INFECTION AND IMMUNITY, May 1982, p. 662–666
0019-9567/82/050662-05$02.00/0

Vol. 36, No. 2

Specificity of a Mycoplasma Mitogen for Lymphocytes from Human and Various Animal Hosts

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Received 3 November 1981/Accepted 20 January 1982

Spleen lymphocytes from Lewis and Buffalo rats and peripheral blood lymphocytes from 10 human donors exhibited high levels of transformation when exposed to *Mycoplasma arthritidis* supernatants. In contrast, spleen lymphocytes from rabbits and guinea pigs and peripheral blood lymphocytes from sheep and calves failed to transform when exposed to *M. arthritidis* supernatants. The lymphocytes from all hosts were transformed in the presence of phytohemagglutinin or concanavalin A or both. Serological studies failed to provide evidence that the responding hosts were presensitized against *M. arthritidis* antigens.

We have demonstrated that *Mycoplasma arthritidis*, an agent of acute or chronic arthritis in rats, mice, and rabbits (8, 10, 11), is mitogenic for normal unsensitized mouse lymphocytes (4, 5) and that the latter develop cytolytic properties for syngeneic and allogeneic target cells (1). Recent studies have shown that lymphocyte activation is dependent upon the mouse strain used and that control of both transformation and lymphocytotoxicity reactions resides within the H2 gene complex (6, 7).

Serological and cultural studies have indicated that the lymphocyte transformation observed was not dependent upon a preexisting sensitized lymphocyte subpopulation. Additional work indicated that viable *M. arthritidis* is predominantly active for the T-cell subpopulation and that the active component of the organisms is a nonsedimentable, heat-labile protein which is present in culture supernatants (Cole et al., J. Immunol., in press). The present studies were undertaken to determine whether this mitogen was specific for murine lymphocytes or whether it was also active for lymphocytes from human and various animal sources.

MATERIALS AND METHODS

Cultures of mycoplasmas. *M. arthritidis* 14124P10 (12) was cultured in modified Hayflick medium consisting of PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented to final concentrations of 15% (vol/vol) heat-inactivated horse serum, 5% (vol/vol) fresh yeast extract, 0.5% (wt/vol) L-arginine–hydrochloride, and 1,000 U of penicillin G per ml (3). *M. arthritidis* supernatants were prepared by centrifuging broth cultures at 27,000 × *g* for 20 min and passing the supernatant through a 0.45-μm and four successive 0.22-μm Millex filters (Millipore Corp., Bedford, Mass.). The supernatants were dialyzed for 24 h against phosphate-buffered saline (pH 7.2), refiltered through a 0.22-μm filter, and cultured on mycoplasma and blood agars to ensure absence of contaminating organisms.

Preparation of lymphocyte suspensions. Spleen lymphocyte suspensions from Lewis and Buffalo rats (Microbiological Associates, Bethesda, Md.), Hartley albino guinea pigs, and New Zealand white rabbits were prepared as described previously for mouse lymphocytes (5). Final lymphocyte suspensions were prepared in RPMI 1640 medium containing 5% heat-inactivated human serum for rat and rabbit lymphocytes and 5% (vol/vol) heated guinea pig serum for guinea pig lymphocytes.

Sheep, bovine, and human lymphocytes were prepared from heparinized blood by Ficoll-Hypaque density gradient centrifugation, using modifications (9) of the procedure of Boyum (2). The bovine lymphocytes received an additional treatment with 0.83% (wt/vol) NH_4Cl to lyse contaminating erythrocytes. Final suspensions were prepared in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum (Hi Clone; Sterile Systems, Logan, Utah), for sheep and bovine lymphocytes and 5% (vol/vol) heat-inactivated human serum for human lymphocytes.

Lymphocyte transformation assays. The basic assay was similar to that described previously (6). In brief, 5×10^5 spleen lymphocytes in RPMI 1640 medium supplemented with 2 mM L-glutamine, 200 U of penicillin G per ml, and appropriate serum were added in 0.2-ml volumes to the wells of microtiter plates. To test for T-cell functions, 0.025 ml of a 1:25 or 1:50 dilution of phytohemagglutinin (PHA) (HA15; Burroughs Wellcome Co., Tuckahoe, N.Y.) was added to triplicate wells to give final concentrations of approximately 45 and 22.5 μg/ml, respectively. Concanavalin A (ConA; Sigma Chemical Co., St. Louis, Mo.) was added in 0.025 ml of medium to give a final concentration of 4.5 μg/ml. *M. arthritidis* supernatants were also added to triplicate wells to give final dilutions of 1:25 to 1:3,125. Wells supplemented with medium alone served as controls for spontaneous uptake of radiolabel (^3H control). After 48 h of incubation in 5% CO_2 and air, 0.025 ml of medium containing 0.125 μCi of

[^{3}H]thymidine (6.7 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added, and the lymphocytes were incubated for an additional 24 h before being harvested on a Skatron cell harvester (Flow Laboratories, Inc., Rockville, Md.). The resulting filter disks were transferred to vials containing Biofluor cocktail and counted to 2% accuracy on a Beckman 8100 liquid scintillation counter. The results were given as the mean counts per minute, minus background, of triplicate determinations. Variability between the triplicate determinations was usually below 10% and rarely above 30%. In no instance was the variance sufficient to negate our conclusions concerning positive or negative responses to the mitogen preparations.

Enzyme-linked immunosorbent assay. The assay is a modification of the micro-enzyme-linked immunosorbent assay described by Ruitenberg et al. (15). It is performed essentially as described in the Public Health Service publication "Procedural Guide for ELISA Microtitration Test" (14), except plates were sensitized overnight at 37°C and stored sealed for no longer than 1 week at 4°C before use. Mycoplasmal antigens were prepared from a whole-cell lysate as described by Horowitz and Cassell (13) and used at a concentration of 10 μg/ml (protein). Test sera were prediluted 1:10 before being tested. Peroxidase-conjugated anti-rat and anti-human immunoglobulin G (heavy and light chain-specific) were purchased from Cappel Laboratories, Cochranville, Pa., and used at dilutions of 1:400 and 1:1,600, respectively. Absorbance was read at 492 nm in a Titertek Multiskan plate reader. We originally standardized the assay for use with rabbit sera by titrating 24 normal rabbit sera (prediluted 1:10). We obtained a mean absorbance reading in the first well of the titration of 0.060 ± 0.033 (standard deviation). We therefore considered as the endpoint in each titration the highest dilution of rabbit serum showing an absorbance 0.16 (mean + 3 standard deviations) (Washburn et al., submitted for publication). On testing 11 normal human and 20 normal rat sera prediluted 1:10, we observed mean absorbances of 0.062 ± 0.037 and 0.074 ± 0.035, respectively, indicating that the previously determined endpoint for rabbits may also be applied to rat and human sera.

RESULTS

The mitogenic responses of rat spleen lymphocytes to *M. arthritidis* supernatants are summarized in Table 1. Lymphocytes from both Lewis and Buffalo rats exhibited a marked increase in uptake of [^{3}H]thymidine when exposed to *M. arthritidis* supernatants. Uninoculated mycoplasma broth used at final dilutions of 1:25 to 1:625 failed to elicit enhanced uptake of [^{3}H]thymidine by lymphocytes, irrespective of the source of the latter (data not shown). The maximum response was obtained when a 1:25 dilution of *M. arthritidis* supernatants was used, and the degree of transformation seen was equal or greater than that obtained with PHA.

Although both guinea pig and rabbit spleen cells responded well to the T-cell mitogens PHA and ConA, no increase in uptake of [^{3}H]thymidine was seen when lymphocytes from either of these hosts were exposed to *M. arthritidis* supernatants (Table 2). Previous work by us on cell-mediated immune responses of rabbits experimentally infected with *M. arthritidis* revealed that, although lymphocytes from sensitized animals were transformed in the presence of *M. arthritidis* antigen, lymphocytes from all control (uninjected) animals failed to exhibit an increase in [^{3}H]thymidine uptake in the presence of *M. arthritidis* (Cole and Washburn, unpublished data).

Peripheral blood lymphocytes from sheep and calves were also assayed for responsiveness to *M. arthritidis* (Table 3). Again, although positive responses were obtained with PHA or ConA or both, the lymphocytes were not responsive to the *M. arthritidis* mitogen. The failure of ovine lymphocytes to transform in response to *M. arthritidis* is of interest in view of our earlier observations which indicated that this myco-

TABLE 1. Mitogenic potential of *M. arthritidis* supernatants for Lewis and Buffalo rat spleen lymphocytes

Source of lymphocytes	Uptake of [^{3}H]thymidine (cpm) in response to[a]:					
	^{3}H control (no mitogen)	PHA (1:50)	ConA (4.5 μg/ml)	*M. arthritidis* supernatant at concn of:		
				1:25	1:125	1:625
Lewis rat spleens						
1	1,491	24,058	37,407	26,531	14,313	5,984
2	2,109	14,220	38,629	28,426	14,220	7,460
3	1,377	18,353	36,615	23,382	11,089	4,341
Buffalo rat spleens						
1	1,666	57,899	37,740	39,079	25,029	8,333
2	850	26,819	37,463	30,510	25,058	8,594
3	1,757	29,527	44,164	30,616	25,285	8,379

[a] Numbers represent the mean value of three determinations.

TABLE 2. Mitogenic potential of *M. arthritidis* supernatants for guinea pig and rabbit spleen lymphocytes

Source of lymphocytes	Uptake of [^{3}H]thymidine (cpm) in response to:					
	^{3}H control (no mitogen)	PHA (1:25)	ConA (4.5 μg/ml)	*M. arthritidis* supernatant at concn of:		
				1:25	1:125	1:625
Guinea pig spleens						
1	804	26,012	27,908	259	222	150
2	955	35,069	32,788	460	217	167
3	754	8,319	5,051	130	231	216
Rabbit spleens						
1	1,095	14,642	26,701	588	1,935	1,664
2	1,455	6,228	19,413	78	1,287	1,036
3	2,036	8,978	24,565	67	619	440

plasma species induced interferon in cultured ovine lymphocytes (9).

In the final experiments, we examined the ability of peripheral blood lymphocytes from 10 healthy human donors to undergo transformation in the presence of *M. arthritidis* supernatants (Table 4). Although there was some variation in the responses of lymphocytes from different donors, all exhibited a marked increase in [^{3}H]thymidine uptake in the presence of the mycoplasma preparations. At the highest concentration of *M. arthritidis* supernatant (1:25), indices of transformation were calculated to range from 9.0 to 50.8, as compared with indices of 18.8 to 183.6 when PHA was used. The low responses obtained with lymphocytes from donor 3 were not confirmed in a repeat experiment with this individual. Uninoculated mycoplasma broth did not promote uptake of [^{3}H]thymidine (data not shown).

The possibility was considered that the positive responses of rat and human lymphocytes to *M. arthritidis* supernatants were due to the presence of the presensitized lymphocyte subpopulation in these hosts. To test for evidence of prior infection with *M. arthritidis*, all rat and human sera were examined for antibodies against this organism by enzyme-linked immunosorbent assay. None of the rat or human lymphocyte donors exhibited significant levels of antibody against *M. arthritidis* antigens. The absorbances at 492 nm of the lowest dilution tested (1:20) were all well below the previously determined level of significance of 0.16 (see Materials and Methods). A positive control serum from a rat experimentally infected with *M. arthritidis* exhibited a titer of 1:640 when 14124P10 antigen was used.

DISCUSSION

We have shown that, despite the uniform reactivity of lymphocytes from all of the animal species studied to PHA and ConA, their ability to respond to the *M. arthritidis* mitogen shows a

TABLE 3. Mitogenic potential of *M. arthritidis* supernatants for sheep and bovine peripheral blood lymphocytes

Source of lymphocytes[a] and expt no.	Uptake of [^{3}H]thymidine (cpm) in response to:					
	^{3}H control (no mitogen)	PHA (1:25)	ConA (4.5 μg/ml)	*M. arthritidis* supernatant at concn of:		
				1:25	1:125	1:625
Sheep peripheral blood						
1	104	13,248	22,996	222	41	NT[b]
2	1,558	34,890	27,226	2,400	459	NT
3	967	13,059	17,460	40	41	NT
4	351	9,407	23,978	329	701	NT
Calf peripheral blood						
1	194	15,155	NT	13	46	72
2	152	7,482	NT	83	42	46

[a] Lymphocytes were collected from the same sheep over a 4-month period. Lymphocytes from two calves were used.

[b] NT, Not tested.

pronounced species specificity. Thus, guinea pig, rabbit, sheep, and bovine lymphocytes were identified as nonresponders, whereas rat and human lymphocytes were positive responders. The degree of proliferation seen with *M. arthritidis* supernatants when rat and human lymphocytes were used was comparable with that previously reported for responding mouse lymphocytes (7).

An important issue to be addressed is whether the positive reactions observed are due to the presence of a preexisting, sensitized lymphocyte population. Previous studies in which mice were used failed to detect the presence of *M. arthritidis* by culture or by serological methodology; furthermore, germ-free animals responded similarly to those reared conventionally (5; Cole et al., in press). In addition, lymphocytes from nonresponder mouse strains experimentally infected with *M. arthritidis* failed to exhibit a transformation response in the presence of *M. arthritidis* supernatants (unpublished observations). In the present study, sera from positively responding donor rats and humans were screened by the sensitive enzyme-linked immunosorbent assay and found to be negative for antibodies against *M. arthritidis*. In contrast, a rat experimentally infected with *M. arthritidis* exhibited a high level of antibody. The issue as to whether humans harbor *M. arthritidis* is a complex one and has been recently discussed in detail elsewhere (18). In brief, the few reported isolations of this species from humans have not been confirmed, and it has been suggested that the original isolate (previously classified as *Mycoplasma hominis* type II) was in fact a laboratory contaminant. We can conclude, however, that if *M. arthritidis* is harbored by humans, its occurrence is very rare.

Previous studies by us have established that the *M. arthritidis* mitogen is strongly active toward mice expressing the k and d haplotypes at the H2 gene complex but is nonreactive toward those expressing the b, s, and q haplotypes at H2 (6, 7). These observations allowed us to map the controlling gene to the IE-IC subregions of the H2 gene complex (7). If the mitogenic responses of rat lymphocytes to the mycoplasma mitogen are under a similar controlling mechanism, it may be possible to identify nonresponding strains. Such a search is currently being undertaken. However, since responsiveness of mouse lymphocytes to *M. arthritidis* supernatants is a dominant trait, it may be difficult to identify nonresponding human individuals.

The response of human lymphocytes to the mitogen may be related to the very close structural similarity of human HLA-DR antigens with antigens coded by the murine IE subregion of the H2 gene complex (17). The occurrence of shared Ia specificities between mice and rats has also been reported (16). Studies to determine more precisely the interaction of the mitogen with these antigens are currently in progress. The negative responses of guinea pig, rabbit, bovine, and sheep lymphocytes to *M. arthritidis* supernatants may be due to a lack of cross-reactivity of Ir gene products with those of rodents and humans. It must also be acknowledged that these negative responses may reflect the particular strain used rather than being characteristic of the species. In any event, it is apparent that the mitogenic capability of *M. arthritidis*, unlike that of PHA or ConA, exhibits a marked specificity for lymphocytes from various hosts.

The pathogenic significance of the mitogen remains to be established. It is of interest that both mice and rats are highly susceptible to the systemic administration of *M. arthritidis*. Rabbits also develop arthritis, but only upon intra-

TABLE 4. Mitogenic potential of *M. arthritidis* supernatants for human peripheral blood lymphocytes

Donor no.	Uptake of [^{3}H]thymidine (cpm) in response to:						
	^{3}H control (no mitogen)	PHA (1:25)	ConA (4.5 μg/ml)	*M. arthritidis* supernatant at concn of:			
				1:25	1:125	1:625	1:3,125
1	384	7,246	NT[a]	10,153	9,294	4,948	831
2	553	10,654	NT	11,236	9,451	5,923	1,036
3	157	11,395	NT	1,664	2,504	1,017	253
4	120	11,733	NT	1,804	3,429	1,967	893
5	106	9,118	14,665	5,395	4,780	2,182	628
6	182	11,560	8,492	7,545	5,352	2,907	221
7	140	10,425	8,696	5,417	4,114	2,119	NT
8	72	13,217	13,719	3,507	3,183	2,246	NT
9	482	19,096	17,190	5,021	3,632	2,042	NT
10	169	20,480	17,937	NT	4,988	4,241	NT

[a] NT, Not tested.

articular injection of the organisms, and these animals are nonresponsive to *M. arthritidis* supernatants. However, the mycoplasmas are rapidly eliminated from these animals, and continued disease appears to be due to persisting immune complexes in cartilaginous tissues rather than to a chronic infectious process (19). The significance of our observations on the stimulation of human lymphocytes by the *M. arthritidis* mitogen remains to be determined, and these findings by themselves in no way imply any pathogenic role for this organism in human disease. Studies to search for soluble mitogens in human mycoplasma species are currently under way. Preliminary experiments have failed to detect activity for human lymphocytes in supernatants from *Mycoplasma pneumoniae*, *Mycoplasma fermentans*, or *Mycoplasma hominis*.

ACKNOWLEDGMENTS

We thank Mary Beth Artz for performance of serological assays. The assistance of Donald B. Olson (Division of Artificial Organs, University of Utah College of Medicine) in securing bovine blood is also gratefully acknowledged.

This work was supported by Public Health Service grants AI 12103 from the National Institute of Allergy and Infectious Diseases and AM 02255 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

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