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Pathogenic Natural Antibodies Propagate Cerebral Injury Following Ischemic Stroke in Mice

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Self-reactive natural Abs initiate injury following ischemia and reperfusion of certain tissues, but their role in ischemic stroke is unknown. We investigated neoepitope expression in the postischemic brain and the role of natural Abs in recognizing these epitopes and mediating complement-dependent injury. A novel IgM mAb recognizing a subset of phospholipids (C2) and a previously characterized anti-annexin IV mAb (B4) were used to reconstitute and characterize injury in Ab-deficient Rag1⁻²/⁻ mice after 60 min of middle cerebral artery occlusion and reperfusion. Reconstitution with C2 or B4 mAb in otherwise protected Rag1⁻²/⁻ mice restored injury to that seen in wild-type (wt) mice, as demonstrated by infarct volume, demyelination, and neurologic scoring. IgM deposition was demonstrated in both wt mice and reconstituted Rag1⁻²/⁻ mice, and IgM colocalized with the complement activation fragment C3d following B4 mAb reconstitution. Further, recombinant annexin IV significantly reduced infarct volumes.

A number of events occur during tissue ischemia, and the pathophysiology of ischemia reperfusion injury (IRI) is complex. Clinical and animal studies have established a causal role for complement in IRI of various organs and tissues (1), including the brain following ischemic stroke (2). Cleavage of complement component C3 is a central event in complement activation, and studies with C3-deficient (3) and inhibited (4) mice have revealed a key role for complement in murine ischemic stroke. Complement can be activated via the classical, lectin, and alternative pathways, and recent data indicate a central role for the lectin pathway in ischemic stroke (5, 6).

In models of intestinal (7, 8), myocardial (9), and skeletal muscle (10) IRI, complement activation is triggered by natural circulating IgM that recognizes cellular neoepitopes that become exposed as a result of ischemia. These pathogenic natural Abs recognize a restricted Ag repertoire and are mainly the product of B-1 lymphocytes in mice and humans (11) and are constitutively expressed throughout life. Although IgM bound to reperfused tissue recruits both C1q and mannose-binding lectin (MBL) (classical and lectin pathway recognition molecules, respectively) (12, 13), IRI has been shown to be dependent on MBL binding to IgM, at least with regard to intestinal and myocardial IRI (14–16). Seminal studies by Zhang et al. (7) showed that Ab-deficient Rag1⁻²/⁻ mice were resistant to intestinal IRI and that natural self-reactive IgM restored IRI. Subsequently, the target of a clonally specific mAb that reconstituted intestinal IRI in Rag1⁻²/⁻ mice was identified as nonmuscle myosin (17), and the same Ab has since been shown to also restore myocardial and skeletal muscle IRI in Rag1⁻²/⁻ mice (9, 18). However, even though a peptide mimic of nonmuscle myosin can block IRI in wild-type (wt) mice (9, 19), it is clear that other targets for self-reactive Abs exist, at least in the postischemic intestine. In this context, intestinal IRI in Rag1⁻²/⁻ mice can also be restored by administration of an anti-annexin IV IgM mAb (8) or the combined administration of anti-phospholipid and anti-β₂–gp1 mAbs (20).

Thus, whereas current evidence indicates that multiple cellular neoepitopes are exposed postischemia, the predominance of their expression in different tissues, as well as the relative contributions of different targets and self-reactive Abs in driving IRI in different tissues, is not known. In this study, we identify a novel IgM mAb that recognizes a subset of phospholipids and show that this mAb, as well as a previously characterized anti-annexin IV mAb, recognizes postischemic neoepitopes in the brain, activates complement, and propagates cerebral IRI.

Materials and Methods

C2 mAb isolation and purification

The C2 mAb hybridoma was isolated following the fusion of spleen cells from unmanipulated wt C57BL/6 mice as described previously (8). Briefly, splenocytes from healthy C57BL/6 mice were fused with the SP2/0-AG14 myeloma cell line by standard protocol to establish...
hybridomas. The hybridomas were then screened by both Western blot analysis using intact epithelial cell lysates and by flow cytometric analysis of isolated intestine epithelial cells. Positive wells were further subcloned until a monoclonal population was obtained. To purify mAbs, Ab from the exhausted supernatants of cultured hybridomas was affinity purified on a column of agarose beads with goat anti-human IgM (Sigma-Aldrich, St. Louis, MO). Bound mAb was eluted with a buffer containing 0.1 M glycine (pH 2.3) and collected into a buffer containing 1.5 M Tris (pH 8.8). Eluted mAb was dialyzed against PBS (pH 7.4) for 48 h and concentrated using centrifugal filtration on Centricon Plus-20 (Millipore, Billerica, MA). Ab concentration was determined by measuring the A_{280} of the sample and purity was confirmed by analysis on a 10% SDS-PAGE gel.

**Characterization of C2 mAb and anti-phospholipid Abs in mouse serum**

ELISAs to determine reactivity of Abs to various phospholipids were performed using microtiter plates (Immulon 1B; Dynatech Laboratories, Chantilly, VA) coated with 100 μl/well 50 μg/ml phospholipid in methanol. The plates were dried under blowing air to allow the organic solvent to evaporate, and the wells were then washed with PBS and blocked with 1% BSA. Supernatant from the mAb hybridoma cell line was added to wells and bound Ab detected by alkaline phosphatase-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). For detection of bound Abs, serial dilutions of mouse serum samples prepared in RPMI 1640 containing 10% FBS were added to wells coated with phospholipids and bound Ab detected with AP-conjugated anti-mouse IgG or anti-mouse IgM Ab, followed by p-nitrophenylphosphate (Sigma-Aldrich) at 1 mg/ml. Relative units of Ab were calculated by comparing OD at 405 nm for individual titrated serum with standard curve of OD measurements of titrated standard high-titer polyclonal antiphospholipid Abs. OD at 405 nm for all sera was expressed as a percentage of the OD of the sample tested. Statistical analysis was done using Prism 4 (Graphpad). Infarct volumes were compared using ANOVA or Student t test, and neurologic deficits were compared using the Kruskal–Wallis or Mann–Whitney U test, as applicable. The p values <0.05 were considered significant.

**Neurological deficit**

Neurological deficit was determined, independent, and blinded, as described (26). Scoring was assigned as follows: 0, normal motor function; 1, torso and contralateral forelimb flexion when lifted by tail; 2, contralateral circling when held by tail on flat surface, though normal at rest; 3, contralateral leaning when at rest; and 4, no spontaneous motor activity.

**Histopathology**

Brains were sectioned using a Rodent Brain Matrix and placed in 4% paraformaldehyde for 48 h at 4°C. Brains were then either processed to paraffin or immersed in 20% sucrose in paraformaldehyde and embedded in OCT medium for cryosectioning. Paraffin sections were stained with Luxol Fast Blue/Nissl stain for morphological analysis, as previously described (4).

**Immunohistochemistry**

Paraffin sections were cut at 5 μm and deparaffinized. Sections were exposed to heated citrate buffer (pH 6) for 2 min to 10 min for Ag retrieval and blocked with normal horse serum (Vector Laboratories, Burlingame, CA). C3d deposition was detected using a goat anti-mouse C3d (1:20; R&D Systems, Minneapolis, MN), and IgM binding was detected using a goat anti-mouse IgM (1:50; Sigma-Aldrich). Primary Abs were detected using the goat-IMPress (Vector Laboratories), and negative controls omitted primary Abs. Slides were coverslipped with Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI) and imaged by light microscopy.

**Immunofluorescence**

Cryosections were cut at 8 μm, fixed in cold acetone, washed in running water, and equilibrated in PBS. Double staining for C3d and IgM deposition was performed. Goat anti-mouse C3d (1:20; R&D Systems) was applied and detected with rat anti-goat IgG Alexa Fluor-555 conjugate (1:200; Invitrogen, Carlsbad, CA). After washing with PBS, anti-mouse IgM FITC conjugate (1:50; Sigma-Aldrich) was applied, followed by ToPro-3 (1:5000; Invitrogen) as a nuclear marker. Slides were coverslipped with Vecta fluorescent hard mount (Vector Laboratories) and imaged on a Leica TCS-SP2 confocal microscope (Leica Microsystems).

**Annexin IV purification and treatment**

Recombinant annexin IV was generated and purified as previously described (8). In one experiment, annexin IV was administered i.v. to C57BL/6 mice (100 μg in 100 μl PBS) 5 min prior to reperfusion. In a second experiment, annexin IV (100 μg in 100 μl PBS) was administered i.v. to Rag1−/− mice 5 min prior to infusion of 300 μl freshly isolated normal mouse serum, which was administered just prior to reperfusion. Briefly, recombinant protein was expressed in transformed Escherichia coli by 0.3 mm isopropyl β-D-thiogalactoside. Bacteria were collected and lysed, and, following centrifugation, the supernatant was adjusted to pH 7.6 and run on a TALON resin column (BD Clontech, Mountain View, CA). A discontinuous urea gradient was used to refold the protein, and an imidazole gradient was used to elute the protein. Coomassie staining confirmed recombinant protein purity.

**In vitro binding of IgM**

Mouse brain endothelial cells (bEnd.3; American Type Culture Collection, Manassas, VA) and human endothelial cells (HUVEC; Lonza, Walkersville, MD) were grown to at least 80% confluence in Endothelial Growth Media (Lonza) on chambered microscope slides (Nunc, Rochester, NY). Slides were washed with PBS and incubated in DMEM serum-free medium for 3 h in a Coy Anaerobic Chamber (Coy Laboratory products, Grass Lake, MI) with O2 monitoring in not >0.1% oxygen. The medium was then supplemented with IgM mAb (15 μg/ml) and incubation continued under normoxic cell-culture conditions for 3 h. The slides were fixed in cold acetone, and IgM was detected with an IgG anti-mouse IgM-FITC (1:100; Sigma-Aldrich). Slides were coverslipped with Vecta fluorescent hard mount (Vector Laboratories) and imaged on a Leica TCS-SP2 confocal microscope (Leica Microsystems). Difficulties in detaching viable cells after hypoxic culture precluded analysis by flow cytometry.

**Statistical analysis**

Statistical analysis was done using Prism 4 (Graphpad). Infarct volumes were compared using ANOVA or Student t test, and neurologic deficits were compared using the Kruskal–Wallis or Mann–Whitney U test, as applicable. The p values <0.05 were considered significant.
Results

**C2 mAb recognizes a subset of phospholipids**

Kulik et al. (8) previously reported a strategy to isolate and identify mAbs that bind to neoeptopes exposed on ischemic cells. The strategy involved the use of isolated intestinal epithelial cells expressing ischemia or apoptosis-associated neoeptopes to screen and identify hybridomas created from B cells of wt C57BL/6 mice (8). In this study, we characterize three of the IgM mAbs isolated by this technique (D5, B4, and C2) in a model of murine ischemic stroke. The specificity of two of these mAbs has been previously determined (8): B4 mAb recognizes annexin IV, and D5 mAb, used in this study as an isotype control Ab, recognizes cytookeratin 19. C2 mAb did not react in Western blots of intestinal epithelial cells (not shown), and because altered phospholipid exposure on apoptotic/ischemic cells has been suggested as a target for pathogenic natural Abs (20, 27), we investigated C2 mAb specificity in antiphospholipid ELISAs. C2 was shown to recognize a subset of phospholipids that included phosphatidylcholine, PE, and cardiolipin (CL), but not PG or PS (Fig. 1). B4 mAb and D5 mAb did not recognize any phospholipid tested (Fig. 1).

**mAbs B4 and C2 restore injury in Rag1−/− mice following ischemic stroke**

C57BL/6 wt and Ab-deficient Rag1−/− mice were subjected to 1 h MCAO-induced cerebral ischemia followed by 24 h reperfusion. Rag1−/− mice showed significantly improved survival at 24 h compared with wt controls, with 100% (18 out of 18) and 59% (10 out of 16) of Rag1−/− and wt mice surviving, respectively (p = 0.006). Infarct size was also significantly reduced 24 h postreperfusion in Rag1−/− mice compared with surviving wt mice (Fig. 2B), and this is in agreement with previous data (21, 22). Rag1−/− mice also displayed an improved neurologic function (Fig. 2C), correlating with the reduction of infarct volume.

It was shown previously that B4 mAb restores intestine IRI in otherwise protected Rag1−/− mice, identifying annexin IV as a postischemic neoeptope expressed in the intestine (8). B4 mAb, but not D5 mAb, also restored cerebral IRI in Rag1−/− mice in terms of infarct size and neurologic outcome, demonstrating that annexin IV is also expressed postischemically in the brain (Fig. 2). C2 mAb also restored cerebral IRI in Rag1−/− mice. To investigate whether there may be a quantitative difference in the postischemic exposure of annexin IV and a subset of phospholipids in terms of IgM-dependent cerebral IRI, we performed mAb dose-response reconstitution experiments. There was no significant difference between the ability of B4 mAb or C2 mAb to restore post-IRF infarct volume or neurologic deficit in Rag1−/− mice (Fig. 2). In a separate experiment, we also demonstrated that C2 mAb restored intestinal IRI in Rag1−/− mice (data not shown).

The effect of Rag1 deficiency and Ab reconstitution on cerebral IRI was further investigated by analysis of demyelination at 24 h postreperfusion. Luxol Fast Blue and Nissl staining of brain sections revealed less myelin loss in the ipsilateral brain of Rag1−/− mice compared with wt mice, with restoration of myelin loss in Rag1−/− mice reconstituted with B4 and C2 mAbs (Fig. 3). The contralateral brain of all groups was unaffected following MCAO or mAb administration (Fig. 3).

As expected, there was no damage observed in the contralateral hemisphere in any group (not shown). Also, to confirm that cerebral blood flow was interrupted by the MCAO procedure, blood flow was measured by laser Doppler before ischemia, during ischemia, and 10 min postischemia (4). There were no significant differences in cerebral blood flow between any of the groups (not shown). Changes in blood pressure and body temperature can significantly influence the outcome post stroke, and we therefore measured blood pressure, heart rate, and temperature before, during, and after ischemia in Rag1−/− and wt mice. There were no differences between the groups (Supplemental Table I).

**Analysis of IgM binding and C3 deposition following ischemic stroke**

The deposition of C3 in postischemic mouse brains has been shown previously, and C3 deficiency or inhibition protects against ischemic stroke (3, 4). To investigate the relationship between postischemic IgM binding and complement activation, we investigated IgM and C3 deposition in infarcted areas of the brain at 24 h postreperfusion (C3 is a cleaved activation fragment of C3). As would be expected, there was no detectable IgM in postischemic mouse brains. We therefore measured blood pressure, heart rate, and temperature before, during, and after ischemia in Rag1−/− and wt mice. There were no differences between the groups (Supplemental Table I).

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epitopes in the postischemic brain with subsequent IgM-mediated activation of complement.

Recombinant annexin IV blocks cerebral IRI in wt mice
Reconstitution of cerebral IRI in \( \text{Rag1}^{+/+} \) mice with either B4 or C2, but not D5, shows that specific IgM is sufficient to mediate cerebral injury. Previous data from Carroll’s group (9, 17) has shown that an IgM mAb recognizing nonmuscle myosin on postischemic tissue is also capable of reconstituting IRI, at least in the intestine, heart, and hind limb. Thus, multiple neoepitopes are expressed on postischemic tissue, and to investigate whether a single Ab reactivity is sufficient to develop cerebral IRI in the context of an entire natural Ab repertoire, we investigated the effect of recombinant annexin IV on cerebral IRI in wt mice. Recombinant annexin IV (100 \( \mu \)g) or vehicle control (PBS) was injected into wt mice 5 min before reperfusion. Recombinant annexin IV significantly reduced infarct volumes, with percentage infarct similar to that seen in Ab-deficient \( \text{Rag1}^{+/+} \) mice (Fig. 5A). There was also a strong trend toward reduced neurologic deficit in annexin IV-treated mice (Fig. 5B), although this did not reach significance.

Normal mouse serum restores injury in \( \text{Rag1}^{+/+} \) mice following ischemic stroke
To address the physiological relevance of the above mAb reconstitution experiments in the context of cerebral IRI, we determined whether normal mouse serum was capable of restoring infarct size and neurologic deficit in \( \text{Rag1}^{+/+} \) mice. After 1 h of MCAO and upon reperfusion, \( \text{Rag1}^{+/+} \) mice were administered 300 \( \mu \)l of freshly isolated pooled C57BL/6 mouse serum via tail vein injection. At 24 h following ischemia, \( \text{Rag1}^{+/+} \) mice that were reconstituted with mouse serum had significantly larger infarct volumes (Fig. 6A) compared with wt C57BL/6 mice (8, 28), and postischemic blockade of annexin IV or phospholipid neoepitopes represent a potential therapeutic strategy to inhibit IRI. Also, direct translation development of B4 and C2 mAb derivatives may be feasible. We therefore determined whether human endothelial cells express B4 and C2 epitopes following exposure to hypoxia. HUVEC were exposed to a period of hypoxia by guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from

FIGURE 2. Effect of \( \text{Rag1} \) deficiency and IgM mAb reconstitution on postischemic infarct size and neurologic deficit. A, Representative 2% triphenyltetrazolium chloride staining of gross brains 24 h following ischemic stroke. B, \( \text{Rag1}^{+/+} \) mice had significantly reduced infarct volume compared with wt C57BL/6 mice following 60 min MCAO and 24 h reperfusion. Reconstitution with C2 or B4 mAb restored infarct size in a dose-dependent manner. D5 mAb did not restore injury in \( \text{Rag1}^{+/+} \) mice, \( n = 8 \) (mAb groups). Results are expressed as mean ± SD. \(^{a}p < 0.0012 \) versus C57BL/6, \(^{b}p < 0.001 \) versus \( \text{Rag1}^{+/+} \). \(^{c}p < 0.0011 \) versus B4 100 \( \mu \)g. C, Neurological scoring at 24 h postreperfusion was significantly improved in \( \text{Rag1}^{+/+} \) mice compared with C57BL/6 wt mice and \( \text{Rag1}^{+/+} \) mice reconstituted with B4 or C2 mAb. \( n = 10 \) (C57BL/6 and \( \text{Rag1}^{+/+} \)) and \( n = 8 \) (mAb groups). Horizontal line represents median score. \(^{#}p < 0.05 \) versus C57BL/6.
showed strong binding of both B4 and C2 mAbs to hypoxic and normoxic mouse brain endothelial cells (bEnd.3). As with HUVEC, both mAbs bound strongly to hypoxic, but not normoxic, cells (Fig. 8B).

**Discussion**

In this study, we identified a novel IgM mAb (C2) that recognizes a subset of phospholipids and that reconstitutes cerebral IRI in Rag1<sup>−/−</sup> mice. The mAb was identified from a panel of mAbs recognizing intestine epithelial cells, a major cellular target of intestine IRI, using the same approach for the previous identification of anti-annexin IV B4 mAb (8). Previous studies have shown that anti-phospholipid and anti-annexin IV Abs are present in normal mouse and human serum (8, 20), and because both C2 and B4 mAbs restored cerebral IRI in Rag1<sup>−/−</sup> mice, the data indicate that distinct neoepitopes and their recognition by natural Abs are involved in the propagation of acute cerebral IRI. To put these findings in a more physiological context, we demonstrated that IgM Abs that recognize a similar phospholipid subset as C2 mAb are present in normal mouse serum and that normal mouse serum restored cerebral IRI in Rag1<sup>−/−</sup> mice. We further showed that IgM is deposited in infarcted areas of wt mouse brains following ischemic stroke, and recombinant annexin IV significantly reduced infarct volumes in wt mice and Rag1<sup>−/−</sup> mice reconstituted with normal mouse serum, demonstrating that a single Ab reactivity is sufficient to develop injury in the context of an entire natural Ab repertoire. Also, an IgM mAb isolated from the same panel that contained C2 and B4 mAbs [D5 mAb, anti-cytokeratin 19 (8)] did not restore cerebral IRI in Rag1<sup>−/−</sup> mice, demonstrating that injury was dependent on specific recognition of self-Ag and not passive deposition of IgM. Postischemic binding of IgM activates complement as indicated by the colocalization of IgM with C3d in wt mice and Rag1<sup>−/−</sup> mice reconstituted with normal mouse serum, demonstrating that a single Ab reactivity is sufficient to develop injury in the context of an entire natural Ab repertoire. 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deficit was not significantly different between groups at 24 h (studies on the role of T cells in cerebral IRI in is not known, but it is not dependent on Ag recognition, TCR (a pathogenic role in IRI. For example, T and B cell-deficient findings in other organs in which T cells have been shown to play mechanisms can contribute to cerebral IRI. There are similar in the current study, we demonstrate Ab-dependent and T cell- mice (21, 22, 29) have demonstrated Ab-independent injury. In the current findings. Although the T cell population is not affected in stroke (22), which does bring into question the role of both B cells shown that B cell-deficient mice are not protected from ischemic substituted in the previous studies. Nevertheless, it has also been shown that the one hand and the production of protective IL-10 by mature B cells on the other (31). Also of interest, B cells and pathogenic Abs have been shown to play an important role in posttraumatic spinal cord injury, a condition involving ischemia and reperfusion of the spinal cord (32). Although it was shown that pathogenic Abs mediated spinal cord pathology, the primary source and specificity of Abs was not determined, and natural Abs produced by B-1 cells are a potential source. Natural Abs contribute to host defense against infection and serve homeostatic functions in the immune system, as well as contribute to pathogenic processes, as described in this study. Anti-

Figure 5. Effect of recombinant annexin IV on posts ischemic infarct size and neurologic deficit in C57BL/6 wt mice. Mice were subjected to 60 min MCAO and 24 h reperfusion with injection of 100 µg recombinant annexin IV immediately after reperfusion. A, Infarct volume was significantly reduced in mice treated with 100 µg Annexin IV (\( p < 0.003 \) versus control, \( n = 4 \)). Results are expressed as mean ± SD. B, Neurological deficit was not significantly different between groups at 24 h (\( p = 0.058, \ n = 4 \)). Horizontal line represents median score.

Figure 6. Postischemic infarct size and neurologic deficit in mice administered normal mouse serum and annexin IV. Rag1\(^{1-/-}\) or wt mice were subjected to 60 min MCAO and 24 h reperfusion, with injection of 300 ul normal mouse serum, with or without coadministration of 100 µg annexin IV, prior to reperfusion. A, Infarct volume was significantly increased in Rag1\(^{1-/-}\) mice treated with normal mouse serum, but the increase in infarct volume was reversed with coadministration of annexin IV. Infarct volumes in wt mice treated with normal mouse serum were similar to Rag1\(^{1-/-}\) mice treated with normal mouse serum, indicating that serum infusion alone does not contribute to injury. There was no infarct in sham-operated wt mice that were treated with normal mouse serum. Results are expressed as mean ± SD. * \( p < 0.001 \) (Rag1\(^{1-/-}\), Rag1\(^{1-/-}\) + annexin IV/normal mouse serum, sham + normal mouse serum, \( n = 4 \)) and (Rag1\(^{1-/-}\) + normal mouse serum, wt + normal mouse serum, \( n = 5 \)). B, Neurological deficit at 24 h postreperfusion was significantly impaired in Rag1\(^{1-/-}\) mice treated with normal mouse serum, but not in Rag1\(^{1-/-}\) mice treated with both mouse serum and annexin IV. Neurological deficit in wt mice treated with normal mouse serum were similar to Rag1\(^{1-/-}\) mice treated with normal mouse serum, indicating that serum infusion alone does not contribute to neurologic deficit, in accordance with data for infarct volume. Horizontal line represents median score. * \( p < 0.05 \) (Rag1\(^{1-/-}\) and Rag1\(^{1-/-}\) + annexin IV/normal mouse serum, \( n = 4 \)) and (Rag1\(^{1-/-}\) + normal mouse serum, wt + normal mouse serum, \( n = 5 \)). NMS, normal mouse serum.

Previous studies have shown that T cells traffic to the posts ischemic brain within 24 h of reperfusion and play a role in the pathophysiology of ischemic stroke. Indeed, reconstitution of Rag1\(^{1-/-}\) mice with either CD4\(^+\) or CD8\(^+\) T cells restores injury after cerebral IRI in the same model of transient ischemic stroke used in this study (22). The mechanism of T cell-dependent injury is not known, but it is not dependent on Ag recognition, TCR costimulation or a prothrombotic effect (21). These and other studies on the role of T cells in cerebral IRI in Rag1\(^{1-/-}\) and SCID mice (21, 22, 29) have demonstrated Ab-independent injury. In the current study, we demonstrate Ab-dependent and T cell-independent injury in Rag1\(^{1-/-}\) mice, indicating compensatory mechanisms can contribute to cerebral IRI. There are similar findings in other organs in which T cells have been shown to play a pathogenic role in IRI. For example, T and B cell-deficient (Rag1\(^{1-/-}\) and/or SCID) mice are also protected from intestinal and myocardial IRI, and injury can be restored by independent reconstitution of either T cells or IgM (reviewed in Ref. 30).

Reconstitution of Rag1\(^{1-/-}\) mice with B cells prior to ischemia in the same model we use in this study does not restore cerebral IRI (21), and although this finding appears to rule out a direct role for B cells in acute murine ischemic stroke, it does not exclude a role for Abs (which must be synthesized by transferred B cells) and, in particular, natural Abs because they are mainly the product of peritoneal B-1 cells and which were not specifically reconstituted in the previous studies. Nevertheless, it has also been shown that B cell-deficient mice are not protected from ischemic stroke (22), which does bring into question the role of both B cells and Abs in cerebral IRI and appears to be in contradiction to the current findings. Although the T cell population is not affected in B cell-deficient mice, the apparent discrepancy may be due to the fact that B cells can have both protective immune suppressive function as well as pathogenic proinflammatory functions. Indeed, a previous study showed that specific depletion of peritoneal B-1 cells did not alter overall circulating levels of IgM, but did reduce renal IgM binding and protected kidney function following renal IR (31), whereas mice completely deficient in mature B cells sustained more severe renal IRI compared with wt mice. The dual role of B cells was ascribed to the production of pathogenic natural IgM by B-1 cells that bound to posts ischemic mesangium on the one hand and the production of protective IL-10 by mature B cells on the other (31). Also of interest, B cells and pathogenic Abs have been shown to play an important role in posttraumatic spinal cord injury, a condition involving ischemia and reperfusion of the spinal cord (32). Although it was shown that pathogenic Abs mediated spinal cord pathology, the primary source and specificity of Abs was not determined, and natural Abs produced by B-1 cells are a potential source.

Natural Abs contribute to host defense against infection and serve homeostatic functions in the immune system, as well as contribute to pathogenic processes, as described in this study. Anti-
phospholipid Abs have long been recognized as a significant component of the natural Ab repertoire, and Abs to PC (a component of phosphatidylcholine) appear to be a dominant specificity (33). Further, natural Abs for annexin IV, a soluble cytosolic Ca\(^{2+}\)-dependent membrane binding protein family that has been identified in extracellular fluids and bound to cellular surfaces (34), represent a potential ischemic injury catalyst. Annexin IV is expressed on early apoptotic cells (35) and elevated in the brain in ethanol-induced injury (36, 37) and ischemic injury (36) and, as previously shown in an intestinal murine IRI model (8), can be bound by specific natural Abs to elicit injury. We demonstrated that the C2 mAb binds to PC, as well as certain other phospholipids, such as CL and PE. Thus, the C2 mAb binding epitope is presented on various phospholipids, and accessibility of the epitope depends on the polar head because negatively charged phospholipids are not bound by C2 mAb. Our data indicate that the epitope recognized by C2 mAb is only exposed on injured or stressed cells. Further, of the antiphospholipid IgM specificities analyzed, anti-PC IgM was present in C57BL/6 sera at the highest relative levels. It is important to note that anti-PC natural Abs do not bind nonoxidized phosphatidylcholine, which explains why they do not interact with healthy cells. The PC headgroup of phosphatidylcholine is exposed following oxidative damage to the polyunsaturated fatty acid side chain in position 2 of the glycerol backbone (38). In addition, apoptotic cell death can also lead to caspase-3 activation of the calcium-independent phospholipase A\(_2\) that can remove the fatty acid at the \(\alpha\)-2 position of phosphatidylcholine to generate lyso-phosphatidylcholine, which is also recognized by PC-specific Abs (27, 39). Thus, the maintenance of a high level of anti-PC Ab can be expected to promote the efficient clearance of injured and apoptotic cells. Significantly, it has been shown that reduction in the level of anti-PC Ab is observed in many pathological conditions, such as Alzheimer’s disease, rheumatoid arthritis, multiple sclerosis, and others (40–44).

IgM can activate both the classical and lectin pathways, and previous studies indicate that IgM-mediated activation of the lectin pathway drives injury after intestinal and myocardial ischemia and reperfusion (14, 16). With regard to ischemic stroke, it has been shown that MBL-deficient mice have reduced infarct following cerebrovascular injury after intestinal and myocardial ischemia and reperfusion (15, 6) and that genetically defined MBL deficiency is associated with improved outcome after acute stroke in humans (5, 38). Additionally, a recent study by Ducruet et al. (45) found that IgM and C3 localized together in the ischemic cerebral vasculature immediately following reperfusion and that MBL is the

**FIGURE 7.** The wt sera from C57BL/6 mice show presence of Ab binding to phospholipids. Ab binding was detected by ELISA as described in the text. Normal serum samples were tested for levels of IgM Ab (A) and IgG Ab (B) recognizing the following panel of phospholipids: PC-BSA, CL, PG, PE, and PS. Each dot represents a single animal.

**FIGURE 8.** Recognition of hypoxic mouse and human endothelial cells by monoclonal Abs in vitro. HUVEC (A) and mouse brain endothelial cells, bend.3 (B), were subjected to 3 h hypoxia followed by 3 h reoxygenation and C2, B4, and D5 mAb binding determined by immunofluorescence (D5 mAb shown only for HUVEC). Normal culture conditions were used as normoxic control. C2 and B4 mAbs bound to hypoxic, but not normoxic human and mouse endothelial cells. D5 control mAb did not bind to either normal or hypoxic HUVEC. Representative images from three experiments. Original magnification \(\times63\).
first complement protein involved in this ischemic complex. The classical pathway does not appear to play a role in murine ischemic stroke (3), and studies using C1-inhibitor (39), which also inhibits the lectin pathway (40), implicates the lectin pathway. Interestingly, Ducruet et al. (45) also observed that the protective effect afforded by genetic MBL deficiency was lost in the subacute phase of stroke. Although further work is needed, these data support a putative role for complement in neurogenesis subsequent to ischemic stroke (46, 47). Thus, together with the data presented in this study, it is likely that complement-dependent injury after cerebral ischemia and reperfusion is mediated by natural Ab-mediated activation of the lectin pathway, but the role of IgM and the lectin pathway in neurogenesis and long-term outcomes after stroke remains unclear.

In summary, natural IgM has been shown to play an important role in the pathogenesis of IRI in certain organs via complement activation, but its role in cerebral IRI was unknown. In this study, we show that pathogenic IgM plays an important role in activating complement and driving cerebral injury after ischemic stroke, and we identify the involvement of two distinct self-reactive Ab specificities. Previous studies have shown that normal human sera contain IgM reactivity to phospholipids recognized by C2 mAb (28) and to annexin IV (8). In this study, we show that hypoxic, but not normoxic, human endothelial cells also bind C2 and B4 mAb, indicating that similar recognition processes occur in mouse and man.

Disclosures

The authors have no financial interests of conflict.

References


