0022-1767/92/1494-1416$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 149, 1416–1423, No. 4, August 15, 1992
Printed in U.S.A.

EARLY RECOGNITION OF A DISCORDANT XENOGENEIC ORGAN BY HUMAN CIRCULATING LYMPHOCYTES[1]

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Cell-mediated immune mechanisms underlying discordant xenograft rejection are poorly characterized. In our study, using a human to rat xenogeneic ex vivo model, we show that a fraction of human lymphocytes, when perfused through the coronary system of a rat heart, rapidly and specifically adheres to the vascular endothelium and infiltrates the myocardium. Lymphocyte phenotypic analysis before and after perfusion, as well as the use of purified cell subpopulations, demonstrate preferential adhesion of $CD3^-$ $CD16^+$ NK cells. NK cell adhesion occurs via xenoreactive antibody-dependent and -independent pathways, because the selective removal of human IgG from the perfusion buffer markedly reduces but does not completely abrogate NK cell sequestration. However, T lymphocytes are retained in the xenoorgan via an antibody-independent pathway, as assessed by the lack of influence of IgG removal. Leukocyte integrins appear to play a crucial role in mediating adhesion of both lymphocyte subsets, because the pretreatment of lymphocytes with anti-CD11a, anti-CD11b, and anti-CD18 antibodies markedly reduces their retention into the xenogeneic organ. Retained human lymphocytes mediate rapid and direct damage of the xenoorgan, as demonstrated by histologic and functional alterations of the endothelium, impaired vascular resistance and in vitro lysis of rat endothelial cells by human NK cells. Taken together, these findings suggest a role for cell-mediated mechanisms in the rapid recognition and rejection of vascularized xenografts.

Rejection of vascularized xenografts is universally observed when discordant species are used as donor and recipient (1). Hyperacute rejection takes place within minutes to hours and is thought to be mediated by the recipient's preformed natural antibodies (2, 3), mostly IgM (4), that recognize a family of membrane glycoproteins expressed by vascular endothelial cells (5) and activate the complement and blood clotting cascades (6, 7). Evidence for these mechanisms is provided by the observation of IgM and complement factors deposited onto xenogeneic endothelial cells (8), by the results of Ig depletion and passive reconstitution experiments (9, 10) and by data showing that hyperacute rejection takes place only after neovascularization of nonvascular xenografts by donor-derived endothelium (11). The tissue specificity of natural antibodies has not been extensively defined. However, several experimental models (human to pig, rat to guinea pig) indicate that natural antibodies preferentially recognize a heterogeneous group of membrane glycoproteins expressed by endothelial cells, RBC, leukocytes, platelets, and kidney tubular and mesangial cells (5, 12).

Due to the hyperacute nature of the antibody-mediated rejection, it is unclear whether cell-mediated recognition of xenogeneic determinants plays a significant role in the rejection process. In this regard, in vitro studies suggest that xenoantigen recognition by T lymphocytes might take place both in concordant and in discordant combinations (13, 16). These studies, however, explore events that need days or weeks to occur, and might not be meaningful in predicting the outcome of the hyperacute rejection. Finally, a role for NK cells in the recognition of vascularized grafts has been recently suggested (17).

To evaluate the early cellular events of xenogeneic recognition, we have introduced a novel ex vivo approach in which an isolated rat heart is perfused with human PBL. We show that a fraction of human lymphocytes is rapidly and specifically retained in the heart; this phenomenon is due to xenoreactive antibody-dependent and independent adhesion of human lymphocytes to vascular endothelial cells and subsequent infiltration of the xenoorgan. Phenotypic and histologic analysis of lymphocyte subpopulations after circulation through the heart, as well as perfusion with selected lymphocyte subsets, reveal a preferential sequestration of NK cells in the xenoorgan. Moreover, morphologic analysis of the perfused organs, evaluation of the vascular resistance and in vitro assays of the NK cell-mediated lytic activity on rat endothelial targets, suggest a role for human lymphocytes in mediating early functional damage of the xenogeneic organ. We propose that cell-mediated effector mechanisms are involved in the rapid recognition and rejection of vascularized xenoorgans.

Received for publication April 8, 1992.
Accepted for publication May 28, 1992.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was partly supported by the BBS CNR Target Project 89.00154.70 and National Institutes of Health (NIH) Grant HL43331-01A2. J. R. B. is the recipient of an NIH Clinical Investigator Award (K08-HL02328-03).
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MATERIALS AND METHODS

Antibodies to human CD Ag. Anti-CD3 (Leu 4; IgG1) and anti-CD16 (Leu 11c; IgG1) were purchased from Becton Dickinson (Mountain View, CA). Anti-CD45 RB (PD7/26 and 2B/11, both IgG1) were purchased from Dako (Hägersten, Denmark). Anti-CD11a (TS 1.22; IgG1) and anti-CD18 (TS1.18; IgG1) were kind gifts of T. A. Springer (The Center for Blood Research, Harvard Medical School, Boston, MA). Anti-CD11b (44a; IgG1) was kindly provided by A. Mantovani (Institutem NEGRI, Milano, Italy). Anti-human β_2-microglobulin (L368; IgG1) was purchased from American Type Culture Collection (Rockville, MA).

Isolation and perfusion of rat hearts. Sprague-Dawley NOS outbred or (for syngeneic perfusion experiments) Lew/Ola inbred rats (250–280 g, Nossan Srl, Milan, Italy) were anesthetized by i.p. injection of heparinized sodium thiopental and the heart was isolated and mounted onto the Langendorff apparatus system (18). The perfusing buffer (144.1 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 1.2 mM Na_2SO_4, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 16.6 mM glucose, and 10 mM HEPES (Sigma Chemical Co., St. Louis, MO) pH 7.4, 37°C) was oxygenated (100% O_2) in a membrane oxygenator and circulated at a flow rate of 15 ml/min by a roller pump. After heart stabilization (20 min), 80 ml of the buffer were recirculated through the system. Upon addition of the cell suspension (final concentration 10^6 cells/ml), 1-ml aliquots of the circulating medium were collected at different times from the efferent limb of the perfusion apparatus for cell count and characterization. Cell counts were performed by two investigators using a standard hemocytometer. Experiments were carried out for up to 60 min. The heart performance was monitored during the whole time course of the experiment by recording coronary pressure by a pressure transducer (Harvard Apparatus model 52-9966, Natick, MA) inserted between the pump and the heart.

Isolation of human PBL and cell subset purification. Human PBL were isolated from buffy coats obtained from the local blood center by centrifugation on density gradient (Lymphoprep, Pharmacia, Uppsala, Sweden). Mononuclear cells were subsequently washed three times in PBS and routinely depleted of macrophages by two rounds of adhesion on plastic Petri dishes and passage through nylon wool column. Macrophage depletion was routinely performed, as it was observed that these cells nonspecifically adhere to the Langendorff apparatus and, therefore, make the evaluation of cell binding to the heart impossible. Cells were resuspended in the perfusion buffer; heat-inactivated serum from different sources was added to the buffer solution at a concentration of 2% v/v. Monocyte-depleted lymphocytes were further purified by negative selection using a panning technique. Briefly, cells were preincubated with either anti-CD16 or anti-CD3 mAb for 30 min on ice, washed in cold PBS, and incubated on Petri dishes that had been previously coated with 10 μg/ml goat anti-mouse Ig antiserum. Nonadherent cells were collected by gently washing the plates and used when indicated. Purified subsets obtained by this technique routinely contained less than 3% of the contaminating population.

Isolation of rat lymphocytes. Rats were killed and the spleen was surgically removed; splenocytes were obtained by perfusing the spleen at high pressure with tissue culture medium (consisting of 10% heat inactivated FCS in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY) through a 21-gauge needle. Cells were pelleted and the E lysed with a brief incubation in ammonium chloride. Splenocytes were then centrifuged and counted. Cell viability after this procedure was always >95%. Macrophages were depleted as described above.

IgG depletion of human serum. In some experiments, pooled human serum was selectively depleted of IgG by affinity chromatography on Sepharose-conjugated protein G. Briefly, human serum was incubated at room temperature over a prepacked column of Sepharose-conjugated protein G (LKB Pharmacia, Uppsala, Sweden) for 30 min at room temperature and eluted with PBS according to the manufacturer's instructions. Electrophoresis of the eluted serum and immunoelectrophoretic analysis confirmed a reduction in the concentration of IgG from an average of 1500 to <5 mg/dl. Serum IgM concentration was only marginally affected by this procedure. The total concentration and the electrophoretic profile of the other main components of the serum were not influenced by such treatment.

Phenotypic analysis. Phenotypic analysis of the recovered lymphocyte subpopulations was performed by direct (using FITC-conjugated antibodies) or indirect (using a FITC-conjugated goat anti-mouse IgG antiserum, Zymed, CA) immunofluorescence. Samples were analyzed on a FACS (FACS IV Becton Dickinson, Emeryville, CA). Forward and 90° scatter profiles were evaluated and no significant difference was recorded between human and rat lymphocytes. Rabbit anti-human IgG and IgM antisera were purchased from Dako and used in the immunohistochemistry studies. The relative percentages of LFA-1^{dull} and LFA-1^{bright} cells were determined by cytofluorographic analysis performed after calibration of the flow cytometer with labeled microspheres as previously described (19).

Antibody pretreatment of human lymphocytes. In some experiments human lymphocytes were pretreated with mAb to assess the role of different molecules in mediating adhesion to xenogeneic endothelium. Briefly, cells were preincubated with saturating concentrations (10 μg/ml) of mAb for 30 min on ice. Cells were subsequently washed in cold PBS and resuspended in the perfusing buffer containing 2% unfractionated or IgG-depleted human serum and used in the perfusion protocol.

Histology and immunohistochemistry. At the end of the perfusion, the heart was removed from the apparatus and samples of the organ were processed either by fixation in 10% paraformaldehyde in PBS or by snap freezing in precooled isopentane and stored at −80°C until used. Conventional histology (hematoxylin-eosin staining) and immunohistochemistry studies were carried out. Tissue sections were incubated with various antibodies and subsequently stained with peroxidase-conjugated goat anti-mouse Ig antiserum or rabbit anti-human IgG or IgM polyclonal antisera (Dako). Sections were developed with 3,3′diaminobenzidine tetrahydrochloride (5 mg in 10 ml Tris buffer, pH 7.4, H_2O_2 0.03%) and analyzed. Tissue specimens from different organs of four outbred rats were collected for immunohistochemical characterization of the binding specificity of human anti-rat natural antibodies. Briefly, specimens of the heart, spleen, liver, lung, kidney, pancreas, and muscle were harvested and snap-frozen as described above. Sections were incubated at room temperature with buffer alone or serial dilutions of pooled human serum (1/50 to 1/500), washed in PBS, stained with peroxidase-conjugated rabbit anti-human IgG or IgM antisera, and developed as described above.

Evaluation of endothelial cell damage. At the end of the various experiments, organs were perfused with colloidal carbon black solution (Rotring, Germany) (1/200 v/v in KRH buffer) for 4 min. Samples were immediately harvested for histology and electron microscopy.

Electron microscopy. Specimens were fixed for 3 h in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embebbed in epoxy resin. Sections were obtained with a Reichert-Jung Ultracut (Vienna, Austria) ultramicrotome and examined with a Zeiss CEM 902 (Carl Zeiss, Oberkochen, Germany) electron microscope.

Endothelial cell cultures. Primary rat endothelial cell cultures were established in vitro. Briefly, animals were killed and aortas were surgically removed. The intimal surface was exposed and thoroughly rinsed with PBS. Collagenase digestion of the endothelial layer was carried out for 30 min at 37°C (Collagenase type VI) (Sigma). The intimal surface was then gently scraped with a scalpel blade and the endothelium pelleted by centrifugation. Cells were gently dissociated by passage through a 21-gauge needle and cultured in 25-cm^2 tissue culture flasks (Bibby-Corning, Staffordshire, UK) in DMEM 20% FCS. Confluent cultures were trypsinized and subcultured in flat bottom 96-well plates for the cytotoxicity assays. Endothelial cell monolayers were identified by morphologic structural (typical cobble-stone appearance) and ultrastructural criteria.

Cell-mediated cytotoxicity. Cell-mediated cytotoxicity was evaluated in a standard ^{51}Cr release assay. Briefly, confluent endothelial cell monolayers were labeled with ^{51}Cr (5 μCi/well) in DMEM 10% FCS for 60 min at 37°C. Unincorporated ^{51}Cr was then removed by washing the monolayers with 200 μl of warm medium four times. Human NK cells were added to the ^{51}Cr labeled endothelial cells to achieve final effector to target ratios of 30:1, 10:1, 3:1, and 1:1. Experiments were carried out in DMEM in the presence of either 10% heat-inactivated pooled human serum or 10% heat-inactivated rat serum. After 4 h of incubation, supernatants were harvested and counted in a γ-counter (Packard Instruments, Downers Grove, IL). Percent cytotoxicity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}) \times 100.$$

Statistical analysis. Student's *t*-test was used in the statistical evaluation of the data.

RESULTS AND DISCUSSION

This study was designed to investigate the earliest stages of cellular immune recognition of discordant xenogeneic vascularized organs. We sought to analyze these events in an experimental system that closely mimics the

in vivo situation, particularly for the hemodynamic and shear forces that affect the recognition of donor capillary endothelium by circulating recipient lymphocytes. For this purpose, we used the Langendorff rat heart perfusion model, which was originally devised to study cardiac contractile performance and metabolism (18). In this model, an oxygenated buffer containing glucose as energy substrate is injected at constant flow through a cannula inserted in the ascending aorta. Retrograde perfusion keeps the aortic valve closed, just as in the in situ heart during diastole, and the perfusate is displaced through the coronary arteries. Coronary pressure is recorded during the experiment as index of the vascular resistance, because the flow is kept constant by means of a pump. The perfusate reaches the coronary sinus and hence the right atrium, drops off the heart, and is collected for a subsequent cycle. We adapted this model to the study of lymphocyte perfusion by using a continuous recirculation apparatus (flow rate 15 ml/min) in which lymphocytes were injected at an initial concentration of 10^6 cells/ml and the perfusing buffer was maintained at constant temperature (37°C).

Human lymphocytes are rapidly and selectively retained in xenogeneic organ: contribution of xenoreactive antibodies. The concentration of lymphocytes in the perfusate was evaluated at 10-min intervals (corresponding to two complete perfusion cycles). When the xenogeneic combination was used (i.e., human lymphocytes in the presence of 2% heat-inactivated human serum), a rapid decrease in the cell concentration in the coronary outflow was recorded (Fig. 1*a*). Lymphocyte sequestration in the heart was maximal after the first two cycles and continued in subsequent cycles, reaching an apparent plateau between the 8th and the 10th passage through the coronary system. Xenogeneic lymphocyte witholding was markedly reduced on removal of IgG from human serum (Fig. 1*a*). The rate of human lymphocyte sequestration in the presence of rat serum or xenogeneic non-human serum (e.g., bovine) was comparable to the one observed using IgG-depleted human serum (not shown).

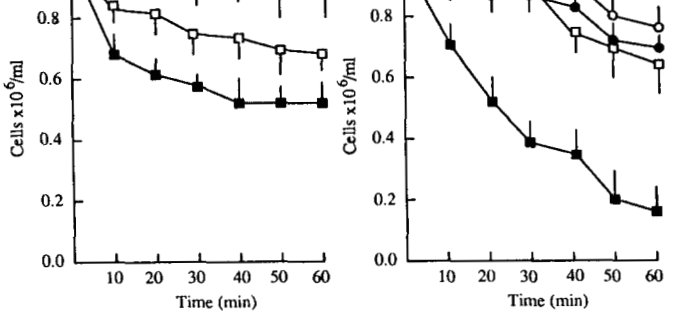

Figure 1. Circulating human lymphocytes are retained into the rat heart. *a*, Xenogeneic human lymphocytes (*square symbols*) or syngeneic rat lymphocytes (*round symbols*) were circulated through the heart at an initial concentration of 10^6 cells/ml in the presence of 2% unfractionated (*filled symbols*) or IgG-depleted (*empty symbols*) heat-inactivated serum homologous to the perfusing lymphocytes; *b*, highly purified $CD16^+CD3^-$ (*square symbols*) or $CD16^-CD3^+$ (*round symbols*) human lymphocytes were circulated through the heart at an initial concentration of 10^6 cells/ml in the presence of 2% unfractionated (*filled symbols*) or IgG-depleted (*empty symbols*) heat-inactivated human serum. Cell counts were performed in the outflow at 10-min intervals. Results are expressed as mean ±SD of four separate experiments.

The importance of the genetic disparity between the perfused organ and the circulating lymphocytes in determining their disappearance from the perfusate was evaluated using lymphocytes syngeneic to the perfused organ. Under these conditions, no cell sequestration was observed, regardless of the source of the serum added to the buffer (Fig. 1*a*). The nonlinear decrease in xenogeneic lymphocyte concentration during perfusion of the rat heart, and the virtual absence of cell sequestration in the syngeneic combination, argue against a nonspecific loss of cells in the system. The lack of sequestration in the syngeneic experiments suggests that a specific recognition of xenodeterminants expressed on vascular endothelium by circulating human lymphocytes is indeed responsible for their disappearance from the perfusate. The presence of IgG homologous to the circulating lymphocytes maximizes their retention into the xenogeneic organ. This evidence argues in favor of a specific interaction between preexisting antibodies and xenogeneic determinants, as the enhancing effect of serum is abrogated by the selective depletion of IgG. Homology between Ig and circulating lymphocytes also appears to be essential, as the use of sera unrelated to both the species under study is devoid of any effect on the rate of lymphocyte sequestration (not shown).

$CD16^+CD3^-$ NK cells are predominant lymphocyte subset infiltrating xenogeneic organ. The enhancing effect of Ig described above can be explained by one of two hypotheses: xenoreactive antibodies mediate a functional modulation or induce a direct damage of endothelial cells, resulting in increased permeability or in the induction of adhesiveness toward circulating lymphocytes; alternatively, endothelial cell-bound Ig opsonize capillary endothelia making them accessible to recognition by lymphocytes bearing surface Ig receptors. The former hypothesis implies a nonselective retention of lymphocytes into the xenogeneic organ. The latter restrains the antibody-mediated enhancement of cell sequestration to lymphocytes expressing membrane FcR. To distinguish between these two possibilities, PBL were separated by negative selection based on the reciprocal expression of the CD16 (low affinity FcγR) and the CD3 markers. The selected subsets were perfused through the coronary system in the presence of unfractionated or IgG-depleted homologous serum. In the presence of homologous IgG, $CD16^+CD3^-$ lymphocytes underwent dramatic sequestration in the xenoorgan, with up to 90% of the input population disappearing from the outflow within 10 perfusion cycles (Fig. 1*b*). $CD16^-CD3^+$ cells showed a less striking, but still significant decrease, with an average 32% reduction after 10 passages in four separate experiments (Fig. 1*b*). Removing IgG from the serum induced a marked decrease in the fraction of $CD16^+CD3^-$ cells witheld in the heart, whereas the sequestration of $CD16^-CD3^+$ cells was only marginally affected. These data suggest a causative role of homologous, xenoreactive IgG in the sequestration of FcγR positive lymphocytes and make it unlikely that vascular endothelium permeability is nonspecifically increased by the binding of heterophylic antibodies, at least in the short term. A significant antibody-independent retention of $CD16^-$- $CD3^+$ lymphocytes was consistently observed, implying that a fraction of FcγR negative T cells is ca-

pable of rapid and specific recognition of the xenogeneic endothelium.

Immunohistochemical analysis was conducted to obtain in situ evidence for the phenomena observed when rat hearts were perfused with unseparated human lymphocytes in the presence of homologous serum. Figure 2 shows human lymphocytes (stained with an anti-human common leukocyte Ag mAb) adhering to the capillary endothelium and infiltrating the myocardium (*A* and *B*). Endothelial cells appear swollen in many microscopic fields, suggesting an anatomical damage of the vascular intimal layer (*B*). A significant proportion of the infiltrating lymphocytes react with an anti-human CD16 antibody (*C*). *D* demonstrates that capillary endothelium of rat hearts perfused with 2% heat-inactivated human serum stains brightly and diffusely with an anti-human IgG antiserum. This suggests that the Ig-dependent cell sequestration observed in the xenogeneic lymphocyte perfusion studies is a consequence of xenoreactive antibodies of the G isotype binding to the endothelial cell lining of coronary vessels. A similar histologic pattern was observed with an anti-human IgM antiserum (not shown). Control experiments consisted of perfusion of rat hearts with buffer and subsequent staining of tissue specimens with rabbit anti-human Ig antiserum. Nonspecific binding of anti-human Ig antiserum on cardiac endothelium was never observed (see below). Myocardial sections obtained from syngeneic lymphocyte perfusion experiments always failed to show rat lymphocytes adhering to the vascular endothelium or infiltrating the myocardial fibers (not shown). Endothelial cells never appeared swollen or anatomically damaged in this syngeneic combination.

Xenoreactive human antibodies preferentially recognize vascular endothelium. To assess whether the binding of human natural antibodies to rat cells is a general phenomenon (i.e., human natural antibodies bind rat cells without any tissue specificity) or it is restricted to structures selectively expressed by vascular endothelial cells, specimens from various tissues were obtained, processed, and stained as described in *Materials and Methods*. Table I shows that reactivity is observed toward small vessels of most organs (lung, kidney, and muscle). The only nonvascular structures specifically recognized by the human natural antibodies appear to be kidney tubules, endocrine pancreas, and lymphoid cells in the spleen. Thus, xenoreactive natural antibodies appear to preferentially, although not exclusively, recognize structures expressed by vascular endothelial cells. High background staining by the anti-human Ig antiserum was observed only in the spleen.

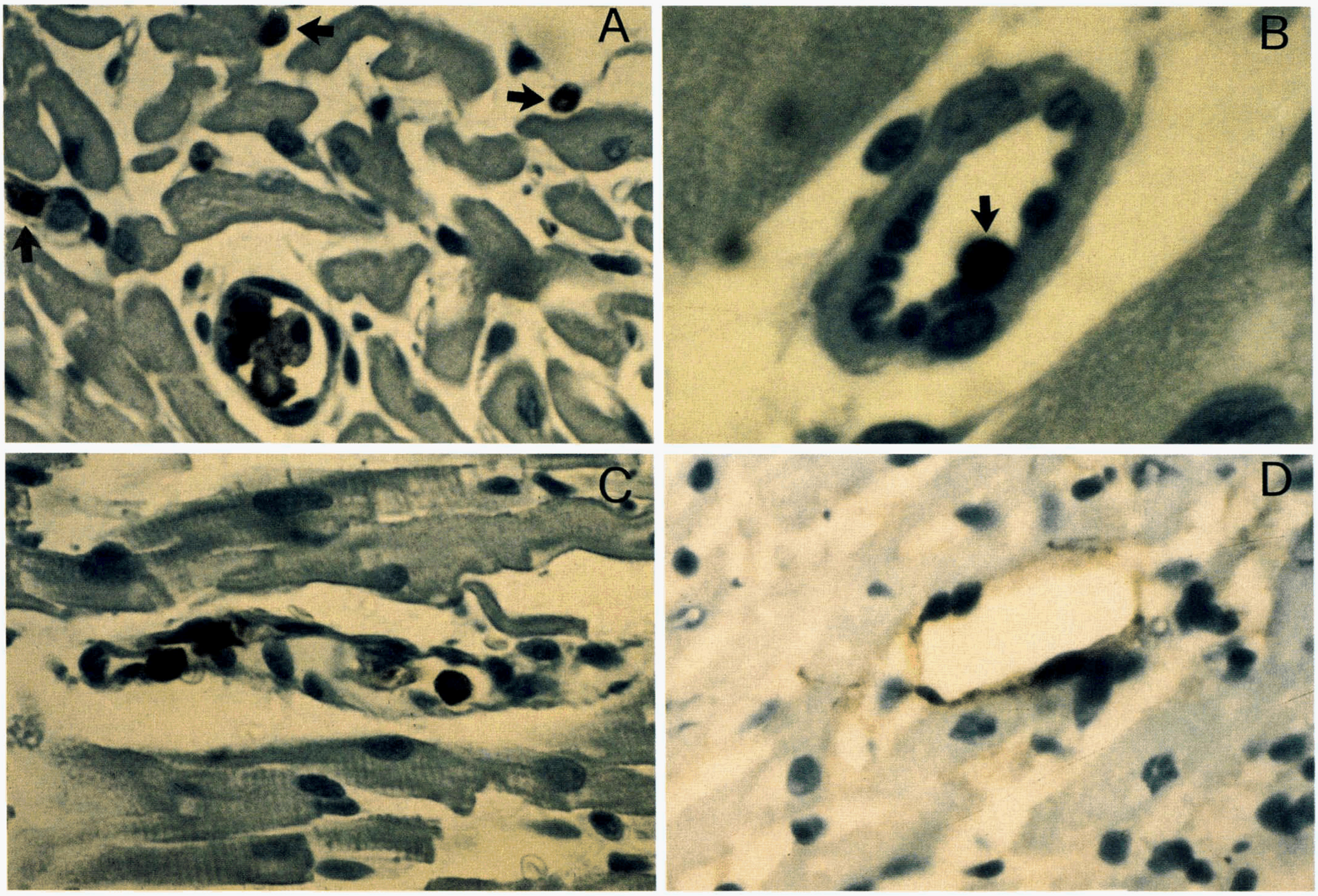

Figure 2. Immunohistochemical analysis of lymphocyte sequestration in the heart. *A*, Immunoperoxidase staining of human lymphocytes (stained with an anti-CD45 mAb) adhering to the vascular endothelial cells and infiltrating the myocardium (*arrows*) after four cycles of perfusion in the presence of 2% heat-inactivated unfractionated human serum (400×); *B*, at a higher magnification (1000×), a CD45 lymphocyte adhering to the capillary endothelium, that appears swollen; *C*, immunoperoxidase analysis of human NK lymphocytes adhering to the xenogeneic endothelium upon perfusion, as described in *A*. Staining was performed with an anti-CD16 mAb; *D*, vascular endothelial cells are stained by an anti-human IgG polyclonal antiserum after a 20-min perfusion with 2% heat-inactivated human serum.

TABLE I

Reactivity of human preformed natural antibodies with xenogeneic rat tissues[a]

Organ	Structures Recognized
Lung	Small vessels, capillaries
Striated muscle	Small vessels, capillaries
Spleen	Lymphoid cells
Heart	Small vessels, capillaries
Kidney	Tubular epithelium, capillaries
Pancreas	*Langerhans islets, capillaries*

[a] Snap-frozen tissue sections were stained with a 1/100 dilution of human serum and a peroxidase-conjugated rabbit anti-human Ig antiserum, as described in *Materials and Methods*.

Leukocyte integrins play major role in lymphocyte adhesion and migration through xenogeneic endothelium. To further explore the molecular interactions underlying lymphocyte infiltration of the xenogeneic organ, purified $CD16^+CD3^-$ or $CD16^-CD3^+$ cells were incubated with a combination of anti-CD11a and anti-CD18 antibodies before injection into the xenogeneic organ. Figure 3*a* shows that the antibody treatment virtually abrogates lymphocyte witholding in the perfused organ in both subsets, demonstrating a major role of leukocyte integrin pathway in the adhesion to and transmigration through xenogeneic endothelium. Pretreatment of either subpopulation with an isotype-matched control antibody (anti-β_2 microglobulin) did not affect cell sequestration. These findings are consistent with previous in vitro observations, showing that human leukocyte integrins can productively interact with their xenogeneic ligands (20). To assess the individual contribution of leukocyte integrins to the sequestration phenomenon, human lymphocytes were pretreated with either anti-CD11a, anti-CD11b, or anti-CD18; Figure 3*b* shows that the antibody to the common β subunit of leukocyte integrins inhibited cell sequestration with the highest efficiency, whereas antibodies to individual α-chains showed significant but less efficient inhibition of cell witholding. These findings indicate a role for both CD11a/CD18 and CD11b/CD18 in mediating the adhesion of NK cells to xenogeneic endothelial cells. It is noteworthy that also in the antibody-mediated sequestration of $CD16^+CD3^-$ cells, leukocyte integrins represent a critical pathway of lymphocyte adhesion to xenogeneic endothelium. It has recently been shown that the avidity of LFA-1 for its ligand can be dramatically up-regulated by triggering the Ag receptor complex in T cells (21). A similar phenomenon occurs in $CD16^+$ cells when the FcγR is cross-linked by specific monoclonal antibodies (R. Pardi and J. R. Bender, unpublished observations). We postulate that the recognition of endothelial cell-bound, xenoreactive IgG by $CD16^+$ lymphocytes may serve as a triggering mechanism to increase their binding efficiency to the endothelium via LFA-1 and possibly, other leukocyte integrins expressed by this subset of lymphocytes.

Comparative phenotypic analysis of pre- and postperfusion lymphocytes (Table II) further emphasizes the importance of LFA-1 in this process. LFA-1^{bright} lymphocytes, which have been shown to comprise the vast majority of NK cells and a subset of $CD8^+$ and $CD4^+$ cells displaying a "memory" phenotype (22, 23), are preferentially retained in the xenogeneic organ. The disappearance of lymphocytes expressing high density of LFA-1 is clearly detectable after the first two perfusion cycles, and it is virtually complete within the 10th passage into the coronary system. The mean relative ratio of LFA-1^{dull} vs LFA-1^{bright} cells in five different experiments increased significantly after two perfusion cycles, and even more dramatically after eight perfusion cycles (Table II). The above data present evidence that rapid and specific lymphocyte adhesion and transmigration through capillary endothelium occur in the xenogeneic human to rat combination. This is mainly due to the capability of human $CD16^+CD3^-$ NK cells, and a subset of $CD16^-CD3^+$ T cells to recognize and traverse rat vascular endothelial cells. This phenomenon takes place both in the presence and in the absence of human IgG (although to a very different extent), indicating that both antibody-dependent and antibody-independent recognition events capable of triggering lymphocyte adhesion and trans-endothelial cell migration are involved. The results also suggest that xenoreactive IgG, in addition to IgM, may have a pathogenetic role in hyperacute recognition and rejection of vascularized xenografts. It is likely that a similar triggering pathway might be used by polymorphonuclear cells, which express the low affinity Fcγ and the C3b receptors and have been shown to bind to xenogeneic endothelium during hyperacute rejection (4).

We have previously shown that, in allogeneic human

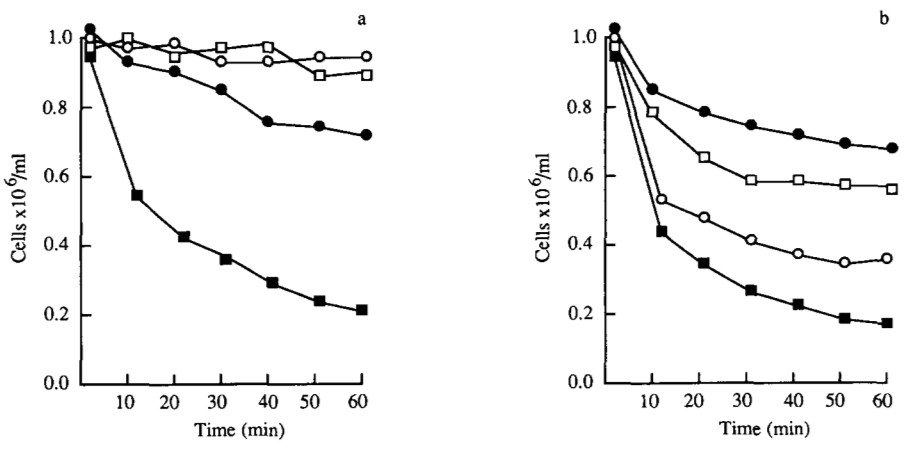

Figure 3. The effect of anti-LFA-1 and anti-Mac 1 antibodies on human T and NK cell recovery after rat heart perfusion. *a*, Purified human $CD16^-CD3^+$ (*round symbols*) or $CD16^+CD3^-$ (*square symbols*) lymphocytes were circulated through the heart in the presence of 2% heat-inactivated human serum. Cells were treated with 10 μg/ml of either a combination of anti-CD11a and anti-CD18 antibodies (*empty symbols*) or an isotype-matched negative control mAb (*filled symbols*) before injection into the heart. Cell counts were performed as described in Figure 1. Results are representative of three separate experiments. *b*, Purified human $CD16^+CD3^-$ lymphocytes were circulated through the heart in the presence of 2% heat-inactivated human serum. Before injection in the heart, cells were treated with 10 μg/ml of the following antibodies: anti-CD11a (*round empty symbols*); anti-CD11b (*square empty symbols*); anti-CD18 (*round filled symbols*), or with an isotype-matched control antibody (*square filled symbol*). Cell counts were performed as described above. Results are representative of three separate experiments.

TABLE II

Relative percentages of LFA-1^{dull} and LFA-1^{bright} lymphocytes upon perfusion of xenogeneic heart[a]

Expt.	Basal		Two Cycles		Eight Cycles	
	% bright	% dull	% bright	% dull	% bright	% dull
1	36	64	23	77	13	87
2	48	52	22	78	12	88
3	44	56	25	75	20	80
4	42	58	24	76	11	89
5	37	63	12	88	10	90
Mean (±SD)	41.4 ± 5	58.6 ± 5	21.2 ± 5	78.8 ± 6	13.2 ± 4	86.8 ± 4
Ratio[b]	1.44 ± 0.29		4.07 ± 1.8[c]		7.02 ± 1.9[d]	

[a] Percentages of LFA1^{dull} and LFA-1^{bright} human PBL were determined by FACS analysis before (basal) or after two and eight perfusion cycles into the xenogeneic rat heart, as described in *Materials and Methods*.
[b] Mean (±SD) ratio of LFA-1^{dull} vs LFA-1^{bright} lymphocytes.
[c] $p < 0.02$.
[d] $p < 0.002$.

systems, the capability of lymphocytes to adhere to vascular endothelial cells correlates with the level of expression of LFA-1 on the effector cells (19, 22, 24). NK cells are homogeneously high expressors of LFA-1 and indeed adhere avidly to allogeneic endothelia. In the xenogeneic system, antibody inhibition experiments have indicated that a similar preferential recognition of endothelial cells by NK cells is evident and the same pathways that are involved in the adhesion to alloendothelia could be involved. Unlike the allogeneic system (25, 26), however, the main triggering event for NK cell adhesion to, and recognition of, xenogeneic endothelium appears to be the presence of antibodies bound to the vascular cells. Antibody-independent recognition of xenoantigens by NK cells, if confirmed by further studies, would support a role for this lymphocyte subset in early cellular rejection of xenoorgans, such as skin, not susceptible to hyperacute humoral rejection. In addition, our data as well as in vitro data from our laboratory show that other leukocyte integrins, such as the CD11b/CD18, play a role in the adhesion of NK cells to vascular xenogeneic endothelium (27).

Human NK cells induce functional damage of xenogeneic organ and lyse xenogeneic endothelial cells in vitro. Functional damage of the heart was monitored by recording the coronary pressure during the experiments. Control experiments, in which hearts were perfused with either buffer alone or any of the syngeneic combinations, showed no significant alterations (not shown). Similarly, perfusion of rat hearts with either human Ig or human Ig together with CD3$^+$CD16$^-$ human lymphocytes induced only a transient rise in the coronary pressure that reverted to baseline values within 30 min (Fig. 4*b* and not shown). However, perfusion of xenogeneic hearts with purified NK cells in the presence of human Ig induced a progressive increase in the coronary pressure during the timecourse of the experiment (Fig. 4*a*).

Functional integrity of the endothelial cell lining was evaluated by perfusing the heart, at the end of the various experiments, with colloidal carbon black, which is a sensitive marker of increased microvascular permeability (28). When the endothelial layer is intact, the dye is exclusively localized inside the vessels. However, when endothelial damage occurs, the dye diffuses through the endothelial cell junctions into the interstitium and surrounding tissues and is readily detected in the microscopic specimens. When either buffer alone or buffer containing human serum were circulated through the heart, no endothelial damage was ever observed, as assessed by the exclusive intravascular localization of colloidal carbon black (Fig. 5*D*). However, when purified NK cells were circulated along with human serum, carbon black extravasation was observed. Figure 5, *A* and *B* shows the extravascular localization of the colloidal dye at the level of the endothelial cell basal membrane and surrounding interstitium; at higher magnification (*C*), the colloidal dye can be seen infiltrating a junction between endothelial cells.

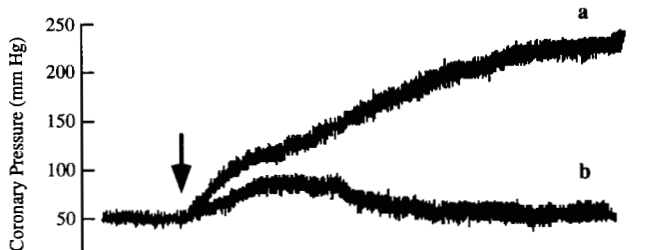

Figure 4. Coronary pressure profiles during heart perfusion. Rat hearts were isolated and perfused with buffer containing 2% heat-inactivated unfractionated human serum with (*a*) or without (*b*) NK cells (at a starting concentration of 10^6 cells/ml). Human Ig alone or human Ig with NK cells were injected at time 0 (*arrow*).

To assess the role of NK cells in mediating direct damage of xenogeneic vascular endothelium, ^{51}Cr-labeled monolayers of rat vascular endothelial cells were cocultured in the presence of human purified NK lymphocytes. As shown in Figure 6, human NK cells efficiently lyse the xenogeneic targets both in the presence of human serum and in the presence of rat serum. Unlike the lysis of the K562 target, however, the amount of xenogeneic endothelial cell lysis is consistently higher when human serum is used in the assay. This argues in favour of a contributory role of antibody-dependent cytotoxicity in the damage of xenogeneic endothelial cells by NK cells.

Taken together, these data strongly suggest a synergistic effect of human lymphocytes and xenoreactive natural antibodies in mediating functional damage of the xenoorgan. Our data point to a possible contribution of NK cells in the early damage of vascular endothelium that occurs in discordant xenograft hyperacute rejection in vivo. The role of NK lymphocytes as cellular effectors of graft rejection is also suggested by previous studies showing early allograft invasion by phenotypic and functional NK cells (29), and by the demonstration that the presence of such cells within the graft is predictive of

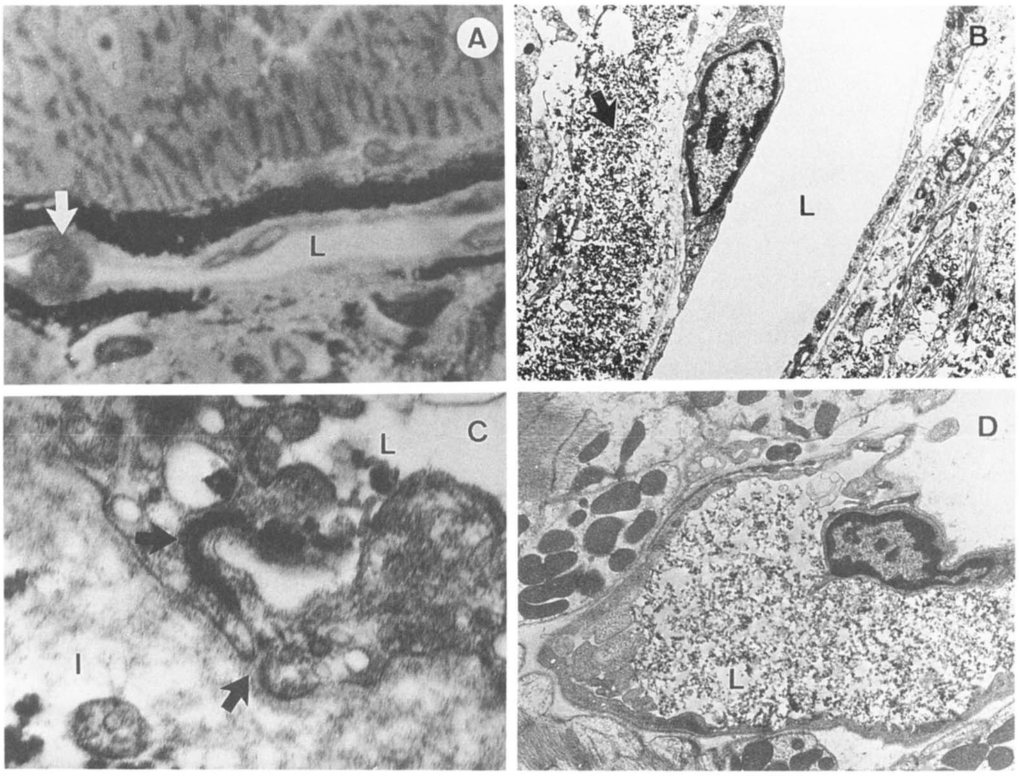

Figure 5. Endothelial damage in the perfused rat heart. Isolated rat hearts were perfused with buffer containing 2% heat-inactivated human serum and NK cells (*A–C*) or buffer containing 2% heat-inactivated human serum (*D*). After 50 min, colloidal carbon black was added to the buffer to achieve a final concentration of 1/200 (v/v). Four min later, organs were removed and samples were immediately fixed in 2.5% glutaraldehyde for electron microscopy. *A* and *B* show the extravasation of the colloidal dye at two different magnification (*A*, 400×; *B*, 3000×). The *white arrow* in *A* indicates an NK cell adhering to the endothelial layer. The *black arrow* in *B* is positioned in the dilated perivascular space that appears filled with carbon black. *C* shows, at higher magnification (30,000×), a junction between endothelial cells that is infiltrated by carbon particles (*arrows*); the dye can also be seen in the surrounding interstitial space. *D* shows the exclusive intravascular localization of the dye in a control experiment (3000×). (*L*, lumen; *I*, interstitium).

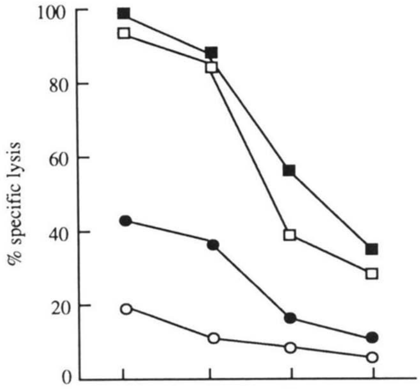

Figure 6. Human NK cells efficiently lyse rat endothelial cells in vitro. Purified human NK cells were coincubated with ^{51}Cr-labeled cultured rat endothelial cells (*round symbols*) or K562 cells (*square symbols*) at the indicated E:T ratios in the presence of either human (*filled symbols*) or rat (*empty symbols*) heat-inactivated serum (10% final concentration). Results are expressed as percent specific lysis, as described in *Materials and Methods*.

subsequent rejection (30).

Interestingly, several reports have described the existence of a clinical and histopathologic syndrome that closely resembles a GVHR[3] when human PBL from normal donors are grafted into immunodeficient rodents (31, 32). Although T and B cells are observed in the sites of GVHR (liver, skin, etc.), no information is given concerning the percentage of NK cells present in the reactive foci. Furthermore, histologic specimens obtained 4 to 8 wk after engraftment, as in the above studies, may be inadequate to detect early events of xenogeneic recognition mediated by NK cells. For these reasons we believe that more focused observations on the fate of NK cells in the early engraftment of immunodeficient rodents would be required to define the role of this subpopulation in the development of GVHR.

In conclusion, our work demonstrates that a fraction of human lymphocytes comprised in the LFA-1^{bright} population specifically recognizes the xenogeneic endothelium and infiltrates the myocardium of the perfused heart. Recent evidence has indicated that the recirculation patterns of naive (LFA-1^{dull}) and memory (LFA-1^{bright}) T lymphocytes are quite different, in that memory cells traffic from blood to peripheral tissues whereas naive cells selectively traffic from blood to lymph nodes through high endothelial postcapillary venules (33). It is therefore likely that circulating lymphocytes capable of recognizing xenodeterminants expressed by vascular endothelium also require a unique array of adhesion molecules, possibly reflecting a discrete stage of differentiation, to efficiently traverse the endothelial cell barrier and migrate into the xenogeneic tissue.

Acknowledgments. The authors thank Hugh Auchincloss, Jr., Robin Geller, Jeffrey Platt, and Henry Winn for helpful discussion. We are grateful to Eugenio Leone for his excellent work with EM analysis.

[3] Abbreviation used in this paper: GVHR, graft vs host reaction.

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