PURIFICATION AND PARTIAL CHARACTERIZATION OF THE NEPHRITIS STRAIN-ASSOCIATED PROTEIN FROM *STREPTOCOCCUS PYOGENES*, GROUP A

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Numerous investigators, most notably Rammelkamp (1) and Stollerman (2), have established that acute poststreptococcal glomerulonephritis (APSGN)[1] results from infection with a limited number of serological types of group A streptococci. However, even within a given nephritogenic type, it has been noted that not all strains cause nephritis, thereby suggesting that only certain strains of these nephritogenic types elaborate a streptococcal product that may be responsible for the initiation of APSGN.

Histological data, in association with immunofluorescent studies of a large number of glomerular biopsies in APSGN have demonstrated the presence of streptococcal antigen(s) in the glomeruli. Seegal et al. (3) have suggested that antigen(s) localized in the glomerular wall of APSGN patients were part of the streptococcal cell wall; whereas Lange et al. (4) have suggested that a cytoplasmic entity known as endostreptosin was the antigen in question. In a similar approach, Ahmed et al. (5) suggested that the putative agent resides also within the streptococcal cell and could be detected only in the supernatant of disrupted streptococci. Vogt et al. (6, 7) reported that cationic, extracellular streptococcal antigens were observed in 8 out of 18 renal biopsies from patients with APSGN and suggested that APSGN was initiated by in situ immune complex formation with these cationic antigens.

Villareal et al. (8) reported that group A streptococci isolated from patients diagnosed as having developed APSGN secreted an extracellular protein that had a subunit of M_r 46,000 as determined by SDS-PAGE. This protein was generally not secreted by streptococcal strains isolated from patients who were diagnosed as not having APSGN. Because of this association between isolate and disease, this protein was called the nephritis strain-associated protein (NSAP). Villareal et al. (8) also showed that several M types of group A streptococci representing both the classical skin and throat strains elaborated this protein. Polyclonal rabbit antibody prepared to this extracellular protein and used as a

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[1] *Abbreviations used in this paper:* APSGN, acute poststreptococcal glomerulonephritis; CDM, chemically defined medium; ESS, endostreptosin; NSAP, nephritis strain-associated protein; TBI buffer, Tris/Brij incubation buffer; TTB buffer, Tris/Tween blocking buffer.

histological probe indicated that the NSAP was present in 14 out of 21 kidney biopsies of patients with APSGN. Ohkuni et al. (9), using an electrophoretically purified NSAP preparation, showed that >95% of APSGN patients exhibited antibody to this protein, while only 20% of either acute rheumatic fever or impetigo sera contained antibody to NSAP.

We have reassessed the relationship between the presence of NSAP in group A streptococcal strains in relation to the source of the strain. Using mAb specific to NSAP and a new solid-phase ELISA, the original observations of Villareal et al. (8) were confirmed. In addition, a method of isolating and purifying NSAP in sufficient amounts for structural and biochemical studies is presented. It became apparent that NSAP is a major extracellular protein of these streptococcal strains isolated from APSGN patients. Preliminary structural and biochemical data on purified NSAP indicated that this protein possesses both structural and biochemical properties identical to streptokinase.

Materials and Methods

Growth Medium. The chemically defined medium of van de Rijn et al. (10) was used throughout this study. The medium was prepared by sequential addition of solubilized amino acids, vitamins, purines and pyrimidines to buffer salts in the order recommended by van de Rijn et al. (10), with the exception that mineral salts were added last. For medium not to be used immediately, glucose, $NaHCO_3$ and cysteine-HCl were added just before use. All solutions, including complete medium were filter sterilized by passage through 0.22 μm cellulose triacetate Metricel membranes (Fischer Scientific, Pittsburgh, PA). All medium components were the best grade possible, purchased from Sigma Chemical Co., St. Louis, MO.

Streptoccal Strains. 24 strains of *Streptococcus pyogenes* group A, originally described by Villareal et al., (8 and Table I) were used in this study. 12 strains had been isolated from patients who had been diagnosed as having APSGN, and 12 strains had been isolated from patients diagnosed as having suppurative and/or nonsuppurative sequelae other than APSGN. All strains were reconstituted from the lyophilized state on sheep blood agar; single colonies were selected and inoculated into the chemically defined medium (CDM). Adaptation to growth in CDM was accomplished by repeated subculture in this medium.

SDS-PAGE. All electrophoretic analyses were studied by SDS-PAGE according to the procedure of Laemmli (11). Electrophoresis was carried out on 10% polyacrylamide gels with a 5% stacking gel, at a constant current of 50 mA. Phosphorylase b (97.4 kD), BSA (68 kD), ovalbumin (45 kD), glyceraldehyde dehydrogenase (36 kD), carbonic anhydrase (29 kD), and β-lactoglobulin (18.4 kD) were used as molecular mass standards. Both samples and standards were mixed with equal portions of sample buffer containing 2% (wt/vol) SDS, 2% (vol/vol) 2-ME, 30% (vol/vol) glycerol, 0.01% (wt/vol) bromophenol blue, and were boiled for 2 min before application to gel.

Immunological Analyses. Samples were analyzed for the presence of NSAP by two procedures. The methods were either Western blot analysis or dot blot analysis. For Western blot analysis, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes (BA85; Schleicher and Schuell, Keene, NH) according to the procedure of Towbin et al. (12), with a transfer time of 1 h at 1 A. After transfer, the nitrocellulose membrane was incubated for at least 1 h at room temperature with two changes of 10 mM Tris-HCl buffer, pH 8.2, containing 0.5% (vol/vol) Tween 20, 0.5 M NaCl, and 0.04% (wt/vol) sodium azide (TTB buffer) to block remaining protein binding sites. Primary antibody, diluted in 10 mM Tris-HCl buffer, pH 8.2, containing 0.15 M NaCl, 0.05% (vol/vol) Brij 35, and 0.02% (wt/vol) sodium azide (TBI buffer) was applied and allowed to react for 2 h at room temperature. The membrane was then washed three times with intermittent 5-min incubations in TTB buffer. Secondary (signal) antibody,

conjugated to bovine calf intestine alkaline phosphatase (Type VII-S; Sigma Chemical Co.) by the procedure of Avrameas et al. (13), diluted in TBI buffer, was applied. After incubation at room temperature for 1 h, the membrane was washed four times at 5-min intervals with TTB buffer, followed by several rinses with 50 mM Tris-HCl buffer, pH 10.0, containing 3 mM $MgCl_2$. The reaction was developed by addition of enzyme substrate solution, which was prepared by adding to 9 ml Tris-HCl buffer, pH 10.0, containing 3 mM $MgCl_2$, 1 ml of 0.1% (wt/vol) nitroblue tetrazolium (Sigma Chemical Co.) in distilled water, and 100 μl of 0.05% (wt/vol) 5-bromo-4-chloro-indolyl phosphate (Sigma Chemical Co.) in anhydrous dimethylformamide (Pierce Chemical Co., Rockford, IL). After development, the reaction was stopped by rinsing with distilled water. Membranes were dried at room temperature and stored between acetate sheets.

For dot blot analysis, 50 μl aliquots of samples to be tested were pipetted onto a nitrocellulose membrane (BA85, Schleicher and Schuell) encased within a 96-well Minifold Apparatus (Schleicher and Schuell). After drying, the membrane was removed from the chamber, blocked, and probed as described above for Western blot analysis.

ELISA was performed according to Voller et al. (14). Purified antigen was immobilized with carbonate buffer to Immulon II polystyrene microtiter wells (Dynatech Laboratories, Alexandria, VA). After binding and blocking with 0.05% Brij 35 (vol/vol) in 10 mM Tris-HCl buffer, pH 7.5, antisera diluted in TBI buffer were added. After incubation for 2 h at 37°C, plates were washed with blocking buffer. Signal antibody, either goat $F(ab)'_2$ anti-rabbit IgG(Fc) antibody or goat $F(ab)'_2$ anti-mouse IgG (H and L chains) antibody, conjugated to bovine intestinal alkaline phosphatase by the method of Avrameas et al. (13), diluted in TBI buffer, was added to appropriate wells and incubated for 2 h at 37°C. After washing with 0.05% Brij 35 (vol/vol) Tris-HCl buffer, pH 7.5, 0.1 M diethanolamine buffer, pH 9.8, containing 1 mM $MgCl_2$ and *p*-nitrophenyl phosphate (1 mg/ml) was added. The reaction mixture was incubated at 37°C for 30 min, at which time the absorbance was recorded at 405 nm using a Titerteck Multiscan, Flow Laboratories, McLean, VA. All assays were performed in triplicate.

Immunological Reagents. Murine mAb against NSAP (mAb-NSAP) isolated from strain A374, was prepared from the procedure described by Buskirk et al. (15).

Rabbit polyclonal antibody against purified NSAP isolated from *Streptococcus pyogenes*, group A, strain A374 was prepared as follows. 200 μg purified (see below) NSAP in CFA was administered intradermally at multiple sites into the scapular region of New Zealand white rabbits. After 30 d, animals were given 100 μg purified NSAP in IFA by intramuscular injection. When antibody titers to NSAP were sufficiently high, as measured by the indirect enzyme immunoassay (see above), animals were bled, and sera processed for Ig isolation and purification by ammonium sulfate precipitation followed by chromatography on protein A–Sepharose 4 B (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Hjelm and Sjoquist (16).

Rabbit polyclonal antibody to purified streptokinase isolated from Group C streptococcus, strain H46 was a generous gift from Professor Stig Holm, University of Umea, Umea, Sweden.

Analysis of Culture Supernatants for Presence of NSAP. Streptococcal strains originally described by Villareal et al. (8) were cultured in 100 ml of CDM in 500 ml nephelometer flasks. Each culture flask was inoculated with organisms to an OD of 0.05 (550 nm) and incubated at 37°C with constant gyratory stirring at 50 rpm. 10-ml aliquots were removed at intervals representing the early log, exponential, late log, early stationary, and late stationary phases of growth. Cells were removed by centrifugation at 10,000 *g* for 15 min at 4°C. Supernatants were filtered through 0.22 μm cellulose triacetate Metricel membranes and protein precipitated by addition of a one-fifth volume of 60% (wt/vol) cold TCA. After centrifugation at 12,000 *g* for 5 min, pellets were washed and neutralized with cold 95% (vol/vol) ethanol containing 5% (vol/vol) 2 M sodium acetate and 0.01% (wt/vol) phenol red. Pellets were resuspended in 50 μl distilled water and an equal volume of SDS-PAGE sample buffer was added. Samples were then analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and subsequently probed with mAb specific for NSAP.

Isolation and Purification of NSAP. Strain A374, adapted to growth in CDM, was inoculated into 2-liter volumes of CDM to an initial OD of 0.05 (550 nm). Cultures were grown at 37°C in roller bottles (3027; Falcon Labware, Oxnard, CA) and rotated at 1 rpm. The pH of the culture was monitored by sterile removal of 10-ml aliquots at hourly intervals; a pH of 6.8 was maintained by addition of sterile 5 N NaOH. At early stationary phase of growth, iodoacetamide (1 mg/ml) and PMSF (1 mM) were added. The culture was centrifuged at 10,000 *g* for 25 min at 4°C; the supernatant was decanted and filtered through a 0.22 μm cellulose triacetate Metricel membrane. Solid ammonium sulfate (Type III; Sigma Chemical Co.) was added to the filtered supernatant to a final concentration of 70% saturation. After stirring at 4°C for 18 h, the suspension was centrifuged at 10,000 *g* at 4°C for 25 min. The pellet was washed twice with 70% saturated ammonium sulfate. The washed precipitate was suspended in 50 mM Tris-HCl buffer, pH 7.5, and dialyzed against several changes of same buffer until all traces of ammonium sulfate were removed. Residual sulfate in dialysate was tested by adding 100 μl 1% (wt/vol) barium acetate, and 200 μl 1 N HCl to 1 ml dialysate.

Dialyzed material was applied to a 1.5 × 20 cm column of DEAE-Sepharose 6 B-Cl (Pharmacia Fine Chemicals) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. After application of sample and elution with four column volumes of 50 mM Tris-HCl buffer, pH 7.5, NaCl gradient (0–0.5 M) in 50 mM Tris-HCl buffer, pH 7.5, was applied. Fractions were collected and assayed for absorbance at 280 nm and NSAP by dot blot analysis using mAb-NSAP. Fractions that were mAb-NSAP–reactive were pooled and dialyzed against 25 mM piperazine-HCl buffer, pH 5.5. After dialysis, the sample was applied to a 0.7 × 50 cm column of Polybuffer Exchanger (PBE 94; Pharmacia Fine Chemicals) previously equilibrated with 25 mM piperazine-HCl buffer, pH 5.5. After application of sample and elution with five vol of start buffer, 12 vol of a 1:10 dilution of Polybuffer 74 (Pharmacia Fine Chemicals), titrated to pH 4.0 with 1 N HCl were added. The column was eluted at a constant flow rate of 15 cm/h. Fractions were monitored at 280 nm and by dot blot analysis using mAb-NSAP as a probe. Fractions containing NSAP were pooled and dialyzed against 0.1 M $NaHCO_3$, pH 8.0, before lyophilization.

Amino Acid Analysis. Quantitative amino acid analysis was performed on 1 nmol of lyophilized purified NSAP. Samples were hydrolyzed in 6 N HCl containing 1% (wt/vol) phenol at 110°C for 22 h in evacuated sealed tubes. Analysis was performed on a Waters Pico-tag HPLC Systems.

Amino-terminal Sequence Analysis. Amino-terminal sequence analysis was performed on 2.4 nmol purified NSAP by automated Edman degradation on an Applied Biosystems Gasphase Sequencer (Model 470A). PTH-amino acids were identified by HPLC as described by Manjula *et al.* (17).

Biochemical Assays. Plasminogen activation was studied by the spectrophotometric method of Jackson et al. (18) as adapted from the method of Radcliffe and Heinze (19). In this method, the plasmin-specific synthetic tripeptide substrate, D-Val-Leu-Lys-*p*-nitroanilide (Sigma Chemical Co.) is cleaved by plasmin to release *p*-nitroanilide, which is measured at an absorbance of 405 nm. Human plasminogen was obtained from Sigma Chemical Co. Assays were performed in 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). To a microwell were added 10 μl of purified NSAP (1 μg/ml) or purified streptokinase isolated from group C streptococcus H46A (Sigma Chemical Co.) (1 μg/ml) in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.5% (wt/vol) BSA, 20 μl 50 mM Tris-HCl buffer, pH 7.5, and 20 μl human plasminogen (0.5 mg/ml) in 50 ml Tris-HCl buffer, pH 7.5, containing 0.5% (wt/vol) BSA. After incubation at 37°C for 15 min, 30 μl of substrate solution were added, which was prepared just before use by mixing 100 μl of D-Val-Leu-Lys-*p*-nitroanilide (5 mg/ml in distilled water) with 150 μl 1.77 M NaCl in 0.032 M Tris-HCl buffer, pH 7.5. The reaction mixture was incubated at 37°C. The reaction was ended by addition of 200 μl of 0.5 M acetic acid. The absorbance was read at 405 mm using a Titerteck Multiscan, Flow Laboratories, McLean, VA. All assays were performed in triplicate.

Results

Analysis of Culture Supernatants with mAb-NSAP. When the culture supernatant of Group A streptococcus strain A374 was analyzed by SDS-PAGE and Western blot, NSAP (46 kD) appeared in the early log phase of growth and continued to be secreted throughout the growth of the culture (Fig. 1). However, as the culture entered late log phase of growth, a second protein appeared (43 kD) that was also recognized by the mAb prepared against the intact 46 kD molecule; both polypeptides possessed the epitope to which the mAb-NSAP was directed. As the culture progressed from the late log phase to the stationary phase of growth, the pH dropped from 7.0 to 5.5. This decrease in pH correlated with emergence of the 43 kD segment. When the pH of the culture was maintained at 6.8 by addition of NaOH the 46 kD molecule did not undergo degradation. When 24 of the streptococcal isolates described by Villareal et al. (8) were analyzed by Western blot analysis, a similar pattern of degradation of the intact NSAP was observed. From Table I, the most interesting observation was that certain strains isolated from APSGN patients would excrete intact NSAP for a period of time, then cease, while others would continue to secrete NSAP as the intact 46 kD species with little or no degradation to the 43 kD (or smaller) fragments.

Isolation of NSAP. When the ammonium sulfate precipitate of a 2-liter culture supernatant of strain A374 grown to early stationary phase of growth was applied to a DEAE–Sepharose 6 B anion-exchange column and eluted with an NaCl gradient (0–0.5 M), several peaks absorbing at 280 nm were observed (Fig. 2). When fractions were analyzed by dot blot analysis using mAb-NSAP, NSAP eluted from the column with 0.13–0.16 M NaCl.

Those fractions containing NSAP were pooled and dialyzed against 25 mM piperazine HCl buffer, pH 5.5, before application to a Polybuffer Exchange 94 chromatofocusing column. Elution of the chromatofocusing column with a 1:10 dilution of Polybuffer 74, pH 4.0, resolved the sample into several discrete fractions (Fig. 3). Dot blot analysis of these fractions using mAb-NSAP showed that NSAP was eluted at a pI of 4.75. Dialysis and lyophilization of NSAP$^+$

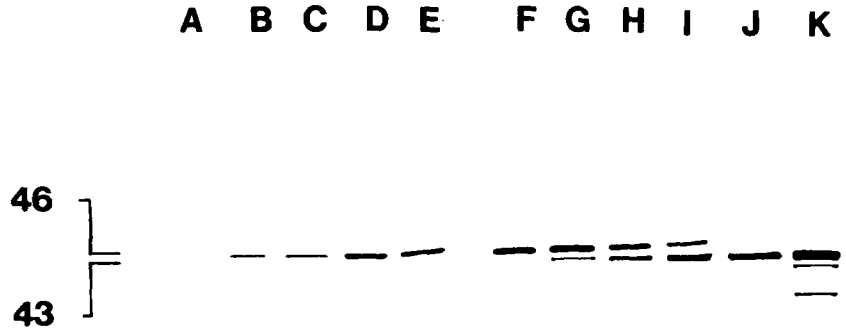

FIGURE 1. Western blot analysis of culture supernatant of group A *Streptococcus pyogenes* strain A374 grown in CDM. 10-ml aliquots were removed at different times in the growth curve: *A*, 0 h; *B*, 1 h; *C*, 2 h; *D*, 3 h; *E*, 4 h; *F*, 5 h; *G*, 6 h; *H*, 7 h; *I*, 8 h; *J*, 12 h; *K*, 18 h. Total protein was precipitated by addition of TCA to 10% (vol/vol). After washing, precipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed using mAb-NSAP as probe (see Materials and Methods).

TABLE I

Presence of NSAP in Streptococcal Strains as Detected by Western Blot Analysis Using mAb-NSAP

Strain	M type	Patient source*	NSAP detected‡ at various times§ (h):			
			2	4	6	8
F203D	1	APSGN$^+$	+	±	±	±
A207	2	APSGN$^+$	+	+	±	±
B512	4	APSGN$^+$	+	±	±	±
B974	4	APSGN$^+$	+	+	+	+
B743	5	APSGN$^+$	+	±	±	±
t12	12	APSGN$^+$	+	±	±	±
B923	12	APSGN$^+$	+	+	+	+
A374	12	APSGN$^+$	+	+	+	+
B438	18	APSGN$^+$	+	±	±	±
Gt8760	49	APSGN$^+$	+	±	±	±
A928	55	APSGN$^+$	+	±	±	±
A995	57	APSGN$^+$	+	±	±	±
D480	1	APSGN$^-$	−	−	−	−
A830	3	APSGN$^-$	−	−	−	−
A964	5	APSGN$^-$	−	−	−	−
D313	12	APSGN$^-$	−	−	−	−
A992	18	APSGN$^-$	−	−	−	−
A456	36	APSGN$^-$	−	−	−	−
A315	50	APSGN$^-$	−	−	−	−
D438	55	APSGN$^-$	−	−	−	−
D438	55	APSGN$^-$	−	−	−	−
B931	2	APSGN$^-$	−	−	−	−
A915	4	APSGN$^-$	−	−	−	−
A552	9	APSGN$^-$	−	−	−	−
A834	49	APSGN$^-$	−	−	−	−

* APSGN$^+$ streptococcal strain obtained from patient diagnosed as having APSGN. APSGN$^-$ streptococcal strain obtained from patient diagnosed as not having APSGN.

‡ + indicates presence of intact 46 kD NSAP; ± indicates major protein is 43 kD, with traces of intact 46 kD; − indicates the absence of NSAP in the supernatant of these cultures.

§ Time of analysis of culture supernatant. 2 h is early exponential, 4 h is late exponential, 6 h is early stationary, and 8 h is late stationary phase of growth.

fractions resulted in a yield of 0.9 mg NSAP per liter of culture supernatant from a culture grown to early stationary phase of growth under controlled pH conditions. Repeated isolations under identical growth conditions resulted in yields of NSAP that varied from 0.8 to 1.2 mg/liter of culture supernatant.

As this purification procedure was highly reproducible, subsequent isolation of NSAP was accomplished by step-wise salt elution of the $(NH_4)SO_4$ precipitate on a DEAE–Sepharose 6B anion-exchange column. When 0.1 M increments of NaCl were applied to the anion-exchange column, all the NSAP was eluted by 0.2 M NaCl (Fig. 4). This was confirmed by Western blot analysis of each fraction eluted with different molarities of salt (Fig. 5).

Figs. 4 and 5 summarize the isolation of NSAP from an $(NH_4)_2SO_4$ precipitate

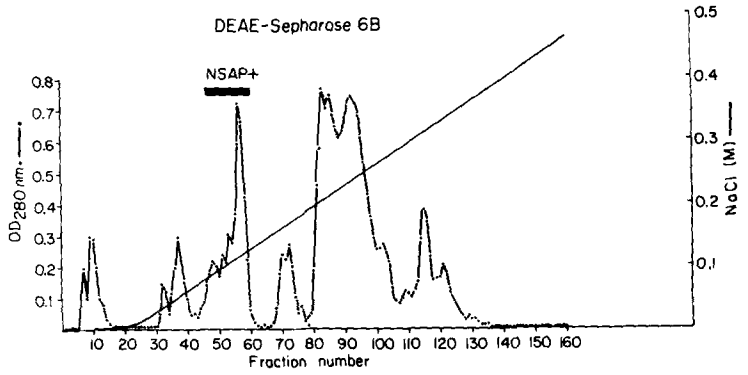

FIGURE 2. DEAE–Sepharose 6 B chromatography of ammonium sulfate precipitate of culture supernatant of group A *Streptococcus pyogenes* strain A374. Column was eluted with 50 mM Tris-HCl buffer, pH 7.5, with an NaCl gradient of 0–0.5 M. Fractions were analyzed for absorbance at 280 nm (●——●), and presence of NSAP by dot blot analysis of individual fractions (▬).

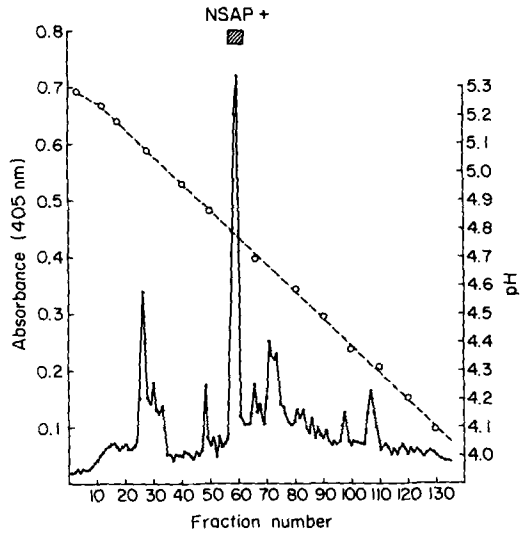

FIGURE 3. Chromatofocusing profile of fraction eluted with 0.2 M NaCl from a DEAE–Sepharose 6 B column (see Fig. 2). Column was monitored for protein at an absorbance of 280 nm (●——●); pH was monitored (– – – –), and fractions were analyzed for the presence of NSAP by dot blot analysis (▨). 2-ml fractions were collected.

of the culture supernatant by step-wise salt elution on a DEAE–Sepharose 6B anion-exchange column, followed by fractionization on a PBE94 chromatofocusing column.

Amino-terminal Sequence and Amino Acid Data on Purified NSAP. Amino-terminal sequence analysis was performed with the purified NSAP. The resultant sequence through residue 21 was compared with all the known sequences of proteins in the Protein Sequence Database using the fast PS program (20). Of the 3384 proteins analyzed, the amino-terminal sequence of streptokinase from group C streptococcus was identical to the amino-terminal sequence of the isolated NSAP (Fig. 6).

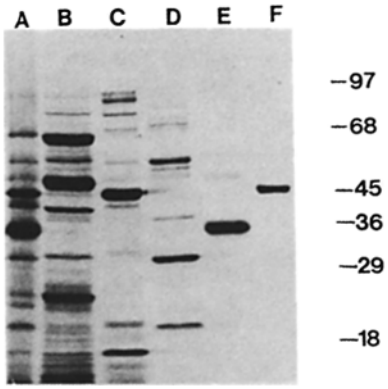

FIGURE 4. SDS-PAGE profile of fractions eluted from DEAE–Sepharose 6B-Cl by batch-wise elution with NaCl in 50 mM Tris-HCl buffer, pH 7.5. Starting material was NH_4SO_4 precipitate of culture supernatant of group A *S. pyogenes* strain A374. *A*, sample before application to column; *B*, fraction eluted with 0.1 M NaCl; *C*, fraction eluted with 0.2 M NaCl; *D*, fraction eluted with 0.3 M NaCl; *E*, fraction eluted with 0.4 M NaCl; *F*, fraction eluted from PBE chromatofocusing column at pH 4.75.

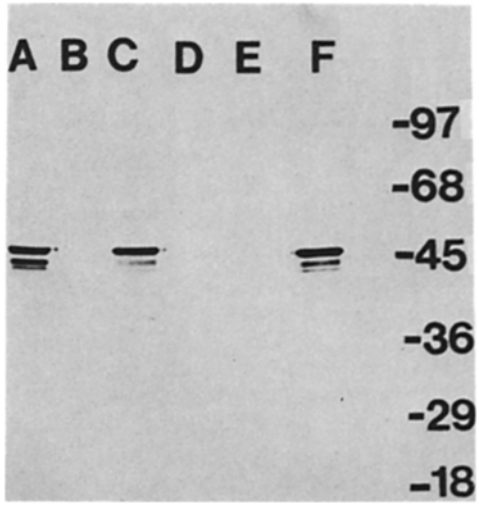

FIGURE 5. Western blot analysis of fractions eluted from DEAE–Sepharose 6 B column and PBE 94 chromatofocusing column. *A*, sample of NH_4SO_4 precipitate of supernatant of strain A374 *S. pyogenes* group A before application to DEAE column; *B*, fraction eluted with 0.1 M NaCl; *C*, fraction eluted with 0.2 M NaCl; *D*, fraction eluted with 0.3 M NaCl; *E*, fraction eluted with 0.4 M NaCl; *F*, fraction from PBE 94 chromatofocusing column eluted at pH 4.75. Nitrocellulose membrane was probed with mAb-NSAP (see Materials and Methods).

	10	20
NSAP:	NH_2-Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser-Val-Asn-Asn-Ser-Gln-Leu-Val-Val-Ser-	
C-Skase:	NH_2-Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser-Val-Asn-Asn-Ser-Gln-Leu-Val-Val-Ser-	

FIGURE 6. *N*-terminal sequence of NSAP isolated from group A *Streptococcus*, strain A374, compared to that of streptokinase isolated from group C *Streptococcus*, strain H46A. Group C streptokinase (Skase) sequence as reported by Jackson and Tang (29).

TABLE II

Amino Acid Composition of NSAP and Streptokinases Isolated from Group A, C, and G Streptococci

Amino acid	NSAP	Amino acid composition of streptokinases isolated from:							
		Group A					Group C		Group G
		1*	2	3	4	5	6	7	8
Asx	55	62	59	57	58	56	57	67	55
Glx	45	43	46	48	48	49	42	45	54
Ser	26	27	25	23	26	22	24	26	22
Gly	22	21	21	24	22	27	20	20	22
His	12	12	10	9	11	9	9	9	8
Arg	19	20	23	19	20	15	18	19	17
Thr	27	27	27	29	27	29	26	28	27
Ala	26	24	25	30	27	30	21	22	35
Pro	17	22	20	20	20	25	21	21	19
Tyr	19	23	21	19	21	18	20	21	16
Val	26	26	28	27	26	25	20	23	30
Met	4	4	5	5	5	6	4	4	5
Cys	0	0	0	0	0	0	0	0	0
Ile	18	19	21	22	20	19	20	22	22
Leu	36	38	37	37	37	32	35	39	37
Phe	15	17	16	16	16	15	14	15	14
Lys	29	29	30	30	31	37	29	34	32
Trp	ND	ND	ND	ND	ND	ND	2	1	ND

Values represent the content of amino acids expressed mole/mole protein based on a molecular weight of 46,000. ND, not determined.

* 1–5, Gerlach and Kohler (30); 6, Brockway and Castellino (31); 7, Morgan and Henschen (32); 8, Gerlach and Kohler (30).

When the amino acid analysis of purified NSAP isolated from group A streptococcus, strain A374, was compared to the published amino acid analysis of streptokinases isolated from groups A, C, and G streptococci, a close similarity was observed (Table II).

Biochemical and Immunological Analyses of Purified NSAP. The amino-terminal sequence and amino acid analysis data indicated that NSAP was a streptokinase-like molecule, and might thus activate plasminogen to produce plasmin. This was tested using the plasmin-specific synthetic substrate, D-Val-Leu-Lys-*p*-nitroanilide. When purified NSAP was incubated in the presence of human plasminogen, the plasmin substrate was cleaved to release *p*-nitroanilide, which could be measured spectrophotometrically. Fig. 7 illustrates the activity of purified NSAP in the presence and absence of human plasminogen. Streptokinase, isolated from group C streptococcus strain H46A also activated plasminogen to form plasmin, which acted upon the synthetic substrate. Both NSAP and streptokinase alone did not cleave the plasmin substrate.

Purified NSAP was also examined for its antigenic relatedness to streptokinase isolated from group C streptococcus strain H46A. Enzyme-linked immunoanalysis (Fig. 8*a*) showed that NSAP isolated from group A streptococcus strain A374 shared antigenic determinants with streptokinase from Group C when tested with rabbit polyclonal antisera prepared to either NSAP or streptokinase.

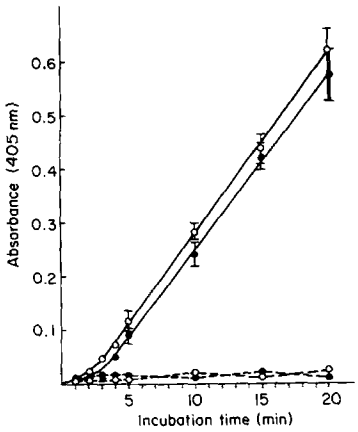

FIGURE 7. Plasminogen activation profile of purified NSAP and streptokinase from group C streptococcus strain H46A. Equivalent amounts (2×10^{-12} mol) of NSAP (●——●) and streptokinase (○——○) were incubated with an equivalent amount of human plasminogen in the presence of the plasmin substrate D-Val-Leu-Lys-*p*-nitroanilide. Identical amounts of NSAP (●- - - -●) and streptokinase (○- - - -○) were also incubated with the substrate in the absence of plasminogen. Activity was monitored for 20 min at 405 nm to measure the release of *p*-nitroanilide from the substrate by the action of plasmin.

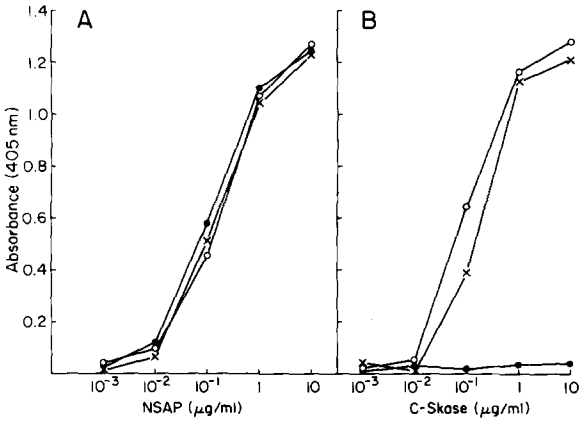

FIGURE 8. Antigenic analysis of purified NSAP and streptokinase from group C streptococcus strain H46A. Varying amounts of NSAP (*A*) and streptokinase (*B*) were immobilized to polystyrene microtiter wells. Wells were incubated with standardized dilutions of rabbit polyclonal antibody to NSAP (×), rabbit polyclonal antibody to streptokinase (○) and mAb-NSAP (●). Alkaline phosphatase–conjugated anti–primary antibody was used as the secondary antibody. Each point represents the mean of triplicate determinations.

However, when the same antibodies were tested against streptokinase isolated from group C streptococcus strain H46A, only the polyclonal antibody raised to NSAP and streptokinase from group C reacted with purified C-streptokinase (Fig. 8*b*); the mAb against NSAP did not recognize C-streptokinase, thus indicating that NSAP was antigenically different from the streptokinase from group C.

Discussion

NSAP was purified from the culture supernatant of group A *Streptococcus pyogenes* strain 374. This strain had been isolated from a patient diagnosed as having APSGN. This protein, of molecular mass 46 kD was a major extracellular protein produced by this strain. From cultures grown to early stationary phase of growth, an average yield of 1 mg/liter of purified NSAP could be obtained.

When the original observations of Villareal et al. (8) were reassessed using immunological methods, it became evident that, as the culture entered stationary phase of growth, a second protein (43 kD) appeared in the supernatant. As the culture supernatants were tested for the presence of NSAP with an mAb raised against this 46 kD protein, it was apparent that the 43 kD species was most likely a cleaved product of NSAP sharing the same epitope. Previous observations by Johnston et al. (21) have indicated that this degradation of the NSAP molecule during growth of a culture was possibly the result of enzymic activity by streptococcal proteinase. It has been known for many years that the majority of group A streptococci elaborate an extracellular zymogen, which, under the reducing conditions of growth and a pH of <6.5, is converted to an active mercaptoproteinase (22–24). If the culture medium is maintained at a pH >6.8, this conversion of zymogen to proteinase is depressed. Thus, for optimum yields of NSAP, streptococci were grown in medium in which the pH was maintained between 6.8–7.0. Furthermore, initial purification procedures used iodoacetamide to inactivate any reduced active form of the proteinase. This lability of NSAP to enzymic degradation may explain the difficulties encountered by other investigators (8) in their attempts to isolate NSAP.

The 46 and 43 kD species of NSAP are reminiscent of other streptococcal proteins implicated in APSGN. Yoshizawa et al. (25) have isolated, from a water soluble fraction of disrupted streptococcal strains isolated from APSGN patients, a protein of molecular mass 43 kD. This protein had a pI of 4.7, and has been associated within a restricted spectrum of group A streptococcal isolates. This protein, known as the preabsorbing antigen, was detected in the glomeruli of kidney biopsies from APSGN patients, and in animals perfused with this antigen (26). In a separate study, Ohkuni et al. (27) reported a pI of 4.7 for NSAP isolated by elution from preparative SDS-PAGE gels. This value corresponds to the value (pI, 4.75) reported here for NSAP isolated by chromatofocusing. Lange et al. (28) have described an antigen, endostreptosin (ESS) derived from the cytoplasm of groups A, C, and G streptococci. This antigen had a molecular mass of 49 kD. All antigens described above have been found in the glomerular tissues of APSGN patients. This correlation may suggest that ESS is the presecreted form of NSAP, that is, NSAP plus a signal peptide; the preabsorbing antigen may be the degraded form of NSAP, and NSAP as described here represents the native extracellular form.

When purified NSAP was subjected to amino acid analysis and the data was compared to that known for other streptococcal extracellular products, it became apparent that NSAP shared an amino acid composition profile with streptokinases isolated from groups A, C, and G streptococci. When the amino-terminal amino acids of NSAP were compared to those of streptokinase isolated from a group C streptococcus (29–32), both molecules possessed the same first 21 amino acid

residues. Thus, it became apparent that NSAP was structurally similar to streptokinase.

Streptokinase is known as a potent activator of plasminogen (33), wherein plasmin, a proteolytic enzyme, is generated. When purified NSAP was incubated in the presence of the plasmin-specific synthetic tripeptide, D-Val-Leu-Lys-*p*-nitroanilide, it activated plasminogen on a mole/mole basis comparable to streptokinase from group C streptococci. Thus, NSAP not only possessed structural but functional similarity to streptokinase.

Numerous investigators, most notably Weinstein (34), and Gerlach and Kohler (30, 35) have shown that streptokinases isolated from groups A, C, and G streptococci are antigenically different from each other. Dillon and Wannamaker (36) found two physically and immunologically different types of streptokinases produced by different group A streptococci, and these were different from streptokinase from group C streptococci. Houba and Hana (37) suggested that one group A streptokinase lacked some antigenic determinant present in group C streptokinase, and differed from those group A streptokinases described by Dillon and Wannamaker (36). With this in mind, NSAP was compared to group C streptokinase using polyclonal antisera to NSAP and to group C streptokinase and an mAb against NSAP. It became evident that NSAP had an antigenic domain (assuming the mAb recognized a single epitope) that differed from that of the group C streptokinase. Dillon and Wannamaker (36) also suggested that streptokinase may share antigenic domain(s) involved in the plasminogen activation site, but distinct variable serological domains. Thus, when NSAP is compared to other group A streptokinases, it may become apparent that NSAP is an unique streptokinase, in that some domains of the molecule are involved in classical streptokinase activities, and other domains may be involved in initiating the nephritogenic process.

When viewed in the context of the human disease, the known biological properties of NSAP (streptokinase) could account for many of the clinical and pathological findings in APSGN. For example, streptokinase activates plasminogen (33, 38), which in turn may stimulate certain cells to proliferate and release cellular inflammatory products (39). In APSGN hypercellularity and endothelial cell proliferation in the glomeruli are pathological hallmarks of the disease (40). The presence of low complement serum levels and renal complement deposition seen in APSGN could represent the known ability of plasmin, resulting from streptokinase-plasminogen interaction, to activate certain components of the complement cascade (41–43). In addition, the low serum complement levels seen in APSGN patients are often associated with disturbances in clotting times, an observation consistent with the known disturbances of the clotting pathways (44) after streptokinase activation of plasminogen. Finally, deposition of fibrin products is commonly seen in the biopsies of APSGN (45), possibly reflecting deposition of fibrin split products after streptokinase activation of plasminogen (46).

Given these observations, how would the molecule exert its effects in view of the common occurence of streptokinase antibodies in the human population? Ohkuni et al. (9) indicated that, while ~95% of APSGN patients had antibody to NSAP, only 15% of rheumatic fever patients had this antibody. More recently, using the more sensitive ELISA method, examination of the sera of 100 normal

Trinidad school children, 5–8 yr of age (the susceptible age group) revealed that ~5% of the children had no antibody to NSAP (unpublished observations). Thus, we propose that these individuals would be at highest risk of contracting APSGN, since the biological activities of NSAP would not be blocked. With increasing age, the number of children without detectable antibody to NSAP would decrease, and thus the risk of contacting APSGN diminish with age. The observation that antibody to NSAP was found in the sera of ~95% of the APSGN patients (9) also correlates quite well with the clinical observation that second attacks of APSGN are extremely rare.

Work now in progress in this laboratory indicates that, when purified NSAP is injected into experimental animals, deposition of complement and NSAP antigen, host antibody, and glomerular hypercellularity are seen in the glomeruli of these animals.

Summary

We report the isolation and purification of the nephritis strain–associated protein (NSAP) first described by Villareal et al. (8). Amino acid analysis, and determination of the first 21 amino-terminal amino acids indicated that this 46 kD protein is a streptokinase. Biochemical analysis confirmed that NSAP could act as a plasminogen activator; immunological investigations indicated that NSAP is antigenically different from streptokinase from group C streptococcus, and possibly represents a unique streptokinase. It is this uniqueness that may contribute to the role of NSAP in the pathogenesis of acute poststreptococcal glomerulonephritis.

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