Patterns of interstitial inflammation during the evolution of renal injury in experimental aristolochic acid nephropathy

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Abstract

Background. Interstitial inflammation is a prominent feature associated with the severity of renal injury and progressive kidney failure. We utilized an animal model of aristolochic acid (AA)-induced nephropathy (AAN) to assess patterns of infiltration and inflammation during the evolution of tubulointerstitial damage and to relate them to the development of fibrosis.

Methods. Male Wistar rats receiving sc daily AA or vehicle were sacrificed between Days 1 and 35. Infiltrating mononuclear cells were characterized by immunohistochemistry. The kidney infiltrating T lymphocytes were phenotyped by flow cytometry. Urinary levels of Th-1/Th-2 cytokines, of monocyte chemoattractant protein-1 and of active transforming growth factor-beta (TGF-β) were measured. Tissue expression of phosphorylated smad 2/3 protein was detected by immunohistochemistry. The kidney infiltrating T lymphocytes were characterized by flow cytometry. Urinary levels of Th-1/Th-2 cytokines, of monocyte chemoattractant protein-1 and of active transforming growth factor-beta (TGF-β) were measured. Tissue expression of phosphorylated smad 2/3 protein was detected by immunohistochemistry.

Results. In AA rats, monocytes/macrophages and T lymphocytes predominantly infiltrated areas of necrotic proximal tubular cells. The coexpressions of ED1 and/or Ki-67/MHCII by infiltrating cells reflected monocyte/macrophage proliferation and their activation, respectively. The accumulation of cytotoxic T lymphocytes was attested by severe signs of CD8+ cell tubulitis. The CD8/E-cadherin containing confirmed intrarenal homing of CD8+CD103+ cells. Urinary levels of proinflammatory cytokines and of active TGF-β significantly increased at Days 10 and 35. An early and persistent nuclear overexpression of phosphorylated smad 2/3 protein was detected in tubular and interstitial compartments.

Conclusion. An early and massive interstitial inflammation characterized by activated monocytes/macrophages and cytotoxic CD8+CD103+ T lymphocytes is demonstrated for the first time during the progression of experimental AAN. The involvement in an interstitial fibrosis onset of active TGF-β is highly suggested, at least via the psmad2/3 intracellular signalling pathway.

Keywords: aristolochic acid; macrophages; renal fibrosis; T lymphocytes; transforming growth factor-β

Introduction

In chronic kidney disease (CKD), the pathological correlate of clinical progression is the relentless development of interstitial fibrosis, associated with cellular inflammation and local release of various cytokines disturbing structure–function relationships among nephrons [1,2]. Actually, the respective involvement of innate (monocytes/macrophages (Mn/Mφ)) and adaptive (T lymphocytes) immunity seems to be crucial in the onset and persistence of renal inflammation, tubular damage and fibrosis [3–5]. Clinical and experimental data have revealed a great pathophysiological heterogeneity in infiltrating Mn/Mφ [6]. These cells have been involved in the scarring as well as in the healing of tubulointerstitial lesions irrespective of the nature of the original renal insult [4,6,7]. Whereas the tubulointerstitial damage is mediated by immunocompetent cells during CKD of immune origin, the pathogenic role of these cells in non-immune nephropathy is still a matter of debate [3,8]. According to in vitro data, phagocytosis of necrotic cells by macrophages could induce differentiation into antigen-presenting cells, suggesting their role in the stimulation of T lymphocyte response [9]. Necrosis of tubular cells could thus represent an important common event to immune and non-immune renal insults [10,11].

We tested this hypothesis in a rat model of renal interstitial fibrosis induced by aristolochic acid (AA). Aristolochic acid nephropathy (AAN) has now replaced the initial term
Animals. Four-week-old Wistar male rats (Elevage Janvier, Le Genest Saint-Isle, France) were used. All animal procedures were approved by the local Ethic Committee for Animal Care and conducted according to all animal procedures and experimental protocols. In the present study, an immunohistochemical approach was used to characterize interstitial inflammatory mononuclear cells during AA intoxication, to further study the time course of this infiltrate and its relationships with PTEC. Intraparenchymal infiltrating lymphocytes were phenotyped by flow cytometry. Moreover, urinary excretion of Th-1/Th-2 cytokines and active transforming growth factor-β (TGF-β) were measured. Activation of the TGF-β-specific pathway was studied by immunohistochemistry by evaluating nuclear expression of a phosphorylated smad 2/3 transcription factor.

Subjects and methods

Experimental protocols

All animal procedures were approved by the local Ethic Committee for Animal Care and conducted according to National Guidelines for the Care and Use of Laboratory Animals. Four-week-old Wistar male rats (Elevage Janvier, Le Genest Saint-Isle, France) were admitted in the animal care facility of the Faculty of Medicine, Université Libre de Bruxelles (Belgium) and housed in a stable environment, with ad libitum access to water and food.

Experimental protocols were performed as described previously [14] and summarized in Figure 1. Briefly, after 1 week of acclimatization, rats were subcutaneously injected with a solution of AA in polyethylene glycol (10 mg/kg body weight) (AA group) or with the solvent of AA alone (control group). The day before being sacrificed, the animals were placed in metabolic cages for 24-h urine collections. Six rats per group were sacrificed after 3, 7, 10, 14, 18 and 35 days of injection. Flow cytometry analyses were performed at Days 10 and 35 of a similar protocol. The kidneys were perfused with saline and removed aseptically, and intrarenal mononuclear cells were isolated. All variables studied and the materials used were presented in Table 1.

Renal immunohistochemistry

The avidin–biotin complex (ABC) enzyme technology immunostaining was performed as previously detailed [20]. All used reagents were provided from Vector Laboratory (Burlingame, USA). The longitudinal frozen sections (7 μm) and FFPE sections (5 μm) were attached to poly-L-lysine pre-treated slides (Sigma-Aldrich, Bornem, Belgium). Briefly, CD4 immunostaining was performed on the frozen sections. After air-drying the slides were fixed in acetone at 4°C (5 min). The paraaffin from FFPE tissue sections was removed (toluene solution). The sections were rehydrated and immersed in a retrieval solution, sodium citrate buffer (pH 6.0), or EDTA buffer (pH 8.0). The microwave oven technique was used (2–3 × 5 min, 650 W). The Tris-buffered saline solution pH 7.6 (TBS; 0.8% Tris–HCl and 0.9% NaCl both from Sigma-Aldrich) was used for all washing steps (3 × 5 min). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in a methanol solution (30 min). Non-specific protein binding sites (background staining due to Fc receptor) were blocked with 20% horse normal serum (blocking buffer). Endogenous avidin-binding activity was suppressed by sequential 15 min incubation of the section first with avidin followed by biotin. Subsequently, the sections were incubated (overnight, 4°C) with the mouse IgG or IgM monoclonal primary antibody diluted in the blocking buffer (Table 2). The optimal dilutions for each used primary antibodies were previously determined in our laboratory. The slides were incubated with the biotinylated horse-specific anti-mouse secondary antibody (rat adsorbed). The extent of the specifically bound primary antibodies was visualized by means of the avidin–biotin peroxidase complex (ABC) method (a kit from Vector Laboratory). The diaminobenzidine/hydrogen peroxide was used as the chromogen substrate producing a brown end product. Counterstaining with haematoxylin completed the processing. After dehydration through alcohol and toluene the slides were immediately mounted in a permanent (organic) mounting medium (DPX, VWR International, Leuven, Belgium) and covered with a glass coverslip.

In order to enhance visualization of antigens detected by using the rabbit polyclonal primary antibody (Table 2), FFPE tissue sections were immersed for 40 min at 98°C
### Table 1. Variables studied in the rat model of aristolochic acid nephropathy

<table>
<thead>
<tr>
<th>Main activity/functions</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype, activation and homing of mononuclear inflammatory cells</td>
<td></td>
</tr>
<tr>
<td>ED1 Monocytes and tissue macrophages</td>
<td>FFPE</td>
</tr>
<tr>
<td>MHC II Activated macrophages and dendritic cells</td>
<td>FFPE</td>
</tr>
<tr>
<td>Ki-67 Expressed by the nuclei of proliferating cells</td>
<td>FFPE</td>
</tr>
<tr>
<td>CD3 T lymphocytes</td>
<td>FFPE</td>
</tr>
<tr>
<td>CD4 CD4+ cell population</td>
<td>Frozen</td>
</tr>
<tr>
<td>CD45RC CD4+ and CD8+ T lymphocytes</td>
<td>FFPE</td>
</tr>
<tr>
<td>CD8a CD8+ subpopulation of mature T lymphocytes including suppressor/cytotoxic cells</td>
<td>FFPE</td>
</tr>
<tr>
<td>CD45 All haematopoetic cells except erythrocytes</td>
<td>+</td>
</tr>
<tr>
<td>CD62L ?-Selectin; mediates lymphocyte binding to endothelium, shed upon activation</td>
<td>+</td>
</tr>
<tr>
<td>αβ TCR T lymphocyte activation and differentiation</td>
<td>+</td>
</tr>
<tr>
<td>CD103 Ligand to E-cadherin, intraepithelial T-cell homing</td>
<td>+</td>
</tr>
<tr>
<td>Tubular injury</td>
<td></td>
</tr>
<tr>
<td>αGST αGST enzymuria reflecting proximal tubule injury</td>
<td>+</td>
</tr>
<tr>
<td>µGST µGST enzymuria reflecting of loop of Henle and distal tubule injury</td>
<td>+</td>
</tr>
<tr>
<td>Pro-inflammatory and pro-fibrosing cytokines, psmad2/3 signalling pathway</td>
<td></td>
</tr>
<tr>
<td>IL-1α Secreted by activated mononuclear cells; involved in inflammatory and immune responses</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α Produced by Mn/Mφ; modulates immune response and metabolic tissue activity</td>
<td>+</td>
</tr>
<tr>
<td>INF-γ Secreted by cytotoxic/suppressor T and Th1 cells; inhibits Th2 cell proliferation</td>
<td>+</td>
</tr>
<tr>
<td>MCP-1 Secreted by renal residents cells, mononuclear cells and activated fibroblasts, involved in recruitment of inflammatory cells in the areas of tissue injury</td>
<td>+</td>
</tr>
<tr>
<td>IL-4 Secreted by T cells; triggers lymphocyte proliferation, TNF-α, IL-1 and IL-6 inhibition</td>
<td>+</td>
</tr>
<tr>
<td>TGF-β Secreted by activated PTEC, Mn/Mφ, T cells; cell growth regulation and differentiation</td>
<td>+</td>
</tr>
<tr>
<td>Psmad2/3 Phosphorylated cytoplasmic smad2/3 proteins activated in TGF-β primed cells</td>
<td>FFPE</td>
</tr>
</tbody>
</table>

IRL = intrarenal lymphocytes; FFPE = formalin fixed paraffin embedded; MHCII = major histocompatibility complex class II; TCR = T-cell receptor; PTEC = proximal tubular epithelial cells; MCP-1 = monocyte chemoattractant protein-1; TGF-β = transforming growth factor beta; α and µ GST = alpha- and mu-isoforms of glutathione S-transferase.

### Table 2. Primary antibodies used for immunohistochemistry procedures

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Dilution</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avidin–biotin complex visualization system for detection of monoclonal mouse anti-human/rat</td>
<td>ED1</td>
<td>1/300</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>MHC II</td>
<td>OX-6</td>
<td>1/100</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CD45RC</td>
<td>OX-22</td>
<td>1/1000</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CD3</td>
<td>1F4</td>
<td>1/300</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CD4</td>
<td>W3/25</td>
<td>1/100</td>
<td>Biognost, Heule, Belgium</td>
</tr>
<tr>
<td>CD8a</td>
<td>OX-8</td>
<td>1/300</td>
<td>Serotec, Oxford, UK</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-5</td>
<td>1/100</td>
<td>DakoCytomation, Heverlee, Belgium</td>
</tr>
<tr>
<td>NEP</td>
<td>56C6</td>
<td>1/300</td>
<td>Lab Vision, Fremont, CA, USA</td>
</tr>
<tr>
<td>Two-step visualization system of high sensitivity (EnVision System) for detection of polyclonal rabbit anti-human/rat</td>
<td>E-cadherin</td>
<td>H-108</td>
<td>1/1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psmad2/3</td>
<td>Ser 433–435</td>
</tr>
</tbody>
</table>

The heat-induced antigen retrieval technique was used for immunohistochemical immunostaining.

In the retrieval solution (EDTA buffer, pH 9.0). After sequential incubations (30 min), first with primary rabbit polyclonal antibodies and then with anti-rabbit secondary antibody conjugated with EnVision⁺ System, HRP-labelled polymer, the HRP activity was detected as described below. All reagents were provided from DakoCytomation (Heverlee, Belgium).

The specificity of antibodies used was established by the producer. According to the technical data sheet, the specificity of antibodies used for infiltrating inflammatory cell identification (ED-1, CD45RC, CD3, CD8 and CD4) was established by FACs of overexpressing cells. For the antibodies detecting Ki-67, neutral endopeptidase (NEP), E-cadherin and pSmad 2/3, the specificity of antibodies used was established by western blot.

We used two negative controls for all labelling. A horse normal serum (5% solution) instead of the primary antibody (used in order to exclude non-specific staining of kit reagents) and mouse IgG, mouse IgM and rabbit IgG immunoglobulin isotypes (used in order to exclude non-specific staining of immunoglobulins binding sites) both showed no staining.
The antigen heating retrieval procedure was necessary to obtain the specific staining of all used primary antibodies on rat spleen and kidney FFPE sections.

The positive immunoreactivity resulted in brown colouration of the cell membrane for CD3, CD8, CD4, CD45 RC, MHCII and nuclei for Ki-67, psmd 2/3 immunostaining, respectively. The NEP and E-cadherin expression were defined as the membrane immunostaining of tubular epithelial cells.

**Double staining–sequential immunoenzymatic technique**
After completion of ED-1 immunoperoxidase staining with DAB (brown end product), the microwave elution step was achieved in order to denature the first primary antibody and to prevent antibody cross-reaction. A second complete immunoperoxidase Ki-67 or MHC II labelling was performed with DAB/Nickel substrate as a second-indicator system producing a violet colour after 1% eosin solution counterstaining.

**Semiquantitative evaluations**
The methodology of semiquantitative evaluations was validated by an estimation of the reproducibility in both rat groups showing a high rate of concordance (>95%) for intra-assay and inter-assay determinations. Evaluations of all immunostainings were performed by one investigator blind to the origin of the rats (AA versus control groups).

Pattern of ED1 (Mn/Mφ) expression was defined as the cell membrane and cytoplasm stainings. Per field, ED1+ cellular clusters (more than two neighbouring ED1+ cells) were independently counted in the cortex, outer strip of outer medulla (OSOM) and inner stripe of outer medulla (ISOM), with a ×20 magnification lens in 30 fields corresponding to 9.66 mm² of an analysed kidney tissue. Results were expressed as an average of ED1+ clusters per field, according to their distribution (cortex, OSOM and ISOM).

CD3+, CD4+, CD45RC+ and CD8+ immunostainings were defined as cellular membrane stainings. Analyses were performed with a ×40 magnification lens. CD3+ as well as CD4+ cell clusters (more than two neighbouring CD4+ cells), CD45RC+ and CD8+-stained cells were counted in 50 fields in the cortex and outer medulla (corresponded to 3.91 mm² of an analysed tissue area). Results were expressed as an average of positive cells or clusters per field.

**Computer-assisted microscopy evaluation of ED1 immunostaining**
The ED1 expression was quantitated by the Histolab computer-assisted microscope system (Alphélys, Plaisir, France) as previously reported [21]. Per each animal, 400 fields (cortex and outer medulla) corresponding to 17.6 mm² were automatically scanned with a ×20 magnification lens in an optical microscope (Olympus, Aartselaar, Belgium) connected to a video camera (Sony Corporation, Tokyo, Japan) and displayed on a colour monitor. The image analysis software detected and measured firstly the ED1-stained area and secondly the renal tissue area (in mm²). The ED1-stained area was calculated as a percentage of the analysed tissue area.

**Isolation of lymphocytes from rat kidney**
The isolation of lymphocytes was adapted from Hammer [22]. After decapsulation, a half kidney was mechanically disintegrated and incubated in a collagenase IV solution (Sigma-Aldrich). Enzymatic reaction was stopped by HBSS–EDTA (PAA Laboratories, Pasching, Austria). Cell suspensions were filtrated (38 µm), suspended in RPMI 1640 (PAA Laboratories) and isolation was performed with ready-to-use Lymphoprep™ (Axis-Shield, Oslo, Norway). Harvested cells were resuspended in a staining buffer (PBS, 1% BSA at pH 7.4, 0.09% sodium azide). The viability of isolated cells was attested by trypan blue staining. Typically, 2.5 × 10⁶ viable cells were isolated per animal.

**Four-colour immunofluorescent staining of isolated cells**
A cell suspension of freshly isolated mononuclear cells was pre-incubated with a purified mouse anti-rat CD32 monoclonal antibody (FcBlock™, BD Biosciences Pharmingen, Erembodegen, Belgium) followed by incubation with a combination of directly conjugated mouse monoclonal anti-rat antibody (Table 3). Fixed cell samples (4% para-formaldehyde) were analysed in FACS caliber (BD Biosciences). Lymphocytes were gated using forward and side scatter to exclude debris and dead cells; then, 2 × 10⁵ events of CD3+ cells were acquired for analysis. Data were expressed as the percentage of total CD3+ cells. Peripheral blood mononuclear cells were used as positive controls and immunoglobulin isotypes as negative controls for renal mononuclear cells. For each cell population, autofluorescence control (unstained cells) served as baseline fluorescence values for instrument settings.

**Quantitative detection of urinary Th1/Th2 cytokines**
Urinary excretion rates of monocyte chemoattractant protein-1 (MCP-1), interleukin-1α (IL-1α), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-4 (IL-4) were measured in duplicate by a multiple fluorescent bead immunoassay (FlowCytomix kit, Bender
Determination of active TGF-β levels in urine

The active form of TGF-β was determined in urine by its transcriptional ability as prior described [23]. Briefly, urine samples (125 μl) were incubated for 18 h in 1 ml of cells (10^6/ml) stably transfected with a reporter plasmid harbouring a synthetic TGF-β-inducible DNA sequence upstream of the luciferase gene. Luciferase activity was determined using a luciferase kit (Promega, Charbonnières, France) according to the manufacturer’s instructions. Levels of TGF-β were determined using the standard curve established in parallel. The same curve was obtained when TGF-β was diluted in rat urine as previously described for human urine [23]. TGF-β specificity was controlled by incubating positive samples with a neutralizing anti-TGF-β antibody (R&D System, Abingdon, UK) before addition to the cells. The urinary excretion rate of active form of TGF-β was expressed in ng/mmol creatinine. TGF-β levels in urine samples were similar when titred again after 2 or 3 thawings, suggesting that active TGF-β was stable when secreted in urine.

Tubular α- and μ-glutathione S-transferase enzymuria

Urinary levels of α- and μ-glutathione S-transferase (GST) enzymes and creatininuria were determined using specific immunoassay kits (Biotrin International, Dublin, Ireland) as previously detailed [15]. Results were expressed in μg/mmol creatinine.

Statistical analysis

Data from six animals per group were expressed as the median values with both interquartile ranges and min–max values. Analysis of immunostaining quantification results in both AA and control groups consisted in comparing data from each time point to the first corresponding time point using the non-parametric Kruskal–Wallis ANOVA and post hoc Dunn tests. At each time point, data from the AA and control groups were compared by means of the non-parametric U (Mann–Whitney) test. Correlations between different variables were determined using the Spearman rank correlation coefficient. A P-value >0.05 was considered to be significant. Graphs were prepared using the Sigma PLOT® 9.0 program (IMARA bvba, Belgium).

Results

The AA subcutaneous administration during 35 days resulted in renal fibrosis mainly located in the medullary rays (OSOM) where interstitial infiltrating cells (Mn/MΦ and T lymphocytes) were also observed (Figure 2).

Phenotyping and activation of monocytes/macrophages

The medullary rays from AA rats were abundantly infiltrated by Mn/MΦ forming clusters around damaged PTEC (Figure 3A). Typical signs of tubulitis were already seen at Day 3 and persisted until Day 35 of the protocol (Figure 3B–C). Immunostaining of NEP inside Mn/MΦ (ED1+ cells) suggested phagocytosis of necrotic debris from PTEC (Figure 3C). Clearly, we observed two different ED1 immunostaining patterns according to the positively stained cell localization; small intravascular ED1+ cells

Statistical analysis

Data from six animals per group were expressed as the median values with both interquartile ranges and min–max values. Analysis of immunostaining quantification results in both AA and control groups consisted in comparing data from each time point to the first corresponding time point using the non-parametric Kruskal–Wallis ANOVA and post hoc Dunn tests. At each time point, data from the AA and control groups were compared by means of the non-parametric U (Mann–Whitney) test. Correlations between different variables were determined using the Spearman rank correlation coefficient. A P-value >0.05 was considered to be significant. Graphs were prepared using the Sigma PLOT® 9.0 program (IMARA bvba, Belgium).
corresponded to the intravascular monocyte phenotype, whereas large interstitial ED1+ cells exhibited both membrane and cytoplasm staining (corresponding to the lysosomes), which was attributed to the macrophage phenotype with phagocytic activity (Figure 3D–F). These modifications in the ED1 expression pattern were interpreted as the result of transendothelial migration of intravascular monocytes. Of note, perivascular clusters contained ED1+ as well as ED1− cells, reflecting a great heterogeneity in the infiltrating cell populations (Figure 3E). A gradual increase in interstitial Mn/Mφ was confirmed by quantitative evaluation of an anti-ED1-stained tissue area (Figure 3G). From Day 7 to Day 14, Mn/Mφ cell clusters were predominant in the renal cortex and in OSOM (Figure 3H–I). From Day 14 to Day 35, they also accumulated in ISOM (Figure 3J).
Fig. 4. ED1/Ki-67 double staining. (A) Absence of Ki-67/ED1 doubly stained cells in controls (Day 10). (B, C and D) Proliferating (Ki-67/ED1 double-stained cells; arrow) in the peritubular areas of injured PTEC after 3, 7 and 35 days of AA injection. Sections were counterstained with eosin. Original magnification ×1000.

Proliferating macrophages (ED1/Ki-67 double staining) were found mainly at Day 7 to Day 14 in AA rats (Figure 4A–C). As compared to controls, only interstitial cells located around injured PTEC expressed the major histocompatibility complex class II (MHCII) molecule (Figure 5A–B). Semiquantitation of MHCII+ interstitial cell clusters showed a progressive increase from Day 7 to Day 35 (Figure 5C) and closely correlated with Mn/Mϕ infiltration (n = 33, r = 0.718, P < 0.0001). Some Mn/Mϕ expressed MHCII as attested by ED1/MHCII costaining (Figure 5D).
Interstitial inflammation in AA nephropathy

Phenotyping, activation and homing of infiltrating T lymphocytes

As compared to controls (Figure 6A–D), an interstitial accumulation of CD3+, CD4+, CD45RC+ and CD8+ T lymphocytes was observed in AA rats (Figure 6E–L).

Double CD8/NEP immunostainings revealed peritubular localization of infiltrating T lymphocytes limited to the areas of medullary rays (Figure 7A–B). As attested by CD8/Ki-67 costaining, numerous peritubular CD8+ cells proliferated (Figure 7C). Typical CD8+ tubulitis was evident in AA intoxicated rats (Figure 7D–F). The CD8/E-cadherin double staining confirmed the intraepithelial presence of CD8+ lymphocytes mainly around distal tubules (Figure 7G–I).

Positive correlations between ED1 quantitative score and respective semiquantitative scores of CD3+, CD4+, CD45RC+ and CD8+ were found (n = 36, r = 0.70,
Fig. 7. Kidney CD8/NEP, CD8/Ki-67, CD8/E-cadherin double stainings and CD8 with PAS counterstaining. (A) After 10 days of AA injections, CD8+ cells (arrow) were preferentially localized around injured NEP+ PTEC (star). (B) At Day 35, CD8+ cells (arrow) were found predominantly around preserved tubules from S1 and S2 segments (NEP−) rather than around degenerated S3 tubules (dramatically decreased NEP staining) (star). (C) In AA rats, proliferating interstitial CD8+ cells (CD8+/Ki-67+ double staining; arrows) around injured PTEC (star). (D) Controls exhibiting no CD8+ staining. (E) CD8+ cell tubulitis (arrow) observed after 10 days and (F) 35 days of AA injections. (G) Absence of CD8-stained cells around E-cadherin-stained tubules (star) in control. (H) Intraepithelial location of CD8+ cells (arrow) in distal tubules expressing E-cadherin (star) observed in AA rats at Day 10 and (I) at Day 35. Sections were counterstained with Mayer’s haematoxylin or eosin. Original magnifications ×400 (A–B and D–I) and ×1000 (C).

Table 4. Urinary excretion of cytokines and growth factors in controls and AA rat groups

<table>
<thead>
<tr>
<th>Variables (ng/mmol Cr)</th>
<th>Day 10</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>AA rats</td>
</tr>
<tr>
<td>IL-1α</td>
<td>8.35 (4.65–126)</td>
<td>47.9*** (23.7–126)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.19 (0.00–0.38)</td>
<td>0.00 (0.00–12.4)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.08 (0.08–1.01)</td>
<td>0.23 (0.00–2.19)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>119 (84.2–145)</td>
<td>219*** (148–2)</td>
</tr>
<tr>
<td>IL-4</td>
<td>3.56 (0.00–6.00)</td>
<td>9.3 (0.00–16.2)</td>
</tr>
<tr>
<td>Active TGF-β</td>
<td>0.81 (0.00–1.91)</td>
<td>56.1*** (3.20–156)</td>
</tr>
</tbody>
</table>

Cr = creatinine; AA = aristolochic acid.

Data from six rats per group are expressed as median with min–max values and are compared with the non-parametric Mann–Whitney test for each time point: *P < 0.05 and ***P < 0.005.

In AA rats, as compared to controls, flow cytometry analyses of isolated intrarenal lymphocytes demonstrated a stable proportion of CD4+ T lymphocytes at Days 10 and 35 (data not shown). A significantly sustained increase in cytotoxic CD8+ T lymphocytes was found at Day 10 and persisted until Day 35 [(percentage) median (min–max); 59.4 (49.7–63.3) versus 39.2 (29.3–45.6), P = 0.01 and 65.8 (60.6–74.3) versus 44.4 (34.6–59.5), P = 0.01, respectively]. Loss of L-selectin (CD62L) from the surface of intrarenal CD8+ T lymphocytes was observed in parallel with an overexpression of CD103 antigen (αEβ7 integrin) (Figure 8A–B).

Urinary markers of tubular injury

Significantly higher urinary levels [µg/mmol creatinine, median value (min–max)] of α-GST were observed at Days 10 and 35 in AA rats versus controls [114 (77.1–388) versus 28.7 (23.5–36.1), P < 0.05 and 89.6 (64.8–108) versus 23.1 (21.9–28.6), P < 0.05, respectively] whereas the increased µ-GST urinary levels were only found at Day 35 [82 (39.6–118) versus 8.7 (3.2–17.6), P < 0.05].
Interstitial inflammation in AA nephropathy

**Inflammatory and fibrosing cytokines; psmad 2/3 signalling pathway**

In AA intoxicated rats, as compared to controls, urinary excretion rates of monocyte chemoattractant protein-1 (MCP-1), interleukin-1α, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) as well as interleukin-4 and active TGF-β were significantly increased at Day 35 in samples obtained from 24-h urine collections (Table 4). The urinary levels of MCP-1 were almost similar to those obtained from fresh urine samples (data not shown). Considering the remaining cytokines (IL-1, IL-4, TNF-α and INF-γ) on Day 10, very low levels of fluorescent activity (located on the lowest part of the standard curve) were obtained in all samples.

During the acute phase, the nuclear expression of phosphorylated smad 2/3 protein was observed in tubular cells. In contrast, during the chronic phase, psmad 2/3 nuclear staining was mainly present in interstitial cells (Figure 9A–C).

**Discussion**

In the present model, our data showed that the early and transient phase of acute PTEC necrosis induced by AA was associated with a sustained interstitial inflammation. Considering the pejorative evolution of tubular injury to tubular atrophy and interstitial fibrosis present in the chronic phase, inflammatory cells might be the physiopathological link between toxic PTEC injury and renal fibrosis. Such an early infiltration process by immunocompetent cells has probably been missed in patients suffering from advanced chronic AAN. The majority of available histological reports were related to the pre-terminal or terminal stage of AAN [16]. However, some patients presented with acute tubulointerstitial nephritis after a short-term AA intoxication [24,25], which can be related to the early acute phase identified in the present model.

This is the first report of the kinetics of evolution of the inflammatory response in the AAN model. A massive interstitial infiltration by activated Mn/Mφ (innate immunity) and cytotoxic CD8+ T lymphocytes (adaptive immunity) was shown around injured PTEC as soon as 3 days after AA intoxication. The damaged PTEC probably triggered mononuclear cell influx into the interstitium [26,27]. Proinflammatory and profibrosing cytokines and chemokines could contribute to this migration, at least MCP-1 according to our data.

As a consequence of effective phagocytosis of necrotic debris promoting cell repair, Mn/MΦ and their secreted products are known to cause renal damage by many different ways [5,6,28,29]. Only a few of them expressed MHCII molecules (ED1/MHCII costaining) reflecting
a typical profile of classically activated macrophages with pro-inflammatory properties [30]. The specific location of Mn/Mφ around injured PTEC might be related to the ‘innate sense of danger’ hypothesis proposed by Matzinger [31]. Applying this hypothesis to the present AAN model, phagocytosis of necrotic PTEC (ED1/NEP costaining) induced Mn/Mφ maturation and differentiation into antigen-presenting cells (ED1/MHCII costaining) able to prime CD4+ T lymphocytes [9,31]. Correlations found between Mn/Mφ and CD4+ T lymphocytes infiltrates suggested such interactions during AAN progression. In the same line, infiltrating immunocompetent cells as well as the expression of HLA class II antigens have extensively been analysed in several forms of glomerulonephritis in human kidney tissue biopsies, by Markovic-Lipkovsk et al. [32,33]. Expression of HLA class II antigens by mononuclear cells and T lymphocytes was associated with the severity of kidney injury (particularly, in cases of rapidly progressive glomerulonephritis) [32]. These findings pointed out the impact of interstitial cell activation on the clinical course of these immunologically mediated kidney lesions. In the present model of toxic tubulointerstitial insult, the observed overexpression of MHC class II antigen and cytotoxic T lymphocytes actually confirms the respective involvement of innate and adaptive immunity in the pathogenesis of AAN.

Obviously, we had to take into account that the anti-CD4 antibody used in immunohistochemistry studies actually recognized antigen expressed not only by CD4+ T cells but also by Mn/Mφ. Flow cytometry analyses of isolated intrarenal lymphocytes solved this ambiguity and clearly showed that the percentage of CD4+ T lymphocytes remained unchanged, while the proportion of cytotoxic CD8+ T lymphocytes significantly increased during the experimental protocol. Their toxicity towards PTEC was attested by marked tubulitis (Figure 7). Such kidney infiltration by CD8+ T lymphocytes was reported in other models of toxic nephropathy such as adriamycine- and cisplatine-induced nephropathy [34,35]. In the adriamycine model of CKD, Harris and co-workers underlined the pejorative role of CD8+ T cells [36].

However, to our knowledge, the presence of an infiltrating CD8+CD103+ T-effector cell subset is reported here for the first time in a toxic nephropathy model (Figure 8). These cells were identified at Day 35. The αEβ7 integrin (CD103) is known to mediate interactions between T cells and epithelium and is upregulated under specific conditions [37,38]. These conditions were found in the model: proliferating lymphocytes (CD8/Ki67 costaining) and the presence of active TGF-β. A critical role of this T lymphocyte subset was mainly reported in acute and chronic renal allograft dysfunction [38,39]. By acting as a specific ligand to epithelial cell-restricted E-cadherin, CD103 was associated with intrarenal CD8+ lymphocyte homing [38]. The observed decrease in the expression of endothelium adhesion molecules (CD62L; L-selectin) measured at the surface of isolated T lymphocytes from AA rats is in agreement with this process. It is likely that the intraparenchymal presence of CD8+CD103+ T cells promoted early PTEC lysis, contributed to the chronicity of inflammation and development of interstitial fibrosis via additional TGF-β secretion. The distal tubules appeared as a preferential site of T-cell homing, as demonstrated by CD8/E-cadherin double staining. Finally, high levels of active TGF-β detected in urine samples derived from many potential sources: injured PTEC, activated Mn/Mφ, CD103+ CD8+ and CD4+ T lymphocytes. This profibrosing cytokine activates resident fibroblasts into myofibroblasts that represent the main source of extracellular matrix production [40]. According to our data, the active form of TGF-β may be regarded as a key cytokine promoting AA-induced fibrosis at least by psmad2/3 intracellular signalling pathway activation [40–42]. From a physiopathological point of view, as AA is now considered as the main etiological factor of the so-called Balkan endemic nephropathy, it could be of interest to investigate the presence and implication of cellular and humoral inflammatory events during the development of the disease [43,44].

In conclusion, the pattern and time course of inflammatory events occurring in this model of CKD of toxic origin underline the crucial interactions between tubular cells and infiltrating immunocompetent cells. Applying this concept to the understanding of mechanisms of tissue injury in acute and chronic renal diseases should be helpful to design more specific therapeutic strategies reducing the severity of renal inflammation, a prerequisite for reducing interstitial fibrosis.

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