An In Vitro Model of T Cell Activation by Autologous Cytomegalovirus (CMV)-Infected Human Adult Endothelial Cells: Contribution of CMV-Enhanced Endothelial ICAM-1

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Cellular immunity is strongly implicated in control of CMV disease; however, many mechanistic details remain unresolved. We previously demonstrated T cell activation responses to CMV-infected allogeneic endothelial cells (EC), suggesting EC as a mediator of CMV response in the transplant recipient. We now test the hypothesis that CMV-specific T cell responses can be directly stimulated by infected EC in an environment free of potentially confounding allogeneic factors. By isolating splenic T cells and gonadal vein endothelial cells (GVEC) from individual cadaveric organ donors, we have developed an in vitro model of T cell interaction with autologous CMV-infected EC. Proliferation assays demonstrated significantly enhanced responses by CMV-seropositive donor-derived T cells cocultured with CMV-infected GVEC, as compared with those elicited by uninfected cells. Similarly, as determined by limiting dilution analysis of IL-2-producing cells, T cell response frequencies to infected GVEC were significantly greater than to uninfected EC. In contrast, responses of CMV-seronegative donor-derived T cells were minimal, regardless of CMV status of stimulator GVEC. Intriguingly, CD4 responses were observed in spite of the fact that CMV-infected EC express no HLA class II. Finally, attenuation of CMV-stimulated T cell proliferation observed in the presence of blocking Ab specific for ICAM-1 suggests a contributing role for CMV-enhanced endothelial ICAM-1 expression in the activation response. These studies demonstrate that EC can stimulate autologous T cell responses to CMV in the absence of accessory APC and suggest potentially novel mechanisms of immune activation. The Journal of Immunology, 1998, 160: 3143–3151.

CMV, although rarely a cause of serious disease in the immunocompetent individual, has achieved considerable notoriety as a major opportunistic pathogen among immunosuppressed populations. Despite substantial progress in antiviral therapy and prophylaxis, CMV remains causally associated with interstitial pneumonia (1, 2), diffuse gastrointestinal mucosal ulceration (3, 4), hepatitis (5), and retinitis (6), as well as destructive inflammatory lesions in a variety of other locations (7). As a member of the herpes group, CMV shares the tendency to establish latency/persistence in the infected individual, a state that remains incompletely understood for this virus (8). Regardless of the specific details defining CMV latency or persistence, it is well established that reactivation frequently follows either pharmacologic immunosuppression in organ transplant recipients or HIV-induced erosion of immune function in AIDS patients.

The vascular endothelium represents the anatomical and functional interface between circulating immune components and all underlying tissues, and is thus uniquely poised to interact with both. Once considered a complacent barrier whose primary assumed function was the inhibition of abnormal intravascular thrombosis, the endothelium has emerged as a dynamically inter-active participant in immunomodulation. Endothelial cells (EC) are now known to regulate leukocyte migration (9), express HLA molecules and present Ag (10, 11), elaborate and respond to immunomodulating cytokines (12), and inducibly express immunoreactive cellular adhesion molecules (12). It is also well documented that EC are a common target for CMV infection in vivo regardless of the organ involved (13–15). Furthermore, EC can serve as fully permissive hosts for CMV in vitro provided the natural endothelial cytopathogenicity of the virus is preserved by propagation in EC (16, 17). Collectively these findings have led us to hypothesize a role for the EC as one mediator of immune responses to CMV.

In support of such a role, we have previously demonstrated that CMV-infected EC powerfully stimulate allogeneic T cells to produce IL-2 and to proliferate (18). In addition, T cell populations thus activated elaborated both IFN-γ and TNF-α (19). These responses appear to be CMV-specific since they are strictly limited to T cell populations isolated from CMV-seropositive donors and since little or no T cell proliferation or cytokine production is elicited by uninfected allogeneic EC regardless of donor serostatus (18, 19). We and others have also shown that CMV-infected human EC neither express detectable levels of HLA class II protein or mRNA nor (unlike their uninfected counterparts) can they be induced to do so by IFN-γ (20–24).

Since these activation responses have been observed in the absence of accessory Ag-presenting monocytes and since we have observed similar responses among purified CD4+ T cell populations (18), the mechanisms by which such interactions are initiated in the absence of HLA class II remain enigmatic. Importantly, all of our earlier studies have been conducted in an allogeneic system,
limiting their relevance to interactions that might occur within the allograft vasculature of the solid organ transplant recipient. Thus, the major objective of the current investigation was to develop a means to model the immunologic impact of CMV-infected EC in the nontransplanted host.

Exploiting the availability of material from a limited number of cadaveric organ donors (following appropriate informed consent according to our approved institutional review board protocol), we have isolated splenic T cells and matched gonadal vein EC (GVEC) from three individuals, generated CMV-infected and uninfected GVEC stocks, and employed various combinations of these cells to compare autologous and allogeneic T cell responses. Furthermore we have employed blocking Ab to identify endothelial surface molecules functionally important in the mediation of these responses. In spite of the small size of the donor pool, this model has provided a unique opportunity to begin to resolve mechanisms of endothelial/T cell interactions in the absence of potentially confounding allogeneic factors.

Materials and Methods

Endothelial cell culture

HUVEC were isolated from vessels by limited collagenase digestion according to a modification (25) of the techniques of Jaffe et al. (26) and Gimbrone et al. (27). Human GVEC were isolated from vessels of cadaveric organ donors and several separate HUVEC stocks from individual umbilical veins. In addition, stocks were similarly prepared from cadaveric organ donors and several separate HUVEC stocks from individual umbilical veins. This method was used to generate separate GVEC stocks from endothelial isolates derived from three different cadaveric organ donors and several separate HUVEC stocks from individual umbilical veins. In addition, stocks were similarly prepared from cor

sponding noninfected cultures. All stock cultures were demonstrated to be free of contaminating mycoplasma.

Phenotypic characterization of stimulator populations

To characterize constitutive and inducible HLA and adhesion molecule expression, samples of stimulator EC stocks were plated in six-well culture plates (Corning Glass Works, Corning, New York) and incubated for 72 h in the presence or absence of 200 IU/ml human rIFN-γ (Chen Environ- mental, Temecula, CA), or 300 IU/ml human rTNF-α (Genzyme, Cambridge, MA) for 4 (E-selectin), 8 (VCAM-1), or 24 h (ICAM-1). Following incubation, cells were suspended by brief trypsinization and reacted for 30 min at 4°C with the following FITC-conjugated mAbs: 1) anti-HLA class I A, B, C (ICN Biomedicals, Costa Mesa, CA); 2) anti-HLA DR (Gen Trak, Plymouth Meeting, PA); 3) anti-ICAM-1 (Gen Trak); or with unlabeled anti-E-selectin (Caltag, San Francisco, CA), or anti-VCAM-1 (generous gift of Dr. M. P. Bevilacqua, V. Brigham and Woman’s Hospital, Boston, MA). The latter two Abs were detected with a secondary FITC-labeled goat anti-mouse Ab (Coulter, Hialeah, FL). Stained cells were washed twice, suspended in cold Seligman’s balanced salt solution (SBSS, Life Technologies), and analyzed (5000 cells) using a Coulter Profile II flow cytometer. As controls for nonspecific binding, cells were reacted with appropriate isotypically matched irrelevant murine Abs.

Lymphocyte culture medium

Culture medium used in responder cell isolations and for experiments, referred to as complete DMEM, consisted of DMEM (Life Technologies), supplemented with 10% pooled human serum (from nontransfused males), 10 mM HEPES buffer, 1 mM sodium pyruvate, 1.5 mM L-glutamine, 0.27 mM sodium selenite, 0.14 mM folic acid, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. To prevent viral replication in assay cultures, medium used in all experiments was additionally supplemented with 300 μM phosphonoformic acid (PFA, Sigma), a specific inhibitor of CMV DNA polymerase (31). Our previous studies have consistently demonstrated this concentration of PFA to effectively reduce background [H1]Thdr incorporation by infected cells (as a consequence of residual viral polymerase activity), with no apparent effect on stimulator/responder interaction or CTL-20 proliferation response (18).

Isolation of human mononuclear leukocyte populations

PBMC were isolated from healthy CMV-seropositive or seronegative individuals by Ficoll-Hypaque density gradient centrifugation by the method of Boyum (32). Mononuclear leukocytes were similarly isolated from CMV-seropositive or seronegative cadaveric organ donor splenocytes (28) and cryopreserved in liquid N2 until use. CD3+ T cells were purified from PBMC freshly isolated from healthy donors or from cryopreserved stocks of cadaveric organ donor splenic mononuclear cells, by two cycles of negative selection with a commercially available mixture of mAbs and complement (T Lympho-kwik, One Lambda, Los Angeles, CA), or through a commercially available mixture of mAbs and complement (Boyum, Boyum, The Netherlands) as previously described (18, 33). For isolation of CD4+ T cells, Helper T Lympho-kwik (One Lambda) was substituted for T Lympho-kwik during the second cycle of purification (18). CD3+ and CD4+ cells prepared in this manner routinely marked 90 to 95% positive for CD3 or CD4, respectively, by immunofluorescence flow cytometry (see below), with undetectable levels of monocyte contamination, as verified by the absence of cells staining for CD14 and unresponsiveness to PHA.

Although purified T cell populations exhibited no obvious signs of monocyte contamination, to increase the stringency of monocyte depletion in selected experiments, additional subsequent purification steps were performed. Isolates were either subjected to plastic adherence or immunomagnetic depletion. For plastic adherence, cells suspended in complete DMEM were incubated 1 to 2 h in culture flasks, followed by recovery of nonadherent cells. For immunomagnetic depletion, cells were first incubated 1 h on ice with 1:25 dilutions of mAbs specific for HLA DR (Chemicon) or CD4 (Gen Trak), then washed three times in SBSS to remove unbound Ab. Cell suspensions were then incubated 30 min on ice with a 1:25 dilution of goat anti-murine IgG-coated Dynabeads (Dynal, Inc., Great Neck, NY) with frequent gentle agitation. Beads and bound cells were immobilized with a magnet while supernatants were carefully transferred to new tubes for a second round of magnetic immobilization. Supernatants containing unbound cells were again carefully removed, pelleted by centrifugation, and resuspended in complete DMEM for use in experiments.

Phenotypic analysis of responder populations

To verify the phenotypic homogeneity of the isolated responder T cell populations, cells were stained with FITC-conjugated Abs specific for CD3, CD4, CD8, and CD14 (Gen Trak), washed twice, suspended in cold

4 Preliminary studies related to this work were presented in poster format at a Keystone Symposium (Inflammation, Growth Regulatory Molecules, and Atherosclerosis, 1994) and published in abstract form (59).
SBSS (Life Technologies), and analyzed (5000 cells) using a Coulter Profile II flow cytometer. As controls for nonspecific binding, cells were reacted with appropriate isotypically matched irrelevant FITC-labeled murine Abs.

Lymphocyte proliferation assay

Stimulator cells (CMV-infected or uninfected GVEC or HUVEC) were drawn from cryopreserved stocks immediately before microculture initiation, thawed rapidly in a 37°C water bath, and washed in complete DMEM. Cells were then pelleted by centrifugation (400 × g, 10 min), resuspended in PFA-supplemented complete DMEM, and γ-irradiated (30 Gy) before microculture initiation. Responder cells (CD3+ or CD4+ T cells) isolated from CMV-seropositive or seronegative cadaveric organ donor splenocytes or from PBMC of healthy volunteers, were likewise suspended in PFA-supplemented complete DMEM. Responder cells (1 × 10^5/well) were then added to microcultures in 50-μl aliquots from cryopreserved stocks were prepared for assay and irradiated as described above, then added to microcultures in 50-μl aliquots at a constant concentration of 1.5 × 10^5 cells/well. Control wells contained stimulator cells alone or responders alone.

Microcultures were incubated at 37°C in a humidified atmosphere of 10% CO2/90% air for 6 days, including an 18-h terminal pulse with [3H]TdR (1 μCi/well). Microcultures were harvested onto glass wool filters, and [3H]TdR incorporation was measured in a β-scintillation counter. Results from triplicate wells were expressed as mean counts (± 1 SD). Statistical significance of differences between paired means was determined by paired t test.

Blocking Ab studies

Proliferation assays were performed essentially as described above but modified by the inclusion of various concentrations of Abs specific for HLA DR and ICAM-1. We have previously shown that Ab L-227, specific for HLA DR, reduces class II-dependent T cell proliferation responses to IFN-γ-treated allogeneic EC by greater than 80% (34). Thus to determine effects of blocking concentrations of the Ab used in the current investigation, anti-HLA DR (IgG2a, Dako, Carpinteria, CA, diahydrated to remove azide) or an irrelevant isotype-matched control (IgG2a, Becton Dickinson, San Jose, CA) were titrated into 6-day cocultures containing IFN-γ-treated HUVEC (5 × 10^5 cells/well) and allogeneic CD4+ T cells (1 × 10^5 cells/well). Microcultures were assayed for [3H]TdR incorporation as described above. The Ab concentration range that resulted in maximal inhibition by anti-DR in the absence of nonspecific interaction by the irrelevant control was used in subsequent experiments.

For blocking studies of T cell responses to CMV-infected EC, microwells contained 1 × 10^5 CD4+ T cells/well and CMV-infected stimulator cells at constant concentrations optimized for maximal response (1–4 × 10^5 cells/well). Abs employed in these experiments included anti-HLA DR (described above) and anti-ICAM-1 (IgG2b, Becton Dickinson) as well as irrelevant isotype-matched controls (Becton Dickinson). Stimulator cells were pretreated for 1 h with Abs before addition of T cells, then incubated, radiolabeled, and harvested as described above. Results from triplicate wells were expressed as mean counts (± 1 SD). Statistical significance of differences between paired means was determined by paired t test.

IL-2-responsive indicator cells

CTL-20, a murine lymphoblastoid, IL-2-dependent cell line was used in limiting dilution analyses (see below) to estimate frequencies of responding T cells. CTL-20 cells were propagated in complete DMEM modified by the substitution of FCS (2%) for human serum, and by the addition of IL-2 in the form of supernatant from Con A-induced rat splenocytes (10%). Cultures were maintained in upright 25-cm² tissue culture flasks (Corning) and passed at 48-h intervals by transfer of 10^6 cells into 10 ml of fresh medium in new flasks.

Limiting dilution microculture conditions

Limiting dilution microculture conditions were adapted from protocols described by Orosz et al. (35) and Clouse et al. (36), as we have described previously (18). Series of eight doubling dilutions of responder cells (CD3+ or CD4+ T cells, concentration range: 20,000–156 cells/well) were prepared in PFA-supplemented complete DMEM in V-bottom microtiter wells (Linbro/Flow), 12 wells/dilution, 25 μl/well. Stimulator cells (CMV-infected or uninfected autologous or allogeneic GVEC) drawn from cryopreserved stocks were prepared for assay and irradiated as described above, then added to microcultures in 50-μl aliquots at a constant concentration of 10% Con A-stimulated rat splenocyte supernatant. This treatment has been shown to expand the CTL-20 population in wells containing IL-2-producing T cells without increasing background activity in negative wells (18, 37). Plates were incubated for an additional 3 days, including a terminal 18-h [3H]TdR pulse (0.5 μCi/well), then harvested onto glass wool discs for scintillation counting as described above.

Limiting dilution analysis

Minimal estimates of responding cell frequencies were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the limiting dilution microwells and the percent replicate microwells that failed to produce detectable IL-2 (35, 38). Limiting dilution microculture wells were considered positive for IL-2 production if [3H]TdR incorporation by CTL-20 cells exceeded that (mean plus 3 SDs) of corresponding control wells containing stimulator cells and CTL-20 cells, but no responders. Calculations, performed as detailed elsewhere (18, 38), generate a frequency estimate (1/f), a corresponding 95% confidence interval, and the χ² estimate of probability (p) for the frequency estimate. In these studies, a p value of >0.05 indicates that the frequency estimate is statistically acceptable.

Results

Phenotypic characteristics of stimulator populations

We have previously demonstrated that all GVEC lines employed in these studies exhibit phenotypic characteristics essentially identical to those of HUVEC (28). Specifically these cells exhibit a typical endothelial cobblestone appearance and constitutively express VWF, CD31, HLA class I, and modest levels of ICAM-1. Treatment of GVEC with IFN-γ induces HLA class II and enhances HLA class I and ICAM-1 expression, while treatment with TNF-α induces VCAM-1 and E-selectin, while enhancing HLA class I and ICAM-1 expression. We have also previously described CMV-mediated perturbations of constitutive and inducible HLA and adhesion molecule expression on infected HUVEC (20, 23, 39). In addition, we have reported CMV-mediated inhibition of HLA class II induction on infected GVEC (23) and EC derived from several human arterial and microvascular beds (24). However to ensure phenotypic homogeneity among stimulator stocks generated specifically for these experiments, it was essential to determine the immunobiologic characteristics of each.

Representative histograms generated by immunofluorescence flow cytometric analysis of stimulator GVEC populations are presented in Figure 1 (donor 3, representative of GVEC isolated from all donors), and corresponding percent-positive and mean fluorescence intensity values are summarized in Table I. These data indicate that HLA class I expression is greatly attenuated in CMV-infected GVEC, as is class II responsiveness to IFN-γ (as well as TNF-α, data not shown). In addition, HLA DR is neither directly induced by the virus, nor are infected cells responsive to IFN-γ, and patterns of HLA DP and DQ expression are identical to those exhibited by DR (data not shown; see Refs. 20, 23). Similarly, VCAM-1 and E-selectin are not expressed on infected GVEC, and these adhesion molecules can no longer be induced by TNF-α (data not shown). ICAM-1, in contrast, is directly induced by CMV infection, and expression progressively increases as cells proceed to cytomegaly.
Lymphocyte proliferation assay

We have previously demonstrated that CMV-infected HUVEC provide a powerful activating stimulus for allogeneic CMV-seropositive donor-derived T cells in the absence of accessory Ag-presenting monocytes (18). A primary objective of this investigation was to develop a model that would control for potentially confounding allogeneic factors to determine whether similar interactions occur in an autologous environment. Hence we initially cocultured purified organ donor splenic CD3$^+$ T cells with CMV-infected or uninfected autologous GVEC or allogeneic HUVEC. Data presented in Figure 2, representative of two to four replicate experiments performed with T cells isolated from each of two seropositive organ donors, confirm that uninfected EC express minimal immunogenicity regardless of whether they are autologous or allogeneic. Furthermore these data show that T cell proliferative responses to CMV-infected autologous GVEC are approximately equivalent to those stimulated by allogeneic HUVEC. Identical experiments performed with splenic T cells isolated from a seronegative organ donor demonstrated no detectable proliferation in response to either infected or uninfected autologous or allogeneic GVEC (data not shown).

Since our previous studies revealed that the CD4$^+$ subset is the major responsive population to CMV-infected allogeneic HUVEC (with respect to IL-2 production and proliferation) (18), we purified CD4$^+$ T cells from donor splenocytes and assessed their proliferative responses to autologous uninfected GVEC. To conserve the limited supplies of donor splenocytes, stimulator cell titrations were abbreviated, or after determination of maximum responses, were reduced to a single concentration. As expected, for all three donors CD4$^+$ T cell proliferative responses to autologous uninfected GVEC were not significantly greater than the minimal activity of T cells cultured alone (Fig. 3). In contrast, significant proliferation was observed in response to CMV-infected GVEC ($p$, 0.01). However, as we have consistently observed in the allogeneic environment, such responses were strictly limited to T cell populations isolated from seropositive donors (Fig. 3).

Estimation of frequencies of IL-2-producing T cells

To more rigorously quantify activation responses to autologous CMV-infected EC, infected or uninfected irradiated GVEC were cocultured with limiting dilutions of autologous CD3$^+$ or CD4$^+$ T cell responder populations. In addition, to compare autologous and allogeneic responses at this level, in a subset of experiments we cocultured seropositive donor-derived CD4$^+$ T cells with CMV-infected or uninfected allogeneic GVEC. Frequencies of responding cells were estimated by limiting dilution analysis with IL-2 production as the endpoint, using a direct indicator cell (CTLL-20) assay. This assay measures the size of the T cell population with

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**Table I. Summary data generated by immunofluorescence flow cytometric analysis of stimulator GVEC treated as described in Figure 1**

<table>
<thead>
<tr>
<th>Population</th>
<th>HLA I</th>
<th>HLA DR</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>100/169</td>
<td>0/0</td>
<td>63/3</td>
</tr>
<tr>
<td>EC/IFN-γ</td>
<td>100/254</td>
<td>87/162</td>
<td></td>
</tr>
<tr>
<td>EC/CMV</td>
<td>34/37</td>
<td>0/0</td>
<td>91/51</td>
</tr>
<tr>
<td>EC/CMV/IFN-γ</td>
<td>36/40</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>EC/TNF-α</td>
<td>100/168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC/CMV/TNF-α</td>
<td>92/54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*% positive and mean fluorescence intensity values were derived by subtracting values generated by irrelevant isotype-matched controls from those generated by specific Ab staining. All mean fluorescence intensity values were generated on an equal scale.*
specificity for the stimulating agent, and with the ability to secrete IL-2 in response to T cell engagement.

Representative data summarizing results of our studies employing splenic CD4+ T cells isolated from CMV-seronegative (donor 1) or CMV-seropositive (donors 2 and 3) cadaveric organ donors measured following 6 days of incubation alone or coculture with CMV-infected or uninfected autologous GVEC. Bars represent mean [3H]Tdr incorporation derived from triplicate wells. Error bars indicate SDs. * denotes statistically significant difference between indicated pairs (p < 0.01).

FIGURE 3. Proliferation responses of splenic CD4+ T cells isolated from CMV-seronegative (donor 1) or CMV-seropositive (donors 2 and 3) cadaveric organ donors measured following 6 days of incubation alone or coculture with CMV-infected or uninfected autologous GVEC. Stimulation effects promote a greater contribution to the cytokine milieu by this discrepancy is not clear, it may be analogous to the well-documented autologous mixed lymphocyte reaction, in which T cells have been observed to exhibit activation markers and to proliferate in response to autologous mononuclear leukocytes (40, 41), albeit to a much greater extent than that we have noted in response to GVEC. Alternatively, but not necessarily mutually exclusively, these apparent self-stimulated responses may be attributable to pretransplantation donor trauma or donor management, donor tissue processing and storage, or culture conditions. In any case, CMV-specific responses were significantly greater (generally by at least an order of magnitude) than the minimal responses to uninfected autologous EC.

Increase in T cell isolation stringency

It is clear from the data presented thus far, as well as from results of our earlier studies with allogeneic HUVEC (18), that the interactions we have observed appear to defy fundamental paradigms in cellular immunology, specifically, self HLA restriction and the requirement for HLA class II in CD4+ T cell activation. Although these responses might be explained by the persistence of monocytes through our T cell isolation procedures, the absence of CD14+ cells demonstrated by immunofluorescence flow cytometry and the lack of PHA response of our purified populations argue against this. Nonetheless, in a subset of experiments, we increased the stringency of purification, either by subjecting T cell populations to plastic adherence, or by immunomagnetic depletion of CD14+ or HLA DR+ cells. Regardless of protocol, the minimal CD4+ T cell proliferation responses to PHA were insignificant compared with T cells cultured in medium alone (Fig. 4). Yet in the absence of PHA responses, CMV-infected GVEC still stimulated significant proliferation in CMV-seropositive donor-derived T cell populations following plastic adherence or immunomagnetic depletion (p < 0.01).

Blocking Ab studies

Since high levels of ICAM-1 are expressed by CMV-infected EC (Fig. 1, Table I) and this molecule has been associated with co-stimulatory function (42, 43), we sought to determine its functional

Table II. Limiting dilution analysis of frequencies of IL-2-producing CD3+ T cells in response to CMV-infected (GVEC/CMV) or uninfected (GVEC) autologous GVEC

<table>
<thead>
<tr>
<th>Donor/Serostatus</th>
<th>CD3+ T Cells (Cells/Million)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GVEC</td>
</tr>
<tr>
<td>1/CMV−</td>
<td>24</td>
</tr>
<tr>
<td>2/CMV+</td>
<td>&lt;5*</td>
</tr>
<tr>
<td>3/CMV+</td>
<td>28</td>
</tr>
</tbody>
</table>

* As determined by χ2 minimization, p > 0.10 for all estimates (p > 0.05 indicates significance).

* Stimulator cell population.

* Below detection limit of assay.

Table III. Limiting dilution analysis of frequencies of IL-2-producing CD4+ T cells in response to CMV-infected (GVEC/CMV) or uninfected (GVEC) autologous or allogeneic GVEC

<table>
<thead>
<tr>
<th>Donor/Serostatus</th>
<th>CD4+ T Cells (Cells/Million)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autologous</td>
</tr>
<tr>
<td></td>
<td>GVEC</td>
</tr>
<tr>
<td>1/CMV−</td>
<td>33</td>
</tr>
<tr>
<td>2/CMV+</td>
<td>12</td>
</tr>
<tr>
<td>3/CMV+</td>
<td>36</td>
</tr>
</tbody>
</table>

* As determined by χ2 minimization, p > 0.10 for all estimates (p > 0.05 indicates significance).

* Stimulator cell population.
significance in the activation responses we have observed. To determine the significance of ICAM-1 in allogeneic activation, we initially titrated anti-ICAM-1 (or an irrelevant isotype-matched control) into series of cocultures containing CMV-infected HUVEC and allogeneic CD4\(^+\) T cells isolated from peripheral blood of healthy CMV-seropositive donors. Data presented in Figure 5, representative of experiments performed with T cells isolated from three donors (two to three replicate experiments/donor), show significant attenuation of proliferation at an Ab concentration of \(1 \text{ mg/ml}\), as compared with responses in the presence of equal concentrations of irrelevant IgG2b (\(p = 0.01\)). We further observed that additional increases in anti-ICAM-1 concentration did not result in greater attenuation (data not shown).

To preserve supplies of donor splenocytes, autologous blocking studies were performed at a single Ab concentration rather than as a complete titration. Thus, CD4\(^+\) T cells isolated from the two CMV-seropositive donors were cocultured with autologous CMV-infected GVEC in the presence of blocking Ab specific for ICAM-1 (2–5 \(\mu\)g/ml) or an irrelevant control at equal concentration. Note that donor No. 1 (CMV-seronegative) was not included in these experiments since CMV-infected EC elicited no response in T cell populations isolated from this individual. With the intent of verifying the insignificance of HLA class II in activation stimulated by CMV-infected EC, we also established cocultures in the presence of anti-HLA DR (2–5 \(\mu\)g/ml, concentrations shown to inhibit T cell proliferation responses to IFN-\(\gamma\)-treated allogeneic EC, data not shown) or its corresponding irrelevant control. As shown in Figure 6 (representative of three replicate experiments), inclusion of anti-ICAM-1 (2 \(\mu\)g/ml) in microcultures resulted in significant attenuation of autologous proliferation responses to IFN-\(\gamma\)-treated allogeneic EC, data not shown) or its corresponding irrelevant control. Although this effect has at times approached, but never achieved, statistical significance, its reproducibility suggests either that 1) infected stimulator EC do indeed express DR, albeit at levels below our limit of detection but sufficient to effectively engage the TCR, or 2) that anti-class II Abs are acting outside the realm of Ag presentation, binding to class II expressed on activated T cells and attenuating their proliferation (see Discussion).
Discussion

Our previous studies, directed toward elucidation of potential CMV-driven immunopathologic interactions that might occur at the allograft endothelial interface, revealed the potential of allogeneic infected EC to elicit cellular immune responses. Specifically, we demonstrated that CMV-infected HUVEC activate allogeneic, CMV-seropositive donor-derived CD3+ and CD4+ T cells to produce IL-2 and proliferate (18), as well as to elaborate inflammatory cytokines IFN-γ and TNF-α (19). The fact that such responses occur in purified, monocyte-depleted populations reinforces the documented ability of EC to act as APC. However the allogenicity of that system, and our concurrent discovery that CMV-infected EC express no HLA class II (18, 20, 23) and greatly reduced levels of HLA class I (or none at all) even following cytokine stimulation, lends an enigmatic twist to our findings.

In the current investigation we sought to eliminate potential allogeneic influence to determine whether similar interactions occur in the autologous environment, and to begin to resolve mechanisms involved in what appears to be a potentially novel activation pathway. To accomplish these objectives, we isolated splenic T cells and matched GVEC from individual cadaveric organ donors. To compare autologous and allogeneic interactions, CD3+ or CD4+ splenic T cells were cocultured with CMV-infected or uninfected irradiated GVEC in various combinations and assayed for proliferation and frequency of IL-2-producing cells.

Data generated by these experiments indicate that autologous CMV-driven activation responses are essentially indistinguishable from those occurring among allogeneic combinations. Specifically, we have shown approximately equivalent levels of proliferation and frequencies of IL-2-producing cells among CMV-seropositive donor-derived CD3+ and CD4+ T cells in response to either autologous or allogeneic CMV-infected donor GVEC. We interpret these responses as CMV specific since they were restricted to T cells isolated from CMV-seropositive donors, and since little or no response was stimulated by uninfected autologous or allogeneic GVEC regardless of donor serostatus. Importantly, these outcomes were unaffected by increasing the rigor of T cell purification (plastic adherence, immunomagnetic depletion of HLA class II+ and CD14+ cells), arguing against the possibility of monocyte contamination. Finally, based upon our observations of CMV-induced endothelial ICAM-1 enhancement, we demonstrated a contributing role for this adhesion/costimulatory molecule in the activation process by significantly attenuating T cell proliferation with anti-ICAM-1.

Although these findings indicate that EC can directly activate autologous T cells in the absence of accessory professional APC, results of these experiments raise several important questions that remain to be resolved. Most prominent of these is the identification of specific components of the intercellular activation signal pathway operational in this system. Several lines of evidence generated herein and by our previous studies argue in favor of mechanisms that are not dependent upon HLA. First, the magnitudes of CMV-driven T cell responses are approximately equivalent regardless of whether the stimulating infected EC are allogeneic or autologous. Such equivalence has also been apparent among over 20 different allogeneic stimulator/responder combinations, part of which have been studied since our first reported ten-donor investigation (18, 19, 22). Second, we and others have demonstrated that CMV-infected human EC express no detectable HLA DR, DP, or DQ even following stimulation with IFN-γ (20–24). Although these studies cannot be taken as absolute proof of complete absence of class II, they do demonstrate that, if present, this molecule is quite scarce on the infected EC. Furthermore, as has been shown by others in infected fibroblasts (44, 45), CMV inhibits HLA class I expression by infected EC (23).

Collectively, these observations argue in favor of an HLA-independent immune activation pathway. Bukowski et al. (46) reported that herpes simplex virus (HSV)-stimulated PBMC exhibited HLA-unrestricted cytolytic activity against Daudi cells and HSV-infected (but not uninfected) targets. These investigators showed that such activity was accountable to the greatly expanded γδ subset in stimulated PBMC populations. Furthermore, HSV-stimulated PBMC exhibited lytic activity against cells infected with vaccinia, a virus unrelated to HSV. Based upon these findings, the authors speculated that their observed responses might be directed against a cellular protein induced as a stress response to viral infection, rather than against a specific viral peptide. Indeed, studies by Pommiah et al. (47) employing a mouse model of influenza virus infection implicate heat shock protein (Hsp60) as such a target. Since we have observed induction of Hsp70 in CMV-infected EC (unpublished observations), as others have observed in fibroblasts (48), it will be of interest to investigate this issue in our model when additional cadaveric organ donor material becomes available.

Also of relevance to the resolution of the intercellular signaling mechanisms operating in our model is our demonstration of anti-ICAM-1-mediated attenuation of T cell proliferation responses to CMV-infected EC. Altmann et al. (43) have shown in cotransfection assays that coexpression of ICAM-1 can restore Ag-presenting capability in mouse L cells expressing suboptimal levels of HLA DR. Although we have been unable to detect the presence of even minimal levels of HLA class II in CMV-infected EC, it is possible that similar mechanisms may be operating in our system. Clearly, however, the activation responses we report herein cannot be accounted solely to enhanced endothelial ICAM since responses are restricted to T cell populations isolated from CMV-seropositive donors and since TNF-α treatment of HUVEC or GVEC (which induces high-level ICAM-1 expression) has little impact upon their ability to stimulate autologous or allogeneic T cell responses (34). Whether overexpression of ICAM-1 simply enhances T cell adhesion to CMV-infected EC, or whether it acts in a costimulatory capacity as well, remains to be determined.

Alternatively (but not mutually exclusively) anti-ICAM-1-mediated attenuation of T cell activity might result from signaling events induced by direct binding of the Ab to ICAM-1 present upon the T cells themselves. Although we cannot presently rule out this possibility, we consider it unlikely since T cells stimulated to proliferate by IL-2 alone are not affected by this Ab even at concentrations exceeding those that attenuate responses to CMV-infected EC (our unpublished observations).

As further support for a novel activation pathway, we note that CMV-infected EC express no B7-1, B7-2, or B7-3 (our unpublished observations), as is also true of their uninfected counterparts. This implies that the B7/CD28 costimulatory pathway does not participate in the interactions we have observed. We also have shown by immunohistochemistry that expression of P-selectin, a contributing factor in monocyte cell adhesion to HSV-infected EC (49), is considerably lower in CMV-infected EC than in uninfected cells (our unpublished observations). Furthermore, although LFA-3 is modestly enhanced upon infected EC, blocking Abs specific for this molecule do not interfere with activation (our unpublished observations), arguing against a role for LFA-3/CD2 interaction in this system. Finally we note that, as expected, blocking Abs specific for HLA class I were also without effect. Thus, it was initially quite curious that anti-HLA DR was found to partially attenuate proliferative responses. Although the explanation for this observation is uncertain, we hypothesize that this Ab is acting
outside the realm of Ag presentation in our experimental system. It is well known that activated T cells express surface HLA class II, and it has been shown that binding of anti-class II Ab initiates a series of signal transduction events in these cells (50). Furthermore, several studies have demonstrated that such Abs attenuate proliferation of a variety of class II+ cells including IL-2-dependent T cell clones (51), EBV-transformed B cells (52), as well as cell lines of malignant B cell and of monocyte/macrophage origins (53). Although the physiologic significance of these phenomena remains to be resolved, these findings indicate that Abs against HLA class II can affect lymphocyte function in ways other than interference with Ag presentation. Thus we hypothesize that in our coculture model, anti-HLA DR is exerting attenuating effects by direct interaction with T cell class II, rather than by interference with TCR engagement with CMV-infected EC. Further studies are in progress to resolve this issue.

We note that the limited number of cadaveric organ donors (n = 3) from which source materials for these studies were available might be construed as a weakness of this investigation. Unfortunately, access to such materials lies beyond our control, depending upon donor availability and donor/family informed consent. Although donor GVEC stocks are replenishable from low passage cultured cells, experimental protocols are strictly limited by the quantity of splenic mononuclear cells initially isolated. However, since the interactions we have observed in the autologous system described in this report are essentially identical with those we have observed in over 20 allogeneic stimulator/responder combinations (18, 19, 22), we feel confident that they are accurately representative. Thus in spite of the small size of the donor pool, this model has provided a unique opportunity to begin to resolve mechanisms of CMV/endothelial/T cell interactions in the absence of potentially confounding allogeneic factors.

In summary, we have shown for the first time that CMV-infected EC can function as a direct activating stimulus for autologous T cells independent of accessory professional APC. Remaining at issue then is the resolution of intercellular signaling mechanisms that promote the activation response in the apparent absence of the traditional array of immunoregulatory surface molecules. CMV is known to code for several proteins with homology to normal cellular components (HLA class I heavy chain (54), G protein-coupled receptors (55), and Fc receptor (56), as examples). Considering that the majority of the over 200 open reading frames present in the CMV genome are presently uncharacterized, it would not be surprising if additional homologous proteins are yet to be discovered. Thus it is tempting to speculate that a CMV protein or protein complex expressed upon the infected cell surface may mimic the HLA/peptide complex sufficiently to engage the TCR, thereby initiating the activation response.

In a larger sense, the observations we report pose a final major question: What evolutionary advantage is conferred upon this virus by induction of ICAM-1 in its host cell and by the ability of such infected cells to activateafferent cellular immune responses? CMV is known to remain tightly cell associated in vivo, being disseminated primarily through cell-to-cell contact. Thus, forces that promote cellular adhesion to infected cells would likewise promote viral dissemination. Indeed we have previously demonstrated that peripheral blood monocytes can become productively infected through adhesion to CMV-infected endothelial monolayers and that monocytes thus infected are capable of transmitting infectious virus to remote endothelia (57). In addition, Söderberg-Naucler et al. have recently shown that the inflammatory environment created by the allogeneic mixed lymphocyte reaction is capable of inducing viral reactivation from latency in monocytes isolated from healthy CMV-seropositive individuals (58). Furthermore, we have observed that maximal viral production is temporally associated with extreme endothelial cytomegaly, maximal surface ICAM-1 expression, complete loss of HLA class I, and resistance to T cell and NK cell-mediated cytolysis. Collectively these observations suggest that the potential risk to the virus posed by afferent immune recognition may be vastly overshadowed by the survival advantage associated with the potential enhancement of dissemination and viral replication.

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