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Monoclonal Antibody Recognizing a Species-Specific Protein from *Streptococcus pneumoniae*

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Monoclonal antibodies (MAbs) against a nonencapsulated strain (R36A) of *Streptococcus pneumoniae* were produced to aid in a search for antigens common to this species. By Western immunoblot analysis, a species-specific 37-kilodalton (kDa) protein was found in lysates of 24 different encapsulated strains of *S. pneumoniae*. Monoclonal antibodies against the 37-kDa antigen did not react with 55 heterologous strains representing 19 genera and 36 species of bacteria that can also cause acute lower respiratory tract disease. Immunogold staining suggests that the antigen is synthesized inside the pneumococcal cell. However, MAbs to the 37-kDa antigen bound whole cells in the enzyme-linked immunosorbent assay and the indirect immunofluorescence assay. Antibody-binding epitopes of the antigen are probably exposed on the outer surface of the pneumococcus cell wall. The effectiveness of the 37-kDa antigen as a useful diagnostic marker is under study.

Disease caused by *Streptococcus pneumoniae* (pneumococcus) is an important cause of morbidity and mortality in the United States and developing countries (37, 40, 41, 44). Pneumococcus disease is very prevalent among the very young, the elderly, and immunocompromised persons. Despite its prevalence, diagnosis of the disease continues to be a problem.

Several tests have been developed to detect pneumococcus antigens and/or antibodies as a means of diagnosing pneumococcus infections (12, 18, 21, 22, 25, 28, 33, 36, 43, 45). The sensitivity of existing antigen detection tests utilizing body fluids such as serum and urine remains very low (1, 2, 7, 10, 11, 13, 14, 16, 30), except for antigen detection in cerebrospinal fluids (17, 21, 27, 38, 45). The measurement of antibody response to pneumolysin by enzyme-linked immunosorbent assay (ELISA) appears to be promising for presumptive etiologic diagnosis (22, 23, 25), but the sensitivity and specificity of the test need improvement.

Although a positive blood culture is diagnostic for pneumococcus disease, most patients with bacterial pneumonia do not have bacteremia (3, 4, 24). The value of sputum cultures has also been questioned because of contamination of the specimens with pharyngeal flora that can include pneumococci (6). Thus, clinical laboratories are rarely successful in establishing a firm bacterial etiology for those patients with respiratory infections diagnosed presumptively as pneumococcus pneumonia. Researchers have been in constant search of immunodiagnostic markers or tests to aid in the early diagnosis of pneumococcus infections. In this report, we describe the production and characterization of monoclonal antibodies (MAbs) that react with a species-specific antigen found in *S. pneumoniae*. We assessed the sensitivity and specificity of the MAbs and the antigens to which they bind as a means of determining their usefulness as immunodiagnostic markers for the etiologic diagnosis of pneumococcal infection.

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MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* R36A was kindly provided by D. E. Briles (University of Alabama at Birmingham). Twenty-four serotypes of *S. pneumoniae* were provided by R. Facklam, Centers for Disease Control (CDC), Atlanta, Ga. These serotypes are 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11F, 11A, 12F, 14, 15B, 18C, 19A, 19F, 20, 22F, 23F, and 33F. *Enterococcus avium*, *Enterococcus casseliflavus*, and *Enterococcus gallinarum* were also provided by R. Facklam. Anaerobic bacteria were obtained from V. R. Dowell, CDC. These included *Bacteroides asaccharolyticus*, *Bacteroides fragilis*, *Bacteroides intermedius*, *Bacteroides thetaiotaomicron*, *Eubacterium lentum*, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, *Peptostreptococcus asaccharolyticus*, *Propionibacterium acnes*, and *Staphylococcus saccharolyticus*. *Branhamella catarrhalis* and *Bordetella parapertussis* were obtained from R. Weaver, CDC. *Mycobacterium tuberculosis* was provided by R. C. Good, CDC. R. Barnes (CDC) provided *Chlamydia pneumoniae*. The following remaining bacteria were from the stock collection of the Immunology Laboratory, CDC: *Bordetella pertussis*, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae* (types a through f), *Legionella micdadei*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus equisimilis*, *Streptococcus pyogenes*, and group G streptococci.

Production of MAbs. Female BALB/c mice were immunized with whole-cell suspensions of *S. pneumoniae* R36A, a rough derivative of the capsular type 2 strain D39 (5). The mice were immunized by intravenous injection three times and intraperitoneal injection one time. The maximum number of cells injected at any time was 10^8. Fusion was done on day 25 by using standard procedures (9). Spleen cells of four mice were fused with Sp2/0-Ag14 myeloma cells (35). Culture fluids of the growing hybridomas were tested for antibodies to *S. pneumoniae* whole cells in an ELISA. A clone designated 1E7A3D7C2 was one of 10 selected for further

study. Further references to MAbs in this article refer to hybridoma clone 1E7A3D7C2.

ELISA. Screening of hybridoma culture supernatants was done by ELISA. U-bottom microtitration plates (Costar, Cambridge, Mass.) were sensitized with 50 μl of *S. pneumoniae* whole-cell suspension (10^9 CFU/ml) diluted 1:4,000 in 0.1 M carbonate buffer, pH 9.6, and kept for 16 h at 4°C. The plates were washed five times with 0.9% NaCl containing 0.05% Tween 20 (NaCl-T). Culture supernatants (50 μl) from the fusion plates were added to 50 μl of a solution containing 2% bovine serum albumin (BSA), 10% normal rabbit serum, 0.3% Tween 20, and 0.02% Merthiolate in phosphate-buffered saline (PBS), pH 7.2 (ELISA diluent) (42), in the plates and were incubated for 30 min at 37°C. The plates were washed five times with NaCl-T. Fifty microliters of goat anti-mouse immunoglobulin horseradish peroxidase conjugate, prepared by one of us (D. E. Wells), diluted in ELISA diluent was added to each well. The plates were incubated for 30 min at 37°C. The plates were washed, and 50 μl of 3,3′,5,5′-tetramethylbenzidine (0.1 mg/ml in 0.1 M sodium acetate–0.1 M citric acid [pH 5.7] with 0.005% hydrogen peroxide) was added to each well and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 4 M H_2SO_4, and the optical density was read on a Dynatech ELISA Reader (Dynatech Laboratories, Inc., Alexandria, Va.) at 450 nm. An optical density of >0.200 was considered positive.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Tsang et al. (39), by using an 8% acrylamide resolving gel. Equal volumes of sample buffer (5% SDS–10% 2-mercaptoethanol–20% glycerol in 0.01 M Tris hydrochloride [pH 8.0]) and cell suspension containing 2.4 μg of protein per μl were mixed and heated at 100°C for 5 min, and a 5-μl portion was applied to 1 of 15 wells. If the final protein content of the portion of sample to be tested was <1.2 μg/μl, a volume up to 10 μl of sample was applied to achieve a final concentration of 6 μg of protein per well. Protein concentrations were determined by the method of Markwell et al. (29), with BSA as the standard.

Proteins separated by SDS-PAGE were either silver stained by the method of Morrissey (31) or electroblotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). The immunoblot procedure was done according to the method of Tsang et al. (39) with slight modifications. The blots were given three 5-min washes with PBS, pH 7.2, containing 0.3% Tween 20 and were gently agitated overnight (16 h) at 25°C. The blots were blocked for 1 h with casein-thimerosal buffer (CTB) (26). After three rinses with CTB, the blots were exposed to MAbs diluted in CTB for 2 h at 25°C. After three rinses with CTB, the blots were exposed to goat anti-mouse immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) for 2 h at 25°C. Conjugate dilutions (1:2,000) were made in CTB. The blots were again rinsed three times with CTB and exposed to 3-3′-diaminobenzidine-4-hydrochloride in PBS, pH 7.2 (0.5 mg/ml), with 0.003% H_2O_2 for 5 min at 25°C. Reactivity was expressed as a visible colored band on the nitrocellulose paper.

Low-molecular-mass protein standards (Bio-Rad) were used in PAGE and immunoblotting. Rabbit antisera to the protein standards were used to develop the standards (8). Molecular masses were calculated by the method of Neville and Glossman (32) by using appropriate molecular mass standards.

IFA. For an immunofluorescence assay (IFA), a bacterial suspension containing approximately 400 to 500 CFU per field (10 μl) was allowed to dry at room temperature on each well of acetone-resistant, 12-well (5 mm in diameter), glass slides (25 by 75 mm) (Cel-Line Associates, Newfield, N.J.). The slides were then immersed in acetone for 10 min and air dried at room temperature. MAbs were added to the slides, which were incubated for 30 min at 37°C. After the incubation, the slides were gently rinsed with PBS and soaked twice at 5-min intervals, blotted on filter paper, and air dried at room temperature. Fluorescein-labeled rabbit anti-mouse immunoglobulin (courtesy of W. F. Bibb, CDC) was then added, and the slides were incubated for 30 min at 37°C. They were then washed twice with PBS and gently blotted on filter paper. Slides were covered with carbonate-buffered mounting fluid, pH 9.0, and cover slips and were then read with a Leitz Dialux 20 fluorescence microscope equipped with an HBO-100 mercury incident light source, an I cube filter system, a 40× dry objective lens, and 6.3× binoculars (E. Leitz, Inc., Rockleigh, N.J.).

Immunoelectron microscopy. Pneumococcal cells were washed two times with PBS and fixed in a mixture of 1% paraformaldehyde–0.1% glutaraldehyde (freshly made) for 20 min at 4°C. The cells were dehydrated in a graded alcohol series and then in a 1:1 mixture of absolute ethanol and Lowicryl K4M (Ladd Research Industries, Inc., Burlington, Vt.) for 1 h at 4°C. The cells were pelleted and suspended in a 1:2 mixture of absolute ethanol and Lowicryl K4M for 1 h at 4°C. They were again pelleted and suspended in Lowicryl K4M (undiluted) for 16 h at 4°C.

The cells were transferred to fresh Lowicryl K4M two times during the next 24-h period. The Lowicryl K4M-treated cells were embedded in gelatin capsules, which were placed inside a box lined with aluminum foil. The capsules were hardened by holding them in the box 35 cm from a shortwave UV light source for 72 h at −20°C. The box was brought to room temperature, and the capsules were allowed to continue hardening for up to 14 days.

Samples of the capsule were cut into 100-μm thin sections and picked up on nickel grids. Grids containing the sample were placed on a droplet of ovalbumin solution in PBS containing sodium azide (E. Y. Laboratories, Inc., San Mateo, Calif.) for 5 min. The grids (wet) were transferred to a solution of primary MAbs diluted in a solution of BSA reagent (1% BSA in PBS containing 0.1% Triton X-100, Tween 20, and sodium azide) (E. Y. Laboratories) and incubated for 1 h at room temperature or 18 to 48 h at 4°C in a moist chamber. For antibody-binding controls, other grids were wetted with MAbs against *L. pneumophila*. The grids were rinsed two times with PBS and incubated on droplets of goat anti-mouse immunoglobulin G-labeled colloidal gold particles (20 μm) (E. Y. Laboratories) for 1 h at room temperature. The grids were rinsed two times and post-stained with osmium tetroxide, uranyl acetate, and lead citrate. The grids were examined with a Philips 410 transmission electron microscope.

RESULTS

MAbs. Hybridoma clone 1E7A3D7C2 produced MAbs that reacted with a 37-kilodalton (kDa) protein antigen found in *S. pneumoniae*. The MAbs reacted with an antigen fractionated in SDS-PAGE, yielding a single immunoblot band. This indicates that the MAb reacted with epitopes found only on the 37-kDa antigen.

Sensitivity and specificity of the MAbs. The MAbs produced by immunization of mice with pneumococcal cells

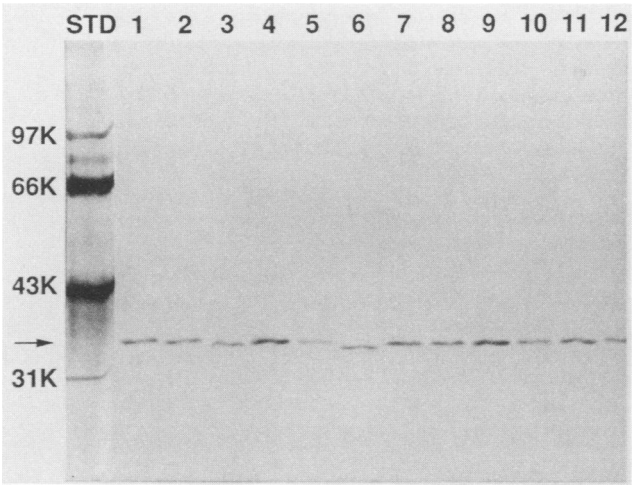

FIG. 1. Immunoblot of *S. pneumoniae* whole-cell antigen preparations with pneumococcus MAbs. Protein standards (STD) (in kilodaltons) and different serotypes of *S. pneumoniae* are shown. Lanes: 1, serotype 3; 2, serotype 6B; 3, serotype 7F; 4, serotype 8; 5, serotype 9V; 6, serotype 10A; 7, serotype 11A; 8, serotype 12F; 9, serotype 15B; 10, serotype 19A; 11, serotype 19F; 12 serotype 22F. The MAbs revealed an antigen at 37 kDa (arrow) in all serotypes tested.

reacted with all pneumococcal strains tested (24 serotypes) to yield a sensitivity of 100%. For specificity, 55 different nonpneumococcus strains of bacteria that can also cause respiratory infections (15) were tested for antigens reacting with the MAbs. The latter strains represented 19 genera and 36 species of bacteria. None of the strains tested reacted with the pneumococcal MAbs, thus yielding a specificity of 100%.

Western blots with human sera. Of 44 patients known to have pneumococcus disease, 34 (77%) had antibodies that reacted with the 37-kDa antigen by Western immunoblot (Fig. 1).

Reactivity of MAbs in the ELISA and IFA. MAbs reacted with whole pneumococcus cells to yield positive test results in both the ELISA and IFA. Figure 2 shows the bright immunofluorescence of whole pneumococcus cells stained by the MAbs and fluorescein-labeled anti-mouse immunoglobulin in the IFA. Results from both the ELISA and the IFA indicate that the antigen has exposed epitopes on the surface of the cell or that the immunoglobulin and other immunologic reagents are able to penetrate the pneumococcus cell walls.

Several strains of group A streptococci were tested for immunofluorescence after reacting with the pneumococcus MAbs. None of the heterologous bacterial cells fluoresced in this test, indicating that the IFA reaction was specific for pneumococcus cells.

Localization of antigen determined by transmission electron microscopy. To further determine the location on the cell of the 37-kDa antigen epitopes reacting with the MAbs, immunolabeling experiments were performed. Figure 3 shows that the cells were typical of gram-negative cocci in the process of division. The figure also shows the reaction of MAbs and colloidal gold-labeled anti-mouse immunoglobulin G with thin sections of whole pneumococcal cells. A large portion of the antigen appeared to be intracellular, since there was no coating or layering of the labeled MAbs around the cell. The large patch of colloidal gold staining indicates that the MAbs bound antigen located inside the cell wall. There was no colloidal gold binding to control pneumococci that were exposed to the MAbs against *L. pneumophila*.

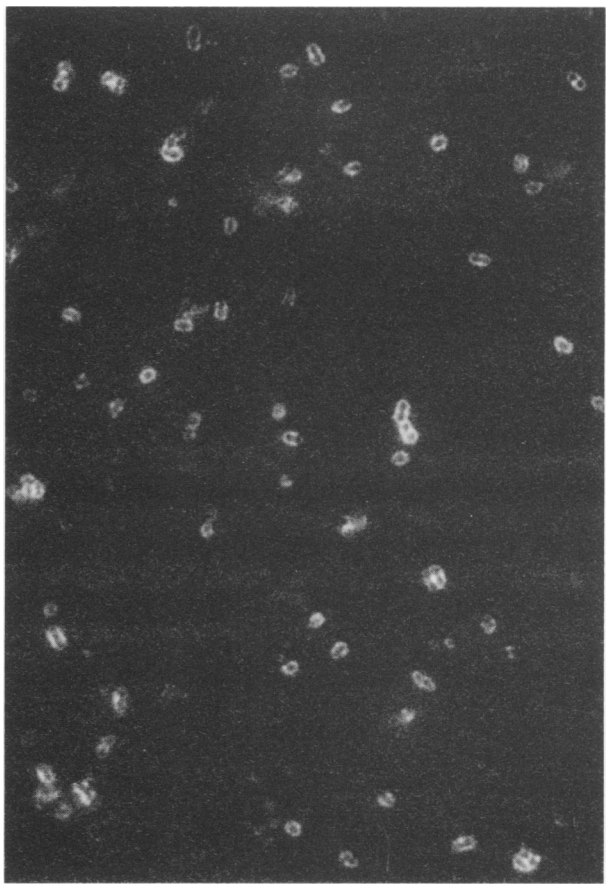

FIG. 2. Immunofluorescence assay staining of *S. pneumoniae* cells with pneumococcus MAbs.

DISCUSSION

In this study, we immunologically identified a species-specific, 37-kDa *S. pneumoniae* protein. The MAbs that reacted with the 37-kDa protein did not react with electrophoretically separated proteins from 55 other strains of bacteria representing 19 genera. These strains were selected for testing because they are known to also cause lower respiratory tract diseases (15) and might interfere in an immunologic assay for detection of the 37-kDa antigen or antibodies to the antigen.

Since the newly identified antigen has a molecular mass of 37 kDa, it was originally thought that it may be homologous to the autolytic enzyme (amidase or *N*-acetylmuramoyl-L-alanine amidase) described by Howard and Gooder (20) and purified by Holtje and Tomasz (19). The molecular mass of the amidase is 35 kDa. However, the anti-37-kDa MAbs did not inhibit the enzymatic activity of the amidase when tested, whereas polyvalent antiamidase serum did inhibit this activity (A. Tomasz, personal communication). To further test for similarity between the antigens, *S. pneumoniae* M31, a mutant strain described by Sanchez-Puelles et al. (34) which contains a complete deletion of the amidase gene (*lytA*), was electrophoresed and the transblotted antigens were reacted with the MAbs. There was a distinct antigen-antibody reaction band on the Western blot. These

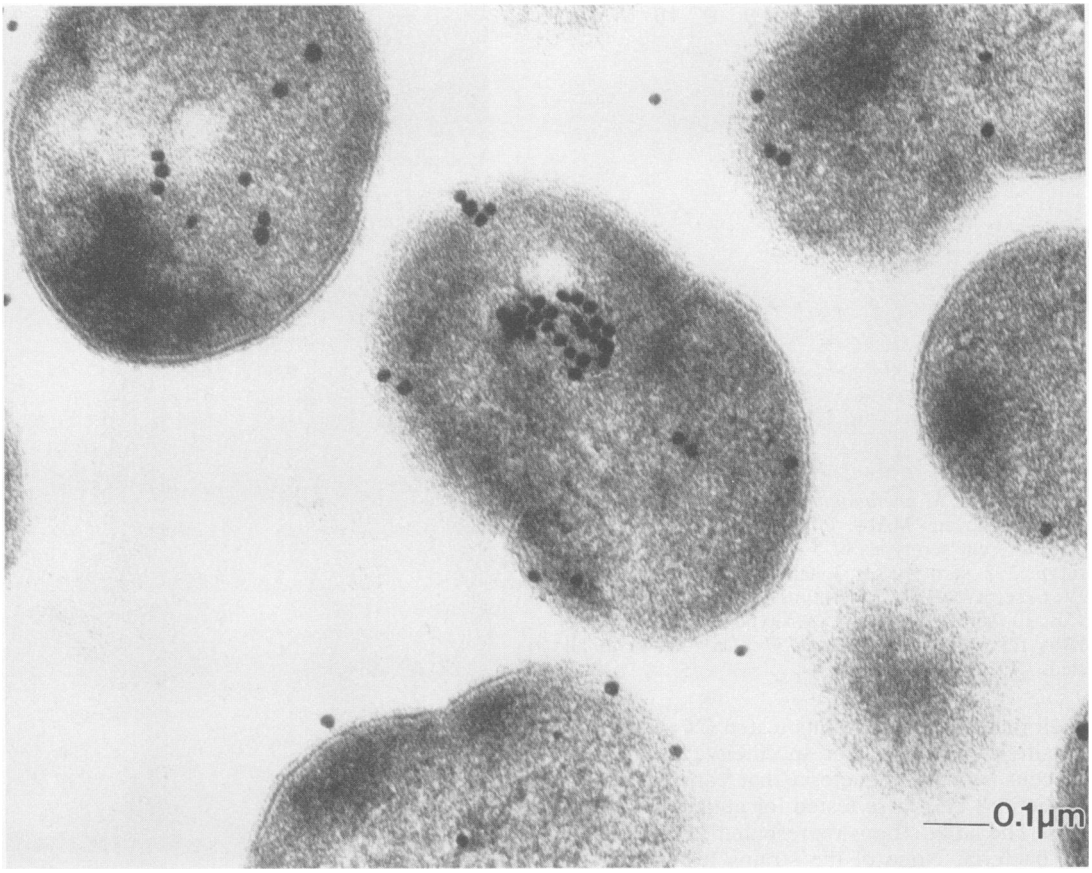

FIG. 3. Transmission electron microscopy of *S. pneumoniae* R36A after embedding, cutting, reacting with MAb, and staining with gold-labeled goat anti-mouse immunoglobulin.

findings provide evidence that the 37-kDa antigen described in this report is distinct from the 35-kDa amidase. Thus, strain M31 does not produce amidase but does produce an antigen that reacts with the MAbs.

The MAbs reacted with whole pneumococcus cells in an ELISA. Immunofluorescence of the bacteria in an IFA did not require any permeabilizing treatment of the pneumococcus cell before staining. Therefore, the determinants or epitopes to which the MAbs bind on the species-specific antigen appear to be exposed or partially exposed on the surface of the cell. The immunogold assay showed that the greater portion of the antibody-binding epitopes are located inside the pneumococcus cell. The antigen is probably synthesized inside the cell and leaches out to the surface. The ELISA and IFA results suggest the latter.

The species-specific antigen was found in all the *S. pneumoniae* isolates tested, including a mutant strain, M31, which was void of the autolytic enzyme. This antigen could therefore be a candidate for a pneumococcus vaccine, since protein antigens are usually more immunogenic in young children than are polysaccharide antigens. The antigen could also be used in the development of a diagnostic test for *S. pneumoniae* infections. Further studies to purify the 37-kDa antigen and determine its usefulness in an antibody capture assay are in progress.

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