Streptokinase as a Mediator of Acute Post-Streptococcal Glomerulonephritis in an Experimental Mouse Model

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Group A streptococcal infections are sometimes followed by the inflammatory kidney disease acute post-streptococcal glomerulonephritis (APSGN). To test the importance of streptokinase in the pathogenesis of this disease, isogenic strains of the nephritis isolate NZ131, differing only in the ability to produce streptokinase of the nephritis-associated ska1 genotype, were used for infection in a mouse tissue cage model for APSGN. Streptokinase production was found to be a prerequisite for the capacity of the strain to induce APSGN in mice. In addition, streptokinase was demonstrated in the kidneys of mice infected with the nephritogenic NZ131 and EF514 strains. After infection with the nonnephritogenic strain S84, neither streptokinase nor C3 deposition were observed. Deposition of streptokinase in the glomeruli was detected as soon as 4 days after infection. These findings provide support for the hypothesis that streptokinase initiates the nephritis process by glomerular deposition, which leads to local activation of the complement cascade. Detection of streptokinase in kidney tissue increased with the degree of glomerular hypercellularity. Thus, the severity of the pathological process may be a reflection of the degree of streptokinase deposition.

Acute post-streptococcal glomerulonephritis (APSGN) sometimes follows skin or throat infections with group A streptococci (GAS). Occasionally, it is also observed after infection with group C or G streptococci (1, 9, 27, 33). The pathogenetic mechanism responsible for this sometimes fatal inflammatory kidney disease is virtually unknown. The nephritogenicity shows a possible connection to certain M serotypes but also appears to be strain dependent, as it has been observed to vary between strains of the same serotype (18, 24). A number of streptococcal products have been suggested to be the nephritogenic factor (6, 23, 29–31, 34), and the nephritis-strain-associated protein (NSAP), later tentatively identified as streptokinase, has received particular attention (2, 15, 22). This protein is polymorphic, with nonidentical residues mainly localized within two major domains referred to as variable region 1 (V1) and variable region 2 (V2). Based on restriction enzyme analysis of PCR-amplified V1s, the streptokinase gene (ska) was grouped into nine different genotypes, of which ska1, ska2, ska6, and ska9 were identified in GAS associated with clinically and experimentally defined APSGN (14). All analyzed strains of groups A, C, and G streptococci were reported to harbor the gene for streptokinase, whereas it was not found in strains of 12 other Lancefield groups (13). The association of the disease with certain ska genotypes was also observed in a genetic analysis of group C streptococcal strains isolated from APSGN patients (33).

Symptoms of APSGN typically appear 10 to 21 days after patient infection. It has therefore been difficult to analyze details of the initial phase of the disease. It is not unusual for the infection to disappear when symptoms arise. Furthermore, due to the high reinfection rate in communities where APSGN is common, it is not certain that the streptococcal isolate was the one which induced the disease in the patient. However, a mouse model was recently presented for the study of the disease where the nephritogenic capacity of a strain could be analyzed (18). In this model, signs of nephritis similar to those observed in humans with APSGN were demonstrated. In the present study, we attempted to clarify whether streptokinase is of relevance for the development of APSGN by using the mouse tissue cage model to study a nephritogenic NZ131 GAS strain from which the streptokinase gene (ska1) was deleted. Furthermore, kidneys of mice infected with these strains, as well as mice infected with the nephritogenic EF514 (ska2) and the nonnephritogenic S84 (ska3) strains, were analyzed for the presence of deposited streptokinase.

MATERIALS AND METHODS

Bacterial strains and growth. The Streptococcus pyogenes GAS nephritis isolate NZ131 (ska1) and EF514 (ska3), the nonnephritogenic isolate S84 (ska3) (18), and an erythromycin-resistant derivative of NZ131 with the streptokinase gene deleted (NZ131 Δska::Emr) through allelic replacement by homologous recombination (25) were used in this study. Trypticase-yeast medium (12) and Todd-Hewitt broth were used for analysis of growth characteristics of the NZ131 wild-type and Δska::Emr strains, at 37°C in 5% CO2. Inoculi of 100, 200, 400, or 800 μl (100 μl was equivalent to 2 × 106 CFU) from overnight or mid-log-phase cultures were added to 10 ml of fresh medium which had been prewarmed to 37°C. Growth was analyzed by determining optical densities at 500 nm, viable counts on blood agar plates, and chain lengths with a light microscope. A longer initial lag phase was observed with NZ131 Δska::Emr than with NZ131.

Both strains reached the same generation time during the exponential growth phase. By doubling the inoculum of NZ131 Δska::Emr, identical growth curves were obtained (data not shown). Supernatants from exponential- and stationary-growth-phase cultures were precipitated overnight with 95% ethanol at −20°C and resuspended in 1 ml of H2O. Samples (2, 4, and 8 μl) were then separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (12% acrylamide), followed by electrotransfer to nitrocellulose filters (28). Detection of SpeB and SpeF was performed with the primary and secondary sera used for their detection in tissue cage fluid (TCF), as described in the section “Sample collection and analyses” below. At the same bacterial density no differences could be detected between the strains regarding the amounts of SpeB and SpeF produced.

Animals and bacterial infection. BALB/c mice (Bomholtgård Breeding & Research Centre A/S, Ry, Denmark), 2 to 3 months old at the time of infection, were kept behind barriers and had free access to drinking water and R36 pellet food for mice and rats (Lactamin AB, Stockholm, Sweden). Steel net cylinders (0.8 by 1.2 cm) were implanted subcutaneously. Before injection of bacteria, a 3-week interval was allowed to elapsed to promote connective tissue infiltration of the net (18). After passage in hepatized mouse blood for 6 h at 37°C, the bacteria were streaked onto blood agar plates, transferred to a Trypticase-yeast
medium (12), and cultivated overnight at 37°C in 5% CO2, followed by reincubation into fresh medium with cultivation for 5 h. The exponentially growing cultures were centrifuged, washed once, and then diluted in NaCl before injection of 0.1 ml into tissue cages at concentrations of 10^9 CFU/ml for the wild-type strain and 10^8 CFU/ml for the NZ131 Δska::Emr strain. The higher inoculum was compensated for the longer initial lag phase of the NZ131 Δska::Emr strain. Comparable bacterial numbers were obtained in TCF during the course of infection. Where administered, benzylpenicillin (ASTRA Lakemedel, Södertälje, Sweden) was given intraperitoneally at 40 mg/kg body weight, twice daily for 6 days, starting on day 7 or day 16 postinfection (p.i.). The animals were sacrificed by exsanguination under anesthesia (Hynorn; Janssen-Cilag Ltd., Saunderton, United Kingdom; and Dormicur; F. Hoffman-La Roche AG, Basel, Switzerland). All animals subjected to antibiotic treatment were sacrificed on day 21 p.i., whereas additional mice, infected with NZ131 and NZ131 Δska::Emr and which did not receive antibiotic treatment, were sacrificed at day 4 p.i. The kidneys were immediately perfused with phosphate-buffered saline in situ via the left heart ventricle until macroscopically free of blood. They were then removed and prepared for histopathological and immunohistochemical evaluation.

Animals used in the evaluation of NZ131 wild-type and Δska::Emr nephritis/genicity. A total of 23 mice were infected with NZ131 wild type and 23 mice were infected with the NZ131 Δska::Emr strain. Penicillin treatment was initiated on day 7 p.i. for 7 of the mice infected with NZ131 Δska::Emr. For 16 wild type-infected and 11 NZ131 Δska::Emr-infected mice, antibiotic treatment was initiated on day 16 p.i. The uninfected group included 46 mice, of which 19 belonged to the same experiment as the NZ131-infected mice of this study, i.e., they were treated and analyzed at the same time. Of these, 15 were injected with 0.1 ml of NaCl and 4 were surgically unmanipulated and unoinoculated. The remaining 27 uninfected mice were surgically unmanipulated and had been analyzed concurrently with mice from another infection experiment (18).

Sample collection and analyses. At days 0, 3, 5, 7, 14, and 21 p.i., urine was collected by abdominal massage and 0.1 ml of TCF was aspired. Blood was obtained from the tail before infection and by retrobulbar punctation at day 21 p.i. Urine, TCF, and blood samples were analyzed on blood agar plates for the presence of bacteria. Increasing amounts of protein in the urine to a concentration of at least 0.1 mg/liter was defined as proteinuria, and hematuria was defined as a hemoglobin concentration corresponding to at least 10 erythrocytes/μl (N-Labtest, Bayer Sverige AB, Gotthenburg, Sweden). Immunoblot procedures for the detection of streptococcal antigens in TCF and of antibodies to streptokinase in sera were performed as described earlier (18). Streptokinase used for antibody analyses was a gift from Kabi Vitrum (Stockholm, Sweden) and was used in amounts corresponding to the 60th percentile of the 190 values for uninfected mice per group. Mice infected with the streptokinase-defective strain served as controls.

Morphological evaluation of glomeruli. Kidney specimens (2 to 3 mm thick) were fixed in 10% buffered formalin, paraffin embedded, cut into 5-μm slices, and stained with hematoxylin and periodic acid Schiff. Ten glomeruli per mouse were evaluated for hypercellularity and morphological changes, such as thickening of the glomerular basement membrane, capsule epithelium, and capillary wall as well as occlusion of capillaries and lobulation of the glomerular tuft. Excluded from calculation were glomeruli close to the edges of the section (20) and those with a diameter below 0.5 times that of the largest glomerular profile (4). Quantitative assessment of glomerular cell density was achieved by calculation of the number of nuclei touching the intersections of a 100-square line pattern (Leitz no. 5040), as described by Weibel (32). The recorded numbers of cells were comparable, as they reflected glomeruli of identical areas. The number of cells corresponding to the 60th percentile of the 190 values for uninfected mice of the same experiment, i.e., the 19 mice treated and analyzed in parallel with the infected mice of this study, was chosen as the limit for glomerular hypercellularity (18). The cell numbers for the 27 uninfected mice that were analyzed with infected mice of a previous study were not included in the calculation of cutoff limits for this report due to the possibility of discrepancies in morphometrical evaluation between different experimental readings. A kidney was designated positive ascription of diffuse hypercellularity when more than 50% of the glomeruli analyzed in a mouse displayed cell numbers above that of the 60th percentile of the uninfected mice of the same experiment, *P < 0.05; **, P < 0.01; ***, P < 0.001; compared to the proportions for the 46 uninfected controls.

Table 1. Number of mice with diffuse hypercellularity after infection with the nephritis GAS isolate NZ131 or its streptokinase-defective isogenic derivative NZ131 Δska::Emr

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Penicillin from day 7</th>
<th>50th</th>
<th>60th</th>
<th>70th</th>
<th>80th</th>
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<tr>
<td>NZ131</td>
<td>7</td>
<td>5/7</td>
<td>3/7</td>
<td>3/7</td>
<td>3/7</td>
</tr>
<tr>
<td>NZ131 Δska::Emr</td>
<td>4/12</td>
<td>1/12</td>
<td>1/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>NZ131 Δska::Emr treated from day 7</td>
<td>16</td>
<td>13/16***</td>
<td>12/16***</td>
<td>11/16***</td>
<td>8/16***</td>
</tr>
<tr>
<td>NZ131 Δska::Emr treated from day 16</td>
<td>16</td>
<td>7/16*</td>
<td>6/11*</td>
<td>5/11*</td>
<td>2/16</td>
</tr>
</tbody>
</table>

Control

<table>
<thead>
<tr>
<th>Unmanipulated</th>
<th>11/31</th>
<th>4/31</th>
<th>0/31</th>
<th>0/31</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl injection</td>
<td>7</td>
<td>7/15</td>
<td>3/15</td>
<td>1/15</td>
</tr>
</tbody>
</table>

Detection of streptokinase in kidneys. Mice analyzed for streptokinase deposits were selected from experimental animals included in another experiment (18). A positive ascription of diffuse hypercellularity occurred when more than 50% of the glomeruli analyzed in a mouse displayed cell numbers above that of the 60th percentile of the uninfected mice of the same experiment, *P < 0.05; **, P < 0.01; ***, P < 0.001; compared to the proportions for the 46 uninfected controls.

RESULTS

Bacterial growth and antigen production. Bacterial growth in TCF was analyzed by sampling and growth on blood agar plates. At day 3 p.i. the counts of NZ131 and NZ131 Δska::Emr were approximately 10^8 CFU/ml of TCF, and at days 5, 7, and 14 p.i. the counts were 10^7 CFU/ml. The bacteria were negative for the presence of SPEB, SPEF, or SPEH production between the NZ131 Δska::Emr strain and NZ131 Δska::Emr (Table 1). Kidney tissues from 13 mice infected with NZ131, 16 mice infected with EF514 (18), 11 mice infected with NZ131 Δska::Emr, and 5 uninfected mice (mice treated with NaCl) were examined for streptokinase production by microwave heating (21), and normal goat serum (DAKO A/S, Copenhagen, Denmark) was used to block unspecific binding. Immunogold-silver staining (7, 10) was used to demonstrate streptokinase in the kidneys. For primary antibody detection, a polyclonal monoclonal rabbit antiserum, obtained by repeated immunizations with streptokinase derived from the wild-type strain H64 (Kabi Vitrum, Stockholm, Sweden), was used. The secondary antibody was gold-labelled goat anti-rabbit IgG (AuroProbe LM GAR), purchased from Amersham (Solna, Sweden). The sections were counterstained with cosin. Sections (approximately every third in depth), which represented a total cortex area of approximately 4 cm^2, were evaluated per mouse. This allowed for the analysis of 70 ± 4.80 glomeruli for each individual. Glomeruli close to the edges of the sections were excluded from analysis. In addition, one section for each mouse was stained with hematoxylin and eosin to confirm the occurrence of glomerular hypercellularity.

Detection of streptokinase in kidneys. Mice analyzed for streptokinase deposits were selected from experimental animals included in another experiment (18). A positive ascription of diffuse hypercellularity would be similar to that found in infection experiments with this strain (Table 1) (18). Kidney tissues from 13 mice infected with NZ131, 16 mice infected with EF514 (18), 11 mice infected with NZ131 Δska::Emr, and 5 uninfected mice (mice treated with NaCl) were examined for streptokinase production by microwave heating (21), and normal goat serum (DAKO A/S, Copenhagen, Denmark) was used to block unspecific binding. Immunogold-silver staining (7, 10) was used to demonstrate streptokinase in the kidneys. For primary antibody detection, a polyclonal monoclonal rabbit antiserum, obtained by repeated immunizations with streptokinase derived from the wild-type strain H64 (Kabi Vitrum, Stockholm, Sweden), was used. The secondary antibody was gold-labelled goat anti-rabbit IgG (AuroProbe LM GAR), purchased from Amersham (Solna, Sweden). The sections were counterstained with cosin. Sections (approximately every third in depth), which represented a total cortex area of approximately 4 cm^2, were evaluated per mouse. This allowed for the analysis of 70 ± 4.80 glomeruli for each individual. Glomeruli close to the edges of the sections were excluded from analysis. In addition, one section for each mouse was stained with hematoxylin and eosin to confirm the occurrence of glomerular hypercellularity.
and the NZ131 wild-type strains. In accordance with the absence of the speA gene in NZ131, SpeA was not detected in TCF. The other antigens were demonstrated in TCF from day 3 p.i. and throughout the infectious process. Streptokinase was produced in the tissue cages of all NZ131 wild-type-infected mice but was not detected in TCF from mice infected with NZ131 Δska::Em'. Antibodies to streptokinase were not detected in sera of these mice but were present in the majority of NZ131 mice but was not detected in TCF from mice infected with NZ131 Δska::Em'. Antibodies to streptokinase were not detected in sera of these mice but were present in the majority of mice infected with the wild-type strain (data not shown).

**Evaluation of nephritogenicity: uninfected control animals.** The parameters chosen to evaluate nephritogenicity of the strains were glomerular C3 and IgG deposition, diffuse hypercellularity, occlusion of capillaries, and lobulation of the tuft of glomeruli, as well as proteinuria and hematuria (18, 26). The uninfected mice used for statistical comparisons included 19 animals analyzed concurrently with the infected mice of this report and 27 animals analyzed prior to this study (18). Data from the latter were used only when no differences in the parameters examined were noted between the two groups (P > 0.05). This precaution was taken to avoid any influence of differences related to reagent batches or time of observation. The only statistical difference noted was occurrence of IgG deposition, an event which was related to the batch of fluorescein isothiocyanate conjugate used. Thus, occurrences of this parameter in groups of infected mice were compared to those for the 19 mice from the same experiment.

**Evaluation of nephritogenicity of the NZ131 wild-type strain.** The NZ131 wild-type strain induced pronounced hypercellularity (Table 1) in groups treated with penicillin from both days 16 and 7 p.i. (Fig. 1). Significantly increased occurrence of capillary occlusion, as determined by its distribution in at least 50% of glomeruli, was demonstrated in the group of animals treated with penicillin from day 16 p.i. (Table 2). Animals infected with this strain revealed C3 deposition after both 7 and 16 days of infection (Fig. 1). Significantly increased occurrence of capillary occlusion, as determined by its distribution in at least 50% of glomeruli, was demonstrated in the group of animals treated with penicillin from day 16 p.i. (Table 2). Animals infected with this strain revealed C3 deposition after both 7 and 16 days of infection (Fig. 1). The deposition was usually heavy and the patterns corresponded to mesangial or starry sky patterns (26). Likewise, proteinuria was induced after both 7 and 16 days of infection. C3 deposition was noted also without concomitant diffuse hypercellularity. Furthermore, diffuse hypercellularity was observed in mice where complement deposition could not be demonstrated. Proteinuria was in most cases accompanied by C3 deposition; however, this result was not significant (P < 0.1).

**Evaluation of nephritogenicity of the NZ131 Δska::Em' strain.** The NZ131 Δska::Em' strain did not induce hypercellularity after 7 days of infection. However, hypercellularity appeared when the infection was allowed to proceed for 16 days. Capillary occlusion, lobulation, C3 or IgG deposition, proteinuria, or hematuria was not induced after infection with the NZ131 Δska::Em' strain. Statistical comparison between the isogenic strains verified the significance of the differences observed regarding the strains’ abilities to induce hypercellularity (80th percentile) and complement deposition (P < 0.05 and P < 0.01, respectively) after 7 days of infection.

**Detection of streptokinase in kidneys.** Of mice treated with penicillin from day 16 p.i., streptokinase was detected in the kidneys of animals infected with the nephritis isolates NZ131 and EF514, whereas it was absent in mice infected with the nonnephritis isolate S84 after the corresponding duration of infection (Table 3). The deposition occurred in both the glo-

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**TABLE 2. Morphological, immunohistopathological, and urinary findings in mice infected with the nephritis GAS isolate NZ131 or its isogenic derivative NZ131 Δska::Em', with the streptokinase gene deleted**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Penicillin from day</th>
<th>No. of mice positive for result/total no. of mice*</th>
<th>Glomeruli</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Occl</td>
<td>Lob</td>
</tr>
<tr>
<td>Exptl NZ131</td>
<td></td>
<td></td>
<td>2/7</td>
<td>0/7</td>
</tr>
<tr>
<td>NZ131 Δska::Em'</td>
<td></td>
<td></td>
<td>1/12</td>
<td>0/12</td>
</tr>
<tr>
<td>NZ131</td>
<td>16</td>
<td></td>
<td>5/16*</td>
<td>0/16</td>
</tr>
<tr>
<td>NZ131 Δska::Em'</td>
<td>16</td>
<td></td>
<td>2/11</td>
<td>1/11</td>
</tr>
<tr>
<td>Control Unmanipulated</td>
<td></td>
<td></td>
<td>1/31</td>
<td>0/31</td>
</tr>
<tr>
<td>NaCl injection</td>
<td>7</td>
<td></td>
<td>0/15</td>
<td>0/15</td>
</tr>
</tbody>
</table>

* For proteinuria, the minimum concentration limit was set at 1.0 g of protein/liter of urine; for hematuria, the minimum concentration was a hemoglobin concentration corresponding to 10 erythrocytes/μl of urine. *, P < 0.05; **, P < 0.01 compared to the proportions for the uninfected control mice. Abbreviations: Occl, occlusion of capillaries; Lob, lobulation; Protein, proteinuria; Hem, hematuria.
meruli and tubuli of EF514-infected mice but occurred primarily in the tubuli of NZ131-infected mice. In glomeruli, streptokinase was demonstrated along the basement membrane, in the mesangium, and in the capsular epithelium layer (Fig. 3). A higher occurrence of deposition of streptokinase in glomeruli was found in the EF514-infected group than in the NZ131-infected group, both after mild ($P < 0.001$) and somewhat harder ($P < 0.01$) fixation of the tissue. In general, the detection sensitivity appeared somewhat lessened when the harder fixation had been used, i.e., 4 days at 4°C in 10% buffered formalin, than when fixation had occurred in 4% buffered paraformaldehyde for 16 h at room temperature. This tendency was significant for the occurrence of tubular deposition, as well as of concomitant tubular and glomerular deposition, in mice infected with EF514 for 16 days. Hence, for statistical comparisons, animal samples treated with different fixation methods were not combined to represent one large group of animals but were evaluated separately. The occurrence of NZ131-infected mice with streptokinase deposition in glomeruli was higher ($P < 0.05$) among animals sacrificed at day 4 p.i. than among those treated with antibiotics after 16 days of infection and sacrificed at day 21 p.i. Apart from tubular deposition, which appeared in hypercellular but not in nonhypercellular animals after infection with EF514, the same deposition patterns were observed in hypercellular and nonhypercellular mice infected with the same strain. However, there was a tendency of streptokinase deposition occurring at a higher frequency among hypercellular than nonhypercellular animals. The significance of this tendency was verified statistically when the findings for the glomeruli and tubuli were combined, i.e., when the kidney as a whole ($P < 0.05$) was evaluated for mice infected with NZ131. In EF514-infected hypercellular mice, the deposition was demonstrated in both glomeruli and tubuli when evaluated separately. Among hypercellular NZ131-infected mice with streptokinase deposition in the glomeruli, 60% of the animals with no concomitant deposition in tubuli. Of mice for which streptokinase was found deposited in tubuli, 66.7% (6 of 9) also had deposition in glomeruli. Among mice sacrificed after 4 days of infection, 60% (3 of 5) of the mice with tubular deposition had streptokinase also in glomeruli, whereas the corresponding figure was 25% (1 of 4) in animals infected with EF514 and treated with antibiotics from day 7 p.i. Streptokinase deposition occurred in both hypercellular and nonhypercellular mice infected with the nephritis isolates and treated with penicillin from day 16 p.i. Apart from tubular deposition, which appeared in hypercellular but not in nonhypercellular animals after infection with EF514, the same deposition patterns were observed in hypercellular and nonhypercellular mice infected with the same strain. However, there was a tendency of streptokinase deposition occurring at a higher frequency among hypercellular than nonhypercellular animals. The significance of this tendency was verified statistically when the findings for the glomeruli and tubuli were combined, i.e., when the kidney as a whole ($P < 0.05$) was evaluated for mice infected with NZ131. In EF514-infected hypercellular mice, the deposition was demonstrated in both glomeruli and tubuli when evaluated separately. Among hypercellular NZ131-infected mice, the deposition was demonstrated in both glomeruli and tubuli when evaluated separately. Among hypercellular NZ131-infected mice treated with penicillin from day 16 p.i., 60% of the animals had streptokinase deposition in both glomeruli and tubuli. Of mice for which streptokinase was found deposited in the glomeruli, 60% (6 of 9) also had deposition in the glomeruli. Among mice sacrificed after 4 days of infection, 60% (3 of 5) of the mice with tubular deposition had streptokinase also in the glomeruli, whereas the corresponding figure was 25% (1 of 4) in animals infected with EF514 and treated with antibiotics from day 7 p.i.

### TABLE 3. Detection of streptokinase in kidneys of selected mice after infection with GAS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Duration of infection (days)</th>
<th>No. of mice with streptokinase deposition/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glomeruli and tubuli (G and T)</td>
<td>Glomeruli (G)</td>
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<tr>
<td>S84</td>
<td>16</td>
<td>0/6</td>
</tr>
<tr>
<td>EF514</td>
<td>7</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>16**</td>
<td>5/10***</td>
</tr>
<tr>
<td>NZ131</td>
<td>4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>16a</td>
<td>1/13</td>
</tr>
<tr>
<td>NZ131 Δaskr::Emr</td>
<td>4</td>
<td>1/11</td>
</tr>
<tr>
<td></td>
<td>16a</td>
<td>0/11</td>
</tr>
</tbody>
</table>

*a Kidney tissue was fixed with 10% buffered formalin for 4 days at 4°C. All other kidney tissues were fixed with 4% buffered paraformaldehyde for 16 h at room temperature. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$ compared to proportions for S84-infected mice.
...After 7 days of infection, no signs of nephritis were seen in mice infected with a nonnephritogenic strain S84. This strain also induced nephritis in mice infected with the nephritogenic strain NZ131. Streptokinase deposition was demonstrated in glomeruli and tubuli of mice infected with the nephritogenic strains NZ131 and EF514. In kidneys of hypercellular mice infected with the nonnephritogenic S84 strain, significant streptokinase deposition was shown only if these two parameters were combined, i.e., in some animals deposition was found only in the glomeruli and in others only in tubuli. Streptokinase was not demonstrated in kidneys of hypercellular mice infected with the nonnephritogenic strain S84 and treated from day 16 with penicillin. There was a tendency toward an increased frequency of mice with streptokinase deposition in the kidneys, with an increased degree of hypercellularity, among mice infected with NZ131 and EF514 for 16 days. Among the mice which were hypercellular, with the 60th percentile as the cutoff limit, 30% showed streptokinase deposition, whereas the corresponding figures were 57.1% with the 70th or 80th percentile as a cutoff limit and 80% with the 100th percentile as the cutoff limit.

**DISCUSSION**

In this study, we show that streptokinase production is required for the GAS nephritis isolate NZ131 to induce APSGN in mice. In addition, streptokinase deposition was demonstrated in kidneys of mice infected with nephritogenic strains but was not seen after infection with a nonnephritogenic strain. The finding of streptokinase in glomeruli as soon as 4 days after infection, and the absence of deposition of streptokinase as well as C3 in mice infected with a nonnephritogenic strain, indicates that the role of the deposited protein may be as an initiator of the disease process. Furthermore, occurrence of streptokinase deposition increased with the degree of hypercellularity. This observation may indicate that the more streptokinase deposited in glomeruli, the more severe the pathological process will be. The mouse model reflects the early stage of APSGN, where C3 is the major immune deposit found in the kidneys (18, 26). Immune complexes may have a role in the later stage of the disease, since C3 deposition is followed by IgG deposition in humans as the disease progresses (26). Such complexes might be composed of antibodies to epitopes of damaged kidney tissue or streptokinase.

In a previous study, strain NZ131 was shown to be nephritogenic in mice (18). This strain also induced nephritis in this study. No signs of nephritis were seen after 7 days of infection with the NZ131 Δska::Em' strain or, for that matter, after prolonged infection to 16 days, except for the appearance of hypercellularity. The corresponding finding was also observed after prolonged infection with strain S84, a nonnephritis isolate (18). Whether this late-appearing hypercellularity reflects a delayed onset of the nephritis process, which would be followed by a fully developed APSGN, or whether it is an effect of high antigen load over an extended time period can only be speculation at this point. There were no detectable differences in expression of other streptococcal factors between the strains during infection. We therefore consider it likely that it was the absence of streptokinase which dramatically affected the nephritogenic capacity of strain NZ131.

Complement has been observed to be deposited in the glomeruli before IgG in the disease process (17, 26). A proposed explanation for the early deposition of C3 is localization of streptokinase to glomeruli through epitopes unique to nephritis-associatedstreptokinases, with subsequent deposition of complement as an effect of the ability of streptokinase to convert plasminogen to plasmin (11). This theory is supported by the finding that streptokinase from a nephritis isolate bound more tightly to isolated human glomeruli than did a non-nephritis-associated streptokinase (22). Consistent with the hypothesis of Holm (11), we here found streptokinase deposited in kidneys of mice infected with the nephritogenic strains EF514 and NZ131, whereas no deposition was detected after infection with the nonnephritogenic S84 strain. A more powerful method was required to demonstrate streptokinase in the tissue than was needed in order to detect C3. Hence, streptokinase appears to be present in smaller amounts. With the proposed mechanism of binding and activation of plasminogen by streptokinase, a very potent protease and complement activator would be trapped in situ. Furthermore, once bound to streptokinase, the proteolytic activity of plasmin cannot be inhibited by α2-antiplasmin (3). Thus, it is not unlikely that deposition of seemingly small amounts of streptokinase may have profound effects on complement activation. The possibility that deposition of streptokinase in the glomeruli may precede and initiate the local inflammatory process of APSGN is further supported by the finding that C3 was not detected in...
glomeruli of mice infected with the streptokinase-deficient mutant strain or with strain S84, which did not cause streptokinase deposition. Statistical analysis verified that the ability to induce hypercellularity (80th percentile) and C3 deposition after 7 days of infection was different between the isogenic NZ131 strains. In addition, deposition occurred at an early stage, i.e., after 4 days of NZ131 infection. In fact, the proportion of mice with glomerular streptokinase deposition was higher after 4 days of infection than after prolonged infection. This finding might reflect antibody masking of bound streptokinase, or decreased presence due to detachment, possibly in conjunction with decreased deposition with time. This might be an explanation for the seemingly contradictory results of attempts to demonstrate the protein in biopsies of renal tissue from APSGN patients (8, 16).

The finding that streptokinase deposition tended to occur more often among hypercellular than nonhypercellular mice, and also increased with the degree of hypercellularity, indicates that the level of streptokinase deposited is coupled to the severity of the pathological process. Additionally, the presence of the molecule in nonhypercellular animals may indicate a lower rate of streptokinase deposition in these mice or that deposition was initiated at a later stage. Thus, the pathological effects may have been initiated but diffuse hypercellularity had not yet appeared. In a previous study, after 16 days of infection with NZ131 or with EF514 diffuse hypercellularity was observed in 50 or in 78.5% of infected mice, respectively (18). In the present study, the corresponding figures were 75.8% for NZ131 infection and 70% for EF514 infection. Combining the results from these reports, EF514 or NZ131 infection for 16 days induced hypercellularity in 75.8% (25 of 33) or 62.5% (20 of 32) of the infected mice. Thus, strain EF514 may have a stronger potential for inducing hypercellularity than strain NZ131. Statistical analysis showed that a larger proportion of EF514-infected animals had streptokinase in the glomeruli than did NZ131-infected animals after 16 days of infection. In addition, there was a tendency toward a higher proportion of mice with streptokinase deposition in the kidney as a whole among EF514-infected mice than among NZ131-infected mice. These findings further suggest the importance of the level of streptokinase deposition for the severity of the disease, as well as indicate that this might lead to different degrees of nephritogenic potential between individual strains.

In APSGN in humans, the pathological process is known to occur preferentially in glomeruli and not in tubuli. However, in mice, we detected the protein at both sites. The tubular presence might reflect a natural effect of passage of streptokinase through the glomerular basement membrane or passage due to detachment, which would then indicate that damage had been induced already after 4 days of infection with NZ131. However, streptokinase, with a pI of 4.7 and a molecular mass of 47 kDa, might also be able to pass through an intact membrane. The tubular presence of streptokinase may be due either to an affinity to tubular structures or to reabsorption of the molecule.

A strain with streptokinase of ska2 genotype was previously found to be nonnephritogenic when tested in our model (18). This finding indicates that, apart from streptokinase, additional factors may also be required in the pathogenetic process of APSGN. Peake et al. have shown that streptokinase of a non-nephritis-associated genotype possessed lower affinity to glomeruli than streptokinase of a nephritis-associated genotype (22). Strains NZ131 and EF514 produce streptokinase of the nephritis-associated genotypes ska1 and ska2, respectively, whereas that of the nonnephritogenic strain S84 is of the non-nephritis-associated genotype ska3. The strains produce approximately the same amount of streptokinase during infection (18). In this study streptokinase was demonstrated in kidneys of mice infected with NZ131 and EF514 but not in kidneys of mice infected with S84. These findings indicate that the requirement for streptokinase for nephritogenicity is likely to be related to the streptokinase genotype. The nephritogenic potential of a strain may be a reflection of the ska genotype in relation to variable degrees of affinity to kidney epitopes, i.e., streptokinase of the ska3 genotype would have the lowest affinity, ska1 a higher affinity, and ska2 the highest affinity of the three. GAS strains varying only with regard to the streptokinase genotype are currently under construction and will be tested for nephritogenicity in the animal model.

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