Cellular Early Immune Recognition of Xenogeneic Vascular Endothelium

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THE clinical outcome of xenografts has been so far very disappointing.[1] In the case of discordant species, rejection of vascularized xenografts occurs within minutes, primarily as a consequence of preformed natural antibodies binding to xenodeterminants expressed by vascular endothelial cells.[2] This event activates the complement and blood clotting cascades and eventually leads to hyperacute rejection.[3] The participation of cellular immune effector mechanisms to xenograft rejection has not yet been clearly defined, but evidence exists suggesting that cell-mediated recognition of xenodeterminants does occur, at least in vitro.[4,5] Furthermore, recent data suggest that natural killer (NK) cells might play a role in the early events of recognition of vascularized xenografts.[6]

We utilized two different models to evaluate the role of immunocompetent cells in the early events of xenogeneic tissue recognition and damage. The first model (ex vivo) is based on the continuous recirculation of human lymphocytes through an isolated and perfused xenogeneic organ (rat heart). The second (in vitro) analyzes the interactions between human lymphocytes and cultured xenogeneic endothelial cells.

The ex vivo system allowed us to establish that human lymphocytes rapidly and specifically adhere to xenogeneic endothelial cells and that this phenomenon is mediated by the engagement of at least two different adhesion pathways, one dependent on and the other independent of the presence of human immunoglobulins of the G isotype. This adhesion appears to be preferentially mediated by NK cells.

The in vitro system, on the other hand, was devised to understand in further detail the molecular basis of this cell–cell interaction. This latter model confirms the prominent role of NK cells in mediating the adhesion phenomenon and shows that this subset of cytotoxic lymphocytes efficiently lyses xenogeneic endothelia; both adhesion and killing are mediated by multiple cell–cell interactions, some of which require the presence of endothelial cell-bound xenoreactive natural antibodies.

MATERIALS AND METHODS

Human peripheral blood lymphocytes (PBL) were separated from random donor buffy coats by centrifugation over a density gradient (Lymphoprep, Pharmacia, Sweden), and routinely depleted of macrophages with two rounds of adhesion on plastic followed by incubation over nylon wool columns. Various subpopulations were purified using a panning technique. The selected populations (T and NK cells) were routinely >95% pure.

Rat hearts (Sprague-Dawley outbred strain) were explanted and mounted onto a Langendorff perfusion apparatus.[7] Human lymphocytes were circulated through the heart at an initial concentration of 1 million cells/mL. The perfusate contained 2% heat-inactivated human serum (HS). In some cases, HS was depleted of IgG by affinity chromatography on Sepharose-conjugated Protein G before its utilization in the perfusion experiments. Syngeneic control experiments in the ex vivo model system were performed with hearts, heat-inactivated serum, and lymphocytes from Lew/Ola inbred rats. Aliquots of the perfusing buffer were harvested at 10-minute intervals from the efferent limb of the Langendorff apparatus and cell counts were performed using a standard hemocytometer.

Bovine adrenal capillary endothelial cells (BACE) were obtained as previously described.[8] Cells were cultured in Dulbecco's modified eagle's medium (DMEM) 10% fetal calf serum (FCS). Cultures were used within 2 days after confluence.

In vitro adhesion of PBL or selected lymphocyte subpopulations to endothelia was evaluated by incubating ^{51}Cr-labeled lymphocytes with BACE in the presence of 10% heat-inactivated HS (untreated or IgG-depleted) or bovine serum; nonadherent cells were removed by gently washing the monolayers, adherent cells were lysed, and the supernatant was harvested and counted in a γ counter. Adhesion was calculated as the percent value of ^{51}Cr incorporation in adherent lymphocytes compared with the total input cpm.

Cellular cytotoxicity was assayed in a ^{51}Cr release assay. BACE were labeled with the isotope and cocultured for 3 hours with different numbers of lymphocytes in the presence of either untreated or IgG-depleted heat-inactivated HS (10%), to achieve effector:target ratios of 30:1, 10:1, 3:1, and 1:1. At the end of the coculture, supernatants were collected and counted in a γ counter. Cytotoxicity was calculated according to the following formula:

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

Inhibition of binding and cytotoxicity was evaluated by preincubating effector cells (30 minutes on ice) with saturating concen-

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Table 1. Cell Sequestration After Perfusion Through Rat Heart

Cells Perfused	Serum	Cell Recovery* ($\times 10^6$/mL)
Rat splenocytes	Rat	0.95 ± 0.02
Rat splenocytes	IgG-depleted rat	0.92 ± 0.05
Human PBL	Human	0.55 ± 0.12
Human T cells	Human	0.70 ± 0.09
Human NK cells	Human	0.10 ± 0.13
Human T cells	IgG-depleted human	0.73 ± 0.15
Human NK cells	IgG-depleted human	0.75 ± 0.08

Note: Cells were perfused through the heart at an initial concentration of 10^6/mL. Cell counts were performed after 40 minutes, corresponding to eight complete perfusion cycles. Heat-inactivated HS from different sources was added to the perfusing buffer at a final concentration of 2%. Where indicated, serum was selectively depleted of IgG prior to the experiment.

*Results are expressed as mean cell concentration (±SD) of four different experiments.

trations (10 μg/mL) of different monoclonal antibodies (MAbs) prior to the assays.

A fluorescence-activated cell sorter was used for lymphocyte phenotypic analysis. Briefly, cells were stained with different fluorescein isothiocyanate (FITC)-conjugated MAbs for 30 minutes on ice, washed, and examined. Alternatively, when unconjugated antibodies were used for the staining, a fluorochrome-conjugated polyclonal antiserum (goat antimouse immunoglobulins) was subsequently added before the assay.

RESULTS AND DISCUSSION

Human Lymphocytes Adhere to and Lyse Xenogeneic Endothelial Cells

Perfusion experiments using unseparated human PBL or selected subpopulations were performed to ascertain whether significant witholding of circulating lymphocytes can occur upon recognition of xenogeneic determinants. Cell counts revealed a significant reduction in the lymphocyte concentration in the outflow when the xenogeneic combination human to rat was used. No retention into the heart was observed when experiments were performed using lymphocytes syngeneic to the perfused organ. A dramatic reduction in the outflow was recorded when NK cells were circulated (up to 90%), while T cells underwent an average 30% reduction (Table 1).

Immunohistochemical analysis showed human lymphocytes adhering to the endothelium and infiltrating the xenogeneic tissue. Staining performed with MAb identifying lymphocyte subsets confirmed that a high percentage of the adherent cells belongs to the NK lineage (data not shown). Phenotypic analysis of human lymphocytes before and after perfusion showed a clear-cut reduction in the percentage of LFA-1^{bright} cells after circulation through the coronary system (data not shown).

In the in vitro system, the adhesion to xenogeneic endothelium was confirmed and so was the preferential adhesion of NK cells to bovine endothelial cells (Fig 1A). Chromium release assays demonstrated efficient killing of BACE by NK cells with up to 90% specific lysis at a 30:1 effector:target ratio, while T cells only marginally affected endothelial cell viability (Fig 1B).

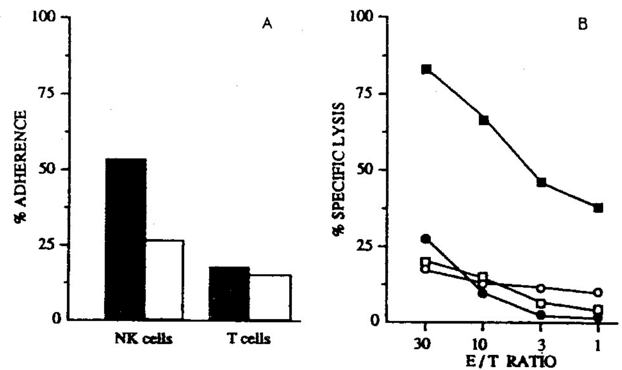

Fig 1. Human lymphocyte subpopulations differently adhere to and lyse cultured BACE. **(A)** Adherence of human purified NK and T lymphocytes to BACE. Purified NK or T cells were incubated on BACE monolayers for 90 minutes in the presence (filled columns) or in the absence (empty columns) of 10% pooled heat-inactivated HS. Percent adhesion was calculated as described in Materials and Methods. **(B)** BACE killing by human NK and T lymphocytes. Purified NK (filled symbols) or T (empty symbols) lymphocytes were incubated with BACE for 4 hours in the presence (square symbols) or in the absence (round symbols) of 10% pooled heat-inactivated HS. Percent cytotoxicity was calculated as described in Materials and Methods. Results are representative of four separate experiments.

Rapid adhesion of lymphocytes to xenogeneic endothelial cells implies recognition of xenodeterminants, expressed on blood vessels, capable of triggering phenomena of adhesion/transmigration; preferential adhesion of the LFA-1^{bright} population (which comprises most of NK cells and memory T cells[9]) suggests a causative role for this molecule in the adhesion process. Finally, efficient endothelial cell killing by NK cells provides an additional pathway of rapid xenogeneic tissue damage not requiring host sensitization. Direct evidence of adhesion to endothelial cells is provided by both experimental systems, while direct evidence of killing is observed only in the in vitro model. On the other hand, the histologic appearance of the endothelium in the perfusion model suggests that a similar phenomenon may occur in vivo.

The Role of IgG in Endothelium Recognition by NK Cells

The in vitro experiments described above were always performed in the presence of either human or bovine serum (10%), while in the ex vivo system either human or rat serum was used. When nonhuman serum was used, adhesion and killing were significantly decreased (Table 1 and Fig 1). To ascertain whether the enhancement of adhesion and killing observed with HS was due to the presence of xenoreactive Ig, experiments were performed in which HS was depleted of IgG and substituted for normal HS.

The removal of IgG indeed profoundly affects adhesion (both in the ex vivo and in the in vitro systems) and killing (Table 1 and data not shown). Reconstitution of the

IgG-depleted serum with pooled human Ig partially reverts such an effect (data not shown). This suggests a direct interaction of NK cells, via surface Fcγ receptors, with endothelial cell-bound xenoreactive antibodies.

NK cell adhesion to BACE is not completely abrogated by IgG removal, suggesting that at least two pathways are involved in mediating this phenomenon. T-cell adhesion to xenogeneic endothelial cells, on the other hand, appears much less influenced by IgG removal.

Adhesion Pathways in the Lymphocyte-Endothelium Interaction

Experiments were performed to elucidate the molecules that are involved in the cell–cell interaction observed in both systems. Ex vivo perfusion and in vitro adhesion and cytotoxicity assays were run with effectors that had been pretreated with MAbs specific for membrane molecules likely to be involved in the xenograft recognition process. Antibodies directed against the leukocyte integrin beta chain (CD18) consistently inhibited adhesion in both systems, while an isotype-matched control antibody (anti β-2 microglobulin) did not. In vitro inhibition experiments performed using MAb specific for individual integrin alpha chains demonstrate that CD11b/CD18 (Mac-1) plays an important role in mediating adhesion when human IgG are present in the assay, while CD11a/CD18 (LFA-1) appears to be involved in the adhesion process both in the presence and in the absence of IgG. These data argue in favor of a crucial role of leukocyte integrins in mediating lymphocyte adhesion to xenogeneic endothelia (Fig 2).

While part of these observations are consistent with the findings in the allogeneic system,[10] differences exist, particularly concerning the role of the CD11b/CD18 heterodimer. Direct involvement of this surface structure in adhesion in the xenogeneic system is also suggested by the different expression of the molecule on xenogeneic endothelium-adherent vs nonadherent NK cells. While other integrins such as LFA-1 and VLA 4 show a similar surface density in both subpopulations, Mac-1 is expressed at a higher density on NK cells that adhere to xenogeneic endothelial cells (data not shown). Complement factors, in addition to other cellular ligands, could be involved in the CD11b/CD18-mediated NK cell adhesion to BACE, since C3b fragments were detected (by FACS analysis) on BACE when IgG were included in the experimental conditions (data not shown).

These observations allow us to conclude that NK cells adhere to xenogeneic endothelium and efficiently lyse it; these phenomena take place both in the presence and in the absence of xenoreactive natural antibodies, although to a different extent, being mediated via at least two different pathways, one dependent on the functional integrity of the leukocyte integrins, the other on the presence of natural antibodies and, possibly, complement factors bound to the vascular endothelium.

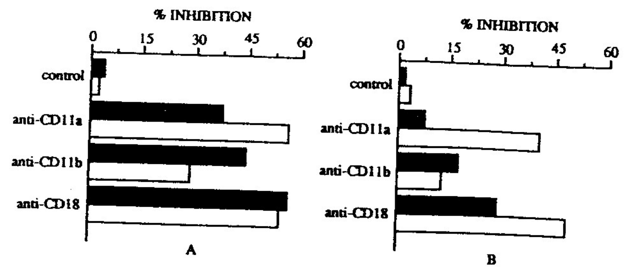

Fig 2. Inhibition of NK cell-mediated binding to and lysis of BACE by MAbs specific for different leukocyte integrin chains. Percent inhibition of NK cell adhesion to **(A)** and killing of **(B)** BACE. Purified human NK cells were treated with the indicated MAbs for 30 minutes on ice, washed, and subsequently coincubated with BACE monolayers in the presence (filled columns) or in the absence (empty columns) of 10% pooled heat-inactivated HS. Basal adhesion and killing (no antibody treatment) were used to calculate the percent inhibition. An isotype-matched irrelevant antibody was used as negative control. Results are representative of three separate experiments.

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