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A New Procedure for the Purification of Streptococcal Pyrogenic Exotoxin A from *Streptococcus pyogenes* Supernatant

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An important role in the pathogenesis of invasive group A streptococcal disease has been ascribed to the production of streptococcal pyrogenic exotoxin A. We present a new technique for the purification of streptococcal pyrogenic exotoxin A from *Streptococcus pyogenes* NY-5 supernate, which is highly efficient with respect to yield (35%), purity (≥99%), and time.

The past decade has seen a worldwide resurgence of severe invasive illness caused by group A streptococcus (GAS) (7, 17–19). In particular, streptococcal toxic shock-like syndrome is associated with a high patient fatality rate. Conspicuously, streptococcal toxic shock-like syndrome occurs predominantly in otherwise healthy persons of any age, although predisposing factors like trauma or varicella infection have been described (17).

The cause of the increasing severity of streptococcal disease remains obscure: no bacterial, host, or environmental factors have been conclusively implicated in invasive GAS infections. Streptococcal pyrogenic exotoxins (SPE) are considered superantigens with potent effects on the host, inducing polyclonal T-cell activation, cytokine responses associated with fever, and eventually a partial depletion of the T-cell repertoire with consequently decreased B-cell responses (1, 10, 13). Four antigenetically distinct pyrogenic exotoxins have been described and were designated SPE-A, -B, -C, and -F. The presence of SPE-A has been implicated in the pathogenesis of severe GAS infections, particularly in combination with the prevalence of certain M-protein types in the bacterial cell wall (4, 7, 16, 18–20, 23). Gene typing revealed the presence of *spe-A* in up to 85% of invasive GAS isolates (6, 21). Four SPE-A types are now known: A2 and A3 differ from the ancient A1 in only one amino acid, whereas 91% homology exists between these alleles and A4 (14, 15).

The goal of our studies is to develop immunochemical tools to measure αSPE-A antibody levels in patients suffering from invasive GAS diseases and SPE-A production by GAS strains in vitro. In addition, we want to know whether there is immunological cross-reactivity between the different SPE-A types. Although methods for the purification of SPE-A have been described by others before, only very small yields were obtained (2, 3). Therefore, the purpose of the present study was to develop an efficient procedure for the isolation of SPE-A starting with SPE-A1 from culture supernatants of GAS strain NY-5. Preliminary data with regard to their immunochemical application are presented.

SPE-A was isolated from GAS strain NY-5 (kindly provided by the National Institute for Environmental and Public Health, RIVM, Bilthoven, The Netherlands), which is known to be a strong producer of SPE-A1. The bacteria were grown overnight at 37°C in 50 ml of chemically defined medium (JRH Biosciences, Kansas, Mich.) supplemented with 0.25% (wt/wt) $NaHCO_3$, 0.05% (wt/wt) L-cysteine, and 0.06% (wt/wt) Todd-Hewitt broth (Difco, Detroit, Mich.). Next, the bacteria were grown for 8 h in a 20-fold volume of fresh medium. The supernatant was concentrated to a volume of 100 ml by ultrafiltration (10-kDa cutoff; Amicon, Lexington, Mass.) and dialyzed twice against 5 liters of PBS at 4°C. After precipitation for 24 h at 4°C with 4 vol of 96% ethanol (precooled to −20°C as described by Kim and Watson [9]), the exotoxin-containing precipitate was dissolved in 10 ml of acetate-buffered saline (0.01 M acetate buffer [pH 4.0] with 0.15 M NaCl) and added to 40 ml of 0.02 M bis-Tris propane buffer (Sigma, St. Louis, Mo.), pH 6.4. This crude extract was separated by fast-protein liquid chromatography (FPLC) anion-exchange chromatography (Mono Q; Pharmacia, Uppsala, Sweden). Elution was performed stepwise with the starting buffer supplemented first with 0.0, 0.200, and then with 1.0 M NaCl.

Fractions were analyzed for the presence of SPE-A by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), performed as described by Laemmli (12), using 12.5% running gels. Aliquots of the concentrated supernatant, the ethanol precipitate, and the FPLC fractions were applied to the gel. The gels were then subjected to silver staining and immunoblotting. The FPLC elution profile is shown in Fig. 1, and the SDS-PAGE mobility pattern of the fractions collected is given in Fig. 2 (top panel). The fractions which did not bind to the column were found to contain a single protein band which was identified as SPE-A by immunoblotting with polyclonal rabbit αSPE-A antibodies diluted 1:20,000 (Fig. 2 [bottom panel]).

For the preparation of SPE-A antiserum, an authentic preparation of SPE-A, purified according to Gerlach et al. (5), was emulsified into complete Freund adjuvant to a final concentration of 100 μg/ml. A total of 0.5 ml was given intramuscularly (im) to rabbits the first week and the same amount in incomplete Freund adjuvant was given on weeks 2, 3, and 4. A booster dose of 50 μg of SPE-A was given in saline after an additional 4 weeks and was followed by exsanguination 10 days later. Similarly, anti-SPE-A antibodies were raised against our purified exotoxin A in two rabbits. Both rabbits became anorectic and one died, indicating that the biological activity of the toxin was retained. Ouchterlony double immunodiffusion of the purified SPE-A with both anti-SPE-A antibody preparations demonstrated that the antibodies raised against the au-

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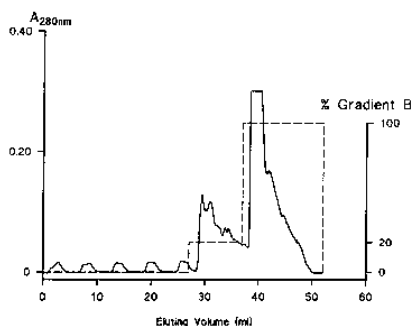

FIG. 1. Chromatogram of resolubilized ethanol precipitate containing SPE-A, showing the protein pattern after FPLC. – – – –, percentage elution buffer used; ——, A_{280} (protein profile).

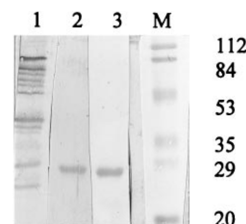

FIG. 3. Immunoblots for estimating SPE-A purity. Lanes: 1, NY-5 culture supernatant blotted and tested with human pooled serum; 2, SPE-A tested with human pooled serum; 3, SPE-A identified by polyclonal rabbit αSPE-A antibodies; M, low-molecular-weight marker proteins. Molecular masses are in kilodaltons.

thentic preparation of SPE-A reacted similarly to antibodies raised to our isolated exotoxin.

In agreement with others (5, 8), we found a molecular mass of 28.8 kDa by SDS-PAGE. Separation elution fractions revealed the presence of a series of proteins. Immunoblotting also identified SPE-A among the series of other proteins eluted from the column. These fractions were not used for further SPE-A isolation. We anticipated that SPE-A would have bound to the Mono Q column. SPE-A, however, bound only partially to the column, with the remainder passing through. In an attempt to explain this finding, we considered the possibility of column overloading. However, since SPE-A was the only passing protein, overloading could be excluded. We speculate, therefore, that after ethanol precipitation and resolubilization, a change in the conformation of SPE-A might take place, resulting in a decreased affinity of SPE-A for the anion-exchange column. Remarkably, there is no information on the secondary and tertiary structure of SPE-A available yet. Till now, only the primary molecular structure has been clarified (22). Another possibility may be that substances coming from NY-5 supernatant may surround SPE-A and thus be able to change the column elution profile. In contrast, Cunningham et al. (3) observed binding of SPE-A to an anion-exchange column. However, they used different columns, different buffers, and a different pH in their experiments.

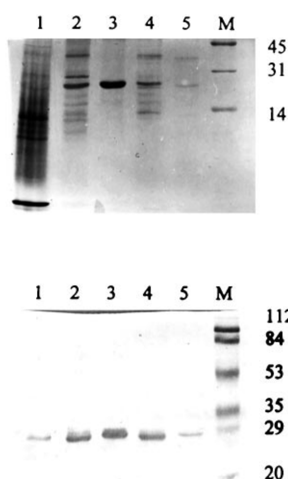

FIG. 2. Gel electrophoresis of the toxin fractions after silver staining (top) and immunoblotting using polyclonal rabbit αSPE-A antibodies (bottom). Lanes: 1, FPLC fractions obtained with 100% eluting buffer; 2, FPLC fractions obtained with 20% eluting buffer; 3, nonbinding FPLC fractions; 4, ethanol precipitate; 5, culture supernatant after ultrafiltration; M, low-molecular-weight marker proteins. Molecular masses are in kilodaltons.

In order to establish SPE-A purity of the nonbinding fractions, another immunoblotting experiment was performed with untreated human pooled serum diluted 1:1,000 as reagent. We observed the presence of a single fat band at 28.8 kDa, suggesting high purity of the SPE-A isolated (≥99% purity) (Fig. 3). We found a pI of 5.2, which is more proof that SPE-A was isolated and SPE-B, which has a reported pI of 8.0 to 9.0 (5, 8), was not.

In a pilot study, αSPE-A antibody levels were determined in serum samples from 20 healthy adult individuals by enzyme-linked immunosorbent assay (ELISA). Round-bottom 96-well microtiter ELISA plates (Costar 2595, Cambridge, Mass.) were coated overnight at 37°C with 100 μl of SPE-A (0.5 μg/ml in saline) purified as described above. Blocking was performed with 1% gelatine in twice-distilled water, and then the plates were incubated with 100 μl of human serum from healthy individuals and serially diluted in washing buffer. In every plate used, a reference serum was included as the control. End-point titers in ELISA at an optical density of 0.500 were calculated by linear regression and expressed as log dilution factor. Antibody contents of the sera tested were expressed in arbitrary units per milliliter, relative to the simultaneously measured titer of the reference serum after reversed log transformation. ELISA results showed a wide variety of αSPE-A immunoglobulin G antibody titers (Fig. 4). It could be speculated that individuals with high anti-SPE-A antibody titers might be protected from developing invasive GAS disease. High-titered sera were selected for experiments on SPE-A production by GAS strains in vitro.

The A_{280} of purified SPE-A was measured and compared with an albumin reference curve. We estimated in this way a total amount of 5.4 mg of SPE-A having been purified from 1 liter of NY-5 culture, which is more than has ever been re-

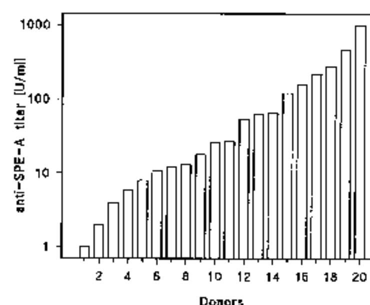

FIG. 4. Antibody titers, in increasing order, against SPE-A in sera from 20 healthy individuals.

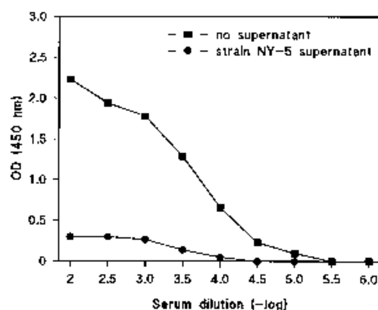

FIG. 5. Detection of SPE-A by inhibition ELISA. High-titered human αSPE-A serum and supernatant from GAS strain NY-5 were incubated in solution, and the concentration of free αSPE-A antibody was determined at equilibrium by ELISA. Todd-Hewitt broth was used as a negative control. Purified NY-5 SPE-A was used as coating reagent. OD (450 nm), optical density at 450 nm.

ported in literature. Correspondingly, Köhler et al. (11) reported that under optimal culture conditions strain NY-5 did not produce more than 16 mg of SPE-A per liter. The yield of the SPE-A purification was determined by inhibition ELISA, which was performed briefly as the ELISA described above: aliquots of serially diluted fractions, obtained after the different isolation steps, were tested in 100-μl volumes. These aliquots were mixed with 50-μl high-titered human serum in a final concentration of 1:1,000 in 96-well flat-bottom microtiter plates (Greiner GmbH, Frickenhausen, Germany) and preincubated for 1 h. Volumes of 100 μl were pipetted onto the coated and subsequently blocked plates (Costar) and incubated at 37°C for 1 h. The relative inhibition of ELISA reactivity was used as a measure of the amount of SPE-A present after the different purification steps. Yields after ultrafiltration, dialysis, and ethanol precipitation were estimated as 94, 83, and 55%, respectively. We estimated a final yield of 35% for our purification procedure after anion-exchange chromatography, which is in agreement with the amount of 16 mg of toxin A per ml produced by GAS strain NY-5.

SPE-A production by GAS strains was also estimated by the inhibition ELISA. Growth curves showed that the tested strains started to produce SPE-A after having reached the mid-log phase after a culture period of 6 h (data not shown). Thus, SPE-A production by the tested strains was estimated after 6 h of culture. GAS strain NY-5, containing the *spe-A1* gene, was found to fully inhibit ELISA reactivity in our assay (Fig. 5). Inhibition of ELISA reactivity was perceived after the addition of supernatant up to a dilution of 1:100.

We conclude that the technique we have developed for SPE-A isolation is highly efficient with respect to yield, purity, and time. Moreover, the purified toxin is useful for the determination of anti-SPE-A antibody levels in serum samples. Whether the procedure described here for SPE-A1 isolation is also suitable for the purification of SPE-A of different allotypes has to be established.

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