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Surface Protein of A *Streptococcus agalactiae* Isolate

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A *Streptococcus agalactiae* isolate of bovine origin was cultured in broth; log-phase cells were washed and radioiodinated and subsequently extracted at low pH in the presence of a nonionic detergent. A protein antigen was purified from concentrated extract by ultracentrifugation, gel filtration, and ion-exchange chromatography. The molecular weight of the protein was estimated at 31,800. The agglutinogenic character of the protein indicated its localization at the cell surface.

Intramammary infections can be established experimentally in lactating cows by intraluminal inoculation of small numbers of *Streptococcus agalactiae* cells (18). Indirect evidence indicates that naturally acquired infections similarly are initiated by small numbers of organisms. Only very small numbers of phagocytes (6) and only low concentrations of immunoglobulins (4) and of complement components (B. De Cueninck, Int. Arch. Allergy Appl. Immunol., in press) are present in normal bovine milk. Preexisting low-grade inflammation, determined by the number of leukocytes in milk, is associated with protection against small numbers of *S. agalactiae* that are infective for normal glands (19); also, an early mild inflammatory response to experimental inoculation of small numbers of *S. agalactiae* is associated with rapid clearance of the infection (18). An antigen-specific inflammatory pathway can be established experimentally in the mammary gland of lactating cows by parenteral sensitization using protein antigens; intraluminal administration of antigen in previously sensitized animals results in transient acute inflammation, i.e., release of leukocytes into the milk (De Cueninck, in press). In counteracting the invasion by *S. agalactiae*, the acquired capacity of the mammary gland to eliminate the infection may depend, at a particular stage, on efficient phagocytosis and may result from an immune-mediated inflammatory response to the bacterial population. Protein antigens exposed at the surface of *S. agalactiae* might elicit inflammation efficiently in specifically sensitized animals during invasion; also, only a cell surface antigen could act simultaneously in other immune phenomena, i.e., in agglutination, chemotaxis, and opsonization and possibly in prevention of adherence of *S. agalactiae* to mammary epithelium (7, 10).

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This working hypothesis motivated the search for a surface protein antigen of *S. agalactiae*. M proteins can be extracted from group A streptococcal cell walls through disruption of noncovalent bonds by nonionic detergent (9). By analogy, detergent extraction of *S. agalactiae* cells was performed, and a protein agglutinogen was isolated from the extract.

MATERIALS AND METHODS

Bacteria. The streptococci used in this study were isolated from a case of bovine mastitis and identified as *S. agalactiae* on the basis of its sero-classification in Lancefield group B (type II) (biological reagents, Communicable Disease Center, Atlanta, Ga.) and as a result of biochemical tests performed by the method of McDonald and McDonald (16); the isolate was positive in the cyclic adenosine 5'-monophosphate test, hydrolyzed hippurate, and produced ammonia from arginine, but did not hydrolyze esculin or starch and did not utilize sorbitol, mannitol, or inulin. A stock culture was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) and was stored in aliquots at −60°C.

Preparation of cells. Sheep blood agar plates were streaked with stock culture and were incubated for 24 h at 37°C. Single colonies were used to inoculate 100-ml volumes of BHI broth; cultures were continuously agitated for 14 h at 37°C; at this time, growth was in the late logarithmic phase. Cells were harvested by centrifugation at 3,000 × *g* and washed three times in sodium phosphate-buffered saline (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.3) (PBS) at 21°C.

Radioiodination of cells. Freshly prepared cells were radioiodinated by using carrier-free ^{125}I (New England Nuclear Corp., Boston, Mass.), H_2O_2, and lactoperoxidase (Calbiochem, La Jolla, Calif.) (5, 14). Reaction mixtures were made of 300-μl cell suspension at an optical density equal to 50 (520 nm, 1 cm) of 100 μCi of ^{125}I in 50 μl of 0.1 M NaOH and of 6 U of lactoperoxidase in 300 μl of PBS; the iodination was initiated by adding 20 μl of 0.03% H_2O_2. Iodination was performed at 21°C, and the reaction was stopped after 2 min by diluting the mixture in a tenfold volume of PBS chilled to 0°C and containing 5 mM 2-mercaptoethanol; near-maximal cell binding of ^{125}I was ob-

tained within this period. Labeled cells were washed four times in PBS at 21°C. The specific radioactivity of labeled cell preparations averaged 0.12 μCi per mg, dry weight.

Preparation of crude cell extract. The extracting medium consisted of a 50 mM sodium acetate buffer at pH 5.5 which contained in addition 0.1 M NaCl and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). Freshly prepared and labeled cells were suspended in the medium to an OD of 10 (520 nm, 1 cm) and stirred at 21°C for 1 h. The extract was harvested after centrifugation of the suspension at 3,000 × *g* for 10 min and clarified by additional centrifugation at 20,000 × *g* for 30 min.

Concentration and dialysis of antigen solutions. Dilute antigen solutions were concentrated by ultrafiltration under nitrogen pressure, using PM-10 Diaflo membranes (Amicon, Lexington, Mass.).

Antigen solutions were dialyzed by using cellulose dialysis tubing (G 20 DM 30 55 OX, Union Carbide, Chicago, Ill.).

Protein determination. Protein determinations were done by a biuret method (3), using bovine serum albumin as standard.

Ultracentrifugation. Ultracentrifugations were performed by using a Beckman L-2 ultracentrifuge with an SW 39 L swinging bucket rotor (Palo Alto, Calif.). Crude extract, in volumes of 4.5 ml, was centrifuged at 85,000 × *g* (R_{avg}) for 120 min at 2°C.

Gel filtration. Columns of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) were equilibrated with 50 mM sodium acetate buffer at pH 5.5 containing 0.1 M NaCl and 0.1% Triton X-100. Gel filtration was carried out at 20°C.

Ion-exchange chromatography. Pool II, which was obtained by gel filtration of concentrated and ultracentrifuged extract, was dialyzed against 50 mM sodium acetate buffer at pH 5.5 containing 50 mM NaCl and 0.1% Triton X-100. It was subsequently applied to a column of diethylaminoethyl-cellulose (DEAE-cellulose; Eastman Organic Chemical Div., Rochester, N.Y.) which was equilibrated with the same buffer. After application, the column was washed exhaustively with equilibrating buffer and eluted with a linear NaCl gradient made with the equilibrating buffer. The chromatography was performed at 20°C.

IE. Immunoelectrophoresis (IE) of concentrated crude extract and of concentrated fractions of extract, obtained by ultracentrifugation, gel filtration or ion-exchange chromatography, were carried out in 1.2% agarose gel (l'Industrie Biologique Française, Seine, France); gel and vessel buffer consisted of sodium acetate (50 mM) at pH 5.5, 0.1 M NaCl, and 0.1% Triton X-100. Electrophoresis was run at 5 V/cm for 30 min.

Preparation of antisera. Hyperimmune anti-whole cell serum was obtained from a rabbit which was immunized by eight subcutaneous injections of 500-μg doses (dry weight) of *S. agalactiae* cells; the cell suspensions in PBS were incorporated in incomplete Freund adjuvant (Difco Laboratories) and were given at 14-day intervals. Serum was obtained at 2 weeks after the last injection.

A second rabbit was sensitized with four 50-μg doses of protein eluted from DEAE-cellulose; eluate obtained between the molarities 0.07 and 0.12 of NaCl was pooled, concentrated, and dialyzed against equilibrating buffer. The preparations were incorporated in complete Freund adjuvant and were injected at 10-day intervals. Antiserum was prepared at 1 week after the fourth injection.

Agglutination tests. Streptococcal cell suspensions were brought to an optical density equal to 1.5 (520 nm, 1 cm) in PBS. Serial twofold dilutions of antisera were made in the same buffer. Equal volumes of cell suspension and serum were mixed in tubes, and agglutination results were read after overnight settling at room temperature. Titers are expressed as the reciprocal of the highest serum dilution at which agglutination occurred.

SDS-PAGE. Gel cylinders for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were made of 8% total acrylamide; 3% of the total was methylenebisacrylamide; polymerization was mediated by *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate; the reagents were obtained from Bio-Rad Laboratories (Richmond, Calif.). The gel buffer consisted of 0.1 M sodium phosphate at pH 7.0 and contained 0.1% SDS. The sample and vessel buffers consisted of 0.01 M sodium phosphate at pH 7.0 and contained 0.1% SDS. Samples of protein in volumes of 50 μl contained also 2 μg of bromophenol blue and 15% sucrose. Gels were either stained for protein with Coomassie blue R250 by the method of Fischbein (8) or sliced in 1-mm sections for measurement of radioactivity.

Preparation of unreduced protein for SDS-PAGE. Protein eluted from DEAE-cellulose between the molarities 0.07 and 0.12 of NaCl was concentrated and dialyzed against a 0.01 M sodium phosphate buffer at pH 7.0 which contained 1% SDS. The solution was then heated in a boiling water bath for 15 min and subsequently dialyzed against a 0.01 M sodium phosphate buffer at pH 7.0 which contained 0.1% SDS. Samples for electrophoresis contained ±50 μg of protein.

Reduction of protein for SDS-PAGE and for molecular weight determination. Protein eluted from DEAE-cellulose between the molarities 0.07 and 0.12 of NaCl was concentrated and dialyzed against 0.55 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 8.2 which contained 6 M guanidine hydrochloride. Subsequently, 2-mercaptoethanol was added to a final concentration of 0.1 M, and the solution was shaken at 25°C for 2 h. After cooling on ice, an equal volume of a cold buffer which contained 0.11 M iodoacetamide, 6 M guanidine hydrochloride, and 1 M Tris-hydrochloride at pH 8.2 was added. The mixture was kept at 0°C for 15 min, after which crystalline SDS was added to a concentration of 0.1%. The solution was dialyzed against a 0.01 M sodium phosphate buffer at pH 7.0 which contained 0.1% SDS. Samples for PAGE contained ±65 μg of protein.

Molecular weight determination. The molecular weight of the reduced polypeptide was determined by SDS-PAGE as outlined by Weber and Osborn (21). Lysozyme soybean trypsin inhibitor, carbonic anhydrase, and bovine serum albumin (molecular weights, 13,000, 21,000, 30,000, and 68,000, respectively) were

used as molecular weight markers. The five proteins were run in duplicate in a parallel manner on different gels. The mean relative mobility of each protein was plotted against the $\log_{10}$ of the molecular weights of the standards. The regression of the $\log_{10}$ molecular weights of the standard proteins on their relative mobilities was calculated by the method of least squares, and the molecular weight of the streptococcal protein was derived by using this equation.

RESULTS

Extraction of antigens. Crude cell extract contained approximately 30 μg of protein per ml; this was equal to ±3.6% of the total dry weight of the cells. IE of concentrated cell extract against hyperimmune anti-whole cell serum revealed only two antigens (Fig. 1); one component migrated toward the anode, and a second component was immobile.

Gel filtration. The elution pattern of radiolabeled extract from Sephadex G-200 is illustrated in Fig. 2. The protein concentration in the eluate was too low to be monitored by the biuret method. The immobile and the mobile antigens detected in unfractionated extract by IE were found in the exclusion volume (peak I) and in peak II, respectively. The radioactive material and the antigen appearing in the exclusion volume could be removed from concentrated crude cell extract by ultracentrifugation.

Ion-exchange chromatography. A single major peak of radioactivity eluted between the molarities of 0.07 and 0.12 of NaCl (Fig. 3). The protein concentration in the eluate was too low to be monitored. In IE, anti-whole cell serum developed a single precipitin line with this component when dialyzed and concentrated (Fig. 4). The antigen migrated anodically. Antiserum, specifically raised against this component, likewise developed an identical precipitin line in IE with unfractionated concentrated cell extract as antigen. This antiserum also agglutinated streptococcal cells to a titer of 256, indicating the superficial localization of the component; hyperimmune anti-whole cell serum agglutinated cells to a titer of 4,096.

SDS-PAGE of unreduced and of reduced protein. A single radioactive band and a single protein-staining band with identical relative mobilities were found in the gel after electrophoresis of unreduced or of reduced protein samples. The relative mobility of the bands did not change significantly upon reduction. The molecular weight of the streptococcal protein was estimated at 31,800 (Fig. 5).

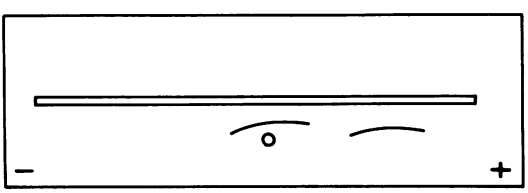

FIG. 1. *IE of concentrated crude S. agalactiae cell extract against hyperimmune anti-whole cell serum.*

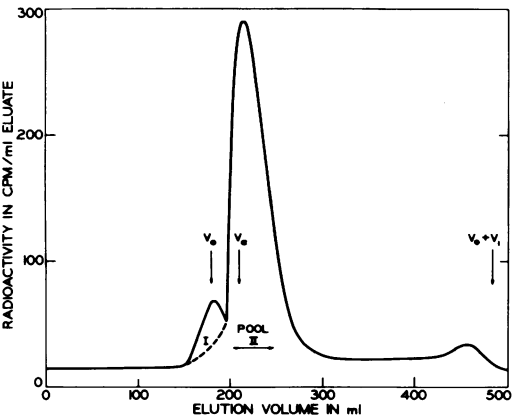

FIG. 2. *Elution pattern of concentrated radiolabeled extract of S. agalactiae from a column (2.5 by 94 cm) of Sephadex G-200, equilibrated and eluted with a 50 mM sodium acetate buffer at pH 5.5 which contained 0.1 M NaCl and 0.1% Triton X-100. Upward flow was at 16 ml/h, and 2.6-ml fractions were collected. The broken line shows the corrected elution pattern for an ultracentrifuged sample. V_o, Exclusion volume; V_e, elution volume; $V_o + V_i$, total volume.*

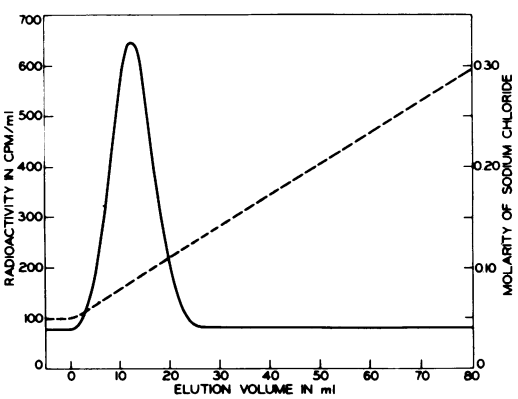

FIG. 3. *Elution pattern of a fraction (gel filtration pool II) of radiolabeled S. agalactiae extract from a column (1.5 by 15 cm) of DEAE-cellulose. Sample and column were equilibrated with a 50 mM sodium acetate buffer at pH 5.5 which contained 50 mM NaCl and 0.1% Triton X-100. The column was eluted with a linear NaCl gradient (50 to 300 mM) in the same buffer.*

DISCUSSION

Generally, nonionic detergents solubilize the cytoplasmic membrane of bacteria effectively

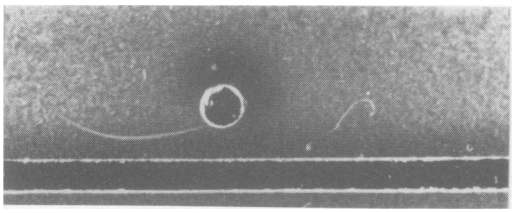

FIG. 4. *IE of an S. agalactiae component eluted from DEAE-cellulose between the NaCl molarities of 0.07 and 0.12. The trough contained hyperimmune anti-whole cell serum.*

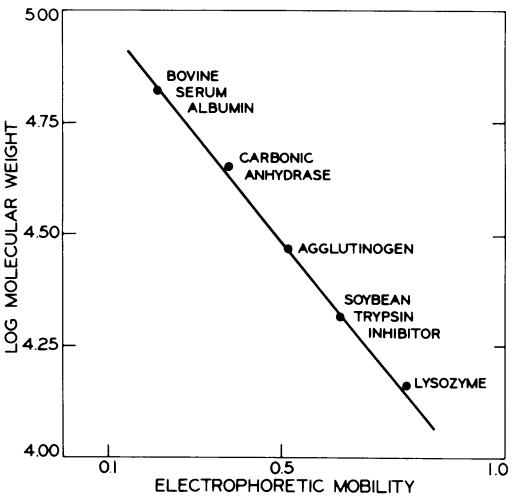

FIG. 5. *Estimation of the molecular weight of the S. agalactiae agglutinogen from a set of eight individual standard gels. The four marker proteins were lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), and bovine serum albumin (68,000). All proteins were run on duplicate gels. The mean mobility of each protein is plotted against the $\log_{10}$ of the molecular weights of the standards. The molecular weight of the streptococcal protein is estimated at 31,800.*

(12); nevertheless, only two antigenic components were detectable in concentrated whole cell extract.

The solubilization of the agglutinogen in the mild conditions described suggests that it is noncovalently bound to the cell wall, possibly in a way comparable to the aggregation of M proteins at the surface of group A streptococci (9, 20). Electron microscopy of thin sections of a group B streptococcus of the Ia subtype (C. B. Cropp, M.S.M. thesis, Colorado State University, Fort Collins, 1973) showed the presence of long pili or fimbriae at the cell surface; these structures, however, were absent or present in a diminished form on other subtypes. Electronmicroscopic studies of group B type III streptococci by different authors (1, 2) showed no evidence of structures on the cell surface comparable to the fimbriae of group A streptococci.

Micelles are spontaneously formed by Triton X-100 above a narrow concentration range (15) which depends on a number of environmental factors. Binding of the agglutinogen to detergent micelles or to mixed micelles, composed of bacterial components and detergent, may have been the basis of the high apparent molecular weight of the protein which was suggested by its early elution from the Sephadex G-200 gel. The large volume of this second gel filtration peak, which suggested heterogeneity in the size of its constituents, may have been caused by the same phenomenon; alternatively, the protein may be arranged naturally in large and heterogeneous quaternary complexes which mild detergents are often unable to dissociate (11, 12).

SDS-PAGE of the reduced preparation revealed a single band of protein and radioactivity, indicating that the protein is made up of a single polypeptide chain. The molecular weight of the protein was estimated at 31,800.

The antigenic relationship of the protein identified in this study to the protein antigen extracts used in the type classification of *S. agalactiae* (13, 17, 22, 23) could be further investigated.

The hypothesis formulated in the introduction can be tested experimentally; a theoretically appropriate antigen is available.

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