ANTIGEN PRESENTATION TO HUMAN T CELLS: ANALYSIS OF ENDOTHELIAL CELL'S INDEPENDENT CAPABILITY TO PRESENT ANTIGEN

by

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This thesis is dedicated to

my family who means everything to me.

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ABSTRACT

Cell-mediated immunity (CMI) is a term used to designate a diverse group of immune reactions including some tumor associated immune responses, certain autoimmune phenomena, and delayed-type hypersensitivity. The unifying link to these reactions is the participation of antigen-specific T cells. In a CMI response circulating T cells must cross the endothelial cells which line the blood vessels and lymphatics in order to reach the extravascular tissue (the potential site of antigen). Endothelial cells (EC) may play a passive role in the development of CMI, or they may play an active role by presenting antigen to T cells. Antigen presentation by EC would provide a specific signal for T cells to become activated and to leave the circulation at the site of antigen, thereby accounting for the initiation of a cellular immune response in an efficient manner. Previous work by our laboratory and others suggests that EC are capable of presenting antigen. In addition to antigen, two other activation signals are required from an antigen-presenting cell. They are the expression of class II major histocompatibility complex (MHC) determinants and interleukin 1 (IL-1) activity. The experiments described in this thesis were designed to determine whether EC (1) are independently capable of presenting antigen to T cells, (2) synthesize class II MHC determinants, and (3) express IL-1 activity. The experiments were

done using human peripheral blood lymphocytes and human umbilical vein EC (and their products) in various in vitro tissue culture systems. The results described hereinafter demonstrate that EC present antigen to T cells in a MHC restricted fashion, that they synthesize class II MHC determinants, and that they secrete a molecule with IL-1 activity. From these results we conclude that endothelial cells are independently-competent antigen-presenting cells. These findings support the hypothesis that endothelial cells play an active role in the development of a cellular immune response by presenting antigen to circulating T cells.

STATEMENT OF THE PROBLEM

Antigen recognition by T cells provides the element of specificity to cellular immune responses. T cells recognize antigen when it is presented by an accessory cell in the context of I-region-associated (Ia) determinants (HLA-D region determinants in man). In addition to antigen and Ia determinants, a third activation signal is required from the accessory cell, namely the soluble mediator interleukin-1 (IL-1). Once T cells recognize antigen, Ia determinants, and IL-1, they become activated leading to the development of a cellular immune response.

T cells circulate within the blood vessels and lymphatics. Since the blood vessels and lymphatics are lined by endothelial cells, T cells must cross the endothelium in order to reach the extravascular tissue (the potential site of antigen). Endothelial cells (EC) may play a passive role in the development of a cellular immune response or they may play an active role. If EC actively participate in cell-mediated immunity (CMI), then conceivably part of their role may be to present antigen to circulating T cells. By presenting antigen on their luminal surface, EC could provide a recognition site for antigen-specific T-cell egression from the blood to the tissue. Another possibility is that the nonspecific inflammatory response induced by antigen alone produces endothelial changes which allows cellular egression. But, if nonspecific inflammatory changes are what lead to the first recognition of

antigen by T cells, thousands of cells would have to enter the tissue before an antigen-specific T cell was present. (In an immune individual, antigen-specific T cells circulate within the vasculature at an approximate frequency of 1 in 1,000 to 1 in 100,000.) However, even if in low frequency, the specific T cell would come in contact with antigen when presented on the lumenal surface of EC. Therefore, endothelial cell presentation of antigen would seem to provide an efficient mechanism to account for the earliest events in recognition. Evidence from our laboratory and others suggests that EC can serve as antigen-presenting cells (APC). The purpose of this thesis was to determine if EC are independently-competent antigen-presenting cells.

The specific aims of this thesis were to determine:

1) whether EC are independently capable of presenting antigen to T cells.

2) whether EC are capable of biosynthesizing and functionally expressing class II major histocompatibility complex determinants (Ia determinants).

3) whether EC express IL-1 activity.

INTRODUCTION

The participation of accessory cells is instrumental to antigenic activation of T lymphocytes. That is, accessory cells are required to present antigen to T cells. Antigen is presented in the context of "self" major histocompatibility complex (MHC) encoded determinants. In addition to antigen, accessory cells provide the T-cell activating mediator interleukin 1 (IL-1). The following is a review of previous investigations of the accessory cell requirement for antigen-specific T-cell activation. Specific aspects of the accessory cell requirement that will be reviewed include MHC restrictions, IL-1, and the various types of cells which are capable of presenting antigen.

A. THE REQUIREMENT FOR AN ACCESSORY CELL:

Antigenic activation of T lymphocytes provides the element of antigen specificity to a cell-mediated immune (CMI) response. One experimental system which allows the requirement for antigen-presenting cells in CMI responses to be studied is the T-cell proliferation assay (1-4). This assay measures the ability of previously sensitized T cells to proliferate and incorporate tritiated thymidine when exposed to appropriately presented antigen in vitro. The level of proliferation in vitro has been correlated

to cellular immunity in vivo (1). The assay is antigen specific, an antigen will only induce proliferation of lymphocytes from donors who have been previously sensitized to the antigen (1-3). Incorporation of tritiated thymidine is dependent on the presence of T cells as treatment of the cell preparations with an anti-T-cell serum and complement (1) or with anti-Thy 1.2 serum and complement (2-4) abolished the response. The presence of B cells was not crucial to the response since the few remaining immune B lymphocytes left after anti-Thy 1.2 treatment failed to reconstitute the response when added to nonimmune T lymphocytes (2), and because nylon wool column-purified T lymphocytes (1% immunoglobulin-positive cells) demonstrated a significant response to the antigen, albeit generally lower than that seen with unfractionated cells (3).

When T lymphocytes are purified by passage through a nylon wool column, macrophages (MΦ) are depleted from the cell preparation (5) which may account for the lower level of proliferation seen with fractionated cells (3). In fact, it has been shown that when MΦ are depleted from the T cell preparations using several different methods including glass-bead columns (6, 7), rayon-wool columns (8), nylon-wool columns (4, 9), or Sephadex G-10 columns (10), that the antigen-specific T-cell response is abrogated. The T-cell response can be reconstituted when adherent mononuclear cells (mainly macrophages) are added back to the cell preparations (8-10). In addition to T-cell responses to antigen, T-cell proliferation induced by mitogens (11-13) and by allogeneic cells (14-17) has also

been shown to be dependent on the presence of adherent mononuclear cells.

The reconstituting adherent mononuclear cells can be termed accessory cells in that they are only required to be a small percentage of the cell preparations (10), and, in that while they must be viable, they do not need to replicate to reconstitute the response (4, 6, 10). Of the cells depleted to give the purified T cell preparations, MΦ consistently were able to serve as accessory cells whereas erythrocytes, thymocytes, fibroblasts, or nonadherent lymph node or peritoneal exudate cells were not (4, 7, 8, 18). Consequently MΦ are considered to be the principal accessory cells.

Microscopic examination of cultures of T cells undergoing blastogenesis in response to antigen showed the formation of MΦ-lymphocyte clusters (19-27). The clusters of cells are formed early in the culture with the majority having been formed by 6-8 hours (20, 21, 24). While a low level of lymphocytes bind nonspecifically to MΦ, the majority of the binding is antigen dependent (21, 24). When the lymphocytes are depleted of immunoglobulin-bearing lymphocytes by passage through a nylon-wool column, antigen-dependent clusters develop implying that it is T cells which interact with the macrophages to form clusters (20-22, 25-27). Formation of the clusters depended upon the lymphocyte donor having been previously sensitized to the antigen, and the number of clusters that developed in culture was dependent upon the number of immune cells present (20). Furthermore, the amount of

radiolabeled thymidine incorporated into lymphocytes in a blast transformation assay was directly proportional to the number of macrophage-lymphocyte clusters produced by the same lymphocytes in a cluster assay (22). When the incorporation of radiolabled thymidine was examined by autoradiography, the cells incorporating the radiolabel were lymphocytes undergoing blast transformation (as evidenced by changed in morphology) which were attached to macrophages (19, 21, 22). (As the culture period was extended beyond 2 days, many transformed lymphocytes were free in suspension, 19). In transformation cultures of guinea pig lymphocytes, the clusters were generally composed of a macrophage to which a central lymphocyte is directly attached through a broad base (the central lymphocyte was the only DNA-synthesizing lymphocyte early in the culture period) and several (8-10) peripheral lymphocytes attached to the central lymphocyte by way of uropods (22, 25). Human MΦ and T cells form clusters, some of which are similar in morphology to those of guinea pig cells, but the majority of which differ in morphology; the human cell aggregate typically consists of one or two adherent cells with several lymphocytes directly attached without interposition of a central lymphocyte (27). Using guinea pig cells Braendstrup et al. have shown that the antigen-specific interaction occurs between the central lymphocyte and the macrophage; the peripheral lymphocytes do not need to be from an immune animal (25). By slope analysis of log-cell number vs. log-response plots the minimum number of interacting cell

populations required to give an antigen-induced T-cell proliferative response is three: accessory cells, antigen-sensitized T cells, and T cells that can be from unprimed donors (28).

When MΦ from either peritoneal exudate or bone marrows were fractionated according to size, it was found that the fraction containing the smallest MΦ correlated to those cells which were the most efficient as accessory cells (29-31). That fraction also contained the highest percentage of macrophages positive for peroxidase which implies that accessory MΦ are relatively immature. When analyzed for the capability to phagocytize sheep erythrocytes, the MΦ with the highest accessory activity were consistently lower in phagocytic activity, as compared to cells of the other fractions. Compared to the other fractions, a higher percentage of the smaller macrophages stained positively for Ia determinants; and when those macrophages were treated with antibody to Ia determinants and complement, the accessory cell activity was lost. Therefore, the macrophages with the highest accessory-cell activity are relatively small and immature, have little phagocytic activity, and are positive for Ia determinants.

When adherent-cell depleted T cells were reconstituted with macrophages, it was possible to have previously exposed the macrophages to antigen, wash away any free antigen, then combine the macrophages and T cells and obtain antigen-specific T-cell activation without any free antigen in the culture. It was possible to get a response as detected by proliferation (6) or by the

formation of macrophage-lymphocyte clusters (20) when the macrophages, but not the T cells were antigen pulsed (unless more free antigen was added to the combined cells). Thus, T cells seemed to recognize antigen only when it was presented by a macrophage; part of the macrophage function was to serve as an antigen-presenting cell.

As antigen-presenting cells (APC), MΦ are thought to "process" antigens. Antigen processing is not completely understood. Its occurence is implied by the fact that antibody to native antigen does not inhibit antigen presentation (32-34) and that T-cell proliferation to antigens such as Listeria monocytogenes requires antigen-pulsed MΦ to be metabolically active for at least a 30 to 60 minute period (35). Shimonkevitz et al. have found that metabolically inactive APC can present the antigen chicken ovalbumin (cOVA) to cOVA-specific T cell hybridomas only when the cOVA has been digested by proteolytic enzymes and not when it is in a native or intact denatured form (36). Similar results were reported by Allen and Unanue regarding the recognition of hen egg-white lysozyne (HEL) by HEL-specific T-cell hybridomas; the T cells recognized a tryptic digest of HEL presented by paraformaldehyde treated MΦ but not native antigen presented by the fixed MΦ (37). These findings suggest that MΦ catabolize the antigen as part of the processing event. Studies on the fate of radiolabeled antigen after uptake by MΦ have revealed that the majority of cell-bound radiolabel is released in a matter of hours in a form not bound to protein while a

small portion (25% or less) is retained by the cell (32, 35, 38, 39). Of the radioactivity that is retained by the cell, 30 to 90% of it is bound to protein which has shifted to a lower molecular weight than that seen by gel eletrophoresis of the antigen originally added to the culture (32, 27).

Using the intracellular pathogenic bacteria Listeria monocytogenes, Ziegler and Unanue have defined some of the events in macrophage-antigen handling required for immune T-cell recognition of Listeria (35). They found that the initial uptake of Listeria was rapid (within a matter of minutes), temperature independent (i.e., similar results were obtained at 4C vs 37C), and independent of both oxidative and glycolytic metabolism (sodium azide and 2-deoxyglucose did not inhibit the uptake of Listeria). Antigen uptake was not sufficient for antigen-specific T-cell binding to MΦ; T-cell antigen recognition required the antigen pulsed MΦ to be incubated for 60 min at 37C. During the 60 min incubation, ingestion of the Listeria occurred as was evidenced by the progressive decrease in the number of macrophage surface-associated bacteria (there was a 50% reduction by approximately 10 min), and the majority of the Listeria was catabolized (approximately 75% of the ^{125}I-labeled Listeria that was originally bound was released from the cell, the majority of it being TCA soluble). Catabolism of the antigen by macrophages was an energy requiring event as it was inhibited at 4C and by sodium azide and 2-deoxyglucose. The requirement for a processing event after antigen uptake was

demonstrated further by the finding that T cells did not bind macrophages treated with 1% paraformaldehyde immediately after antigen uptake while a substantial number of T cells (approximately 60% of control) bound MΦ treated with paraformaldehyde after a 50 min exposure to Listeria at 37C. Thus, Ziegler and Unanue's work suggests a two-step mechanism for MΦ presentation of immunologically relevant antigen to T cells: temperature-and-energy independent uptake of antigen followed by antigen processing which requires the MΦ to be metabolically active. This two-step mechanism is supported by the similar results of Waldron et al. (40).

The uptake and processing of antigens has been further examined using pharmacological reagents. For example, cytochalasin B, an inhibitor of a variety of membrane functions including microfilament assembly and pinocytosis, does not affect the uptake of the antigen purified protein-derivative of tuberculin (PPD) (41). The lysosomotropic compounds, ammonia and chloroquine, which disrupt normal lysosome function by increasing lysosomal pH with subsequent depression of the activity of acid hydrolases, do not affect the uptake and ingestion of heat killed Listeria organisms while they do inhibit the catabolism and subsequent presentation of Listeria to T cells by MΦ (42). From these studies it appears that antigen uptake and processing does not depend upon macropinocytosis but does involve phagolysosome handling of the antigen. The conclusions from these results may be restricted to the particular antigens used in the experiments. For example, a study by Lee et al. (43)

demonstrated that the inhibitory effect of chloroquine on antigen processing was related to the size of the antigen; chloroquine had little affect on MΦ presentation of PPD (MW 2,000 to 9,000), some affect on the presentation of monomeric flagellin from Salmonella (MW 40,000) while it almost completely inhibited presentation of polymeric flagellin from Salmonella (MW many millions).

Thus, while antigen-presenting cells (typically MΦ) are known to be required to T-cell recognition of antigen, the steps in the uptake and processing of antigen by APC are not completely understood. Furthermore, the required processing functions of the APC may differ depending upon the antigen. Additional aspects of antigen presentation to T cells, not addressed above are genetic restriction and production of the soluble mediator interleukin-1, both of which will be reviewed below.

B. HISTOCOMPATIBILITY REQUIREMENT FOR ANTIGEN PRESENTATION:

In antigen-induced T-cell proliferation assays such as those described above, the MΦ had to be histocompatible to the T cells in order for T cells to proliferate in response to antigen. Rosenthal and Shevach demonstrated the requirement for histocompatible MΦ and lymphocytes using cells from inbred strain 2 and strain 13 guinea pigs (44, 45). They found that lymphocyte proliferation to antigens such as PPD or 2,4-Dinitrophenyl-guinea pig albumin (DNP-GPA) occurred with antigen-presenting MΦ from the

same strain or from F_1 guinea pigs but not with allogeneic MΦ. Examination of ^{3}H-thymidine incorporation for 72 hours after the addition of antigen showed that the failure to detect antigen presentation by allogeneic MΦ was not due to a shift in kinetics. The possibility that the allogeneic MΦ were inducing a mixed-leukocyte response (MLR) that was suppressive to the antigen response was disproved by showing that the presence of allogeneic MΦ did not inhibit the response to antigen by histocompatible T cells and MΦ. When alloantisera directed against the histocompatibility antigens present on both the MΦ and T cells was added to the culture, it inhibited the response to the antigen. Inhibition was not seen when the alloantisera did not recognize determinants on either cell type or when it recognized determinants only on the MΦ.

Rosenthal and Shevach also showed the effect of gene dosage on T-cell proliferation to antigen (44). That is, when (2 X 13)F_1 lymphocytes respond to the same antigen when it is presented by either parental MΦ (2 or 13), the response is approximately half of that seen when F_1 MΦ serve as the antigen-presenting cells. Paul et al. (46) used both positive and negative-selection techniques to demonstrate two subpopulations of T cells responsive to the same antigen. If (2 X 13)F_1 guinea pig cells from an animal immune to ovalbumin (OVA) are cultured for a week in the presence of OVA and parental MΦ (either 2 or 13), when restimulated with OVA the T cells will only respond if MΦ

syngeneic to those in the first culture are used. Thus it was possible to select positively from (2 X 13)F_1 T cells one subpopulation that would respond to OVA presented by strain 2 MΦ and another subpopulation that would respond to OVA presented by strain 13 MΦ. When (2 X 13)F_1 cells were cultured with P_1 MΦ plus OVA, and then exposed to bromodeoxyuridine and fluorescent light 24 hr later (to kill all of the replicating cells), in a second culture the remaining T cells will proliferate in response to OVA but only when it is presented by P_2 MΦ. Therefore, Paul's work along with that of Shevach and Rosenthal demonstrated that the presenting MΦ must be histocompatible for T cells to recognize antigen and that there are subpopulations of antigen-responsive T cells in heterozygous donors.

Histocompatibility restriction of T cell responses was also found to occur in vivo. For example, Zinkernagel reported that it was possible to transfer immunity to Listeria monocytogenes when the donor of the immune spleen cells and the recipient were either histocompatible or semiallogeneic but not when they were allogeneic to each other (47). Miller et al. (48) also reported the ability to transfer CMI (measured by delayed-type hypersensitivity to antigen-challenged ears) when murine lymphocytes sensitized to either fowl (chicken) gamma globulin (FGG) or dinitroflurobenzene (DNFB) were transferred into either syngeneic or semiallogeneic mice. Thus, histocompatibility restriction of T-cell responses occurs both in vivo as well as in vitro.

Studies using murine cells have proven helpful in elucidating which genes of the MHC are responsible for the restriction of antigen-induced T-cell proliferation. By combining immune T cells and MΦ from different inbred strains of mice, Yano et al. demonstrated that identity at the I-A subregion of the MHC, in the face of differences in all other MHC regions, is sufficient for the activation of T-cell proliferative responses to DNP-OVA (49). Similar to the guinea pig experiments of Rosenthal and Shevach (44), Yano et al. found that the lack of antigen presentation by allogeneic MΦ was not due to a shift in kinetics and that MLR suppression cannot account for the genetic differences observed in the ability of antigen-pulsed spleen cells to activate T cells from primed donors.

Anti-histocompatibility antisera and monoclonal antibodies have also been used to define which regions of the murine MHC restrict T-cell proliferation (50-52). Schwartz et al. demonstrated that antisera against H-2K or H-2D antigens did not inhibit T-lymphocyte proliferative responses to PPD whereas antisera to H-2I region antigens (Ia antigens; I-region-associated antigens) inhibited the response to several different antigens (50). In particular, antibodies to I-A and I-C/E antigens (class II MHC antigens) were sufficient to block the response (51, 52).

In experiments using F_1 hybrids between responder and nonresponder strains, the anti-Ia antisera showed haplotype-specific inhibition suggesting that the mechanism of anti-Ia antisera

inhibition was to block antigen stimulation of the cell surface rather than cell killing or a nonspecific turning off of the cells (50). Anti-Ia antibodies do not appear to interfere with antigen processing as they have been reported to not effect the uptake and catabolism of the antigen Listeria (35). Furthermore, anti-Ia antibodies must be present throughout the culture period, not just during the period where the APC are pulsed with antigen, in order to block the proliferation (53). Anti-Ia antibodies, present throughout the culture period, also inhibit the formation of MΦ-lymphocyte clusters (26). When used to treat adherent spleen cells in a cytotoxic protocol, antibodies to I-A and I-C/E subregion antigens completely inhibited the antigen-presenting function of the cells (10). Thus, the binding of APC Ia antigens by antibodies appears to inhibit the T cell-APC cell-cell interaction that occurs during antigen presentation.

The importance of Ia determinants to antigen presentation has also been examined by transfecting cells with the genes for Ia determinants and looking to see if the transfected cells acquire the ability to present antigen. Germain et al. and Ben-Nun et al. have transfected the cloned $I\text{-}A^{k}_{\beta}$ gene into the antigen-presenting, Ia-antigen positive BALB/c ($I\text{-}A^{d}$, $I\text{-}E^{d}$) derived, B-cell lymphoma M12.4.1 (54, 55). Once transfected the B-cells expressed the $I\text{-}A^{k}\,\beta$ chain on their surface. Germain et al. reported that the expression of the $I\text{-}A^{k}_{\beta}$ gene conferred on the cells the capacity for both allostimulation and antigen-dependent activation of an

$I\text{-}A^k$-restricted T-cell clone (54). (The T-cell clone failed to respond to the antigen, L-glutamic-acid^{60}-L-alanine^{30}-L-tyrosine^{10}, when presented by nontransfected M12.4.1 cells.) The T-cell proliferation to the antigen presented by the transfected cells could by blocked by anti-$I\text{-}A^k$, but not by anti-$I\text{-}A^d$, antibodies. Ben-Nun et al. also reported that the transfected B cells were functional in stimulating allospecific T cells, but, in contrast to Germain et al. finding, they could not get the transfected cells to present antigen to any of several different T-cell clones and hybridomas (each specific for a different antigen though all restricted by $I\text{-}A^k$ determinants) (55). As part of their discussion Ben-Nun et al. suggest that, for the transfected cells to present antigen, the responding T cells would have to be restricted by the heterozygous α^d/β^k dimer. While it may be that the T-cell clone used by Germain et al. was restricted by such a heterodimer, it is more likely that the clone was restricted by a determinant which the $I\text{-}A^k$ β chain could express when associated with either $I\text{-}A^k\alpha$ or $I\text{-}A^d\alpha$ chains. Interestingly, when Malissen et al. transfected both $I\text{-}A^k_\alpha$ and $I\text{-}A^k_\beta$ genes into either mouse L cells or hamster B cells, the transfected L cells and B cells could present Keyhole limpet hemocyanin to $I\text{-}A^k$ restricted T-cell hybridomas but could not present chicken ovalbumin to $I\text{-}A^k$ restricted T-cell hybridomas (56). From these different groups' results it appears that, while expression of the correct restricting Ia determinants allows cells to present antigens which they could

not have otherwise presented, there are also additional factors which ultimately decide whether a cell can present any one antigen, such as the capability to correctly process different antigens.

In man the structural equivalents of the murine Ia antigens are those encoded for by the D region of the human MHC locus, HLA (57-62). The HLA-D region contains at least 3 loci: DR, DS(DC), and SB. All three loci encode for heterodimers with structures typical of class II MHC determinants; the D-region determinants consist of two non-covalently linked glycoprotein chains with molecular weights of approximately 33000 (α chain) and 28000 (β chain). Comparison of partial amino acid sequences suggests that DR antigens are equivalent to the murine I-E antigens (63, 64). SB (secondary B cell) antigens have been demonstrated to also be I-E homologues but, nevertheless, distinct from HLA-DR antigens (65-67). DS is a D-region "second locus" which codes for determinants equivalent to murine I-A antigens (58, 64, 68-70).

Several groups have reported that human-T-cell proliferation in response to antigen is restricted by HLA-D-region-encoded determinants but not by HLA-A,-B, or-C-region determinants (71-75). The lack of antigen presentation by HLA-D-region-incompatible MΦ was not due to suppression by the MLR (71, 75). Similar to the results of Rosenthal and Shevach using guinea pig cells (44), a HLA-D-gene-dosage effect was found using heterozygote cells (76-78). For example, Ford and Burger showed by using HLA-DR 3/5 heterozygous KLH-primed T cells that elimination of cells responsive

to antigen-pulsed HLA-DR 3/3 MΦ by thymidine suicide techniques left intact responsiveness to antigen-pulsed HLA-DR 5/5 MΦ and vice versa (78). Therefore, within a heterozygous T-cell donor there are at least two T-cell populations responsive to the same antigen though restricted by the different HLA-D-region haplotypes.

Genetic restriction of antigen presentation in humans has also been examined using antibodies to determinants coded for by different regions of the HLA locus. Antisera to HLA-A or-B encoded antigens had an inhibitory effect only when reactive with the responding T lymphocytes, irrespective of their reactivity with the cooperating MΦ (79). Monoclonal antibodies and antisera to HLA-DR antigens inhibit T-cell proliferation when directed towards monomorphic (common) antigens or a polymorphic determinant shared by the donors of the T cells and macrophages (78-80). Thus, it appeared that it was the HLA-DR-encoded determinants which were important in the interaction between T cells and MΦ during antigen presentation.

When antigen-specific T cells were cloned, it became evident that not all antigen-induced T-cell proliferative responses are restricted by HLA-DR determinants. For example, a study done by Eckels et al. (81) shows that from one donor that there are T-lymphocyte clones directed at strain A influenza virus which appear to be restricted by SB determinants. From the same donor they also found both SB-and-DR-restricted clones to herpes simplex virus. Other groups have also found T-cell clones whose restriction

pattern correlates the best with class II HLA determinants other than DR (82, 83). Therefore it appears that there are subpopulations of T cells responsive to one antigen which are restricted by different class II HLA determinants within a given individual.

As part of Koide and Yoshida's study on the clonal distribution of Ia-restricted T cells, they examined the phenotypic markers of the T-cell clones that were restricted by different class II HLA determinants (83). All of their clones were essentially 100% positive for the determinant bound by the monoclonal antibody OKT4. Initally it was thought that the human T lymphocytes which were bound by OKT4 antibody represented the helper/inducer subset of T cells (84, 85) vs the cytotoxic/suppressor subset (recognized by the monoclonal antibodies OKT5 and OKT8) (86, 87). Recent experiments suggest that expression of the determinant recognized by OKT4 antibody more closely correlates with antigen recognition being restricted by class II HLA determinants than with the function of the cells (88-91). (In contrast, antigen recognition by cells positive for the determinant bound by OKT8 antibody are restricted by class I HLA determinants, (88-90)). This fits with the finding that in T-cell proliferation cultures where the stimulus is a nominal antigen such as herpes-zoster that the proliferating cells are recognized by OKT4 antibody (84). Thus in cultures of human lymphocytes, proliferation in response to nominal antigen only occurs when the antigen is presented by a HLA class II region

histocompatible accessory cell; the proliferating cells are composed of subsets of cells restricted by the different class II loci and their haplotypes; and the proliferating cells are positive for the determinant bound by the monoclonal antibody OKT4.

C. REGULATION OF THE EXPRESSION OF CLASS II MHC DETERMINANTS:

Ia antigens which are coded for by class II MHC genes (HLA-D region in man and H-2I region in mice) are found predominantly on the surface of B lymphocytes and macrophages, but have also been detected on other cells such as antigen-activated T lymphocytes (92, 93). Studies on the expression of Ia antigens on murine macrophages have shown that different populations of macrophages bear different amounts of Ia antigens on their membranes (94, 95). For example, peritoneal macrophages express very few Ia determinants (approximately 5% are Ia^+) whereas greater than 50% of spleen macrophages have Ia determinants on their surface (94). Beller, Kiely, and Unanue reported that peritoneal exudates from mice infected with Listeria monocytogenes or injected with hemocyanin contained a marked increase in MΦ expressing Ia determinants compared to the peritoneal exudates from normal mice or mice injected with inflammatory materials such as mineral oil, peptone, or thioglycollate (96). When heat-killed Listeria organisms or KLH was injected intraperitoneally into mice, they were the most effective at increasing the percentage of peritoneal MΦ expressing

Ia determinants when the recipient had been previously immunized with the same antigen. Heat-killed Listeria organisms were also very efficient inducers of MΦ expressing Ia determinants when the organisms were injected intraperitoneally with Listeria-immune T cells. Thus, the expression of Ia determinants seems to be not only restricted to certain cell types but, within one cell type, the expression of Ia determinants can vary and may be dependent upon an immune process.

Beller et al. finding of an increased MΦ population expressing Ia determinants within immunologically induced exudates does not by itself distinguish between the possibilities of their being two MΦ populations (one expressing Ia determinants and the other not) and MΦ not expressing Ia determinants being induced to express Ia determinants. To determine whether all MΦ are capable of expressing Ia antigens, Calamai, Beller, and Unanue studied the expression of Ia determinants on MΦ colonies derived from single bone marrow-precursor cells (97). They found that all of the MΦ colonies contained cells that could be induced to express Ia antigens when exposed to an antigen-stimulated T-cell supernatant. Uptake of Listeria organisms during exposure to the T-cell supernatant produced a synergistic increase in Ia antigen expression. In contrast, the presence of L cell-conditioned medium, a selective growth stimulator, reduced the level of Ia determinant expression induced by the antigen-stimulated T-cell supernatant. Therefore, growth and differentiative stimuli appear to have

opposing effects on Ia antigen induction but all MΦ stem cells have the potential to give rise to MΦ expressing Ia antigens under the appropriate stimulation.

Several groups have reported that supernatant from antigen-stimulated T cells can induce Ia antigen synthesis and expression by macrophages (98-100). Scher et al. entitled the soluble mediator MIRF for macrophage-(Ia-positive) recruiting factor (100). They characterized MIRF as a protein of greater than 10,000d whose action was not restricted by the H-2 gene locus. Steeg et al. (101) characterized the macrophage-Ia-antigen-regulatory mediator as sharing antigenic and biochemical characteristics with immune interferon (IFN-γ). Furthermore, IFN-γ, purified to 10^7 u/mg protein specific activity, both induced and maintained MΦ Ia antigen expression in vitro whereas other lymphokines such as colony-stimulating factor, interleukin-2, and epidermal cell-derived thymocyte-activating factor did not. Additionally, antiserum to mouse IFN-γ neutralized both the MΦ Ia antigen regulatory and IFN-γ bioactivities in the activated T cell supernatant. Finally, murine IFN-γ obtained free of other lymphokines by recombinant DNA technology induces the expression of both I-A and I-E/C antigens (102).

In contrast to IFN-γ induction of MΦ expression of Ia antigens, inhibitors of Ia antigen expression have also been reported. For example, murine α-fetoprotein, a major protein in amnionic fluid and perinatal sera, has been shown in vitro to

inhibit macrophage expression of cell surface Ia antigens (103). The environmental agents E. coli endotoxin and Saccharomyces cerevisiae zymosan A have also been reported to be inhibitors of MΦ-Ia antigen expression (104, 105). Additionally, prostaglandins of the E series have been described as potent inhibitors of Ia antigen expression (105, 106). The finding that PGE_1 and PGE_2 can inhibit Ia antigen expression (105, 106) supports the suggestion of Snyder et al. that a murine neonatal spleen suppressor cell, which appeared to be an early precursor of the phagocytic lineage, inhibited MΦ-Ia antigen expression by way of metabolites of arachidonic acid since indomethacin and aspirin blocked the suppression (107). Based on their findings that indomethacin, a cyclooxygenase inhibitor, abrogates the Ia antigen inhibitory effect of LPS, Steeg et al. suggested that LPS inhibits MΦ-Ia antigen expression by stimulating MΦ PGE_2 production (105). Thus, there appear to be two major negative regulatory signals for MΦ expression of Ia antigens: α-fetoprotein and prostaglandins of the E series.

While in the mouse not all macrophages express Ia antigens, human macrophages predominantly have Ia antigens on their surface though the level of Ia antigen expression may vary (108). Though the absolute presence of HLA-DR determinants on human MΦ may not be regulated by soluble mediators or environmental agents, the number of HLA-DR determinants is regulated. For example, E. coli endotoxin and Saccaromyces cerevisiae zymosan A have been reported

to induce a loss of surface Ia antigen (104) while recombinant IFN-γ has been reported to enhance surface Ia antigen (109, 110). Recombinant IFN-γ has also been reported to induce HLA-DR antigen expression on the surface of fibroblasts and endothelial cells (111). The capability of recombinant IFN-γ to induce Ia antigen expression has also been studied with regard to its ability to enhance HLA-DS antigens on macrophages and was found to be a potent stimulant of HLA-DS-antigen expression (110). In summary, the expression of class II MHC antigens appears to be limited to certain cell types; and, while some of those cells may express Ia antigens constitutively, the presence of Ia antigens on other cells is regulated by soluble mediators and environmental agents.

D. INTERLEUKIN 1:

In 1972, Gery et al. reported that LPS-stimulated human and murine adherent cells (presumably macrophages) produced a factor which was mitogenic for thymocytes (112, 113). This factor, termed "lymphocyte-activating factor" (LAF) was able to synergize with suboptimal concentrations of the T-cell mitogens phytohemagglutinin (PHA) or concanavalin A (Con A) in the induction of thymocyte proliferation. Several immunological and inflammatory effects were attributed to LAF resulting in many different acronyms for the factor (114-116). In 1979 a standardized monokine and lymphokine nomenclature was introduced resulting in LAF being renamed

interleukin 1 (IL-1) (116). Of the many biological activities attributed to IL-1, one of its major functions is with regard to T-cell activation. For in addition to presenting antigen in the context of Ia determinants, macrophages are also required to provide IL-1 in order to stimulate antigen-specific T-cell proliferation.

Evidence for the requirement of macrophages to provide IL-1 in order for optimal antigen-specific T-cell proliferation to occur comes from experiments like those of Scala and Oppenheim (117). Scala and Oppenheim showed that monocytes treated with paraformaldehyde after being pulsed for 2 hours with either streptolysin-O or S. aureus protein A were not able to stimulate T-cell proliferation while monocytes prepared identically except for paraformaldehyde fixation did stimulate the T cells. Treatment of the MΦ with paraformaldehyde prevented the cells from synthesizing any proteins (as evidenced by their failure to incorporate significant amounts of radiolabeled leucine) and from producing any detectable IL-1. When partially-purified IL-1 was added to cultures of monocytes which had been treated with paraformaldehyde after pulsing with antigen, the monocytes regained the ability to stimulate a proliferative response by the T cells. Similar experiments reported by Defreitas et al. also demonstrated the requirement for IL-1 in order for T cells to proliferate (118). Defreitas et al. showed that ultraviolet (UV) irradiation of adherent spleen cells reduced by 82% the amount of detectable IL-1 from the spleen cells when stimulated with lipopolysaccharide. The

UV-irradiated spleen cells were not capable of stimulating cells to proliferate in response to antigen unless IL-1 was supplied exogenously, thereby demonstrating a requirement for IL-1. Chu et al. also found that UV irradiation of tetanus toxoid-pulsed MΦ abolished their capacity to support T-cell proliferation unless IL-1 was added to the culture (119). They concluded that IL-1 was not necessary for T cells to recognize the immunogeneic moiety presented by MΦ because monolayers of UV-irradiated tetanus toxoid-pulsed MΦ were equivalent to monolayers of unirradiated tetanus toxoid-pulsed MΦ in their capacity to absorb specifically tetanus toxoid-reactive T cells. Chu et al. finding that IL-1 does not appear to be necessary for T-cell recognition of the immunogenic moiety presented by MΦ is supported by the work of Shimonkevitz et al. (36) and by that of Allen and Unanue (37). Both groups measured T-cell proliferation in response to antigenic peptides presented by paraformaldehyde or gluteraldehyde-fixed antigen-presenting cells. In these experiments IL-1 did not have to be supplied exogenously for the T cells to proliferate. These findings are most likely not a contradiction to those discussed above since the responding T cells in both Shimonkevitz et al. and Allen and Unanue's experiments were T-cell hybridomas (in contrast to either freshly-isolated T cells or T-cell lines used in the other experiments). The T-cell hybridomas (T-cell blasts fused with BW514 T-cell lymphoma cells) did not require IL-2 (T-cell growth factor) to be maintained in culture whereas the antigen-primed T cells that were maintained in

vitro for the experiments reported by DeFreitas et al. did require IL-2. The fact that the T-cell hybridomas do not require either IL-1 or IL-2 in order to proliferate fits with the hypothesis that the reason for the requirement for IL-1 (along with antigen presented in the context of the correct Ia determinant) to stimulate T-lymphocyte proliferation is that IL-1 functions to facilitate the production of IL-2.

Several groups have reported that IL-1 functions to promote T-cell proliferation by stimulating the production of T-cell-derived growth factor (interleukin 2, IL-2) (120-122). Larsson et al. reported that in order for resting T cells to proliferate and produce IL-2 in response to Con A stimulation that a soluble factor with an approximate molecular weight of 20,000 from WEHI-3 cells was required (120). In the discussion of their results Larsson et al. noted that WEHI-3 cells (a murine cell line with functional markers characteristic of macrophages) were known to secrete IL-1 but not IL-2. Due to the closeness in molecular weight of the soluble factor required for Con A-induced elaboration of IL-2 (approximately 20,000) compared to that of IL-1 (approximately 15,000), Larsson et al. concluded that it was most likely WEHI-3 derived IL-1 which supported the production of IL-2 by the T cells. Using partially-purified IL-1, Smith et al. also reported that both IL-1 and lectin are required for optimal IL-2 production and T-cell proliferation (121). Based on their previous observation that lymphoid cells from nude mice were incapable of releasing detectable

IL-2 activity yet the lymphoid cells proliferated vigorously to lectin provided IL-2 was supplied exogenously, Smith et al. reasoned that if IL-1 promoted T-cell proliferation via the facilitation of IL-2 release from T cells, that IL-1 should have no effect on nude-mouse splenocytes. In fact, they found that in contrast to the results from experiments using normal lymphoid cells, neither human or murine IL-1 promoted a detectable response from nude-mouse thymocytes cultured with lectin (121). Farrar et al. have found that purified IL-1 from human monocytes induced IL-2 production by the murine EL-4 thymoma cell line without the addition of mitogen (122). Their results along with the other results discussed above support the conclusion that IL-1 promotes the production of IL-2 as part of an immunologic process whereby T cells become activated.

The ability of IL-1 to faciliate the production of IL-2 is the basis for IL-1 assays. The murine-thymocyte proliferation assay is the most commonly used assay for IL-1 activity (123, 124). In the thymocyte-proliferation assay, IL-1 is added to cultures of thymocytes and suboptimal concentrations of either PHA or Con A. The cultures are incubated for 72 hrs and then harvested. Four hours before harvesting ^{3}H-thymidine is added to the cultures to allow the level of cell proliferation to be determined. Thymocytes cultured with both IL-1 and mitogen show enhanced proliferation compared to those cells cultured either alone or with only mitogen or IL-1. In the thymocyte-proliferation assay, the presence of IL-1 results in the production of IL-2 which in turn results in the high

level of thymocyte proliferation (124). As would be expected from the finding that it is IL-2 vs. IL-1 which ultimately facilitates the proliferative response, IL-2 will also enhance thymocyte proliferation to suboptimal doses of mitogen. Consequently, supernatants being analyzed for IL-1 activity must also be tested for IL-2 by way of IL-2-dependent cell lines (124). While the thymocytes used in IL-1 assay are typically of murine origin, IL-1 will also enhance the response of human thymocytes to mitogen (125).

In addition to the murine-thymocyte proliferation assay, another biologic assay useful in the measurement of IL-1 activity is IL-1 conversion of a murine tumor cell line, LBRM-33-IA5, to an IL-2 producer (126, 127). LBRM-33-IA5 cells secrete IL-2 only when stimulated with both mitogen and IL-1 (126). An IL-2-dependent cell line such as CTLL-2 can then be used to detect the IL-2, thereby measuring the amount of IL-1 added to the LBRM-33-IA5 cells. Conlon has calculated that the LBRM-33-IA5 assay is 1,000 to 10,000 times more sensitive than the traditional thymocyte-proliferation assay (127) which could be very benefical in trying to detect IL-1 production by cells producing low levels of IL-1.

Assays for IL-1 activity have allowed for the purification of IL-1. Many biochemical characteristics of murine and human IL-1 have been determined as has been reviewed elsewhere (114, 115). Some of the basic findings include that both murine and human IL-1 are single chain polypeptides, both have approximate molecular weights of 15,000, and both display microheterogeneity in that human

IL-1 has three isoelectric points (ranging between 5.2 and 7.2) and murine IL-1 displays 3 charge species which are resolved by Tris-glycinate-discontinous polyacrylamide gel electrophoresis (114, 115, 128). While murine and human IL-1 are slightly different biochemically, their biological properties are species unrestricted (114-116).

Most of the IL-1 that has been biochemically and biologically analyzed has been obtained from freshly-isolated macrophages or the murine macrophage cell line $P388D_1$ (129-134). Stimulants for MΦ secretion of IL-1 include phorbol myristic acetate (129), lipopolysaccharide, latex microspheres, antigen-antibody complexes, and barium sulfate (130). Silica and glucocerebroside (GL_1) which damage cultured macrophages have been reported to act synergistically with LPS on MΦ to cause the release of high levels of IL-1 (131). Colony stimulating factor from L cells has been reported to stimulate IL-1 secretion (132) as has C5a (133) and IFN-γ (134).

In addition to MΦ and $P388D_1$, other sources of IL-1 have been reported. For example, leukemia cells from acute monocytic and myelomonocytic patients undergoing leukophoresis have been used to prepare large quantities of IL-1 (135). A human acute-monocytic-leukemia cell line has also been described as a continuous source of IL-1 (136). Other cell types have been reported to produce soluble factors which have IL-1 activity. These include keratinocytes (137), glomerular mesangial cells (138),

glioblastoma cells (139), and large granular lymphocytes (140). Consequently, IL-1 may be released by many different cell types in the body, and because of the importance of IL-1 to T-cell stimulation, the fact that these other cells secrete a factor with IL-1 activity suggests that they may influence immune responses in a previously unrecognized manner.

E. ANTIGEN-PRESENTING CELLS:

As was reviewed above, in order for T cells to proliferate in response to antigen the antigen must be presented by an antigen-presenting cell (APC). In almost all experiments where purified T cells were reconstituted with cells from which they were separated, the reconstituting APC was an adherent cell type, primarily macrophages (4, 7-10, 18). Subsequently, macrophages were considered to be the principal APC. As Cowing et al. suggested, the adherent cells could contain a minor population of another cell type such as dendritic cells which could be potent APC (10). The possibility of other cell types being APC will be discussed below.

The suggestion by Cowing et al. that dendritic cells may be APC appears to be correct. VanVoorhis et al. have shown that human peripheral blood adherent cells which are depleted of macrophages to give a preparation that is 70-80% dendritic cells are capable of presenting Candida albicans and tetanus toxoid antigens to T cells (141). This is in following with a report by Forre et al. that

dendritic cells isolated from the synovial tissues of patients with rheumatoid arthritis can present antigen (142). There have also been reports that dendritic cells can serve as accessory cells for mitogenic responses (141, 143) and that they are potent stimulators of mixed leukocyte reactions (144).

Langerhan's cells, a minor cell population in the mammalian epidermis with dendritic cell morphology, have also been studied for their antigen-presenting capability. Due to the location, Langerhan's cells may play a critical antigen-presenting role in the induction of contact hypersensitivity. The importance of Langerhan's cells to contact hypersensitivity is demonstrated by the Toews et al. experiments demonstrating that contact hypersensitivity to dinitroflurobenzene can not be induced through the skin of mice either naturally depleted of Langerhan's cells (eg. the tail) or depleted of Langerhan's cells via exposure to short course ultraviolet-light irradiation (145). They also found that as the number of Langerhan's cells recovered in skin previously exposed to ultraviolet light, that the capability to induce contact hypersensitivity also recovered. In vitro, Braathen demonstrated that T cells from patients with contact allergic dermatitis and a positive epicutaneous patch test to nickel gave a strong proliferative response to nickel presented by Langerhan's cells (146). Langerhan's cells have also been reported to present PPD to T cells in both guinea pig and human in vitro cell cultures (147, 148).

Endothelial cells have also been tested for the capability to present antigen. Endothelial cells are of interest because they are in continuous contact with circulating lymphocytes and therefore may be important in signaling lymphocytes to egress from the circulation into tissue where antigen is present. Human endothelial cells isolated from umbilical cord veins, pulmonary arteries, and ovarian arteries have been shown to serve as accessory cells in T-cell mitogen responses (149) as have human endothelial cells from aortic or vena caval vessels (150). Endothelial cells have also been shown to stimulate and serve as accessory cells in mixed-leukocyte reactions (150-152). Using endothelial cells isolated from the veins of human umbilical cords Hirschberg et al. has reported that endothelial cells can present PPD, varicella-zoster virus antigen, and herpes simplex virus antigen to T cells in a HLA-DR restricted fashion (152, 153). Burger et al. (154) as well as Nunez et al. (155) have also shown that endothelial cells can present antigen to T cells. One group of investigators, that of Roska, Johnson, and Lipsky, has concluded that while endothelial cells can serve as accessory cells to mitogen responses that they do not function as antigen-presenting cells (156). The reason for the discrepancy in the results of Roska et al. vs. the others mentioned above is not clear. It may be a simple difference in culture conditions or cell collection and purification techniques, or it may be a result of Roska et al. using guinea pig aortic endothelial cells while all of the other investigators used human umbilical vein endothelial

cells. That there is a difference beyond that of species in the two types of endothelial cells is suggested by the preliminary findings that guinea pig aortic endothelial cells can not be induced to express Ia antigens by either supernatant from activated T cells or by IFN-γ (156) while Ia antigens are inducible on human umbilical vein endothelial cells (111, 157, 158).

Since antigen presentation is restricted by class II MHC determinants, antigen-presenting cells would be expected to express Ia antigens. This is the case for the APC discussed above; macrophages (92, 93, 108), dendritic cells (159), Langerhan's cells (160), and endothelial cells (111, 157, 158) all express Ia antigens. While resting T cells do not express Ia antigens and are not capable of presenting antigen (as evidenced by the need for APC), activated T cells do express Ia determinants and therefore may be APC. Brown et al. have found that when conjugated with trinitrophenyl (TNP) irradiated human cloned T cells (which express Ia determinants) can stimulated a primary proliferative response by freshly-isolated T cells (161). Consequently antigen presentation by activated T cells may occur in some in vivo immune processes conceivably as an autostimulatory positive-feedback circuit for activation of naive T cells or for interaction between different T-cell subsets. In the same paper Brown et al. examined the capability of TNP-conjugated B cells to stimulate a primary proliferative response by T cells. Since B cells express Ia antigens it is possible that they may present antigen to T cells.

Yet Brown et al. could not detect any T-cell proliferation due to B-cell presentation of antigen. Bergholtz and Thorsby also reported that TNP-conjugated B cells did not induce a primary T-cell response but that they could induce a secondary TNP-specific response of in vitro-sensitized T cells (162). B cells have also been reported to stimulate proliferation of normal-rabbit-gamma globulin-Fab_2 (NRGG-Fab_2)-primed T cells when the antigen that they are presenting is rabbit anti-mouse immunoglobulin-Fab_2 (RAMIG-Fab_2) (163, 164). RAMIG-Fab_2 is taken up by the murine B cells by binding to their surface immunoglobulin thereby concentrating the antigen and activating the B cells. Surface immunoglobulin uptake of the antigen appeared to be crucial to the B-cells' capability to present antigen as B cells could not efficiently present NRGG-Fab_2 which is only bound by a very few antigen-specific B cells. Kammer and Unanue reported that B cells isolated from nonadherent spleen cells using fluorescein-labeled polyvalent anti-immunoglobulin antibody and a fluorescence-activated cell sorter are capable of presenting keyhole limpet hemocyanin (KLH) to T cells from KLH-immunized mice (165). B-cell surface immunoglobulin uptake of KLH may not be important to B-cell presentation of antigen in Kammer and Unanue's experiments as B cells from KLH-immunized mice were not more efficient at presenting KLH than B cells from unimmunized mice. Thus, depending upon the antigen and whether the T-cell response is a primary or secondary response, B cells in some instances do seem to have the capability to present antigen to T

cells. B cells can then be added to what is most likely a growing list of cells capable of presenting antigen to T cells.

F. SUMMARY:

T-cell recognition of antigen provides the antigenic specificity to cell-mediated immune responses. The T-cell proliferation assay is an experimental system used to study the requirements for T-cell recognition of antigen. In order for T cells to proliferate in response to antigen an accessory cell is required to present the antigen. Consequently the accessory cell is called an antigen-presenting cell. The most commonly used antigen-presenting cell in proliferation assays is the macrophage. Microscopic examination of cultures of T cells undergoing blastogenesis in response to antigen shows the formation of MΦ-lymphocyte clusters. Cluster formation and proliferation occur when antigen is either continuously present in the culture or when the macrophages are pulsed with the antigen. Metabolic studies have shown that macrophages catabolize the antigen. Macrophages must be metabolically active for approximately an hour after exposure to antigen before they can present the antigen to T cells. The requirement for metabolic activity can be bypassed if the macrophage is pulsed with proteolytic-digestion fragments of the antigen. These findings suggest that the macrophage in some way "processes" the antigen though the phenomenon is not well understood.

Macrophages then present the antigen in the context of class II major histocompatibility determinants. That is, the macrophages and T cells must be histocompatible at their class II major histocompatibility loci for presentation to occur. Macrophages also provide the soluble-mediator interleukin 1. Interleukin 1 facilitates T-cell production of interleukin 2, a factor which promotes T-cell proliferation. Studies on the capability of macrophages to present antigen have led to the conclusion that antigen-presenting cells provide at least three signals to T cells: antigen, class II major histocompatibility determinants, and interleukin 1.

Various cell types have been examined for their antigen-presenting capability. While macrophages have consistently been shown to be antigen-presenting cells, erythrocytes, thymocytes, fibroblasts, and nonadherent lymph-node or peritoneal-exudate cells do not seem to be capable of antigen presentation. Yet, several other cell types have been shown to present antigen to T cells. These include dendritic cells, epidermal Langerhan's cells, endothelial cells, activated T cells, and B cells.

Many of the experiments examining the capability of different cell types to present antigen are complicated with the problem of obtaining pure cell populations. Therefore, it is difficult to know whether a cell is independently able to present antigen to T cells. The major goal of the following studies was to determine if endothelial cells are fully-capable independent-antigen-presenting

cells. The results obtained in the course of these studies will be discussed in the summary section along with those of other investigators which have asked similar questions.

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SUBCULTURED HUMAN ENDOTHELIAL CELLS CAN FUNCTION INDEPENDENTLY AS FULLY COMPETENT ANTIGEN PRESENTING CELLS

ABSTRACT

Recent evidence has suggested that dendritic cells, epidermal Langerhan's cells, and endothelial cells (EC) as well as macrophages fulfill the requirements of antigen-presenting cells. Despite a variety of controls, one weakness in the evidence that these latter cell types can independently serve as antigen-presenting cells is that the cell preparations may contain small numbers of contaminating macrophages or other cell types. The experiments described in this paper are directed towards providing firm evidence that human EC are independently capable of presenting antigen to T cells. EC were isolated from human umbilical veins and maintained continuously by serial subculture for periods of up to eight months. The subcultured EC displayed classic EC morphology and uniform immunofluorescent staining for Factor VIII-related antigen. The subcultured EC (tested to the 18th subculture) presented both particulate and soluble antigens to macrophage-depleted T cells with an efficiency equivalent to freshly isolated cells. Monoclonal antibodies to HLA-DR and HLA-DS determinants inhibited antigen presentation by either autologous macrophages or EC. In addition, antigen presentation by the subcultured EC was not affected by the macrophage-specific monoclonal antibody Mac-120, which inhibited antigen presentation by autologous macrophages in the same

experiments. These results are consistent with human EC being able to independently function as fully competent antigen-presenting cells.

INTRODUCTION

T lymphocytes are thought to recognize three signals from accessory cells for optimal activation: antigen, a restricting Ia determinant (HLA-D region determinants in man), and interleukin 1 (1-6). Although traditionally macrophages (MΦ) are considered to be antigen-presenting accessory cells (7-8), recent evidence has suggested that other cell types such as epidermal Langerhan's cells (9-11), dendritic cells (12-14), and endothelial cells (15-19) can also serve as antigen-presenting accessory cells.

Endothelial cells line the blood vessels and lymphatics, and therefore provide a barrier between circulating T lymphocytes and extravascular tissue. Consequently, as antigen-presenting accessory cells, endothelial cells may be important not only in activating T lymphocytes but also in triggering mononuclear cells to egress from blood to tissue at the site of antigenic insult.

Previous work has shown that freshly isolated human umbilical vein endothelial cells are capable of serving as accessory cells in T-cell mitogenic responses (20) and as antigen-presenting accessory cells (15-19). Similar to macrophage presentation of antigen, endothelial cell presentation was shown to be HLA-DR restricted (15-19). Since the endothelial cells used in these experiments were principally from primary cultures, it was difficult to exclude the possibility of contaminating cells participating in the

reconstitution of the MΦ-depleted T-cell responses.

As a means to study the independent antigen-presenting capability of endothelial cells, we have pursued long term endothelial cell culturing and used extensively subcultured cells to reconstitute antigen-specific proliferative responses by MΦ-depleted T lymphocytes. The subcultured endothelial cells display classical cobblestone morphology and uniformly display immunofluorescent staining for Factor VIII-related antigen, a unique marker for endothelial cells (21, 22). We found that the subcultured endothelial cells were capable of presenting both particulate and soluble antigens, and that they retained this capability for a period of eight months (approximately 18 subcultures). Monoclonal antibodies to HLA-DR and HLA-DS determinants blocked presentation of antigen by autologous macrophages and by the subcultured endothelial cells. In additional experiments, we found that the macrophage-specific monoclonal antibody, Mac-120, inhibited antigen presentation by autologous MΦ but did not affect antigen presentation by the subcultured endothelial cells. These results support the conclusion that subcultured endothelial cells can independently provide the necessary signals for optimal antigen activation of T lymphocytes.

MATERIALS AND METHODS

Isolation of blood mononuclear cells (MNC).

Mononuclear cells were separated from heparinized (10 u/ml) peripheral blood from normal donors by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation (23). Interface cells were subsequently washed twice with RPMI 1640 (GIBCO, Grand Island, N.Y.) and counted.

Macrophage isolation.

Cells of the mononuclear phagocytic series (MΦ) were isolated from MNC by adherence to plastic. Tissue culture flasks (No. 25100, Corning, Corning, N.Y.) were pretreated for 30-60 min at 4C with RPMI-50% AB serum and rinsed. MNC (40-60 X 10^6 cells) were added to the pretreated flasks in RPMI with 5% AB serum. After a 1-2 hr incubation at 37C and 5% CO_2, the flasks were rinsed with medium to remove nonadherent cells. Subsequently, RPMI with 5% AB serum and 0.2% EDTA was added and the flasks were held for 10-20 min at room temperature. The macrophages were removed by shaking and vigorous pipetting, washed twice, and counted.

Macrophage depletion.

Macrophage depletion was accomplished by two sequential steps: adherence to plastic and passage over Sephadex G-10. The MNC, routinely containing 5-15% monocytes, were first subjected to adherence on AB serum pretreated plastic flasks as described above. After 1-2 hr the nonadherent cells were washed from the flasks and resuspended in 2 ml with 5% AB serum. Sephadex G-10 (Pharmacia Fine Chemicals) was resuspended to 2-3 times its bed volume in RPMI and autoclaved for 20 min. A column consisting of a plastic 20 ml syringe barrel was prepared and used as previously described (24). Briefly, the column was packed by gravity flow with 15 ml swollen Sephadex G-10 and washed with 2 volumes of RPMI with 5% AB serum. Cells (2 ml) were added to the top of the column and followed by 1 to 2 ml RPMI (5% AB serum) to ensure entry of cells into the column. The column was then incubated for 30 min at 37C and 5% CO_2. The cells were eluted with RPMI (5% AB serum) in 5 to 10 ml and pelleted (10 min at 250 X g) in a 15-ml conical centrifuge tube. The isolated cells characteristically contained 0.1% to 0.4% monocytes by esterase staining and would not support antigen-induced lymphocyte proliferation without macrophage addition.

Endothelial cells.

Endothelial cells were isolated from the vein of umbilical cords

by a modification of the technique described by Jaffe et al. (25). The fresh cords were cannulated, flushed with saline, and then filled with 0.2% collagenase (Sigma Chemical Co., No. C-0131, St. Louis, MO). After 20 min incubation at 37C, the endothelial cells were flushed out with 20 ml RPMI and washed (10 min at 250 X g). The cells were dispersed into wells of 24-well culture plates (approximately 10^4 cells/well) and cultured in RPMI with 20% heat inactivated fetal calf serum and endothelial cell growth factor (ECGF) (26) at 37C and 5% CO_2. Approximately 10% of the cultures grew vigorously becoming monolayers within 4 to 6 days. The cells were released from the plastic by a brief treatment (3 min) with trypsin-EDTA (GIBCO) at room temperature, centrifuged (10 min at 250 X g), and replated into human fibronectin coated (4 ug/ml, Collaborative Research Products, Lexington, MA) 25 cm^2 culture flasks. The endothelial cell cultures were expanded by repetitive subculturing (splitting the cells 1:4 within a week of the cells reaching a monolayer). To confirm that the cultured cells were endothelial cells, cytocentrifuged cells were stained with monoclonal antibody to Factor VIII-related antigen (Cappel Labroatories, West Chester, PA) followed by FITC conjugated goat anti-mouse IgG (Cappel Laboratories) and then examined by fluorescent microscopy.

Standard lymphocyte proliferation assay.

MΦ-depleted MNC were diluted to 1 X 10^6 cells/ml in RPMI supplemented with antibiotics and 30% AB serum and 100 ul (1 X 10^5 cells) distributed in flat-bottom, 96-well microplates (cat. no. 76-003-05, Linbro, Flow Laboratories, McLean, VA). Additional 100 ul preparations containing antigen dilutions, other cells, or media alone were added in quadruplicate to appropriate wells depending upon experimental design. The antigens used were Measles (30-850J), Mumps - viral and soluble (30-848J), and Varicella (30-149J), all complement fixation antigens from M. A. Bioproducts, Walkersville, MA, and Tuberculin PPD from Connaught Laboratories Limited, Willowdale, Ontario, Canada. The microplates were incubated in a 5% CO_2, humidified atmosphere for 4 days, the last 24 hr with 0.1 uCi ^{3}H-thymidine (specific activity 6.7 Ci/mmole, New England Nuclear, Boston, MA). The cultures were harvested and counted by commonly used automated techniques (27).

HLA-DR typing of MNC.

Cell donors were healthy volunteers whose HLA-DR phenotypes were established by standard serological techniques (28) in a commercial laboratory (Epitope Incorporated, Portland, OR).

Antisera/monoclonal antibodies

Monoclonal antibodies to monomorphic HLA-DR determinants: NEI-011 (clone 7.2), was obtained from New England Nuclear, Boston, MA; and BRL DR.1, was obtained from Bethesda Research Laboratories, Rockville, MD. Genox 3.53, a monoclonal antibody to HLA-DS 1, was the gift of Frank Bodmer, Imperial Cancer Research Fund, London. Monoclonal antibody specific for human monocytes, Mac-120, was a gift of Howard Raff, Department of Microbiolgy, University of Washington, Seattle, WA. Monoclonal antibody to HLA-B8 was kindly provided by Epitope Incorporated. All antibodies used in culture were extensively dialysed against RPMI prior to use.

RESULTS

Characterization of subcultured endothelial cells.

Human endothelial cells were isolated from umbilical veins and propagated in vitro as previously described (15). To maintain the endothelial cells in culture for extended periods the cell culture system was modified in two ways: 1) pretreatment of the culture flasks with human fibronectin, and 2) addition of ECGF to the endothelial cell medium. Approximately 10% of the primary endothelial cell isolates formed monolayers of cells which were essentially free from smooth muscle cell and fibroblast contamination. As the endothelial cells were subcultured, any undetected contaminating macrophages would be progressively lost. Rapidly dividing contaminating cells (eg. fibroblasts) could be easily detected by the third or fourth subculture and such cultures were discarded. The subcultured endothelial cells displayed classical cobblestone morphology as can be seen in Figure 1. This morphology was retained as the endothelial cells were subcultured up to 8 months (approximately 18 subcultures). The uniformity of the subcultured endothelial cells was confirmed by immunofluorescent staining with monoclonal antibody to the endothelial cell specific marker, Factor VIII-related antigen. As can be seen in Figure 2,

the subcultured endothelial cells stained in a perinuclear granular pattern typical of that seen in endothelial cells (21, 22). The subcultured endothelial cells routinely failed to thrive after a period of 8-12 months (approximately 20 subcultures) and could not be further subcultured.

Endothelial cells retain the capability to present antigen after extensive subculturing.

The subcultured endothelial cells were tested for their capability to present antigen at different culture intervals. T-cell donors were chosen based on 2 criteria: (1) that they were either HLA-DR compatible or haplocompatible with the EC; and, (2) that their T cells were capable of significant antigen-specific proliferation in a 4 day culture. Macrophage-depleted mononuclear cells (prepared from donors who met the criteria given above) and endothelial cells were cultured with antigen for 4 days and T-cell activation assessed. The experiments were harvested on day 4, even though maximal T-cell proliferation to antigen presented by autologous MΦ was typically on day 6, in an attempt to avoid proliferation due to the allogenicity of the endothelial cells (T-cell proliferation to allogeneic endothelial cells without antigen present was maximal on day 6). The results from cultures where endothelial cells served as the APC were analyzed by direct comparison to cultures done simultaneously where autologous

macrophages served as the APC. Tables 1 and 2 contain results from subcultures where endothelial cells isolated from the same umbilical cord vein were used to present antigen to T cells from two different individuals responsive to different antigens. The results demonstrate that human endothelial cells retain the capability to present antigen after extensive subculturing (17th and 18th subcultures, respectively). For example at subculture 5, T-cell proliferation to varicella presented by EC resulted in a Δ CPM of 6067 (autologous MΦ presentation of antigen resulted in a Δ CPM of 5370), and at subculture 17 varicella presented by EC gave a Δ CPM of 1522 compared to a Δ CPM of 1392 when the APC was autologous MΦ (Table 1). At subculture 5 the EC also efficiently presented PPD to give a Δ CPM of 11274 (vs. 17532 when MΦ served as the APC) as they did at subculture 18 (when EC served as the APC the Δ CPM was 7021 compared to 5246 when MΦ were the APC, Table 2). While statistically significant, these Δ CPM values are lower than values from traditional antigen induced proliferation cultures harvested on day 6. It was not possible to obtain T-cell donors who were completely HLA compatible to the EC. The consequence of using T-cell donors only matched to the EC for HLA-DR determinants was that the cultures had to be harvested on day 4, two days prior to the maximal antigen response, in order to avoid the mixed leukocyte response which could mask antigen-specific proliferation. Additional factors which contributed to the low rate of proliferation was that the T-cell donors were not recently exposed

to the antigen and the T cells were not primed to the antigens in vitro before using them in these experiments. In some experiments endothelial cells were more effective than autologous MΦ in reconstituting the T-cell response, whereas in other experiments the reverse was true. Additional experiments suggested that the variation in responses was due to problems with the precise enumeration of the number of APC added to the cultures and not to changes in their antigen-presentation efficiency. The subcultured endothelial cells presented both particulate and soluble antigens (Tables 1 and 2, respectively) and served as accessory cells for mitogenic responses (24 experiments, results not shown). It was also possible to recover endothelial cells after storage for up to a year in liquid nitrogen and then use them in antigen-presentation experiments (Table 1). For example, when endothelial cells which had been maintained continuously in culture for 8 subcultures were used to present antigen the result was a Δ CPM of 2099. When endothelial cells from the same isolate culture were used after recovery from liquid nitrogen storage and culturing the same final number of subcultures, the Δ CPM in response to presentation of the same antigen was 2273.

Endothelial cells present antigen vs. serving as a feeder layer.

Our laboratory and others have previously reported that antigen presentation by primary and secondary cultures of endothelial cells

is genetically restricted at the HLA-DR locus (15-19). The finding that EC presentation of antigen is restricted can then be used to demonstrate further that the EC are actually presenting the antigen vs. augmenting proliferation induced by presentation of antigen by contaminating cells. That is, if the EC are simply serving as a feeder layer then "presentation of antigen" by the subcultured EC should be seen regardless of the HLA-DR phenotype of the T-cell donor. As can be seen in Table 3 that is not the case; the addition of subcultured EC only resulted in antigen specific proliferation in the cultures where the T-cell phenotype was HLA-DR 4 and/or 7. Since the subcultured EC used in the experiments shown in Table 3 have been typed as being HLA-DR 4, 7 the results are what would be expected if the subcultured EC presented the antigen in a restricted fashion rather than served as a feeder layer in an unrestricted fashion.

An anti-macrophage monoclonal antibody, Mac-120, was used to confirm that subcultured endothelial cells and the MΦ-depleted T cells did not contain any MΦ responsible for the antigen presentation attributed to the endothelial cells (Table 4). Mac-120 antibody has been previously shown to block lymphocyte proliferation resulting from MΦ presentation of antigen (29). The results in Table 4 further demonstrate the capability of Mac-120 to block antigen presentation by autologous MΦ (50% to 95%). In contrast to the results with MΦ, Mac-120 did not inhibit antigen presentation by cultured EC ever more than 26% and generally less

than 20%. Table 4 also shows the high background proliferation that can occur when the APC are not entirely HLA compatible (but are at least HLA-DR haplocompatible). For example, in experiment 2 of Table 4 the T-cell proliferation to EC without antigen present is 2952 CPM. Addition of antigen results in 5461 CPM a Δ CPM of 2509. Note however that, even in experiments with such high backgrounds, Mac-120 did not inhibit the EC presentation of antigen.

Inhibition of antigen presentation by monoclonal antibodies to HLA-D region determinants.

If antigen presentation by subcultured EC is HLA-DR restricted then antibodies directed against Ia determinants should inhibit the capability of EC to present antigen. When monoclonal antibodies directed against monomorphic HLA-DR determinants (NEI-011 clone 7.2 and BRL DR.1) were added to cultures of MΦ-depleted lymphocytes with either autologous MΦ or subcultured EC serving as the APC, antigen-induced proliferation was inhibited (Table 5). For example, in experiment 1 of Table 5, antigen presented by autologous MΦ resulted in 2867 CPM and antigen presented by subcultured EC resulted in 6323 CPM. Upon adddition of monoclonal BRL DR.1 antibody the CPM dropped to 624 and 1331 respectively. As the amount of BRL DR.1 antibody in the cultures was reduced the proliferation recovered toward the level of that without antibody present.

In order to examine whether determinants encoded for by the HLA-D region second locus, HLA-DS, were important to EC presentation of antigen, the monoclonal antibody Genox 3.53 has been tested for its capability to block presentation of antigen. Genox 3.53 has been reported to have a specificty for MB1/DS1 (30). When used in a noncytoxic protocol Genox 3.53 also blocked antigen presentation by both subcultured EC and autologous MΦ (approximately 90% inhibition was seen with the highest amount of antibody present which diminished to roughly 20% inhibition when the amount of antibody was diluted 1000 fold, Table 6).

Discussion

Endothelial cells line the vessels and lymphatics, forming a barrier between circulating T cells and extravascular tissue (the potential site of antigen). The fact that this barrier must be transgressed in a cell-mediated immune (CMI) response has stimulated our interest in the role of EC in CMI. Burger and Vetto have recently published a hypothesis suggesting that the vascular endothelium may be a major participant in T-lymphocyte immunity (31). They suggest that circulating T cells recognize antigen on the surface of EC, resulting in the activation of EC such that the EC then release the key mediators of a CMI response. The EC mediators would then lead to lymphocyte activation and egression into the extravascular tissue. This theory would account for the brief cell-cell exposure which occurs during ordinary hemodynamics, the low frequency of antigen-specific cells in blood, and the rapid dilution of factors by the blood flow, as the EC could become activated after brief contact with antigen-specific T cells and then the activated EC could focally release mediators. Thus, while antigen-specific T cells would still provide the specificity to the CMI response, it would be the EC instead of the T cells that would be the central participant in the CMI response.

According to this hypothesis, part of the central role that EC would play in a CMI response is to present antigen to circulating T

lymphocytes. Experiments reported by Hirschberg et al. (17, 19) and Nunez et al. (18), along with those of our laboratory (15, 16), have demonstrated that freshly isolated human umbilical vein endothelial cells present antigen in a HLA-DR restricted fashion. One criticism of those experiments is that the freshly isolated EC population may have contained contaminating cell types which either were totally responsible for the antigen presentation or whose presence was necessary for the EC to present antigen.

As part of a series of experiments to demonstrate that EC can independently provide all of the necessary signals for antigen-specific T-cell activation, we examined the capability of extensively subcultured EC to present antigen. The basic culture system was initally modified in two ways in order to be able to maintain EC for extended periods of time: the culture flasks were pretreated with human fibronectin, and ECGF was added to the medium. During the course of these experiments, two additional modifications to the culture system have been made: the EC are grown in "Primaria" tissue culture labware (Falcon, Oxnard, CA), and 90 ug/ml of heparin has been added to the culture medium. Both of these latter modifications appear to enhance further the growth of EC in vitro.

The subcultured EC displayed the classical cobblestone morphology of EC (Figure 1) and by immunofluorescent staining to Factor VIII-related antigen (Figure 2) contained only endothelial cells. By subculturing endothelial cells it was possible to obtain

an endothelial cell preparation that was cleaner than was used in experiments involving primary and secondary cultures of endothelial cells. T-cell donors were then chosen who were either HLA-DR compatible or haplocompatible to the subcultured EC. Since T-cell donors completely compatible for all HLA determinants were not available, the culture system had to allow for the ability to detect antigen-specific proliferation over that resulting from the mixed leukocyte response. Consequently the cultures were harvested on day 4. Preliminary experiments demonstrated that in some individuals it was possible to get an antigen-specific response by their mononuclear cells on day 4. These responses were low compared to those seen in the same experiment harvested on day 6, but when semiallogeneic EC were used to present the antigen the day 6 results were difficult to interpret because of the MLR response. As the endothelial cells were subcultured they were tested for their continued ability to present antigen. The results in Tables 1 and 2 demonstrate that extensively subcultured EC retained the capability to present antigen after having been maintained continuously in culture or after recovery from liquid nitrogen storage. Since any small percentage of nonproliferating or slowly proliferating contaminating cells in early subcultures would only become a smaller percentage to the point of being lost as the EC were subcultured, the fact that the EC continued to present antigen after 18 subcultures (approximately 8 months in culture) is strongly supportive of the conclusion that EC are fully-competent independent

antigen-presenting cells.

Another possible source of undetectable contaminating accessory cells would be the T-cell preparations. If the T-cell preparations contained enough macrophages or any other type of APC (eg. dendritic cells) so that the subcultured EC were only augmenting antigen presentation by any contaminating cells, then the "EC antigen presentation" should be detectable regardless of HLA-DR compatibility between the T cells and EC. In fact that is what Roska et al. found in experiments where EC were used to augment MΦ presentation of antigen (32): EC augmentation of MΦ presentation of antigen is not MHC restricted. We used this evidence to demonstrate that the subcultured EC were not simply augmenting antigen presentation by cells in the T-cell preparations. Table 3 shows an example of where one endothelial cell culture, EC-38, was used to present antigen to several different T-cell populations. Only in the experiments where the T-cell donor was HLA-DR 4 and/or 7 did EC-38 present the cells. This correlated with the EC-38 cells being HLA-DR 4, 7. These results make it very unlikely that the EC are simply serving as a feeder layer.

To insure further that any contaminating MΦ (eg. from the MΦ-depleted lymphocyte preparation) did not contribute to the antigen presentation, the MΦ-specific monoclonal antibody Mac-120 was tested for its effect on EC presentation of antigen. Mac-120 has been shown to block MΦ antigen presentation (29). These observations were confirmed in our culture system (Table 4). When

added to cultures where subcultured EC served as the APC, Mac-120 did not inhibit the EC presentation of antigen. Thus MΦ were not responsible for the T-cell proliferation when EC served as the APC. To extend this observation we have recently cloned human vascular EC and used cloned EC to present antigen to either freshly-isolated T cells or cloned T cells. Preliminary experiments show that in both cases the cloned EC are equally efficient as autologous MΦ at presenting antigen.

Experiments in progress suggest that within the population of responsive T cells there are subpopulations that have a preference for antigen presented by particular APC (i.e., either EC or MΦ). One possible explanation might be that different T-cell clones recognize antigen in the context of different HLA-D region determinants and that the combination of antigen and the various Ia molecules (HLA-DR vs. HLA-DS) may be preferentially found on the different APC. Such a mechanism could add an additional element of regulation to T-cell activation. To test whether T cells responded to antigen in the context of different HLA-D region determinants when presented by EC vs. MΦ, we examined the capability of monoclonal antibodies which recognize HLA-DR and HLA-DS determinants to block antigen presentation by EC or MΦ. As can be seen in Tables 5 and 6, antibodies to HLA-DR determinants (BRL DR.1 and 7.2) are equally efficient at blocking antigen presentation by both MΦ and EC as is Genox 3.53, an antibody to the HLA-DS molecule. Thus, based on the results presented here, presentation of antigen in the

association of different HLA-D region determinants does not seem to account for there being different T-cell clones which respond to the same antigen but only when presented by a particular type of APC. The results were also a surprise from the standpoint that, within the population of cells responsive to one antigen, separate populations of the T cells would be expected to be restricted by HLA-DR vs. HLA-DS determinants. Thus, antibodies to HLA-DR determinants should only block 50% of the response while antibodies to HLA-DS determinants should block the other 50% of the response so that the sum of the blocking should be complete (ignoring the contribution of SB-restricted T cells). Why we found greater than 50% inhibition with antibodies to HLA-DR and HLA-DS determinants is a matter of speculation. Conceivably the antibodies which have been characterized using B cells and MΦ are somehow crossreactive between the HLA-D region determinants on EC. More likely is the possibility that the culture system is not sensitive enough for antibody blocking experiments to detect separate clones responsive to the same antigen but restricted by different HLA-D region determinants.

In summary, we have reported evidence that extensively subcultured human vascular endothelial cells can present antigen to T lymphocytes in vitro. Since the EC retained the capability to present antigen for up to approximately 8-12 months after isolation (18-20 subcultures), they are capable of providing all of the necessary signals for antigen activation of T cells. This conclusion is further supported by preliminary observations showing

that cloned EC can present antigen to either freshly-isolated T cells or cloned T cells. This work along with that previously done suggests that vascular endothelial cells are capable of independently processing and presenting antigen to antigen-specific T cells in vivo. If that is the case, then endothelial cell presentation of antigen may be a crucial feature of T-cell-mediated immune responses.

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Table 1

PRESENTATION OF VARICELLA ANTIGEN BY SUBCULTURED HUMAN ENDOTHELIAL CELLS[a]

Endothelial Cell Subculture Number	Lymphocyte Proliferation to Varicella Presented by: No APC[b]	Autologous MΦ[c]	Subcultured EC[c]
		Δ CPM	
5	-76	5370	6067
6	11	569	7126
7	-278	768	2509
8	201	750	2099
8[d]	351	1320	2273
9	218	1975	1983
9[d]	216	1379	694
12[d]	200	3171	1320
17	38	1392	1522

[a] MΦ-depleted freshly isolated lymphocytes from donor C. W. were cultured with or without varicella, freshly isolated autologous MΦ, or subcultured EC (cells cultured in RPMI 1640 with 15% AB serum added, see Methods).

[b] Δ CPM = (mean CPM lymphocytes + antigen) - (mean CPM lymphocytes)

[c] Δ CPM = (mean CPM lymphocytes + antigen + APC) - (mean CPM lymphocytes + APC)

[d] The EC used in these experiments were from the same umbilical cord vein as the EC used in the other experiments in this table except that the EC were stored in liquid nitrogen for 5 months, thawed, subcultured, and then used in the antigen presentation experiments.

Table 2

PRESENTATION OF PPD BY SUBCULTURED HUMAN ENDOTHELIAL CELLS[a]

Endothelial Cell Subculture Number	Lymphocyte Proliferation to PPD Presented by:		
	No APC[b]	Autologous MΦ[c]	Subcultured EC[c]
		Δ CPM	
5	2430	17532	11274
6	193	9449	3393
7	5122	36576	21027
11	21	869	2181
18	409	5246	7021

[a] MΦ-depleted freshly isolated lymphocytes from donor R. S. were cultured with or without PPD, freshly isolated MΦ, or subcultured EC (cells cultured in RPMI 1640 with 15% AB serum added, see Methods).

[b] Δ CPM = (mean CPM lymphocytes + antigen) - (mean CPM lymphocytes)

[c] Δ CPM = (mean CPM lymphocytes + antigen + APC) - (mean CPM lymphocytes + APC)

Table 3

ENDOTHELIAL CELL LINE 38

ONLY PRESENTS ANTIGEN TO HLA-DR 4 AND/OR 7 T CELLS[a]

Lymphocyte Donor	Lymphocyte HLA-DR Phenotype	Lymphocyte Proliferation to Antigen Presented by		
		No APC[b]	Autologous MΦ[c]	EC-38[c]
			Δ CPM	
M. H.	7	-296	1005[d]	1432
D. B.	5, 9	38	2788	161
R. J.	8	16	2556	-26
S. S.	5	-17	1651	158
S. O.	5, 9	18	1836	-135
T. T.	2, 5	23	1173	346
C. W.	4, 7	122	1681	1431
P. F.	w6	-40	823	162
R. S.	4, 5	193	9449	3200
F. R.	3, 4	-31	5284	4539

[a] MΦ-depleted freshly isolated lymphocytes from various donors were cultured with or without antigen, freshly isolated autologous MΦ, or subcultured EC-38 (the EC used in this set of experiments were a single isolate, culture 38 typed as HLA-DR 4, 7). (See Materials and Methods.)

[b] Δ CPM = (mean CPM lymphocytes + antigen) - (mean CPM lymphocytes)

[c] Δ CPM = (mean CPM lymphocytes + antigen + APC) - (mean CPM lymphocytes + APC).

[d] Underlined values are those where the addition of antigen resulted in at least a 2x increase in the mean CPM.

Table 4

MAC-120 INHIBITS MΦ PRESENTATION OF ANTIGEN

BUT NOT EC PRESENTATION OF ANTIGEN

Experimental Conditions					Experiment		
T	Antigen	MΦ	EC	Mac-120[a]	1	2	3
+	–	–	–	–	2233±2375[b]	523± 392	156± 13
+	+	–	–	–	7355±3472	245± 62	607± 204
+	–	+	–	–	373± 100	659± 581	204± 105
+	+	+	–	–	36949±6128	1427± 365	3375± 896
+	+	+	–	1:40	N.D.	N.D.	1487± 322 (56)
+	+	+	–	1:400	4493±1752 (88)[c]	521± 472 (63)	N.D.
+	+	+	–	1:4000	2248± 813 (94)	519± 331 (64)	2399± 172 (29)
+	+	+	–	1:40000	N.D.	N.D.	2818± 420 (16)
+	–	–	+	–	5718±1119	2952± 730	489± 109
+	+	–	+	–	26745±5206	5461± 681	1809± 142
+	+	–	+	1:40	N.D.	N.D.	1330±101 (26)
+	+	–	+	1:400	25213±1804 (6)	5083± 586 (7)	N.D.
+	+	–	+	1:4000	21080±5895 (21)	4883± 525 (10)	2087± 256 (0)
+	+	–	+	1:40000	N.D.	N.D.	2013± 635 (0)

[a]The monoclonal antibody Mac-120 was employed in a noncytotoxic protocol. The dilutions given are the final dilutions of the antibody in culture.

[b]Responses shown are mean CPM ± SD. N. D. indicates experiment not done.

[c]( ) indicates % reduction of response.

Table 5

MONOCLONAL ANTIBODIES AGAINST HLA-DR DETERMINANTS INHIBIT ANTIGEN PRESENTATION BY MΦ AND SUBCULTURED EC

Experimental Conditions								
T	Antigen	MΦ	EC	Anti-DR[a]	Exp 1	Exp 2	Exp 3	Exp 4
+	–	–	–	–	522± 149[b]	145± 43	712± 76	213± 29
+	+	–	–	–	740± 600	156± 6	913± 624	855± 354
+	–	+	–	–	892± 475	205± 41	422± 106	224± 27
+	+	+	–	–	2867± 421	774± 752	1172± 920	3430± 997
+	+	+	–	1:40	624± 275	203± 67	549± 223	682± 265
+	+	+	–	1:400	840± 300	391± 267	474± 271	1477± 332
+	+	+	–	1:4000	1200± 759	151± 53	513± 172	1092± 204
+	+	+	–	1:40000	1611± 463	211± 192	289± 41	1125± 196
+	–	–	+	–	4340± 296	13139±3156	8398±1603	1242± 92
+	+	–	+	–	6323± 952	20265± 393	10497± 685	9786± 802
+	+	–	+	1:40	1331± 574	5785± 586	1430± 292	3328± 747
+	+	–	+	1:400	5075±1515	11802±2248	7265±1569	4293± 670
+	+	–	+	1:4000	4018±1113	15135±2683	9303±1477	5386± 367
+	+	–	+	1:40000	5133±1158	17180±1400	9611±2995	5543± 284

[a]The monoclonal antibodies were employed in a noncytotoxic protocol. The dilutions given are the final dilutions of the antibody in culture. BRL DR.1 was used in experiments 1-3 while NEI-011 was used in experiment 4. Control monoclonal antibodies (eg. anti-HLA B8) did not inhibit antigen presentation.

[b]Responses shown are mean CPM ± SD.

Table 6

GENOX 3.53, A MONOCLONAL ANTIBODY TO HLA-DS,

INHIBITS ANTIGEN PRESENTATION BY MΦ AND SUBCULTURED EC

Experimental Conditions					Experiment			
T	Antigen	MΦ	EC	Genox 353[a]	1	2	3	4
+	–	–	–	–	156± 13[b]	299± 44	655± 206	370± 124
+	+	–	–	–	607± 204	388± 226	353± 173	218± 71
+	–	+	–	–	204± 105	153± 56	102± 13	119± 34
+	+	+	–	–	3375± 896	12209±2659	3546± 202	3488±1432
+	+	+	–	1:40	185+ 45	232+ 26	309± 32	194± 7
+	+	+	–	1:400	1142+ 257	3300± 341	1000± 268	473± 187
+	+	+	–	1:4000	3011± 564	9813+ 430	1994± 347	1299± 62
+	+	+	–	1:40000	3811± 238	15968± 871	3648± 174	3699± 430
+	–	–	+	–	489± 109	692± 63	471± 101	721± 85
+	+	–	+	–	1809± 142	5222±1592	3314± 356	4788±1165
+	+	–	+	1:40	206± 39	140± 37	276± 117	152± 61
+	+	–	+	1:400	874± 99	942± 66	768± 264	803± 159
+	+	–	+	1:4000	2024± 598	4615± 609	1985± 510	2935±1397
+	+	–	+	1:40000	1884± 292	4209± 856	2441± 496	5315± 888

[a]The monoclonal antibody Genox 353 was employed in a noncytotoxic protocol. The dilutions given are the final dilutions of the antibody in culture. Control monoclonal antibodies (eg. anti-HLA B8) did not inhibit antigen presentation.

[b]Responses shown are mean CPM ± SD.

Figure 1

Photomicrograph of subcultured human umbilical vein endothelial cells (subculture 12). Subcultured endothelial cells reach a confluent monolayer with classical cobblestone morphology 10-14 days after splitting the cells 1:4. (100X).

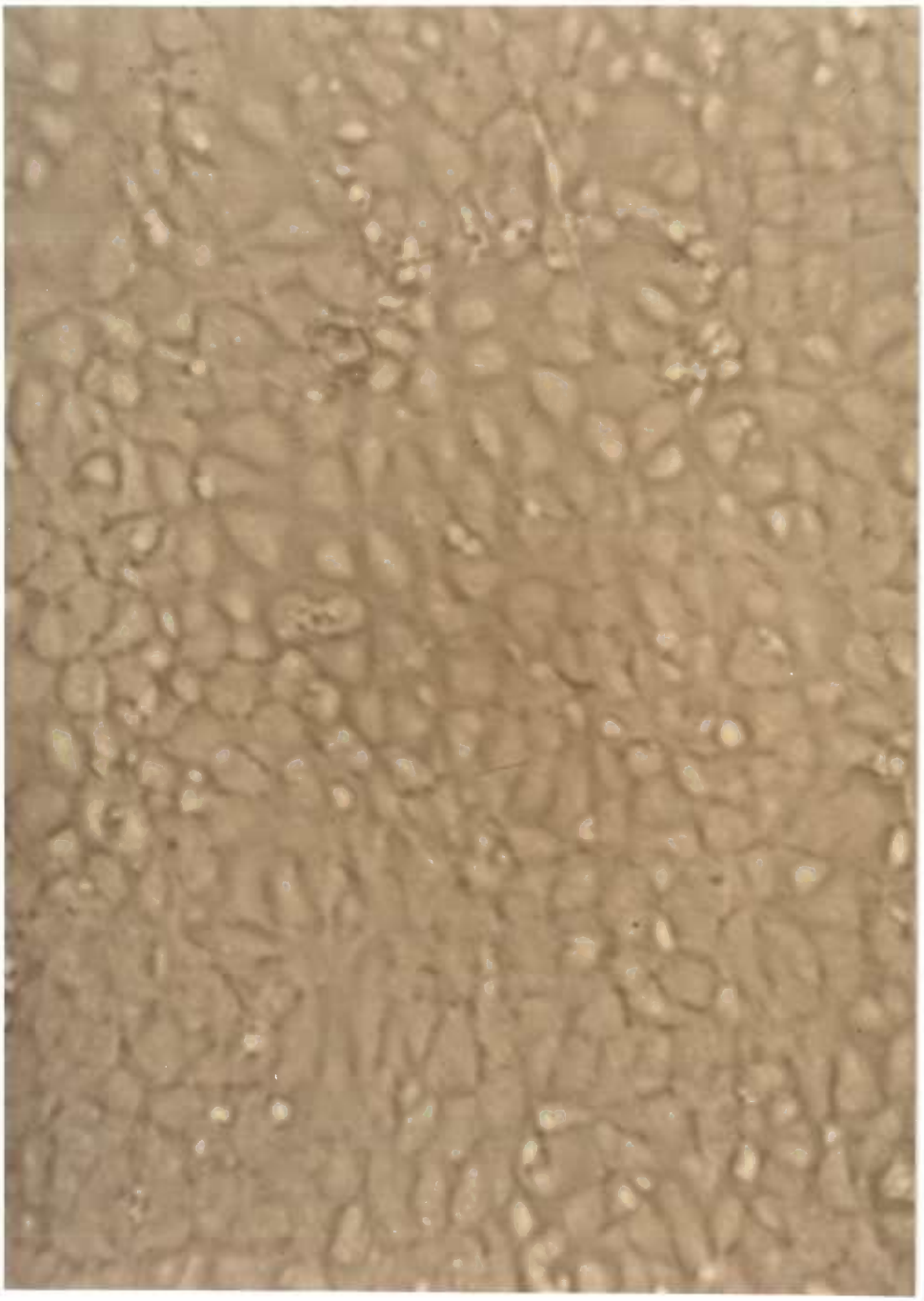
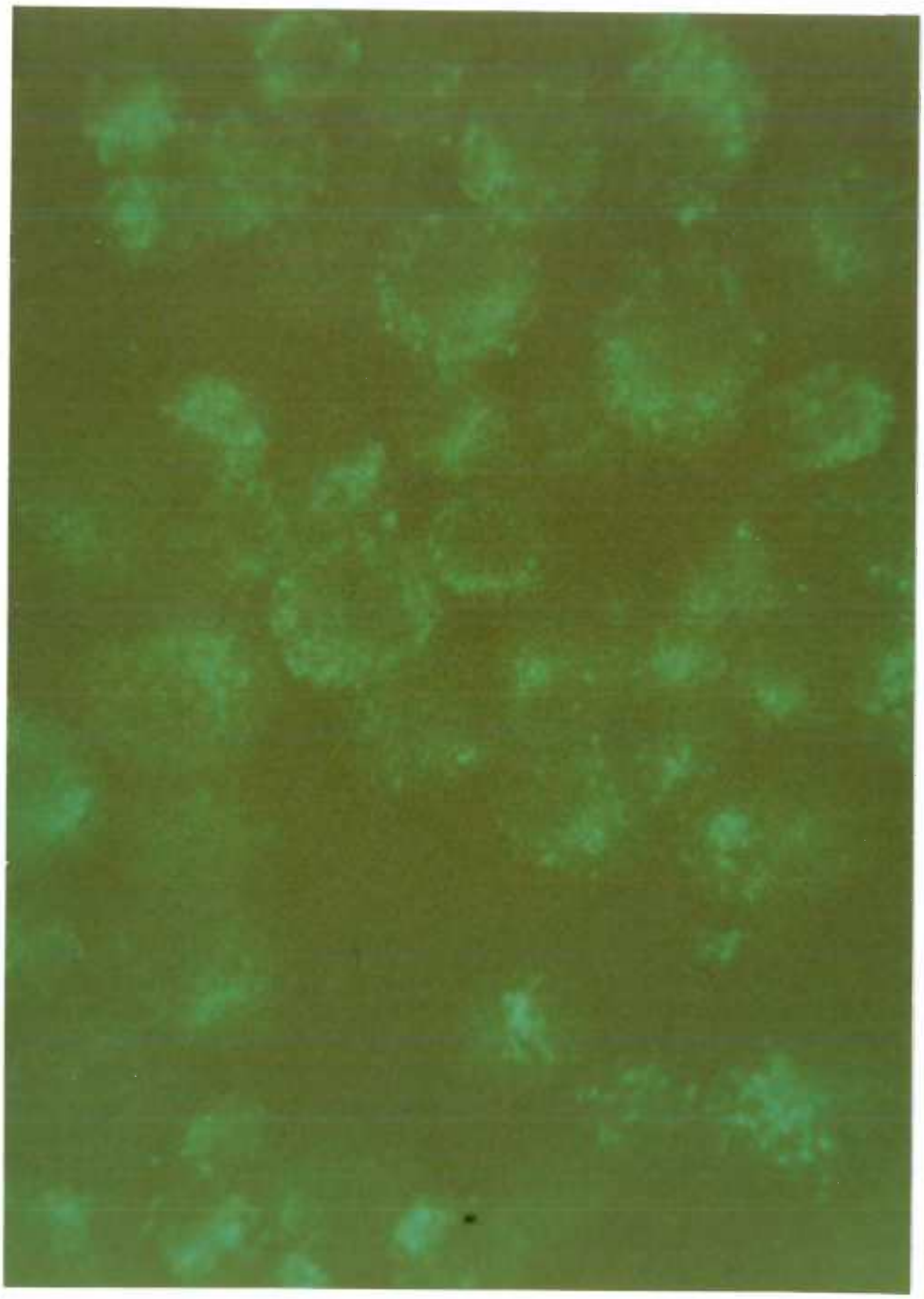


Figure 2

Immunofluorescent staining of subcultured human umbilical vein endothelial cells with monoclonal antibody to Factor VIII-related antigen (subculture 14). Greater than 99% of the subcultured endothelial cells stain with antibody to the endothelial cell specific marker, Factor VIII-related antigen. (400X).



EXPRESSION OF Ia AND INTERLEUKIN 1

BY SUBCULTURED HUMAN ENDOTHELIAL CELLS

ABSTRACT

Activation of T cells requires three signals from an antigen presenting cell: antigen, Ia determinants (HLA-D region determinants in man), and interleukin 1 (IL-1). Recent evidence has suggested that macrophages, dendritic cells, epidermal Langerhan's cells, and endothelial cells can each function as antigen-presenting cells (APC). If these cell types can independently function as APC, they should synthesize both Ia determinants and secrete IL-1. To determine if endothelial cells fulfill these requirements, we have propagated human umbilical vein endothelial cells by serial subculture for extended periods of time and assessed Ia expression and IL-1 secretion. The endothelial cells were subcultured for 8 months (approximately 20 subcultures) and were found to display classic morphology and immunofluorescent staining for the endothelial cell-specific marker Factor VIII-related antigen. In a separate paper we have shown that these subcultured endothelial cells can present antigen to T cells in a HLA-D region restricted fashion.[1] In this paper we present evidence demonstrating that extensively subcultured endothelial cells biosynthesize both HLA-DR and HLA-DS molecules after exposure to T cells and antigen or to a supernatant from antigen-activated T cells. Evidence is also presented that when endothelial cells are cultured in the presence

of lipopolysaccharide they secrete a molecule(s) with IL-1 activity as assayed by LBRM-33-IA5 cell line production of IL-2.

INTRODUCTION

Activation of human T cells requires presentation of antigen by antigen presenting cells (APC) which traditionally have been thought to be cells of the mononuclear phagocytic series (macrophages, MΦ) (1, 2). Antigen presenting cells provide three activation signals to T cells: antigen, Ia determinants (HLA-D region determinants in man), and interleukin 1 (IL-1) (3-10). Recently Langerhan's cells (11-13), dendritic cells (14-16), and endothelial cells (17-21) have also been implicated as antigen-presenting cells. If these latter cell types are capable of antigen presentation, then their expression of Ia determinants and secretion of IL-1 might be expected. The experiments presented here were designed to determine whether human vascular endothelial cells (EC) synthesize Ia determinants and express IL-1 activity.

Since T cells must negotiate the endothelial barrier to egress into extravascular tissues during immune reactions, presentation of antigen by EC could provide an important signaling sequence in the development of cell-mediated immune responses. Using freshly isolated EC, several laboratories have shown that EC can present antigen (17-21). One criticism of those experiments is that the EC or T-cell populations may have contained a small number of MΦ (or other APC) which provided the Ia determinants and IL-1 that allowed presentation of antigen. In order to study rigorously the antigen-presenting capability of EC, we have used extensively

subcultured human umbilical vein endothelial cells to reconstitute antigen-specific proliferative responses by MΦ-depleted T cells. The subcultured EC presented antigen to T cells in a HLA-D region restricted manner[1].

In this paper we have directly analyzed the capability of EC to synthesize Ia determinants and secrete IL-1. Membrane extracts of endothelial cells grown in the presence of ^{14}C-labeled amino acids were immunoprecipitated with monoclonal antibodies to HLA-D region determinants and the resulting immunoprecipitates analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, immunofluorescent staining was used to determine what percentage of the subcultured EC expressed Ia determinants. Two different assays were used to determine if EC secrete IL-1; murine thymocyte mitogenesis and IL-1 dependent LBRM-33-IA5 cell conversion to IL-2 production. Our results show that when EC are stimulated they synthesize both Ia determinants and express IL-1 activity.

MATERIALS AND METHODS

Endothelial cells.

Endothelial cells were isolated from the vein of umbilical cords by a modification of the technique described by Jaffe et al. (22). The fresh cords were cannulated, flushed with saline, and then filled with 0.2% collagenase (Sigma Chemical Co., No. C-0131, St. Louis, MO). After 20 min incubation at 37C, the endothelial cells were flushed out with 20 ml RPMI 1640 (GIBCO, Grand Island, N.Y.) and washed (10 min at 250 X g). The cells were dispersed into 24-well culture plates (approximately 10^4 cells/well) and cultured in RPMI with 20% heat inactivated fetal calf serum and endothelial cell growth factor (23) at 37C and 5% CO_2. Approximately 10% of the cultures grew vigorously becoming monolayers within 4 to 6 days. The cells were released from the plastic by a brief treatment (3 min) with trypsin-EDTA (GIBCO) at room temperature, centrifuged (10 min at 250 X g), and replated into human fibronectin coated (4 ug/ml, Collaborative Research Products, Lexington, MA) 25 cm^2 culture flasks (No. 25100, Corning, Corning, N.Y.). The endothelial cell cultures were expanded by repetitive subculturing (splitting the cells 1:4 within a week of the cells reaching a monolayer). To confirm that the cultured cells were endothelial cells, cytocentrifuged cells were stained with monoclonal antibody to

Factor VIII-related antigen (Cappel Laboratories, West Chester, PA) followed by FITC-conjugated goat anti-mouse IgG (Cappel Laboratories) and then examined with a fluorescent microscope. The endothelial cells were maintained continuously by serial subculture for periods of up to eight months (approximately 20 subcultures). The subcultured endothelial cells displayed classical cobblestone morphology and uniform immunofluorescent staining for Factor VIII-related antigen (an EC specific marker) (24, 25).

Stimulation of EC synthesis of Ia determinants.

Two different protocols were used to stimulate EC synthesis of Ia determinants. (1) Mononuclear cells (MNC) were separated from heparinized (10 u/ml) peripheral blood from normal donors by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation (26). Interface cells were subsequently washed two or three times with RPMI. The MNC were then depleted of MΦ by adherence to plastic followed by passage over Sephadex G-10 as previously described (27). The MΦ-depleted cells characteristically contained 0.1% to 0.4% monocytes by esterase staining and would not support antigen-induced lymphocyte proliferation without macrophage addition. 20 X 10^6 HLA-DR compatible, MΦ-depleted MNC were added to a 75 cm^2 tissue culture flask with 2 X 10^6 EC in a total of 10 ml of RPMI 1640 containing 15% AB serum and antigen. After 48 hrs of incubation at 37C and 5% CO_2 the culture flask was shaken

and the media containing the nonadherent cells recovered. The recovered media was centrifuged (10 min at 250 X g) to remove the cells, and then the cell-free media was added back to the flask. The EC were then cultured for another 24 hours at which time they were used in either an immunoprecipitation experiment or in an immunofluorescent staining experiment. (2) MNC were prepared as described above. The MNC were then cultured 48 hours at a concentration of 2 X 10^6/ml in RPMI 1640 containing 15% AB serum and antigen. At the end of the culture period the media was recovered and centrifuged to remove the cells. The resulting activated T-cell supernatant was then added directly to cultures of EC (approximately 10 ml of supernatant per 2 X 10^6 EC). The EC were then cultured for another 48 hours at which time they were used in either an immunoprecipitation experiment or in an immunofluourscent staining experiment.

Immunoprecipitation of Ia determinants from biosynthetically radiolabeled EC.

Subcultured EC were stimulated to synthesize Ia determinants using the protocols described above. During the last 4-6 hrs of the incubation period the EC were cultured in the presence of 1 mCi L-[^{14}C(u)]-amino acid mixture (NEC-445E, New England Nuclear, Boston, MA) and stimulant (i.e. an activated T-cell supernatant that had been extensively dialyzed to remove free unlabeled amino acids),

both of which were suspended in RPMI 1640 minus amino acids (without the amino acids in the ^{14}C-labeled mixture, RPMI 1640 select-amine kit, GIBCO). The exception to this labeling procedure was in some preliminary experiments where MΦ-depleted T cells and antigen were added directly to the EC (Ia stimulation protocol 1) and the ^{14}C-amino acids were present throughout the 72 hr incubation. The internally radiolabeled EC were recovered from the culture flask with the aid of a cell scraper. The EC were washed twice with RPMI. The final cell pellet was extracted with 2.0 ml of 2% Nonidet P-40 (Sigma) in 25 mM NaCl/25 mM Tris-HCl, pH 7.4/0.1 mM phenylmethylsulfonyl fluoride (Sigma)/50 ug/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (Sigma)/50 ug/ml N-p-tosyl-L-lysine chloromethyl ketone (Sigma) for 30 min at 4C and the detergent extract was clarified by centrifugation. Nonspecifically precipitating material was removed by incubation with prewashed Protein A Sepharose-CL-4B (Sigma) to which goat anti-mouse IgG (Cappel) had been bound. The supernatant was then mixed with monoclonal antibody. After 2 hrs the immune complexes were adsorbed with Protein A Sepharose-CL-4B-goat anti-mouse IgG by mixing overnight at 4C. The complexes were washed 5 times with buffer (0.2 M NaCl, 0.5% NP-40, 0.0125 M K-PO_4, pH 7.5). The immunoprecipitated proteins were eluted from the complex with 30 mg DTT in 150 ul of sample buffer (0.5 M Tris-Cl, pH 6.8: Glycerol: 10% (w/v) SDS: 2-ME, 1:1:4:1), 30 min, 100C. The supernatants were then loaded on top of 10% SDS-polyacrylamide disc gels and

electrophoresed as described below.

The immunoprecipitated products were analyzed by SDS-PAGE under reducing conditions following the procedure of Laemmli (28) (disc gel electrophoresis apparatus, Bio-Rad, Rockville Centre, N.Y.). The radiolabeled molecular weight standards: bovine serum albumin (69,000d), ovalbumin (46500d), carbonic anhydrase (30,000d), lactoglobulin (18,000d), and cytochrome C (12400d) (New England Nuclear), were electrophoresed simultaneously in a separate gel. The disc gels were mechanically sliced. The individual slices were placed in glass counting vials and the dissolved in 0.2 ml of 30% hydrogen peroxide solution (12 hrs, 56C). Aquasol (New England Nuclear) was then added and radioactivity in the slices measured using a Prias Tri-Carb Beta Counter (Packard Instruments, Downers Grove, IL).

The following murine monoclonal antibodies were used to immunoprecipitate human class II MHC determinants: BRL DR.1 (Bethesda Research Laboratories, Gaithersburg, MD); NEI-011, clone 7.2 (New England Nuclear); and Anti-Leu 10 (Becton-Dickinson, Sunyvale, CA). A monoclonal antibody directed against the RBC-M antigen (Epitope, Inc., Portland, OR) was used as a negative control. Human B cells were used as a positive control for Ia bearing cells.

Immunofluorescent staining of Ia determinants on EC.

Subcultured EC were stimulated to synthesize Ia determinants using the protocols described above. The stimulated EC were recovered from the culture flask by a brief exposure (1-3 min.) to trypsin-EDTA. The recovered cells were immediately centrifuged (10 min, 250 X g) and then resuspended in RPMI. The cells were then cytocentrifuged onto slides which had been pretreated by soaking (30 min, room temperature) in a 1% BSA/PBS (0.01M, pH 7.2) solution. The EC slides were then fixed by immersion into 95% ethanol for 10 min. at room temperature. The slides were then washed with water followed by PBS and the EC covered with the primary antibody solution (primary antibodies are listed above). After an incubation period of 45 min at room temperature the slides were washed again and then the EC were covered with a solution of FITC conjugated goat anti-mouse IgG (Cappel Laboratories). Again after an incubation period of 45 min at room temperature, the slides were thoroughly washed. The slides were then examined with a fluorescent microscope.

Preparation of endothelial cell supernatants containing IL-1 activity.

Subcultured EC were stimulated to secrete IL-1 by exposure to 5-50 ug/ml of Lipopolysaccharide (E. coli 055:B5, cat. no. 3923-25, Difco Laboratories, Detroit, MI) in RPMI with 20% fetal calf serum

for either 24 or 48 hours. The media was aspirated and centrifuged (10 min, 250 X g) to remove any cells. The supernatant was extensively dialyzed against saline and finally RPMI. After dialysis, the supernatant was filter sterilized and stored at -70C. Postive control supernatants were similarly generated by exposing plastic-adherent human peripheral blood lymphocytes (i.e., macrophages) to lipopolysaccharide (LPS). Negative control supernatants consisted of RPMI containing serum and LPS treated in the same manner as the other supernatants. The EC supernatants and the control supernatants were tested for the presence of IL-1 activity by either a thymocyte proliferation assay or by IL-1 dependent LBRM-33-IA5 production of IL-2 as described below. Since both IL-1 assays give a false positive in the presence of IL-2, the EC and control supernatants were also analyzed for IL-2 activity by measuring HT-2 cell line (29) incorporation of ^{3}H-thymidine in the presence of the supernatants.

Thymocyte proliferation assay.

Thymocytes prepared from C57BL/6N mice (Simonsen Laboratories, Gilroy, CA) were cultured at 7.5 X 10^{6} thymocytes/ml in flat-bottomed 96-well microplates containing various dilutions of EC and control supernatants added in quadruplicate. The cultures were incubated 72 hrs at 37C and 5% CO_2. Six hours before harvesting, the wells were pulsed with 0.1 uCi ^{3}H-thymidine (specific activity

6.7 Ci/mMole, New England Nuclear) and were subsequently harvested with the aid of a multiple automated sample harvester (Titertek cell harvester, Flow Laboratories, Rockville, MD). Samples were counted by liquid scintillation in a Prias Tri-Carb Beta Counter.

IL-1-dependent LBRM-33-IA5 production of IL-2.

Assessment of IL-1 by the use of IL-1 dependent LBRM-33-IA5 production of IL-2 was performed by Steven Hefeneider (Immunex Corp., Seattle, WA). One hundred microliters of LBRM-33-IA5 cells (5 X 10^5 cells/ml) inactivated by mitomycin C (50 ug/ml, 1 hr., 37C), were cultured in 96-well flat bottomed plates in the presence of tissue culture medium alone, PHA (Phytohemmagglutinin M; 0.1% final concentration by volume, GIBCO), endothelial cell or control supernatants (at several dilutions), or both PHA and supernatant. After 24 hrs of incubation at 37C and 5% CO_2, IL-2 activity was determined by the direct addition of 50 ul of CTLL-2 cells (8 X 10^4 cells/ml). The microwell cultures were incubated for an additional 20 hrs, followed by a 4 hr pulse with 0.5 uCi of ^{3}H-thymidine (specific activity 20 Ci/mMole, New England Nuclear. The thymidine-pulsed cultures were harvested onto glass fiber filter strips with the aid of a multiple automated sample harvester (MASH II; Microbiological Associates, Bethesda, MD). ^{3}H-thymidine incorporation was measured by liquid scintillation counting.

Units of IL-1 activity were determined by using the reciprocal

dilution of the sample that caused 50% maximum indicator cell line proliferation. One unit of activity was thus equivalent to the number of microliters present in the culture that caused 50% of maximal proliferation. For example, if a 1:10 dilution of the sample caused 50% maximal ^{3}H-thymidine incorporation, the microliter value associated with that dilution, i.e., 1/10 of 200 ul or 20 ul was said to contain 1 unit. A 1 ml aliquot of that test sample would therefore contain 50 units of activity/ml.

RESULTS

Synthesis of HLA-D region determinants by subcultured endothelial cells.

Our laboratory (17, 18) and others (19, 21) have reported that antigen presentation by endothelial cells is HLA-D region restricted. This implied that endothelial cells expressed class II MHC determinants. Yet, when subcultured human endothelial cells (EC) were examined for HLA-DR determinants by immunofluorescent staining they appeared negative. Based upon experience with macrophages (MΦ) suggesting that synthesis of Ia determinants is inducible, we studied EC expression of HLA-DR determinants after induction by (a) T cells and antigen or (b) supernatants from antigen-activated T cells. Under both conditions EC expressed HLA-DR determinants. As can be seen in Figure 1, after stimulation with a supernatant from antigen-activated T cells, subcultured EC stained uniformly with monoclonal antibody to a monomorphic HLA-DR determinant. Detection of Ia determinants was possible after 24 hrs of exposure to supernatants from activated T cells and was maximal after 48 hrs of exposure.

The EC Ia determinants could have been synthesized by the cells or acquired cytotropically from the T-cell supernatant. In order to select between these alternatives, we attempted to demonstrate

directly the synthesis of class II MHC determinants by endothelial cells. The endothelial cells were cultured with stimulants as in the staining experiments described above, only in these experiments 4-6 hrs prior to harvesting the EC ^{14}C-amino acids were added to the culture media. The EC membranes were then extracted and the class II MHC determinants specifically immunoprecipitated with monoclonal antibodies. When such endothelial cell membrane extracts were exposed to monoclonal antibodies to HLA-DR determinants, SDS-PAGE of the precipitates resulted in peaks of radioactivity at 32 Kd and 29 Kd (Figure 2), typical of the HLA-DR alpha and beta chains, respectively, that have been isolated from macrophages and B cells (30). It was also possible to immunoprecipitate the internally radiolabeled alpha and beta chains (31 Kd and 29 Kd, respectively) of HLA-DS molecules (31-34) from endothelial cells (Figure 3). When HLA-DR and HLA-DS molecules were immunoprecipitated from the same EC membrane extract, the alpha chain of the HLA-DS molecule had a lower molecular weight than that of the HLA-DR molecule (eg. 31 Kd in Figure 3 vs. 32 Kd in Figure 2).

Subcultured endothelial cells secrete IL-1 activity.

Synthesis of IL-1 by the antigen presenting cell is thought to be essential for antigen-induced T-lymphocyte proliferative responses (6-10). To examine whether endothelial cells secrete IL-1, they were cultured in the presence of lipopolysaccharide (LPS)

for 24 or 48 hrs and the media harvested from the culture flasks and tested for the presence of IL-1. This procedure was selected since LPS is known to stimulate human macrophages to secrete IL-1 (35, 36). When the endothelial cell supernatants were tested in a murine thymocyte proliferation assay for the presence of IL-1, thymocyte proliferation was only slightly enhanced by the EC supernatants compared to the control media (Table 1). For example, thymocyte proliferation in the presence of the EC supernatants resulted in S.I. from 1.5 to 3.7 in the 6 experiments (essentially all negative results except for experiment 2 with a S.I. of 3.67). Adding suboptimal doses of either phytohemaglutinin or concanavalin A along with the EC supernatants to the thymocyte proliferation assay (a costimulation assay) did not result in higher S.I. (results not shown). Supernatants from LPS-stimulated adherent human mononuclear cells (macrophages) were used as a positive IL-1 control in these experiments and typically gave a mean CPM at least two times greater than that from the EC. Since IL-2 will also induce thymocyte mitogenesis (37), supernatants from LPS-stimulated endothelial cells and macrophages were also tested for the presence of IL-2 using the IL-2 dependent cell line, HT-2 (29). Neither type of supernatant ever gave a positive response in an IL-2 assay (results not shown).

While the supernatants from the LPS-stimulated endothelial cells did enhance thymocyte proliferation, the results were not demonstrative. A second IL-1 assay was used to improve upon the thymocyte proliferation results. LBRM-33-IA5 cells have been

characterized as a cell line that produces IL-2 when exposed to IL-1 and PHA (38, 39). The presence of IL-1 then is detected indirectly by assaying the amount of IL-2 produced using an IL-2 dependent cell line, CTLL-2 (39). Endothelial cell supernatants assayed in this manner were clearly positive for IL-1 activity (Table 2). That is, at a 1:10 dilution the endothelial cell supernatants induced IL-2 production that resulted in a level of proliferation ranging from 30,000 to 40,000 CPM vs. 2,730 CPM with the control media. Supernatants were collected from LPS-stimulated endothelial cells as the EC were subcultured. As can be seen in Table 2, subcultured EC (from the 9 to 12 subculture) retained the ability to express IL-1 activity.

While endothelial cells clearly secreted IL-1 activity, supernatants from EC did not contain as much activity as supernatants from approximately the same number of macrophages. Figure 4 shows dilution response curves for endothelial cell and macrophage supernatants in both the thymocyte proliferation assay (panel A) and LBRM-33-IA5 assay (panel B). In both types of assays the macrophage supernatants consistently contained higher levels of IL-1 activity than the EC supernatants. By comparing the two panels it is also evident that the LBRM-33-IA5 assay is much more sensitive than the thymocyte assay.

DISCUSSION

Endothelial cells line the blood vessels and lymphatics forming a barrier that circulating lymphocytes must transgress in order to reach antigens within the tissue. Burger and Vetto have published a hypothesis (40) suggesting that endothelial cells may play an active role in signaling lymphocytes to leave the circulation near sites of extravascular antigen. In particular they suggested that circulating T cells recognize antigen on the surface of EC, resulting in activation of EC such that the EC then release the key mediators of a CMI response. The EC mediators would then lead to lymphocyte activation and cellular egression into the extravascular tissue. This theory would account for the brief cell-cell exposure which occurs during ordinary hemodynamics, the low frequency of antigen-specific cells in the blood, and the rapid dilution of factors by blood flow, as the EC could become activated after brief contact with antigen-specific T cells and then the activated EC could focally release mediators. Therefore, while antigen-specific T cells would still provide the specificity to the CMI response, it would be the EC instead of the T cells that would be the central participant in the CMI response.

According to this hypothesis, part of the central role that EC would play in a CMI response is to present antigen to circulating T lymphocytes. We (17, 18) and others (19-21), have demonstrated that

freshly isolated human umbilical vein endothelial cells present antigen in a HLA-D region restricted fashion. One criticism of those experiments is that the freshly isolated endothelial cell preparations may have contained contaminating cell types which were either totally responsible for the antigen presentation or whose presence was necessary for the EC to present antigen.

By maintaining human umbilical vein endothelial cells in culture for extended periods of time (8-12 months, 18-20 subcultures) we were able to obtain homogeneous preparations of EC that displayed uniform staining for Factor VIII-related antigen (an endothelial cell specific marker) (24, 25) but could not survive in culture indefinitely. When used to present antigen to T cells, we found that extensively subcultured endothelial cells retained the capability to present antigen in a HLA-D region restricted manner[1].

Our finding that subcultured human endothelial cells can independently function as fully competent antigen presenting cells[1] implies that EC can provide all of the necessary signals for antigen-specific T-cell activation. Since Ia determinants and interleukin 1 are thought to be signals involved in presentation of antigen (3-10), one would expect EC to express both Ia determinants and interleukin 1.

The implication that EC synthesize Ia determinants is supported by the findings of Hirschberg et al. that human endothelial cells can be destroyed by treatment with complement and anti-DRw antisera (41, 42). Yet, as stated above, immunofluorescent staining of

resting subcultured EC with monoclonal antibodies to monomorphic HLA-DR determinants was negative. Pober et al. reported similar results using primary cultures of pooled human umbilical vein EC (43).

Synthesis and expression of class II MHC determinants by macrophages is inducible by supernatants of activated T cells (44, 45) or γ interferon (46). Using monoclonal antibody binding techniques along with immune precipitation of radioiodinated EC surface proteins, Pober et al. have shown that primary cultures of pooled human umbilical vein EC express Ia determinants when exposed to either PHA (in the presence of contaminating T cells), medium conditioned by PHA-activated peripheral blood mononuclear cells, allogeneic T cells, or recombinant human γ interferon (47). Gamma interferon has been reported to activate multiple class II MHC genes in endothelial cells as measured by detection of mRNA for HLA-DR, -DS, and -SB heavy and light chains (48). Additionally, Pober et al. have reported that recombinant γ interferon induces the expression of Ia determinants by multiply-passaged human foreskin capillary EC and human umbilical vein EC that had been transformed by Simian virus 40 viral DNA (49). In the same publication they also reported that γ interferon induces biosynthesis of Ia antigens by human foreskin capillary EC, and that Ia antigens induced by interferon on human umbilical vein EC can be functionally recognized by a human cytotoxic T lymphocyte line.

Using extensively subcultured human umbilical vein EC we have

examined the capability of EC to synthesize HLA-D region determinants. Subcultured EC provide a population of EC which are free of any contaminating cells (i.e., they are free of cells such as macrophages that may contaminate early subcultures), and their cellular characteristics and functions should be representative of normal EC (i.e., they have not been transformed). Immunofluorescent staining was used to define the culture conditions which allowed detection of EC Ia determinants. It was found that after exposure to an activated T-cell supernatant cytocentrifuged preparations of EC stained positively for HLA-DR determinants (Figure 1). When endothelial cells were similarly stimulated with an activated T-cell supernatant or by exposure to T cells and antigen, the subcultured endothelial cells were found to synthesize both HLA-DR and HLA-DS determimants as demonstrated by immunoprecipitation of biosynthetically radiolabeled EC proteins (Figures 2 and 3, respectively) . Anti-Leu-10 antibody has been shown by serial immunoprecipitations of B cell lines expressing HLA-DR types 1-6, 8, or 9 to react with non-HLA-DR molecules that comigrate with the DS1 molecule (34). While serial immunoprecipitations of the EC lysates were not done, the findings from serial immunoprecipitations done on the B-cell lysates would suggest that BRL DR.1 and NEI-011, clone 7.2 are binding different EC molecules than anti-Leu-10. Also when the same EC lysate was immunoprecipitated simultaneously with BRL DR.1 and anti-Leu-10 it was observed that the α chain precpitated by anti-Leu-10 migrated faster than that precipitated by BRL DR.1,

suggesting that different EC molecules were bound by the antibodies to HLA-DR vs. HLA-DS. That the class II MHC determinants synthesized by the subcultured EC are functional is demonstrated by EC presentation of antigen in an HLA-D region restricted manner[1].

Although we have not identified the mediators that are responsible for the induction of EC Ia, a likely candidate in our experiments is γ interferon. Gamma interferon as the responsible mediator is suggested by the findings of Steeg et al. that the MΦ Ia regulatory mediator in a Con A supernatant shares antigenic and biochemical characteristics with γ interferon (50). Moreover, as mentioned above, γ interferon is known to induce MΦ (46) and EC (47, 49) expression of Ia.

In addition to presenting antigen in the context of Ia determinants, antigen presenting cells are thought to provide a third activation signal to T cells, IL-1 (6-10). Endothelial cells have been reported to augment T-lymphocyte responses to mitogen or antigen in cultures supported by macrophages (51). It is likely that the EC augmented the T-lymphocyte responses by secreting IL-1, especially since EC supernatants also augmented mitogen-induced T-cell proliferation in the presence of macrophages (51). If EC can independently function as antigen presenting cells (i.e., in the absence of MΦ or any other APC), then because of the T-cell requirement for IL-2 it is important that EC produce a mediator that facilitates IL-2 production (eg. IL-1). To show that EC secrete such a mediator, supernatants from EC free of any contaminating cell

types were tested in IL-1 assays. Extensively subcultured EC were used to produce supernatants from EC free of any other cell types. Using lipopolysaccharide at doses previously reported to stimulate macrophage secretion of IL-1 (35, 36), subcultured endothelial cells were stimulated to secrete IL-1 into the culture medium. Supernatants from LPS-stimulated EC were assayed for IL-1 activity using both the traditional murine thymocyte proliferation assay (37) and the LBRM-33-IA5 cell line (39). When the supernatants were assayed by thymocyte proliferation marginal IL-1 activity was detected (Table 1). Consequently a more sensitive IL-1 assay was employed; namely IL-1-dependent LBRM-33-IA5 cell production of IL-2. Figure 4 shows dilution response curves for MΦ and EC supernatants containing IL-1 activity and for an IL-2 containing supernatant for both types of IL-1 assay systems. Comparison of the two assay systems confirms the greater sensitivity of the LBRM assay (39), while comparison of the dilution response curves for the MΦ and EC supernatants demonstrates the higher level of IL-1 activity from MΦ supernatants. LPS-stimulated EC supernatants contained about 1/100 of the activity found in supernatants from approximately the same number of MΦ (500 U/ml of IL-2 induced by the EC supernatant vs. 50000 U/ml of IL-2 induced by the MΦ supernatant in the LBRM-33-IA5 assay). The higher level of MΦ secretion of IL-1 seems to be a function of the cells used as the same pattern of results were obtained when the dose of LPS was varied (5-50 ug/ml) and when the supernatants were collected at different time intervals

(results not shown). Again, it is extremely unlikely that the IL-1 activity in the EC supernatants is from contaminating MΦ; the EC preparations stained uniformally for a cytoplasmic EC-specific marker and any undetected MΦ would not be expected to multiply, resulting in their loss after only a couple of subcultures. As was shown in Table 2, EC retain the capability to produce IL-1 activity after extensive subculturing. This finding was anticipated based upon the continued ability of the EC to present antigen after 18-20 subcultures[1]. Therefore, while we have not directly shown that EC synthesize IL-1 (i.e., we have not isolated biosynthetically labeled EC IL-1), the continued ability of EC to secrete IL-1 after extensive subculturing is strongly suggestive that EC do synthesize IL-1.

The IL-1 activity secreted by EC is likely attributable to a molecule very similar to MΦ IL-1. This has been shown to be the case for keratinocyte (52) and glomerular mesangial cell (53) thymocyte-activating factors. Biochemical confirmation of the similarity between endothelial cell and macrophage IL-1 remains to be done. Even if the two molecules are not identical, what is probably of greater importance is that an EC mediator facilitates the production of IL-2 as is evidenced by the LBRM-33-IA5 assay. It would be of importance because facilitation of IL-2 production by IL-1 is thought to be necessary for T-cell proliferation (6-8).

The results of the experiments reported in this paper substantiate the antigen presenting capability of EC by

demonstrating that EC can synthesize Ia determinants and express IL-1 activity, both of which are signals from antigen presenting cells that are required for antigen-activation of T cells. These findings further support the hypothesis that endothelial cells may be an important regulatory element of cell-mediated immunity.

FOOTNOTE

[1]Wagner, C. R., R. M. Vetto, and D. R. Burger. Subcultured human endothelial cells can independently function as fully competent antigen presenting cells. Manuscript submitted for publication.

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TABLE 1

PRODUCTION OF IL-1 ACTIVITY BY SUBCULTURED HUMAN ENDOTHELIAL CELLS AS DETECTED BY A MURINE THYMOCYTE PROLIFERATION ASSAY[a]

	CONDITIONS FOR IL-1 PRODUCTION		
	MEDIA	EC	
EXPERIMENT	with LPS	with LPS	S.I.
1	741 ± 161[b]	1906 ± 204	2.57[c]
2	2995 ± 715	10996 ± 364	3.67
3	1363 ± 118	2015 ± 426	1.48
4	487 ± 453	1056 ± 226	2.17
5	3714 ± 481	6762 ± 1131	1.82
6	444 ± 24	858 ± 181	1.93

[a] Subcultured EC were cultured in RPMI containing 25 ug/ml LPS for 48 hours at which time the supernatants were collected, dialyzed against fresh RPMI, and then tested at final dilutions of 1:2 and greater for the capability to induce thymocyte proliferation (C57BL/6N mice, 7.5 X 10^6 thymocytes/ml) (see Materials & Methods). The results given above are for the final dilution of the supernatant that gave the largest S.I. (generally seen at a final dilution of 1:4). Supernatants from plastic adherent PBL stimulated with 25 ug/ml LPS generally gave mean CPM values at least 2X greater than those from EC stimulated with LPS.

[b] The results are given as the mean CPM ± SD.

[c] S.I. = (mean CPM EC+LPS) ÷ (mean CPM media+LPS).

TABLE 2

IL-1 PRODUCTION BY SUBCULTURED HUMAN ENDOTHELIAL CELLS AS DETECTED BY IL-1 DEPENDENT LBRM-33-IA5 CELL LINE PRODUCTION OF IL-2

FINAL SUPERNATANT DILUTION	CULTURE MEDIA WITH LPS	MΦ CONDITIONED MEDIA[a]	ENDOTHELIAL CELL CONDITIONED MEDIA Sc9[b]	Sc10	Sc11	Sc12
1:3	3456	10330	34762	N.D.	N.D.	N.D.
1:10	2730	13772	33144	39470	35100	31854
1:50	2314	23578	26304	26008	16916	22752
1:200	3578	29806	15464	21062	15162	23856
1:700	8424	27806	13010	20772	13918	17246
1:3000	4242	30650	5970	18412	16940	18412

[a] MΦ or EC were exposed to 5 ug/ml LPS (E. coli 055:B5, Difco Laboratories) for 48 hrs prior to collection of cell supernatants. The supernatants were dialyzed against saline followed by RPMI and then assayed for IL-1 activity using the LBRM-33-IA5 cell line as described in Materials and Methods.

[b] Subculture of the EC stimulated to generate the supernatant tested (i.e., Sc9 stands for subculture 9).

Figure 1

Immunofluorescent staining of subcultured human EC with NEI-011, a monoclonal antibody to a HLA-DR monomorphic antigen. Endothelial cells were exposed to a supernatant from antigen-activated T cells for 48 hrs, were cytocentrifuged, and then stained with NEI-011 followed by FITC-conjugated goat anti-mouse IgG. (400X).

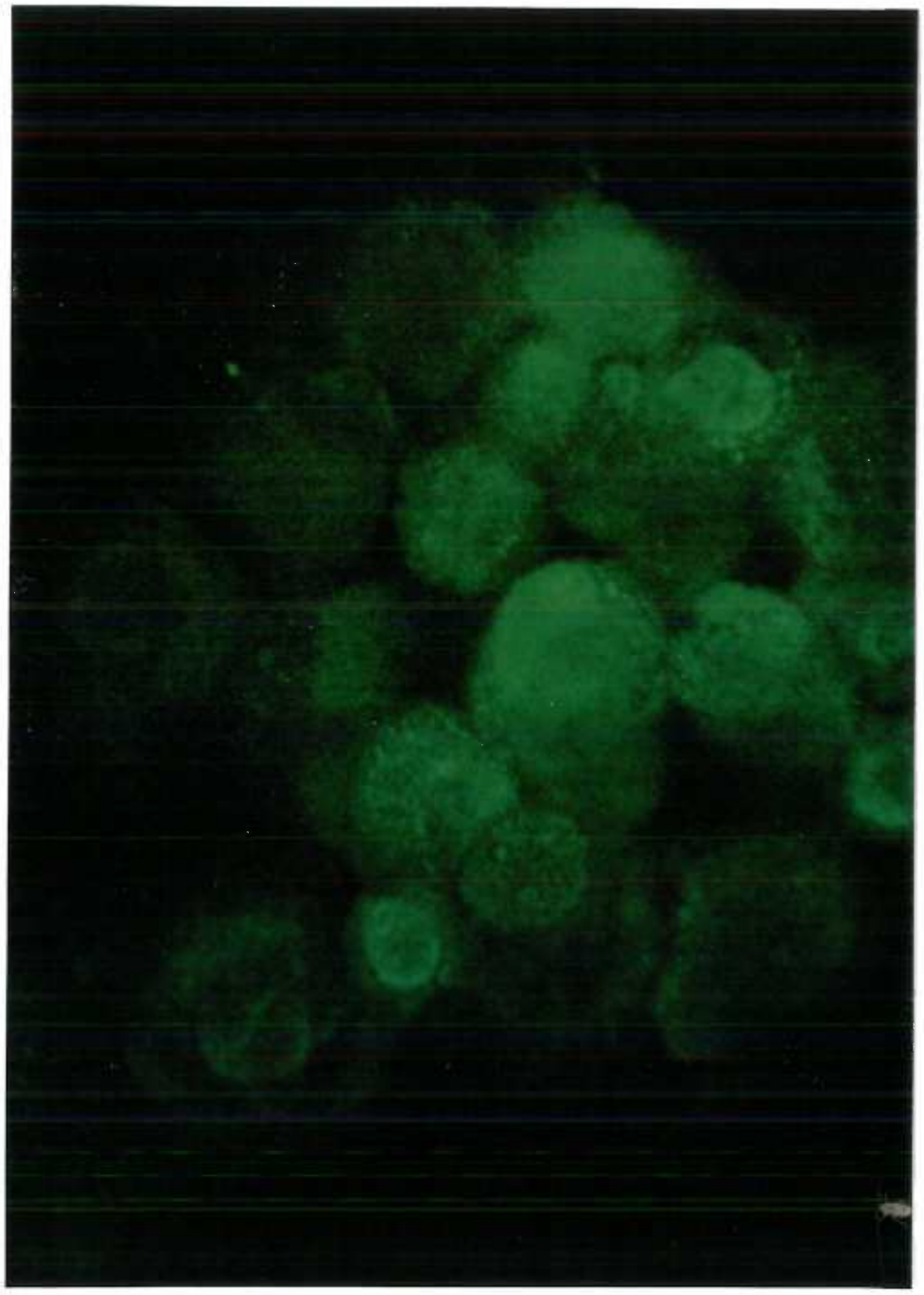


Figure 2

Synthesis of HLA-DR molecules by subcultured human endothelial cells. Endothelial cells were exposed to a supernatant from antigen-activated T cells for 48 hrs, the last 4 hrs in the presence of ^{14}C-amino acids. Data points (●-●) represent CPM per gel slice of ^{14}C-amino acids incorporated into proteins immunoprecipitated by BRL DR.1 monoclonal antibody following SDS-PAGE. The positions of the molecular weight standards (listed in Materials and Methods) following electrophoresis are also shown (●-●).

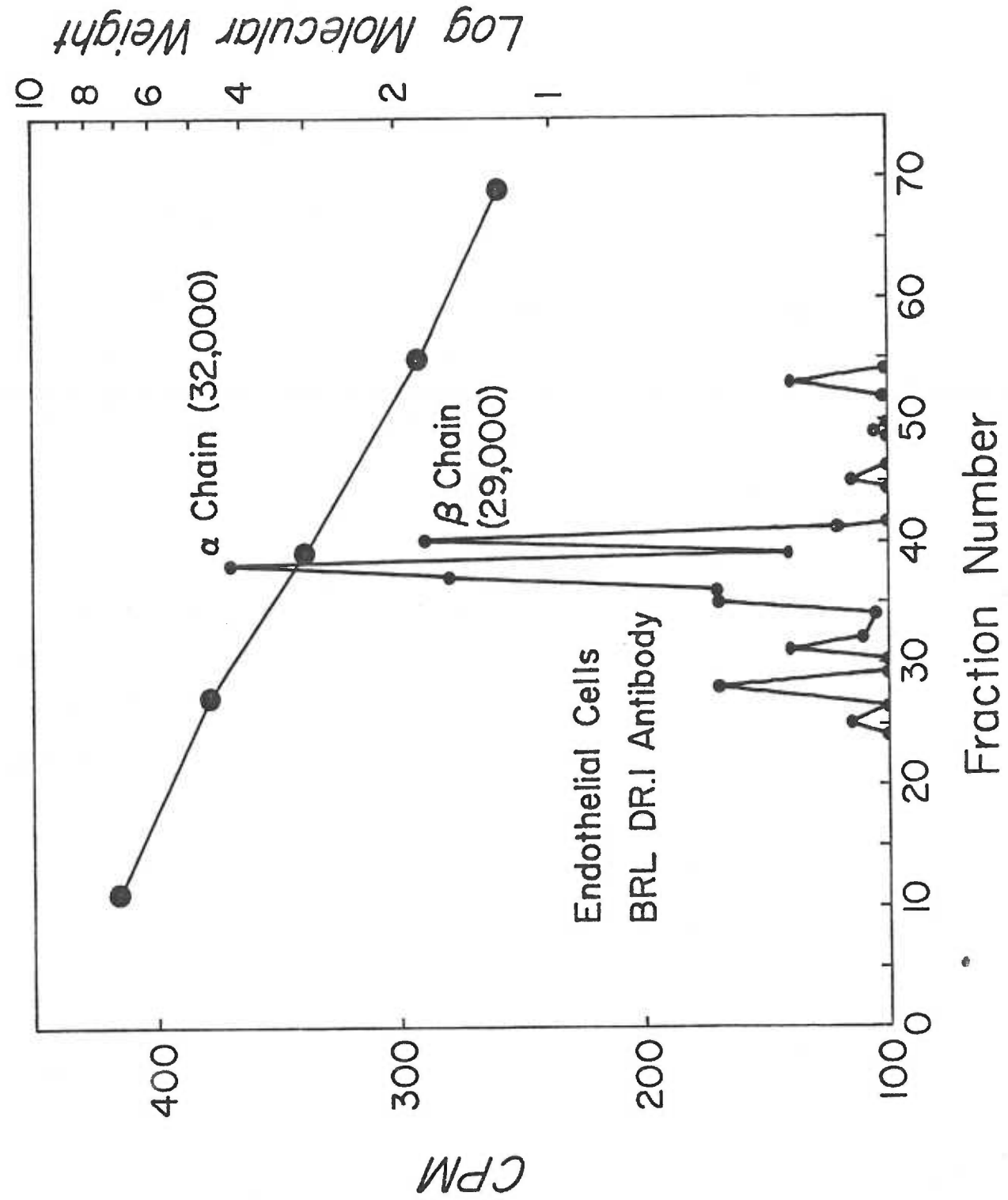

Log Molecular Weight
10
8
6
4
2
1
α Chain (32,000)
β Chain
(29,000)
Endothelial Cells
BRL DR.I Antibody
CPM
400
300
200
100
0
10
20
30
40
50
60
70
Fraction Number

Figure 3

Synthesis of HLA-DS molecules by subcultured human endothelial cells. Endothelial cells were exposed to MΦ-depleted T cells and antigen for 48 hrs, the nonadherent cells were removed, and the EC were cultured for an additional 24 hrs, the last 4 hrs in the presence of ^{14}C-amino acids (see Materials and Methods). Data points (●-●) represent CPM per gel slice of ^{14}C-amino acids incorporated into proteins immunoprecipitated by Leu 10 monoclonal antibody following SDS-PAGE. The positions of the molecular weight standards (listed in Materials and Methods) following electrophoresis are also shown (●-●).

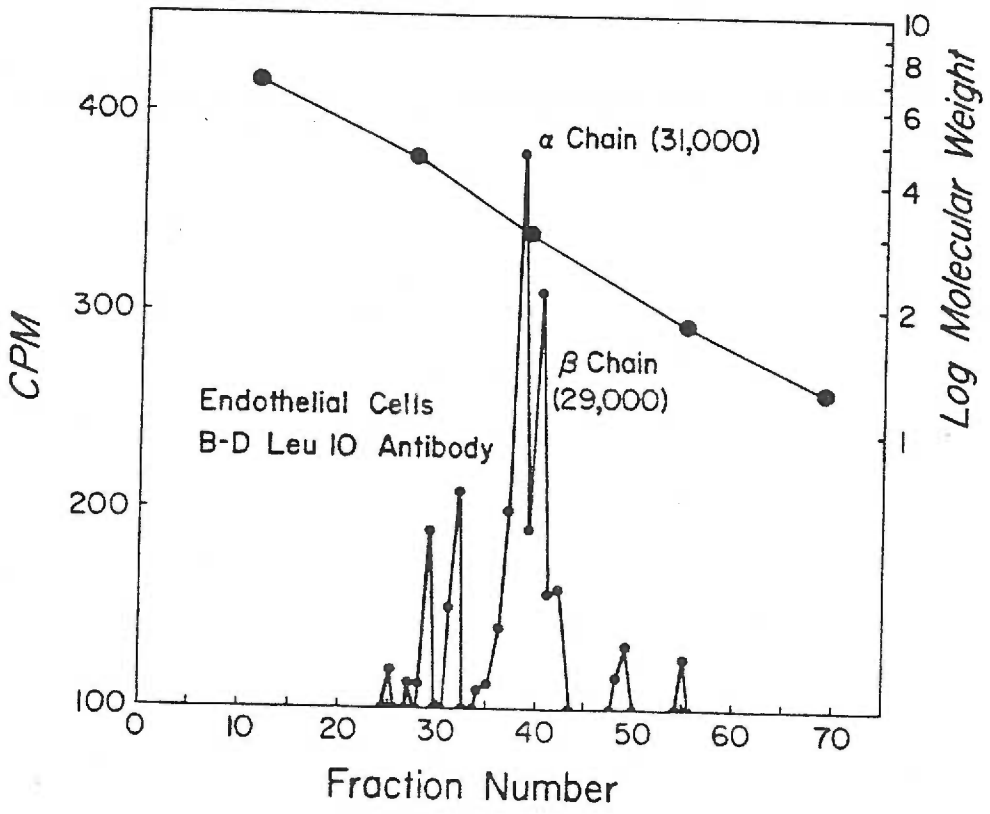
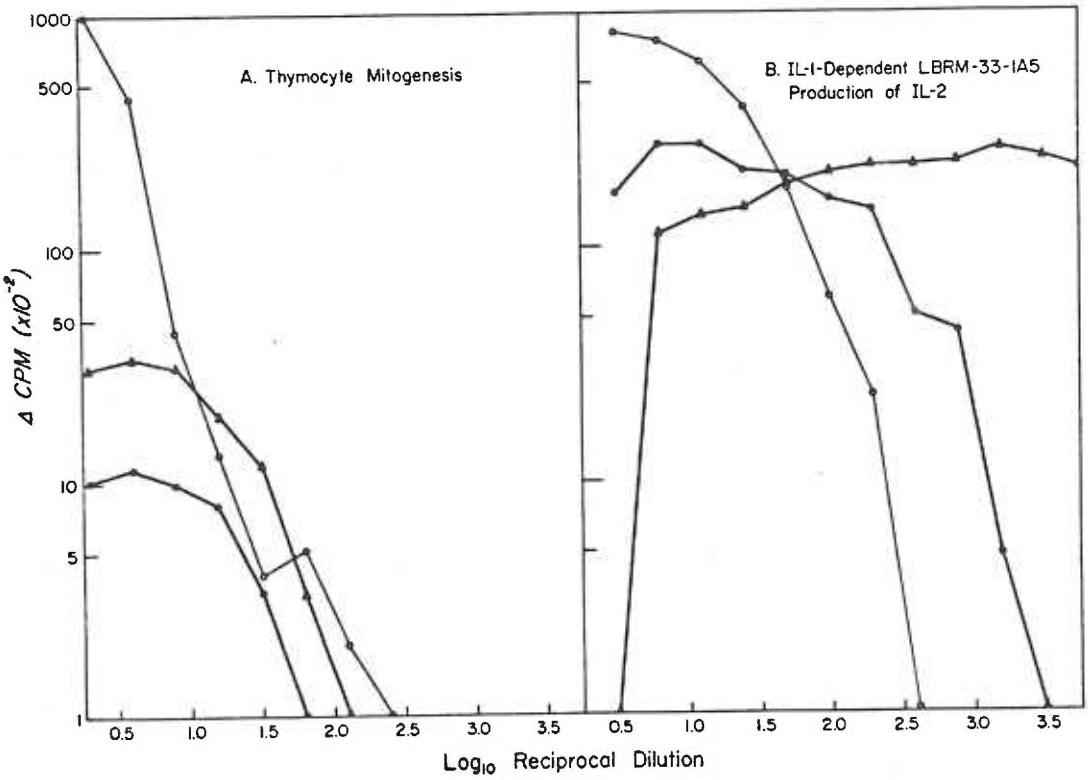

CPM
400
300
200
100
0
10
20
30
40
50
60
70
Fraction Number
α Chain (31,000)
β Chain
(29,000)
Endothelial Cells
B-D Leu 10 Antibody
10
8
6
4
2
1
Log Molecular Weight

Figure 4

Comparison of the dose-response curves of human endothelial cell IL-1 activity on thymocyte proliferation (panel A) and LBRM-33-IA5 cell conversion to IL-2 production (panel B). (A) multiple dilutions of supernatants from either LPS-stimulated MΦ (▲-▲) or LPS-stimulated EC (●-●) were cultured with murine thymocytes for 72 hours. Thymocyte proliferation was quantified by the incorporation of ^{3}H-Tdr in a 6-hr pulse period. Dilutions of a Con A-stimulated rat spleen cell supernatant containing IL-2 (O-O) were also tested as a positive control. The results are expressed as the difference between the CPM incorporated with supernatant from LPS-stimulated cells minus the CPM incorporated with media plus LPS cultured in the absence of cells. (The mean CPM ± SD for the media controls were: media alone = 335 ± 91; media + LPS = 648 ± 140.) (B) Multiple dilutions of supernatants from either LPS-stimulated MΦ (▲-▲) or LPS-stimulated EC (●-●) were cultured with mitomycin C-inactivated LBRM-33-IA5 cells. The IL-1-dependent conversion of LBRM-33-IA5 cells to IL-2 production was assessed by the direct addition of the IL-2 dependent cell line CTLL-2. CTLL-2 proliferation was quantified by the incorporation of ^{3}H-Tdr in a 4 hr pulse period. As in panel A, rat IL-2 (O-O) was used as a positive control. The results are expressed in the same manner as those in panel A. (The mean CPM ± SD for the controls were: media alone = 370 ± 174; IL-1 = 248 ± 47; PHA = 3539 ± 85; IL-1 + PHA = 22878 ± 2427.)


A. Thymocyte Mitogenesis
B. IL-1-Dependent LBRM-33-1A5 Production of IL-2
Δ CPM (x10⁻²)
1000
500
100
50
10
5
1
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Log10 Reciprocal Dilution

THE MECHANISM OF ANTIGEN PRESENTATION

BY ENDOTHELIAL CELLS

ABSTRACT

Endothelial cells line the vessels and lymphatics forming a barrier between circulating T cells and the extravascular tissue site of antigen. We have suggested that circulating T cells recognize antigen on the surface of endothelial cells, resulting in the activation of the endothelium such that the endothelial cells then release the key mediators of a cell-mediated immune response. To test this hypothesis we have evaluated the extent to which endothelial cells can signal antigen-specific T-cell activation. We have shown that cultured endothelial cells are as effective as macrophages in lymphocyte activation and that this activation is HLA-DR restricted. In additional experiments we have established that endothelial cells synthesize Ia determinants and secrete IL-1 early in the signaling process. To eliminate any possible contribution of other cell types participating in the T-cell-endothelial-cell interaction, we have shown that cloned endothelial cells present antigen to cloned T cells. Moreover, there appeared to be a preference of selected T-cell populations for different types of antigen-presenting cells. These experiments document that endothelial cells are independently competent antigen presenting cells.

INTRODUCTION

Endothelial cells (EC) line the vessels and lymphatics, forming a barrier between circulating T cells and the extravascular tissue site of antigen. The fact that this barrier must be trangressed in a cell-mediated immune response (CMI) has stimulated our interest in the role of the endothelium in CMI. We have recently published a hypothesis suggesting that the vascular endothelium may be a major participant in T-lymphocyte immunity (1). We suggested that circulating T cells recognize antigen on the surface of EC, resulting in the activation of endothelium such that the EC then release the key mediators of a CMI response. These mediators would then promote lymphocyte activation and egression into the extravascular tissue. This theory would account for the brief cell-cell exposure which occurs during ordinary hemodynamics and for the rapid dilution of factors by blood flow. That is, the EC would become activated after brief contact with antigen-specific T cells and then the activated EC could focally release mediators. Thus, while antigen-specific T cells would still provide the specificity of the CMI response, the EC would be the central participant in the maturation of this response.

A crucial feature for the validity of this model is the extent to which endothelial cells can signal T cells. Recently we (2, 3)

and others (4, 5) have provided preliminary evidence that endothelial cells fulfill the requirements that lead to the expression of T-cell activation. We have have previously shown that cultured endothelial cells from umbilical veins are as effective as macrophages in signaling lymphocyte activation and that this activation is genetically restricted at the HLA-DR locus (2, 3). The experiments presented here are designed to evaluate the mechanism by which endothelial cells fulfill the antigen presentation requirements and activate antigen-specific T cells.

Endothelial cells are obtained by collagenase treatment of umbilical veins and are cultured in fibronectin-treated flasks in media containing EC growth factor (ECGF) (6). As a result of these culture conditions we have been able to maintain EC in culture for at least 20 subcultures and recover EC from liquid nitrogen storage. The capability to maintain EC in culture for extended periods has facilitated the experiments described below. In this report we document that (a) endothelial cells synthesize and express Ia antigens upon contact with T cells or T-cell factors, (b) endothelial cells produce IL-1 activity upon external stimulation, and (c) cloned endothelial cells present antigen to cloned T cells thus eliminating concerns that additional cell types participate in these activation events. Moreover, we present suggestive evidence that T cells contain subsets of responsive cells that have a

preference for antigen presented on different types of antigen presenting cells (i.e., macrophages vs. endothelial cells).

MATERIALS AND METHODS

Isolation of blood mononuclear cells.

Mononuclear cells (MNC) were separated from heparinized (10 u/ml) peripheral blood from normal donors by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation (2). Interface cells were subsequently washed twice with RPMI 1640 (GIBCO, Grand Island, N.Y.) and counted.

Macrophage isolation.

Cells of the mononuclear phagocytic series (MΦ) were isolated from MNC by adherence to plastic. Tissue culture flasks (No. 3013, Falcon, Oxnard, CA) were pretreated for 30-60 min at 4C with RPMI-50% AB serum and rinsed. MNC (40-60 X 10^6 cells) were added to the pretreated flasks in RPMI with 5% AB serum. After a 1-2 hr incubation at 37C and 5% CO_2, the flasks were rinsed with medium to remove nonadherent cells. Subsequently, RPMI with 5% AB serum and 0.2% EDTA was added and the flasks were held for 10-20 min at room temperature. The macrophages were then removed by shaking and vigorous pipetting, washed twice, and counted.

Macrophage depletion.

Macrophage depletion was accomplished by two sequential steps: adherence to plastic and passage over Sephadex G-10 (Pharmacia Fine Chemicals). The MNC, routinely containing 5-15% monocytes, were first subjected to adherence on serum-pretreated plastic flasks as described above. After 1-2 hr, the nonadherent cells were washed from the flasks and resuspended in 2 ml of RPMI with 5% AB serum. Sephadex G-10 was resuspended to 2-3 times its bed volume in RPMI and autoclaved for 20 min. A column consisting of a plastic 20 ml syringe barrel was prepared and used as previously described (7). Briefly, the column was packed by gravity flow with 15 ml swollen Sephadex G-10 and washed with 2 volumes of RPMI with 5% AB serum. Cells (2 ml) were added to the top of the column and followed by 1 ml RPMI (5% AB serum). The column was then incubated for 30 min at 37C and 5% CO_2. The cells were eluted with RPMI (5% AB serum) in 5 to 10 ml and washed (10 min at 250 X g). The isolated cells characteristically contained less than 0.2% monocytes by esterase staining and would not support antigen-induced lymphocyte proliferation without macrophage addition.

Endothelial cells.

Endothelial cells were isolated from the vein of umbilical cords by a modification of the technique described by Jaffe et al.

(8). The fresh cords were cannulated, flushed with saline, and then filled with 0.2% collagenase (Sigma Chemical Co., No. C-0131, St. Louis, MO). After a 20 min incubation at 37C, the endothelial cells were flushed out with 20 ml RPMI and washed (10 min at 250 X g). The cells were dispersed into culture plates (approximately 10^4 cells/well) and cultured in RPMI with 20% heat inactivated fetal calf serum and 4 ug/ml of ECGF (6) at 37C and 5% CO_2. Approximately 10% of the cultures grew vigorously becoming monolayers within 4 to 6 days. The cells were released from the plastic by a brief treatment (3 min) with trypsin-EDTA (GIBCO) at room temperature, centrifuged (10 min at 500 X g), and replated into human fibronectin-coated (4 ug/ml, Collaborative Research Products, Lexington, MA) 25 cm^2 culture flasks. The endothelial cell cultures were expanded by repetitive subculturing (splitting the cells 1:4 within a week of reaching a monolayer). To confirm that the cultured cells were endothelial cells, cytocentrifuged cells were stained with monoclonal antibody to Factor VIII-related antigen (Cappel Laboratories, West Chester, PA) followed by FITC conjugated goat anti-mouse IgG (Cappel Laboratories) and then examined by fluorescent microscopy.

Standard lymphocyte proliferation assay.

MΦ-depleted MNC were diluted to 1 X 10^6 cells/ml in RPMI supplemented with antibiotics and 30% AB serum and 100 ul (1 X 10^5

cells) distributed in flat-bottom, 96-well microplates (cat. no. 76-003-05, Linbro, Flow Laboratories, McLean, VA). Additional 100 ul preparations containing antigen dilutions, other cells, or media alone were added in quadruplicate to appropriate wells depending upon the experimental design. The antigens used were Measles (30-850J), Mumps - viral and soluble (30-848J), and Varicella (30-149J), all complement fixation antigens (M. A. Bioproducts, Walkersville, MA), and Tuberculin PPD (Connaught Laboratories Limited, Willowdale, Ontario, Canada). The microplates were incubated in a 5% CO_2, humidified atmosphere for 4 days, the last 24 hr with 0.1 uCi ^{3}H-thymidine (specific activity 6.7 Ci/mmole, New England Nuclear, Boston, MA). The cultures were then harvested and counted by commonly used automated techniques (9).

HLA-DR typing of MNC.

Cell donors were healthy volunteers whose HLA-DR phenotypes were established by standard serological techniques (10) in a commercial laboratory (Epitope Incorporated, Portland, OR).

Antisera/monoclonal antibodies.

Monoclonal antibodies to monomorphic HLA-DR determinants: NEI-011 (clone 7.2), was obtained from New England Nuclear; and BRL DR.1, was obtained from Bethesda Research Laboratories, Rockville,

MD. Monoclonal antibodies to HLA-DS determinants: Genox 3.53, was the gift of Frank Bodmer, Imperial Cancer Research Fund, London; and Anti-Leu-10, was obtained from Becton Dickinson, Mountain View, CA. Monoclonal antibody specific for human monocytes, Mac-120, was a gift of Howard Raff, Department of Microbiology, University of Washington, Seattle, WA. All antibodies used in culture were extensively dialysed against RPMI prior to use.

T-cell cloning.

T cells were expanded in continuous culture by alternate exposure to IL-2 and antigen-pulsed MΦ for 7-10 day periods. After three rounds, the cells were cloned by limiting dilution on irradiated monocyte monolayers as previously described (11). Antigen-responsive clones were recloned in IL-2 containing media without monocytes at least once prior to use in these experiments.

Thymidine-suicide technique.

The thymidine-suicide technique for elimination of dividing cells was a modification of that described by Hirschberg et al. (12). The MNC were cultured in tissue culture tubes (1×10^6 cells/ml, 2-5 ml) in RPMI supplemented with 15% heat-inactivated (56C for 30 min) fetal calf serum with and without antigen added in the optimal dose-response range. Monocytes isolated from the MNC

(described above) were distributed into microplates (10^4 cells/100 ul/well) and the plates held at 37C until needed. After 2-5 days of incubation (37C, 5% CO_2) the tube cultures received 10 uCi ^{3}H-thymidine (sp. act. 44 Ci/mmole, The Radiochemical Centre, Amersham, England) for an additional 24 hr. The tube-cultured cells were washed (two to three times), diluted with RPMI so that 50 ul dispensed 1 X 10^5 cells into each well of the microplate which had been set aside with monocytes previously added. An additional 50 ul containing antigen dilutions was then distributed to quadruplicate wells and the microplate was incubated for 2-4 days, pulsed with 0.1 uCi ^{3}H-thymidine (sp. act. 6.7 Ci/mmole) and harvested 24 hr later.

EC cloning.

Endothelial cells from the 14th subculture were cloned twice by limiting dilution in the presence of ECGF. Less than 2% of the cloning attempts survived to yield the cell numbers required for a single experiment. None produced more than 2 X 10^6 cells before exhibiting failure to thrive.

IL-1 assessment.

IL-1 assessment was accomplished by the use of IL-1 dependent LBRM-33-1A5 production of IL-2 (13) performed by Steven Hefeneider (Immunex Corp., Seattle, WA.). One hundred microliters of

LBRM-33-1A5 cells (5 X 10^5 cells/ml) inactivated by mitomycin C (50 ug/ml, 1 hr, 37C), were cultured in 96-well flat bottomed plates in the presence of equal volumes of tissue culture medium alone, PHA (Phytohemmagglutinin M; 0.1% final concentration by volume, GIBCO), IL-1 (at several diluations), or both PHA and IL-1. After 24 hrs of incubation, IL-2 activity was determined by the direct addition of 50 ul of CTLL-2 cells (8 X 10^4 cells/ml). The microwell cultures were incubated for an additional 20 hr, followed by a 4 hr pulse with 0.5 uCi of ^{3}H-thymidine, and then harvested.

RESULTS

Antigen presentation by subcultured endothelial cells.

T cells respond to antigen when it is presented by autologous macrophages (MΦ). Therefore, in order to study EC presentation of antigen it was first necessary to deplete the T-cell preparation of any MΦ. The MΦ-depleted T cells were then reconstituted with endothelial cells and subsequently asayed for antigen-specific proliferation. Figure 1 depicts this basic experimental design. The results from an analysis of MΦ-depletion of T cells is shown in Figure 2. The T cells in unseparated mononuclear cell preparations gave a strong response to the antigen PPD at three different doses (2, 5, and 25 ug/ml). When the MΦ were depleted from the T cells, the T cells no longer responded to the PPD at any dose. Reconstituting the T cells with MΦ also reconsituted the response to antigen. When instead of MΦ, HLA-DR compatible endothelial cells (EC) were used to reconsitute the T-cell preparations, the T cells gave a proliferative response to the antigen (Figure 3). That is, macrophage-depleted T cells do not respond to antigen alone (Figure 3, column one) whereas addition of either macrophages or endothelial cells fully reconstitutes the response (Figure 3, columns 2 and 3 respectively). In these experiments the endothelial cells were from the second to third

subculture. In control experiments we showed that fibroblasts or smooth muscle cells cultured from the same umbilical veins as the endothelial cells could not reconstitute these responses (Figure 4, column 2). Moreover, the reconstitution of the response with endothelial cells was not due to macrophage contamination of either cell preparation since it was not blocked by monoclonal antibodies (14) against macrophages (Figure 4, column 3) but was blocked by endothelial-specific monoclonal reagents (Figure 4, column 4). In previous experiments (2) we have shown that the anti-macrophage monoclonal antibody completely blocked macrophage presentation of antigen. The presentation of antigen by EC was HLA-DR restricted since polymorphic antisera against HLA-DR blocked presentation in a restricted fashion (Figure 5).

When endothelial cell cultures were maintained by serial subculture, the cells continued to be endothelial by morphologic criteria, general growth patterns, and specific marker analysis (eg. Factor VIII-related antigen). The results presented in Figure 6 demonstrate that endothelial cells from the fifth to eighteenth subculture were effective at antigen presentation. Additional experiments suggested that the variation in responses seen when subcultured EC were used is due to problems with the precise enumeration of the number of EC added to the cultures and not changes in presentation efficiency (Figure 6, tuberculin response, subcultures 5, 6, 7, and 18). Overall, these results support the

idea that EC can themselves fulfill all of the requirements of antigen presenting cells.

MHC class II determinants on endothelial cells.

It is currently thought that T cells recognize antigen in the context of Ia (MHC class II) determinants on the surface of APC. Therefore, if subcultured EC are capable of presenting antigen without the aid of other "accessory" cells, EC should be able to synthesize Ia determinants (HLA-D region determinants in man). After exposure of subcultured EC to either T cells (depleted of APC) and antigen or T-cell factors, we have been able to immunoprecipitate radiolabeled EC membrane proteins with antibody to HLA-DR (BRL DR.1). By SDS-PAGE (reducing conditions), the immunoprecipitated proteins were found to have the characteristic molecular weights of traditional human class II MHC determinants with an alpha chain of 32,000 and a beta chain of 29,000 (Figure 7).

Recently an additional human class II locus, HLA-DS has been recognized (15). Using the same protocol as above it was possible to immunoprecipitate EC membrane proteins (with molecular weights of 31,000 and 29,000) using Anti-Leu-10 antibody to HLA-DS (data not shown). Consequently, it appears that EC are capable of synthesizing both HLA-DR and HLA-DS determinants.

Interleukin 1 and signals for activation.

In addition to antigen and Ia determinants, macrophage derived IL-1 is thought to be a requirement for T-cell activation. MΦ IL-1 activity has been assayed using the LBRM cell line as described by Conlon et al. (13). In the presence of IL-1, LBRM cells secrete IL-2 which can then be assayed on IL-2 dependent cells. As can be seen in Table 1, our results show that when subcultured EC are stimulated with bacterial lipopolysaccharide (5ug/ml, E. coli 055:B5, Difco Laboratories) they produce an activity that is positive in this IL-1 assay. For example, with a 1:50 dilution of a 48 hr EC supernatant the CTLL-2 cells gave 26,304 CPM compared to 23,578 CPM with a MΦ supernatant (a positive control supernatant) while the CTLL-2 cells only gave 3,214 CPM with the LPS/media control (Table 1). These results are consistent with the conclusion that EC secrete functional IL-1, although isolation and characterization of this activity will be required to conclude that it has the same structure as MΦ IL-1. (In preliminary experiments macrophage IL-1 activity and endothelial cell IL-1 activity co-chromatograph on reverse phase HPLC.)

Antigen presentation using cloned cells.

One of the subcultured endothelial cell lines (EC-38) was cloned by limiting dilution and the clones tested for their capacity

to present antigen. Five EC clones survived two rounds of limiting dilution cloning and were available for these experiments. All cells from each of the clones tested were positive for Factor VIII-related antigen. Since these EC clones were derived from a line that was HLA-DR 4,7 positive, antigen presentation was evaluated on both histocompatible (HLA-DR 4) and histoincompatible (HLA-DR 6) T cells. EC line 38 and the derived clones presented antigen only to the histocompatible T cells (Table 2). That is, histocompatible T cells gave approximately 2,000 CPM in the presence of antigen and cloned EC while histoincompatible T cells gave approximately 650 CPM (compared to background counts of about 300 CPM when antigen was not present) (Table 2). These results eliminated any contribution from other cell types in the EC preparations. In order to exclude any contribution from non-T cells in the T-cell preparation, we established several T-cell clones from T-cell lines maintained by alternate cycles of IL-2 and antigen-pulsed MΦ stimulation (11). The T-cell lines were cloned by limiting dilution and shown to be responsive in antigen presentation experiments (Table 3). The response of two of the T-cell clones (M6 and M18) to presentation of antigen by EC was significant. When antigen was presented by EC-38 cells, M6 gave 3,850 CPM and M18 gave 6,220 CPM (without antigen present the CPM were 540 and 310, respectively) (Table 3). However each of the T-cell clones reponded better to antigen presented by MΦ than antigen presented by EC. For example, T-cell clone M18 gave 16,950

CPM in response to antigen presented by MΦ while it gave 6,220 CPM when the antigen was presented by EC-38 (Table 3). When T-cell clone M18 was used in additional experiments to test presentation of antigen by the EC clones, antigen-specific activation occurred with each of the five EC clones evaluated (Table 4).

Clonal nature of the response of lymphocytes to antigen presented by macrophages or endothelial cells.

We were surprised by the apparent preference of cloned T cells for antigen presented by MΦ since this had not been observed with fresh T cells. Additional experiments showed that as few as 10 days in culture with antigen-pulsed MΦ prejudiced T cells for the antigen re-presented on the priming cell type. The reciprocal was also observed; T cells cultured with antigen-pulsed endothelial cells seemed to prefer antigen presented on EC rather than MΦ for secondary responses (data not shown). In order to ask if within the T-cell population there were two populations of responding cells (one with a preference for EC and the other for MΦ), we conducted a proliferation suicide experiment as previously described (12). MΦ and EC were cultured from the same umbilical vein and used to present antigen to histocompatible T cells. At the peak of the proliferative response, responding cells were eliminated by lethal addition of high specific activity tritiated thymidine. The remaining T cells were re-exposed to the alternate APC. When

responsive T cells were eliminated by this procedure they could not respond to antigen presented by the homologous APC (as evidence by a stimulation index of approximately 1, Table 5). However they did respond to the other type of APC (MΦ vs. EC) (stimulation index values of approximately 4 to 5, Table 5). This suggested that there are two populations of responsive T cells, one with a preference for each type of APC.

If this premise were correct, T-cell clones derived in the presence of EC should respond well to EC plus antigen and show an "EC preference" over antigen presented by MΦ. Three T-cell clones were derived by alternate exposure to IL-2 and antigen-pulsed EC. All three of these clones responded well to antigen presented by EC (the CPM ranged from 9,000 to 14,000 greater than control CPM, Table 6). Two of the three (E13 and E32) showed a preference for the antigen on EC, that preference being most dramatic in the case of clone E32 (T-cell clone E13 gave 14,620 CPM in response to antigen presented by EC compared to 8,333 CPM when it was presented antigen by MΦ, and T-cell clone E32 gave 13,535 CPM when the antigen was presented by EC-38 vs. 1,121 CPM when the antigen was presented by MΦ). In one case, clone E15, the responses were approximately equal. When T-cell clone E13 was further evaluated for responsiveness, we found that all five of the EC clones presented antigen to it (Table 4). These data confirm the observations that T-cell populations contain more than one responsive subset in regards to APC preference.

DISCUSSION

T cells are responsible for a diverse group of immune reactions all of which fall under the heading of cell-mediated immunity (CMI). T cells are thought to provide the specificity of the CMI response while their mediators stimulate cellular exudates and the histologic findings at the site of antigen. T cells require the antigen to be displayed on Ia-bearing antigen presenting cells (APC) and require the involvement of IL-1 and IL-2 for maximal activity. Antigen-specific T cells in an immune individual circulate within the vasculature at an approximate frequency of 10^{-3} to 10^{-5} (16). Some of these cells must be triggered for activation and development of the CMI response. To account for the involvement of the low frequency, antigen-specific T cell, we have considered two basic alternatives; first the nonspecific inflammatory response induced by antigen alone produces endothelial changes which allow thousands of cells to egress from blood to tissue. When enough antigen-specific cells are present at the extravascular site of antigen, the specific T cells become activated and via mediators induce and/or direct the development of classic CMI pathology.

A second possibility is that antigen is taken up by the endothelium and presented on the lumenal surface of capillaries. An intravascular display of antigen could provide the recognition site for triggering of antigen-specific T cells. Even if in low

frequency, the specific T cell would eventually come in contact with the antigen at this intravascular site. These two alternatives are distinguished by where the T cell first encounters antigen. We favor the second hypothesis since it seems to account in an efficient manner for the earliest events in recognition.

After specific T-cell activation, the accumulation of mononuclear cells in the tissue can be accounted for in at least two ways. The activated T cell could release mediators which would affect endothelium inducing changes in vascular permeability, or the endothelium could be activated by the T-cell contact to undergo structural changes and to elaborate EC mediators which would lead to classic CMI histology. This latter theory (1) would acount for the brief cell-cell exposure which occurs during ordinary hemodynamics, the low frequency of antigen-specific T cells in blood, and for the rapid dilution of factors by the blood flow. In this model EC could become activated after brief contact with antigen-specific T cells and then the activated EC could focally release mediators (Figure 8). Thus, while antigen-specific T cells would still provide the specificity to the CMI response, the EC would be the central participant in the CMI response.

Several findings support the above theory. First, EC are known to express MHC determinants including Ia (5, 17). Secondly, EC have been shown to a) activate lymphocytes in allogeneic mixed-cell culture reactions (18, 19), b) present conventional antigen to antigen-specific T cells in an HLA-DR restricted manner (2, 3), and

c) function as accessory cells for mitogen-induced responses (20). There is also the suggestion that EC and T cells interact in the development of delayed-type hypersensitivity (DTH) in that histological examination of DTH sites showed lymphocytes attached to vascular endothelium within the lumen of the venule at the DTH site (21). Also lymphocytes have been shown to adhere to cultured endothelium and migrate through the EC if sensitized (21). Finally, EC are known to produce metabolites of arachidonic acid such as prostacyclin, a mediator of vasodilation (22, 23).

While the above findings support a major role for EC in CMI responses, the experiments are not conclusive. For example, the requirement of other cell types for EC presentation of antigen has not been previously excluded. Also, while Ia determinants have been found on the surface of EC, there has only been preliminary evidence that EC themselves synthesize Ia determinants. Moreover, EC have not been previously shown to produce interleukin 1 activity. These questions have been the central theme of this work.

A significant factor in all of our experiments has been the long term serial culture of endothelial cells. We have been able to demonstrate that extensively subcultured EC (up to 18 subcultures) present antigen as effectively as fresh EC (Figure 6) and do so in an HLA-DR restricted manner (Figure 5). Additional experiments confirmed our earlier observations that other cell types (macrophages, fibroblasts, and smooth muscle cells) were not participating in these presentation experiments (Figures 3, 4).

Moreover, we have now provided formal evidence that EC express the two critical signals required by antigen presenting cells. We have shown that when exposed to appropriate stimuli they synthesize Ia determinants (Figure 7) and secrete IL-1 activity (Table 1).

To eliminate any final concern that either the T cell or endothelial cell populations still contained a small number of contributory cell components, we cloned both cell populations. The cloned EC presented antigen to the cloned T cells in a restricted fashion (Tables 2, 3, 4). This further clarified the independent presentation capacity of EC.

We observed in these latter experiments a functional T-cell preference for the APC type used in initial stimulation. That is, T cells selected or primed in the presence of MΦ responded better to antigen presented by MΦ than EC, and T cells primed on EC preferred antigen presented by EC upon restimulation (Tables 4, 5, 6). This suggests that the T-cell population to any antigen is made up of subsets of responding cells, some with a preference for different APC. The mechanism for this preference remains to be elucidated. It could be rather trivial or biologically significant. It could simply represent a subtle difference in antigen processing by different APC, preference for different Ia loci, or even anatomical considerations, to name a few possibilities. If this phenomenon is biologically relevant it could provide an additional level of T-cell sophistication in honing in on or responding to certain stimuli.

Based on the available data and some of our thoughts expressed above, it is possible to speculate on a signaling sequence for T-cell-endothelial cell interaction. We have detailed such a theoretical sequence below (Table 7). It provides us with a working model until new information requires its modification.

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TABLE 1
ASSAY OF CELL CULTURE SUPERNATANTS FOR IL-1
USING LBRM-33-1A5 CELLS

CELL SUP. DILUTION	CULTURE MEDIA WITH 5ug LPS	MΦ CONDITIONED MEDIA (5ug LPS)[a]	EC CONDITIONED MEDIA (5ug LPS) 24 hr	EC CONDITIONED MEDIA (5ug LPS) 48 hr
1:3	3456[b]	10330	24594	34762
1:6	4390	12960	23612	30470
1:50	3214	23578	17718	26304
1:400	2854	30722	4064	12602
1:3000	4242	30650	2430	5970

Assay Controls:

Media alone	= 521 (LBRM + CTLL-2).
IL-1 alone	= 562
PHA alone	= 2062
IL-1 + PHA	= 36100
IL-2	= 85846

[a]The length of MΦ or EC exposure to 5 ug/ml LPS (E. coli 055:B5, Difco Laboratories) prior to collection of the conditioned media was 48 hr.

[b]Mean CPM of ^{3}H-thymidine uptake.

TABLE 2
ANTIGEN PRESENTATION BY ENDOTHELIAL CELL CLONES

T CELLS	ANTIGEN PRESENTING CELLS[a]	VARICELLA −	VARICELLA +
PBL (MΦ-DEPLETED) HISTOCOMPATIBLE	NONE	186[b]	176
	MΦ	211	2308
	EC-38	286	1811
	EC-38 CLONE 1A5	256	1921
	2A7	288	2640
	2F3	412	3111
	3C3	196	1796
	3F6	241	1899
PBL (MΦ-DEPLETED) HISTOINCOMPATIBLE	NONE	202	189
	MΦ	313	675
	EC-38	322	667
	EC-38 CLONE 1A5	381	711
	2A7	295	582

[a]MΦ-depleted T cells were reconstituted with either MΦ, subcultured EC, or cloned EC. The T cell proliferation either with or without antigen present was then determined (see Methods).
[b]Mean CPM of ^{3}H-thymidine uptake.

TABLE 3
RESPONSE OF T-CELL CLONES TO ANTIGEN PRESENTED BY ENDOTHELIAL CELLS

T CELLS	ANTIGEN PRESENTING CELLS[a]	VARICELLA	
		-	+
PBL (MΦ-DEPLETED)	NONE	156[b]	161
	MΦ	138	2183
	EC-38	206	1454
CLONE M6	MΦ	421	6240
	EC-38	540	3850
CLONE M18	MΦ	386	16950
	EC-38	310	6220
CLONE M32	MΦ	510	7151
	EC-38	538	1062

[a] The same basic protocol was used as was described for the standard lymphocyte proliferation assay in Methods.
[b] Mean CPM of ^{3}H-thymidine uptake.

TABLE 4
RESPONSE OF T-CELL CLONES TO ANTIGEN PRESENTED BY CLONED ENDOTHELIAL CELLS

T CELLS	ANTIGEN PRESENTING CELLS[a]	VARICELLA	
		-	+
CLONE M18	NONE	169[b]	211
	MΦ	561	14510
	EC-38	610	5850
	EC-38 CLONE 1A5	658	6150
	2A7	586	3650
	2F3	313	3280
	3C3	621	5400
	3F6	606	4280
CLONE E13	NONE	136	141
	MΦ	388	14600
	EC-38	422	22650
	EC-38 CLONE 1AJ	512	21860
	2A7	826	14582
	2F3	588	23466
	3C3	436	22831
	3F6	581	26510

[a]The same basic protocol was used as was described for the standard lymphocyte proliferation assay in Methods.
[b]Mean CPM of ^{3}H-thymidine uptake.

TABLE 5
CLONAL NATURE OF THE RESPONSE OF LYMPHOCYTES TO PPD
PRESENTED BY MACROPHAGES OR ENDOTHELIAL CELLS

ANTIGEN-PULSED APC USED FOR[a]		RESPONSE IN SECONDARY CULTURE	
SUICIDE	RESTIMULATION	MEAN CPM ± S.D.	S.I.[b]
NONE	MΦ (NO Ag)	484 ± 82	
NONE	MΦ	4620 ± 510	9.5[b]
MΦ	MΦ	586 ± 120	1.2
MΦ	EC	2320 ± 420	4.8
NONE	EC	3915 ± 386	8.1
EC	EC	615 ± 310	1.3
EC	MΦ	2187 ± 186	4.5

[a]See Methods for a description of the thymidine suicide technique.
[b]S.I. = stimulation index (mean CPM ÷ 484 CPM).

TABLE 6
RESPONSE OF T CELL CLONES TO ANTIGEN PRESENTED BY ENDOTHELIAL CELLS

T CELLS	ANTIGEN PRESENTING CELLS	VARICELLA	
		-	+
PBL (MΦ-DEPLETED)	NONE	262	241
	MΦ	281	3210
	EC-38	355	3640
CLONE E13	MΦ	538	8333
	EC-38	561	14620
CLONE E15	MΦ	642	8890
	EC-38	612	9622
CLONE E32	MΦ	386	1121
	EC-38	422	13535

[a]The same basic protocol was used as was described for the standard lymphocyte proliferation asay in Methods.
[b]Mean CPM of ^{3}H-thymidine uptake.

TABLE 7
T CELL - ENDOTHELIAL CELL SIGNALING SEQUENCE

STEP	INTERACTION	RESULT
1	Antigen interacts with EC	Antigen "armed" EC* Induction of IL-1 & Ia*
2	T-cell clonotypic receptor recognizes antigen on EC in conjunction with Ia	EC alterations, mediator secretion
3	T cell and monocyte egress across EC	T cells, monocytes in extravascular spaces
4	IL-1 interacts with IL-1 receptor positive cells	IL-2 production
5	IL-2 interacts with IL-2 receptor	T-cell expansion, recruitment
6	T cells, mediators, MΦ, EC factors	CMI pathology

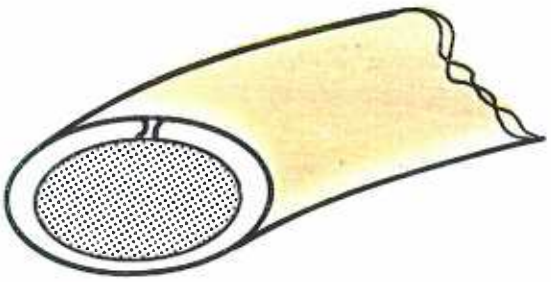
* The signal for Ia expression may be lymphocyte interaction with Ia negative EC or with low-density Ia positive EC.

Figure 1

Experimental design for experiments evaluating the capability of endothelial cells to present antigen. Cultured EC isolated from umbilical veins are added to 96-well microplates along with mononuclear cells (previously depleted of antigen presenting cells) and antigen. The microplates were then cultured for 4 to 6 days (the last 24 hrs in the presence of ^{3}H-thymidine) and harvested.

Experimental Design

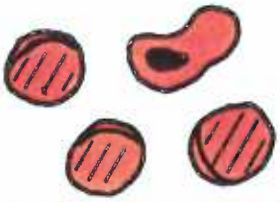
HEPARINIZED BLOOD



UMBILICAL VEIN

ISOPAQUE-FICOLL CENTRIFUGATION

COLLAGENASE

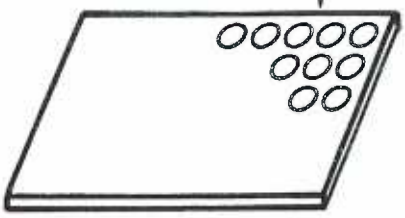


MONONUCLEAR CELLS

ENDOTHELIAL CELL CULTURE

MACROPHAGE DEPLETION

TRYPSIN-EDTA



PROLIFERATION ASSAY

^{3}H-Thymidine incorporation in response to antigen stimulation (measured at 4-6 days)

Figure 2

Demonstration that lymphocyte proliferation is dependent on macrophages for optimal responses. Macrophage-depleted T cells are cultured at 10^5 cells/well. Macrophages are added back at either 10^3, 3.3 X 10^3, or 10^4 cells/well. Antigen-specific T-cell proliferation is measured by ^{3}H-thymidine incorporation.

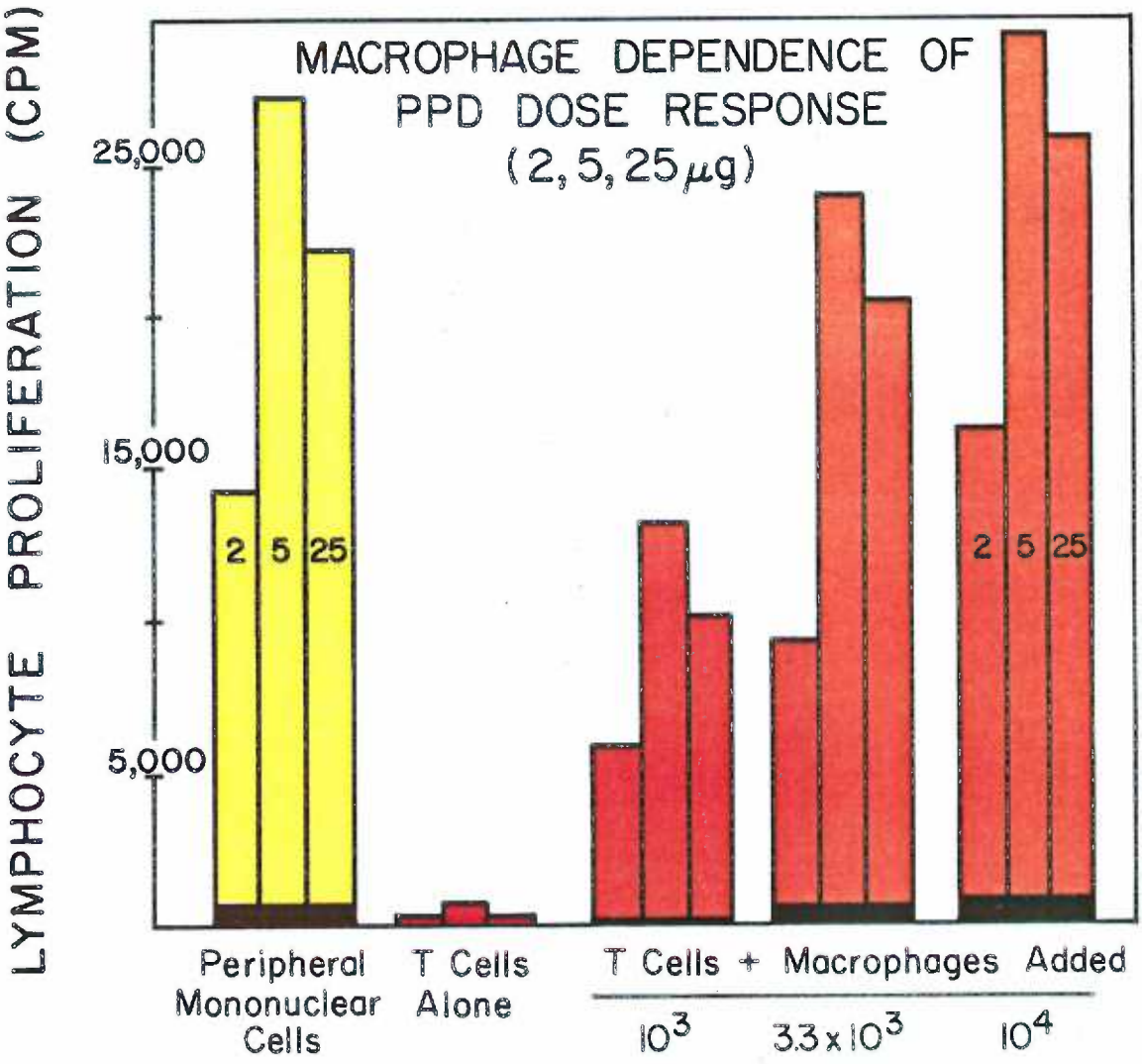

MACROPHAGE DEPENDENCE OF
PPD DOSE RESPONSE
(2, 5, 25 μg)
LYMPHOCYTE PROLIFERATION (CPM)
25,000
15,000
5,000
2
5
25
2
5
25
Peripheral Mononuclear Cells
T Cells Alone
T Cells + Macrophages Added
10^3
3.3 x 10^3
10^4

Figure 3

Scatter diagram comparing presentation of antigen by endothelial cells to presentation of antigen by macrophages. The proliferation response is expressed as a percentage of the response obtained with mononuclear cells (prior to macrophage depletion).

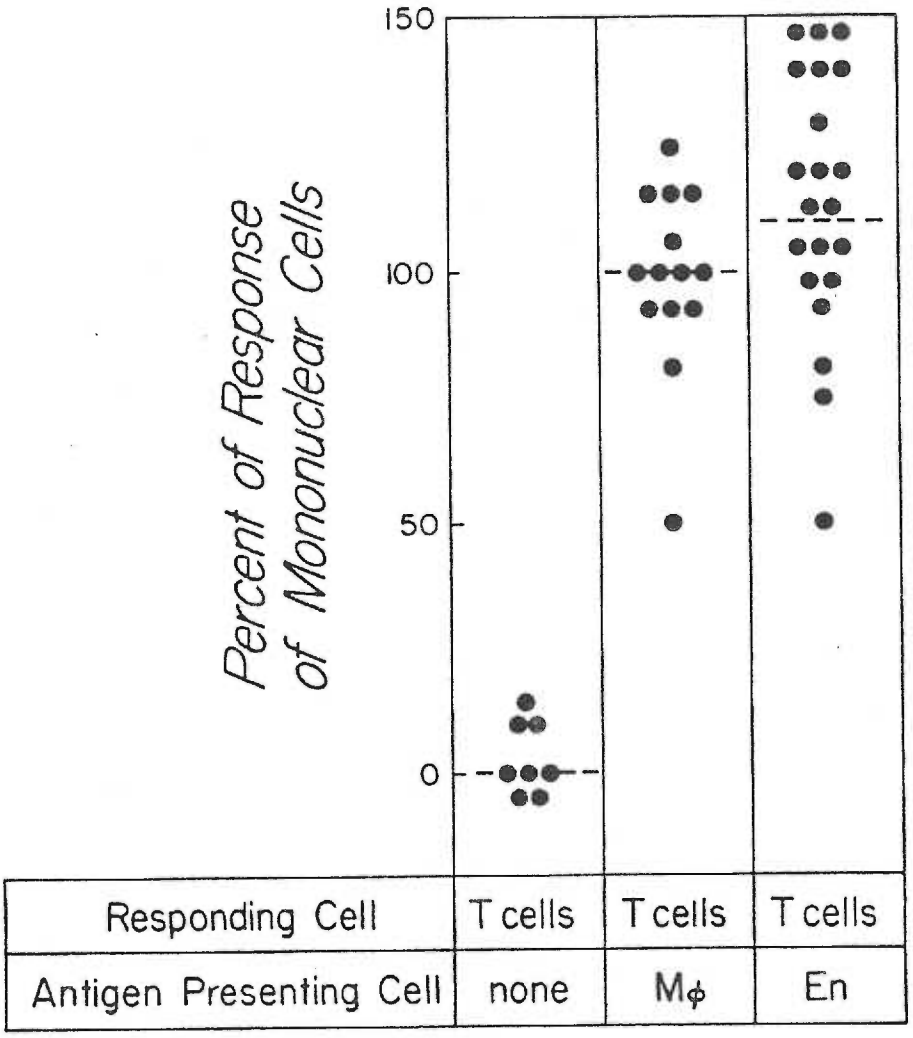

Percent of Response of Mononuclear Cells
150
100
50
0
Responding Cell
T cells
T cells
T cells
Antigen Presenting Cell
none
Mϕ
En

Figure 4

Scatter diagram depicting the effects of a monoclonal anti-macrophage antibody or monoclonal anti-endothelial cell antibody on lymphocyte proliferation induced by antigen-pulsed endothelial cells (EC). The effects of fibroblasts and smooth muscle cells (F/SM) as antigen-presenting cells are also shown. The 100% response was the proliferation obtained with mononuclear cells, antigen, and a control monoclonal antibody added to cultures. (-- represents the mean response.)

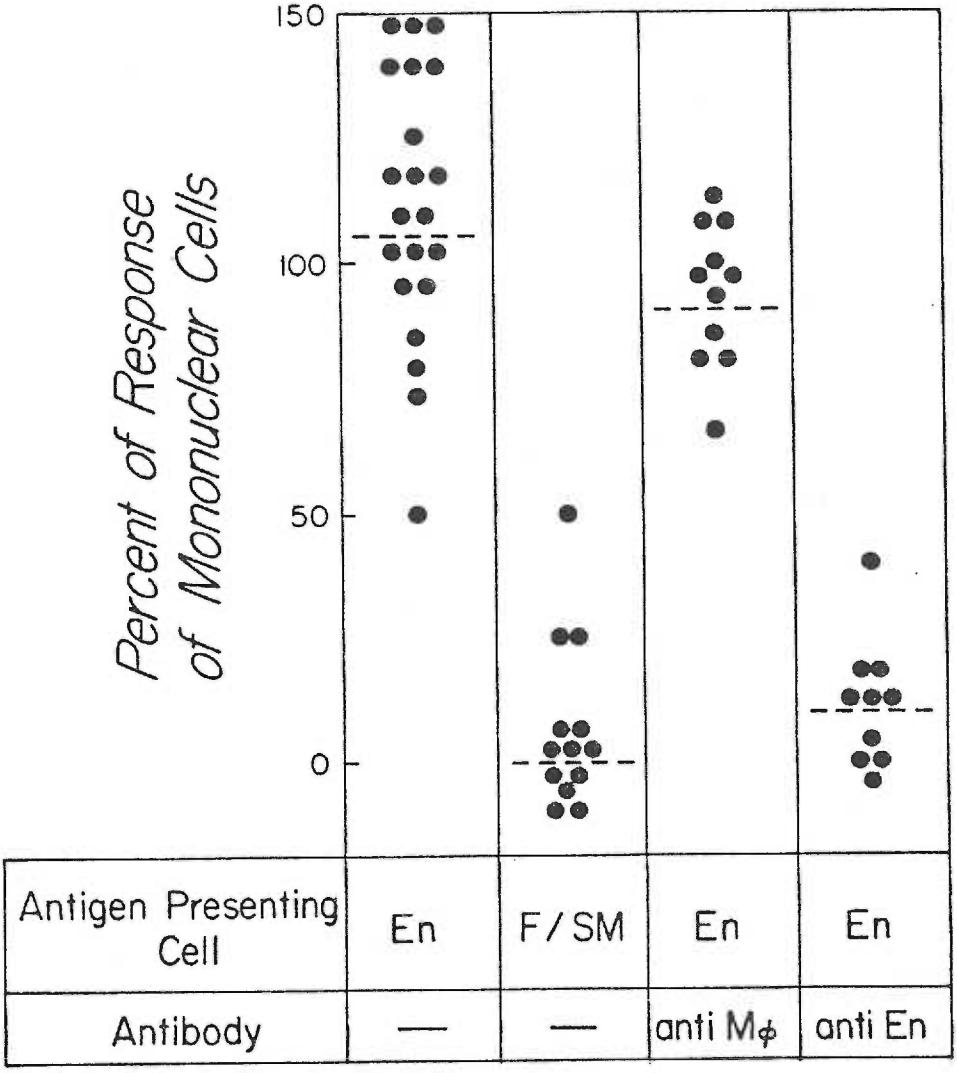

Percent of Response of Mononuclear Cells
150
100
50
0
Antigen Presenting Cell
En
F/SM
En
En
Antibody
—
—
anti Mφ
anti En

Figure 5

Scatter diagram depicting the effects of polymorphic HLA-DR antisera and monoclonal monomorphic HLA-DR antibody on lymphocyte proliferation in cultures induced by antigen-pulsed endothelial cells. The maximum response was the proliferation obtained with antigen-pulsed endothelial cells and normal human sera added to cultures rather than the alloantisera. (-- represents mean response.)

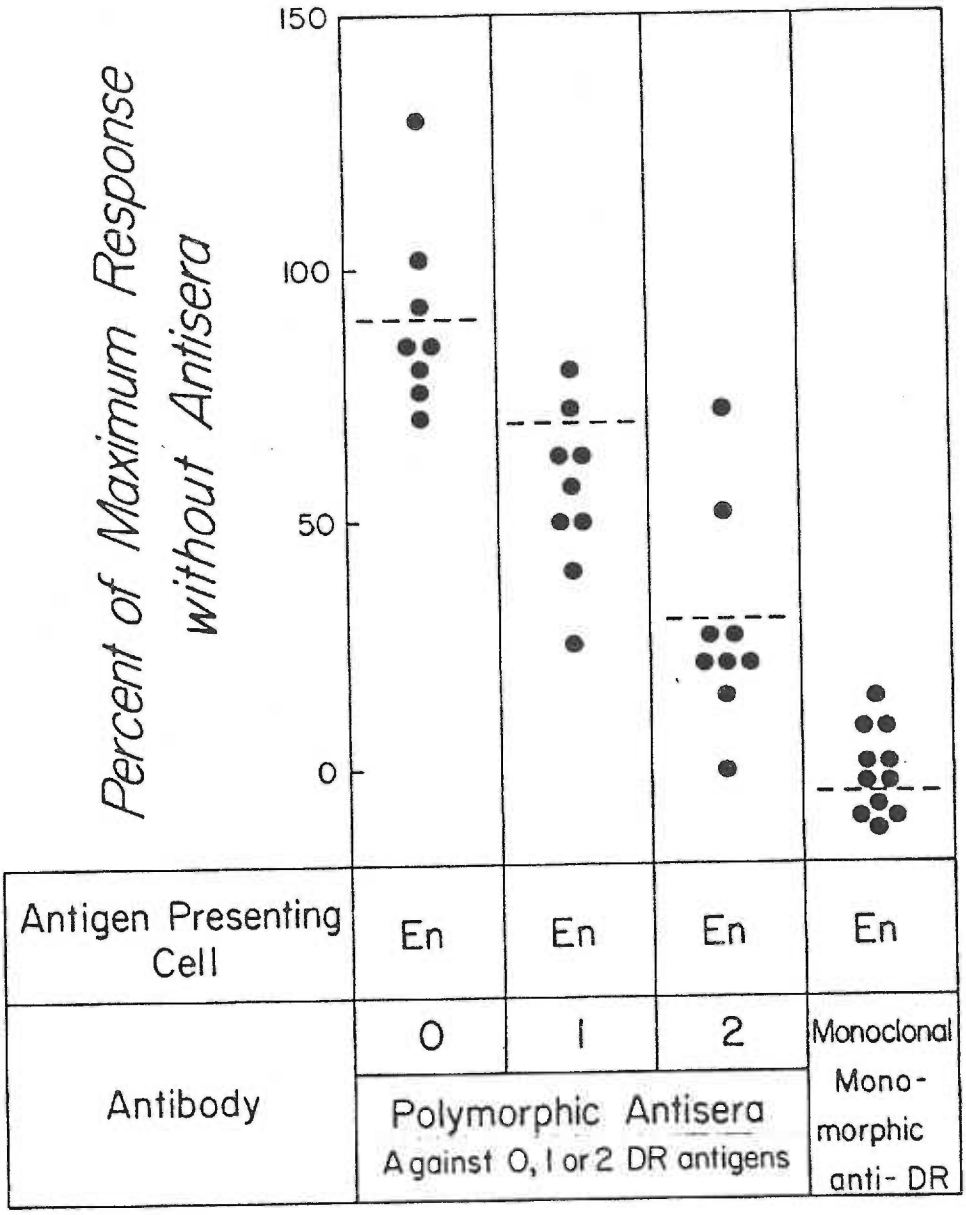

Percent of Maximum Response without Antisera
150
100
50
0
Antigen Presenting Cell
En
En
En
En
Antibody
0
1
2
Monoclonal Mono-morphic anti-DR
Polymorphic Antisera
Against 0, 1 or 2 DR antigens

Figure 6

Comparison of antigen presentation by various subcultures of EC to antigen presentation by fresh autologous macrophages.

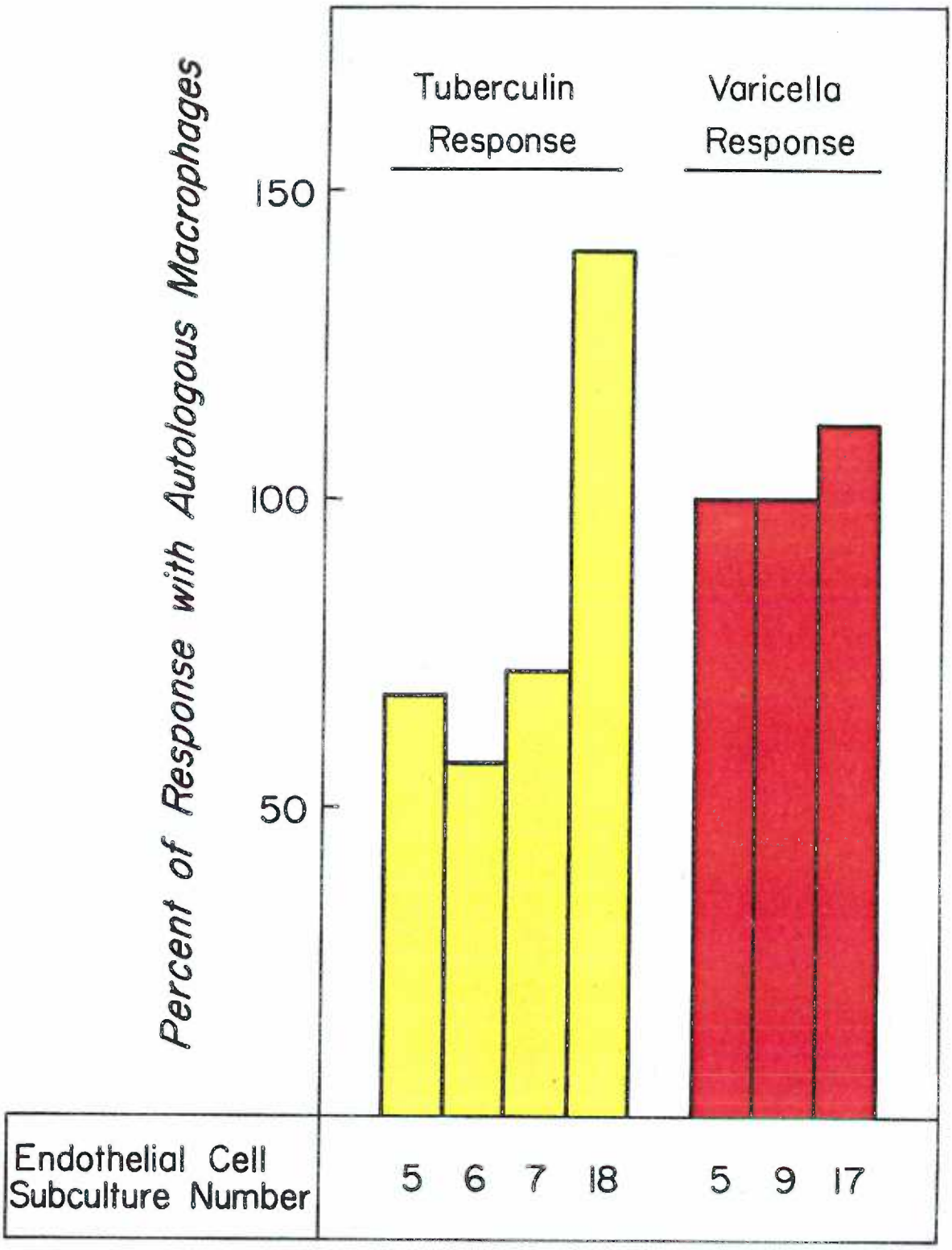

Tuberculin Response
Varicella Response
Percent of Response with Autologous Macrophages
150
100
50
Endothelial Cell Subculture Number
5 6 7 18
5 9 17

Figure 7

Synthesis of HLA-DR antigen by human endothelial cells. Data points (●–●) represent CPM of ^{14}C-amino acids incorporated into immunoprecipitated proteins in SDS-polyacrylamide gel following electrophoresis. The positions of the molecular weight standards (listed in Materials and Methods) following electrophoresis are also shown (●–●).

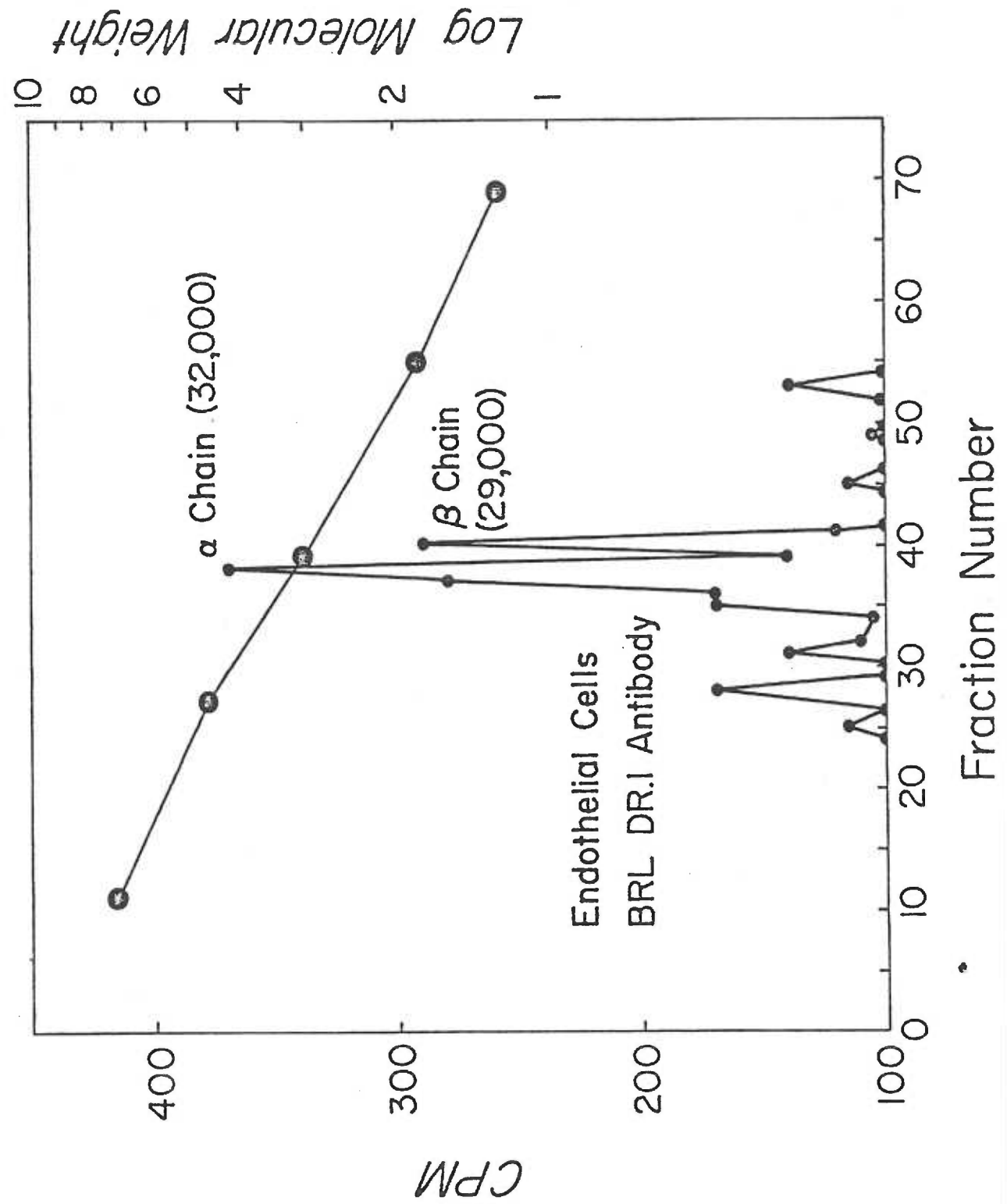
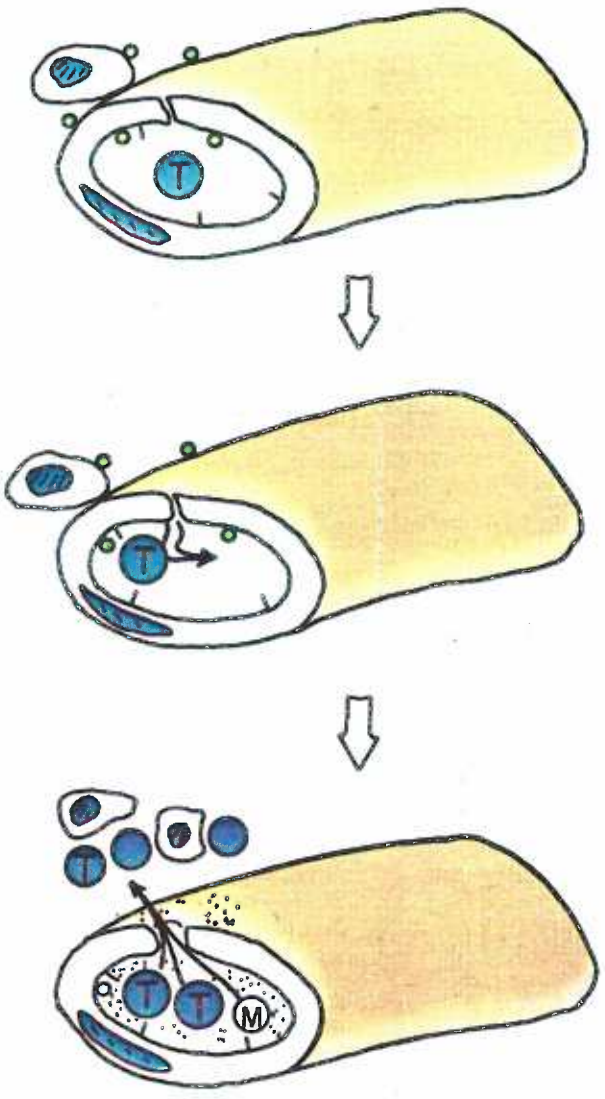

Log Molecular Weight
10
8
6
4
2
1
α Chain (32,000)
β Chain
(29,000)
Endothelial Cells
BRL DR.I Antibody
CPM
400
300
200
100
0
10
20
30
40
50
60
70
Fraction Number

Figure 8

General model for the T-cell-endothelial-cell interaction leading to mononuclear cell egress into the extravascular tissue.



Antigen - Armed Endothelium

"Activation" of Endothelium by T cell

Release of mediators by Endothelium leading to accumulation of mononuclear cells in the tissue

DISCUSSION

T-cell recognition of antigen provides the antigen specificity to cell-mediated immune responses. As was discussed in the introduction section, T cells recognize antigens when they are presented by antigen-presenting cells (APC). Traditionally, macrophages (MΦ) have been considered to be the APC (1-7). Recently Langerhan's cells (8-10), dendritic cells (11-13), and endothelial cells (14-18) have also been implicated as APC. In order to demonstrate that any cell type can independently present antigen to T cells, it is necessary to perform experiments using cell preparations free of other APC. If a cell type can independently present antigen, then, based on what is known about MΦ presentation of antigen (19-26), it should express class II MHC determinants and interleukin 1 (IL-1) activity. The experiments described in this thesis were designed to examine whether EC can independently present antigen to T cells, and whether EC can synthesize and functionally express class II MHC determinants and IL-1 activity.

Endothelial cells line the blood vessels and lymphatics, forming a barrier between circulating T cells and the extravascular site of antigen. In a cell-mediated immune (CMI) response the endothelial cell (EC) barrier is transgressed by circulating cells resulting in a cellular exudate (27). Endothelial cells may simply play a

passive role in the development of a CMI response; leukocyte products may affect EC to cause increased vascular permeability leading to nonspecific cellular infiltration of extravascular tissue. Alternatively EC may play a central role in the development of a CMI response as hypothesized by Burger and Vetto (28). The model proposed by Burger and Vetto consists of the following elements: [1] antigen presentation regionally by capillary EC, [2] EC "activation" by sensitized circulating T cells, and [3] a focal EC response such that the EC produce factors which recruit nonspecific leukocytes, allow extravascular-cell migration and subsequently lead to the development of cell-mediated responses. This theory would account for the brief cell-cell exposure which occurs during ordinary hemodynamics and the rapid dilution of factors by the blood flow. Also, it would account for antigen recognition by low-frequency antigen-specific cells in blood. In an immune individual, antigen-specific T cells circulate within the vasculature at an approximate frequency of 1 in 10,000 (29). If nonspecific inflammatory changes are what leads to the first recognition of antigen by T cells, thousands of T cells would have to enter the tissue before an antigen-specific T cell was present. However, even if in low frequency, the specific T cell would come in contact with antigen when presented on the lumenal surface of EC. Therefore, EC presentation of antigen would seem to provide an efficient mechanism to account for the earliest events in antigen recognition.

Several findings support the above theory in which EC play a major role in CMI responses. First, EC are known to express MHC determinants (30) including Ia determinants (31, 32) which are thought to be one of the signals required for T-cell activation. Secondly, EC have been shown to a) present HLA determinants, in that they activate lymphocytes in allogeneic mixed-cell culture reactions (33-35), b) present conventional antigen (non-HLA) to antigen-specific T cells in an HLA-DR-restricted manner (14-18), and c) function as accessory cells for mitogen-induced responses (36, 37). There is also the suggestion that EC and T cells interact in the development of delayed-type hypersensitivity (DTH) in that histological studies showed lymphocytes attached to the vascular endothelium within the lumen of the venule at the DTH site (27). Also, lymphocytes have been shown to adhere to cultured endothelium and migrate through the EC if sensitized (38, 39). Finally, EC are known to produce metabolites of arachidonic acid such as prostacyclin, a mediator of vasodilation (40, 41). This supports the above suggestion that activated endothelial cells may release the key mediators of CMI response.

A crucial element of the model proposed by Burger and Vetto is that of antigen presentation by EC. Even if after presenting the antigen EC do not become activated to produce the key mediators of a CMI response, the capability of EC to present antigen to circulating T cells may be an important step in the development of CMI responses (eg. EC presentation of antigen could provide an efficient mechanism

for early antigen recognition as discussed above). Several different groups of investigators have examined the capability of EC to present antigen. Burger et al. have demonstrated that primary or secondary subcultures of human umbilical vein EC can present either purified-protein derivative of tuberculin (PPD), streptokinase-streptodornase, Candida, or Keyhole limpet hemocyanin to MΦ-depleted T cells (14, 15). Hirschberg et al. has also published a series of papers (16, 18, 42) demonstrating the capability of primary subcultures of human umbilical vein endothelial cells to present antigen to T cells. The endothelial cells presented several different antigens including herpes simplex virus antigen (HSV), PPD, and varicella-zoster virus antigen (VZV). Using human umbilical vein EC immediately after isolation or after culturing for 2-7 days, Nunez et al. showed that EC could present antigen (mumps virus) to antigen-primed T-cell lines (17). All three groups of investigators found that the EC presented antigen in a HLA-DR-restricted manner.

Roska et al. (43) has published the results of experiments using guinea pig aortic EC to present antigen to T cells, but the conclusion they made contrasted from that of the three groups mentioned above. That is, Roska et al. concluded that EC do not function as APC. To explain the discrepancy between their results and those of the others, Roska et al. suggested that because the human umbilical vein EC used by the other groups had not been passaged in culture (or at the most had only been subcultured

twice), it was possible that the EC preparations contained contaminating cells which were the active antigen-presenting cells. Another possibility was that the T-cell preparations contained enough MΦ (or other APC) such that the EC only augmented the presentation of antigen by the MΦ. In support of their latter suggestion, Roska et al. showed the results of experiments where the guinea pig aortic EC augmented primed-lymphocyte responses to antigen-pulsed syngeneic MΦ. The capability of the EC to augment MΦ presentation of antigen was not MHC restricted.

Burger et al., Hirschberg et al., and Nunez et al. reported the results of experiments designed to examine the possibility that the EC were simply augmenting antigen presentation by other contaminating cell types. Both Burger et al. (14) and Nunez et al. (17) did experiments using a total of five different anti-macrophage antibodies showing that none of the antibodies inhibited antigen presentation by the EC. To discount the possibility that fibroblasts or smooth muscle cells from the subendothelial layers of the umbilical vein were not actually presenting the antigen, Burger et al. (14) and Hirschberg et al. (18) directly tested the ability of those cells to present antigen. In both sets of experiments the fibroblasts and smooth muscle cells could not present antigen. In Burger et al. experiments the fibroblasts and smooth muscle cells were isolated from the same umbilical vein as the EC so that they would have the same MHC haplotypes. In experiments where the EC did present antigen, fibroblasts and smooth muscle cells did not. This

ruled out the possibility that the fibroblasts and smooth muscle cells did not present antigen because they were not HLA compatible with the T cells. In the experiments of Roska et al. (43) showing the capability of EC to augment MΦ presentation of antigen only the MΦ had to be MHC compatible to the T cells; the EC-mediated augmentation of antigen responsiveness was not genetically restricted. In contrast, Burger et al. (14, 15), Hirschberg et al. (16, 17, 42), and Nunez et al. (17) demonstrated that EC presentation of antigen is HLA-DR restricted, as would be expected if the EC were presenting the antigen vs. augmenting antigen presentation by APC in the T-cell preparations. The fact that the responding lymphocytes in the experiments of Nunez et al. (17) were mumps-specific T-cell lines (maintained in culture for 1 to 6 months by alternate exposure to IL-2 and antigen-pulsed irradiated adherent cells) gives additional support to the conclusion that the EC were not augmenting antigen presentation by cells contaminating the T-cell preparations. Therefore, the discrepancy between the results of Roska et al. and the other groups of investigators mentioned above is still not clear. It may be a result of Roska et al. using guinea pig aortic endothelial cells while all of the other investigators used human umbilical vein endothelial cells. That there is a difference beyond that of species in the two types of endothelial cells is suggested by the preliminary findings that guinea pig aortic EC can not be induced to express Ia antigens by either supernatant from activated T cells or by IFN-γ (43) whereas

Ia antigens are inducible on human umbilical vein EC (44-46). While the reason for the contradictory findings of Roska et al. is still a matter for speculation, the results do point out the need for additional experiments to confirm that EC are fully capable independent APC.

One of the major criticisms of experiments designed to demonstrate that EC present antigen is that the EC preparations may contain contaminating cells. Burger et al., Hirschberg et al., and Nunez et al. isolated EC from human umbilical veins by similar modifications of the technique described by Jaffee et al. (47). When EC are isolated from umbilical veins by the methodology of Jaffe et al. the primary candidates for contaminating cells are fibroblasts and smooth muscle cells from the subendothelial layer of the vein and cells from the cord blood. As discussed above, fibroblasts and smooth muscle cells from umbilical veins have been directly tested by Burger et al. for their capability to present antigen, and they were found to not present antigen to T cells (14). Therefore, even if they do contaminate some of the primary EC cultures, fibroblasts and smooth muscle cells do not seem to be responsible for the antigen presentation. Prior to collecting the EC, the umbilical vein is flushed with saline to remove cord blood cells (14). Still it is possible that some cord blood cells may be in the primary EC culture. Since endothelial cells are adherent, normally as part of the process of changing their growth medium the culture flask is actually rinsed. Consequently, any nonadherent

contaminating cells (eg. erythrocytes) are removed when the endothelial cell's medium is changed. If present, cord blood cells such as granulocytes, monocytes, and dendritic cells will initially adhere to the plastic along with the EC. After approximately two days in culture the granulocytes die and are no longer adherent (42). The dendritic cells and the majority of macrophages are no longer adherent after 24 to 48 hrs of culturing, and during that period the EC undergo significant proliferation, greatly increasing their number compared to the non-proliferating macrophages and dendritic cells (11, 42). Consequently, as the period of time that the EC can be maintained in culture lengthens, the percentage of any contaminating cord blood cells should decrease.

In order to maintain EC in culture for an extended period of time several modifications of the basic culture system are required. The basic culture system consists of growing the EC in untreated plastic tissue-culture vessels and using medium (199 or RPMI-1640, GIBCO) with 20% fetal calf serum. In 1979 Maciag et al. reported the discovery of an EC growth factor derived from bovine hypothalmi (48). The factor was termed endothelial cell growth factor (ECGF) and was found to be a protein with a molecular weight of approximately 75,000. Another modification to the basic culture system is to pretreat the culture vessels with human fibronectin. Maciag et al. reported that human umbilical vein EC could be grown for 15 to 21 passages at a split ratio of 1:5 (at least 27 population doublings) on a human fibronectin matrix in Medium 199

supplemented with fetal bovine serum and ECGF (49). The number of population doublings can be increased even further to a median level of 58 by adding heparin along with ECGF to the culture medium (50). Consequently by growing EC in fibronectin-coated tissue-culture flasks in medium containing serum, ECGF, and heparin it is possible to maintain a single isolate of EC in culture for several months.

By using the modified culture system described above we were able to maintain human umbilical vein endothelial cells in culture for approximately eight months (18-20 subcultures). If the original EC isolates were contaminated with fibroblasts, the fibroblasts became the predominate cell type after only a couple of subcultures due to their fast rate of replication. Cultures contaminated with fibroblasts could then be easily detected and discarded. Endothelial cells subcultured approximately five times had been in culture for 1-2 months. Any contaminating non-proliferating or non-adherent cells would no longer be expected to be present. As was anticipated, the subcultured EC stained uniformly for the EC-specific marker Factor VIII-related antigen (51, 52) (Manuscript 1, Fig. 2). The subcultured EC were then assayed for their capability to present antigen and were found to be able to do so (Manuscript 2, Tables 1 and 2). The possibility of any undetected contaminating cells decreases as EC are further subcultured: any other cells replicating faster than the EC would be more readily detected and any cells dividing at a slower rate than the EC progressively would become an insignificant percentage of the cells

if not nonexistent. As the EC were further subcultured they did retain the capability to present antigen (the EC were assayed through the 18th subculture) (Manuscript 1, Tables 1 and 2). Similar to the experiments using primary and secondary subcultures of EC (14), anti-MΦ monoclonal antibody which inhibited MΦ presentation of antigen did not inhibit presentation of antigen by extensively subcultured EC (Manuscript 1, Table 4). While the continued capability of subcultured EC to present antigen makes it extremely unlikely that any cells contaminating the EC preparations were critical to the antigen-activation of the T cells, one further step to assure the purity of the EC preparations was taken, namely the EC were cloned. The cloned EC had a morphology characteristic of EC and stained uniformally for Factor VIII-related antigen. When the cloned EC were used to present antigen to T cells they did so in an HLA-D-region restricted manner (Manuscript 3, Tables 2 and 4).

After subculturing and then finally cloning the EC, the only remaining possible source of contaminating APC would be the T cell preparations. For experiments where subcultured EC were used as the APC, the T cell preparations consisted of MΦ-depleted peripheral-blood lymphocytes (Manuscript 1, Tables 1-6). The major criteria in those experiments for determining that the T-cell preparations were free of APC was a functional one: the T cells were tested for their capability to proliferate to antigen without any APC being added to the culture and, if they were depleted of APC, the results were equal to the T-cell proliferation without any

antigen present. The majority of the T-cell preparations did not proliferate in response to antigen without APC being added, making it unlikely that the T-cell preparations contained cells required for EC presentation of antigen. To eliminate further the possibility of contaminating cells, the T cells were cloned. Using cloned T cells, it was found that subcultured and cloned EC could present antigen (Manuscript 3, Tables 3 and 4).

The finding that cloned EC can present antigen to cloned T cells implies that EC are fully capable independent APC. Based on what is known about MΦ presentation of antigen, it would then be expected that EC can synthesize Ia determinants and interleukin 1 (IL-1) (19-26). The fact that EC presentation of antigen is HLA-D-region restricted (14-18), and the fact that it is possible to lyse freshly isolated EC with anti-HLA-DR antisera and complement (31, 32), suggests that EC can synthesize and express class II MHC determinants. Yet it is difficult to detect HLA-DR determinants on subcultured human umbilical vein EC. Pober and Gimbrone reported that treatment of primary cultures of human umbilical vein EC with the lectin phytohemagglutinin induced the expression of Ia antigens (45). They found that the addition of lectin uniformally affected all of the EC in culture, did not depend on cell division, and was associated with a cell-shape change (the EC assumed a more spindle-shaped morphology). Expression of HLA-DR determinants was detected in those experiments by monoclonal antibody binding and by immunoprecipitation of radioiodinated proteins. Pober and Gimbrone

suggested that the mechanism of action by the PHA was to stimulate contaminating T cells so that the T cells then induced the expression of EC Ia determinants. Their hypothesis was supported by their preliminary observation that the dose of PHA used in their experiments was stimulatory for human T lymphocytes, and that treatment of the primary EC cultures with an anti-human T-cell monoclonal antibody and complement before the addition of PHA abrogated the induction of Ia antigens without altering the EC viability. In a subsequent paper they confirmed those preliminary observations and they showed that a supernatant from activated T cells or human γ interferon (an activated T cell product) could induce the expression of HLA-DR antigens (46). Furthermore, they found that it was not only possible to induce the expression of HLA-DR determinants but also that of HLA-DS and -SB determinants (46).

The Pober and Gimbrone experiments described above looked at the expression of Ia antigens by primary cultures of human umbilical vein EC. The primary cultures of EC could have been contaminated with other cell types such as MΦ that were responsible for the synthesis of the HLA-D-region determinants. The fact that there were enough contaminating T cells remaining in their EC cultures so that the addition of PHA resulted in Ia antigen expression demonstrates that the EC cultures were not pure cultures. Since activated-T-cell supernatants and γ interferon have been reported to induce MΦ expression of Ia antigens (53-56), it is possible

that contaminating MΦ synthesized the HLA-D-region determinants that were then acquired cytotropicly by the EC. As was discussed above, there is little likelihood that extensively subcultured EC are contaminated with other cell types. We therefore examined the capability of subcultured EC to synthesize HLA-D-region determinants. When subcultured human umbilical vein EC were grown in medium containing radiolabeled amino acids, it was possible to stimulate them with an activated-T-cell supernatant so that EC HLA-D-region determinants could be immunoprecipatated (Manuscript 2, Figures 2 and 3). Monoclonal antibodies to both HLA-DR determinants (BRL DR.1 and NEI-011 clone 7.2) and HLA-DS determinants (Anti-Leu-10) immunoprecipitated proteins from biosynthetically labeled NP-40 lysates of EC with molecular weights typical of class II MHC molecules (i.e., they immunoprecipitated two chain structures with an alpha chain of approximately 32 kd and a beta chain of approximately 29 kd). While we did not formally demonstrate that the anti-HLA-DS antibody, Anti-Leu-10, did not cross-react with EC HLA-DR determinants, Anti-Leu-10 antibody has been shown to be non-cross-reactive using B-cell lines (57). The class II determinants synthesized by the EC were functional in that similar cultures of subcultured EC presented antigen in an HLA-D-region restricted fashion (i.e. they only presented antigen to HLA-D-region compatible T cells) (Manuscript 1, Table 3). Also, antibodies to both HLA-DR and HLA-DS determinants blocked EC presentation of antigen (Manuscript 1, Tables 5 and 6, respectively). From these

results we concluded that EC can synthesize and functionally express HLA-D-region determinants.

Collins et al. has used subcultured human umbilical vein EC (passages 5-10) to study the induction of EC class II MHC determinants by recombinant human γ interferon (58). Using cDNA probes they found that IFN-γ induces the de novo appearance of transcripts of multiple class II antigen genes, including the α and β chain genes of HLA-DR, -DS, and -SB, as well as transcripts of the invariant (γ) chain. This correlated with their finding that multiply-passaged human umbilical vein EC transformed by Simian virus 40 DNA could be induced to express HLA-DR antigens which were specifically recognized by cloned cytotoxic T cell lines (44).

All of the reported experiments designed to analyze the capability of EC to synthesize and functionally express HLA-D-region determinants agree that EC do have that capability when they are appropriately stimulated (eg. by IFN-γ). If subcultured EC do not express Ia determinants as measured by immunoflorescence, immunoprecipitation, or the presence of class II MHC gene transcripts, then the question arises as to how subcultured EC can present antigen to T cells when it is necessary for T cells to recognize antigen in the context of Ia determinants (leading to T-cell activation and secretion of IFN-γ). Simultaneous recognition of both antigen and Ia determinants supposedly is the basis for the phenomenon of MHC restriction of antigen presentation (59) and subcultured EC do present antigen in a HLA-D-region

restricted manner. One possible explanation for why Ia antigen-negative EC can stimulate T cells to secrete mediators that in turn induce EC expression of Ia determinants is that the EC may not be truly negative for class II MHC determinants; the level of expression of Ia determinants may simply be below that of detection. Another possibility is that some T-cell recognition of antigen occurs independently of Ia determinants resulting in a T-cell signal such that EC begin to synthesize Ia determinants leading to T-cell recognition of and proliferation in response to antigen that is measured by ^{3}H-thymidine incorporation. Whether either of these possibilities is correct is unknown. Thus, the sequence of induction of Ia determinants vs. T-cell recognition of antigen is still a matter of speculation.

In addition to presenting antigen in the context of Ia determinants, APC are thought to provide a third activation signal, namely interleukin 1 (22-26). Interleukin 1 (IL-1) appears to be necessary for T-cell proliferation subsequent to T-cell recognition of antigen (60-64). APC production of IL-1 is thought to promote T-cell proliferation by stimulating the production of T-cell-derived growth factor (interleukin 2, IL-2) (22-24).

Endothelial cells have been reported to augment T-lymphocyte responses to mitogen or antigen in cultures supported by MΦ (43). It is likely that the EC augmented the T-cell proliferation by secreting IL-1, since EC supernatants also augmented mitogen-induced T-cell proliferation in the presence of MΦ (43). To test whether

EC free of contaminating cells release a mediator(s) with IL-1 activity, we exposed extensively subcultured EC to lipopolysaccharide (LPS) and then 24-48 hours later collected supernatants to be analyzed for IL-1 activity. Macrophages were the first cells described to be producers of IL-1 (65, 66) and most of the IL-1 that has been biochemically and biologically analyzed has been obtained from freshly isolated macrophages or the murine cell line $P388D_1$ (67-72). Consequently supernatants from freshly isolated human MΦ were used as positive controls. LPS was used to stimulate EC and MΦ secretion of IL-1 as it is a known stimulant for MΦ production of IL-1 (68, 73).

Both the MΦ and EC supernatants were tested for IL-1 activity in two different assay systems: murine thymocyte proliferation and IL-1-dependent LBRM-33-IA5 production of IL-2. In the murine thymocyte proliferation assay IL-1 is added to cultures of thymocytes with or without suboptimal doses of phytohemagglutinin or concanavalin A (74, 75). The cultures are incubated for 72 hrs (the last 4 hrs in the presence of ^{3}H-thymidine) and then harvested. The presence of IL-1 results in the production of IL-2 which in turn results in increased thymocyte proliferation (75). When EC supernatants were tested in the thymocyte assay the results were marginal; the EC supernatants enhanced the proliferation but only very slightly (Manuscript 2, Table 1). The same equivocal results were obtained whether or not mitogen was added along with the EC supernatant to the thymocytes. Conlon has calculated that the

LBRM-33-IA5 assay is 1,000 to 10,000 times more sensitive than the traditional thymocyte proliferation assay (76). Therefore the EC supernatants were tested using the LBRM-33-IA5 cell line in an attempt to unambiguously ascertain whether IL-1 activity was present. In the presence of IL-1 and mitogen, LBRM-33-IA5 cells secrete IL-2 (75). An IL-2 dependent cell line such as CTLL-2 can then be used to detect the IL-2, thereby indirectly measuring the amount of IL-1 added to the LBRM-33-IA5 cells. The EC supernatants assayed using the LBRM-33-IA5 cells were definitely positive for IL-1 activity (Manuscript 2, Table 2). Since IL-2 will also give positive results in both the thymocyte and LBRM-33-IA5 assays, the EC supernatants were tested for IL-2 activity using an IL-2-dependent cell line. While the EC supernantants were positive for IL-1 activity, they were negative for IL-2 activity (results not shown).

The LBRM-33-IA5 assay demonstrated that the EC supernatants did contain IL-1 activity, though they contained approximately 100 times less than supernatants from roughly the same number of MΦ. The difference in levels of activity is probably why the EC gave marginal results while the MΦ supernatants were positive in the less sensitive thymocyte assay. EC may produce less IL-1 than MΦ or it may be that the EC were not maximally stimulated to secrete IL-1. Haq et al. have recently reported that when human monocytes are cultured for 2-6 days, that coincidently with transforming to become macrophages that the cells lose their ability to secrete IL-1

when stimulated by LPS (77). Interestingly the macrophages were as effective if not more effective than monocytes at supporting concanavalin A-driven T-cell proliferation. When the macrophages were stimulated with LPS and then lysed, their lysates were found to contain high levels of IL-1 activity (as did lysates of LPS stimulated monocytes). The mediator(s) responsible for the IL-1 activity in the macrophage lysates was not biochemically characterized, but a previous report by Mizel and Rosenstreich (78) demonstrating and characterizing the presence of $P388D_1$ intracellular IL-1 would support the contention that the macrophage lysate's biologic activity was due to IL-1. Endothelial cells may be similar to the macrophages described by Haq et al. in that the majority of EC IL-1 may not be secreted but on the EC surface membrane or within the EC. Consequently, it is possible that the total EC IL-1 activity (secreted plus cellular) may be equal to that from macrophages.

The EC mediator(s) responsible for the IL-1 activity has not been biochemically characterized. It is likely the EC mediator(s) is very similar to IL-1. This has been shown to be the case for keratinocyte (79) and glomerular mesengial cell (80) thymocyte activating factors. Even if the EC mediator(s) is not identical to MΦ IL-1, what is probably of greater importance is that an EC mediator(s) facilitates the production of IL-2 as evidenced by the LBRM-33-IA5 assay. It is of importance because facilitation of IL-2

production by IL-1 is thought to be necessary for maximal T-cell proliferation (22-24).

To summarize to this point, human umbilical vein EC, free of contaminating cell types as evidenced by extensive subculturing or by cloning, are capable of presenting antigen to either adherent-cell-depleted T cells or cloned T cells. Presentation of antigen by EC is HLA-D-region restricted. Since EC presentation of antigen is restricted, then as independent APC, EC must be able to synthesize Ia antigens. This has proven to be the case: human umbilical vein EC synthesize HLA-DR, -DS, and -SB molecules. Finally, EC secrete a mediator(s) (presumably IL-1) which facilitates the production of IL-2.

When subcultured EC were examined for their capability to present antigen to cloned T cells (Manuscript 3, Table 3), in addition to concluding that the EC could present the antigen, the observation was made that the cloned T cells gave a higher response when the antigen was presented by freshly-isolated autologous MΦ vs. the subcultured EC. Since the cloned T cells were derived and maintained by alternating exposure to antigen-pulsed MΦ and IL-2, possibly a subpopulation of the antigen-responsive T cells that recognized the antigen presented by MΦ had been selected. In order to determine if subpopulations of antigen-responsive T cells with a preference for a particular APC type do exist, two different types of experiments were undertaken. One approach was to derive and clone T cells using antigen-pulsed EC (vs. antigen-pulsed

MΦ). When those cloned T cells were tested for their response to antigen presented by MΦ vs. EC, two of the three clones (E13 and E32) gave higher proliferative responses when the antigen was presented by EC, while the third clone did not show a preference (Manuscript 3, Table 6). The second approach was to do a proliferation suicide experiment (Manuscript 3, Table 5). In this experiment MΦ-depleted T cells were cultured with antigen and either MΦ or EC. (The MΦ and EC were cultured from the same umbilical cord and were histocompatible to the T cells.) At the peak of the proliferative response, lethal high-specific-activity ^{3}H-thymidine was addded to the cultures, killing all of the responding T cells. The remaining viable T cells were then restimulated by the same antigen presented by the same type of APC and, in a separate culture, the same antigen presented by the alternate APC. When the same APC was used in both the suicide and restimulation cultures, the T cells did not give a proliferative response. In contrast, when the antigen was alternately presented by MΦ and EC , the T cells did give a proliferative response and the response was approximately equal for both sequences of APC (EC in the first culture followed by MΦ in the second culture and vice versa). Interestingly, the response of the whole T-cell population (i.e., T cells not exposed to antigen in the suicide culture) to antigen presented by either the EC or the MΦ in the restimulation culture was the same in both cases (a mean CPM of approximately 4,000) and was equal to the sum of the responses by the two

subpopulations (2,320 CPM from the "EC-preferring" T cells + 2,187 CPM from the "MΦ-preferring" T cells), suggesting that the two subpopulations overlap. Thus, the results of the experiments using the cloned T cells along with the results from the suicide experiment show that T cells may have a preference for antigen presented by EC or MΦ but that the preference is not absolute (they can respond to the antigen when it is presented by either EC or MΦ).

Hirschberg et al. have also commented on whether identical populations of lymphocytes respond to antigen on either MΦ or EC (42). They performed an experiment where T cells were cultured in the presence of antigen and autologous MΦ for twelve days, the T cells were recovered, and then in a secondary culture the T cells were restimulated by the same antigen but presented by EC. This protocol differs from that of the suicide experiments discussed above in that the proliferating cells in the first culture were not killed. The results showed that T cells primed to antigen presented by MΦ can respond to the same antigen presented by EC. Hirschberg et al. concluded that the same population of T cells respond to antigen regardless of the type of accessory cells used in the secondary culture. Unfortunately, they did not also determine what the results would be if the same APC type was used in both the first and second cultures. If they had, our results would lead to the prediction that the T-cell proliferation would be greater than that when different APC types were used. Without those additional

results it is impossible to completely evaluate the experiments of Hirschberg et al. in relation to ours. From the results published by Hirschberg et al. it is only possible to say that they agree with ours in that T cells do not seem to have an absolute preference for antigen presented by EC vs. MΦ.

One example of T cells having an absolute preference for an APC type has reported by Braathen (81). T cells were obtained from patients with contact allergic dermatitis and a positive epicutaneous patch test to nickel. The T cells were cultured with various dilutions of nickel sulphate and either autologous Langerhan's cells or autologous MΦ. The patient's T cells gave a strong Langerhan's cell-dependent T-cell response to nickel sulphate, while the T cells cultured with peripheral blood adherent cells and nickel sulphate showed no response. Braathen along with Thorsby has reported that, with a different antigen (PPD) T cells respond equally well when the PPD is presented by either Langerhan's cells or adherent peripheral blood lymphocytes (10). Therefore, whether there are different subpopulations of T cells responsive to the same antigen may be dependent on the antigen involved.

All of the reported experiments testing the capability of endothelial cells to present antigen have been done in vitro. In our laboratory we have developed an in vivo protocol for the adoptive transfer of delayed-type hypersensitivity (DTH) in rats using antigen-conditioned cells (82). We are in the process of applying that protocol to ask questions regarding the importance of

EC presentation of antigen in vivo. The transfer of DTH is genetically restricted: PPD responsive cells from one rat will only transfer cellular immunity to a recipient rat with the same MHC haplotypes as the donor rat. In this system DTH is measured by the swelling of the recipient's ear upon antigenic challenge. The positive responses have been shown to be antigen specific, and histologic examination of a positive ear revealed a cellular infiltration typical of a DTH response (82). We have begun to do experiments testing the ability to transfer DTH into chimera recipients. The chimeras are generated by reconstituting lethally irradiated P_1 rats with a $(P_1 \times P_2)$ F_1 bone marrow. Antigen primed cells from a P_2 donor are then transferred into the chimera and three days later the chimera is tested for antigen-specific DTH. In that experiment the T cells are MHC haplocompatible to the bone marrow derived cells (eg. antigen presenting MΦ and dendritic cells), but are MHC incompatible with the recipient's non-bone-marrow-derived cells. Consequently, the T cells are incompatible with the recipient's EC. Preliminary results from such experiments demonstrate that P_2 cells cannot transfer DTH to a recipient P_1 chimera rat [reconstituted with $(P_1 \times P_2)$ F_1 bone marrow] whereas P_1 T cells (which are MHC compatible to the recipient chimera's EC) can transfer DTH to the P_1 chimeric rat. These results suggest that a MHC-restricted event between the T cells and the recipient's non-bone-marrow derived cells is crucial to the development of a DTH response. While the experiments thus

far do not prove that the restricting non-bone-marrow-derived cells are EC, it is likely that the "missing" restricting cells are EC based on in vitro experiments showing that EC present antigen in a MHC restricted manner. Therefore, our preliminary in vivo experiments suggest that antigen presentation by EC is necessary for the development of a DTH response.

The results of both in vitro and in vivo experiments are consistent with Burger and Vetto's hypothesis that EC play a central role in the development of cell-mediated immune responses. The in vitro experiments studying the antigen presenting capability of human umbilical vein endothelial cells demonstrate that EC can serve as fully-capable independent antigen-presenting cells. The results of preliminary in vivo experiments are strongly suggestive that antigen presentation by EC is required for the development of delayed-type hypersensitivity, a cell-mediated immune response. In addition to hypothesizing that EC present antigen to circulating T cells, Burger and Vetto have suggested that subsequent to presenting the antigen EC release the key mediators of a cellular immune response. While this aspect of their hypothesis remains to be tested, the experimental results thus far imply that EC are active participants of cell-mediated immune responses.

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ABBREVIATIONS

APC	Antigen presenting cells
CMI	Cell-mediated immunity
Con A	Concanavalin A
cOVA	Chicken ovalbumin
DNFB	Dinitroflurobenzene
DNP-GPA	2,4-Dinitrophenyl-guinea pig albumin
DTH	Delayed-type hypersensitivity
EC	Endothelial cells
ECGF	Endothelial cell growth factor
FGG	Fowl gamma globulin
H-2	The murine MHC locus
HEL	Hen egg-white lysozyme
HLA	The human MHC locus
HSV	Herpes simplex virus antigen
Ia	I-region associated
IL-1	Interleukin 1
IL-2	Interleukin 2
KLH	Keyhole limpet hemocyanin
LAF	Lymphocyte-activating factor
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIRF	Macrophage-(Ia positive) recruiting factor

MLR	Mixed leukocyte response
MNC	Mononuclear cells
MΦ	Macrophages
NRGG-Fab_2	Normal-rabbit-gamma globulin-Fab_2
OVA	Ovalbumin
PHA	Phytohemaglutinin
PPD	Purified protein derivative of tuberculin
RAMIG-Fab_2	Rabbit anti-mouse immunoglobulin-Fab_2
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	Stimulation index
TNP	Trinitrophenyl
UV	Ultraviolet
VZV	Varicella-zoster virus antigen