and T-cell-enriched preparations was determined by the SRBC (E) rosette test (17).

Antibody tests. Sera were examined for antibodies to *M. fermentans* and *M. pneumoniae* by the complement fixation (CF) test, as described previously (2). The *M. fermentans* antigen used in the CF test was prepared in the same way as the antigen used in the stimulation tests. The optimal antigen concentration for the CF test was determined by a checkerboard titration against a rabbit anti-*M. fermentans* serum (Microbiological Associates) kindly obtained from E. A. Freundt.

RESULTS

Stimulation of DNA synthesis. Figure 1 shows dose response curves to *M. fermentans* and *M. pneumoniae* antigens of blood lymphocytes from each of two healthy subjects. There was a significant stimulation response even to antigen diluted 10^{-5}.

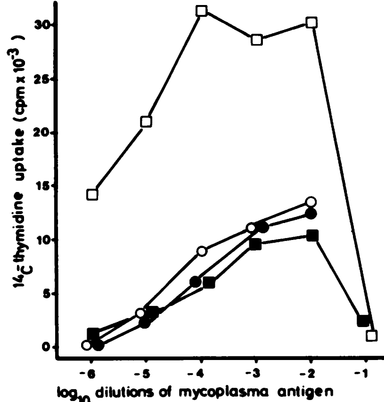

FIG. 1. *Dose response curves of lymphocytes from two healthy subjects (H.L. and A.P.) cultured in the presence of M. fermentans or M. pneumoniae antigen. Symbols:* (●) *response of H.L. lymphocytes to M. fermentans;* (○) *response of H.L. lymphocytes to M. pneumoniae;* (■) *response of A.P. lymphocytes to M. fermentans;* (□) *response of A.P. lymphocytes to M. pneumoniae.*

Examination of the stimulation response after 2, 3, 4, and 5 days of culture showed that the [^{14}C]thymidine uptake was highest after 5 days (data not shown).

The stimulation response of blood lymphocytes to *M. fermentans* and *M. pneumoniae*, respectively, was examined in 20 unselected healthy adults. The results obtained in 15 of these subjects are shown in Table 1; results from the other 5 subjects are shown in Table 2. Lymphocytes from all 20 donors responded to *M. fermentans* as well as to *M. pneumoniae* by increased DNA synthesis. Sera from 15 of 18 donors tested had CF antibodies to *M. pneumoniae*, but only 1 had CF antibodies to *M. fermentans*. In 9 subjects the stimulation response to *M. fermentans* was of the same magnitude as the response to *M. pneumoniae*, whereas in the other 11 subjects the response was significantly lower to *M. fermentans* than to *M. pneumoniae* (Tables 1 and 2). The variation in the degree of lymphocytic responsiveness to *M. fermentans* between the donors might partly be due to technical differences in the stimulation test. Lymphocyte preparations which had been treated with carbonyl iron to remove phagocytic cells usually gave a stronger stimulation response to mycoplasma than lymphocyte preparations which had not been treated with iron. Lymphocytes from three donors showed a slight stimulation response to the complete myco-

TABLE 1. *Maximal lymphocyte stimulation response to M. fermentans or M. pneumoniae antigen (diluted 1/100 or 1/1,000) in 15 healthy subjects*[a]

Subject	DNA synthesis (cpm)			CF antibody titer against:	
	No additive	*M. fermentans*	*M. pneumoniae*	*M. fermentans*	*M. pneumoniae*
A.A.	345[b]	16,053[b]	15,070[b]	<4	16
B.A.	724[b]	15,958[b]	19,663[b]	<4	4
H.L.	155[b]	13,032[b]	12,593[b]	<4	128
E.B.	328[b]	11,148[b]	11,818[b]	<4	16
I.G.	350[b]	11,891[b]	15,773[b]	<4	16
P.K.	304[b]	18,851[b]	35,006[b]	<4	64
G.G.	198[b]	18,040[b]	31,246[b]	<4	8
G.P.	562[b]	17,254[b]	25,951[b]	<4	<4
L.A.	258[b]	14,503[b]	23,188[b]	<4	4
K.E.	183[b]	13,523[b]	25,651[b]	<4	16
K.H.	275	10,850	35,144	<4	<4
A.P.	639[b]	10,424[b]	31,640[b]	<4	16
G.B.	712	8,213	17,714	<4	8
S.F.	174	7,368	18,879	ND[c]	ND
M.O.	336	4,788	9,837	<4	<4

[a] Lymphocytes from these donors were also cultured in the presence of complete mycoplasma broth medium. Lymphocytes from three donors showed a slight response to mycoplasma medium diluted 1/100 (A.P., 2,449 cpm; G.B., 1,961 cpm; S.F., 947 cpm).

[b] Lymphocyte preparations treated with carbonyl iron to remove phagocytic cells.

[c] ND, Not determined.

plasma broth medium at a 1/100 dilution but not at higher dilutions (see Table 1).

B- and T-cell-enriched fractions of blood lymphocytes from five donors were also examined (Table 2). T cells and nonfractionated cells from all donors were stimulated to DNA synthesis by both *M. fermentans* and *M. pneumoniae*. B cells from two subjects only (L.B. and L.Å., Table 2) showed a weak response to *M. fermentans*.

Stimulation tests were performed with adenoid lymphocytes from 22 children. All adenoids except one showed lymphocyte responsiveness to *M. fermentans*, whereas only eight of these adenoids responded to *M. pneumoniae* (see examples in Table 4). *M. fermentans* stimulated both B- and T-cell-enriched preparations from all of nine adenoids examined, as shown in Table 3 (see also Table 4, adenoid 5). It is possible, though, that part of the stimulation response of some adenoid T-cell fractions was due to B cells, since the adenoid T-cell preparations sometimes were rather incompletely purified. *M. pneumoniae* stimulated T cells from four of nine adenoids and B cells from two adenoids.

B and T cells from one human spleen were available for study. *M. fermentans* stimulated both B (3-day culture) and T spleen cells, whereas *M. pneumoniae* stimulated only T cells (Table 3).

Activation of antibody-forming cells. Lymphocytes from 11 adenoids were examined by the hemolytic plaque assay for mycoplasma-induced, non-antigen-specific antibody production. In all of these experiments *M. fermentans* as well as *M. pneumoniae* was shown to activate production of increased numbers of PFC to SRBC (Table 4); *M. fermentans* induced a stronger PFC response than did *M. pneumoniae* ($P < 0.05$). *M. fermentans* also stimulated lymphocytes from 10 of these adenoids to increased DNA synthesis, whereas lymphocytes from only 4 adenoids showed a DNA-synthetic response to *M. pneumoniae* (Table 4).

Lymphocytes cultured in the presence of dilutions of the complete broth medium used for cultivation of *M. fermentans* did not show any increase of PFC above the background.

To exclude that the PFC-activating effect of *M. fermentans* and *M. pneumoniae* was due to contamination with bacterial lipopolysaccharide, the mycoplasma antigens were tested for the presence of endotoxin by the limulus assay (10) (kindly done by Lars Falksveden, National Bacteriological Laboratory). *M. fermentans* and *M. pneumoniae* antigens diluted 1/100 in RPMI medium (as used in the lymphocyte cultures) were negative by the limulus assay. The assay detected as little as 0.5 ng of endotoxin.

DISCUSSION

The finding that *M. fermentans* stimulated DNA synthesis in blood, adenoid, or spleen lymphocytes from virtually all individuals examined (cells from one adenoid did not respond) indicates that the organism is mitogenic for human lymphocytes. In adenoid and spleen cultures, both B and T cells showed a proliferative response to *M. fermentans*, whereas in blood lym-

TABLE 2. *Stimulation response to M. fermentans or M. pneumoniae (diluted 1/100) of B- and T-cell-enriched fractions of human blood lymphocytes from five healthy donors*

Donor	CF antibody titer against:		No. of cells per culture	Stimulus	DNA synthesis (cpm)						% E rosettes	
					3-day culture			5-day culture				
	M. fermentans	*M. pneumoniae*			Nonfractionated cells	B cells	T cells	Nonfractionated cells	B cells	T cells	B cells	T cells
L.B.	<4	16	6 × 10^5	No additive	1,141 ± 48	3,360 ± 100	187 ± 21	3,489 ± 201	2,293 ± 49	386 ± 91	6	93
				M. fermentans	9,210 ± 181	8,254 ± 51	1,906 ± 260	19,873 ± 707	9,943 ± 275	6,609 ± 167		
				M. pneumoniae	4,502 ± 91	4,738 ± 156	1,320 ± 115	14,535 ± 1211	3,054 ± 194	12,057 ± 870		
L.Å.	4	16	7 × 10^5	No additive	226 ± 22	1,323 ± 25	217 ± 14	525 ± 38	3,483 ± 452	656 ± 190	9	92
				M. fermentans	2,702 ± 362	3,697 ± 41	1,381 ± 62	23,973 ± 1003	3,789 ± 214	15,424 ± 1556		
				M. pneumoniae	2,692 ± 203	1,441 ± 71	3,871 ± 135	27,750 ± 1140	2,898 ± 2898	25,257 ± 2040		
B.P.	<4	8	8 × 10^5	No additive	580 ± 13	3,123 ± 31	1,009 ± 33	3,647 ± 287	2,672 ± 255	6,016 ± 774	5	95
				M. fermentans	5,659 ± 104	6,156 ± 37	3,582 ± 314	22,115 ± 310	4,972 ± 22	23,201 ± 1125		
				M. pneumoniae	4,471 ± 96	3,147 ± 1	5,941 ± 258	23,990 ± 248	2,283 ± 77	22,790 ± 1618		
K.K.	<4	4	8 × 10^5	No additive	206 ± 7	1,517 ± 27	180 ± 7	261 ± 4	1,671 ± 116	247 ± 45	6	75
				M. fermentans	1,087 ± 94	2,636 ± 79	542 ± 113	4,999 ± 599	1,397 ± 147	1,655 ± 160		
				M. pneumoniae	1,320 ± 108	1,649 ± 34	1,533 ± 24	9,698 ± 411	1,291 ± 69	11,278 ± 572		
S.S.	ND	ND	5 × 10^5	No additive	150 ± 31	1,677 ± 31	183 ± 24	283 ± 5	5,412 ± 436	290 ± 2	ND[a]	94
				M. fermentans	755 ± 461	2,318 ± 50	450 ± 64	6,351 ± 302	2,332 ± 39	1,932 ± 659		
				M. pneumoniae	605 ± 15	ND	320 ± 27	8,261 ± 567	ND	1,787 ± 34		

[a] ND, Not determined.

TABLE 3. *Stimulation response of B- and T-cell-enriched fractions of human adenoid or spleen lymphocytes to M. fermentans or M. pneumoniae antigen diluted 1/100*

Source of lymphocytes	Stimulus	DNA synthesis (cpm, means ± standard error)						% E rosettes	
		3-day culture			5-day culture				
		Nonfractionated cells	B cells	T cells	Nonfractionated cells	B cells	T cells	B cells	T cells
Adenoid from a 5-year-old child	No additive	4,399 ± 1333	969 ± 348	3,489 ± 247	2,843 ± 580	922 ± 124	4,151 ± 124	1.5	71
	M. fermentans	9,536 ± 26	13,415 ± 512	13,847 ± 123	29,334 ± 1223	18,896 ± 126	29,028 ± 659		
	M. pneumoniae	4,413 ± 1566	1,107 ± 10	3,669 ± 573	10,607 ± 1152	715 ± 33	12,555 ± 1078		
Adenoid from a 13-year-old girl	No additive	2,890 ± 421	279 ± 36	148 ± 21	2,781 ± 388	317 ± 23	269 ± 61	3	96
	M. fermentans	8,429 ± 333	3,466 ± 218	1,425 ± 117	30,405 ± 636	13,679 ± 1228	5,572 ± 1010		
	M. pneumoniae	3,411 ± 701	727 ± 57	469 + 25	3,420 ± 347	1,650 ± 269	450 ± 65		
Spleen from a cadaveric kidney donor	No additive	503 ± 63	2,213 ± 29	484 ± 138	1,197 ± 512	2,637 ± 404	4,450 ± 388	1	88
	M. fermentans	14,234 ± 281	12,295 ± 661	5,896 ± 1055	17,007 ± 853	6,558 ± 714	36,829 ± 3893		
	M. pneumoniae	6,945 ± 294	3,716 ± 228	3,248 ± 722	29,714 ± 594	5,455 ± 570	38,243 ± 2711		

phocyte cultures T cells responded preferentially. *M. fermentans*, like *M. pneumoniae*, activated adenoid lymphocytes to nonspecific antibody production, but *M. pneumoniae* usually did not stimulate adenoid B lymphocytes to DNA synthesis. It has been shown in the mouse system that various B-cell activators stimulate subpopulations of B lymphocytes at various stages of differentiation, resulting in proliferation, antibody production, or both (14, 15). In mouse spleen cultures, however, both *M. pneumoniae* (5) and *M. fermentans* (G. Biberfeld and G. Möller, unpublished data) stimulate DNA synthesis as well as nonspecific, polyclonal antibody production.

The *M. pneumoniae*-induced proliferation of human T cells has been interpreted as an antigen-specific response (3). It has been shown that subjects with recent *M. pneumoniae* infection show a stronger lymphocyte stimulation response to the organism than healthy controls lacking CF and metabolism-inhibiting antibodies (4, 12, 21). *M. fermentans*, however, appears to have a mitogenic effect on human T cells as judged by the present results. Both *M. fermentans* and *M. pneumoniae* stimulated DNA synthesis in blood lymphocytes from all donors examined, but only one donor had CF antibodies to *M. fermentans* whereas most donors had CF antibodies to *M. pneumoniae*, suggesting that they had experienced an *M. pneumoniae* infection some time in the past. Recently, Kirchner et al. (18) reported that another nonpathogenic mycoplasma, *Acholeplasma laidlawii*, which was shown to be mitogenic for murine B cells, stimulated human blood T cells from healthy donors. Since both *M. fermentans* and *A. laidlawii* appear to be mitogenic for human T cells, it is possible that *M. pneumoniae* also may have a mitogenic effect on human blood T cells in addition to the antigen-specific stimulating effect. *M. pneumoniae* does not seem to have any mitogenic effect on adenoid and tonsil T cells (see reference 3).

It has been reported that serum components from the broth medium used for cultivation of mycoplasma can be concentrated in mycoplasma pellets (23), but it is unlikely that the lymphocyte stimulation response obtained with *M. fermentans* and *M. pneumoniae* antigens was due to reactivity against some component in the medium. In most instances there was no lymphocyte reactivity to broth medium. Lymphocytes from a few donors showed a slight response to medium diluted 1/100, but not to higher dilutions, whereas when dose response curves with mycoplasma antigens were performed, there was a response even to antigen diluted 10^{-5}.

TABLE 4. *PFC response to SRBC and DNA synthetic response of human adenoid lymphocytes cultured in the presence of M. fermentans antigen (final dilution, 1/100) or M. pneumoniae antigen (final dilution, 1/100)*

Adenoid no.	Age of donor (yrs)	PFC per 10^6 viable lymphocytes (mean ± SE[a])			DNA synthesis (cpm, mean ± SE)		
		BG[d]	*M. fermentans*	*M. pneumoniae*	BG[d]	*M. fermentans*	*M. pneumoniae*
1	4	6 ± 3	544 ± 31	447 ± 39	146 ± 13	2,337 ± 159	1,997 ± 841
2	8	12 ± 4	138 ± 7	208 ± 33	515 ± 60	4,917 ± 633	2,329 ± 248
3	4	13 ± 4	156 ± 4	176 ± 42	666 ± 129	5,223 ± 255	1,748 ± 209
4	4	46 ± 10	472 ± 110	156 ± 10	4,838 ± 152	12,725 ± 94	7,910 ± 969
5	14	70 ± 7	1,122 ± 310	620 ± 17	582 ± 51	3,284 ± 98[b]	733 ± 71[b]
					1,637 ± 269	12,865 ± 1,007[c]	28,130 ± 1,772[c]
6	2	17 ± 5	340 ± 44	92 ± 16	1,516 ± 91	2,198 ± 176	971 ± 128
7	4	23 ± 9	105 ± 3	88 ± 21	848 ± 91	2,945 ± 203	371 ± 95
8	4	46 ± 3	830 ± 90	325 ± 6	3,669 ± 507	19,515 ± 1,115	7,235 ± 569
9	12	62 ± 18	473 ± 92	303 ± 104	3,386 ± 297	25,928 ± 1,287	6,904 ± 696
10	4	63 ± 32	361 ± 28	192 ± 20	583 ± 57	2,470 ± 265	828 ± 292
11	5	67 ± 17	888 ± 79	234 ± 56	959 ± 104	2,905 ± 110	829 ± 7
Mean PFC ± SE		38 ± 7	494 ± 332	258 ± 159			

[a] SE, Standard error.
[b] DNA synthetic response of the B-cell-enriched fraction.
[c] DNA synthetic response of the T-cell-enriched fraction.
[d] BG, Background.

Four glucose-metabolizing mycoplasmas, *M. fermentans*, *M. pneumoniae*, *A. laidlawii*, and *M. pulmonis*, have so far been found to be mitogenic for human, mouse, or rat lymphocytes (5, 13, 18). Mitogenicity might be a common property of many mycoplasmas. Arginine-metabolizing mycoplasmas, however, inhibit lymphocyte stimulation (8) by depletion of arginine (1).

We do not know if the mitogenic potential of *M. fermentans* is of any importance in vivo. It is conceivable, though, that when *M. fermentans* occurs in an arthritic joint (19) it may induce mitogenic activation of synovial lymphocytes. Preliminary studies have shown that *M. fermentans* has the ability to stimulate DNA synthesis in synovial fluid lymphocytes (E. Nilsson and G. Biberfeld, unpublished data).

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ADDENDUM

After this manuscript had been submitted for publication, two studies of myoplasma-induced activation of mouse and rat lymphocytes, respectively, were reported. Cole et al. (B. C. Cole, K. E. Aldrich, and J. R. Ward, Infect. Immun. **18**:393–399, 1977) showed that a wide variety of mycoplasma species, including *M. fermentans*, were mitogenic for mouse lymphocytes. Naot et al. (Y. Naot, J. G. Tully, and H. Ginsburg, Infect. Immun. **18**:310–317, 1977) found that murine mycoplasmas but not human mycoplasmas were mitogenic for rat lymphocytes.

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