CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 1996, p. 746–752 Vol. 3, No. 6
1071-412X/96/$04.00+0

Monoclonal Antibodies to Surface-Exposed Proteins of *Mycoplasma mycoides* subsp. *mycoides* (Small-Colony Strain), Which Causes Contagious Bovine Pleuropneumonia

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Received 26 January 1996/Returned for modification 2 May 1996/Accepted 24 July 1996

Outbreaks of bovine pleuropneumonia caused by small-colony strains of *Mycoplasma mycoides* subsp. *mycoides* occur in Africa, and vaccination is used for control. Since protein subunits are needed to improve multivalent vaccines, monoclonal antibodies (MAbs) were made to facilitate protein identification and isolation. Eleven immunoglobulin M MAbs derived from mouse spleen donors immunized with disrupted whole organisms bound periodate-sensitive epitopes on externally exposed polysaccharide. Seven of these MAbs caused in vitro growth inhibition of *M. mycoides* subsp. *mycoides*; however, reaction with carbohydrate epitopes prevented their use in identifying proteins. Ten additional MAbs from mouse spleen donors immunized with Triton X-114-phase integral membrane proteins reacted with periodate-insensitive, proteinase K-sensitive epitopes. These MAbs were classified into three groups based on immunoblots of Triton X-114-phase proteins. One group reacted with 96-, 16-, and 15-kDa proteins. Another group reacted with 26-, 21-, and 16-kDa proteins, while a third group reacted only with 26- and 21-kDa proteins. One MAb from each group reacted with trypsin-sensitive epitopes on live organisms, yet none caused in vitro growth inhibition. Representative MAbs reacted with all small-colony strains in immunoblots and did not react with large-colony strains. However, these MAbs were not specific for small-colony strains, as proteins from two other *M. mycoides* cluster organisms were identified. Nevertheless, MAbs to surface-exposed epitopes on integral membrane proteins will be useful for isolation of these proteins for immunization, since one or more might induce growth-inhibiting antibodies or other protective responses.

Mycoplasma mycoides subsp. *mycoides* small-colony strains cause contagious bovine pleuropneumonia (CBPP) in cattle and water buffalo (9, 32). In Africa, CBPP is second only to rinderpest in economic significance in cattle (27). The disease is established in Portugal and Spain and may occur in the Middle East and Asia (27). Transmission is by contact with infected cattle and occurs by droplet inhalation. Approximately 50% of exposed cattle develop clinical signs following a variable incubation period. Acute CBPP is characterized by severe exudative pleuropneumonia that progresses to necrotic lobules and thickened pleura. The mortality rate in cattle with clinical signs varies between 10 and 90%, and those surviving may have chronic pleuropneumonia or recover. In some cattle that appear to recover, infected foci become encapsulated but later release organisms that are transmitted to other cattle (19). Cattle that actually recover develop protective immunity to reinfection (13, 26, 41).

The potential methods for controlling CBPP include antibiotic treatment, diagnosis and quarantine measures, and vaccination. Antibiotics, including tetracyclines, can be used for treatment of CBPP. However, antibiotic use is not recommended because treatment does not completely eliminate *M. mycoides* subsp. *mycoides* colonization, resulting in carriers that can infect susceptible cattle (19). Quarantine based on detection of infected animals can be used to limit the spread of CBPP, but livestock movement makes implementation difficult. Detection requires measurement of serum antibodies (7) or isolation and identification of *M. mycoides* subsp. *mycoides* from infected organs (2). Identification is difficult because of complex cross-reactions with other *Mycoplasma* strains within the *M. mycoides* cluster (27, 30, 38). Vaccination with either inactivated (10, 15) or attenuated (5, 11, 18, 25) vaccines has been used to prevent CBPP. A lyophilized T1 broth vaccine induces effective immunity and is very stable, but most diluents used before lyophilization result in a loss of potency (32), making it difficult to produce organisms for vaccines. Even though the T1 vaccine strain reverted to virulence after six successive endobronchial inoculations in cattle (8), there is no evidence of reversion during normal vaccine use. The attenuated CBPP vaccine has been used in combination with the rinderpest vaccine (31), demonstrating its use in multivalent vaccines. In addition, the success of inactivated CBPP vaccines (10, 15) suggests that subunit vaccine development is possible.

While protective immunity against *M. mycoides* subsp. *mycoides* can be induced, the responsible antigens have not been identified. Surface-exposed proteins are targets for investigation because antibody is one mechanism of protective immunity. This is evidenced by the conferring of protective immunity on recipient calves following passive transfer of sera from cattle recovered from CBPP (24, 26). Surface-exposed proteins on live organisms are accessible to antibody binding and antibody-dependent immune mechanisms.

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The purpose of this study was to develop monoclonal antibodies (MAbs) to surface-exposed epitopes of *M. mycoides* subsp. *mycoides* that would identify potential antigens for later development of a subunit vaccine. The study focused on identifying MAbs to surface-exposed protein epitopes of *M. mycoides* subsp. *mycoides* rather than carbohydrate epitopes for two reasons. First, immunization with a galactan preparation of *M. mycoides* subsp. *mycoides* did not protect cattle against challenge but enhanced the severity of disease following challenge (6). Second, a MAb that kills *Mycoplasma hyorhinis* binds to an integral membrane protein (16), and isolated membranes of *Mycoplasma gallisepticum* elicit agglutinating antibodies that inhibit growth and metabolism (23). One group of MAbs described in this paper bound surface-exposed carbohydrate epitopes, while another group bound surface-exposed integral membrane protein epitopes of *M. mycoides* subsp. *mycoides*. Some of the MAbs binding carbohydrate epitopes caused in vitro growth inhibition, while none of the MAbs binding surface-exposed protein epitopes caused in vitro growth inhibition. The MAbs to membrane proteins will facilitate isolation of *M. mycoides* subsp. *mycoides* surface proteins for immunization trials and to make polyclonal antisera for expression screening of recombinant DNA libraries.

MATERIALS AND METHODS

In vitro culture and harvesting of *Mycoplasma* strains. Fifteen *Mycoplasma* strains were cultured at 37°C in modified Newing's tryptose broth (12). The strains were from three sources: the National Veterinary Research Centre, Magugu (NVRC-M), Kenya; the National Veterinary Research Centre, Kabete (NVRC-K), Kenya; and R. H. Leach, National Collection of Type Cultures (NCTC), Corrindale, England. The strains (origins in brackets) included five small-colony strains (T419 [NVRC-M], Gladysdale [NCTC], T_1M_{44} [NVRC-M], B613/87 [NCTC], and B467/92 [NVRC-K]), three large-colony strains (VR1/3172.LB2 [NCTC], 78/441 [NCTC], and Y-goat [NCTC]), one *M. mycoides* subsp. *mycoides* strain of undefined colony type (NVRC-K), two *Mycoplasma capricolum* strains (74/3220 [NCTC] and ZT [NCTC]), two bovine group 7 strains (L2917 [NCTC] and 4055 [NCTC]), one *M. capricolum* subsp. *capripneumonia* strain (F38 [NVRC-K]), and one *M. mycoides* subsp. *capri* strain (Pendik [NCTC]). Cultures for detergent partitioning and immunoblotting were harvested by centrifugation (12,000 × *g*, 4°C, 20 min) and washed with phosphate-buffered saline (PBS) (pH 7.4). The protein concentration in the resuspended pellets was determined (35), and they were stored at −70°C. Intact organisms for surface proteolysis were harvested and used immediately without freezing. Growth inhibition was performed as described previously with minor modifications (40).

Triton X-114 separation of integral membrane proteins. Organisms were treated with Triton X-114 (3) as described for *M. gallisepticum* (1). One milligram of *M. mycoides* subsp. *mycoides* protein per ml was mixed with ice-cold Triton X-114 to a final concentration of 1.0% (vol/vol) in Tris-saline buffer (10 mM Tris, 150 mM NaCl, pH 7.4) and incubated at 4°C for 30 min. Insoluble components were removed by centrifugation (13,000 × *g*, 4°C, 15 min). The supernatant containing Triton X-114-solubilized material was incubated at 37°C for 8 min to induce rapid condensation of the Triton X-114 and centrifuged (10,000 × *g*, room temperature, 5 min). The aqueous phase was adjusted to 1% (vol/vol) Triton X-114, while the detergent phase was adjusted to the original volume with Tris-saline buffer, and phase partitioning was repeated five times as described above to remove any aqueous phase remaining in the Triton X-114 phase. The proteins were removed from Triton X-114 by ethanol precipitation, except for the detergent-phase antigen used for immunizing mice.

Immunization of mice. For fusion 4, each of six BALB/c mice was injected intraperitoneally with 100 μl containing 40 μg of *M. mycoides* subsp. *mycoides* integral membrane protein mixed with an equal volume of Freund's complete adjuvant. Four weeks later, the mice were injected intraperitoneally with the same antigen concentration in Freund's incomplete adjuvant. A month later, antigen was given intraperitoneally without adjuvant. The mouse with the highest serum antibody titer as determined by enzyme-linked immunosorbent assay (ELISA) (described below) was selected as a spleen donor. Three days prior to spleen collection, mice were injected intravenously with 100 μl containing 40 μg of antigen without adjuvant. For fusion 72, six mice were immunized with disrupted whole *M. mycoides* subsp. *mycoides* proteins by a procedure similar to the one described above for fusion 4.

Screening of mouse serum antibodies. Washed *M. mycoides* subsp. *mycoides* was disrupted by 10 freeze-thaw cycles and 5 1-min cycles of ultrasonication and used in ELISA (22). Flat-bottomed 96-well plates (Immunlon 2; Dynatech Laboratories, Chantilly, Va.) were coated with (per well) 50 μg (100 μl) of protein suspended in 0.1 M carbonate buffer (pH 9) containing 0.0025% glutaraldehyde and were incubated for 18 h at 4°C. After three washes with PBS containing 0.1% Tween 20, the plates were blocked with PBS containing 0.1% Tween 20 and 5% powdered milk for 3 h at room temperature. Immune sera (100 μl per well) diluted 1:10, 1:100, 1:1,000, and 1:10,000 were incubated for 1 h at 37°C. Antibodies (1:500) to mouse serum conjugated to horseradish peroxidase (100 μl per well) were incubated for 1 h at 37°C. The substrate 2,2-azino-bis-3-ethyl-benzthiazoline (100 μl per well) was added and incubated in the dark for 30 min at room temperature, and the optical density at 490 nm was measured with a microplate ELISA reader.

Production and propagation of hybridomas. Mouse spleen cells were washed in Dulbecco's modified Eagle's medium and combined with nonsecreting P3X63.Ag8.653 myeloma cells and spleen cells in a ratio of 1:10. Fusion was performed by adding 0.5 ml containing 41.6% polyethylene glycol and 15% dimethyl sulfoxide to the cell pellet and mixing by gentle rocking for 1 min (21). Then, 0.5 ml of 25% polyethylene glycol was added, and the mixture was rocked for 3 min; this was followed by the addition of 4 ml of 20% fusion medium. Twenty-five milliliters of fusion medium was added, and the cells were distributed to a 48-well culture plate and incubated at 37°C with 5% CO_2. Cultures were fed with medium containing 5×10^{-3} M hypoxanthine, 2×10^{-5} M aminopterin, and 8×10^{-4} M thymidine. Supernatants were screened by ELISA, and limiting dilution was done on selected hybridomas. MAb from cloned hybridomas was precipitated with 50% saturated ammonium sulfate and dialyzed with PBS. Antibody isotyping was done with a commercial kit (Sigma, St. Louis, Mo.).

SDS-PAGE and Western immunoblotting. *M. mycoides* subsp. *mycoides* antigen preparations were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.5 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% glycerol, 5% 2-beta-mercaptoethanol, and 0.01% bromophenol blue), boiled, and separated on a 7.5 to 15% gradient gel. Separated polypeptides were transferred to nitrocellulose membranes (39), blocked with PBS containing 0.1% Tween 20 and 5% powdered milk for 45 min, air dried, and stored at −20°C until used. For immunoblots, the strips were incubated with diluted MAb at room temperature overnight, and peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:1,000) was used as a second antibody. Bound antibodies were visualized with 3,3-diaminobenzidine and hydrogen peroxide.

Periodate and proteinase K treatment. Treatment of *M. mycoides* subsp. *mycoides* antigens was done following transfer to nitrocellulose strips (42). Briefly, strips were rinsed with 50 mM sodium acetate buffer (pH 4.5) and incubated with 20 mM periodate in sodium acetate buffer for 1 h at room temperature in the dark. The strips were then incubated in 50 mM sodium borohydride for 30 min, and immunoblotting was carried out as described above. Other strips were treated with 25 μg of proteinase K per ml in reaction buffer (0.01 M Tris [pH 7.8], 0.5% SDS) for 1 h at 50°C. Proteinase K was then inhibited by addition of EDTA to a final concentration of 10 mM. The strips were rinsed three times in PBS containing 0.1% Tween 20 and immunoblotted as described above.

Trypsin treatment of intact *M. mycoides* subsp. *mycoides*. A freshly grown culture of *M. mycoides* subsp. *mycoides* was centrifuged (12,000 × *g*, 4°C, 10 min), washed three times in PBS, and resuspended in PBS. Trypsin (0, 0.1, 0.4, and 0.6 mg/ml) was added and incubated at 37°C for 30 min (16). Organisms were pelleted by centrifugation (12,000 × *g*, 4°C, 5 min), washed twice with PBS, and resuspended to the original volume. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with MAbs. *M. mycoides* subsp. *mycoides* from the pellets was plated on agar to assay viability following trypsin treatment.

RESULTS

MAbs to *M. mycoides* subsp. *mycoides* carbohydrate epitopes. Hybridoma supernatants derived from a mouse immunized with disrupted whole *M. mycoides* subsp. *mycoides* organisms were screened by ELISA. Hybridomas from 11 wells with antibody to *M. mycoides* subsp. *mycoides* were cloned twice by limiting dilution, and the resulting MAbs (Table 1) were further evaluated. All 11 MAbs were IgM, and 7 of them caused in vitro growth inhibition of *M. mycoides* subsp. *mycoides* (Table 1). Immunoblots were done with whole *M. mycoides* subsp. *mycoides* to identify the antigens being recognized by the MAbs. The 11 MAbs reacted with a diffuse region at the top of and extending into the resolving gel, and examples of three MAbs which caused growth inhibition are shown in Fig. 1, lanes 1, 3, and 5. The binding to periodate-treated *M. mycoides* subsp. *mycoides* antigens was then evaluated, because the diffuse band was similar to the surface-exposed polysaccharide described for *M. capricolum* subsp. *capripneumoniae* F38 (34) and because *M. mycoides* subsp. *mycoides* has a well-defined

TABLE 1. In vitro growth inhibition of *M. mycoides* subsp. *mycoides* by fusion 72 MAbs

MAb	Growth inhibition[a] with dilution of:			
	1:1	1:2	1:4	1:16
72/27.9.9	+	+	+	+
72/5.8.4	−	−	−	−
72/16.2.14	+	+	+	−
72/12.3.9	−	−	−	−
72/18.11.7	+	+	+	+
72/22.6.11	+	+	+	−
72/25.4.14	−	−	−	−
72/26.1.1	+	+	−	−
72/11.13.6	+	+	+	−
72/6.4.11	+	NT	NT	NT
72/8.2.1	−	NT	NT	NT

[a] +, a zone of inhibition of organism growth which extended radially from the well containing the MAb was observed; −, no zone of inhibition was detectable around the MAb well; NT, not tested.

carbohydrate capsule (14, 29). Periodate treatment destroyed binding by all 11 MAbs (Fig. 1, lanes 2, 4, and 6). In addition, treatment with 5 mM periodate completely destroyed binding by three of nine MAbs tested in ELISA, while 20 mM periodate destroyed binding by eight of nine MAbs and reduced binding of the ninth MAb by 73%. On the basis of these results, it was concluded that the MAbs from fusion 72 reacted with periodate-sensitive epitopes on an *M. mycoides* subsp. *mycoides* surface-exposed polysaccharide.

Triton X-114-phase protein partitioning. To generate MAbs to membrane protein epitopes, *M. mycoides* subsp. *mycoides* was partitioned with Triton X-114. Figure 2 shows a comparison of protein profiles of *M. mycoides* subsp. *mycoides* aqueous-phase (lane 1), Triton X-114-phase integral membrane (lane 2) and whole-organism (lane 3) proteins following SDS-PAGE and Coomasie blue staining. A small number of the total proteins partitioned exclusively into the Triton X-114 phase (Fig. 2, lane 2), while the majority were in the aqueous phase (lane 1).

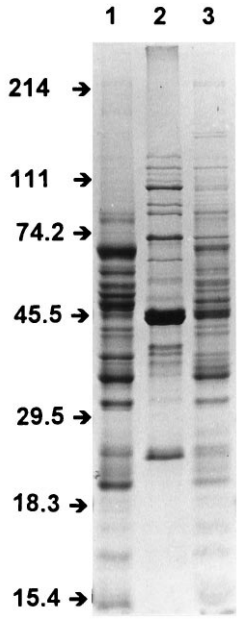

FIG. 2. Coomasie blue stain of SDS-PAGE-separated *M. mycoides* subsp. *mycoides* proteins before and after Triton X-114 phase partitioning. Lane 1, aqueous-phase *M. mycoides* subsp. *mycoides* proteins; lane 2, detergent-phase *M. mycoides* subsp. *mycoides* proteins; lane 3, whole *M. mycoides* subsp. *mycoides* proteins. Molecular mass standards (in kilodaltons) are shown on the left.

An immunoblot of Triton X-114-partitioned proteins with serum antibodies from an *M. mycoides* subsp. *mycoides*-infected cow is shown in Fig. 3. Antibodies bound to aqueous-phase, detergent-phase, and whole-organism proteins, while serum from an uninfected cow did not have antibodies to these proteins (Fig. 3). Comparison of the Coomasie blue-stained pro-

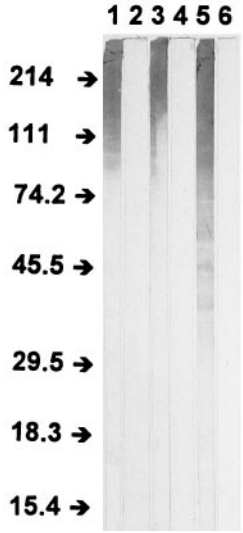

FIG. 1. Immunoblot of *M. mycoides* subsp. *mycoides* with fusion 72 MAbs. Lanes 1, 3, and 5 were loaded with whole *M. mycoides* subsp. *mycoides*, while lanes 2, 4, and 6 were loaded with the same antigen treated with periodate. Lanes 1 and 2 were reacted with MAb 72/16.2.14, lanes 3 and 4 were reacted with MAb 72/18.11.7, and lanes 5 and 6 were reacted with MAb 72/11.13.6. Molecular mass standards (in kilodaltons) are shown on the left.

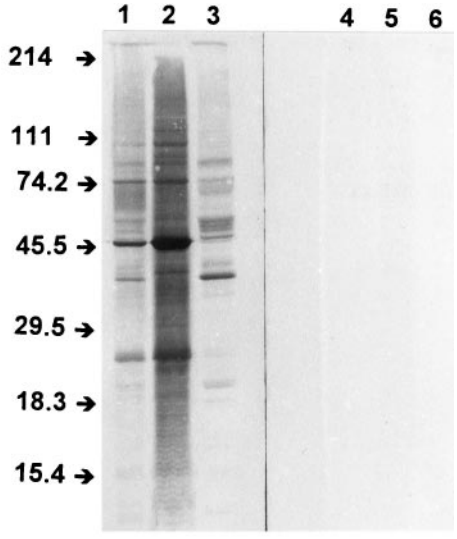

FIG. 3. Immunoblot of *M. mycoides* subsp. *mycoides* proteins after Triton X-114 phase fractionation. Lanes 1 and 4, whole *M. mycoides* subsp. *mycoides* proteins; lanes 2 and 5, detergent-phase proteins; lanes 3 and 6, aqueous-phase proteins. Lanes 1, 2, and 3 were reacted with serum from an *M. mycoides* subsp. *mycoides*-infected cow, and lanes 4, 5, and 6 were reacted with serum from a uninfected cow. Molecular mass standards (in kilodaltons) are shown on the left.

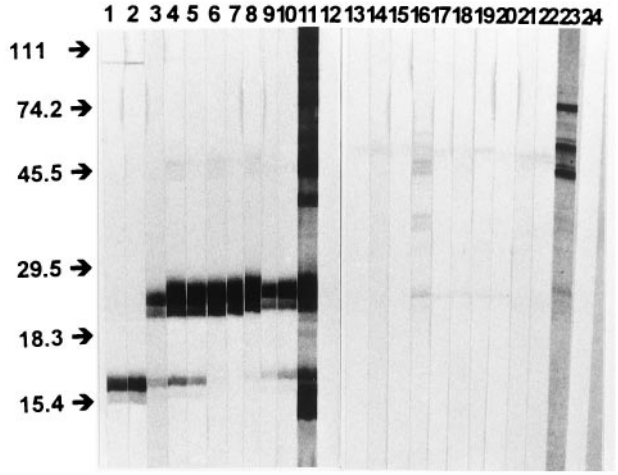

FIG. 4. Immunoblot of *M. mycoides* subsp. *mycoides* Triton X-114-phase proteins with fusion 4 MAbs. Lanes 1 to 12, detergent-phase proteins; lanes 13 to 24, aqueous-phase proteins. Lanes from each group were blotted with MAbs 4/13.71.28 (lanes 1 and 13), 4/13.71.27 (lanes 2 and 14), 4/13.71.24 (lanes 3 and 15), 4/27.68.2 (lanes 4 and 16), 4/27.68.12 (lanes 5 and 17), 4/27.68.15 (lanes 6 and 18), 4/27.68.62 (lanes 7 and 19), 4/27.68.36 (lanes 8 and 20), 4/30.69.72 (lanes 9 and 21), and 4/30.69.14 (lanes 19 and 22). Lanes 11 and 23 were reacted with mouse antiserum to *M. mycoides* subsp. *mycoides* detergent-phase proteins, and lanes 12 and 24 were reacted with nonimmunized mouse serum. Molecular mass standards (in kilodaltons) are shown on the left.

teins in Fig. 2 with the proteins detected in the immunoblot (Fig. 3) demonstrated that the polyclonal antibodies reacted with many, but not all, of the proteins in each fraction.

MAbs to integral membrane proteins. Hybridoma supernatants derived from mice immunized with Triton X-114-phase proteins were screened by ELISA with disrupted *M. mycoides* subsp. *mycoides* antigen. Supernatants from 48 positive wells had antibody which also bound to Triton X-114-phase proteins by immunoblotting (data not shown). Hybridomas secreting antibody that reacted with epitopes insensitive to periodate treatment were cloned by two limiting dilutions. Ten MAbs were selected for further studies; two were IgG1 (4/13.71.28 and 4/13.71.27), and eight were IgG2b (4/13.71.24, 4/27.68.2, 4/27.68.12, 4/27.68.15, 4/27.68.36, 4/27.68.62, 4/30.69.72, and 4/30.69.14).

Reactivity of MAbs with *M. mycoides* subsp. *mycoides* proteins. Figure 4 shows an immunoblot of the 10 selected MAbs reacted with *M. mycoides* subsp. *mycoides* detergent-phase (lanes 1 to 10) and aqueous-phase (lanes 13 to 22) proteins. MAbs 4/13.71.28 and 4/13.71.27 bound strongly to a 16-kDa protein in the Triton X-114 phase (Fig. 4, lanes 1 and 2) and weakly to 96- and 15-kDa proteins. These two MAbs did not bind with aqueous-phase proteins (Fig. 4, lanes 13 and 14). MAbs 4/13.71.24 (Fig. 4, lane 3), 4/27.68.2 (lane 4), 4/27.68.12 (lane 5), 4/27.68.36 (lane 8), 4/30.69.72 (lane 9), and 4/30.69.14 (lane 10) bound strongly to 26- and 21-kDa detergent-phase proteins and weakly to a 16-kDa protein. Only MAb 4/27.68.2 also bound weakly to a 26-kDa protein and several larger proteins in the aqueous phase (Fig. 4, lane 16). MAbs 4/27.68.15 and 4/27.68.62 bound strongly to 26- and 21-kDa proteins in the detergent phase (Fig. 4, lanes 6 and 7). Lanes 11 and 23 of Fig. 4 were reacted with an *M. mycoides* subsp. *mycoides*-immunized mouse serum, and lanes 12 and 24 were reacted with a nonimmunized mouse serum.

Proteinase K, but not periodate, treatment abolished MAb reactivity with Triton X-114-phase proteins. Detergent-phase proteins were separated by SDS-PAGE, transferred to nitrocellulose, treated with either proteinase K or periodate, and then reacted with MAbs. There was no difference between the reactivities of MAbs to proteins treated with periodate (Fig. 5, even-numbered lanes 2 to 20) and those of MAbs to untreated proteins (Fig. 5, odd-numbered lanes 1 to 19). The efficacy of the periodate reaction was demonstrated by the destruction of the *M. mycoides* subsp. *mycoides* epitope recognized by MAb PK-2 (compare untreated lane 21 with treated lane 22 of Fig. 5), which reacted with a carbohydrate epitope (33). However, the binding of MAb FGJ-B7, which reacted with a proteinase K-sensitive protein epitope partitioning in the aqueous phase following Triton X-114 treatment which was not affected by trypsin treatment of whole organisms (33), was unaltered (compare lanes 24 and 23 of Fig. 5).

Immunoblot analysis of MAb binding to *M. mycoides* subsp. *mycoides* Triton X-114-phase proteins following proteinase K treatment is illustrated in Fig. 6. The binding of nine MAbs from fusion 4 with untreated proteins (odd-numbered lanes 1 to 17 of Fig. 6) was completely abolished by treatment with proteinase K (Fig. 6, even-numbered lanes 2 to 18). Binding by MAb FGJ-B7 to an aqueous-phase protein epitope was also destroyed by proteinase K treatment (compare untreated lane 19 with treated lane 20 of Fig. 6).

Proteolysis of surface-exposed *M. mycoides* subsp. *mycoides* proteins. To determine if the proteins bound by MAbs were exposed on the surface of live *M. mycoides* subsp. *mycoides* organisms, freshly harvested organisms were treated with trypsin. The treated organisms were centrifuged, and the supernatant and pellet were analyzed by immunoblotting (Fig. 7).

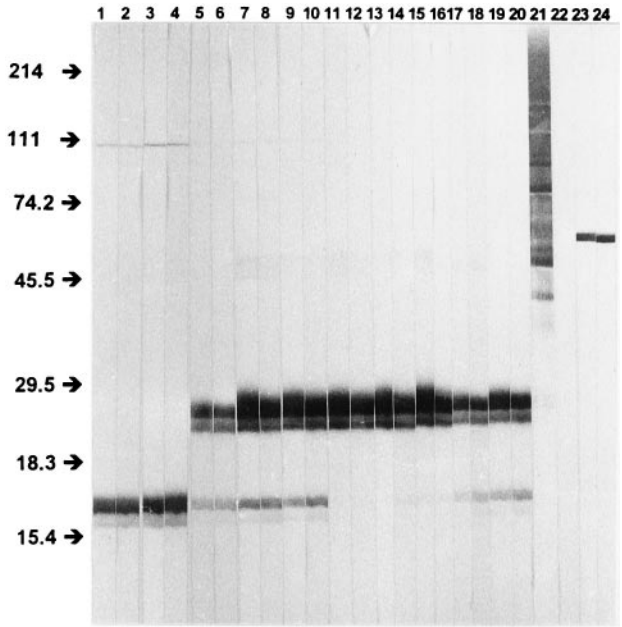

FIG. 5. Immunoblot of periodate-treated and untreated *M. mycoides* subsp. *mycoides* Triton X-114-phase proteins with fusion 4 MAbs. Lanes 1 to 20, detergent-phase proteins; lanes 21 and 22, whole-organism proteins; lanes 23 and 24, aqueous-phase proteins. The odd-numbered lanes were treated with periodate, and the even-numbered lanes were not treated. MAb 4/13.71.28 was reacted with lanes 1 and 2; 4/13.71.27 was reacted with lanes 3 and 4; 4/13.71.24 was reacted with lanes 5 and 6; 4/27.68.2 was reacted with lanes 7 and 8; 4/27.68.12 was reacted with lanes 9 and 10; 4/27.68.15 was reacted with lanes 11 and 12; 4/27.68.62 was reacted with lanes 13 and 14; 4/27.68.36 was reacted with lanes 15 and 16; 4/30.69.72 was reacted with lanes 17 and 18; 4/30.69.14 was reacted with lanes 19 and 20; PK-2 was reacted with lanes 21 and 22; and FGJ-B7 was reacted with lanes 23 and 24. Molecular mass standards (in kilodaltons) are shown on the left.

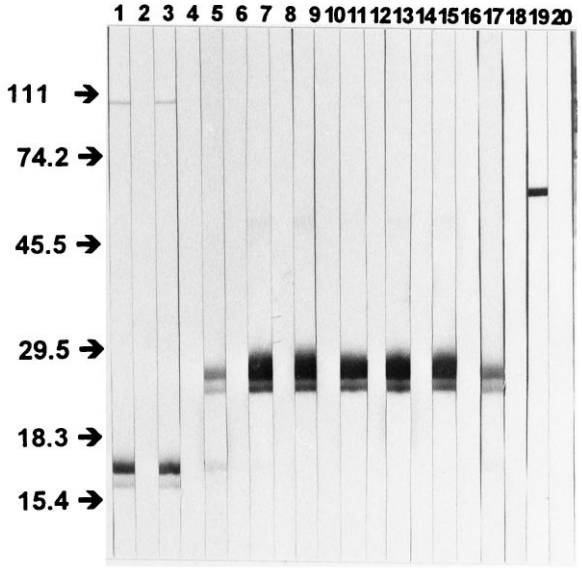

FIG. 6. Immunoblot of proteinase K-treated and untreated *M. mycoides* subsp. *mycoides* Triton X-114-phase proteins with fusion 4 MAbs. Lanes 1 to 18, detergent-phase proteins either treated with proteinase K (even-numbered lanes) or not treated (odd-numbered lanes). Lane 19, untreated aqueous-phase proteins; lane 20, aqueous-phase proteins treated with proteinase K. MAb 4/13.71.28 was reacted with lanes 1 and 2; 4/13.71.27 was reacted with lanes 3 and 4; 4/13.71.24 was reacted with lanes 5 and 6; 4/27.68.2 was reacted with lanes 7 and 8; 4/27.68.12 was reacted with lanes 9 and 10; 4/27.68.15 was reacted with lanes 11 and 12; 4/27.68.62 was reacted with lanes 13 and 14; 4/27.68.36 was reacted with lanes 15 and 16; 4/30.69.72 was reacted with lanes 17 and 18; and FGJ-B7 was reacted with lanes 19 and 20. Molecular mass standards (in kilodaltons) are shown on the left.

Binding of MAb 4/27.68.15 to 26- and 21-kDa proteins in the pellet was almost abolished by treatment with 0.4 mg of trypsin per ml (Fig. 7, lane 5) and was completely abolished with 0.6 mg/ml (lane 7). No reaction occurred with the treated supernatant. However, there was antigen present in the untreated supernatant (Fig. 7, lanes 2 and 15), possibly released by the live organisms. Results for MAbs 4/27.68.36 and 4/13.71.27 were similar to those described for 4/27.68.15 (data not shown). In contrast, binding of MAb FGJ-B7, which recognized an intracellular protein epitope, was not affected by trypsin treatment even at a concentration of 0.6 mg/ml (Fig. 7, lane 10). The viability of trypsin-treated and untreated organisms was not affected as determined by growth on agar plates.

Reactivity of MAbs with different *Mycoplasma* strains. MAbs (4/13.71.27, 4/27.68.12, and 4/27.68.36) representing the three different patterns of immunoblot reactivity were tested against *Mycoplasma* strains to determine specificity. Immunoblots were done on five *M. mycoides* subsp. *mycoides* small-colony and three large-colony strains, two *M. capricolum* strains, two bovine group 7 strains, one *M. capricolum* subsp. *capripneumonia* strain, and one *M. mycoides* subsp. *capri* strain. MAb 4/27.68.12 reacted strongly with a 26-kDa protein band of all small-colony strains (Fig. 8, lanes 1 to 3, 5, and 6) and weakly with a 21-kDa protein. This MAb did not react with the large-colony strains tested (Fig. 8, lanes 7, 9, and 12) but did bind a 49-kDa protein of *M. capricolum* (Fig. 8, lane 13). MAb 4/13.71.27 bound 96-, 16-, and 15-kDa proteins of the small-colony organisms tested (Fig. 8, lanes 16 to 18, 20, and 21) but did not bind large-colony strains (Fig. 8, lanes 22, 24, and 27). This MAb did bind 96- and 16-kDa proteins of one *M. capricolum* strain (Fig. 8, lane 28) and one bovine group 7 strain (lane 29) but did not react with another strain of *M. capricolum* (Fig. 8, lane 25) and of bovine group 7 (lane 26). The reactivity of MAb 4/27.68.36 with the various strains was similar to that of 4/27.68.12 (data not shown).

Evaluation of growth inhibition by MAbs to protein epitopes. None of the fusion 4 MAbs to integral membrane protein epitopes inhibited growth of *M. mycoides* subsp. *mycoides*. This was in contrast to the results obtained with fusion 72 MAbs, where seven MAbs recognizing carbohydrate epitopes caused growth inhibition (Table 1).

DISCUSSION

All of the MAbs made to disrupted whole *M. mycoides* subsp. *mycoides* organisms were IgM isotype and reacted with periodate-sensitive and proteinase K-insensitive epitopes, and seven caused in vitro growth inhibition. Even though growth inhibition was a desirable characteristic, reaction with carbohydrate epitopes prevented the potential use of these MAbs for identifying and isolating candidate proteins for a subunit vaccine. Therefore, proteins of *M. mycoides* subsp. *mycoides* that were soluble in Triton X-114 were used to immunize mice to generate additional MAbs against protein epitopes. This was done because integral membrane proteins, including p23 and p18 of *M. hyorhinis* (16) and those of other *Mycoplasma* spp. (20, 23, 28), partition selectively into the Triton X-114 detergent phase (3, 17, 36). The resulting fusion 4 MAbs all bound to Triton X-114-phase proteins, and binding was lost following proteinase K treatment of *M. mycoides* subsp. *mycoides* antigen, while periodate treatment had no effect. This was in contrast to the results obtained with the fusion 72 MAbs and control MAb PK-2 (anticarbohydrate), with which reactivity was completely abolished with periodate treatment.

Fusion 4 MAbs divided into three groups based on the molecular mass of the Triton X-114-phase proteins bound in immunoblots. It remains to be determined whether MAbs in the same group bind similar or different epitopes. It is anticipated that MAbs in different groups will recognize different epitopes, since (i) group 1 and 2 MAbs bound a 16-kDa protein, while only group 1 MAbs bound proteins of 96 and 15

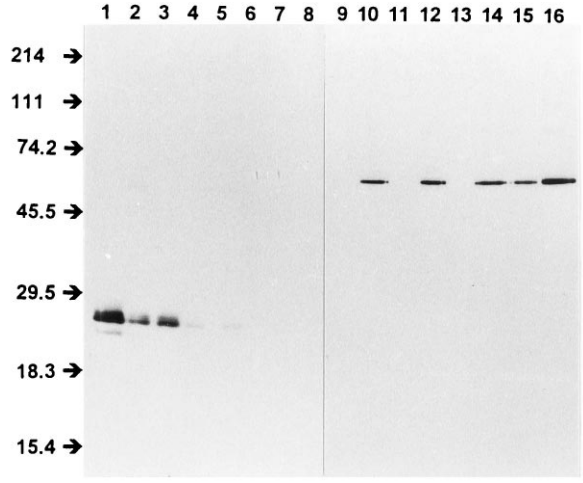

FIG. 7. Immunoblot of *M. mycoides* subsp. *mycoides* proteins following treatment of intact organisms with trypsin. Lanes 1 to 8 were reacted with MAb 4/22.68.15, and lanes 9 to 16 were reacted with MAb FGJ-B7. Organisms were treated with 0 (lanes 1, 2, 15, and 16), 0.1 (lanes 3, 4, 13, and 14), 0.4 (lanes 5, 6, 11, and 12), and 0.6 (lanes 7, 8, 9, and 10) mg of trypsin per ml and centrifuged before separation by SDS-PAGE. Lanes 1, 3, 5, 7, 10, 12, 14, and 16 contain the pellets; lanes 2, 4, 6, 8, 9, 11, 13, and 15 contain the supernatants. Molecular mass standards (in kilodaltons) are shown on the left.

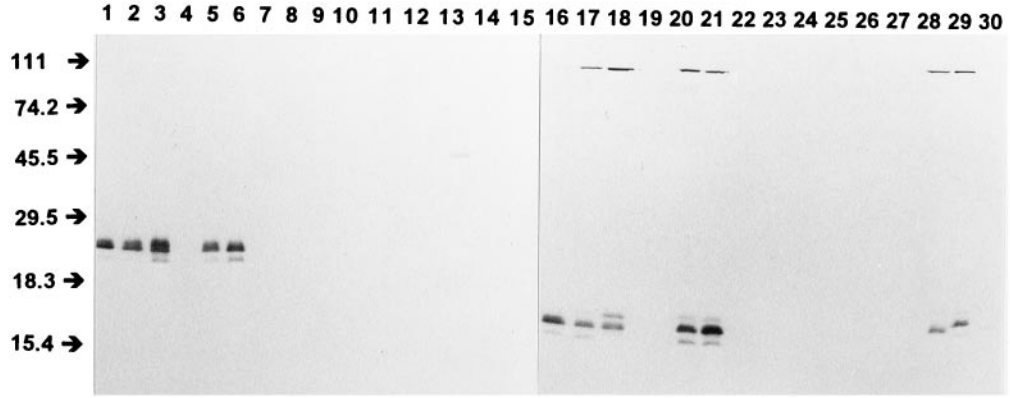

FIG. 8. Immunoblot of *M. mycoides* cluster strains (20 μg per lane) with fusion 4 MAbs. Lanes 1 to 3, 5, 6, 16 to 18, 20, and 21, *M. mycoides* subsp. *mycoides* small-colony strains; lanes 7, 9, 12, 22, 24, and 27, large-colony strains; lanes 4 and 19, *M. mycoides* subsp. *mycoides* unknown colony type; lanes 8 and 23, *M. mycoides* subspecies *capri*; lanes 10, 13, 25, and 28 *M. capricolum* strains; lanes 11, 14, 26, and 29, bovine group 7 strains; lanes 15 and 30, *M. capricolum* subsp. *capripneumonia*. Lanes 1 to 15 were reacted with MAb 4/27.68.12, and lanes 16 to 30 were reacted with MAb 4/13.71.27. Molecular mass standards (in kilodaltons) are shown on the left.

kDa; (ii) group 2, but not group 1, MAbs bound 26- and 21-kDa proteins; and (iii) group 2 and 3 MAbs bound 26- and 21-kDa proteins, and only group 2 MAbs bound a 16 kDa protein. All of the MAbs bound to more than one protein, and a likely explanation is that the proteins are derived by proteolysis from the largest protein recognized. However, other explanations are possible, including MAb binding to similar epitopes on multiple distinct proteins.

The question of whether the epitopes on integral membrane proteins were exposed on the surface of *M. mycoides* subsp. *mycoides* was answered by trypsin treatment of intact organisms. Binding of fusion 4 MAbs from each of the three groups was completely abolished by trypsin treatment. Reactive fragments were not recovered, indicating that either the epitope was completely destroyed or the resulting peptides were too small to detect in the immunoblot procedure. The trypsin treatment was selective for surface-exposed proteins, because neither the reaction of an intracellular protein epitope recognized by MAb FGJ-B7 or the viability was affected by treatment. Although this indicates that surface proteins are not important in vitro, they may be important for survival of the organism in vivo. For instance, surface proteolysis did not affect survival of *M. hominis* in vitro even though the surface proteins were found to be involved in organism attachment to fibroblasts (28).

When proteins of *M. mycoides* subsp. *mycoides* large- and small-colony strains were immunoblotted with selected fusion 4 MAbs, only the small-colony strains reacted. Thus, it appeared that these MAbs could be used to differentiate small- from large-colony strains. Similar differentiation with protein dot blots (30) and DNA probes (37) has been reported. In addition, six MAbs to a 70-kDa protein reacted specifically with small-colony strains (4). However, when the three MAbs in this study were reacted with other strains from the *M. mycoides* cluster (27), binding to one strain each of *M. capricolum* and bovine group 7 occurred. This is not unusual, as one-way cross-reactivity between bovine group 7 and *M. mycoides* subsp. *mycoides* strains has been reported (30). It was unexpected that the MAbs in this study reacted with only one strain, but not the other, of *M. capricolum* and bovine group 7.

It was concluded that the fusion 4 MAbs reacted with epitopes on surface-exposed integral membrane proteins of *M. mycoides* subsp. *mycoides* that were heat stable at 100°C, proteinase K sensitive, and periodate insensitive. Heat stability was demonstrated by reactivity with MAbs after boiling in SDS-PAGE sample buffer. Even though these MAbs did not cause in vitro growth inhibition, they will be useful to isolate the identified proteins for animal immunization, since polyclonal antisera to one or more of the proteins might have growth-inhibiting or other protective immune functions.

ACKNOWLEDGMENTS

The technical assistance of Pat Mason, Anderson Wambugu, Abuu Oriko, Alphonso Ndirangu, and John Thiongo is gratefully acknowledged.

This work was supported by U.S. Agency for International Development grants COM-5542-G-00-0014-00 and DAN-1328-G-00-0046-0, the Kenya Agricultural Research Institute, and Washington State University.

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