

Nephritis-Associated Plasmin Receptor and Acute Poststreptococcal Glomerulonephritis: Characterization of the Antigen and Associated Immune Response

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Abstract. The role of nephritis-associated antigen as a virulence factor for acute poststreptococcal glomerulonephritis (APSGN) remains to be fully clarified. Nephritis-associated plasmin receptor (NAPlr) was previously isolated from group A streptococcus (GAS) and shown to bind plasmin(ogen). The nucleotide sequence of the *naplr* gene from GAS isolates obtained from patients with APSGN was determined. The sequence of the putative open reading frame (1011 bp) showed 99.8% identity among isolated strains. Homology screen revealed an exact match with streptococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH). NAPlr exhibited GAPDH activity in zymography, and it activated the com-

plement pathway *in vitro*. In APSGN kidney biopsy specimens, NAPlr was observed mainly in the early stage of the disease (1 to 14 d after onset) but was not colocalized with either C3 or IgG as assessed by double immunofluorescence staining. Sera of patients with APSGN, patients with GAS infection without renal involvement, nonrenal pediatric patients, and healthy adults as controls were assayed for anti-NAPlr antibody titers. Anti-NAPlr antibodies were present most frequently in APSGN sera, and antibody titers were also significantly higher than in patients with GAS infection alone or in other control patients. Moreover, antibody titers remained elevated during the entire 10-yr follow-up period.

Group A streptococcus (GAS) causes various levels of infection ranging from mild pharyngitis to severe streptococcal toxic shock syndrome. One sequela of GAS infection is acute poststreptococcal glomerulonephritis (APSGN), which is associated with long-term renal dysfunction in some patients (1). However, only certain strains appear to cause APSGN (2), and only these strains produce nephritis-associated antigens (3).

A number of streptococcal proteins, including nephritis strain-associated protein, streptococcal pyrogenic exotoxin B (SPEB), preabsorbing antigen, and NAPlr, are involved in the pathogenesis of APSGN (4–7). Nephritogenic antigens are expressed by so-called nephritis-associated serotypes, accumulate in the glomeruli of patients with APSGN, and induce high antibody titers in these patients. NAPlr is such a nephritogenic

antigen; it is expressed by streptococcus strains historically associated with APSGN, it is highly antigenic, and it is localized in affected glomeruli (7). However, we cannot exclude the possibility that NAPlr is identical to previously described nephritogenic antigens because some other streptococcal proteins also exhibit plasmin(ogen)-binding activity (4,8,9). Only a partial amino acid sequence has been available for NAPlr (7); however, the protein may be homologous to streptococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10,11) and may show GAPDH activity. Thus, additional analysis of NAPlr is needed.

APSGN-related antibodies against putative nephritogenic antigens have been identified (12–14). High levels of anti-SPEB antibody were present in patients with APSGN (6), and subsets of APSGN kidney specimens were positive for anti-SPEB antibody. In addition, increased levels of anti-zymogen antibody appear to be a marker of APSGN (15,16). Glomeruli from patients with early APSGN can be stained with IgG obtained from the sera of convalescing patients (17). Reactivity is typically observed on the endothelial side of the glomerular basement membrane (GBM) and in the mesangial matrix (18). The streptococcal antigen, such as preabsorbing antigen, has also been detected in the glomeruli of patients in relatively early stages of APSGN (5).

In the study presented here, we determined the amino acid sequence of NAPlr purified from GAS strains in patients with

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APSGN. We also examined GAPDH activity, complement activation, and immune responses to NAPlr in patients with APSGN.

Materials and Methods

GAS and Preparation of NAPlr

Strains of group A β -hemolytic streptococci belonging to T types 1, 4, and 12 and M types 12 and 49 were isolated from the pharynx of five patients with APSGN. Growth conditions and purification of NAPlr were as described previously (7). We had prepared NAPlr in the presence of protease inhibitors and had confirmed the absence of proteolytic activity in the purified fraction (7).

DNA Sequencing

Genomic DNA of GAS T types 1, 4, and 12 and M types 12 and 49 were purified with SepaGene (Sanko Junyaku, Tokyo, Japan) and used as templates to amplify a fragment of *naplr* by PCR. The following primers were used: forward primers, 5'-AAGTTAAA-GAAGGTGGAT-3', 5'-AGCTGCTTCAAACGATAG-3', and 5'-TATATTTGGTGGGTTTTG-3'; reverse primers, 5'-CAGCT-TCTTCTTCTAG-3', 5'-GAATGCATCGTGAAGAGC-3', and 5'-CCCCTCCATCTTAGCCTTTTTGTGA-3' at a concentration of 1 μ M each. Primers were designed from the results of partial amino acid sequencing of purified NAPlr (7) and preliminary DNA sequence analysis. The PCR temperature profile was carried out as follows: consisting of an initial denaturation step of 95°C for 5 min, followed by 99 cycles of a denaturation step of 95°C for 30 s, a primer annealing step at 55°C for 20 s, and an extension step at 60°C for 4 min. The amplified DNA fragments were sequenced with a BigDye Terminator Ready Reaction Kit and an ABI PRISM 377 XL DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences were analyzed with the Query GenBank Database (NCBI, <http://www.ncbi.nlm.nih.gov/GenBank/index.html>) and GENETYX-MAC software (Software Development, Tokyo, Japan).

GAPDH Activity Assay

Similarity of NAPlr to GAPDH was assessed by Western blot analysis (7). After SDS-PAGE and transfer of purified NAPlr to PVDF membranes (Millipore, Billerica, MA), proteins were reacted with mouse anti-*Bacillus* GAPDH antibody (1:1000 in PBS containing 0.1% Tween 20; Chemicon, Temecula, CA) followed by incuba-

tion with an horseradish peroxidase-labeled goat anti-mouse IgG (1:2000 in PBS containing Tween 20; BioSource, Camarillo, CA). An ECL kit (Amersham Biosciences, Piscataway, NJ) was used to visualize immunocomplexes. *Bacillus* GAPDH (Sigma, St. Louis, MO) was included in each assay as a control.

For zymographic analysis of GAPDH activity, purified NAPlr (1 μ g protein) was separated by electrophoresis on 8% polyacrylamide gels in Tris-glycine (pH 8.9) at 4°C. After electrophoresis, gels were gently washed with 0.1 M Na₂HPO₄ (pH 8.5) for 10 min, then with 0.05 M Na₂HPO₄ (pH 8.5) for 10 min. Gels were incubated for 20 to 30 min at room temperature in substrate buffer containing 2.5 mM glyceraldehyde-3-phosphate (Sigma), 0.5 mM NAD⁺, 300 μ g/ml nitroblue tetrazolium, and 20 μ g/ml phenazine methosulfate (Wako Pure Chemical Industries, Osaka, Japan) in 50 mM Na₂HPO₄ (pH 8.5). GAPDH activity was detected as a blue band. *Bacillus* GAPDH (Sigma) was also included in the assay as a positive control.

Complement Activation by NAPlr

To analyze complement activation by NAPlr, we incubated normal human serum (50 μ l) for 1 h at 37°C with NAPlr (10 μ g/50 μ l in physiologic saline). In some samples, 10 μ l of 0.1 M EGTA and/or 0.1 M EDTA was added before incubation to differentiate between the two complement activation pathways. The reactions were separated by electrophoresis on 1.1% agarose gels in veronal buffer (pH 8.6) with an ionic strength of 0.05 (Wako). Conversion of C3 was examined with anti-human C3 antibody (ICN, Costa Mesa, CA). Zymosan (Sigma) was used as a control for complement activation.

The product of NAPlr cleavage of C3, iC3b, was assayed by ELISA. For sample preparation, 50 μ l of human serum, diluted 1:40 with physiologic saline, was incubated with 50 μ l of NAPlr (ranging from 0.01 μ g to 6.25 μ g) at 37°C for 1 h. The level of iC3b in each sample was measured with a commercial iC3b EIA kit (Quidel, San Diego, CA) according to the manufacturer's instructions.

Patients and Control Subjects

Sera from 50 patients with APSGN (27 men and 23 women), diagnosed by renal biopsy, (Table 1) were tested for levels of anti-NAPlr antibody. Serum samples were obtained at the time of biopsy (1 to 90 d after disease onset); anti-NAPlr antibody levels were determined and taken as the initial antibody titers. Samples were collected over a 10-yr follow-up period and used to monitor antibody levels in each patient. The diagnosis of APSGN was confirmed by the

Table 1. Clinical and laboratory features of patients with APSGN

From Onset to Biopsy	n	Mean Age in yr ^a (range)	ASO ^b (U)	ASK ^c (U)	CH50 ^d (U)	C3 ^e (mg/dl)	Urine		GFR (ml/min)	BUN (mg/dl)	S-cr (mg/dl)	BP (mmHg)
							Protein	RBC/HPF				
1–14d	25	30.8 (8–51)	584	4632	16.2	30.8	±~+++	1~many	66	23.8	1.4	149/88
15–30d	18	26.1 (8–75)	550	8533	17.1	42.0	–~+++	0~many	72	22.0	1.4	131/83
31–90d	7	31.4 (5–66)	865	4480	27.0	60.0	–~+++	0~many	75	18.1	0.9	122/74
Total	50	29.3 (5–75)	612	5952	18.1	44.3	–~+++	0~many	70	22.3	1.3	139/84

^a Per group.

Normal values in our hospital:

^b ASO-adults, < 240 U; children, < 320 U.

^c ASK-adults, < 2560 U; children, < 5120 U.

^d CH50, 30–40 U.

^e C3, 55–120 mg/dl.

Table 2. Clinical and laboratory features of patients with group A streptococcal infection without renal involvement, children, and normal adults

	n	Mean age in yr ^a (range)	ASO (U)	ASK (U)	CH50 (U)	Urine	
						Protein	Occult Blood
Streptococcal infection without renal involvement	50	29.0 (8–64)	499	4764	37.0	—	—
Pediatric I	50	7.2 (0.2–10)	177	451	30.9	—	—
Pediatric II	50	14.1 (11–20)	187	554	28.5	—	—
Normal adults I	50	30.0 (25–35)	186	576	36.0	—	—
Normal adults II	50	53.2 (52–59)	80	401	37.8	—	—

^a Per group.

presence of proteinuria, hematuria, hypocomplementemia, history of antecedent streptococcal infection with titers of anti-streptolysin O (ASO) and/or anti-streptokinase (ASK), and renal biopsy. Fifty age-matched patients with GAS upper respiratory tract infection without detectable renal involvement (26 men and 24 women) (Table 2) were included as subjects. GAS upper respiratory tract infection was diagnosed on the basis of clinical sign with significant elevation of ASO and/or ASK titers. The control groups included 100 nonrenal pediatric patients and 100 healthy adults. Pediatric patients were categorized by age into two groups: pediatric I (age 0.2 to 10 yr, n = 50, 27 boys, 23 girls) and pediatric II (age 11 to 20 yr, n = 50, 23 boys and 27 girls).

Healthy adults were also categorized by age into two groups: adult I (age 25 to 35 yr, n = 50, 25 men and 25 women, age matched with patients with APSGN), and adult II (age 52 to 59 yr, n = 50, 25 men and 25 women). These subjects showed no signs of recent streptococcal infection. Informed consent was obtained from all subjects in each group.

Measurement of Serum Anti-NAPlr Antibody

Serum anti-NAPlr antibody was measured by Western blot analysis as described previously (7). Affinity-purified NAPlr (7) was separated

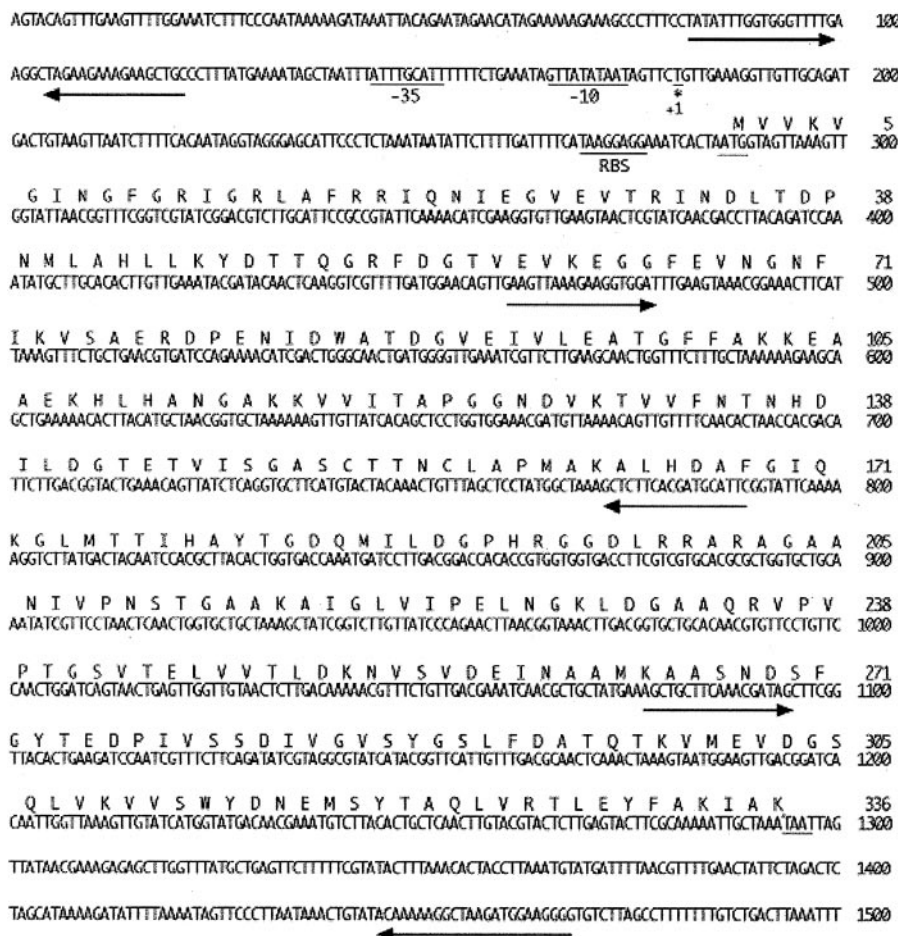


Figure 1. Nucleotide sequence and predicted amino acid sequence of the *naplr* gene in group A streptococci T type 12. Putative conserved promoter sequences (–35 and –10) and ribosome-binding site sequence (RBS) are indicated by bars. The predicted transcription start site, +1, is denoted by an asterisk. The predicted ATG start codon and TAA stop codon are indicated by bars. Positions of the PCR primers and their orientations are indicated by arrows.

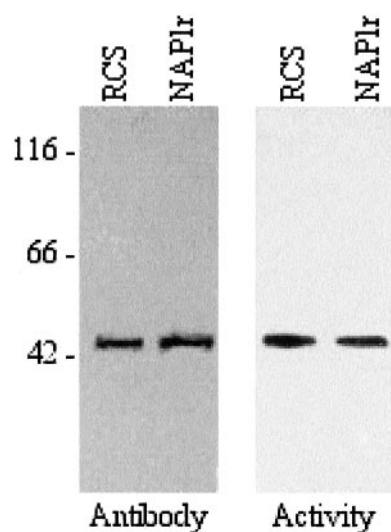


Figure 2. Antigenic and functional similarities between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and NAPlr. The Western blot profile of RCS and NAPlr shows that both proteins reacted with anti-*Bacillus* GAPDH antibody (left). The zymogram profile indicates that both RCS and NAPlr have GAPDH activity (right). The positions of protein standards are shown (kD) on the left of panel. RCS, ruptured streptococcal cell supernatant, which is the starting material for the isolation of NAPlr.

by SDS-PAGE (10% polyacrylamide gel) and the proteins were transferred to PVDF membranes (Millipore) at 0.8 mA/cm² for 50 min in a semidry transfer cell (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% nonfat milk in 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl and 0.1% Tween 20 (TBS-Tween) for 1 h. The membranes were incubated with each serum sample (1:500 to 1:2000 in nonfat milk/TBS-Tween) for 1 h. Membranes were washed with TBS-Tween and then incubated with horseradish peroxidase-conjugated anti-human IgG antibody (1:2000 in nonfat milk/TBS; American Qualex, San Clemente, CA) for 1 h. Immune complexes were visualized by development with ECL (Amersham). Pooled sera from convalescing patients were included as positive controls, and pooled sera from age-matched healthy donors were used as negative controls. NAPlr bands were quantified with a Densitometry System and Imaging Software (ATTO, Tokyo, Japan). The level of anti-NAPlr antibody was determined relative to the density of the positive control band (titer: 1000 units) and that of the age-matched healthy control band (titer: 60 to 140 units).

Immunofluorescence Microscopy

Direct and indirect immunofluorescence microscopy, FITC-conjugated rabbit anti-NAPlr antibody, and monoclonal antibody to recombinant Plr were as described by Yamakami *et al.* (7). Briefly, direct immunofluorescence was used for the detection of NAPlr, complement components (C3, C1q, C4, P), immunoglobulins (IgG, IgA, IgM), fibrinogen, and plasminogen (ICN, Irvine, CA). Indirect immunofluorescence was used to detect other complement components (C5, C9, S, MAC) (ICN). As a negative control, sections were pretreated with either unlabeled rabbit anti-NAPlr antibody or serum from a convalescing patient with APSGN. NAPlr-C3 and NAPlr-IgG colocalization assays were performed with double staining for NAPlr and C3 or IgG in renal sections from several NAPlr-positive patients. To examine colocalization of NAPlr and C3, we labeled anti-NAPlr

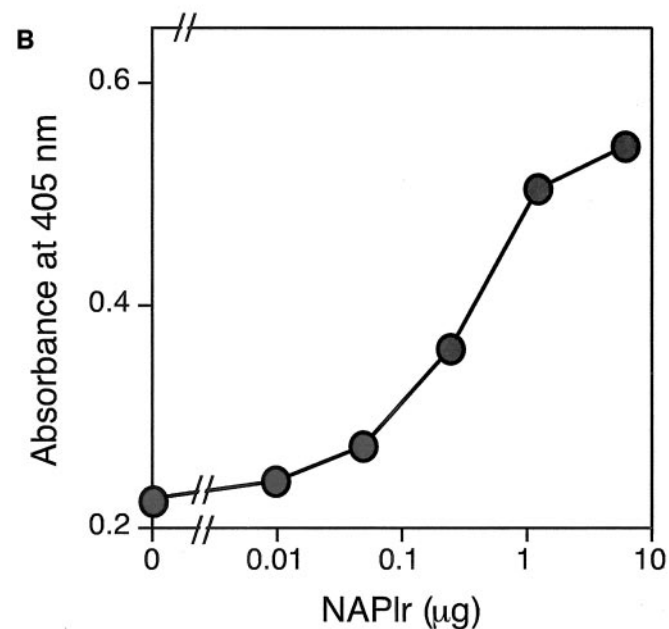
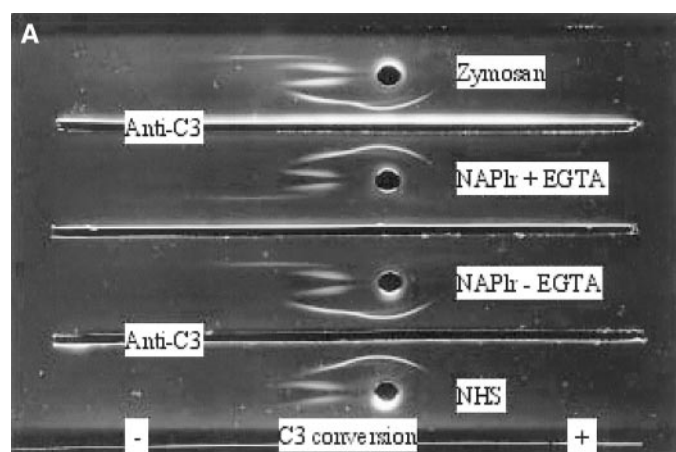


Figure 3. Complement activation by NAPlr. (A) Immunoelectrophoresis shows conversion of C3 after incubation of normal human serum (NHS) with NAPlr with or without Mg²⁺ and EGTA (middle). As a positive control, zymosan was added to NHS and indicates activated C3 (top). NHS not incubated with NAPlr shows a single arc of C3 (bottom). (B) Formation of iC3b from C3 in NHS incubated with various amounts of NAPlr. The plots use average values from triplicate assays.

antibody (1 mg protein) with Alexa Fluor 594 (Molecular Probes, Eugene, OR), according to the manufacturer's instructions and applied the labeled antibody with FITC-labeled anti-C3 antibody (ICN). For NAPlr-IgG colocalization experiments, Alexa Fluor 594-labeled goat anti-human IgG antibody (Molecular Probes) and FITC-labeled anti-NAPlr antibody were applied simultaneously to the sections.

Statistical Analyses

Statistical analyses of ASO titers and anti-NAPlr antibody titers in the present report were performed by unpaired *t* test. Two-tailed *P* values of less than 0.05 were considered statistically significant.

Table 3. Anti-NAPlr antibody in patients with APSGN, patients with group A streptococcal infection without renal involvement, children, and normal adults

	Age in yr, range	Anti-NAPlr Antibody (positive rate) ^a	Anti-NAPlr antibody titers ^b
APSGN	5–75, mean 29.3	46/50 (92%)	566.0 ± 106.1 ^c
Streptococcal infection	8–64, mean 29.0	30/50 (60%)	227.1 ± 51.2
Pediatric I	0.2–10, mean 7.2	13/50 (26%)	138.9 ± 23.4
Pediatric II	11–20, mean 14.1	18/50 (36%)	166.0 ± 25.7
Normal adults I	25–35, mean 30.0	24/50 (48%)	100.1 ± 18
Normal adults II	52–59, mean 53.2	36/50 (72%)	186.0 ± 17.3

^a The presence of anti-NAPlr antibody was determined by Western blot analysis.

^b Values of anti-NAPlr antibody titers are expressed as mean ± SEM.

^c *P* < 0.05 for APSGN versus streptococcal infection, pediatrics, and normal adults.

Results

naplr Gene Sequences

The full-length nucleotide sequence of the *naplr* gene of T type 12 is shown in Figure 1. Among sequences from the five-nephritogenic strains analyzed, only two nucleotides in the open-reading frame (ORF) differed; however, the predicted NAPlr amino acid sequence was identical among strains. The predicted *naplr* ORF is 1011 bp long, and the putative promoter contains a conserved TATA box at –10 and a CAT box (TTGCAT) at –35. In addition, a potential ribosome binding site (TAAGGAGG) is located nine nucleotides upstream from the predicted ATG start codon. Guanine at position 1066 of *naplr* was substituted for thymine in comparison to the nucleotide sequence of the *plr* gene encoding plasmin receptor (Plr), which is identified as GAPDH of GAS strain 64/14 (10; GenBank Database accession number M95569). Thus, NAPlr and Plr showed 99.8% identity at nucleotide and 99.7% identity at amino acid levels.

The *naplr* ORF encodes a 336 amino acid polypeptide with a predicted isoelectric point of 5.2 and a predicted molecular mass of 35.8 kD. The predicted molecular mass was lower than that determined by SDS-PAGE (43 kD), which may reflect the amino acid compositions of NAPlr. The *N*-terminal amino acid sequence of purified NAPlr contained the following five residues: VVKVG. The *N*-terminal amino acid sequence of native NAPlr was homologous to the deduced *N*-terminal sequence of NAPlr, with the exception of an additional *N*-terminal methionine.

Functional Analysis of NAPlr

On the basis of the *naplr* nucleotide sequence, which was homologous to the *GAPDH* sequence (11), NAPlr was tested for reactivity with anti-GAPDH antibody and for GAPDH activity. Western blot analysis revealed that NAPlr reacted with anti-*Bacillus* GAPDH antibody (Figure 2, left). In addition, zymographic analysis showed that both purified NAPlr and crude extract each contained activity in single bands that had identical migration profiles (Figure 2, right).

The ability of NAPlr to activate complement was measured as conversion of C3 (Figure 3A). C3 conversion was observed in the presence or absence of chelating reagent. Thus, NAPlr

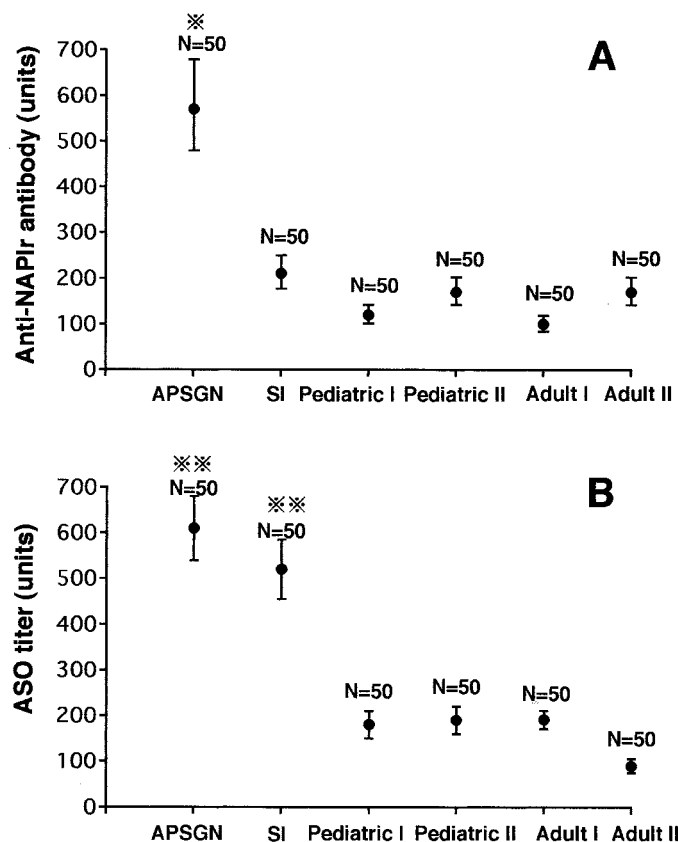


Figure 4. (A) Levels of anti-NAPlr antibody titers in acute poststreptococcal glomerulonephritis (APSGN), streptococcal infection without renal involvement (SI), nonrenal pediatric patients, and normal adults. Values are mean ± SEM. ※ *P* < 0.05 for APSGN versus SI, pediatrics, and normal adults by *t* test. (B) Levels of ASO titers from the same group of patients compared with anti-NAPlr antibody titers. The APSGN and SI groups show significantly elevated ASO titers in comparison to those in nonrenal pediatric patients and normal adults. Values are mean ± SEM. ※※ *P* < 0.001 for titers in APSGN and SI versus those in other groups by *t* test.

activated the alternate complement pathway. In addition, we found that NAPlr induced the formation of iC3b in a dose-dependent manner (Figure 3B).

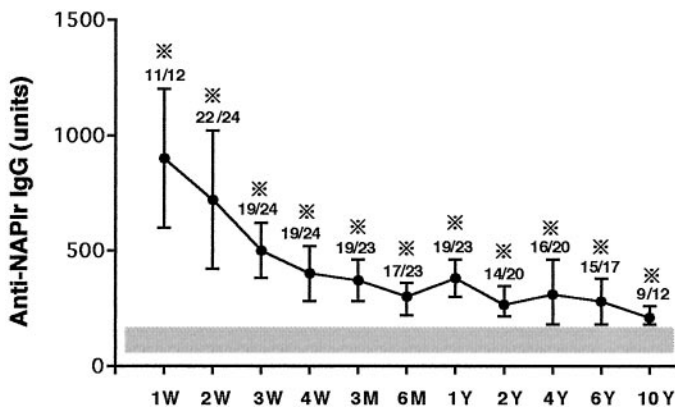


Figure 5. Serial anti-NAP1r antibody levels in the sera of patients with acute poststreptococcal glomerulonephritis (APSGN). Values are mean ± SEM, and the figure indicates the rate of anti-NAP1r antibody detection at each time point. The shaded area is the mean ± SEM of normal adults (mean age 30 yr). * $P < 0.05$ for titers of the serial sera of patients with APSGN versus age-matched controls by *t* test.

Measurement of Serum Anti-NAP1r Antibody

Sera from patients with APSGN, pediatric and adult patients with streptococcal infection without renal involvement, and control subjects were tested to determine the titers of anti-NAP1r antibody. Anti-NAP1r antibody was detected more frequently in the sera of patients with APSGN than in sera from other subjects (Table 3), and significantly increased levels of anti-NAP1r antibody were found in the sera of patients with APSGN (Figure 4A). It is noteworthy that as much as 72% of the adult II group (mean age 53.2 yr) possessed anti-NAP1r antibody, whereas only 26% of the nonrenal pediatric patients (mean age 7.2 yr) possessed anti-NAP1r antibody.

ASO titers compared with the anti-NAP1r antibody titers are shown on Figure 4B. ASO titers were significantly higher in the APSGN and streptococcal infection groups than in the control groups, indicating a serologic response to a streptococcal product, regardless of renal involvement. Over the 10 yr that the sera of the 50 patients with documented APSGN were monitored, the anti-NAP1r antibody titers tended to increase during the acute phase of the disease. After the acute phase, titers decreased but remained significantly higher in patients with APSGN than in age-matched control adults (Figure 5). The rate of anti-NAP1r antibody positivity was also higher than that of controls during the 10-yr follow-up period.

Immunofluorescence Studies of Kidney Biopsy Specimens

Thirty-six (72%) of 50 APSGN renal biopsy specimens were positive for glomerular NAP1r with anti-NAP1r antibody (Table 4). All 25 renal biopsy specimens obtained in the early disease stage (1 to 14 d after APSGN onset) and 11 (61%) of 18 biopsy specimens obtained in the middle disease stage (15 to 30 d after onset) were positive for glomerular NAP1r. The antigen was localized mainly to the mesangium and part of the GBM, and infiltrating leukocytes were observed in a ringlike pattern (Figure 6A). However, no staining was observed 31 d after onset.

Table 4. Immunofluorescence studies in patients with APSGN^a

Time from Onset to Biopsy	n	NAP1r	Plasminogen	Fibrinogen	C3	IgG	IgA	IgM	P	Clq	C4	C5	C9	S	MAC
1–14 d	25	25/25 (100)	10/25 (40)	15/25 (60)	25/25 (100)	16/25 (64)	11/25 (44)	10/25 (40)	23/25 (92)	7/25 (28)	8/25 (32)	25/25 (100)	24/25 (96)	24/25 (96)	25/25 (100)
15–30 d	18	11/18 (61)	5/18 (28)	11/18 (61)	18/18 (100)	11/18 (61)	8/18 (44)	9/18 (50)	16/18 (89)	6/18 (33)	3/18 (17)	18/18 (100)	17/18 (94)	17/18 (94)	18/18 (100)
31–90 d	7	0/7 (0)	0/7 (0)	4/7 (57)	6/7 (86)	3/7 (43)	3/7 (43)	3/7 (43)	5/7 (71)	2/7 (29)	1/7 (14)	6/7 (86)	5/7 (71)	5/7 (71)	6/7 (86)
Total	50	36/50 (72)	15/50 (30)	30/50 (60)	49/50 (98)	30/50 (60)	22/50 (44)	22/50 (44)	44/50 (88)	15/50 (30)	12/50 (24)	49/50 (98)	46/50 (92)	46/50 (92)	49/50 (98)

^a P, properdin; S, S protein (vitronectin); MAC, membrane attack complex; Percentages are reported in parentheses.

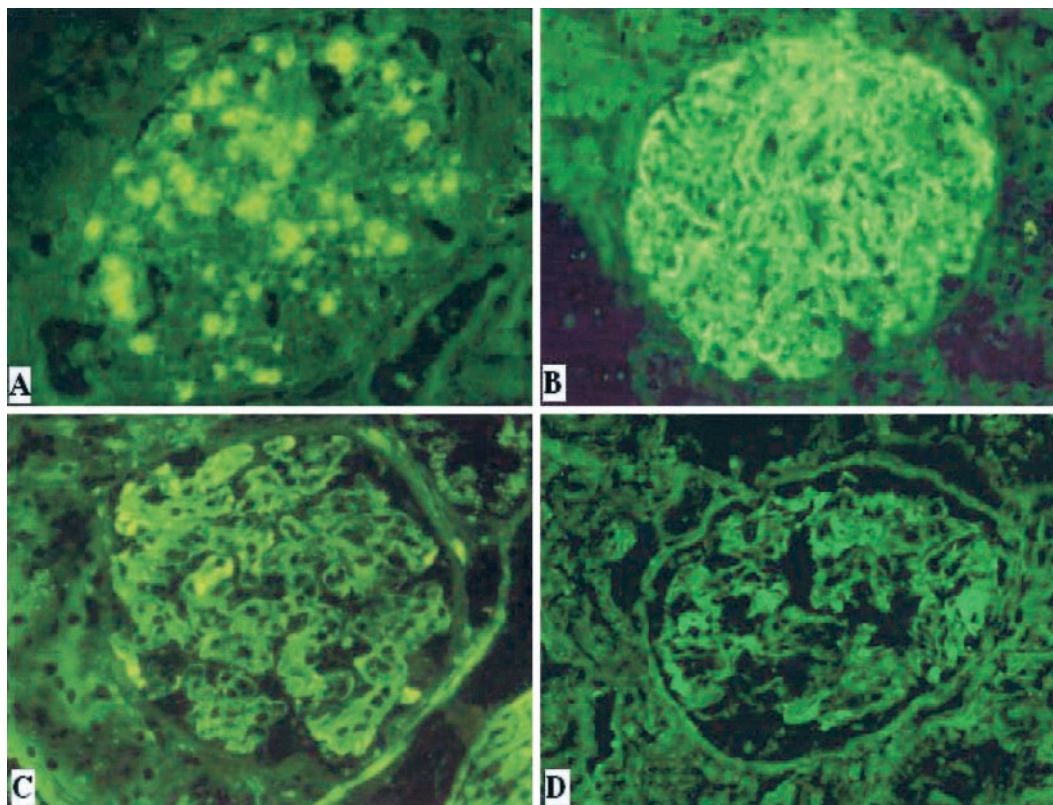


Figure 6. Immunofluorescence microscopy of glomeruli from a patient with acute poststreptococcal glomerulonephritis (APSGN) 11 d after onset. (A) Localization of NAPlr. Staining sites, which are thought to represent free antigen, are localized primarily in the mesangium and part of the glomerular basement membrane (GBM), and infiltrating leukocytes show a ring-like granular pattern (original magnification, $\times 200$). (B) Deposition of C3. Intense, diffuse, fine granular deposition of C3 is seen mainly along the GBM (original magnification, $\times 200$). (C) Deposition of fibrinogen. Fibrinogen is deposited primarily along the inner side of the GBM (original magnification, $\times 200$). (D) Deposition of plasminogen. Plasminogen is deposited predominantly in the mesangium and along part of the GBM (original magnification, $\times 200$).

Pretreatment of sections with unlabeled anti-NAPlr antibody or with serum from a convalescing patient abolished the staining with FITC-labeled anti-NAPlr-antibody. In addition, preabsorption of FITC-labeled anti-NAPlr antibody with recombinant streptococcal Plr abolished glomerular staining of NAPlr. All APSGN renal biopsy specimens obtained within 30 d of onset showed intense and extensive deposition of C3 along the GBM and/or in the mesangium (Figure 6B). IgG staining was present in the glomeruli of 64% and 61% of the sections representing the early and middle disease stages, respectively, and it was not always colocalized with C3. Staining of IgA and IgM ranged from blush to faint. Colocalization studies of NAPlr with C3 or IgG revealed that the distribution of NAPlr differs from that of C3 or IgG (Figure 7).

Fibrinogen was stained intensely in the glomeruli in 15 (60%) of 25 patients 1 to 14 d after onset. Fibrinogen was observed mainly on the endothelial side of the GBM (Figure 6C), and the frequency of staining was relatively consistent throughout the course of the disease. In contrast, plasminogen was observed in the glomeruli in 10 (40%) of 25 patients in the early stage and less frequently in the later stage. The localization of plasminogen was predominantly in the mesangium and part of the GBM (Figure 6D), and when present, it was always

colocalized with NAPlr. Most complement components, except C1q and C4, were detected frequently in glomeruli. We observed intense staining of C3, P, C5, C9, S, and MAC, particularly in the early stage (Table 4). Staining of C1q and C4 was weak and infrequent.

Discussion

Characterization of NAPlr has been incomplete (7). The available partial amino acid sequence for purified NAPlr was identical to that of streptococcal Plr (10) and similar to that of streptococcal GAPDH, suggesting that NAPlr has GAPDH activity (11). Thus, NAPlr is implicated as a virulence factor. However, the role of cytoplasmic GAPDH in APSGN was not clear. In the study presented here, we found that purified NAPlr has GAPDH activity on zymograms. Characteristics of NAPlr that are similar to characteristics of GAPDH include adhesion to fibronectin, myosin, and actin and plasmin receptor activity (10,19,20). Thus, NAPlr is expected to interact with these molecules in the pathogenesis of APSGN.

We previously detected anti-NAPlr antibody in sera at a relatively early stage of APSGN (7). In the study presented here, analysis of sera from patients with APSGN showed a significantly higher frequency of anti-NAPlr antibody than in

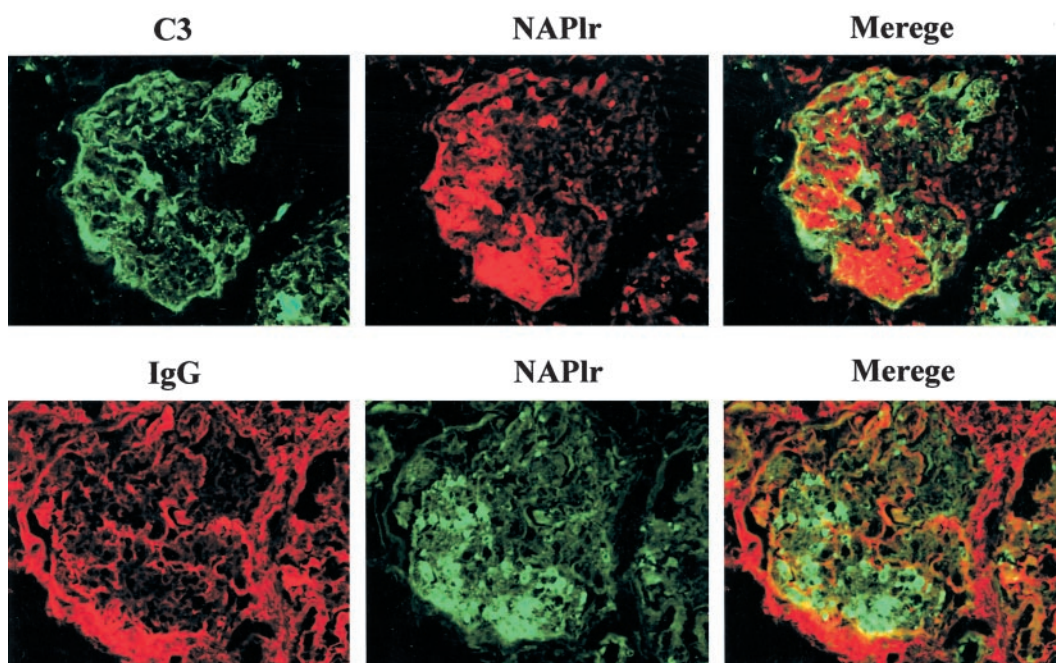


Figure 7. Immunofluorescence microscopy of glomeruli from a patient with acute poststreptococcal glomerulonephritis (APSGN) 18 d after onset. The distributions of NAP1r (Alexa Fluor 594, red) and C3 (FITC, green) (top panels) and of NAP1r (FITC, green) and IgG (Alexa Fluor 594, red) (bottom panels) are essentially different, as shown by the respective double immunofluorescence stainings (original magnification, $\times 200$).

other subjects, including those with streptococcal infection alone. In addition, anti-NAP1r antibody titers tended to be highest during the first week of infection and decreased thereafter. However, the titers did not decrease to the baseline levels of age-matched controls, and they remained significantly higher than those of the control subjects over the 10-yr follow-up period. These findings suggest that recurrence of APSGN is rare and that a single infection confers life-long immunity. Anti-NAP1r antibody was present in only 26% of subjects in the youngest control group (age 0.2 to 10 yr), and the rate increased to 72% (age 52 to 59 yr). This may explain why younger children have a greater tendency to suffer from this disease. Individuals appear to acquire immunity gradually through repeated streptococcal infection and thus, older people seldom develop APSGN (21).

As we reported previously, immunohistochemistry showed that NAP1r was present in glomeruli in APSGN renal biopsy specimens (7). In the study presented here, all specimens obtained 1 to 14 d after APSGN onset were positive for glomerular NAP1r, whereas no specimen obtained 31 to 90 d after onset was positive for the antigen. Thus, glomerular NAP1r tended to decrease over time in patients with APSGN. Furthermore, the difference in the localization of NAP1r in comparison to that of IgG or C3 indicated that NAP1r exists as a free antigen with or without plasmin(ogen). We suspect that during the early phase of APSGN, the antigenic sites are not fully saturated and can interact with anti-NAP1r antibody, whereas later in the course of the disease, the sites are saturated.

We previously reported that complement components were deposited in affected glomeruli 1 to 32 d after onset of APSGN (7). In the study presented here, the majority of biopsy specimens showed frequent and intense staining for C3, P, C5, C9, S, and MAC, particularly at 1 to 90 d after onset. NAP1r was

deposited in 100% of the specimens obtained from early in the disease course (1 to 14 d after onset). Thus, NAP1r as well as complement components are associated with APSGN (22–24). In the study presented here, the deposition of C3 without IgG in glomeruli in 9 of 25 patients 1 to 14 d after APSGN onset and the lack of circulating anti-NAP1r antibody in 4 of 50 patients suggest that complement components are associated with the initial inflammatory reaction (25–27). In addition, NAP1r cleaved C3 to C3b in human serum *in vitro*. Thus, NAP1r may activate the complement cascade in circulation (28,29).

NAP1r was detected in glomeruli of all early APSGN biopsy specimens, and anti-NAP1r antibody was detected in the majority of serum samples from patients with APSGN. Because NAP1r has plasminogen-binding activity (7,19), NAP1r on the mesangial matrix and GBM is expected to interact with plasmin(ogen). Plasmin may induce glomerular damage by degrading the GBM through activation of matrix metalloproteinase precursors. In fact, we recently observed significant glomerular plasmin activity that reflected the distribution of NAP1r deposition in the early phase of APSGN (T. Oda *et al.*, unpublished data). Circulating immune complexes may readily pass through the altered GBM and accumulate in the subepithelial space (30). Taken together, our findings suggest that NAP1r is a virulence factor for APSGN and that the presence of a high titer of anti-NAP1r antibody should prevent autoimmune sequelae. Further studies regarding the role of NAP1r will allow us to better understand the pathology of APSGN.

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