Mycoplasma hyorhinis Molecules That Induce Tumor Necrosis Factor Alpha Secretion by Human Monocytes

DAVID A. KOSTYAL,^{1*} GARY H. BUTLER,² AND DONALD H. BEEZHOLD¹

Laboratory of Macrophage Biology, Guthrie Foundation for Medical Research, Sayre, Pennsylvania 18840,¹ and
Department of Microbiology, Coriell Institute for Medical Research, Camden, New Jersey 08103²

Received 27 February 1995/Returned for modification 24 April 1995/Accepted 5 July 1995

Mycoplasma hyorhinis has been shown to induce the secretion of tumor necrosis factor alpha (TNF- α) from monocytes. To identify the molecules responsible for this activity, we separated sonicated *M. hyorhinis* lysate material by centrifugation at 100,000 $\times$ g into soluble (S) and particulate (P) fractions. The fractions were assayed for TNF- α -inducing activity by the L929 bioassay. Both the soluble and particulate fractions were able to induce TNF- α in roughly equal amounts. The optimum dose for both fractions was 1 μ g/ml. Proteinase K treatment of either fraction eliminated the activity, suggesting that a protein component is involved in induction. Phase partitioning into Triton X-114 aqueous (A) and detergent (D) phases showed that the soluble fraction was composed of 80% aqueous-phase proteins, while the particulate fraction was >75% detergent-phase proteins. All four fractions (SA, SD, PA, and PD) were able to induce TNF- α release. Treatment with NaIO₄ to remove carbohydrate reduced the inducing activity of the SA phase by 80%, whereas that of the other fractions was unaffected by this treatment. The *M_s* of the inducing activity were determined by the monocyte Western (immunoblot) technique. The SA phase activity was associated with a single periodate-sensitive peak of 69 to 75 kDa. The two detergent phases had similar profiles of inducing activity, containing four peaks of activity. These peaks corresponded to 48 to 52, 43 to 45, 39 to 40, and 31 to 32 kDa. The PA fraction also contained four peaks of activity, 69 to 75, 55 to 57, 48 to 52, and 39 to 40 kDa. Thus, both a protein and glycan moiety from *M. hyorhinis* are capable of inducing TNF- α release from human monocytes.

The *Mollicutes* have a number of effects on immune cells in culture. For instance, some species have been shown to possess B- and T-cell mitogens (10, 12), chemotactic factors (27), and immunosuppressive factors (3, 37) and induce major histocompatibility complex (MHC) class II expression in monocyte-macrophage cell lines (34, 35). One of the most common effects of members of the *Mollicutes* on immune cells is their ability to induce the release of cytokines (for reviews, see references 10, 21, and 28). Previous studies have shown that tumor necrosis factor alpha (TNF- α)-inducing activity is associated with the whole mycoplasma (4, 8, 14, 20) or the membrane fraction (29–31). A set of specific membrane lipoproteins from *Mycoplasma arginini* have been implicated as a source of cytokine induction (15), as well as a low-molecular-weight carbohydrate-containing moiety (41). *Mycoplasma fermentans* inducing activity has been associated with a 48-kDa membrane protein (18) and a lipid containing periodate-sensitive material (22, 23). *Spiroplasma* membranes contain at least two TNF- α -inducing proteins of 68 and 15 kDa (30).

Mycoplasma hyorhinis also has a number of effects on immune cells. For example, it induces cell proliferation and immunoglobulin secretion from B cells (26), suppression of T-cell cytotoxicity (37), and release of the cytokines interleukin-1 (IL-1), IL-6, and TNF- α from monocytes (21). A 37-kDa surface protein (p37) has also been shown to increase the in vitro invasiveness of a tumor cell line (11). Recently, we used the monocyte Western (immunoblot) technique to identify a 48-kDa (p48) protein from the membrane of *M. fermentans* that induces the secretion of TNF- α from human monocytes (18). In this study, we used the monocyte Western blot assay to

characterize the proteins of *M. hyorhinis* that are responsible for the induction of TNF- α release. Activity was found in both the soluble and particulate fractions and in both Triton X-114 phases. The activity associated with the soluble aqueous (SA) phase was sensitive to periodate treatment.

MATERIALS AND METHODS

Cell culture. *M. hyorhinis* BTS-7 (ATCC 27717) was obtained from the American Type Culture Collection, Rockville, Md. Organisms were grown aerobically in PPLO broth (BBL Microbiology Systems) containing 10% horse serum, 4% fresh yeast extract, and 100 U of penicillin G per ml for 4 days at 37°C. Mycoplasmas were pelleted at 12,000 $\times$ g, washed with 250 ml of 0.25 M NaCl–0.02 M Tris (pH 7.2) and then briefly in 30 ml of H₂O, and finally resuspended in 50 ml of H₂O. Cells were sonicated (Branson Sonifier model 250; 3-mm microtip probe) three times with 10-s bursts at 4°C. Following sonication, the lysates were centrifuged at 100,000 $\times$ g for 2 h. Floating lipids were removed, and the supernatant or soluble (S) and pellet or particulate (P) fractions were collected.

L929 cells for the TNF- α bioassay were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 IU of penicillin per ml, and 100 μ g of streptomycin per ml.

Triton X-114 phase separation. The soluble and particulate fractions of mycoplasmas were separated into Triton X-114 aqueous and detergent phases by the partitioning method of Bordier (7). Samples were solubilized in lysis buffer, consisting of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-114 (Sigma, St. Louis, Mo.), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 10,000 $\times$ g for 10 min at 4°C. Triton-insoluble material was not assayed. The supernatant (450 μ l) was layered onto a 300- μ l cushion consisting of 6% sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.06% Triton X-114, incubated for 3 min at 37°C, and then centrifuged at 300 $\times$ g for 5 min. The aqueous phase was removed and adjusted to 0.5% Triton X-114, chilled to 0°C, placed over the original sucrose cushion, and recentrifuged. The final aqueous phase was removed, and both aqueous and detergent fractions were brought to equal volumes in phosphate-buffered saline (PBS). Triton X-114 was removed by precipitating proteins by the addition of 10 volumes of cold acetone. Proteins were recovered by centrifugation at 16,000 $\times$ g for 10 min at 4°C. Protein pellets were dissolved in PBS, and the protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Purification and stimulation of monocytes. Monocytes were isolated from peripheral blood as previously described (17). Briefly, venous blood drawn from healthy volunteers was collected into heparin (10 U/ml), and the peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-

* Corresponding author. Mailing address: Guthrie Foundation for Medical Research, 1 Guthrie Square, Sayre, PA 18840. Phone: (717) 882-4619. Fax: (717) 882-4666.

TABLE 1. TNF induction by *M. hyorhinitis* fractions as a function of protein concentration

Fraction	Protein concn ($\mu\text{g/ml}$)	TNF (U/ml) $\pm$ SEM ($n = 3$)		
		Expt 1	Expt 2	Expt 3
Soluble	0.0	3.8 $\pm$ 2.6	5.2 $\pm$ 3.6	8.8 $\pm$ 5.0
	0.01	2.6 $\pm$ 2.0	4.7 $\pm$ 2.5	7.9 $\pm$ 2.9
	0.1	3.5 $\pm$ 2.2	4.6 $\pm$ 2.3	13.9 $\pm$ 5.0
	1.0	57.2 $\pm$ 18.7	50.1 $\pm$ 15.9	77.1 $\pm$ 25.2
	2.5	112.2 $\pm$ 43.5	39.1 $\pm$ 13.1	81.0 $\pm$ 30.2
	5.0	ND ^a	86.5 $\pm$ 31.0	ND
Particulate	0.01	7.6 $\pm$ 4.7	9.0 $\pm$ 4.7	11.1 $\pm$ 5.2
	0.1	24.0 $\pm$ 6.0	11.7 $\pm$ 4.8	18.8 $\pm$ 6.0
	1.0	45.0 $\pm$ 9.2	79.7 $\pm$ 17.3	57.5 $\pm$ 11.9
	2.5	32.8 $\pm$ 6.4	65.7 $\pm$ 15.2	105.1 $\pm$ 23.2
	5.0	41.6 $\pm$ 8.5	54.6 $\pm$ 13.1	86.7 $\pm$ 17.5

^a ND, not done.

Hypaque (1,000 $\times$ g, 20 min). The PBMC were washed three times with Hanks' balanced salt solution (HBSS) and resuspended in RPMI 1640 containing 10% FCS. The PBMC ($2 \times 10^6/\text{ml}$) were plated in 24-well tissue culture plates (Linbro; Flow Laboratories) and allowed to adhere for 2 h at 37°C. Nonadherent cells were removed by washing three times with HBSS, and the adherent cells were cultured in serum-free or 10% FCS-RPMI. The adherent cells were 93 to 95% monocytes, as determined by Giemsa staining and phagocytosis of latex beads. The monocytes were stimulated with mycoplasma fractions for 18 h, and supernatants were collected, centrifuged (10,000 $\times$ g, 2 min), and assayed for TNF- α activity. Polymyxin B (10 U/ml) was added to all mycoplasma fractions and incubated at room temperature for 30 min before addition to monocyte cultures to neutralize any contaminating endotoxin. TNF- α activity was determined by the L929 assay as previously described (5).

Monocyte Western blot analysis. The monocyte Western blot assay was performed by the described method (1, 39). Samples were mixed with Laemmli reducing sample buffer (19) and heated to 60°C before being applied to a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and electrophoresed at 20 mA until the dye front reached the bottom of the gel. Molecular size markers (Pharmacia, Piscataway, N.J.) were used as standards. The gels were either silver stained (Bio-Rad, Richmond, Calif.) or equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol) for 5 min and transferred to nitrocellulose membranes (0.1 μm ; Schleicher & Schuell, Keene, N.H.) at 150 mA for 3 h (38). Strips (0.1 or 0.5 by 1.0 cm) were cut from the nitrocellulose blot, placed in a tube, and dissolved in 1 ml of dimethyl sulfoxide. Antigen-coated particles were then formed by the dropwise addition of 3 ml of carbonate buffer (pH 9.6), with constant mixing. The precipitate was washed twice with HBSS and resuspended in 1 ml of serum-free RPMI 1640. Fifty microliters of each sample was used to stimulate the monocytes. After harvesting of the supernatants, monocyte viability was determined by trypan blue staining. Monocytes were found to be greater than 95% viable in all samples.

Proteinase K digestion. Twenty-five micrograms of mycoplasma soluble or particulate fraction proteins was adjusted to 50 mM Tris (pH 8.8) and 150 mM NaCl. Samples were then incubated with 25 μg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) per ml at 37°C for 12 h (9). Samples were then mixed with SDS sample buffer, separated by SDS-10% polyacrylamide gel electrophoresis (PAGE), blotted to nitrocellulose, and assayed in the monocyte Western blot assay.

Periodate treatment. Soluble and particulate proteins were concentrated by acetone precipitation, resuspended in 0.01 M NaIO₄ (42) and 100 μM PMSE, and incubated at 37°C for 30 min. Samples were again acetone precipitated, resuspended in PBS, and Triton X-114 partitioned as described above.

RESULTS

TNF- α -inducing activity of soluble and particulate fractions. *M. hyorhinitis* lysate material was fractionated into the soluble or cytoplasmic (100,000 $\times$ g supernatant) and particulate or membrane (100,000 $\times$ g pellet) fractions and assayed for TNF- α induction. The optimum protein concentration necessary to induce TNF- α release was determined. Table 1 shows that concentrations of less than 1 $\mu\text{g}/\text{ml}$ induced little TNF- α release. At 1 $\mu\text{g}/\text{ml}$, significant TNF- α secretion was observed. However, increasing the concentration beyond 1 $\mu\text{g}/\text{ml}$ did not result in a proportionate increase in the amount of TNF- α

released. In some instances, higher protein concentrations actually resulted in a decrease in the amount of TNF- α released.

Protein required for TNF- α induction. The particulate and soluble fractions were treated with proteinase K to determine if a protein moiety was necessary for the induction of TNF- α activity. The monocyte Western blot assay was then used to assay fractions for their ability to induce TNF- α . Figure 1 shows that proteinase K treatment removed essentially all of the TNF- α -inducing activity from both the soluble (Fig. 1A) and particulate (Fig. 1B) fractions, while untreated samples retained significant inducing activity. This activity is spread over a wide range, from 30 to 83 kDa. The gel insets in Fig. 1 show that proteinase K treatment removed essentially all of the protein from the particulate fraction and all but one 120-kDa protein from the soluble fraction.

Effects of periodate treatment on TNF- α induction. To further characterize the cytokine-inducing activity of *M. hyorhinitis*, the soluble and particulate fractions were phase separated with Triton X-114. Insoluble material was pelleted, and the supernatant was partitioned into detergent and aqueous phases. As expected, the soluble fraction consists primarily of aqueous-phase proteins (80%), while the majority of proteins of the

FIG. 1. Monocyte Western blot analysis of *M. hyorhinitis* soluble (A) and particulate (B) fractions in the presence (dashed line) and absence (solid line) of proteinase K. Soluble and particulate fraction material (12.5 μg) was digested overnight with proteinase K. Samples were then separated by SDS-10% PAGE and assayed in the monocyte Western blot. Each value shown represents the mean $\pm$ standard error of the mean (SEM); samples were assayed in triplicate. The sizes of the samples (in kilodaltons) are as follows: 1, >150; 2, 112 to 150; 3, 85 to 112; 4, 64 to 85; 5, 52 to 64; 6, 42 to 52; 7, 35 to 42; 8, 30 to 35; 9, 25 to 30; and 10, <25. (Insets) Silver-stained 10% gel of the representative fractions. Lanes: a, undigested control; b, proteinase K-digested sample. The figure is representative of three experiments.

TABLE 2. Effects of periodate treatment on TNF induction by Triton X-114 phases^a

Sample and expt no.	TNF (U/ml) $\pm$ SEM (<i>n</i> = 3)				% Change ^b		
	Medium control	No treatment		Periodate treatment		Aqueous phase	Detergent phase
		Aqueous phase	Detergent phase	Aqueous phase	Detergent phase		
Soluble							
1	0.73 $\pm$ 0.4	9.6 $\pm$ 3.3	19 $\pm$ 5	1.4 $\pm$ 0.4	21 $\pm$ 5	−85	10
2	0.73 $\pm$ 0.4	13 $\pm$ 3.3	30 $\pm$ 7.6	2.4 $\pm$ 0.7	30 $\pm$ 7	−82	0
3	3.5 $\pm$ 1.4	40 $\pm$ 7.9	95 $\pm$ 21	18 $\pm$ 4.2	68 $\pm$ 14	−55	−30
4	3.5 $\pm$ 1.4	48 $\pm$ 10	99 $\pm$ 25	12 $\pm$ 3.6	79 $\pm$ 16	−75	−20
Particulate							
1	0.73 $\pm$ 0.4	17 $\pm$ 5.3	16 $\pm$ 4	18 $\pm$ 5.3	15 $\pm$ 3.8	6	−7
2	0.73 $\pm$ 0.4	21 $\pm$ 4.8	25 $\pm$ 7.1	19 $\pm$ 4.6	23 $\pm$ 5.2	−10	−8
3	3.5 $\pm$ 1.4	77 $\pm$ 15	76 $\pm$ 15	92 $\pm$ 19	67 $\pm$ 13	19	−12
4	3.5 $\pm$ 1.4	68 $\pm$ 14	110 $\pm$ 22	82 $\pm$ 18	91 $\pm$ 18	20	−18

^a All samples were tested at 1 μ g/ml.^b Percent change = [(treated/untreated) - 1] $\times$ 100.

particulate fractions are found in the detergent phase (74%). Both Triton X-114 phases of the soluble and particulate fractions were assayed for their TNF- α -inducing activity. Table 2 shows that all untreated samples have activity. Taking into account the standard error, the total activity for each fraction is approximately equal to the sum of the other fractions (SA + SD = PA + PD). Mild periodate treatment had little if any effect on three of the four phases, SD, PA, and PD. The change observed was not significant by analysis of variance ($P > 0.1$). One phase however, the SA, was particularly sensitive. The activity of this phase was reduced by 55 to 85% after treatment ($P \ll 0.01$).

Determining the molecular mass of the inducing molecules.

To determine the molecular mass of the TNF- α -inducing molecules, samples were assayed by the monocyte Western blot technique. Blots were cut into 0.5-cm strips before assaying. Figure 2 shows the TNF- α -inducing activity of both periodate-treated and untreated fractions of the SA phase. The data in Fig. 2 demonstrate that the activity of this phase is associated with a periodate-sensitive fraction of 69 to 85 kDa. Further fractionation of this fraction into 1-mm strips showed that the activity was 69 to 75 kDa (data not shown). The other phases were assayed as 1-mm strips (Table 3) and showed no sensitivity to periodate treatment (Fig. 3). The two detergent-phase fractions have similar TNF- α -inducing profiles. Activity is associated with species of 48 to 52, 43 to 46, 39 to 40, and 31 to 33 kDa. There is some additional activity associated with fractions 38 to 40. This material migrated at the dye front, and therefore the sizes of the activating molecules may not be accurate. The PA phase has four peaks of inducing activity, corresponding to 69 to 75, 55 to 57, 48 to 52, and 39 to 40 kDa.

DISCUSSION

M. hyorhinis, like other mycoplasmas, has the ability to induce TNF- α from human monocytes (4, 21). Monocytes stimulated with 1 μ g of *M. hyorhinis* proteins of either cytosolic or particulate proteins per ml released significant amounts of TNF- α . However, increasing the protein concentration above 1 μ g/ml did not induce a proportionate increase in the amount of TNF- α released and, in some cases, resulted in a decrease in activity. This probably reflects the maximal stimulation of monocytes and possibly the presence of an inhibitor in the mycoplasma preparation that exerts its effect upon reaching a certain level. Similar findings have been reported for human

monocytes (13), mouse macrophages (14), and astrocytes (8) stimulated with high doses of mycoplasmal proteins. These results differ from earlier reports that did not find TNF- α -inducing activity associated with the cytosolic fraction of *Mycoplasma capricolum* (29) and *Spiroplasma* sp. strain MQ1 (31). Other reports of TNF- α induction by mycoplasmas used either whole cells or cell lysates and did not specifically look at the contribution by cytosolic proteins (4, 8, 14, 20). Different species of *Mollicutes* have been shown to induce different levels of TNF- α release (14, 30). It is possible that some of the differences seen in the TNF- α -inducing properties of mycoplasma species may be due to soluble inducing molecules.

A protein moiety is important in the induction of TNF- α release, as proteinase K digestion eliminates the TNF- α -inducing activity. These results are consistent with those showing that treatment of *Spiroplasma* membranes with proteinase K or pronase reduced the amount of TNF- α released by bone marrow-derived macrophages by 60 to 80% (30), and that proteinase K removed all TNF- α -inducing activity associated with a *Mycoplasma fermentans* membrane fraction (18). It is possible that the inducer is dependent upon a protein moiety for binding to the nitrocellulose and that treatment with proteinase K

FIG. 2. Effects of periodate treatment on the TNF- α -inducing activity of the SA phase. Proteins from the SA phase were precipitated with acetone and resuspended in either 0.01 M NaIO₄ or 50 mM Tris (pH 7.5), both with 100 μ M PMSF added, and incubated at 37°C for 30 min. Protein (12.5 μ g) was then separated by SDS-10% PAGE and blotted, and 0.5-cm strips were assayed in the monocyte Western blot. Each value shown represents the mean $\pm$ SEM; samples were assayed in triplicate. The sizes of the samples (in kilodaltons) are as follows: 1, >85; 2, 69 to 85; 3, 57 to 69; 4, 47 to 57; 5, 40 to 47; 6, 35 to 40; 7, 31 to 35; and 8, <31. Solid line, untreated sample; dashed line, periodate-treated sample. The figure is representative of three experiments.

TABLE 3. Molecular mass of strips assayed in Fig. 3

Strip no.	Molecular mass (kDa)
1.....	110+
2.....	98-110
3.....	92-98
4.....	89-92
5.....	85-89
6.....	82-85
7.....	78-82
8.....	75-78
9.....	72-75
10.....	69-72
11.....	67-69
12.....	64-67
13.....	61-64
14.....	59-61
15.....	57-59
16.....	55-57
17.....	52-55
18.....	50-52
19.....	48-50
20.....	46-48
21.....	45-46
22.....	43-45
23.....	42-43
24.....	41-42
25.....	40-41
26.....	39-40
27.....	38-39
28.....	37-38
29.....	36-37
30.....	35-36
31.....	34.5-35
32.....	34-34.5
33.....	33-34
34.....	32-33
35.....	31-32
36.....	30.5-31
37.....	30-30.5
38.....	29-30
39.....	28.5-29
40.....	28-28.5

results in the loss of binding and not the destruction of the inducer. *M. hyorhinis* contains a proteinase K-resistant protein (9) that migrates at 120 kDa when the sample is not heated before loading onto the gel. This protein is found exclusively in the soluble fraction and therefore is most likely cytosolic in origin.

Like those of other bacteria, mycoplasma membranes are composed of primarily hydrophobic proteins, and many of these proteins are lipid modified. Mycoplasmas are estimated to have twice as many acylated proteins as other bacteria (24). Bacterial lipoproteins are strong inducers of TNF- α from monocytes and macrophages (16). Thus, the TNF- α activity associated with the detergent phase of the particulate fraction may be due to acylated proteins. *M. hyorhinis* possesses a family of acylated surface proteins known as variant lipoproteins (VLPs) (40). These proteins vary in size because of the integration of a repetitive element into the gene coding for the protein. The sizes of three of the inducing molecules of the PD phase vary by a constant mass. It is possible that the activity that we see associated with the detergent-phase proteins includes these lipoproteins. Further characterization is needed to determine whether the inducing proteins are indeed lipid modified and that they belong to the VLP family. Reports characterizing *M. arginini* proteins that induce cytokine pro-

duction describe a set of five membrane-bound aryl proteins with molecular masses of 60 to 80 kDa, which differ by a constant mass (15, 28).

The masses of the inducing molecules of the SD phase are the same as those of the PD phase and may represent a contamination of the SD with PD proteins. It is possible that sonication produces very small vesicles that are difficult to sediment and may thus contaminate the soluble fraction. Conversely, these proteins may be similar to two recently described *Acholeplasma laidlawii* cytoplasmic proteins which are lipid modified and partition into the Triton X-114 detergent phase (25). The PA contains four inducing molecules, two of which are similar in size to the active peaks in the SD and PD phases, 48 to 52 and 39 to 40 kDa. One peak is shared with the SA phase (69 to 72 kDa), and one is unique, with a size of 55 to 57 kDa. Because of the nature of the monocyte Western blot, it is possible that this peak of activity is an extension of the 48- to 52-kDa peak.

The SA phase contains a single peak of activity associated with a size of 69 to 72 kDa. A similar peak of activity was observed in the PA phase; however, we believe that these two molecules are different. In addition to being sensitive to proteinase K treatment, the SA activity is also sensitive to periodate treatment, while the activity in the PA phase is not. *M. hyorhinis* is not known to contain lipoglycans or glycoproteins

FIG. 3. Determination of sizes of inducing activity in the SD (A), PA (B), and PD (C) phases. Periodate-treated protein (12.5 μ g) from each fraction was separated by SDS-10% PAGE and assayed in the monocyte Western blot assay. The molecular masses of the fractions are given in Table 3. Each value shown represents the mean $\pm$ SEM; samples were assayed in triplicate. The peaks of inducing activity are labeled a through f. Each graph is representative of three separate experiments.

(33), although these results suggest that the TNF- α activity may be due to an uncharacterized glycan moiety. *M. fermentans* contains TNF- α -inducing material with a molecular mass of 14 kDa. This material contains a polyol structure, as it is also sensitive to periodate (22). *M. arginini* also contains a low-molecular-mass molecule of 2 to 4 kDa that is capable of activating murine macrophages to become tumoricidal (41). This partially characterized molecule is thought to contain a carbohydrate moiety as well. The TNF- α -inducing activity associated with *Mycoplasma gallisepticum* has avoided characterization but does not appear to be lipid, protein, sugar, or nucleic acid (36). We do not believe that this activity is due to contaminating lipopolysaccharide because all samples were negative in the *Limulus* test and were treated with polymyxin B before addition to monocyte cultures. In addition, lipopolysaccharide has been shown to partition into the Triton X-114 detergent phase (2).

One inducing fraction found in both the soluble and particulate detergent phases (48 to 52 kDa) of *M. hyorhinae* is similar in size to a 48-kDa protein found in *M. fermentans*. This protein was also found associated with the Triton X-114 detergent phase fraction (18). It is possible that this is a single protein common to both species of mycoplasmas. We did not test molecules with a molecular mass of less than 30 kDa or the floating lipids, and it is possible that additional TNF- α -inducing molecules exist in *M. hyorhinae*. Furthermore, the monocyte Western technique involves SDS-PAGE, and denaturing the proteins may result in the loss of activity of other stimulating molecules. This technique has been used successfully in identifying cytokine-inducing molecules from mycobacteria (1, 39).

While many studies have shown that mycoplasmas can induce TNF- α release, the identification of a specific inducer remains elusive. Identification of the masses of the molecules responsible for TNF- α induction should help in ultimately identifying and characterizing the molecules responsible. *M. hyorhinae* is an important pathogen in swine, in which it retards the growth rate and causes a form of arthritis (32). The retardation of growth may be due to *M. hyorhinae*'s inducing high levels of TNF- α , which is known to cause cachexia or wasting syndrome (6). Thus, by identifying the proteins responsible for the induction of TNF- α release, it is possible to test whether they are indeed responsible for the retardation of growth rate. If this is so, then an ideal vaccine would be one which induced neutralizing antibodies to these proteins.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant CA53482 and funding through the Guthrie Foundation for Education and Research.

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