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Characterization of the Metabolism Inhibition Antigen of *Mycoplasma arthritidis*

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The *Mycoplasma arthritidis* antigen(s) responsible for eliciting metabolism-inhibiting antibodies in rabbits has been partially characterized. Metabolism-inhibiting activity was absorbed from rabbit antisera by intact *M. arthritidis* cells and membranes but much less so by the soluble cytoplasmic fraction, indicating that the antigen is located on the outer membrane surface. It was stable to periodate and lipid extraction but labile to heat and proteolytic enzymes, indicating that it is protein in nature. Finally, it is most likely a tightly bound integral rather than a peripheral membrane protein, since it was not extracted by low-ionic-strength solutions or by the nonionic detergents Triton X-100, Nonidet P-40, and Tween 20. It was solubilized by both the anionic agent sodium deoxycholate and the zwitterionic detergent Zwittergent$_{3\text{-}14}$. Two monoclonal antibodies with metabolism-inhibiting activity were produced. One recognized a 45,000-dalton surface protein; however, the other recognized an antigen which is probably of cytoplasmic origin, indicating that more than one cell component may be involved in the metabolism-inhibiting antibody response.

Metabolism-inhibiting (MI) antibodies are considered protective in some mycoplasmal diseases; however, in the case of *Mycoplasma arthritidis* (8), *Mycoplasma pulmonis*, *Mycoplasma neurolyticum* (9, 10), *Mycoplasma synoviae* (21), *Mycoplasma gallisepticum* (45), and *Mycoplasma hyosynoviae* (50) MI antibodies are either absent or difficult to demonstrate in the natural host. In the case of at least three of these organisms, *M. arthritidis*, *M. pulmonis*, and *M. neurolyticum*, the defect does not appear to reside with the parasite, since guinea pigs and rabbits both produce MI antibodies in high titers against these mycoplasmas (10). This apparent lack of expression by the natural host of antibodies directed against what are generally considered major membrane antigens remains unexplained. Cahill et al. (6) proposed that biological mimicry may prevent rats from recognizing the *M. arthritidis* "MI antigen(s)" as foreign. However, this remains to be confirmed. To study rat responses to the *M. arthritidis* antigens responsible for eliciting MI antibodies, it was first necessary to characterize them in greater detail. We shall use the term MI antigen to refer to the cellular components responsible for eliciting MI antibodies, with the understanding that more than one antigenic determinant may be involved in this reaction.

MATERIALS AND METHODS

Mycoplasmas and membrane preparations. *M. arthritidis* 158p10 (15) and 158p10p9 (11) were grown in a dialysate medium modified from Kenny (26) and Washburn et al. (L. R. Washburn, W. J. Cathey, B. C. Cole, and J. R. Ward, Clin. Exp. Rheumatol., in press) or in a modified Hayflick-type medium (7) containing 70 g of PPLO Broth (Difco Laboratories, Detroit, Mich.) per liter and supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) fresh yeast extract, 0.5% (wt/vol) L-arginine-HCl, and 250 U of penicillin per ml. Membranes were prepared by alkaline lysis of whole cells from 1- or 2-liter 18- to 24-h cultures (13) and washed and separated from unlysed cells by sucrose gradient ultracentrifugation, as described by Razin and Rottem (37). The soluble fraction was dialyzed against 0.01 M phosphate-buffered 0.15 M NaCl (pH 7.4; PBS) and water and lyophilized. Before use, the lyophilized residue was reconstituted with approximately 1 ml of water per liter of original culture medium. Protein determinations were by the method of Lowry et al. (33).

Preparation of rabbit antisera. Two rabbits, designated R2 and L2, were immunized with *M. arthritidis* 158p10. Rabbit R2 received 8×10^8 CFU of viable *M. arthritidis* by intra-articular injection and was killed by exsanguination under pentobarbital anesthesia 8 weeks later. Rabbit L2 received 1×10^9 CFU intra-articularly and at 5 weeks was boosted with 3×10^9 CFU intravenously. L2 was killed by exsanguination 1 week after the booster injection. A third rabbit was immunized against 158p10p9 as done for rabbit L2. All mycoplasmas for rabbit injections were grown in the dialysate medium.

Biochemical and detergent treatments of mycoplasma membranes. One-milligram (protein) quantities of *M. arthritidis* membranes were treated with 0.005 M $NaIO_4$ for 1 h at room temperature or with 0.2% (wt/vol) trypsin, 0.1% (wt/vol) protease (type VI, *Streptomyces griseus*), or 0.1% (wt/vol) chymotrypsin for 1 h at 37°C. These reagents were purchased from Sigma Chemical Co., St. Louis, Mo.; all solutions were made in PBS. The treated membranes were washed twice with PBS to remove the reagents before use in immunoblotting and absorption experiments.

Membranes (5 mg of protein) were extracted with cold butanol as described by Rodwell et al. (38). The butanol (lipid-enriched) phase was dried under N_2 and stored at 4°C, and the aqueous (lipid-depleted) phase was dialyzed exhaustively against PBS and stored lyophilized until use.

For detergent extraction of membrane proteins, 1-mg quantities of membranes were treated for 30 min at 37°C with 1% (wt/vol) sodium deoxycholate (NaDOC; Sigma), 0.5% (vol/vol) Triton X-100 (Sigma), 0.5% (vol/vol) Nonidet P-40 (NP-40; BDH, Poole, England), 1.0% (vol/vol) Tween 20 (J. T. Baker Chemical Co., Phillipsburg, N.J.), or 1.2%

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(wt/vol) Zwittergent$_{3\text{-}14}$ (Calbiochem-Behring, La Jolla, Calif.). These solutions also were made in PBS. After treatment, the undissolved membrane residues were washed twice with PBS to remove the detergents. The NaDOC- and Zwittergent$_{3\text{-}14}$-extracted membrane proteins were treated twice to remove the detergent by batch extraction with SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, Calif.) by the procedure described by Holloway (18), followed by exhaustive dialysis against PBS. They were stored lyophilized. Samples of the extracts were removed for electrophoresis before removal of detergent.

Quantities (100 μg) of strain 158p10 membranes were electrophoresed on 10% (wt/vol) polyacrylamide slab gels containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS). The gels were then stained for protein with Coomassie blue and for glycoproteins with the periodic acid Schiff stain as described by Segrest and Jackson (41).

Hybridoma and mouse antiserum production. A BALB/c mouse which had been injected intravenously with 2.6×10^9 CFU of *M. arthritidis* 158p10p9 22 weeks previously was challenged intravenously with 1 mg of strain 158p10p9 membranes. Viable mycoplasmas for the first injection were grown in the dialysate medium; the membranes were prepared from mycoplasmas grown in the Hayflick-type medium. The fusion technique used for the generation of monoclonal antibody-producing hybridoma cell lines was based on the methods described by Kohler and Milstein (28) and Kennett (25). Briefly, 3 days after challenge splenocytes from the mouse were fused with P3X63.Ag8.6.5.3 hypoxanthine-aminopterin-thymidine-sensitive myeloma cells at a cell ratio of 10:1 by incubation with polyethylene glycol (molecular weight, 1,000; J. T. Baker Chemical Co.). Fused cells were grown in 96-well flat bottom microtiter plates at approximately 1.5×10^5 cells per well. Hypoxanthine-aminopterin-thymidine medium (hypoxanthine-aminopterin-thymidine-containing Dulbecco modified minimal essential medium; Dutchland Laboratories, Inc., Denver, Penn.) was added to the cultures 1 and 4 days after the cell fusion. Fourteen days later, the supernatants from wells containing hybridoma cells were screened for antibody activity by the MI assay described below. Positive hybridomas were selected, expanded to 1-ml cultures in 24-well plates, and cloned on thymocyte feeder layers by the limiting dilution technique (25). Monoclonal antibody-containing ascites fluids were obtained from BALB/c mice which had been inoculated with 2×10^6 hybridoma cells 14 days after intraperitoneal injection of 0.5 ml of pristane.

Mouse anti-158p10p9 antiserum was collected after membrane challenge from a BALB/c mouse immunized as described for the mouse from which the hybridomas were derived. This antiserum had an MI titer of 320.

Immunological techniques. The MI assay was performed by the method of Purcell et al. (35) as modified by Washburn (48).

For immunoblotting experiments, the treated residues from 20 μg of membranes or 20-μg quantities of untreated membranes were electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred electrophoretically overnight to nitrocellulose paper in a Bio-Rad Transblot apparatus. For certain experiments, antigens other than mycoplasma membranes were used; these also were electrophoresed in 20-μg quantities. Blots were soaked in PBS containing 0.05% (vol/vol) Tween 20 for 1 h at 37°C and then treated with rabbit or mouse anti-*M. arthritidis* antiserum or with monoclonal antibody-containing ascites fluids diluted 1:40 in PBS containing 0.05% Tween 20 for 2 h at room temperature. Sheets were rinsed six times for five min each in PBS containing 0.05% Tween 20, exposed to peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) or sheep anti-mouse IgG (heavy and light chain specific) diluted 1:1,600 (Cappel Laboratories, Cochranville, Penn.), rinsed as before, and finally developed in a solution containing 10 ml of 3% (wt/vol) 4-chloro-1-naphthol (Sigma) in methanol, 50 ml of 0.05 M Tris-buffered 0.2 M NaCl (pH 7.4), and 19.8 μl of H_2O_2 (16) until bands were clearly visible (usually 10 to 20 min). The blots then were rinsed with water, dried between sheets of filter paper under a glass plate, and photographed. This technique was modified from Towbin et al. (47) and Batteiger et al. (3). For experiments in which horse serum and medium components were used as antigens, the peroxidase-labeled anti-IgG second antibody was absorbed twice with equal volumes of whole horse serum before use.

For radioimmunoprecipitation (RIP) experiments, surface proteins were labeled with ^{125}I by the lactoperoxidase-glucose oxidase method as modified from Hubbard and Cohn (20). *M. arthritidis* 158p10p9 (2×10^9 CFU) was washed once with Hanks balanced salt solution and then suspended in 200 μl of PBS in a 1-dram vial containing a microstir bar. To this were added 100 μCi of $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.), 10 μl of lactoperoxidase (1 mg/ml; Sigma), 10 μl (10 mU) of glucose oxidase (8 μg/ml; Sigma), and 10 μl of 2 M glucose. The enzyme and glucose solutions were prepared with 0.05 M phosphate buffer (pH 7.4). After 10 min of incubation at room temperature, the reaction was stopped with 10 μl of 0.1 M sodium azide. The labeled cells then were lysed by adding 2 ml of 1% (wt/vol) NaDOC in 0.01 M Tris-buffered 0.15 M NaCl (pH 8.3) (1% DOC-Tris), and the mixture was stirred for an additional 10 min. It then was desalted in a disposable Sephadex G25 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and concentrated by negative-pressure dialysis to 1 ml. For one experiment, cells were lysed with NaDOC before labeling to permit labeling of interior proteins as well as surface proteins.

For RIP, 10^6 cpm of labeled proteins were added to 10 to 20 μl of antiserum and incubated overnight at 4°C. A 100-μl volume of 10% (vol/vol) *Staphylococcus aureus* Cowan I, prepared by the method of Cullen and Schwartz (12), was added, and the suspension was incubated for an additional 2 h at 4°C. The staphylococci containing the immune complexes were washed five times with 0.5% DOC-Tris and boiled for 5 min in 100 μl of a solution containing 0.15% (wt/vol) dithioerythritol, 18% (vol/vol) glycerol, 0.07 M SDS, and 0.05 M Tris buffer (pH 6.8). The supernatants were electrophoresed by SDS-PAGE, and the dried gels were examined by autoradiography on Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). Optex high-speed X-ray intensifying screens (MCI Optonix, Inc., Cedar Knolls, N.J.) were used, with exposure times of 18 to 24 h.

RESULTS

Cellular location and biochemical nature of the MI antigen. To determine the cellular location of the MI antigen, we absorbed 0.25-ml quantities of 1:10-diluted anti-158p10 antiserum (L2) with 1 mg (protein) of 159p10 membranes and 1 mg (protein) of the soluble fraction. In addition, we absorbed 0.25 ml of 1:5-diluted anti-158p10 antiserum (R2) with approximately 5×10^9 CFU of 158p10 intact cells (Table 1). Reaction mixtures were incubated overnight at 4°C. Most of the MI antibody was absorbed by intact cells and membranes (titers were reduced 64- and 128-fold, respectively),

TABLE 1. Effect of absorption of rabbit antisera with membranes, cytoplasmic fraction, and intact cells on MI titer

Antiserum	Absorbed with:	MI titer
Anti-158p10 (L2)	Nothing (unabsorbed)	2,560
	Cytoplasmic fraction	640
	Membrane fraction	20
Anti-158p10 (R2)	Nothing (unabsorbed)	320
	Intact cells	5

whereas the soluble fraction reduced the MI titer only fourfold.

To determine the effect of enzymatic digestion and lipid and protein depletion on membrane antigens, we prepared immunoblots of proteolytic enzyme-treated and lipid- and protein-depleted membranes in comparison with untreated membranes. Most of the membrane antigens of strain 158p10 were removed by protease and, to a lesser extent, by trypsin; chymotrypsin was the least effective (Fig. 1). Similar results were obtained with strain 158p10p9 and the original parent strain 158 (not shown). In addition, the lipid-depleted 158p10p9 membrane fraction retained all the major antigens identifiable by immunoblotting, whereas very few were present in the lipid fraction (Fig. 2).

To determine the effect of these treatments on the MI antigen, we absorbed 0.25-ml quantities of 1:20-diluted anti-158p10p9 antiserum with the residues from 1-mg quantities of enzyme- and peroidate-treated and lipid- and protein-depleted 158p10p9 membranes. In addition, we absorbed 0.25-ml quantities of 1:5-diluted anti-158p10 antiserum (R2) with untreated intact 158p10 cells and cells which had been heated for 5 min in a boiling-water bath. The MI antigen was sensitive to heat and the proteolytic enzymes but not to periodate, and it was present in the lipid-depleted but not in the protein-depleted membrane fraction (Table 2). Repeated experiments (not shown) indicated that protease was the most effective and chymotrypsin was the least effective in destroying the MI antigen.

Periodic acid Schiff staining of SDS-PAGE-separated

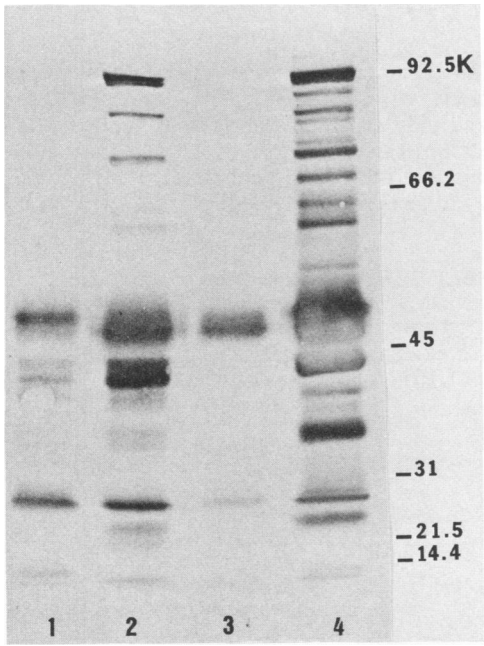

FIG. 1. Immunoblot of enzyme-treated and untreated membranes. *M. arthritidis* 158p10 membranes were treated with trypsin (lane 1), chymotrypsin (lane 2), protease (lane 3), and PBS (lane 4), electrophoresed by SDS-PAGE, transferred to nitrocellulose, and exposed to rabbit anti-158p10 antiserum (L2) and peroxidase-labeled anti-rabbit IgG. Positions of molecular weight markers are shown.

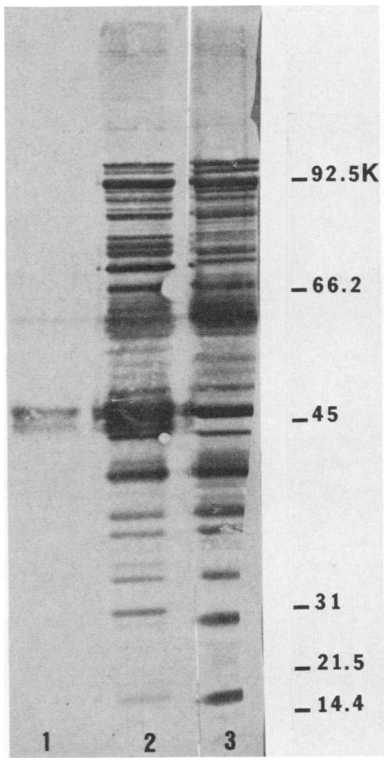

FIG. 2. Immunoblot of lipid-depleted (butanol-extracted) membrane proteins (lane 2) and the protein-free lipid extract (lane 1) in comparison with unextracted membranes (lane 3). The protein and lipid fractions from butanol-extracted *M. arthritidis* 158p10p9 membranes were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and exposed to rabbit anti-158p10p9 antiserum and peroxidase-labeled anti-rabbit IgG. Positions of molecular weight markers are shown.

TABLE 2. Effect of absorption of rabbit antisera with intact cells and membranes exposed to heat and various biochemical agents on MI titer

Antiserum	Absorbed with:	MI titer
Anti-158p10 (R2)	Nothing (unabsorbed)	320
	Intact cells	5
	Heat-treated cells	320
Anti-158p10p9	Nothing (unabsorbed)	1,280
	Membranes	40
	Membranes treated with:	
	Trypsin	640
	Protease	640
	Chymotrypsin	160
	$NaIO_4$	40
	Lipid-depleted membranes	40
	Protein-depleted membranes	1,280

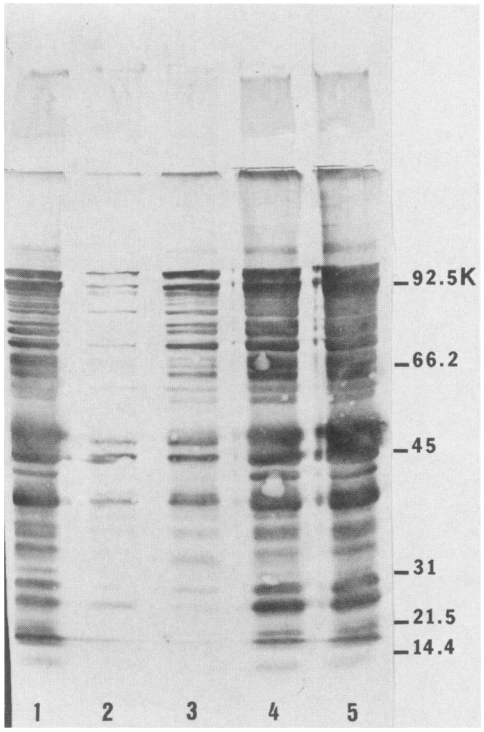

FIG. 3. Immunoblot of detergent-extracted membranes. *M. arthritidis* 158p10 membranes were treated with PBS (lane 1), Zwittergent$_{3-14}$ (lane 2), NaDOC (lane 3), NP-40 (lane 4), and Tween 20 (lane 5), electrophoresed by SDS-PAGE, transferred to nitrocellulose, and treated with rabbit anti-158p10 (L2) and peroxidase-labeled anti-rabbit IgG. Positions of molecular weight markers are shown.

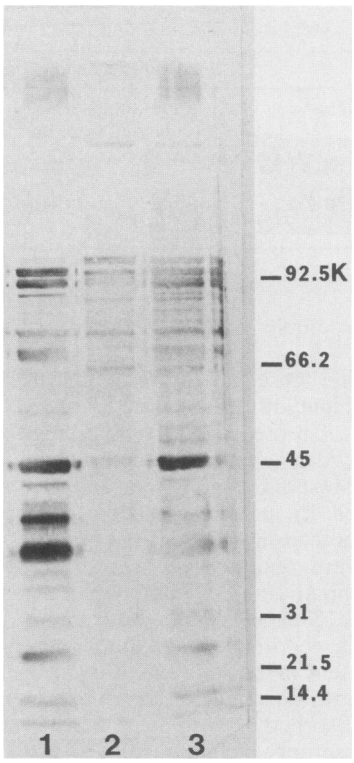

FIG. 4. Immunoblot of detergent-extracted and insoluble membrane proteins. *M. arthritidis* 158p10 membranes were treated with NaDOC, and the solubilized membrane fraction (lane 1), the insoluble residue (lane 2), and untreated membranes (lane 3) were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and treated with rabbit anti-158p10 antiserum (L2) and peroxidase-labeled anti-rabbit IgG. Positions of molecular weight markers are shown.

membrane components showed no evidence of glycoproteins (not shown).

Detergent solubility of *M. arthritidis* membrane antigens. To determine the effect of detergent extraction of *M. arthritidis* membrane antigens, we prepared immunoblots of the insoluble residues from Tween 20-, NP-40-, NaDOC-, and Zwittergent$_{3-14}$-treated membranes. We omitted Triton X-100 from this experiment since it is identical to NP-40. The nonionic detergents Tween 20 and NP-40 did not alter the antigenic profile, whereas NaDOC was much more effective at solubilizing membrane proteins, and the zwitterionic detergent Zwittergent$_{3-14}$ was even more so (Fig. 3). Figure 4 shows more clearly the effect of NaDOC. Most of the membrane antigens were present in the NaDOC extract, which showed an antigenic profile similar to that of unextracted membranes. In this experiment, NaDOC extraction was somewhat more effective than that in the experiment illustrated in Fig. 3. Membrane proteins extracted by Zwittergent$_{3-14}$ also showed an antigenic profile identical to that of unextracted membranes (not shown).

Next, we absorbed 0.25-ml quantities of 1:20-diluted anti-158p10 (L2) and anti-158p10p9 antisera with the residues from detergent-extracted membranes. As expected from the immunoblotting results, membranes treated with Triton X-100, NP-40, and Tween 20 still retained the MI antigen and strongly absorbed MI antibody, whereas the NaDOC- and Zwittergent$_{3-14}$-treated membranes did not. We removed NaDOC from 158p10p9 solubilized proteins as described above and absorbed the anti-158p10p9 antiserum with this extract. The solubilized proteins absorbed as much MI activity as did intact membranes (Table 3). We repeated this experiment with both NaDOC- and Zwittergent$_{3-14}$-solubilized 158p10 proteins. This time, the extracts reduced the anti-158p10 (L2) MI titer by 16-fold (not shown), thus confirming that these detergents left the MI antigen at least partially antigenically intact.

TABLE 3. Effect of absorption of rabbit antisera with membranes treated with various detergents on MI titer

Antiserum	Absorbed with:	MI titer
Anti-158p10 (L2)	Nothing (unabsorbed)	1,280
	Untreated membranes	40
	Membranes treated with:	
	Triton X-100	40
	NP-40	40
	Tween 20	20
	NaDOC	640
	Zwittergent$_{3-14}$	1,280
Anti-158p10p9	Nothing (unabsorbed)	1,280
	Untreated membranes	<20
	Membranes treated with:	
	Triton X-100	<20
	NP-40	<20
	Tween 20	<20
	NaDOC	320
	NaDOC-extracted proteins	<20

Monoclonal antibody-defined MI antigens. Two IgG monoclonal antibodies with MI activity were produced from a 158p10p9-immunized BALB/c mouse as described above. These antibodies were designated E11.6 and F11.10 and had MI titers of 80 and 640, respectively. The E11.6 antibody reacted specifically by RIP and immunoblotting with a 158p10p9 surface protein migrating by SDS-PAGE in approximately the 45,000 (45K)-dalton range (Fig. 5). This antibody recognized an identical antigen on the 158p10 membrane (not shown). We also tested this antibody by RIP against ^{125}I-labeled whole cells which had been lysed by NaDOC before labeling, so that cytoplasmic and inner surface membrane proteins were labeled as well. It did not react with any labeled *M. arthritidis* cell component other than the 45K-dalton membrane protein (results not shown).

The other MI-active monoclonal antibody, F11.10, did not react with membrane proteins by RIP or immunoblotting (Fig. 5), but it did react by immunoblotting with a 77K-dalton peptide present in both whole cells and the soluble fraction (Fig. 6). Interestingly, both intact cells and isolated membranes absorbed MI activity equally well from F11.10.

Effect of medium contamination of mycoplasma membranes on immunoblotting results. The presence of serum and other medium components in mycoplasma immunogen and antigen preparations has been a problem in our laboratory (L. R. Washburn, unpublished data) and for numerous other investigators studying immunological aspects of mycoplasma infection (4, 27, 32, 34, 40, 46, 49). To minimize interference by anti-medium antibodies in the present study, we used mycoplasmas grown only in the dialysate medium for rabbit immunizations. The resulting antisera showed titers by enzyme immunoassay against whole horse serum of 20 for the R2 anti-158p10 and anti-158p10p9 antisera and <20 for the L2 anti-158p10 antiserum. We tested the two rabbit antisera used in the immunoblotting experiments described in this report (L2 anti-158p10 and anti-158p10p9) by immunoblotting against whole horse serum, the boiled agamma fraction, and the complete dialysate medium. In addition, we compared the reactivity of these antisera for *M. arthritidis* membranes prepared from organisms grown in the Hayflick-type and the dialysate media (Fig. 7). Neither of these antisera reacted significantly with either of the horse serum preparations or with the medium used to grow their respective immunogens, even though the antisera were tested against concentrations of medium antigens far in excess of those amounts which might be expected to contaminate membrane preparations. In addition, the immunoblots of Hayflick-type and dialysate media-grown membranes were nearly identical (Fig. 7). These results indicated to us that the antigens recognized in the immunoblotting experiments reported here were not of medium origin and that medium contamination of mycoplasma antigen preparations did not affect the results of this study.

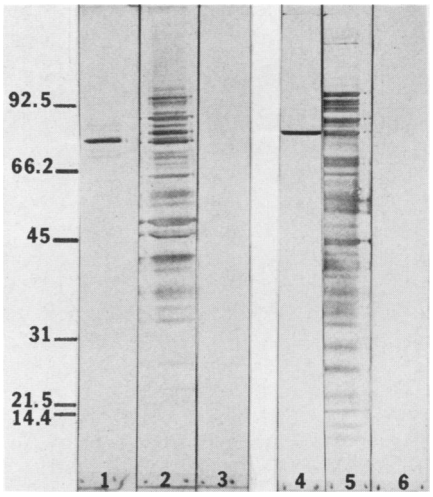

FIG. 6. Immunoblot of *M. arthritidis* 158p10p9 soluble fraction (lanes 1 to 3) and whole-cell lysate (lanes 4 to 6) exposed to MI active monoclonal antibody F11.10 (lanes 1 and 4), mouse anti-158p10p9 (lanes 2 and 5), and a normal BALB/c mouse serum (lanes 3 and 6) and to peroxidase-labeled sheep anti-mouse IgG. Positions of the molecular weight markers (K) are shown.

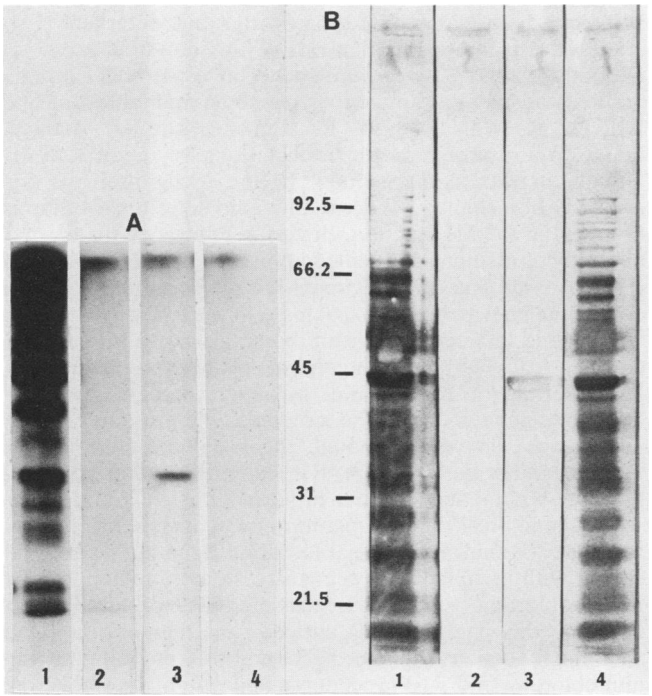

FIG. 5. Reactivity of monoclonal antibodies with *M. arthritidis* membrane antigens. (A) Autoradiograph of ^{125}I-labeled *M. arthritidis* 158p10p9 surface antigens precipitated by rabbit anti-*M. arthritidis* 158p10p9 antiserum (lane 1), MI active monoclonal antibodies F11.10 (lane 2) and E11.6 (lane 3), and a normal mouse serum (lane 4). The single peptide in lane 3 precipitated by E11.6 is approximately 45K daltons. (B) Immunoblot of *M. arthritidis* 158p10p9 membranes exposed to rabbit anti-158p10p9 (lane 1), MI active monoclonal antibodies F11.10 (lane 2) and E11.6 (lane 3), and mouse anti-158p10p9 (lane 4). Peroxidase-labeled second antibodies were goat anti-rabbit IgG (lane 1) and sheep anti-mouse IgG (lanes 2 to 4). Positions of molecular weight markers (K) are shown for B.

DISCUSSION

Host contact with most of the pathogenic mycoplasmas occurs first with antigens on the limiting plasma membrane. Therefore, these antigens take on a significance as great as those of bacterial cell walls in host-parasite interaction. In addition, *Mycoplasma* species are obligate parasites and reside in close cell-to-cell contact with their hosts. Some species attach through receptors which can be blocked by specific antibodies (19, 29). The lack of a rigid cell wall plus this very close mycoplasma-host cell interaction make the study of mycoplasma membrane antigens a critical part of any study of mycoplasma pathogenesis. We have begun to

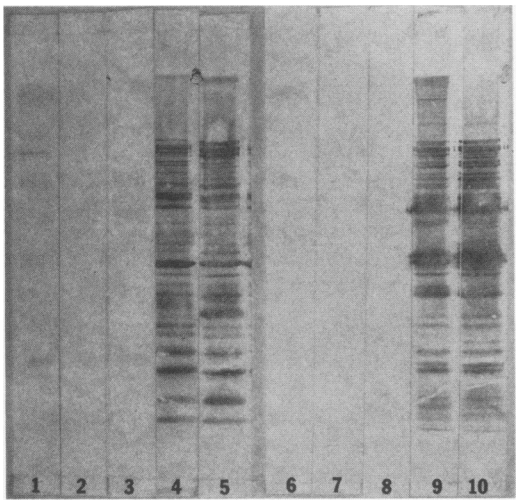

FIG. 7. Immunoblot comparing reactivity of two rabbit anti-*M. arthritidis* antisera with medium components and with membranes prepared from *M. arthritidis* grown in the Hayflick-type and the dialysate media. Lanes 1 to 5 were exposed to rabbit anti-158p10p9, and lanes 6 to 10 were exposed to L2 rabbit anti-158p10; the second antibody was horse serum-absorbed peroxidase-labeled anti-rabbit IgG. Antigens were whole horse serum (lanes 1 and 6), the dialysate medium (lanes 2 and 7), the boiled agamma horse serum fraction (lanes 3 and 8), and *M. arthritidis* membranes prepared from strain 158p10p9 grown in the dialysate medium (lane 4) and in the Hayflick-type medium (lane 5) and from strain 158p10 grown in the dialysate medium (lane 9) and the Hayflick-type medium (lane 10).

examine the role of antigenic recognition in *M. arthritidis* host-parasite interaction, and as a first step in this process, we undertook the present analysis of those major membrane antigens responsible for eliciting neutralizing, or MI, antibodies.

Most mycoplasma membranes consist of about three parts protein and one part lipid, although some may contain small amounts of carbohydrate, and at least one *Mycoplasma* species, *M. neurolyticum*, contains a lipopolysaccharidelike material (36, 42). Major membrane antigens can reside in any of these fractions. For *Mycoplasma pneumoniae* the specific complement-fixing and growth-inhibiting antibodies are directed against glycolipid antigens, which, although highly antigenic, are only weakly immunogenic in a purified state and function primarily as haptens (31, 43). The lipids of a number of other mycoplasmas also are antigenic (39, 44). For *Mycoplasma mycoides* the major antigens are carbohydrate (5, 22). In the case of at least three mycoplasmas, *Mycoplasma fermentans*, *Mycoplasma hominis*, and *M. pulmonis*, the major membrane antigens are protein (2, 17). The present study indicates that *M. arthritidis* may be counted among this group as well. Glycoproteins are rare among procaryotes; however, one has been identified in *M. pneumoniae* (23, 24) and tentatively in *M. gallisepticum* (14), although the latter has not been confirmed (1). We did not detect glycoproteins in *M. arthritidis* by periodic acid Schiff staining, but since not all glycoproteins stain with Schiff reagent, our observation cannot be considered conclusive.

As expected from the nature of the MI antibody, the *M. arthritidis* MI antigen appears to be located on the outer surface of the cell membrane, since both membranes and intact cells absorbed most of the MI antibody from rabbit antisera, whereas the soluble fraction absorbed only a small amount. This is consistent with reports by other investigators that the MI antigens of other mycoplasmas also are located on the membrane, as reviewed by Kenny (27). The lability of this antigen to heat and proteolytic enzymes as well as its stability to periodate treatment and butanol extraction indicate that it is protein in nature and probably does not contain antigenically important carbohydrate or lipid components. It thus resembles the major antigens of *M. hominis*, to which *M. arthritidis* is antigenically related (30).

The MI antigen is apparently an integral part of the membrane, hydrophobically rather than covalently bound, since it persisted through the numerous washes with low-ionic-strength solutions that are part of the membrane preparation procedure, and it was difficult to extract with nonionic detergents. We found the zwitterionic detergent Zwittergent$_{3\text{-}14}$ to be the most successful among those tested at solubilizing *M. arthritidis* membrane antigens, and solubilization in both NaDOC and Zwittergent$_{3\text{-}14}$ left the MI antigen at least partly intact. Since these agents can be removed by adsorption with polystyrene beads, followed by dialysis, it should be possible to prepare highly purified *M. arthritidis* membrane antigens, starting with NaDOC or Zwittergent extraction.

An IgG monoclonal antibody with MI activity reacted by immunoblotting and RIP with a single 45K-dalton membrane protein. The RIP results indicated that the MI antigen defined by this monoclonal antibody was located on the cell surface, since the lactoperoxidase-glucose oxidase iodination procedure labels only surface proteins. This major antigen was susceptible to proteolytic enzymes (Fig. 1) and soluble in both NaDOC and the zwitterionic detergent (Fig. 3 and 4). It is interesting that rats, which do not express MI antibody against *M. arthritidis*, nevertheless produce an IgG antibody against this antigen, as seen by immunoblotting and RIP (L. R. Washburn, J. R. Ramsay, and D. Asbury, manuscript in preparation). Explanations for the lack of MI activity in rats might include: (i) the recognition by this antibody of a slightly different antigenic determinant than is involved in the MI reaction, (ii) steric hindrance by another antibody or medium-derived component during the course of the MI assay, or (iii) the inability of this antibody to fix complement, which may participate in the MI reaction (27).

A second IgG monoclonal antibody, also with MI activity, did not react with any membrane proteins by either immunoblotting or RIP. It did, however, recognize a 77K-dalton antigen which may be a cytoplasmic protein, perhaps an essential enzyme, although the possibility that it is a loosely bound membrane protein cannot be ruled out. Preliminary results suggest that rats recognize this antigen as well. The ability of intact membranes to absorb MI activity from this second monoclonal antibody does not seem consistent with its inability to recognize membrane antigens by immunoblotting or RIP. It is possible that two components make up this particular MI antigen, one membrane bound and inactivated by denaturing conditions in both the immunoblotting and RIP procedures and one more stable and oriented toward the interior of the cell. It also is possible that the membrane-associated portion of this MI antigen is not protein in nature and thus is not detected by these techniques.

Further investigation into the role of the antigens recognized by these two monoclonal antibodies in the MI reaction and in host-parasite interaction is clearly required, although it is apparent from the present study that the most important MI antigens are proteins located at the outer surface of the membrane. Since these are among the antigens with which the host first comes into contact upon infection with *M.*

arthritidis, they may have considerable significance in pathogenesis.

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LITERATURE CITED

1. **Archer, D. B., and A. W. Rodwell.** 1982. Membrane proteins of *Mycoplasma gallisepticum*. J. Bacteriol. **151**:1598–1601.
2. **Argaman, M., and S. Razin.** 1969. Antigenic properties of mycoplasma organisms and membranes. J. Gen. Microbiol. **55**:45–58.
3. **Batteiger, B., W. J. Newhall V and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
4. **Bradbury, J. M., and F. T. W. Jordan.** 1972. Studies on the adsorption of certain medium proteins to *Mycoplasma gallisepticum* and their influence on agglutination and haemagglutination reactions. J. Hyg. Camb. **70**:267–278.
5. **Buttery, S. H.** 1970. Hapten inhibition of the reaction between *Mycoplasma mycoides* polysaccharide and bovine antisera. Immunochemistry **7**:305–310.
6. **Cahill, J. F., B. C. Cole, B. B. Wiley, and J. R. Ward.** 1971. Role of biological mimicry in the pathogenesis of rat arthritis induced by *Mycoplasma arthritidis*. Infect. Immun. **3**:24–35.
7. **Chanock, R. M., L. Hayflick, and M. F. Barile.** 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. U.S.A. **48**:41–49.
8. **Cole, B. C., J. F. Cahill, B. B. Wiley, and J. R. Ward.** 1969. Immunological responses of the rat to *Mycoplasma arthritidis*. J. Bacteriol. **98**:930–937.
9. **Cole, B. C., L. Golightly-Rowland, and J. R. Ward.** 1975. Arthritis of mice induced by *Mycoplasma pulmonis*: humoral antibody and lymphocyte responses of CBA mice. Infect. Immun. **12**:1083–1092.
10. **Cole, B. C., L. Golightly-Rowland, J. R. Ward, and B. B. Wiley.** 1970. Immunological response of rodents to murine mycoplasmas. Infect. Immun. **2**:419–425.
11. **Cole, B. C., J. R. Ward, R. S. Jones, and J. F. Cahill.** 1971. Chronic proliferative arthritis of mice induced by *Mycoplasma arthritidis*. I. Induction of disease and histopathological characteristics. Infect. Immun. **4**:344–355.
12. **Cullen, S. E., and B. D. Schwartz.** 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein-A-bearing staphylococci. J. Immunol. **117**:136–142.
13. **Goel, M. C.** 1973. New method for the isolation of membranes from *Mycoplasma gallisepticum*. J. Bacteriol. **116**:994–1000.
14. **Goel, M. C., and R. M. Lemcke.** 1975. Dissociation of *Mycoplasma gallisepticum* membranes with lithium diiodosalicylate and isolation of a glycoprotein. Ann. Microbiol. (Paris) **126B**:299–312.
15. **Golightly-Rowland, L., B. C. Cole, J. R. Ward, and B. B. Wiley.** 1970. Effect of animal passage on arthritogenic and biological properties of *Mycoplasma arthritidis*. Infect. Immun. **1**:538–545.
16. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
17. **Hollingdale, M. R., and R. M. Lemcke.** 1972. Membrane antigens of *Mycoplasma hominis*. J. Hyg. Camb. **70**:85–97.
18. **Holloway, P. W.** 1973. A simple procedure for removal of Triton X-100 from protein samples. Anal. Biochem. **53**:304–308.
19. **Hu, P. C., R. M. Cole, Y. S. Huang, J. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde, Jr.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216**:313–315.
20. **Hubbard, A. L., and Z. A. Cohn.** 1972. The enzymatic iodination of the red cell membrane. J. Cell Biol. **55**:390–405.
21. **Jordan, F. T. W.** 1975. Avian mycoplasmas and pathogenicity—a review. Avian Pathol. **4**:165–174.
22. **Jones, A. S., J. R. Tittensor, and R. T. Walker.** 1965. The chemical composition of the nucleic acids and other macromolecular constituents of *Mycoplasma mycoides* var. *capri*. J. Gen. Microbiol. **40**:405–411.
23. **Kahane, I., and H. Brunner.** 1977. Isolation of a glycoprotein from *Mycoplasma pneumoniae* membranes. Infect. Immun. **18**:273–277.
24. **Kahane, I., and V. T. Marchesi.** 1973. Studies on the orientation of proteins in mycoplasma and erythrocyte membranes. Ann. N.Y. Acad. Sci. **225**:38–45.
25. **Kennett, R. H.** 1979. Cell fusion. Methods Enzymol. **58**:345–349.
26. **Kenny, G. E.** 1967. Heat-lability and organic solvent-solubility of mycoplasma antigens. Ann. N.Y. Acad. Sci. **143**:676–681.
27. **Kenny, G. E.** 1979. Antigenic determinants, p. 351–384. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. I, Cell biology. Academic Press, Inc., New York.
28. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
29. **Krause, D. C., and J. B. Baseman.** 1983. Inhibition of *Mycoplasma pneumoniae* hemadsorption and adherence to respiratory epithelium by antibodies to a membrane protein. Infect. Immun. **39**:1180–1186.
30. **Lemcke, R. M.** 1964. The serological differentiation of mycoplasma strains (PPLO) from various sources. J. Hyg. Camb. **62**:199–219.
31. **Lemcke, R. M., B. P. Marmion, and P. Plackett.** 1967. Immunochemical analysis of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. **143**:691–702.
32. **Lin, J. S.-L.** 1982. Complement-mediated killing of *Ureaplasma urealyticum* by antibody directed against components of the growth medium. J. Med. Microbiol. **15**:135–140.
33. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
34. **Nicolet, J., P. Paroz, and B. Kristensen.** 1980. Growth medium constituents contaminating mycoplasma preparations and their role in the study of membrane glycoproteins in porcine mycoplasmas. J. Gen. Microbiol. **119**:17–26.
35. **Purcell, R. H., D. Taylor-Robinson, D. C. Wong, and R. M. Chanock.** 1966. A color test for the measurement of antibody to the non-acid-forming human mycoplasma species. Am. J. Epidemiol. **84**:51–66.
36. **Razin, S.** 1979. Isolation and characterization of mycoplasma membranes, p. 213–229. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. I, Cell biology. Academic Press, Inc., New York.
37. **Razin, S., and S. Rottem.** 1976. Techniques for the manipulation of mycoplasma membranes, p. 3–26. *In* A. H. Maddy (ed.), Biochemical analysis of membranes. John Wiley & Sons, Inc., New York.
38. **Rodwell, A. W., S. Razin, S. Rottem, and M. Argaman.** 1967. Association of protein and lipid in *Mycoplasma laidlawii* membranes disaggregated by detergents. Arch. Biochem. Biophys. **122**:621–628.
39. **Ryan, M. D., P. Noker, and L. L. Matz.** 1975. Immunological properties of glycolipids from membranes of *Acholeplasma laidlawii*. Infect. Immun. **12**:799–807.
40. **Sayed, I. A., and G. E. Kenny.** 1980. Comparison of the proteins and polypeptides of the eight serotypes of *Ureaplasma urealyticum* by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Int. J. Syst. Bacteriol. **30**:33–41.
41. **Segrest, J. P., and R. L. Jackson.** 1972. Molecular weight determination of glycoproteins by polyacrylamide gel electro-

phoresis in sodium dodecyl sulfate. Methods Enzymol. **28**:54–63.

42. **Smith, P. F.** 1979. The composition of membrane lipids and lipopolysaccharides, p. 231–257. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. I, Cell biology. Academic Press, Inc., New York.
43. **Sobeslavsky, O., B. Prescott, W. D. James, and R. M. Chanock.** 1967. Serological and immunogenic activities of different fractions of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. **143**:682–690.
44. **Sugiyama, T., P. F. Smith, T. A. Langworthy, and W. R. Mayberry.** 1974. Immunological analysis of glycolipids and lipopolysaccharides derived from various mycoplasmas. Infect. Immun. **10**:1273–1279.
45. **Taylor-Robinson, D., and D. M. Berry.** 1969. The evaluation of the metabolic-inhibition technique for the study of *Mycoplasma gallisepticum*. J. Gen. Microbiol. **55**:127–137.
46. **Thorns, C. J., and E. Boughton.** 1980. Studies on the effect of growth medium composition on the antigenicity of *Mycoplasma bovis*. J. Hyg. Camb. **84**:29–36.
47. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
48. **Washburn, L. R.** 1983. Spectrophotometric method for determining titers of antimycoplasma metabolism inhibition antibody. Appl. Environ. Microbiol. **45**:343–346.
49. **Williams, M. H.** 1972. Pathogenic mycoplasmas in rheumatoid arthritis?, p. 251–257. *In* K. Elliott and J. Birch (ed.), Pathogenic mycoplasmas. Associated Scientific Publishers, New York.
50. **Zimmerman, B. J., and R. F. Ross.** 1977. Modified metabolic-inhibition test for detection of antibodies to *Mycoplasma hyosynoviae* in swine serum. Am. J. Vet. Res. **38**:2075–2076.