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Research Article

The effects of interferons (IFNs) on lymphocyte adhesion to cultured human vascular endothelial cells (EC) were investigated using an in vitro assay. Endothelial cells obtained from umbilical vein were first cultured at a low density with a conditioned medium (CM) from 12-O-tetra decanoylphorbol 13-acetate-concanavalin A (TPA-Con A) stimulated human peripheral blood lymphocytes (PBL), or with recombinant (r) gamma interferon (IFN-gamma) or r alpha interferon (IFN-alpha), and then were incubated with freshly isolated PBL. Natural IFN-gamma in the TPA-Con A CM and rIFN-gamma (12.5-500 U/ml) induced major histocompatibility complex-class II antigens (HLA-DR, HLA-DP, and HLA-DQ) and significant lymphocyte adhesion to the EC, whereas rIFN-alpha did not. The lymphocyte adhesion to the EC and the expression of DR antigens on the EC were well correlated in terms of both kinetics and the dose-response pattern of rIFN-gamma. When EC expressing I region associated (Ia) antigen were preincubated with monoclonal anti-DR antibody before the addition of lymphocytes, the lymphocyte adhesion was significantly inhibited in both allogeneic and syngeneic combinations, whereas anti-HLA-DP, anti-HLA-DQ, and anti-HLA-ABC antibodies did not inhibit the binding at all. Cell fractionation experiments indicated that the majority of lymphocytes adhering to Ia-expressed EC were Leu-3+ T cells, whose binding was again almost completely inhibited by anti-DR antibody. Moreover, anti-Leu-3a, but not anti-Leu-2a, antibody effectively inhibited the T cell adhesion to the EC. [...]

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Mechanisms of Lymphocyte Adhesion to Human Vascular Endothelial Cells in Culture

T Lymphocyte Adhesion to Endothelial Cells through Endothelial HLA-DR Antigens Induced by Gamma Interferon

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Abstract

The effects of interferons (IFNs) on lymphocyte adhesion to cultured human vascular endothelial cells (EC) were investigated using an *in vitro* assay. Endothelial cells obtained from umbilical vein were first cultured at a low density with a conditioned medium (CM) from 12-O-tetra decanoylphorbol 13-acetate-concanavalin A (TPA-Con A) stimulated human peripheral blood lymphocytes (PBL), or with recombinant (r) gamma interferon (IFN- γ) or r alpha interferon (IFN- α), and then were incubated with freshly isolated PBL. Natural IFN- γ in the TPA-Con A CM and rIFN- γ (12.5–500 U/ml) induced major histocompatibility complex-class II antigens (HLA-DR, HLA-DP, and HLA-DQ) and significant lymphocyte adhesion to the EC, whereas rIFN- α did not. The lymphocyte adhesion to the EC and the expression of DR antigens on the EC were well correlated in terms of both kinetics and the dose-response pattern of rIFN- γ . When EC expressing I region associated (Ia) antigen were preincubated with monoclonal anti-DR antibody before the addition of lymphocytes, the lymphocyte adhesion was significantly inhibited in both allogeneic and syngeneic combinations, whereas anti-HLA-DP, anti-HLA-DQ, and anti-HLA-ABC antibodies did not inhibit the binding at all. Cell fractionation experiments indicated that the majority of lymphocytes adhering to Ia-expressed EC were Leu-3⁺ T cells, whose binding was again almost completely inhibited by anti-DR antibody. Moreover, anti-Leu-3a, but not anti-Leu-2a, antibody effectively inhibited the T cell adhesion to the EC. These results strongly suggest that the interaction of the Leu-3(T4) receptor of T cells with IFN- γ -induced DR antigens on EC plays a central role in the selective adhesion of Leu-3⁺ T cell to EC.

Introduction

Accumulation and *in situ* activation of sensitized T cells in non-lymphoid tissues where triggering antigens are localized, is considered to be the initial event in local cell-mediated immune responses, such as delayed-type hypersensitivity, allograft rejection, and allergic neuritis (1, 2). In these lesions, the lymphocyte traffic dramatically increases from blood through the inflammatory site into afferent lymph (3–5). However, the lymphocyte

migration from blood is not random, and distinct populations of lymphocytes can be directed selectively to sites of local immune responses (6–9). For example, a recent study in human skin allografts has shown that helper/inducer T cells predominantly infiltrate the graft dermis and graft bed and have a different distribution pattern from that of suppressor/cytotoxic T cells (6).

The selective migration of lymphocytes to particular lymphoid organs or sites of chronic inflammation raises two basic questions: first, whether or not the selectivity depends on a specific recognition between lymphocytes and vascular endothelial cells (EC)¹ in the adhesion process, which is the important initial step of lymphocyte migration through microvessels (10), and second, what controls the increased lymphocyte adhesion on the surface of microvessels in the site of chronic inflammation. As for the former, accumulated evidence shows that the binding between organ-specific determinants on EC of postcapillary high endothelial venules (HEV), in particular, lymphoid organs and corresponding receptor molecules of lymphocytes, was involved in the selective migration of lymphocytes into lymph nodes (11–13). Recently, lymphocyte receptors specific for the endothelial determinants in lymph nodes have been elucidated by direct biochemical isolation (14) as well as by monoclonal antibody (15). As for the latter, little is known about the exact mechanisms involved in the lymphocyte entry through microvessel into chronic inflammatory sites.

In this study, we tried to analyze the mechanisms of lymphocyte adhesion to EC using cultured human umbilical EC. The results clearly indicated that Leu-3⁺ (helper/inducer) T cells recognized the major histocompatibility complex (MHC) class II antigens, especially DR antigens, on EC induced by gamma interferon (IFN- γ) and adhered tightly onto the EC.

Methods

Isolation and culture of EC. The EC were obtained by treating a fresh human umbilical vein with 0.1% collagenase (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS), as described by Jaffe et al. (16). The EC were then suspended in medium 199 (M199) (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% unheated human serum (HS) (pooled in our laboratory), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 15 mM HEPES (Sigma Chemical

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1. *Abbreviations used in this paper:* Ab, antibody; A.I., adhesion index; BRBC, bovine red blood cells; Con A, concanavalin A; CM, conditioned medium; E, erythrocytes; EAE, experimental allergic encephalitis; EC, endothelial cells; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HEV, high endothelial venules; HS, human serum; IFN, interferon; IFN- α , alpha interferon; IFN- γ , gamma interferon; M199, medium 199; MHC, major histocompatibility complex; r, recombinant; SRBC, sheep erythrocytes; TPA, 12-O-tetra decanoylphorbol 13-acetate.

Co.), and were cultured in 60-mm culture dishes (Nunc, Denmark). The EC thus obtained were morphologically homogeneous and showed positive immunofluorescence with a rabbit anti-Factor VIII antiserum (Behring Diagnostic, Somerville, NJ). The cultures were incubated at 37°C in a humidified 5% CO₂, 95% air environment. The culture medium was changed every 2–3 d. The EC were serially passed by brief exposure to 0.25% trypsin (Difco Laboratories, Inc., Detroit, MI) and 0.04% EDTA (Sigma Chemical Co.). In this study, EC were used from four passages only.

Preparation of lymphocytes. Human peripheral blood lymphocytes (PBL) were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. The PBL were washed twice in PBS and resuspended in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 5% fetal calf serum (FCS) (Flow Laboratories, Inc., McLean, VA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The monocytes were removed by being passed through a Sephadex G-10 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as described by Ly and Mishell (17). The nonadherent cells contained <1% monocytes as determined by peroxidase staining.

Separation of T and non-T cells. T cell-enriched fraction and T cell-depleted fraction were separated by the sheep erythrocyte-rosetting method as described elsewhere (18). Briefly, 5 × 10⁶ G-10 passed lymphocytes and 2 × 10⁸ neuramidase-treated sheep erythrocytes (SRBC) were mixed in 1 ml of RPMI 1640 supplemented with 50% FCS. The mixture was centrifuged for 5 min at 1,000 rpm and incubated at 4°C for 90 min. Using the Ficoll-Hypaque gradient technique, the T cell-enriched fraction and T cell-depleted fraction were isolated from the bottom and the interface layers, respectively. After the SRBC had been lysed with distilled water, each fraction of lymphocytes was suspended in M199. The T cell-enriched fraction contained >85% erythrocyte (E) rosette-forming cells and the T cell-depleted fraction contained <5% E rosette-forming cells. In some experiments, T cell fraction was also prepared by being passed through a nylon-wool column (19).

Isolation of T cell subsets. Nylon-wool nonadherent lymphocytes served as a source of cells for the isolation of T cell subsets. Helper/inducer T cells and suppressor/cytotoxic T cells were prepared with monoclonal antibodies (Becton-Dickinson & Co., Oxnard, CA) specific for each subset; Leu-2a antibody for suppressor/cytotoxic T cells and Leu-3a for helper/inducer T cells. The T cell subsets were separated by using the method of Van Epps et al. (20). Briefly, lymphocytes were incubated at 4°C for 20 min with 1 µg of anti-Leu-2a or anti-Leu-3a monoclonal antibodies per 10⁶ cells. The lymphocytes were then washed three times and resuspended in RPMI 1640 supplemented with 5% FCS (preabsorbed with bovine erythrocytes [BRBC]) to 1 × 10⁷ cells/ml. Equal volumes of these cells were mixed with BRBC that had been coated with purified goat anti-mouse IgG antibody (Cappel Laboratories, Cochranville, PA) in a chromic chloride treatment. This suspension was centrifuged for 2 min at 1,000 rpm, and the cells were resuspended, placed over Ficoll-Hypaque gradient, and centrifuged for 30 min at 400 g to separate rosetting and nonrosetting cells. Rosetting cells and free BRBC were obtained from the cell pellet and nonrosetting cells were obtained from the interface layer. The isolated subsets were washed, resuspended in M199, and used in lymphocyte-EC adhesion assay. The purity of isolated T cell subsets was assessed by a FACS analyzer (Spectrum III; Ortho Diagnostic Systems, Inc., Raritan, NJ). In this study, only cell preparations with >90% purity were used.

Preparation of 12-O-tetra decanoylphorbol 13-acetate (TPA) concanavalin A (Con A) conditioned medium (CM). Freshly isolated human PBL (3 × 10⁶ cells/ml) were preincubated with 2 ng/ml of TPA (Sigma Chemical Co.) at 37°C for 4 h. After being washed three times with RPMI 1640, the cells were cultured with Con A (Sigma Chemical Co.), 5 µg/ml, in RPMI 1640–5% FCS at 37°C in 5% CO₂/air. The TPA–Con A CM was harvested after a 72-h incubation, and immediately added to the EC cultures for the lymphocyte-EC adhesion assay and endothelial DR expression assay. The interferon (IFN) activity in the TPA–Con A CM was determined by microcytopathic effect inhibition assay using encephalomyocarditis virus and FL cells (human amnion-derived cell line) as described elsewhere (21). Acid treatment of TPA–Con A CM was done by dialysis against a pH 2.0 glycyl-glycine buffer at 4°C for 48

h using Spectrapor membrane tubing ([mol wt cutoff, 8,000] Spectrum Medical, Los Angeles, CA). The acid-treated CM was passed through a 0.22-µm millipore filter (Millipore Corp., Bedford, MA). For some experiments, TPA–Con A CM and rIFN-γ (10³ U/ml) were treated with monoclonal anti-IFN-γ antibody (diluted 1 in 10) (Biogen Co., Ltd., Geneva, Switzerland) for 30 min in a 37°C waterbath.

Indirect immunofluorescence and flow cytometry. The MHC class II antigens on the surface of the EC were examined with indirect immunofluorescence assay using a variety of monoclonal antibodies and fluorescein-conjugated rabbit F(ab')₂ anti-mouse IgG (Cappel Laboratories) as the second antibody. The mouse monoclonal antibodies used were PTF29.12 (IgG1, purified antibody from ascites, against an HLA-DR monomorphic determinant [22], a gift from Dr. Guido Damiani, Genova University, Italy), anti-HLA-DR (IgG1, purified antibody from ascites, against a monomorphic determinant of HLA-DR, Cat. 0201-2007, obtained from Cappel Laboratories), B7/21 (IgG1, ammonium sulfate precipitated fraction from ascites, against an HLA-DP monomorphic determinant, originally published as anti-FA [23], a gift from Dr. Takeo Juji, University of Tokyo, Tokyo, and from Dr. Joan E. Shaughnessy and Dr. F. H. Bach, University of Minnesota, Minneapolis, MN), anti-Leu-10 (IgG1, purified antibody, against an HLA-DC/DS common polymorphic determinant, obtained from Becton-Dickinson & Co., Monoclonal Center, Inc.), anti-HLA-ABC (IgG1, purified antibody against an HLA-ABC monomorphic determinant, Cat. 0201-2027, obtained from Cappel Laboratories), and anti-Leu-2a, -3a (IgG1, purified antibody, obtained from Becton-Dickinson & Co.). P3 × 63 mouse IgG1 myeloma protein obtained from Dr. Damiani was used as the negative control. Ion exchange chromatography-purified IgG fractions from ascites were used as antibody preparations, unless otherwise specified. IgG concentration of B7/21 and P3 × 63 was determined by a standard method of enzyme-linked immunosorbent assay.

Endothelial cells (2 × 10⁵/well) were cultured with TPA–Con A CM, recombinant human IFN (rIFN)-α (RO 22-8181, obtained from Ohtsuka Pharmaceutical Co., Osaka, Japan), or recombinant gamma interferon (rIFN-γ) (S-6819, obtained from Shionogi Pharmaceutical Co., Osaka) at various concentrations in Costar 24-well tissue culture clusters (Costar, Cambridge, MA). The EC were then gently rinsed with M199, and incubated with 0.1% trypsin-0.02% EDTA/PBS for 2 min at 37°C. The EC were detached by gentle pipetting, and cold M199-10% HS was added to stop trypsinization. The single cell suspension of the EC was washed twice with 1% bovine serum albumin (Sigma Chemical Co.) in PBS and stained with saturating amounts of monoclonal antibodies and the second antibody. Analysis of fluorescence intensity and the percentage of positive cells were determined by flow microfluorometry using FACS analyzers, Spectrum III (Ortho Diagnostic Systems, Inc.) and FACS 302 (Becton-Dickinson & Co.).

Assay of lymphocyte-EC adhesion. Lymphocyte-EC adhesion was studied by the method of Hoover et al. (24) with modification. A sterile coverslip (Thermanox tissue culture coverslip 5408; Miles Laboratories, Inc., Elkhart, IN) was cut to the appropriate size, and placed into each well of a 24-well tissue culture plate (Costar). 1 ml of the EC suspensions in M199-10% HS containing 1.5 × 10⁶ cells was added to each well. The plate was incubated at 37°C for 4–6 h until the EC were fully attached on the coverslip. Then 50 µl of either TPA–Con A CM, rIFN-γ, or rIFN-α in appropriate dilutions or titers were added to each well. After an incubation of 1–3 d, 1-ml aliquots of freshly isolated lymphocyte suspensions in serum-free M199 containing 5 × 10⁵ cells were added to each well. In the inhibition experiments of lymphocyte-EC adhesion, the EC were pretreated for 30 min with either monoclonal antibodies, or control IgG1 and were washed three times with M199 before the addition of lymphocytes suspended in M199-5% HS. After the addition of the lymphocytes, the coverslip was incubated at 37°C for varying periods, and washed in PBS on the magnetic stirrer to remove loosely bound lymphocytes. The coverslips were then fixed in ethanol and Giemsa-stained.

In the experiments of syngeneic combination, EC and umbilical cord blood mononuclear cells were obtained from the same umbilical cord. The EC harvested by collagenase treatment were further exposed to 0.25% trypsin with 0.04% EDTA, washed twice with PBS, and suspended in

M199-10% HS. Subsequent procedures were the same as described above. In all experiments, cultures were set up in duplicate.

Adhesion-positive EC (EC with two or more lymphocytes) were counted in three microscopic fields (2–3 mm²/field) near the center of each coverslip, and the adhesion index (A.I.) was determined as follows: A.I. = Number of adhesion-positive EC/Number of total EC/field. Results were expressed as the mean A.I.±SD of six determinations, since no significant differences were observed between the duplicate cultures in separate statistical analysis. Inhibition of lymphocyte-EC adhesion with monoclonal antibody (Ab) was determined as follows: percent inhibition = A.I. of no Ab treatment – A.I. of Ab treatment/A.I. of no Ab treatment – A.I. of control × 100.

Direct characterization of adherent lymphocyte subsets by an immunohistochemical method. The subsets of G-10–passed PBL adhering to rIFN-γ–treated EC on coverslips were directly stained with anti-Leu series (Becton-Dickinson & Co.) by an indirect immunoperoxidase method. In brief, the coverslips were fixed in cold ethanol and were incubated sequentially in normal goat serum, monoclonal antibody, peroxidase conjugated goat anti-mouse Ig (Cappel Laboratories), 3,3'-diaminobenzidine, tetrahydrochloride (DAB) substrate, after which they were counterstained with methyl green.

Enzyme treatment of lymphocytes. Freshly isolated and G-10–passed lymphocytes were suspended in PBS (2 × 10⁶ cells/ml) and incubated in a 37°C waterbath for 10 min with trypsin (25 μg/ml, Type XIII, Sigma Chemical Co.) in the presence or absence of trypsin soybean inhibitor (25 μg/ml, Type I-S, Sigma Chemical Co.). After the treatment, the lymphocytes were washed in cold RPMI-5% FCS to remove free enzymes and were resuspended in M199 at a density of 5 × 10⁵ cells/ml. Part of the trypsinized lymphocytes was further incubated for 12 h in a culture medium.

Statistical analysis. Probability (*P*) values were determined by using Student's *t* test.

Results

Effect of TPA-Con A CM on lymphocyte-EC adhesion. When freshly isolated and G-10–passed lymphocytes were incubated with untreated EC, no significant attachment of lymphocytes to the EC was observed. However, once the EC were precultured for 3 d in the presence of 1% TPA-Con A CM, the EC became elongated in shape and significant lymphocyte-EC adhesion was observed within 1 h (Table I). The control medium containing Con A (5 μg/ml) had no such effects.

Since TPA-Con A CM contained IFN activity that was acid labile and neutralized by monoclonal anti-IFN-γ antibody, the majority of IFN in the TPA-Con A CM was thought to be of γ type. When EC were cultured with 1% TPA-Con A CM depleted of IFN activity by either acid (pH 2) treatment or monoclonal anti-IFN-γ antibody treatment, the adhesion index was found to be significantly, if not completely, reduced (Table I). Therefore, it was suggested that IFN-γ was one of the factors in the CM responsible for the lymphocyte adhesion to EC. This was supported by the similar experiments with rIFN-γ.

Recently, it was reported by Pober et al. (25, 26) that IFN-γ was a potent inducer of endothelial DR antigens. In fact, the HLA-DR antigens were expressed on 62 and 100% of the EC cultured for 3 d with 1% TPA-Con A CM and 100 U/ml of rIFN-γ, respectively. In contrast, no DR antigens were expressed in the absence of IFN-γ activity (Table I). However, it is also clear from the data presented in Table I that TPA-Con A CM, which had been depleted of IFN activity by either acid treatment or treatment with anti-IFN-γ antibody, still increased the adhesion index substantially, which indicates that there are other mechanisms of lymphocyte-EC binding irrelevant to DR antigens. One such mechanism may be the induction of EC surface

Table I. Effect of TPA-Con A CM and rIFN-γ on Lymphocyte-EC Adhesion*

| Treatment | Change in shape‡ of EC | Adhesion index | IFN titer§ | | HLA-DR expression on EC |
|--------------------|------------------------|----------------|------------|---------|---------------------------------------|
| | | | Lab. U/ml | percent | |
| Medium alone | – | 0.09±0.02 | <2 | <5 | |
| TPA-Con A CM† (1%) | | | | | |
| Untreated | + | 0.47±0.04 | 32 | 62 | |
| Anti-IFN-γ–treated | + | 0.31±0.03** | <2 | <5 | |
| Acid-treated | + | 0.33±0.05** | <2 | <5 | |
| rIFN-γ (100 U/ml) | | | | | |
| Untreated | + | 0.32±0.05 | 64 | >99 | |
| Anti-IFN-γ–treated | – | 0.10±0.03 | <2 | <5 | |
| Acid-treated | – | 0.08±0.02 | <2 | <5 | |

* 2 × 10⁴ cells of endothelial cells (EC) obtained from human umbilical vein were placed on a cover slip in each well of a 24-multiwell dish, and were incubated in M199 containing 10% human serum, or in the culture medium supplemented with 1% conditioned medium of PBL stimulated with 12-O-tetra decanoylphorbol 13-acetate and concanavalin A (TPA-Con A CM), or with recombinant gamma interferon (rIFN-γ). After 3-d incubation, 5 × 10⁵ freshly isolated and G10–passed PBL were added to each well and were cultured for 1 h at 37°C. Then, nonadherent or loosely adhered lymphocytes were washed out with PBS and the coverslips were stained with 8% Giemsa solution. Lymphocyte adhesion to EC was determined under a light microscopy. All experiments were performed in duplicate. Adhesion index indicates the ratio of adhesion-positive EC (EC with two and more adherent lymphocytes) to total EC. Values indicate mean±SD. See Methods in detail.

‡ Observation under a light microscopy.

§ IFN activity in the TPA-Con A CM was determined by a microcytopathic effect inhibition assay using encephalomyocarditis virus and FL cells. The IFN titer indicates laboratory unit.

|| HLA-DR antigens expressed on the EC were stained with mouse monoclonal anti DR antibody (PTF29.12) and FITC-F (ab)₂ rabbit antimouse IgG, and determined by a FACS analyzer (Spectrum III, Ortho Diagnostic Systems, Inc.).

† TPA-Con A CM was prepared from the supernate of PBL stimulated with Con A (5 μg/ml) for 72 h after pretreatment with TPA (2 ng/ml) for 4 h. The TPA-Con A CM was then treated with either monoclonal anti-IFN-γ antibody or pH 2 glycine buffer. See Methods in detail.

** The adhesion index was significantly reduced as compared with that of EC that had been cultured with untreated TPA-Con A CM (*P* < 0.001 by nonpaired *t* test).

adhesiveness by interleukin 1 for several leukocyte classes, including peripheral blood lymphocytes, as reported by Bevilacqua et al. (27). At the moment, we have no clue as to the function of these mechanisms; therefore, subsequent experiments were focused on the role of IFN-γ and DR antigens in the lymphocyte-EC adhesion, using rIFN-γ.

Effect of rIFN-γ on lymphocyte-EC adhesion. The role of IFN-γ in the lymphocyte-EC adhesion was further investigated using rIFN-γ. After EC had been treated with rIFN-γ (500 U/ml) for 3 d, lymphocytes were added to the EC and incubated for varying periods. As shown in Fig. 1, significant lymphocyte adhesion to the EC occurred within 15 min after the addition of the lymphocytes and reached a plateau in 1 h.

Under microscopic observation, the EC became elongated in shape when cultured in the presence of rIFN-γ, and the lymphocyte-binding property of the EC apparently correlated with their morphological changes. It is clearly shown in Fig. 2 that many lymphocytes bound tightly to rIFN-γ–treated EC (Fig. 2 B) in contrast to untreated EC (Fig. 2 A). The lymphocytes bound to the EC also exhibited changes in cell shape that are characteristic of motile lymphocytes (Fig. 2 C). In contrast, when EC

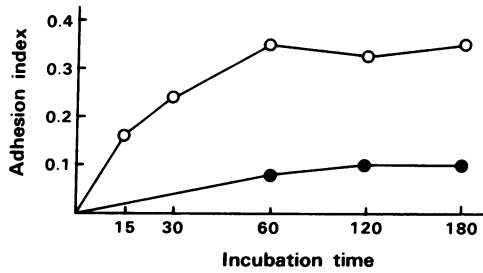


Figure 1. Time course (min) of adhesion index after addition of G-10 passed lymphocytes to rIFN- γ -treated EC. The experimental conditions and methods were the same as described in Table I. Significant increase in lymphocyte adhesion to rIFN- γ (500 U/ml)-treated EC (○) as compared with untreated EC (●) occurred within 15 min and reached a plateau in 1 h.

were cultured in the presence of rIFN- α even up to 10^4 U/ml, neither a change of EC shape nor adhesion of lymphocytes was observed.

Comparison of dose dependency on rIFN- γ and kinetics of adhesion index and DR expression on EC. To elucidate the possible relationship between lymphocyte adhesion to EC and DR expression on EC, the dose responses on rIFN- γ were first compared. As shown in Fig. 3, both adhesion index and intensity of DR expression on the EC increased in a linear fashion as the doses of rIFN- γ increased up to 500 U/ml. The percentage of DR positive cells also increased dose-dependently on rIFN- γ

but reached a plateau at 100 U/ml. It thus appeared that adhesion index more closely correlated with DR intensity on EC rather than percentage of DR positive cells. In contrast, rIFN- α induced no apparent increases in either adhesion index or DR expression even up to a dose of 10^4 U (data not shown). As shown in Fig. 4, adhesion index closely correlated with the mean number of lymphocytes bound per rIFN- γ (500 U/ml)-treated EC. Therefore, we used adhesion index in this study because it was more convenient to assess.

Since both the adhesion index and the DR expression on the EC showed similar dose-dependent fashion on rIFN- γ , we then performed the comparative study of kinetics for both. EC were cultured with 50 U/ml and 200 U/ml of rIFN- γ for varying periods, and then adhesion index and DR expression were simultaneously assayed. As shown in Fig. 5, the adhesion index showed essentially the same pattern of kinetics as the DR expression. These results indicated that the capacity of EC to bind lymphocytes was closely correlated with the expression of DR antigens on them in terms of both dose dependency on rIFN- γ and the kinetics in culture.

Effect of rIFN- γ on the expression of other MHC class II antigens on EC. Recent studies have shown that rIFN- γ induces the surface expression of not only DR antigens, but also other class II antigens (HLA-SB and DC) as well as an increase in class I antigens on EC (25, 26, 28). We thus investigated the induction of DR, DP(SB), and DQ(DC) antigens on the EC by rIFN- γ , and their role in lymphocyte adhesion. After EC were cultured with 500 U/ml of rIFN- γ for 4 d, the surface expression

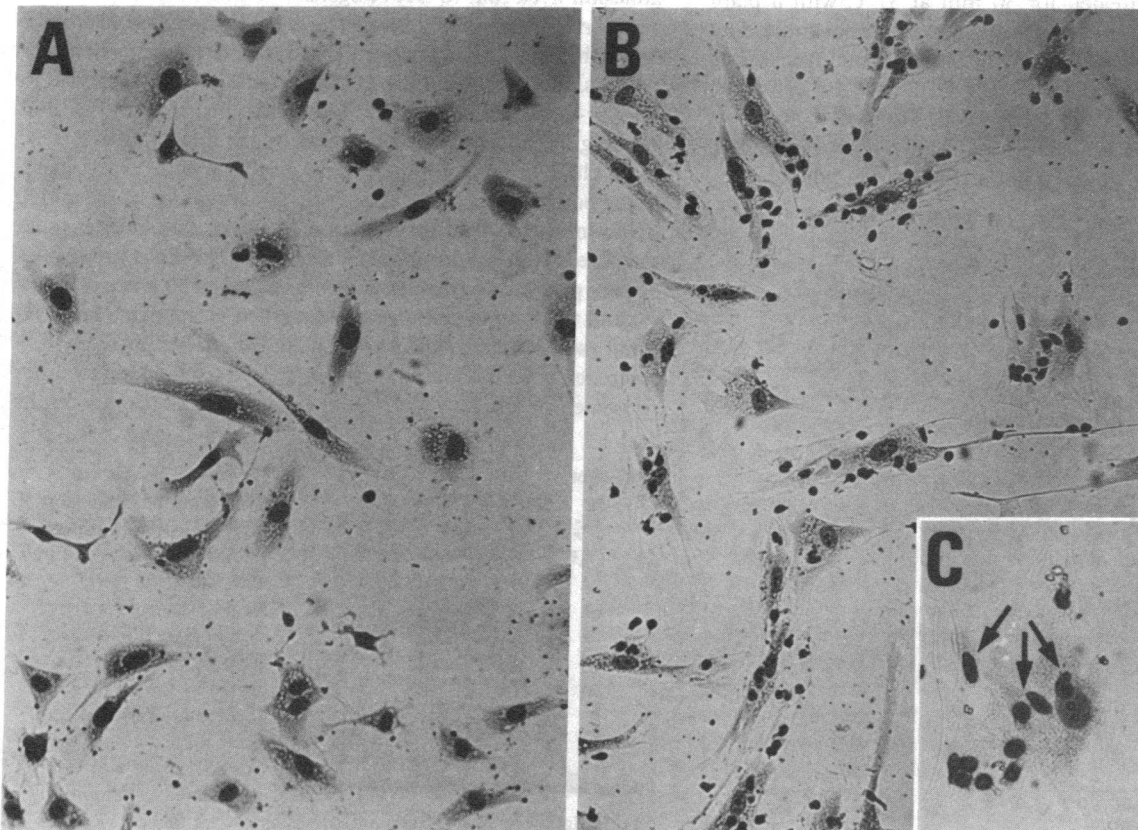


Figure 2. Light microscopies of lymphocyte adhesion to rIFN- γ -treated EC. Many lymphocytes tightly adhered to rIFN (500 U/ml) -treated EC (B) compared with untreated EC (A). Giemsa staining. $\times 100$. Note that the lymphocytes on EC with arrow exhibit changes in the cell shape, which are characteristic of motile lymphocytes as shown in C at a higher magnification, $\times 200$.

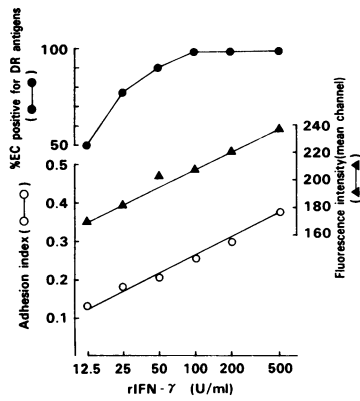


Figure 3. Dose-response relationships for adhesion index, and the percentage and fluorescence intensity of DR expression of EC. After EC were incubated for 72 h with varying concentrations of rIFN- γ , these assays were performed at the same time. Expression of DR antigens on EC was examined by a FACS analyzer (Spectrum III; Ortho Diagnostic Systems, Inc.).

of three class II antigens on EC was examined by fluorescence flow cytometry (FACS 302) using monoclonal antibodies and a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. The results showed that rIFN- γ induced all three sets of class II antigens on almost all of the EC (Fig. 6). However, the intensity of DR expression was clearly much greater than that of the other two class II antigens. Both rIFN- γ and rIFN- α increased the surface expression of class I antigens on the EC (not shown).

Effects of monoclonal anti-class I and class II antibodies on the adhesion of T cells to rIFN- γ -treated EC. To definitely prove if class II antigens on EC were involved in lymphocyte-EC adhesion, we directly examined the effect of various anti-class II antibodies at varying concentrations on lymphocyte-EC adhesion. Endothelial cells were cultured for 3 d with 500 U/ml of rIFN- γ , rinsed, and then treated for 30 min at 37°C with a plain medium or medium containing various monoclonal antibodies. After the treated EC were rinsed with M199 three times, freshly isolated nylon-nonadherent allogeneic PBL (5×10^5 cells) suspended in 1 ml of 5% HS-M199 were added to the EC. As shown in Fig. 7, only anti-DR antibodies significantly, if not completely, inhibited the adhesion in a dose-dependent fashion, whereas anti-

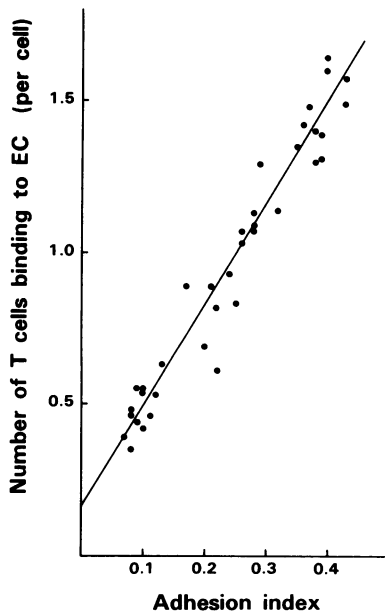


Figure 4. Relationship of adhesion index with the mean number of lymphocytes bound per EC. Endothelial cells were cultured with 500 U/ml of rIFN- γ for 3 d. $Y = 0.16 + 3.29X$; $r^2 = 0.95$; $P < 0.01$.

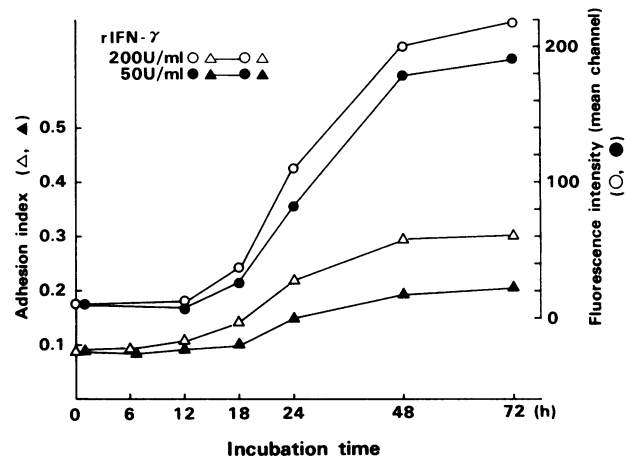


Figure 5. Kinetics of adhesion index (Δ , \blacktriangle) and DR expression on EC (\circ , \bullet). EC were incubated with 50 U/ml (\bullet , \blacktriangle) and 200 U/ml (\circ , Δ) of rIFN- γ for varying periods. These assays were performed at the same time. The mean fluorescence intensity of total EC (DR negative and positive EC) was used in this experiment.

DP, anti-DQ, and anti-ABC antibodies did not affect the adhesion at all, as well as a nonspecific mouse IgG1. From these results, DR antigens on the EC appeared to be responsible for the lymphocyte adhesion to the EC. However, note that the increase in lymphocyte adhesion induced by TPA-Con A CM depleted of IFN- γ was not inhibited by anti-DR antibody (Table II). Thus, it was clear that there are other mechanisms of such adhesion irrelevant to DR antigens.

T cell-EC adhesion in syngeneic and allogeneic combinations. Since the EC and T cells used thus far were allogeneic combinations, the possibility existed that the lymphocyte adhesion to rIFN- γ -treated EC merely represented the recognition and binding of endothelial DR antigens by the alloreactive T cells. To exclude this possibility, we performed the same experiments using both syngeneic and allogeneic combinations of EC and T cells. In a syngeneic combination, EC and lymphocytes were prepared from the same umbilical cord. The EC were immediately placed on coverslips and cultured with 500 U/ml of rIFN- γ , and the lymphocytes were maintained in a culture medium until use. Three days later, nylon-nonadherent lymphocytes were prepared from the maintained lymphocytes and added to either syngeneic or allogeneic EC. As shown in Table III, similar results

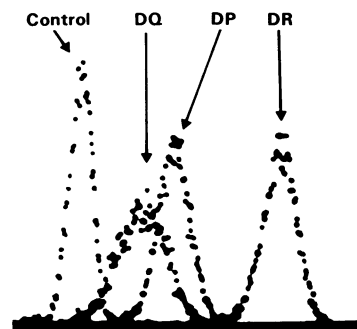


Figure 6. The effect of rIFN- γ on the expression of MHC-class II antigens on EC. After EC were cultured with 500 U/ml of rIFN- γ for 4 d, the surface expression of class II antigens (DP, DQ, and DR) was analyzed by the FACS 302 (Becton-Dickinson & Co.) using mouse monoclonal antibodies and an FITC-conjugated rabbit anti-mouse IgG. The monoclonal antibodies used were PTF29.12 (anti-DR), B7/21 (anti-DP, originally anti-FA), and anti-Leu-10 (anti-DQ). Nonbinding mouse myeloma protein of the same isotype (IgG1) as these antibodies was used as the control antibody. Three photographs superimposed on this photograph.

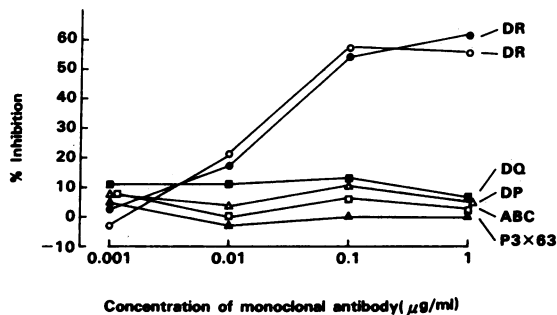


Figure 7. The effect of anti-class I and class II antibodies on T cell-EC adhesion. The experimental conditions and methods were the same as described in Table II. The mouse monoclonal antibodies used were PTF29.12 (anti-DR, ○), Cat. No. 0201-2007, Cappel Laboratories (anti-DR, ●), B/27 (anti-DP, △), anti-Leu-10 (anti-DQ, ■), Cat. No. 0201-2027, Cappel Laboratories (anti-ABC, □), and P3 × 63 (non-binding mouse myeloma protein, ▲).

were obtained in both syngeneic and allogeneic combinations, and again the binding was significantly inhibited by anti-DR antibody (PTF29.12). Thus, it seems unlikely that T cell-EC adhesion merely reflects the response of T cells to allo-DR antigens on EC.

Effects of trypsin treatment of lymphocytes on lymphocyte-EC adhesion. In an attempt to elucidate if cell surface structures of lymphocytes are involved in lymphocyte adhesion to EC, we studied the effect of proteolytic enzyme treatment of lymphocytes on the binding. Nylon-nonadherent lymphocytes were briefly incubated at 37°C with trypsin (25 µg/ml) in the presence or absence of a trypsin soybean inhibitor, washed, and then overlaid onto rIFN-γ-treated EC. The results showed that the trypsin treatment of the lymphocytes completely destroyed their ability to adhere to EC (Table IV). When a trypsin soybean inhibitor was used with trypsin, no inhibition was observed on the lymphocyte-EC adhesion, indicating the specificity of the treatment. However, if the trypsin-treated lymphocytes were further incubated for 12 h in a culture medium in the absence of the enzyme, the binding capacity of the lymphocyte was again completely restored. The results thus suggested that trypsin-sensitive

Table II. Inhibitory Effect of Monoclonal Anti-DR Antibody on T Cell-EC Adhesion Induced by TPA-Con A CM*

| Additives | Adhesion index, pretreatment of EC | | |
|-----------------------|------------------------------------|-------------|-----------|
| | None | anti-DR Ab‡ | P3 × 63‡ |
| Medium alone | 0.09±0.02 | 0.09±0.02 | 0.10±0.01 |
| TPA-Con A CM (1%) | | | |
| Untreated | 0.51±0.03 | 0.36±0.05§ | 0.53±0.04 |
| Anti-IFN-γ Ab treated | 0.35±0.04§ | 0.32±0.04 | 0.37±0.03 |
| Acid-treated | 0.34±0.02§ | 0.33±0.02 | 0.35±0.02 |

* The experimental conditions and methods were the same as described in Table I, except that T cells were prepared using a nylon-wool column.

‡ Before the addition of T cells, EC were pretreated for 30 min with anti-DR antibody (PTF29.12, 0.1 µg/ml) or P3 × 63 (nonbinding mouse IgG1, 0.1 µg/ml) and were washed three times with M199.

§ The adhesion index was significantly reduced as compared with that of EC that were cultured with untreated TPA-Con A CM ($P < 0.001$ by nonpaired *t* test).

structures on lymphocytes were in some way critically involved in the binding to DR-expressed EC, which could be spontaneously regenerated on the surface of lymphocytes within 12 h if freed from the enzyme.

Lymphocyte subsets adhering to Ia-expressed EC. Our next question was then to decide which subset of lymphocytes was preferentially responsible for the adhesion to EC. To examine this point, T and non-T cells separated by E-rosette formation were added to EC treated with rIFN-γ (500 U/ml) for 3 d. The adhesion index of T cells (E-rosette positive cells) was comparable to that of unseparated G-10 passed lymphocytes, whereas few non-T cells (E-rosette negative cells) bound to the EC (Table V). T cell fraction was then positively separated into Leu-2⁺ and Leu-3⁺ T cells as indicated in Methods, and each population was incubated with EC. The results clearly showed that Leu-3⁺ T cells rather preferentially bound to rIFN-γ-treated EC, whereas Leu-2⁺ T cells did so only marginally (Table V). The binding of Leu-3⁺ T cells, but not Leu-2⁺ T cells, was strongly inhibited by anti-DR antibody (PTF29.12).

The proportion of adherent T cells in the added T cells was estimated to be 2–3%. To negate that contaminant non-T cells selectively adhered to EC, direct staining of the adherent lymphocyte subsets on coverslips was performed with anti-Leu series by an indirect immunoperoxidase method. When G-10-passed lymphocytes were added to rIFN-γ-treated EC, most of the adherent lymphocytes was Leu-4 positive cell as shown in Fig. 8. In preliminary observations, Leu-4⁺, Leu-3⁺, and Leu-2⁺ lymphocytes on the EC were ~85, 65, and 2% of the total adherent lymphocytes, respectively, whereas the proportions of the added lymphocytes were ~75, 50, and 20%, under light microscopic observation. The results obviously showed a preference in adhesion for Leu-3⁺ T cells over Leu-2⁺ T cells.

It was shown that anti-T4 (Leu-3) antibody inhibits the cytolysis of class II-restricted T4⁺ cytotoxic T lymphocyte clones by blocking the T cell recognition of the nonpolymorphic Ia epitope on target cells (29–31). To examine whether the T cell surface structure, T4 (Leu-3), is also involved in the lymphocyte adhesion to EC, adhesion assay was performed using nylon wool nonadherent lymphocytes in the presence of anti-Leu-2a or anti-Leu-3a antibody which were extensively dialyzed to remove sodium azide affecting to increase lymphocyte adhesion nonspecifically. As shown in Fig. 9, the lymphocyte adhesion to the EC was strongly blocked by anti-Leu-3a antibody, but not by anti-Leu-2a antibody, at concentrations of 0.2–5 µg/ml, thus suggesting that T4 antigens are indeed involved in the process of recognizing class II molecules expressed on EC.

Discussion

In this study, we demonstrated that unstimulated T cells, predominantly Leu-3⁺ (helper/inducer) T cells, selectively adhered to EC that had been treated with rIFN-γ, but not with rIFN-α, in both syngeneic and allogeneic combinations. Several lines of evidence indicate that the class II antigens, especially DR antigens, expressed on EC play a central role in the selective adhesion of Leu-3⁺ T cells; that is, first, the kinetics as well as the dose response pattern of both lymphocyte-EC adhesion and endothelial DR expression induced by rIFN-γ are essentially parallel and well correlated; second, the adhesion was significantly inhibited by the treatment of Ia-expressed EC with monoclonal anti-DR antibody; third, Leu-3⁺ T cells, which are known to interact with class II antigens, selectively adhered to Ia-expressed

Table III. Inhibitory Effect of Monoclonal Anti-DR Antibody on T cell-EC Adhesion in both Syngeneic and Allogeneic Combinations*

| rIFN- γ U/ml | Antibody μ g/ml | Syngeneic EC | | Allogeneic EC | |
|------------------------|------------------------|-----------------|--------------------|-----------------|--------------------|
| | | Adhesion index | Percent inhibition | Adhesion index | Percent inhibition |
| 0 | 0 | 0.07 \pm 0.02 | — | 0.06 \pm 0.02 | — |
| 500 | 0 | 0.40 \pm 0.05 | — | 0.41 \pm 0.05 | — |
| 500 | 0.1 | 0.24 \pm 0.04 | 48 | 0.22 \pm 0.02 | 54 |

* In syngeneic combination, EC and lymphocytes were harvested from the same umbilical vein. Then, the EC were immediately cultured with rIFN- γ on a coverslip, and the lymphocytes were maintained in a culture medium until use as a source of T cells for an adhesion assay. Simultaneously, allogeneic EC were also cultured with rIFN- γ . After 3 d, T cells prepared from the maintained lymphocytes were cultured with the syngeneic and allogeneic EC. Anti-DR antibody used for blocking of the T cell-EC adhesion was PTF29.12.

Table IV. Effect of Trypsin Treatment of Lymphocytes on EC Adhesion

| Treatment of lymphocytes* | Adhesion index | | | |
|---|-------------------------|---------------------------|---------------------------------|---------------------------|
| | Just after treatment | | 12-h incubation after treatment | |
| | 0 rIFN- γ , U/ml | 500 rIFN- γ , U/ml | 0 rIFN- γ , U/ml | 500 rIFN- γ , U/ml |
| Untreated | 0.07 | 0.45 | 0.15 | 0.52 |
| Trypsin (25 μ g/ml) | <0.01 | 0.02 | 0.13 | 0.55 |
| Trypsin (25 μ g/ml) + soybean trypsin inhibitor (25 μ g/ml) | 0.09 | 0.49 | 0.16 | 0.56 |

* Freshly isolated and G10-passed lymphocytes were incubated for 10 min at 37°C with trypsin (25 μ g/ml) in the presence or absence of soybean trypsin inhibitor (25 μ g/ml). These treated lymphocytes were washed three times in cold RPMI-5% FCS and incubated with rIFN- γ -treated EC. Part of the trypsinized lymphocytes was further incubated for 12 h in a culture medium.

EC, whereas the adhesion of Leu-2⁺ T cells was only negligible. The comparable degree of T cell-EC adhesion was observed in syngeneic combination to that in allogeneic combination, which suggests that such T cell-EC interaction is not merely the reflection of T cell-alloreactivity against class II antigens.

Recently, Collins et al. (28) reported that rIFN- γ induces HLA-DR, HLA-SB(DP), and HLA-DC(DQ) in human umbilical EC and dermal fibroblasts by transcription of apparently silent genes. In this study, we confirmed that almost all EC expressed the three sets of class II antigens on their surface when cultured

Table V. Lymphocyte Subsets Adhering to Ia-expressed EC

| Lymphocytes | Adhesion index of cells cultured with rIFN- γ | | | Percent inhibition |
|--|--|-------------------------|---|--------------------|
| | 0 U/ml | 200 U/ml | | |
| Unseparated lymphocytes | 0.09 \pm 0.02 | 0.33 \pm 0.03 | | — |
| T cells (E-rosette ⁺)* | — | 0.30 \pm 0.02 | | — |
| Non-T cells (E-rosette ⁻)* | — | 0.13 \pm 0.03 | | — |
| | Adhesion index of cells cultured with: | | | |
| | 0 U/ml rIFN- γ | 100 U/ml rIFN- γ | 100 U/ml rIFN- γ plus 0.1 μ g/ml PTF29.12 | |
| Unseparated T cells | 0.08 \pm 0.02 | 0.28 \pm 0.04 | 0.17 \pm 0.03 | 55 |
| Leu-2 ⁺ T cells‡ | 0.07 \pm 0.02 | 0.19 \pm 0.02 | 0.18 \pm 0.02 | 8 |
| Leu-3 ⁺ T cells‡ | 0.06 \pm 0.01 | 0.46 \pm 0.08 | 0.15 \pm 0.01 | 78 |

* T cells and non-T cells were separated by the sheep erythrocyte-rosetting method. The T cell-enriched fraction contained >85% E rosette-forming cells and the T cell-depleted fraction contained <5% E rosette-forming cells. ‡ Leu-2⁺ and Leu-3⁺ T cells were separated with the indirect antiglobulin rosetting method using monoclonal anti-Leu-2a and anti-Leu-3a antibodies, respectively. The purity of isolated subsets was assessed by a FACS analyzer and only cell preparations that were >90% pure were used. Experiments were done three times with essentially the same results.

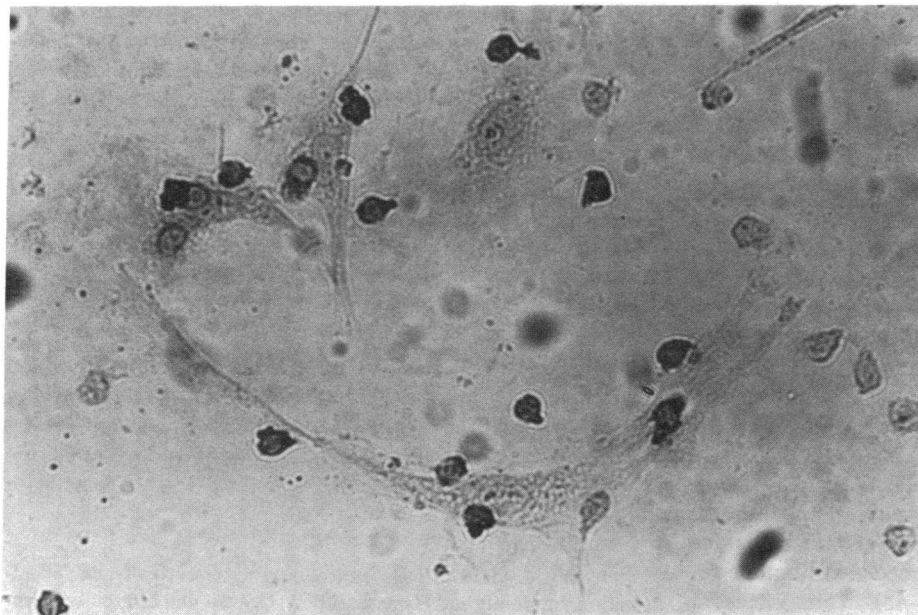


Figure 8. Direct staining of the endothelial-adherent lymphocytes with anti-Leu-4 by an immunoperoxidase method. After G-10-passed lymphocytes were cultured with rIFN- γ -treated EC, direct staining was performed by an immunoperoxidase method. See Methods in detail. Leu-4 positive cells preferentially adhered to rIFN- γ -treated EC, whereas Leu-4 negative cells except one were peripheral to the EC, $\times 200$.

in the presence of rIFN- γ . Among them, the DR antigen expression was by far the highest in intensity. The efficiency of lymphocyte adhesion to EC seemed to depend on the density of DR antigens expressed on the EC. Thus, adhesion index increased in proportion to the increase in mean DR intensity on the EC even after 100% of the EC expressed DR antigens (Fig. 3). Such DR density-dependency of the interaction between T cell and EC seemed to be comparable to that of helper T cell and B cell interaction where density of Ia antigens on B cells is critical (32). However, it remained unclear whether apparent lack of involvement of other class II antigens (HLA-DP and HLA-DQ) in lymphocyte-EC adhesion was due to their quantitative insufficiency of expression.

It is now well established that the Leu-3⁺ T subset interacts with class II antigens, whereas the Leu-2⁺ T subset interacts with class I antigens. It is generally considered that an antigen-specific helper T cell receptor, in association with an antigen, recognizes a polymorphic determinant of Ia antigens, and that the interaction of a T4 (Leu-3) antigen on a helper T cell surface with a nonpolymorphic determinant of Ia antigens stabilizes and strengthens the receptor/ligand interactions (29–31, 33–35). Janeway (36) recently proposed a more complex role of L3T4 molecules, in that the interaction of the murine L3T4 molecule

with a nonpolymorphic portion of Ia antigens is an initial step in T cell activation. If the Ia-bearing cell is not also antigen-bearing, a negative signal via aggregation of L3T4 is sent to the T cell, causing it to detach from this cell. If an antigen is present on the Ia-bearing cell and the T cell's receptor is specific for the antigen/Ia complexes borne by that cell, both the initial interaction of the T cell with Ia-bearing cell and subsequent receptor/ligand interactions are stabilized by L3T4, leading to activation of the T cells.

In our preliminary investigations, the nonbound T cells harvested in both syngeneic and allogeneic EC adhesion experiments did not adhere to a second substrate in the syngeneic and allogeneic combinations. This, in turn, indicated that T cells adhering to either syngeneic or allogeneic EC are involved in the same subpopulation (~2–3% of added T cells) that recognizes a nonpolymorphic DR portion. The interaction between a T4 molecule and a nonpolymorphic DR portion may be an important initial event in T cell adhesion and motility on EC.

Indeed, if Leu-3⁺ T cells adhere to EC by the interaction of the receptor on T cells with the class II antigens on EC, it would be somewhat puzzling why normal T cells do not adhere tightly to other class II antigen-bearing cells, such as B cells and macrophages. At least two explanations would be possible. First, class II antigens on B cells and macrophages may differ from those on EC quantitatively. As already noted, the intensity of DR antigen expression was the major factor determining the adhesiveness (Fig. 3), and our preliminary results using a FACS analyzer indicated that the intensity of DR antigens on EC cultured with optimal doses of rIFN- γ was much higher than normal B cells and monocytes. Second, the extracellular matrix (ECM) of EC may be relevant to the T cell adhesion in addition to DR antigens because a change in the cell shape of the EC was always observed whenever lymphocytes adhered to the EC as shown in Table I. It is now clear that the ECM (e.g., fibronectin, several types of collagen, or glycosaminoglycans), plays an important role in cell adhesion, shape, and metabolic functions. For example, fibronectin was shown to mediate neutrophil adhesion to cultured EC (37, 38). Recent studies also showed that IFN- γ stimulated cultured EC to produce glycosaminoglycans (39), and

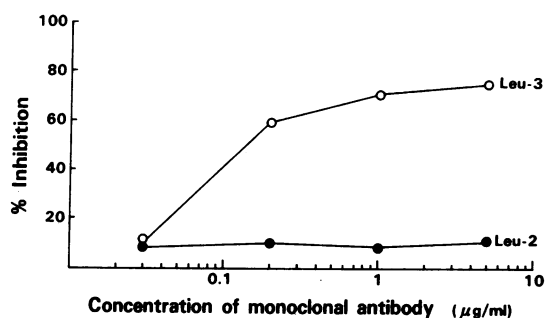


Figure 9. The effect of anti-Leu-2a and anti-Leu-3a antibodies on the adhesion of T cells to rIFN- γ -treated EC. T cells were cultured with rIFN- γ (500 U/ml)-treated EC in the presence or absence of either anti-Leu-2a or anti-Leu-3a antibody that was free of sodium azide.

that both increased fibronectin and Ia antigens were found on the EC in the target organ in acute experimental allergic encephalitis (EAE) (40). Therefore, not only the expression of class II antigens, but also the supportive action of the ECM on EC may be required for the T cell adhesion to EC.

Although a small part of Leu-2⁺ cells or Leu-4⁻ cells seemed to adhere to Ia-expressed EC, it remains unclear whether or not the adhesion was due to specific interactions with the EC.

In this study, we showed that lymphocytes treated with trypsin lost their capacity to bind to Ia-expressed EC. These findings were in accord with previous studies that examined the effects of trypsin on lymphocyte homing in vivo and lymphocyte binding to HEV in vitro (41, 42). It was suggested that trypsin cleaved the surface receptors on T cells interacting with the Ia antigens on EC, and that the receptors re-expressed on the lymphocyte surface after an additional incubation of 12 h.

Direct interaction of T cell and vascular EC as shown here have important physiological as well as pathological implications. Accumulating evidence shows that vascular endothelial Ia antigens may play important roles in cell-mediated immune responses, such as antigen presentation (43–45). In fact, increased Ia expression on EC in particular organs has been demonstrated in skin allograft rejection (6, 46) and in EAE (47). However, the precise role of Ia-expression of EC in the target organ is still not completely understood. Considering the observations that Ia antigens were expressed on the involved vascular EC with predominant accumulation of T4⁺ (helper/inducer) T cells in the graft dermis and graft bed in human skin allograft (6), it seems quite possible that a direct interaction of T4⁺ cells with Ia molecules on EC may bring about such selective migration and accumulation. IFN- γ is known to be secreted by activated T cells, and since the action of IFN- γ is supposed to be short-ranged, it would induce Ia antigens on surrounding vascular EC of a limited area, in addition to other types of cells such as macrophages, thus creating "Ia-positive focus" around antigens. Ia-expressed EC in such foci would be expected to function in three aspects. First, they would provide sufficient antigenic presentation to antigen-reactive T cells to further initiate immune responses. Second, Ia-expressed EC would be damaged by the attack of allogeneic cytolytic T lymphocytes in allograft rejection (26, 34). Third, Ia antigens would be expressed on the internal surface of microvessels with quantitative variations depending on the local concentration of IFN- γ produced in the foci. If Ia antigens on EC are strongly expressed, it should effectively facilitate the selective migration and accumulation of Ia-reactive T cells to the foci. Consequently, a sufficient amplification of local immune responses would be led.

The hypothesis mentioned above may explain one of the mechanisms operating in the following phenomenon. Steinman et al. (48) observed a decrease in the accumulation of lymphocytes in the central nervous system of an EAE mouse injected with monoclonal anti-Ia antibody. As a result, anti-Ia antibodies blocked the adhesion of Ia-reactive T cells to Ia-expressed EC, resulting in the inhibition of T cell migration from blood into the target organ.

It seems unlikely that such tight T cell-EC adhesion as demonstrated in this study occurred due to only the binding force between DR molecules and the complementary receptors. The T cell recognition of DR molecules may be the signal for the initiation of subsequent adhesion processes in which complementary adhesion surface molecules become engaged. Although certain cell surface molecules are regarded as being involved in

lymphocyte adhesion reactions (11–15, 49), it remains to be defined whether these molecules are also related to the T cell adhesion to Ia-expressed EC. At present, we do not know the changes of cellular membrane or metabolism that occur in the process of recognition and adhesion between T cells and EC, as well as the characteristics of acid-stable cytokines (other than IFN- γ) such as those that enhance lymphocyte-EC binding as shown in Table II. The experiments to define these points are now in progress.

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