Signal Requirements for the Generation of CD4⁺ and CD8⁺ T-Cell Responses to Human Allogeneic Microvascular Endothelium

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Microvascular endothelium has been implicated as a major target in the rejection of vascularized allografts. In an attempt to dissect the stepwise generation of the T-cell–mediated immune response to microvascular endothelial cells (ECs), we analyzed the requirements for the two major T-cell subsets, CD4⁺ and CD8⁺, in the triggering of proliferative and cytotoxic responses to allogeneic ECs in vitro. Results demonstrate that resting ECs are unable to stimulate a functional response by purified T, CD4⁺, and CD8⁺ cells in the absence of costimulatory signals. T cells and CD8⁺ cells develop both proliferative and cytotoxic anti-EC responses by the addition of as little as 2 units/ml interleukin-2, 10 units/ml interleukin-1, or irradiated monocytes autologous to the responder lymphocytes, whereas only autologous monocytes are capable of triggering CD4⁺ T-cell precursors to proliferate and become anti-EC–specific effector cytotoxic T lymphocytes. CD8⁺ cell–mediated anti-EC cytotoxicity is directed toward allogeneic major histocompatibility complex (MHC) class I determinants on ECs and involves recognition by the CD3/T-cell receptor complex and the CD8 molecule on the effecter T cells. CD4⁺ cells can be driven to proliferate, produce interleukin-2, and become anti-EC–specific cytotoxic T lymphocytes despite the lack of detectable membrane MHC class II determinants on the target cells. Chloroquine inhibition experiments demonstrate that autologous monocytes/macrophages are required by CD4⁺ T-cell precursors for the processing of EC-derived alloantigens and their subsequent presentation in the context of self-MHC molecules. These results are in agreement with the adoptive transfer experiments in experimental allograft models and suggest that the coordinate engagement of cells of the CD4, CD8, and monocyte/macrophage series is more effective than the individual cell subsets in the generation of a functional response to allogeneic nonlymphoid tissues. (Circulation Research 1991;69:1269–1279)

The continuous exposure of vascular endothelium to circulating lymphocytes, together with its ability to express pleiotropic major histocompatibility complex (MHC) antigens in pathological conditions,1–3 imparts this tissue with the ability to greatly amplify the immune response toward genetically incompatible grafts, or self-antigens, in certain autoimmune diseases.4 Accumulating evidence also supports the notion that microvascular endothelium can be involved as a primary target of functional rejection in vascularized allografts. Endothelial cell (EC) injury has been demonstrated in early stages of rejection, with perivascular mononuclear cell infiltrates and interendothelial cell gaps rapidly progressing to blood vessel lining disruption.5 The immunohistological demonstration of EC involvement in allogeneic as well as self-directed immune response has prompted a number of in vitro studies focused on the immunogenicity of vascular endothelium. Our group6 and other groups7 have shown that cultured ECs, either constitutively or after exposure to lymphocyte-derived cytokines, express functionally normal MHC determinants, in addition to polymorphic surface antigens largely restricted to this tissue.8–11 In agreement with these findings, ECs have been shown to elicit proliferative and cytotoxic responses by allogeneic lymphocytes,12 to present soluble antigens to T cells,13,14 and to secrete soluble factors with immunoregulatory activity, such as interleukin (IL)-1,15 IL-6,16 and granulocyte/macrophage colony stimulating factor.17
In an attempt to investigate the stepwise generation of the T-cell–mediated immune response toward allogeneic microvascular endothelium, we studied the signal requirements for the two major T-cell subpopulations, CD4+ and CD8+, in the induction of a functional response to ECs in vitro. Results presented here demonstrate that resting microvascular ECs are poorly immunogenic to purified T cells in vitro and that a complex interaction between various T-cell subsets and autologous accessory cells (ACs) is required to trigger a T-cell–mediated immune response toward this allogeneic tissue. Furthermore, the data suggest that CD4+ cytotoxic T cells, recognizing processed EC alloantigens, may play a role in the cascade of events leading to EC damage in vivo in vascularized allograft rejection.

**Materials and Methods**

**Microvascular EC Cultures**

Stable cultures of human microvascular ECs were established as previously described. Briefly, fresh discarded preputial skin from randomly selected anonymous newborns was treated with 0.3% trypsin and 1.0% EDTA for 1 hour at 37°C, after which the microvascular ECs were extruded, plated in microvascular EC culture medium, and grown to confluence. On reaching confluence, the cultures were passaged and used for functional assays within the fifth passage. Cells were identified as ECs by standard light microscopy and immunofluorescence techniques as previously described and discarded if they contained >1% contaminating cell types. Experimental ECs were considered in resting conditions based on 1) the lack of surface class II HLA expression or monolayer reorganization and 2) background [3H]thymidine incorporation levels. Stable cultures of autologous fibroblasts were established by plating a small aliquot of cells obtained in the EC isolation procedure into a 35-mm dish in the presence of 10% peripartum serum but without EC selection components.

**Monoclonal Antibodies**

Anti–Leu-2a (clone SK.1, immunoglobulin [Ig] G1) binds to the CD8 antigen present on cytotoxic/suppressor T cells; anti–Leu-3a (clone SK.3, IgG1) reacts with the CD4 antigen on helper/inducer T cells; anti–Leu-4 (clone SK.7, IgG1) reacts with CD3 complex present on mature T cells; anti–Leu-2a, -3a, and -4 monoclonal antibodies were purified over Sepha-rose protein A columns from crude ascites; W6/32, an IgG2a reacting with a framework-specific determinant on HLA-A,-B, and -C molecules was provided by Dr. Peter Parham, Stanford University; CA-141, an IgG1 that reacts with a monomorphic determinant on HLA-DR, was provided by Dr. H.O. McDevitt, Stanford University; L243 is an IgG2a that reacts with a different HLA-DR framework-specific epitope than CA-141 and was provided by Dr. R. Levy, Stanford University; anti–lymphocyte function associated (LFA)-1 antigen (CD11a) (clone TS.1.22) and anti-LFA-3 antibodies, both IgG1, react with broadly distributed antigens on hematopoietic tissues (LFA-1) and on both hematopoietic and nonhematopoietic tissues (LFA-3) and were provided by Dr. A. Krensky, Stanford University; anti–Leu-M3 (clone MOP9, IgG2b) reacts with monocytes/macrophages and was purchased from Becton Dickinson, Mountain View, Calif. Anti–Leu-10 (clone SK.10, IgG1), which reacts with a polymorphic determinant of HLA-DQ expressed in linkage disequilibrium with DR-2, -4, and -6, and anti–HLA-DP (clone B7/21, IgG1), which reacts with a framework-specific determinant of HLA-DP, were purchased from Becton Dickinson.

**Reagents**

Recombinant human interleukin (IL)-1α and -1β were gifts from S. Gillis, Immunex Corp., Seattle, Wash. Recombinant human interleukin (IL-2) was obtained from Cetus Corp., Emeryville, Calif. Chloroquine was purchased from Sigma Chemical Co., St. Louis, Mo.

**Flow Cytometry**

Immunofluorescent staining was performed as described elsewhere in detail. All dilutions, washing, and incubations were performed in cold phosphate-buffered saline (0.1 M, pH 7.3) containing 0.1% NaN3. Cytofluorographic analysis was performed by means of an Ortho Cytofluorograph System 50H and a FACStar (Becton Dickinson), using fluorescein isothiocyanate–conjugated or phycoerythrin-conjugated monoclonal antibodies.

**Isolation of Mononuclear Cell Subpopulations**

Leukocyte-rich fractions from healthy Red Cross blood donors were randomly selected. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation, after which they were resorted by a single-step rosetting technique using neuraminidase-treated sheep erythrocytes to obtain erythrocyte-rosette–forming cells. Erythrocyte–rosette–forming cells were further depleted of monocytes and B cells by plastic adherence and passage over a nylon wool column. This yielded a T-cell population that was >95% CD3+ and <0.3% Leu-M3+ when analyzed by flow cytometry. These T-cell–enriched populations were also functionally depleted of monocytes, as demonstrated by the lack of proliferative response to 1 μg/ml phytohemagglutinin (not shown), which effectively induces [3H]thymidine incorporation if the peripheral blood mononuclear cells are not monocyte-depleted.

The T cells were used to isolate CD4+ (>95% CD8+) and CD8+ (>95% CD4+) subsets by negative selection using a panning technique. Both subsets contained <0.1% Leu-M3+ cells.

To obtain monocyte-enriched ACs, the nonresetting fraction from the erythrocyte-rosette–forming cell isolations was adhered to plastic flasks (60 minutes at 37°C) and recovered with 1% EDTA (10
minutes at 4°C) and gentle scraping. The resultant population was >85% Leu-M3* and had a characteristic monocyte appearance by light microscopy and cytofluorographic light scatter.

### CD4+ Cell Clone Generation

Peripheral blood mononuclear cells obtained from a donor were rosetted and monocyte-depleted as described above. CD4+ cells (1×10^6) were plated in each well of a 24-well plate (Costar Corp., Cambridge, Mass.) containing confluent monolayers of either the unstimulated MHC class II negative EC30 line or the γ-interferon–treated (60 units/ml) MHC class II positive EC45 line in medium containing 10% pooled human serum, 10% lymphoblastoid cell line–conditioned medium, 20 units/ml IL-2, and 2×10^5 irradiated autologous (30 Gy) ACs. The T cells were refed with fresh medium every 3–4 days and added to fresh IA negative or positive EC monolayers at weekly intervals, at which time they also received fresh, irradiated autologous ACs. After 28 days, the initial populations had grown through a 30–40-fold expansion and were >95% CD4+. Cells were then cloned by limiting dilution into microtiter wells at 0.5 cells/well and propagated under identical culture conditions. For functional analyses, cloned cells were “rested” in medium without IL-2 or stimulator cells for 18–24 hours before the assay.

### Proliferation and T-Cell Growth Factor Production Assays

Lymphocyte proliferation was evaluated on the basis of [³H]thymidine incorporation. Briefly, 5×10^4 lymphocytes were cultured in microtiter well plates with or without irradiated (30 Gy) allogeneic stimulator ECs (1×10^4/well), irradiated autologous ACs (1×10^4/well), and IL-1 or IL-2 at the indicated concentrations. Proliferation was assessed at day 7 by the addition of 1 μCi/well [³H]thymidine 18 hours before harvest on glass fiber filters. When testing proliferation of cloned cells, identical cell ratios were used, including allogeneic ACs in one combination tested, but the cultures were carried out for 72 hours and pulsed with [³H]thymidine 12 hours before harvest. All samples were performed in triplicate.

T-cell growth factor activity was quantitated using [³H]thymidine incorporation during dose-dependent proliferation of the IL-2–dependent murine T-cell line HT-2, as described elsewhere.22

### T-Cell–Mediated Cytotoxicity Assay

T, CD4+, or CD8+ cells were cultured in 12-well plates (1×10^6 cells/well) containing confluent EC monolayers (2×10^5 cells/well), with or without irradiated autologous ACs, 2 units/ml IL-2, or 10 units/ml IL-1 in 2 ml medium containing 10% pooled human serum. In some experiments, culture supernatants from ECs or 72-hour CD4+/EC cocultures were added at a 1:1 ratio to the indicated coculture media. After 7 days, the suspension cells, which were always >90% viable (both CD4+ and CD8+ cells), were recovered and used as effectors in ⁵¹Cr release assays. These assays were performed in flat-bottomed microtiter wells with confluent EC monolayers, either autologous or allogeneic to the stimulating EC line, which had been labeled with 2 μCi/well ⁵¹Cr for 2 hours at 37°C. Spontaneous and maximum release was determined by incubating labeled targets in culture medium or 0.7% Triton X-100 in glycerol/water, respectively. Mean corrected percent lysis in triplicate culture was calculated as follows: cytotoxicity (%)=(mean experimental cpm−spontaneous release cpm)/(maximum release cpm−spontaneous release cpm)×100. Spontaneous release was always <20% of maximum release.

In some experiments, monoclonal antibodies (10 μg/ml) were added either directly to the 4-hour assay or used to pretreat either the effector or target cells for 30 minutes at 37°C, after which unbound antibody was washed out and the assay was performed. In other experiments, ACs were treated with 500 μM chloroquine at the onset of the 7-day stimulation period. In preliminary experiments, 500 μM chloroquine gave the most efficient functional inhibition without affecting cell viability (not shown) and was therefore used in subsequent experiments. ACs were suspended at 1×10^6/ml in RPMI medium containing 500 μM chloroquine for 30 minutes at 37°C, after which they were flooded with 10% fetal calf serum, washed twice, irradiated (30 Gy), and added to the responder cells for the 7-day culture. Alternatively, ACs were pulsed overnight with the relevant ECs at a 1:10 (AC:EC) ratio and chloroquine-treated the next day before the initiation of the 7-day culture. In other experiments, the natural killer (NK)-sensitive line K562 was ⁵¹Cr-labeled (50 μCi/10^6 cells) and used as a target in cytotoxicity assays.

### Results

#### Basal Requirements for the Generation of Anti-EC T-Cell–Mediated Immune Response

Primary sensitization in vitro of highly purified T cells or T-cell subsets with allogeneic microvascular ECs is not sufficient to stimulate a detectable anti-EC cytotoxic response (Figure 1). This is not dependent on the particular allogeneic combination used or the time required to generate cytotoxic T lymphocytes (CTLs), as identical results were obtained using multiple genetically unrelated EC lines as stimulators or prolonging the primary lymphocyte/EC coculture for as long as 3 weeks (not shown).

These results are at variance with the classical mixed lymphocyte reaction assay, in which mononuclear lymphoid cells are used as stimulators. We reasoned that either unstimulated ECs did not display immunogenic MHC antigens or that they were unable to provide additional signals required for triggering the proliferation and differentiation of allospecific anti-EC precursor T cells. To address these points, primary lymphocyte/EC cocultures were performed in the presence of low concentrations of
recombinant lymphokines or irradiated AC-enriched populations autologous to the responder T cells. Our results demonstrate that these additional signals are indeed capable of driving a functional anti-EC cytotoxic response by T cells (Figure 1). Moreover, they indicate that the minimal requirements for an anti-EC response by CD8+ and CD4+ T-cell subsets are distinct, since the former subset can be induced to become CTLs by the addition to the cultures of 2 units/ml IL-2, 10 units/ml IL-1, or, to a lesser extent, autologous ACs (Figure 1), whereas only the presence in the primary culture of irradiated autologous accessory cells can stimulate an anti-EC cytotoxic response by purified CD4+ T lymphocytes (Figure 1). As previously shown, 23 IL-2 concentrations >5 units/ml, although more efficient in driving anti-EC CD8+ T-cell-mediated cytotoxicity, would increase the amount of nonspecific killing, as detected by using the K562 target cell line (not shown). In contrast, IL-2 could not generate an anti-EC CD4+ CTL response even at concentrations as high as 50 units/ml (not shown). EC culture or CD4+/EC coculture supernatants had no effect on the ability to derive EC-specific CD4+ cytotoxic T cells in the presence of autologous ACs or EC-specific CD8+ cytotoxic T cells in the presence of IL-2, ruling out that the inability of CD4+ T cells to respond to ECs in the absence of ACs was due to the elaboration of relevant suppression of inhibitory factors in the experimental conditions (not shown).

When the T-cell or T-cell subset proliferation was evaluated under the same experimental conditions, similar results were observed (Table 1). IL-2 (2 units/ml) was the most efficient costimulatory signal for CD8+ lymphocytes, whereas autologous ACs were essential for driving an anti-EC proliferative response by purified CD4+ cells (Table 1).

**Specificity of the Anti-EC Cytotoxic Response by CD4+ and CD8+ T Lymphocytes**

To further investigate whether the costimulatory signals required to generate anti-EC cytotoxic T cells were nonspecifically triggering lytic mechanisms by the effector cells and whether EC alloantigens were actually involved in the generation of the response, multiple genetically unrelated EC lines were used as targets in the final cytotoxicity assay, in addition to the NK-sensitive line, K562. The results (Figures 2a and 2b) demonstrate that the two most efficient pathways for generating an anti-EC cytotoxic response by T-cell subsets (i.e., CD8+ cells plus 2 units/ml IL-2 and CD4+ cells in the presence of irradiated autologous ACs) display a high degree of target-related specificity. This is further demonstrated by crossover killing experiments, where CD8+ (Figure 2c) or CD4+ (Figure 2d) cells from another donor were cocultured with a different EC line (EC44) than that shown in Figures 2a and 2b. The absence of reciprocal killing (CD8 or CD4 anti-EC45 lyses EC45 better than EC44, and CD8 or CD4 anti-EC44 lyses EC44 better than EC45) indicates that the stimulating EC alloantigens are, in fact, responsible for target specificity and that differential target sensitivity is not a major component of the noted phenomenon. Furthermore, fibroblasts syngeneic to the target EC44 line are also lysed by the CD8+ and CD4+ effector cells (Figures 2c and 2d), suggesting that the target antigens are shared by ECs and fibroblasts. With fibroblast targets, the percent cytotoxicity is lower at each effector to target cell (E/T) ratio. A greater or more homogeneous expression of the putative allogene-
neic target molecule(s) may be present on ECs, although it is possible that fibroblasts are relatively more resistant to T-cell–mediated lysis.

**Cell Surface Antigens Involved in T-Cell–Mediated Anti-EC Cytotoxicity**

To identify the surface molecules involved in the anti-EC cytotoxic response by T-cell subsets, soluble monoclonal antibodies directed toward a number of lymphocyte or EC membrane antigens were added to the final cytotoxic assay. Figure 3a shows that antibodies recognizing the CD3–T cell receptor complex, the CD8 antigen, and MHC class I framework determinants were effective, to a variable extent, in blocking the CD8+ cell–mediated anti-EC cytotoxic activity. Experiments performed by pretreating either the effector or the target cells with the indicated antibodies demonstrated that MHC class I determinants on the surface of ECs, but not of lymphocytes, were involved in the recognition process leading to EC lysis by CD8+ T cells (not shown).

When the same panel of antibodies was added to CD4+ cells that had been cultured with ECs, only antibodies against the CD3/TcR complex or the CD4 molecule could effectively inhibit the effector function of this cell subset. Anti–class I, anti–HLA-DR, anti–HLA-DQ, and two anti–HLA DR antibodies recognizing distinct epitopes of the heterodimer did not block this activity at E/T ratios as low as 6:1 (Figure 3b, data not shown). ECs used as stimulators in the generation of EC-specific T cells or T-cell subsets were, in fact, always MHC class II antigen positive at the end of the 7-day coculture period (data not shown). However, to further confirm the lack of involvement of EC Ia antigens in the CD4+ T-cell–mediated anti-EC response, resting ECs, in conditions identical to the assay targets, were stained with antibodies recognizing monomorphic HLA-DP, -DQ, and -DR epitopes. Figure 4 shows that, as previously demonstrated with large vessel–derived ECs,1 microvascular ECs used as targets in the cytotoxicity assay do not express detectable levels of membrane MHC class II antigens, whereas surface MHC class I determinants are constitutively present at a high density. When induced, EC membrane HLA class II is detectable on the cell surface (Figure 4d).

**Role of Autologous ACs in the Generation of CD4+ T-Cell–Mediated Anti-EC Response**

The above results demonstrate an essential role for irradiated autologous ACs in the generation of an anti-EC response by CD4+ lymphocytes, but their precise role is uncertain. Conceivably, these cells produce soluble factors, distinct from IL-1, that act as costimulatory signals in the expansion and differentiation of CD4+ allospecific T-cell precursors. Alternatively, ACs might process and present EC-derived membrane alloantigens in an immunogenic manner to autologous CD4+ lymphocytes. To explore these possibilities, ACs were pulsed overnight with the stimulator EC line and then treated with the weak base chloroquine, an agent known to block exogenous antigen processing by inhibiting the catabolism of peptides in intracellular acidic vesicles.24,25 Chloroquine-treated alloantigen-pulsed ACs were then used as costimulators in the CD4+ lymphocyte/EC primary culture. The results (Figure 5) demonstrate that treatment of autologous ACs with chloroquine (500 μM) before the addition of ECs abrogated the subsequent generation of anti-EC cyto-

### Table 1. Alloimmune Endothelial Cell–Induced Proliferative Response and Interleukin-2 Production by T Cells and T-Cell Subsets

<table>
<thead>
<tr>
<th>Responders</th>
<th>Cofactors*</th>
<th>Proliferation (cpm)</th>
<th>HT-2 cell response† (cpm)</th>
</tr>
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<tbody>
<tr>
<td>T cells</td>
<td>None</td>
<td>399±78</td>
<td>310±40</td>
</tr>
<tr>
<td>T cells</td>
<td>IL-2</td>
<td>9,911±799</td>
<td>5,310±617</td>
</tr>
<tr>
<td>T cells</td>
<td>IL-1</td>
<td>9,002±786</td>
<td>6,601±788</td>
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<tr>
<td>T cells</td>
<td>Auto ACs</td>
<td>19,566±2,100</td>
<td>8,356±1,105</td>
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<tr>
<td>CD4+ cells</td>
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<td>206±46</td>
<td>602±90</td>
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<td>CD4+ cells</td>
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</tr>
<tr>
<td>CD8+ cells</td>
<td>IL-1</td>
<td>3,700±601</td>
<td>2,902±456</td>
</tr>
</tbody>
</table>

*Values are mean±SEM of triplicate cultures. HT-2, interleukin (IL)-2–dependent murine T-cell line; Auto ACs, autologous accessory cells; ND, not done. Purified T cells or CD4+ and CD8+ T cells (1×10⁵) were cocultured with irradiated allogeneic endothelial cells for 6 days, after which 1 μCi [³H]thymidine was added, and cultures were harvested 18 hours later.

†Lymphokine concentrations were 2 units/ml for IL-2 and 10 units/ml for IL-1. Monocyte-enriched populations autologous to the responder lymphocytes (Auto ACs) were irradiated and added to the cultures at a monocyte to T-cell ratio of 0.2:1. T-cell response to Auto ACs, as measured by [³H]thymidine incorporation, never exceeded 2,000 cpm.

Experiments performed by pretreating either the effector or the target cells with the indicated antibodies demonstrated that MHC class I determinants on the surface of ECs, but not of lymphocytes, were involved in the recognition process leading to EC lysis by CD8+ T cells (not shown).
toxic CD4⁺ T cells, whereas the drug was ineffective when used after the ACs had been pulsed overnight with allogeneic ECs. This strongly suggests that an intracellular alloantigen processing step, carried out by ACs, is a prerequisite for the sensitization of anti-EC allospecific CD4⁺ precursor T lymphocytes.

Analysis of the CD4⁺ T-Cell–Mediated Anti-EC Response at the Clonal Level

The restriction elements responsible for the induction of anti-EC-specific, CD4⁺ CTLs were further analyzed by generating CD4⁺ cytotoxic T-cell clones, under limiting dilution conditions, using MHC class II negative allogeneic EC lines as stimulators in the presence of ACs autologous to the responder lymphocytes. Data obtained with one representative clone, designated AT.30.1, are illustrated in Table 2. This clone is a CD8⁻, CD4⁺, and α/β⁺ T-cell clone, as demonstrated by its reactivity with the monoclonal antibody WT31, which recognizes an epitope on the antigen receptor complex of α/β⁺ T cells. Also, the AT.30.1 clone does not kill K562, nor does it express classical natural killer cell markers; thus, it is not a natural killer or natural killer–like T-cell clone (not shown). The data demonstrate that the same cloned cell line is capable of EC-specific, self-MHC–restricted proliferation in the presence of autologous monocytes as a source of ACs and of cytotoxicity to the relevant EC line. IL-2 also is extremely efficient in triggering a proliferative response by cloned CD4⁺ cells. Of note, ECs alone were extremely inefficient in driving a proliferative response by the AT.30.1 clone, although they could serve as a sensitive target in the cytotoxic assay.

Three additional clones (out of 36 anti-EC CD4⁺ clones tested) had functional properties similar to the AT.30.1 clone; the remainder were not capable of anti-EC–specific cytotoxic activity (not shown). Anti-

![Graph showing microvascular endothelial cell (EC) line 45 cocultured for 7 days with CD8⁺ T cells in the presence of 2 units/ml interleukin-2 (panel a) or CD4⁺ T cells in the presence of irradiated autologous accessary cells (accessory cell to lymphocyte ratio, 0.2:1) (panel b). E/T ratio, effector to target cell ratio. After 1 week, the lymphocytes were recovered and used as effectors in a 4-hour cytotoxicity assay, with the relevant or irrelevant EC lines as targets, as well as the natural killer target K562. The identical cocultures and cytotoxicity experiments were performed with CD8⁺ T cells (panel c) or CD4⁺ T cells (panel d) obtained from a different donor than for panels a and b and stimulated with EC line 44. Fib 44 is syngeneic to EC44.

![Graph showing microvascular endothelial cell (EC) line 45 cocultured for 7 days with CD8⁺ T cells in the presence of 2 units/ml interleukin-2 (panel a) or CD4⁺ T cells in the presence of irradiated autologous accessary cells (accessory cell to lymphocyte ratio, 0.2:1) (panel b). E/T ratio, effector to target cell ratio. After 1 week, the lymphocytes were recovered and used as effectors in a 4-hour cytotoxicity assay, with the relevant or irrelevant EC lines as targets, as well as the natural killer target K562. The identical cocultures and cytotoxicity experiments were performed with CD8⁺ T cells (panel c) or CD4⁺ T cells (panel d) obtained from a different donor than for panels a and b and stimulated with EC line 44. Fib 44 is syngeneic to EC44.
T-Cell Alloreactivity to Microvascular Endothelium

body inhibition studies of this clone confirmed the results obtained at the bulk culture level, demonstrating the involvement of the CD3/T-cell receptor complex and the CD4 molecule in both the inductive and effector phases of the anti-EC response by CD4+ lymphocytes (Table 2). Anti-MHC class I and two anti–HLA-DR antibodies recognizing nonoverlapping epitopes of the DR molecule were again unable to inhibit the allogeneic EC-specific cytotoxicity mediated by cells of the CD4+ sublineage. When γ-interferon-treated, MHC class II–expressing ECs were used as stimulators to generate allospecific CD4+ CTL clones, the recognition pattern of the effector cells was at variance with the results observed using MHC class II negative stimulators. Under these circumstances, the majority of the CTL clones tested appeared to recognize MHC class II determinants, as shown by the absence of killing of 1a negative EC targets and by the inhibition of class II+ EC target lysis observed using anti-class II monoclonal antibodies (Table 3).

Discussion

Donor microvascular endothelium is likely to be involved both as a stimulus and as a target in

![Figure 3](image)

**Figure 3.** Bar graphs showing microvascular endothelial cells cocultured for 7 days with CD8+ T cells in the presence of 2 units/ml interleukin-2 (panel a) or CD4+ T cells in the presence of irradiated autologous accessory cells (accessory cell to lymphocyte ratio, 0.2:1) (panel b). Irrel. G1 and G2a, irrelevant immunoglobulin G1 and G2a. After 1 week, the lymphocytes were recovered and used as effectors in a 4-hour cytotoxicity assay with the relevant endothelial cell target, in the presence of saturating concentrations (10–25 pg/ml) of the indicated monoclonal antibodies. Results are representative of four separate experiments, all performed at effector to target cell ratios of 25:1.

![Figure 4](image)

**Figure 4.** Cell-sorter immuno-fluorescent analysis of microvascular endothelial cells cultured in standard medium*8* (panels A–C and E) or medium containing 100 units/ml γ-interferon (panel D), comparing irrelevant immunoglobulin G1 staining with specific endothelial cell surface major histocompatibility complex expression. Monoclonal antibodies used were fluorescein isothiocyanate (FITC)–CA-141 (anti–HLA-DR, panel A), FITC–Leu-10 (anti–HLA-DQ, panel B), FITC–anti-DP (anti–HLA-DP, panel C), FITC–CA-141 (panel D), and FITC-W6/32 (anti–HLA class I, panel E). Ten thousand cells were analyzed for each sample.
vascularized allograft rejection, since it forms a contiguous barrier, bearing surface donor alloantigens, to circulating recipient lymphocytes. Immunohistological data in human vascularized grafts undergoing rejection support this notion, because they frequently demonstrate widespread microvascular damage, which is initially seen in those venules, arterioles, and small veins enveloped by lymphocytes. However, analysis of mononuclear cell infiltrates in rejected allografts is not sufficient to determine which lymphocyte subset is primarily responsible for damaging the donor tissue, nor does it clarify the sequence of events underlying the rejection process. For this reason, we analyzed the signals and restriction elements used by unprimed lymphocyte subsets to generate a functional response in vitro to allogeneic ECs of microvascular origin. The results demonstrated that resting microvascular ECs are unable to trigger a functional response by allogeneic monocyte-depleted T cells. These observations confirm the data obtained using ECs derived from large veins as stimulators and suggest that T cells, unlike their functional response to genetically unrelated lymphoid cells, require additional signals to proliferate and differentiate into allospecific effectors. Because ECs express surface class I MHC determinants with a density comparable to lymphoid cells, it is likely that their inability to stimulate allogeneic T cells reflects either defective “accessory” cell function (i.e., soluble factor production or antigen processing capabilities) or insufficient expression of MHC class II alloantigens to be recognized by allospecific T cells. The induction of alloantigen-specific T-cell proliferation and cytotoxicity to allogeneic ECs in the presence of low concentrations of IL-1 or IL-2 (Figure 1 and Table 1) would suggest that surface alloantigens displayed by ECs are normally recognized by precursor T cells, whereas the IL-1 mediated activation pathway involved in the afferent limb of the T-cell-dependent immune response is defective.

This conclusion is supported by the experiments performed with CD8+ cells (Figure 1 and Table 1), which were induced to lyse the sensitizing EC line if as little as 2 units/ml IL-2 were present in the 7-day cultures. This result suggests that CD8+ cells express

![Figure 5](http://circres.ahajournals.org/)

**FIGURE 5.** Graphs showing purified CD4+ T cells cocultured for 7 days with allogeneic endothelial cells and autologous accessory cells that had been pretreated with medium (○), 500 μM chloroquine (●), or 500 μM chloroquine after overnight pulsing with the relevant endothelial cell line (□). E/T ratio, effector to target cell ratio. After 1 week, the lymphocytes were recovered and used as effectors in a 4-hour cytotoxicity assay with the relevant endothelial cell target.

**TABLE 2.** Proliferative and Cytotoxic Activity of Allogeneic Endothelial Cell–Specific CD4+ T-Cell Clone (AT.30.1)

<table>
<thead>
<tr>
<th>Cofactors</th>
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<td>...</td>
<td>2,812</td>
<td>...</td>
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<td>IL-2 (2 units/ml)</td>
<td>+</td>
<td>...</td>
<td>66,332</td>
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<td>...</td>
<td>6,002</td>
<td>...</td>
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<tr>
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<td>...</td>
<td>25,331</td>
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<tr>
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<td>44.0</td>
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<tr>
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<td>Anti-CD3</td>
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<tr>
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<td>Anti-CD4</td>
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<td>+</td>
<td>Anti-LFA-1</td>
<td>ND</td>
<td>...</td>
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<tr>
<td>Auto ACs</td>
<td>+</td>
<td>Anti-LFA-3</td>
<td>ND</td>
<td>71.4</td>
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</table>

*The indicated antibodies (10 μg/ml) were added at the beginning of the culture or during the 4-hour cytotoxic assay.
†AT.30.1 clone was recovered, rested for 18 hours in the absence of stimulating antigen or IL-2, and cultured (5x10⁴) for 72 hours in the presence of the indicated cofactors, with or without irradiated endothelial cells (1x10⁴) from the line originally used as stimulator. Irradiated autologous (auto AC) or allogeneic (allo AC) monocytes were added at a monocyte to T-cell ratio of 0.2:1. Twelve hours before harvesting the cultures, 1 μCi [³H]thymidine was added.
‡Cloned AT.30.1 cells were washed and added to [⁵¹Cr]-labeled endothelial cells from the original stimulator line at a T-cell to endothelial cell ratio of 50:1, with or without 10 μg/ml of the indicated antibodies. Four hours later, [⁵¹Cr] release was evaluated in the supernatants, and specific lysis was calculated as described in “Materials and Methods.”

**MAB, monoclonal antibody; IL-2, interleukin-2; Auto ACs, autologous accessory cells; Allo ACs, allogeneic accessory cells; LFA, lymphocyte function associated; ND, not done.**
The presence of IL-1 or autologous ACs could, therefore, provide the additional signals required for the production of IL-2 by CD8+ cells,29 thus driving an autocrine allospecific response by this lymphocyte subset. The demonstration of an autocrine loop, driven by allogeneic class I MHC antigens and entirely mediated by CD8+ lymphocytes, has preceded, as "helper-independent" cytotoxic lymphocytes have been described both in murine and human experimental systems.23,30 Blocking experiments (Figure 3a) carried out in the final lytic assay using allogeneic EC primed CD8+ cells as effectors demonstrated that, as expected, membrane class I MHC allodeterminants on ECs represent the major target and presumed stimulus for this lymphocyte subset. The CD3/TcR and CD8 molecular complexes on the effector cells appear to be involved in the recognition and subsequent lysis of the stimulating EC line. These findings confirm observations made by other investigators14 and indicate that class I MHC antigens displayed by ECs are fully capable of triggering allospecific CD8+ CTL precursors. This functional correlation between CD8+ cells and EC class I MHC alloantigens is consistent with the molecular constraints originally described in the classical mixed lymphocyte reaction model31 and subsequently confirmed by functional studies performed on lymphocytes infiltrating class I MHC incompatible grafts.32-35

The analysis of the CD4+ cell–mediated anti-EC response provided interesting and somewhat unexpected results. The existence of CD4+ cells capable of differentiating into cytotoxic effectors has been clearly demonstrated when allogeneic class II antigen positive cells have been used as stimulators,36 and it was confirmed in the present study using γ-interferon–treated Ia-positive ECs as stimulators (Table 3). Nonetheless, the likelihood of detecting such activity in CD4+ lymphocytes cultured for 1 week with allogeneic ECs seemed low, since HLA-DP, -DQ, and -DR antigens are not detectable on microvascular ECs under these conditions (Figure 4). Clonal analysis of the CD4+ cell–mediated anti-EC cytotoxic response demonstrates that, at least in some instances, the same cell can be induced to proliferate in response to the relevant EC alloantigen and to differentiate toward the final effector CTLs (Table 2). The presence in the sensitizing culture of autologous ACs appears to be a condition necessary and sufficient for generating a detectable response by CD4+ cells in our experimental model. The activity of autologous ACs in the culture system is not replaceable by IL-1 or IL-2 (Figure 1 and Table 1), but it is completely abrogated by the treatment of ACs with 500 μM chloroquine before alloantigen pulsing (Figure 5). This strongly suggests that intracellular processing of allogeneic EC-derived antigens is required to trigger the recognition of allogeneic EC determinants by CD4+ lymphocytes. Results obtained with an allogeneic EC-specific CD4+ clone (Table 2) suggest that self-MHC class II-expressing autologous ACs, in addition to processing EC-derived antigens, provide the necessary restriction elements for CD4+ cells, as shown by the inhibition observed with anti-Ia antibodies and by the inability of ACs bearing MHC antigens unrelated to those of the responding T cells to stimulate a proliferative response to the sensitizing EC line. The most likely explanation for these findings is that alloantigen processing and presentation in the context of self-MHC membrane molecules by ACs is a requirement for the stimulation of allospecific responses by CD4+ lymphocytes. This interpretation is further supported by other studies using cell-free minor histocompatibility antigens as stimulators of a T-cell–mediated cytotoxic response in the murine system37-39 and, more recently, by the elegant demonstration that peptide fragments derived from the hypervariable domain of MHC molecules physically associate with membrane Ia antigens on antigen-presenting cells and effectively stimulate an allospecific T-cell response.40,41

### Table 3. Cytotoxic Activity of CD4+ T-Cell Clones Induced by Major Histocompatibility Complex Class II+ Allogeneic Endothelial Cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Blocking MAb*</th>
<th>Ia+ EC45† Lysis (%)</th>
<th>Inhibition (%)</th>
<th>Ia+ EC45† Lysis (%)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>AT.45.3</td>
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<td>0</td>
<td>8.1</td>
<td>0</td>
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<td>56.8</td>
<td>7.5</td>
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<tr>
<td></td>
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<td>1.9</td>
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<tr>
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<tr>
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<td>66.9</td>
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<td>60.0</td>
<td>0</td>
<td>2.6</td>
<td>0</td>
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</tbody>
</table>

*The indicated Mabs (10 μg/ml) were added during the 4-hour chromium release assay.
†To induce major histocompatibility complex II expression, EC45 cells were pretreated for 72 hours with 60 units/ml γ-interferon before the cytotoxicity assay.
responses remain elusive, as resting ECs do not express detectable surface HLA-DP, -DQ, or -DR antigens, which are typically involved in the triggering of CD4+ lymphocyte–mediated activities (Figure 4 and Reference 33). Particularly difficult to understand is how antigen presentation by autologous ACs leads to recognition and lysis of allogeneic ECs. Since no monocytess were added to the cytotoxicity assays and since the resting EC targets lack detectable class II MHC determinants, the possibility exists that cytolysis by the CD4+ CTLs is not specific for classical MHC class II (or class I) molecules. This is surprising in light of the generally accepted view that all or nearly all CD4+ T-cell functions are MHC class II restricted.42 On the other hand, since we have not yet tested the effects on cytotoxicity of a large panel of MHC class II–specific antibodies, the possibility remains that ECs may express quantities of MHC class II antigens that are sufficient to trigger cytotoxicity but insufficient to generate an immunofluorescent signal or to stimulate clonal expansion. Alternatively, class II MHC determinants shed either from the activated CD4+ effectors or autologous monocytes may have passively adsorbed onto allogeneic EC targets during the 4-hour chromium release assay and combined with EC alloantigens in a manner recognized by CD4+ CTL. There is precedent for passive adsorption of Ia molecules onto T cells that have been incubated with allogeneic non-T mononuclear cells.43 Finally, it is possible that non-MHC–encoded EC antigens might play a major role in the sensitization of an allospecific CD4+ CTL precursor. This concept is supported by the existence of EC–specific alloantigens apparently responsible for allograft rejection in HLA-identical individuals44 and by recent reports demonstrating a role for CD4+ cytolytic45,46 and noncytolytic47 cells specific for non-MHC–encoded determinants. All of these possibilities remain to be explored.

In conclusion, our results indicate that the generation of a functional immune response to allogeneic vascular endothelial cells, which are defective in AC activity, requires a complex interaction between different cell types having specialized functions. In addition to the role of CD4+ and CD8+ precursor lymphocytes capable of clonal expansion, cytokine production, and allo-EC–specific cytotoxicity, our data underscore the importance of ACs of host origin in the inductive phase of the response to allogeneic tissues. ACs autologous to the responder lymphocytes appear to be critical not only as a source of soluble factors, such as IL-1, involved in the clonal expansion and subsequent differentiation of allo-EC–specific precursors but also as elements capable of uptaking and processing the alloantigens required to sensitize CD4+ precursors in association with the appropriate restriction elements.48 These data lend support to the observations made in vivo in experimental allograft rejection models, showing that adoptive transfer of whole mononuclear cell populations to a sublethally irradiated host is consistently more effective in promoting allograft rejection than selected lymphocyte subsets of the helper or cytotoxic lineage (for review, see Reference 26). Moreover, they provide evidence for the existence of a sequential activation process, involving ACs and CD4+ and CD8+ T cells, in the response to nonlymphoid allogeneic tissues, as suggested by histological data that shows the serial appearance of activated cells belonging to these lineages in cellular infiltrates associated with graft rejection.49,50

**Acknowledgments**

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**References**


Key Words: cytotoxic T lymphocytes, microvascular endothelium, alloreactivity, allograft rejection
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R Pardi and J R Bender

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