Xenogeneic Serum Promotes Leukocyte-Endothelium Interaction under Flow through Two Temporally Distinct Pathways: Role of Complement and Nuclear Factor-κB

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Abstract. Endothelial cell activation and mononuclear cell infiltration are consistent features of discordant xenograft rejection. This study evaluated whether xenogeneic serum—as a source of xenoreactive natural antibodies and complement—induced endothelial activation with consequent leukocyte adhesion and transmigration under flow conditions. Porcine aortic endothelial cells (PAEC) were incubated for 30 min, 1 h 30 min, or 5 h with 10% human serum or 10% porcine serum and then perfused with human leukocytes in a parallel plate flow chamber under flow (1.5 dynes/cm²). Adherent and transmigrated cells were counted by digital image analysis. Results showed that human serum significantly (P < 0.01) increased over time the number of adherent leukocytes compared with porcine serum. Stimulation of PAEC with human serum also promoted a progressive increase in leukocyte transmigration that reached statistical significance (P < 0.01) at 1 h 30 min and at 5 h compared with porcine serum. Studying the role of complement in leukocyte-endothelium interaction in xenogeneic conditions, a marked complement C3 deposition on PAEC exposed to human serum was shown by immunofluorescence, whereas cells incubated with porcine serum were negative. Next, it was documented that human serum decomplemented by heating and C3-deficient human serum failed to promote both leukocyte adhesion and transmigration, results that were comparable to porcine serum. To elucidate the intracellular mediators involved in endothelial cell activation by xenogeneic serum, this study focused on transcriptional factor nuclear factor-κB (NF-κB), a central regulator for the induction of different genes, including adhesive molecules and chemotactants. Positive nuclear staining of NF-κB (p65 subunit) was found by confocal fluorescence microscopy of PAEC exposed to human serum that was taken to reflect NF-κB activation. NF-κB was instead strictly localized in the cell cytoplasm in PAEC incubated with the homologous serum. Heat-inactivated human serum failed to activate NF-κB. Electrophoretic mobility shift assay of nuclear extracts from PAEC exposed to human serum revealed an intense NF-κB activation that was inhibited by the NF-κB inhibitor pyrrolidinedithiocarbamate. The NF-κB inhibitor inhibited pyrrolidinedithiocarbamate and tosyl-phe-chloromethylketone did not affect the number of adherent and transmigrated leukocytes in PAEC exposed to human serum for 30 min and 1 h 30 min. Both inhibitors instead significantly reduced leukocyte adhesion and transmigration induced by human serum at 5 h. Confocal fluorescence microscopy studies showed that human serum induced an increase in the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Functional blocking of these adhesive molecules with the corresponding antibodies significantly inhibited xenogeneic serum-induced leukocyte adhesion. These data suggest that leukocyte adhesion and transmigration are directly dependent on complement deposited on PAEC in the early phase of cell activation (30 min and 1 h 30 min) induced by xenogeneic serum, whereas leukocyte adhesive events observed after 5 h of incubation of endothelial cells with xenogeneic serum are possibly regulated by transcription of NF-κB-dependent genes. The finding that xenogeneic serum promotes leukocyte-endothelial interaction depending on NF-κB activation might be relevant for designing future therapeutic strategies intended to prolong xenograft survival.

Xenotransplantation of pig organs into humans is regarded as an attractive strategy to meet the increasing organ demand for human transplantation (1–4). However, when transplanted into primates, pig organs are subject to a hyperacute rejection mediated by the binding of xenoreactive natural antibodies to the carbohydrate structure Galα1–3Gal on porcine endothelial cells, with consequent activation of the recipient’s complement (1,2,5,6). The activation of complement on xenogeneic cell surface is critically controlled by C3 convertase, which catalyzes the cleavage of C3 into C3a and C3b fragments (6,7). C3b may covalently bind to cell surface as iC3b or participate in the formation of the lytic membrane attack complex (MAC). The formation of these active components leads to perturbation of endothelial cell function and morphology, which results in loss of vascular integrity and development of edema, hemorrhages, and microthrombi. Complement-mediated injury is characterized by the release from endothelial surface of heparan sulfate (8), which contributes importantly to the barrier and...
anticoagulant function of endothelial cells and provides protection from oxidant injury by anchoring superoxide dismutase (9).

Another mechanism that determines the loss of endothelial cell functions is the alteration in cytoskeleton architecture and the cell shape changes, recently attributed to the assembly of C5b67 complexes on endothelial cells and accelerated by MAC, which lead to the formation of intercellular gaps (10). The presence of the gaps allows the escape of vascular contents, and may provide a site for adhesion and aggregation of platelets and leukocytes (2,11). During the activation of complement cascade, the generation of proteins with chemotactic properties such as C3a, C4a, and C5a helps to amplify the recruitment and the adhesion of inflammatory cells into the xenografts (7,12). There is also evidence that the deposition of iC3b on xenogeneic endothelium triggers adhesion of polymorphonuclear leukocytes through their CD11b/CD18 receptors (13).

Recently, a novel pathway through which complement activates endothelial cells has been proposed. The assembly of MAC on the endothelium triggers the synthesis and release of interleukin-1α, and it is in response to this cytokine that the endothelium becomes “activated,” adopting a procoagulant and proinflammatory posture with the elaboration of tissue factor, adhesion molecules, cytokines, and chemokines (2,14).

It is well known that endothelial cells play an active role in the process of adhesion of circulating cells to vascular vessel wall and their subsequent extravasation. Compelling evidence is available that the events that modulate adhesion and infiltration of leukocytes into the endothelium are critically dependent on hemodynamic flow conditions. The flow velocity gradient near the vessel wall induces tractive forces tangential to endothelial surface (shear stress) that oppose leukocyte adhesion. The effects of flow on leukocyte-endothelium interaction have been mimicked in vitro using a parallel plate perfusion chamber and video microscopy (15,16). Using the in vitro replica of these dynamic conditions, it has been documented that before adhering firmly, leukocytes roll on the endothelial surface. Early molecules involved in the process of leukocyte rolling belong to the selectin family (17,18). Firm leukocyte adhesion and consequent transmigration suggest an interaction of their β1 and β2 integrin receptors with specific endothelial ligand, i.e., intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), belonging to the Ig gene superfamily, whose levels are regulated by humoral mediators and flow (16,19,20).

In the present study, we sought to: (1) evaluate whether human serum—as a source of xenoreactive natural antibodies and complement—induced leukocyte rolling, adhesion, and transmigration in porcine endothelial cells under flow conditions; and (2) assess the role of complement and nuclear factor-κB (NF-κB)-dependent adhesive molecules that regulate leukocyte-endothelial interaction in the above experimental setting.

**Materials and Methods**

**Endothelial Cell Culture and Incubation**

Primary cultures of pig aortic endothelial cells (PAEC) were prepared by using a modification of the method of Ohbayashi et al. (21).

Briefly, the aorta was washed in phosphate-buffered saline with calcium and magnesium (D-PBS) containing antibiotics. After removing the connective and adipose tissue, the aorta was cut into 2- to 3-cm-long pieces that were opened longitudinally and laid endothelium side down onto 130 U/ml collagenase-coated tissue culture dishes (collagenase type I; Sigma Chemical Co., St. Louis, MO) in D-PBS for 20 min at 37°C. At the end of incubation, the luminal surface of tissue fragments was rinsed with 40 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY) plus 5% porcine serum (Life Technologies), and the cell suspensions were then centrifuged (200 × g for 10 min). The cell pellet was resuspended in growth medium: Dulbecco’s modified Eagle’s medium supplemented with 15% porcine serum plus glutamine (2 mM, Life Technologies), penicillin (200 U/ml, Life Technologies), streptomycin (200 μg/ml, Life Technologies), and fungizone (500 ng/ml, Life Technologies), and plated on 6-well tissue culture plates coated with 0.2% porcine gelatin (Sigma Chemical). Cultures were grown at 37°C in 5% CO₂/95% air. Confluent PAEC were routinely used for experiments between the first and fifth passage. Cultured cells were identified as endothelial by their morphology, the presence of E-selectin evaluated by fluorescence-activated cell sorter analysis, and their ability to take up acetylated LDL (13,22).

For the adhesion experiments, PAEC were plated on 60-×-20 mm plastic coverslips (Thermoxan; Nunc, Naperville, IL) coated with porcine gelatin and used 2 d after reaching confluence. Before adhesion assay, PAEC were incubated for 30 min, 1 h or 30 min, or 5 h with 10% homologous porcine serum, as control, or with 10% xenogeneic human serum (prepared from a pool of plasma from five healthy volunteers, obtained at the Transfusion Center). According to a previous study (13), 10% human serum had no cytopathic effect on porcine endothelium as evaluated by cell count and morphology (Figure 1, a and b). PAEC stimulated for 5 h with recombinant human tumor necrosis factor-α (TNFα) (100 U/ml; 69 × 10⁶ U/mg protein; a kind gift of Dr. G. Paul; Knoll AG, Ludwigshafen, Germany) were used as positive control.

The involvement of complement was assessed by: (1) studying C3 deposition on PAEC exposed to porcine serum, human serum, or heat-inactivated human serum (30 min, 56°C) using confocal fluorescence microscopy; and (2) evaluating leukocyte adhesion and transmigration on PAEC treated with 10% heat-inactivated human serum or with 10% complement C3-deficient human serum (Sigma Chemical).

**Figure 1.** Photomicrographs of porcine aortic endothelial cells (PAEC) exposed to 10% porcine serum (a) or human serum (b) for 5 h. Human serum had no cytotopic effect on porcine endothelium. Magnification, ×70.
To study a possible role of NF-κB in xenogeneic human serum-induced leukocyte adhesion, we performed the following steps. (1) PAEC were seeded on plastic coverslips and, when confluent, were exposed for 30 min, 1 h 30 min, or 5 h to porcine serum, xenogeneic human serum, heat-inactivated human serum, or TNFα; then PAEC were fixed and stained for NF-κB by indirect immunofluorescence. (2) NF-κB activity was also evaluated by electrophoretic mobility shift analysis (EMSA) in nuclear extracts from PAEC exposed for 1 h 30 min to human serum, human serum plus the antioxidant and reported NF-κB inhibitor pyrrolidinedithiocarbamate (PDTC) (25 μM; Sigma Chemical) (23) added 1 h before the addition of serum, or heat-inactivated human serum. (3) PAEC were preexposed for 1 h to PDTC (25 μM) or to the protease inhibitor tosyl-phe-chloromethylketone (TPCK) (25 μM; Sigma Chemical) (24), which prevents the proteolytic degradation of IkB, the cytoplasmic inhibitor of NF-κB (25), before the addition for 30 min, 1 h 30 min, or 5 h of porcine serum or xenogeneic human serum. Then cells were used for adhesion experiments.

Confocal fluorescence microscopy experiments were performed to study the expression of adhesive molecules after 5 h exposure to xenogeneic serum, as described below. The involvement of adhesive molecules in xenogeneic serum-induced leukocyte adhesion was evaluated using adhesion-blocking mouse anti-human ICAM-1 monoclonal antibody (mAb), which recognized porcine ICAM-1 (26) (5 μg/100 μl clone BBA-3; R&D Systems, Minneapolis, MN) and anti-porcine VCAM-1 antibody YT11.1 (5 μg/100 μl; Celltech, Berkshire, United Kingdom), or with mouse isotype-matched mAb (X40 Becton Dickinson) for 20 min before the adhesion assay.

Leukocyte Isolation

Before adhesion experiments, a leukocyte suspension was prepared from human fresh venous blood collected from healthy volunteers on ethylenediaminetetra-acetic acid (EDTA) (final concentration, 5 mM) and diluted with an equal volume of cold saline solution as described previously (16). The blood samples were centrifuged at 200 × g for 10 min at 4°C, the cell pellet was resuspended in 4 vol of Emagel (Behringwerke, Marburg, Germany), and erythrocytes were sedimented at 4°C for 40 min. The supernatant was removed and centrifuged at 500 × g for 7 min at 4°C, and the pellet was washed twice by centrifugation with saline. Remaining erythrocytes were removed by ammonium chloride lysis at 4°C and centrifugation. After this procedure, the cell viability, measured by trypan blue exclusion, was greater than 95%. Cells were then resuspended in culture medium at a final concentration of 10^6 cells/ml.

Adhesion Assay under Flow Conditions

For adhesion experiments, we used a parallel-plate flow chamber and a perfusion system that have been described previously (15,16). Briefly, the chamber is composed of two parallel surfaces: a coverslip coated with PAEC at confluence and a flat surface machined from polymethylmethacrylate. The two surfaces are separated by a 250-μm-thick silicon rubber gasket, leaving a rectangular surface of 30 × 13 mm exposed to flow. An inlet and outlet channel distribute the fluid uniformly along the entrance side of the adhesion surface. Shear stress level as a function of medium flow rate was calculated as described previously (15), assuming fully developed laminar flow between the two parallel plates. After assembling with the PAEC monolayer, the chamber is placed on the stage of an inverted phase-contrast microscope with a thermostated hood to maintain the temperature at 37°C. The microscope is connected with a video recording system (Panasonic, Osaka, Japan).

Leukocyte suspension was pumped through the chamber at controlled flow rates, using a syringe pump (Harvard Apparatus, South Natick, MA). After initial perfusion with cell-free medium at 0.6 dynes/cm² for 2 min for equilibration, the leukocyte suspension was perfused through the chamber at 1.5 dynes/cm² and images were recorded thereafter. After 10 min, cell-free medium was perfused at a flow rate of 3.0 dynes/cm² for evaluation of the number of leukocytes rolling on the PAEC surface. At this flow rate, leukocytes rolling are easily distinguishable from cells freely flowing in the suspension that move much faster. After 3 min of perfusion at 3.0 dynes/cm², several fields (>10) were observed for evaluation of the number of firmly adherent cells. Images acquired during the perfusion experiments were digitized and processed on a personal computer using general purpose image processing software (NIH Image, version 1.59; National Institutes of Health, Bethesda, MD). Adherent leukocytes were identified and counted at the end of the 13-min perfusion as described previously in detail (15,16). The number of rolling leukocytes was evaluated by superimposition of 16 consequent frames so that rolling cells could be identified from their wake.

The number of cells that transmigrated across the PAEC monolayer during the perfusion experiment was investigated as described previously (27). Briefly, the number of transmigrated leukocytes was quantified during the 10 min of perfusion in each experiment by visual inspection of videotape, since adherent cells that transmigrate under endothelium change their color from white to black.

In selected experiments, after adhesion assay cells were stained by May–Grunwald/Giems. Cells were fixed in methyl alcohol for 3 min at room temperature and stained with 1:2 dilution of May–Grunwald solution in PBS for 1 min. After two washings with distilled water, cells were incubated for 10 min with 1:10 dilution of Giems solution in PBS. Coverslips were washed twice and examined by microscope.

Fluorescence Confocal Microscopy

PAEC grown on gelatin-coated coverslips were incubated with 10% porcine serum, heat-inactivated human serum (1 h 30 min), or human serum (30 min, 1 h 30 min, and 5 h). Then cells were fixed with 2% paraformaldehyde in PBS, pH 7.4, for 10 min at 37°C. After three washings with PBS, cells were incubated with 0.1% bovine gelatin in PBS for 5 min to prevent nonspecific antibody binding. Complement deposition on endothelial cells was evaluated by using FITC-conjugated rabbit anti-human C3 antibodies (13.6 μg/100 μl; Dako, Glostrup, Denmark).

To evaluate NF-κB activation, PAEC were treated for 30 min, 1 h 30 min, and 5 h with porcine serum, xenogeneic human serum, heat-inactivated human serum, or TNFα (100 U/ml), as positive control. Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature and then permeabilized with Triton X-100 (0.1% in PBS; Sigma Chemical) for 4 min before incubation with 1 μg/100 μl affinity-purified rabbit polyclonal anti-human NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by exposure to 0.7 μg/100 μl Cy3-conjugated affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Negative control experiments were carried out with Cy3-conjugated antibody alone.

To study E-selectin, VCAM-1, and ICAM-1 expression after 5 h of exposure to porcine serum, human serum, or TNFα, PAEC (fixed in paraformaldehyde) were incubated with mouse anti-porcine E-selectin mAb 1.2B6 (1 μg/100 μl; Celltech), mouse anti-porcine VCAM-1 antibody YT11.1 (1 μg/100 μl; Celltech), and anti-human ICAM-1 mAb (1 μg/100 μl clone BBA3; R&D Systems), respectively, and then with 2 μg/100 μl Cy3-conjugated affinity-purified donkey anti-
mouse IgG (Jackson Immunoresearch Laboratories). Negative control experiments with Cy3-conjugated antibody were performed. Coverslips were washed, mounted in 1% N-propyl-gallate in 50% glycerol, 0.1 M Tris-HCl, pH 8, and examined under a confocal inverted microscope (InSight Plus, Meridian Instruments, Okemos, MI). Representative fields of resting or stimulated PAEC were digitized with 256 gray levels and printed using a film printer (Montage, FR2 film recorder; Presentation Technologies, Sunnyvale, CA).

Preparation of Nuclear Extracts and EMSA

Nuclear extracts were prepared according to Satriano and Schlodorf with minor modifications (28). After washing with PBS, PAEC were harvested and resuspended in 1.5 ml of hypotonic buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1.0 mM dithiothreitol [DTT; Sigma], and 0.2% Nonidet P-40 [Boehringer Mannheim, Germany]). After incubation for 5 min on ice, the homogenate was centrifuged at 650 × g, and the pellet was then washed once with buffer A. The resulting nuclear pellet was resuspended by gentle pipetting in 100 µl of buffer C (25 mM Hepes, 50 mM KCl, 0.1 mM EDTA, pH 8.0, 1.0 mM DTT, 10% [vol/vol] glycerol) and 0.4 M NaCl. This suspension was incubated for 45 to 60 min at 4°C followed by centrifugation at 18,000 × g for 10 min. The supernatant collected (nuclear extract) was divided into aliquots and stored at −70°C for subsequent use. Protein concentrations were determined by the Bradford assay using the Bio-Rad protein assay reagent.

The κB DNA sequence of the Ig gene was used for EMSA (5′-CCGTCAGAGGGGACTTTCCGAGACT). The core κB sequence is underlined. Oligonucleotides were synthesized (Life Technologies BRL, San Giuliano Milanese, Italy) and annealed. Probe DNA (with 5′ overhangs) was end-labeled by the kleenow enzyme with α-32P dCTP, and separated from unincorporated nucleotides over a G-50 Sephadex column (Pharmacia Biotech, Uppsala, Sweden).

Nuclear extracts (2 µg) were incubated with 50 × 10³ counts per min of 32P-labeled NF-κB oligonucleotide in binding reaction mixture (10 mM Tris-HCl, pH 7.5, 80 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1.5 µg of poly(dI-dC)) in a final volume of 15 µl. After 30 min on ice, the protein-DNA complexes were resolved on a nondenaturing 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer and run at 200 V for 1.5 h at room temperature. Gels were then dried and subjected to autoradiography for analysis. In competition studies, a 1000-fold molar excess of unlabelled oligonucleotide was added to the binding reaction mixture as indicated, before the addition of the labeled κB probe.

Statistical Analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Tukey test for multiple comparisons, as appropriate (29). Statistical significance was defined as P < 0.05.

Results

Human C3 Deposition on PAEC Exposed to Xenogeneic Serum

We confirmed in our experimental setting human C3 deposition on PAEC exposed to xenogeneic human serum (Figure 2). PAEC treated with 10% human serum after 30 min displayed on their surface a positive staining for C3 as granular

Figure 2. Confocal micrographs of C3 deposition on PAEC exposed to 10% porcine serum (1 h 30 min, a), heat-inactivated (H.I.) human serum (1 h 30 min, b), or xenogeneic human serum (30 min, c; 1 h 30 min, d; 5 h, e). Magnification, ×1000.
deposits. After 1 h 30 min, a more marked fluorescence for C3 was observed. At 5 h, C3 staining became almost completely undetectable at the cell surface. Most of the cells revealed weak intracellular staining at this time, mainly concentrated in the perinuclear region and with a finely granular distribution. Endothelial monolayers incubated with porcine serum were not positive for human C3 staining at all incubation times. Cells exposed to heat-inactivated human serum were not reactive.

**Effect of Xenogeneic Serum on Leukocyte Adhesion to PAEC under Flow Conditions**

We studied the adhesion of human leukocytes to porcine endothelium as an in vitro model of vascular xenograft under laminar flow conditions. Before the adhesion assay, PAEC were exposed for 30 min, 1 h 30 min, or 5 h to 10% homologous porcine serum, as control, or 10% xenogeneic human serum. As shown in Figure 3, a limited number of leukocytes adhered to endothelial cells exposed to porcine serum at all time points considered, whereas a significant increase in leukocyte adhesion was observed over time on PAEC incubated with human serum. Thus, a 2.8-fold increase in the number of adhering leukocytes was measured on endothelial cells after 30 min of exposure to xenogeneic serum (44 ± 1 versus 124 ± 7 leukocytes/mm², \( P < 0.01 \)). When PAEC were incubated with human serum for 1 h 30 min or 5 h, a more marked increase (4.7- and 5.4-fold, respectively) in leukocyte adhesion was observed with respect to porcine serum (1 h 30 min: 36 ± 3 versus 170 ± 19; 5 h: 36 ± 5 versus 195 ± 28 leukocytes/mm², \( P < 0.01 \)). This response was similar to that elicited by TNFα, one of the most potent inducers of endothelial cell adhesive properties (5 h: 229 ± 22 leukocytes/mm²). Leukocyte adhesion on PAEC maintained in serum-free medium was comparable to that observed on PAEC exposed to homologous serum (5 h: 34 ± 12 versus 33 ± 15 leukocytes/mm²).

To evaluate a possible role of complement on xenogeneic serum-induced leukocyte adhesion, PAEC were incubated with human serum that was decomplemented by heating. As shown in Figure 3, at all incubation times heat-inactivated human serum failed to promote leukocyte adhesion, which was comparable to that induced by porcine serum. Similar data were obtained when PAEC were exposed to complement C3-deficient human serum (Table 1).

By May Grunwald/Giemsa staining (Figure 4), we identified polymorphonuclear cells as the major subset (68%) of leukocytes that adhered to PAEC treated for 5 h with xenogeneic serum. Mononuclear cells (25%) and granulocytes (7%) were also present.

**Effect of Xenogeneic Serum on Leukocyte Rolling**

The number of rolling leukocytes on PAEC exposed to xenogeneic or homologous serum was evaluated at the end of the perfusion at 3 dynes/cm². The number of rolling leukocytes on human serum-treated PAEC was almost negligible at all time points considered (30 min: 0.8 ± 0.8; 1 h 30 min: 1.6 ± 0.8; 5 h: 1 ± 0.7 cells/mm²) and was absent on PAEC incubated with porcine serum. TNFα activation, as expected, induced important leukocyte rolling on the endothelial surface (23.1 ± 3.3 cells/mm²).

**Table 1. Effect of complement C3-deficient human serum on leukocyte adhesion on PAEC under flow**

<table>
<thead>
<tr>
<th>Category</th>
<th>Adherent Leukocytes/mm²</th>
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<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>10% human serum</td>
<td>137 ± 13</td>
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<tr>
<td>10% C3-deficient human serum</td>
<td>34 ± 5b</td>
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<tr>
<td>10% H.I. human serum</td>
<td>38 ± 5b</td>
</tr>
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PAEC, porcine aortic endothelial cells; H.I., heat inactivated human serum.

* Data are expressed as mean ± SEM.

\(^b\) \( P < 0.01 \) versus human serum.
Effect of Xenogeneic Serum on Leukocyte Transmigration

When PAEC were exposed to porcine serum, only a few adherent leukocytes migrated across the endothelial monolayer (Figure 5). In contrast, stimulation of PAEC with human serum promoted a progressive increase in leukocyte transmigration that became statistically significant ($P < 0.01$) at 1 h 30 min and at 5 h compared with porcine serum (1 h 30 min: 15 ± 2 versus 4 ± 1; 5 h: 23 ± 2 versus 4 ± 1 cells/mm$^2$). A similar transmigration was observed after endothelial cell activation with TNF$\alpha$ (5 h: 24 ± 7 cells/mm$^2$).

Heat-inactivated human serum (Figure 5) (30 min: 5 ± 1; 1 h 30 min: 2 ± 2; 5 h: 5 ± 1 cells/mm$^2$) as well as C3-deficient human serum did not induce transendothelial migration (30 min: 4 ± 1; 1 h 30 min: 3 ± 2; 5 h: 4 ± 2 cells/mm$^2$).

Role of NF-κB in Leukocyte-Endothelium Interaction Induced by Xenogeneic Serum

To elucidate possible mechanisms underlying endothelial cell activation by xenogeneic serum, we studied the transcription factor NF-κB in PAEC treated with human serum. By confocal fluorescence microscopy after 30 min of cell exposure to human serum, NF-κB weakly stained the nucleus of a few cells, while a complete activation of NF-κB was evident after 1 h 30 min (Figure 6b). After 5 h, NF-κB nuclear staining decreased. PAEC exposed to porcine serum did not show evidence of NF-κB activation, as documented by NF-κB staining exclusively localized in the cell cytoplasm (Figure 6a).

When PAEC were incubated for 1 h 30 min with human serum decomplemented by heating, NF-κB was not activated (Figure 6c). This indicates that complement derived from human serum is instrumental to NF-κB activation in xenogeneic conditions. TNF$\alpha$, a strong inducer of NF-κB activation, was used as positive control (Figure 6d). Experiments carried out in the absence of the primary antibody showed no fluorescence on TNF$\alpha$-stimulated PAEC.

That xenogeneic serum directly induces NF-κB activation is also supported by electrophoretic mobility shift assay experiments performed in nuclear extracts from PAEC incubated for 1 h 30 min with human serum, human serum plus PDTC (25 μM), or heat-inactivated human serum. As shown in Figure 7, two bands with specific NF-κB binding activity were detected in nuclear extracts from human serum-treated cells. Treatment of cells with the NF-κB inhibitor PDTC resulted in inhibition of human serum-induced NF-κB activation. Nuclear extracts from cells treated with decomplemented human serum displayed only faint bands. The specificity of the binding reaction was confirmed by the ability of excess unlabeled NF-κB oligonucleotide to inhibit binding (not shown).

To evaluate the possible role of NF-κB on human serum-induced leukocyte adhesion and transmigration, PAEC were pretreated with the NF-κB inhibitors TPCK and PDTC 1 h before the addition of human serum. As shown in Figure 8, xenogeneic serum-induced leukocyte adhesion was significantly ($P < 0.01$) inhibited by TPCK and PDTC at 5 h, but not at 30 min and 1 h 30 min. Similarly, transmigration of leukocytes across PAEC exposed to xenogeneic serum was significantly ($P < 0.01$) reduced by NF-κB inhibitors only at 5 h (Figure 8).
These results suggest two temporally distinct pathways that cooperate in the adhesion and transmigration of leukocytes induced by xenogeneic serum. Although the early adhesive events we observed at 30 min and 1 h 30 min were not dependent on NF-κB activation, it seems plausible that upregulation of adhesive molecules on the endothelial surface, which occurred after a more prolonged exposure to xenogeneic serum, actually requires activation of NF-κB.

**Endothelial Surface Adhesive Proteins Involved in Leukocyte Adhesion**

To define the precise molecular determinants of the adhesive interaction of human leukocytes with porcine endothelium, we studied by confocal fluorescence microscopy endothelial expression of E-selectin, VCAM-1, and ICAM-1. As shown in Figure 9, PAEC treated for 5 h with xenogeneic serum showed a weak positive staining for E-selectin (Figure 9b) compared with homologous serum, which appeared negative (Figure 9a). VCAM-1 expression was upregulated on the luminal surface of PAEC exposed to human serum (Figure 9e) compared with cells incubated with porcine serum that faintly stained for VCAM-1. TNFα, used as positive control, remarkably increased the surface expression of E-selectin and VCAM-1. In xenogeneic serum-treated PAEC, staining for ICAM-1 resulted in a strong diffuse granular pattern (Figure 9h), which was quite comparable to that observed in TNFα-stimulated cells (Figure 9i). No fluorescence was observed when PAEC exposed to TNFα were processed in the absence of primary antibody.

The functional significance of the upregulation of VCAM-1 and ICAM-1 expression was investigated further by incubating porcine endothelium with the adhesion blocking anti-VCAM-1 and anti-ICAM-1 antibody. As shown in Figure 10, both anti-VCAM-1 and ICAM-1 antibody significantly (P < 0.01) inhibited (by 55 and 60%, respectively) leukocyte adhesion to human serum-treated PAEC. Treatment with irrelevant antibody did not modify human serum-induced leukocyte adhesion.

**Discussion**

The endothelium is the first site of contact between a vascularized xenograft and the recipient’s immune system. We studied the interaction of human leukocytes with porcine endothelium, as an in vitro model of vascular xenograft, under laminar flow conditions that mimic postcapillary venule circulation.

Confirming and extending our previous preliminary observations (30), here we documented that human serum, as a source of xenoreactive natural antibodies and complement, at a
conceivable that at this time, complement deposited on the endothelial surface is either shed or internalized into the cell cytoplasm. That complement plays an active role in xenogeneic serum-induced leukocyte adhesion is consistent with findings that human decomplemented serum, as well as C3-deficient human serum, failed to enhance the number of adherent leukocytes to porcine endothelium even under flow. In this context, a recent study showed that endothelial cells isolated from pigs transgenic for decay-accelerating factor, a human complement regulatory protein that inhibits the formation of C3b, are protected against neutrophil adhesion in the presence of human xenoreactive antibodies and complement (34). A considerable amount of in vivo evidence is available in models of discordant xenograft rejection (guinea pig hearts into rats) showing that complement blocking by cobra venom factor or soluble complement receptor type I treatment determined less or no C3 deposition with marked reduction in the number of infiltrating neutrophils and macrophages, which resulted in prolongation of xenograft survival (32,35). On the other hand, in a swine to primate model of hyperacute xenograft rejection, the depletion of circulating natural antibodies in the recipient prevented complement deposition, formation of platelet and fibrin thrombi, and neutrophil infiltration, suggesting that the fixation of complement on the blood vessels is a critical factor in the activation of the coagulation cascade and in the adhesion and infiltration of neutrophils (31).

\textbf{In vivo} hemodynamic shear forces strictly regulate the interaction of leukocytes with the endothelium through a series of coordinated steps regulated by different families of adhesive proteins. Rolling of leukocytes on the endothelium is mediated by selectins and precedes the process of stable adhesion, regulated by the Ig superfamily adhesive proteins ICAM-1 and VCAM-1, which are then responsible for leukocyte transmigration into the extravascular space (19,20). In our study, xenogeneic human serum induced massive adhesion and transmigration of leukocytes in porcine endothelium, but failed to promote leukocyte rolling. Rolling was instead remarkable on porcine endothelial cells activated with TNFα. Consistent with this finding, we observed by confocal fluorescence microscopy a weak staining for E-selectin on endothelial cells exposed to human serum for 5 h, whereas E-selectin expression was greatly upregulated upon TNFα challenge. At variance, the expression of VCAM-1 appeared upregulated on the luminal surface of cells treated with human serum compared with porcine serum. TNFα-stimulated cells were stained for VCAM-1 to a greater extent. Human serum also induced a strong intensity staining for ICAM-1 quite comparable to the effect of TNFα. These data, together with the evidence that the functional blocking of VCAM-1 and ICAM-1 with specific antibodies significantly reduced the number of leukocytes adhering to human serum-treated cells, can be taken to document that xenogeneic conditions strongly promote endothelial cell adhesive properties under flow.

Several studies indicate that NF-κB, a ubiquitously expressed transcription factor, plays a regulatory role in endothelial cell activation (36–38). In resting endothelial cells, NF-κB exists in an inactive form in the cytoplasm, bound to the

\textbf{Figure 8.} Effect of NF-κB inhibitors on xenogeneic serum-induced leukocyte adhesion (top panel) and transmigration (bottom panel) under flow conditions. Before the adhesion assay, PAEC were treated with NF-κB inhibitors PDTC (25 μM) and tosyl-phe-chloromethylketone (TPCK) (25 μM) 1 h before the addition of human serum for 30 min, 1 h 30 min, and 5 h. Data are expressed as percentage of adherent or transmigrated leukocytes with xenogeneic human serum considered as 100%. *P < 0.01 versus human serum.
inhibitory protein IκB. Cell activation by triggers such as cytokines and oxidants leads to proteolytic degradation of IκB, thus allowing NF-κB translocation into the nucleus where it binds to κB sites in the promoter regions of several proinflammatory genes, including adhesive molecules and chemoattractants (24,37–39). Our data show clearly by fluorescence microscopy as well as by EMSA that xenogeneic serum activated NF-κB in porcine endothelial cells. Therefore, we sought evidence for a functional role of NF-κB on human serum-induced leukocyte adhesion and transmigration by using two NF-κB inhibitors that by virtue of their anti-oxidant and protease inhibitory activities prevent the proteolytic degradation of IκB (23–25). We found that PDTC and TPCK did not affect the number of adherent and transmigrated leukocytes in porcine endothelial cells exposed to human serum for 30 min and 1 h. By contrast, at 5 h both inhibitors significantly reduced adhesion and transmigration. From these observations, we propose that two temporally distinct pathways are involved in leukocyte-endothelial interaction in xenogeneic conditions under flow. Thus, at early times C3 once deposited on endothelial surface directly promotes adhesion and transmigration of leukocytes possibly through their CD11b/CD18 receptor (13). On the other hand, our data that decomplemented human serum failed to activate NF-κB indicate that complement triggers

Figure 9. Confocal micrographs of PAEC treated for 5 h with porcine serum (a, d, and g), xenogeneic serum (b, e, and h), or TNFα (c, f, and i) and stained for E-selectin (a through c), vascular cell adhesion molecule-1 (VCAM-1) (d through f), and intercellular adhesion molecule-1 (ICAM-1) (g through i). PAEC exposed to human serum showed a weak staining for E-selectin (b) compared to cells treated with porcine serum that were negative (a). Expression of VCAM-1 and ICAM-1 increased in human serum-treated PAEC (e and h) compared to control cells (d and g). TNFα-treated cells were used as positive stimulus for adhesive proteins. Magnification, ×1500.

Figure 10. Effect of anti-VCAM-1 or anti-ICAM-1 antibody on xenogeneic serum-induced leukocyte adhesion under flow. Before the adhesion assay, PAEC treated for 5 h to human serum were exposed for 20 min with anti-VCAM-1 or anti-ICAM-1 antibody. Data are expressed as percentage of leukocyte adhesion with xenogeneic human serum considered as 100%. *P < 0.01 versus human serum.
intracellular events leading to NF-κB activation and consequent upregulation of adhesive molecules accounting for the adhesion and transmigration we observed at 5 h. Exactly how complement causes NF-κB activation remains to be elucidated. On the basis of the recent demonstration that activation of human complement cascade with assembly of membrane attack complex on porcine endothelium induced the synthesis and release of interleukin-1 (14), we speculate that this cytokine might be responsible in part for NF-κB activation and consequent induction of genes involved in the adhesive phenomena. On the other hand, there is evidence that in pulmonary artery endothelial cells C5a triggers the generation of radical oxygen O2· (40), which is a well known intracellular intermediate for NF-κB activation (41). Thus, it is possible that also in our experimental setting oxidant stress consequent to complement activation in xenogeneic conditions is responsible for the activation of NF-κB.

In conclusion, our results indicate that: (1) xenogeneic serum is a potent promoter of leukocyte adhesion and transmigration in porcine aortic endothelial cells under flow; (2) complement deposited on porcine endothelium is responsible for the early adhesion and transmigration of leukocytes; and (3) the adhesive events are regulated later by endothelial activation of NF-κB dependent genes.

The finding that xenogeneic serum promotes leukocyte-endothelial interaction depending on NF-κB activation may be relevant for designing future therapeutic strategies aimed at prolonging xenograft survival.

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