Induction of glomerulonephritis in rats with staphylococcal phosphatase: New aspects in post-infectious ICGN

YEHIA YOUSIF, KANAME OKADA, STEPHEN BATSFORD, and ARNOLD VOGT

Department of Immunology, Institute of Medical Microbiology, Freiburg University, Freiburg, Germany

Induction of Glomerulonephritis in rats with staphylococcal Phosphatase: New aspects in post-infectious ICGN. Staphylococcal neutral phosphatase (NPtase) is a highly cationic bacterial surface-bound protein. It has significant affinity for human and rat immunoglobulins in vitro and an electrostatic interaction may be involved. Radioisotopic studies showed that NPtase had a high affinity for the poly-anionic structures of the rat renal glomerulus. When the left kidneys of germ-free or naive (non-immune) Wistar rats were perfused with 80 μg of 125I NPtase, 21 μg of NPtase were found in the left kidneys and 11 μg in the isolated glomeruli 15 minutes after perfusion. Deposits of autologous immunoglobulin and C3 were seen in the glomeruli of rats immediately after perfusion with NPtase (15 min) and persisted throughout the 14-day observation period. Histologically, neutrophil influx into the glomerulus was seen at 15 minutes and increased until three hours; subepithelial electron-dense deposits were found after three days and were still visible on day 14. Proteinuria started within the first 24 hours after a decline of an immune response at this time and was still present on day 14. Similar results were observed in immune deficient athymic nude rats in the early phase. Perfusion of heparin after NPtase inhibited the deposition of IgG and C3 and prevented proteinuria in naive but not in actively immunized rats. This result provides further evidence that specific antibodies to NPtase were not involved in the immune complex-like deposits seen in the early phase. NPtase is a novel molecule, as it reveals both high affinity for the GBM and binding of circulating immunoglobulins, by a non-antigen-antibody mechanism, to form IC-like deposits on the GBM. These deposits are capable of activating the complement system, thus triggering a series of events leading to glomerulonephritis. These results delineate an additional pathway for the pathogenesis of ICGN related to bacterial infection.

The connection between staphylococcal infection and glomerulonephritis is well established [1–8], although it is not a frequent complication of staphylococcal infection. The pathogenetic pathways involved have not yet been clarified.

Staphylococci are known to produce a number of cationic products, and some of these, for example a neutral phosphatase, reveal high affinity for the rat glomerular basement membrane (GBM) [9]. In addition, an in vitro affinity of certain cationic proteins for immunoglobulin has been reported [10, 11]. In particular, we reported an affinity of cationic neutral phosphatase for human polyclonal and myeloma immunoglobulin, especially the F(ab')2 portion, and rat polyclonal immunoglobulin [12]. These two properties suggest an interesting possibility in post-infectious complications of staphylococcal infections, and in the current report the nephritogenic potential of staphylococcal neutral phosphatase (NPtase) in non-immunized rats was tested. NPtase was able to induce glomerular injury via its affinity for both the GBM and autologous immunoglobulin. This observation pinpoints a new pathomechanism which may be involved in cases of glomerulonephritis related to bacterial infections.

Methods

Animals

Experiments were performed in 6- to 8-week-old germ-free, or naive, Wistar male rats, or 4- to 6-week-old athymic nude rats (HAN-RNU nu/nu) (Zentrahtier-zuchtere, Hannover, Germany).

NPtase preparation

NPtase was prepared as previously described [12]. The Staphylococcus aureus strain ATCC 25923 was grown at 37°C in 10 liter volumes of a chemically defined medium (CDM) [13]. In a typical preparation 34.5 g (wet weight) of cells were harvested from a 24-hour culture by centrifugation (6,000 g for 30 min), washed with 0.05 M Tris-HCl buffer pH 8.0, centrifuged again and resuspended in 44 ml of 1.0 M KCl in 0.10 M Tris-HCl buffer pH 8.5. A crude surface bound protein fraction was eluted by gentle shaking for 60 minutes at 25°C. After centrifugation as above, the supernatant was ultra-centrifuged (45,000 rpm, Ti 60 rotor, Beckman) for 14 hours. The supernatant was applied, in starting buffer (0.32 M NaCl, 0.03 M phosphate buffer pH 7.0), to a Mono S HRS/S cation exchange column (Pharmacia LKB, Freiburg, Germany) previously equilibrated in starting buffer. After 17 ml of buffer had been passed, a linear gradient up to 1.0 M NaCl in starting buffer was run, NPtase eluted at 0.58 M NaCl.

Properties of NPtase

Properties of NPtase are described in detail elsewhere [12] and are summarized here. It consists of a non-covalently linked dimer and appears as a double band (31/32 kDa) in SDS-PAGE; its pI was > 10. It reveals maximal phosphatase activity at a neutral pH (7.2 to 7.5). No close similarity between NPtase and other known bacterial proteins in respect of their N-terminal amino acid sequences was found.
Protein concentration

Total protein concentrations were determined with the protein assay reagent (BCA) (Pierce, Germany).

Radiolabeling of proteins

Proteins were labeled with $^{125}$I (Amersham Buchler GmbH, Germany) using the chloramine-T method [14].

Anti-NPase antibody

Rabbits were immunized with NPase in incomplete Freund’s adjuvant (Sigma). Rats were immunized twice with 100 µg NPase in incomplete Freund’s adjuvant at a two week interval.

Renal perfusion technique in rats, processing of organs

After opening the peritoneal cavity, a number 20 needle was inserted into the aorta and tied in place so that the tip was level with the left renal artery. The aorta was ligated above the left and below the right renal artery, and the left kidney was injected with 0.3 ml of PBS to remove blood, followed by 0.8 ml of $^{125}$I-NPase (80 µg, labeled with $1 \times 10^6$ cpm) at a flow rate of 1 ml/min, followed again by 0.3 ml of PBS. The ligature was then removed to re-establish normal blood flow. At sacrifice one ml of blood was taken from the jugular vein and then both kidneys were perfused with 2 ml of PBS via the aorta after clamping the aorta above the arteries and severing both renal veins. Both kidneys were removed and the total radioactivity was counted, and then they were divided longitudinally. One half was taken for light, immunofluorescent and electron microscopy. Glomeruli were isolated from the other half and the radioactivity was measured. The number of glomeruli isolated was estimated by direct counting of aliquots. For further calculation it was assumed that a single kidney contained 38,000 glomeruli [15]. In addition, liver, spleen, lung and heart were removed, weighed and the radioactivity was counted.

Autoradiography of glomerular preparations

After isolation and counting of the radioactivity, an aliquot of the glomeruli was dissolved in SDS-PAGE sample buffer, containing 2% SDS and run in 12.6% SDS-PAGE; the gel was then vacuum dried and autoradiographed at $-70^\circ$C using a Kodak x-Omat X-ray film and a Kodak x-Omat regular intensifying screen (Eastman Kodak, Rochester, NY, USA).

Histological examination

Renal tissue was fixed with buffered formalin, embedded in paraffin, and 4 µm sections were stained with hematoxylin eosin (HE) or periodic acid shiff (PAS). A second portion was snap-frozen in precooled n-Hexane, and then 4 µm frozen sections were directly stained with fluorescein-labeled goat anti-rat IgG, IgM, IgA and C3 (Nordic, The Netherlands). Deposition of NPase was detected by an indirect immunofluorescence test: sections were incubated with specific rabbit anti-NPase antisera, then with fluorescein-labeled goat anti-rabbit IgG (Nordic). Normal rat sera were used as a control. The intensity of fluorescent staining was evaluated on a negative to 3+ scale. A third piece of renal tissue was fixed in 2% glutaraldehyde and embedded in epon for electron microscopic examination.

Detection of autoantibody

Screening for serum autoantibodies to renal antigens was performed by incubating frozen sections of normal (unreated) rat kidney with dilutions of rat anti-NPase sera, followed by fluorescein-labeled goat anti-rat IgG (Nordic).

Experimental groups

Groups of three rats (naive or immunized) were examined histologically at 15 minutes, one hour, three hours, six hours, 24 hours, three days, seven days and 14 days after renal perfusion of 80 µg NPase as described above. Measurements of bound radioactive antigen were only performed in naive rats up to 24 hours (early stage groups). In two groups of three naive or three immunized rats perfusion with NPase was followed by 0.5 ml of heparin-natrium (2500 IU liquemin N; Hoffmann-La Roche, Germany). Germ-free Wistar rats, 3 rats per group, were examined at 15 minutes, three hours and 24 hours after perfusion of 80 µg NPase. Athymic nude rats were perfused with 80 µg NPase and three animals were examined at 15 minutes, six hours, 7 and 14 days. These rats were included since they were not able to produce antibody to NPase. Control groups of three rats each, given renal perfusion of $^{125}$I-ovalbumin (80 µg, labeled with $10^6$ cpm) were examined at 15 minutes, six hours and seven days.

Estimation of proteinuria

Eighteen-hour urine samples were collected one day before perfusion, then every day after perfusion, centrifuged and the protein content was measured by the biuret method [16] after acid precipitation using human serum albumin as the standard.

Statistical analysis

A KWIKSTAT software program was used. All values are expressed as means ± 1 sd.

Results

Disappearance kinetics of radioactive NPase

Table 1 shows the organ distribution of $^{125}$I-NPase after renal perfusion of 80 µg into naive Wistar rats. Approximately one half of the total radioactive NPase found in the left kidney at 15 minutes (21 µg) was localized in the glomeruli (11 µg). Radioactivity in the left kidney was significantly higher than in the right kidney up to 24 hours. The blood level of $^{125}$I-NPase remained relatively constant until 24 hours, indicating that NPase was still circulating. Under similar experimental conditions, only 0.2 µg (from 80 µg) ovalbumin was found in glomeruli isolated from control rats (Fig. 1). SDS-PAGE autoradiography of isolated glomeruli from left kidneys of rats previously perfused with radioactive NPase (Fig. 2) demonstrated the affinity of NPase to glomerular structures. Both characteristic NPase forms (31/32 kDa bands in SDS-PAGE) [12] showed a significant affinity to glomerular structures.

Immunofluorescence studies

Immunofluorescence findings in the left kidneys of Wistar naive rats are summarized in Table 2. Deposition of IgG and C3 was seen as early as 15 minutes in a diffuse segmental granular pattern in the glomerular capillaries; a weak capillary deposition of NPase was also present. By six hours, IgG and C3 were deposited in a linear pattern along the capillary wall (Fig. 3). After six hours
Table 1. Disappearance kinetics of $^{125}$I-NPase from various organs after renal perfusion

<table>
<thead>
<tr>
<th>Organs</th>
<th>15 min</th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left kidney (perfused)</td>
<td>21.60 ± 6.04</td>
<td>13.09 ± 0.93</td>
<td>5.53 ± 1.88</td>
<td>1.37 ± 0.61</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>Right kidney</td>
<td>1.75 ± 0.48</td>
<td>1.25 ± 0.09</td>
<td>1.01 ± 0.52</td>
<td>0.35 ± 0.05</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>25.90 ± 5.97</td>
<td>24.05 ± 3.48</td>
<td>3.21 ± 0.16</td>
<td>1.90 ± 0.36</td>
<td>0.67 ± 0.13</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.83 ± 0.39</td>
<td>0.99 ± 0.69</td>
<td>0.53 ± 0.07</td>
<td>0.25 ± 0.09</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Lung</td>
<td>1.46 ± 0.14</td>
<td>1.20 ± 0.16</td>
<td>0.17 ± 0.13</td>
<td>0.42 ± 0.06</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>0.23 ± 0.09</td>
<td>0.14 ± 0.06</td>
<td>0.11 ± 0.02</td>
<td>0.09 ± 0.002</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Blood (1 ml)</td>
<td>0.21 ± 0.02</td>
<td>0.99 ± 0.69</td>
<td>0.30 ± 0.08</td>
<td>0.24 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Isolated glomeruli (left kidney)</td>
<td>11.23 ± 5.12</td>
<td>5.92 ± 1.16</td>
<td>3.15 ± 1.38</td>
<td>0.49 ± 0.13</td>
<td>0.16 ± 0.11</td>
</tr>
</tbody>
</table>

Three Wistar rats were used in every group.

* Left kidney was perfused with 80 μg NPase via the left renal artery

b All values of binding of NPase are in μg ± sd

![Fig. 1. Binding of $^{125}$I-labeled Staphylococcal phosphatase to glomeruli of left kidney of naive wistar rat after perfusion of 80 μg (10$^6$ cpm) into left renal artery. Symbols are: (□) phosphatase; (○) ovalbumin.](image)

![Fig. 2. Autoradiography of glomerular eluates after separation on 12.6% SDS-PAGE under reducing conditions. Lane S, $^{125}$I-NPase (10$^6$ cpm); lane M, radioactive labeled molecular weight marker; lane one hour, glomerular eluate of kidney isolated from a Wistar rat one hour after perfusion; lane 15 minutes, glomerular eluate of kidney isolated 15 minutes after intrarenal perfusion. Characteristic double bands of NPase are seen (arrows).](image)

![Table 2. Summary of immunofluorescent findings of glomeruli of left kidneys of rats perfused with 80 μg NPase](image)

- Neutrophils in immunized rats was more significant after 15 minutes. The heparin-treated naive rats and ovalbumin control group showed no neutrophil influx.

**Light microscopy**

A neutrophil influx was evident at 15 minutes, this increased till three hours in germ-free and naive Wistar rats (Fig. 6) and was still visible at day 14 in naive rats. No significant proliferative changes were observed. A similar influx of neutrophils was also visible in the athymic nude rats up to six hours; the influx of neutrophils in immunized rats was more significant after 15 minutes. The heparin-treated naive rats and ovalbumin control group showed no neutrophil influx.

**Electron microscopy**

A neutrophil influx into the glomeruli of the left kidneys of germ-free and naive Wistar rats was seen at 15 minutes, one hour and three hours. An influx of mononuclear cells was sometimes seen in the glomeruli of the six hours and one day groups. Subendothelial electron dense deposits were found in naive Wistar and nude rats from six hours onward.
By day 3 subepithelial electron-dense deposits had appeared in Wistar rats, which was associated with a partial loss of epithelial foot processes (Fig. 7). Thereafter, subepithelial deposits decreased in number, but were still visible at day 14. Weak subepithelial-dense deposits were also visible in nude rats at day 3. More intensive subepithelial deposits were seen in immunized rats. There were no changes in the glomeruli of the heparin-treated naive rats and the ovalbumin control group.

**Proteinuria**

Proteinuria started within 24 hours after perfusion of NPtase in germ-free and naive, immunologically intact rats and was still significant at day 14 in naive rats (Fig. 8). The nude rat group showed a similar level of proteinuria, but only in the early phase (until day 2). The active model (immunized group) showed a higher level of proteinuria than the naive group especially in the late phase (86 ± 0.06 mg/day). Protein excretion in the urine of the heparin-treated naive rats and ovalbumin control group was within the normal limit (< 12 mg/day).

**Discussion**

Recently we characterized a highly cationic protein isolated from the staphylococcal cell surface, a staphylococcal neutral phosphatase [12]. Evidence shows that it possesses an affinity for human and rat immunoglobulin, mainly through the Fab part of the immunoglobulin molecule. An electrostatic interaction seems to be an important factor in the underlying mechanism, as was previously reported for other cationic proteins with affinity for immunoglobulin [10, 11]. It is also well known that cationic antigens and other polycations have a high affinity to polyanionic glomerular structures [17, 18]. This is also the case with NPtase, as demonstrated in a preliminary study [9].

As a native, highly cationic protein with a considerable molecular weight, 65 kDa [12], derived from a pathogenic bacteria NPtase obviously represents an interesting molecule, it not only has affinity for the GBM but it also reveals nephritogenic potential via subsequent binding of circulating immunoglobulins to form IC-like deposits along the GBM. This suggests that there is more than one binding domain on the NPtase molecule that is responsible for simultaneous GBM and immunoglobulin binding.

On testing a large number of rats, all animals showed similar renal lesions after renal perfusion of NPtase in the early stages. After perfusion of a single bolus of NPtase into the left kidney of non-immunized rats, an immediate deposition of immunoglobulins and C3 was seen along the GBM in all animals, including germ-free Wistar rats and immune deficient athymic nude rats. The nude rats showed the same renal lesion as Wistar rats in the early phase, although they are not able to produce specific antibody to NPtase. Heparin perfusion after NPtase inhibited the deposition of IgG and C3 in naive rats but not in active, immunized rats. This result is compatible with in vitro heparin inhibition studies [12]. Heparin, a highly anionic molecule, obviously blocks the cationic binding sites on the NPtase molecule and inhibits the deposition of IgG and the formation of IC-like
structures and prevents proteinuria. These results extend the theoretical basis for the use of heparin in the treatment of some types of glomerulonephritis [19] and indicate that we are dealing, in the early phase of the lesion, with an immunoglobulin-binding mechanism, not simply a phenomena due to fixation of specific antibody onto its target antigen.

Wistar rats immunized with NPtase before perfusion (active model), as could be predicted, showed a much more intensive renal lesion than that seen in the non-immunized naive model, especially during late phase of proteinuria (Fig. 8). In the various models used the levels of proteinuria in later stages were correlated with the level of specific anti-NPtase antibody. The levels increased gradually in late stages after perfusion in non-immunized rats, and were much higher in the active model (data on antibody levels not shown) and absent in nude rats. This supports the importance of a specific antibody for the maintainance of renal injury after triggering of the initial events. The early phase of our model may be important in explaining how an immunological series of events are initially triggered and maintained, until a specific immune response appears. The relatively constant level of NPtase in blood would allow circulating NPtase to stimulate the specific immune response. In a preliminary screening study a number of sera from patients infected with Staphylococcus aureus recognized NPtase (76%, N = 42). In this model we were able to

Fig. 5. Fluorescence micrograph of glomerulus from the left kidney of an immunized Wistar rat seven days after perfusion of 80 μg NPtase. Deposition of IgG (a) and C3 (b) in a diffuse global granular pattern (original magnification ×200).
show the nephrogenicity of NPtase after only a single injection of the antigen. Prolonged exposure to the antigen may be essential for chronicity.

Complement activation is a very important factor in causing renal damage and induction of proteinuria [20, 21]. Immunoglobulin binding proteins, like protein A, can activate the complement system through binding to the Fab portion of the immunoglobulin molecule, which is an alternative binding pathway of protein A
Further studies are required to elucidate the nature of such IC deposits on the GBM.

We did not see any significant proliferative changes in glomeruli by light microscopic examination at any time after perfusion of NPTase. One explanation is that proliferative changes may need repeated, longer exposure to the antigen than that presumed to occur here in a one shot model. However, a modest influx of inflammatory cells was seen in both the early and the late phase, data which speak for inflammatory changes triggered by the IC-like deposition in the early phase and by genuine IC formation in the late phase.

If similar mechanisms are involved in human post-infectious GN, this could explain the fact that antigens could only be detected in glomeruli in a very few cases and only in the early phase after infection, for example in post-streptococcal GN [23].

Methicillin resistant Staphylococcus aureus (MRSA) infection has become a serious problem throughout the world [24]. Furthermore, glomerulonephritis after MRSA infections was recently reported [25]. Moreover, a role for Staphylococcal aureus infection in the pathogenesis of primary glomerulonephritis also has been proposed [1, 2, 26]. Staphylococcus aureus, which has replaced Streptococcus viridans as the predominant etiologic agent of fatal bacterial endocarditis, is reported to be associated with glomerulonephritis, especially in parenteral drug abusers [27-29].

Our model shows how a cationic bacterial protein could play a role in the pathogenesis of infection related GN, and at the same time may represent a new conceptualization of the pathomechanisms involved in some cases of idiopathic ICGN that are preceeded by subclinical bacterial infections.

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