Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes

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Received 5 September 2001; accepted in final form 14 September 2001

Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes. Am J Physiol Renal Physiol 282: F352–F357, 2002; 10.1152/ajprenal.00284.2001.—Ischemia-reperfusion injury (IRI) is a complex and incompletely understood process involving a cascade of events that culminates in apoptotic and/or necrotic cell death. Natural IgM antibodies and complement have been implicated in the pathogenesis of IRI in a variety of organ systems as have T lymphocytes in renal IRI. To investigate the role of Ig and T lymphocytes in renal IRI, recombination-activating gene (RAG)-1-deficient mice were studied. RAG-1(−/−) mice were not protected from acute renal failure induced by 27.5 min of bilateral renal ischemia and subsequent reperfusion (serum urea nitrogen levels 30 h after reperfusion, 155.2 ± 5.6 and 152.8 ± 11.4 mg/dl in RAG-1(−/−) and wild-type mice, respectively; n = 13 each). Histological examination showed acute tubular necrosis and neutrophil infiltration with no significant differences between groups. In contrast with other organ systems, Igs were not found in kidneys at time points ranging from 1 min to 30 h after ischemia. Thus Igs and mature T lymphocytes do not appear to play a significant role in the pathogenesis of IRI in the kidney.

A series of complex events occurs during tissue ischemia and reperfusion including cell-surface expression of intracellular P-selectin as well as antigens that are normally hidden and upregulated expression of a number of proteins such as E-selectin and intercellular adhesion molecule (ICAM)-1 (12, 31). Once reperfusion occurs, the access of inflammatory mediators such as antibodies, complement, and blood cells sets off an inflammatory reaction in which neutrophils and molecular oxygen are key mediators (27, 32). Not surprisingly given its direct exposure to blood-borne inflammatory elements, the endothelial surface appears to be the key cell type in the early events that occur in ischemia-reperfusion injury (IRI), as it produces multiple factors such as adhesion molecules, cytokines, leukotrienes, endothelin, and platelet-activating factor, all of which contribute to the inflammation (31, 36).

An elegant series of experiments performed by Carroll, Hechtman, and colleagues (33, 35) has defined events in skeletal muscle and intestinal IRI. Mice deficient in complement components C3 and C4 as well as in Igs were protected from IRI. Reconstitution of IgM in Ig-deficient mice restored IRI. Taken together, these data indicate that IgM natural antibody-mediated activation of the classical pathway on endothelium is a proximate event in IRI in these two organs.

In renal IRI, tubular cells are also prominently injured, which leads to the pathological picture of acute tubular necrosis. This complicated process involves complement activation on endothelial cells, endothelial cell P- and E-selectin (but not L-selectin) engagement of neutrophils, and an ensuing inflammatory reaction that ultimately leads to necrotic and apoptotic tubular cell death through a yet incompletely characterized mechanisms (8, 12, 25, 31). A role for membrane attack complex C5b-9-mediated tubular injury has also been proposed through the use of mice with both targeted and natural deficiencies of various complement proteins (37). In addition, mice lacking T lymphocytes were also protected from IRI, owing in part to decreased neutrophil influx into the kidneys (23). These data also suggested that T lymphocytes may have direct toxicity to renal tubular epithelia.

To evaluate the roles of Igs and T lymphocytes in renal IRI, we have used mice deficient in recombination-activating gene (RAG)-1. RAG-1 and RAG-2 encode for the lymphocyte-specific V(D)J recombinase (19, 26). As a consequence, RAG-1(−/−) mice have no mature B or T lymphocytes and fail to produce either Ig or T cell receptor proteins. Contrary to our expectations, we show here that these Ig and T lymphocyte-deficient mice are not protected from renal IRI.

METHODS

Mice. All work with animals was approved by the University of Chicago Animal Care and Use Committee and was

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27.5-min period of ischemia, the clamps were released and thereafter leaving the renal veins unoccluded. Cessation of automatic vascular clamp was applied directly to the arteries. After a 30 h of reperfusion. Of the RAG-1(−−) animals, the absence of IgG was confirmed by ELISA. Similarly, all wild-type controls were verified to have IgG in plasma. Microtiter plates were coated overnight with either mouse sera diluted 1:100 in 0.1 M sodium carbonate buffer, pH 9.6, or varied concentrations of a known IgG1 standard (MOPC21, Sigma Aldrich, St. Louis, MO). Wells were blocked with 1% BSA in PBS and washed three times with 0.1% Tween-PBS. Biotinylated goat anti-mouse IgG (Sigma) was added for 30 min at room temperature and wells were washed with 0.1% Tween-PBS. Horse-radish peroxidase-conjugated streptavidin (Calbiochem, San Diego, CA) was then added for 30 min at room temperature. Wells were again washed with 0.1% Tween-PBS, and plates were developed with o-phenylenediamine dihydrochloride (Sigma). The resultant reaction product was quantified at an optical density of 450 nm.

Experimental protocol. One hour before IRI, animals were given 5 U of subcutaneous heparin (9, 14). Mice anesthetized with inhalational isoflurane had baseline blood samples drawn for serum urea nitrogen (SUN) and creatinine determinations before undergoing laparotomy. Core body temperatures were maintained between 37 and 38°C. Both renal arteries were isolated by blunt dissection, and a nontraumatic vascular clamp was applied directly to the arteries thereby leaving the renal veins unoccluded. Cessation of blood flow was documented by visual inspection and by Doppler ultrasound (Koven Technology, St. Louis, MO). After a 27.5-min period of ischemia, the clamps were released and reflow was verified by visual inspection of the kidneys and by Doppler ultrasound. Fluid resuscitation was via 1 ml of normal saline administered subcutaneously after closure of the abdomen.

To study the effects of Ig deficiency on IRI, RAG-1(−−) and wild-type control animals (n = 13 each) were studied. Blood was collected from the retroorbital plexus at 10, 20, and 30 h after ischemia, and SUN and serum creatinine levels were measured using a Beckman Autoanalyzer. Mice were then killed, and both kidneys were obtained for histological studies (see Renal histology).

To examine whether Ig was deposited in the kidneys early in the course of IRI, normal C57BL/6 mice (susceptible to IRI; see DISCUSSION) were studied at time points ranging from 1 to 60 min postreperfusion. As this time frame is too early to discern a rise in SUN or an alteration in histology, kidneys were removed at these time points and studied for Ig and complement deposition by immunofluorescence (IF) microscopy.

Renal histology. Sagittal sections of kidneys were fixed in buffered formalin. Sections (4 μm) were stained with periodic acid-Schiff base. The extent of epithelial necrosis and neutrophil infiltration was graded according to the schema of Kelly and colleagues (12). In this, the percentage of tubules in the outer medulla and corticomedullary junction that had epithelial cell necrosis and/or necrotic debris was estimated, and a score was assigned as follows: 0, none; 1+, <10%; 2+, 10–25%; 3+, 26–75%; and 4+ >75%. The extent of neutrophil infiltration was derived from the estimated mean number of neutrophils per high-power field (×400) in 5–10 consecutive fields from the outer medulla and corticomedullary junction starting at the most-involved area and proceeding in the direction of greatest involvement. Scores were assigned based on these counts as follows: 0, 0–1; 1+, 2–10; 2+, 11–20; 3+, 21–40; and 4+, >40 or too many to count. In some instances where scoring was borderline between two scores, an average value was assigned (e.g., 2.5+). All sections were provided as coded slides so the observer (M. Haas) was blinded as to treatment group and duration of ischemic injury.

IF microscopy. For IF microscopy, 4-μm cryostat sections of frozen tissue were processed for direct microscopy as described previously (21). For Ig staining, a dual-labeling technique was performed in which sections were incubated with heavy-chain-specific fluorescein isothiocyanate-conjugated rabbit anti-mouse IgM (Cappel Laboratories, Durham, NC) and rhodamine-conjugated anti-mouse IgG (Dako, Carpente- ria, CA). C3 deposition was analyzed with fluorescein isothio cyanate-conjugated anti-mouse C3 (Cappel). As a second measure of renal neutrophil infiltration, sections were stained with monoclonal anti-mouse neutrophil antibody 7/4 (Serotec, Oxford, UK) as previously described (22). The number of neutrophils in the corticomedullary junction was counted, and the average from 5–10 high-power fields was recorded for each animal.

![Fig. 1. Renal ischemia-reperfusion injury (IRI) occurs independently from Ig and mature T lymphocytes. Shown are serum urea nitrogen (SUN) values from individual recombination-activating gene (RAG)-1(−−) and wild-type control mice after 27.5 min of ischemia and subsequent 30 h of reperfusion. Of the RAG-1(−−) mice, animals on a mixed B6129 background (○) vs. a C57BL/6 background (●) are indicated. Horizontal lines show group means.](image1)

![Fig. 2. Time course of renal functional impairment in mice subjected to IRI. There was no difference between RAG-1(−−) animals lacking Ig and mature T lymphocytes and wild-type controls.](image2)
Table 1. Histological data in mice subjected to ischemia-reperfusion injury

<table>
<thead>
<tr>
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<th>ENS</th>
<th>NIS</th>
<th>PMNs By IF</th>
<th>C3 by IF</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>3.1 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td>19.6 ± 3.0</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>RAG-1 (−/−)</td>
<td>3.2 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>19.0 ± 2.0</td>
<td>1.2 ± 0.3</td>
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Values are means ± SE; n, no. of mice. ENS, epithelial necrosis score; NIS, neutrophil infiltration score; C3 by IF, C3 staining score by immunofluorescence microscopy. Each of these scores was assigned a score of 0–4 (as defined in Methods). PMNs by IF, number of neutrophils stained with monoclonal antibody 7/4 per high-power field. There were no statistical differences between groups in any of the measures.

Statistics. Statistical analyses were performed with Minitab software (State College, PA). All data are expressed as means ± SE. Two-sample t-testing and one-way ANOVA with subsequent Tukey’s pairwise comparisons were used. Correlations among variables were examined by regression analysis.

RESULTS

Effects of Ig- and T-lymphocyte deficiencies on renal function after IRI. In our hands, renal injury in IRI is dependent on the length of ischemia. In this study, we used 27.5 min of ischemia, which leads to reliable renal failure upon reperfusion and corresponds to conditions used in similar studies of mouse IRI (12, 16, 25, 28). In initial studies, IRI was induced in RAG-1(−/−) mice on a mixed B6129 background (n = 5). All animals developed acute renal failure (SUN 30 h after ischemia, 161.4 ± 6.5 mg/dl). To ensure that the observed findings were not dependent on background strain (3), further studies were done using RAG-1(−/−) mice on a pure C57BL/6 background (n = 8). As with the mixed-background mice, all animals developed acute renal failure (SUN, 151.3 ± 8.2 mg/dl). Figure 1 shows SUN values 30 h after ischemia for individual RAG-1(−/−) animals as well as control C57BL/6 mice in which IRI was induced. SUN values at baseline were no different between groups. Similar results were obtained using serum creatinine as a marker of renal function [serum creatinine at 30 h of reperfusion, 3.2 ± 0.1 and 2.7 ± 0.1 mg/dl in RAG-1(−/−) mice on B6129 and C57BL/6 backgrounds, respectively]. Figure 2 illustrates the course of renal failure in wild-type and RAG-1(−/−) mice. Thus renal IRI occurs independently from the presence of Ig.

Histology. The histological indices of injury measured in this study were epithelial cell necrosis (ENS) and extent of neutrophil infiltration (NIS). As expected, both of these measures were remarkably elevated in all groups of animals subjected to IRI, and there were no differences among any of the groups (Table 1). Figure 3A shows a representative field from a wild-type control animal, and Fig. 3B shows one from a RAG-1(−/−) mouse. SUN levels and the values for ENS and NIS were markedly elevated. As such, there was not a significant correlation between these histological measures and the functional measure of renal failure as assessed by SUN values.

IF findings. There was no IgG or IgM in the vasculature or the tubulointerstitium of wild-type or RAG-1(−/−) mice after 27.5 min of ischemia with subsequent 30 h of reperfusion (data not shown). Complement was activated equally on the basolateral tubular surfaces in the kidneys of both strains with no statistical differences between the two (see Table 1 and Fig. 4). As with the studies by Zhou and colleagues (37) in which C3 was deposited in C4-deficient mice, these data support the concept that any complement activation that takes place in renal IRI must be occurring through the alternative pathway. As with histological estimates of neutrophil infiltration, histochemical enumeration of neutrophils indicated there was marked influx of these inflammatory cells into the kidneys (Fig. 5) with no difference between RAG-1(−/−) and wild-type mice (see Table 1).

The possibility that Ig was deposited at earlier time points after reperfusion was addressed in studies in which wild-type C57BL/6 mice were subjected to ischemia and then kidneys were harvested at time points ranging from 1 to 60 min after reperfusion. As with the later time points, no IgG or IgM was present in renal vessels or tubules. Glomerular IgM was present to a variable degree in mesangial regions of glomeruli (Fig. 5).
6). This is a normal finding in mice and is unlikely to be of pathogenic importance as glomeruli were histologically normal (see Fig. 3A).

**DISCUSSION**

In the last decade, a clear role for the complement system in IRI involving a number of organ systems has emerged. Specifically, inhibiting complement activation lessens IRI in the heart (34), intestine (10), liver (5), skeletal muscle (15, 20), and lung (9). Similarly, preventing normal complement regulation in stomach worsens IRI in that organ (11). As C3, C4, and RAG(-/-) mouse strains were resistant to IRI in skeletal muscle and intestine, a compelling explanation for the involvement of complement in IRI involves natural IgM antibodies binding to antigens newly expressed on endothelia as a result of ischemia; these endothelia-bound IgM antibodies can then lead to subsequent complement activation (33, 35). Given this underlying rationale, it was our expectation that Ig deficiency would attenuate classic pathway activation of complement and thus limit IRI in the kidney. In addition to preventing complement activation through the classic pathway, the absence of IgG would similarly eliminate potential interactions with Fc receptors on infiltrating leukocytes, the presence of which is key in IRI (13, 36). Surprisingly, our results unequivocally show that renal IRI proceeds independently from Ig, as RAG-1(-/-) mice were equally susceptible to IRI as wild-type controls.

Given the finding of intense deposition of IgM in organs subjected to IRI such as skeletal muscle and intestine (33, 35), we investigated whether similar deposition was occurring in kidney. After 30 h of reperfusion, no IgM or IgG deposits were found in kidney, other than for the variable presence of IgG and IgM in the glomerular mesangium, which is a normal finding in unmanipulated wild-type mice (22). As the deposition of natural IgM antibodies to antigens newly expressed during ischemia is likely to occur quickly after reperfusion, the possibility was investigated that the antibody-antigen complexes on the endothelial surfaces were shed (1) before this 30-h time point. Even at times of 1-60 min of reperfusion, no IgG or IgM was found in the kidney, either in the vasculature or in the cells of the tubulointerstitium. Thus by functional and immunohistochemical criteria, Ig does not appear to be playing a role in renal IRI.

The data of Zhou and colleagues (37) implicate the complement system in renal IRI. Their studies using mice deficient in C3, C4, C5, and C6 allowed dissection of the roles of various complement-activation products and showed that C5b-9-mediated tubular cell damage was etiologic in their model. From their results and our studies presented here, classical pathway activation does not appear to be relevant in the kidney, as renal IRI can proceed independently from C4 and Ig. In cultured human umbilical vein endothelial cells made

Fig. 4. Tubular complement deposition was present in animals after ischemia-reperfusion independent of the presence of Ig. Shown is staining for C3 in wild-type (A) and RAG-1(-/-) (B) mice subjected to 27.5 min of ischemia and subsequent 30 h of reperfusion. Arrows depict the basolateral staining for C3 in both groups of animals.

Fig. 5. Marked neutrophil infiltration in IRI as illustrated by immunohistochemical staining with the neutrophil-specific monoclonal antibody 7/4. Shown is a high-power field of the corticomedullary junction from a RAG-1(-/-) mouse subjected to 27.5 min of ischemia and subsequent 30 h of reperfusion.

Fig. 6. IgM deposition at early time points after reperfusion. No specific tubulointerstitial IgM staining was present; glomeruli stained in a mesangial pattern, which is typical of normal mice. For illustration purposes, the figure is slightly overexposed and contains a glomerulus in the center of the field.
hypoxic and subsequently reoxygenated, complement activation occurred. This activation was originally considered to occur through the classic pathway (6), but with the development of more specific reagents it was actually found to be via the lectin pathway (7). However, activation through the lectin pathway also necessitates the involvement of C4, which does not appear to be involved in renal IRI (37). Taken together, if complement activation is truly etiologic in renal IRI, this must be occurring through the alternative pathway. The site of activation could be on the apical surface of tubules (because this site lacks complement regulators (2)) or on the basolateral surface (a site that is clearly susceptible to spontaneous complement activation (17) as is illustrated by the finding of C3 deposits in this location in normal animals (22)).

A recent interesting finding by Rabb and co-workers (23) concerns the role of T lymphocytes in renal IRI. Mice lacking CD4- and CD8-bearing lymphocytes had accelerated recovery from renal IRI. Pathologically, there was less tubular necrosis and neutrophil infiltration. In contrast, we observed no recovery from renal failure nor any difference in histological scores for tubular epithelial cell damage or neutrophil infiltration between RAG-1(-/-) mice lacking mature T lymphocytes and wild-type controls. An explanation for the discrepancy between the two studies is likely related to the distinct proteins targeted to result in the T lymphocyte-deficient phenotype. In the CD4- and CD8-deficient mice, other T lymphocytes are likely to be present such as γδ- and coreceptor-independent T lymphocytes. These may be considerably altered by the induced deficiency of CD4- and CD8-bearing lymphocytes (29). In contrast, the RAG-1(-/-) phenotype is a purer (or, less “leaky”) phenotype for T lymphocyte deficiency. Nonetheless, the contrasting results in the two studies support further investigation into the role of T lymphocytes in renal IRI.

Renal IRI has been examined by a number of groups. From these studies, there are several candidate mediator systems that originate from endothelial, tubular epithelial, and infiltrating inflammatory cells (27, 32). Renal vascular endothelial cells are stimulated in IRI to express adhesion molecules, which results in recruitment and activation of inflammatory cells, particularly neutrophils. In addition to expressing adhesion molecules, endothelial cells also produce phospholipid products such as leukotriene B4 and platelet-activating factor, which can stimulate inflammatory cells (4). Therefore, blockade of platelet-activating factor as well as the adhesion molecules, ICAM-1 (12, 24), P- and E-selectins (28, 31) [but not L-selectin (25)], and β2-integrins (30) all reduce the extent of renal IRI. The resulting situation in which endothelial cells and leukocytes are activated results in production of reactive oxygen species including superoxide (4). Nitric oxide is also clearly involved in IRI, as mice deficient in inducible nitric oxide synthase are protected from renal IRI (16). Tubular epithelial cell alterations in IRI include cell adhesion molecule alterations (38) and cellular death. Although commonly termed acute tubular necrosis, the tubular cell death in renal IRI clearly can proceed through the process of apoptosis (8, 18). Thus renal IRI involves a number of mediator systems; however, our data support that Ig and T lymphocytes are not involved in the pathogenesis of renal IRI.

In summary, here we show that renal IRI can proceed independently of the presence of the RAG-1 protein. Its absence translates into a deficiency of T and B lymphocytes and secreted Ig proteins, none of which appear to be necessary for renal IRI. In addition to susceptibility to renal IRI by functional and morphological criteria, we provide evidence that Ig deposition in the kidney is not a feature at any time after reperfusion. These results are in contrast to other organ systems such as the intestine and skeletal muscle, in which Ig and in particular natural IgM antibodies are key for IRI to occur.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants R01-DK-41873 and R01-DK-55357, and by a Biomedical Sciences Grant from the National Arthritis Foundation. F. Park and P. N. Cunningham were supported by NIDDK Training Grant T32-DK-07510.

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