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Effects of Interferon-γ on Primary Cultures of Human Brain Microvessel Endothelial Cells

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Primary cultures of human brain microvessel endothelial cells were used to study the effects of human recombinant interferon-γ (IFN-γ) on cerebral endothelium in vitro. Incubation of monolayers with various concentrations of IFN-γ (10 to 200 U/ml) for 12 to 96 hours induced surface expression of class II major histocompatibility complex (Ia) antigen in a time- and concentration-dependent manner. In immunogold-stained cultures, labeling was observed as early as 12 hours, was maximal after 48 hours, and persisted at plateau levels in the continuous presence of the cytokine. Expression was blocked by coincubation with anti-IFN-γ antibody and was reversed 4 days following removal of IFN-γ from the culture media. Endothelial cells treated with IFN-γ for 3 to 4 days became spindle-shaped, extensively overlapped, and frequently formed cellular whorls. These changes did not occur in the presence of anti-IFN-γ antibody and reversed upon removal of IFN-γ from the media. The morphological alterations were associated with increased permeability of confluent monolayers to macromolecules as compared with untreated cultures. The results of these studies indicate that human brain microvessel endothelial cells respond to in vitro cytokine stimulation by undergoing profound morphological, functional, and permeability changes. We conclude that cerebral endothelium may play an important role in the initiation and regulation of lymphocyte traffic across the blood-brain barrier in inflammatory disorders of the human central nervous system. (Am J Pathol 1993, 142:1265–1278)

Inflammatory and autoimmune demyelinating diseases of the central nervous system (CNS), such as multiple sclerosis (MS) and its prime animal model, experimental autoimmune encephalomyelitis (EAE), are characterized by increased permeability of the blood-brain barrier (BBB) and migration of lymphocytes from blood into brain through the cerebral vasculature that normally excludes circulating inflammatory cells from the brain. Since brain endothelial cells are the first native cells of the CNS to encounter circulating lymphocytes, membrane interactions between these two cell types may be important in initiating a localized immune response. The specific mechanisms that mediate such interactions and are responsible for the selective adhesion and transmigration of lymphocytes across the cerebral endothelial barrier have not yet been fully investigated. A large body of evidence indicates that endothelial cells lining extracerebral blood vessels may be induced to express class II major histocompatibility complex (MHC II or Ia) antigens following *in vitro* or *in vivo* stimulation with interferon-γ (IFN-γ) and may thus act as antigen-presenting cells for circulating $CD4^+$ T lymphocytes.[1–8] In addition, IFN-γ-treated endothelial cell monolayers increase lymphocyte adhesion and transmigration as compared with untreated monolayers.[9–13]

Endothelial cells lining the cerebral blood vessels are morphologically and functionally different from endothelial cells of other vascular beds. The presence of interendothelial tight junctions that restrict the paracellular movement of macromolecules, a paucity of cytoplasmic vesicles, and specialized membrane transport systems endow these cells with unique barrier properties. The role of the cerebral endothelium in CNS inflammation remains ill defined and rather controversial. Recently, molecular changes on the ce-

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rebral endothelium have been increasingly implicated as important modulators of the initial stages of the localized immune response. Thus, rat and guinea pig brain microvessel endothelium can be induced to express Ia antigen by IFN-γ *in vitro*[14,15], and isolated mouse brain endothelial cells, treated with concanavalin A–conditioned media, are able to present antigen to myelin basic protein-sensitized lymph node cells.[16] Other studies, however, report expression of Ia antigen by brain microvascular smooth muscle or microglia rather than endothelial cells[17,18] and indicate that smooth muscle cells can present antigen to specific T-cell lines to a much greater extent than endothelial cells.[19] *In vivo* immunohistochemical studies have demonstrated Ia antigen expression on endothelial cells, in addition to astrocytes, microglia, and macrophages in acute, active chronic, and silent chronic MS lesions,[20,21] in graft-*versus*-host disease,[22] and in simian immunodeficiency virus encephalitis.[23] Similar studies in EAE, however, are rather conflicting, since Ia antigen expression by brain endothelium has been reported by some[24,25] but not other investigators.[26,27]

In the present study, the induced expression of Ia antigen by human cerebral endothelium was investigated in primary cultures of human brain microvessel endothelial cells (HBMEC) treated with recombinant human IFN-γ, a cytokine known to specifically induce class II MHC antigen expression. We have previously reported that HBMEC in culture form confluent monolayers of Factor VIII and *Ulex europaeus* agglutinin–positive cells that retain important morphological characteristics of their *in vivo* counterparts, namely, a paucity of cytoplasmic vesicles and the presence of tight junctions that restrict the transendothelial passage of macromolecules.[28] Treatment of HBMEC with IFN-γ resulted in *de novo* expression of Ia antigen in a time- and concentration-dependent manner and induced changes in the morphology, organization, and permeability of the monolayers. Our results indicate that IFN-γ induces profound morphological and functional alterations on human cerebral endothelial cells that may be important for antigen presentation and initiation of inflammatory responses within the human CNS.

Materials and Methods

Isolation and Culture of Human Brain Microvessel Endothelial Cells

Primary cultures of endothelial cells were established from normal brains at autopsy and temporal lobectomy specimens as previously described.[28] The isolated clumps of endothelial cells were seeded onto fibronectin-coated plastic wells (Corning Plastics, Corning, NY) and maintained in culture in minimum essential medium α (αMEM) (Gibco, Burlington, Ontario) supplemented with 10% horse plasma–derived serum (HyClone Laboratories, Logan, UT), 25 mM (*N*-2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.4), 20 μg/ml endothelial cell growth supplement, 100 μg/ml heparin (all from Sigma Chemical Co., St. Louis, MO), and penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml) (Gibco) at 37 C in a humidified 2.5% CO_2/97.5% air atmosphere. The endothelial origin of the cells was confirmed by their strongly positive, perinuclear immunofluorescence for Factor VIII-related antigen and their binding of *U. europaeus*-I lectin as described previously.[28] Culture media were changed every other day. Confluent monolayers of contact-inhibiting cells were obtained 5 to 7 days after plating. Cultures were used as soon as they reached confluency. There were no differences in the growth pattern and cell morphology between cells derived from postmortem brains and temporal lobectomy specimens.

Antibodies

Mouse monoclonal antibody against human recombinant IFN-γ (KM48) and mouse anti-human HLA-DR IgG (DK22) were obtained from Dimension Laboratories, Inc. (Missisauga, Ontario). Goat anti-mouse IgG coupled to 5-nm gold particles (Auroprobe LMGAM IgG) was obtained from Janssen/Cedarlane Labs, Ltd., (Hornby, Ontario), and mouse anti-human pituitary follicle-stimulating hormone IgG was from Biogenex Laboratories (Dublin, CA).

Treatment of Primary HBMEC Cultures

Human recombinant IFN-γ (Collaborative Research, Inc., Bedford, MA) was diluted in complete media to a final concentration of 10, 20, 50, 100, 150, and 200 U/ml. Confluent monolayers of HBMEC grown in replicate wells were incubated with different concentrations of IFN-γ for 4 days and with 200 U/ml for 12 hours to 4 days at 37 C. Cultures used for these experiments were derived from endothelial cells isolated from several different brains and temporal lobectomy specimens. The specificity of Ia Ag induction by IFN-γ was tested in cultures coincubated with IFN-γ (200 U/ml) and anti-IFN-γ monoclonal antibody (optimal concentration, 10 μg/ml) for 4 days. In order to study the reversibility of Ia Ag expression, monolayers previously treated with

IFN-γ (200 U/ml) for 4 days were thoroughly washed with αMEM to remove the cytokine, then placed in complete media and returned to the incubator for another 4 days prior to Ia Ag detection.

Immunocytochemical Localization of Ia Ag

Light Microscopy Immunocytochemistry

Following incubation with IFN-γ, the monolayers were washed 3 times with buffer containing phosphate-buffered saline (PBS) (10 mM, pH 7.2), 1% bovine serum albumin (BSA), and 1% normal goat serum (PBS/BSA/NGS) and incubated for 40 minutes at room temperature with mouse anti-human HLA-DR monoclonal antibody at 1:30 dilution in carrier buffer containing PBS, 5% BSA, and 4% NGS. Following brief washing with PBS/BSA/NGS, the monolayers were incubated with the secondary antibody (Auroprobe LMGAM IgG coupled to 5-nm gold particles) diluted 1:40 in carrier buffer for 60 minutes at room temperature. At the end of the incubation period, the cells were washed with PBS/BSA/NGS, fixed in fresly prepared buffered formaldehyde-acetone fixative (20 mg Na_2HPO_4, 100 mg KH_2PO_4, 30 ml distilled H_2O, 25 ml 37% formaldehyde, and 45 ml acetone) for 30 seconds, washed with distilled H_2O, and incubated in silver enhancing solution (IntenseM, Janssen/Cedarlane) for 25 to 35 minutes. After washing with distilled H_2O, the monolayers were counterstained with Giemsa and coverslipped using JB-4 plus (Polysciences/Analychem, Markham, Ontario) as the mounting medium.

Controls included intact monolayers grown in the absence of IFN-γ and cultures incubated with 1) normal mouse IgG at the same concentration as the primary antibody (5.9 μg/ml IgG), 2) carrier buffer, and 3) irrelevant antibody (anti-human pituitary follicle-stimulating hormone IgG) instead of the primary antibody.

Stained monolayers were examined under a Nikon Labophot light microscope. Quantitation of Ia Ag expression was performed by counting one central and four peripheral randomly selected fields of each culture well with an ocular grid under ×200 magnification. All counts were performed blindly. Data are expressed as the mean ± SEM.

Immunoelectron Microscopy

Confluent monolayers of HBMEC, treated with 200 U/ml IFN-γ for 4 days, were washed with buffer containing PBS, 1% BSA, and 0.2% NaN_3 (PBS/BSA) and incubated with mouse anti-human HLA-DR monoclonal antibody at 1:30 dilution in carrier buffer containing PBS, 5% BSA, and 4% NGS for 30 minutes at room temperature. After washing with PBS/BSA, the cells were incubated with 5-nm gold particle-conjugated secondary antibody (Auroprobe LMGAMIgG) at 1:40 dilution in carrier buffer for 45 minutes, washed, and fixed in periodate-lysine-paraformaldehyde fixative[29] overnight at 4 C. Following fixation, the cells were washed in PBS, postfixed in 1% buffered OsO_4, stained *en bloc* with uranyl magnesium acetate overnight at 4 C, dehydrated in graded series of methanol, and embedded in Epon-Araldite. Blocks cut from the embedded cultures were re-embedded for cross-sectioning. Thin sections were examined in a Philips EM400 without heavy metal staining. Controls consisted of cells maintained in IFN-γ-free growth media, and monolayers incubated with normal mouse IgG or carrier buffer instead of the primary antibody.

Scanning Electron Microscopy

Confluent HBMEC monolayers treated with 200 U/ml IFN-γ for 3 to 4 days and monolayers incubated for the same period of time with 200 U/ml IFN-γ with the addition of 10 μg/ml anti-IFN-γ monoclonal antibody, as well as intact control cultures, were processed for scanning electron microscopy as described by Schroeter et al.[30] Cultures were washed in Hanks' balanced salt solution and fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 1 hour at 4 C. Following washing in cacodylate buffer, the cells were post-fixed in buffered 1% OsO_4 for 1 hour, washed in buffer, and treated with 1% tannic acid for 1 hour. After further washing in cacodylate buffer, the monolayers were dehydrated in graded series of methanol up to 70% and block stained with 4% uranyl acetate overnight at 4 C. The cells were then dehydrated with methanol up to 100%, critical point dried, gold coated, and viewed with a Cambridge Stereoscan 250T scanning electron microscope.

Permeability Studies

Confluent HBMEC monolayers treated with IFN-γ (200 U/ml) for 4 days were washed with serum-free αMEM and incubated in αMEM containing 1 mg/ml horseradish peroxidase (HRP) (Sigma Type VI) for 5 to 10 minutes at 37 C as previously described.[31] At the end of the incubation period, the cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for

1 hour at 4 C. Following washing with buffer, the monolayers were incubated with 3,3'-diaminobenzidine (Sigma) for 1 hour at 4 C, washed with cacodylate buffer, postfixed in 1% buffered OsO_4, stained *en bloc* with uranyl magnesium acetate, dehydrated with graded series of methanol, and embedded in Epon-Araldite. Thin plastic sections were examined in a Philips EM400 without heavy metal staining. Controls consisted of identical age-matched primary cultures grown to confluence in IFN-γ-free media.

Quantitation of the junctional permeability and pinocytotic activity of endothelial cells was performed by counting the number of permeable and impermeable intercellular contacts and the number of HRP-labeled and -unlabeled cytoplasmic vesicles in IFN-γ-treated and in untreated cultures.

Growth Studies

Freshly isolated endothelial cells were plated in replicate wells of Corning 24-well plates at a density of 1×10^5 cells/cm^2 on day 0. On day 1, all experimental wells were refed with complete medium containing IFN-γ (150 U/ml). Medium was changed every other day. Control cultures were maintained in growth medium in the absence of IFN-γ. The cells were viewed with a Nikon Diaphot TMD inverted microscope, and photographs of one central and four peripheral fields of each well were taken daily at ×100 magnification. The number of cells in each photograph was counted, and the data are expressed as the mean ± SEM. Student's *t*-test was used to determine significant differences between IFN-γ-treated and untreated cultures.

Results

Induction of Ia Ag Expression

Treatment of cultures with IFN-γ induced expression of Ia Ag by endothelial cells, which was dependent upon the concentration and length of exposure to IFN-γ. Surface labeling was observed as early as 12 hours following incubation with 200 U/ml in a small cell population (9.24 ± 0.99%), increased up to 88.35 ± 0.18% after 24 hours, and reached 100% after 48 hours (Fig. 1). Ia Ag expression reached plateau levels after 2 days and persisted for 4 days in the continuous presence of the cytokine. Expression was maximal with 100 to 200 U/ml IFN-γ (100% of cells) and minimal with 10 U/ml (25.76 ± 7%) (Fig. 2). Incubation with 20 U/ml of IFN-γ induced Ia Ag expression in 68.73 ± 18.5%

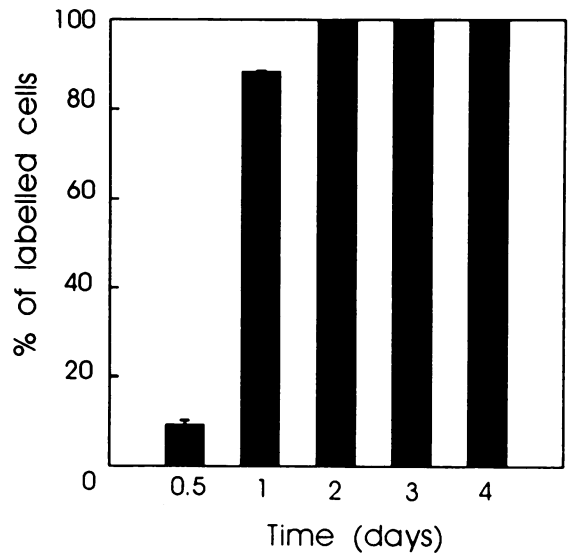

Figure 1. *Time course of Ia antigen induction on human cerebral endothelium. Confluent HBMEC cultures were incubated with 200 units/ml IFN-γ for 0.5 to 4 days and then stained with the immunogold technique for the immunohistochemical demonstration of Ia antigen. Results are expressed as percentage of labeled cells in treated cultures. Untreated cells were not labeled. Bars represent the mean ± SEM of duplicate wells of two separate experiments.*

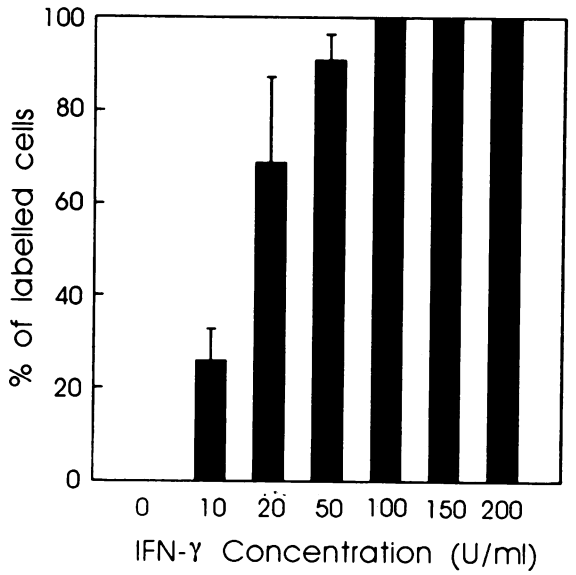

Figure 2. *Dose response of Ia antigen induction by IFN-γ on HBMEC. Confluent monolayers were incubated for 4 days with 10 to 200 units/ml IFN-γ and then immunostained for the demonstration of Ia antigen. Results are expressed as percentage of labeled cells in treated and untreated cultures. Bars represent the mean ± SEM of duplicate wells of three seperate experiments.*

of cells, while 90.85 ± 5.5% of cells were labeled after treatment with 50 U/ml. Endothelial cells expressing Ia Ag showed diffuse surface staining in the form of dark brown-black, granular deposits (Fig. 3A). In marked contrast, untreated endothelial cells invariably lacked Ia Ag expression, as indicated by their consistently negative staining with

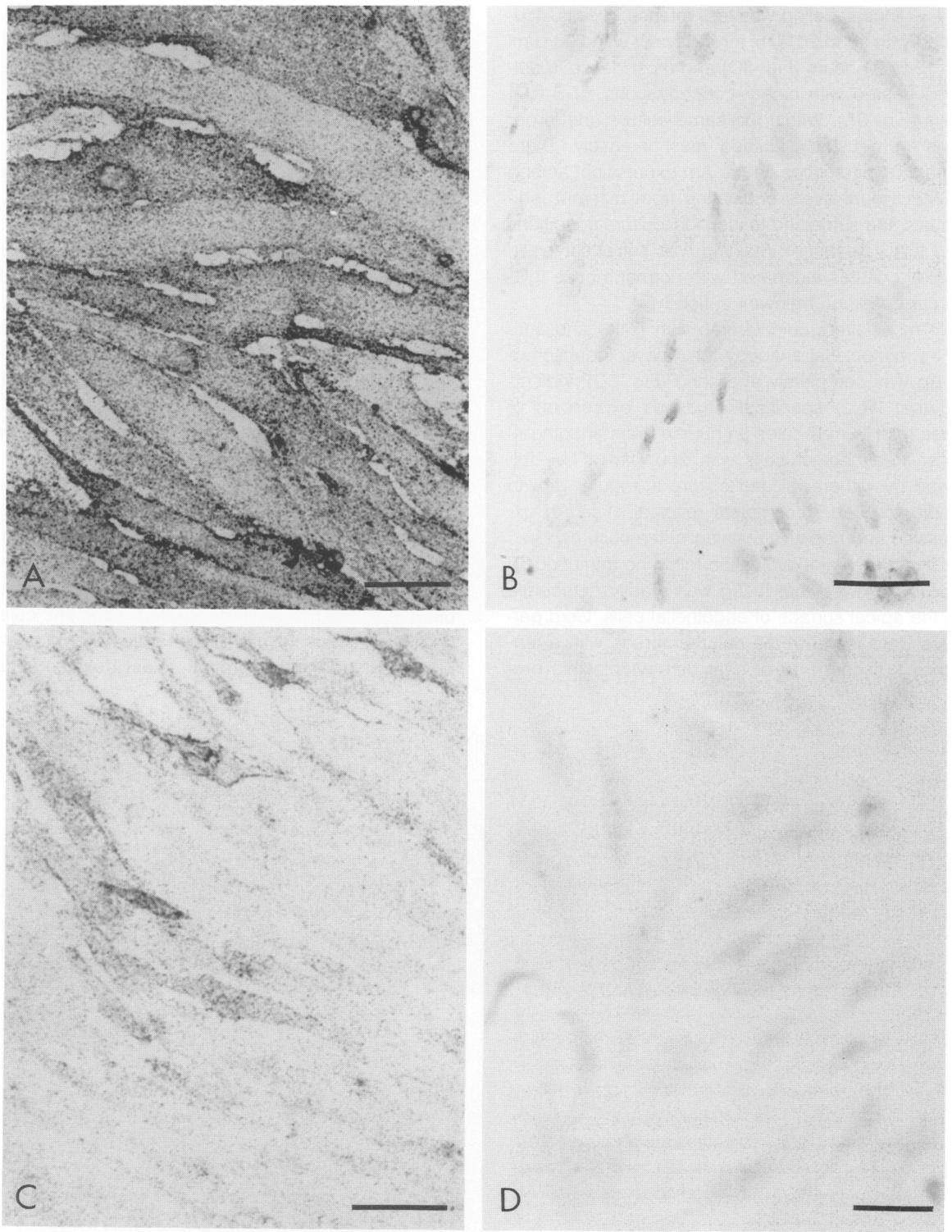

Figure 3. *Ia antigen expression by HBMEC detected by immunogold silver staining.* A, *endothelial cells incubated with 200 units/ml IFN-γ for 4 days demonstrating intense granular surface staining for Ia antigen.* B, *control untreated monolayers not expressing Ia antigen.* C, *endothelial cells treated with 200 units/ml IFN-γ for 24 hours exhibiting less dense labeling. Individual cell variation in staining intensity is apparent in* A *and* C. *In* D, *cultures coincubated with anti-IFN-γ antibody failed to label with the immunogold reagent. Bars = 20 μm.*

immunogold (Fig. 3B). The staining intensity varied with the concentration and length of incubation with IFN-γ. Thus, labeling was less intense in cells incubated with 10 to 20 U/ml for 4 days or with 200 U/ml for 12 to 24 hours (Fig. 3C) and most dense in cultures treated with higher concentrations for 3 to 4 days (Fig. 3A). Within the same culture, the larger cells were usually stained most intensely. There were no differences in Ia Ag expression among HBMEC monolayers originating from different individuals and subjected to identical culture conditions and IFN-γ treatment. Staining was not observed in control cultures incubated with normal mouse IgG, carrier buffer, or irrelevant antibody.

In monolayers coincubated with IFN-γ and anti-IFN-γ monoclonal antibody for 4 days, induction of Ia Ag was completely abolished (Fig. 3D), indicating that IFN-γ specifically induces expression of class II MHC molecules on human brain endothelial cells. Treatment of cells with 200 U/ml IFN-γ followed by withdrawal and culture in regular growth media resulted in complete reversal of Ia Ag expression and negative staining of the cultures.

Ultrastructural examination following immunogold labeling showed that Ia Ag was readily detectable on the apical surface of endothelial cells. Gold particles were found at the cell membrane with a tendency to localize on or near thin cytoplasmic processes (Fig. 4A). The basal cell surface was not labeled. No labeling was seen in untreated control cultures (Fig. 4B).

Effects of IFN-γ on Cell Morphology, Organization, and Growth

Primary cultures of HBMEC grown in regular media in the absence of IFN-γ formed highly ordered confluent monolayers of elongated, closely associated, contact-inhibiting cells (Fig. 5A). Endothelial cells treated with 200 U/ml IFN-γ for 3 to 4 days acquired a spindlelike shape and long attenuated processes. These markedly elongated cells frequently arranged themselves in ill-defined whorls and exhibited prominent overlapping, thus contributing to the unique appearance of the monolayers (Fig. 5B). These changes were most conspicuous under scanning electron microscopic examination. Under normal culture conditions, elongated HBMEC grow in close contact to each other and display distinct marginal folds in areas of cell-to-cell contact (Fig. 6A). In contrast, endothelial cells treated with IFN-γ became attenuated, and their long, thin processes often extended over and covered adjacent cells (Fig. 6B). As a result of this rearrangement, intercellular contacts and marginal folds became less

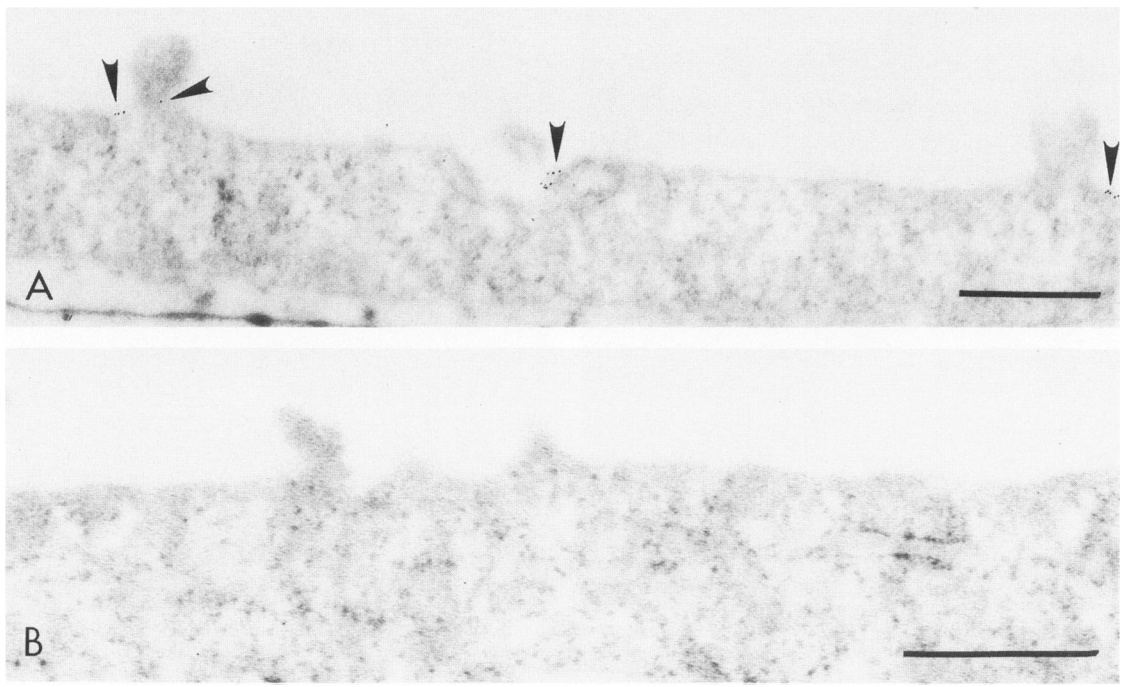

Figure 4. *Immunogold staining of HBMEC for the demonstration of Ia antigen.* **A**, *endothelial cells incubated with 200 units/ml IFN-γ for 4 days. Five-nanometer gold particles focally decorate the apical surface of endothelial cells (arrowheads) with a tendency to localize close to fingerlike cytoplasmic folds. The basal cell surface is not labeled.* **B**, *staining absent in untreated cells. Bars = 0.5 μm.*

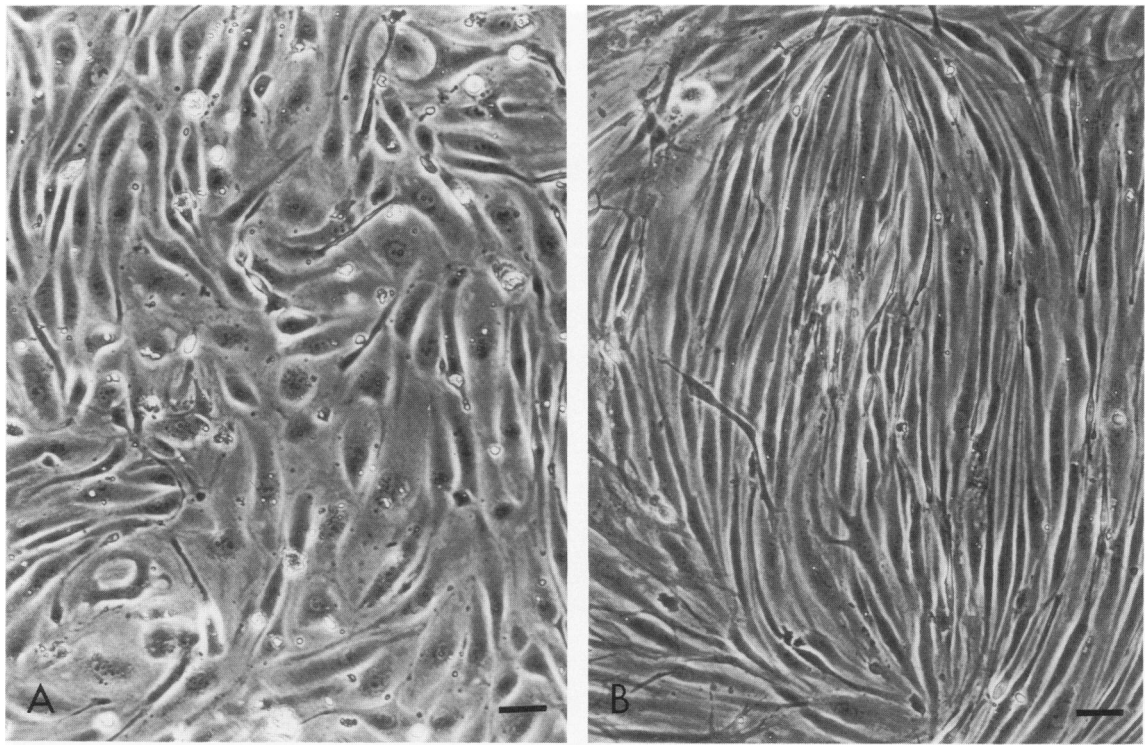

Figure 5. **A**, *HBMEC maintained under standard culture conditions forming highly organized, confluent, contact inhibiting monolayers composed of elongated cells.* **B**, *endothelial cells incubated with IFN-γ (200 units/ml) for 4 days have become spindle shaped, overlap, and focally arrange themselves into whorls. Bars = 10 μm.*

prominent, and the monolayers lost their highly organized appearance. The above morphological changes were reversed 4 days following withdrawal of the cytokine from the culture media and were not observed in cultures coincubated with IFN-γ and anti-IFN-γ antibody.

The effect of IFN-γ on the growth of HBMEC was less profound. Thus, the number of cells in primary HBMEC cultures treated with 150 U/ml IFN-γ from day 1 was slightly less than that in control cultures (Fig. 7). This slight inhibitory growth effect of IFN-γ provides further support to the observation that rearrangement and overlapping of HBMEC are the direct effect of the cytokine and not the result of cell overgrowth.

Permeability of HBMEC Monolayers

In order to examine the effect that the IFN-γ-induced morphological changes might have on the permeability of the monolayers to macromolecules, confluent treated and untreated cultures were incubated with HRP, and the labeling of intercellular contacts and cytoplasmic vesicles was assessed ultrastructurally. Intercellular contacts that impeded the tracer entirely or were penetrated for a short distance from either the apical or basal cell surface by HRP were considered impermeable. In untreated cultures, 75.2% of interendothelial junctions prevented the passage of HRP, in contrast to 36.6% of impermeable junctions in cultures incubated with the cytokine for 4 days (Table 1). In untreated monolayers, endothelial cells formed a single cell layer and were bound together by junctions, most of which were not labeled with the tracer (Fig. 8A). Focally, HRP penetrated an intercellular contact for a short distance from the basal aspect of the monolayer before being arrested at a junctional complex of an otherwise intact cleft (Fig. 8B). In treated cultures, interendothelial clefts were often penetrated by the tracer throughout their entire length (Fig. 8C). Overlapping of endothelial cells resulted in the formation of two or more layers. HRP often penetrated the intercellular clefts between endothelial cells at the top layer, and extensive deposits were found between adjacent cells at the lower layers (Fig. 8D). The number of cytoplasmic vesicles labeled with HRP was equally low in control and experimental cultures (Table 1), indicating that, contrary to the prominent conformational and organizational changes, the pinocytotic activity of HBMEC is not affected by IFN-γ treatment.

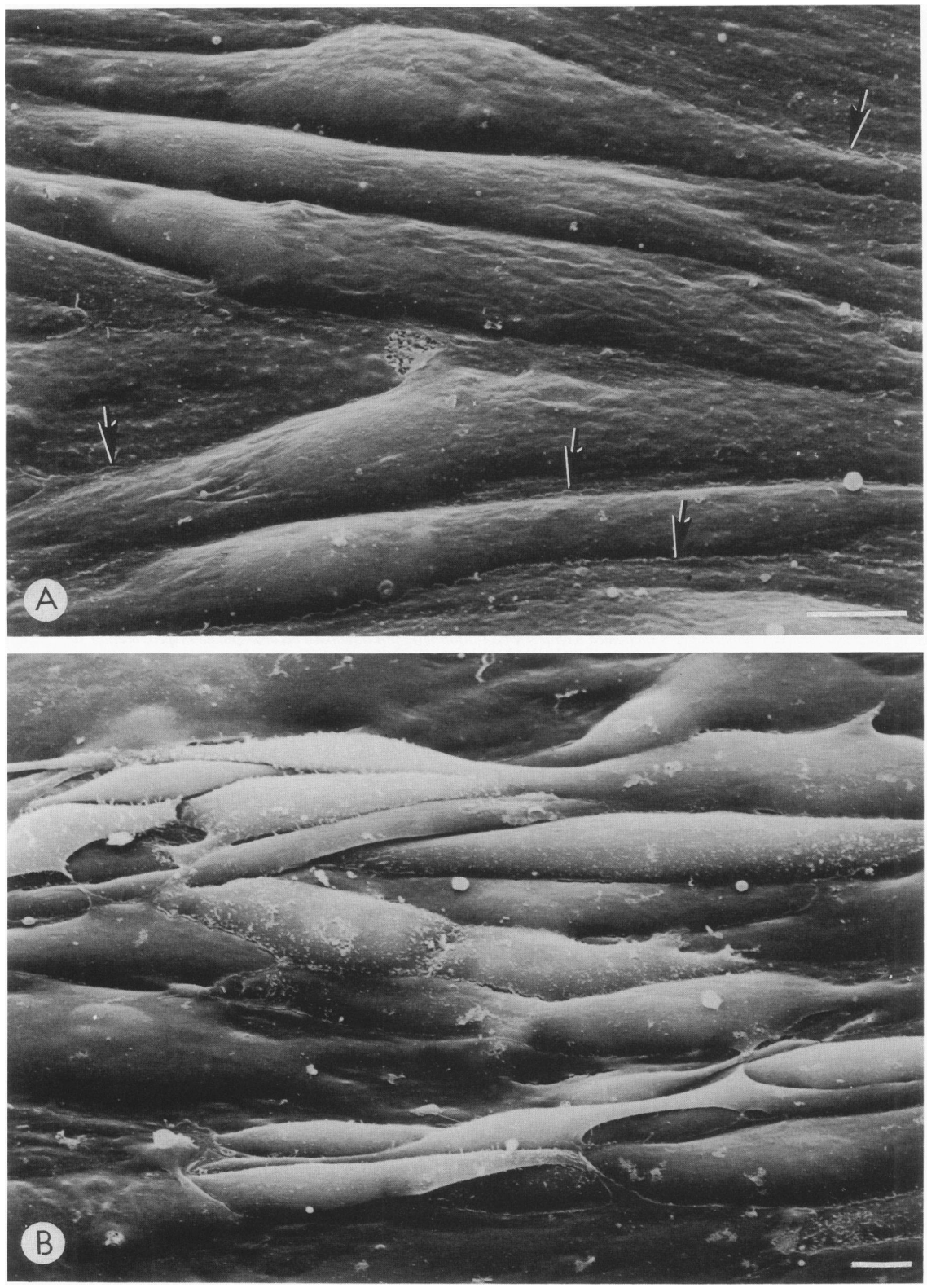

Figure 6. *Scanning electron micrograph of HBMEC grown in the absence* **(A)** *or presence* **(B)** *of IFN-γ in the culture media.* **A**, *endothelial cells closely packed without apparent intercellular spaces. Marginal folds (arrows) are present in areas of cell-cell contact. In* **B**, *incubation with IFN-γ (200 units/ml) for 3 days induces marked attenuation of cell cytoplasm and disorganization of the monolayer due to the tendency of endothelial cell processes to extend over and under adjacent cells. Bars = 20 μm.*

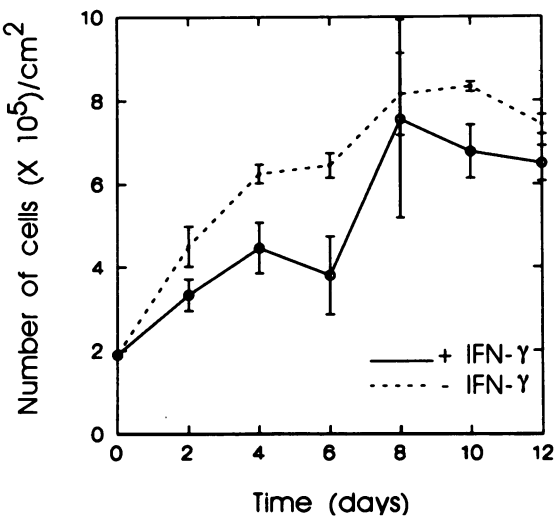

Figure 7. *Effect of IFN-γ upon the growth of primary cultures of HBMEC. Cells were cultured under standard conditions (– – –) or in the continuous presence of IFN-γ (150 units/ml) in the culture media (——). Values represent the mean ± SEM of two experiments, each done in duplicate. P = 0.035 for days 4 and 6. Differences are not significant after day 6.*

Discussion

The present studies demonstrate that human recombinant IFN-γ induces *de novo* expression of class II MHC antigen by human brain microvessel endothelial cells in primary culture in a time- and concentration-dependent manner. In addition, cytokine-treated endothelial cells undergo unique changes in their morphology and organization, which coincide with a considerable increase in the permeability of confluent cultures to macromolecules.

Unstimulated HBMEC grown under standard culture conditions do not constitutively express Ia antigen, as indicated by the lack of immunogold staining on light and electron microscopy. Previous *in vivo* immunohistochemical studies have demonstrated the absence of Ia antigen expression by endothelial cells within the normal human CNS with low levels of reactivity detected in blood vessels of patients with brain neoplasms, abscesses, autoimmune connective tissue disease, and cerebral infarcts and in older patients without identifiable CNS lesions.[20,32–34] Although the endothelial cells used in our studies were isolated from normal brains of several donors with a wide age distribution, expression of Ia antigen was not observed in any of the untreated cultures. A similar lack of constitutive expression of class II molecules has been observed in primary cultures of rat brain endothelium[14] and in freshly isolated[3,5] as well as serially passaged[6] human umbilical vein endothelial cells (HUVEC) maintained under normal culture conditions. Contrary to these reports, endothelial cells of normal guinea pig CNS display surface MHC *in vivo* and *in vitro,*[15] and minimal basal expression has been reported in primary cultures of rhesus monkey cerebral endothelium,[23] while cultured rat heart vascular endothelial cells constitutively express considerably higher levels of Ia Ag.[2] It is apparent, from the above studies, that the presence of Ia antigen on normal, unstimulated vascular endothelium may vary among different species and vascular beds.

Previous studies on Ia antigen induction by IFN-γ on HUVEC report a rapid increase of MHC class II mRNA that precedes surface expression by 1 to 2 days and rapidly declines to almost undetectable levels following withdrawal of the cytokine, while surface expression declines slowly after 4 days.[1] In HBMEC, removal of IFN-γ from the media results in uniform loss rather than a decrease to lower levels of class II MHC surface expression after 4 days. Rat heart endothelium, however, behaves in a much different way, since withdrawal of IFN-γ is not followed by return of the Ia antigen expression to basal levels after 3 days.[2]

Previous immunohistochemical studies on MS and EAE have demonstrated that surface expression of Ia antigen on endothelial cells is discontinuous along the microvessel lumen, so that Ia^+ cells are interposed between endothelial cells lacking Ia antigen expression.[20,25] A similarly variable expression of Ia antigen was observed *in vitro* when HBMEC were treated with low concentrations of IFN-γ or with higher concentrations for less than 2 days. Taken together with the *in vivo* studies, these observations may indicate individual cell variation in the regulation of class II MHC molecule expression.

Induction of Ia antigen expression on HBMEC was restricted to the apical portion of the cell membrane. Immunogold particles were not identified on the lateral or basal cell surfaces. Our findings correlate with previous immunohistochemical studies in acute EAE demonstrating Ia expression on the luminal but not abluminal surface of cerebral microvessel endothelial cells[25] and with similar observations in a variety of epithelial cells in mice treated with IFN-γ.[35] Although the mechanisms responsible for the asymmetrical presentation of Ia antigen on the cell membrane are not known, polarization of expression is probably of functional significance since it would enable circulating T lymphocytes to recognize antigen in association with class II MHC molecules on the luminal surface of the cerebral endothelium and then migrate to sites of inflammation.

The antiproliferative effect of IFN-γ on primary cultures of HBMEC correlates with previous studies demonstrating inhibition of cell growth by IFN-γ induced on extracerebral large and small vessel endothelial cultures in a dose-dependent manner[36,37–39] and possibly through modulation of the endothelial cell growth factor receptors.[38] Lower concentrations of IFN-γ (10 to 100 U/ml), however, appear to have a stimulating effect on cultured HUVEC in both the absence and presence of endothelial cell growth factor.[40] In addition, IFN-γ significantly inhibits the formation of endothelial tubular structures in *in vitro* models of angiogenesis.[41,42]

Treatment of HBMEC with IFN-γ induces marked elongation of endothelial cells, prominent overlapping, and frequent arrangement in a whorled pattern. A similar alteration of the morphological phenotype and monolayer organization has been previously reported in cultures of HUVEC[38,39] and human dermal microvascular endothelial cells[36] treated with IFN-γ for 3 to 4 days and has been shown to be associated with reorganization of the cytoskeletal filaments and considerable loss of the fibronectin matrix.[39]

Cerebral microvessel endothelial cells in primary culture are bound together by tight junctions and have a paucity of cytoplasmic vesicles, two important morphological characteristics of their *in vivo* counterparts.[28,31] Under standard culture conditions, the great majority of interendothelial junctions restrict the passage of HRP. In cultures incubated with IFN-γ, an increase in the permeability of the monolayers was observed that coincided temporally with changes in morphology and rearrangement of the cells. The number of labeled cytoplasmic vesicles was not increased in IFN-γ-treated monolayers, indicating that increased junctional permeability is primarily responsible for the permeability changes of the monolayers. The mechanism(s) responsible for the increased junctional permeability are not known at present. Recent *in vitro* studies have demonstrated that tumor necrosis factor–treated aortic endothelial cell cultures undergo prominent cytoskeletal changes similar to those induced by IFN-γ alone or in combination with tumor necrosis factor, which are temporally related to an increase in the permeability of the monolayers to macromolecules and are regulated by G protein.[43] The fact that leakiness of intercellular contacts appears concomittantly with the morphological changes of the endothelium following IFN-γ treatment may indicate that physiologically "tight" junctional complexes fail to form during the extensive rearrangement of the cells and their cytoskeleton. However, other mechanisms, such as modulation of regulatory proteins or cell surface molecules by IFN-γ, cannot be excluded. Disruption of the BBB has been previously described as an early and critical event in the evolution of EAE.[44–47] Recent electron microscopic studies indicate that increased junctional permeability as well as increased interendothelial space and migration of inflammatory cells are primarily responsible for the increased permeability of the BBB to macromolecules in this disease.[48] The functional significance of the *in vitro* morphological and permeability changes of HBMEC, observed in our studies, is currently unknown. If, however, similar changes are induced *in situ* on cerebral endothelial cells by cytokines released locally by activated T lymphocytes, they would provide an additional mechanism for the opening of the BBB and could facilitate the transmigration of inflammatory cells from blood into brain across the endothelial barrier.

Expression of Ia antigen *in situ* by cerebral vascular endothelium has been previously demonstrated in autoimmune demyelinating CNS disorders. Thus, class II MHC molecules have been localized on the surface of endothelial cells lining microvessels at the edge of demyelinating plaques as well as within the adjacent white matter in acute, active, and silent chronic MS lesions.[20,21] The presence of Ia-positive endothelial cells has also been documented in acute EAE,[24,25] while expression of Ia antigen by cerebral endothelium in chronic relapsing EAE appears to coincide with the appear-

Table 1. *Permeability of HBMEC Monolayers to HRP*

	No. of labeled cytoplasmic vesicles*	No. of interendothelial tight junctions†	
		Permeable	Impermeable
Control	2.0 ± 1.7 vesicles/cell	24.8 ± 2.7%	75.2 ± 2.7%
Experimental	2.4 ± 2.1 vesicles/cell	63.4 ± 5.2%	36.6 ± 5.2%

* Numbers represent mean ± SD of labeled vesicles in 100 control and 100 IFN-γ-treated cells from one experiment. $P > 0.05$.
† Numbers represent mean ± SD of 400 junctions (200 treated and 200 untreated) from two experiments using two different isolates. $P < 0.05$.

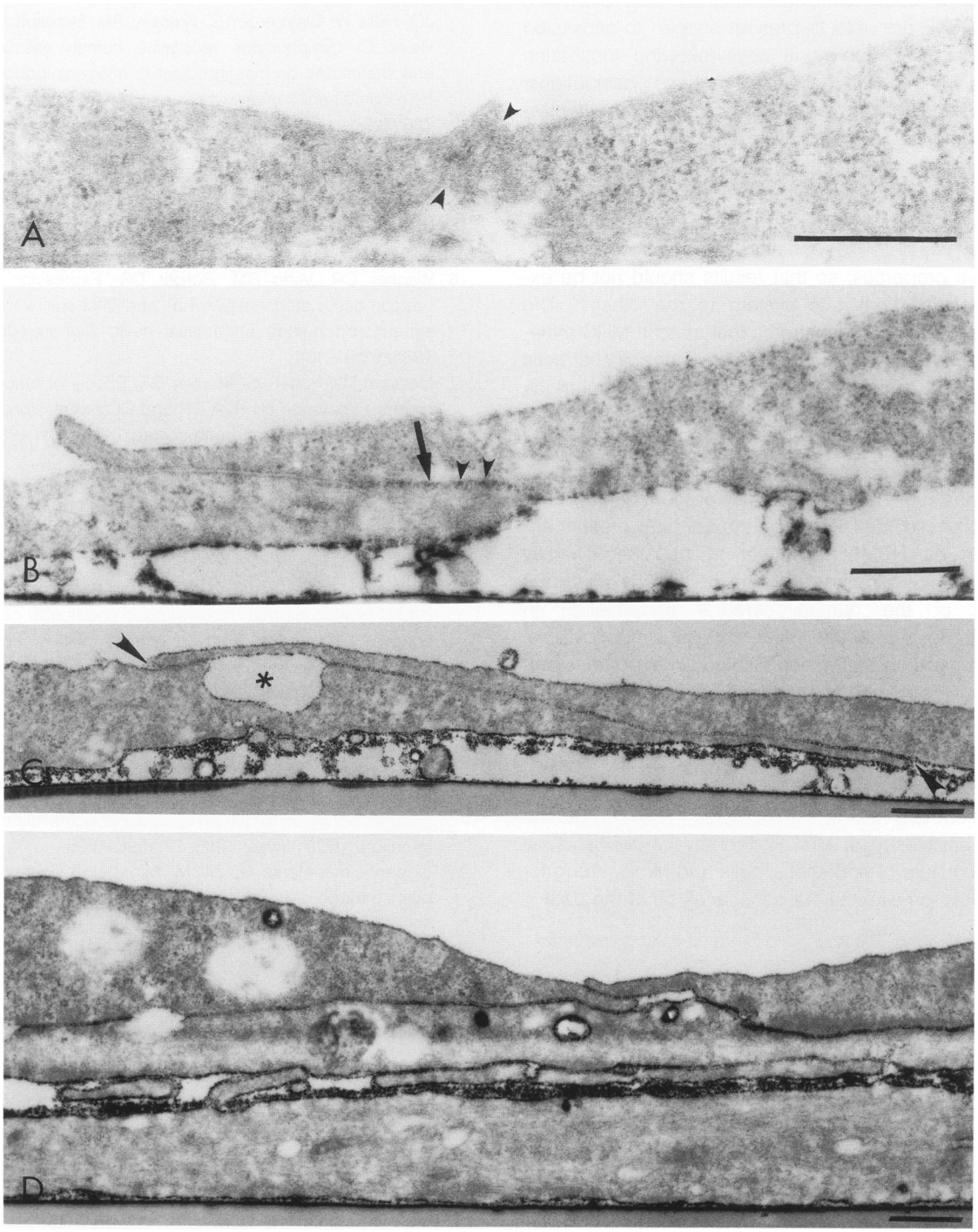

Figure 8. *HRP localization in untreated* (A) *and* B) *and IFN-γ-treated* (C *and* D) *confluent HBMEC monolayers.* A, *under standard culture conditions, tight junctions at intercellular contacts (between arrowheads) impeding the passage of HRP.* B, *HRP penetrating a short segment of an intercellular cleft from the basal cell surface, forming small deposits at the basal aspect of the cleft (arrowheads) and stopping at a junctional complex (arrow). The remaining interendothelial cleft is free of HRP. In* C, *following 4 days' incubation with IFN-γ (200 units/ml), heavy deposits of HRP are seen under the basal cell surface, and the tracer permeates the entire length of a long intercellular cleft. The proximal portion of the cleft is focally dilated (*). There is no increase in the pinocytotic activity of the endothelium.* D, *in monolayers treated with IFN-γ, HRP penetrating the intercellular clefts and forming extensive deposits between the layers of overlapping cells. Bars = 0.5 μm.*

ance of inflammatory cell infiltrates and diminishes when inflammation subsides.[49] In addition, murine cerebral endothelial cells isolated from SJL mice with EAE are able to present antigen to sensitized syngeneic lymph node cells following incubation with IFN-γ *in vitro*.[50] Contrary to these observations, cultured rat brain endothelial cells are not effective at stimulating T-cell division and therefore have not been considered important as antigen-presenting cells.[51] It is now well accepted that endothelial cells derived from different species and vascular beds vary greatly in their function, morphology, and antigenic properties, so that results should not be extrapolated from one system to the other.[52] The present work demonstrates that class II MHC molecules are not detectable on intact endothelial cells lining human brain microvessels by the methods employed in our study but can be specifically induced *in vitro* by human recombinant IFN-γ in association with prominent alterations in the morphology, organization, and permeability of the monolayers to macromolecules. Although the ability to present antigen by HBMEC has yet to be unequivocally proved, our findings indicate a possibly important role of the human cerebral endothelium in lymphocyte-endothelial interactions, lymphocyte recruitment, and alteration of blood-brain barrier permeability in immune-mediated CNS inflammation.

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References

1. Collins T, Korman AJ, Wake CT, Boss JM, Kappes DJ, Fiers W, Ault KA, Gimbrone MA Jr, Strominger JL, Pober JS: Immune IFN activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc Natl Acad Sci USA 1984, 81: 4917–4921
2. Ferry B, Halttunen J, Leszczynski D, Schellekens H, Meide PHVD, Hayry P: Impact of class II major histocompatibility complex antigen expression on the immunogenic potential of isolated rat vascular endothelial cells. Transplantation 1987, 44:499–503
3. Pober JS, Gimbrone MA Jr: Expression of Ia-like antigens by human vascular endothelial cells is inducible *in vitro*: Demonstration by monoclonal antibody binding and immunoprecipitation. Proc Natl Acad Sci USA 1982, 79:6641–6645
4. Pober JS, Collins T, Gimbrone MA Jr, Cotran RS, Gitlin JD, Fiers W, Clayberger C, Krensky AM, Burakoff SJ, Reiss C: Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. Nature 1983, 305: 726–729
5. Pober JS, Gimbrone MA Jr, Cotran RS, Reiss CS, Burakoff SJ, Fiers W, Ault KA: Ia expression by vascular endothelium is inducible by activated T cells and by human γ interferon. J Exp Med 1983, 157:1339–1353
6. Wagner CR, Vetto RM, Burger DR: Expression of I-region-associated antigen (Ia) and interleukin 1 by subcultured human endothelial cells. Cell Immunol 1985, 93:91–104
7. Messadi DV, Pober JS, Murphy GF: Effects of recombinant γ interferon on HLA-DR and DQ expression by skin cells in short term organ culture. Lab Invest 1988, 58:61–67
8. Munro JM, Pober JS, Cotran RS: Tumor necrosis factor and interferon-γ induce distinct patterns of endothelial activation and leukocyte accumulation in skin of Papio anubis. Am J Pathol 1989, 135:121–133
9. Haskard DO, Cavender D, Fleck RM, Sontheimer R, Ziff M: Human dermal microvascular endothelial cells behave like umbilical vein endothelial cells in T-cell adhesion studies. J Invest Dermatol 1987, 88:340–344
10. Issekutz TB: Effects of six different cytokines on lymphocyte adherence to microvascular endothelium and *in vivo* lymphocyte migration in the rat. J Immunol 1990, 144:2140–2146
11. Masuyama J-I, Minato N, Kano S: Mechanisms of lymphocyte adhesion to human vascular endothelial cells in culture. J Clin Invest 1986, 77:1596–1605
12. Oppenheimer-Marks N, Ziff M: Migration of lymphocytes through endothelial cell monolayers: augmentation by interferon-γ. Cell Immunol 1988, 114:307–323
13. Thornhill MH, Kyan-Aung U, Lee TH, Haskard DO: T cells and neutrophils exhibit differential adhesion to cytokine-stimulated endothelial cells. Immunology 1990, 69:287–292
14. Male DK, Pryce G, Hughes CCW: Antigen presentation in brain: MHC induction on brain endothelium and astrocytes compared. Immunology 1987, 60:453–459
15. Wilcox CE, Baker D, Butter C, Willoughby DA, Turk JL: Differential expression of guinea pig class II major histocompatibility complex antigens on vascular endothelial cells *in vitro* and in experimental allergic encephalomyelitis. Cell Immunol 1989, 120:82–91
16. McCarron RM, Kempsi O, Spatz M, McFarlin DE: Presentation of myelin basic protein by murine cerebral vascular endothelial cells. J Immunol 1985, 134:3100–3103
17. Pardridge WM, Yang J, Buciak J, Tourtellotte WW: Human brain microvascular DR-antigen. J Neurosci Res

1989, 23:337–341
18. Sethna MP, Lampson LA: Immune modulation within the brain: recruitment of inflammatory cells and increased major histocompatibility antigen expression following intracerebral injection of interferon-γ. J Neuroimmunol 1991, 34:121–132
19. Fabry Z, Waldschmidt MM, Moore SA, Hart MN: Antigen presentation by brain microvessel smooth muscle and endothelium. J Neuroimmunol 1990, 28:63–71
20. Traugott U, Scheinberg LC, Raine CS: On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. J Neuroimmunol 1985, 8:1–14
21. Traugott U, Raine CS: Multiple sclerosis. Evidence for antigen presentation *in situ* by endothelial cells and astrocytes. J Neurol Sci 1985, 69:365–370
22. Kajiwara K, Hirozane A, Fukumoto T, Orita T, Nishizaki T, Kamiryo T, Ito H: Major histocompatibility complex expression in brain of rats with graft-*versus*-host disease. J Neuroimmunol 1991, 32:191–198
23. Beilke MA, Riding In D, Hamilton R, Stone GA, Jordan EK, Brashears G, Nusbaum W, Huddleston D, Gibbs CJ Jr, Gravell M: HLA-DR expression in macaque neuroendothelial cells *in vitro* and during SIV encephalitis. J Neuroimmunol 1991, 33:129–143
24. Craggs RI, Webster HdeF: Ia antigens in the normal rat nervous system and in lesions of experimental allergic encephalomyelitis. Acta Neuropathol (Berl) 1985, 68:263–272
25. Sobel RA, Natale JM, Schneeberger EE: The immunopathology of acute experimental allergic encephalomyelitis. J Neuropathol Exp Neurol 1987, 46:239–249
26. Vass K, Lassmann H, Wekerle H, Wisniewski HM: The distribution of Ia antigen in the lesions of rat acute experimental allergic encephalomyelitis. Acta Neuropathol (Berl) 1986, 70:149–160
27. Butter C, O'Neill JK, Baker D, Gschmeissner SE, Turk JL: An immunoelectron microscopical study of the expression of class II major histocompatibility complex during chronic relapsing experimental allergic encephalomyelitis in Biozzi AB/H mice. J Neuroimmunol 1991, 33:37–42
28. Dorovini-Zis K, Prameya R, Bowman PD: Culture and characterization of microvascular endothelial cells derived from human brain. Lab Invest 1991, 64:425–436
29. McLean IW, Nakane PK: Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J Histochem Cytochem 1974, 22:1077–1083
30. Schroeter D, Spiess E, Paweletz N, Benke R: A procedure for rupture-free preparation of confluently grown monolayer cells for scanning electron microscopy. J Electron Microsc Tech 1984, 1:219–225
31. Dorovini-Zis K, Bowman PD, Betz AL, Goldstein GW: Hyperosmotic urea reversibly opens the tight junctions between brain capillary endothelial cells in cell culture. J Neuropathol Exp Neurol 1987, 46:130–140
32. Sobel RA, Ames MB: Major histocompatibility complex molecule expression in the human central nervous system: immunohistochemical analysis of 40 patients. J Neuropathol Exp Neurol 1988, 47:19–28
33. Lampson LA, Hickey WF: Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from "histologically normal" to that showing different levels of glial tumor involvement. J Immunol 1986, 136:4054–4062
34. Frank E, Pulver M, de Tribolet N: Expression of class II major histocompatibility antigens on reactive astrocytes and endothelial cells within the gliosis surrounding metastases and abscesses. J Neuroimmunol 1986, 12:29–36
35. Skoskiewicz MJ, Colvin RB, Schneeberger EE, Russell PS: Widespread and selective induction of major histocompatibility complex-determined antigens *in vivo* by γ interferon. J Exp Med 1985, 162:1645–1664
36. Ruszczak Z, Detmar M, Imcke E, Orfanos CE: Effects of recombinant IFN Alpha, Beta and Gamma on the morphology, proliferation, and cell surface antigen expression of human dermal microvascular endothelial cells *in vitro*. J Invest Dermatol 1990, 95:693–699
37. Heyns A du P, Eldor A, Vlodavsky I, Kaiser N, Fridman R, Panet A: The antiproliferative effect of interferon and the mitogenic activity of growth factors are independent cell cycle events. Exp Cell Res 1985, 161: 297–306
38. Friesel R, Komoriya A, Maciag T: Inhibition of endothelial cell proliferation by γ interferon. J Cell Biol 1987, 104:689–696
39. Stolpen AH, Guinan EC, Fiers W, Pober JS: Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. Am J Pathol 1986, 123:16–24
40. Saegusa Y, Ziff M, Welkovich L, Cavender D: Effect of inflammatory cytokines on human endothelial cell proliferation. J Cell Physiol 1990, 142:488–495
41. Maheshwari RK, Srikantan V, Bhartiya D, Kleinman HK, Grant DS: Differential effects of interferon γ and α on *in vitro* model of angiogenesis. J Cell Physiol 1991, 146:164–169
42. Tsuruoka N, Sugiyama M, Tawaragi Y, Tsujimoto M, Nishihara T, Goto T, Sato N: Inhibition of *in vitro* angiogenesis by lymphotoxin and interferon-γ. Biochem Biophys Res Commun 1988, 155:429–435
43. Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D: Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J Exp Med 1989, 169:1977–1991
44. Vulpe M, Hawkins A, Rozdilsky B: Permeability of cerebral blood vessels in experimental allergic encephalomyelitis studied by radioactive iodinated bovine albumin. Neurology 1960, 10:171–177

45. Hirano A, Dembitzer HM, Becker NH, Levine S, Zimmerman HM: Fine structural alterations of the blood-brain barrier in experimental allergic encephalomyelitis. J Neuropathol Exp Neurol 1970, 29:432–440
46. Kristensson K, Wisniewski HM: Chronic relapsing experimental allergic encephalomyelitis. Studies in vascular permeability changes. Acta Neuropathol (Berl) 1977, 39:189–194
47. Juhler M, Barry DI, Offner H, Konat G, Klinken L, Paulson OB: Blood-brain and blood-spinal cord barrier permeability during the course of experimental allergic encephalomyelitis in the rat. Brain Res 1984, 302: 347–355
48. Claudio L, Kress Y, Factor J, Brosnan CF: Mechanisms of edema formation in experimental autoimmune encephalomyelitis. Am J Pathol 1990, 137: 1033–1045
49. Sakai K, Tabira T, Endoh M, Steinman L: Ia expression in chronic relapsing experimental allergic encephalomyelitis induced by long-term cultured T cell lines in mice. Lab Invest 1986, 54:345–352
50. McCarron RM, Spatz M, Kempski O, Hogan RN, Muehl L, Mc Farlin DE: Interaction between myelin basic protein-sensitized T lymphocytes and murine cerebral vascular endothelial cells. J Immunol 1986, 137: 3428–3435
51. Pryce G, Male D, Sedgwick J: Antigen presentation in brain: brain endothelial cells are poor stimulators of T-cell proliferation. Immunology 1989, 66:207–212
52. Zetter BR: Endothelial heterogeneity: influence of vessel size, organ localization and species specificity on the properties of cultured EC. Endothelial Cells, vol 2. Edited by US Ryan. Boca Raton, FL, CRC Press, Inc, 1988, pp 63–79