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Immunosuppressive Properties of the *Mycoplasma arthritidis* T-Cell Mitogen In Vivo: Inhibition of Proliferative Responses to T-Cell Mitogens

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We have previously shown that *Mycoplasma arthritidis* produces a soluble T-cell mitogen (MAM) which is active for most mouse strains that express the alpha chain of the I-E molecule (E_α) encoded within the murine major histocompatibility complex. The lymphocytes from mice injected intravenously with the MAM exhibited a marked decrease in their ability to respond in vitro to MAM, to phytohemagglutinin, or to concanavalin A T-cell mitogens. Suppression could only be induced in MAM-responsive mouse strains and was most marked 1 to 4 days postinjection. Splenic and node cells and, to a lesser extent, thymic cells from MAM-injected mice could inhibit the ability of lymphocytes from normal mice to respond to MAM and lectin mitogens. A minimum of 2.5×10^4 viable cells was required for significant transfer of suppression, and no major histocompatibility complex restrictions were seen. Unlike concanavalin A-induced suppressor cells, which consist of a $CD4^-$, $CD8^+$ T-cell subset, suppressor cells induced by MAM were due to a $CD4^+$ $CD8^-$ subset. We hypothesize that MAM may play a role in *M. arthritidis*-mediated disease by both its inflammatory and immunosuppressive properties.

Many of the mycoplasma species appear to have the ability to bypass or suppress host defense mechanisms, thus leading to establishment of chronic disease of the respiratory tract (7), the urogenital tract (34), or the joints (20). *Mycoplasma arthritidis*, an agent of murine arthritis (19), is particularly interesting owing to its failure to induce neutralizing (8, 35) or opsonizing (18) antibodies in rodents and the recurrent and chronic nature of the destructive joint lesions which develop (19, 20). The reason for these defective immune responses and the mechanisms for chronic recurrent disease remain largely unexplained.

Unlike other mycoplasmas tested to date, *M. arthritidis* secretes a soluble T-cell mitogen (MAM) into culture supernatants (10). This mitogen is unique in a number of ways. First, it is haplotype restricted (10) and must be presented to T cells via the I-E molecule (E_α), which appears to be the receptor site for MAM on accessory cell surfaces (4, 11). Second, T-cell recognition of MAM is not restricted by the major histocompatibility complex (MHC) but does require the presence of I-E in association with MAM (26, 36). Third, MAM–I-E is recognized by T cells via their V_β α/β T-cell receptor products. Specific receptors include the $V_\beta 8$ gene family (12) and the $V_\beta 6$ products (B. C. Cole et al., J. Immunol., in press). Fourth, the mitogen has some unusual physical properties in that it is a very potent substance, being half optimally active at $\leq 10^{-11}$ M, and is a low-molecular-weight, acid-labile protein with an isoelectric point of >9.0 (2).

The role of MAM in *M. arthritidis* disease remains unknown, although two symptoms induced by the organisms, namely, dermal necrosis (14) and systemic toxicity (17), occur with much greater severity in mouse strains whose lymphocytes are responsive to MAM. The present report describes studies which were conducted to investigate the effects of MAM in vivo with a view to eventually defining the role of MAM in mycoplasma-induced disease and evaluating the potential role of MAM as a new immunomodulatory agent.

(Preliminary results have been reported previously [B. C. Cole and D. J. Wells, Abstr. Ann. Meet. Am. Soc. Microbiol. 1989, G24, p. 152].)

MATERIALS AND METHODS

MAM. The MAM used in these studies consisted of cell-free dialyzed supernatant (MAS) of a culture of *M. arthritidis* or of a partially purified preparation (MAS-P) made in a manner similar to the procedure described previously (2). Mitogen for injection was diluted with phosphate-buffered saline (PBS) just before use.

Mice. Mice (C3H/HeJ, C3H.SW, BALB/c, C57BL/10, and CBA) were purchased from Jackson Laboratory (Bar Harbor, Maine) and used at 8 to 12 weeks of age.

Lymphocyte transformation assay. Spleen, thymus, or lymph nodes were individually minced in RPMI 1640 medium and passed through 80-mesh screens to remove clumps. These suspensions were centrifuged at $200 \times g$ for 5 min, washed with 0.83% (wt/vol) Tris-buffered NH_4Cl (pH 7.2) to lyse erythrocytes, centrifuged, and suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 U of penicillin G per ml, 50 μg of streptomycin sulfate per ml, and 5% (vol/vol) heat-inactivated human serum.

Viable cells were quantitated by trypan blue exclusion, and cell suspensions were diluted to the appropriate concentration. Cell suspensions were added to the wells of microdilution plates which were then supplemented in triplicate with 0.05 ml of RPMI 1640 as a control for spontaneous uptake of radiolabeled thymidine or with 0.05 ml of an inducer. Inducers for T-cell activity consisted of wells containing 5 and 1.5 μg of phytohemagglutinin (PHA) (HA16; Burroughs Wellcome Co., Tuckahoe, N.Y.) per ml, 5 and 1.5 μg of concanavalin A (ConA) (Sigma Chemical Co., St.

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Louis, Mo.) per ml, and 1:10,000 and 1:50,000 dilutions of MAS-P.

After 48 h of incubation at 37°C in 5% CO_2, 0.025 ml of medium containing 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) (2 Ci/mM; Dupont, NEN Research Products, Boston, Mass.) was added and the lymphocytes were incubated for an additional 24 h before being harvested on a cell harvester (Skatron; Flow Laboratories, Inc., McLean, Va.). The resulting filter disks were transferred to minivials (Beckman Instruments, Inc., Fullerton, Calif.) containing Ready-Solve EP liquid scintillation cocktail (Beckman) and counted on a Beckman LS7800 counter. The results were expressed as the mean counts per minute, minus background, of triplicate determinations for which replicates were within a 30% range.

Antibodies. The GK 1.5 (L3T4/CD4), ADH 4.15 (Lyt2.2/CD8), and 30-H12 (Thy1.2) monoclonal antibody (MAb)-producing cell lines were obtained courtesy of Barbara Araneo (Department of Medicine, University of Utah).

Depletion of lymphocyte subsets. A 10^7-cells per ml concentration of splenic lymphocytes was incubated in RPMI 1640 medium at 37°C in the presence of complement alone, with a 1:10 dilution of Cedarlane Low-Tox-M rabbit complement (Accurate Chemical Corp., Westbury, N.Y.), or with complement plus antibody to Thy1.2, CD4, or CD8 markers. After the incubation, the cells were washed three times in medium and each individual cell suspension was resuspended to 2.5×10^5 cells per well based on the cell counts of the complement control.

Flow cytometry. After complement depletion of lymphocyte subsets, portions from each sample were used in flow cytometry analysis for determination of the effectiveness of subset elimination. After quantitation of cells, 2×10^6 cells were washed by centrifugation in 0.2% sodium azide in PBS. The pellets of the individual samples were suspended in 50 μl of goat serum (GIBCO Laboratories, Grand Island, N.Y.) and incubated for 30 min at room temperature to minimize nonspecific binding of the goat anti-mouse or goat anti-rat immunoglobulin G conjugated with fluorescein isothiocyanate. Cells were then washed three times, and the pellets were suspended in 200 μl of a 1:50 dilution of culture supernatant containing MAb to CD4 or Thy1.2 or ascites fluid containing MAb to CD8. After incubation at room temperature for 30 min followed by three washes, a 1:50 dilution of goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate (Sigma) was added to the MAb-treated cells and allowed to incubate for 30 min at 4°C. The samples were then washed three times and suspended in 1 ml of 1% paraformaldehyde–PBS solution and stored at 4°C. Immunofluorescence was measured with an Ortho Cytofluorograf IIs cell sorter with a 448-nm argon laser for fluorescence excitation. To exclude cell debris, cells were selected and gated according to their forward versus right-angle light scatter characteristics.

RESULTS

Inhibition of mitogen responses by MAM. In preliminary experiments, we found that the intravenous (i.v.) injection of mice with undiluted MAS-P resulted in a toxic syndrome similar to that observed after injection of large doses of viable *M. arthritidis* (17). The visible symptoms, consisting of ruffled fur and lethargy, disappeared after 3 to 4 days. All MAM-responsive mice receiving systemic administration of MAS-P exhibited pronounced splenomegaly and lymphadenopathy even at lower nontoxic doses. MAM-nonresponsive mice failed to develop clinical toxic symptoms or splenomegaly. We next investigated the effect of MAM on lymphocyte responses to lectin mitogens.

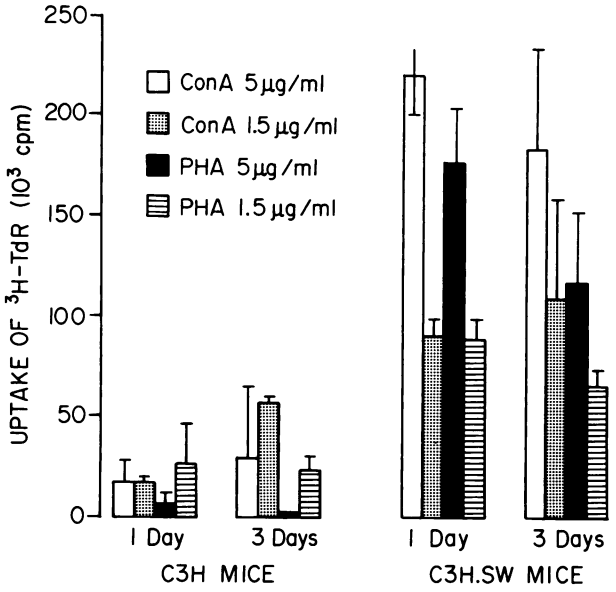

FIG. 1. Inhibition of lymphocyte responses to lectin mitogens mediated by MAM. C3H and C3H.SW mice were injected i.v. with MAS-P, and after 1 and 3 days, splenic lymphocytes were collected and tested for proliferative responses to ConA and PHA.

MAM-responsive C3H (*H-2^k*) mice and congenic, MAM-nonresponsive C3H.SW mice (*H-2^b*) were injected i.v. with MAS-P. Mice injected with PBS served as controls. At 1 and 3 days later, spleens were removed and cell suspensions were tested for their ability to respond to the lectin mitogens ConA and PHA (Fig. 1). Despite the pronounced splenomegaly, spontaneous uptake of [^{3}H]TdR by splenocytes from MAS-P-injected mice was not substantially enhanced compared with uptake by splenocytes from PBS-injected mice (also, see data in Tables 1 to 4). Lymphocytes from C3H mice exhibited impaired responses to ConA and PHA compared with those from C3H.SW mice. For example, at day 1, C3H cell responses to 5 μg of ConA per ml were 92.5% less than the responses of C3H.SW cells. Splenocytes from C3H mice injected with PBS gave similar high responses to mitogens, as did C3H.SW cells (data not shown). An experiment comparing MAM-responsive CBA (*H-2^k*) splenocytes with the MAM-nonresponsive C57BL/10 (*H-2^b*) splenocytes gave similar results, in that an injection of MAS-P inhibited the ability of CBA (*H-2^k*) splenocytes to respond to lectin mitogens, whereas it had no significant effect on the responses of C57BL/10 (*H-2^b*) splenocytes to lectin mitogens (data not shown). The results suggest that MAM-induced in vivo suppression of lymphocyte responses to T-cell mitogens requires recipient expression of an MAM-responsive haplotype (*H-2^k*). Administration of MAS-P by intraperitoneal injection was somewhat less effective in inducing suppression than that seen for i.v. injection; subcutaneous administration failed to induce any suppression.

The splenocytes from BALB/c mice injected with MAM also exhibited a marked suppression of their in vitro responses to crude (MAS) and to partially purified (MAS-P) mitogen (Fig. 2). In this experiment, MAM-responsive BALB/c (*H-2^d*) mice were injected i.v. with various doses of MAS-P or with PBS. The degree of suppression of prolifer-

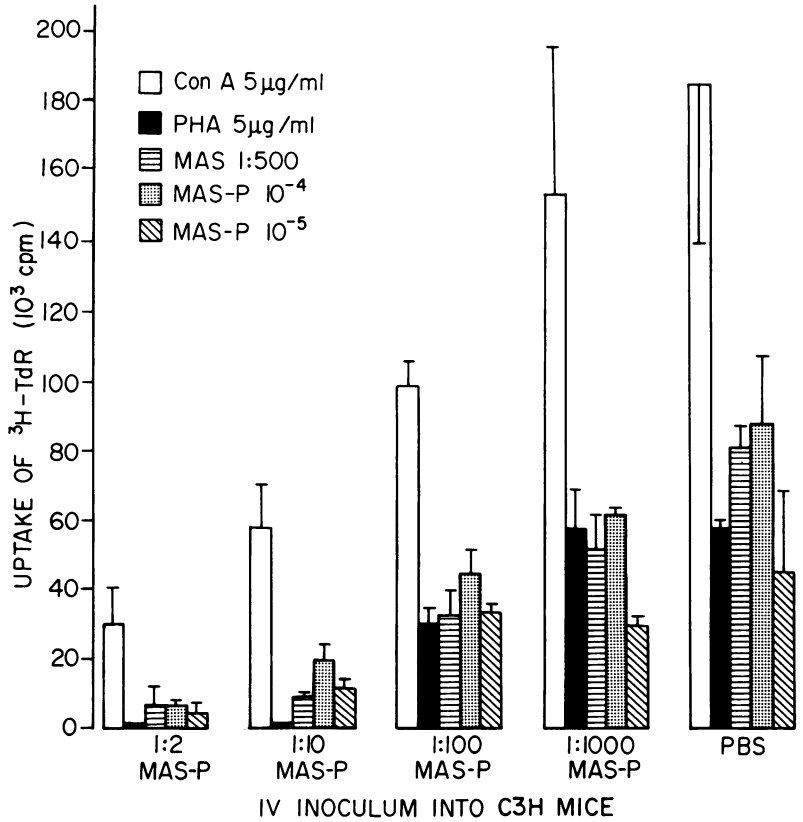

FIG. 2. Inhibition of lymphocyte responses to MAM and lectin preparations induced by a primary i.v. injection of MAS-P. BALB/c mice were injected i.v. with a 1:2 to 1:1,000 dilution of MAS-P or with PBS. After 48 h, splenocytes were tested for responsiveness to ConA, PHA, MAS, and MAS-P. Spontaneous uptake of [^{3}H]TdR by splenocytes from mice injected with MAS-P was 3.9×10^3 cpm for 1:2, 8.1×10^3 cpm for 1:10, 2.8×10^3 cpm for 1:100, and 4.7×10^3H]TdR cpm for 1:1,000. Uptake of [^{3}H]TdR by cells from PBS-treated mice was 2.1×10^3 cpm.

ative responses to the mitogens depended on the dose of MAS-P given. Maximum inhibition was seen with 1:2 to 1:10 dilutions, although significant suppression (approximately 50%) was still seen at a 1:100 dilution. Boiled MAS-P, which lost >90% of its mitogenic activity, failed to induce significant suppression when injected into mice (data not shown). In subsequent experiments, a dose of 0.2 ml of 1:10 or 1:20 MAS-P was used.

We next examined the duration of the lymphocyte hyporeactive state to mitogens after a single i.v. injection of MAS-P. The results obtained with lymphocytes from MAS-P-injected mice were expressed as a percentage of values

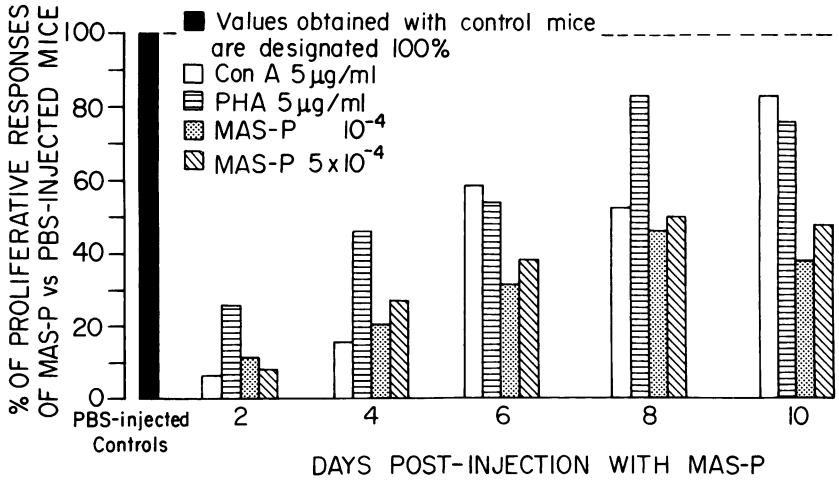

FIG. 3. Duration of suppression induced in vivo by MAM. BALB/c mice were injected i.v. with MAS-P or with PBS, and mice from each group were sacrificed at indicated times and splenocytes were tested for responsiveness to MAS-P and lectin mitogens. Spontaneous uptake of [^{3}H]TdR by splenocytes from MAS-P- and PBS-injected mice was similar.

TABLE 1. Transfer of suppression to normal lymphocytes

Treatment of mouse spleen donors[a]	Uptake of [^{3}H]TdR (10^3 cpm)[b] in response to:			
	^{3}H control (no mitogen)	MAS (10^{-3})	MAS-P (10^{-4})	MAS-P (5×10^{-4})
Expt 1 (BALB/c)				
PBS	4.2 ± 0.2	NT[d]	109.5 ± 5.3	96.7 ± 15.2
MAS-P	2.2 ± 0.6	NT	34.0 ± 5.5 (68.9[c])	12.2 ± 2.5 (87.4[c])
PBS + MAS-P	2.4 ± 0.6	NT	18.7 ± 0.8 (90.2[c])	12.1 ± 1.1 (86.8[c])
Expt 2 (C3H)				
PBS	0.6 ± 0.2	110.5 ± 5.5	NT	116.7 ± 2.7
MAS-P	0.7 ± 0.1	6.6 ± 1.1 (94.0[c])	NT	11.3 ± 1.3 (85.0[c])
PBS + MAS-P	3.1 ± 1.1	13.2 ± 1.9 (84.1[c])	NT	17.5 ± 0.6 (83.9[c])

[a] Mice were injected with 0.2 ml of PBS or 1:10 MAS-P. Animals were sacrificed 48 h later, and splenic cells (2×10^5 per well) of each alone or combined together were tested for proliferative responses to MAS and MAS-P.
[b] Mean counts per minute of three determinations ± standard deviation.
[c] Percent reduction in counts per minute compared with response for same number of cells from PBS-injected mice.
[d] NT, Not tested.

obtained with lymphocytes from PBS-injected control mice (Fig. 3). Maximum inhibition for all mitogens was seen 2 days postinjection, after which there was a gradual restoration of responses. By 8 days, the responses to PHA were essentially normal, and by 10 days, responses to ConA were near normal. The proliferative responses to MAS-P were still only 40 to 45% of control values at 10 days (Fig. 3).

In vitro transfer of suppression. The results observed above might be due to a direct toxic effect of MAM on immune cell functions or to the active induction of MAM-mediated suppressor cells. To distinguish these possibilities, we examined the ability of MAM-induced hyporeactive cells to transfer suppression to normal cells.

BALB/c or C3H mice were injected i.v. with MAS-P or PBS, and after 48 h, splenocytes were collected and tested alone or in combination for their responses to MAS and MAS-P (Table 1). In both experiments, lymphocytes from MAS-P-injected animals not only exhibited depressed responses to MAS or MAS-P but also actively suppressed the mitogenic responses of lymphocytes from PBS-injected controls by 68.9 to 94%. In a separate experiment, lymphocytes from MAM-nonresonder C3H.SW mice previously injected with MAS-P failed to suppress the response of normal C3H splenocytes to MAS-P (data not shown). Subsequently (Fig. 4), we demonstrated that at least 2.5×10^4 suppressor splenocytes or node cells were required for significant inhibition of MAS-P responses of normal splenocytes. Thymocytes collected from MAS-P-injected mice were largely ineffective in suppressing splenocyte responses subsequent to exposure to MAS-P. Similar results were obtained when we examined the effect of MAS-P-induced splenic or nodal suppressor cells on the responsiveness of normal cells to ConA (Fig. 5). However, in this case, thymocytes at 2.5×10^5 per well resulted in a 70% suppression of ConA-induced activation of normal cells, but 2.5×10^4 thymocytes were ineffective.

Although both MAM and antigen-induced activations of T cells are absolutely dependent on accessory cells, only antigen-induced activation is MHC restricted. The next experiments were designed to determine whether MAM-

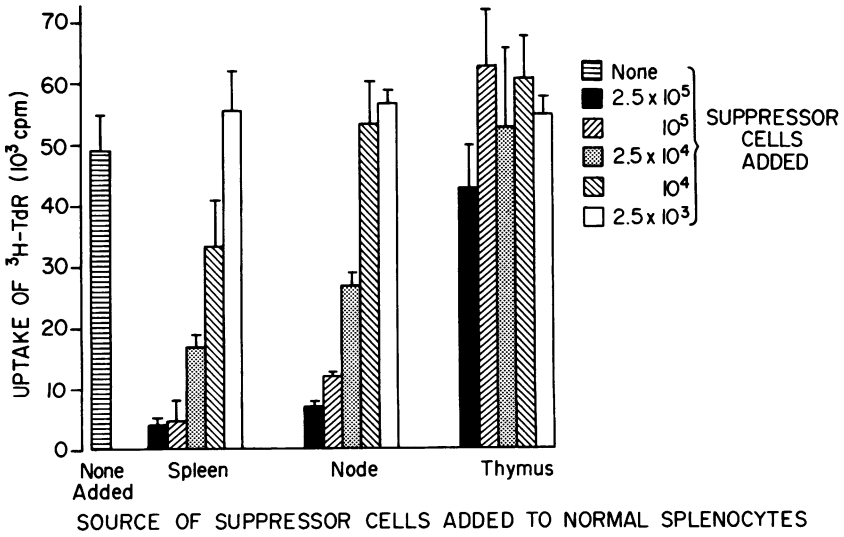

FIG. 4. Comparative efficacy of MAM-induced spleen, node, and thymic suppressor cells. BALB/c mice were injected i.v. with MAS-P, and 48 h later, splenic, nodal, and thymic lymphocytes were collected and assayed at various concentrations for their ability to suppress the proliferative response of normal splenocytes to MAS-P. Spontaneous uptake of [^{3}H]TdR by splenocytes from MAS-P- and PBS-injected mice was similar.

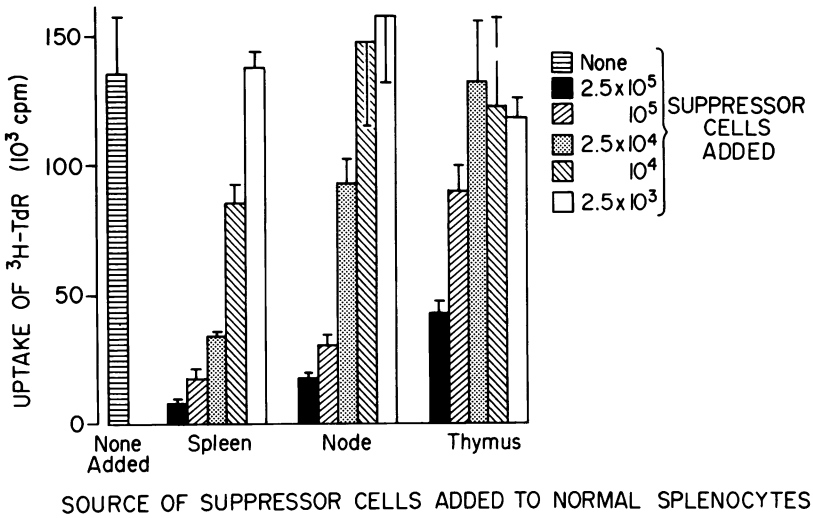

FIG. 5. Inhibition of ConA responses by various concentrations of splenic nodal and thymic lymphocytes taken from mice injected with MAS-P.

induced suppression was MHC restricted. C3H (*H-2*^{k}) mice were injected with MAS-P or with PBS. After 48 h, splenocytes were collected and tested for responsiveness to PHA and ConA and for their ability to suppress the responses of C3H (*H-2*^{k}), C3H.SW (*H-2*^{b}), and BALB/c (*H-2*^{d}) splenocytes to PHA and ConA (Table 2). As seen before, splenocytes from MAS-P-injected C3H mice responded poorly to lectin mitogens in comparison with those from mice injected with PBS. Splenocytes from MAS-P-injected C3H mice but not those from PBS-injected mice suppressed the responses of normal C3H, C3H.SW, and BALB/c splenocytes to PHA and ConA. We conclude that suppressor splenocytes induced by MAM are not MHC restricted (Table 2).

We next tested whether viable functional cells were required to transfer suppression. BALB/c mice were injected with MAS-P or with PBS, and splenocytes were harvested 48 h later. These splenocytes were untreated or treated with 2,500 rads γ of irradiation or fixed with 1% paraformaldehyde for 5 min prior to addition to splenocytes from normal untreated mice. Splenocytes from MAS-P-injected mice exhibited impaired responses to T-cell mitogens and significantly suppressed the ability of normal splenocytes to respond to mitogens (Table 3). Treatment of MAS-P-induced suppressor cells with γ irradiation or with paraformaldehyde abolished their ability to transfer suppression.

We next examined whether MAM-induced suppressor cells acted on the early or late events required for DNA synthesis mediated by mitogens. Suppressor cells collected from BALB/c mice injected with MAS-P were added to normal BALB/c splenocytes at 0.5, 6, 12, 18, and 24 h after the addition of ConA and MAS-P mitogens. For each time period, a separate donor mouse was used which had been injected with MAS-P 48 h prior to sacrifice. The results were expressed as the percentage of [^{3}H]TdR uptake of normal cells in the presence of suppressor cells compared with [^{3}H]TdR uptake by normal cells alone (Fig. 6). MAS-P-induced suppressor cells exhibited marked inhibition of mitogen responses when added up to 12 h after the addition of mitogens. MAS-P responses were still inhibited by 50% when suppressor cells were added 24 h after exposure of normal cells to mitogen. ConA responses could not be inhibited 24 h after the interaction of normal cells and mitogen. In another experiment, suppressor cells were added 0.5 h before mitogens, at the same time as mitogens, and at 0.5, 2, and 6 h after mitogen addition. Marked inhibition of responses to ConA and MAS-P was seen at all

TABLE 2. Suppression of lectin responses by MAM

Treatment of splenocytes used[a]	Uptake of [^{3}H]TdR (10^3 cpm) in response to:				
	^{3}H control (no mitogen)	PHA (μg/ml)		ConA (μg/ml)	
		5	1.5	5	1.5
PBS	0.6 ± 0.3	85.1 ± 18.0	76.5 ± 13.8	165.2 ± 9.1	143.2 ± 22.0
MAS-P	1.1 ± 0.3	0.4 ± 0.3	2.5 ± 0.5	5.7 ± 1.7	13.3 ± 0.8
PBS[b] +					
Untreated C3H	7.7 ± 0.5	106.1 ± 12.7	46.7 ± 8.3	181.1 ± 70.1	125.1 ± 43.9
Untreated C3H.SW	4.7 ± 1.2	140.8 ± 1.0	52.6 ± 5.0	216.7 ± 31.8	237.8 ± 23.7
Untreated BALB/c	5.6 ± 1.2	90.9 ± 15.4	36.1 ± 3.1	189.8 ± 27.6	126.6 ± 15.0
MAS-P[b] +					
Untreated C3H	2.8 ± 0.6	1.8 ± 0.2	9.6 ± 6.6	7.5 ± 2.0	12.2 ± 1.5
Untreated C3H.SW	10.8 ± 1.5	0.3 ± 0.3	2.9 ± 0.8	18.7 ± 1.5	40.7 ± 1.5
Untreated BALB/c	8.8 ± 0.9	0.4 ± 0.1	6.8 ± 0.9	25.6 ± 8.3	31.4 ± 3.0

[a] C3H mice were injected i.v. with 0.2 ml of PBS or MAS-P, and after 48 h, splenocytes (2.5×10^5 cells per well) were tested for proliferative responses to lectin mitogens.

[b] Splenocytes (2.5×10^5 per well) from mice injected with PBS or MAS-P were added to splenocytes (2.5×10^5 per well) from normal untreated mice.

TABLE 3. Viable cells are required for suppression

Treatment of mouse spleen donors and cells	Uptake of [^{3}H]TdR (10^3 cpm) in response to:		
	^{3}H control (no mitogen)	MAS-P (10^{-4})	ConA (5 μg/ml)
Untreated[a]	0.4 ± 0.1	88.8 ± 15.7	195.1 ± 5.1
PBS[a]	0.4 ± 0.1	81.5 ± 10.7	151.7 ± 18.6
MAS-P[a]	0.6 ± 0.1	13.8 ± 1.5	28.0 ± 2.9
Untreated cells[b] +			
PBS	1.2 ± 0.1	64.5 ± 15.1	281.1 ± 7.7
MAS-P	1.3 ± 0.4	15.1 ± 2.0	57.2 ± 12.4
γ-irradiated MAS-P	2.8 ± 0.4	80.2 ± 1.7	246.9 ± 26.5
Fixed MAS-P	0.5 ± 0.2	93.3 ± 7.9	174.0 ± 1.2

[a] BALB/c splenocytes (2.5×10^5 per well) from uninjected mice or from mice injected i.v. with PBS or MAS-P.

[b] Splenocytes (2.5×10^5) from normal mice were added to 2.5×10^5 viable splenocytes from PBS- or MAS-P-injected mice or to splenocytes from MAS-P-injected mice which had been γ irradiated (2,500 rads) or fixed with 1% paraformaldehyde for 5 min.

time intervals of suppressor cell addition (data not shown). We conclude from these two experiments that the suppressor cells induced by MAM act on the late events which occur during induction of proliferative responses to mitogens.

Lymphocyte subset responsible for suppression. Suppressor cells induced by antigens or ConA are typically T cells which are CD4 (L3T4) negative but CD8 (Lyt2) positive (T_S). We next investigated the effect of depletion of T cells with anti-Thy1 plus complement or of T_H cells with anti-CD4 plus complement or of T_S cells with anti-CD8 plus complement on suppressor activity of splenocytes collected from BALB/c mice injected 48 h previously with MAS-P.

In Table 4, we demonstrate that the suppressor activity of splenocytes from mice injected with MAS-P is eliminated by treatment with anti-Thyl and complement and also with anti-CD4 and complement, suggesting that suppression is mediated by means of T cells, more specifically T_H cells. Anti-Thy1 antibody treatment of suppressor cells restored activity as before, but treatment with anti-CD8 antibody had no effect on the suppressor activity (Table 5). Flow cytometry established that treatment of splenic cells with anti-Thyl and anti-CD8 antibodies plus complement virtually eliminated cells expressing these markers. The results confirm that suppression is mediated by a CD4-positive, CD8-negative helper T-cell subpopulation.

DISCUSSION

Mycoplasmal infections are frequently characterized by chronic recurrent disease owing to the inability of the host to eliminate the causative organisms. The invasiveness of mycoplasmas may be due to failure of the host to recognize these organisms as foreign, to the localization of the organisms at sites protected from immunological responses, or to the active suppression of host defenses by the organisms.

The data presented here demonstrate that the systemic administration to mice of a T-cell mitogen (MAM) derived from *M. arthritidis* results in a profound inhibitory effect on lymphocyte proliferative responses to the plant lectin mitogens ConA and PHA, as well as to MAM. Lymphocyte suppression was limited to mouse strains whose lymphocytes are responsive to MAM in vitro. Thus, congenic MAM-nonresponder mouse strains exhibited normal lymphocyte responses to lectin mitogens after administration of MAM. The duration of the suppressive effect is relatively long lived since in vitro lymphocyte responses to MAM were still 50% suppressed 10 days after administration of MAM. However, responses to PHA and ConA were restored to normal 8 and 10 days postinjection, respectively. Although high doses of MAM resulted in toxicity to the mice, immunosuppression was still seen at apparently nontoxic doses of MAM.

Despite the marked splenomegaly seen in MAM-injected mice, there was no marked difference in most experiments between the levels of spontaneous [^{3}H]TdR uptake by splenocytes from MAM-injected mice compared with those from PBS-injected mice. This could be due in part to the presence of large numbers of blast cells. In addition, the suppressor

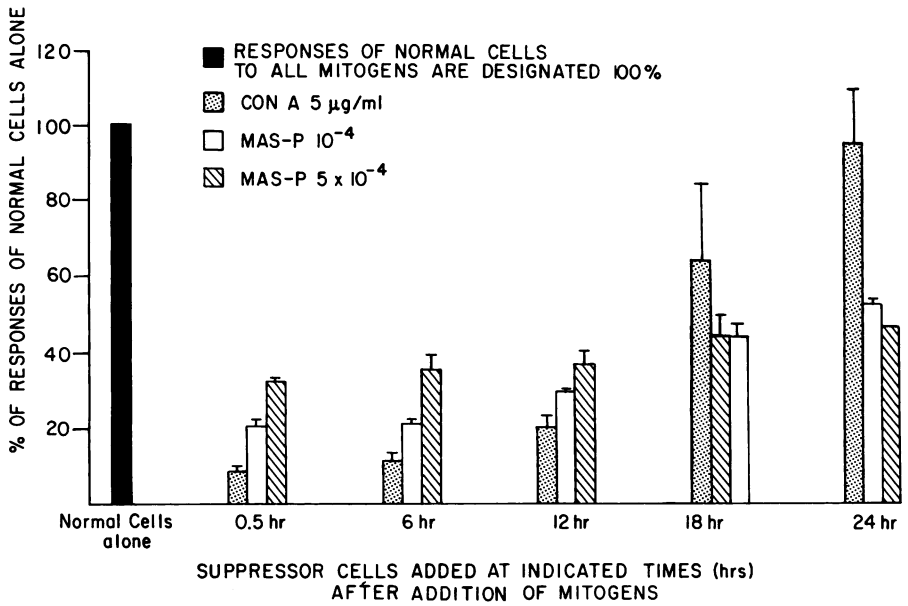

FIG. 6. Kinetics of MAM-induced suppression. MAS-P-induced suppressor cells were added to normal lymphocytes 0.5 to 24 h after the addition of MAS-P. Proliferative responses were recorded 72 h after the addition of MAS-P.

TABLE 4. Lymphocyte subpopulation responsible for suppressor activity

Treatment of mouse spleen donors and cells	Uptake of [^{3}H]TdR (10^3 cpm) in response to:				
	^{3}H control (no mitogen)	ConA (μg/ml)		MAS-P	
		5	1.5	10^{-4}	5×10^{-4}
Untreated[a]	0.7 ± 0.2	134.3 ± 11.0	99.3 ± 7.0	51.0 ± 18.7	51.8 ± 5.3
PBS + complement + untreated[b]	1.6 ± 0.2	83.4 ± 4.7	40.5 ± 3.0	32.0 ± 4.6	44.2 ± 3.0
MAS-P + complement + untreated[b]	1.8 ± 0.6	14.8 ± 0.4	18.3 ± 0.6	10.0 ± 1.6	17.5 ± 5.4
MAS-P + complement + Thy1[c] + untreated	2.2 ± 0.2	104.0 ± 11.5	58.7 ± 6.4	34.0 ± 8.8	41.7 ± 4.6
MAS-P + complement + CD4[c] + untreated	1.8 ± 0.2	112.3 ± 14.3	97.8 ± 13.2	38.4 ± 9.0	35.7 ± 9.2

[a] Freshly prepared BALB/c splenocytes from untreated animals.
[b] BALB/c mice were injected i.v. with PBS or MAS-P, and 48 h later, spleen cell suspensions were prepared, treated with complement, and added (2.5×10^5) to microdilution wells containing 2.5×10^5 untreated cells.
[c] Splenocytes from MAS-P-injected mice were treated with complement and anti-Thy1.2 antibody or complement and anti-CD4 antibody. The treated splenocytes were then added to normal untreated BALB/c splenocytes (2.5×10^5 per well).

circuit would be well established after the 1 to 3-day in vivo interaction of MAM with lymphocytes followed by the 3-day in vitro incubation to detect uptake of [^{3}H]TdR.

The suppressive effects induced by MAM appear not to be related to the in vitro inhibition of lymphocyte proliferation mediated by live mycoplasmas or mycoplasmal extracts (3, 21). In these cases, the inhibition is due to arginine depletion from culture media by the action of arginine deiminase, which is produced by many mycoplasmas including *M. arthritidis* (3, 21, 31, 32).

The mechanism of the suppressive effect of in vivo MAM administration remains to be defined. First, it is unlikely that inhibition of lymphocyte responses is due to a direct toxic effect of MAM on host cell functions since suppressor effector cells were detected which could transfer hyporeactivity to normal untreated cells. Furthermore, only viable suppressor cells exhibited this property. The marked lymphadenopathy and splenomegaly seen suggested the occurrence of an active phase of lymphocyte proliferation. Such a marked activation could lead to liberation of lymphotoxins or suppressor factors which might be toxic for or regulate normal T-cell functions.

It is not unlikely that the suppressor cells generated in MAM-injected mice have cytotoxic potential. Earlier studies in our laboratory demonstrated that *M. arthritidis* organisms (1) and MAS (B. C. Cole and G. J. Sullivan, unpublished observations) could induce mouse splenocytes to become cytotoxic for syngeneic or allogeneic fibroblast target cells. We showed later that the cytotoxicity reaction was haplotype specific as for lymphocyte proliferation (9). During this work, we also found that supernatants from splenocytes activated by *M. arthritidis* exhibited weak cytotoxic effects, suggesting the presence of a lymphotoxin.

Suppression induced by MAM might also be mediated by a soluble suppressor factor. Antigen-specific suppressor factors have been well documented (23, 33), as have the nonspecific factors generated by ConA-induced suppressor cells (29, 30) termed SIRS (soluble immune response suppressor). Although we showed here that MAM-induced suppressor cells are nonspecific in that they are not MHC restricted, we failed to consistently demonstrate soluble suppressor factors in the serum of mice injected with MAM or in supernatants of lymphocyte cultures activated by MAM.

ConA mediates suppression by activation of $CD4^-$ $CD8^+$ T cells. In contrast, MAM-induced suppression appears to be mediated by a $CD4^+$ $CD8^-$ T-helper subset since CD4 MAb and complement treatment of suppressor cells abolished their ability to inhibit mitogen responses of normal lymphocytes. CD8 MAb and complement was ineffective in abrogating suppressor activity. Our observation that thymocytes failed to develop significant suppressor activity is consistent with the suppressor cell population being a mature $CD4^+$ cell. Recent reviews by Bottomly (5) and Mosman and Coffman (28) described growing evidence for the existence of two subsets of $CD4^+$ $CD8^-$ murine T-helper cells, termed T_H1 and T_H2 cells. T_H1 or "inflammatory cells" produce interleukin-2, gamma interferon, and lymphotoxin upon stimulation. These cells also participate in delayed-type hypersensitivity responses, exhibit cytotoxic properties, and can also mediate suppression of antibody responses. In contrast, T_H2 cells fail to produce interleukin-2, gamma interferon, or lymphotoxin but characteristically produce interleukin-4 and supply B-cell help. The properties of MAM-responsive cells most closely resemble T_H1 cells in their production of interleukin-2, gamma interferon, and lymphotoxin and cytotoxic activities (1, 16, 25, 36). In addition, we have recently demonstrated that MAM induces a transient inflammatory response when administered intraarticularly into rats (6).

TABLE 5. Suppressor activity is not due to $CD8^+$ cells

Treatment of mouse spleen donors and cells[a]	Uptake of [^{3}H]TdR (10^3 cpm) in response to:				
	^{3}H control (no mitogen)	ConA (μg/ml)		MAS-P	
		5	1.5	10^{-4}	5×10^{-4}
Untreated	0.1 ± 0.2	86.9 ± 8.7	40.9 ± 1.2	29.3 ± 3.2	15.3 ± 2.3
PBS + complement + untreated	0.4 ± 0.1	167 ± 5.5	104.0 ± 50.2	42.6 ± 5.9	ND[b]
MAS-P + complement + untreated	0.4 ± 0.1	25.5 ± 1.2	14.8 ± 1.7	7.0 ± 0.9	6.9 ± 0.8
MAS-P + complement + Thy1 + untreated	0.6 ± 0.1	135.9 ± 8.1	74.1 ± 8.7	37.6 ± 3.8	24.1 ± 5.1
MAS-P + complement + CD8 + untreated	0.8 ± 0.2	31.4 ± 0.9	18.0 ± 5.0	10.7 ± 2.2	8.8 ± 1.9

[a] For description of treatment, see Table 4 footnotes.
[b] ND, Not determined.

Mycoplasmas are very successful host-specific parasites, and the observations presented here may well relate to the ability of *M. arthritidis* to invade its host. Previous studies have established that the antibody response of rodents to *M. arthritidis* is abnormal in that neutralizing, growth-inhibiting, metabolic-inhibiting, and opsonizing antibodies either fail to develop or are produced only in low titer (20). These antibodies are produced by rodents in response to other nonmurine mycoplasma species, and *M. arthritidis* is capable of evoking these antibodies when injected into guinea pigs and rabbits (20). There is also evidence that infection with *M. arthritidis* can directly suppress the induction of humoral antibodies to other antigens (24). We have also documented that *M. arthritidis* can inhibit the in vivo interferon response of mice to viral challenge (13). Studies are now being conducted to determine whether MAM is responsible for these suppressive effects on immune functions.

MAM may also contribute to the pathogenesis of *M. arthritidis*-mediated disease. We demonstrated that MAM-responsive mouse strains develop a toxic syndrome after injection with *M. arthritidis* which is not seen in congenic MAM-nonresponsive strains (17). Furthermore, *M. arthritidis* organisms are cleared from the peripheral circulation more slowly in MAM-responsive mouse strains than in congenic MAM-nonresponsive strains. Further studies indicated that *M. arthritidis* induced severe necrotic lesions when injected subcutaneously into MAM-responsive mouse strains, yet produced only encapsulated abscesses when administered to congenic MAM-nonresponsive mouse strains (14). These studies suggest that MAM indeed plays a role in the disease pathology induced by *M. arthritidis* and that this process is mediated by rendering the host more susceptible to invasion by the organisms by virtue of the immunosuppressive property of MAM.

It remains to be established whether the suppressive effects of MAM on murine T-cell functions have any clinical application. It should be noted, however, that the human HLA.DR molecule is the equivalent of the murine I-E molecule and that MAM induces proliferation of and interferon induction by human lymphocytes (15, 22, 27).

Susceptibility to human disease has been clearly linked to expression of MHC and non-MHC genes which code for lymphoid cell surface proteins. We propose that *M. arthritidis* infection offers a unique model to study the genetic influence of MHC (E_α) and non-MHC (T-cell receptor) gene products in disease pathogenesis.

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