# Human endothelial cell-lymphocyte interaction. Endothelial cells function as accessory cells necessary for mitogen-induced human T lymphocyte activation in vitro.

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**Research Article**

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# Human Endothelial Cell-Lymphocyte Interaction

## ENDOTHELIAL CELLS FUNCTION AS ACCESSORY CELLS NECESSARY FOR MITOGEN-INDUCED HUMAN T LYMPHOCYTE ACTIVATION IN VITRO

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ABSTRACT Mitogen-stimulated human T cell activation is absolutely dependent on the participation of a nonresponding accessory cell. In populations of human peripheral blood mononuclear cells, monocytes function as the requisite accessory cells. The possibility that cultured endothelial cells (EC) might also function as accessory cells was studied by examining the potential of endothelial cells to restore mitogen responsiveness to monocyte-depleted human T cells. Highly purified T cells were prepared by isolating cells rosetting with sheep erythrocytes and removing monocyte contamination by glass adherence and nylon wool column passage. When cultured at low cell density, T cells failed to respond to stimulation with various mitogenic lectins, whereas co-culture with monocytes restored responsiveness. Similarly, EC obtained from umbilical vein, pulmonary artery, and ovarian vein restored the capacity of T cells to respond to mitogens. Mitogen-stimulated T cell activation required viable endothelial cells. Moreover, effective endothelial T cell cooperation appeared to involve the establishment of cell-to-cell contact between EC and responding T cells. Accessory cell function was not a nonspecific property of all tissue culture cells as evidenced by the finding that human foreskin fibroblasts, lung fibroblasts, and HeLa cells were unable to restore responsiveness to monocyte-depleted T cells. These observations indicate that endothelial cells can support the induction of mitogen-induced T cell activation and suggest that cells lining blood vessels may play an active role in the initiation of immune responses in vivo.

## INTRODUCTION

Antigen- and mitogen-induced T lymphocyte activation is absolutely dependent on the participation of an accessory cell. In many responses, mononuclear phagocytes (M$\phi$)[1] serve as the requisite accessory cells (1). Only a subpopulation of M$\phi$ is able to function in this capacity. Thus, some but not all M$\phi$ synthesize and express Ia antigens on their surface membranes. The presence of these glycoproteins, which are encoded by genes of the major histocompatibility complex, identifies those M$\phi$ able to function as antigen presenting cells or as accessory cells required for mitogen-induced T cell activation (2, 3). Recent evidence indicates that other Ia-bearing cells such as Langerhans cells of the skin (4) and dendritic cells of various lymphoid organs (5, 6) may also function as accessory cells.

A number of observations suggest the possibility that endothelial cells (EC) may also function as accessory cells required for T cell activation. Thus, freshly isolated EC and endothelium *in situ* express Ia antigens (7–9) and cultured EC appear to be capable of stimulating mixed leukocyte reactions in vitro (10, 11). The idea that EC can function as accessory cells is supported by histological examinations of delayed type hypersensitivity reactions. These frequently reveal activated lymphocytes, some of which are in mitosis, attached to vascular endothelium within the lumen of the venule (12). Such observations suggest that initial lymphocyte activation may occur intravascularly, supported by the vascular endothelium.

The following studies were undertaken to examine the potential of EC to function as accessory cells necessary for T cell activation. Mitogen-induced human T cell DNA synthesis was used as an in vitro model system to determine whether EC could provide a sufficient

[1] *Abbreviations used in this paper:* EC, endothelial cells; EC$_{OV}$, ovarian vein EC; EC$_{PA}$, pulmonary artery EC; EC$_{UV}$, umbilical vein EC; FBS, fetal bovine serum; HFF, human foreskin fibroblasts; M$\phi$, mononuclear phagocytes; NG, neuraminidase and galactose oxidase; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

accessory cell signal to allow T cell triggering. The data indicate that EC are able to function as accessory cells and support the idea that cells lining blood vessels may play an active role in the induction of immune responses in vivo.

## METHODS

### Culture medium

Except when noted, cell cultures were carried out in medium RPMI 1640 (Microbiological Associates, Walkersville, Md.), supplemented with 10% fetal bovine serum (FBS, Microbiological Associates), penicillin G (200 U/ml), gentamicin (10 $\mu$g/ml), and L-glutamine (0.3 mg/ml).

### Cell preparation

*Peripheral blood mononuclear cells (PBM).* PBM were prepared from normal adult volunteers by centrifugation of heparinized venous blood on sodium diatrizoate/Ficoll cushions (Isolymph, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) as previously described (13).

*Monocytes (M$\phi$).* Two techniques were used to prepare M$\phi$-enriched populations. The first method was based on the capacity of M$\phi$ to adhere to glass surfaces. PBM were suspended in medium and 20-ml aliquots containing $2 \times 10^6$ cells/ml were incubated on glass petri dishes (100 mm Diam) for 1 h at 37°C (13). Loosely adherent cells were removed by washing the petri dishes with warm Hanks' balanced salt solution. The dishes were then flooded with phosphate-buffered saline (pH 7.2) and incubated for 1 h at 4°C, after which the adherent cells were dislodged with a rubber policeman. This adherent cell population contained >85% monocytes.

In some experiments, M$\phi$ were prepared from PBM by centrifugation on discontinuous gradients of polyvinylpyrrolidone-coated colloidal silica particles (Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden) (14, 15). PBM were aliquoted into 15-ml centrifuge tubes, each holding 20–30 million cells, pelleted and overlaid with a discontinuous (100, 50, 40%) Percoll gradient, The gradients were centrifuged at 400 $g$ for 20 min at room temperature after which the M$\phi$-enriched population (>90% M$\phi$) was harvested from the 40–50% interface. M$\phi$ prepared by either technique had similar functional activities and, therefore, data obtained using M$\phi$ obtained from both methods have been combined.

*T lymphocytes.* T cells were prepared from PBM as previously described (16). First, adherent cells were removed by two sequential incubations on glass petri dishes. A T cell-enriched population was then prepared from the nonadherent cells by rosetting with neuraminidase-treated sheep erythrocytes. Following this, the rosette-forming cells were separated by centrifugation on diatrizoate/Ficoll cushions. The pelleted cells were treated with NH$_4$Cl to lyse the sheep erythrocytes and then passed over a nylon wool column. The T cell population eluted from the column contained <0.1% M$\phi$ and no detectable surface immunoglobulin positive cells.

*Endothelial cells.* EC were prepared from umbilical cord veins, pulmonary arteries and ovarian arteries, and veins as previously described in detail (17, 18). Briefly, umbilical veins were filled with 0.25% collagenase (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), clamped and incubated for 15 min at 37°C. Vessels from cadaver lungs (3–5 h postmortem) were dissected free of connective tissues and placed on moist gauze in sterile dishes. The vessel lumen was filled with collagenase (0.25%) and the vessel was covered with moist gauze. The vessels from ovarian tissues were dis-

sected from the ovarian ligament and treated with collagenase in a similar manner. After incubation for 15–30 min at 37°C, detached cells were washed from the vessels with culture medium containing 10% FBS.

The suspended cells were washed twice, resuspended in fresh medium 199, supplemented with 20% FBS, 10% human serum, and antibiotics (penicillin G, streptomycin, and fungizone) and cultured in 25-cm tissue culture flasks or petri dishes. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. A confluent monolayer of cells was obtained within 1–2 wk. When confluent, the cells were detached using Puck's EDTA and 0.25% trypsin (Gibco Laboratories) and then transferred to fresh culture vessels. The cells were used for experiments after two to seven passages in culture.

Each culture of EC used in these experiments was examined for two endothelial markers, angiotensin I converting enzyme and Factor VIII antigen (18). All of the cultures had angiotensin I converting enzyme activity as determined by the hydrolysis of [$^3$H]hippuryl glycylglycine and Factor VIII antigen was detected in all cells by indirect immunofluorescence. The EC populations contained no M$\phi$ as judged morphologically or by examining for Fc receptor-mediated phagocytosis using IgG-opsonized sheep erythrocytes as test particles.

*Fibroblasts.* Fibroblasts from human foreskin and embryonic lung were cultured in medium 199 supplemented with 10% FBS and antibiotics. Cells from foreskin were initiated from 1-mm$^3$ blocks of tissue. The tissue explants were maintained undisturbed until cell outgrowth began (~21 d). At that time, the tissue blocks were discarded and the cells were allowed to replicate to confluency in small flasks. Lung fibroblasts, IMR 90, were obtained from the Institute of Medical Research (Camden, N. J.). All fibroblast cultures were used between the 3rd and 15th passage.

*HeLa cells.* HeLa cells were obtained from American Type Culture Collection (Rockville, Md.) and cultured in Dulbecco's medium (Gibco Laboratories), supplemented with 10% FBS.

### Treatment of cells

*Preparation of single cell suspensions.* EC, fibroblasts, and HeLa cells were detached from the culture vessels with Puck's EDTA and 0.25% trypsin (Gibco Laboratories) for use in experiments. The cells were then washed extensively and suspended in medium RPMI 1640 supplemented with 10% FBS.

*Mitomycin C treatment.* Before use, all potential accessory cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) to prevent replication. The cells were incubated with 40 $\mu$g/ml mitomycin C for 30 min at 37°C, washed extensively, and suspended in fresh medium RPMI 1640 supplemented with 10% FBS for culture.

*Chemical modification of cells.* For the generation of cell surface aldehydes, endothelial cells, M$\phi$ or T cells were treated with neuraminidase and galactose oxidase (NG) as previously described (19). Briefly, T cells or mitomycin C-treated M$\phi$ or EC were suspended in medium and incubated with 50 U/ml Vibrio cholera neuraminidase (Gibco Laboratories) and 0.5 U/ml galactose oxidase (Sigma Chemical Co.) for 30 min at 37°C. Control cells were incubated in a similar manner in medium alone. The cells were then washed twice before culture.

### Cultures for analysis of mitogen responsiveness

*Tritiated thymidine incorporation.* T cells were cultured alone or with mitomycin C-treated accessory cells in micro-

titer plates with flat-bottomed wells (Microtest II 3040, Falcon Labware Div., Becton, Dickinson & Co., Oxnard, Calif.). Phytohemagglutinin (PHA, 0.5 μg/ml, Wellcome Research Laboratories, Beckenham, England), concanavalin A (Con A, 5 μg/ml, Pharmacia Fine Chemicals), pokeweed mitogen (PWM, 10 μg/ml, Gibco Laboratories), or an equivalent volume of saline as control was added. Alternatively, control or NG-modified Mϕ or EC were co-cultured with control or NG-modified T cells. Each experimental point was carried out in triplicate. The cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. 1 μCi of tritiated thymidine (6.7 Ci/mM, New England Nuclear, Boston, Mass.) was added to each well 8 h before harvesting. After a total incubation of 96 h, the cells were harvested onto glass fiber filter paper with a multiple sample automated harvester (Mash II, Microbiological Associates). Tritiated thymidine incorporation was determined by liquid scintillation spectroscopy. All data are expressed as mean total counts per minute of triplicate culture wells or as the difference in counts per minute between the means of triplicate mitogen-stimulated and control cultures (Δcpm).

*Analysis of cellular DNA content.* Cultures were set up in 17 × 100-mm polypropylene tubes with each tube containing a total of 2 ml of cell suspension. T cells (1 × 10⁵) were cultured alone or supplemented with 1 × 10⁵ mitomycin C-treated Mϕ or other potential accessory cell population. PHA (0.5 μg/ml) or an equal volume of saline was added to each tube. Each experimental point was obtained from 10 replicate tubes. The cultures were incubated for 48 h in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C. After incubation, the cells were pooled, washed, and stained for 20 min at 20°C in a solution containing mithramycin (Mithracin, 100 μg/ml, Dohme Division, Miles Laboratories, Inc., West Haven, Conn.), 25% ethanol, and 15 mM $MgCl_2$ (20). The samples were then analyzed with a fluorescence activated cell sorter (FACS III, Becton, Dickinson & Co., Mountain View, Calif.). In this assay, the fluorescence intensity of each cell is directly related to the cellular DNA content.

## RESULTS

*Accessory cell dependence of mitogen-induced T cell activation.* A number of studies have shown that mitogen-induced T cell activation as assayed by [³H]thymidine incorporation is dependent on the active participation of nonlymphoid accessory cells. In human PBM, Mϕ subserve this function (16, 21). To examine the potential accessory function of other cell types, it was necessary, therefore, to prepare a population of responding T cells that was free of Mϕ contamination. The approach used involved purifying T cells by rosetting with neuraminidase-treated sheep erythrocytes and depleting contaminating Mϕ by glass adherence and passage over nylon wool columns. This method yielded a population of responding cells that contained <0.1% Mϕ. Despite this minimal Mϕ contamination, mitogen responsiveness was still observed when this population was cultured at high cell density. Although this was most marked for PHA stimulation, a small response to Con A and PWM was also usually observed.

A modified limiting dilution technique was used to eliminate the residual mitogen responsiveness supported by the small number of Mϕ in the cultures. As can be seen in Fig. 1, mitogen-induced [³H]thymidine incorporation was not observed in low density cultures (<2.5 × 10⁴ T cells/well). The reducing agent, 2-mercaptoethanol, which can partially substitute for the accessory function of Mϕ in some in vitro lymphocyte responses (22) or augment the function of small numbers of Mϕ (23), did not augment mitogen responsive-
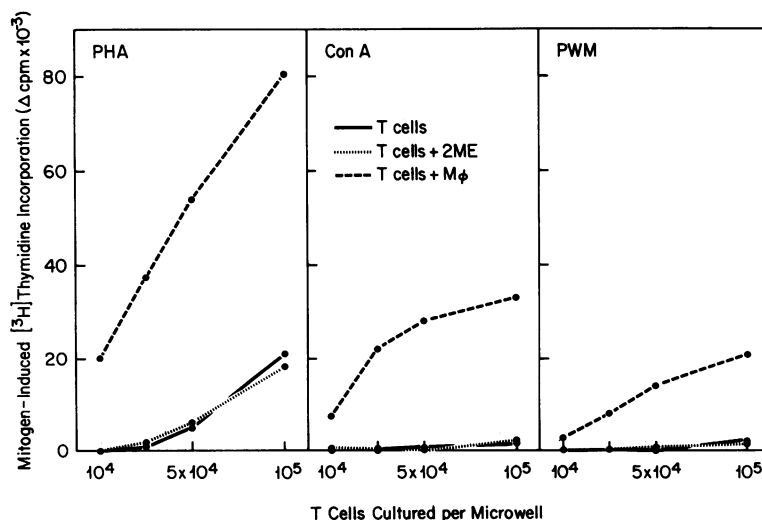


FIGURE 1 Accessory cell dependence of mitogen-induced human T cell activation. Varying numbers of T cells were cultured in the wells of flat-bottomed microtiter plates with PHA (0.5 μg/ml), Con A (5 μg/ml), PWM (10 μg/ml), or Hanks' balanced salt solution as control. T cells were incubated alone or supplemented with 2-mercaptoethanol (2-ME) (50 μM) or 1 × 10⁴ mitomycin C-treated Mϕ per well. [³H]thymidine incorporation was determined after a 96-h incubation.

ness in these low density cultures. Moreover, Mφ supernates that contained interleukin I activity (24) were also unable to restore mitogen responsiveness to these Mφ-depleted T cells (data not shown). It should be noted that Mφ could not be detected in these cultures by direct observation using inverted phase contrast microscopy either initially or after the 96-h incubation period. Supplementation of low density cultures with Mφ routinely restored mitogen responsiveness as shown in Fig. 1. The magnitude of mitogen-induced T cell [3H]thymidine incorporation was directly related to the number of supplemental Mφ (Fig. 2). These experiments confirmed that mitogen-triggered human T cell activation depends on the participation of accessory cells. In addition, they established conditions for examination of the potential of various populations to function as accessory cells in T cell cultures free of Mφ contamination. In all subsequent cultures, T cells were therefore cultured at a density of $1 \times 10^4 \rightarrow 2.5 \times 10^4$ per flat-bottomed microwell.

*Umbilical vein endothelial cells support PHA-induced T cell activation.* The capacity of endothelial cells to function as accessory cells was examined in a similar manner. Mitomycin C-treated umbilical vein endothelial cells ($EC_{UV}$) were compared to Mφ with regard to their capacity to restore mitogen responsiveness to T cells. When cultured alone, T cells did not respond to PHA, Con A, or PWM, whereas co-culture with Mφ restored responsiveness (Table I). Umbilical vein endothelial cells were also able to restore mitogen responsiveness to purified T cells. These results suggest that Mφ may not be unique in their capacity to function as accessory cells but that other nonlymphoid cells, such as endothelial cells, may be equally effective in this regard. When T cells were supplemented with both Mφ and $EC_{UV}$, mitogen responsiveness was greater than that seen when cultures were supplemented with either accessory cell alone, suggesting that there may be a synergistic interaction between the two accessory cell populations.

*Accessory function of endothelial cells obtained from adult tissues.* To determine whether accessory function was a general property of endothelial cells, EC from pulmonary artery and ovarian vein were examined. Both EC populations restored PHA-induced [3H]thymidine incorporation to Mφ-depleted T cell cultures as shown in Table II. These EC populations also supported Con A and PWM responsiveness as shown in Table III. In additional experiments, ovarian artery EC also supported mitogen-induced T cell responses (data not shown). These data indicate that arterial and venous EC obtained from both adult and neonatal tissues are able to function as accessory cells for the induction of T cell activation.
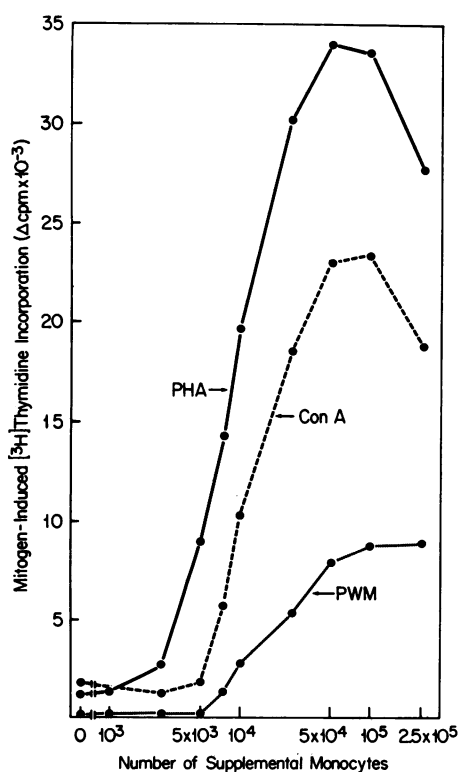


FIGURE 2 Accessory cell dependence of mitogen-stimulated human T cell activation. T cells ($1 \times 10^4$/well for PHA stimulation and $2.5 \times 10^4$/well for Con A and PWM stimulation) were cultured with varying numbers of mitomycin C-treated Mφ and [3H]thymidine incorporation determined after a 96-h incubation.

TABLE I
Accessory Cell Function of Umbilical Cord
Vein Endothelial Cells

| Accessory cell | Mitogen-induced T cell [3H]thymidine incorporation* | | |
|---|---|---|---|
| | PHA (n = 9) | Con A (n = 7) | PWM (n = 8) |
| | Δcpm × 10⁻³ | | |
| Nil | 0.3±0.1 | 0.4±0.1 | 0.6±0.2 |
| Mφ | 17.2±2.8 | 11.4±1.7 | 4.3±1.0 |
| $EC_{UV}$ | 19.6±3.2 | 7.2±1.7 | 6.2±1.4 |
| Mφ + $EC_{UV}$ | 26.4±3.7 | 14.5±3.5 | 7.6±1.8 |

* T cells were incubated at a density of $1 \times 10^4$ per well for PHA stimulation and $2.5 \times 10^4$ per well for Con A or PWM stimulation. These cultures were supplemented, where indicated, with either mitomycin C-treated Mφ or umbilical cord vein endothelial cells ($1 \times 10^4$/well), or a mixture of Mφ and $EC_{UV}$ ($5 \times 10^3$ of each per well). Data represent the mean±SEM of n experiments. [3H]thymidine incorporation in cultures containing either mitomycin C-treated Mφ or mitomycin C-treated $EC_{UV}$ alone was less than 300 cpm, irrespective of the presence of mitogen. [3H]thymidine incorporation in cultures containing T cells and mitomycin C-treated $EC_{UV}$ but no mitogens was 353±88 cpm (mean±SEM, n = 19).

## TABLE II
### Accessory Cell Function of Endothelial Cells

| Accessory cell | No. of experiments | PHA-induced [³H]thymidine incorporation ($\Delta$cpm $\times 10^{-3}$)* |
|---|---|---|
| Nil | 9 | $0.3\pm0.1$ |
| M$\phi$ | | $20.6\pm3.2$ |
| EC$_{PA}$ | | $17.3\pm4.1$ |
| Nil | 10 | $0.4\pm0.1$ |
| M$\phi$ | | $14.8\pm5.6$ |
| EC$_{OV}$ | | $11.1\pm2.9$ |

* T cells ($1 \times 10^4$/well) were cultured alone or supplemented with mitomycin C-treated M$\phi$ or EC ($1 \times 10^4$/well). Data represent the mean$\pm$SEM of the number of experiments indicated. [³H]thymidine incorporation in cultures containing T cells and EC$_{PA}$ with no mitogen was $324\pm105$ cpm and [³H]thymidine incorporation in those containing T cells and EC$_{OV}$ was $100\pm53$ cpm. [³H]thymidine incorporation by EC$_{PA}$ or EC$_{OV}$ cultured alone was <100 cpm, irrespective of mitogen.
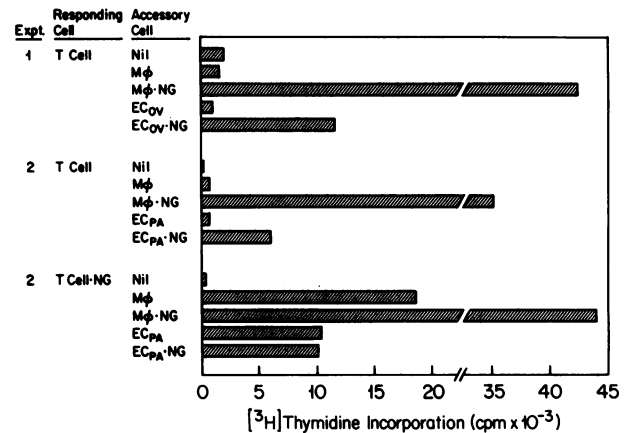


FIGURE 3   EC support NG stimulation of T cells. Control or NG-treated T cells were cultured with control or NG-treated M$\phi$ or EC and [³H]thymidine incorporation determined after a 96-h incubation. Each EC population had been maintained in culture for >4 wk and transferred three to four times before use.

*Accessory function of endothelial cells in T cell activation induced by NG.*   Treatment of T lymphocytes with the oxidative mitogen, NG, leads to accessory cell-dependent T cell blastogenesis (6, 19). Moreover, NG-treated M$\phi$ are capable of triggering activation of untreated T cells when the two populations are cocultured. In this system, stimulation is related to biochemical alteration of cell surface glycoproteins and does not require the continuous presence of mitogen. It was, therefore, of interest to determine whether EC could support responsiveness of NG-modified T cells and whether NG-modified EC could trigger responses in unmodified T cell populations. As shown in Fig. 3,

EC were able to accomplish both accessory cell functions, although not as efficiently as M$\phi$.

*Heat-killed M$\phi$ and EC do not support T cell activation.*   The accessory function of M$\phi$ requires viable and metabolically intact cells (21, 24). In order to determine whether the accessory function of EC similarly required living cells, M$\phi$ and pulmonary artery EC were incubated at 60°C for 45 min. Staining with fluorescein diacetate and ethidium bromide established that these cells had been completely killed. A typical experiment is shown in Table IV. Viable M$\phi$

## TABLE III
### Accessory Cell Function of Endothelial Cells

| Accessory cell | Mitogen-induced T cell [³H]thymidine incorporation* | |
|---|---|---|
| | Con A ($n = 4$) | PWM ($n = 3$) |
| | $\Delta$cpm $\times 10^{-3}$ | |
| Nil | $0.0\pm0.2$ | $0.0\pm0.1$ |
| M$\phi$ | $11.3\pm3.0$ | $5.3\pm1.6$ |
| EC$_{PA}$ | $10.2\pm3.1$ | $5.5\pm1.9$ |
| Nil | $0.0\pm0.2$ | $0.0\pm0.2$ |
| M$\phi$ | $11.7\pm2.2$ | $4.6\pm1.5$ |
| EC$_{OV}$ | $13.8\pm4.8$ | $2.8\pm0.6$ |

* T cells ($2.5 \times 10^4$/well) were cultured alone or supplemented with mitomycin C-treated M$\phi$ or EC ($1 \times 10^4$/well). Data represent the mean$\pm$SEM of the number of experiments indicated. [³H]thymidine incorporation in cultures containing T cells and EC but no mitogen was <300 cpm.

## TABLE IV
### Heat-killed Endothelial Cells Do Not Support Mitogen-induced T Cell Activation

| Accessory cells | | Mitogen-induced T cell [³H]thymidine incorporation* | |
|---|---|---|---|
| Untreated | Heat-killed | PHA | Con A |
| | | $\Delta$cpm $\times 10^{-3}$ | |
| — | — | 0.3 | 0 |
| M$\phi$ | — | 27.1 | 14.9 |
| EC$_{PA}$ | — | 10.1 | 4.5 |
| — | M$\phi$ | 3.3 | 2.0 |
| — | EC$_{PA}$ | 0.1 | 1.4 |
| M$\phi$ | EC$_{PA}$ | 21.3 | 11.4 |
| EC$_{PA}$ | M$\phi$ | 12.2 | 5.8 |
| M$\phi$ | M$\phi$ | 10.4 | 8.7 |
| EC$_{PA}$ | EC$_{PA}$ | 11.1 | 5.6 |

* T cells ($1 \times 10^4$/well) were cultured alone or supplemented with untreated or heat-killed M$\phi$ or EC$_{PA}$ ($1 \times 10^4$/well) or a mixture of M$\phi$ and EC ($5 \times 10^3$ of each population per well). Data represent the means of triplicate determinations.

or EC were able to support mitogen-induced T cell activation, but heat-killed cells were unable to carry out this accessory cell function. Viable accessory cells added to either heat-killed Mφ or EC restored responsiveness of T cells indicating that the heat-killed cells were not suppressive.

*Lack of accessory function of other tissue culture cells.* To determine whether the enhanced mitogen responsiveness observed in T cell cultures supported by EC resulted from a nonspecific "feeder-layer effect," a number of other tissue culture cells were tested. As shown in Table V, human foreskin fibroblasts (HFF) were unable to enhance mitogen-induced T cell [3H]-thymidine incorporation and in some experiments (experiment 3, Table V) suppressed responsiveness supported by Mφ. Human lung fibroblasts (IMR 90) were also found to be ineffective accessory cells (data not shown). HeLa cells also were unable to enhance mitogen responsiveness in Mφ-depleted T cell cultures but did not inhibit responses supported by Mφ (Table VI). These data indicate that accessory cell function is not shared by all tissue culture cells but rather appears to be unique for endothelial cells.

### TABLE V
*Fibroblasts Are Ineffective at Supporting Mitogen-induced T Cell Activation*

| Expt. | Accessory cell | Mitogen-induced T cell [3H]thymidine incorporation* | | |
|---|---|---|---|---|
| | | PHA | Con A | PWM |
| | | $\Delta cpm \times 10^{-3}$ | | |
| 1 | Nil | 0.4 | 0 | 0.4 |
| | Mφ | 29.3 | 9.7 | 1.7 |
| | HFF | 0.8 | 0.1 | 0 |
| | HFF + Mφ | 20.9 | 9.0 | 1.1 |
| 2 | Nil | 1.0 | 0.4 | 1.7 |
| | Mφ | 33.0 | 14.9 | 6.5 |
| | HFF | 1.7 | 1.9 | 0.3 |
| | HFF + Mφ | 20.0 | 12.9 | 2.8 |
| 3 | Nil | 0.8 | 0 | 0.1 |
| | Mφ | 53.8 | 33.2 | 28.7 |
| | HFF | 0.3 | 2.3 | 0.7 |
| | HFF + Mφ | 13.2 | 11.6 | 6.7 |

* T cells were incubated at a density of $1 \times 10^4$ per well for PHA stimulation and $2.5 \times 10^4$ per well for Con A or PWM stimulation. Cultures were supplemented, where indicated, with either mitomycin C-treated Mφ, mitomycin C-treated human foreskin fibroblasts ($1 \times 10^4$/well), or a mixture of Mφ and HFF ($5 \times 10^3$ of each per well). Data represent the means of triplicate determinations. [3H]thymidine incorporation in cultures of T cells and HFF with no added mitogen was <400 cpm. In cultures of HFF alone, [3H]thymidine incorporation was <40 cpm irrespective of the presence of mitogen.

### TABLE VI
*HeLa Cells Are Ineffective at Supporting Mitogen-induced T Cell Activation*

| Experiment | Accessory cell | Mitogen-induced T cell [3H]thymidine incorporation* | | |
|---|---|---|---|---|
| | | PHA | Con A | PWM |
| | | $\Delta cpm \times 10^{-3}$ | | |
| 1 | Nil | 0 | 0.7 | 0 |
| | Mφ | 33.5 | 29.9 | 14.0 |
| | HeLa | 0.5 | 1.0 | 0.2 |
| | Mφ + HeLa | 34.8 | 33.3 | 11.6 |
| 2 | Nil | 0.5 | 0 | 0 |
| | Mφ | 14.7 | 8.9 | 3.8 |
| | HeLa | 0.1 | 0.2 | 0 |
| | Mφ + HeLa | 12.9 | 12.8 | 3.6 |

* T cells were incubated at a density of $1 \times 10^4$ per well for PHA stimulation and $2.5 \times 10^4$ per well for Con A or PWM stimulation. Cultures were supplemented, where indicated, with either mitomycin C-treated Mφ, mitomycin-C-treated HeLa cells ($1 \times 10^4$/well), or a mixture of Mφ and HeLa cells ($5 \times 10^3$ of each per well). Data represent the means of triplicate determinations. [3H]thymidine incorporation in cultures of T cells and HeLa cells with no mitogen was <200 cpm.

*Accessory function of endothelial cells: induction of lymphocyte DNA synthesis.* DNA synthesis by lymphocytes can be detected by staining the cells with mithramycin and analyzing the cell associated fluorescence by flow cytofluorometry (20). In this technique, cell-associated fluorescence is directly related to the DNA content of the cell.

The percentage of cells entering the DNA synthetic phase of the cell cycle in response to mitogenic stimulation can be determined by calculating the number of cells with a greater than diploid intracellular DNA content. This allows a population to be analyzed for the actual number of cells stimulated to replicate DNA. In addition, it obviates the potential errors inherent in the measurement of [3H]thymidine incorporation as an estimate of lymphocyte DNA synthesis (25).

The ability of Mφ and EC to support mitogen-induced T cell activation was examined in this way. As shown in Fig. 4, very few T cells in control or PHA-stimulated cultures were found to be in S or $G_2$-M in the absence of supplemental accessory cells. Mitomycin C-treated Mφ or EC reconstituted the PHA response of T cells as evidenced by the marked increase in the number of cells with a greater than diploid DNA content. Similar results were obtained in three additional experiments that tested the accessory function of pulmonary artery and ovarian artery EC. These findings support the conclusion that endothelial cells are able to function as accessory cells necessary to trigger mitogen-induced entry of T cells into the cell cycle.
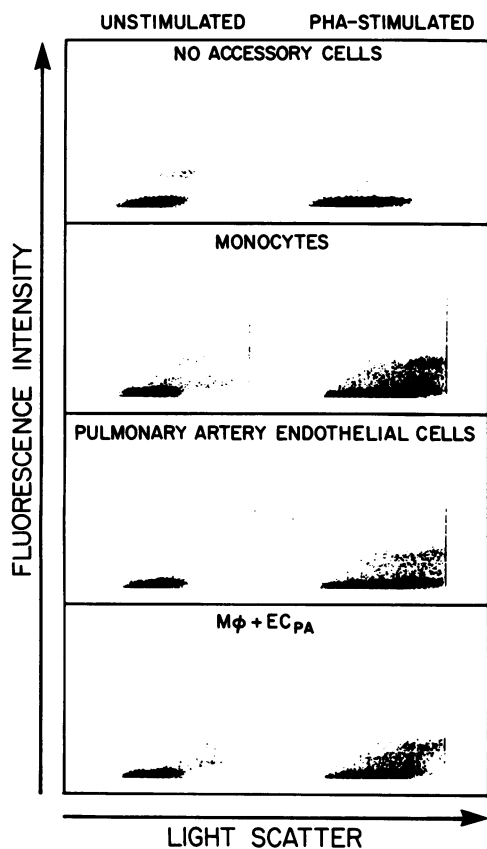
UNSTIMULATED    PHA-STIMULATED

NO ACCESSORY CELLS

MONOCYTES

PULMONARY ARTERY ENDOTHELIAL CELLS

$M\phi + EC_{PA}$

FLUORESCENCE INTENSITY

LIGHT SCATTER

FIGURE 4   EC support mitogen-induced T cell DNA synthesis. T cells were cultured with or without PHA either alone or supplemented with $M\phi$ or EC as indicated. After a 48-h incubation, the cells were stained with mithramycin. Ten thousand cells were analyzed for cell-associated fluorescence and results depicted on each dot plot. Fluorescence intensity is directly related to cellular DNA content. Percentage of cells in S or $G_2$-M: T cells alone, control = 5.0%, PHA-stimulated = 6.9%;   T cell + $M\phi$,   control = 12.7%,   PHA-stimulated = 39.1%;   T cells + $EC_{PA}$, control = 10.6%, PHA-stimulated = 29.8%.

*Cell-to-cell contact between endothelial cells and lymphocytes during T cell activation.* Cell-to-cell contact between $M\phi$ and T cell develops during T cell activation triggered by mitogens (26, 27). Similarly, mitogenic stimulation results in the establishment of physical interactions between T cells and EC. T cells were cultured with PHA and pulmonary artery endothelial cells ($EC_{PA}$) for 48 h, then the cells were fixed with glutaraldehyde and examined by interference phase contrast microscopy. As can be seen in Fig. 5, clustering of lymphocytes about adherent EC was observed. While lymphocytes were bound to most of the EC, there was considerable heterogeneity with some EC associated with only a few lymphocytes, whereas others were surrounded by large clusters. In control cultures containing T cells and EC but without PHA no cell-to-

cell contact developed and PHA did not cause the aggregation of T cells in the absence of accessory cells.

## DISCUSSION

Antigen- and mitogen-induced T cell activation depend on the function of accessory cells (1). In populations of human PBM, monocytes subserve this accessory cell function (13, 16, 21). In the current studies, mitogen responsiveness of human T cells was also found to require the participation of an accessory cell. This was documented by showing that mitogen-induced T cell activation was abolished as a result of monocyte depletion and that co-culture with an appropriate accessory cell population restored responsiveness. Both monocytes and endothelial cells but not fibroblasts or HeLa cells functioned as effective accessory cells in this system.

The aim of the current study was to determine whether EC alone were sufficient to support mitogen-induced T cell activation. Therefore, it was essential to ensure that both the responding T cells and the EC were free of contamination with other potential accessory cells. Otherwise, the possibility that EC functioned as amplifying cells, but not as completely sufficient accessory cells could not be eliminated. Indeed, an amplifying role was suggested by the finding that T cell responses were greater in cultures supported by both $M\phi$ and $EC_{UV}$ than in those supported by either $M\phi$ or $EC_{UV}$ alone (Table I).

It was unlikely that the endothelial cells were contaminated with $M\phi$ or other potential accessory cells. Although freshly isolated EC may contain small numbers of other cell types including $M\phi$ (8), these are lost with prolonged incubation and successive transfer. All of the EC tested in the current study had been cultured for at least 2 wk and transferred once before use and many had been maintained for as long as 7 wk and transferred several times. Before use or transfer, EC were removed from the plastic culture vessels with trypsin. Since trypsinization does not detach $M\phi$ (8), any contaminating $M\phi$ would be left on the initial tissue culture vessel. No cells with typical $M\phi$ morphology could be detected in the EC populations. Moreover, no cells with Fc receptors for IgG could be detected using IgG opsonized sheep erythrocytes as test particles. Finally, a monoclonal anti-human $M\phi$ antibody (28) identified no cells in the EC population upon analysis by indirect immunofluorescence using a fluorescence activated cell sorter. Thus, the cells used in the current study were uniform populations with the morphologic and biochemical characteristics of EC (17, 18), with no contaminating $M\phi$.

The responding T lymphocytes were prepared by isolating cells that formed rosettes with sheep erythrocytes and depleting $M\phi$ by glass adherence and nylon
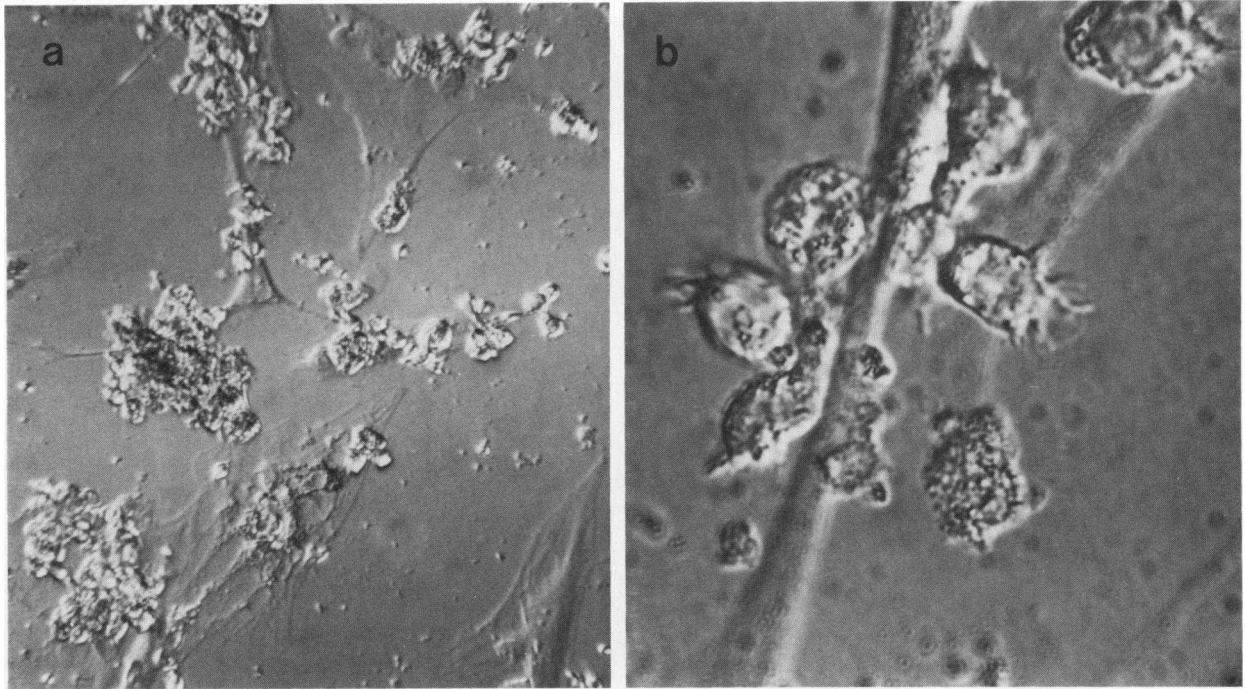
FIGURE 5   EC-T cell interaction in mitogen-stimulated cultures. $EC_{PA}$ and T cells were cultured with PHA for 48 h, fixed with glutaraldehyde and observed by interference phase contrast microscopy. Lower power view shows clustering of T cells about EC with few free lymphocytes, whereas higher power view shows the intimate association of lymphocytes with EC.

wool column passage. In some experiments, $M\phi$ were additionally removed by Percoll density gradient centrifugation, although this step did not appear to enhance the purity of the final T cell population. The T cell population routinely contained less than 1 $M\phi$ per $10^3$ T cells as determined by the criteria of morphology, esterase activity or phagocytosis of latex particles, or IgG-opsonized erythrocytes. This minimal degree of $M\phi$ contamination was confirmed using the monoclonal anti-$M\phi$ antibody (29). Two additional steps were used to ensure that the small number of contaminating $M\phi$ was not contributing to the mitogen responses supported by EC. First, the T cells were cultured at very low cell density ($1 \rightarrow 2.5 \times 10^4$/well) to dilute out the few contaminating $M\phi$. Secondly, the functional activity of any residual $M\phi$ was limited by culturing the cells in microtiter plates with flat-bottomed wells. This technique, which disperses the responding cells and thus minimizes the opportunities for the development of cell-to-cell contact, effectively restricts the accessory function of small numbers of contaminating $M\phi$ (16, 26).

The use of these techniques to deplete $M\phi$ from the T cell population and then limit the activity of any residual $M\phi$ causes mitogen responsiveness to be dependent on the accessory function of the population cocultured with the T cells. EC alone were sufficient accessory cells to support mitogen-induced T cell

activation. This conclusion is supported by the photomicrographic observations presented in Fig. 5 showing activated T cells clustered about EC, with no $M\phi$ evident.

It has generally been accepted that $M\phi$ are the major accessory cell population involved in antigen- and mitogen-induced T cell activation (1). Indeed, only a subpopulation of $M\phi$, those that express Ia antigens, appears to be capable of effectively interacting with T cells to initiate these responses (2, 3). Recently, other Ia-antigen bearing populations such as Langerhans cells and dendritic cells have been shown to share some accessory functions with $M\phi$ (4-6). Earlier work, however, suggested that still other cell types may have some accessory functions. For example, fibroblasts isolated from 14-d old mouse embryos were able to bind adequate haptenated proteins to stimulate secondary antihapten antibody responses in cultures of murine spleen cells (30). Similarly, fibroblasts obtained from 15-16-d-old mouse embryos restored the primary antibody response induced by sheep erythrocytes in murine spleen cell cultures, depleted of adherent cells (31). Interpretation of these studies is complicated, however, because of the possible $M\phi$ contamination of the responding population. "Fibroblasts" obtained from fresh kidneys could restore mitogen responsiveness to adherent cell-depleted guinea pig lymph node T cells (26). Although both responding cells and "fibroblasts" in this study appeared to be free of $M\phi$, the exact nature

of the accessory cell population was not well described. Finally, murine embryonic fibroblasts, but not a variety of other mouse cell lines, restored mitogen responsiveness to murine lymph node cells that had been rigorously depleted of Mφ (32). Again, the nature of the fibroblasts was not well defined. Despite the difficulties inherent in interpreting these studies, they suggest the possibility that cells other than Mφ may function as accessory cells under certain circumstances.

A number of observations suggested the possibility that EC may function as accessory cells involved in lymphocyte stimulation. First, EC *in situ* and, freshly isolated, express Ia antigens (7–9). Secondly, EC are capable of stimulating a mixed leukocyte reaction (10, 11). Thirdly, human EC$_{UV}$ and monocytes share a system of antigens that is distinct from HLA-A, B, C, and DR antigens (7) suggesting that they may also share certain functional characteristics. Indeed, recent evidence has suggested that EC$_{UV}$ may function as antigen presenting cells (33). In view of these results, it seems reasonable that the vascular endothelium, which forms the interface between the blood and tissues, could play a role in actively selecting subpopulations of circulating lymphocytes and thus promoting their egress into appropriate tissue sites. This possibility is supported by the observation that porcine lymphocytes adhere to cultured endothelial cells and, if appropriately sensitized, are stimulated to migrate through EC monolayers (34, 35).

In the current studies, EC from several different vessels supported mitogen-induced T cell activation. Somewhat surprisingly, EC that had been cultured in vitro for as long as 7 wk and transferred five times before use were also able to function as accessory cells. This was unexpected because Hirschberg et al. (8) could no longer detect HLA-DR antigens with a complement-mediated microcytotoxicity assay on EC$_{UV}$ that had been cultured in vitro for 14 or 21 d. HLA-A and B antigens were still found on these cells. In preliminary studies, carried out with G. Nunez-Ollero and P. Stastny, we were also unable to detect Ia antigens on EC after prolonged in vitro incubation using a monoclonal anti-HLA-DR antibody (L 243, Becton, Dickinson & Co.) and analysis by indirect immunofluorescence with a fluorescence-activated cell sorter. These results suggest that cultured EC may express too small a quantity of surface Ia antigens to be detected by the current methodology. Alternatively, Ia-negative EC may serve as accessory cells for mitogen-induced T cell activation. Further delineation of this issue should provide additional insight into the role of Ia antigens in T cell-accessory cell interactions.

## ACKNOWLEDGMENTS

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