

Anti-P-Selectin Monoclonal Antibody Attenuates Reperfusion Injury to the Rabbit Ear

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Abstract

Neutrophil adherence and/or aggregation has been implicated in ischemia reperfusion injuries. We examined the role of P-selectin in PMN-mediated injury after reperfusion of the rabbit ear. The ear was partially amputated, and then reattached leaving the central artery and vein intact. To induce ischemia the central artery was then occluded. Treatment was at reperfusion with either saline or one of two murine P-selectin mAbs, designated PB1.3 and PNB1.6. mAb PB1.3 cross-reacts with rabbit P-selectin and prevents histamine-induced leukocyte rolling, whereas PNB1.6 does not. Using a peroxidase-antiperoxidase system P-selectin was detected in the ischemic ear, but not in the nonischemic ear. Ear volume increased to 5.3 times baseline in the saline-treated animals ($n = 8$), 6.6 times baseline in the nonblocking mAb PNB1.6-treated animals ($n = 2$), and 3.7 times baseline in the blocking mAb PB1.3-treated animals ($n = 8$). Estimated tissue necrosis of the combined saline- and PNB1.6-treated animals was 46 vs. 2.7% for the mAb PB1.3-treated animals. We conclude that: (a) P-selectin is expressed in ischemia reperfusion; (b) P-selectin participates in PMN-endothelial cell interactions in ischemia reperfusion; and (c) inhibiting P-selectin adhesion significantly reduces reperfusion injury. (*J. Clin. Invest.* 1993. 92:2042–2047.) Key words: selectins • CD11/CD18 • CD62 • GMP-140 • adhesion molecules

Introduction

Neutrophil (PMN) migration into tissue is essential for host defense against microorganisms and for tissue repair. A critical step in their emigration and accumulation at sites of inflammation is adherence to endothelial cells. Adherence occurs after activation of PMNs and/or endothelial cells in response to a variety of signals that cause them to become proadhesive (1). Leukocyte adherence is followed by migration along the endothelial surface, diapedesis between endothelial cells, and migration through the interstitial space to the extravascular site (2).

Neutrophils are potent effector cells in host defense generating many products with microbicidal activity. However, release of products such as oxidants and proteases into extracellular environment can cause blood vessel and tissue injury (3, 4).

Ischemia followed by reperfusion can result in significant cellular injury to tissues and is important in a variety of clinical disorders, including stroke, myocardial infarction, organ transplantation, and organ hypoperfusion. Clearly, if an organ is ischemic for a sufficient time cellular necrosis will occur and the only protection from this type of injury is prompt restoration of blood flow. However, significant injury can occur as a result of reperfusion of previously ischemic tissue (5–7). Reperfusion injury frequently is associated with activation of the inflammatory system and enhanced leukocyte-endothelial adhesiveness. We and others have shown that blocking neutrophil adherence and/or aggregation with mAbs to CD11/CD18 glycoprotein complex can ameliorate the reperfusion injury (8–13).

Several adhesion molecules involved in PMN adherence to endothelium have been defined. The leukocyte β_2 integrin receptor complex (CD11/CD18) interacts with the endothelial ligand intercellular adhesion molecule-1 (ICAM-1). The selectin receptors, E-, P-, and L-selectin, recognize carbohydrate counter-structures, including sialyl Lewis x (SLe^x)¹ (reviewed in references 14 and 15). The current model of the contribution of integrins and selectins to PMN-endothelial cell interaction is based on studies in vivo and in vitro. Observations by intravital microscopy have defined the sequence leading to leukocyte emigration at inflammatory sites. There is an initial slowing and rolling of leukocytes followed by firm sticking and diapedesis (16). Selectins appear to mediate the initial transient adhesion, which is manifested by rolling under conditions of shear forces (17–19). The CD11/CD18 complex is responsible for sticking and transendothelial migration (16). Consequently, defects in either integrin or selectin function will impair PMN emigration as evidenced by leukocyte adherence deficiency type I (LAD I), the congenital absence of functional CD11/CD18, and leukocyte adherence deficiency type II (LAD II), the congenital absence of SLe^x (20). The model also proposes that inhibition of selectin as well as integrin function should attenuate PMN-mediated vascular and tissue injury. In this regard, mAbs to E-selectin have been shown to reduce lung injury associated with the deposition of IgG immune complexes (21). Also, antigen-induced acute airway inflammation and late-phase airway obstruction in monkeys were reduced by

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1. Abbreviations used in this paper: LAD, leukocyte adherence deficiency; MPO, myeloperoxidase; SLe^x, sialyl Lewis x.

mAbs to E-selectin (22). In addition, mAbs to P-selectin have been shown to attenuate complement-induced lung injury (23) and ischemia reperfusion injury to the heart (24).

The experiments described herein tested the hypothesis that P-selectin is expressed on the surface of endothelial cells after ischemia and reperfusion, and this expression promotes neutrophil adherence to endothelial cells leading to a neutrophil-mediated injury. This hypothesis was formed from the synthesis of data in separate reports. Granger et al. (6) have shown that oxidants are produced in ischemia reperfusion injuries and scavengers of these oxidants ameliorate the injury. Patel et al. (25) reported that oxidants caused luminal expression of P-selectin, thus, P-selectin expression is predicted in ischemia reperfusion injury. This leads to the hypothesis that the initial leukocyte rolling results from their interaction with P-selectin. This hypothesis was tested using mAbs directed to a functional epitope of P-selectin to block the interaction of neutrophils with endothelial P-selectin.

Methods

New Zealand White rabbits were prepared as previously described (11). Briefly, they were anesthetized with intravenous ketamine plus xylazine and nerve block at the base of the left ear with local lidocaine. Using sterile procedures, the left ear was partially amputated, leaving the central artery, central vein, and a narrow bridge of cartilage intact. Care was taken to insure that all blood vessels in the cartilage bridge were cut. All nerves were carefully divided to render the ear completely anesthetic. The ear was then reattached and a microvascular clip was placed across the artery to produce total ischemia. Rabbits were kept at an ambient temperature of 23.5°C for 6 h, then the microvascular clamp was removed and the ear allowed to reperfuse. Treatment was given immediately before reperfusion with either saline, or an adherence-blocking P-selectin mAb (mAb PB1.3) or a nonblocking P-selectin mAb (mAb PNB1.6). mAbs were given by intravenous injection of 2 mg/kg, and the saline treatment group was given an equal volume of saline.

Immunocytochemistry. Punch biopsies were taken from the anesthetic ear for evaluation of P-selectin expression. Samples were taken, embedded in OTC compound (Miles, Inc.), and rapidly frozen in ethanol cooled with dry ice. P-Selectin is normally found in the Weibel-Palade bodies of the endothelial cells and is expressed on plasma membrane after endothelial cell activation (26). Thus, routine staining with mAbs on cut tissue might stain positive. Therefore, samples for immunohistochemistry were taken only after the animal was given P-selectin mAbs intravenously. The circulating murine mAb could attach only to luminal P-selectin and was subsequently detected with anti-murine antibodies.

Cryosections were cut at 6 μm , collected onto sialinized slides, and allowed to air dry before fixation in ice-cold 4% paraformaldehyde for 10 min. Slides were washed in PBS after fixation, then incubated for 30 min with 20% normal rabbit serum in PBS to block nonspecific staining. Detection of bound mAb was performed using reagents provide in a peroxidase-antiperoxidase immunostaining kit (Zymed Labs., Inc., S. San Francisco, CA). Briefly, this involved removal of the blocking solution, its replacement by rabbit anti-mouse Ig (bridging antibody) for 1 h, washing in PBS, then treatment with peroxidase-antiperoxidase-soluble complex for 1 h. Sections were then washed in PBS and developed with 0.03% diaminobenzidine that was sometimes followed by faint staining with hematoxylin. Concurrently run controls included replicate sections where bridging antibody was substituted with dilute normal rabbit serum and analysis of sections of ears from untreated rabbits.

ELISA. Plasma concentration of mAb PB1.3 was determined in four normal rabbits after injection of 2 mg/kg. 96-well plates were incubated at 4°C for 24 h with unlabeled goat anti-mouse IgG1 and

washed three times with PBS/Tween. Test samples and standards were placed in the wells and incubated at 4°C for 24 h, then horseradish peroxidase-labeled goat anti-mouse IgG1 was added and incubated for 1 h. Plates were washed three times in PBS/Tween buffer, then chromogen was added and incubated for 15 min, and the reaction stopped with 1 N H₂SO₄. Plates were immediately analyzed by microtiter plate reader at 450/650 nm. Standards were determined in quadruplicate and samples in duplicate.

Ear volume and necrosis were measured as previously described (11). Briefly, the ear was submerged in a beaker of water up to the suture line and its volume determined by the amount of water displaced. Necrosis was estimated from gross observation of the ear surface and expressed as a percentage of total area. Ear volume was measured daily for 7 d by water displacement to quantify tissue edema. On day 7, necrosis was estimated as a percentage of total surface area. The protocol of these experiments was approved by the University of Washington Animal Care Committee and complies with the National Institutes of Health guidelines for care and uses of laboratory animals.

Myeloperoxidase (MPO) assay. The enzyme MPO is found in the granules of neutrophils and has been used as an indicator of PMN emigration into tissue (21, 27–29). We used the previously described technique to determine MPO concentration (30). Both ears of rabbits were used to measure MPO activity. The skin from 18 rabbit ears (9 saline treated and 9 mAb PB1.3 treated) was dissected off the cartilage at 24 h after the start of ischemia, weighed, and homogenized in PBS containing 0.5% hexadecyltrimethylammonium bromide (HTAB). 1 ml of the PBS solution was added to 500 mg of tissue before homogenization. The homogenized solutions were freeze thawed three times and sonicated twice to disrupt the cells, then the mixture was centrifuged at 10,000 g for 10 min. The supernatant was decanted and heated at 60°C for 120 min to inactivate inhibitors of MPO activity (31). The optical density has been shown to be proportional to MPO concentration (30).

PMN infiltration. A measure of total PMN infiltration was determined from multiplying the MPO activity of the sample by the 24-h ear volume and dividing by the initial ear volume. Multiplication by total ear volume results in total MPO activity in the ear and correction for different initial size was made by normalization to initial ear volume. MPO content of normal ears (never ischemic) was determined in a separate set of measurements and was subtracted from the values determined in ischemic ears. The resulting MPO content represents the increased MPO activity resulting from ischemia and reperfusion of the ear.

Statistics. Data were expressed as mean \pm SEM. Statistical significance of the differences between ear volume was determined by analysis of variance for repeated measures. Differences in the percent necrosis between the combined control groups (mAb PNB1.6 treated and saline treated) and the mAb PB1.3-treated animals were examined by Mann-Whitney U test. Differences of $P < 0.05$ were considered to be statistically significant.

mAb. The mAb PB1.3 is a murine-derived IgG1 directed to human P-selectin and cross-reacts with rabbit P-selectin. Also, it blocks rolling in the cat mesentery after preparation for viewing through intravital microscopy (32). mAb PNB1.6 is a nonblocking murine-derived IgG1 also directed to human P-selectin, but does not block rolling in vivo. The intravenous injection of mAbs did not have any acute effects on circulating white blood cell counts when given to normal rabbits.

Results

Ear Volume. 6 h of ischemia at 23.5°C resulted in considerable edema in the saline-treated and mAb PNB1.6-treated rabbits with the average ear volume exceeding five and six times baseline in the two groups, respectively. There were only two animals in the PNB1.6-treated rabbits, too few for statistical analysis, and thus, the saline- and PNB1.6-treated rabbits were com-

bined in Fig. 1. The blocking mAb PB1.3 prevented some of the edema formation but the ear volume reached a peak of ~ 3.7 times baseline. The combined control animals were statistically different from the mAb PB1.3-treated animals by analysis of variance for repeated measures.

Necrosis. Tissue necrosis was estimated for seven of the combined control animals and for six of the mAb PB1.3-treated animals (Fig. 2). There was considerable variability in both groups with four of the six mAb PB1.3-treated and one of the control group having no necrosis. Maximum necrosis in the control group and the mAb PB1.3-treated group was ~ 85 and 15%, respectively. Differences among groups were significant by Mann-Whitney U test.

Myeloperoxidase. MPO content of ears from saline-treated rabbits averaged more than five times that of the mAb PB1.3-treated group. These differences were statistically significant when compared by *t* test ($P < 0.01$). These results are consistent with the hypothesis that protection afforded by the administration of P-selectin mAbs resulted from a reduced PMN-mediated injury after ischemia and reperfusion.

Immunocytochemistry. Tissues stained for detection of P-selectin from animals given mAb PB1.3 at the time of reperfusion are shown in Fig. 3. A nonischemic control ear is shown in Fig. 3 A. Very faintly staining vessels were occasionally seen in control ears. An ear made ischemic and allowed to reperfuse for 4 h before tissue sampling is shown in Fig. 3, B and C. P-Selectin staining is clearly observed as the brown oval and linear outlines in Fig. 3 B. Fig. 3 C is a higher power view of a section of positively stained vessels where faint staining of individual endothelial cells was seen. A linear section of a capillary is shown next to the larger vessel that stained positive for P-selectin. Surface expression of P-selectin was observed as early as 30 min after reperfusion.

ELISA (PB1.3). Injection of 2 mg/kg of mAb PB1.3 resulted in a plasma concentration of $46 \pm 13.6 \mu\text{g/ml}$ at 1 h. Concentration of mAb slowly decayed over the next 24 h to a concentration of $23 \pm 6.3 \mu\text{g/ml}$. Thus, the plasma half-life of this mAb was ~ 24 h in normal rabbits.

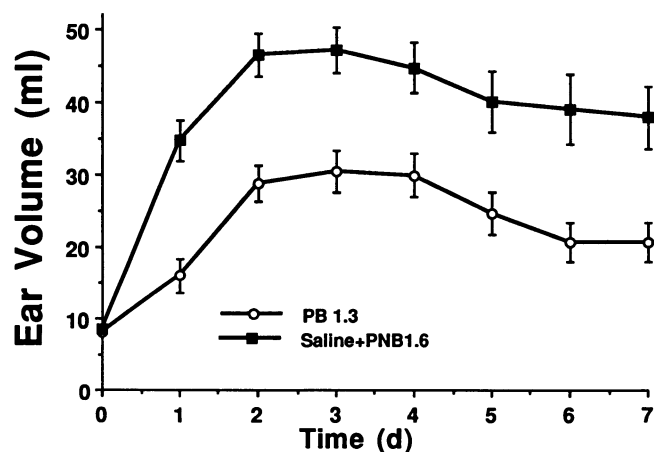


Figure 1. Ear volume measured by displacement of water at baseline and after a 6-h ischemic period followed by reperfusion. Rabbits were treated with mAbs directed to P-selectin adhesion molecules or with saline. The mAb PB1.3 blocks leukocyte rolling in vivo. The mAb PNB1.6 recognizes a nonfunctional epitope of P-selectin and does not block rolling. The control group consists of saline ($n = 8$) plus PNB1.6-treated ($n = 2$) animals. Values are mean \pm SEM.

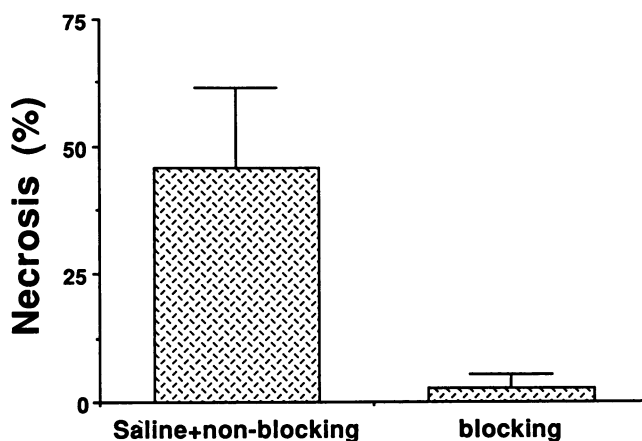


Figure 2. Tissue necrosis estimated 7 d after ischemia reperfusion in mAb PB1.3 ($n = 6$) and control rabbits. Control animals consist of the combined saline-treated ($n = 5$) and mAb PNB1.6-treated ($n = 2$) rabbits. Control animals were compared with mAb PB1.3-treated ($n = 5$) animals. Necrosis was not estimated in all animals. Values are mean \pm SEM.

Discussion

The P-selectin molecules are normally found in the α granules of platelets and in Weibel-Palade bodies of endothelial cells. Upon stimulation, these structures fuse with the cell membrane releasing their contents and exposing P-selectin to the luminal surface (33). Endothelial cell surface expression of P-selectin after stimulation with histamine or thrombin is rapid but short lived in vitro, lasting from 30 to 60 min (26, 34). Deposition of the complement proteins C5b-C9 cause surface expression, however, the time course of expression was not determined (35). Continuous in vitro stimulation of endothelial cells with oxygen radicals results in P-selectin expression lasting at least 4 h (25). This expression also corresponded to adherence of PMNs to the stimulated endothelial cells and was blocked by mAbs to P-selectin (25). More recently, P-selectin was shown to be induced by TNF in murine endothelial cells (36).

The selectin molecules (L-, P-, and E-selectin) all have similar molecular structures. These consist of a cytoplasmic domain, a transmembrane domain, a variable number of regulatory protein repeats, an EGF domain, and a lectin domain (reviewed in reference 33). The counter-structure for P-selectin on leukocytes has not been completely defined, but it recognizes the oligosaccharide SLe^x (37, 38) and a recently described glycoprotein on myeloid cells (39). Earlier reports showed that binding of neutrophils to P-selectin was sensitive to sialidase treatment of neutrophils, suggesting that sialic acid is necessary for adherence (38, 40, 41). Neutrophils lacking SLe^x as well as other fucose-containing carbohydrate moieties from a patient with LAD II syndrome failed to bind to histamine-treated endothelial cells or purified P-selectin in vitro (Phillips, L., B. Schwartz, A. Etzioni, R. Bayer, H. Ochs, J. Paulson, and J. Harlan, manuscript in preparation), indicating that a fucose-containing carbohydrate structure is essential for binding.

PMN adherence or aggregation was shown in previous studies to mediate a major component of ischemia reperfusion injury since injury was markedly reduced by injection of mAbs to

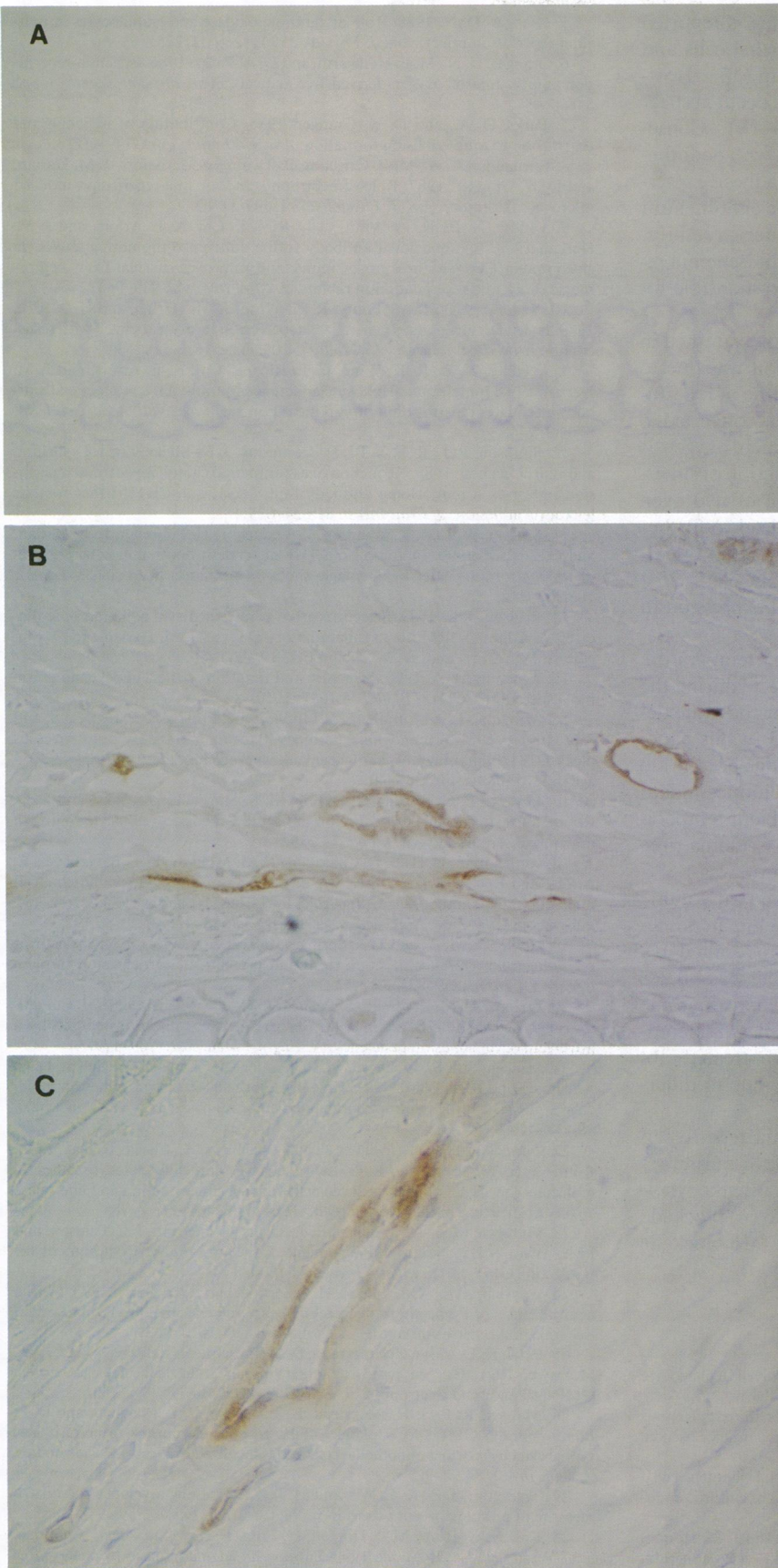


Figure 3. P-Selectin expression determined by immunocytochemistry in the rabbit ear. Photomicrographs of tissue from rabbit ears show the immunohistochemical findings from a rabbit receiving mAb PB1.3. (A) Section of control ear that was stained for presence of mAb PB1.3. The cartilaginous pinna is evident in the lower half. The highly vascular zone between the mid-dermis and pinna exhibits essentially no staining ($\times 100$). (B) Brown oval and linear outlines represent positively staining vessels in the ischemic ear of a mAb PB1.3-treated rabbit. These small vessels are located just above the cartilaginous pinna. Lumina lack erythrocytes because the cryopreservation process results in their lysis ($\times 200$). (C) A higher power view of an elliptical section of a positively stained vessel is shown here. Individual endothelial cells are faintly visible. An adjacent linear section of a capillary is also staining ($\times 500$).

CD11/CD18 (8–13). The CD11/CD18 complex is reported to support firm leukocyte adherence to endothelial cells and their subsequent emigration into the interstitium (18). However, CD11/CD18-dependent adhesion does not occur at shear forces observed in the microcirculation (16–18). Consequently, it cannot provide the initial interaction with endothelial cells. This event is thought to result from the adhesive interaction of selectins on leukocytes (L-selectin) or endothelium (E- and P-selectin) with carbohydrate counter-structures. Thus, the effectiveness of anti-P-selectin mAbs in ischemia reperfusion injury suggests that this molecule is responsible for the initial leukocyte–endothelial interaction.

The mediator of P-selectin expression was not examined in this study; however, it is possible that expression was due to generation of oxygen radicals beginning at the time of reperfusion. During ischemia, xanthine dehydrogenase is converted to xanthine oxidase, and upon reperfusion molecular oxygen is used as an electron donor, resulting in the production of superoxide anion radical and subsequent generation of hydrogen peroxide (5). Activation and adherence could lead to further generation of oxygen radicals by the granulocytes and additional expression of P-selectin as described by Patel et al. (25). TNF is another potential mediator of P-selectin expression in this setting (36).

Ischemia reperfusion injury is known to be temperature dependent. A lowering of temperature by 1 or 2°C during the ischemic period allows longer ischemic times for the same degree of injury (42). The experiments described in this paper show that the rabbit ear is no exception. Vedder et al. (11) showed an average maximum increase in ear volume equal to 4.3 times baseline in saline-treated rabbits. Ambient temperature in those experiments was ~ 22°C and the ischemia time was 10 h. By comparison, the ambient temperature was 1.5°C warmer in experiments described in this study, and ear volume increased to an average maximum 5.3 times baseline in saline-treated rabbits. Clearly, the increased temperature resulted in a more severe injury even with an ischemic time that was 4 h less than that of Vedder et al. (11). The percentage increase in the P-selectin mAb-treated group was ~ 3.7 times baseline compared with 3.0 times baseline for the CD18 mAb-treated rabbits. Difference in these two groups is probably due to differences in temperature.

We conclude that in rabbit ear: (a) ischemia reperfusion causes endothelial surface expression of P-selectin during reperfusion; (b) P-selectin is involved in the initial leukocytes–endothelial cell interaction after reperfusion; and (c) blockade of P-selectin provides protection equivalent to CD18 blockade after ischemia and reperfusion.

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