Oxygen-dependent and -independent mechanisms of renal injury in experimental ascending pyelonephritis

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Abstract

Pyelonephritis is the most common urinary tract infection affecting females of all age groups. Despite concerted efforts the mechanism of renal injury in pyelonephritis is not clearly understood. In the present study we have made an attempt to characterise the mediators of inflammatory insult in an experimental model of ascending pyelonephritis. Mice infected with Escherichia coli O6:K13:H1 were sacrificed at 2, 7 and 14 days post-infection. Luminol-dependent chemiluminescence response, NADPH oxidase, acid phosphatase, β-glucuronidase and N-acetyl-β-D-glucosaminidase activities were monitored in circulating as well as renal phagocytic cells in order to determine the role of reactive oxygen species and lysosomal enzymes in genesis of renal injury. We have demonstrated that reactive oxygen species are generated at the initiation of infection and the levels increase progressively during the course of infection. While intracellular release of lysosomal enzymes was seen in all groups, extracellular release was primarily observed at 7 and 14 days post-infection only. The results indicate that while reactive oxygen species play a significant role in tissue injury during all stages of infection, lysosomal enzyme release in extracellular milieu augments tissue destruction at later stages only.

Keywords: Reactive oxygen species; Acid phosphatase; β-Glucuronidase; N-Acetyl-β-D-glucosaminidase; Pyelonephritis

1. Introduction

Pyelonephritis has been defined as a pathological state of kidney, where following bacterial invasion, pelvocalyceal and parenchymal inflammation is seen. It is one of the most common infections affecting females and has been recognised as the major cause of end stage renal failure and death [1].

The pathogenic mechanism of renal inflammatory process leading to interstitial involvement has not been given much attention till date. It has been shown that virulent clones of bacteria are less frequently present in patients with increased renal scarring [2]. More recently, it has been observed in subclinical pyelonephritis, that bacterial invasion was not associated with gross or histologic changes within...
renal parenchyma [3]. Both of these studies indicate that renal injury is not due to bacterial colonisation but perhaps due to inflammatory reaction taking place inside the infected kidneys. It has been demonstrated that in response to rapid increase in bacterial number, a characteristic inflammatory infiltrate containing lymphocytes, mononuclear cells and neutrophils accumulate at the site of infection [4,5]. To kill invading microbes, these cells are equipped with an arsenal of bactericidal molecules involving both oxygen-independent and -dependent mechanisms, characterised by increased generation of reactive oxygen species (ROS), release of intracellular proteins and generation of pro inflammatory mediators [6–8]. There is considerable evidence that ROS and proteases released by phagocytic cells during inflammatory response can contribute directly to tissue damage [9,10].

While contribution of ROS towards renal injury has been well documented [11–13], role of lysosomal enzymes in pyelonephritis has not been given much attention. In the present study, we have tried to find the possible interplay of ROS and lysosomal enzymes in pathogenesis of pyelonephritis.

2. Materials and methods

2.1. Bacterial strain

*Escherichia coli* O6:K13:H1 was procured from Central Research Institute, Kasauli (HP, India). Bacteria were maintained on Brain Heart Infusion stabs. Everytime at least five colonies were biochemically tested to confirm the strain purity.

2.2. Animal model

Femal balb/c mice (12–14 weeks old) weighing 30 ± 5 g were used as animal model. Mice were kept in sterile polypylene cages and fed meat pellet diet and water ad libidum. Mice with sterile urine culture were used for experimentation.

2.3. Induction of pyelonephritis

Ascending pyelonephritis was induced in mice with no bacteriuria by the method described by O’Hanley et al. [14]. Mice were anaesthetised with anaesthetic ether and a sterile French Fogarty’s #2 Catheter (non radiopaque, outer diameter 0.61 mm, Clay Adams, USA) with tip cut off, was inserted via the urethral meatus into the bladder. Bladder was voided with gentle compression of lower abdomen. 10⁸–⁹ *E. coli* cells/100 µl of normal saline was injected. Catheter was removed and no further manipulation of the urinary tract was done. Infection was confirmed with positive urine culture after 24 h. The control mice were catheterised and 100 µl of normal saline was injected. Mice were sacrificed at 2, 7 and 14 days post-infection.

2.4. Isolation of circulating phagocytic cells

Circulating phagocytic cells (monocytes and neutrophils) were obtained by the method of Boyum [15]. Cells from 3 animals were pooled to get sufficient number of cells for each experiment.

2.5. Isolation of renal phagocytic cells

Renal phagocytic cells were isolated by the method of Mann et al. [16]. The procedure has been described in our earlier report [13]. Cells from 3–4 animals were pooled to get sufficient number for each experiment.

2.6. Measurement of luminol-dependent chemiluminescence

1–2 × 10⁶ cells were seeded in polyethylene cuvettes for 30 min at 37°C in 5% CO₂ atmosphere, as described previously [17]. Background counts were taken in Berthold luminometer (BioLumat, LKB 9500C) set at integeration mode at 37°C. 20 µl of luminol was added and counts were taken after 60 s. Cells were then stimulated with latex (20 µl, 0.8 µm diameter. Difco Labs, USA) and counts taken every 60 s, till a peak was achieved. Results are expressed as peak counts min⁻¹ 10⁶ cells.

2.7. NADPH oxidase activity

NADPH oxidase activity was measured by the method of Bhatnagar et al. [18]. 1–2 × 10⁶ cells were suspended in 1 ml of Kreb’s Ringer phosphate
buffer (pH 7.4) and incubated for 30 min at 37°C. Cells were centrifuged at 3000 rpm and pellet was suspended in 1 ml of phosphate buffer (33 mM, pH 5.5). Cells were then lysed with three cycles of freezing/thawing followed by sonication (3 cycles, 5 s each) at 4 mA with 5 s intervals. Clear supernatant was obtained after centrifugation at 2000 rpm for 30 min at 4°C. Protein was estimated by Lowry's method \cite{19} and concentration was adjusted to approximately 70 µg ml⁻¹ of supernatant. Assay was done by mixing 100 µl of sample with 100 µl of NADPH (0.1 mM in KRP buffer, pH 7.4), in total assay volume of 1 ml (made by phosphate buffer) in quartz cuvettes maintained at 25°C. Decrease in the absorbance was recorded at 340 nm for 3 min using double beam spectrophotometer (Uvikon 860). Concentration of NADPH oxidised was calculated by using extinction coefficient of NADPH as 6.22 × 10³ M⁻¹ cm⁻¹. Results have been expressed as µmol of NADPH oxidised (mg protein⁻¹) min⁻¹.

2.8. Measurement of lysosomal enzyme release

Extracellular as well as intracellular release of N-acetyl-β-D-glucosaminidase (NAG), β-glucuronidase (BG) and acid phosphatase was measured in different phagocytic cells during the course of infection. NAG and BG were measured fluorimetrically by the method of Bowers et al. \cite{20}, while AP was measured by colorimetric method described by Baggiolini et al. \cite{21}. Cells (2 × 10⁶ ml⁻¹ of MEM) were incubated for 2 h at 37°C in 5.0% CO₂ and centrifuged at 2000 rpm for 10 min at 4°C. Supernatant was used to measure the extracellular release. Pellet was resuspended in MEM and subjected to repeated cycles of freezing and thawing. Cell lysates were used to estimate the intracellular activity of lysosomal enzymes. Assays of NAG or BG were done by incubating 100 µl of sample with 100 µl of 10 mM 4-methyl umbelliferyl-β-D-acetamido β-glucuronide trihydrate or 10 mM 4-methyl umbelliferyl-β-D-glucuronide (in 0.2 M sodium citrate or 0.2 M sodium acetate buffers, pH 4.5, respectively), for NAG or BG, respectively. After incubation for 60 min at 37°C, reaction was stopped with 3 ml of 50 mM Glycine NaOH buffer (pH 10.4 with 5 mM EDTA). Fluorescence was measured at 365 nm excitation and 460 nm emission. Results have been expressed as (nmol 4-methyl umbelliferrone released) min⁻¹ 10⁶ cells. For acid phosphatase, 100 µl of sample was incubated with 100 µl of p-nitrophenyl phosphate (5 mM in 0.1 M sodium acetate buffer, pH 4.5) for 2 h at 37°C. Reaction was stopped by adding 1 ml of 1 N NaOH and absorbance read at 450 nm. Results have been expressed as (µmol p-nitrophenol released) min⁻¹ 10⁶ cells.

2.9. Statistical analysis

One-way analysis of variance and Student’s t-test was applied using SPSS/PC + version 3.1, a statistical package, wherever required.

3. Results

3.1. Chemiluminescence response

LDCL is widely accepted as a sensitive indicator for ROS generation. Phagocytic cells undergo respiratory burst as they come in contact with stimulating agent. As a result they release various ROS, which emit light when they come down from exited to ground state. Mammalian oxidation produce chemi-

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>528.01 ± 10.97</td>
<td>90.13 ± 4.64</td>
<td>550.35 ± 49.55</td>
<td>212.71 ± 15.31</td>
</tr>
<tr>
<td>2 Days P.I.</td>
<td>509.57 ± 57.00</td>
<td>207.56 ± 8.60</td>
<td>565.36 ± 144.44</td>
<td>693.47 ± 81.86</td>
</tr>
<tr>
<td>7 Days P.I.</td>
<td>5901.7 ± 397.84</td>
<td>673.17 ± 121.10</td>
<td>9095.09 ± 444.99</td>
<td>4823.05 ± 263.10</td>
</tr>
<tr>
<td>14 Days P.I.</td>
<td>6189.56 ± 184.32</td>
<td>2581.81 ± 118.45</td>
<td>9444.44 ± 183.30</td>
<td>5409.26 ± 324.90</td>
</tr>
</tbody>
</table>

Data represent Mean ± S.E. of six different observations. Results are expressed as peak counts/min/10⁶ cells.

a vs. control; b vs. 2 days post-infection (P < 0.001).
luminescence which is < 100 photons $s^{-1}cell^{-1}$, therefore luminol (5 amino, 2,3 dihydro-1,4 phthalalazinedine) is used as chemilumogenic probe for amplification of luminescence to $10^{3-4}$ times by its conversion to pathalate ions. Latex acts as non specific stimulant. Based on this principle, extracellular release of ROS was monitored in various phagocytic cells during the course of infection. LDCL was found to increase significantly as the infection progressed from 2 to 14 days, in all cell populations. There was no appreciable change at 2 days post-infection as compared to LDCL in control mice; however, a sharp and significant rise was observed at 7 days ($P < 0.001$) as compared to control and 2-day infected mice. Inconsequential increase was further observed at 14 days post-infection.

Levels in circulating phagocytic cells were significantly less ($P < 0.001$) as compared to LDCL of renal cells at later stages of infection; however at 2 days, LDCL of only renal neutrophils (RN) was significantly higher ($P < 0.001$) as compared to blood neutrophils (BN) (Table 1).

### 3.2. NADPH oxidase activity

NADPH oxidase is a membrane bound flavoprotein which catalyzes partial reduction of $O_2$ to $O_2^-$. NADPH is favoured over NADH as reducing equivalent by a factor of 10–30 ($K_m$ values for NADPH and NADH are around 30 $\mu$M and 0.5 mM, respectively). Continuous increase in the activity was observed in all cell populations during the course of infection (Table 2). In blood monocytes (BM), no significant alteration was observed at 2 days, however at 7 and 14 days post-infection marked increase was observed as compared to controls ($P < 0.05$, $P < 0.01$ respectively) and 2 days post-infection levels ($P < 0.05$, $P < 0.001$ respectively). Oxidase activity was found to be increased significantly in all infected groups ($P < 0.01$) as compared to controls in BN. In renal macrophages (RM), while no change was observed at 2 days, at 7 days a significant increase was observed ($P < 0.05$) as compared to controls and 2 days post-infection levels. Further, no significant rise in the oxidase activity was observed at 14 days post-infection as compared to oxidase activity at 7 days post-infection. Similar observations were made with RN, where marked increase ($P < 0.001$) was observed at 7 and 14 days post-infection as compared to levels in control and 2-day post-infected mice.

In controls and 2-day post-infected mice, oxidase levels were significantly high ($P < 0.05$) only in renal neutrophils as compared to blood neutrophils.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.21 ± 0.22</td>
<td>0.32 ± 0.06</td>
<td>1.82 ± 0.53</td>
<td>1.28 ± 0.22</td>
</tr>
<tr>
<td>2 Days P.I.</td>
<td>1.54 ± 0.19</td>
<td>0.61 ± 0.02</td>
<td>2.28 ± 0.17</td>
<td>2.15 ± 0.07</td>
</tr>
<tr>
<td>7 Days P.I.</td>
<td>2.46 ± 0.34</td>
<td>1.44 ± 0.46</td>
<td>5.28 ± 0.88</td>
<td>4.86 ± 0.31</td>
</tr>
<tr>
<td>14 Days P.I.</td>
<td>3.12 ± 0.37</td>
<td>1.39 ± 0.47</td>
<td>5.70 ± 0.31</td>
<td>5.90 ± 1.34</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as nmol NADPH oxidised min$^{-1}$ mg$^{-1}$ protein.

* vs. control; † vs. 2 days post infection ($P < 0.05$; * $P < 0.01$; † $P < 0.001$).
Table 4
Extracellular release of β-D-glucuronidase in various phagocytic cells during the course of infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26 ± 0.09</td>
<td>0.24 ± 0.06</td>
<td>0.25 ± 0.11</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td>2 Days P.I.</td>
<td>0.29 ± 0.07</td>
<td>0.28 ± 0.05</td>
<td>0.28 ± 0.07</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>7 Days P.I.</td>
<td>0.36 ± 0.06</td>
<td>0.34 ± 0.13</td>
<td>0.40 ± 0.18</td>
<td>0.42 ± 0.17</td>
</tr>
<tr>
<td>14 Days P.I.</td>
<td>0.41 ± 0.18</td>
<td>0.40 ± 0.16</td>
<td>0.54 ± 0.09</td>
<td>0.49 ± 0.18</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as nmol 4-methylumbelliferone released/10^6 cells ml^{-1}. * vs. control; † vs. 2 days post-infection (P < 0.05; ‡ P < 0.001).

however at later stages of infection, the levels in renal cells were markedly high (P < 0.001) as compared to circulating phagocytic cells.

3.3. β-Glucuronidase activity

A continuous increase in the intracellular release of BG was observed in all cell populations. Significant increase (P < 0.001) was observed only from 7 days post-infection onwards, except in renal neutrophils, where significant increase was observed at 2 days post-infection also (P < 0.05). In BM and RM, levels at 14 days post-infection were also markedly higher (P < 0.01) than at 2 days post-infection. While no significant increase at 14 days post-infection was observed in BN, intracellular release of BG was significantly high (P < 0.01) as compared to 2 days in RN (Table 3).

No significant change in extracellular release (Table 4) was observed during the course of infection in circulating phagocytic cells. However, in RM, extracellular release at 14 days was markedly higher than levels in controls and 2-day post-infected mice (P < 0.001), while in RN, significant increase was observed from 7 days post-infection onwards (P < 0.001 vs. control). Intracellular levels in renal phagocytic cells were also significantly high (P < 0.05) as compared to circulating cells at 7 and 14 days post-infection; however no change in the extracellular release of BG by various cell populations was observed during the course of infection.

3.4. N-acetyl-β-D-glucosaminidase activity

A significant rise in intracellular release of NAG activity in circulating phagocytic cells was observed from 7 days post-infection onwards (P < 0.001), which further increased at 14 days in BM (P < 0.001) and BN (P < 0.01) as compared to levels at 7 days post infection. In RM intracellular release at 2 and 7 days post-infection was significantly higher than controls (P < 0.001), while at 14 days maximum activity was observed (P < 0.001 vs. control and 2-day post-infection levels and P < 0.01 vs. 7-day post-infection levels). In RN, however, a marked increase was observed only at 7 days (P < 0.05 vs. control), which further increased at 14 days (P < 0.001 vs. control and 2 day and P < 0.05 vs. 7 days post-infection). Results are shown in Table 5.

Marked elevation in extracellular release was observed at 7 and 14 days (P < 0.001) as compared to controls in BM, while in RM a significant change (P < 0.001) as compared to extracellular levels in controls was observed only at 14 days post-infection.

Table 5
Intracellular release of N-acetyl-β-D-glucosaminidase in various phagocytic cells during the course of infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.99 ± 0.71</td>
<td>2.91 ± 0.48</td>
<td>4.89 ± 0.57</td>
<td>3.45 ± 0.75</td>
</tr>
<tr>
<td>2 Days P.I.</td>
<td>3.63 ± 0.59</td>
<td>3.22 ± 0.37</td>
<td>6.95 ± 0.63</td>
<td>4.33 ± 0.46</td>
</tr>
<tr>
<td>7 Days P.I.</td>
<td>6.44 ± 0.52</td>
<td>4.93 ± 0.55</td>
<td>7.50 ± 0.47</td>
<td>5.27 ± 0.61</td>
</tr>
<tr>
<td>14 Days P.I.</td>
<td>8.83 ± 0.30</td>
<td>6.53 ± 0.46</td>
<td>8.81 ± 0.13</td>
<td>6.98 ± 0.95</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as nmol 4-methylumbelliferone released/10^6 cells ml^{-1}. * vs. control; † vs. 2 days post-infection; ‡ vs. 7 days post-infection (P < 0.05; ‡ P < 0.01; ‡ P < 0.001).
No alteration in extracellular release was observed in BN and RN (Table 6). No statistical difference in the levels of circulating and renal phagocytic cells was observed.

3.5. Acid phosphatase activity

Unlike BG and NAG, a significant rise in intracellular release of AP was observed from 2 days post-infection onward in circulating as well as renal phagocytic cells ($P < 0.001$). While no significant rise at 7 days was observed in BM and RM, levels were markedly higher in BN ($P < 0.001$) and RN ($P < 0.05$) as compared to 2-day post-infection levels. A further increase at 14 days post-infection was only observed in BM ($P < 0.001$) as compared to levels at 7 days (Table 7).

Extracellular release of AP was found to increase markedly ($P < 0.01$) at 2 days in BM and RM. At 7 days, the extracellular release in circulating phagocytic cells were markedly higher as compared to controls ($P < 0.001$) and 2 days post-infection ($P < 0.001$ for monocytes, $P < 0.05$ for neutrophils), whereas levels in renal phagocytic cells were significantly higher ($P < 0.001$) as compared to controls only. At 14 days, however, the levels were markedly higher than controls ($P < 0.001$) and 2 days ($P < 0.001$) for all cell types except renal neutrophils (Table 8). Intracellular as well as extracellular levels in renal phagocytic cells were significantly higher

### Table 6
Extracellular release of N-Acetyl-β-D-glucosaminidase in various phagocytic cells during the course of infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.16</td>
<td>0.42 ± 0.36</td>
<td>0.46 ± 0.19</td>
<td>0.66 ± 0.17</td>
</tr>
<tr>
<td>2 Days PI</td>
<td>0.39 ± 0.16</td>
<td>0.62 ± 0.36</td>
<td>0.61 ± 0.27</td>
<td>0.73 ± 0.26</td>
</tr>
<tr>
<td>7 Days PI</td>
<td>0.55 ± 0.06</td>
<td>0.72 ± 0.34</td>
<td>0.81 ± 0.33</td>
<td>0.81 ± 0.31</td>
</tr>
<tr>
<td>14 Days PI</td>
<td>0.64 ± 0.13</td>
<td>0.78 ± 0.43</td>
<td>1.09 ± 0.49</td>
<td>0.95 ± 0.18</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as nmol 4-methylumbelliferone released/10⁶ cells ml⁻¹. a vs. control; b vs. 2 days post-infection ($P < 0.001$).

### Table 7
Intracellular release of acid phosphatase in various phagocytic cells during the course of infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.28 ± 0.05</td>
<td>0.29 ± 0.01</td>
<td>0.48 ± 0.06</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>2 Days PI</td>
<td>0.42 ± 0.02</td>
<td>0.33 ± 0.01</td>
<td>0.78 ± 0.05</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>7 Days PI</td>
<td>0.47 ± 0.10</td>
<td>0.68 ± 0.06</td>
<td>0.81 ± 0.12</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td>14 Days PI</td>
<td>0.81 ± 0.10</td>
<td>0.67 ± 0.10</td>
<td>0.94 ± 0.16</td>
<td>0.99 ± 0.13</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as μmol p-nitrophenol released/10⁶ cells ml⁻¹. a vs. control; b vs. 2 days post-infection; c vs. 7 days post-infection ($P < 0.05$; * $P < 0.001$).

### Table 8
Extracellular release of acid phosphatase in various phagocytic cells during the course of infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood monocytes</th>
<th>Blood neutrophils</th>
<th>Renal macrophages</th>
<th>Renal neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14 ± 0.05</td>
<td>0.19 ± 0.01</td>
<td>0.33 ± 0.07</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>2 Days PI</td>
<td>0.26 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.40 ± 0.06</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td>7 Days PI</td>
<td>0.60 ± 0.09</td>
<td>0.43 ± 0.08</td>
<td>0.66 ± 0.21</td>
<td>0.78 ± 0.16</td>
</tr>
<tr>
<td>14 Days PI</td>
<td>0.76 ± 0.40</td>
<td>0.45 ± 0.10</td>
<td>0.72 ± 0.23</td>
<td>0.81 ± 0.23</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. of six different observations. Results are expressed as μmol p-nitrophenol released/10⁶ cells ml⁻¹. a vs. control; b vs. 2 days post-infection ($P < 0.05$; * $P < 0.01$; ** $P < 0.001$).
than circulating cells \( (P < 0.001) \) in controls, 2- and 7-day post-infected mice.

4. Discussion

The role of bacterial properties in the induction of renal scarring has been a matter of debate. The formation of scars during pyelonephritis has been shown to result from the inflammatory tissue reactions which involves infiltration of lymphocytes, mononuclear cells and neutrophils at the site of infection [4,5].

Although the role of ROS in genesis of renal injury in pyelonephritis has been well documented [5,11–13], very few studies are available which have demonstrated the association of lysosomal enzymes with pathology of pyelonephritis. The phagocytic cells have a well defined population of intracellular enzymes which are released from distinct classes of cytoplasmic granules in response to particulate or soluble stimuli [7,22]. The primary (1) granule contains a number of acid hydrolases and neutral proteases as well as myeloperoxidase. These enzymes are discharged into the phagocytic vacuole and are believed to be responsible for microbial killing through the action of various proteases and by generation of \( \text{H}_2\text{O}_2 \) and other ROS [23]. The secondary (2) granule contains lysozyme, lactoferrin, vitamin \( \text{B}_12 \), binding proteins and neutral proteases. These proteins limit chemotaxis and increase neutrophil adhesiveness [24]. Tertiary granules (3) containing NAG, BG etc. have also been recognised as important for microbial killing [22].

Extracellular release of NAG, BG and AP has been linked to tissue injury in end stage renal failure [25]. In pyelonephritis, recently Ivanyi et al. [26] demonstrated increased lysosomal consumption in macrophages containing \( E. \text{coli} \) while Harton et al. [27] observed increased release of NAG from human polymorphonuclear leukocytes stimulated with Tamm Horsfall protein.

In order to characterise the role of ROS and lysosomal enzymes in renal injury seen in pyelonephritis, we studied the extracellular release of ROS and release of lysosomal enzymes inside the phagocytic cells as well as in extracellular medium. Increased LDCL response and NADPH oxidase activity in all infected animals clearly indicate, specific role of ROS in renal injury which we have further documented by increased levels of lipid peroxidation and DNA damage in renal tissue (data not shown). While increased intracellular release of lysosomal enzymes indicates a synergy with ROS in microbial killing, extracellular release of BG, NAG and AP during various stages of infection demonstrate that lysosomal enzymes must be playing a significant role in tissue destruction, in particular, late in the disease progression.

From these observations, we propose that reactive oxygen species are the key mediators of inflammatory insult in pyelonephritis, while lysosomal enzymes have the potential to further augment the renal injury at later stages of acute infection. Therefore, the role of extracellular release of lysosomal enzymes, as critical factor in tissue injury in conjunction with ROS, specially in chronic renal scarring, needs to be carefully and critically investigated in order to have a better understanding of pathogenesis of renal injury in pyelonephritis.

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