Significant Frequencies of T Cells With Indirect Anti-Donor Specificity in Heart Graft Recipients With Chronic Rejection

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**Background**—The purpose of this study was to determine whether T cells with indirect allospecificity could be detected in heart transplant recipients with chronic rejection.

**Methods and Results**—Human T-cell clones were used to determine the most effective way to deliver major histocompatibility complex alloantigens for indirect presentation. Seven allograft recipients with evidence of progressive, chronic rejection were selected. Four heart graft recipients with no evidence of chronic rejection were used as controls. Peripheral blood T cells and antigen-presenting cells from the recipients were cultured with frozen/thawed stored donor cells or major histocompatibility complex class I–derived synthetic peptides in limiting dilution cultures and then compared with controls using tetanus toxoid and frozen/thawed third-party cells with no human leukocyte antigens in common with the donor. In 5 of 7 patients analyzed who had chronic rejection, elevated frequencies of T cells with indirect, anti-donor specificity (iHTLf) were detected. No such elevated iHTLf were detected in recipients without chronic rejection.

**Discussion**—iHTLf can be obtained from human transplant recipients, which supports the contention that the indirect pathway is involved in chronic transplant rejection. (*Circulation*. 2000;101:2405-2410.)

Key Words: transplantation ■ immunology ■ rejection ■ immune system ■ lymphocytes

Chronic rejection is manifest by the development of an accelerated form of atherogenesis known as transplant coronary artery disease (TxCAD).

Two pathways contribute to the allorecognition of donor major histocompatibility complex (MHC) molecules.1,2 Direct allorecognition refers to the recognition of donor alloantigens present on donor cells. Its vigor, which seems to violate the rules of self-MHC restriction, is driven by antigenic cross-reactivity.3,4 Models of transplantation demonstrated that the activation of T cells via this pathway is the prerogative of donor dendritic cells, which are transplanted with the graft, because of their potent immunogenicity. In some strains, the depletion of donor dendritic cells leads to such a reduction of immunogenicity that the allograft can be accepted without the need for any immunosuppression.5,6 However, once the donor dendritic cells are lost from the graft, recipient T cells can be activated by the second, indirect pathway, whereby donor antigens are shed, internalized, processed, and presented as peptides by recipient MHC molecules. This corresponds to the mechanisms by which all other antigens are recognized by T cells. In other strain combinations, it seems that the indirect pathway is sufficiently vigorous to provoke graft rejection, albeit at a slower tempo than that seen when the direct pathway is operative.2 On the basis of these observations, we proposed that the indirect pathway might play a key role in chronic graft rejection in humans.7-9

We previously observed significant reductions in the frequencies of T cells with direct anti-donor allospecificity in patients with TxCAD.9,10 These findings suggested that T cells with direct anti-donor reactivity are not responsible for driving chronic rejection. The hypothesis tested in this study was that the indirect pathway of allorecognition contributes to the progression of TxCAD.

Human T-cell clones were used to determine the most effective way to deliver MHC alloantigens for indirect presentation. Patients with and without angiographic evidence of TxCAD were selected for this study. Peripheral blood T cells and antigen-presenting cells (APCs) from the patients were cultured with cell-free, membrane-bound, frozen/thawed stored donor cells or MHC class I–derived peptides in limiting dilution cultures.
Methods

Determination of the Most Efficient Strategy for Delivery of Alloantigen for Indirect Presentation

A human T-cell clone, EL26, with indirect allospecificity for HLA-A2 and restricted by HLA-DR15, was used. This clone was generated from a DR15-positive, A2-negative, renal transplant recipient whose A2-positive transplant failed because of chronic rejection. The clone was generated using a cocktail of synthetic peptides spanning the 3 polymorphic regions of A2 and autologous peripheral blood mononuclear cells (PBMCs); the clone was specific for the peptide corresponding to residues 92 to 120 of HLA-A2.11

A variety of sources of A2 were compared for their capacity to stimulate the EL26 T-cell clone in proliferation assays.

Synthetic Peptide

A peptide corresponding to residues 92 to 120 of HLA-A2 was synthesised by the Immunology Unit of the Imperial Cancer Research Fund, London, UK. The final concentration of peptide in the culture wells was 10 μg/mL.

Soluble HLA-A2 Molecule

Drosophila SC-2 cells, cotransfected with truncated constructs for soluble HLA-A2 with a C-terminal histidine tag and β2 microglobulin, were provided by Dr Andreu Brunmark of the R.W. Johnson Research Institute (La Jolla, Calif). Secreted soluble HLA-A2 was purified from the supernatant of SC-2 cells as previously described.12 The purity of soluble HLA-A2 was confirmed by SDS-PAGE and Coomassie blue staining. The final concentration of soluble molecules in the culture wells was 10 μg/mL.

Frozen/Thawed Cells

This approach has previously been used in the context of indirect xenorecognition by Yamada et al.13 PBMCs were isolated from heparinized blood from individuals who were HLA-A2-positive but DR15-negative. Mononuclear cells were isolated by density-gradient centrifugation on Lymphoprep at 1.077 g/mL (Nyomed Pharma DR15-negative. Mononuclear cells were added to each well. For all experiments using intact cells were visible microscopically. The equivalent of 5×10^4 intact cells were added to each well.

Serum Derived From HLA-A2–Positive Cells

PBMCs were isolated from HLA-A2-positive individuals by density-gradient centrifugation and depleted of HLA class II cells to avoid direct alloactivation. After the depletion process, the HLA-A2-positive cells were γ-irradiated with 100 Gy using a ^137cesium source. A total of 5×10^4 cells were added to each well.

Serum Derived From HLA-A2–Positive Whole Blood

Soluble HLA alloantigens released from donor tissue may be a source of indirect allostimulation. Aliquots of 50 μL of serum from HLA-A2-positive individuals were assessed for their ability to stimulate the T-cell clone in the presence of the restriction element provided by DR15.

Sonicated HLA-A2–Positive Cells

PBMCs were isolated from HLA-A2-positive individuals by density-gradient centrifugation and depleted of HLA class II cells (as above). The cells were then sonicated for 20 minutes, after which no intact cells were visible microscopically. The equivalent of 5×10^4 intact cells were added to each well.

Controls

Positive controls for the stimulation of the T-cell clone were APCs derived from an individual who was HLA-A2- and DR15-positive because peptides derived from MHC molecules represent a substantial fraction of the naturally processed peptides displayed by MHC class II molecules.15,16 Negative controls included the use of HLA-A2-positive, DR15-negative and HLA-A2-negative, HLA-DR15–positive APCs.

Proliferation Assays

A total of 5×10^4 DR15-positive and DR15-negative APCs were prepulsed overnight with the HLA-A2 and non-A2 antigenic preparations and then γ-irradiated with 35 Gy. The T-cell clone EL26 was then added at 1×10^4 cells per well. The cells were plated out in a total volume of 200 μL in complete medium. After 48 hours, wells were pulsed with 1 μCi of tritiated thymidine (³H-Tdr), and the cultures were harvested onto glass fiber filters 18 hours later. Proliferation was measured as ³H-Tdr incorporation by liquid scintillation spectrometry. Background counts were obtained for each experiment in the absence of the clone and then subtracted from these readings. The results are expressed as the mean of triplicate cultures.

Estimation of iHTLf in Recipients of Heart Grafts With and Without Chronic Rejection

Patients

Seven graft recipients developed chronic rejection in their first year after transplantation (mean age, 39.4 years). The control group was composed of 4 recipients who had no angiographic evidence of chronic rejection (mean age, 40.25 years). All recipients were receiving maintenance cyclosporine and azathioprine immunosuppression. In 3 of these 4 recipients, limiting dilution analyses (LDA) were performed 2 weeks after angiography; in the final recipient, it was done 6 months after a clear angiogram. The length of graft residence for all recipients at the time of iHTLf estimation varied from 1 to 10 years. TxCAD was diagnosed using established angiographic criteria17 and, in the case of the recipient of the renal graft, by biopsy. For heart recipients with chronic rejection, progression of TxCAD was demonstrated by yearly angiography. No recipients were diabetic or hypertensive, and none had any evidence of previous cytomegalovirus infection (clinical evidence and detection of early antigen fluorescent foci). For all recipients, post-transplant hyperlipidemia was treated and corrected.

HLA Typing

Recipients and donors were typed using conventional serological methods. Since April 1993, HLA-DR typing has been performed by polymerase chain reaction amplification with sequence-specific primers.

Preparation of Frozen/Thawed Stimulator Cells

The organ retrieval yielded donor spleen cells. Cell suspensions were released by injecting cold, sterile RPMI medium into the splenic material with a syringe; mononuclear cells were then enriched on Lymphoprep gradients and cryopreserved in a freezing mixture composed of serum from AB donors and 30% dimethyl sulfoxide. AB serum was added at a volume of 1:3 to give a final concentration of 7.5% dimethyl sulfoxide and 75% AB serum. Samples were subsequently stored in liquid nitrogen. When required, spleen cells were thawed rapidly and washed twice in RPMI 1640. The preparation of membrane-bound, cell-free donor and third-party antigens was then accomplished by freeze/thawing as described above.

The A2 synthetic peptide (residues 92 to 120) was used as described above in the 2 recipients who were HLA-A2–negative but who received an HLA-A2–positive graft.

Preparation of T Cells and APCs From Recipient PBMCs

Preparation of T-cell depleted APCs: Cryopreserved PBMCs were thawed rapidly and washed twice in RPMI 1640. CD4^+ and CD8^+ T-lymphocyte depletion was performed by adding anti-CD4^+ and anti-CD8^+–coated immunomagnetic beads (Dynabeads, Dynal AS) to the mixture at a ratio of 2:1 at 4°C. After gentle mixing for 45
minutes, a magnet was then applied to the outside wall of the test tube to collect the bound cells and free beads. The stimulator cell suspension was then subjected to a further 2 rounds of depletion, this time at a ratio of 1:1.

**Preparation of APC-depleted T cells from recipient PBMCs:** Cryopreserved responder cells were thawed rapidly and washed twice in RPMI 1640. Adherent cells were removed from PBMCs by incubation for 2 hours at 37°C on tissue culture–grade petri dishes.

**Flow cytometry:** To determine the efficiency of depletion, cells were stained with directly conjugated anti-CD3 fluorescein isothiocyanate and anti-DR-phycocerythrin (Simultest, Becton Dickinson). They were then washed and fixed with 1% paraformaldehyde in PBS. Residual cells were subsequently analyzed using an EXCEL flow cytometer (Coulter Electronics). In all experiments, T cell–depleted stimulators contained 6% CD3+ cells, and HLA class II-depleted responders, 5% DR+ cells.

**LDA Assays to Estimate Frequencies of IL-2–Secreting Recipients in iHTLf**

**LDA assays:** The limiting dilution culture system was used to determine all recipient/anti-donor indirect frequencies. For recipients 1 and 2, the antigen used was HLA-A2 peptide because both were HLA-A2-negative recipients of HLA-A2-positive grafts. For the other recipients, frozen/thawed membrane-bound cell-free donor antigens were used.

The response to tetanus toxoid served as a positive control for the indirect pathway. To control for the AMLR, which was previously identified as a potential confounding factor in LDA, replicate wells were set up containing APC and T cells, with no added antigen, for the 2 patients tested against the A2 peptide. For the remainder of the patients who were tested against frozen/thawed donor cells, frequencies were compared with those measured in response to third-party frozen/thawed cells that shared no HLA antigens with the donor. In addition, to ensure that the frozen/thawed cells were unable to provoke a direct alloresponse, they were cultured with third-party responder T cells. In all experiments, no direct proliferative response was observed.

After the depletion steps, the stimulator and responder cells were resuspended in medium, and graded numbers (1.56×10^5 through 10^3 cells) were added to 24 replicate wells of U-bottom, 96-well microtiter plates (Flow laboratories). Equal volumes containing stimulator cells were incubated with A2 peptide at a final concentration of 10 μg/mL (recipients 1 and 2); frozen/thawed, membrane-bound, cell-free donor antigens (recipients 3 to 7); and tetanus toxoid at a final concentration of 0.01 U/mL. All APC preparations were then γ-irradiated with 35 Gy, and 5×10^4 cells were added in 100 μL to each of the wells. Plates were incubated at 37°C in 5% CO2 and 95% air for 72 hours.

After incubation, the plates were γ-irradiated with 25 Gy (8 MeV linear accelerator, Philips MEL). The presence or absence of IL-2 production in each well was assessed by adding 1×10^5 cytotoxic T-lymphocyte line-2 (CTLL-2) cells in 25 μL of medium. Eight hours later, 1 μCi of H-TdR in 25 μL of medium was added to each well. After a further 16-hour incubation, the cells were harvested on glass fiber filter mats, and the H-TdR incorporation by CTLL-2 was evaluated by liquid scintillation spectrophotometry.

Control wells for the calculation of background activity consisted of 24 wells containing irradiated stimulator cells alone. Wells were classified as positive for IL-2 production if the H-TdR incorporation exceeded the mean±3SD of these control wells.

**Maintenance of the IL-2–dependent indicator cell line, CTLL-2:** The continued proliferation of CTLL-2 (European Collection of Animal Cell Cultures) was dependent on the presence of human or murine IL-2 or murine IL-4.19,20 The line was maintained in culture medium with the addition of human recombinant IL-2 (10 U/mL; Boehringer Mannheim) and 10% FCS. The cells were cultured in 25-cm² flasks (Costar) and subcultured every 3 days. Before use in a limiting dilution assay, the CTLL-2 cells were washed twice and cultured overnight in normal culture medium without recombiant IL-2. A total of 1×10^3 cells were added to all wells of each assay.

**Statistical Analysis**

Frequencies of alloreactive T cells were calculated using a maximum-likelihood statistical program with GLIM software (NAG Ltd) that was based on the method of Finney. The proportion of negative wells in each sample size of responder cells was linearly related to the frequency of responder cells according to the Poisson distribution, as follows:

\[-\log_x P_{neg} = fX\]

where \(P_{neg}\) is the proportion of negative wells, \(f\) is the frequency of responder cells, and \(X\) is the number of responder cells per well.

The 95% confidence limits of the frequencies and \(x^2\) estimates of probability were calculated. From the \(x^2\) values and degrees of freedom (the number of responder dilutions minus one), probability estimates of the data conforming to single-hit kinetics were calculated. Assays with a \(P=0.05\) are likely to conform to single-hit kinetics, ie, that a single cell type (in this case, the indirectly sensitized alloreactive IL-2-producing T cell) is limiting. Assays with \(P<0.05\) that do not conform to single-hit kinetics provide no evidence for the existence within the sample of a significant iHTLf and are consequently regarded as invalid. Frequencies are regarded as different if their 95% confidence limits (>2SD) do not overlap.

We consider the limit of sensitivity of the iHTLf assay as 1:500 000. Accordingly, anti-donor iHTLf levels were regarded as significant if they exceeded 1:500 000 and followed single-hit kinetics. Anti-donor frequencies were regarded as elevated if they were significantly different from those estimated in the AMLR or against third-party controls.

**Results**

Frozen/thawed donor cells provide the most efficient delivery of alloantigens for indirect presentation. Initially, we wished to establish the most efficient way to deliver alloantigens for indirect presentation. This was achieved using a T-cell clone with indirect allospecificity for human leukocyte antigen (HLA)-A2. The peptide of HLA-A2 recognized by this T-cell clone was amino acid residues 92 to 120, which corresponds to the hypervariable region of the molecule.

A variety of sources of HLA-A2 were compared to determine which would provoke the strongest proliferative response by the clone. The results are shown in Figure 1. Three positive controls were used in these experiments: a synthetic form of the A2 peptide (Figure 1E), a soluble HLA-A2 (Figure 1F) molecule prepared from transfected insect cells, and stimulator cells coexpressing HLA-A2 and the restriction element for this clone, HLA-DR15 (Figure 1A). All the controls induced a strong proliferative response by the clone compared with the negative controls. Three experimental sources of HLA-A2 were compared: 3 involved HLA-A2-positive, DR15-negative cells (Figures 1D, 1G, and 1H), and the fourth was serum from an HLA-A2–positive individual (Figure 1I). As seen in Figure 1, the only source of A2 that induced clonal proliferation was frozen/thawed HLA-A2–positive cells (Figure 1D), and this preparation induced the same level of response from the clone as in any of the positive controls. On the basis of these findings, frozen/thawed donor cells were used in the limiting dilution assays for the measurement of frequencies of T cells with indirect anti-donor allospecificity.

**T Cell Frequency and Donor Alloantigens**

Elevated frequencies of T cells with indirect specificity for donor alloantigens were detected in 5 of 7 patients with
chronic allograft rejection and in 0 of 4 patients without chronic rejection.

A total of 11 transplant patients were included in this study; 6 had angiographic evidence of chronic cardiac allograft rejection. One patient was a renal transplant recipient whose graft had failed due to chronic rejection. Four patients who had received a cardiac allograft and showed no angiographic evidence of chronic rejection served as controls. Limiting dilution assays were performed on all subjects.

As a positive control for each patient, the frequency of T cells secreting interleukin-2 (IL-2) in response to the recall antigen, tetanus toxoid, was measured. As expected, the frequencies of tetanus toxoid–specific T cells were in the range of \( \approx 1:10 \,000 \) to \( 1:60 \,000 \) and followed “single-hit” kinetics, indicating that the APCs and responder cells were fully functional. As outlined in Methods, negative controls included the autologous mixed lymphocyte response (AMLR; when peptide was used) and the frequency generated with frozen/thawed cell-free, membrane-bound third-party cells.

As presented in Figure 2, in patients with chronic rejection, the frequencies against donor alloantigens were significantly higher than those against third-party antigens in 5 of the 7 patients with chronic rejection. The actual frequencies measured ranged from \( \approx 1:80 \,000 \) to \( 1:300 \,000 \) for donor-specific T cells with indirect, anti-donor specificity (iHTLf) and \( \approx 1:350 \,000 \) to \( 1:4 \,500 \,000 \) for third-party/AMLR. For patients without chronic rejection, either no statistically valid iHTLf were enumerated or no difference existed between donor and third-party controls.

**Discussion**

Elevated frequencies of T cells with indirect anti-donor allospecificity have previously been measured in patients...
with acute heart transplant and chronic renal transplant rejection.\textsuperscript{22,23} In addition to class I–derived peptides, we chose to use whole donor cells as a source of donor alloantigen because this then makes no assumptions about the nature of the indirectly recognized antigens and proffers the full repertoire of donor MHC epitopes. The use of whole donor cells in the measurement of indirect allosponses has the advantage that all donor alloantigens can be presented, thereby maximizing the likelihood of detecting significant responses.

A T-cell clone was used to establish the most effective means of delivering alloantigen for indirect presentation. This was done by freeze/thawing the donor cells. This approach has the advantage of excluding viable cells from the stimulator population, so that direct allorecognition and “back stimulation” cannot lead to extraneous IL-2 production and distorted frequencies.\textsuperscript{24}

The potential complication of having to coculture recipient T cells with recipient APCs is the occurrence of an AMLR. This response may generate frequencies that follow single-hit kinetics.\textsuperscript{18} For this reason, controls were included in each experiment that cultured recipient T cells with recipient APC in the absence of donor antigen (for the experiments using peptide) or that measured the frequency against an equally mismatched third-party population of frozen/thawed spleen cells of donor origin. This limb of the experiments controlled for the AMLR (due to the presence of responder T cells and APC), in addition to testing the specificity of any observed anti-donor frequency.

On the basis of the statistical criteria outlined in Methods, 2 of 2 recipients had high anti-donor frequencies when using HLA class I–derived peptide. Using membrane-bound, cell-free, frozen/thawed cells, 3 of 5 recipients had high anti-donor frequencies. In patient 6, the frequencies seemed to be raised against both donor and third-party cells, possibly due to cross-reactive recognition of the third-party antigens or the influence of the AMLR; in patient 7, no frequency to donor or third-party cells was detectable, although the frequency estimated against tetanus toxoid was in the expected range. In patients 8 to 11 (controls), who showed no evidence of chronic rejection, either statistically valid iHTLfi were not generated or the anti-donor frequency overlapped with that measured against third-party cells.

These data provide the first indication that measurable frequencies of IL-2–secreting T cells with indirect recipient/anti-donor specificity can be detected in the peripheral circulation in patients with chronic rejection. Indirect allorecognition may, therefore, continue to drive the rejection process once donor-derived APCs have left the graft, either allowing for or complementing other nonantigenic mechanisms of chronic graft destruction.\textsuperscript{25,26}

If indirect-pathway T cells represent one of the driving factors of chronic graft rejection, 2 final points merit discussion. (1) The assays using frozen/thawed cells (which allow the use of the full repertoire of donor antigens to estimate indirectly sensitized T helper cells) have the potential for clinical usefulness in monitoring the effectiveness of different drug regimens and tolerance-promoting strategies that prevent indirect allorecognition. (2) It is difficult to suppress indirect allorecognition. The direct alloreponse is stimulated by donor dendritic cells in the early weeks after transplantation. Once these APCs have been lost from the graft, continuing alloantigen presentation by the parenchymal cells of the graft itself likely induces unresponsiveness, not the activation of these T cells by the lack of expression of B7 costimulatory molecules.\textsuperscript{27–30} This suggestion is supported by the fall in direct anti-donor frequencies that we and others have reported previously.\textsuperscript{9,10,31–33} In contrast, indirect-pathway T cells are continuously stimulated by recipient APCs and, in the absence of true HLA-DR matching, the graft has no means of turning indirect-pathway T cells off.

The challenge generated by data such as these is to devise strategies for the induction of tolerance in T cells with indirect allospecificity. Arguably, until this is achieved, the lifespan of organ allografts is unlikely to change significantly.

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