# Immunological Studies of Post-Streptococcal Sequelae

## Evidence for Presence of Streptococcal Antigens in Circulating Immune Complexes

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**Abstract.** Since elevated levels of circulating complexes have been noted to occur in the sera of patients with post-streptococcal sequelae, the possibility that these complexes contained streptococcal antigens within the complex was investigated. Sera from these patients were precipitated with polyethylene glycol to extract a fraction rich in these complexes, which was then injected into rabbits. The rabbit sera were then reacted with both cellular and extracellular fractions obtained from streptococcal strains associated with either acute post-streptococcal nephritis (APSGN) or acute rheumatic fever (ARF) by using immunoelectrophoresis and ELISA techniques. The data demonstrate that both ARF and APSGN complexes contain streptococcal antigens. However, APSGN complexes react uniquely to certain extracellular antigens present in those strains associated with nephritis, while ARF complexes react specifically to certain streptococcal extracellular antigens excreted by strains associated with rheumatic fever. Neither of the two groups of complexes appear to contain streptococcal antigens related to any cellular antigens derived from the group A streptococcus. Additionally, a rabbit serum immunized with streptococcal extracellular products reacted directly with complexes isolated from nephritis patients. Removal of the gamma globulin by absorption with an anti-human Fc serum resulted in the concomitant loss of reactivity with the anti-streptococcal serum, strongly suggesting an intimate association of the streptococcal antigen with these complexes. The presence of streptococcal antigens within the circulating immune complex of patients with APSGN coupled with their specific presence in those strains associated with post-streptococcal glomerulonephritis argues strongly for a causal role of these antigens in the disease process.

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## Introduction

On the basis of the clinical course coupled with evidence of the depositions of immunoglobulins and complement components in the glomeruli, acute post-streptococcal glomerulonephritis (APSGN)[1] has been characterized as an immune complex disease (1–7). Furthermore, decreased values of serum complement have been recorded on numerous occasions (8), and more recently, circulating complexes have been clearly documented in the sera of these patients during acute and convalescent phases of the disease (9).

The main controversy has focused on the presence or absence of streptococcal antigens in the renal biopsies of these patients. Early work by Seegal and co-workers (6) demonstrated the presence of streptococcal products in renal biopsies that appeared to be related to the cell wall complex. Reports by other investigators failed to confirm the presence of these streptococcal antigens (2, 5). Later work implicated a streptococcal cytoplasmic component as the relevant antigen in the biopsy tissue (10–12). More recently, Villarreal et al. noted (13) that the majority of group A streptococcal strains directly isolated from patients with APSGN excreted a protein of 46,000 D; the Nephritic Strain Associated Protein (NSAP). Antibody prepared against this extracellular protein was present

1. *Abbreviations used in this paper:* APSGN, acute post-streptococcal glomerulonephritis; ARF, acute rheumatic factor; CIE, crossed immunoelectrophoresis; ECP, extracellular products; ELISA, enzyme-linked immunosorbent assay.

in 14/21 biopsies of patients with APSGN. Furthermore, the protein was antigenically identical in all strains isolated from nephritic patients.

The observations by Villarreal et al. coupled with previous reports of elevated circulating complexes in patients with both acute glomerulonephritis and acute rheumatic fever (ARF) prompted us to examine the question of whether or not streptococcal antigens, particularly extracellular products, were also present in the circulating complexes of these patients.

## Methods

*Patients.* Sera obtained from 10 patients with well-documented APSGN from Trinidad were chosen for the study. The common site of the preceding streptococcal infection was the skin. All patients had skin lesions culture positive for group A beta hemolytic streptococci. Clinical signs included edema, hypertension, oliguria, proteinuria, and hematuria. Laboratory data included third component of complement levels < 70 mg/100 ml and all patients had elevated anti-streptolysin titers and elevated blood urea nitrogen levels. The average level of circulating immune complexes by complement component 1q solid-phase assay was 44 μg/ml aggregated IgG equivalents (normal range < 4 μg/ml) (9). Because of ethical considerations, biopsies were not performed in these patients but the cases were similar in all respects to those cases studied and biopsied by Villarreal et al. (13).

The sera for the ARF study were drawn from four patients who met the American Heart Association-modified Jones' Criteria for the diagnosis of ARF (14). Laboratory tests demonstrated positive C-reactive protein, elevated anti-streptolysin titers, and erythrocyte sedimentation rates. The average immune complex level was 42.3 μg/ml aggregated IgG equivalents (9). A third group consisted of two subjects who had no evidence of streptococcal infection or post-streptococcal sequelae, and three patients with culture-positive impetigo, but with no laboratory or clinical evidence of renal complications or elevated levels of circulating immune complexes.

*Preparation of immune complexes.* Serum fractions were prepared in the following manner: First, a pooled serum fraction was collected by mixing aliquots of sera obtained from five patients with documented APSGN. Additionally, serum fractions obtained from early serial bleedings of five individual APSGN patients and four individual acute rheumatic fever patients were prepared and treated in the same manner as the pooled serum fractions. As a final control an "immune complex" preparation obtained by mixing the sera of two impetigo patients and two normal control sera was also included in the study.

The precipitation of the immune complexes of all human sera was carried out according to the method of Creighton et al. (15). The fractions were analyzed for gamma globulin by immunoelectrophoresis against rabbit immunoglobulins to human immunoglobulins (Dako Immunoglobulins, Copenhagen, Denmark) and for immune complexes by the Raji cell radioimmune assay (kindly performed by Dr. N. K. Day of the Memorial Sloan-Kettering Cancer Research Institute, New York). Those fractions found to have elevated titers of immune complexes (>6,400 μg/ml aggregated IgG equivalents) were inoculated into rabbits.

*Preparation of antisera to human immune complexes.* Samples of each immune complex preparation were aliquoted into 120–410 μg of protein, mixed with a 1.5:1 vol of Bacto Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI), and injected both intramuscularly and intradermally into rabbits weekly for 8 wk (see Table I).

The rabbits were bled 3 wk after the final injection and tested for serological activity as described below. The gamma globulin fraction was isolated from the serum of each rabbit by precipitation with a 25% saturation of ammonium sulfate according to the method of Harboe and Ingild (16). After repeated dialyses as described, the materials were finally dialyzed against 0.1 M NaCl with 0.02% sodium azide and stored at −70°C until use.

*Crossed immunoelectrophoresis (CIE).* Rabbit globulin fractions were tested for their response to human serum components and to streptococcal antigens by CIE after one-dimensional rocket electrophoresis ascertained the optimal ratio of antigens to antibodies. CIE was conducted as described (17). To achieve constant experimental conditions, all antisera was electrophoresed in parallel against antigens from the same preparation of each culture. Tandem CIE was performed by placing two antigens in separate wells 8-mm apart in the first dimension as described in the method of Weeke (18).

*Streptococcal strains.* A streptococcal strain is referred to as "nephritogenic" if it has been isolated directly from an APSGN patient at the onset of disease. These strains were chosen from those previously investigated by Villarreal et al. (13). The antigens used in the described experiments were isolated from the following streptococcal strains of the Rockefeller University collection. Extracellular products from nephritogenic strains: strain A374, nephritogenic type 12; strain F301, nephritogenic type 49; strain A995, nephritogenic type 57. Extracellular products from nonnephritogenic strains: strain L02407, pharyngitis type 12; strain B737, impetigo type 12; strain D480, pharyngitis type 1; strain A964, rheumatic fever type 5. Cell walls and cytoplasmic components: strain F300, nephritogenic type 12; strain A374, nephritogenic type 12; strain F301, nephritogenic type 49; strain S43, pharyngitis type 6.

*Preparation of streptococcal antigens.* Extracellular products (ECP) from the growth of up to 60 liters of streptococci was obtained for each strain. A 1% inoculum of a given streptococcal strain at exponential phase of growth was placed in the desired quantity of a chemically defined medium for group A streptococci (19). After overnight growth, the bacteria were removed from the extracellular supernatant by centrifugation through a continuous flow high speed centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, PA). The supernatant was then brought to 80% saturation with ammonium sulfate and stirred for 18 h at 4°C. The ammonium sulfate precipitate was then collected by centrifugation at 27,000 *g* for 50 min. The pellet was resuspended in 0.01 M ammonium bicarbonate, placed in dialysis tubing (Spectrapor 2, Spectrum Medical Industries, Inc., Los Angeles, CA) and dialyzed extensively against 0.01 M ammonium bicarbonate with 0.02% sodium azide over a period of 48 h at 4°C. The material was centrifuged at 12,100 *g* for 15 min to remove insoluble material. The solution was then lyophilized and stored at −70°C until use. A concentration of 10 mg/ml dry weight was used in the CIE experiments.

Streptococcal cell walls were prepared as previously described (20, 21), with the slight modification that the cell walls were not trypsinized after treatment with RNase and DNase. This preparation was then solubilized by group C phage-associated lysin (22), centrifuged at 9,600 *g* for 15 min, and the supernatant was obtained for experimentation. A protein concentration of 10 mg/ml was used for CIE experiments.

Cytoplasmic components were isolated from the supernatant of the cell wall preparation as follows: The cytoplasmic fraction was centrifuged at 30,000 *g* for 1 h to remove any residual membrane contamination and dialyzed against 0.01 M Tris buffer, pH 8.6 (Sigma Chemical Co., St. Louis, MO). Cell membranes were solubilized as previously described (23) and used at a concentration of 3.5 mg/ml in CIE.

*Absorption experiments.* To remove all free or complexed gamma globulin from the immune complex preparation, an antiserum to human Fc fragments (graciously provided by Dr. N. Chiorazzi, The Rockefeller University) was used in the following manner. The antiserum was coupled to CNBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) (24) and 1 ml of Sepharose-coupled antibody to Fc fragments was mixed with 0.5 ml of the polyethylene glycol precipitate of pooled nephritic sera (4 mg/ml). After incubation for 2 h at 37°C and overnight at 4°C, the suspension was centrifuged and the supernatant was tested for residual gamma globulin in rocket immunoelectrophoresis against anti-human immunoglobulin antibody (Dako Immunoglobulins). The absorption was repeated until gamma globulin could no longer be detected in the supernatant by rocket immunoelectrophoresis.

*Production of antiserum to streptococcal ECP.* Antiserum to streptococcal ECP was prepared by serially injecting a rabbit with type 57 crude ECP (500 μg protein/injection) and equal volumes of Freund's incomplete adjuvant weekly for a total of five injections. Reactivity of this serum to streptococcal extracellular products was verified by immunodiffusion.

*Enzyme-linked immunosorbent assay (ELISA).* The ELISA assay for antibody binding to streptococcal antigens was carried out according to the method of Engrall and Perlmann (25) with the following modifications: Microtiter Plates (Falcon Labware, Oxnard, CA) were coated with the streptococcal antigen preparation to be used diluted appropriately in 0.1 M Tris buffer (pH 9.8) and left overnight at room temperature. After serial washes in a buffer containing 0.01 M Tris 0.05% BRIJ detergent (Pierce Chemical Co., Rockford, IL) and 0.15 M NaCl, the appropriate antibody was diluted in the buffer and placed in the microtiter wells for 2 h at 37°C. After a series of washes, goat or anti-human anti-rabbit IgG coupled to alkaline phosphatase (Sigma Chemical Co.) was added to the wells and incubated for another hour at 37°C and then washed again. The substrate, *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.), was added at a concentration of 1 mg/ml in 1 M Tris buffer and the reaction allowed to proceed for 1 h at room temperature. The yellow color reaction was then read at 405 nm on a Titer Tek Multi-scanner (Flow Laboratories, Inc., Mclean, VA). The results were expressed as the experimental reading after substraction of antibody and antigen control values.

*Protein determination.* The protein quantitations of the immune complex preparations and streptococcal antigens used in these experiments were determined by the method of Lowry et al. (26) or the Coomassie Blue protein quantitation (27).

*Immunoblotting techniques.* The electrophoresis, immunoblotting, and staining with alkaline phosphatase-conjugated species-specific antibodies was carried out according to the method of Blake et al. (28).

## Results

*Detection of streptococcal antigens in immune complexes from post-streptococcal sequelae.* To determine whether rabbit sera prepared against any of the immune complex preparations from APSGN patients would react with streptococcal ECP, the ECP of three nephritogenic strains (types 12, 49, and 57, respectively) were tested in CIE with different rabbit sera. Fig. 1 is representative of these CIE experiments and demonstrates that serum from a rabbit immunized with pooled APSGN complexes (R643—group II) elicited a positive response of one or more peaks when reacted against the ECP obtained from three different nephritogenic streptococcal strains. Closer examination of Fig. 1, *A–C* also suggests that there was at least one antigen peak (arrows) that was common to the ECP of all three strains tested. Positive results were obtained with six other rabbit sera (7/8) irrespective of whether or not they are immunized with either individual or pooled APSGN complexes (rabbits from groups I and II, Table I). Only one rabbit failed to produce a response against the ECP of the various streptococcal strains.

As elevated titers of immune complexes were also manifest in the sera of patients with rheumatic fever (9), complexes obtained from ARF patients were studied in a similar manner to determine if streptococcal antigens were also present in these complexes. Rabbit antisera raised to complexes isolated from individual rheumatic fever patients were tested in CIE. The response to the ECP obtained from a type 5 strain, isolated from a case of ARF, was investigated. The multiple peaks observed, depicted in Fig. 1 *D*, suggest the presence of streptococcal extracellular antigens within the circulating complexes of ARF sera. A comparison with the arcs precipitated against the ECP of nephritogenic strains indicated that the pattern of the reactions of the rabbit sera raised against ARF complexes were different. The ECP from three other streptococcal strains, unrelated to either nephritis or rheumatic fever, did not produce any precipitation arcs.

To further ascertain whether APSGN immune complexes contained a streptococcal antigen common to the ECP of different streptococcal strains, rabbit serum (640—group I) immunized with complexes obtained from a single APSGN patient was run in tandem CIE with the ECP obtained from type 12 and type 57 streptococci. As seen in Fig. 2, a line of identity was observed between the two preparations, which indicates that both type 12 and type 57 ECP contained a common antigen recognized by a rabbit serum that had been immunized with complexes isolated from a single acute nephritic patient. Pretreatment of the ECP of all three strains with trypsin abolished the CIE reaction seen in Fig. 1, which indicated the immunodeterminants of the ECP in the CIE reaction were protein in nature.

The specificity of these reactions was confirmed by the fact that all the preimmune rabbit sera (14) tested produced no response in CIE. In addition, both rabbit sera (group IV) immunized with a pool of normal and impetigo "complexes" preparation also failed to elicit a response to the ECP of any of the streptococcal strains. Extracellular products from three nonnephritogenic strains were also tested in CIE. In contrast to the nephritogenic ECP, these strains, isolated from either skin or pharynx but not associated with nephritis, failed to produce any reaction with the antiserum to APSGN complexes.

*Absence of streptococcal cell fractions in APSGN complexes.* In view of the observations by Tresor and colleagues (4) that the relevant streptococcal antigen in APSGN was within the streptococcal cell, purified cell walls from nephritogenic strains

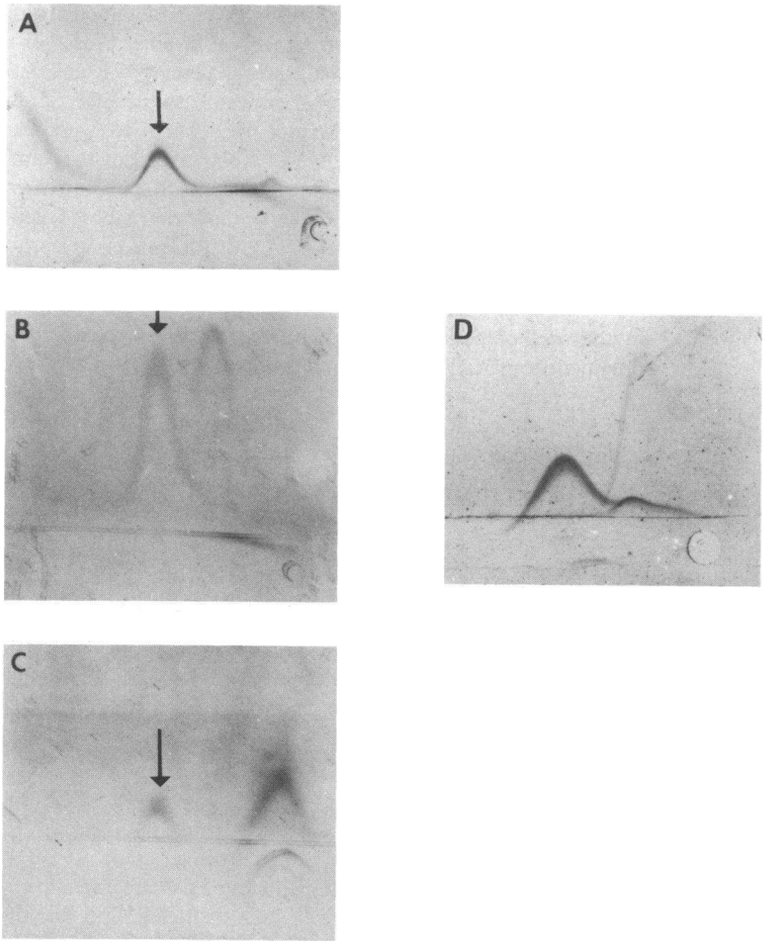


*Figure 1.* CIE demonstrating the reactivity of rabbit antiserum (R643—group II) raised against pooled APSGN immune complexes to extracellular products obtained from three nephritogenic strains; *A*, type 12 (strain A374); *B*, type 49 (F 301); *C*, type 57 (A 995). Note the presence of a peak that appears to be common to all strains tested (arrows). For purposes of comparison, *D* demonstrates the reaction where rabbit serum (R674—group III) raised against ARF complexes was reacted with the ECP of strain A964, a type 5 rheumatogenic strain. Note the differences in the CIE pattern of *D* when compared with *A–C*.

were solubilized by phage-associated lysin and tested in CIE against rabbit sera immunized with either nephritic or rheumatic immune complexes. Fig. 3 is illustrative of this type of reaction. It can be seen that both a rabbit serum (R-678—group II) prepared against pooled nephritic complexes (Fig. 3 *A*) and rabbit serum (R-676—group III) raised against rheumatic fever complexes (Fig. 3 *B*) elicited only a small flat band of precipitation close to the origin in all streptococcal cell walls

*Table I. Immunization Protocols of Rabbits Injected with Various Human Polyethylene Glycol Fractions*

| Group I | Group II | Group III | Group IV |
|---|---|---|---|
| R639 | R643 | R674 | R638 |
| R640 | R678 | R675 | R637 |
| R641 | R679 | R676 | |
| R642 | | R677 | |
| R918 | | | |

Group I, individual APSGN complexes; group II, pooled APSGN complexes; group III, individual ARF complexes; group IV, pooled impetigo and normal complexes.

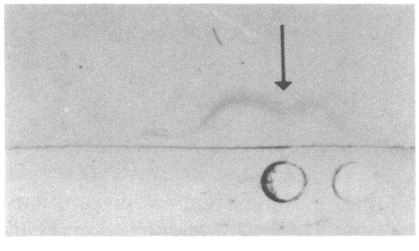

*Figure 2.* Tandem CIE demonstrates shared antigenic determinants denoted by the continuous peak (arrow) between extracellular products of nephritogenic type 12 (right well) and type 57 (left well) when tested against rabbit serum (R 640—group I), which was immunized with immune complexes isolated from a single patient with APSGN.

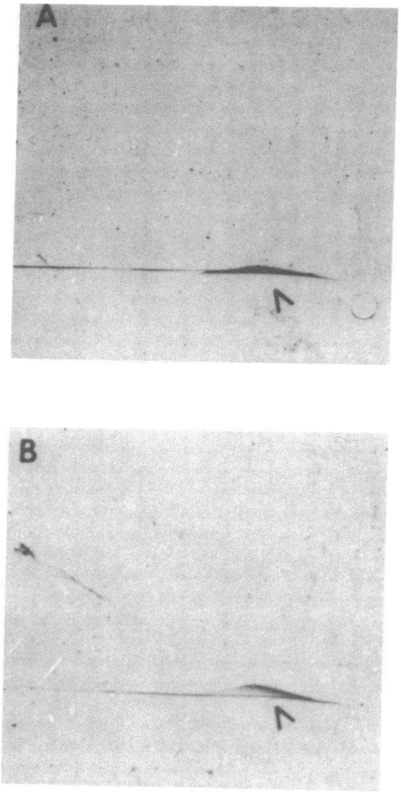


*Figure 3.* A CIE experiment using solubilized type 12 cell walls and rabbit serum (R-640—group I) immunized with nephritic complexes is demonstrated in *A*. *B* demonstrates the reactivity against the same cell wall preparation with rabbit serum (R 676—group III) immunized with ARF complexes. The precipitin arcs denoted by the arrows were elicited with all rabbit serum tested, including preimmune, indicating the presence of nonspecific antibody binding moieties in the cell wall preparation.

tested. This response was elicited with all rabbit sera tested, including preimmune sera, which suggests the presence of streptococcal cell wall components capable of nonspecific antibody-binding activity as reported previously by Schalen et al. (29) and Holm et al. (30). Furthermore, streptococcal cell membranes and cytoplasmic components obtained from a number of strains representing several streptococcal types gave no reaction with any rabbit serum produced against APSGN immune complexes.

As the human serum components in the complexes also produced a humoral response in the rabbit sera, it was critical to investigate the possibility of cross-reactivity between the human serum components and streptococcal antigens, as previously described by Bisno et al. (31). When rocket immunoelectrophoresis was conducted with antiserum to whole human serum components (Dako Immunoglobins) against streptococcal extracellular products, no precipitation peak appeared.

*An ELISA comparison of anti-immune complex rabbit sera with ECP of APSGN- and ARF-associated streptococcal strains.* As further proof that rabbit sera prepared against ARF and APSGN complexes differed in their reactivity to the ECP of nephritogenic- or ARF-associated strains, the immunoreactivity of rabbit sera raised against various complexes was compared in an ELISA system. Three sera from each disease category were selected for the test. As can be seen in Table II, A, rabbit sera raised to nephritic complexes exhibited significantly higher titers when reacted with the ECP of a nephritogenic streptococcal strain as compared with the sera of rabbits immunized with ARF complexes. Table II, B demonstrates that the converse was true. Antisera to ARF complexes had higher titers when tested with the ECP obtained from a rheumatic fever-associated streptococcal strain. The fact that both groups of rabbit sera exhibited some reaction with the ECP of either a nephritis- or a rheumatic fever-associated strain suggests that these complexes contained more than one streptococcal antigen, some of which were common to the ECP of both strains. However, the activity was always highest when the appropriate antisera was reacted against the extracellular products of those strains associated with each disease. A rabbit sera raised against impetigo complex (data not shown) exhibited minimal reaction when tested with either A314 or the A694 extracellular products.

*Immunoblotting experiments.* While the ELISAs showed differences in reactivity depending on the rabbit serum and the particular streptococcal ECP used, the use of immunoblotting techniques demonstrated that depending on the particular

*Table II. ELISA Reactivity of Anti Immune Complex Rabbit Sera with ECP (1 μg/ml)*

| | | Rabbit serum dilutions | | | |
|---|---|---|---|---|---|
| Immunization | Rabbit | 1:100 | 1:200 | 1:400 | 1:800 |
| A. Nephritogenic strain A374 ECP | | | | | |
| AGN complexes | R641 | 1.647*‡ | 1.250 | 0.958 | 0.366 |
| AGN complexes | R918 | 0.304 | 0.354 | 0.307 | 0.205 |
| AGN complexes | R642 | 1.557 | 0.349 | 0.229 | 0.120 |
| ARF complexes | R676 | 0.154 | 0.113 | 0.106 | 0.060 |
| ARF complexes | R677 | 0.339 | 0.169 | 0.094 | 0.101 |
| ARF complexes | R675 | 0.132 | 0.050 | 0.020 | 0.010 |
| B. Rheumatic fever strain A964 ECP | | | | | |
| ARF complexes | R675§ | 0.303 | 0.423 | 0.274 | |
| ARF complexes | R676 | 0.322 | 0.239 | 0.350 | |
| ARF complexes | R677 | 0.404 | 0.306 | 0.300 | |
| Nephritic complexes | R640 | 0.197 | 0.099 | 0.078 | |
| Nephritic complexes | R642 | 0.141 | 0.084 | 0.050 | |
| Nephritic complexes | R641 | 0.195 | 0.090 | 0.008 | |

* Optical density of cell samples read at 405 nm.
‡ Antibody control, 0.10; antigen control, 0.007.
§ Antibody control, 0.068; antigen control, 0.002.

rabbit antisera used, streptococcal extracellular antigens of differing molecular weights were recognized by these antisera. As seen in Fig. 4, lanes A and B represent the immunoblots obtained with the sera of two rabbits immunized with AGN complexes that reacted against the ECP of strain A374. The immunoprecipitation patterns obtained with these two sera differ markedly from the antigen antibody reactions seen in two rabbit sera raised against ARF complexes that reacted with the ECP of strain A964. Particularly evident is the high molecular antigen-antibody reactions seen in lanes C and D when compared with lanes A and B.

*Demonstration of the intrinsic association of streptococcal antigens within the immune complex.* To verify that streptococcal antigens were indeed an intrinsic part of the complex, and not just present in free form in the sera of these patients, the following absorption experiment was performed. Rabbit antibody to human Fc fragments was coupled to Sepharose beads and used to absorb 500 $\mu$l of pooled nephritic immune complexes (4 mg/ml). After two serial absorptions with 1 ml of the Sepharose-coupled antibodies, the supernatant was then tested for residual reactivity with an anti-human immunoglobulin serum and with a rabbit serum raised against nephritogenic extracellular products. As seen in Fig. 5, the left wells of each panel demonstrate the reactivity of nephritic immune complexes to an anti-human gamma globulin serum (*A*) and anti-streptococcal ECP serum (*B*). The right wells of each slide (see arrows) demonstrate the loss of reactivity to these antibodies after two serial absorptions of the complexes with Sepharose-coupled anti-human Fc fragment serum. These results strongly suggest that streptococcal antigen(s) was bound to the gamma globulin within the nephritic complex and that removal of the immune complex resulted in the concomitant removal of the streptococcal antigen(s).

## Discussion

When CIE, ELISA, and immunoblotting techniques are used, the evidence presented above strongly suggests that immune complexes isolated from the sera of either APSGN or ARF patients contain streptococcal extracellular antigens. The patterns exhibited on CIE indicated that more than one streptococcal extracellular product was present within the complexes of each group of patients and that some of the ECP antigens were common to the complexes of both groups. However, at least one of the streptococcal extracellular products excreted by those nephritogenic strains was associated with APSGN but not ARF complexes. The converse was also true in that ARF complexes contained streptococcal antigens not present in APSGN complexes. Immunoblotting demonstrated the different molecular weights of streptococcal antigens reactive to the anti-immune complex sera of each disease. The specificity of the reaction was ascertained in the following manner. Similar reactions were not seen with a variety of cellular components of the group A streptococcus. None of the preimmune sera exhibited any reactions to these ECP and preparations obtained from impetigo sera (without elevated levels of complexes) failed to demonstrate the presence of streptococcal antigens in these complexes.

Reports of the detection of microbial antigens in immune complexes is not new and several investigators have reported the presence of these antigens (32–37) in the sera of patients with a variety of disease states. However, in many instances,

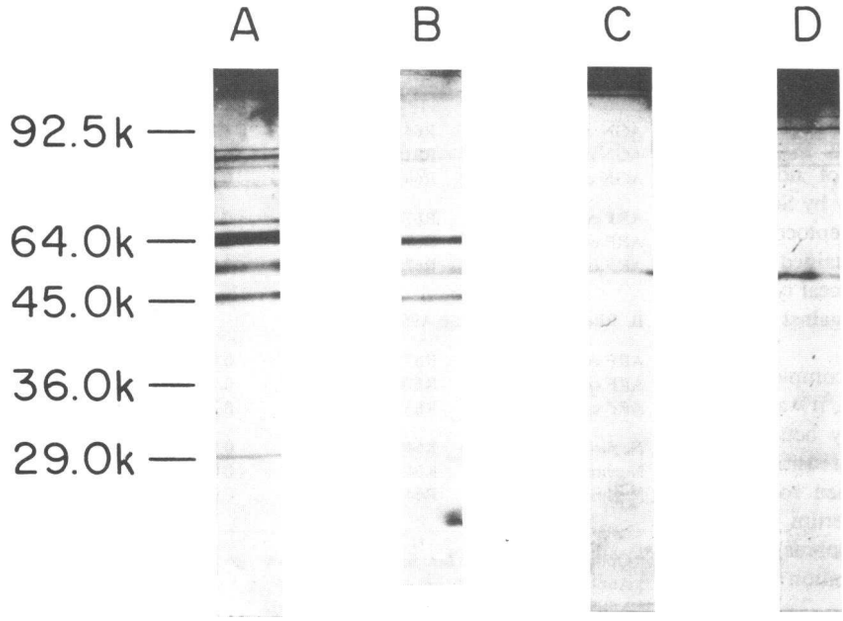


*Figure 4.* Immunoblotting experiment using rabbit antisera raised against AGN or ARF complexes and the ECP of strains A374 and A964. Lane *A* serum R642 (group I) and lane *B* serum R643 (group II) are reactions against ECP of strain A374 (nephritis strain). Lane *C* serum R674 and Lane *D* serum R675 (group III) are reactions against the ECP of strain A964. Note the differences in the molecular weights of the antigens in the reactions of the rabbit sera raised against AGN complexes (lanes *A* and *B*) when compared with the rabbit sera directed against ARF complexes and the A964 strain.

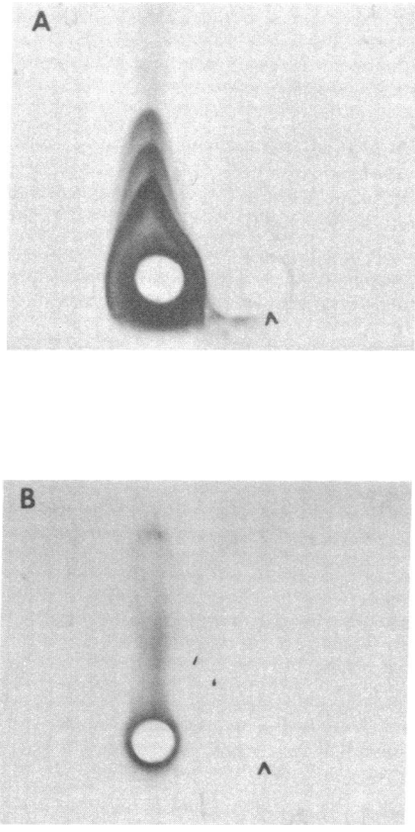


*Figure 5.* Rocket immunoelectrophoresis experiment in which isolated nephritic complexes were reacted against either an anti-human Fc serum or a rabbit anti-streptococcal ECP serum. *A*, left well, shows the reactivity with an anti-gamma globulin serum before absorption. The right well of *A* (arrow) shows loss of reactivity after absorption of the nephritic complexes with sequential addition of Sepharose beads coupled with an anti-human Fc serum. *B*, left well, shows the reactivity of the nephritic complexes with an anti-streptococcal ECP serum before the absorption. The right well of *B* (arrow) shows a parallel loss of reactivity of the nephritic complex with an anti-ECP antiserum after absorption of the complexes with the Sepharose beads. These results indicate that streptococcal antigens were bound within the nephritic complex.

the question of whether or not the antigen was actually an integral part of the complex was not answered. In contrast our results clearly demonstrate that these streptococcal antigens were an integral part of the complex since reactivity to the streptococcal ECP disappeared after absorption of the immune complex with Sepharose-bound anti-human Fc preparations. The possibility that these reactions were a result of cross-reactions between streptococcal antigens and human serum components as suggested by Bisno et al.'s studies (31) was negated by the finding that antiserum raised against whole human serum components did not react with the ECP of nephritogenic streptococcal strains.

Viewed in the general context of previous studies of the identification of streptococcal antigens either in biopsy material, or eluted products thereof in APSGN patients, our studies of streptococcal antigens in the circulating complex are most similar to those described by Villarreal et al. (13). The antigen(s) within the APSGN complex appears to be present in the extracellular products of all strains associated with nephritis that we tested. Furthermore, the antigen was not detected in streptococcal cytoplasm or membrane preparations prepared from a number of strains tested, suggesting it is not the endostreptosin described by Lange and his colleagues (11). The fact that Rodriguez-Iturbe et al. (38, 39) could not find streptococcal antigens from eluted biopsy material taken from nephritic patients is perhaps best explained by the increased sensitivity of the CIE method. Perhaps the most difficult observations to reconcile with our data have been the previous reports by Seegal et al. (6) that the streptococcal antigen found in their biopsy material appeared to be a cell wall component. Since the cases were exclusively patients with "throat" nephritis, the most plausible explanation for these discrepancies is that nephritogenic strains differ in their capacity to secrete the nephritogenic protein or sequester it in the cell wall of the organism.

The exact relationship of the antigens within the circulating complexes to the disease process is at present unanswered and further purification of the antigens detected by the anti-immune complex sera will be needed to determine their exact role in each of the post-streptococcal sequelae. However, the finding that the ECP of those strains associated with nephritis contain a protein that has been detected in the biopsies of patients with APSGN (13) coupled with the observation that antisera raised to the immune complexes of nephritis patients react specifically with the ECP of these same strains lends strong support for a causal role of this streptococcal antigen in post-streptococcal nephritis. One might next investigate the possibility that this antigen has unique biological properties that either alone or in combination with circulating serum components of the host favor its deposition in the human glomerulus. The possibility that a streptococcal antigen(s) within the ARF complex also plays an important role in the pathogenic process of this disease merits further investigation.

## References

1. McCluskey, R. S. 1970. Evidence for immunological mechanisms in several forms of human glomerular disease (review). *Bull. NY Acad. Med.* 46:769–787.

2. McCluskey, R. S., P. Vassali, G. Gallo, and D. S. Baldwin. 1966. An immunofluorescent study of pathogenic mechanism in glomerular disease. *N. Engl. J. Med.* 274:695–701.

3. Michael, A. F., Jr., K. M. Drummond, R. A. Good, and R. L. Vernier. 1966. Acute post streptococcal glomerulonephritis. *J. Clin. Invest.* 45:237–248.

4. Tresor, G., M. Seman, M. McVicar, M. Franklin, A. Ty, I. Sagel, and K. Lange. 1969. Antigenic streptococcal components in acute glomerulonephritis. *Science (Wash. DC).* 163:676–671.

5. Feldman, J. O., M. K. Mardiney, and S. E. Shuler. 1966. Immunology and morphology of acute post-streptococcal glomerulonephritis. *Lab. Invest.* 15:283–301.

6. 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.* 24:100–107.

7. Zabriskie, J. B. 1971. The role of streptococci in human glomerulonephritis. *J. Exp. Med.* 134(3, Pt. 2):180s–192s.

8. Fischel, E. E. 1957. Immune reaction in glomerulonephritis. *J. Chronic Dis.* 5:34–44.

9. van de Rijn, I., H. Fillit, W. E. Brandeis, H. Reid, T. Poon-King, M. McCarty, N. K. Day, and J. B. Zabriskie. 1978. Serial studies on circulating immune complexes in post-streptococcal sequelae. *Clin. Exp. Immunol.* 34:318–325.

10. Ahmed, U., G. Treser, J. J. Jackson, D. G. Carlo, and K. Lange. 1978. The relation of a recently identified streptococcal antigen (endostreptosin) to renal disease. *In* Pathogenic Streptococci. M. T. Parker, editor. Reedbooks, Surrey, England. 137–139.

11. Lange, K., U. Ahmed, H. Kleinberger, and G. Treser. 1976. A hitherto unknown streptococcal antigen and its probable relation to acute post-streptococcal glomerulonephritis. *Clin. Nephrol.* 5:207–215.

12. Treser, G., M. Semar, A. Ty, I. Sagel, M. A. Franklin, and K. Lange. 1970. Partial characterization of antigenic streptococcal plasma membrane components in acute glomerulonephritis. *J. Clin. Invest.* 49:762–768.

13. Villarreal, H., Jr., V. A. Fischetti, I. van de Rijn, and J. B. Zabriskie. 1979. The occurrence of a protein in the extracellular products of streptococci isolated from patients with acute glomerulonephritis. *J. Exp. Med.* 149:459–472.

14. American Heart Association. 1965. Jones criteria (revised) for guidance in the diagnosis of rheumatic fever. *Circulation.* 32:664–668.

15. Creighton, D. W., P. H. Lambert, and P. A. Miescha. 1973. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. *J. Immunol.* 111:1219–1227.

16. Harboe, N., and A. Ingild. 1975. A Manual of Quantitative Immunoelectrophoresis. N. H. Axelson, J. Kroll and B. Weeke, editors. Universitetsforlaget, Oslo. 161–164.

17. Weeke, B. 1975. A Manual of Quantitative Immunoelectrophoresis. N. H. Axelson, J. Kroll and B. Weeke, editors. Universitetsforlaget, Oslo. 47–56.

18. Weeke, B. 1975. A Manual of Quantitative Immunoelectrophoresis. N. H. Axelson, J. Kroll and B. Weeke, editors. Universitetsforlaget, Oslo. 57–64.

19. van de Rijn, I., and R. E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. *Infect. Immun.* 27:444–448.

20. Krause, R., and M. McCarty. 1961. Studies on the chemical structure of the streptococcal cell wall. *J. Exp. Med.* 114:127–140.

21. Bleiweis, A., W. Karakawa, and R. Krause. 1964. Improved technique for the preparation of streptococcal cell walls. *J. Bacteriol.* 88:1198–1200.

22. Fischetti, V. A., E. C. Gotschlich, and A. W. Bernheimer. 1971. Purification and physical properties of Group C streptococcal phage-associated lysin. *J. Exp. Med.* 133:1105–1117.

23. Kessler, R. E., and I. van de Rijn. 1979. Quantitative immunoelectrophoretic analysis of *Streptococcus pyogenes* membrane. *Infect. Immun.* 26:892–902.

24. Pharmacia Fine Chemicals AB. 1979. Affinity chromatography principles and methods. Ljungföretagen AB, Orebro:15.

25. Engrall, E., and P. Perlmann. 1971. Enzyme linked immunosorbent assay (ELISA). *Immunochemistry.* 8:874–879.

26. Lowry, O. H., W. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193:265–275.

27. Spector, T. 1978. Refinement of the Coomassie Blue method of protein quantitation. *Anal. Biochem.* 86:142–146.

28. Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid sensitive method for detection of alkaline phosphatase conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175–179.

29. Schalen, C., P. Christiansen, and R. Grubb. 1979. Streptococcal immunoglobulin receptors: restricted specificity. *In* Pathogenic Streptococci. M. T. Parker, editor. Reedbooks, Surrey, England. 69–70.

30. Holm, S. E., P. Christensen, and C. Schalen. 1979. Alternative interpretations of cross-reactions between streptococci and human tissues. *In* Pathogenic Streptococci. M. T. Parker, editor. Reedbooks, Surrey, England. 72–74.

31. Bisno, A., J. Wood, J. Lawson, S. Roy, E. Beachey, and G. Stollerman. 1978. Antigens in urine of patients with glomerulonephritis and in normal human serum which cross-react with group A streptococci: identification and partial characterization. *J. Lab. Clin. Med.* 91(3):500–513.

32. Anh-Tuan, N., and E. Novak. 1980. Detection and quantitation of hepatitis-B surface antigen immune complexes (HBsAg-ICs by an antigen-specific method.) I. Detection and quantitation of *in vitro* prepared HBsAg-ICs. *J. Immun. Meth.* 33:293–300.

33. Yoshinoya, S., R. Cox, and R. Pope. 1980. Circulating immune complexes in coccidiomycosis. Detection and characterization. *J. Clin. Invest.* 66:655–663.

34. Stass, S. A., T. Phillips, O. Weislow, E. Perlin, and H. Schumacher. 1980. Antigen-antibody complexes related to the baboon endogenous virus in humans with Acute Lymphoblastic Leukemia-Hand Mirror Variant (ALL-HMC). *Blood.* 56:661–666.

35. Pagarelli, R., and R. J. Levinsky. 1979. Immune complexes containing food proteins in normal and atopic subjects after oral challenge and effect of sodium cromoglycate on antigen absorbtion. *Lancet.* II:1270–1272.

36. Milgrom, F., Z. Maide Tuggae, and W. A. Campell. 1965. Analysis of antigen-antibody complexes by immunoelectrophoresis. *Immunology.* 8:406–410.

37. Inman, R. D., P. B. Redecha, S. J. Knechtle, E. S. Schned, I. van de Rijn, and C. L. Christian. 1982. Identification of bacterial antigens in circulating immune complexes of infective endocarditis. *J. Clin. Invest.* 70:271–280.

38. Rodriguez-Iturbe, B., D. Rabideau, R. Garcia, L. Rubio, and R. C. McIntosh. 1980. Characterization of the glomerular antibody in acute post-streptococcal glomerulonephritis. *Ann. Int. Med.* 92:478–481.

39. Rodriguez-Iturbe, B., R. Garcia, L. Rubio, R. J. Carr, J. E. Allen, D. Rabideau, and R. M. McIntosh. 1980. Etiologic, pathogenic, clinical, and immunopathologic consideration in post-streptococcal glomerulonephritis. *In* Streptococcal Diseases and the Immune Response. S. Read and J. Zabriskie, editors. Academic Press, Inc., New York. 537–552.