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Characterization of *Borrelia coriaceae* Antigens with Monoclonal Antibodies

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Three monoclonal antibodies (F6F3, F6B11, and F6B3) were developed against *Borrelia coriaceae* antigens. All three antibodies appeared to be specific for this species and did not cross-react with *Borrelia burgdorferi* (strains B31 and IRS), *Borrelia hermsii*, *Borrelia anserina*, *Leptospira interrogans* serovar hardjo, or *Treponema hyodysenteriae*, as determined by indirect fluorescent antibody staining, enzyme-linked immunosorbent assay, and Western immunoblot analysis. Only one of these antibodies, F6B3, bound to spirochetes present in organ smears from the argasid tick, *Ornithodoros coriaceus*. The antigens recognized by F6F3, F6B11, and F6B3 have apparent molecular weights of ca. 37,000, 35,000, and 16,000, respectively, as determined by Western blot analysis. Antigens were analyzed by immune electron microscopy as well as Western blot and indirect fluorescent antibody staining analysis of spirochetes after enzyme (trypsin and protease K) and detergent (Triton X-100) treatments. These studies suggest that all three antigens are integral membrane proteins. The characteristics of the 37K and 35K proteins are consistent with the outer surface proteins of *B. burgdorferi* (OSP A and OSP B) described by Barbour et al. (A. G. Barbour, S. L. Tessier, and S. F. Hayes, Infect. Immun. 45:94–100, 1984), while data regarding the 16K protein are less conclusive but may suggest a cytoplasmic membrane location. We suggest that the 37K, 35K, and 16K antigens be designated integral membrane proteins A, B, and C, respectively, as a result of these studies.

The spirochete *Borrelia coriaceae* was first reported in 1985 after it was isolated from the argasid tick, *Ornithodoros coriaceus* (7). Through immunological and electron micrographic techniques, this organism was determined to belong in the taxonomical classification *Borrelia* and was distinct from any other previously described spirochete (7).

B. coriaceae has been suggested as the causal agent of epizootic bovine abortion (EBA) in cattle (7, 8). This disease manifests itself in the abortion of 6- to 8-month-gestation fetuses. The lesions are characteristic and include subcutaneous edema, ascites, generalized hemorrhage, and hepatopathy (4). Little is known regarding the etiology of the disease, but *O. coriaceus* has been shown to be a biological vector (18). Circumstantial evidence connecting this spirochete to EBA has been outlined previously (7) and includes (i) the high percentage of adult *O. coriaceus* infected with *B. coriaceae* in a recognized EBA area, (ii) the presence of the spirochete in the salivary glands of most ticks examined, and (iii) the finding that prophylactic treatment of cattle with chlortetracycline reduces the rate of EBA under field conditions (9) and suggests the agent is antibiotic sensitive (a trait characteristic of spirochetes). Direct evidence of *B. coriaceae*'s role in this disease is lacking, and *B. coriaceae* has never been isolated from the bovine.

A spirochete with a morphology similar to that of *B. coriaceae* was observed in thick blood smears of several deer (6), a known host of *O. coriaceus*. The organisms in the smears reacted with polyvalent *Borrelia* antisera as well as a genus-specific *Borrelia* monoclonal antibody (H9724) but not with *Borrelia burgdorferi*-specific monoclonal antibody (H5332) (6). Investigators were unable to culture these organisms or otherwise confirm their identity.

A spirochetelike organism has been reported in EBA fetuses (12, 13), but the morphology of this organism appeared to differ from that of *B. coriaceae*. It has been reported to have a length similar to that of *B. coriaceae* and to be approximately half the width of that spirochete. However, dark-field and electron micrographs provided in the original papers describing each of these organisms (7, 13) list magnifications of the spirochetelike organisms as being two to five times larger than those provided with *B. coriaceae* micrographs. Neither paper provided scale bars with the micrographs, so resolution of this discrepancy is difficult.

Effective reagents are needed to facilitate investigation of the possible connection between *B. coriaceae* and EBA. The difficulty in isolating spirochetes exacerbates this need, not only for localizing spirochetes in tissue samples but also for identifying organisms that cannot be propagated. Polyclonal antibodies typically produce unacceptable background levels for examining tissues and body fluids of both mammals and ticks and are ineffective in species identification because of extensive cross-reactivity. Production of *B. coriaceae*-specific monoclonal antibodies would greatly enhance efforts directed toward diagnostics and pathogenesis studies.

This paper describes the specificity of three monoclonal antibodies and characterizes the *B. coriaceae* antigens to which they react. The monoclonal antibodies were mixed with different spirochete species and examined by indirect fluorescent antibody staining (IFA), Western immunoblot, and/or enzyme-linked immunosorbent assay (ELISA) techniques to determine species specificity. The apparent molecular weights of these antigens were determined by Western blot analysis. IFA and Western blotting were used in conjunction with protease and detergent treatments of the spirochetes to gain insight as to antigen location. Immune electron microscopy (IEM) was also utilized for this latter purpose.

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MATERIALS AND METHODS

Organisms. The *Borrelia* species utilized for this study were as follows: *B. coriaceae* Co53 (ATCC 43381; *O. coriaceus* isolate), *B. burgdorferi* B31 (ATCC 35210; *Ixodes dammini* isolate), *B. burgdorferi* IRS (ATCC 35211; *Ixodes ricinus* isolate), *B. hermsii* (ATCC 35209; *Ornithodoros hermsi* isolate), and *B. anserina* (clinical chicken isolate; R. Walker, California Veterinary Diagnostic Laboratory, University of California, Davis). All *Borrelia* spp. were grown in modified Barbour-Stoener-Kelley medium (1) with 7% rabbit serum at 34°C. Six- to eight-day cultures were used for all procedures. *Borrelia* spp. were pelleted in a microcentrifuge (Eppendorf 5415; Brinkman Instruments, Inc., Westbury, N.Y.) at 12,000 × *g* for 3 min at room temperature and washed three times in 1/15 M phosphate-buffered saline (pH 7.4) with 5 mM $MgCl_2$ (PBS/Mg) (3), unless otherwise indicated.

Leptospira interrogans serovar hardjo (reference strain, hardjoprajitno) was provided by R. LeFebvre, University of California, Davis. The cells had been grown in EMJH medium (19a) at 29°C. These cells were harvested in the same manner as were the *Borrelia* isolates.

Treponema hyodysenteriae was a clinical swine isolate (provided by R. Walker, California Veterinary Diagnostic Laboratory). The culture had been grown on blood agar plates under anaerobic conditions. Organisms were harvested and washed as with *Borrelia* species.

Tick dissection. *O. coriaceus* ticks were collected by using CO_2-baited traps from EBA-endemic areas. Females were utilized exclusively, as they are the most likely to harbor the organism (7). Tick organs were removed, suspended in PBS/Mg, and macerated. Five microliters of sample was placed on 12-well Teflon-coated slides (Cel-Line Associates Inc., Newfield, N.J.), allowed to air dry, fixed in acetone, and stained by IFA.

Monoclonal antibody production. *B. coriaceae* cultures were washed and resuspended to a concentration of ca. 10^{10}/100 μl in PBS/Mg, boiled for 5 min, and suspended in an equal volume of Freund's incomplete adjuvant. Preparations were injected subcutaneously into BALB/c mice, on two occasions, 3 weeks apart. A final intraperitoneal injection without adjuvant was given 3 days before fusion of mouse spleen cells with P3X63-Ag8.653 myeloma cells at a ratio of 1:1. Monoclonal antibodies were produced as previously described (11). Antibodies were screened by IFA, ELISA, and Western blot. Polyclonal antiserum to all *Borrelia* species was prepared by this same immunization schedule. Blood from hyperimmunized mice was recovered by heart puncture.

ELISA. Antigen preparation procedures were adapted from the method of Russell et al. (17). *Borrelia* cultures were washed, suspended in PBS/Mg, and sonicated (Sonifier cell disrupter; Heat Systems Co., Melville, N.Y.) in an ice bath for 10 min by using a Microtip (model 420; Heat Systems Co.) at 40% output. Protein concentration was determined with a bicinchoninic acid reagent kit (Pierce Chemical Co., Rockford, Ill.) per the manufacturer's instructions. Antigen suspensions were diluted in carbonate buffer (45 mM $NaHCO_3$, 18 mM Na_2CO_3; pH 9.6) to a final concentration of 5 μg/ml. Fifty microliters of this preparation was used to sensitize each well of 96-well Probind plates (Becton Dickinson Labware, Oxnard, Calif.) overnight at 4°C. Undiluted monoclonal antibody supernatants were tested with the Mono Ab-Screen ID Kit (Zymed Laboratories Inc., South San Francisco, Calif.) by following the manufacturer's instructions. This same kit was used in isotype determination of the antibodies. Controls consisted of irrelevant mouse immunoglobulin G1(κ) [IgG1(κ)] (MOPC21)-clarified ascitic fluid (mouse IgG1 ascitic fluid; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 μg/ml, negative mouse serum diluted 1:100, and serially diluted mouse polyvalent antisera. Those samples with optical density values three times greater than those of the negative controls were considered positive.

IEM. IEM was performed on *B. coriaceae* as previously described (21), except for minor alterations. Briefly, live spirochetes were pelleted at 9,500 × *g* in microcentrifuge tubes and washed twice in PBS/Mg. Pellets were resuspended in 100 μl of monoclonal antibody supernatant, incubated for 1 h at 4°C, and washed three times in PBS/Mg. The pellet was resuspended in 100 μl of a 1:3 dilution of 10-nm colloidal gold-labeled goat anti-mouse immunoglobulin (Auroprobe; Janssen Life Science Products, Olen, Belgium) with 1% bovine serum albumin and incubated for 1 h at 4°C. Spirochetes were washed as before and suspended in 100 μl of PBS, and a 10-μl sample was placed on Formvar-coated grids. Excess fluid was wicked off after 10 min. A drop of 0.15% ammonium molybdate was added to the grid and wicked off after 30 s. Samples were examined with a Zeiss EM-109 electron microscope at 80 kV. Controls included irrelevant mouse IgG1-clarified ascitic fluid and mouse anti-*B. coriaceae* polyvalent antiserum.

IFA. Spirochetes were prepared as described by Russell et al. (17) and applied to 48- and 12-well Teflon-coated slides (Cel-Line Associates Inc.), by using 3 and 10 μl of washed (three times) spirochetes per well, respectively. All slides were fixed in acetone for 10 min and allowed to air dry before immediate use or being stored at −20°C until needed. Forty-eight-well slides contained untreated *Borrelia* preparations and were used to screen fusion supernatants. Twelve-well slides contained untreated as well as enzyme- and detergent-treated samples (described below) for studying specific antibody interactions. The slides were stained in a procedure similar to that described previously (17), with the following exceptions. Undiluted monoclonal antibody supernatants were added in volumes of 5 and 20 μl to wells of 48- and 12-well slides, respectively. The secondary conjugate was fluorescein isothiocyanate-labeled goat anti-mouse IgG (Zymed) diluted 1:30. The slides were mounted with 70% glycerol in PBS with 2% *n*-propyl gallate and examined by fluorescent microscopy. Controls consisted of irrelevant mouse IgG1-clarified ascitic fluid and mouse polyvalent antisera diluted 1:100.

Protease treatments. Protease treatments were performed similarly to a previously described procedure (2) with some variation. Briefly, trypsin and protease K were prepared at concentrations of 3 and 4 mg/ml, respectively. Cells were washed twice and resuspended in 475 μl of PBS/Mg with 25 μl of the appropriate protease. Protease-treated samples were incubated for 20 min at room temperature, after which 5 μl of 0.2 M phenylmethylsulfonyl fluoride (PMSF; solubilized in isopropanol; Sigma) was added to neutralize the enzyme activity. Pellets were washed once in PBS/Mg with 2 mM PMSF and resuspended in 50 μl of PBS/Mg–2 mM PMSF. Two microliters of suspension was removed from the resuspended pellets and diluted in PBS/Mg for IFA preparations (see above); the remaining suspension was reserved for polyacrylamide gel electrophoresis (PAGE) analysis (see below).

Detergent treatments. Triton X-100 (TX; Sigma) extractions of outer envelope proteins were performed on pellets of

ca. 2×10^9 cells (14). Pellets were washed, resuspended in 50 μl of 0.2% TX–2 mM PMSF, incubated for 30 min at 37°C, and pelleted at 16,000 × *g* for 10 min at room temperature. The supernatant was removed, mixed with sample application buffer that included 10% 2-mercaptoethanol, and retained for PAGE. The pellet was washed once in PBS/Mg–2 mM PMSF, resuspended in 50 μl of PBS/Mg–2 mM PMSF, and prepared for PAGE (see below). To test the susceptibility of dissociated antigens to in situ proteolysis, pellets were initially treated with 0.5% TX in the same manner as the 0.2% TX samples, but without PMSF. After the supernatant was removed from these pellets, the pellets were washed and resuspended in 50 μl of PBS/Mg. Five microliters of enzyme was added to both supernatant and pellet samples. These were incubated for 20 min at room temperature, and then 1 μl of 0.2 M PMSF was added. Sample application buffer was then added, and the samples were prepared for PAGE.

Control pellets containing ca. 2×10^9 organisms were washed, resuspended in 50 μl of PBS/Mg–2 mM PMSF, and handled in the same manner as TX-treated samples were.

PAGE and Western blot analysis. Discontinuous sodium dodecyl sulfate (SDS)-PAGE was performed as described by Laemmli (5) with gels containing either 12 or 15% acrylamide under denaturing conditions. All samples (described above) to be loaded onto gels for blotting were mixed with equal volumes of sample application buffer and boiled for 5 min. Loading of samples on the gel varied as to the gel application and was as follows. (i) For screening blots, pellets contained ca. 10^{10} cells and were washed, resuspended in 150 μl of PBS/Mg with an equal volume of sample application buffer, and loaded into a well made with a single-well comb. (ii) For PAGE gels stained with Coomassie brilliant blue R-250, the entire volume of sample from treated and control preparations was loaded into wells (15-well comb). (iii) For gels for blotting, wells (15-well comb) were loaded with either the entire sample volume (chloro-naphthol detection system) or 1/5 volume (enhanced chemiluminescence detection system) of treated and control preparations. Molecular weight standards were run with each gel (low-range molecular weight protein standards; Bio-Rad Laboratories, Richmond, Calif.; or Rainbow Markers; Amersham Corp., Arlington Heights, Ill.).

The proteins from all gels used for blotting purposes were transferred from SDS-PAGE gels to Immobilon P (Millipore, South San Francisco, Calif.) via a Milliblot-SDE electroblotting unit (Millipore) for 30 min at an initial setting of 2.5 mA/cm^2 of membrane as suggested by the manufacturer.

Two blotting techniques were utilized to visualize antigen. A conventional chloro-naphthol substrate for membrane development was used to visualize F6F3 and F6B11 reactions. A second detection system, enhanced chemiluminescence (Amersham), was required to analyze F6B3 binding, as this antibody did not blot with the chloro-naphthol detection system.

Though the two blotting methodologies were similar, some variation existed. Membranes to be developed with chloro-naphthol were blocked with 1% gelatin (derived from fish skin; Sigma) and 0.5% Tween 20 (Sigma) in Tris-buffered saline (TBS-Tween; 20 mM Tris, 500 mM NaCl, pH 7.5) at room temperature for 1 h. Blots were washed in TBS-Tween and incubated for 1 h with a 1:2 dilution of monoclonal antibody in blocking reagent. After washing, the membrane was incubated for 1 h at room temperature with a 1:500 dilution of biotin-labeled goat anti-mouse IgG (Zymed). Membranes were again washed and incubated with a 1:1,000 dilution of streptavidin-horseradish peroxidase (Zymed) for 30 min. Following a final washing, the substrate (100 ml of TBS, 60 μl of H_2O_2, and 60 μg of 4-chloro-1-naphthol in 20 ml of cold methanol) was added and allowed to develop for up to 45 min. Membranes analyzed by the enhanced chemiluminescence detection system were blotted in the same manner as for chloro-naphthol development, but the blocking reagent and the concentration of some reagents varied. Membranes were blocked in 5% dry milk with 0.5% Tween in TBS. Monoclonal antibodies were diluted 1:6, and the secondary conjugate was diluted 1:10,000. The streptavidin-horseradish peroxidase was used in the same manner as before. Membranes were developed with enhanced chemiluminescence per the manufacturer's instructions and visualized by exposing the blots to X-OMAT ARS film (Eastman Kodak Co., Rochester, N.Y.). Blotting steps were usually performed in tubes (35 by 300 mm) with a hybridization incubator (Robbins Scientific, Sunnyvale, Calif.). In cases in which multiple antisera were analyzed against the same antigen, blotting was performed in either a miniblotter (Immunetics, Cambridge, Mass.) or an incubation tray (Bio-Rad).

RESULTS

Monoclonal antibodies. Hybridoma culture supernatants were initially screened by IFA. The fluorescent patterns observed with F6F3, F6B11, F6B3, and polyvalent mouse anti-*B. coriaceae* are illustrated in Fig. 1. All monoclonal antibodies were determined to be IgG1 isotypes by ELISA.

The specificity of the monoclonal antibodies for *B. coriaceae* was determined by reacting these antibodies with preparations of spirochetes (the organisms described in Materials and Methods) in one or more of the following systems: IFA, ELISA, and Western blot. All three monoclonal antibodies reacted positively and exclusively with *B. coriaceae* in any of the systems tested.

Adult female ticks were dissected and examined for the presence of spirochetes by IFA. Spirochetes were detected in smears incubated with both polyvalent antiserum and F6B3. No spirochetes were detected in any tick smear preparations after incubation with either F6B11 or F6F3. It was noted that incubation of smears with monoclonal antibodies resulted in preparations with low background levels. These smears could be examined more quickly and with less difficulty than those incubated with polyvalent antiserum (Fig. 2).

IEM. The apparent subcellular location of the antigenic determinants, for which a monoclonal antibody was specific, could be visualized by IEM. All three monoclonal antibodies recognized antigens that appeared to be present on the cell surface (Fig. 3). Gold-labeled F6B3 and F6B11 (indirect) bound to structures previously described as the outer membrane and membrane blebs (2) (Fig. 3a and b). The antigen recognized by F6F3 (Fig. 3c) appeared to be loosely associated with the cells, as many of the gold particles were attached to small membrane fragments dissociated from the organism. Antigens recognized by F6B3 and F6B11 appeared to bind to structures more intimately associated with the cell. Negative controls (Fig. 3d) show little binding of gold particles to either the grid or the organism.

IFA. Monoclonal antibody reactions to *B. coriaceae* after protease and detergent treatments are presented in Table 1. All antibodies bound to untreated spirochetes. Once treated with enzymes, little fluorescent activity remained with either F6F3 or F6B11, but binding of F6B3 was not effected. TX treatment did not prevent binding of any of the antibodies to

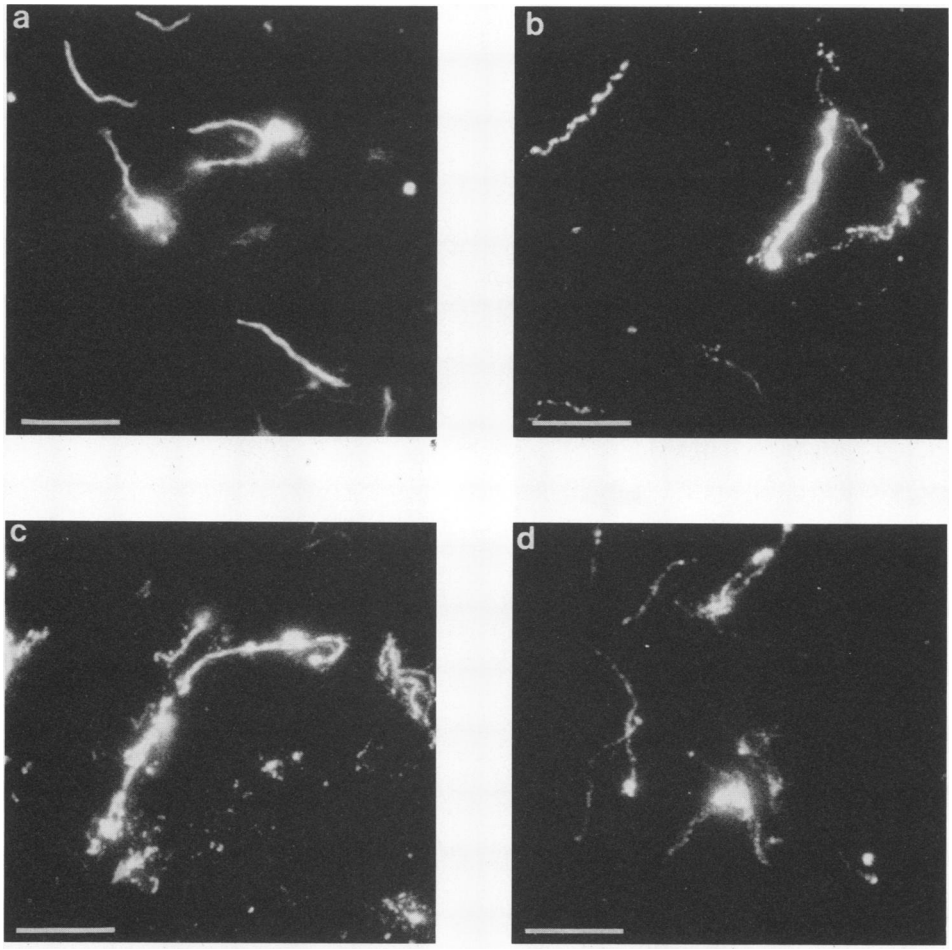

FIG. 1. IFA of *B. coriaceae* Co53 with monoclonal antibodies. The antibodies used were polyvalent mouse anti-*B. coriaceae* (positive control) (a), F6B3 (b), F6B11 (c), and F6F3 (d). Fluorescein isothiocyanate-labeled goat anti-mouse IgG was used as a secondary conjugate. Bars, 10 μm.

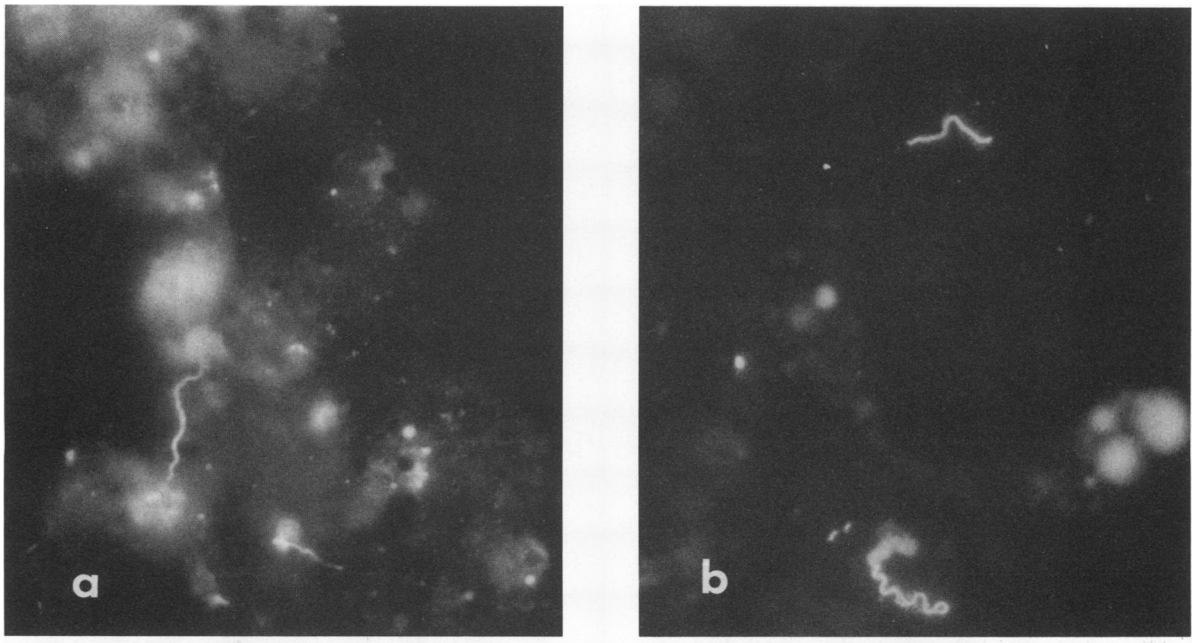

FIG. 2. IFA of tick organ preparations with mouse anti-*B. coriaceae* antibody (a) and monoclonal antibody F6B3 (b). Magnification, ×1,125.

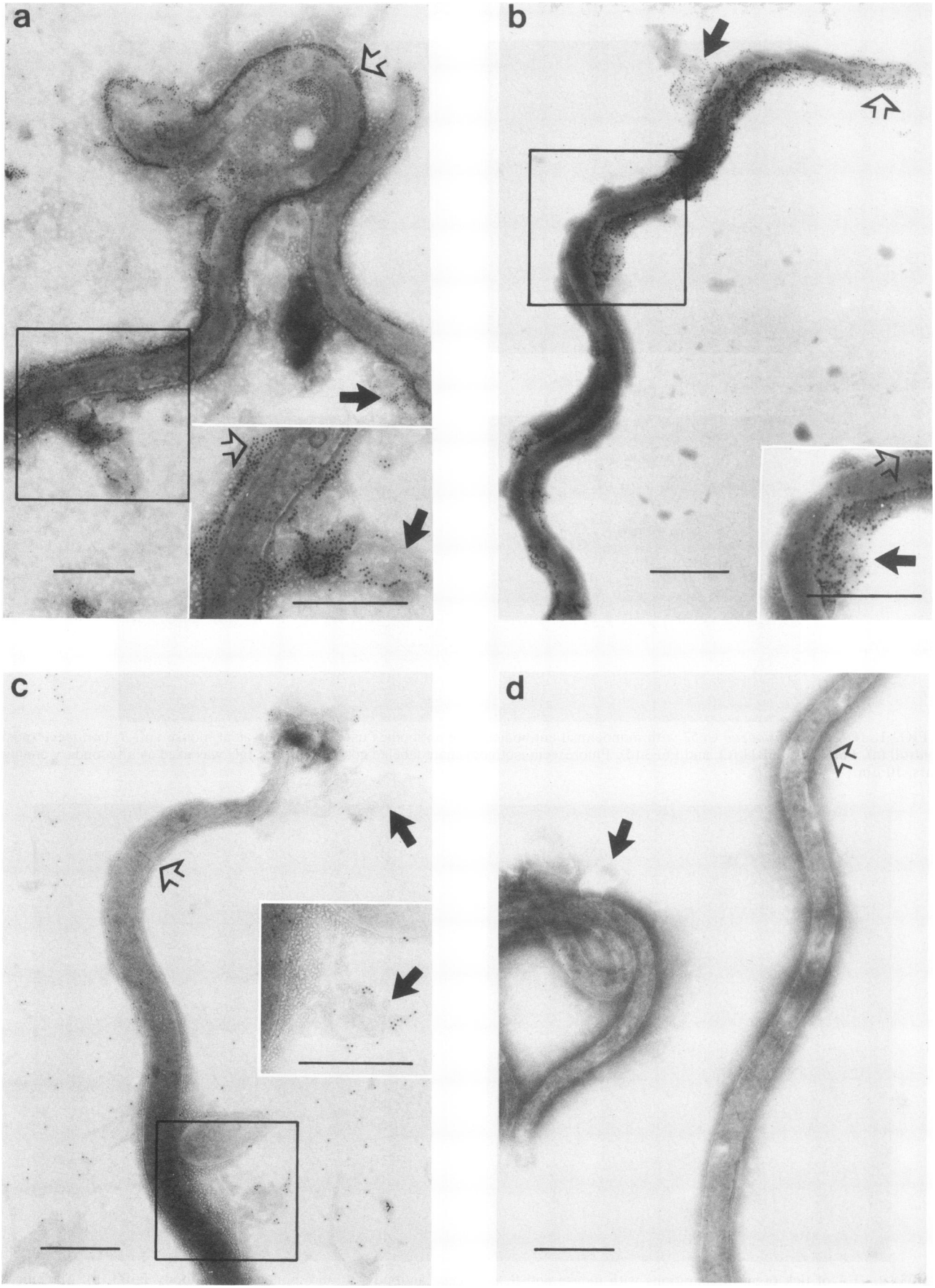
a
b
c
d

FIG. 3. IEM of *B. coriaceae* Co53 with colloidal gold-labeled anti-mouse IgG bound to monoclonal antibodies. The antibodies used were F6B3 (a), F6B11 (b), F6F3 (c), and irrelevant mouse IgG1(κ) (MOPC21)-clarified ascitic fluid (negative control) (d). Filled arrows indicate membrane blebs; open arrows indicate outer membrane. Bars, 0.5 μm.

the spirochete. The results correlate well with Western blot data (see Fig. 5 and 7).

PAGE. Untreated *B. coriaceae* whole-cell lysates, as well as those subjected to protease and detergent treatments, were subjected to SDS-PAGE analysis and stained with Coomassie brilliant blue (Fig. 4). Incubation of the live spirochete with either trypsin or protease K resulted in partial in situ proteolysis of two major proteins, with M_rs of ca. 37,000 and 35,000. The 16,000-M_r (16K) protein did not appear to be affected by any protease treatment. TX treatment removed, partially or completely, a number of major and minor proteins, as evidence of their presence in the supernatant. The 37K, 35K, and 16K proteins are present in both pellet and supernatant portions of TX-treated organisms.

Western blotting. The apparent molecular weights of antigens recognized by the monoclonal antibodies were determined by Western blot analysis. Western blot profiles of the antigenic determinants before and after enzyme and detergent treatments are illustrated in Fig. 5 and 7. The antigen bound by F6F3 had an M_r of ca. 37,000 (Fig. 5a). This antigen underwent in situ proteolysis when treated with either trypsin or protease K. A significant portion of the F6F3-recognized antigen was detected in the supernatant from control pellets (incubated with PBS/Mg only), and a majority of this antigen was removed from the cell by TX treatments. The F6B11 bound to an antigen with an M_r of ca. 35,000 (Fig. 5b). This antigen also underwent in situ proteolysis after enzyme treatments. The majority of antigen was removed from the intact cell by TX, though there was some variation from run to run as to the proportion of antigen removed. When these antigens were separated by SDS-PAGE (15% acrylamide) and blotted with a pool of F6F3 and F6B11 (Fig. 6), it became clear that the two antigenic determinants were located on different protein bands. Finally, a protein with an M_r of ca. 16,000 was bound by F6B3 (Fig. 7). This antigen was not affected by enzyme treatments performed on either live organisms or organisms pretreated with 0.5% TX. Treatment of spirochetes with 0.2% TX partially removed this antigen from the intact organism.

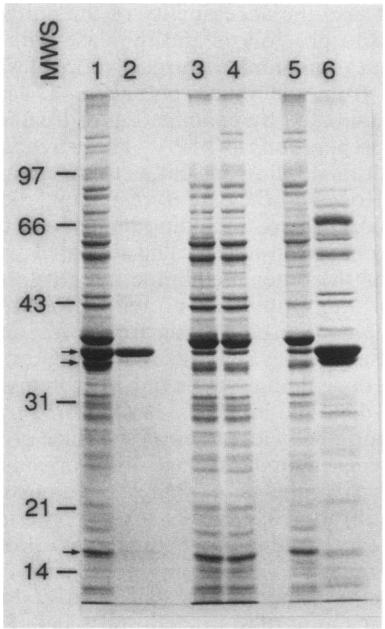

FIG. 4. Coomassie brilliant blue-stained proteins separated by SDS-PAGE of *B. coriaceae* lysates. Lanes: 1, whole-cell lysate (control; incubated with PBS/Mg) pellet; 2, whole-cell lysate supernatant; 3, trypsin (150 μg/ml)-treated pellet; 4, protease K (200 μg/ml)-treated pellet; 5, TX (0.2%)-treated pellet; 6, TX (0.2%)-treated supernatant. Arrows indicate the positions of the 37K, 35K, and 16K proteins. Molecular weight standards (MWS) (in thousands) are indicated at the left of the figure.

DISCUSSION

Despite its recognition as a separate disease entity in 1956 (4), EBA is still a disease with no known etiological agent. The isolation of the spirochete *B. coriaceae* from the known vector has given a new focus to the study of this elusive agent. There has been only one isolation of *B. coriaceae* to date, despite many unsuccessful attempts to isolate spirochetes resembling *B. coriaceae* from deer blood (6) and from additional ticks. The lack of isolations hampers rapid progress in this research. The sole isolate, which was first reported in 1985, has been inoculated directly into bovine fetuses (19) and has produced no clinical disease. The passage number of these spirochetes was unknown, but the organism had been maintained in culture for a considerable length of time. The lack of disease has two possible explanations. First, *B. coriaceae* may not be the etiologic agent of EBA, or secondly, perhaps the length of time this organism was in culture effected its capacity for virulence. Loss of virulence due to repeated passage in culture has been demonstrated for *B. burgdorferi* (10). The fact that only one monoclonal antibody (F6B3) reacted with native spirochetes (found in ticks) suggests that in vitro cultivations can influence antigen expression. This variation of antigen expression may be involved in a loss of virulence in cultured organisms.

Attempts in our laboratory to examine EBA fetal tissues with polyvalent antisera raised to *B. coriaceae* proved discouraging because of high background levels; many tick structures brightly fluoresce and have morphology resembling that of a spirochete. An obvious approach to pursuing

TABLE 1. IFA reactions of monoclonal antibodies F6B3, F6B11, and F6F3 against protease- and detergent-treated spirochetes (*B. coriaceae*)

Treatment	Reaction[a] of:		
	F6B3	F6B11	F6F3
No treatment	+	+	+
Trypsin (150 μg/ml)	+	±	−
Protease K (200 μg/ml)	+	±	−
TX (0.2%)	+	+	+

[a] +, fluorescence; ±, fluorescence present but minimal; − no fluorescence.

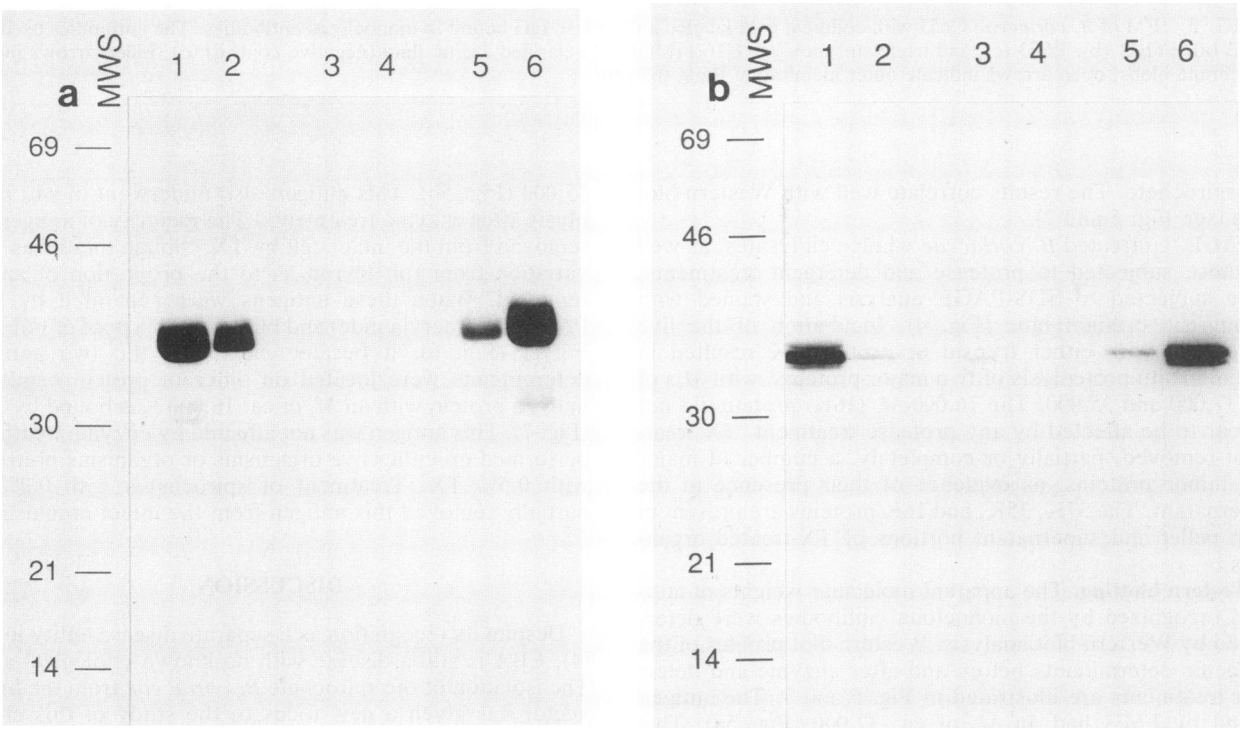

FIG. 5. Western blots of antigens recognized by monoclonal antibodies in various *B. coriaceae* lysates. Lanes: 1, whole-cell lysate pellets (controls; incubated with PBS/Mg); 2, whole-cell lysate supernatants; 3, trypsin (150 μg/ml)-treated pellet; 4, protease K (200 μg/ml)-treated pellets; 5, TX (0.2%)-treated pellets; 6, TX (0.2%)-treated supernatants. (a) Blotted with F6F3; (b) blotted with F6B11. Blots were developed with chloro-naphthol substrate. Molecular weight standards (MWS) (in thousands) are indicated at the left of the figure.

an association between *B. coriaceae* and EBA was development of better reagents, specifically monoclonal antibodies.

The three antibodies described herein appeared to recognize species-specific antigenic determinants, as they did not cross-react with four different *Borrelia* isolates, one *Leptospira* isolate, or a representative from the genus *Treponema*. Without testing these antibodies against representatives from all known *Borrelia* species, it is difficult to categorically state that these antibodies are species specific, but the data to date suggest this conclusion. With only one *B. coriaceae* isolate, it was not possible to determine how universal the epitopes of the monoclonal antibodies were for this species. In an attempt to answer this question, *O. coriaceus* ticks were dissected and examined by both polyvalent anti-*B. coriaceae* antiserum and the monoclonal antibodies. Of the three monoclonal antibodies, only F6B3 recognized antigens on the tick spirochetes. Smears examined with F6B3 had low background levels, and spirochetes could be identified quickly and easily. These data suggest that F6B3 will be useful for further diagnostic studies and may prove invaluable in the examination of fetal tissues and mammalian blood samples. The remaining two antibodies should continue to be tested, particularly in connection with spirochetes in mammalian hosts. It may be that different environments affect the expression of different antigens and that antigens expressed on spirochetes living in ticks differ from those seen after growth in mammalian hosts.

These monoclonal antibodies have proven to be efficacious in characterizing *B. coriaceae* antigens. Through Western blot analysis, it has been possible to examine the antigenic determinants against which the monoclonal antibodies were directed. This technology has allowed us to determine not only the apparent molecular weights of the antigens but also the accessibility of the antigenic determinants to in situ proteolysis and the ease with which a low concentration of nonionic detergent (0.2% TX) can remove the antigen from the intact organism. These results are further substantiated by examination of treated and control spirochete preparations by IFA. These reactions, in conjunction with IEM, allow insight as to the antigen's location on the cell. However, the extensive manipulation required to study these organisms, including high-speed centrifugation required to pellet spirochetes and repeated washings, may have affected the outer membrane integrity, leading to the exposure of more integral proteins. Recent work with *Treponema pallidum* employing freeze fraction and phase partitioning techniques suggests that the outer membrane of this species, once thought to be rich in proteins, is composed of a protein-deprived lipid bilayer (15, 16, 20). Only one of the proteins originally identified as a surface protein (47 kDa) is actually exposed on the surface of the organism (15). Since three other spirochetes examined demonstrate a much higher concentration of exposed surface proteins, these data do not suggest that all spirochetes have a protein-deprived outer membrane. However, they do suggest caution in the interpretation of experimental data relative to determining the cellular location of spirochete proteins. To our knowledge, no similar studies have been performed with any representative from the genus *Borrelia*. Though results from our study suggest that at least two of the proteins identified here are analogous to those described as outer surface proteins in *B. burgdorferi* (2), we shall identify our antigens as integral membrane proteins (IMPs) until their cellular location can be more precisely identified.

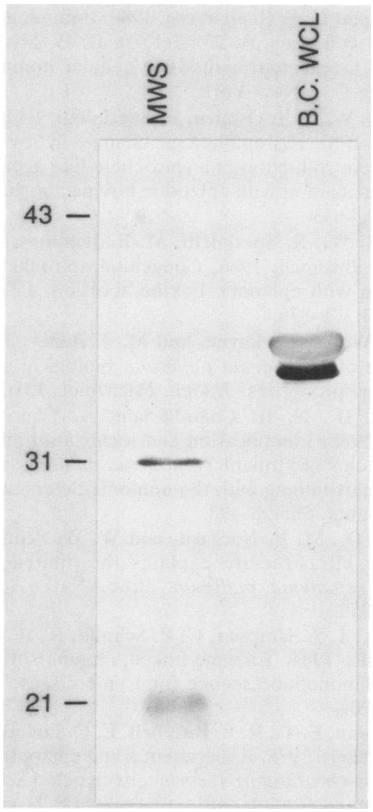

FIG. 6. Western blot prepared by transfer of *B. coriaceae* whole-cell lysates (B.C. WCL) separated by SDS-PAGE (15% acrylamide) to an Immobilon P membrane and incubated with a pool of F6F3 and F6B11. The blot was developed with chloro-naphthol substrate. Molecular weight standards (MWS) (in thousands) are indicated at the left of the figure.

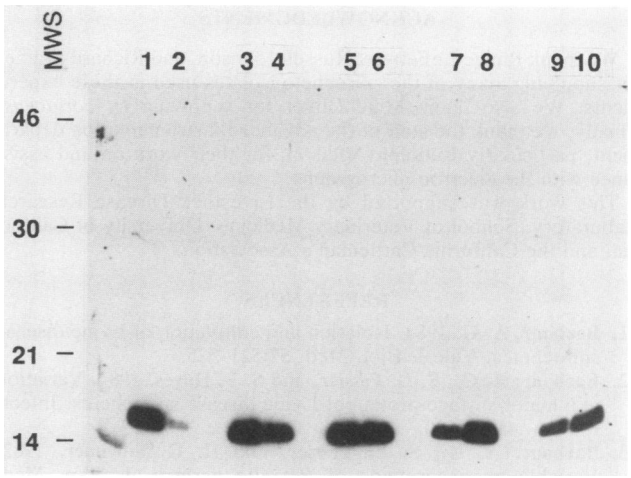

FIG. 7. Western blot of antigens recognized by F6B3 in various *B. coriaceae* lysates. Lanes: 1, whole-cell lysate pellets (controls; incubated with PBS/Mg); 2, whole-cell lysate supernatants; 3, trypsin (150 μg/ml)-treated pellet; 4, protease K (200 μg/ml)-treated pellets; 5, TX (0.2%)-treated pellets; 6, TX (0.2%)-treated supernatants; 7, pretreated TX (0.5%)–trypsin (300 μg/ml)-treated pellet; 8, TX–trypsin-treated supernatants; 9, pretreated TX (0.5%)–protease K (400 μg/ml)-treated pellet; 10, TX–protease K-treated supernatants. This blot was developed with an enhanced chemiluminescence substrate. Molecular weight standards (MWS) (in thousands) are indicated at the left of the figure.

The 37K protein of *B. coriaceae* contains the antigenic determinant recognized by F6F3. Several pieces of evidence suggest that this antigen is located on or close to the surface of the cultured cell. These include (i) the attachment of gold-labeled anti-mouse immunoglobulin to membrane blebs and membrane fragments loosely associated with spirochetes after incubation with F6F3 (Fig. 3c), (ii) the effective removal of the 37K antigenic determinants from the cell by proteases (Table 1 and Fig. 5a), and (iii) the effective removal of the 37K antigen by TX (Fig. 5a), as well as the sloughing of this antigen into the supernatant of control (untreated) samples (Fig. 4 and 5a). Coomassie brilliant blue-stained gels (Fig. 4) indicate the presence of a 37K protein after both enzyme treatments, which is not consistent with results from Western blot and IFA analysis. These bands probably represent an unrelated protein with the same molecular weight as the protein recognized by F6F3. Relative to the IEM (Fig. 3c), most gold particles were scattered throughout the grid on membrane fragments and the majority of gold directly associated with spirochetes was on membrane-associated blebs, not the cell surface. This is consistent with an antigen that is easily dissociated from the cell surface by not only detergents, but also labeling and washing procedures. We conclude that these micrographs provide evidence for specific binding, since the negative control, an irrelevant IgG1 molecule, showed little binding to either the grid or the cell (Fig. 3d). The IEM results are substantiated by IFA (Fig. 1d), in which the most brightly fluorescing areas are found in discrete blebs on the organism. This evidence supports the hypothesis that the 37K antigen is an IMP, with characteristics that are consistent with *B. burgdorferi* outer surface proteins, and the designation of IMP A is suggested.

The 35K protein recognized by F6B11 appears to be an IMP on the basis of the following data: (i) a high concentration of gold particles attached to the outer membrane and to closely associated outer membrane blebs after incubation with the F6B11 (Fig. 3b), (ii) in situ protease treatments removed this antigenic determinant (Table 1 and Fig. 5b), and (iii) F6B11-recognized antigen was present in the supernatant after TX treatment (Fig. 4 and 5b). These results are consistent with the characteristics of *B. burgdorferi* outer surface proteins A and B. Electron micrograph results suggest that this antigen may be more integrally associated with the cell than IMP A. We suggest a designation of IMP B for this antigen.

Finally, a 16K band was recognized by F6B3. This small antigen appeared refractory to both trypsin and protease K treatment, possibly because of the absence of cleavage sites for either of these enzymes. Exposure of the dissociated antigen (supernatant of 0.5% TX-treated cells) to either enzyme had no apparent effect (Fig. 7), suggesting that the inability of the enzymes to cleave this protein was not due to antigen inaccessibility. Therefore, enzyme assays on living cells were of little help in determining the antigen's subcellular location. This antigen was partially removed from the intact cell by TX, suggesting membrane association. Examination of the IEM data (Fig. 3a) suggests that this antigen is an IMP, on the basis of the high concentration of gold particles bound to the cell surface and blebs (Fig. 3a). This antigen appears to be very tightly associated with the cell and may be a component of the cytoplasmic rather than the outer membrane. Thus, we suggest that this 16K antigen be designated IMP C.

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