THE OCCURRENCE OF A PROTEIN IN THE EXTRACELLULAR PRODUCTS OF STREPTOCOCCI ISOLATED FROM PATIENTS WITH ACUTE GLOMERULONEPHRITIS*

By HERMAN VILLARREAL, Jr.,‡ VINCENT A. FISCHETTI,§ IVO van de RIJN,‖ and JOHN B. ZABRISKIE

From The Rockefeller University, New York 10021

The relationship of infections with certain types of group A streptococci (nephritogenic streptococci) to the subsequent development of acute poststreptococcal glomerulonephritis (APSGN)[1] has been clearly established (1). However, this is not a constant phenomenon since the incidence of APSGN has varied considerably in those individuals infected with nephritogenic types of streptococci (2). These observations have suggested as one possible explanation that only certain strains within these nephritogenic types have the capability of inducing this disease.

The mechanisms by which only a limited number of nephritogenic streptococcal strains exert their noxious effects upon the glomerulus have not been clearly elucidated. One school of thought postulates that streptococcal antigen-antibody interactions initiate the immunological damage (3, 4). Others propose that an endogenous immune complex system initiated by the streptococcus could be of pathogenic significance; either streptococcal antigens cross-reactive with components of the human glomerulus (5) or alteration by streptococcal products of human immunoglobulin molecules (6). A third point of view suggests that direct complement activation by streptococcal components without the participation of immunoglobulins is of primary importance in some cases of APSGN (7, 8).

In an effort to determine the streptococcal antigen(s) involved, two main avenues of investigation have been explored. Using immunofluorescence techniques, Seegal et al. (3) have suggested that the antigen in question is part of the streptococcal cell wall complex and limited only to nephritogenic types of streptococci. In contrast, Lange et al. (4) have reported that the antigen lies within the streptococcal cell and is unrelated to the serological type of the streptococcus. The second approach has been the examination of structural and biological differences between nephritogenic and nonnephritogenic strains of streptococci. In general, these studies have been inconclusive or controversial (9). The possible reasons for these discrepancies might be the

[1] *Abbreviations used in this paper:* APSGN, acute poststreptococcal glomerulonephritis; BSA, bovine serum albumin; NSAP, nephritis strain-associated protein; SDS-PAGGE, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis; SEP, streptococcal extracellular proteins.

complex nature of the streptococcal cell (10), the excretion of many different extracellular products (11), and the marked variation in the production of these extracellular proteins by a given strain (12).

Although aware of the difficulties inherent in comparing multiple extracellular products in various streptococcal strains, we decided to re-explore the question of whether nephritis-producing strains of streptococci preferentially excreted an antigen(s) unique to these strains for the following reasons. First we were encouraged by the appearance of new biochemical methods capable of detecting minute differences in the protein composition of microbial products (13). These techniques could provide a powerful tool with which to examine the differences in the extracellular proteins of those strains which did or did not cause APSGN. Second, an analysis of the antigen(s) in its naturally occurring form might provide valuable insight into the biological and structural properties of the protein in question. This assumes some importance because many of the current methods of protein extraction from streptococcal cells (14) could destroy the antigen under study or produce a larger molecule in which the relevant antigenic sites were buried (15).

The present report demonstrates that the extracellular products of streptococcal strains isolated from patients with APSGN possess a protein which is unique to these strains. This protein is generally not produced by streptococci recovered from patients without APSGN. It appears to be similar in those strains of streptococci obtained from patients with APSGN irrespective of the serotype and can be demonstrated by immunofluorescent techniques in the kidney biopsies of these patients.

Materials and Methods

Streptococcal Strains. 46 streptococcal strains from The Rockefeller University collection were used for this study (Table I in Results section). 21 were isolated from patients with clinical and/or pathological diagnoses of APSGN and 25 were isolated from patients with suppurative and nonsuppurative sequelae other than APSGN. Five additional group A streptococcal strains recovered from patients with APSGN were also employed. Three of these were mouse passed after the initial isolation: strains T12/126/4 (type 12), A995/91/1 (type 57), and A928/73/1 (type 49). Strains A374 and A547 originally isolated from patients with APSGN were repeatedly subcultured in rabbit blood neopeptone broth containing 4% rabbit blood and Todd-Hewitt dialysate medium (Difco Laboratories, Detroit, Mich.).

Culture Broth. Todd-Hewitt dialysate medium was prepared following methods previously described from this laboratory (16) and sterilized by autoclaving. The dialysate medium was chosen in an effort to eliminate contaminating broth proteins greater than 10,000 daltons in the analysis of the streptococcal extracellular proteins.

Preparation of Streptococcal Extracellular Proteins (SEP). Lyophilized streptococcal strains were grown in 5 ml of neopeptone broth with 4% rabbit blood for 18 h at 37°C. 0.1 ml of this culture was subcultured twice in 250 ml of Todd-Hewitt dialysate media for 18 h at 37°C with the purpose of diluting and eliminating the contaminating rabbit blood proteins. 10 ml of the second 250 ml stationary phase culture were transferred to three flasks each containing 2 liters of Todd-Hewitt dialysate medium. The individual flasks were allowed to grow at 37°C for varying periods (5, 18, and 48 h) to study the SEP during the exponential, early stationary, and late stationary phases of growth, respectively. In some experiments the optical density was measured (560 nm) at frequent intervals on a Beckman Quartz spectrophotomer (National Technical Laboratories, South Pasadena, Calif.) to obtain cultures with similar growth density and to compare their SEP. The purity of each culture was assessed by rabbit blood agar plating. The organisms were removed from each of the 2-liter cultures by centrifugation at 10,000 g for 30 min at 4°C (Sorvall RC2B, Dupont Instruments, Newtown, Conn.). The supernates were filtered through a 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass.) and brought to 80% saturation (560 g/liters) with ammonium sulfate (AR, Mallinckrodt Inc., St. Louis, Mo.).

This material was stirred overnight at 4°C and then centrifuged at 10,000 g for 30 min at 4°C. The pellet was dissolved in 10 ml of 0.005 M sodium phosphate buffer (approximately 200-fold concentrated), placed in dialysis tubing with mol wt cutoff of 6,000–8,000 (Spectrum Medical Industries, Inc., Los Angeles, Calif.) and dialyzed for 18 h against 32 liters of the same buffer. The protein content in each of the samples was measured by the method of Lowry et al. (17) and adjusted to appropriate concentrations by either dilution with 0.005 M phosphate buffer or concentration through an A25 Minicon (Amicon Corporation, Scientific Sys. Div., Lexington, Mass.).

Polyacrylamide Gel Electrophoresis.   SEP of all strains were studied by sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS-PAGGE) by using the method of Maizel (13) with a discontinuous Tris-glycine buffer system. Electrophoresis was carried out on 7–30% gradient slab polyacrylamide gels at 50 V for 18 h. Bovine serum albumin, carbonic anhydrase, cytochrome C, and ovalbumin standards, were run concurrently on the gels for molecular weight determinations. Samples and standards were mixed with equal portions of a solution containing 2% SDS, 2% mercaptoethanol, and 30% glycerol and boiled for 2 min in a waterbath before loading to the gels.

Isolation of the Nephritis Strain-Associated Protein (NSAP).   60 liters of Todd-Hewitt dialysate medium were sterilized by filtration into a Biogen Fermentor (American Sterilizer Co., New York) through a 0.22-μm Millipore filter as previously described from this laboratory (16). The medium was then inoculated with 1.5 liters of a stationary phase group A, type 49, streptococcal culture (Gt 8760) isolated from a patient with APSGN. The culture was incubated for 5 h, and the streptococcal cells were removed at 4°C by using a Sharples high-speed centrifuge (Sharples-Stock Division, Pennwalt Corporation, Warminster, Penn.). The supernate was then filtered and treated with ammonium sulfate as described in the preparation of SEP section. The precipitate was collected at 4°C by Sharples high-speed centrifugation, dissolved in 60 ml of 0.005 M sodium phosphate buffer pH 5 (approximately 1,000-fold concentrated), and dialyzed extensively against the same buffer. This material was stored at 4°C with sodium azide at a final concentration of 0.02% added to the solution.

Purification of the NSAP.   Purification of the NSAP was accomplished as follows. The crude SEP of strain Gt 8760 were loaded into a column (15 × 2.7 cm) of carboxy methyl cellulose (CM 52, Whatman Ltd., Maidstone, Kent, England) previously equilibrated with 0.005 M sodium phosphate buffer pH 5. The same buffer was used to remove unbound material from the column until the effluent conductivity (Radiometer, Copenhagen) and OD at 280 μm were similar to that of the eluting buffer. A linear gradient from 0.005 M to 0.5 M (550 ml) of sodium phosphate buffer pH 5 at a rate of 25 ml/h was used to elute the SEP. 3-ml fractions were collected. The different fractions were dialyzed and then assayed by SDS-PAGGE to ascertain the presence of the NSAP.

Fractions containing the NSAP were pooled, dialyzed against 0.1 M ammonium bicarbonate buffer, and lyophilized. These were then resuspended in 1.0 ml of 0.1 M ammonium bicarbonate buffer and loaded on a column (2.5 × 90 cm) of Sephadex G200 (Pharmacia Fine Chemicals, Piscataway, N.J.) previously equilibrated with the same buffer. 2-ml fractions were eluted at a flow rate of 8 ml/h and tested for the NSAP as previously described. The fractions containing the NSAP were then pooled, lyophilized, and resuspended in 1 ml of 0.1 M ammonium bicarbonate and loaded onto a column (1.5 × 90 cm) of Sephadex G75 (Pharmacia Fine Chemicals) equilibrated with the above buffer. The proteins were eluted with 0.1 M ammonium bicarbonate, collecting 1.2-ml fractions. The different fractions were again analyzed by SDS-PAGGE for the presence of the NSAP. The final product contained three major and eight minor proteins by SDS-PAGGE.

Further purification of the partially purified NSAP from the G75 Sephadex chromatography step was accomplished by preparative electrophoresis from polyacrylamide gels. The partially purified NSAP was lyophilized and resuspended in sample loading buffer and separated by SDS-PAGGE as described previously in the SDS-PAGGE section. The position of NSAP in the gel was determined by staining the two end slots by using Coommassie Blue. The gels were then lined up, and the NSAP band was cut from the remainder of the gel. Separated bands from many gels were pooled and cut into 5-cm pieces. The NSAP was then electrophoresed from the polyacrylamide gel slices by using 0.01 M Tris-glycine buffer pH 8.6 containing 0.1% SDS at 25 mA for 24 h into small chambers (18). The NSAP was assayed for its purity by SDS-

PAGGE. This process of electrophoresing the NSAP from polyacrylamide gels was repeated until only a single polypeptide was detected when analyzed by SDS-PAGGE.

Preparation of Antisera to the SEP.   Antisera specific for the NSAP was prepared by two methods. The first method consisted of inoculating three 2-kg New Zealand Red rabbits with 300 μg of the purified NSAP in 1 ml incomplete Freund's adjuvant (Difco Laboratories) subcutaneously into three different locations. Similar injections were administered 2 and 3 wk after the first inoculation. The rabbits were bled 1 wk after the final injection, and the gamma globulin fraction was isolated by the caprylic acid technique (19) and dialyzed (three times) against 10 vol of 0.02 M sodium phosphate buffer pH 7.0. The dialyzed gamma globulin was then loaded to a column of DEAE cellulose (DE 52, Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England) and equilibrated with the above buffer. The tubes containing only gamma globulin as determined by cellulose acetate electrophoresis (20) were pooled and concentrated by using an Amicon stirred cell with a XM 50 membrane (Amicon Corp.).

In the second method, 500 μg of the crude SEP of strain A995 containing significant amounts of NSAP was mixed with 1 ml of incomplete Freund's adjuvant and injected into two New Zealand Red rabbits (2 kg). The injections were given subcutaneously three times weekly for 5 wk. The animals were bled 1 wk after the last inoculation and the gamma globulin fraction was obtained as in the first method.

To remove antibodies to streptococcal extracellular proteins unrelated to the NSAP, the antiserum to the SEP of strain A995 was then absorbed with pooled crude SEP not containing the NSAP. SEP from strains A995/91/1, A834, and B434 were used for the absorptions. Absorptions were performed after coupling the pooled SEP to N-hydroxy succinamide-activated Sepharose (21) (Bio-Rad Laboratories, Richmond, Calif.). The gamma globulin fraction of the antiserum to the crude SEP of strain A995 was then added to the SEP coupled beads and incubated at 25°C for 2 h followed by incubation at 4°C for 18 h. The antiserum specific for the NSAP produced in this manner was then tested by immunodiffusion analysis.

For control experiments, antisera to crude SEP not containing NSAP (strain A995/91/1) was prepared by injecting two New Zealand Red rabbits (2 kg) with 500 μg of protein in 1 ml of incomplete Freund's adjuvant. These injections were given subcutaneously three times a week for 5 wk. The animals were bled 1 wk after the last inoculation, and the gamma globulin fraction was prepared as previously outlined.

Immunodiffusion Studies.   Slides for double diffusion precipitin tests were prepared by placing a solution (3 ml) of 1% agarose (Matheson, Coleman and Bell, Norwood, Ohio) in 0.1 M barbital buffer, pH 8.6, onto microscope slides. Wells were cut immediately before use and then filled with the appropriate antigen solutions and antisera. The slides were then placed in a moist chamber and read 24 and 48 h later.

Immunofluorescence Studies.   Isolated gamma globulin fractions of the rabbits described above were adjusted to a concentration of 10 mg/ml with 0.5 M sodium carbonate buffer pH 9.5, conjugated with fluorescein isothiocyanate (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co., Cockeysville, Md.) and separated from the free dye by methods previously described (16). The conjugated gamma globulin was aliquoted into 1-ml samples and stored at −70°C until used.

For preparation of the goat anti-rabbit gamma globulin, inocula and immunization schedules were made according to the method of van de Rijn et al. (16).

For the immunofluorescence assay, desiccated 4 μm frozen kidney sections were prepared by the method of Koffler et al. (22) and then incubated with various dilutions of: (*a*) fluoresceinated rabbit anti-human IgG, IgM, C3, fibrinogen (Behring Diagnostics, American Hoechst Corp., Somerville, N.J.), (*b*) fluoresceinated rabbit antiserum to the crude SEP containing the NSAP, (*c*) antisera to the purified NSAP obtained by both methods, and (*d*) antisera to the crude SEP not containing the NSAP. The slides were analyzed for the presence of immunofluorescent staining using a Zeiss Universal ultraviolet microscope (Carl Zeiss, Inc., New York) with BG12 and 440 μm filters. The fluorescence was graded on a 0–4+ scale with 4+ representing the greatest intensity of staining. The indirect immunofluorescence assay was performed as previously described (16).

Kidney Biopsies.   Percutaneous kidney biopsies used in this study were longitudinally divided for light, electron, and immunofluorescence microscopy. The tissue for immunofluorescent
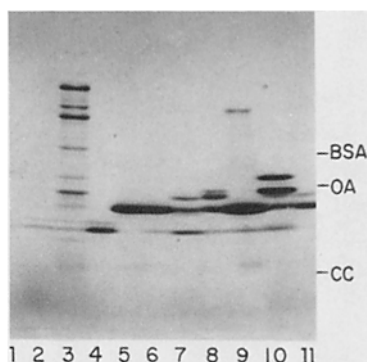
FIG. 1.   SEP patterns of 11 group A strains of different serotypes. 100 μl of each preparation isolated from 5-h grown cells were loaded onto an SDS gel as described in Materials and Methods. Note the marked variation in the number of proteins produced by the different types of streptococci. Strains: 1, A547; 2, A456; 3, B281; 4, A992; 5, B434; 6, 1GL1362; 7, A552; 8, A928; 9, B737; 10, A207; 11, D480. The position of the migration of the protein standards ([BSA], ovalbumin [OA], and cytochrome C [CC]) are noted.

studies was immediately embedded in 7% gelatin (Knox, St. Paul, Minn.), frozen in a mixture of dry ice-isopropanol, and stored at −70°C until shipment. For transportation, the biopsies were placed in a sealed container with dry ice. Kidney biopsies of patients with APSGN were provided by Dr. R. Mahabir from the San Fernando General Hospital in Trinidad, West Indies. Kidney biopsies of patients with acute rheumatic fever were supplied by Dr. Villarreal, Sr., from the National Institute of Cardiology of Mexico, and kidney biopsies of patients with other glomerulopathies, such as lupus nephritis, minimal change disease, membranous nephritis, as well as normal human autopsy kidney, were provided by Dr. C. Becker from the New York Hospital, Cornell University Medical Center.

Absorption Studies with Antiserum to the NSAP.   For absorption studies, 20 μl of fluoresceinated or untagged rabbit anti-NSAP antisera were placed in microfuge tubes containing 20 μl of the following preparations: (a) crude SEP containing the NSAP (over 60 protein bands on SDS-PAGGE), (b) crude SEP that did not possess the NSAP, (c) a partially purified preparation containing the NSAP isolated from G75 chromatography, (d) and the purified NSAP. The contents were allowed to incubate at 37°C for 2 h and then overnight at 4°C. Before use, the samples were centrifuged for 5 min at 23°C in a microfuge 152 (Beckman Instruments, Inc., Cedar Grove, N. J.) and the supernatant fluid was used in the immunofluorescence assay. Unabsorbed antiserum to the purified NSAP which was diluted in the same proportions and incubated for the same periods of time was used as the 4+ staining control. All dilutions were done with 0.01 M phosphate buffer pH 7:6.

Results

Examination of the SEP from Various Strains by SDS-PAGGE.   When the SDS gels containing the SEP of 44 group A strains were compared, a marked variability in the number of protein bands was observed. Fig. 1 shows a representative gel where it can be seen that SEP of some strains showed numerous protein bands while in others only a few were detected. The mol wt of these protein bands ranged from about 80,000 to 10,000 daltons. It was not possible to assess the presence of SEP with mol wt below 10,000 daltons due to the presence of contaminating low molecular weight media peptides.

Because no consistent differences were observed in a wide variety of random streptococcal strains examined, experiments designed to look at the extracellular products from nephritogenic versus nonnephritogenic serotypes were carried out. The
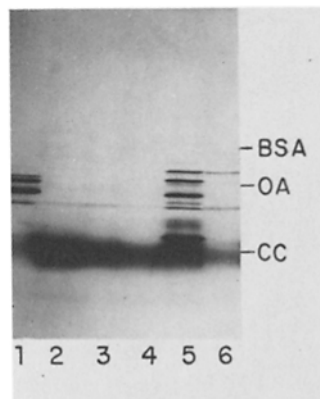
FIG. 2.   SEP of six group A type 12 strains. 100 µl of each preparation isolated from a constant culture density was loaded onto an SDS gel as noted in Materials and Methods. Note again the marked variation in the number of proteins excreted by these strains. Strains: 1, T12; 2, T12 M+; 3, T12 M−; 4, T12/126/4; 5, B923; 6, A374. The position of migration of the protein standards are noted.

SEP of strains known to be capable of inducing APSGN, such as types 2, 12, 18, 49, 55, 57, were compared to those strains not previously described as causing this disease; i.e., types 3, 9, 19, 30, 36. In general, the nephritogenic SEP tended to possess more protein bands than the nonnephritogenic ones, but on occasion the opposite was observed.

To determine whether variation existed within a single streptococcal serotype, several group A strains of similar serotypes were compared. Eight type 12 strains, five type 4 strains, and five type 49 strains were examined. Fig. 2 shows the SEP of six type 12 strains. Once again, marked differences in the number of protein bands present was observed. Strain-to-strain variation of the SEP persisted even when the numbers of streptococci in the cultures as measured by the optical density was kept constant or the protein concentration of each strain studied was equalized. These differences also occurred in samples taken at specific times (5, 18, 48 h) after inoculation.

Examination of the SEP of Strains Isolated from Patients with and without APSGN.   Although it was hot possible to demonstrate that strains commonly labeled nephritogenic excreted proteins that were different from nonnephritogenic strains, the possibility arose that consistent differences might exist in strains directly isolated from patients with APSGN irrespective of the serotype. Fig. 3 illustrates the results of these experiments. While the variation in protein bands between these group A strains is again apparent, an examination of the gels showed that a protein band with mol wt of ≈ 46,000 daltons (NSAP) (arrows) occurred mainly in the SEP of the strains recovered from patients with APSGN. This band was not present in the SEP of those strains of similar serotypes recovered from patients without this disease.

Table I shows that of 21 strains isolated from patients with APSGN, 19 had the NSAP; the only exceptions were one type 5 and one type 55 strain obtained from patients with clinical diagnoses of APSGN. Of 25 group A strains recovered from patients without APSGN (16 of which were similar in serotype to those isolated from patients with APSGN), only two showed the NSAP, and both were type 12 strains. 5 h of culture were usually sufficient for the NSAP to be detected by SDS-PAGGE in
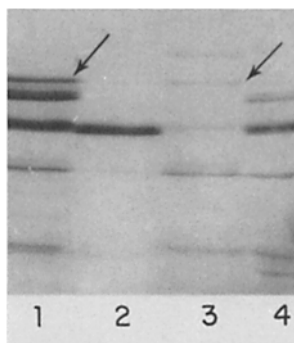
FIG. 3.   SEP of four group A streptococcal strains. 100 µl of each preparation was loaded onto an SDS gel as noted in Materials and Methods. Sections 1 (type 2) and 3 (type 12) are extracellular products obtained from strains isolated from patients with APSGN. Sections 2 (type 2) and 4 (type 12) are SEP recovered from strains obtained from patients without APSGN. Note (arrow) the presence of a protein band with mol wt of 46,000 daltons (NSAP) only in the strains isolated from patients with APSGN.

TABLE I

Presence of the NSAP in Various Streptococcal Strains

Group type		Source of strains			
		APSGN patients		Non-APSGN patients	
		NSAP+	NSAP−	NSAP+	NSAP−
A	1	F203D			D480
	2	A207			B931
	3				A830, A868
	4	B512, B905, B974			A915, A990
	5		B743		A964, B434
	9				A552, A728
	12	A374, B281, B923		D313	D897
		Gt7940, Gt7899		L92541	L02407
		t12			L01596
	18	B438			A992
	19				1GL362
	30				1GL22
	36				A456
	49	B915, B920, Gt8760			B737, A834
	50				A315
	55		A928		D438
	57	A995			
	NT*	B515, A218, A547			
D					B272, B443
Strains		19	2	2	23

* Nontypable strains.

the SEP of the throat strains studied. Further incubation (up to 48 h) generally did not result in an increased production of the NSAP. However, the prolonged incubation up to 48 h resulted in the appearance of new protein bands. The NSAP was not detected in the negative strains even when the SEP were concentrated 2,000-fold at various times of growth (up to 48 h).
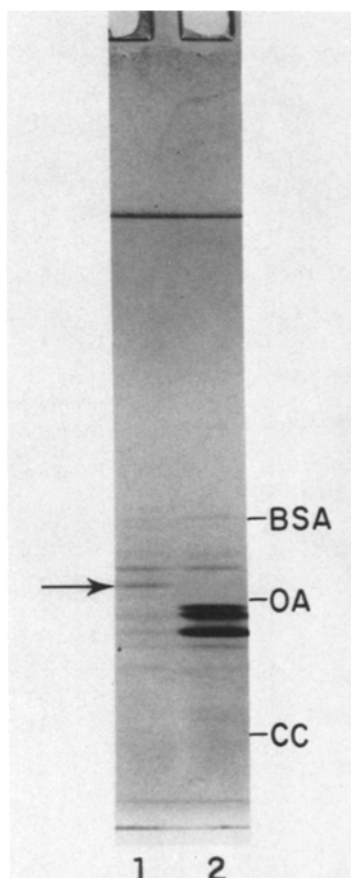
It was of interest that the skin strains obtained from patients with APSGN also showed the NSAP. However, they differed from the throat strains in that this protein was not, or only faintly apparent, in 5-h cultures. Incubation for 18 or 24 h was necessary for this band to be detected.

Loss of the NSAP after Mouse Passage or Subcultures. It has been well established that mouse passage results in the selection of high producers of type-specific M protein (23). To determine if this procedure also increased the production of the NSAP, three streptococcal strains were mouse passed. These experiments resulted in the disappearance of the NSAP (Fig. 4). In contrast, certain other proteins increased after mouse passage. In a similar manner, repeated subcultures in the growth medium also resulted in the disappearance of the NSAP.

Gel Analysis of the Purified NSAP. Although the protein band was clearly visible in each of the SEP of the strains isolated from patients with APSGN, many other protein bands were also present. Therefore, purification steps were carried out in an effort to further characterize the NSAP and to prepare antisera against the purified protein.
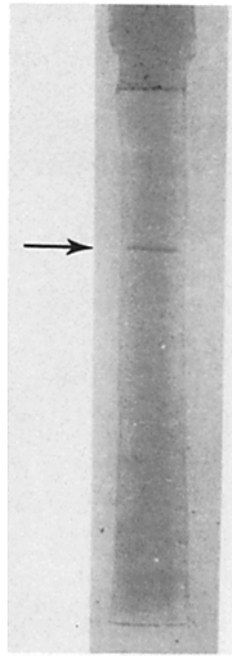
Fig. 5. SDS polyacrylamide gel electrophoresis of the purified NSAP. An aliquot of the NSAP isolated by electrophoresis from acrylamide gels was rerun on SDS-PAGGE to determine its purity. Only a single polypeptide with wt of 46,000 was resolved when the gel was stained with Coomassie Blue.

By using ion exchange, molecular sieve chromatography, and the electrophoresis procedures outlined in the Materials and Methods section, it was possible to obtain the purified protein. Fig. 5 is an SDS gel pattern of this protein and shows only a single band. This material was then used for production of antisera and the absorption studies to be described below.

Examination of the SEP of the Strains Studied by Double Diffusion Analysis.   To assess the specificity of the antiserum to the NSAP, the following double diffusion studies were carried out. When antiserum to the NSAP obtained by the first method was reacted with the purified NSAP or with crude SEP from strains isolated from patients with APSGN (NSAP present), a faint and broad precipitin line was formed. However, a single, more distinct precipitin line was observed when antiserum specific to the NSAP (obtained by absorptions with crude SEP from strains without NSAP) was reacted with crude SEP containing NSAP (strain L02541). This precipitin line disappeared when this antiserum to the NSAP was absorbed with the purified NSAP (Fig. 6). To determine whether the small amount of SDS contaminant present in the purified NSAP preparation could be responsible for the inhibition of the precipitin line, purified NSAP was added to a rabbit antiserum to bovine serum albumin (BSA). The addition of the NSAP to this antiserum did not prevent its reaction in double diffusion with BSA.

Whether or not the NSAP present in each of the SEP of strains of various serotypes isolated from patients with APSGN was the same protein was determined in the following experiment. Antiserum to the NSAP obtained by the absorptions was
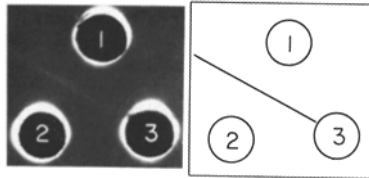
FIG. 6. A photograph of an immunodiffusion slide in which well 1 contains the SEP (NSAP present) of strain LO2541 (group A, type 12). Well 2 contains antiserum to the NSAP absorbed with the SEP of strains in which the NSAP is not present. Well 3 contains the antiserum to the NSAP which was absorbed with the purified NSAP. Note that a line of precipitation only appears between the crude SEP and the antiserum to the NSAP not absorbed with the purified protein.
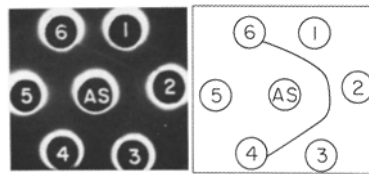

FIG. 7. A photograph of an immunodiffusion slide in which the center well contains antiserum specific to the NSAP. Wells 1–3 contain the SEP of three group A strains isolated from patients with APSGN. Well 4 contains the SEP of a mouse passed strain. Wells 5 and 6 contain the SEP of strains obtained from patients without APSGN. Note the line of identity formed only by those strains isolated from patients with APSGN (for details, see text).

reacted with crude SEP of strains isolated from patients with and without APSGN. Fig. 7 is illustrative of one of these double diffusion studies. Antiserum specific for the NSAP was added to the center well. In wells 1–3 the crude SEP of three group A streptococcal strains isolated from patients with APSGN was placed. These SEP were obtained from strains B281 (type 12), Gt 8760 (type 49), and A995 (type 57), respectively. Wells 4–6 contained the crude SEP of three group A strains obtained from patients without APSGN or mouse passed strains (no NSAP present), strains A995/91/1 (type 57), A 834 (type 49), and D897 (type 12), respectively. A line of identity was formed by the SEP of the three strains isolated from APSGN patients. No precipitin line formed with the SEP of strains recovered from patients without APSGN or mouse passed strains.

Immunofluorescent Studies in Kidney Biopsy Specimens. Because the NSAP was primarily found in the extracellular products of streptococci isolated directly from patients with APSGN, we decided to examine 21 kidney biopsies of patients with APSGN for the presence or absence of this protein. These were also studied for the presence of IgG, IgM, C3, and fibrinogen.

14 of 21 biopsies from patients with APSGN showed moderate fluorescence deposits when fluoresceinated antiserum specific to the NSAP was employed. The fluorescence increased markedly when an indirect immunofluorescence method was used. The deposits were finely granular and found both along the glomerular basement membrane and mesangium (Fig. 8). The positive biopsies of patients with APSGN were usually those that also had deposits of IgG. In contrast, 5 kidney biopsies of patients with acute rheumatic fever and 11 from other nonstreptococcal glomerulonephritis patients, such as membranous nephritis, lupus nephritis, and minimal change disease, were all negative for the NSAP (Table II). The antiserum to crude SEP containing the NSAP produced similar fluorescence in the kidney biopsies of patients with APSGN as did the antiserum to the purified NSAP. In contrast, antiserum to crude
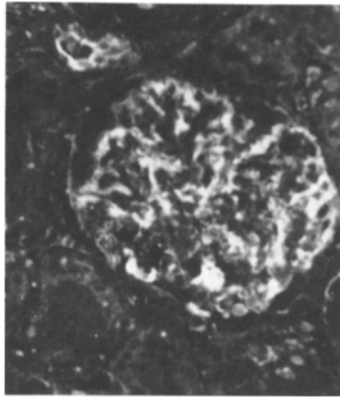
Fig. 8.   Glomerulus of a patient with early well-documented APSGN stained with antiserum specific to the NSAP. Note the presence of granular deposits of this antigen along the glomerular basement membrane and mesangium ×384.

TABLE II

Results of Immunofluorescence Studies in Kidney Biopsies

Antiserum to:	Acute post-streptococcal glomerulone-phritis	Acute rheu-matic fever	Other nonstrep-tococcal glo-merulonephritis
Crude SEP of strain A995 (NSAP present)	7/12*	0/5	0/10
Purified NSAP strain Gt8760	14/21	0/5	0/11
Crude SEP of strain A995/91/1 (no NSAP present)	0/21	0/5	0/9

* Positive/total.

TABLE III

Absorption Studies in Kidney Biopsies of Patients with Acute Poststreptococcal
Glomerulonephritis with Antiserum Specific to the NSAP

Absorption with:	Fluorescence posi-tive/total
None	11/11
Crude SEP containing NSAP (strain Gt8760)	0/11
Partially purified SEP containing NSAP (strain Gt8760)	0/11
Purified NSAP (strain Gt8760)	0/11
Crude SEP not containing NSAP (strain A995/91/1)	11/11
Concentrated Todd-Hewitt media dialysate	5/5

SEP without the NSAP failed to exhibit fluorescence in the same biopsies. The fluorescence was abolished from the APSGN biopsies when the rabbit anti-NSAP gamma globulin was absorbed with crude, partially purified or purified NSAP. Absorptions with crude SEP which did not contain NSAP or lyophilized Todd-Hewitt media did not abolish the fluorescence (Table III).

Discussion

The analysis of the extracellular proteins of streptococci by SDS-PAGGE revealed marked qualitative and quantitative differences in their excreted proteins. These differences were noted even when the time of harvest, mass of organisms, or total

extracellular proteins were equalized. The variations in the extracellular proteins occurred not only among the different types of streptococci but also in strains of similar serotypes. Furthermore, in those strains tested it was found that mouse passage or repeated subcultures of a particular strain resulted in changes in the amount and number of proteins excreted.

At first, these variations prevented us from identifying consistent differences among types of streptococci commonly associated with APSGN and those strains which rarely cause the disease. It was only after analysis of the SEP of a large number of strains of the same nephritogenic type that it was possible to identify a single protein band (NSAP) which was predominantly present in the strains isolated directly from patients with APSGN. The unique production of this protein by those streptococcal strains isolated from patients with APSGN was attested to by the fact that the majority of strains isolated from patients without APSGN did not exhibit the NSAP. This was true even when the SEP of strains of similar serotypes were analyzed or when they were concentrated ≈ 2,000-fold.

The common occurrence of the NSAP in different types of streptococci isolated from APSGN patients was confirmed by immunodiffusion studies. Only the SEP of strains obtained from patients with APSGN formed a line of identity with antiserum specific to the NSAP. Strains isolated from patients without APSGN or mouse passed strains failed to exhibit this antigen-antibody reaction.

The fact that antiserum specific for the NSAP bound to the kidney sections obtained from patients with documented APSGN suggests a possible causal relationship of this antigen to the disease process. The positive biopsies were, with few exceptions, those that were obtained early in the course of this disease and also had deposits of IgG. The specificity of the immunofluorescent localization of the antiserum to the NSAP to the glomeruli of patients with APSGN was confirmed by absorption studies employing purified NSAP. These results were further strengthened by the fact that the antiserum to the NSAP failed to detect antigens in kidney biopsies from patients with diseases other than APSGN.

Although we still do not know whether the NSAP is to be found only in the extracellular products of streptococci or is also present in the bacterial cell, it is appropriate to consider its possible relationship to the pathogenesis of APSGN. The presence of the NSAP in the SEP of serotypes commonly associated with APSGN (1), coupled with its production predominantly by strains isolated from patients with this disease, could explain why APSGN follows infection with only a limited number of streptococcal strains even within the same serotype. For example, many group A type 12 strains induce pharyngitis, but only those producing the NSAP would be of potential pathogenic significance in poststreptococcal glomerulonephritis.

With respect to the discrepancies noted in different immunofluorescent studies, attempts to detect streptococcal antigens in the kidney biopsies of patients with APSGN could have failed for the following reasons (24, 25). First, the antisera prepared against streptococcal strains which did not produce the NSAP (even of nephritogenic types) would not have contained antibodies to the NSAP and therefore would have failed to detect this streptococcal antigen in the kidney biopsy specimens. Second, because mouse passage or repeated subcultures appear to result in the loss of the NSAP, antisera raised against these strains (commonly used in immunization procedures) might not contain antibodies to the NSAP. Finally, it is conceivable that more than one streptococcal antigen could be involved in the pathogenesis of APSGN.

This might explain the apparently controversial results of different investigators (3, 4) with respect to the localization of the antigens in the streptococcal cell.

The limited production of the NSAP in the extracellular products of these strains, coupled with the technical difficulties inherent in isolating a single protein from the many others that form the extracellular products of streptococci, have hindered our efforts in characterizing the biological properties of this antigen. Specifically, it would be important to know whether the NSAP has a peculiar affinity for specific components of the glomerulus (26), whether it binds to serum proteins (27), whether it can activate the alternate pathway of complement (7), and finally whether immunoglobulin molecules are specifically altered by this antigen (6). These studies are currently underway.

Summary

The present report compares the extracellular proteins of streptococci by sodium dodecyl sulfate polyacrylamide electrophoresis. A marked variation in the streptococcal extracellular proteins (SEP) of different strains was detected, even in strains of similar serotypes. It was possible, however, to identify a single protein band that occurred predominantly in the SEP of strains isolated from patients with acute poststreptococcal glomerulonephritis (APSGN). This protein was generally not produced by streptococci obtained from patients without this disease. It appears to be immunologically similar in the various serotypes of streptococci isolated from patients with APSGN and can be demonstrated by immunofluorescence techniques to be present in the glomeruli of these patients.

References

1. Stollerman, G. H. 1971. Rheumatogenic and nephritogenic streptococci. *Circulation.* **43:**915.
2. Rammelkamp, C. H., Jr. 1954. Acute hemorrhagic glomerulonephritis. *In* Streptococcal Infections. M. McCarty, editor. Columbia University Press, New York. 197–207.
3. Seegal, B. C., G. A. Andres, K. C. Hsu, and J. B. Zabriskie. 1965. Studies on the pathogenesis of acute and progressive glomerulonephritis in man by immunofluorescence and immunoferritin techniques. *Fed. Proc.* **24:**100.
4. Lange, K., U. Ahmed, H. Kleinberger, and G. Treser. 1976. A hitherto unknown streptococcal antigen and its probable relation to acute poststreptococcal glomerulonephritis. *Clin. Nephrol.* **5:**207.
5. Markowitz, A. S., and C. F. Lange, Jr. 1964. Streptococcal related glomerulonephritis. *J. Immunol.* **92:**565.
6. McIntosh, R. M., W. R. Griswold, W. B. Chernack, G. Williams, J. Straus, D. B. Kaufman, M. N. Koss, J. R. McIntosh, R. Cohen, and R. Weil, III. 1975. Cryoglobulins. III. Further studies on the nature, incidence, clinical, diagnostic, prognostic and immunopathologic significance of cryoproteins in renal disease. *Q. J. Med.* **44:**285.
7. Tauber, J. W., M. J. Polley, and J. B. Zabriskie. 1976. Non-specific complement activation by streptococcal structures. II. Properdin independent initiation of the alternate pathway. *J. Exp. Med.* **143:**1352.
8. Zabriskie, J. B., V. Utermohlen, S. E. Read, and V. A. Fischetti. 1973. Streptococcus-

related glomerulonephritis. *Kidney Int.* **3**:100.

9. Noble, R. C., and K. L. Vosti. 1973. Biological and immunologic comparison of nephrito-genic and non-nephritogenic strains of group A, M-type 12 streptococcus. *J. Infect. Dis.* **128**: 761.

10. McCarty, M. 1964. The streptococcal cell wall and its biologic significance. *In* The Streptococcus, Rheumatic Fever, and Glomerulonephritis. J. Uhr, editor. Williams and Wilkins Company, Baltimore, Md. 3-24.

11. Halbert, S. P., and S. L. Keatinge. 1961. The analysis of streptococcal infections. VI. Immunoelectrophoretic observations on extracellular antigens detectable with human antibodies. *J. Exp. Med.* **113**:1013.

12. Wannamaker, L. W. 1958. The differentiation of three distinct desoxyribonucleases of group A streptococci. *J. Exp. Med.* **107**:797.

13. Maizel, J. B. 1971. Polyacrylamide gel electrophoresis of viral proteins. *In* Methods in Virology. K. Maranorosh and Koprowski, editors. Academic Press, Inc. New York. **5**:179.

14. Fox, E. N. 1974. M proteins of group A streptococci. *Bacteriol. Rev.* **38**:57.

15. Krause, R. M. 1958. Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage associated lysin. *J. Exp. Med.* **108**:803.

16. van de Rijn, I., J. B. Zabriskie, and M. McCarty. 1977. Group A streptococcal antigens cross-reactive with myocardium. Purification of heart-reactive antibody and isolation and characterization of the streptococcal antigen. *J. Exp. Med.* **146**:579.

17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.

18. Chua, N., K. Matlin, and P. Bennoun. 1975. A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. *J. Cell. Biol.* **67**:361.

19. Steinbuch, M., and R. Audran. 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys.* **134**:279.

20. Osterland, C. K., E. J. Miller, W. W. Karakawa, and R. M. Krause. 1966. Characteristics of streptococcal group specific antibody isolated from hyperimmune rabbits. *J. Exp. Med.* **123**:599.

21. Cuatracasas, P., and I. Parikh. 1972. Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose. *Biochemistry.* **11**:2291.

22. Koffler, D., P. H. Shur, and H. G. Kunkel. 1967. Immunological studies concerning the nephritis of systemic lupus erythematosus. *J. Exp. Med.* **126**:607.

23. Todd, E. W., and R. C. Lancefield. 1928. Variants of hemolytic streptococci; their relation to type specific substance, virulence and toxin. *J. Exp. Med.* **48**:751.

24. McCluskey, R. S., P. Vassali, G. Gallo, and D. S. Baldwin. 1966. An immunofluorescent study of pathogenic mechanisms in glomerular diseases. *N. Engl. J. Med.* **274**:695.

25. Feldman, J. O., M. R. Mardiney, and S. E. Shuler. 1966. Immunology and morphology of acute poststreptococcal glomerulonephritis. *Lab. Invest.* **15**:283.

26. Wilson, C. B., S. M. Golbus, T. J. Neale, and A. J. Woodroffe. 1978. Nephritogenic immune responses involving antigens in or of the glomerulus. *In* Streptococcal Diseases and the Immune Response. S. E. Read, and J. B. Zabriskie, editors. Academic Press, Inc. New York.

27. Kantor, F. S. 1965. Fibrinogen precipitation by streptococcal M protein. I. Identity of reactants and stoichiometry of the reaction. *J. Exp. Med.* **121**:849.