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NEUTROPHIL ADHESION TO XENOGENEIC ENDOTHELIUM VIA iC3b[1]

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Neutrophils are thought to play an important role in the pathogenesis of hyperacute rejection, a dramatic form of tissue injury caused by the reaction of antigraft antibodies with endothelial cells of an organ allograft or xenograft. We asked whether the interactions of human polymorphonuclear leucocytes (PMN) with xenogeneic endothelium might be promoted by the binding of natural anti-endothelial antibodies and complement by using porcine aortic endothelial cells (PAEC), human serum, and human PMN in an in vitro model of hyperacute rejection. Pretreatment of PAEC with 10% human serum followed by washing markedly increased PMN adhesion from 15.7 ± 1.8% to 62.5 ± 3.6% ($p < 0.001$). Complement and anti-endothelial antibodies were necessary for the increase, because heat-inactivated serum or serum depleted of IgM did not significantly increase PMN adhesion to treated endothelium. The induction of increased PMN adhesion to PAEC by human serum was observed within 1 min. The essential role of complement was defined using complement-depleted serum. Ten percent C2-deficient serum did not increase PMN adhesion whereas 10% C5-depleted or 10% C8-depleted serum caused the same increase in PMN adhesion as observed with normal human serum. These results suggested that C3 might play a critical role in enhanced neutrophil adhesion. In fact, PAEC treated with 10% human serum for 15 min and incubated with an $F(ab')_2$ antihuman C3 for 10 min completely abolished the enhanced adhesion. PAEC treated with 10% human serum or C5-depleted serum displayed fluorescence of iC3b whereas monolayers treated with heat-inactivated serum or C2-deficient serum were nonreactive. The enhanced PMN adhesion to serum-treated PAEC was mediated through neutrophil receptors binding iC3b because mAb directed against CD11b/CD18 inhibited the serum-enhanced adhesion of PMN. We conclude that PMN adhesion to endothelium can be significantly enhanced by the endothelial deposition of iC3b.

Neutrophils (PMN[3]) have been shown to play a critical role in the pathogenesis of tissue injury in a variety of disease models, particularly those mediated by humoral factors (1–5). The ability of PMN to mediate such injury is dependent at least in part on adherence to endothelial cells (6–8). PMN adherence is augmented by both neutrophil- and endothelial cell-dependent processes. For example, when stimulated by agonists such as C5a or the oligopeptide FMLP, neutrophils up-regulate the receptor CD11b/CD18 (also known as CR3, or iC3b receptor) which increases neutrophil adherence to vascular endothelial cells (9–12). Similarly, stimulation of endothelial cells by thrombin, cytokines, or endotoxin triggers expression of GMP140, ELAM-1, and ICAM-1 which support neutrophil adhesion (13–15). Recently Marks et al. (16) demonstrated the rapid induction of neutrophil adhesion to endothelial cells by fixation of complement on endothelial surfaces (16).

Neutrophils are thought to play an important role in the pathogenesis of hyperacute rejection, a dramatic form of tissue injury caused by the reaction of antigraft antibodies in the circulation of a recipient with endothelial cells of an organ allograft or xenograft (17–20). Such reactions lead to the activation of complement and rejection of the graft within minutes to a few hours. Because PMN constitutively express receptors for the complement fragment iC3b (CD11b/CD18), we asked whether binding of naturally occurring anti-endothelial cell antibodies and generation of endothelial cell surface iC3b would promote adhesion of human PMN to porcine aortic endothelial cells in vitro.

MATERIALS AND METHODS

Materials. DMEM, FCS, trypsin-EDTA (0.5% trypsin + 0.53 mM EDTA), and HBSS were from GIBCO (Grand Island, NY). RPMI 1640, L-glutamine, and penicillin/streptomycin were obtained from Hazelton Biologics, Inc. (Lenexa, KS). Percoll (1.075) was from Sigma Chemical Co. (St. Louis, MO). Albumin (human, USP 25% solution) was from Baxter Health Care Corp., Island Division (Glendale, CA). Hespan 6% (hydroxyethylstarch in 0.9% sodium chloride) was from Dupont Pharmaceuticals (Wilmington, DE). Sodium heparin was from Lyphomed, Inc. (Melrose Park, IL). Sodium chloride was from Fisher Scientific (Fairlawn, NJ). Sodium ^{51}chromate (sp. act. 1 mCi/ml) was obtained from Amersham Corp. (Arlington Heights, IL). Human sera immuno-depleted of the complement components C5 or C8 were obtained from Cytotech (San Diego, CA).

Preparation of PMN. Blood samples (40 ml) from human volunteers (after receiving informed consent under the guidelines of the Committee on the Use of Human Subjects in Research at the University of Minnesota) were obtained in plastic syringes containing

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leucocytes; PAEC, porcine aortic endothial cells.

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20 ml of hydroxyethyl starch and 200 U preservative-free heparin. The mixture was allowed to sediment at room temperature and the supernatant was collected and centrifuged at 400 × g for 5 min. The pellet was suspended in 0.2 ml ice-cold HBSS containing 100 mg/100 ml glucose and residual erythrocytes were lysed by addition of 15 ml ice-cold water for 25 s, after which isotonicity was reconstituted by addition of 5 ml of 3.6% sodium chloride. The suspension was centrifuged in the cold at 400 × g for 5 min, the pellet resuspended in 5 ml of HBSS, carefully layered on top of Percoll made to a density of 1.075 g/ml, and centrifuged at 20,000 × g for 30 min at 4°C. The resulting PMN (>95% purity with <1% platelet contamination) were washed once and resuspended in 10 ml of HBSS containing 0.5% human albumin. Viability of PMN was assessed by Trypan blue exclusion and exceeded 95%.

Endothelial cell cultures. Porcine aortic endothelial cells were grown from pig aortae and cultured according to the method of Ryan (21). Cells were grown in DMEM containing 10% FCS with L-glutamine and penicillin/streptomycin. Cells were plated in 24-well, 16-mm diameter tissue culture plates (Costar, Cambridge, MA). The cells were identified as endothelial cells by their morphology and their ability to take up acetylated LDL (22). Cells were used in passages 3 to 10. Some studies used human umbilical vein or bovine aortic endothelial cells, cultured as previously described (23, 24).

Neutrophil adhesion to endothelial cells. PMN were labeled with ^{51}Cr by adding 60 μCi of sodium ^{51}chromate to 5 × 10^7 PMN in HBSS for 60 min at 37°C with constant, gentle rocking. Subsequently, PMN were washed × 2 with 10 ml HBSS and once with 10 ml HBSS with 0.5% human albumin. The labeled PMN were resuspended at a concentration of 4 × 10^6 in HBSS containing 0.5% human albumin, so that the assayed volume of 0.25 ml contained 1 × 10^6 PMN. In the typical experiment, porcine aortic endothelial cells were pretreated with human serum diluted to either 10 or 25% in RPMI 1640 with a total volume of 0.5 ml/well. After various time intervals (1 to 60 min), the cells were washed twice with 0.5 ml warm HBSS. Subsequently, 0.25 ml of labeled PMN (approximately 1 × 10^6/well) were added and incubated for 15 min at 37°C. The ratio of PMN to PAEC is 5:1. After the incubation period, the non-adherent PMN were removed and the wells washed three times with 0.5 ml HBSS and the unattached cells were pooled for counting in a gamma-counter. The wells were inspected using an inverted phase microscope and graded for the degree of adhesion of PMN to the monolayers. The adherent cells were then lysed with 1 N sodium hydroxide, the lysates removed and the wells wiped with cotton swabs to remove all counts. Percent adherent PMN were calculated as:

$$\frac{\text{cpm, adherent cells}}{\text{cpm, adherent cells + non-adherent cells}} \times 100\%$$

All studies were done in triplicate. All studies included a control monolayer that was treated with RPMI 1640 and washed in a similar fashion before addition of PMN. In some studies, heat-inactivated serum (56°C × 30 min) or complement-deficient serum was used in the designated concentrations. IgM-depleted serum was prepared by two cycles of affinity chromatography of pooled human serum containing 0.01 M EDTA (25). The IgG fraction of goat anti-human IgM (Cappel, Westchester, PA) was conjugated to Affi-gel 10 (Bio-Rad, Richmond, CA) at 10 mg of protein/ml of gel. The depleted serum was dialyzed against PBS, pH 7.4, containing 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}, and concentrated to the initial volume. Purified C1q was added to a concentration of 75 μg/ml. Total hemolytic complement (CH50) and IgM content of the IgM-depleted serum were 70% and less than 3% of the initial serum, respectively.

In some studies mAb were added to PMN for 5 min before incubating the PMN with the monolayer. The mAb used in these studies include 60.3 (10 μg/ml) (IgG 2a), an anti-CD18 antibody (a kind gift from Dr. John M. Harlan, University of Washington-Harborview Medical Center) (26), Mol 17 (IgM) and Mol 44 (IgG 2a) (both anti-CD11b antibodies used as ascites in a 1/50 dilution), a kind gift from Dr. Robert Todd, University of Michigan, Ann Arbor, MI) (27). A murine mAb to human neutrophil lactoferrin, 9.1 (IgG 2b) (used as ascites 1/50), was obtained from Dr. Keith Skubitz (University of Minnesota) (28) and a murine mAb to human CR2, HB-5 (IgG 2a) (10 μg/ml), was obtained from Becton Dickinson (Mountain View, CA). In some experiments, a rabbit (IgG) $F(ab')_2$ to human C3 (Organon Teknika Corporation, West Chester, PA) was added to endothelial cells (50 μg/ml) in HBSS/0.5% Hanks' albumin after treatment of the cells with human serum. The cells were washed and the ^{51}Cr-labeled PMN added for assessing adhesion as described above.

Immunofluorescence studies. Porcine aortic endothelial cells were grown to confluence on eight-chamber polystyrene slides (Nunc, Inc., Naperville, IL). The cells were treated with various sera as outlined. Cells were washed twice with PBS and fixed in cold 95% ethanol. Slides were then washed twice with PBS for 3 min. A murine mAb to iC3b (IgG2b) (Cytotech, San Diego, CA) (20 μg) was then added to each slide and incubated for 30 min at 37°C. Slides were rinsed three times with PBS and FITC anti-mouse IgG was added and the slides incubated for 45 min. Slides were washed again three times with PBS, the coverslip mounted, and the cells observed using a Karl Zeiss III RS epifluorescence microscope. Photographs of the adherent neutrophils were taken using a Nikon Diaphot inverted microscope.

Statistics. Data are expressed as the mean ± the SEM. Statistical analysis utilized Student's t-test with paired data when appropriate.

RESULTS

We have previously shown that human serum contains natural antibodies that are reactive with porcine aortic endothelial cell monolayers in vitro and cause their activation (20, 29, 30). Treatment of porcine endothelial cells with human serum for 30 min significantly increased the adhesion of ^{51}Cr-labeled PMN from 15.7 ± 1.8% to 62.5 ± 3.6%, $p < 0.001$ (Fig. 1). As seen in Figure 2*B*, there is a marked increase in PMN adhesion to serum-treated endothelial cells compared to cells treated with heat-inactivated serum-treated cells (Fig. 2*A*). Pretreatment of porcine aortic endothelial cells with heat-inactivated serum did not significantly increase PMN adhesion above control (18.9 ± 3.0%). It is important to note that treatment of PAEC for 30 min had minimal effect on the confluence of the endothelial cell monolayer or its viability as assessed by trypan blue exclusion. Furthermore, treatment of naked plastic tissue culture wells with human serum or heat-inactivated serum caused no increase in PMN adhesion. In fact, treatment with human serum or heat-inactivated serum decreased PMN adhesion to plastic compared to treatment with RPMI (RPMI treated, 75 ± 2%; 10% human serum treated, 53.1 ± 7%; 10% heat-inactivated serum treated, 51.5 ± 8%). Similar results were obtained when bovine aortic endothelial cells were treated with human serum. However, when human umbilical vein endothelial cells were treated with human serum, PMN adhesion (13.1%) was not increased compared with RPMI-treated human cells (13.2 ± 1%).

We have previously shown that IgM is the primary Ig deposited on blood vessels in hyperacute xenograft rejection (20, 29, 30). When we used human serum depleted of IgM, there was no increase in PMN adhesion (25.8 ± 10%) compared to heat-inactivated serum (Fig. 1). The IgM-depleted serum contained approximately 70% of the hemolytic activity, i.e., was not complement deficient. Thus, the presence of IgM was necessary for enhanced PMN adhesion.

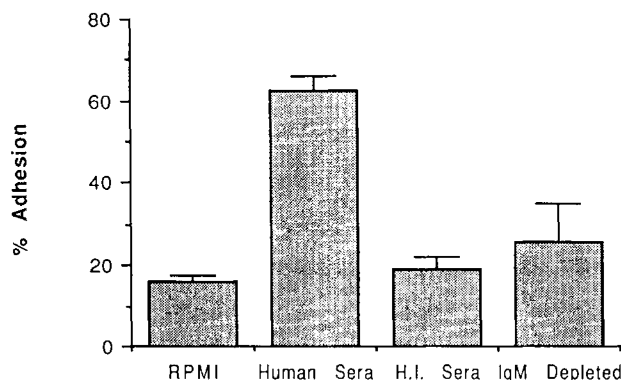

Figure 1. The effects of serum on neutrophil adherence to porcine endothelial cells. Endothelial cells were treated with RPMI 1640, 10% human serum, 10% heat-inactivated serum, or 10% IgM-depleted serum for 30 min, washed, and neutrophil adhesion performed as described in *Materials and Methods*. Data are expressed as mean neutrophil adhesion ± SEM representing 10 experiments done in triplicate except for the IgM depleted serum that was done three times in triplicate.

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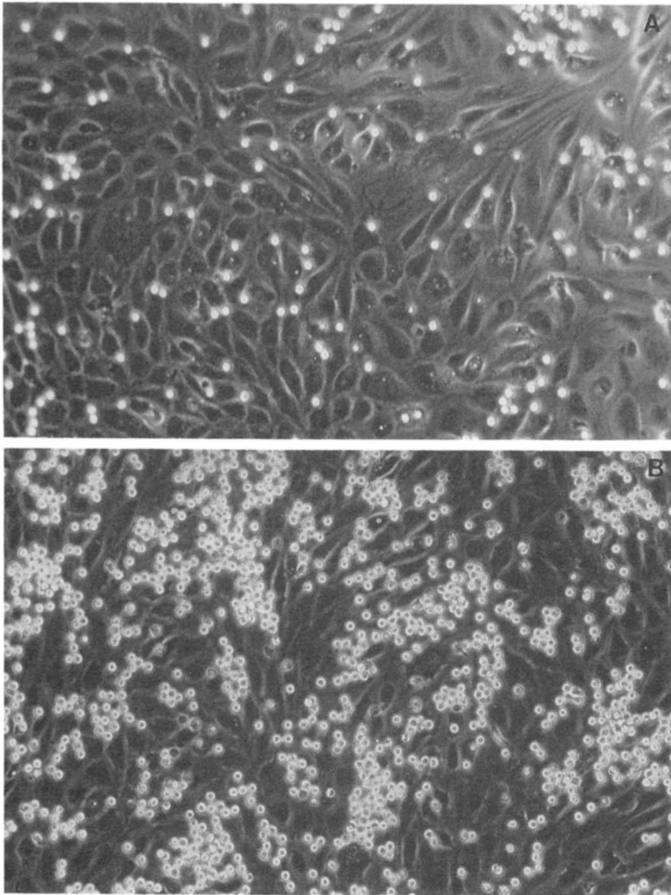

Figure 2. Photomicrograph of neutrophils adhering to porcine aortic endothelial cells treated with 10% heat-inactivated serum (*A*) or 10% normal human serum for 30 min (*B*).

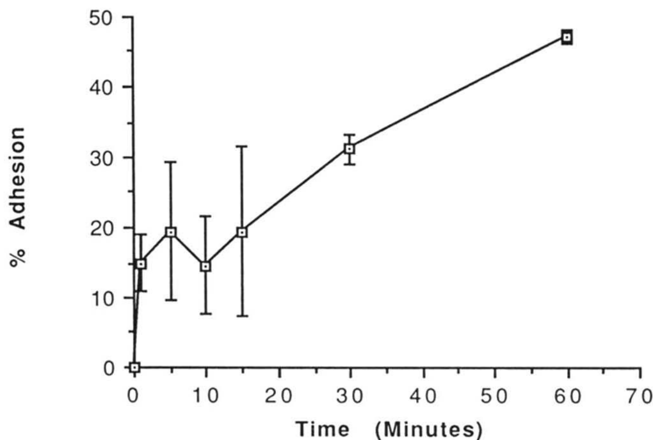

Figure 3. Time course of enhanced neutrophil adhesion to serum-treated endothelial cells. Porcine aortic endothelial cells were treated with 10% human serum for the times listed and neutrophil adhesion performed as in *Materials and Methods.* Data represent the mean and SE absolute percent increase of neutrophil adhesion relative to PMN adhesion to RPMI-treated cells done twice in triplicate.

The induction of increased PMN adhesion by human serum was observed within 1 min of treatment with xenogeneic serum and continued to increase up to 60 min (Fig. 3). Treatment for greater time periods did further increase adherence (data not shown). The essential role of complement in this system was also defined using complement-depleted sera. Porcine aortic endothelial cells were treated with 10% heat-inactivated serum for 10 min to provide fixation of natural antibodies. Subsequently the cells were washed and then exposed to 10% C2-deficient serum for 15 min (obtained from a patient congenitally deficient in C2) (31). C2-deficient serum did not allow enhanced PMN adhesion (Table I). However, when purified C2 (DiaMedix, Miami, FL) (2000 U/ml of serum) was added to the C2-deficient serum, enhancement of PMN adhesion was similar to that observed with normal serum. In contrast, 10% C5-depleted serum or 10% C8-depleted serum caused the same increase in PMN adhesion as observed with normal serum (Table I). These results suggested that C3 might play a critical role in the enhanced neutrophil adhesion. To test that hypothesis, PAEC were treated with 10% human serum for 15 min, washed, and incubated with $F(ab')_2$ anti-human C3 for 10 min. The endothelial cells were then washed and PMN adhesion assayed. The antibodies directed against C3 completely abolished the enhanced adhesion seen with normal serum (Table I). PAEC treated with 10% human serum or C5-depleted serum displayed fluorescence for iC3b (Fig. 4), whereas monolayers treated with heat-inactivated serum or with C2-deficient serum were non-reactive.

We next evaluated whether binding of PMN to serum-treated porcine endothelial monolayers was mediated by the neutrophil receptor for iC3b (CD11b/CD18). mAb directed against CD11b/CD18 inhibited the serum-enhanced adhesion of PMN. Antibodies to CD11b, Mol 17, and Mol 44 inhibited the increase of adhesion by 90 and 78%, respectively (Fig. 5). A mAb directed against CD18, 60.3, inhibited serum-enhanced adhesion by 82%. In contrast, mAb against human neutrophil lactoferrin and antibodies against CR2 had no effect on serum-enhanced PMN adhesion.

DISCUSSION

This study demonstrates that porcine endothelial cells treated with human serum become an adhesive surface for neutrophils. The enhanced neutrophil adhesion to serum-treated endothelial cells appears to require C3, but not the terminal complement components. Treatment of endothelial cells with C2-deficient serum did not increase subsequent neutrophil adhesion, whereas endothelial cells treated with serum depleted of C5 or C8 showed enhanced neutrophil adhesion. Consistent with these findings is the observation that $F(ab')_2$ anti-C3 also blocked enhanced neutrophil adhesion to serum-treated endothelial cells. The activation of complement was triggered by antibody binding because human serum depleted in IgM did not cause an increase in PMN adhesion to PAEC and increased binding was only observed for xenogeneic combinations of serum and cells.

Neutrophil adherence to vascular endothelial cells is an early event in inflammation. A variety of agonists, including C5a, bacterial oligopeptides such as FMLP,

TABLE I

Role of complement on PMN adhesion to porcine endothelial cells[a]

Endothelial Treatments	Adhesion (Percent of Human Sera)
Human sera	100.0
C2 deficient	6.1 ± 2.73
C2 deficient + C2	86.2 ± 14.00
C5 depleted	82.4 ± 7.85
C8 depleted	100.0 ± 4.0
HS + $F(ab')_2$	10.6 ± 13.20

[a] Data are expressed as percentage of neutrophil adhesion with complement-depleted serum compared to control adhesion with normal human serum (100%). In each case, the endothelial cells were treated as described in *Materials and Methods.*

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Figure 4. iC3b deposition on serum-treated endothelial cells. *A*, iC3b deposition on porcine aortic endothelial cells treated with heat-inactivated serum for 30 min. *B*, 10% human serum. *C*, 10% C2-deficient serum. *D*, 10% C5 depleted serum as described in *Materials and Methods*.

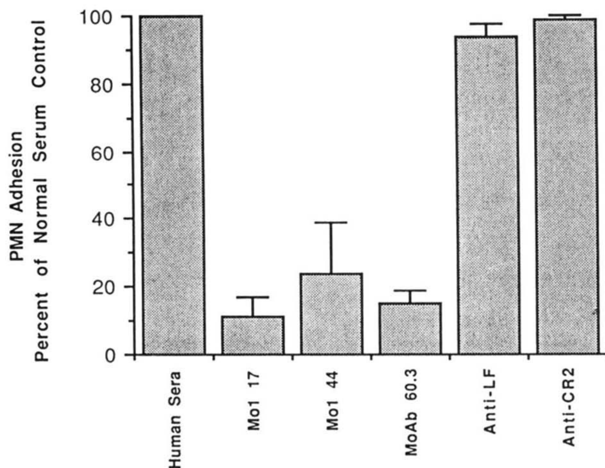

Figure 5. Effect of mAb to neutrophil CD11b/CD18 on neutrophil adhesion. Porcine aortic endothelial monolayers were treated with 10% human serum for 30 min and neutrophil adhesion performed. As described in *Materials and Methods*, neutrophils were pre-treated with Mol 17, Mol 44, 60.3, anti-lactoferrin, and anti-CR2. A total of 100% control adhesion was defined as the adhesion of HBSS-treated neutrophils to serum-treated endothelial cells. The data are expressed as the percentage of this control for each antibody treatment. The data represent the mean of three experiments done in triplicate.

platelet-activating factor, leukotrene B_4, LPS, IL-1, granulocyte-macrophage-CSF, and TNF can directly activate neutrophils and increase their adhesiveness both to themselves (aggregation) and to endothelial cells (32–38). This enhanced adhesion is in part associated with an increase in the expression of CD11b/CD18 on the neutrophil surface. Neutrophils from individuals genetically deficient in CD11b/CD18 do not become more adherent in response increase to chemoattractants, and this failure may underlie the increased susceptibility to infection seen in these individuals (39–42). Endothelial cells can reciprocally increase the adhesiveness for neutrophils by increasing the surface expression of GMP-140, platelet activating factor, ICAM-1, and ELAM-1 (43, 44). These endothelial molecules are up-regulated by agonists such as thrombin, TNF, LPS, or IL-1.

Several investigators have demonstrated that activated complement can promote neutrophil endothelial cell interactions (2, 3, 9). Tonnesen (9) demonstrated that human C5a and C5a des-arg act on neutrophils to enhance adhesion to human endothelial cells. Similarly, bovine neutrophils increase their adherence for bovine endothelium in response to complement-activated bovine serum. Marks et al. (16) demonstrated the rapid induction of neutrophil-endothelial adhesion by the fixation of complement to endothelial cell surfaces. These studies used human endothelial cells and human serum complement activated by cobra venom factor and attached to endothelial cells by Ulex Europaeus-1. Increased human neutrophil adhesion to the treated endothelial cells was inhibited by mAb to the CD11b/CD18 complex, suggesting that neutrophil adhesion was mediated by binding to C3 deposited on the endothelium.

We were interested in the mechanism by which PMN

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accumulate on endothelial cells during the hyperacute rejection that takes place when vascularized organs are transplanted from one species to a phylogenetically distant recipient species. It seems likely that the deposition and activation of the PMN in such situations contributes to endothelial activation, which in turn would potentially aggravate the severity of hyperacute rejection. Previous studies have suggested neutrophil depletion can prolong xenograft survival (45). In the context of such a model, our studies suggest that blockade of neutrophil CD11b/CD18 might prolong xenograft survival by preventing neutrophil adhesion. Inasmuch as close approximation of the neutrophil with the endothelium is necessary for the toxic oxygen species and lysosomal contents to activate/damage the endothelium, blockade of adhesion should promote vessel wall integrity.

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