Ia EXPRESSION BY VASCULAR ENDOTHELIUM IS
INDUCIBLE BY ACTIVATED T CELLS
AND BY HUMAN γ INTERFERON*

By JORDAN S. POBER,‡ MICHAEL A. GIMBRONE, Jr., RAMZI S. COTRAN,
CAROL S. REISS, STEVEN J. BURAKOFF, WALTER FIERS, AND
KENNETH A. AULT§

From the Departments of Pathology and Medicine, Brigham and Women's Hospital and Harvard Medical School, and the Department of Pediatric Oncology, Sidney Farber Cancer Institute, Boston, Massachusetts 02115; and the Laboratory of Molecular Biology, State University of Ghent, Ghent, Belgium

T helper cells recognize exogenous antigens in association with class II histocompatibility antigens (Ia antigens) on the surface of an antigen-presenting cell (APC)[1] (reviewed in reference 1). The best-studied APC (mononuclear phagocytes, dendritic cells, Langerhans cells) are derived from bone marrow precursors. In mouse, the ability of a population of macrophages to present antigen correlates with the proportion of macrophages that expresses Ia (2). Similar results have been noted with macrophage cell lines (3). T helper cells, when activated, secrete a lymphokine(s) that recruits Ia-positive macrophages from the bone marrow in vivo (4) and induces Ia-negative macrophages to express Ia in vitro (5–7). Thus, T cells can recruit additional functional APC to amplify the immune response. Two preliminary reports have suggested that human mononuclear phagocytic cells may be under similar control (8, 9).

Recent data (10, 11) have suggested that cultured human vascular endothelial cells may substitute for bone marrow-derived APC, presenting antigen to immunized T cells in vitro in an Ia-restricted manner. In these experiments, contaminating bone marrow-derived APC, especially dendritic cells, could have accounted for the results. Furthermore, Ia antigens could not be demonstrated on cultured human umbilical vein endothelial cells by indirect immunofluorescence using a mouse monoclonal antibody (8, 12). Recently (13), we have shown that although primary isolates of human umbilical vein endothelial cells do not express Ia antigens under standard culture conditions, they may be induced to do so by treatment of the cultures with phytohemagglutinin (PHA). In this paper, we report that the induction of endothelial Ia can be mediated by activated T cells as shown by transfer of medium conditioned by activated T cells, by co-cultivation with allogeneic T cells, and by addition of γ

[1] *Abbreviations used in this paper:* APC, antigen-presenting cells; Con A, concanavalin A; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HUVE, human umbilical vein endothelial; LSM, lymphocyte separation medium; M199-FBS, Medium 199 with fetal bovine serum; PHA, phytohemagglutinin.

interferon, an activated T cell product. Thus, this similarity of T cell regulation of Ia expression on endothelial cells and classic APC supports the hypothesis that vascular endothelium may indeed function to present antigen.

Materials and Methods

Cells.   Human umbilical vein endothelial (HUVE) cells were harvested by collagenase treatment of two to six normal term umbilical cord segments and pooled in Medium 199 containing 20% (vol/vol) heat-inactivated fetal bovine serum supplemented with 125 U/ml penicillin, 125 μg/ml streptomycin, and 2 mM L-glutamine (M199-FBS; all components from M. A. Bioproducts, Walkersville, MD) as described elsewhere (14). Replicate primary cultures were plated (day 0) in either Costar 96 flat-bottomed microtiter wells (0.32 cm^2/well) or Costar 24 flat-bottomed wells (2.0 cm^2/well, both from M. A. Bioproducts). The cultures were washed (day 1) to remove unattached blood and endothelial cells and re-fed with M199-FBS. Cultures normally reached visual confluence ($\sim$0.75 $\times$ 10^5 cells/cm^2) by day 3.

Peripheral blood mononuclear cells were sterilely isolated from heparinized (5% vol/vol heparin, 1,000 USP U/ml; Elkins-Sinn, Inc., Cherry Hill, NJ) adult peripheral venous blood by density step gradient centrifugation (15) using lymphocyte separation medium (LSM; Litton Bionetics, Inc., Kensington, MD). T cells and T cell subsets were also sterilely purified from adult peripheral venous blood. To prepare T cells, blood was defibrinated with glass beads, depleted of red cells by 1 *g* sedimentation through 3% dextran in HEPES-buffered Hanks' balanced saline solution (HBSS) and the erythrocyte-poor fractions were further purified by density step gradient centrifugation as above. The mononuclear cells from the interface were incubated on plastic for 1 h to remove adherent cells, washed, and then incubated with neuraminidase-treated sheep erythrocytes (Flow Laboratories, Inc., McLean, VA) for 15 min at 37°C in suspension and 1 h at 4°C as a pellet to form rosettes. Rosettes were collected by sedimentation through ice-cold LSM and washed in HEPES-buffered HBSS with 5% FBS; the sheep erythrocytes were lysed with buffered ammonium chloride. Finally, the T cells were further depleted of adherent cells by an additional overnight incubation on plastic. This population stained >95% positive with mouse monoclonal antibody Leu-1 (Becton, Dickinson & Co., Sunnyvale, CA). T cell subsets (16) were prepared by cell sorting of cells stained by indirect immunofluorescence using mouse monoclonal antibody OKT4 (Ortho Diagnostic Systems Inc., Westwood, MA) diluted 1:100 in HBSS with 4% wt/vol bovine serum albumin and fluorescein-conjugated rabbit anti-mouse immunoglobulin, F(ab')$_2$ fragment at 0.5 mg/ml in the same buffer. Staining and sorting were performed at ice temperature using a FACS II cell sorter (B-D FACS Systems, Becton, Dickinson & Co.). The purity of the sorted cells was assessed by reanalyzing the sorted population using the same criteria used for sorting.

Treatment of Primary HUVE Cultures.   HUVE cell cultures were treated by addition of 4 μg/ml PHA (type IV leucoagglutinin; Sigma Chemical Co., St. Louis, MO) or conditioned medium in M199-FBS on either day 1 (subconfluent) or on day 4 (at confluence). Medium conditioned by activated peripheral blood mononuclear cells was produced by incubating peripheral blood mononuclear cells in M199-FBS containing 4 μg/ml PHA at 2 $\times$ 10^6 cells/ml for 24 h at 37°C in a humidifed 5% CO$_2$ environment. This medium was used undiluted in place of fresh M199-FBS. Media conditioned by clones derived from the Jurkat T cell line were gifts of Dr. Jack Strominger (Sidney Farber Cancer Institute, Boston, MA) and Dr. Terry Strom (Brigham and Women's Hospital, Boston, MA). These were used at concentrations from 0.1 to 10% (vol/vol) in M199-FBS and were compared with matched control media not incubated with cells. Finally, media containing human γ interferon were produced by a cloned Chinese hamster ovary cell line transfected with a plasmid containing the human γ interferon gene (17). Characterization of the cloned cell line is to be described in detail elsewhere. This cell line constitutively secretes human γ interferon. Matching medium from a cloned Chinese hamster ovary cell line not containing the γ interferon gene was used as a control. These conditioned media were added at concentrations of 0.2–10% (vol/vol) in M199-FBS. None of the mitogens or conditioned media showed any cytopathic effect upon the HUVE cells at the concentrations used in these experiments.

Co-Culture of HUVE Cells with Peripheral Blood Cells.   Confluent HUVE cell monolayers in

Costar 24 wells were washed and re-fed with M199-FBS containing peripheral blood mononuclear cells or subsets of peripheral blood mononuclear cells, isolated as described above. Cell ratios ranged from <1 peripheral blood cell to 20 peripheral blood cells per HUVE cell. In some experiments, either the HUVE culture, the peripheral blood cells, or both were given 1,200 rad of γ irradiation from a cesium source (Gammacell 40; Atomic Energy of Canada Ltd., Commercial Products, Ontario, Canada) prior to co-culture. Finally, in some experiments, 10% (vol/vol) heat-inactivated noncytotoxic human serum (Bio Bee, Boston, MA) was used instead of 20% FBS.

Monoclonal Antibody Binding. Mouse monoclonal antibody binding to the surface of HUVE cells was examined by double antibody radioimmunoassay, by immunocytochemistry, and by fluorescence flow cytometry (all described below). The mouse monoclonal antibodies used were W6/32 (IgG_{2a}, directed against an HLA-A,B monomorphic heavy chain determinant [18], a gift of Dr. Jack Strominger); LB3.1 (IgG_{2b}, against an HLA-DR monomorphic determinant, characterization unpublished, a gift of Dr. Peter Knudsen and Dr. Jack Strominger); I2 (IgG_{2a}, also against an HLA-DR monomorphic determinant, [19]), I-LR1 (IgG_{2b}, against an SB polymorphic determinant [19, 20]), I-LR2 (IgG_2, against a polymorphic human Ia determinant [19], all three gifts of Dr. Lee Nadler, Sidney Farber Cancer Institute), and Genox 3.53 (IgG_1, against the DC-1 human Ia antigen [21], gift of Dr. Frances Brodsky, Stanford University, Stanford, CA). K1210 (IgG_1), MPC11 (IgG_{2b}), and UPC10 (IgG_{2a}) were nonbinding mouse immunoglobulins used as negative controls, obtained from Dr. Donna Mendrick (Brigham and Women's Hospital), Dr. Abul Abbas (Brigham and Women's Hospital) and Walgene R & D Laboratories, Inc. (Arcadia, CA), respectively. Second antibodies were ^{125}I-labeled sheep anti-mouse immunoglobulin, F(ab')2 fragment from New England Nuclear (Boston, MA); horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin, a gift of Dr. Donna Mendrick; or fluorescein-conjugated rabbit anti-mouse immunoglobulin from Miles Laboratories Inc., Elkhart, IN.

Double antibody radioimmunoassay was performed using confluent HUVE cultures in Costar 96 microtiter wells as described elsewhere (13). Immunocytochemistry at the electron microscope level (22) was performed using HUVE cultures or co-cultures of HUVE cells and blood cells in Costar 24 wells. In brief, cells were stained as for radioimmunoassay with saturating amounts of mouse monoclonal antibodies in 500 μl and detecting antibody in 250 μl of 1% (wt/vol) bovine serum albumin (Sigma Chemical Co.) in HBSS (with calcium; M. A. Bioproducts); the detecting antibody was rabbit anti-mouse immunoglobulin coupled to horseradish peroxidase. After the final wash, the cell monolayer was lightly fixed with 1.25% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 2 h at 4°C and then washed three times with 0.05 M Tris-HCl, pH 7.56 (Tris buffer). The cells were incubated with diaminobenzidine and hydrogen peroxide (5 mg diaminobenzidine, 0.1 ml of 1% H_2O_2/10 ml Tris buffer) for 40 min at room temperature, and excess substrate was removed by three washes with Tris buffer. Each well was post-fixed with 2% aqueous osmium tetroxide for 30 min at room temperature and then dehydrated and embedded in Epon, and the monolayer was re-embedded at right angles to the original plane of the culture dish to obtain cross-sections. These sections were examined by electron microscopy using a Philips 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Immunofluorescence flow cytometry was performed using HUVE cultures or co-cultures of HUVE cells plus blood cells in Costar 24 wells. Cells were suspended by two washes with calcium-free HBSS followed by incubation with trypsin-versene mixture (M. A. Bioproducts) for 5 min at 37°C. After gentle tituration with a pasteur pipette, the trypsin was quenched by addition of M199-FBS. This protocol yields a nearly monodisperse suspension (a few lymphocyte-endothelial couplets persist) of trypan blue dye-excluding cells and does not alter staining for Ia antigens. The suspension was washed twice with bovine serum albumin-Dulbecco's phosphate-buffered saline using the clinical centrifuge and labeled as for the radioimmunoassay with saturating amounts of monoclonal antibodies and detecting antibody (fluorescein isothiocyanate [FITC]-rabbit anti-mouse immunoglobulin). The final washed cell pellets were fixed by suspension in freshly made 1% paraformaldehyde (Fisher Scientific Co., Pittsburgh, PA). Fixed cells were stored for up to several days in the dark at 4°C prior to analysis.

Analysis was performed by flow cytometry using a FACS Analyzer (B-D FACS Systems,

Becton, Dickinson & Co., Sunnyvale, CA) with a 100-μm orifice to permit entry of endothelial-sized cells. Data were obtained on both electronic cell volume and immunofluorescence of each cell passing through the orifice. Endothelial-sized cells form a distinct cluster on the volume vs. fluorescence intensity plot either when analyzed alone or when mixed with blood mononuclear cells. Blood mononuclear cells, analyzed alone, are much smaller and do not overlap with the endothelial cells. Gates were empirically set to exclude small cells (e.g., lymphocytes) and subcellular debris from analysis. Data was normally collected and displayed as a histogram of cell number (y axis) vs. log fluorescence intensity (x axis); anti-Ia was always compared with nonspecific binding of an unreactive mouse monoclonal antibody of the same immunoglobulin class.

Treatment of HUVE Cultures with OKT3 plus Complement. HUVE monolayers, confluent or subconfluent, were washed once with L15 medium (M. A. Bioproducts) and incubated for 30 min at room temperature in mouse monoclonal antibody diluted in L15; for OKT3 (Ortho Diagnostic Systems Inc., Westwood, MA), the effective dilution was 1:10. Further dilutions (e.g., 1:50) have given variable results. Excess antibody was removed and the cells were incubated in pooled baby rabbit complement (Pel Freez Biologicals, Rogers, AR) diluted 1:1 with L15 for 30 min at 37°C in a humidified, 5% CO_2 incubator. The complement was removed and replaced by fresh complement (1:1 with L15) for a second 30-min incubation. The cells were then washed and re-fed with M199-FBS with or without specific effectors (e.g., PHA). The complement treatment did not appear cytopathic to the endothelial cells.

Proliferation of T Cells. T cell proliferation to allogeneic endothelial cells, to allogeneic peripheral blood cells, or to mitogenic lectin have all been assessed by [^{3}H]thymidine incorporation into DNA. T cells were incubated with allogeneic irradiated stimulator cells (1,200 rad γ irradiation from a cesium source) in triplicate in Costar 96 flat-bottomed microtiter wells in 200 μl vol. HUVE cells were present as a confluent monolayer ($\sim$1.5 $\times$ 10^4 cells/well) and allogeneic peripheral blood mononuclear cells were added in suspension (1 $\times$ 10^5 cells/well). In some experiments, the effect of the number of stimulator peripheral blood mononuclear cells was assessed, adding as few as 1 $\times$ 10^4; no stimulation was observed below 3 $\times$ 10^4. Peripheral blood mononuclear cells, purified T cells, and T cell subsets have been used as responder cells varying from 1.5 $\times$ 10^4 to 3 $\times$ 10^5 cells/well; most assays use 3 $\times$ 10^5 cells. Incubations were conducted in medium M199 containing 10% (vol/vol) heat-inactivated human serum (noncytotoxic), but 20% FBS may be substituted without apparent effect. The onset of co-culture marks the beginning of day 1; proliferation was assessed by adding 1 μCi [^{3}H]thymidine (New England Nuclear) 18 h before harvest and is numbered by the day on which incubation started (e.g., day 3 proliferation is harvested 72 h after the onset of co-culture). Incorporation of radioactivity into DNA was measured by automated harvest (mini-MASH; M. A. Bioproducts) with collection of macromolecules on a filter and subsequent liquid scintillation counting. Each point was determined in triplicate and corrected for incorporation into the unstimulated cell population(s) incubated separately, also determined in triplicate.

Results

Endothelial Cells May Be Induced to Express Ia Antigens. We have previously reported that HUVE cells in primary culture, under standard conditions, do not express Ia antigens, but that treatment of the cultures with the plant lectin PHA induces the expression of endothelial cell Ia (13). The expression of Ia by the culture was quantified by monoclonal antibody binding, measured in a double antibody radioimmunoassay; the increase in binding was confirmed to be an increase in Ia antigen expression by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of radiolabeled cell surface protein. We have now determined the percentage of Ia-positive cells by fluorescence flow cytometry, using an FITC-conjugated anti-mouse Ig immunoglobulin as the second antibody. LB3.1 binding to endothelial cells in untreated cultures (Fig. 1 A, large dots) is indistinguishable from nonspecific binding with a mouse myeloma protein (small dots); in contrast, in the
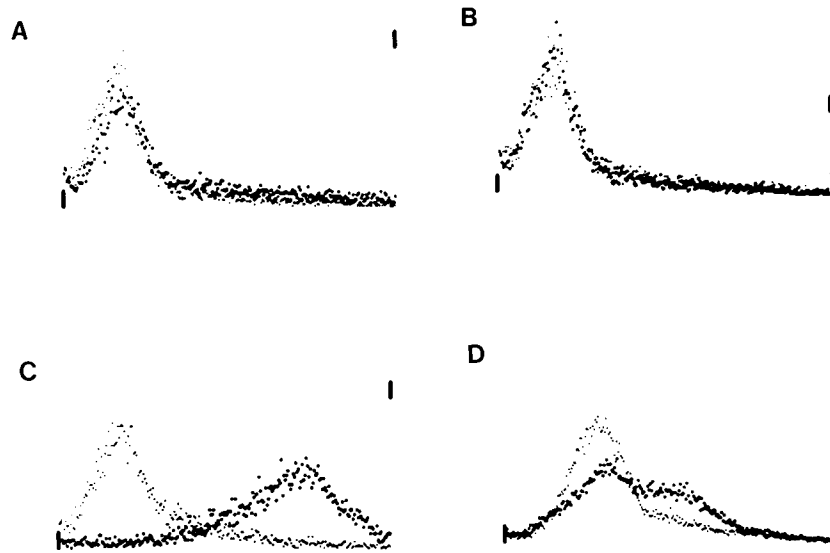
A

B

C

D

FIG. 1.   Mouse monoclonal anti-human Ia antibody binding to endothelial cells analyzed by fluorescence flow cytometry. The data are presented as histograms, plotting cell number (*y* axis) vs. log fluorescence intensity (*x* axis). The binding of LB3.1 (monomorphic anti-HLA-DR large dots, panels A and C) and I-LR1 (polymorphic anti-SB, large dots, panels B and D) are compared with a nonspecific mouse immunoglobulin (small dots) in PHA-treated (C and D) and untreated (A and B) cultures. Note that binding of LB3.1 and I-LR1 is indistinguishable from control in untreated cultures, that every endothelial cell is HLA-DR-positive in the treated cultures, and that ~40% of these cells from pooled donors bind I-LR1 after PHA treatment. We interpret this to mean that every cell expresses SB antigens but that only 40% of the cells genetically encode for an SB antigen recognized by I-LR1.

PHA-treated cultures, the entire population has become HLA-DR-positive (Fig. 1 B). An incidental but reproducible finding is that Ia-positive endothelial cells appear slightly smaller as judged by electronic volume than Ia-negative cells. This change probably correlates with the change in cell shape noted previously (13).

Several human Ia antigens in addition to HLA-DR have been recently described. The mouse monoclonal antibody I-LR1 is a polymorphic reagent that recognizes a subset of allelic products of the SB locus (20). Endothelial cells in untreated cultures derived from several pooled donors appear SB-negative (i.e., do not bind I-LR1, Fig. 1 C) and upon PHA treatment, ~40% of the endothelial cells bind I-LR1 (Fig. 1 D), whereas ~60% of the cells remain indistinguishable from the untreated control. This result has two implications. First, it shows that more than one Ia antigen (DR and SB) are coordinately induced. Similar results by radioimmunoassay have been obtained for mouse monoclonal antibodies Genox 3.53 and I-LR2 polymorphic reagents that recognize products of other human Ia loci (not shown). Second, the fact that ~40% of the cells in these pooled cultures become discretely I-LR1 binding, whereas ~60% remain nonbinding (a bimodal distribution), suggests that each endothelial cell synthesizes its own Ia. According to this interpretation, every cell has become SB antigen-positive, but only 40% of the cells genetically encode for SB antigens recognized by I-LR1. If, on the other hand, Ia were being shed into the medium and passively bound by endothelium, it would be expected that every cell should have

become I-LR1 reactive to some degree, as was seen with the monomorphic anti-HLA-DR antibody LB3.1 (Fig. 1, A and C).

The Induction of Endothelial Ia by PHA Is Mediated by T Cells. PHA could act directly upon endothelial cells to induce Ia expression or could activate a minor cell population in the culture, which in turn would act upon endothelial cells or could do both. The most likely candidate for a mediator cell is the T lymphocyte. T cells were not detectable in the primary HUVE cultures by morphology or by monoclonal antibody binding assays with the anti-human T cell mouse monoclonal antibodies OKT3 or Leu-1. Furthermore, the cultures were multiply washed, a condition not expected to retain T cells, a nonadherent (to plastic) cell type. Nevertheless, T cells could be involved because (*a*) T cells, although nonadherent to plastic, bind tenaciously to cultured vascular endothelium (23, and personal observations); (*b*) in general, very few T cells can produce enough lymphokine to affect many target cells; and (*c*) both PHA and concanavalin A (Con A), another polyclonal T cell activator, induce endothelial Ia expression at doses that activate T cells.

Direct evidence for the role of T cells in mediating endothelial Ia expression was obtained by the following experiment. Primary HUVE cultures were treated either with complement alone (mock-treated) or with anti-human T cell mouse monoclonal antibody OKT3 plus complement before the addition of PHA. Endothelial cells in cultures treated with OKT3 plus complement no longer express Ia in response to PHA, whereas mock-treated cultures respond as usual (Fig. 2). Similar results were obtained with Con A (data not shown). In other experiments, use of mouse myeloma
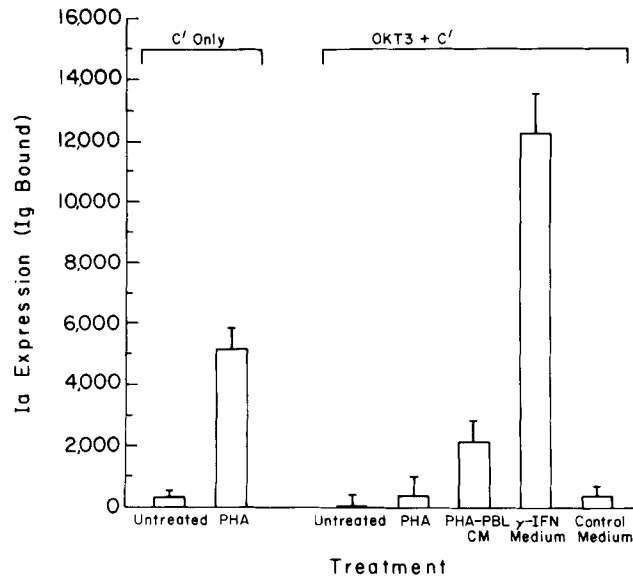

Fig. 2. Effect of OKT3 plus complement pretreatment upon the induction of endothelial Ia. Replicate HUVE primary cultures were plated on day 0, treated with OKT3 plus complement or complement alone on day 1, and immediately treated with fresh M199-FBS (untreated), M199-FBS containing 4 µg/ml PHA, M199-FBS conditioned for 24 h by 2 × 10⁶ peripheral blood mononuclear cells/ml in 4 µg/ml PHA (PHA-PBL CM), and M199-FBS containing 1% Chinese hamster ovary cell conditioned medium by cells expressing human γ interferon (γ-IFN medium containing 5 × 10⁴ U/ml assayed on FS4 cells) and not expressing human γ interferon (control medium). Cultures were assayed for Ia expression by double antibody radioimmunoassay on day 4.

proteins or mouse monoclonal antibodies not reactive with human T cells of the same immunoglobulin class as OKT3 plus complement also had no effect upon the PHA response (data not shown). There was no evidence of a cytopathic effect upon the endothelial cells after treatment with OKT3 plus complement. Most importantly, medium conditioned by human peripheral blood mononuclear cells treated with the same dose of PHA was effective in inducing endothelial cell Ia expression even after treatment with OKT3 plus complement (Fig. 2). The efficacy of different samples of conditioned medium was variable, which suggests that a labile factor was involved. The role of a T cell factor in the induction of endothelial Ia was further suggested by the following two types of experiments.

Co-Culture with Allogeneic T Cells Induces Endothelial Ia in the Absence of Lectin. When peripheral blood mononuclear cells from an adult donor are co-cultured with an allogeneic HUVE cell monolayer, the T cells proliferate (24, and Fig. 3), with a peak of proliferation on day 6. The time course of this response is similar to that of the response to allogeneic blood cells. Although the primary HUVE cultures contain some blood cells, these do not appear to be present in sufficient quantity to account for the proliferation. Specifically, the HUVE cultures morphologically contain $<1 \times 10^3$ blood mononuclear cells and 1×10^4 blood mononuclear cells do not stimulate proliferation. By trypsin treatment, we have been able to disperse the cell cultures at various times after initiation of co-culture and have tested for Ia expression by the endothelial-sized cells, using the fluorescence flow cytometer. Very few endothelial-sized cells express Ia at the onset of co-culture (i.e., at 30 min); however, increasing
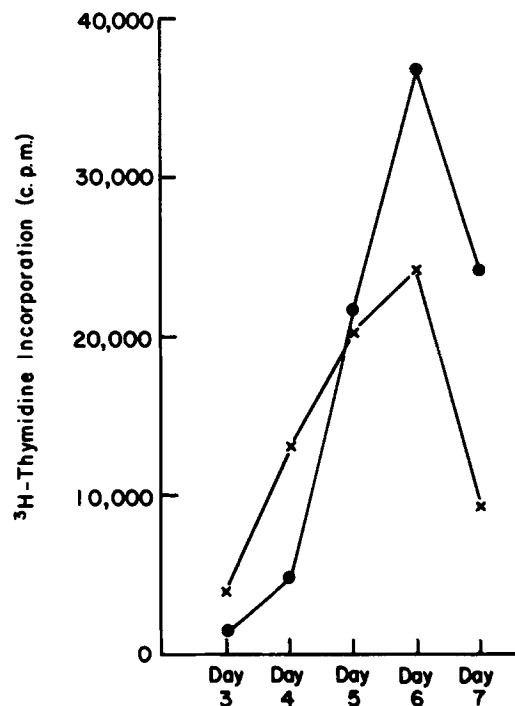

Fig. 3.   Proliferation of 3×10^5 peripheral blood mononuclear cells in response to 1×10^5 allogeneic peripheral blood mononuclear cells (●) or 1.5×10^4 HUVE cells in primary culture (×). Assay conditions are described in Materials and Methods.
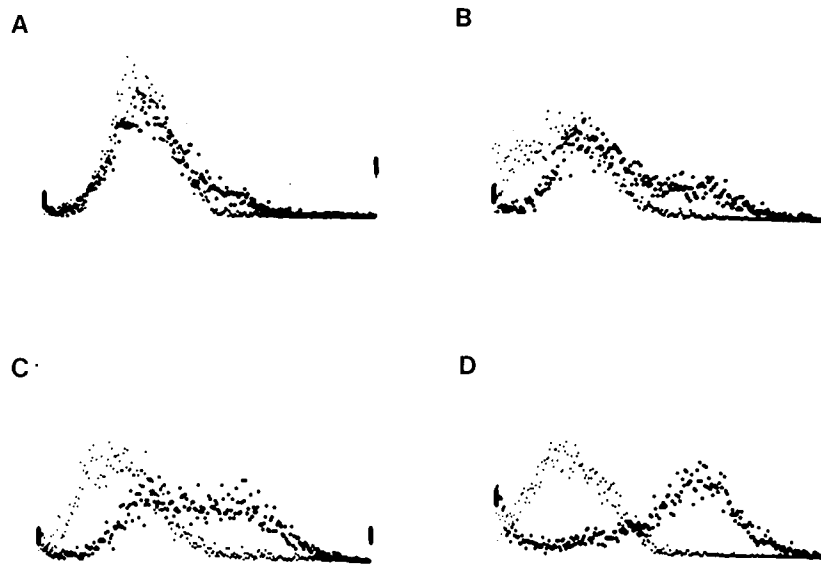
A   B

C·   D

Fig. 4. Time course of the induction of endothelial Ia induced by co-culture with allogeneic peripheral blood mononuclear cells, assayed by fluorescence flow cytometry. The data are presented as histograms, plotting cell number (y axis) vs. log fluorescence intensity (x axis). LB3.1 binding (mouse monoclonal anti-human HLA-DR, large dots) is compared with nonspecific antibody binding (small dots) after (A) 15 min, (B) 24 h, (C) 48 h, and (D) 72 h of co-culture.

numbers of cells are positive at 24, 48, and 72 h. In fact, by 72 h, almost all of the endothelial-sized cells are Ia-positive (Fig. 4). By immunocytochemistry, we have confirmed that the Ia-positive large cells are morphologically identifiable endothelial cells (Fig. 5). By fluorescence flow cytometry and immunocytochemistry, most of the small cells remain Ia-negative.

These results have four implications. First, incubation with allogeneic peripheral blood mononuclear cells induces Ia in the absence of lectin. The exogenous T cells appear to be the important effectors because OKT3 plus complement pretreatment of the HUVE cultures has no effect upon Ia induction (data not shown). Second, endothelial Ia expression precedes T cell proliferation, which suggests that T cell activation but not proliferation is needed for Ia induction. In fact, γ irradiation (1,200 rad) of the blood cells to prevent proliferation does not alter the induction of endothelial Ia (data not shown). Third, the presence of discretely Ia-negative and positive endothelial cells in the culture at 24 and 48 h again suggests that endothelial cells synthesize Ia rather than absorb Ia shed by other cells into the medium. Finally, Ia appears sufficiently early in the co-culture that it could be a major stimulator of allogeneic T cell proliferation in the response to endothelium, as has been shown in the mixed lymphocyte reaction (reviewed in reference 25).

The peripheral blood mononuclear cells added to HUVE cells in the previous experiment are predominantly T cells, but contain several other cell types. To better establish a role for T cells in this phenomenon, we further fractionated the blood mononuclear cells. Fractions enriched for T cells by E-rosette formation retained Ia-inducing activity (data not shown). After overnight adherence to plastic, the T cell-enriched population was further separated into the OKT4-positive (>96% OKT4-
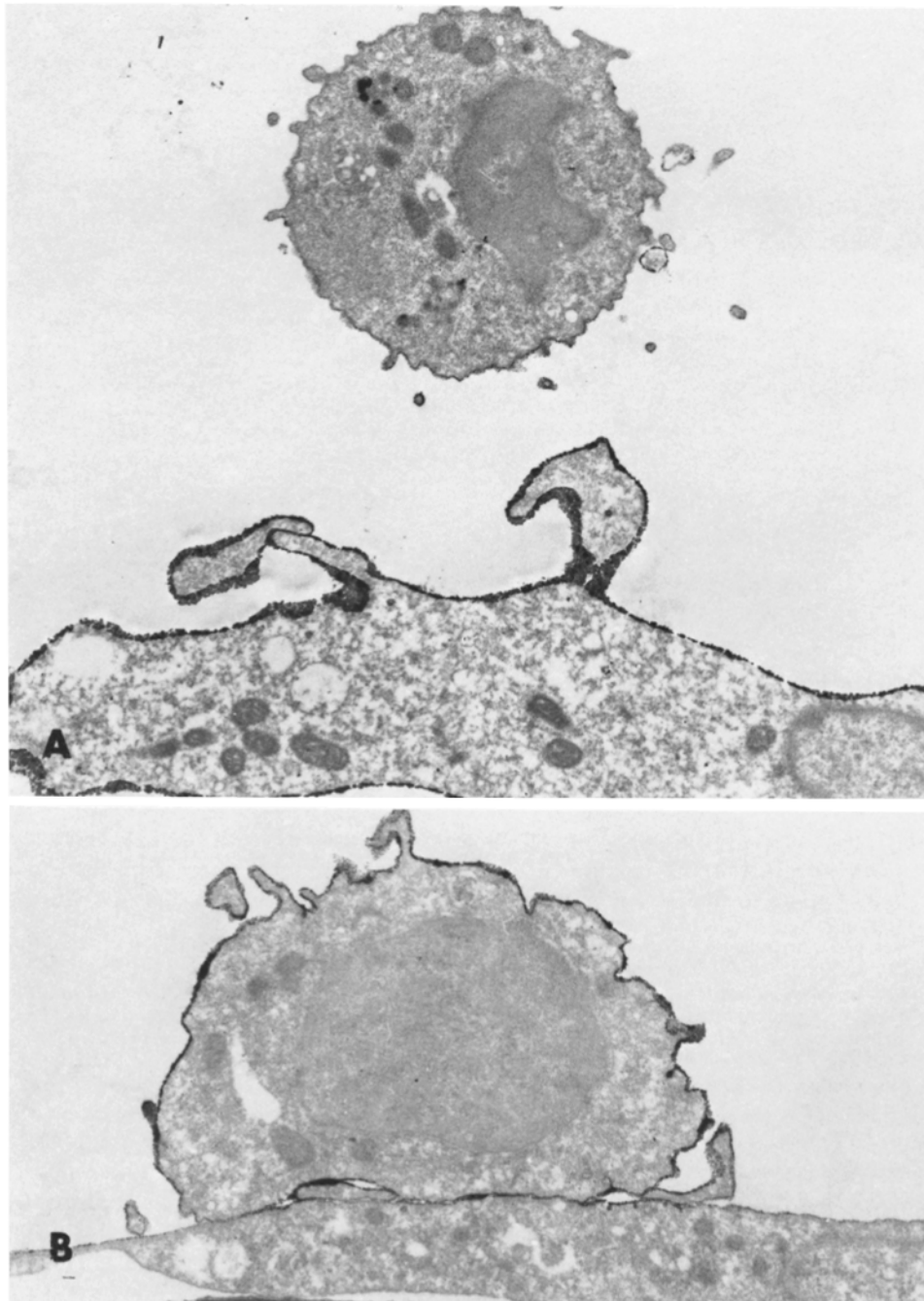
Fig. 5. Immunocytochemical localization of mouse monoclonal (A) anti-human Ia (LB3.1) and (B) anti-human T cell (Leu-1) antibodies to cells in co-culture of human endothelium with peripheral blood mononuclear cells after 72 h. Most of the morphologically identified endothelial cells (lower cells in both panels) were Ia-positive, whereas most of the T cells (upper cells in both panels) were Ia-negative. This result confirms that the Ia-positive large cells analyzed by flow cytometry (Fig. 4) represent endothelial cells.

TABLE I

Lymphocytes added	Endothelial Ia expression (Relative fluorescence intensity)
None	1.0
1×10^6 OKT4-positive	2.6
3×10^5 OKT4-positive	2.0
1×10^5 OKT4-positive	1.1
1×10^6 OKT4-negative	1.9
3×10^5 OKT4-negative	1.0
1×10^5 OKT4-negative	1.1

OKT4-positive or -negative T cells, purified by cell sorting as described in Materials and Methods, were placed in co-culture with 1.5×10^5 HUVE cells for 72 h. Endothelial cells were harvested, fluorescently labeled for HLA-DR antigens with LB3.1, and analyzed by fluorescence flow cytometry, also as described in Materials and Methods. The data are presented as the mode of the fluorescence intensity relative to a nonreactive mouse myeloma protein.
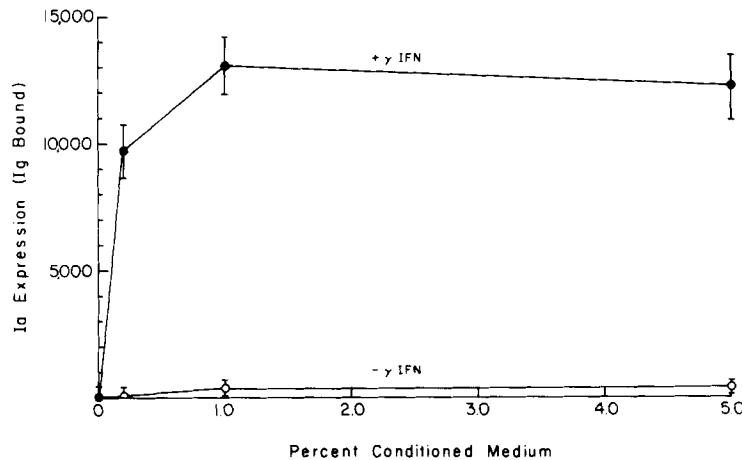

FIG. 6.   Dose-response curve for the induction of endothelial Ia by conditioned media from Chinese hamster ovary cell lines that do ($+\gamma$-IFN) or do not ($-\gamma$-IFN) secrete human γ interferon (5×10^4 U/ml assayed on FS4 cells). Replicate primary HUVE cultures were plated on day 0, treated with M199-FBS containing various concentrations of control or γ interferon-containing conditioned medium on day 1, and assayed for Ia expression by double antibody radioimmunoassay on day 4. The maximum amount of Ia expressed, in other experiments, appears to be about one-fifth that expressed per JY human B lymphoblastoid cell.

positive) and OKT4-negative (<2% OKT4-positive) subsets by means of the fluorescence-activated cell sorter. Both of these cell populations still proliferated in response to PHA or to an allogeneic B cell lymphoblastoid line, which suggests that some residual APC remained. Whereas whole peripheral blood mononuclear cells fail to induce endothelial Ia below a 10:1 ratio (1.5×10^6 mononuclear cells per 1.5×10^5 HUVE cells in a flat-bottomed Costar 24 well, surface area 2.0 cm^2), both the OKT4$^+$ and OKT4$^-$ subsets were effective endothelial Ia inducers at 1.0×10^6 cells/well and the OKT4$^+$ subset was still active at 3.0×10^5 cells (Table I). Thus, T cell-enriched fractions are more potent endothelial Ia inducers than total peripheral blood mononuclear cells and monocyte-enriched fractions do not have discernible Ia-inducing activity. These data suggest the activity resides in the T cell population.

γ *Interferon Induces Endothelial Cell Ia Expression.* As shown earlier, conditioned medium from PHA-activated peripheral blood mononuclear cells induced endothelial cell Ia expression in primary HUVE cultures treated with OKT3 plus complement. To establish that T cells were the source of the factor(s), we have examined several preparations of known human lymphokines. Conditioned medium from some clones of the Jurkat T cell line, a known producer of lymphokines (26), were effective Ia inducers, whereas medium conditioned by other clones did not work; this activity did not correlate with measurable interleukin 2 (data not shown).

Recently, the gene for human γ interferon has been cloned in a plasmid (17) and introduced into a Chinese hamster ovary cell line, and clones have been isolated that constitutively secrete γ interferon into the medium. We compared the activity of γ interferon-containing conditioned medium with that of conditioned medium from a matching Chinese hamster ovary cloned cell line not containing the γ interferon gene. Whereas the control conditioned medium had no Ia-inducing activity, the γ interferon preparations have extremely potent endothelial cell Ia-inducing activity (Figs. 2 and 6). Thus, γ interferon, a protein produced by activated T cells, induces endothelial Ia.

Discussion

Recent studies (10, 11) have suggested that human vascular endothelial cells in culture can act as immune accessory cells, presenting antigen to immunized T cells in an Ia-restricted manner. Since contaminating bone marrow-derived APC could be present, a demonstration that the endothelial cells in these cultures were, in fact, Ia-positive is necessary to support the interpretation that endothelial cells can function as APC.

HLA-DR typing sera and rabbit anti-human Ia xenosera have been shown to be cytotoxic for cultured HUVE cells (27–29). However, these sera may cross-react with alloantigens other than Ia (e.g., endothelial-monocyte alloantigens not expressed on lymphocytes). More recent reports (8, 12) using binding of mouse monoclonal antibodies reactive with human Ia analyzed by fluorescence flow cytometry have reported that human endothelial cells in culture are Ia-negative. Our earlier results (13) demonstrated that HUVE cells do not bear Ia under standard culture conditions, but that Ia could be induced in culture. Here we extend these observations to show that every HUVE cell synthesizes and expresses Ia and that T cells, activated by lectin or alloantigen, mediate the induction of endothelial Ia. T cells appear to work, at least in part, by release of soluble mediators (i.e., conditioned medium induces Ia) and that γ interferon, a protein secreted by activated T cells, acts directly to induce endothelial Ia. Therefore, experiments that have used HUVE cell cultures to support antigen- or lectin-dependent T cell proliferation (10, 11, 30) may have started with Ia-negative endothelial cell populations, but our results predict that once T cell activation has begun, perhaps with residual contaminating monocytes in the T cell population acting as APC, the activated T cells will induce endothelial Ia expression, recruiting additional APC in vitro.

There are two previous reports that suggest that some endothelial cell functions may be under lymphocyte control. First, medium conditioned by the human lymphoblastoid cell line RPMI 8392 or by activated (by lectin or antigen) peripheral blood mononuclear cells inhibit the migration of bovine endothelial cells in culture (31). Second, supernatants from mixed lymphocyte cultures appear mitogenic for bovine

endothelial cells (32). Human leukocyte (α) interferon has also been shown to inhibit the migration of bovine endothelial cells (33), but immune (γ) interferon has not been tested.

Endothelial Ia arises early in the course of co-culture with T cells; about one-third of the cells are Ia-positive by 24 h. For this reason, we have suggested that endothelial Ia could be a major stimulator of the subsequent T cell proliferation, just as it is in the response to allogeneic blood mononuclear cells (25). This hypothesis needs to be tested. What activates exogenous allogeneic T cells before Ia is induced? It is possible that a small number of Ia-positive cells are present in the primary HUVE cultures, either a subpopulation of endothelium or, more likely, a contaminating population of bone marrow-derived APC (monocytes, tissue macrophages, or dendritic cells). Although these cells are few, they could trigger an occasional responding T cell to release an endothelial cell Ia-inducing factor, perhaps γ interferon. Alternatively, the exogenous T cells have been added with their own APC, even in the case of our enriched T cell subsets, and these APC can function to present foreign HLA-A,B antigens that are clearly expressed in untreated cultures. Presentation of alloantigens by syngeneic APC has been seen in the murine mixed lymphocyte reaction (34).

The induction of endothelial Ia by activated T cells has a striking parallel in the induction of murine macrophage Ia by activated T cells. The factor(s) responsible for macrophage Ia induction may be γ interferon, but the identity of the Ia-inducing factor has not been established. Preparation of partially purified murine γ interferon increases the percentage of Ia-positive thymocytes, but the authors of this report could not distinguish between induction of Ia or selection for Ia-positive cells (35). Human monocyte Ia may be regulated (8, 9), but these cells are uniformly Ia-positive upon isolation and Ia quantitatively increases in culture without additional perturbations (36). Therefore, induction of Ia in human monocytes has been difficult to study. The human endothelial cell system thus has two clear experimental advantages: (a) human γ interferon, through the techniques of molecular genetics, is available free of all other contaminating human proteins, whereas mouse γ interferon of similar purity is not yet available; and (b) untreated endothelial cells in culture are uniformly Ia-negative and may be uniformly induced to express Ia. The present report offers evidence that γ interferon does induce endothelial cell Ia and thus accounts for at least some of the Ia-inducing activity of medium conditioned by activated lymphocytes or T cell lines. These results differ from those recently reported using cloned human γ interferon to treat a B lymphoblastoid cell line; HLA-A,B antigens were increased but Ia antigens were not affected (37).

A biological role for endothelial Ia expression in vivo is not clear. Endothelial cells could have the specific task of presenting Ia plus foreign antigen to the circulation, serving to recruit antigen-specific T helper cells into the site of an immune response. Ia expression would be induced when needed. The consequences of inducible Ia on vascular endothelium may extend to allograft rejection. Ia-bearing cells are markedly more immunogenic than Ia-negative cells (38), and this observation has led to the theory of Ia-positive passenger leukocytes as being important determinants of the rejection of kidney and other organ grafts (39). Our data suggest that it may be important to suppress endothelial Ia expression in conjunction with depletion of passenger leukocytes in order to improve graft survival.

Summary

We have used monoclonal antibody binding, measured by radioimmunoassay, fluorescence flow cytometry, and ultrastructural immunocytochemistry, to measure expression of Ia antigens on cultured human umbilical vein endothelial (HUVE) cells. Under standard culture conditions, HUVE cells do not express Ia antigens. However, treatment of primary HUVE cultures with phytohemagglutinin induces the expression of Ia antigens. Every endothelial cell in the culture becomes Ia-positive and endothelial cells appear to synthesize Ia. HLA-A,B is concomitantly increased. The expression of Ia appears to be mediated by T cells because (a) pretreatment of primary HUVE cultures with OKT3 plus complement blocks the action of the lectins but not of medium conditioned by lectin-activated peripheral blood mononuclear cells; (b) co-culture of endothelial cells with allogeneic T cells, in the absence of lectin, also induces endothelial Ia; and (c) human immune (γ) interferon, produced by Chinese hamster ovary cells transfected with the human γ interferon gene, directly induces endothelial Ia. During co-culture with lymphocytes, about one-third of the endothelial cells are Ia-positive after 24 h and all of the endothelial cells are Ia-positive by 72 h. Proliferation of allogeneic T cells starts by 96 h and peaks at 144 h. Thus, endothelial Ia appears sufficiently early to be a determinant for the proliferation of allogeneic T cells. Inducible expression of Ia by endothelium may be important both for allograft rejection and for recruitment of circulating T cells into the site of an immune response.

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