ENDOTHELIAL CELLS AUGMENT T CELL INTERLEUKIN 2 PRODUCTION BY A CONTACT-DEPENDENT MECHANISM INVOLVING CD2/LFA-3 INTERACTION

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Activation of resting CD4+ Th cells depends upon two processes: (a) acquisition of responsiveness to low levels of IL-2, mediated by de novo synthesis and expression of the p55 subunit of the IL-2-R; and (b) biosynthesis and secretion of IL-2, the principal autocrine cell cycle progression factor for T cells (1). T cells require at least two signals for optimal IL-2 synthesis. The first signal is generated by crosslinking of the TCR by its specific antigen complexed to an appropriate self MHC molecule, an event that can be mimicked by antibodies directed at nonpolymorphic regions of the TCR or at the associated CD3 complex. Mitogenic lectins such as PHA are also thought to deliver a signal via the TCR/CD3 complex. Second, or “accessory” signals can be delivered in three major ways: (a) binding of soluble cytokines such as IL-1, IL-6, or TNF to their specific receptors (2–6), (b) binding of antibodies to T cell surface molecules such as CD2, CD4, CD28, or CD44 (7–11); or (c) direct activation of second messenger pathways by drugs such as phorbol esters (e.g., PMA [12]). The need for a second signal appears inversely related to the strength of the primary signal delivered via the TCR. In the presence of a strong primary signal such as high concentrations of immobilized anti-CD3, secondary signals may not further enhance proliferation (13). However, even in a system undergoing maximal proliferation, secondary signals such as PMA (12) or antibodies to CD28 (14) may further augment IL-2 synthesis to “supra-optimal” levels.

Accessory cells are the physiological source of many of the secondary signals described above either by the secretion of cytokines or by direct cell contact involving interactions between specific ligand pairs. Previous work has demonstrated that endothelial cells (EC)¹, like classical APC such as lymphoid dendritic and Langerhans cells, can activate resting T cells in a primary allogeneic response (15–19). Fibroblasts and smooth muscle cells, in contrast, are not able to elicit primary responses (16–18), although fibroblasts, induced to express MHC class II molecules with IFN-
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γ, can present nominal antigen to cloned T cell lines (20). The failure of fibroblasts to present antigen to resting T cells can be overcome by the addition of exogenous IL-2 (21). Moreover, the addition of endothelial cells or mAb to CD28 also allows fibroblasts to present antigen to resting T cells (19). We have shown previously that endothelial cells, but not fibroblasts or macrophages, can markedly augment the production of IL-2 by optimally PHA-stimulated PBMC or purified CD4+ cells (22). The augmented concentrations of IL-2 are physiologically functional since T cells proliferate to a greater extent in the presence of EC than in the presence of fibroblasts when the PHA concentration is suboptimal. We have proposed that EC-mediated augmentation of IL-2 synthesis underlies the accessory role of this cell type.

The aim of this project was to establish the nature of the signal delivered by the EC to the T cell that leads to augmented IL-2 production. Using cultures of PBMC or purified CD4+ cells optimally stimulated with PHA, we compared the ability of EC to deliver the appropriate signal with the ability of soluble cytokines or conditioned medium. The use of PHA to drive maximally T cell proliferation allows the dissection of the role of secondary signals in T cell activation without the complication of different proliferative rates between treatments. Cytokines that modulate T cell responses and are produced by EC failed to mimic the effect of intact cells, and furthermore, neither conditioned medium from resting nor cytokine-treated EC were effective. Our results further demonstrate that there is an absolute requirement for cell-cell contact for augmentation of IL-2 production to occur and that antibodies to lymphocyte function-associated antigen 3 (LFA-3) and CD2 block this effect. In addition, we demonstrate in PBMC-EC cocultures, in which T cell proliferation and IL-2 utilization are prevented by means of blocking antibody to the p55 subunit of the IL-2-R, that a similar contact-dependent, CD2/LFA3 pathway is used to augment IL-2 synthesis in response to anti-CD3 antibody. Moreover, the CD2/LFA-3 pathway is also used in both the augmented proliferative responses of PBMC to anti-CD3 antibody and the primary proliferative response of purified CD4+ T cells to class II MHC-expressing allogeneic EC. We conclude that EC deliver costimulatory signals to T cells via the CD2/LFA-3 pathway and suggest that this pathway is important in the initial activation of T cells that recognize antigen or alloantigen on the surface of vascular endothelium.

Materials and Methods

EC Culture. EC from human umbilical vein were isolated and serially passaged as previously described (23, 24). Cells from subcultures two to five were used for these experiments. In all experiments involving EC, wells were precoated with fibronectin (24).

Isolation of PBMC. Heparinized venous blood from healthy human volunteers was centrifuged over Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) according to the manufacturer's instructions. PBMC were removed, washed three times, and resuspended in assay medium.

Isolation of CD4+ T Cells. PBMC, prepared as above, were depleted of monocytes and B cells by adhesion to fibronectin-coated culture plates for 1 h at 37°C. Nonadherent cells were decanted and depleted of CD8+ T cells and CD16+ NK cells by panning using antibodies OKT8 (CRL 8014; American Type Culture Collection, Rockville, MD) and 3G8 (a gift from Jay Unkeless, Mt. Sinai School of Medicine, New York, NY), respectively. Panning plates were coated with goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). The purity of the enriched CD4+ population was checked by direct immunofluorescent labeling with Leu-3 (anti-CD4), Leu-2 (anti-CD8), Leu-11 (anti-CD16), and Leu-M3 (anti-CD14 for
monocytes) (Becton Dickinson & Co., Mountain View, CA) and flow cytometry using a FACS analyzer (Becton Dickinson & Co.).

**Culture of PBMC or CD4⁺ Cells with EC, Cytokines, or Conditioned Medium.** Freshly isolated PBMC (10⁶) were suspended in 100 μl assay medium (RPMI 1640 containing 10% heat inactivated FCS, 2 mM glutamine, 125 U/ml penicillin/streptomycin [all Gibco Laboratories, Grand Island, NY] and 50 μM 2-ME [Sigma Chemical Co., St. Louis, MO]) and added either to control wells or to wells preplated 18 h earlier with EC (1.2 × 10⁶ cells/well) in fibronectin-coated 96-well U-bottomed plates (Linbro Chemical Co., Hamden, CT). Wells containing EC were washed three times before PBMC were added. Medium containing PHA (PHA-L; Sigma Chemical Co.) was then added either alone, with cytokines at the indicated concentrations, or with conditioned medium to a final volume of 200 μl. The final concentration of PHA was always 10 μg/ml, a concentration previously demonstrated to produce maximal T cell proliferation. In some experiments, PHA was replaced with anti-CD3 mAb (OKT3; Ortho Diagnostic Systems Inc., Raritan, NJ) at the indicated concentrations. When OKT3 was used as the stimulus for IL-2 production, blocking antibody to the p55 subunit of the IL-2-R (anti-Tac; gift of Thomas Waldmann, National Institutes of Health, Bethesda, MD) was also included at a final dilution of 1:1,000 to prevent proliferation and IL-2 utilization. The conditioned media used in these experiments were generated by culture of EC in assay medium for 2 d in the presence of cytokines or PBMC. Recombinant cytokines used were IL-1β (5 × 10⁵ U/mg; gift of Alan Shaw, Glaxo, Geneva), IL-6, TNF (1.68 × 10⁸ U/mg and 2.5 × 10⁸ U/mg, respectively; both gifts of Walter Fiers, Ghent, Belgium), and IFN-γ (2.5 × 10⁵ U/mg; Biogen, Cambridge, MA). Conditioned media were collected from the EC cultures and freeze-thawed before use. Plates were incubated for 2 d in a humidified CO₂ incubator at 37°C, after which supernatants were collected and frozen before IL-2 and/or IL-6 quantitation.

**Culture of EC and PBMC in Transwells.** EC were grown to confluence in fibronectin-coated 24-well plates (Costar, Cambridge, MA) and washed three times before use. PBMC (3 × 10⁵/well) were suspended in assay medium containing PHA and added either directly to the EC in the wells or to Transwells (Costar) suspended above the EC. In parallel wells, PBMC were added in a similar geometry but to wells lacking EC. The final concentration of PHA was 10 μg/ml. OKT3 was also used in some experiments at a final concentration of 25 ng/ml. When OKT3 was used as the stimulus for IL-2 production, the cultures were concomitantly supplemented with anti-Tac antibody (1:1,000). Plates were incubated for 2 d and supernatants collected as above.

**Culture of EC with PBM or CD4⁺ Cells in the Presence of Antibodies.** Freshly isolated PBMC or CD4⁺ T cells were suspended in assay medium containing PHA (10 μg/ml final) or OKT3 (25 ng/ml) and added in triplicate to EC in 96-well U-bottomed plates as described above. Antibodies were added in assay medium to the concentrations indicated to a final volume of 200 μl. mAbs were used as ammonium sulphate precipitations of ascites and were: TS 2/9 (anti-LFA-3), TS 2/18 (anti-CD2), TS 1/18 (anti-CD18), and RRIA (anti-ICAM-1) (all IgGl; gifts of Timothy Springer, Center for Blood Research, Boston, MA). A nonbinding antibody, K16/16 (IgGl; gift of Donna Mendrick, Brigham and Women's Hospital, Boston, MA), was used as a control in all experiments.

**Culture of CD4⁺ T Cells with Allogeneic EC.** Confluent EC, in flat-bottomed C96 plates (~10⁴/well), were cultured for 3 d in the presence of IFN-γ (1,000 U/ml) to induce expression of MHC class II molecules. Continued EC proliferation was inhibited by treatment with mitomycin C (10 μg/ml; Sigma Chemical Co.) for 1 h, and HBSS three times for 1 h) immediately before addition of T cells. Purified CD4⁺ T cells (3 × 10⁴/well) and the indicated antibodies were added and the plates incubated for 6 d.

**Cell Proliferation.** For all experiments except those involving Transwells, duplicate plates were run and assayed for T cell proliferation by addition of 1.0 μCi/well [³H]thymidine (New England Nuclear, Boston, MA) to triplicate wells for the final 6 h of culture. In cultures involving allore cognition, proliferation was assessed by [³H]thymidine incorporation during the final 18 h of culture. Cells were harvested using a cell harvester (PHD Technology Inc., Cambridge, MA), and incorporation was measured by liquid scintillation counting.

**Cytokine Bioassays.** The concentration of cytokines in the supernatants was measured using the IL-2-dependent murine cell line HT-2 (reference 25; gift of Abul Abbas, Brigham and
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Women's Hospital) and the IL-6-dependent line B9.9 (reference 26; gift of David Raulet, Massachusetts Institute of Technology, Cambridge, MA). Indicator cells were washed and added to flat-bottomed 96-well plates (10^4/well for HT-2, 2 x 10^5/well for B9 in 50 µl). At least five serial dilutions of each supernatant were then added to a final volume of 100 µl. Plates were incubated for 24 h (HT-2) or 3 d (B9), and then assayed for cell proliferation by a colorimetric method (27) using the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (Sigma Chemical Co.). Standard curves were run on all plates using rIL-2 (2.2 x 10^6 U/mg; Biogen) or IL-6. Concentrations of IL-2 are shown as weighted means (28). Weighted variances were consistently <5% of the weighted mean.

mRNA Quantitation. Freshly isolated PBMC (3 x 10^6) were cultured with or without EC (3 x 10^5) in the presence of PHA (10 µg/ml) for the indicated times. Where PBMC were cultured without EC, parallel plates of EC were grown and the cell lysates pooled. Whole cell RNA was prepared by guanidinium isothiocyanate lysis and CsCl centrifugation. RNA (25 µg/lane) was subjected to electrophoresis in 1.2% agarose containing formaldehyde and blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH). Monitoring of the 18S and 28S ribosomal bands on the nitrocellulose, by ethidium bromide staining and UV illumination, confirmed equal loading and transfer of samples. The pSt1 fragment from the cDNA IL-2 clone pTCGF11 (ATCC no. 39673) was separated on low-melt agarose and 32P labeled by random oligo priming (Boehringer Mannheim Biochemicals, Indianapolis, IN) to a specific activity of 1-2 x 10^9 dpm/µg. Blots were hybridized overnight at 43°C and then washed to a final stringency of 0.5 x SSC/0.5% SDS at 65°C. Autoradiography was for 12-72 h at -70°C.

Results

EC Augment T Cell IL-2 Synthesis. PBMC or CD4+ T cells were cultured with EC or soluble cytokines in the presence of a supraoptimal concentration of PHA. Cell proliferation, as measured by [3H]thymidine uptake in each experiment, is maximal at 10 µg/ml PHA and is not increased further by any of the treatments reported (data not shown). Thus, within each experiment the number of T cells is constant among treatment conditions. In experiments where PHA was omitted, there was no detectable proliferation or IL-2 production. In the presence of EC, both PBMC and CD4+ cells produce considerably more IL-2 than when cultured alone (Fig. 1). The degree of augmentation varies among donors and experiments, but is usually between 3- and 10-fold. A similar augmentation occurs in the level of IL-2 mRNA (Fig. 2). Message levels were maximal by 6 h and were declining by 12 h. At both 6 and 12 h, more specific mRNA was detectable when the PBMC had been cultured in the presence of EC. These observations establish that the augmented IL-2 concentration detected in the medium results largely from an EC-mediated increase in IL-2 synthesis.

Conditioned Medium from EC or Recombinant Cytokines Does not Augment T Cell IL-2 Production. Two cytokines were tested for their ability to mimic the effect of EC. In the presence of supraoptimal PHA, neither IL-1β nor IL-6, both secreted by EC (29-32), is able to augment IL-2 production from PBMC (Table I, Exp. 1). Likewise, TNF and IFN-γ are also without effect (data not shown). To test the possibility that some other signal was being produced by the EC, either constitutively or in response to a PBMC-derived factor, medium from resting or treated EC was added to PBMC cultures. Medium from resting EC or EC treated with 200 U/ml TNF has no effect on IL-2 production, although in some experiments, medium from IFN-γ (100 U/ml)-treated EC does produce a slight augmentation (Table I, Exp. 2). However, the augmentation was never comparable with that produced by coculture with EC, and adding more conditioned medium (50% final) was no more effective (data
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FIGURE 1. EC augment IL-2 concentrations secreted by both PBMC and CD4+ T cells. PBMC or purified CD4+ T cells were cultured for 2 d with or without EC in the presence of supraoptimal concentrations of PHA as described in Materials and Methods. Supernatants were then assayed for IL-2 by HT2 bioassay. Each symbol represents a separate experiment comparing PBMC with CD4+ T cells purified from the same donor.

not shown). In a separate series of experiments, EC were cultured with IL-1β (100 U/ml), TNF (1,000 U/ml), or PBMC without PHA. Again, medium from cytokine-treated cultures fails to augment IL-2 production when added to PHA-stimulated PBMC (Table I, Exp. 3). Medium from EC grown with non-PHA-stimulated PBMC has only a small effect, much less than that of viable EC. The cumulative result of these experiments is that none of the soluble signals examined can replace EC as a means of augmenting IL-2 synthesis.

EC Augmentation of T Cell IL-2 Production Requires Cell-Cell Contact. To investigate whether cell contact is necessary for augmentation and to determine whether a short-lived soluble signal was missed in medium transfer experiments, cells were grown either in contact with EC, separated from EC by a Transwell membrane, or in the absence of EC, either above or below the membrane. These experiments establish that cell contact is required. Only when PBMC are in direct contact with the EC is there significant IL-2 production (Fig. 3). As evidence that the lack of augmentation was not due to the inability of a soluble signal to cross the membrane, EC IL-6 production is induced by PHA-activated PBMC to comparable levels whether the cells were in contact or separated. This experiment also confirms that the presence of IL-6 in high concentration is not sufficient to induce augmentation.

To investigate further the nature of the interactions between EC and T cells involved in this phenomenon, various mAbs were tested for their ability to inhibit aug-
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TABLE I

| Exp. | Additions                        | IL-2* (U/ml) | Stimulation index
|------|----------------------------------|--------------|-------------------|
| 1    | No additions                     | 1.5          | 1.0               |
|      | 10⁴ EC                           | 9.5          | 6.4               |
|      | IL-1β (10 U/ml)                  | 1.4          | 0.9               |
|      | IL-6 (400 ng/ml)                 | 2.4          | 1.6               |
|      | (2,000 ng/ml)                    | 3.1          | 2.0               |
| 2    | No additions                     | 3.9          | 1.0               |
|      | 10⁴ EC                           | 11.2         | 2.9               |
|      | 25% EC-conditioned medium        |              |                   |
|      | Untreated EC                     | 3.2          | 0.8               |
|      | + 100 U/ml IFN-γ                 | 5.4          | 1.4               |
|      | + 200 U/ml TNF                   | 3.5          | 0.9               |
|      | + 100/200 U/ml IFN-γ/TNF         | 4.6          | 1.2               |
| 3    | No additions                     | 3.5          | 1.0               |
|      | 10⁴ EC                           | 10.0         | 2.9               |
|      | 25% EC-conditioned medium        |              |                   |
|      | Untreated EC                     | 3.5          | 1.0               |
|      | + 10⁵ PBMC                       | 5.0          | 1.4               |
|      | + 100 U/ml IL-1β                 | 2.7          | 0.8               |
|      | + 1,000 U/ml TNF                 | 2.8          | 0.8               |

* IL-2 concentrations were measured by HT-2 bioassay as described in Materials and Methods.

Stimulation index is defined as: U/ml IL-2 produced in presence of EC per U/ml IL-2 produced in absence of EC.

§ Each experiment was repeated at least twice with similar findings.

mentation. Antibodies that block interaction between the CD2/LFA-3 ligand pair markedly reduce the degree of augmentation seen: in some experiments by >80% (Fig. 4). In contrast, antibodies blocking interaction between the LFA-1/ICAM-1 ligand pair have little or no effect on IL-2 production in response to PHA (Fig. 4). To confirm that these antibodies are blocking a direct interaction between the EC

FIGURE 3. EC augmentation of PBMC IL-2 synthesis requires cell contact. PBMC were cultured for 2 d, either with EC, in contact or separated by a Transwell membrane, or without EC, in a similar geometry. All wells contained PHA (10 μg/ml). Supernatants were collected and assayed for IL-2 or IL-6 by HT-2 or B9 bioassay, respectively. No IL-2 was detectable in the absence of PHA. EC alone produced 6 ng/ml IL-6. Shown is one of three similar experiments.
and the IL-2-producing cells (mostly CD4+), and not an interaction involving an intermediary cell, the experiments were repeated using purified populations of CD4+ cells. FACS analysis showed these populations to be effectively free of monocytes, NK cells, and CD8+ cells (data not shown). As also shown in Fig. 4, EC-augmented IL-2 production by purified CD4+ T cells can be inhibited by antibodies to CD2 or LFA-3 but not by antibodies to LFA-1 or ICAM-1. Antibodies to CD4 and MHC class II also do not inhibit augmented IL-2 synthesis, consistent with a system in which PHA stimulates the TCR-CD3 complex and EC provide accessory signals. The failure of antibodies to block augmentation in these cultures cannot be explained by their degradation or clearance from the medium as supernatants collected at the end of the culture still contained saturating concentrations of antibody as measured by indirect immunofluorescence and FACS analysis on fresh PBMC (data not shown).

**EC-augmented T Cell Responses to OKT3 Involves CD2/LFA3 Interaction.** The costimulation assay using supraoptimal concentrations of PHA was developed to analyze costimulation under conditions of uniform (i.e., maximal) T cell proliferation. However, it is possible that the signals provided by PHA or other mitogenic lectins are different
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from more specific stimuli delivered to the T cell through the TCR-CD3 complex. We therefore investigated the response of PBMC to an anti-CD3 antibody in the presence or absence of EC. Only PBMC were examined in these experiments because purified CD4+ T cells do not respond to soluble OKT3 antibody in the absence of Fc receptor-bearing cells (data not shown). At all concentrations of OKT3 tested, EC conferred a proliferative advantage on the T cells (Table II), leading to an approximately threefold increase in [3H]thymidine incorporation in the presence of EC. This proliferative advantage could be abrogated to a large extent by antibodies to LFA-3 and CD2 (Table III). IL-2 could not routinely be detected in the supernatants of these cultures, presumably because it is consumed as rapidly as it is produced. Therefore, to avoid differences in T cell proliferative rates and to prevent IL-2 utilization, we added mAb to the p55 subunit of the IL-2-R (anti-Tac) to our PBMC and PBMC + EC cultures. In the presence of anti-Tac antibody, augmented levels of IL-2 were detectable in cultures containing EC, and these could be inhibited to >80% by antibodies to LFA-3 and CD2 (Table IV). In addition, experiments with Transwells established that there is also a requirement for cell contact in EC-mediated augmentation of IL-2 levels when OKT3 is used as the primary stimulus (Table V).

Thus, the same phenomenon of EC-mediated IL-2 augmentation may be observed in response to the more specific but weaker stimulus provided by OKT3 as it was

### Table II

| Exp. | Additions | Response on day 2 |
|------|-----------|-------------------|
|      |           | + EC | - EC |
|      | ng/ml     | cpm x 10^3 |     |
| 1    | Control*  | 4.7 ± 0.6 | 0.5 ± 0.1 |
|      | Anti-CD3  | 14.8 ± 0.4 | 2.4 ± 0.2 |
|      | (0.05)    | 41.4 ± 6.5 | 13.0 ± 1.4 |
|      | (0.5)     | 59.6 ± 4.3 | 16.6 ± 1.5 |
|      | (5.0)     | 41.3 ± 3.1 | 13.5 ± 0.6 |
|      | (50.0)    | 44.9 ± 2.1 | 13.5 ± 1.1 |
|      | (500.0)   | 55.4 ± 0.3 | 0.7 ± 0.1 |
| 2    | Control   | 5.5 ± 0.3 | 0.7 ± 0.1 |
|      | Anti-CD3  | 17.0 ± 1.1 | 2.1 ± 0.1 |
|      | (0.05)    | 49.7 ± 1.4 | 20.5 ± 1.3 |
|      | (0.5)     | 77.0 ± 2.6 | 24.4 ± 1.5 |
|      | (50.0)    | 77.0 ± 2.6 | 24.4 ± 1.5 |
|      | (500.0)   | 58.4 ± 4.5 | 31.7 ± 2.0 |

* Control antibody was K16/16 (1.250 ng/ml).

1 Proliferation was measured by [3H]thymidine incorporation (1 μCi/well, 4 h). The data are expressed as the mean of triplicate determinations ± SEM. Incorporation by EC alone was 6.1 ± 10^3 ± 0.2 cpm (Exp. 1) and 3.7 x 10^3 ± 0.4 cpm (Exp. 2). With PHA (10 μg/ml) as the stimulus, cells incorporated 94.1 x 10^3 ± 5.6 cpm and 88.3 x 10^3 ± 5.7 cpm with and without EC (Exp. 1) and 125.8 x 10^3 ± 0.7 cpm and 109.3 x 10^3 ± 0.6 cpm with and without EC (Exp. 2).
TABLE III

**Effect of Antibodies to LFA-3 and CD2 on PBMC Proliferation to Anti-CD3 Antibody in the Presence of EC**

| Exp. | mAb added | Response on day 2 (cpm × 10⁻³) | Percent inhibition of augmented response |
|------|-----------|-------------------------------|----------------------------------------|
| 1    | - EC      | Control: 21.6 ± 1.4             | -                                      |
|      | + EC      | Control: 82.7 ± 5.7             | -                                      |
|      |           | Anti-LFA-3: 51.5 ± 4.5          | 51                                     |
|      |           | Anti-CD2: 46.6 ± 4.5            | 59                                     |
| 2    | - EC      | Control: 31.8 ± 1.9             | -                                      |
|      | + EC      | Control: 96.1 ± 3.7             | -                                      |
|      |           | Anti-LFA-3: 58.6 ± 1.2          | 60                                     |
|      |           | Anti-CD2: 83.0 ± 2.8            | 23                                     |

All wells contained OKT3 at 25 ng/ml.
* Control antibody was K16/16 (60 µg/ml). TS 2/9 (anti-LFA-3) and TS 2/18 (anti-CD2) were used at a saturating concentration of 60 µg/ml.

in response to the powerful mitogen PHA. In addition, these experiments demonstrate that EC provide a proliferative advantage to OKT3-stimulated T cells that is inhibitable by antibodies to LFA-3 and CD2.

**CD4⁺ T Cell Proliferation to Allogeneic EC Involves CD2/LFA-3 Interaction.** To investigate further the role of EC in T cell activation, we established cultures in which purified alloreactive CD4⁺ T cells proliferate in response to IFN-γ-treated EC. As has been previously reported, we found that EC stimulate proliferation of allospecific T cells whereas fibroblasts do not. Moreover, IFN-γ pretreatment of EC to induce expression of class II MHC molecules was necessary in order to see a proliferative response. Our data indicate that this proliferative response can be significantly inhibited by antibodies to LFA-3 and CD2 (Table VI), providing evidence of a role for this ligand pair in EC-supported T cell alloproliferation.

TABLE IV

**Effect of Antibodies to LFA-3 and CD2 on PBMC IL-2 Production in Response to Anti-CD3 Antibody**

| mAb added | Exp. 1 | Exp. 2 |
|-----------|--------|--------|
|           | IL-2 U/ml | % inhibition of augmented response | IL-2 U/ml | % inhibition of augmented response |
| - EC      | 1.2     | -      | 0.4     | -        |
| + EC      | 2.7     | -      | 0.8     | -        |
| Anti-LFA-3| 1.4     | 87     | 0.6     | 50       |
| Anti-CD2  | 1.4     | 87     | 0.6     | 50       |

All wells contained OKT3 (25 ng/ml) and anti-Tac (1:1,000 of ascites). No IL-2 was detectable in the absence of anti-Tac antibody.

* Cells were incubated for 24 h and supernatants assayed for IL-2 by HT-2 bioassay.

1 Control antibody was K16/16 (60 µg/ml). TS 2/9 (anti-LFA-3) and TS 2/18 (anti-CD2) were used at 60 µg/ml.
**Table V**

*Production of IL-2 from OKT3-stimulated PBMC in Contact with or Separated from EC*

|                      | Exp. 1 | Exp. 2 |
|----------------------|--------|--------|
|                      | IL-2 U/ml | IL-2 U/ml |
|                      | Stimulation index | Stimulation index |
| PBMC/EC contact      |         |         |
| + EC                 | 6.7     | 1.8     |
| - EC                 | 1.1     | 0.4     |
| No PBMC/EC contact   |         |         |
| + EC                 | 1.1     | 0.6     |
| - EC                 | 1.4     | 0.8     |

PBMC (3 x 10⁵) were cultured either in or below Transwells, in the presence or absence of EC cultured below the Transwell, and stimulated with OKT3 (25 ng/ml). All wells contained anti-Tac antibody (1:1,000).

* Supernatants were collected at 24 h and assayed for IL-2 using HT-2 cells.

**Discussion**

In this report, we have examined the mechanism by which EC cause PHA-stimulated PBMC or CD4⁺ T cells to greatly increase the amounts of IL-2 secreted into the medium. Quantitation of mRNA indicates that augmentation is mediated at the level of IL-2 synthesis. IL-1 and IL-6 can both modulate T cell responses and proliferation in vitro, and both are produced by EC (29-32). Neither cytokine, even at supraoptimal concentrations, was able to mimic the ability of EC to augment IL-2 production. Furthermore, IFN-γ, TNF, IL-1, and resting PBMC all failed to induce a soluble signal from EC that could mediate the effect. Coculture of EC separated from PBMC by a permeable membrane similarly failed to produce augmented IL-2 synthesis. We conclude that EC transmit a signal only via a contact-dependent mechanism, although an extremely short-lived soluble signal cannot be completely ruled out. Antibody-blocking experiments demonstrated involvement of the receptor-ligand pair CD2/LFA-3 in this interaction. In contrast, antibodies to ICAM-1 or LFA-1, which can inhibit other lymphocyte functions requiring cell contact, had little or no effect. When the more specific signal OKT3 was used as the primary stimulus, our findings were comparable with those with PHA. That is, EC augmented IL-2 production in a contact-dependent manner involving a CD2/LFA-3 interaction. The CD2/LFA-3 pathway is also used to provide a proliferative advantage to T cells stimulated with OKT3 in the presence of EC, and may function similarly in the recognition of allogeneic EC by resting CD4⁺ T cells.

Although we have used allogeneic EC as T cell stimulators in the PHA- and OKT3-stimulated cultures (a restriction imposed by the use of neonatal EC), we do not believe that recognition of EC alloantigens is participating in the increased IL-2 production. First, as we have previously shown, only allogeneic EC and not allogeneic fibroblasts or monocytes increase IL-2 production from PBMC (22). Second, we only see alloreponses (in the absence of mitogen) after 4-7 d in culture, whereas the PHA and OKT3 experiments reported here were run for 1-2 d. Finally, when EC from pooled donors were compared in the same experiment with two lines of EC from...
TABLE VI
Effect of Antibodies to LFA-3 and CD2 on Proliferation of
CD4+ T Cells to Allogeneic EC

| Exp. | mAb added | Response at day 6 | Percent inhibition |
|------|-----------|-------------------|--------------------|
|      |           | cpm x 10^3        |                    |
| 1    | + EC      | Control* 23.7 ± 2.4 | -                  |
|      |           | Anti-LFA-3 2.7 ± 0.1 | 88                 |
|      |           | Anti-CD2 3.9 ± 2.0 | 84                 |
| 2    | + EC      | Control 48.3 ± 5.9 | -                  |
|      |           | Anti-LFA-3 23.2 ± 3.2 | 52                 |
|      |           | Anti-CD2 9.7 ± 1.2 | 80                 |

CD4+ T cells (3 x 10^5/well) were cultured with allogeneic EC (IFN-γ pretreated, 1,000 U/ml, 3 d) or alone. EC proliferation was inhibited by treatment with mitomycin C (10 μg/ml for 1 h) immediately before addition of T cells. [3H]Thymidine (1 μCi/well) was included for the final 18 h of culture. Proliferation of T cells alone was 0.5 x 10^3 ± 0.1 cpm (Exp. 1) and 2.1 x 10^3 ± 0.9 cpm (Exp. 2). No proliferation was seen on EC not pretreated with IFN-γ.

* Control antibody was K16/16 (60 μg/ml). TS 2/9 (anti-LFA-3) and TS 2/18 (anti-CD2) were used at 60 μg/ml.

single donors using the same allogeneic PBMC, an equivalent degree of augmentation and absolute level of IL-2 production were found (data not shown). Thus, the degree of alloantigenic difference between the lymphocytes and EC is not important in cultures stimulated by PHA or OKT3.

The requirement for contact between EC and T cells for delivery of an activation signal is reminiscent of the finding of Inaba et al. (33) that dendritic cells require contact to stimulate T cell proliferation, an effect that was similarly LFA-1 independent. Our antibody-blocking experiments suggest an important role for CD2/LFA-3 interaction. We do not know if the observed augmentation is being mediated by direct signaling through CD2. Fibroblasts express comparable levels of LFA-3 (unpublished results) and yet fail to induce augmented IL-2. However, novel forms of LFA-3, possibly involving differences in glycosylation, or a second CD2 ligand present only on EC, may represent alternative explanations for the different capabilities of these two cell types. An additional possibility is that strengthening of cell contact via these molecules may allow signaling through other surface structures (34). However, use of the CD2/LFA-3 pathway simply to strengthen adhesion seems unlikely in the cultures stimulated by PHA because the LFA-1/ICAM-1 adhesive interaction is apparently unimportant. Furthermore, PHA is a multivalent ligand that can directly crosslink T cells to EC, probably bypassing the need for adhesion molecules. Under more physiological conditions, especially in vivo where T cell interactions may occur in the vascular lumen in the presence of disruptive shear forces, adhesive ligands are likely to be much more important. Indeed, preliminary observations suggest that even in our in vitro experiments involving OKT3 or alloantigen stimulation, antibody to LFA-1 is partly inhibitory.

The CD2/LFA-3 interaction may not be the only signaling pathway utilized in
EC accessory functions. Geppert and Lipsky (19) have demonstrated that either EC or anti-CD28 antibody (9.3) can provide the necessary signal to allow fibroblasts to present antigen. It has also been shown that 9.3 stabilizes mRNAs of several cytokines in activated T cells, including the mRNA for IL-2 (35). Our results are consistent with the possibility of EC signaling via CD28, although preliminary experiments suggest that the effect of 9.3 is more than additive with that of EC. A recent report (11) presents evidence for CD44 delivering activation signals to T cells. This is particularly interesting as CD44 mediates adhesion of T cells to high endothelial venules and may deliver an early priming signal to emigrating cells. Finally, the cell contact may involve delivery of signals via pathways other than through membrane proteins; we have shown previously that EC actually appear to form gap junctions with clustered T cells (36).

The observation that in vitro, EC share with dendritic cells the ability to activate resting T cells, suggest the possibility that in vivo, there is more than one pathway of initiating immune reactions depending on which accessory cell presents the antigen. Dendritic cells, resident in epithelia, are believed to pick up environmental antigens and carry them, via draining lymphatics, to lymph nodes where the antigens are presented to antigen-specific T cells (37). A systemic immune reaction may ensue. In contrast, we propose that EC pick up foreign antigens in tissues and present them on their luminal surface to circulating T cells, leading to immune reactions localized to the site of antigenic challenge. In this context, T cell activation in the presence of suboptimal concentrations of antigen may depend upon the contact-dependent EC-mediated augmentation of IL-2 synthesis we have described in this report. In addition, increased IL-2 production by T cells may have a regulatory effect on the production of various other cytokines involved in cell-mediated immune reactions. For example, superinduction of IL-2 by PMA increases transcription and release of IFN-γ (12), a cytokine that can convert an ineffective immune reaction to a successful one in the skin of leprosy patients (38). Finally, if our hypothesis is correct that EC augmentation of T cell IL-2 synthesis underlies initiation of cell-mediated immunity at peripheral sites, then use of antibodies or drugs that disrupt CD2/LFA-3 interaction may be of therapeutic benefit in ameliorating local hypersensitivity reactions or acute allograft rejection.

Summary
We have demonstrated that endothelial cells (EC) augment IL-2 production by PHA-stimulated PBMC or purified CD4+ T cells and that the increase is apparent both in the amount of soluble IL-2 secreted and in the level of specific mRNA detectable by Northern blot hybridization. The ability of EC to affect levels of IL-2 cannot be reproduced by soluble factors, including the cytokines IL-1, IL-6, IFN-γ, or TNF, conditioned medium from resting EC or IL-1, IFN-γ- or TNF-treated EC, or from resting PBMC + EC cultures. Separation of the EC and PBMC by a Transwell membrane demonstrated that cell contact was required for augmentation of IL-2 synthesis and that this effect was unlikely to be mediated by a short-lived soluble signal. The cell-cell interaction required the ligand pair CD2/LFA-3, since augmentation could be inhibited by antibodies to these structures. Antibodies to ICAM-1, LFA-1, CD4, and MHC class II were without effect. A contact-dependent pathway involving CD2/LFA-3 interactions also may be used by EC to augment IL-2 production.
from T cells stimulated more specifically through the TCR/CD3 complex with antibody OKT3. This pathway provides a proliferative advantage to T cells stimulated with OKT3 in the presence of EC and may also be involved in the proliferative response of resting T cells to allogeneic class II MHC-expressing EC. We propose that EC augmentation of T cell IL-2 synthesis may be critical in the ability of EC to elicit primary T cell antigen responses and may have consequences for the development of localized cell-mediated immune reactions.

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