Intestinal reperfusion injury is mediated by IgM and complement

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Williams, Julian P., Taine T. V. Pechet, Martin R. Weiser, Russell Reid, Les Kobzik, Francis D. Moore, Jr., Michael C. Carroll, and Herbert B. Hechtman. Intestinal reperfusion injury is mediated by IgM and complement. J. Appl. Physiol. 86(3): 938–942, 1999.—Intestinal ischemia-reperfusion injury is dependent on complement. This study examines the role of the alternative and classic pathways of complement and IgM in a murine model of intestinal ischemia-reperfusion. Wild-type animals, mice deficient in complement factor 4 (C4), C3, or Ig, or wild-type mice treated with soluble complement receptor 1 were subjected to 40 min of jejunal ischemia and 3 h of reperfusion. Compared with wild types, knockout and treated mice had significantly reduced intestinal injury, indicated by lowered permeability to radiolabeled albumin. When animals deficient in Ig were reconstituted with IgM, the degree of injury was restored to wild-type levels. Immunohistological staining of intestine for C3 and IgM showed colocalization in the mucosa of wild-type controls and minimal staining for both in the intestine of Ig-deficient and C4-deficient mice. We conclude that intestinal ischemia-reperfusion injury is dependent on the classic complement pathway and IgM.

Three recombinant extramembranous portion of CR1 binds and degrades both complement factor 4b (C4b) and C3b (21). Other work, using transgenic mice deficient in complement components or immunoglobulin to investigate skeletal muscle ischemia and reperfusion, has shown that the injury-related increases in muscle vascular permeability results from activation of the classic pathway via natural antibody (20). Thus mice genetically deficient in C4 or C3 or immunoglobulin were protected from muscle injury. Consonant with these findings are those of Czurco and Nashino (3), who demonstrated in reperfused rat brain the localization of immunoglobulin in the same sites where C3 was deposited. In contrast, work with sCR1 and murine cremaster ischemia-reperfusion has suggested a role for alternative pathway, non-antibody-dependent scheme of complement activation (13). To test the role of complement and immunoglobulin in intestinal reperfusion injury, we examined strains of mice deficient in either C3 or C4 or immunoglobulin in a new murine model of jejunal ischemia-reperfusion.

METHODS

Animals. Mice deficient in either C4 (C4−/−) or C3 (C3−/−) were constructed by one of the authors (M. C. Carroll; 4, 22) by using the technique of homologous recombination in embryonic stem cells. Both strains had undetectable levels of serum C4 or C3, respectively, as measured by ELISA (4, 22). Mice totally deficient in immunoglobulins but with normal levels of complement components, i.e., recombination-activating gene-1-deficient (RAG1−/−) (C57BL/6J — RAG1tm1Mom) and control (F1[B6/129]) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 85–23, Revised 1985).

Animal model. Rommel tourniquets were fashioned from 100-cm loops of 3–0 Prolene suture (Ethicon, Somerville, NJ) and 1-cm lengths of PE-90 Intramedic polyethylene tubing (Becton-Dickinson, Parsippany, NJ), which were used as snuggers. Mice, 6–8 wk old, were anesthetized with intraperitoneal pentobarbital (90 mg/kg). A laparotomy was performed, and the tourniquet was applied to the most proximal 6-cm segment of jejunum. Tourniquets were placed to occlude the mesenteric vessels and jejunal lumen and were secured with a microvascular clippers. Ischemia was confirmed by the lack of pulsation in the mesentery and by intestinal pallor. The laparotomy incision was closed and then was reopened after 40 min. The tourniquet was removed, and reperfusion of the mesenteric vasculature was confirmed by the return of pulsa-
tion to the vascular arcade. The incision was again closed, and all animals were kept anesthetized in a supine position and warm with electric heating pads at 38°C for 3 h of reperfusion. Sham control animals underwent laparotomy without the application of tourniquets. All animals received 1 μCi 125I-labeled bovine serum albumin (in 0.25 ml normal saline) intravenously 5 min before reperfusion. The group treated with sCR1 (T Cell Sciences, Needham, MA) received 20 mg/kg of this complement antagonist intravenously, mixed with the 125I-labeled albumin in 0.25 ml PBS. Those animals reconstituted with IgM received 400 μg intravenously 30 min before the initial laparotomy. Each animal received the same amount of intravenous fluid before reperfusion (0.60 ml in total). All animals received 0.2 ml saline intravenously 1 h after the start of reperfusion. Approximately 0.2–0.5 ml of blood was obtained by cardiac puncture before euthanasia by intraperitoneal pentobarbital injection. The ischemic segment of jejunum was then harvested and washed with 10 ml of saline to remove excess stool. The intestinal segment was then passed through a 0.45-µm syringe filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) and applied to a 2-ml protein G column (GammaBind Sepharose, Pharmacia Biotech, Uppsala, Sweden). After successive rounds of sample binding, washing, and elution (0.1 M glycine-HCl, 0.15 M NaCl, pH 2.5), the protein G flow through was adjusted to 0.5 M NaCl with 10× buffer and applied to a 2-ml anti-IgM agarose column (Sigma Chemical). Elution was achieved with 0.1 M glycine buffer. Protein-containing fractions were pooled, dialyzed, and concentrated and assessed by SDS-PAGE (2) and dialyzed, and concentrated and assessed by SDS-PAGE (2), isotype-specific ELISA (Southern Biotechnologies, Birmingham, AL), and limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) to confirm lack of lipopolysaccharide contamination. IgM was found to be pure and devoid of contaminating IgG, Clq, or lipopolysaccharide.

Reconstitution of antibody-deficient mice. RAG1−/− mice were reconstituted by intravenous administration of 400 μg of purified IgM in 0.35 ml PBS 30 min before initial laparotomy. Serum levels of IgM were measured at harvest by isotype-specific ELISA (Southern Biotechnologies).

Immunohistological analysis. Immunoperoxidase labeling of IgM, IgG, and C3 was performed on paraformaldehyde-fixed cryostat serial sections of intestine by using goat anti-mouse IgM (Sigma Chemical), goat anti-mouse IgG (Sigma Chemical), or goat anti-mouse C3 (5 μg/ml; Organon Teknia, Durham, NC) and a standard avidin-biotin protocol (8). Semi-quantitative estimation of staining intensity was performed by a pathologist (L. Kozib) in a blinded fashion.

Statistics. Groups were subjected to analysis of variance, and, when significance was found, a Student’s t-test with the Bonferroni correction for multiple comparisons was applied.

RESULTS

Reperfusion edema is mediated by the classic pathway of complement (Fig. 1). Reperfusion of ischemic intestine resulted in endothelial cell injury characterized by increased vascular permeability and edema. The intestinal vascular PI of injured wild-type mice F1(B6/129) (n = 12) was 3.26 ± 0.30 compared with 0.70 ± 0.04 in sham animals (n = 5). In contrast, C3−/− (n = 8) animals had a significant reduction in permeability with a 2.25 ± 0.27 (P < 0.05). Thus deficiency in C3, which impairs both classical and alternative pathways of complement, resulted in a 40% reduction in permeability. Similarly, sCR1 (n = 5), which binds and degrades both C3b and C4b, thus inhibiting the alternative as well as the classical pathway, led to a 66% reduction in permeability, with a PI of 1.58 ± 0.19 (P < 0.05). C4−/− mice (n = 8), which have an intact alternative pathway but cannot form the classical pathway C3 convertase (C2aC4b), were also protected to a degree similar to that for C3−/− mice, with a PI of 2.20 ± 0.28, a 42% reduction (P < 0.05). Thus the permeability increases caused by ischemia-reperfusion are mediated by activation of the classical and not the alternative pathway.

Classic complement activation is dependent on IgM (Fig. 2). RAG1−/− mice (n = 4), deficient in IgM and IgG but with normal levels of complement, were protected from changes in permeability with a 75% reduction in PI of 1.34 ± 0.20 (P < 0.05). RAG1−/− mice reconstituted with IgM (n = 5) had injuries similar to those in control mice with a PI of 2.86 ± 0.13 (Fig. 2). Levels of IgM after reconstitution were 468 ± 7 μg/ml compared with levels in normal mice of 437.5 ± 5 μg/ml as measured by isotype-specific ELISA. RAG1−/− unreconstituted mice did not have detectable levels of IgM.
Immunohistochemistry. Macroscopic examination showed marked differences in appearance of the ischemic segments of bowel, with more evidence of infarction and hemorrhage in control animals compared with those treated with sCR1. There was also an apparent, although less marked, reduction in injury in the complement-deficient animals compared with control rats. Histologically, all specimens were graded 0–5 according to the system of Chiu et al. (1), where 0 corresponded to normal mucosal villi and 5 corresponded to digestion and disintegration of lamina propria with hemorrhage and ulceration. Those animals with less severe injury (scored in a blinded fashion by L. Kobzik) corresponded to those with reduced PI (correlation coefficient of 0.92, Table 1). Sections of reperfused and sham control intestine were stained for mouse IgM. IgM was deposited on mucosa from reperfused mice but was absent from sham controls (Fig. 3, A and E, respectively). Thus IgM binds to reperfused tissue. Immunostaining of intestine for C3 demonstrated colocalization with IgM on mucosa of reperfused complement-sufficient mice but was absent from sections of intestine from sham animals (Fig. 3, B and F, respectively).

### Table 1. Intestinal vascular permeability and microscopic injury modified by IgM and complement

<table>
<thead>
<tr>
<th>Groups</th>
<th>Permeability Index</th>
<th>Injury Score</th>
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<tr>
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<tr>
<td><strong>Complement</strong></td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>3.26 ± 0.22</td>
<td>3.75 ± 0.18</td>
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<tr>
<td>Wild-type + sCR1</td>
<td>1.58 ± 0.19</td>
<td>1.80 ± 0.20</td>
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<tr>
<td>C3−/−</td>
<td>2.27 ± 0.19</td>
<td>2.50 ± 0.27</td>
</tr>
<tr>
<td>C4−/−</td>
<td>2.20 ± 0.28</td>
<td>2.71 ± 0.20</td>
</tr>
<tr>
<td>Sham</td>
<td>0.65 ± 0.03</td>
<td>0.20 ± 0.20</td>
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<tr>
<td><strong>IgM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.50 ± 0.30</td>
<td>3.88 ± 0.23</td>
</tr>
<tr>
<td>RAG1 wild-type</td>
<td>1.34 ± 0.20</td>
<td>1.75 ± 0.25</td>
</tr>
<tr>
<td>RAG1 + IgM</td>
<td>2.86 ± 0.13</td>
<td>3.80 ± 0.20</td>
</tr>
<tr>
<td>Sham</td>
<td>0.65 ± 0.30</td>
<td>0.20 ± 0.20</td>
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Values are means ± SD. C3−/−, C3 knockout; sCR1, soluble complement receptor 1; RAG1, recombination-activating gene-1; C4−/−, C4 knockout.
tively). Reperfused intestine from C4- and C3-deficient animals showed less staining for IgM and minimal staining for C3 compared with control-complement-sufficient mice (Fig. 3, C and D). Similarly, reperfused intestine from RAG1−/− animals had minimal staining for C3 and IgM (Fig. 4, A and B), whereas RAG1−/− animals reconstituted with IgM showed increased staining and colocalization of C3 with IgM (Fig. 4, C and D). Staining for IgG deposition was minimal in all groups.

**DISCUSSION**

The dependence of intestinal ischemia-reperfusion injury on complement is confirmed in these studies, with significant reduction in injury in animals treated with sCR1. This is consistent with previous reports from several laboratories that have looked at amelioration of injury in rat intestine (7), cardiac muscle (21), and skeletal muscle (13) by prevention of complement activation. The observation that C4-deficient mice are protected from injury to a degree similar to that for C3-deficient mice supports the thesis that this injury is mediated by the classic pathway of complement. The primary mechanism for activation is fixation of serum antibody to antigen. Animals deficient in immunoglobulin but sufficient in complement components were protected from permeability changes associated with intestinal ischemia-reperfusion. This is similar to our findings in hindlimb ischemia and reperfusion (20). Further similarity is the observation that IgM but not IgG localized to the reperfused, injured endothelium. In the present study, reconstitution of immunoglobulin-deficient animals with IgM completely restored the change in intestinal permeability to levels seen in wild-type animals undergoing ischemia. These results indicate that fixation of natural IgM antibody, normally present in mouse serum, is responsible for the complement activation that then leads to jejunal injury and increased vascular permeability.

Antibody triggers inflammation by both cellular and humoral mechanisms. Binding of C1q to antigen bound antibody initiates the humoral classic cascade, whereas binding of antibody to leukocyte Fc receptors initiates the cellular response. IgG and IgE but not IgM have been shown to initiate the cellular mechanism (14). Thus, after jejunal ischemia, in the absence of complement, binding of IgM would not be expected to result in an inflammatory reaction characterized by vascular leakage. This is confirmed in the present study, in which complement-deficient animals have reduced levels of intestinal vascular permeability after reperfusion, despite normal levels of immunoglobulin. Conversely, those animals deficient in immunoglobulin have reduced vascular permeability after reperfusion, despite normal levels of complement.

The mechanism by which reperfused tissue acquires IgM-binding capacity and subsequent complement deposition is not known. Hypoxia is known to result in cell synthesis of membrane-associated proteins (11), which may function as antigens for natural antibody. Furthermore, natural antibodies are known to circulate in normal individuals and are often implicated in autoimmune vasculitis (15). Anti-endothelial cell antibodies are found in a variety of human diseases as well as in normal sera (9, 12, 17). A mechanism of injury that starts with neoantigen exposure, allowing IgM binding followed by complement activation by the classic pathway, would be expected to demonstrate control levels of IgM binding to reperfused tissue in C4-deficient animals. However, levels of IgM staining in reperfused C4-deficient animals were reduced compared with those in complement-sufficient animals. This may be because the amount of IgM binding reflects the overall injury and neoantigen exposure, which are greater in the complement-sufficient mouse.

Binding of natural antibody to injured endothelial cells could allow the initiation of the complement cascade via the classic pathway. Activation of complement may mediate local injury by several routes. Integration of the terminal components of complement C5b-C9 in the membranes of vascular endothelium may lead to ionic flux through the pore. In addition, release of anaphylatoxins C3a and C5a induces vascular leakage and enhances infiltration and activation of neutrophils. Adhesion and activation of neutrophils are also promoted by covalent deposition of iC3b at the site of reperfusion (10). Our prior studies conducted in rats have shown the intestinal injury to be relatively independent of neutrophils (18). However, in the present mouse study, which is a different model, it is possible that neutrophil-mediated injury is occurring. This might explain the residual vascular leakage in the C3- and C4-deficient mice.

These data indicate that the local inflammatory injury after intestinal ischemia is largely dependent on activation of the classic complement pathway. The initiating event may be a reperfusion-induced alteration of the endothelial membrane, allowing a neoantigen to be expressed. Thereafter, IgM natural antibody binds to this antigen, thereby triggering injury via the classic complement cascade.

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