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Anti-Phospholipid Antibodies Restore Mesenteric Ischemia/Reperfusion-Induced Injury in Complement Receptor 2/Complement Receptor 1-Deficient Mice

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Complement receptor 2-deficient (Cr2−/−) mice are resistant to mesenteric ischemia/reperfusion (I/R) injury because they lack a component of the natural Ab repertoire. Neither the nature of the Abs that are involved in I/R injury nor the composition of the target Ag, to which recognition is lacking in Cr2−/− mice, is known. Because anti-phospholipid Abs have been shown to mediate fetal growth retardation and loss when injected into pregnant mice, we performed experiments to determine whether anti-phospholipid Abs can also reconstitute I/R injury and, therefore, represent members of the injury-inducing repertoire that is missing in Cr2−/− mice. We demonstrate that both murine and human monoclonal and polyclonal Abs against negatively charged phospholipids can reconstitute mesenteric I/R-induced intestinal and lung tissue damage in Cr2−/− mice. In addition, Abs against β2 glycoprotein I restore local and remote tissue damage in the Cr2−/− mice. Unlike Cr2−/− mice, reconstitution of I/R tissue damage in the injury-resistant Rag-1−/− mouse required the infusion of both anti-β2-glycoprotein I and anti-phospholipid Ab. We conclude that anti-phospholipid Abs can bind to tissues subjected to I/R insult and mediate tissue damage. The Journal of Immunology, 2004, 173: 7055–7061.

Tissue injury due to ischemia and subsequent reperfusion events is a common pathology that occurs in multiple clinical conditions. Organ ischemia induces immediate cellular injury that is significantly magnified by the return of blood flow and can lead to a systemic inflammatory response that damages remote organs as well (1). Due to the sensitivity of the mucosal surfaces, ischemia/reperfusion (I/R)1-induced injury is exceptionally prominent in the intestine and is frequently followed by liver and lung damage (2).

Mesenteric I/R-induced tissue injury is mediated by at least two components, neutrophil infiltration and complement activation (3–5). Initial studies showed protection from I/R-induced injury after neutrophil depletion (4, 5). However, the presence of neutrophils is not sufficient for tissue damage, and a critical role for complement activation has also been demonstrated (6). Depletion or inhibition of complement activation products prevents I/R-induced local and remote organ injury in response to I/R (7–10).

The classical complement activation pathway has been implicated in I/R-induced tissue damage (11–13). Mice that lack Abs, Rag-1−/− mice, are resistant to mesenteric I/R-induced damage (12, 13). Infusion of IgM, purified from the natural Ab pool, into Rag-1−/− mice restored tissue injury and resulted in colocalization of IgM and complement deposition within the intestine (12, 13). Complement receptor 2/complement receptor 1-deficient (Cr2−/−) mice are also resistant to I/R-induced injury (11, 14), and infusion of Abs from wild-type mice, but not Cr2−/− mice, restores intestinal injury. The experiments in the Cr2−/− mice suggest that only a portion of the natural Ab repertoire is critical to intestinal damage (11). Despite the recent identification of a mAb that is pathogenic in the mesenteric I/R model, the specificity and diversity of the critical natural Abs that are absent in Cr2−/− mice remain unknown (13).

Intestinal I/R results in TUNEL-positive epithelial cells that can be inhibited by treatment with a caspase inhibitor or in Bcl-2 transgenic mice (15, 16), indicating that apoptosis plays a role in I/R-induced damage. During cellular apoptosis, phosphatidylserine is translocated to the cell surface, exposing the negatively charged phospholipid (PL) to natural Ab binding (17–19). The anti-PL Ab recognition of apoptotic cells is dependent on β2-glycoprotein I (20). Others have also shown that natural IgM Abs specific for lysophosphatidylcholine activate complement on apoptotic cells (21).

In the presence of serum cofactors, such as β2-glycoprotein I, anti-PL Abs bind negatively charged PL and, specifically, cardiolipin (CL) (22, 23). Injection of anti-PL Abs into pregnant mice results in fetal growth restriction and abortion, which is mediated by complement activation triggered by Abs bound to trophoblasts (24, 25). Similar to mesenteric I/R, fetal loss requires activation of the classical complement pathway (26–28).

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1 Abbreviations used in this paper: I/R, ischemia/reperfusion; CL, cardiolipin; Cr, complement receptor; LTBI, leukotriene B4; pAb, polyclonal Ab; PL, phospholipid.
In an effort to identify the type of naturally occurring Abs that initiate mesenteric I/R injury, we hypothesized that infusion of Abs with specificities against PL can reconstitute mesenteric I/R-induced tissue damage in Cr2−/− mice. Such reconstitution should provide insights on the nature of the Ags that are expressed by I/R-injured tissues. We show in this work that Cr2−/− mice infused with mouse or human anti-PL mAbs reconstituted mesenteric I/R-induced intestinal and lung tissue damage.

Materials and Methods

Mice

Adult male and female Cr2−/− and control Cr2+/+ mice were maintained and bred as two sublines. Adult male Rag-1−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained for at least a 7-day acclimation period following shipment, in the Uniformed Services University for the Health Sciences animal facility.

Experimental protocol

Mice were anesthetized with ketamine (16 mg/kg) and xylazine (8 mg/kg) administered i.p. All procedures were performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water-circulating heating pad. All procedures were reviewed and approved by the Institute’s Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Experiments were performed according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication 85-23).

Animals were subjected to I/R, as previously described (6, 11). Briefly, a midline laparotomy was performed, the superior mesenteric artery was identified and isolated, and a small vascular clamp (Roboz Surgical Instruments, Rockville, MD) was applied for 30 min. After removal of the clamp, the intestine was allowed to reperfuse for 2 h. In some experiments, 30 min before laparotomy, animals were given 50 µg of purified Ab (poly- or monomodal). Additional mice received control Ab in an equal volume of normal saline. Sham animals were subjected to the same surgical intervention without superior mesenteric artery occlusion. To control for the effects of Ab injection, Igs were administered to sham-treated mice as well. The laparotomy incisions were sutured, and the animals were monitored during the reperfusion period. Additional ketamine and xylazine were administered by i.m. injection immediately before euthanasia. After euthanasia, the small intestine 10–20 cm distal to the gastroduodenal junction was removed for histologic and immunohistochemical analysis and for the measurement of inflammatory mediators, as described below. There was no significant difference in survival between treatment and control groups.

Histology and immunohistochemistry

Immediately after euthanasia, segments of small intestine specimens were fixed in 10% buffered Formalin, embedded in paraffin, sectioned transversely in 5-µm sections, and stained with Giemsa. Mucosal injury was graded on a six-tiered scale in a blinded manner, as described previously (11, 29). Briefly, the average of villi damage in a ~2-cm intestinal section (50–100 villi) is determined after grading each villus in the section on a 0–6 scale. Score 0 is assigned to a normal villus; score 1 to villi with tip erosion; score 2 is assigned to villi with exposed but intact lamina propria; score 3 is assigned to villi with disrupted but intact lamina propria with epithelial cell sloughing; when the lamina propria is exuding a score of 5 is assigned; last, score 6 is assigned to villi that display hemorrhage or to villi that are denuded.

Additional tissue sections were fixed for 2 h in cold 4% paraformaldehyde in PBS before transfer to PBS for paraffin embedding and preparation of transverse sections. Following removal of paraffin from sections, non-specific Ab binding sites were blocked by treatment with a solution of 10% donkey serum in PBS for 30 min. After washing in PBS, the tissues were incubated with goat anti-mouse C3 (Valeant Pharmaceuticals, Costa Mesa, CA) or appropriate control IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) Ab overnight at 4°C. After incubation with a 7-amino-4-methylcoumarin-3-acetic acid-labeled secondary donkey anti-goat Ab (Jackson ImmunoResearch Laboratories), the slides were then mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). A blinded observer examined the slides by fluorescent microscopy using a Leica DM RXA fluorescence microscope (Leica Microsystems, At- lanta, GA) with SPOT diagnostic computer software (Diagnostic Instruments, Sterling Heights, MI).

Immediately after harvest, additional intestinal tissue was placed into OCT compound, frozen, and stored at ~80°C until slides were cut. Immunohistochemistry for β2-glycoprotein I was performed using frozen tissue sections fixed in acetone at 4°C. Non-specific binding sites were blocked with 20% normal donkey serum in 1% BSA. Sections were incubated with goat anti-human β2-glycoprotein 1 IgG (United States Biological, Swampscott, MA) at 4°C overnight with fluorescein-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories). Control sections were incubated with normal goat sera replacing the primary Ab. After washing, the slides were mounted and examined, as indicated above.

Eicosanoid determination

The ex vivo generation of eicosanoids by small intestine tissue was determined, as described previously (6, 11). Briefly, immediately after collection, mid-jejenum sections were minced, washed, and resuspended in 37°C oxygenated Tyrode’s buffer (Sigma-Aldrich, St. Louis, MO). After incubating for 20 min at 37°C, supernatants were collected and stored at ~80°C until assay. The concentration of leukotriene B4 (LTB4) was determined using an enzyme immunooassay (Cayman Chemical, Ann Arbor, MI). The tissue protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) adapted for use with microtiter plates. LTB4 levels were expressed as pg protein per 20 min.

Preparation of anti-PL Abs

The murine anti-PL mAbs were purified from culture supernatant by affinity chromatography on recombinant protein G-agarose. The monoclonal clone F93 is a mouse anti-PL IgG3 Ab, whereas clone FC1 is a mouse anti-β2-glycoprotein I mAb of the IgG1 isotype. Both mouse mAbs have been characterized previously (32). Isotype control Abs (IgG1, clone MOPC-21 and IgG3, clone FLOPC-21) were obtained from Sigma-Aldrich and diluted to exchanged the buffer. Human IgG-containing polyclonal Ab (pAb) were obtained from patients with anti-PL syndrome (characterized by high titer pAb, thromboses, and/or pregnancy losses) (30). IgG was purified by affinity chromatography using protein G-Sepharose chromatography columns (Amersham Biosciences, Piscataway, NJ). All IgG samples were treated to deplete endotoxin with Centriprep ultracentrifugation devices (Millipore, Bedford, MA) and determined to contain <0.03 endotoxin U/ml using the Limulus amebocyte lysate assay. The generation, structure, and specificity of the human IgG1, anti-CL mAb (mAb 519), were previously described (31).

Statistical analysis

Data are presented as mean ± SEM and were compared by one-way ANOVA with post hoc analysis using Newman-Keuls test (GraphPad/Instat Software, Philadelphia, PA). The difference between groups was considered significant when p < 0.05.

Results

Murine mAbs against negatively charged PL or β2-glycoprotein I can restore mesenteric I/R injury in Cr2−/− mice

To determine whether anti-PL Abs can reconstitute mesenteric I/R-induced tissue damage, Cr2−/− mice wereinfused i.v. with 50 µg of a murine anti-PL mAb. Murine anti-PL mAb binds CL and negatively charged PL; it does not cross-react with β2-glycoprotein I or PL. In addition, the murine anti-β2-glycoprotein I mAb of the IgG1 isotype control Ab into Cr2−/− mice restored tissue damage similar to wild-type controls (Fig. 1A). In addition, the murine anti-PL mAb induced LTB4 production (Fig. 1B) and subsequent organ injury. In mice, intestinal I/R induces mucosal injury to the villi (Fig. 1A). In addition, the murine anti-PL mAb induced LTB4 production (Fig. 1B) and subsequent organ injury. In addition, the murine anti-PL mAb induced LTB4 production (Fig. 1B) and subsequent organ injury. In addition, the murine anti-PL mAb induced LTB4 production (Fig. 1B) and subsequent organ injury.
controls. These data indicate a role for anti-PL Abs in I/R-induced local and remote organ damage.

Abs directed against negatively charged PL form complexes with the serum factor, β2 glycoprotein I. To determine the involvement of β2 glycoprotein I in I/R-induced tissue damage, Cr2−/− mice were infused with anti-β2 glycoprotein I Abs (murine anti-β2-glycoprotein I mAb) before the I/R procedure. Similar to the anti-PL Ab, murine anti-β2-glycoprotein I mAb induced intestinal damage accompanied by significantly enhanced LTB4 production and increased cell numbers in the bronchoalveolar lavage fluid (Fig. 2).

To establish that β2-glycoprotein I is expressed in intestinal tissue in response to I/R, we performed immunohistochemistry using tissue sections from sham- and I/R-treated mice. Compared with isotype control-stained sections (Fig. 3, A and D), no significant staining for β2-glycoprotein I was detected in sections from animals subjected to sham treatment (Fig. 3, B and E). In contrast, β2-glycoprotein I was expressed in large quantities in the intestinal tissue of either Cr2+/+ or Cr2−/− mice that had been subjected to I/R treatment (Fig. 3, C and F).

There is ample published evidence that mesenteric I/R-induced tissue damage is mediated by complement activation as well as inflammation (5, 8, 9, 12, 33–36). To determine whether complement is activated in response to I/R treatment after infusion of either of these Abs, C3 deposition on the intestinal tissue was determined. As expected, the presence or absence of the Abs did not affect complement deposition in the intestine of sham-treated animals (Fig. 4A, and data not shown), and similar to previous studies (11–13), complement is deposited in intestinal tissue from wild-type mice (Fig. 4B). Infusion of murine anti-PL mAb resulted in significant deposition of C3 on the intestinal tissue after I/R treatment (Fig. 4C). Treatment with the IgG1 murine anti-β2-glycoprotein I mAb resulted in less complement deposition on the injured tissue, suggesting that the severity of tissue damage is influenced by the large quantities of LTB4 production.

**FIGURE 1.** Murine anti-PL mAb restores I/R-induced injury in Cr2−/− mice. One hour after injection of murine anti-PL mAb (m-PL mAb), isotype control Ab (IgG3), or PBS, wild-type Cr2+/+ or Cr2−/− mice were subjected to mesenteric I/R or sham treatment. Immediately after 2-h reperfusion, intestinal tissue was Formalin fixed and paraffin embedded, and Giemsa-stained sections from each treatment group were scored for mucosal injury (A), as described in Materials and Methods. Additional tissue sections were collected, and ex vivo LTB4 production was determined (B). In addition, the number of white blood cells in the lavage fluid was determined for each animal (C). Each bar is the average ± SEM with three to six mice/group. Using ANOVA with Newman Keuls post hoc test, * indicates significant difference from sham treatment group, p < 0.05.

**FIGURE 2.** Murine anti-β2 glycoprotein I mAb restores I/R-induced injury in Cr2−/− mice. One hour after injection of murine anti-β2 glycoprotein I mAb (m-β2GPI mAb) or PBS, Cr2−/− mice were subjected to mesenteric I/R or sham treatment. Immediately after 2-h reperfusion, intestinal tissue was Formalin fixed and paraffin embedded, and Giemsa-stained sections from each treatment group were scored for mucosal injury (A), as described in Materials and Methods. Additional tissue sections were collected, and ex vivo LTB4 production was determined (B). In addition, the number of white blood cells in the lavage fluid was determined for each animal (C). Each bar is the average ± SEM with three to six mice/group. Using ANOVA with Newman Keuls post hoc test, * indicates significant difference from sham treatment group, p < 0.05.

**Human polyclonal anti-PL autoantibodies restore I/R-induced tissue injury**

The mouse mAbs against negatively charged PL and the associated binding protein, β2 glycoprotein I, used in the previous experiments bind Ags similar to the autoantibodies present in patients...
with anti-PL syndrome (22, 23, 37). Infusion of autoantibodies from patients with anti-PL syndrome causes complement-mediated fetal loss in a murine model of anti-PL syndrome (28, 38). We therefore asked whether similar patient-derived Abs were pathogenic in the mesenteric I/R-induced tissue damage. 

Cr2/H11002/H11002/H11002 mice were infused with human polyclonal anti-PL Abs before the I/R procedure. As indicated in Figs. 5 and 4, the pAbs induced significant intestinal and lung damage 2 h after I/R. Infusion of human anti-PL pAb, before subjecting the Cr2/H11002/H11002/H11002 mice to I/R, also induced significant C3 deposition (Fig. 4). In contrast to the murine IgG mAbs, the human pAbs did not induce significant LTB4 production at 2 h postreperfusion (Fig. 5B).

**Human anti-CL mAbs restore local and remote lung injury in Cr2/H11002/H11002/H11002 mice**

Although the pAbs are known to bind anionic PL, the preparation contains additional Abs as well (38). To determine the involvement of anti-PL Abs in the human pAbs, a human anti-CL mAb was studied. After infusion of the human anti-CL mAb, the I/R-induced intestinal damage was restored in Cr2/H11002/H11002/H11002 mice (Fig. 6A). The significant increase in remote organ damage, as indicated by increased cell numbers in the bronchoalveolar lavage (Fig. 6C), was similar to I/R-induced damage in wild-type control mice (Fig. 1A). As with the polyclonal anti-PL Abs, significant amounts of C3 were deposited on the injured intestinal tissue (Fig. 4F), but there was no significant increase in LTB4 production (Fig. 6B).

**Rag-1/H11002/H11002/H11002 mice require the infusion of multiple Abs to restore I/R-induced intestinal injury**

In systemic autoimmune diseases such as anti-PL syndrome and systemic lupus erythematosus, anti-PL and anti-β2 glycoprotein I Abs are believed to form a complex and bind the endothelium. To determine whether I/R-induced tissue damage required interaction between the anti-PL and anti-β2 glycoprotein I Abs, Rag-1/H11002/H11002/H11002 mice, which are known to be resistant to mesenteric I/R injury, were infused with murine anti-PL mAb, murine anti-β2-glycoprotein I mAb, or both Abs before I/R-induced tissue damage. As shown previously, Rag-1/H11002/H11002/H11002 mice, which are known to be resistant to mesenteric I/R injury, were infused with murine anti-PL mAb, murine anti-β2-glycoprotein I mAb, or both Abs before I/R-induced tissue damage. As shown previously, Rag-1/H11002/H11002/H11002 mice were protected from I/R-induced injury (Fig. 7). In contrast to Cr2/H11002/H11002/H11002 mice, neither murine anti-PL mAb nor murine anti-β2-glycoprotein I mAb were able when injected alone to restore damage in these Ab-deficient mice. However, a combination of both Abs restored injury similar to that seen in wild-type animals.
Additional tissue sections were collected, and ex vivo LTB4 production in cells (15, 16, 21) we predicted that Abs present in the sera of patients with anti-PL syndrome and Abs that recognize charged PL can initiate I/R-induced tissue damage as well. In this study, we show that Abs directed against negatively charged PL and Abs from patients with anti-PL syndrome can cause intestinal and lung damage in Cr2<sup>−/−</sup> mice following mesenteric I/R. In addition, we show that Abs directed against β2 glycoprotein I can induce damage in the Cr2<sup>−/−</sup> mice, but that to achieve I/R injury in Rag-1<sup>−/−</sup> mice, the presence of both anti-β2 glycoprotein I and anti-PL Abs is required.

The role of Abs in the expression of I/R-induced tissue injury has been shown in studies using Rag-1<sup>−/−</sup> and in Cr2<sup>−/−</sup> mice that are both resistant to mesenteric I/R. Passive Ab transfer from normal mice to Rag-1<sup>−/−</sup> or Cr2<sup>−/−</sup> restores I/R injury, suggesting strongly a role for Ig in the expression of this injury (11–13). The fact that sera from normal, but not Cr2<sup>−/−</sup> mice are able to restore I/R injury suggests that Cr2<sup>−/−</sup> mice lack part of the repertoire that has tissue injury-inducing features (11). Cr2<sup>−/−</sup> mice express normal total levels of Ig, but have decreased production of IgG3, and subsequently, reduced Ab production in response to Streptococcus pneumoniae and other Ags (39–42). This suggested that the tissue injury-inducing component of the repertoire may be produced in part in response to bacteria such as S. pneumoniae. In addition, the fact that murine anti-PL mAb that restores I/R-induced intestinal injury in Cr2<sup>−/−</sup> mice is an IgG3 Ab (Figs. 1 and 4) further supports this position.
Anti-PL Abs form frequently complexes with β2-glycoprotein I on the endothelial membrane. Interestingly, binding of the IgG1 murine anti-β2-glycoprotein I mAb can also induce tissue injury in Cr2−/− mice. Similar to previous studies (11), this Ab appears to induce tissue damage by a different mechanism than the murine anti-PL mAb. Treatment with murine anti-β2-glycoprotein I mAb results in very high amounts of LTB₄ production (Fig. 2B) and sparse complement deposition. Recent studies indicate that in vitro binding of other anti-β2-glycoprotein I Abs to the surface of endothelial cells similarly induces an inflammatory response, as indicated by NF-κB translocation and subsequent E-selectin and proinflammatory cytokine production (43). In this study (Fig. 3), we show that β2-glycoprotein I is expressed in intestinal tissue of animals subjected to I/R, but not sham treatment. It is well established that anti-PL Abs are critical to the expression of pathology in patients with anti-PL syndrome. Recent studies have shown that similar to I/R-induced injury, complement activation is required (27, 28). Further studies have identified roles for neutrophils, the anaphylatoxin, C5a, and the alternative complement cascade (38, 44) in the expression of anti-PL Ab-associated pathology. Because each of these components is involved in mesenteric I/R (5, 6, 33, 35, 45), it is possible that Abs with anti-PL specificities are involved. In this study (Figs. 5 and 6), we show that Abs known to induce fetal loss are also pathogenic in this complement-dependent mouse model of tissue injury.

Previous studies of mesenteric I/R have shown that LTB₄ production is associated with the recruitment of neutrophils into the intestine (8, 11). Data presented in Figs. 5B and 6B indicate lack of LTB₄ production following the injection of human pAbs and human anti-CL mAb. The significant increase in neutrophil recruitment into the intestine (data not shown) may be due either to LTB₄ produced at earlier time point or to the production of an unidentified chemotactic molecule. In vitro studies demonstrated that similar Abs induced MCP-1 production by endothelial cells (46), suggesting that this and other chemotactic agents may also be produced.

In contrast to the Cr2−/− mice, neither murine anti-PL mAb nor murine anti-β2-glycoprotein I mAb alone is sufficient to induce tissue injury in the Rag-1−/− mice. This observation suggests that both anti-PL and anti-β2 glycoprotein I Abs may exist in the sera of Cr2−/− mice at very low concentrations, but sufficient to complement the action of each other when one of them is infused in sufficient amounts. The exact measurement of the levels of these Abs in wild-type and Cr2−/− mice has been difficult with the available ELISA (data not shown).

What is the nature of the neoantigen(s) in I/R-induced tissue damage? In this study, we have identified at least one set of Ags (β2-glycoprotein I and/or negatively charged PL) that is recognized by pathogenic Abs. It is likely that additional Abs present in normal mice, but not in Cr2−/− and Rag-1−/− mice, with yet unknown specificities can bind to tissues exposed to I/R and induce damage. This is consistent with previous results indicating that infusion of normal mouse IgG and IgM into Cr2−/− mice results in more severe damage than either isotype alone. Recent studies have identified an IgM clone that is pathogenic in mesenteric ischemia (13). Although the specific Ag for this Ab is not known, it appears to recognize a large protein of ~250 kDa. Therefore, multiple Ags are involved in this process, and the identification of additional specific neoantigens recognized by the pathogenic Abs will aid in the development of specific therapeutics for treatment of ischemic conditions. Our studies do not exclude the possibility that additional Ags are involved because it is possible that the used Abs may recognize additional, yet not identified, Ags. We propose that multiple neoantigens are expressed in response to I/R on the endothelial and epithelial surfaces and are recognized by natural Abs/autoantibodies that can fix complement and result in tissue damage.

References


