Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM

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**Background.** Murine hindlimb reperfusion injury (I/R), is initiated by activation of the classical pathway of complement. Complement receptor-2 knockout mice (Cr2−/−) are protected from I/R injury due to defective B-1 cells with a resulting deficient natural immunoglobulin M (IgM) repertoire. Cr2−/− and wild type (WT) mice were studied to isolate the antibody or antibodies responsible for initiation of I/R.

**Methods.** IgM-secreting B-1 cell clones were produced with hybridoma technology from WT cells. Of 21 clones tested in murine I/R models, only 1 clone, CM22, was found to restore injury in protected mice. Cr2−/− mice reconstituted with IgM from individual clones, WT serum, or saline were subjected to 2 hours hindlimb ischemia and 3 hours reperfusion and compared with WT.

**Results.** Muscle injury in Cr2−/− mice reconstituted with CM22 was similar to injury in WT mice reconstituted with saline and Cr2−/− mice reconstituted with WT serum. This injury was 137% greater (P < .05) than in both Cr2−/− mice reconstituted with saline and those reconstituted with a different IgM clone, CM31. IgM and C3 deposition was found only on injured muscle of WT mice or Cr2−/− mice reconstituted with CM22 or WT serum.

**Conclusion.** A single clone of self-reactive IgM, CM22, can initiate complement-dependent I/R injury. (Surgery 2004;136:401-6.)

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**Ischemia/Reperfusion** (I/R) injury occurs after an ischemic event and subsequent restoration of blood flow. The majority of this injury is caused by the inflammatory response to a hypoxic insult. I/R often marks and defines the severity of numerous clinical events, such as cardiovascular ischemia, intestinal ischemia, and stroke. It also profoundly influences the outcome and morbidity of vascular, transplant, plastic, and trauma surgery.

Although many different inflammatory mediators have been implicated in I/R, we and others have championed the pivotal role of the complement system. The observation that complement components are deposited on reperfused tissue preceded attempts to abrogate injury by blocking specific components of this innate inflammatory system. Pretreatment of rodents with sCR1, a potent global inhibitor of complement, resulted in profound protection from I/R injury in myocardial, intestinal, and skeletal muscle ischemia.

By further studying mice genetically deficient in complement components, we have more specifically interpreted the role of complement. We found that knockout mice deficient in C4 were as protected from local injury as mice deficient in C3 in the hindlimb and intestinal model of I/R; all complement-deficient groups were protected from injury similarly to sCR1-treated wild type (WT) animals. These experiments demonstrate that the dependence on complement is via the classical or lectin pathways.

Others have suggested that this classical pathway activation could be an antibody-independent process and that intracellular moieties exposed by ischemia may bind C1q directly. However, recombinant activating gene-1-deficient (RAG- 1−/−) mice deficient in immunoglobulin M (IgM) were protected from intestinal I/R injury, whereas RAG-1−/− animals reconstituted with pooled WT IgM had...
a pronounced reduction in injury in models of intestinal I/R. This protection is due to a lack of specific IgM because in vivo reconstitution of either strain with WT IgM or peritoneal engraftment with an enriched, congenic fraction of B-1 cells restores injury. RAG-1-/- mice reconstituted with IgM from Cr2-/- mice, however, remained protected from intestinal I/R injury although their littermates receiving WT IgM were not protected. Thus, these studies demonstrated that initiation of an I/R injury was not an inherent property of all IgM but suggested that it was specific to B-1 cell IgM product.

In order to isolate an I/R-specific natural IgM-producing cell or cells, hybridomas were constructed from B-1-enriched peritoneal cells. Resultant IgM was screened in vivo for reconstitution of injury in our antibody-deficient model of I/R protection, using RAG-1-/- mice. A single IgM-producing clone, CM22, was identified that restored significant I/R injury. In this study we confirm that a single clone, CM22, can activate the classical pathway-mediated induction of I/R injury in antibody-sufficient animals that are otherwise protected from I/R injury.

METHODS

Animals. The construction and generation of Cr2-/- mice was achieved through embryonic stem cell gene targeting as described. To verify deficiency, we tested the parents of all mice by Southern blot analysis of tail DNA. Male mice 8-12 weeks old were used in all I/R experiments. WT control mice represent C57BL-6 Cr2+/+ littermates generated by crossing C57BL-6 Cr2 +/- heterozygotes. C57BL-6 were purchased from the Jackson Laboratories (Bar Harbor, Me) and bred under specific pathogen-free conditions. Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Department of Health, Education and Human Services, Publication no. 85-23 [National Institute of Health], revised 1985).

B-1 cell hybridomas. To generate hybridomas, peritoneal cells, which are enriched in B-1 cells, were obtained via peritoneal lavage of 8-week-old to 12-week-old C57BL-6 mice. Recovered cells were pooled, washed, and activated with lipopolysaccharide (LPS) overnight, then fused with S/P 20 myeloma cells followed by selection in HAT medium (Cell Essential, Cambridge Mass). IgM-producing restoration of intestinal injury. This evidence effectively eliminated the lectin pathway as the primary pathway (although amplification of the initial activation by any of the pathways is possible) and confirmed that no novel process of classical pathway activation was involved.

The classical pathway is most potently activated by IgM bound to antigen. This led us to conclude that I/R might be activated by recognition and binding of preexisting natural IgM to neoantigen expressed by hypoxic cells. Natural IgM is well known to be a first line of defense against foreign pathogens. Recent work has demonstrated that natural IgM may play an active role in autoreactivity. B-1 cells that reside primarily within the peritoneal and pleural cavities of humans, mice, and other animals represent a major source of preexisting or natural antibodies. They are distinguished from conventional B-2 cells by their restricted germline repertoire, their surface receptors, and their capacity for self-renewal. Recent I/R work has supported the suggestion that these B-1 lymphocytes are a source of I/R-activating IgM. Cr2-/- mice are deficient in both complement receptors 1 and 2, which are important for lymphocyte differentiation. Two different laboratories, studying 2 different strains of Cr2-/- mice, have shown that both strains have normal serum concentrations of IgM. One strain demonstrated a reduction in B-1 cell number when compared with WT, and this is presumed to be the case in the other strain. Both strains have
hybridomas were screened by enzyme-linked immunosorbent assay. Subcloning of hybridomas was carried out by limiting dilution, and clonality was confirmed by sequencing the VDJ regions of Ig. IgM from hybridoma supernatant or WT mouse serum (Accurate Chemical, Westbury, NY) was isolated as described. The pentameric conformation of IgM was assessed by analysis on nonreducing SDS PAGE compared with a high molecular weight marker (Amersham Pharmacia, Piscataway, NJ). Analysis of both cell surface markers and partial sequence confirmed these to be of B-1 cell origin.

**Screening of B-1 clones for activity.** Among an initial set of 80 viable hybridomas, 21 secreted appreciable levels of IgM. Immunoglobulin was purified from the supernatant of each clone, and equal amounts were combined into a single pool for in vivo analysis using an injury-protected intestinal I/R model using antibody-deficient mice, as previously described. Antibody-deficient RAG-1−/− mice were reconstituted with 0.5 mg of pooled IgM before surgery. Intestinal permeability index of treated mice indicated that the total pool of hybridoma IgM could restore injury comparable to serum IgM. After 4 rounds of subsequent IgM pool division (50 μg IgM/clone in each pool), a single hybridoma clone, CM22, was identified that when injected, alone restored injury based on increased vascular permeability. The remaining clones, including CM31, had no activity.

**Hindlimb ischemia reperfusion.** Male mice 8 weeks old to 12 weeks old were anesthetized with intraperitoneal pentobarbital (90 mg/kg), and subjected to 2 hours of hindlimb ischemia followed by 3 hours of reperfusion, as previously described. Upon complete sedation, the hindlimbs were elevated for 2 minutes to minimize retained blood, and then bilateral rubber bands (Latex O-Rings; Miltex Instruments, York, Pa) were applied above the greater trochanter, using the McGivney Hemorrhoidal Ligator (Miltex). Sham mice did not undergo band placement. Hydration was maintained by intravenous infusion of 0.1 ml of 0.9% saline during each hour of reperfusion. Mice were maintained in a supine

![Fig 2. Histology of gastrocnemius muscle harvested after 2 hours of ischemia and 3 hours of reperfusion. A, Cr2−/− mice reconstituted with NS demonstrate minimally detectable injury comparable to D, Cr2−/− mice reconstituted with CM31. B, Cr2−/− reconstituted with CM22 are severely injured and comparable to E, Cr2−/− mice reconstituted with WT IgM, and F, WT mice injected with NS. C, WT sham demonstrates normal muscle morphology. Masson Trichrome stain, original magnification ×200.](image-url)
position and kept anesthetized by intermittent intraperitoneal injections. They were covered throughout the experiment to maintain body temperature. After the mice were humanely killed by anesthetic overdose, muscle was harvested from both hindlimbs. All animal groups had an n = 5.

**Histology and histochemistry of hindlimb skeletal muscle.** Tissues from each animal were fixed for 8 hours in 4% paraformaldehyde, dehydrated, embedded in JB-4 glycolmethacrylate, sectioned at 1.5-micron thickness, and picked up on coated glass slides for staining. After fixation of the intact hindlimb, two 1 cm longitudinal and two 0.5 cm cross-sectional portions of gastrocnemius muscle were harvested. For histologic assessment of muscle fibers, sections were stained with Masson Trichrome. Fifty individual muscle fibers were counted in each histologic section, and the injury was reported as the number of damaged fibers per 50 fibers counted. Reviewers were blinded to specimen type. All groups were subjected to analysis of variance followed by the Student t test with Bonferroni adjustment for multiple comparisons.

**Immunohistologic analysis.** Labeling of IgM and C3 was performed on paraformaldehyde-fixed cryostat serial sections of hindlimb muscle using goat antimouse IgM (Sigma Chemical Company, St. Louis, Mo) and goat antimouse C3 (5 μg/ml) (Organon Teknika, Durham, NC) and a standard avidin-biotin protocol.21

**RESULTS**

Injury score in Cr2−/− mice reconstituted with clone CM22 (28 ± 4) was similar to injury score in B6 mice reconstituted with saline (30 ± 6) and Cr2−/− mice reconstituted with B6 serum (37 ± 3) (Figs 1 & 2). This injury score was 137% greater (P < .05) than either Cr2−/− mice reconstituted with saline (12 ± 4) or those reconstituted with a different IgM clone, CM31 (12 ± 1), a clone that was also inactive in the mesenteric I/R screening assessment.

To assess whether there was also the expected classical pathway of complement activation in this model, immunoperoxidase staining was performed on all harvested muscle (Figs 3 & 4). IgM and C3 deposition was colocalized and was found only on injured muscle fibers of WT mice (Fig 3, G, J) or Cr2−/− mice reconstituted with B6 serum (37 ± 3) (Figs 1 & 2). The injury score was 137% greater (P < .05) than either Cr2−/− mice reconstituted with saline (12 ± 4) or those reconstituted with a different IgM clone, CM31 (12 ± 1), a clone that was also inactive in the mesenteric I/R screening assessment.

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**Fig 3.** G, J, Deposition of IgM and C3 on injured muscle is seen in WT mice. H, K, WT sham animals do not stain, nor do Cr2−/− mice injected with NS (I, L). Immunoperoxidase labeling of IgM and C3 was performed on paraformaldehyde-fixed cryostat serial sections of hindlimb muscle using goat antimouse IgM and C3 (5 μg/ml) and a standard avidin-biotin protocol (G-I, anti-IgM; J-L, anti-C3). Results were similar in 3 mice per group.
results are consistent with our hypothesis that CM22 specifically binds to ischemic tissue and activates the classical pathway of complement.

DISCUSSION

Previous experiments confirm that complement-mediated murine reperfusion injury is initiated by specific natural IgM and that B-1 cells are a potential source. The most recent reports have been based on experiments with Cr2−/− mice, which have a specific defect in B-1 lymphocytes and a resulting failure to develop a full repertoire of antigen-binding specificities for natural IgM. These experiments also suggested that not all natural IgM species were capable of initiating reperfusion injury. This observation in turn indicated that the initiation of reperfusion injury might be caused by binding of specific natural IgM species to specific cell surface ischemia-induced or injury-induced antigens.

In order to test whether specific natural IgM species were responsible for reperfusion injury, we developed IgM-producing hybridoma clones from peritoneal B cells. The peritoneal and chest cavities are the primary anatomic sites where B-1 cells are located and represent a self-replicating population. In the present study we have found that IgM produced by a single B-1 cell hybridoma clone, CM22, was capable of restoring reperfusion injury in Cr2−/− mice in a hindlimb I/R model. In addition, the injury to hindlimb appeared to be identical to that seen in WT animals or in Cr2−/− animals reconstituted with WT serum. The degree of injury was similar, as was the pattern of injury and the pattern of C3 and IgM deposition on injured muscle fibers. Furthermore, injury was not restored with a different B-1-cell-derived IgM, CM31. Thus, the property of initiating reperfusion injury in the hindlimb is not shared by all natural IgMs. Finally, the active IgM clone, CM22, was identified for testing after we screened for the ability of multiple clones to cause reperfusion-induced tissue edema in an entirely different tissue, the gut. In hindlimb I/R, this antibody clearly causes tissue disruption in addition to edema. These data support the hypothesis that self-reactive natural IgMs might interact with neoantigens common to damaged tissue. The IgM-antigen complex on the injured cell surface then initiates I/R injury by activating the classical pathway of complement as the first step.

Fig 4. M,P, Cr2−/− reconstituted with WT IgM has deposition of IgM and C3 comparable to Cr2−/− reconstituted with active clone CM22 (N,Q). O,R, mice reconstituted with another clone, CM31, had no significant deposition of IgM or C3. Results were similar in 3 mice per group.
We have tested only 21 IgM-producing clones in murine models of I/R. The “library” of natural IgM specificities would be expected to be much greater than 21 antigens. Also, natural IgM antibodies are known to be polyreactive. It is possible that there are other active IgM clones of differing specificity or that CM22 binds to more than 1 ischemia neoantigen. It is also possible that engagement of only 1 of many neoantigens by IgM is capable of producing the entire range of injury but that all IgMs binding to any neoantigens have to be blocked to prevent injury. Thus, the identification of a single active clone may in the end lead to the discovery of significant redundancy in this mechanism that renders blockade difficult. Yet the simultaneous identification of an inactive clone, CM31, means that this injurious property of natural IgM must be antigen dependent. That CM22 also seems to have activity in gut I/R in turn suggests the presence of ischemia neoantigens that are common to different tissues and therefore more likely to be restricted in number.

The identification of CM22 as a self-reactive antibody should greatly assist in the search for such neoantigens.

REFERENCES