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Inhibition of leukocyte adherence by anti-CD18 monoclonal antibody attenuates reperfusion injury in the rabbit ear

(neutrophil/endothelium/ischemia)

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ABSTRACT **Tissue injury resulting from ischemia and reperfusion forms the basis of several important disorders including myocardial infarction, stroke, and circulatory shock. To examine the role of neutrophils in this process and to determine the extent to which injury is a consequence of reperfusion, we utilized the monoclonal antibody 60.3, directed to CD18, the human leukocyte adherence glycoprotein, to block intravascular neutrophil aggregation and neutrophil adherence to endothelium in a rabbit model of tissue ischemia and reperfusion. Antibody treatment either before ischemia or after ischemia, but prior to reperfusion, resulted in the same degree of significant protection against endothelial, microvascular, and tissue injury. We conclude that neutrophils and increased neutrophil adhesiveness are important in the development of microvascular and tissue injury after ischemia and reperfusion and that under these circumstances, injury is primarily a consequence of reperfusion.**

Ischemia-related cellular, tissue, and organ injury form the basis of many important clinical disorders, including myocardial infarction, stroke, vascular disease, organ transplantation, and circulatory shock. Much progress has been made in the area of early restoration or perfusion. However, there is evidence that under certain circumstances, a significant proportion of the injury associated with ischemia may be a consequence of events associated with reperfusion of ischemic tissues, i.e., "reperfusion injury" (1, 2). This concept has clinical implications since specific therapy usually cannot be instituted until after the ischemic event, whereas it may be feasible to administer therapy prior to reperfusion.

Oxygen-derived free radicals, generated within the tissues at the time of reperfusion, have been identified as potentially important mediators of this reperfusion injury (3). Another important source of free radicals is the neutrophil. Activated neutrophils can also release proteases and phospholipase products, all of which are capable of causing significant cellular and tissue injury (4, 5). Neutrophil-mediated endothelial injury has been demonstrated to result in loss of vascular integrity, edema, hemorrhage, thrombosis, and tissue necrosis. An important role for neutrophils in ischemia-reperfusion injury is suggested by studies that have shown a close association between neutrophil accumulation and tissue injury in this setting (6) and by studies that have demonstrated significant injury reduction by depletion of circulating neutrophils (7, 8).

Increased neutrophil adhesiveness is a critical early step in the sequence of events leading to neutrophil-mediated injury (4). An alternative approach to the investigation and perhaps to therapy of neutrophil-mediated injury has developed from studies that have identified a human neutrophil membrane glycoprotein heterodimer, designated CD11b/CD18 (Mac-1, Mol), which plays a major role in mediating neutrophil adhesiveness (9). We have shown that specific monoclonal antibodies (mAbs) directed to these glycoproteins effectively inhibit stimulated neutrophil aggregation, adherence to endothelium, and neutrophil-mediated endothelial injury *in vitro* and *in vivo* (10, 11). mAb-mediated inhibition of leukocyte adherence functions is effective at reducing injury in intestinal (12), myocardial (13), and whole-animal (14) models of ischemia–reperfusion. In all of these models, however, neutrophil adherence was inhibited far in advance of reperfusion, making it difficult to determine whether the injury was occurring during ischemia or with reperfusion.

To further examine the role of neutrophils in endothelial and tissue injury after ischemia and reperfusion, we utilized anti-CD18 mAb 60.3 to block intravascular neutrophil aggregation and neutrophil adherence to endothelium in a rabbit ear model of ischemia–reperfusion. To determine the extent to which the neutrophil-mediated injury is a result of reperfusion, we compared the effect of blocking neutrophil adherence functions prior to ischemia versus the effect of inhibition after ischemia but prior to reperfusion.

METHODS

New Zealand White rabbits (1.1–1.5 kg) were anesthetized i.v. with ketamine (30 mg/kg) plus xylazine (2 mg/kg) and underwent sterile operation with the aid of the operating microscope to transect one ear at its base, leaving intact only the central artery and vein (Fig. 1). All nerves were divided, rendering the ear completely anesthetic. A microvessel clip was placed across the artery to produce complete ischemia, and the ear was reattached with the clip exiting through the wound. The ear remained completely ischemic at an ambient temperature of 22°C for 10 hr at which time the clip was removed to effect reperfusion. Pretreatment animals ($n = 5$) received mAb 60.3 (2 mg/kg) i.v. 5 min prior to ischemia and again prior to reperfusion; post-treated animals ($n = 5$) received mAb 60.3 (2 mg/kg) immediately prior to reperfusion; control-ischemic animals ($n = 8$) received saline. Sham-operated animals ($n = 3$) underwent the same operation but were not made ischemic.

Tissue was examined by obtaining 3-mm-punch biopsies from the anesthetic ear. These were then fixed in formalin, stained with hematoxylin and eosin, and photographed at ×132. Ear volume was measured by water displacement to quantify edema. Necrosis was expressed as a percent of total ear skin surface, measured at 6 days. The experimental protocol was reviewed and approved by the University of Washington Animal Care Committee.

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Abbreviation: mAb, monoclonal antibody.

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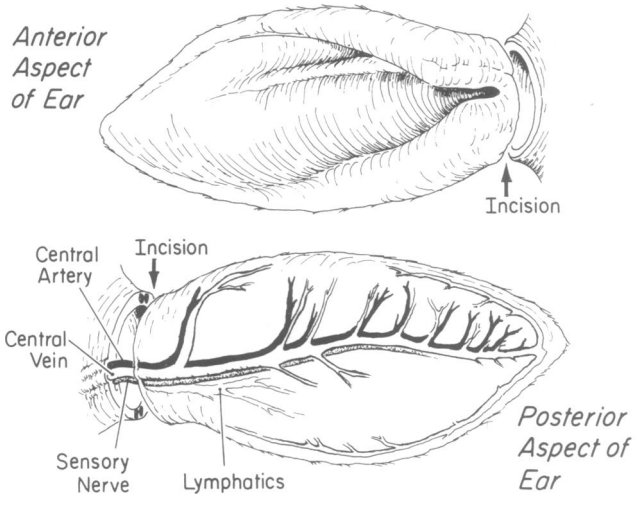

FIG. 1. Technique of producing complete ischemia in the rabbit ear. Careful microscopic dissection left only the central artery and vein intact (see text). A microvessel clip was placed across the artery for 10 hr to produce ischemia.

Statistics. Data were expressed as mean ± SEM and significance determined by analysis of variance for repeated measures or nonparametric methods.

mAb. mAb 60.3 is a murine IgG2a antibody that recognizes a functional epitope on CD18. mAb 60.3 was prepared as described (9) and used at 3 mg/ml in sterile 0.15 M NaCl (saline). This solution had no detectable endotoxin contamination by limulus assay (Sigma). This dose of mAb 60.3 had been determined by immunofluorescence to completely saturate CD18 binding sites and to prevent stimulated neutrophil immigration into experimental skin lesions for 24 hr (15). Saline was used as a control in these studies rather than an immunoglobulin. This was chosen because available isotype-matched control antibodies do not bind to rabbit neutrophils and all batches of nonspecific murine IgG tested were contaminated with endotoxin, producing fever and leukopenia in rabbits. Prior studies in rabbits using a nonbinding mAb (CJ7F3) demonstrated no nonspecific blocking effect of control antibody compared to mAb 60.3 (15).

RESULTS

Ten hours of ischemia followed by reperfusion produced severe microvascular injury in the control-ischemic animals. Loss of endothelial barrier function was manifested by significant edema formation with a 5-fold increase in ear volume at 24 hr (Figs. 2 and 3*A*). Microscopic evaluation of the tissue at 24 hr confirmed neutrophil-associated vascular injury with dense intra- and extravascular neutrophil accumulation and evidence of vascular destruction with parenchymal hemorrhage (Fig. 3*C*). Some of the smaller vessels were occluded by neutrophil aggregates and thrombus. The end result of this microvascular injury was significant tissue necrosis in several animals.

Inhibition of neutrophil adherence by administration of mAb 60.3 prior to ischemia prevented tissue accumulation of neutrophils at 24 hr and dramatically attenuated all aspects of the injury. Microvascular endothelium remained morphologically and functionally intact. Edema formation was significantly less than in control-ischemic animals (Fig. 2) and, in fact, not statistically different from sham-operated animals, suggesting that much of the initial injury observed was a result of the surgical procedure rather than the ischemic insult. Eventual tissue necrosis was similarly reduced (3.0 ± 2.0% versus 23.8 ± 11.1% in control-ischemic animals; $P < 0.05$ by Mann–Whitney U test).

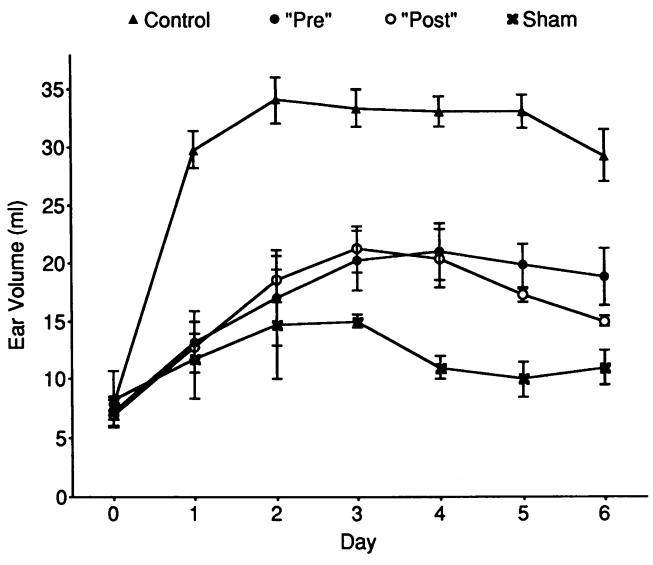

FIG. 2. mAb 60.3 attenuates edema formation after 10 hr of ischemia and then reperfusion. Days after reperfusion are indicated on the abscissa and ear volume is indicated on the ordinate. Edema was quantified by volume displacement. Mean values for sham-operated animals ($n = 3$) are represented by an "x". Values for control-ischemic animals ($n = 8$) are represented by closed triangles. Values for animals that received antibody prior to ischemia and again prior to reperfusion ("Pre"; $n = 5$) are represented by closed circles. Values for animals that received antibody only prior to reperfusion ("Post"; $n = 5$) are represented by open circles. Error bars represent ±SEM. Ear volumes in both antibody-pretreated and antibody-posttreated animals were significantly less than in control-ischemic animals ($P < 0.005$ by analysis of variance), but not significantly different from sham-operated animals ($P > 0.05$). Ear volumes in antibody-pretreated and antibody-posttreated animals were not different from each other ($P > 0.05$).

To determine the extent to which this neutrophil-mediated injury was a consequence of reperfusion of ischemic tissue (i.e., "reperfusion injury"), we examined the effect of antibody administration after ischemia but prior to reperfusion. We found that the degree of protection provided by blocking neutrophil adherence at the time of reperfusion was equal to the protection provided by treatment before ischemia and again at reperfusion. Edema formation, as in the pretreated animals, was significantly less than control-ischemic animals and not significantly different from sham-operated animals (Figs. 2 and 3*B*). Morphologically, neutrophil accumulation and cellular injury were absent (Fig. 3*D*). Overall tissue necrosis was also significantly less than control-ischemic animals (0 ± 0% versus 23.8 ± 11.1% in controls; $P < 0.05$ by Mann–Whitney U test). Tissue necrosis was not significantly different from antibody pretreated animals ($P > 0.05$).

DISCUSSION

An association between leukocytes and ischemic tissue injury has been known for many years. Until recently, however, leukocytes were thought to have a role only in the healing process that follows ischemic injury (16). Studies in myocardium (17), liver (18), and intestine (6) have all shown a dramatic increase in tissue leukocytes after ischemia and reperfusion. Evidence implicating leukocytes in the actual pathogenesis of ischemia–reperfusion injury has come from studies demonstrating significant injury reduction in neutrophil-depleted animals (19).

There are several mechanisms by which neutrophils can cause injury in the setting of ischemia–reperfusion. Being relatively large viscoelastic cells, neutrophils have a slow transit time through the microcirculation under normal con-

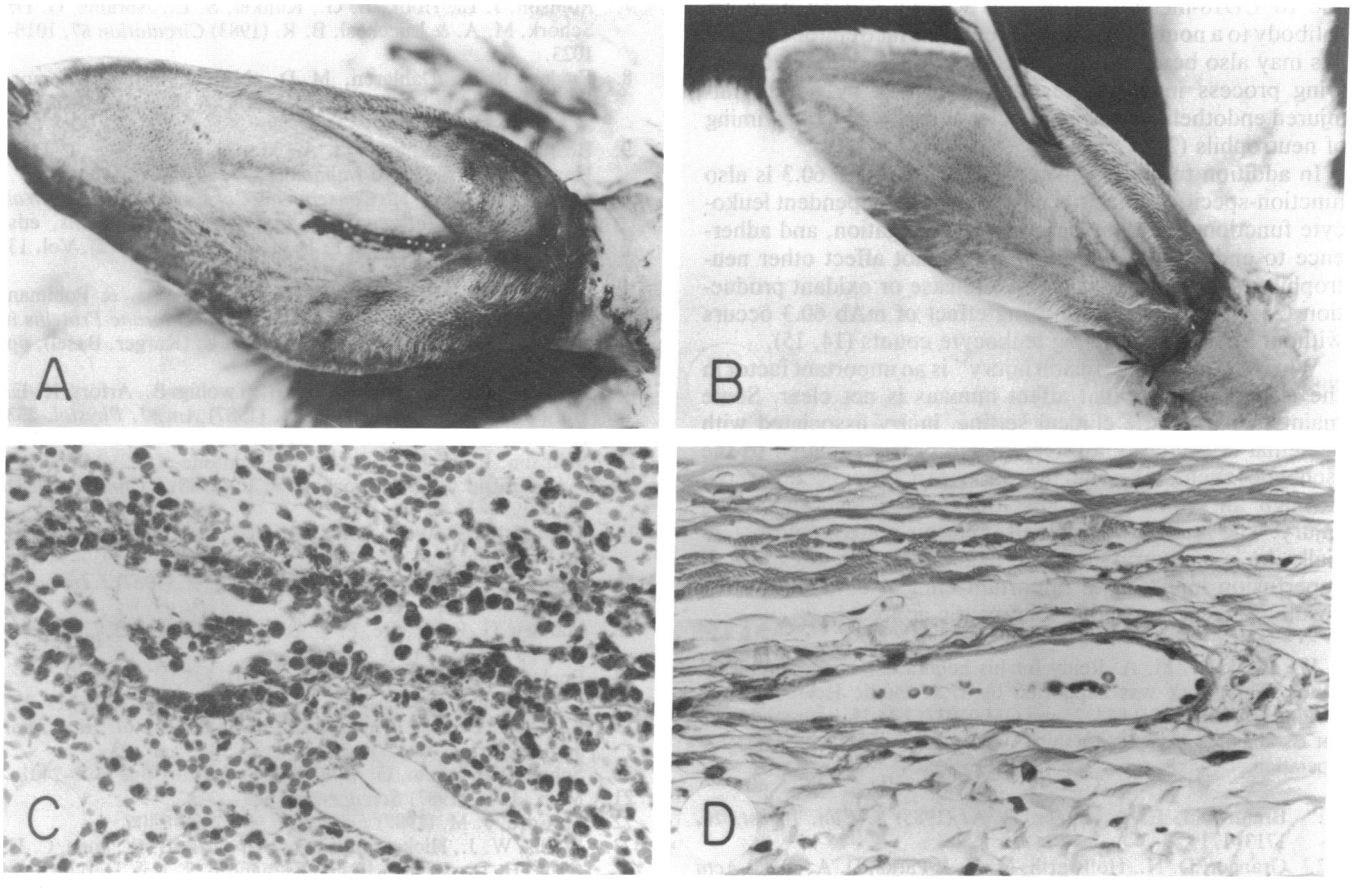

FIG. 3. Comparison of tissue injury in rabbits treated with saline (control-ischemic) (*A* and *C*) or mAb 60.3 (Post) (*B* and *D*) just prior to reperfusion after 10 hr of complete ischemia. This comparison was made 24 hr after initial ischemia. *C* and *D* are representative of similar-sized venules in the two animals.

ditions. When perfusion pressure drops during ischemia, neutrophils can occlude the microvasculature, contributing to the "no-reflow phenomenon" (20). This circumstance is aggravated by increased neutrophil adhesiveness, as neutrophils adhere tightly to endothelium and to each other. Once adherent to endothelium, neutrophils can release proteases and oxidants, causing significant cellular and tissue injury with destruction of the normal endothelial barrier. Thus, a progressive downward spiral may result in which ischemia induces leukocyte activation and adherence, leading to leukocyte accumulation and endothelial injury, which results in further ischemia and eventually complete cessation of flow. This may be the basis of what has been called the "no-reflow phenomenon," which more precisely might be called a "diminishing-reflow" phenomenon. Another important consequence of neutrophil–endothelial adherence is the formation of a "protective microenvironment" beneath the adherent neutrophil wherein its destructive proteases and oxidants can act, protected from the effects of circulating antioxidants and antiproteases (4). Inhibiting the adherence step, therefore, should be a very effective method of blocking neutrophil-mediated injury.

The approach of using anti-CD11b/CD18 mAbs to block neutrophil adherence functions has proved to be very effective at preventing neutrophil-mediated injury. We and others have demonstrated substantial effectiveness in intestinal (12), myocardial (13), and whole-animal (14) models of ischemia–reperfusion. In all of these models, however, antibody was administered far in advance of reperfusion. As a result, it has not been clear whether the injury was occurring during ischemia or with reperfusion. The results of the current study clearly demonstrate that the neutrophil-mediated injury in this setting is indeed a reperfusion injury. This study demonstrates that leukocyte-mediated tissue injury associated with ischemia and reperfusion is primarily a reperfusion injury.

The concept of reperfusion injury was introduced over a decade ago and has since received considerable attention. Much of this work has focused on the role of oxygen-derived free radicals generated at the time of reperfusion or reoxygenation (3). Since these reactive oxygen species clearly are destructive and since free radical scavengers attenuate injury in experimental models of reperfusion, they seem a likely culprit (21). Neutrophils may be linked to this process in two ways. Neutrophils are a major source of toxic oxygen radicals. In addition, there is evidence that free radicals, such as those generated by reoxygenation of tissue, can elicit the production of neutrophil chemoattractants in plasma and cause massive tissue accumulation of neutrophils, thereby setting the stage for significant neutrophil-mediated injury (22).

In interpreting these results, it is important to recognize the highly specific nature of mAbs. In this case, mAb 60.3 binds to a function-related idiotype of CD18 (23) that has been found only on leukocytes. Because of this, any differences between treatment and control-ischemic groups must be attributed to leukocytes. Since neutrophils are the primary leukocyte involved in the acute phase of injury and were the predominant cell type seen in our histologic sections, it is likely that neutrophils are responsible for this leukocyte-mediated injury. Our results, however, do not rule out the possibility that other leukocytes may also be involved, as CD18 is present on all leukocytes. Furthermore, we cannot conclude unequivocally that this leukocyte involvement is

due to CD18-mediated adhesion without having used an antibody to a nonfunctional neutrophil surface epitope. Platelets may also be involved in this injury in a mutually amplifying process involving platelet activation by neutrophil-injured endothelium and by platelet-activating factor priming of neutrophils (24).

In addition to being leukocyte-specific, mAb 60.3 is also function-specific, blocking only adherence-dependent leukocyte functions such as chemotaxis, aggregation, and adherence to endothelium. mAb 60.3 does not affect other neutrophil functions such as granule release or oxidant production (25). This specific blocking effect of mAb 60.3 occurs without altering circulating leukocyte counts (14, 15).

Whether true "reperfusion injury" is an important factor in the ischemic states that affect humans is not clear. Some maintain that in the clinical setting, injury associated with ischemia is primarily an irreversible process related to the ischemic event (1). This question can only be answered through an approach of specifically blocking reperfusion injury. The method we have used in this study could potentially be a powerful tool for examining this question. If reperfusion injury is an important clinical entity, then our results suggest a possible therapeutic approach.

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