A murine skeletal muscle ischemia-reperfusion injury model: differential pathology in BALB/c and DBA/2N mice

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Carter, W. O., C. Bull, E. Bortolon, L. Yang, G. J. Jesmok, and R. H. Gundel. A murine skeletal muscle ischemia-reperfusion injury model: differential pathology in BALB/c and DBA/2N mice. J. Appl. Physiol. 85(5): 1676–1683, 1998.—Ischemia-reperfusion injuries can occur with diseases such as myocardial infarction and stroke and during surgical procedures such as organ transplantation and correction of aortic aneurysms. We developed a murine model to mimic abdominal aortic aneurysm repair with cross-clamping of the aorta distal to the renal artery. After model development, we compared the normal complement BALB/c mouse with the C5-deficient DBA/2N mouse. To assess quantitative differences, we measured neuromuscular function up to 72 h after ischemia with a subjective clinical scoring system, as well as plasma creatine phosphokinase, lactate dehydrogenase, and muscle histopathology scores of the cranial tibialis and quadriceps. Muscle histopathology scores of BALB/c mice compared with those in DBA/2N mice and sham-surgery mice. Muscle histopathology scores of the cranial tibialis and quadriceps correlated well with clinical signs, creatine phosphokinase, and lactate dehydrogenase, and indicated the greatest pathology in these muscle groups. We developed a murine model of skeletal muscle ischemia-reperfusion injury that can utilize the benefits of murine genetic and transgenic models to assess therapeutic principles of this model. Additionally, we have shown a significant reduction in clinical signs, plasma muscle enzyme concentrations, and muscle pathology in the C5-deficient DBA/2N mouse in this model.

C5: cobra venom factor; creatine phosphokinase; muscle pathology

AORTIC ANEURYSMS ARE A SERIOUS, potentially life-threatening disease for which the only effective treatment is surgical replacement of the aorta (13, 17). The etiology may be related to atherosclerosis, yet the aneurysm results from a weakening and disruption of the aortic wall layers with subsequent dilatation and the potential for rupture (13, 40). Abdominal aortic aneurysms are the most common type and account for ~75% of all aortic aneurysms. In abdominal aortic aneurysms, the dilatation typically occurs caudal to the renal artery. Cross-clamping of the aorta during the surgical repair induces ischemia of tissues distal to the clamp followed by reperfusion when the clamp is removed after surgical correction of the aneurysm.

In addition to abdominal aortic aneurysms, ischemia-reperfusion (I/R) injuries occur in many disease processes, including myocardial infarction, trauma, stroke, and organ transplantation. Skeletal muscle I/R injuries have been studied extensively in many disease models (23, 27, 29, 35, 38, 39, 44, 47). Whereas the ischemic injury itself is pathogenic, tissue reperfusion has been shown to significantly contribute and exacerbate the cellular injury (7). At least three main hypotheses describe the mediators involved in the reperfusion injury: 1) free radical generation, 2) calcium overloading, and 3) loss of sarcoplasmic phospholipids (7). These theories are supported by many publications demonstrating the therapeutic benefit of free radical scavengers (31, 47, 49), calcium-channel blockers (6, 16), and phospholipase inhibitors and membrane stabilizers (9, 34, 45). Several investigations have demonstrated the role of complement in I/R injuries and the therapeutic benefit of complement blockade in various injuries (1, 30, 36, 41, 42).

We developed a murine model of abdominal aortic aneurysm surgical repair that induced a skeletal muscle I/R injury. The only existing murine skeletal muscle I/R model is a cremaster model for the purpose of microvascular investigations (36). The value of a murine model is the availability of genetic and transgenic mice to assess therapeutic principles for this model. After development, we evaluated this model in C5-deficient DBA/2N mice.

MATERIALS AND METHODS

Animals. Experiments were performed on 20- to 25-g BALB/c or DBA/2N mice that were fed commercial chow ad libum and maintained on a normal light-dark cycle.

Experimental protocol. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (90 mg/kg). The ventral abdomen was shaved and prepared for sterile surgery with chlorhexidine soap and methyl alcohol. Sterile ophthalmic ointment was placed in each eye. Mice were placed on isothermo pads to maintain a constant body and muscle temperature. The internal temperature of the semitendinosus muscle was measured every 5 min during ischemia with a needle fast-response microprobe (1 cm, 29 gauge; Harvard Apparatus, South Natick, MA) and a portable thermocouple thermometer (Harvard Apparatus). The ischemic muscle was maintained at 34 ± 1°C. A ventral midline incision was made through the skin, and linea alba and abdominal organs were gently retracted to expose the descending aorta. The aorta was isolated from surrounding fat and fascia, and a 4-mm micro-serrefine (Fine Science Tools, Foster City, CA) was clamped on the aorta distal to the renal arteries. The abdominal incision was temporarily closed with a clamp during the ischemia to reduce evaporative loss during the surgical procedure. The micro-serrefine clamp was removed after an ischemia time of 105 min. Anesthesia was maintained for extended surgical times by dripping pentobarbital sodium into the peritoneal cavity as needed. One milliliter of 0.9% sterile saline was instilled into the abdomen before the abdominal incision was closed in two layers with 4–0 prolene.

For the purpose of complement depletion in five BALB/c mice, cobra venom factor (CVF; Quidel, San Diego, CA) was...
administered into the peritoneal cavity at a dose of 100 U/kg, 24 h before the ischemic injury.

The animals were scored clinically at 6, 24, 48, and 72 h after the start of reperfusion. The clinical scoring was blinded (performed by E. Bortolon and W. O. Carter) and consisted of a subjective neuromuscular evaluation that ranged from 0 to 8 (Table 1). Separate clinical scores were determined for each rear leg, and the summation was used for statistical analysis. Moribund mice were then euthanized, and all mice were euthanized at 72 h.

Tissue and blood collection. Three hours after the start of reperfusion, 150–200 µl of heparinized whole blood were collected by retroorbital bleed. One milliliter of heparinized whole blood was collected from the caudal vena cava when the mice were euthanized 72 h after the start of reperfusion. Tissue samples collected at the time of death included those from liver, spleen, kidney, heart, lung, small and large intestine, pancreas, spinal cord, and skeletal muscle (semimembranosus, quadriceps, cranial tibialis, and gastrocnemius). All tissues were fixed in 10% neutral buffered formalin, and the lung was infused with 10% neutral buffered formalin at 40 cm H2O. Additionally, cranial tibialis and gastrocnemius muscles were flash frozen in optimal cutting temperature medium (Sakura Finetek, Torrance, CA) and stored at −70°C for succinic dehydrogenase (SDH) histochemical analysis (4). A subjective histopathology score was recorded (performed blinded by W. O. Carter) for each muscle group and ranged from 0 to 10 (Table 2). A total muscle histopathology score (MHS) was recorded as the summation of all skeletal muscle group scores for one mouse.

Whole blood samples were used to measure total leukocytes, monocytes, lymphocytes, neutrophils, and platelets 72 h after the start of reperfusion. Plasma was used to measure lactate dehydrogenase (LDH), creatine phosphokinase (CPK), alanine transaminase, and urea nitrogen (UN) 3 and 72 h after the start of reperfusion and to measure creatinine, glucose, and albumin 72 h after the start of reperfusion.

Statistical analysis. The experimental results were evaluated by using ANOVA, unpaired t-tests, and simple and polynomial regression analyses utilizing StatView software. Numerical and graph values are expressed as means ± SE.

RESULTS

The survival rate for BALB/c sham-surgery mice (n = 13) and DBA/2N mice (n = 9) with I/R injury was 100%. The survival rate for BALB/c mice (n = 21) with I/R injury was 90%, and this was not significantly different from that of the other groups.

The clinical score for the BALB/c mice with I/R injury was significantly greater (P < 0.0001) than that for either the BALB/c sham-surgery mice or DBA/2N mice with I/R injury at 6, 24, 48, and 72 h (Fig. 1). The clinical score for the BALB/c mice with I/R injury was highest at 6 h after ischemia (11.61 ± 0.69) and gradually decreased with each time point to the lowest value at 72 h (8.90 ± 0.83). The clinical score for DBA/2N mice 6 h after ischemia (2.43 ± 0.48) was significantly higher (P < 0.01) than that of BALB/c sham-surgery mice (1.0 ± 0.16). There were no significant differences in clinical scores between DBA/2N mice and BALB/c sham mice at any other time points.

The plasma UN 3 h after ischemia was significantly higher (P < 0.01) in BALB/c mice with I/R injury (28.67 ± 1.81 mg/dl) than in DBA/2N mice with I/R injury (19.50 ± 1.50 mg/dl) and BALB/c sham-surgery mice (15 ± 1.13 mg/dl) (Fig. 2). The UN 3 h after ischemia was also significantly higher (P < 0.05) in DBA/2N mice than in sham-surgery mice. The UN 72 h after ischemia was significantly higher (P < 0.05) in BALB/c mice with I/R injury at 6, 24, 48, and 72 h (Fig. 1). The clinical score for the BALB/c mice with I/R injury was highest at 6 h after ischemia (11.61 ± 0.69) and gradually decreased with each time point to the lowest value at 72 h (8.90 ± 0.83). The clinical score for DBA/2N mice 6 h after ischemia (2.43 ± 0.48) was significantly higher (P < 0.01) than that of BALB/c sham-surgery mice (1.0 ± 0.16). There were no significant differences in clinical scores between DBA/2N mice and BALB/c sham mice at any other time points.

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Table 2. Histopathology scoring system

<table>
<thead>
<tr>
<th>Numerical Score</th>
<th>Skeletal Muscle Histopathology</th>
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<tbody>
<tr>
<td>0</td>
<td>No abnormal findings</td>
</tr>
<tr>
<td>1</td>
<td>Mild localized mononuclear cell infiltration</td>
</tr>
<tr>
<td>2</td>
<td>Mild multifocal mononuclear cell infiltration, rare necrotic fibers</td>
</tr>
<tr>
<td>3</td>
<td>Moderate generalized mononuclear cell infiltration, occasional necrotic fibers</td>
</tr>
<tr>
<td>4</td>
<td>Moderate cell infiltration including PMN, mild multifocal necrosis</td>
</tr>
<tr>
<td>5</td>
<td>Marked cell infiltration including PMN, moderate multifocal fiber necrosis</td>
</tr>
<tr>
<td>6</td>
<td>Marked cell infiltration including PMN, moderate generalized fiber necrosis</td>
</tr>
<tr>
<td>7</td>
<td>Severe cell infiltration including PMN, marked multifocal fiber necrosis</td>
</tr>
<tr>
<td>8</td>
<td>Severe cell infiltration, hemorrhage possible with severe fiber necrosis</td>
</tr>
<tr>
<td>9</td>
<td>Massive cell infiltration, hemorrhage possible with severe generalized fiber necrosis</td>
</tr>
<tr>
<td>10</td>
<td>Massive cell infiltration, and complete loss of tissue architecture</td>
</tr>
</tbody>
</table>

PMN, polymorphonuclear cells.

Table 1. Clinical scoring system

<table>
<thead>
<tr>
<th>Numerical Score</th>
<th>Clinical Signs</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No abnormal findings</td>
</tr>
<tr>
<td>1</td>
<td>Mild weakness or pain</td>
</tr>
<tr>
<td>2</td>
<td>Mild CP deficits, no paresis</td>
</tr>
<tr>
<td>3</td>
<td>Moderate CP deficits, no paresis</td>
</tr>
<tr>
<td>4</td>
<td>Moderate CP deficits, mild paresis</td>
</tr>
<tr>
<td>5</td>
<td>Severe CP deficits, moderate paresis</td>
</tr>
<tr>
<td>6</td>
<td>Severe CP deficits, severe paresis</td>
</tr>
<tr>
<td>7</td>
<td>Complete paralysis, nociception present</td>
</tr>
<tr>
<td>8</td>
<td>Complete paralysis, nociception absent</td>
</tr>
</tbody>
</table>

CP, conscious proprioception.
BALB/c mice with I/R injury (27.47 ± 1.91 mg/dl) than in BALB/c sham-surgery mice (23.46 ± 1.15 mg/dl). There were no significant differences in plasma creatinine levels 72 h after surgery among any of the groups. The plasma CPK 3 h after ischemia was significantly higher (P < 0.0001) in BALB/c mice with I/R injury (110,923 ± 25,055 IU/l) than in DBA/2N mice with I/R injury (12,907 ± 2,836 IU/l) and sham-surgery BALB/c mice (5,024 ± 525 IU/l) (Fig. 3). The plasma CPK 3 h after ischemia was also significantly higher (P < 0.05) in the DBA/2N mice after I/R injury than in sham-surgery BALB/c mice. The plasma LDH 3 h after ischemia was significantly higher (P < 0.0001) in BALB/c mice with I/R injury (9,867 ± 1,329 IU/l) than in DBA/2N mice with I/R injury (1,842 ± 278 IU/l) and in sham-surgery BALB/c mice (1,212 ± 143 IU/l) (Fig. 3). The plasma LDH 3 h after ischemia was also significantly higher (P < 0.05) in the DBA/2N mice after I/R injury than in sham-surgery BALB/c mice. The plasma LDH 72 h after ischemia was significantly higher (P < 0.01) in BALB/c mice with I/R injury (210 ± 47 IU/l) than in sham-surgery BALB/c mice (56 ± 13 IU/l). There was no significant difference in plasma CPK 72 h after surgery between DBA/2N mice and either BALB/c mice group. The plasma LDH 3 h after ischemia was significantly higher (P < 0.0001) in BALB/c mice with I/R injury (9,867 ± 1,329 IU/l) than in DBA/2N mice with I/R injury (1,842 ± 278 IU/l) and in sham-surgery BALB/c mice (1,212 ± 143 IU/l) (Fig. 3). The plasma LDH 3 h after ischemia was also significantly higher (P < 0.05) in the DBA/2N mice after I/R injury than in sham-surgery BALB/c mice. The plasma LDH 72 h after ischemia was significantly higher (P < 0.01) in BALB/c mice with I/R injury (210 ± 47 IU/l) than in sham-surgery BALB/c mice (56 ± 13 IU/l). There was no significant difference in plasma CPK 72 h after surgery between DBA/2N mice and either BALB/c mice group.

There were no significant differences in glucose, albumin, or alanine transaminase 72 h after surgery among any of the three animal groups. Additionally, there were no significant differences in absolute leukocyte count or monocyte count among any of the three animal groups. The absolute lymphocyte count was significantly reduced (P < 0.01) in BALB/c mice 72 h after ischemia compared with that in DBA/2N mice (1,261 ± 110 cells/µl) compared with that in sham-surgery BALB/c mice (1,829 ± 164 cells/µl) (Fig. 4). The absolute lymphocyte count was significantly reduced (P < 0.05) in BALB/c mice after ischemia compared with that in sham-surgery mice. Platelet count in BALB/c and DBA/2N mice 72 h after ischemia was significantly decreased (P < 0.01) compared with that in sham-surgery mice.
sham-surgery BALB/c mice. The absolute neutrophil count was significantly increased ($P < 0.05$) in BALB/c mice 72 h after ischemia (1,102 ± 118 cells/µl) compared with that in sham-surgery BALB/c mice (765 ± 106 cells/µl). There were no significant differences in absolute neutrophil count between DBA/2N mice and either BALB/c mice group. The total platelet count was significantly reduced ($P < 0.001$) in BALB/c mice 72 h after ischemia (660 ± 179×10³/µl) and DBA/2N mice with I/R injury (781 ± 32×10³/µl) compared with that in sham-surgery BALB/c mice (970 ± 33×10³/µl). The platelet count in sham-surgery DBA/2N mice (796 ± 32×10³/µl; n = 4 mice) was not significantly different from that in DBA/2N mice after I/R injury.

There were no significant differences between sham-surgery BALB/c mice and sham-surgery DBA/2N mice in any parameters except platelet count and murine C5 concentration. The platelet count was significantly increased ($P < 0.01$) in sham-surgery BALB/c mice compared with that in sham-surgery DBA/2N mice. The C5 concentration in five BALB/c mice ranged from 157 to 183.9 µg/ml, whereas C5 was undetectable in the five DBA/2N mice.

The histopathology score for the gastrocnemius muscle was significantly increased ($P < 0.05$) in BALB/c mice 72 h after ischemia (1.58 ± 0.46) compared with that in sham-surgery BALB/c mice (0.5 ± 0.15) (Fig. 5). There were no significant differences between DBA/2N mice and either BALB/c mice group. The semitendinosus MHS for BALB/c mice (3.32 ± 0.59) and DBA/2N mice (2.43 ± 0.53) 72 h after ischemia was significantly increased ($P < 0.001$) compared with that in sham-surgery BALB/c mice after ischemia (1.15 ± 0.41) and was significantly increased ($P < 0.01$) compared with that in DBA/2N mice after I/R injury (5.71 ± 1.97). The total MHS in DBA/2N mice was significantly increased ($P < 0.01$) compared with that in sham-surgery BALB/c mice. No other significant histopathology was identified in other tissues 72 h after the ischemia.

Linear regression analyses of the gastrocnemius MHS and clinical scores at 6 and 72 h, CPK at 3 h, and LDH at 3 h were $r^2 = 0.054, 0.077, 0.001, and 0.007$, respectively. Linear regression analyses of the semitendinosus MHS and clinical scores at 6 and 72 h, CPK at 3 h, and LDH at 3 h were $r^2 = 0.371, 0.432, 0.177, and 0.312$, respectively. Linear regression analyses of the quadriceps MHS and clinical scores at 6 and 72 h, CPK at 3 h, and LDH at 3 h were $r^2 = 0.649, 0.648, 0.587, and 0.628$, respectively. Linear regression analyses of the quadriceps MHS and clinical scores at 6 and 72 h, CPK at 3 h, and LDH at 3 h were $r^2 = 0.771, 0.762, 0.584, and 0.688$, respectively.

Histochemical analysis with SDH of the cranial tibialis muscle in BALB/c and DBA/2N mice showed heterogeneous and variable staining in various regions of the muscle. The ratio of type I to type II fibers ranged from 1:20 to 1:1 for BALB/c mice and from 1:10 to 1:1 for DBA/2N mice. No remarkable differences were evident with SDH staining.

There were no significant differences in clinical signs, hematology, plasma chemistry, or histopathology between CVF-treated BALB/c mice and untreated BALB/c mice after the I/R injury. The baseline murine C3 and C5 concentrations in BALB/c mice ranged from 79.4 to 87.6 and 157 to 183.9 µg/ml, respectively. Twenty-four hours after treatment with CVF, there was a significant reduction ($P < 0.001$) in murine C3 and C5 concentrations, and the concentrations ranged from 6.5 to 11.8 and 45.6 to 70.7 µg/ml, respectively.

**DISCUSSION**

The purpose of these experiments was to develop a murine model to mimic the ischemic damage associated...
with surgical correction of abdominal aortic aneurysms. Many animal models of skeletal muscle I/R injuries have been published (23, 27, 29, 35, 38, 39, 44, 47). However, the only murine I/R skeletal muscle model that we could identify was for microvascular observation of the cremaster muscle (36). The value of a murine model is that it allows investigators to utilize existing genetic and transgenic models to assess proof of principle experiments for therapeutic intervention. We utilized the C5-deficient DBA/2N mouse to evaluate the role of complement and specifically C5 in the I/R injury. We utilized the BALB/c mouse as a normal complement mouse for model development and comparison (30a).

Our initial experiments in BALB/c mice revealed relatively large variations in clinical responses to the I/R injury. We were able to eliminate most of this variation after we designated one breeding site from a single vendor to supply our mice. The cause for the variation was not determined. We also observed clinical signs and muscle damage that were directly proportional to the muscle temperature. Other investigators have reported a direct effect between muscle temperature and posts ischemic pathology (2, 29, 37). We used an intramuscular probe sensitive to \pm 0.1°C to maintain the muscle temperature in the ischemic legs at 34 \pm 1°C. This experimental modification also reduced relatively large variations among animals to acceptable values.

The clinical signs were assessed at 6, 24, 48, and 72 h after ischemia to closely monitor the health status of the mice. The clinical assessment score was a subjective neuromuscular assessment and included evaluations of nociception, conscious proprioception, weakness, paresis, and paralysis. The sham-surgery mice occasionally had clinical scores of 1, even though they had no direct muscle damage. A clinical score of 1 was consistent with mild muscle weakness in one leg and potentially reflected the postsurgical pain from the abdominal incision or reaffirmed the subjectivity and potential variability of the clinical assessment. The clinical score in DBA/2N mice (2.43 \pm 0.48) was significantly increased in comparison with that in sham-surgery mice for only the clinical assessment 6 h after ischemia. A typical clinical assessment in DBA/2N mice at 6 h would have consisted of mild weakness in one leg and mild conscious proprioception deficits in the contralateral leg. Yet the clinical score for BALB/c mice after ischemic injury was significantly and markedly increased at all time points measured compared with that in both the DBA/2N and sham-surgery mice. A typical clinical assessment in BALB/c mice 6 h after the ischemic injury would have consisted of severe paresis or complete paralysis in both legs. Seventy-two hours after the ischemic injury, there was some clinical improvement; however, a typical clinical assessment for BALB/c mice was still mild-to-moderate paresis in both legs. Although the investigators were blinded to the treatment groups, they could not be blinded to the color of the mice, which may have influenced the clinical scoring results between the BALB/c and DBA/2N mice. Nevertheless, the magnitude of the clinical results was very remarkable and could only partially be depicted by the results in Fig. 1. The BALB/c mice had a 10% mortality rate after the ischemic injury compared with no mortality in the DBA/2N and sham-surgery mice; however, the difference was not significant.

Plasma UN was measured 3 and 72 h after the ischemic injury. At both time points, the UN was significantly increased in the BALB/c mice compared with that in sham-surgery mice. Additionally, the UN in the DBA/2N mice was significantly increased at only the 3-h time point compared with that in the sham-surgery mice. Many factors can influence UN concentrations, including changes in renal filtration and dietary nitrogen and catabolism of tissues. In this particular model, two explanations were likely. A degree of pre-renal azotemia probably developed that was associated with the tissue trauma and fluid redistribution. However, because of the significant muscle necrosis that occurred in BALB/c mice, muscle catabolism was the most likely explanation for the increase in UN. Creatinine is a better measure of renal filtration because it is not readily affected by diet or catabolism, although the substantial muscle trauma could still slightly impact the creatinine, which is a nonenzymatic product of phosphocreatine (11). Creatinine was not measured 3 h after ischemia because of the small quantity of blood collected from the mice. However, 72 h after the ischemia, the creatinine concentration was not significantly different among any of the three groups, which suggested that the elevation in UN 72 h after the ischemia was related to muscle catabolism and not a direct effect on renal filtration or function.

Plasma CPK is a cytosolic enzyme found primarily in striated muscle and is used as an indicator of muscle damage. CPK has a short half-life and, in our model, had a peak plasma concentration between 2 and 3 h after the start of the reperfusion. The CPK concentrations in BALB/c mice 3 h after ischemic injury were significantly increased, \sim 10-fold above those in the DBA/2N mice and 20-fold above those in the sham-surgery mice. The CPK concentrations in DBA/2N mice were also significantly elevated above those in the sham-surgery mice; however, there was only slightly more than a twofold elevation. The plasma CPK concentrations 3 h after ischemic injury had very good linear correlations to quadriceps, cranial tibialis, and total MHS. This was expected because, as more muscle was damaged, as indicated by the MHS, more CPK was released from the damaged myocytes. Seventy-two hours after the ischemic injury, the CPK concentration in BALB/c was still significantly increased compared with that in sham-surgery mice; however, the increase was greatly reduced from the peak concentration 3 h after ischemic injury and was poorly correlated with MHS. Because of the short half-life of CPK, the mild increase 72 h after ischemic injury was probably associated with continued muscle inflammation and membrane leakage.

Plasma LDH is also a cytosolic enzyme found in muscle, but, additionally, it can be found in liver,
erythrocytes, and most tissues of the body (30a). Accordingly, it lacks tissue specificity but can still be a good assessment of tissue damage. Tissue isoenzymes of LDH and CPK can be used to increase tissue specificity but were not used in these studies. Plasma LDH concentrations in BALB/c mice 3 h after ischemic injury were significantly increased approximately fivefold above those in the DBA/2N mice and eightfold above those in the sham-surgery mice. There was a very significant linear correlation between plasma LDH and CPK concentration 3 h after ischemia, and, in general, all the CPK results were quite similar to LDH results. Accordingly, the LDH that was measured in these experiments was presumably from muscle tissue. The sham-surgery mice had significant elevations in CPK and LDH 3 h after surgery compared with 72 h after surgery. This was likely due to the abdominal surgical procedure and the placement of the muscle temperature probe.

Hematologic parameters were measured 72 h after the ischemic injury only because a sufficient amount of blood could not be collected from the mice 3 h after the ischemia. The absolute lymphocyte count was significantly reduced in BALB/c mice compared with that in sham-surgery mice and DBA/2N mice. This reduction was most likely a stress-induced lymphopenia. Additionally, circulating neutrophils increased significantly in BALB/c mice compared with those in sham-surgery mice. This increase was probably induced from increased tissue demand for phagocytosis of the cellular debris from the I/R injury. Many investigators have published results demonstrating a role for neutrophils and free radicals in I/R injuries (14, 19, 25, 36, 43). There was a significant reduction in circulating platelets in BALB/c mice and DBA/2N mice after the ischemic injury compared with those in sham-surgery BALB/c mice. The thrombocytopenia in BALB/c mice was likely associated with I/R-induced platelet sequestration and microvascular adhesion mediated by P-selectin (3, 12, 22). The platelet count in the DBA/2N mice after ischemic injury was not significantly different from that in the DBA/2N sham-surgery mice. Except for undetectable C5 concentrations, this was the only hematomic, chemical, or tissue pathological difference that we identified between the sham-surgery BALB/c mice and sham-surgery DBA/2N mice. Clinically, the difference between a platelet count of 1,000,000 and 800,000 cells/µl was not relevant. However, platelet function tests would need to be performed to thoroughly assess any potential differences in the mouse strains.

In our initial experiments, we observed striking differences in muscle pathology that existed among different muscle groups. Many investigators have reported conflicting results regarding the susceptibility of various muscle groups and fiber types to I/R injury (2, 18, 20, 21, 26, 32, 37, 47, 50). It is inviting to assume that susceptibility to I/R pathology is related to such distinct differences in fiber physiology. However, given the variability in the literature, one must assume that many factors, such as ischemia time, temperature, maintenance of blood flow during reperfusion, and animal variation, were not adequately controlled among these different experimental models. We evaluated four distinct muscle groups to assess and correlate MHS to a variety of parameters. Of the four muscle groups evaluated, the degree of pathology or MHS increased from gastrocnemius, semitendinosus, and quadriceps to cranial tibialis. Accordingly, as described above, plasma CPK and LDH had high linear correlations to quadriceps and cranial tibialis muscle groups, indicating that these muscle groups likely leaked the majority of the myocyte enzymes into the plasma. The total MHS, derived by the summation of the individual groups, had a slightly lower linear correlation to CPK and LDH than did the cranial tibialis muscle group. This was merely an averaging effect of the higher and lower correlations into one value. However, the total MHS still had a relatively high and significant linear correlation and was presumably the best assessment of the total muscle pathology.

We also assessed the correlation between clinical scores 6 and 72 h after ischemia and the MHS. The findings were very similar to the CPK and LDH results. In general, the highest linear correlations existed between the cranial tibialis MHS and clinical scores; however, the total MHS had relatively high and significant linear correlations. On the basis of the clinical neuromuscular assessment, neurological damage was expected in addition to muscular damage. However, 72 h after the ischemic injury, no light microscopic neuropathology was evident. On the basis of previous investigations, ultrastructural changes likely existed in the peripheral nerves or lumbar spinal cord (24, 28, 33, 50).

One of the remarkable findings of these experiments was the significant protection afforded DBA/2N mice compared with BALB/c mice. This protection included significant reductions in clinical scores at all time points, reductions in plasma CPK and LDH concentrations, and reductions in muscle pathology as indicated by the MHS. The purpose for selection of the DBA/2N mouse for this model development, along with the BALB/c mouse, was to utilize a mouse strain that was deficient in C5. We confirmed the presence of murine C5 in BALB/c mice and the absence of C5 in DBA/2N mice. To investigate the possibility that a difference between the BALB/c and DBA/2N in muscle-fiber composition could cause the difference in clinical response or pathology, we used the histochemical stain, SDH. SDH is a mitochondrial enzyme and provides a relative proportion of mitochondrial quantity in the cell. Accordingly, type I muscle fibers (slow oxidative) with more mitochondria stain dark, and type II muscle fibers (fast glycolytic) stain light. The SDH staining of the cranial tibialis muscle in BALB/c and DBA/2N mice revealed relatively similar distributions of type I and type II muscle fibers. To test whether complement was the critical factor, we attempted to deplete BALB/c mice of complement, specifically C5, with CVF. BALB/c mice pretreated with CVF demonstrated no significant difference in clinical signs, hematology, plasma chemistry,
and histopathology compared with untreated BALB/c mice after I/R injury. The C5 concentrations were significantly reduced in the CVF-treated mice, whereas DBA/2N mice had undetectable concentrations of C5. If C5 depletion was the therapeutic difference between DBA/2N mice and BALB/c mice, we must assume that more complete or total removal of C5 would be necessary to render a significant difference. To test this effect, a monoclonal antibody against murine C5 could validate the role of C5 in this model.

Various papers have reported other differential immune responses of BALB/c and DBA/2N mice. DBA/2 mice were shown to have reduced mucosal clearance of Candida compared with BALB/c mice, which was thought to be related to differential priming of T-cell subsets (5). Another group showed a 3- to 30-fold increase in interleukin-12 production in antigen-primed lymph-node cells from DBA/2 mice compared with that from BALB/c mice (15). DBA/2 mice were also shown to develop a fulminating lethal infection with Plasmodium compared with BALB/c mice, which developed a low or undetectable parasitemia (10). These results represent some immunologic differences between DBA/2 and BALB/c mice, but they fail to reveal a possible explanation for the differences observed in our results. It is tempting to assume that all the benefit realized by the DBA/2N in this model was because of the deficiency of C5 in this mouse strain. However, because this and essentially all mouse strains used in research today are excessively inbred, the possibility exists that a separate genetic mutation may be rendering the benefit in this model.

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