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Characterization of an Immunoreactive 17.5-Kilodalton Outer Membrane Protein of *Haemophilus somnus* by Using a Monoclonal Antibody

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A single outer membrane protein (OMP) of *Haemophilus somnus*, with an apparent molecular mass of 17.5 kDa, was identified in the sodium dodecyl sulfate (SDS)-insoluble fraction after extraction with 1% SDS–0.5 M NaCl–0.1% β-mercaptoethanol. A hybridoma derived from mice immunized with *H. somnus* OMP fractions produced a monoclonal antibody (MAb), designated 20-3-5, that bound to the 17.5-kDa OMP of *H. somnus*. The MAb 20-3-5 epitope was present on 45 of 45 strains of *H. somnus* tested. MAb 20-3-5 cross-reacted with *Haemophilus agni*, *Histophilus ovis*, and *Haemophilus haemoglobinophilus* but not with 13 other species and subspecies of gram-negative bacteria. Immunoelectron-microscopic and antibody absorption studies revealed that the MAb 20-3-5 epitope is exposed on the surface of bacteria. In an immunoblot analysis, convalescent-phase sera obtained from calves with experimental *H. somnus* pneumonia contained antibodies to the 17.5-kDa OMP of *H. somnus*. Future studies will be directed toward examining the role of the 17.5-kDa OMP in immunity to *H. somnus* infections.

Haemophilus somnus is an important bovine pathogen which causes several diseases, including thromboembolic meningoencephalitis, septicemia, pneumonia, abortion, and arthritis (5). To understand the pathogenesis of *H. somnus* infections and to develop a new vaccine and an immunodiagnostic tool for the diseases it causes, several studies have examined the antigenic properties of *H. somnus* surface structures, including lipopolysaccharide (LPS) and outer membrane proteins (OMPs) (2, 6, 7, 10, 15–18, 21). Of these surface components, the 37-kDa heat-modifiable OMP, the 39-kDa OMP, the 40-kDa lipoprotein, the 40- to 41-kDa major OMP (Fc receptor protein), and the 78-kDa OMP are immunogenic to animals, antigenically conserved among strains, and surface exposed (2, 7, 15, 16, 21). The 40-kDa lipoprotein has been shown to be protective in a passive-protection experiment (4).

In this paper, we describe the production and characterization of a monoclonal antibody (MAb) that reacts with an antigen epitope that is located on a 17.5-kDa OMP of *H. somnus*, is conserved among all strains of *H. somnus* tested, and is exposed on the bacterial surface. We also examine the presence of antibodies to the 17.5-kDa OMP in calves with experimental *H. somnus* pneumonia.

MATERIALS AND METHODS

Bacterial strains. *H. somnus* strains used in this study have been described previously (15, 17). Other bacteria used to test MAb reactivity have been described previously (15). Bacterial culture media and growth conditions have been described previously (15, 17).

Preparation of OMP fractions. Sarkosyl-insoluble OMP fractions were prepared from *H. somnus* 8025, D1238, NT2301, and 540 as described previously (17).

Preparation of LPS. LPS was prepared from *H. somnus* 8025 by the hot-phenol method of Westphal and Jann (20), as described previously (17).

Isolation of an SDS-insoluble fraction. A sodium dodecyl sulfate (SDS)-insoluble fraction was isolated by a modification of the methods of Munson and Granoff (8) and Murphy et al. (9). The Sarkosyl-insoluble OMP fraction prepared from strain 8025 was used as the starting material. It was suspended in buffer A (0.5% Zwittergent 3-14, 50 mM Tris, 10 mM EDTA [pH 8.0]) and incubated at room temperature for 30 min. The insoluble residue was harvested by centrifugation at 105,000 × *g* for 60 min, suspended and incubated in buffer A, and recovered. The insoluble residue was then suspended in buffer A containing 0.5 M NaCl and treated as above. The Zwittergent 3-14–NaCl-insoluble residue was suspended in 1% SDS–20 mM sodium phosphate buffer (pH 7.5). After incubation at room temperature for 30 min, the insoluble residue was recovered by centrifugation as described above. The SDS-insoluble residue was suspended in buffer B (1% SDS, 0.1 M Tris, 0.5 M NaCl, 0.1% β-mercaptoethanol [pH 8.0]) and incubated at 37°C for 30 min. The insoluble residue was recovered by centrifugation as described above and then treated once more with buffer B under the same conditions. The SDS-insoluble fraction was washed twice with water and stored at −70°C.

MAb production. An MAb was produced by fusing spleen cells from BALB/c mice immunized with a mixture of the OMP preparations from four strains of *H. somnus* with P3U1 mouse myeloma cells, as described previously (17). MAbs in the form of cell culture supernatant fluids were used for all experiments except the antibody absorption experiment, for which an MAb in the form of mouse ascitic fluid was used.

Sera from calves with experimental *H. somnus* pneumonia. We used sera collected from three Japanese black male calves (calves 9001, 9007, and 9111) which were inoculated intrabronchially with 10^9 CFU of *H. somnus* NT2301 as described previously (15).

SDS-PAGE. Samples were heated at 100°C for 5 min in sample buffer as described previously (17) and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE)

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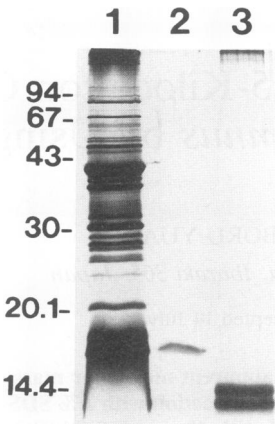

FIG. 1. Silver-stained SDS-PAGE gel (14% acrylamide) showing isolation of the SDS-insoluble fraction prepared from *H. somnus* 8025. Lanes: 1, Sarkosyl-insoluble OMP fraction (5 μg of protein); 2, SDS-insoluble fraction (0.6 μg of protein); 3, LPS preparation (15 μg). Molecular mass standards (in kilodaltons) are noted on the left.

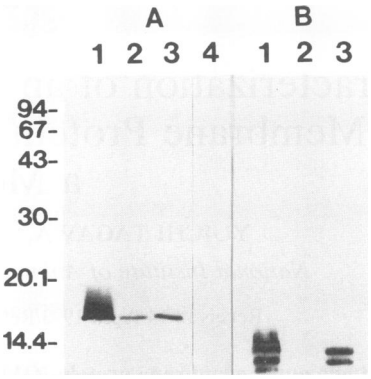

FIG. 2. Immunoblot analysis of OMP fraction, whole-cell lysate, SDS-insoluble fraction, and LPS preparation from *H. somnus* 8025 probed with MAbs 20-3-5 (A) and 2-15-5 to *H. somnus* LPS (B). MAbs 20-3-5 and 2-15-5 were diluted 1:10 and 1:20, respectively. (A) Lanes: 1, Sarkosyl-insoluble OMP fraction (2.5 μg of protein); 2, whole-cell lysate (3 μg of protein); 3, SDS-insoluble fraction (0.1 μg of protein); 4, LPS preparation (3 μg). (B) Lanes: 1, whole-cell lysate (3 μg of protein); 2, SDS-insoluble fraction (0.1 μg of protein); 3, LPS preparation (3 μg). Molecular mass standards (in kilodaltons) are noted on the left.

with 14% separating gels. The gels were subjected to Coomassie brilliant blue stain (17), silver stain by using a commercial kit (Daiichi Pure Chemicals Co.), or immunoblot analysis.

Protein determination. The protein concentration was measured by the bicinchoninic acid method (13) as described previously (17).

Immunological procedures. Enzyme-linked immunosorbent assay (ELISA) with OMP and LPS preparations as the antigens was performed as described previously (17). ELISA values were expressed as the A_{492}s (means ± standard deviations) for two experiments. Immunodot assay with boiled whole-cell antigens (16) and immunoblot analysis with OMP and LPS preparations and whole-cell lysates as the antigens (17) were performed as described previously. Immunoelectron microscopy with freshly prepared bacteria and goat anti-mouse immunoglobulin G conjugated to colloidal gold spheres (15) and the antibody absorption experiment (15) were also performed as described previously. An MAb prepared as a mouse ascitic fluid was diluted 1:40,000 and then absorbed with log-phase live bacterial cells of *H. somnus* 8025. After absorption, the MAb fluid was diluted to a final concentration of 1:400,000 and then examined for antibody reactivity by immunoblot analysis.

RESULTS

Isolation of an SDS-insoluble fraction. A 17.5-kDa OMP was the only protein detected in the SDS-insoluble fraction by silver staining (Fig. 1). The 17.5-kDa OMP band could also be stained with Coomassie brilliant blue but only faintly (data not shown). The yield from 43 mg (protein content) of OMP fraction was approximately 80 μg because the 17.5-kDa OMP is not completely insoluble in 1% SDS buffer at 37°C since a band at 17.5 kDa is visible when 1% SDS buffer-soluble fraction is subjected to SDS-PAGE (data not shown).

Epitope specificity of MAb. ELISA with undiluted MAb culture supernatants against OMP and LPS preparations from strain 8025 as the antigens demonstrated that an MAb, designated 20-3-5 (isotype immunoglobulin G2b), reacted with the OMP preparation (A_{492}, 0.850 ± 0.026) but not with the LPS preparation (A_{492}, 0.041 ± 0.001). The control LPS-specific MAb 2-15-5 reacted with both the OMP preparation (A_{492}, 1.261 ± 0.144) and the LPS preparation (A_{492}, 2.178 ± 0.132). Immunoblot analysis with the OMP fraction, whole-cell lysate, SDS-insoluble fraction, and separated LPS prepared from strain 8025 as the antigens revealed that the antibody reactivity of MAb 20-3-5 was monoclonal and was directed to the 17.5-kDa OMP but not to LPS (Fig. 2A). A control experiment with MAb 2-15-5 showed that this MAb bound to double bands corresponding to the LPS components from strain 8025 in a lower-molecular-mass range than 17.5 kDa (Fig. 2B).

Reactivity of MAb 20-3-5 with *H. somnus* strains and other bacterial species. In immunodot assays, MAb 20-3-5 reacted with 45 of 45 strains of *H. somnus* tested and with strains from three other species: *Haemophilus agni*, *Histophilus ovis*, and *Haemophilus haemoglobinophilus* (Table 1). It

TABLE 1. Reactivity of MAb 20-3-5 with a variety of gram-negative bacteria in the immunodot assay

Species	No. of strains:	
	Tested	Positive
Haemophilus somnus	45	45
Haemophilus agni	1	1
Histophilus ovis	3	3
Haemophilus haemoglobinophilus[a]	1	1
Haemophilus influenzae[a]	1	0
Haemophilus parainfluenzae[a]	1	0
Actinobacillus lignieresii[a]	1	0
Actinobacillus seminis	1	0
Pasteurella multocida[a]	3	0
Pasteurella haemolytica[a]	3	0
Brucella abortus	1	0
Moraxella bovis[a]	1	0
Campylobacter species[b]	3	0
Escherichia coli	2	0
Salmonella dublin	2	0

[a] The type strain was tested, or the test strains included the type strain.
[b] One strain each of *Campylobacter fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and *C. sputorum* subsp. *bubulus*.

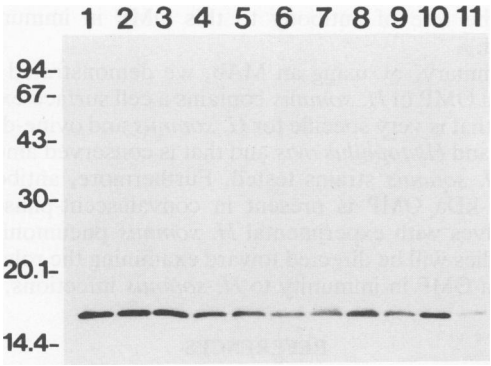

FIG. 3. Immunoblot analysis of whole-cell lysates (3 μg of protein per lane) probed with MAb 20-3-5. Lanes: 1 to 8, *H. somnus* 8025, D1238, NT2301, 540, ATCC 25836, 2359, 43, and 719, respectively; 9, *H. agni* 1344; 10, *Histophilus ovis* H989; 11, *H. haemoglobinophilus* NCTC 1659. MAb 20-3-5 was diluted 1:10. Molecular mass standards (in kilodaltons) are noted on the left.

gave no detectable reaction with strains from the other 13 gram-negative species and subspecies tested. In immunoblot analysis with whole-cell lysates prepared from all of the *H. somnus*, *H. agni*, *Histophilus ovis*, and *H. haemoglobinophilus* strains tested, MAb 20-3-5 reacted with a protein with an apparent molecular mass of 17.5 kDa in all of the 50 strains tested. Figure 3 shows a representative immunoblot analysis result. These data indicate that the antigen epitope recognized by MAb 20-3-5 is conserved among strains of *H. somnus* and is shared by *H. agni*, *Histophilus ovis*, and *H. haemoglobinophilus*. They further indicate that the molecular mass of the reactive antigens is identical among all strains from the above four species within the experimental accuracy of SDS-PAGE.

Surface exposure of the MAb 20-3-5 epitope. To determine whether MAb 20-3-5 reacts with a surface-exposed epitope of the 17.5-kDa OMP of *H. somnus*, we examined a freshly prepared bacterial suspension by immunoelectron microscopy. MAb 20-3-5 showed positive staining on the bacterial cells, but P3U1 cell culture supernatant (as a negative control) showed no staining (Fig. 4). To confirm the surface exposure of the MAb 20-3-5 epitope, we absorbed MAb with log-phase whole cells and tested the resulting absorbed MAb preparation in immunoblot analysis. In this experiment, absorption of MAb 20-3-5 resulted in a decrease in the intensity of the band at 17.5 kDa compared with unabsorbed MAb (Fig. 5). From these results, we conclude that the MAb 20-3-5 epitope is exposed on the bacterial surface.

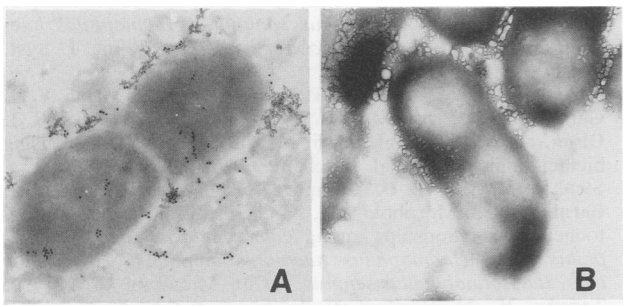

FIG. 4. Immunoelectron micrographs of *H. somnus* 8025 whole cells incubated with MAb 20-3-5 (A) and a P3U1 cell culture supernatant (as a negative control) (B) and then with goat anti-mouse immunoglobulin G conjugated to colloidal-gold spheres.

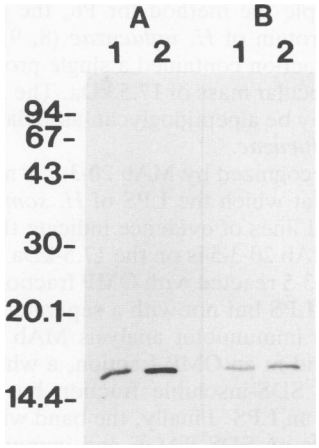

FIG. 5. Immunoblot analysis of the reactivity of MAb 20-3-5 absorbed with log-phase whole cells of *H. somnus* 8025. (A) Unabsorbed MAb 20-3-5. (B) Absorbed MAb 20-3-5. Lanes: 1, whole-cell lysate (6 μg of protein) from *H. somnus* 8025; 2, SDS-insoluble fraction (0.1 μg of protein) from *H. somnus* 8025. Molecular mass standards (in kilodaltons) are noted on the left.

Reactivity of sera from calves with experimental *H. somnus* pneumonia. To determine whether sera from calves with experimental *H. somnus* pneumonia contain antibodies to the 17.5-kDa OMP, we performed immunoblot analysis with a whole-cell lysate and an SDS-insoluble fraction as the antigens. Convalescent-phase sera (1:400), which were collected at 2 weeks (calf 9001) and 6 weeks (calves 9007 and 9111) after infection, reacted strongly with the 17.5-kDa OMP (Fig. 6A to C). Preinfection sera (1:400) from the three calves reacted with an antigen band at 43 kDa but not with other antigen bands, as shown for calf 9007 in Fig. 6D. These results indicate that the 17.5-kDa OMP is antigenic and elicits an antibody response in *H. somnus*-infected animals.

DISCUSSION

In this study, resistance to solubilization with SDS was used in the isolation of the 17.5-kDa OMP of *H. somnus*.

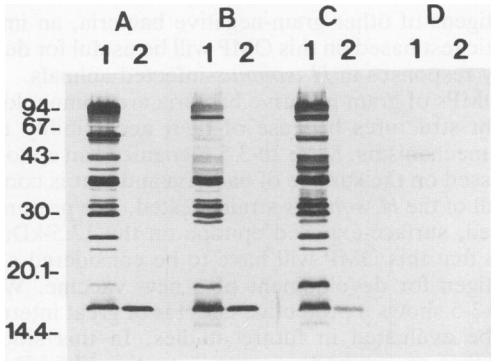

FIG. 6. Immunoblot analysis of the reactivity of sera from calves with experimental *H. somnus* pneumonia. Convalescent-phase sera from calves 9001 (A), 9007 (B), and 9111 (C) and preinfection serum from calf 9007 (D) were diluted 1:400. Lanes: 1, whole-cell lysate (6 μg of protein) from *H. somnus* 8025; 2, SDS-insoluble fraction (0.1 μg of protein) from *H. somnus* 8025. Molecular mass standards (in kilodaltons) are noted on the left.

This is in principle the method for P6, the peptidoglycan-associated lipoprotein of *H. influenzae* (8, 9). The isolated SDS-insoluble fraction contained a single protein band with an apparent molecular mass of 17.5 kDa. The 17.5-kDa OMP of *H. somnus* may be a peptidoglycan-associated lipoprotein like P6 of *H. influenzae*.

The antigen recognized by MAb 20-3-5 is near the molecular mass range at which the LPS of *H. somnus* separates; however, several lines of evidence indicate that the epitope recognized by MAb 20-3-5 is on the 17.5-kDa OMP. First, in ELISA MAb 20-3-5 reacted with OMP fractions that contain both OMPs and LPS but not with a separated LPS preparation. Second, in immunoblot analysis MAb 20-3-5 reacted with a single band in an OMP fraction, a whole-cell lysate, and an isolated SDS-insoluble fraction but not with any determinants on an LPS. Finally, the band with a molecular mass of 17.5 kDa on SDS-PAGE and immunoblot analysis was apparently distinct from double bands corresponding to LPS components. Taken together, these observations indicate that the epitope recognized by MAb 20-3-5 resides on the 17.5-kDa OMP.

The cross-reactivity of MAb 20-3-5 with a variety of gram-negative bacteria was examined because of the importance of specificity in immunodiagnostic assays for detecting *H. somnus* antigens in clinical specimens and antibodies to *H. somnus* in sera from *H. somnus*-infected animals. None of the other bacteria tested with MAb 20-3-5 reacted in an immunodot assay, except *H. agni*, *Histophilus ovis*, and *H. haemoglobinophilus*, which possessed an antigen with a molecular mass similar to that of the 17.5-kDa OMP of *H. somnus* in immunoblot analysis. This may account for the antigenic similarity of *H. somnus*, *H. agni*, *Histophilus ovis*, and *H. haemoglobinophilus* (1, 14). DNA-DNA and DNA-rRNA hybridization studies indicate that *H. somnus* and ovine-derived *H. agni* and *Histophilus ovis* constitute a single species (3, 12, 19). *H. haemoglobinophilus* has been isolated from preputial secretions in dogs and has occasionally been implicated in vaginitis in dogs (11). This organism is thought to be an unusual pathogen for cattle and sheep. Therefore, the cross-reactivity among these bacteria should not lead to confusion in immunodiagnostic assays. MAb 20-3-5 will be useful for rapid detection and identification of *H. somnus* because this MAb recognizes an antibody-accessible, surface-exposed epitope. Furthermore, if polyclonal antibodies to the 17.5-kDa OMP show no cross-reactivity with antigens of other gram-negative bacteria, an immunodiagnostic test based on this OMP will be useful for detecting antibody responses in *H. somnus*-infected animals.

The OMPs of gram-negative bacteria are immunologically important structures because of their accessibility to host defense mechanisms. MAb 20-3-5 recognized an epitope that is expressed on the surface of bacteria and that is conserved among all of the *H. somnus* strains tested. The presence of a conserved, surface-exposed epitope on the 17.5-kDa OMP suggests that this OMP will have to be considered a candidate antigen for development of a new vaccine. Whether MAb 20-3-5 shows a protective effect is of great interest and should be evaluated in future studies. In this study, we showed that an antibody response to the 17.5-kDa OMP occurred in calves during experimental *H. somnus* infection. The presence of antibody to the OMP in convalescent-phase serum suggests that this OMP is potentially important with regard to the bovine antibody response to *H. somnus*. Further studies should examine the antibody response to the 17.5-kDa OMP in naturally H. somnus-infected calves and assess the role of antibody to this OMP in immunity to infection.

In summary, by using an MAb, we demonstrated that a 17.5-kDa OMP of *H. somnus* contains a cell surface-exposed epitope that is very specific for *H. somnus* and ovine-derived *H. agni* and *Histophilus ovis* and that is conserved among all of the *H. somnus* strains tested. Furthermore, antibody to the 17.5-kDa OMP is present in convalescent-phase sera from calves with experimental *H. somnus* pneumonia. Future studies will be directed toward examining the role of the 17.5-kDa OMP in immunity to *H. somnus* infections.

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