Role for the Alternative Complement Pathway in Ischemia/Reperfusion Injury

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The terminal complement components play an important role in mediating tissue injury after ischemia and reperfusion (I/R) injury in rats and mice. However, the specific complement pathways involved in I/R injury are unknown. The role of the alternative pathway in I/R injury may be particularly important, as it amplifies complement activation and deposition. In this study, the role of the alternative pathway in I/R injury was evaluated using factor D-deficient (−/−) and heterozygote (+/−) mice. Gastrointestinal ischemia (GI) was induced by clamping the mesenteric artery for 20 minutes and then reperfused for 3 hours. Sham-operated control mice (+/+ versus −/−) mice had similar baseline intestinal lactate dehydrogenase activity (P = ns). Intestinal lactate dehydrogenase activity was greater in −/− mice compared to +/+ mice after GI/R (P = 0.02) thus demonstrating protection in the −/− mice. Intestinal myeloperoxidase activity in +/+ mice was significantly greater than −/− mice after GI/R (P < 0.001). Pulmonary myeloperoxidase activity after GI/R was significantly higher in +/+ than −/− mice (P = 0.03). Addition of human factor D to −/− animals restored GI/R injury and was prevented by a functionally inhibitory antibody against human factor D. These data suggest that the alternative complement pathway plays an important role in local and remote tissue injury after GI/R. Inhibition of factor D may represent an effective therapeutic approach for GI/R injury. (Am J Pathol 2003, 162:449–455)

Several reports have indicated that complement activation mediates ischemia/reperfusion (I/R) injury of the myocardial, skeletal, cerebral, and intestinal circulations.1–5 Therapeutic inhibition of complement with scCr1, C1 esterase inhibitor, or monoclonal antibodies (mAbs) directed against specific complement components (eg, C5 or C5a) have demonstrated decreased tissue injury, improved organ function, and increased survival in many different animal models of human disease.1,4–8 Recent studies have demonstrated the important contribution of the terminal complement components (ie, C5a and C5b-9) in I/R injury.5,9–11 However, the specific contributions of the individual complement pathways to the disease process are unknown.

At least three separate pathways (the lectin, classical, and alternative pathways) can activate complement. The three pathways merge at the level of C3, where two similar C3 convertases cleave C3 into C3a and C3b. The alternative pathway is a self-amplifying pathway and is important in the clearance and recognition of pathogens in the absence of antibodies. The alternative pathway can also amplify complement activation after initial complement activation by either the lectin and/or classical pathway. The rate-limiting step of activation of the alternative pathway in humans is the enzymatic action of factor D on the cleavage of factor B to form the alternative pathway C3 convertase, C3bBb.

C3 and C4 knockout (KO)/deficient mice have suggested an important role of the classical and/or lectin pathway in initiation of complement activation during gastrointestinal or skeletal I/R injury.2,3 In contrast, renal I/R injury appears to involve the initial activation of the alternative complement pathway, but a supporting role for the other two pathways was not ruled out.9 It is important to understand the mechanism/means by which complement activation occurs after I/R injury, so that specific and effective therapeutic approaches may be designed to limit the inflammatory process and tissue injury and retain necessary biological activity of complement in host defense.

We recently demonstrated a novel model of murine gastrointestinal (G) I/R injury that induces local and re-
molt complement-dependent tissue injury mediated by the terminal complement components. In the present study, we investigated the specific role of the alternative pathway in GI/R injury by using recently described factor D-deficient mice. Although the alternative complement pathway has been demonstrated to amplify complement activation and deposition in vitro, to date it has not been possible to investigate directly the role of this pathway in vivo after I/R, because specific molecular tools and transgenic animals were not available.

Materials and Methods

Animals

Homozygous factor D-deficient mice were derived by homologous recombination in embryonic stem cells as described previously. Heterozygous littermates were used as controls. All animals were from the F3 progeny.

Ischemia Protocol

The methods for the model have been published previously. Briefly, male mice (22 to 25 g) were anesthetized with isoflurane. Body temperature was maintained by a heated OR table. After a midline laparotomy, intestinal ischemia was produced by occlusion of the superior mesenteric artery with surgical microvascular clips (Roboz, Rockville, MD) for 20 minutes. Reperfusion was achieved by removing the clips, the surgical site closed in layers, and mice were allowed to wake up and reperfuse for 3 hours. Sham-operated controls underwent the same surgical procedures without clamping. Mice were euthanized by heart removal under deep anesthesia with isoflurane.

Lactate Dehydrogenase (LDH) Activity in Intestinal Homogenates

Intestinal LDH activity from tissue homogenates was measured as an index for tissue injury. LDH activity (Lactate Dehydrogenase 500; Sigma, St. Louis, MO) was expressed as U/mg of protein [measured using a protein assay kit (Bio-Rad, Hercules, CA)] and its loss from the tissue is a biochemical marker of cellular injury.

Myeloperoxidase (MPO) Activity

MPO activity, an index of neutrophil infiltration, was measured as described previously. One unit of MPO activity was defined as the quantity of enzyme that hydrolyzed 1 μmol of H₂O₂/minute at 25°C. MPO activity was expressed as U/100 mg of wet tissue.

Histological Sections

Tissue samples for histological staining were taken and fixed in 10% formalin-phosphate-buffered saline at 4°C overnight. The samples were dehydrated and embedded in paraffin. Sections (7 μm) were cut and stained with hematoxylin and eosin (H&E). A pathologist evaluated the slides in a blinded manner.

Reconstitution of Factor D in Factor D KO Mice

Factor D was purified from the urine of a patient with Fanconi’s syndrome by successive chromatography as described. Purified human factor D (100 or 300 μg, i.p.) was administered to 8-week-old mice of either sex. Serum was collected by retro-orbital bleeding at various intervals. Serial dilutions of serum were incubated in 96-well plates precoated with monoclonal anti-factor D antibody FD10-1 at 5 to 10 μg/ml at 4°C for overnight. Carpylic acid-purified rabbit anti-factor D IgG (5 μg/ml) and affinity-purified peroxidase-conjugated goat anti-rabbit IgG were used as the detection reagents. The assay was developed as described previously. The concentration of factor D was calculated from a standard curve constructed with purified factor D of known concentration. In additional animals, a functionally inhibitory mAb against human factor D (clone 166-32) or an isotype control mAb was given (intraperitoneally) at 1.5 molar excess to inhibit human factor D.

Statistical Analysis

All values in the text and figures are presented as mean ± SEM of n independent experiments. All data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls post hoc test using SigmaStat software (SPSS Science, Chicago, IL). Differences were considered significant at P ≤ 0.05. A Student’s t-test was used to evaluate the intestinal LDH data for the factor D reconstitution study.

Results

Alternative Pathway Activation Contributes to Gastrointestinal Injury

As illustrated in Figure 1 (top panels) and previously demonstrated in this model, 20 minutes of gastrointestinal ischemia followed by 3 hours of reperfusion resulted in a loss and shortening of villi and marked epithelial cell denudation in +/- mice after GI/R compared to sham-operated controls. Factor D-deficient mice (KO; +/-) displayed less loss of villi height and tissue injury after GI/R compared to the heterozygotes (HE; +/-).
Confocal micrographs of C3 deposition in the gut (Figure 2, top) and lung (Figure 2, bottom) from mice undergoing sham GI/R (Figure 2, A and C) or after GI/R (Figure 2, B and D) are presented in Figure 2. Complement C3 deposition was increased in the gut and lung after GI/R in HE mice compared to sham-operated controls (Figure 2, B and A, respectively). C3 deposition in the gut and lung was attenuated after GI/R in KO mice compared to HE mice (Figure 2, D compared to B, respectively). C3 deposition in the gut from KO mice after GI/R did not appear to increase much above sham-operated control mice (Figure 2, top, D and C, respectively). In contrast, pulmonary C3 deposition in KO mice after GI/R was slightly elevated compared to sham GI/R mice (Figure 2, bottom, D and C, respectively). These data demonstrate that amplification of C3 deposition via alternative pathway activation occurs in the gut and lung after GI/R in the mouse.

Biochemical analysis of gastrointestinal tissue demonstrated a significant ($P = 0.004$) loss of LDH in heterozygotes compared to their respective sham-operated controls (Figure 3A). The factor D-deficient animals had slightly lower LDH activity after GI/R compared to their sham-operated controls but this was not statistically significant ($P = 0.086$). Factor D-deficient mice had significantly ($P = 0.019$) higher tissue LDH activity compared to the heterozygotes after GI/R, thus demonstrating a role for alternative pathway activation after I/R.

One of the hallmarks of GI/R injury and the resulting inflammation is the infiltration of neutrophils. The terminal complement components (i.e., C5a and C5b-9) play a key role in the activation, adherence, recruitment, and accumulation of polymorphonuclear leukocytes (PMNs) into ischemic/reperfused organs in this model. The intestinal MPO activity of sham-operated control and ischemic-reperfused mice is presented in Figure 3B. We observed a significant increase in intestinal MPO activity of HE mice compared with sham-operated mice ($P < 0.001$). Factor
D-deficient mice had significantly lower MPO activity than +/− mice after GI/R (P = 0.002). There was no significant difference in intestinal MPO activity between ischemic-reperfused and sham-operated factor D-deficient mice. Intraperitoneal administration of LTB4 (1 μg) into the abdomen of sham mice induced PMN transmigration that was significantly greater in factor D-deficient compared to HE mice (45 ± 5 and 25 ± 4 × 10⁶ total cells, respectively; n = 6 per group; P = 0.006, Student’s t-test). Thus, decreased PMN infiltration into the intestine is likely not a result of altered PMN chemotaxis. These data demonstrate that neutrophil infiltration in the intestine after GI/R is significantly attenuated after disruption of the alternative complement pathway.

Second Organ Injury

There is a propensity for pulmonary inflammation after major gastrointestinal injury. We have recently characterized this process in the mouse after GI/R.¹¹ In the present study, pulmonary congestion and moderate PMN infiltration were observed in the lung after GI/R in the heterozygotes (Figure 1, bottom). A reduction in pulmonary congestion and PMN infiltration was observed in the factor D-deficient mice after GI/R. Quantitative analysis of pulmonary MPO activity confirmed these histological findings (Figure 4). A significant increase in MPO activity was observed in the heterozygotes after GI/R compared to the sham-operated controls. Further, a significant (P = 0.017) increase in MPO activity was also observed in the factor D-deficient animals after GI/R, but this increase was significantly (P = 0.002) less than that observed in the heterozygotes after GI/R. Thus, at least part of the pulmonary PMN infiltration is a result of alternative complement pathway activation.

Reconstitution of Factor D-Deficient Mice

To verify that these results are manifested by a loss of factor D and not another cellular or molecular mechanism, we reconstituted the alternative complement pathway in factor D-deficient mice. Figure 5A demonstrates that a single intraperitoneal injection of human factor D restores serum factor D to a physiological level in a dose-related manner. This serum factor D concentration was shown previously to restore alternative pathway activation in vitro.¹² Using these data, we gave factor D-deficient mice a single injection of human factor D (200 μg, i.p.) 20 minutes before anesthesia and another 200 μg immediately after reperfusion (i.e., before abdominal closure). During each of these factor D administrations, a 1.5-molar excess of a functionally inhibitory mAb against human factor D (clone 166-32) or an isotype control mAb was also given (intraperitoneally). Total volume of these injections was 200 μl or less. Sera samples collected at the end of reperfusion revealed average factor D concentration of 6.5 and 7.0 μg/ml for the isotype control and factor D-deficient groups, respectively. Biochemical analysis of the gastrointestinal LDH activity demonstrated that reconstitution of factor D-deficient mice with human factor D resulted in a significant loss of LDH from the gastrointestinal tissue compared to sham-operated controls (Figure 5B compared to sham KO mice in Figure 3A). Administration of anti-human factor D mAb 166-32 or an isotype control mAb was also given (intraperitoneally). These data demonstrate that alternative complement pathway activation alone results in signifi-
factor D with a potent anti-factor D neutralizing mAb.

**Discussion**

It is well established that complement plays an important role in the inflammation and tissue injury associated with I/R injury in many animal models of human disease. Experimental data and results from a clinical trial suggest that inhibition of complement at C5 (eg, elimination of C5a and C5b-9 production) significantly attenuates tissue injury and the inflammatory process after oxidative stress.5,6,8,11,18,19 However, cleavage of C5, in most biological situations, is determined by the formation of two similar C5 convertases that are formed after activation of any of the three currently known complement pathways. Thus, to understand the biological sequelae responsible for activation of the terminal complement components, one must understand what complement pathways are activated during the disease process. In the present study, we have examined the role of the alternative complement pathway during intestinal I/R injury.

A previous study using C3- and C4-deficient mice suggested that activation of the classical pathway of complement plays an important role in GI/R injury.3 The study clearly demonstrated an important role of C4 in the initiation of complement activation after GI/R. However, the potential role of the alternative pathway in the amplification of the classical and lectin pathways was not studied. Thus, to evaluate the importance of the alternative pathway in a disease process, in which complement activation occurs initially via the classical or lectin complement pathway, a specific alternative pathway inhibitor must be used. The use of factor D-deficient mice allows such an investigation and evaluation to take place. C3 deposition present within the tissues of ischemic/reperfused factor D-deficient animals extends the observations of others and supports that complement activation occurs initially via the classical and/or lectin pathway.5 A reduction in C3 deposition and tissue injury in factor D-deficient mice compared to the heterozygotes further demonstrates for the first time that the alternative pathway amplifies complement activation during the inflammatory process *in vivo*. Thus, effective complement inhibition during I/R conditions may require inhibition of all complement pathways.

The magnitude of the decrease in gastrointestinal injury in factor D-deficient mice was similar to that observed in C5-deficient mice, as well as wild-type mice treated with anti-C5 mAb.11 C5a and/or C5b-9 play a major role for increased chemokine and adherence molecule after pulmonary insults and lead to pulmonary PMN recruitment.11,20 Pulmonary PMN recruitment was not completely inhibited in factor D-deficient mice, whereas inhibition of pulmonary PMN recruitment is observed in mice treated with anti-C5 mAb after GI/R.11 Gut barrier dysfunction and systemic bacterial translocation are often a consequence of GI/R. Because C4 and terminal complement components are important for increased gastrointestinal permeability,2,3,21 bacterial translocation after GI/R may still occur in factor D-deficient mice. Further, the alternative complement pathway plays an early and important role in the opsonization and ultimate clearance of bacteria via host defense.12 Thus, it is possible that whereas factor D-deficient mice are protected from local intestinal injury, gut barrier dysfunction may lead to bacterial translocation to the lung and less opsonization via the alternative pathway, resulting in increased pulmonary PMN translocation. This scenario is likely because we still observed C3 deposition in the lung after GI/R, whereas C3 deposition in the gut was greatly decreased. C3 deposition in the lung after GI/R thus appears to result from lectin and/or classical pathway activation in addition to the alternative pathway. Collectively, these data suggest that pulmonary injury after gastrointestinal injury is dependent on local complement activation and the production of C5a and C5b-9 and minimally on C3a production.11 Additional studies in classical and lectin pathway transgenic mice are needed to investigate the role of these pathways in this model.

Although a majority of experimental animal models have demonstrated the importance of adaptive immunity, Ig deposition, and activation of the classical complement
pathway, recent data suggest that one should not overlook the specific role of the other complement pathways. We have demonstrated an important role for the alternative pathway in renal ischemia and reperfusion. Moreover, recent evidence suggests that an adaptive immune response can result in alternative complement pathway activation and the development of rheumatoid arthritis. Thus, despite an adaptive immune response and Ig deposition, an inflammatory process in a disease can be solely dependent on the alternative pathway, terminal complement components, and not classical pathway activation.

Recent data from our group suggests that the lectin complement pathway is initially activated after myocardial ischemia and reperfusion. Anti-MBL-A mAb not only decreased tissue injury and infarct size, but decreased proinflammatory gene expression and inhibited C3 deposition in the reperfused myocardium. Moreover, recent data demonstrate that mice deficient in MBL-A are protected against the lethal effects of sepsis, whereas C1q-deficient mice quickly succumb to death. Thus, the obvious proinflammatory role of the classical pathway in human disease based on previous data within the literature cannot be fully explained by recent results/publications. In our study, the lectin and classical complement pathways were still intact, yet we observed protection from I/R injury. Thus, our study is the first to demonstrate conclusively an important role of the alternative complement pathway in I/R in vivo. The novelty of these findings plus the unexpected findings of the proinflammatory role of the lectin pathway demonstrates the need for careful dissection of the individual complement pathways and components in models of human disease. The availability of novel molecular tools and transgenic mice will allow the careful evaluation of the complement system and a better understanding of the inflammatory process in multiple different experimental models.

Our data clearly demonstrate a pathophysiological role for alternative complement pathway activation after GI/R. A significant reduction in gastrointestinal injury, as well as decreased PMN accumulation in the gut and lungs, was observed in factor D-deficient mice after GI/R. Reconstitution of the alternative pathway of factor D-deficient mice with human factor D restored the ability of these mice to display significant tissue injury after GI/R. Further, the injury in this setting was significantly attenuated with a neutralizing mAb against human factor D. These data suggest that inhibitors of the alternative complement pathway may become important therapeutics for a variety of inflammatory diseases.

References

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