Increased Permeability of Microcarrier-Cultured Endothelial Monolayers in Response to Histamine and Thrombin

A Model for the In Vitro *Study of Increased Vasopermeability*

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The permeability response of endothelial monolayers to some "direct-action" type mediators of vasopermeability were studied *in vitro*. Endothelial cells, cultured to confluence on denatured collagen-coated dextran microcarriers or gelatin microcarriers, prevented staining of the microcarriers with Evans blue dye. Increases in staining, as determined by the spectrophotometric quantitation of the dye after extraction from the microcarriers with formamide, occurred after treatment of human umbilical vein endothelium with histamine (10^{-5} M) or thrombin (0.1 U/ml). These increases in monolayer permeability were reversible. Neither bradykinin nor serotonin had any effect in this system. Endothelial monolayers cultured this way consistently stained with silver nitrate at the cell junction areas. Monolayer response to histamine was characterized morphologically by small openings which occurred randomly along the cell junctions; while with thrombin, the spaces, which had developed at junctions, occurred to a greater extent. Prostaglandin E_1 (30 μM) and isoproterenol (10 μM), in the presence of 3-isobutyl-1-methylxanthine (1 mM), partially inhibited histamine- and thrombin-mediated changes in permeability. This model responds to certain vasopermeability-altering agents in a manner similar to that of the microcirculation. These studies support the concept that the vasopermeability enhancing effect of histamine *in vivo* results, in part, from a direct effect on the endothelium. (Am J Pathol 1986, 122:50–61)

ONE OF THE five cardinal signs of inflammation, tumor or swelling, results from a change in microvascular permeability which leads to the extravasation of protein-rich fluid from the vascular compartment into the interstitium.[1] The mechanisms of changes in vascular permeability, which lead to swelling, are incompletely understood. There are mediators of permeability which act *via* polymorphonuclear leukocytes (PMNs).[2] In contrast to these PMN-dependent mediators of vasopermeability, the "direct-action" mediators have effects in neutropenic animals. This group of mediators, which includes histamine, serotonin, bradykinin, and the intact molecules of the anaphylatoxins C3a, C4a, and C5a,[3,4] is thought to interact directly with the endothelium to cause permeability changes.

One potentially useful method for elucidating the role of the endothelium in controlling changes in vasopermeability involves the use of cultured cells. The effects of histamine and bradykinin on the permeability of the vascular endothelium have been investigated here with the use of an *in vitro* model in which confluent endothelial monolayers, cultured on either denatured collagen-coated dextran microcarriers or gelatin microcarriers, exclude Evans blue dye, preventing staining of the microcarriers. The measure of increased permeability in this model is the increased staining of the microcarriers after treatment of the endothelium with biologically active agents. This model is analogous to *in vivo* techniques for assessing changes in permeability in response to local mediator administration by meas-

Supported by the Medical Research Council of Canada (MA-7925, to M.G.J.) and the Ontario Heart and Stroke Foundation (T082 to H.Z.M.). Dr. John J. F. Killackey is a Postdoctoral Fellow supported by the Medical Research Council of Canada (MT-1251). Dr. Miles G. Johnston is a Scholar of the Medical Research Council of Canada. Dr. Henry Z. Movat is a Career Investigator of the Medical Research Council of Canada.

Accepted for publication July 31, 1985.

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uring the leakage of systemically administered Evans blue from the vascular compartment into the surrounding tissue.[5] A preliminary report of this procedure has been published in abstract form,[6] and a model based on similar principles has recently been published by Boiadjieva et al.[7] The aims of this study were to characterize the functional and morphologic changes in the permeability of endothelial monolayers in response to these "direct action" agents in light of their effects *in vivo* and also to compare these changes with those of thrombin, an enzyme reported to cause alterations in endothelial cells *in vitro*.[8]

Materials and Methods

Materials

Medium 199 (with Earle's salts, 2.5 mM HEPES buffer, and L-glutamine), penicillin-streptomycin, amphotericin B, neomycin, collagenase, trypsin-EDTA solution, and Nunclon 24-well tissue culture dishes were purchased from GIBCO (Burlington, Ontario). Fetal bovine serum (FBS) was from Bocknek (Toronto, Ontario). Thrombin (both human, T4393, 80% α-thrombin, 3000 NIH units/mg, and bovine, T7513, 2000 NIH units/mg), histamine, bradykinin, serotonin, Evans blue dye, 3-isobutyl-1-methylxanthine (MIX), and ($\pm$) isoproterenol (IPN) were from Sigma (St. Louis, Mo). Bovine serum albumin was purchased from Sigma (St. Louis, Mo) or Miles Scientific (Toronto, Ontario). Formamide was obtained from Fisher (Toronto, Ontario) or Aldrich (Montreal, Quebec). Mepyramine maleate was from Rhône-Poulenc (Montreal, Quebec). Tissue culture dishes were from Falcon Plastics (Oxnard, Calif) unless otherwise indicated. Hemacolor Stain Kits were from Harleco (Gibbstown, NJ). Cytodex 3 microcarriers were from Pharmacia (Dorval, Quebec), and Gelibeads were from K. C. Biological (Lenexa, Kans). Nifedipine, used in the culture of human umbilical artery cells, was a gift from Miles Laboratories (Toronto, Ontario). Captopril (SQ 14225) was a gift from E. R. Squibb and Sons, Inc. (Princeton, NJ). Prostaglandin E_1 (PGE_1) was from Upjohn (Kalamazoo, Mich).

Endothelial Cell Culture

Endothelial cells from human umbilical veins (HUV) were cultured by the method described by Jaffe et al.[9] with the use of Medium 199 supplemented with antibiotics (penicillin 5 U/ml, streptomycin 50 U/ml, neomycin 5 μg/ml, amphotericin B 2.5 μg/ml) and 20% FBS. These cultures are positive for Factor VIII antigen when tested by immunofluorescence.[10] Permeability studies involved human umbilical endothelial cells that were in the first passage only. When cultured under these conditions for such short time periods, very few cultures contained cells with the morphologic features of fibroblasts or smooth-muscle cells; but if any were found, the cultures were discarded. In our experience, smooth-muscle cells and fibroblasts proliferate much more slowly on microcarriers than endothelial cells, further diminishing the risk of nonendothelial cell contamination of the cultures.

Endothelial cells from bovine superior mesenteric arteries, veins, and lymphatics were also studied in this assay with the use of vessels obtained from a local abattoir. Bovine mesenteric artery and vein endothelial cells were cultured as described above but were subcultured 5 to 8 times before innoculation onto the microcarriers. Lymphatic endothelial cells were cultured by the method of Johnston and Walker.[10]

Microcarriers (600 mg) were swollen and hydrated in calcium and magnesium-free phosphate-buffered saline (PBS), autoclaved, and then equilibrated in the endothelial cell culture medium in a 5% CO_2 atmosphere under stirring conditions (30 rpm) according to the manufacturer's recommendations. Cells (1–2 $\times$ 10^7) from 3–10 umbilical veins were added to the spinner bottle (Techne, Cambridge) in a final volume of 100 ml. Cultures were stirred at 30 rpm for 3 minutes every 30 minutes for the first 6 hours, for inoculation of the microcarriers with cells, and then at 30 rpm continuously afterwards. The volume was increased to 200 ml on Day 2, and then 10% of the medium was replaced every other day.

Most studies described here involve HUV endotheiial cells. However, for some studies, other endothelial cells were examined as well. Primary cultures of human umbilical artery, vein, and bovine mesenteric vein and lymphatic endothelial cells on microcarriers were obtained by a different method and were monitored for morphologic changes in response to thrombin. Approximately 100–200 microcarriers were added to primary cultures of the various cells, which would in turn grow onto the microcarriers. After 2 days the microcarriers were transferred to new wells, and the attached cells were allowed to grow to confluence on the microcarriers. The cultures were washed (as described below), treated with mediators, and then fixed and stained with the Hemacolor Stain Kit.[10] Human umbilical artery endothelial cells were isolated by the method of Mano et al.[11]

In Vitro Permeability Assay

Endothelial monolayers reached confluence in 5–11 days after subculture onto the microcarriers and were used immediately for assay. Microcarriers (approxi-

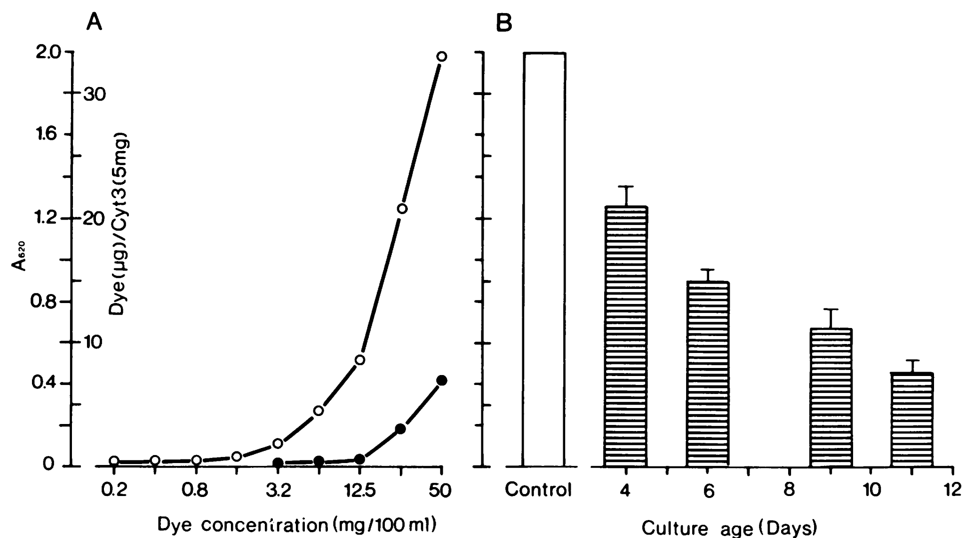


Figure 1—Dye adsorption by microcarriers. **A**—Microcarriers (5 mg dry weight), not covered with endothelial monolayers (*open circles*) or cell covered (*closed circles*) were incubated under the conditions of the assay with various concentrations of Evans blue dye and then assayed for dye adsorption. A final dye concentration of 500 µg/ml (50 mg/100 ml) was used in subsequent assays. Outer ordinate: absorbance reading at 620 nm. Inner ordinate: the conversion of absorbance to "micrograms dye per 5 mg microcarriers (Cytodex 3)." **B**—Monolayers of human umbilical vein endothelial cells at various days after the start of culture on microcarriers prevent dye adsorption to microcarriers. The control represents cell-free microcarriers. Detectable inhibition of dye adsorption occurred on Day 4, and maximum inhibition occurred on Day 11. Each point or bar represents the mean and standard deviation of triplicate determinations.

mately 90 mg dry weight of microcarriers—30 ml volume) were removed from the culture vessel, placed into a siliconized Erlenmyer flask prewarmed to 37 C, washed three times with Tyrode's solution (with 2 mM calcium and 1 mM magnesium) containing 0.86% bovine serum albumin (Tyrode's-BSA) at 37 C, and finally resuspended in 8.1 ml of the same buffer. At this concentration, 450 µl of buffer contains 5 mg of microcarriers, and this portion of resuspended microcarriers was placed into 18 wells of a 24-well dish. The washed cultures were incubated at 37 C for 15 minutes at which time drugs, in a final volume of 50 µl, were mixed with the cultures and allowed to incubate at 37 C for, usually, 15 minutes. Evans blue dye (50 µl, 0.5% in Tyrode's-BSA) was added, mixed, and allowed to incubate for 2 minutes. A short incubation period was used to minimize the contribution of the normal intracellular transport of dye to the increased permeability effects caused by the inflammation-related mediators. Cultures were then washed, first with Tyrode's-BSA and then up to 8 times with PBS, for removal of all traces of unbound dye. All PBS was then removed from the microcarriers, and they were dried overnight at 40 C. Dye was extracted from the microcarriers by incubating them with formamide (1 ml/well) at room temperature for 6–8 hours. With this extraction procedure, the dye reaches a point of equilibrium between the microcarriers and the formamide. Because the microcarrier volume is small relative to the formamide, it was considered negligible. The dye concentration was quantitated spectrophotometrically at 620 nm.[5] Statistical significance was determined by analysis of variance and the Student *t* test for unpaired data.

Silver Nitrate Staining of the Endothelium

HUV endothelial monolayers, cultured on Cytodex 3 or Gelibeads and treated with thrombin, histamine, or saline, were stained with silver nitrate by the method of Furie et al[12] except that the cultures were not counterstained with Wright's stain. This involves the incubation of the cells in 24-well dishes with rinse with 5% glucose for 30 seconds, 0.25% $AgNO_3$ for 30 seconds, rinse with 5% glucose, 1.0% NH_4Br for 30 seconds, rinse with 5% glucose, 3.0% $CoBr_2$ for 30 seconds, and rinse with 5% glucose. The cells were examined immediately and hence were not fixed with formalin. In some cases the endothelium was counter-stained with the use of a cell-staining kit (Hemacolor).

Preparation of Semithin Sections

HUV endothelial monolayers on Gelibeads, treated with thrombin, histamine, or saline, were fixed in 0.1 M phosphate-buffered (pH 7.4) 2% glutaraldehyde and 10% formaldehyde, dehydrated with the use of a series of ethanol solutions, and then embedded in hydroxyethyl methacrylate for 2 days before polymerization. The sections were cut with glass knives on a du Pont-Sorvall JB4 microtome and stained with Azure II. Be-

cause of the difficulty in thin-sectioning Cytodex 3, all morphologic data demonstrated are from Gelibead-cultured endothelial monolayers.

Results

Evans Blue Staining of Endothelial Cell-Covered Microcarriers

Cytodex 3 microcarriers, incubated in endothelial cell culture medium (with FBS) in the absence or presence of cells, were used in control studies. Detectable dye absorption by 5 mg portions of cell-free microcarriers occurred in final dye concentrations of 16 μg/ml, and concentrations up to 500 μg/ml were associated with further increases in microcarrier dye adsorption (Figure 1A). The maximum difference in dye adsorption by microcarriers coated with confluent layer of endothelial cells versus cell-free microcarriers occurred at a final dye concentration of 500 μg/ml, and this dye concentration was used in all subsequent studies.

Endothelial monolayers inhibited microcarrier staining in a manner related to the time after cell inoculation onto the microcarriers (Figures 1B and 2). Significant inhibition of staining occurred usually by Day 4, and maximal inhibition occurred by at least Day 11, when cells were cultured as described (Materials and Methods). Cultures usually deteriorate rapidly in our system after this time, because no exogenous growth factors, other than FBS, are used. All endothelial monolayers caused the same maximum degree of inhibition of staining.

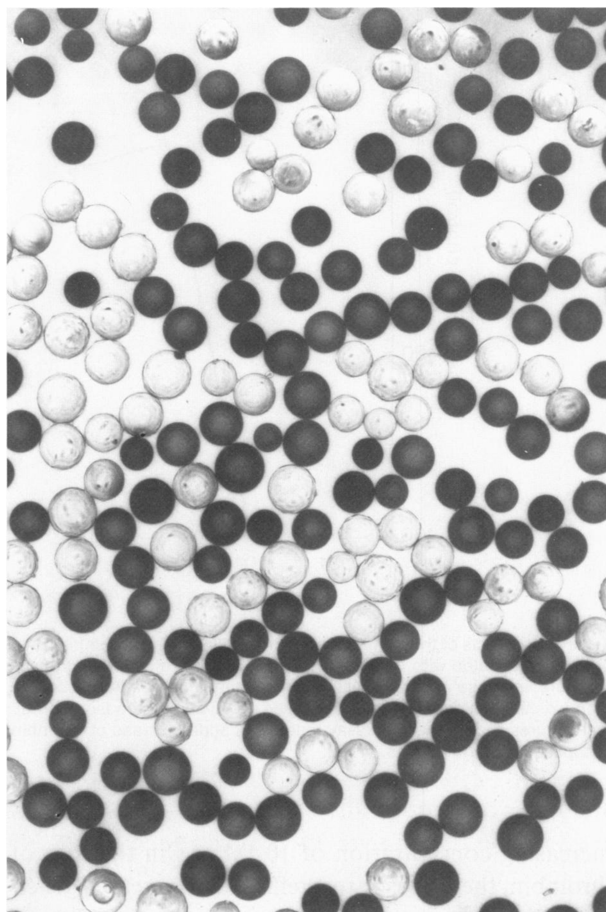


Figure 2—Photomicrograph of mixtures of human umbilical vein endothelial cell-covered and noncovered Cytodex 3 microcarriers after staining for 2 minutes with Evans blue (500 μg/ml) and subsequent wash. Uncovered microcarriers stain dark, while cell-covered microcarriers do not stain. (×45)

Mediator-Induced Changes in Endothelial Monolayer Permeability

Both human and bovine thrombin caused changes in HUV endothelial monolayer permeability. Maximum effects occurred at 0.1 U/ml, while concentrations less than 0.01 U/ml caused no detectable changes (Figure 3A). To assess the reversibility of the thrombin effect, HUV endothelium was treated with 1 U/ml of thrombin for 15 minutes, washed free of thrombin, and then incubated with dye for 2 minutes at 0. 0.5, 1, or 2 hours after thrombin washout. The increased permeability was reversed in a time-dependent manner despite the fact that no serum was present (Figure 3B). This indicates that the endothelial monolayer regained its capacity to exclude dye over a 1–2-hour period after thrombin washout.

Thrombin caused no alterations in the permeability of bovine mesenteric artery, vein, or lymphatic endothelium. These cells were studied under conditions identical to those of the HUV endothelial cells except that they had been subcultured five to eight times, compared with one time for the latter. Preliminary studies indicate that histamine also has no effect on nonhuman endothelium. Because of the difficulty in obtaining large numbers of nonhuman endothelial cells in primary culture, the influence of passage number on the response of different endothelia to thrombin was examined morphologically and not by microcarrier staining. The HUV and artery endothelial cell responses to thrombin (1 U/ml) were characterized by rounding and protrusion of the cells from the surface of the microcarrier. In contrast, bovine mesenteric artery, vein, and lymphatic endothelial cells in primary culture did not change shape in response to thrombin. Changes to any of these cell types after treatment with histamine were difficult to see morphologically.

Histamine also caused a dose-dependent increase in HUV endothelial permeability (Figure 4). The histamine dose-response curve was characterized by a threshold concentration of 10^{-5}M and a peak permeability-

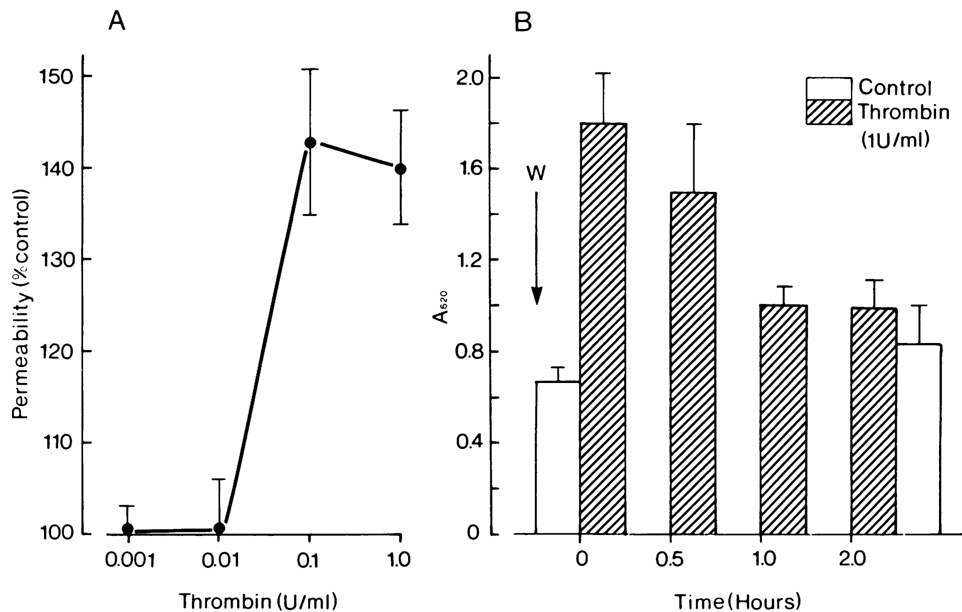


Figure 3—Effects of thrombin on the permeability of human umbilical vein endothelium cultured on Cytodex 3. **A**—Various concentrations of thrombin were incubated with monolayers for 15 minutes, after which the cultures were assayed for permeability. Thrombin was most effective at 0.1 U/ml. Ordinate: increase in permeability expressed as a percentage of control. **B**—Monolayers were treated with thrombin (1.0 U/ml) for 15 minutes and then washed. The cultures were assayed for Evans blue adsorption at various times after removal of thrombin. Controls were treated exactly as the test cultures except that the assay buffer was added instead of thrombin in assay buffer. Each point or bar represents the mean and standard deviation of triplicate determinations.

increasing concentration of 10^{-4}M. As in the case of thrombin, these effects were reflected in increased generalized staining of the microcarriers, as opposed to an increase in the number of microcarriers staining. Higher concentrations of histamine were not associated with further increases in permeability of endothelial monolayers. Both thrombin and histamine caused negligible differences from controls in dye uptake by endothelial cells cultured on plastic dishes. Therefore, the microcarrier dye staining in response to these agents was not an artifact of dye uptake by the cells. The effect of histamine on endothelial permeability increased with time over a 20-minute period (Figure 5A). The reversibility of the histamine effect was examined by incubating monolayers with histamine (10^{-4} M) for 15 minutes, washing the monolayers in Tyrode's-albumin buffer, and then staining the microcarriers as described in the thrombin washout experiment above. The histamine effects were completely reversible in 0.5 hour after washout, in contrast to the thrombin effects, which required 1–2 hours (Figure 5B). This reversibility occurred in a buffer environment, and serum was not required, but it was essential that the stimulating agent was removed. Although no studies were performed with an H_2 histamine receptor antagonist, mepyramine maleate (5×10^{-6} M), an H_1-blocker, completely inhibited the effects of histamine. Serotonin, which is another biogenic amine, was tested over the same concentration range as histamine as a control. Serotonin had no effect on the endothelial cells in this assay.

Bradykinin had no significant effect on monolayer permeability at any concentration tested. Bradykinin was also tested in the presence of captopril (SQ 14225) (Figure 4), a compound which inhibits the endothelial-associated, bradykinin-degrading, angiotensin-convert-

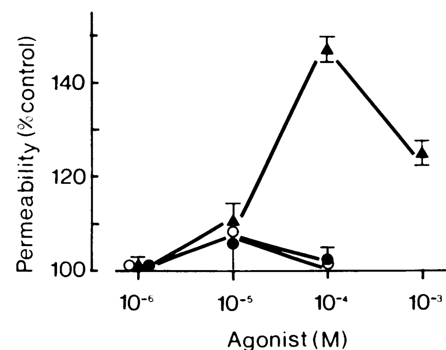


Figure 4—Comparison of the effects of histamine with the effects of other mediators. Various concentrations of histamine (*closed triangles*) bradykinin (*open circles*), or bradykinin and captopril (10_3 M) (*closed circles*) were incubated with Cytodex 3-cultured human umbilical vein endothelial cell monolayers for 15 minutes. The cultures were then assayed for Evans blue adsorption. Histamine typically had a maximum effect of 10^{-4} M. Each point represents the mean and standard deviation of triplicate determinations. Ordinate: increase in permeability expressed as a percentage of controls.

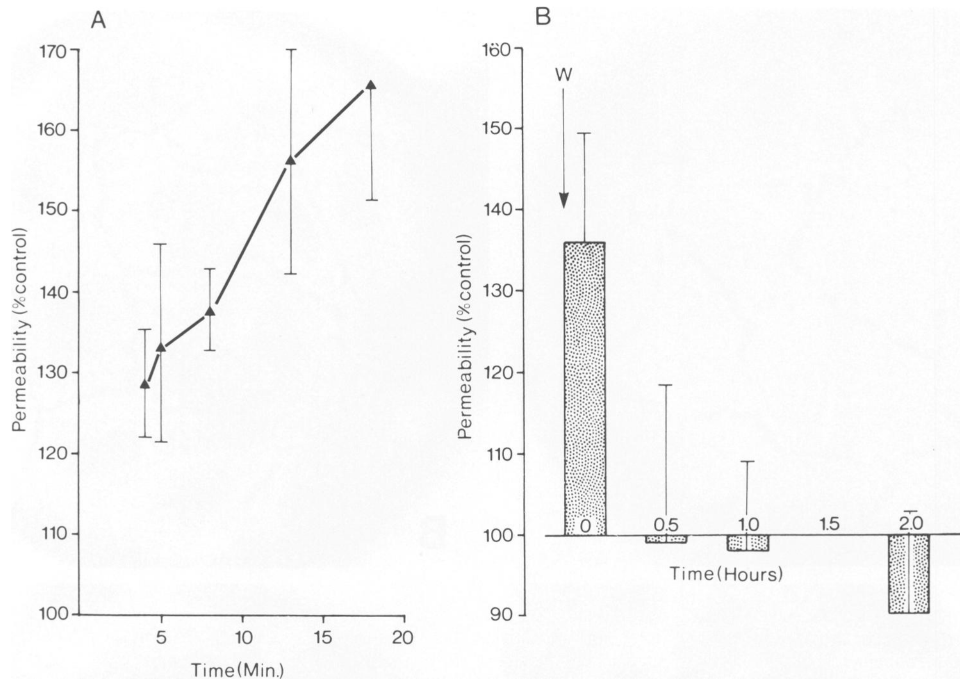


Figure 5—Characterization of histamine-mediated increases in permeability. **A**—Histamine (10^{-4} M) was incubated with Cytodex 3-cultured human umbilical vein endothelial monolayers for various times, after which the cultures were assayed for permeability. Permeability increased over a 20-minute period. **B**—Cytodex 3-cultured monolayers were treated with histamine (10^{-4} M) for 15 minutes and then washed. The cultures were assayed for permeability at various times after washing. Each bar represents the mean and standard deviation of triplicate determinations. Ordinate: increase in permeability expressed as a percentage of control.

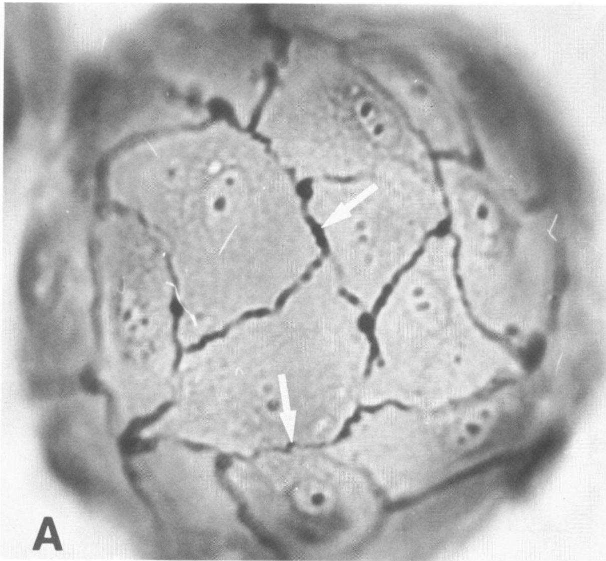
ing enzyme or kininase II.[13] Captopril caused no change in permeability, nor did it modify the effects of bradykinin. Bradykinin was proven biologically active by bioassay for effects on permeability after intradermal injection into rabbit skin.

Morphologic Changes in Thrombin and Histamine-Treated Endothelium

Confluent monolayers of microcarrier-cultured HUV endothelial cells stained consistently along the cell junctions with $AgNO_3$ (Figure 6A and B). Similar effects were found with Cytodex 3 or Gelibead microcarriers; however, $AgNO_3$ staining did not occur in plastic dish-cultured cells. Thrombin treatment of the microcarrier-cultured cells (0.1 U/ml, 15 min) resulted in cell retraction with severe disruption of cell junctions (Figure 6C and D). Histamine (10^{-4}) caused a consistent but less severe effect (Figure 6E and F). Small opening occurred, but only among some junctions; and cell shape change was much less evident. Semithin sections of control, cell-covered microcarriers demonstrated that although the microcarriers were completely covered, the cell covering in some areas consisted of a very thin layer of cytoplasm, especially in areas where nuclei were widely spaced (Figure 7A). Severe shape changes and clear disruption of cell–cell contacts occurred after thrombin treatment (Figure 7B). Cell-free areas of the microcarrier perimeter were also seen with histamine treatment, even in areas where two nuclei were relatively close together (Figure 7C).

Pharmacologic Inhibition of Mediator-Induced Permeability

The effects of histamine were examined on human umbilical vein endothelial monolayers preincubated with prostaglandin E_1 (PGE_1) (30 μM) or isoproterenol (IPN) (10 μM). Both of these agents cause alterations in the permeability effects of histamine *in vivo* (see Discussion). In addition, PGE_1 and IPN stimulate endothelial cell adenylate cyclase,[14] leading to elevations of intracellular cyclic AMP. Therefore, studies were also carried out in the presence of 3-isobutyl-1-methylxanthine (MIX) (1 mM), an agent which causes elevations of cyclic AMP by inhibiting cyclic nucleotide phosphodiesterase enzyme activity. There was a trend to inhibition, of histamine-mediated increases in permeability after preincubation of the cultures with PGE_1 or IPN, however, with PGE_1 the inhibition did not reach statistical significance (Figure 8A and B). MIX, alone, also significantly inhibited histamine-mediated increases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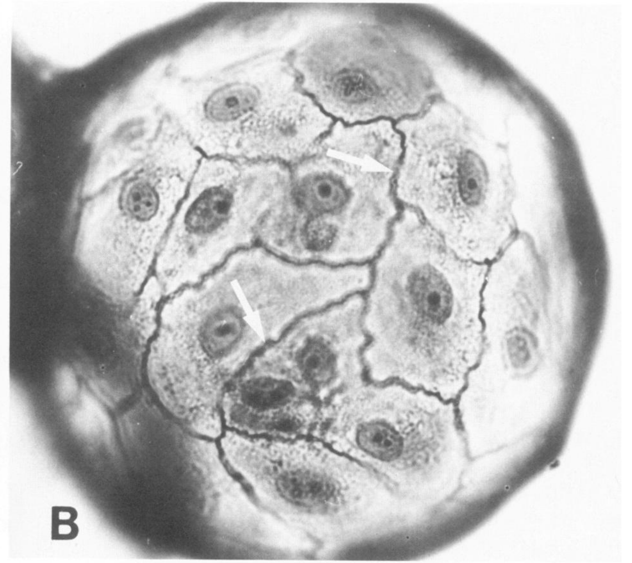
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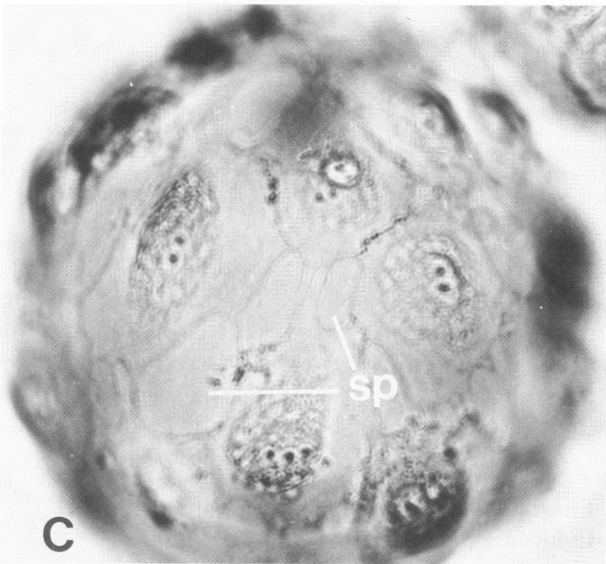
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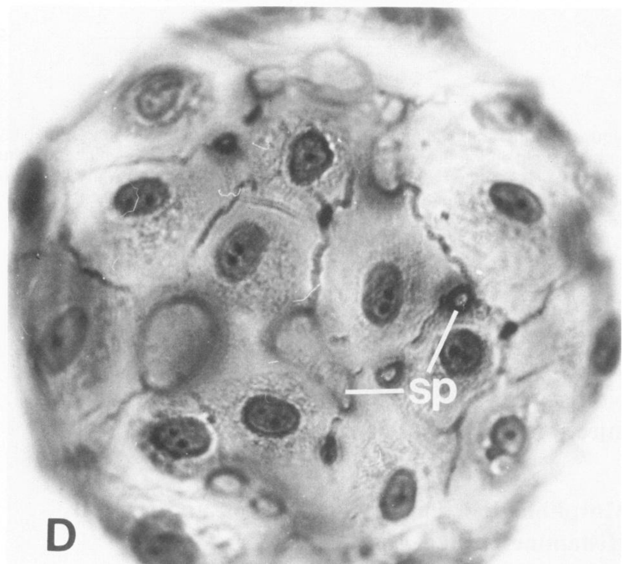
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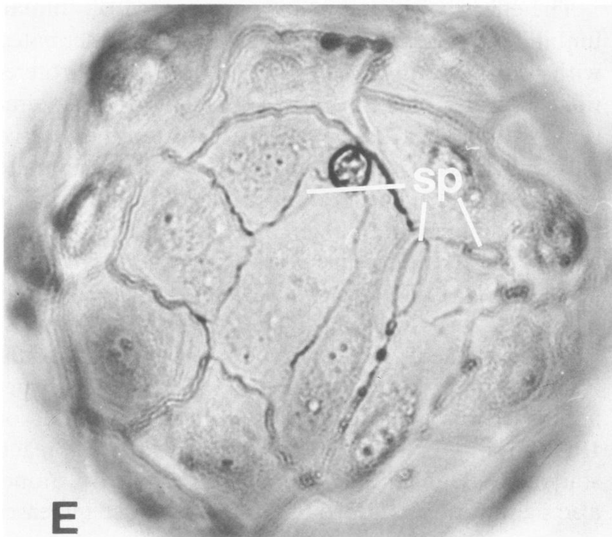
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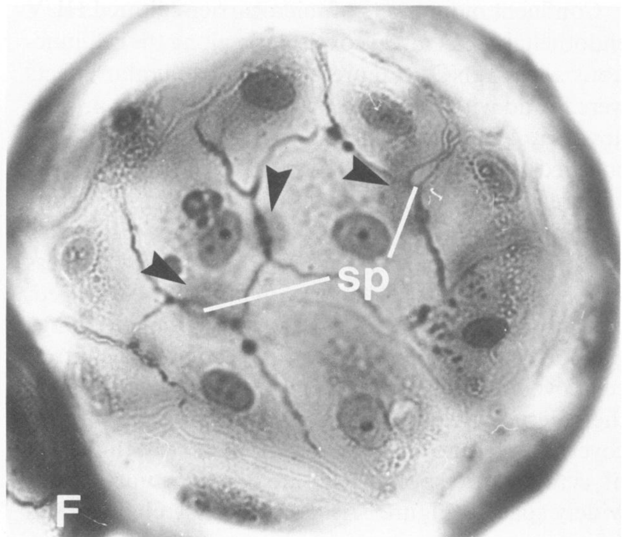
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in permeability. The greatest degree of inhibition always occurred with combinations of PGE_1 or IPN and MIX. These compounds also had the same spectrum of activities on thrombin-mediated increases in permeability.

Discussion

Physiologic experiments have demonstrated indirectly that histamine[15] and bradykinin[16] increase permeability by two mechanisms, one of which is independent of intravascular pressure. Electron-microscopic studies of vessels treated with "histamine-type" mediators *in vivo* have revealed interendothelial gaps in certain vessels,[17] and these have been attributed to endothelial-cell contraction,[18] although this last point is controversial.[19] Simionescu et al[20] reported that endothelial cells contain both H_1 and H_2 histamine receptors. The implication of these studies is that histamine and other such mediators cause increases in permeability by interacting directly with the endothelium.

We have clearly demonstrated that thrombin and histamine can cause increases in the permeability of first-passage, HUV endothelial-cell monolayers to Evans blue dye in a dose-dependent manner. The mechanism of increased microcarrier staining appears to be through disruption of cell–cell junctions, and no other specialized transport mechanism such as vesicular transport of Evan's blue dye–BSA complex is necessarily stimulated. Because the endothelial effects of thrombin occur at a concentration which is below that detectable with the synthetic substrate benzoyl-arginine-ethyl ester and within the range by which thrombin stimulates human blood platelet aggregation, these effects may be receptor-mediated. This interesting issue must be examined in other studies, which, along with ultrastructural studies, are currently being pursued. Our present study confirms the work of Laposata et al,[8] who demonstrated the formation of large "holes" between cells in primary cultures of HUV endothelial cells when incubated with these agents. Although histamine is a potent mediator of vascular permeability, thrombin has only weak effects *in vivo*.[21] Thrombin has been shown, however, to cause the release of platelet-activating factor (PAF) from endothelial cells in culture,[22,23] and PAF does cause increases in vascular permeability *in vivo*,[24–26] possibly directly. Human umbilical artery endothelial cells in primary culture also respond to thrombin, although cells from bovine vessels, even in primary culture, do not. Harland and co-workers[27] have found similar variations in the response of cultured endothelial cells from various sites and species of origin with regard to sensitivity to lipopolysaccharide-mediated injury.

The characteristic of dye-adsorbing capacity of microcarriers made of dextran or gelatin, together with the permeability barrier effect of endothelial cells cultured on these microcarriers, constitutes the basis for establishing this system for studying the role of the endothelium in response to some "direct-action" mediators of permeability. The human umbilical vein endothelial cell[28] is particularly suitable for initial studies with this model. These cells reversibly change shape in response to thrombin[8,29–31] and also are available in large numbers in primary culture. At present it would be impossible to assay cells from other important vessels, such as postcapillary venules, in primary culture or first passage. This is important, since evidence exists that the capacity for some receptor-mediated events, including bradykinin- and thrombin-induced prostacyclin production by endothelial cells, diminishes significantly after early passages.[32] There were no permeability nor morphologic differences between Cytodex 3 or Gelibead-cultured endothelia, regardless of the treatment or staining procedure.

Bradykinin has no effects on permeability in this system despite the high degree of activity *in vivo*. It is possible that postcapillary venules are lined with a highly specialized endothelium and that this model is not completely representative. We know, however, that HUV endothelial cells contain receptors for bradykinin because of evidence that bradykinin stimulates prostacyclin production in both primary cultures and early passage cells.[32,33] Since HUV endothelial cells have the capacity to change shape *in vitro* and the endothelium becomes permeable by histamine treatment, it may be that bradykinin does not cause increases in permeability by interacting directly with the endothelium. There is some precedent for this concept, because bradykinin has been shown to mediate mast-cell histamine release.[34,35] Alternatively, bradykinin may stimulate increases in permeability and prostacyclin release by different receptors, the former not expressed in this cell type or system or at least not coupled to the intracellular effector system.

Beta adrenoceptor agonists have been shown to in-

Figure 6—Photomicrographs of confluent monolayers of Gelibead microcarrier cultured-HUV endothelial cells, stained with $AgNO_3$ (**A**, **C**, and **E**) or $AgNO_3$ and Hemacolor Stain Kit (**B**, **D**, and **F**) after treatment with saline (**A** and **B**), thrombin (**C** and **D**) or histamine (**E** and **F**). The *arrows* point to silver deposits at cell junctions (**A** and **B**). Spaces (*sp*), seen between some of the adjacent endothelial cells, are larger after thrombin treatment (**C** and **D**) than those resulting from histamine treatment (**E** and **F**). The microcarrier marked **F** shows staining around the spaces (*arrowheads*). (×550)

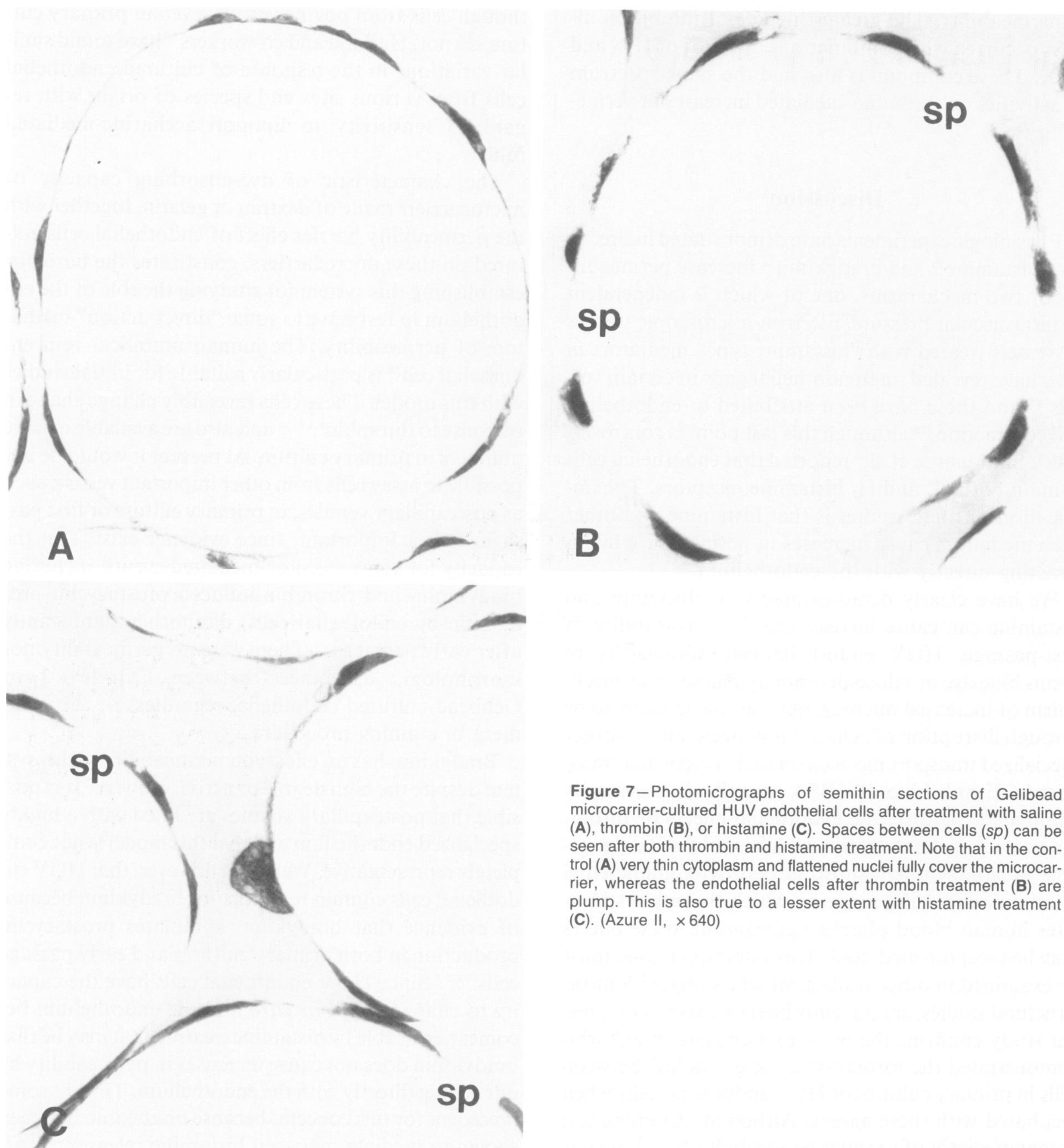

Figure 7—Photomicrographs of semithin sections of Gelibead microcarrier-cultured HUV endothelial cells after treatment with saline (A), thrombin (B), or histamine (C). Spaces between cells (*sp*) can be seen after both thrombin and histamine treatment. Note that in the control (A) very thin cytoplasm and flattened nuclei fully cover the microcarrier, whereas the endothelial cells after thrombin treatment (B) are plump. This is also true to a lesser extent with histamine treatment (C). (Azure II, ×640)

hibit histamine- and bradkinin-mediated increases in vasopermeability *in vivo*,[36,37] supposedly by direct effects on the endothelium. IPN, in the presence of MIX,[14,38] was an effective inhibitor of histamine-mediated increases in permeability here. Thus, the *in vivo* observation of beta agonist inhibition of "direct-action" mediator effects on vascular permeability may result, at least in part, from the inhibition of the permeability response of the endothelium itself. Elevations of intracellular cyclic AMP have been associated with the inhibition of thrombin-induced shape changes of endothelial cells *in vitro*,[8] and MIX augments IPN-mediated increases in endothelial cell cyclic AMP.[14,38] PGE_1, like IPN, is a stimulator of endothelial adenylate cyclase[14]; and the concept that the inhibition of histamine-mediated increases in permeability seen here is through elevation of endothelial cell cyclic AMP is supported by the increased effect observed when PGE_1

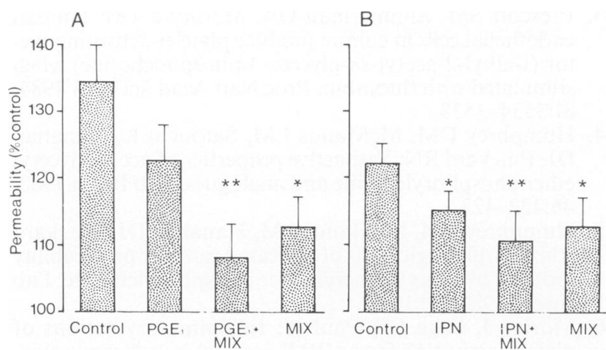

Figure 8—Representative experiments of the effects of preincubation with PGE_1, isoproterenol, or control buffer alone and in the presence of MIX on histamine-mediated increases in permeability. Cytodex 3-cultured HUV endothelial monolayers were preincubated for 5 minutes with **(A)** PGE_1 (30 μM), MIX (1 mM), PGE_1 plus MIX, or buffer control or **(B)** isoproterenol (10 μM), MIX (1 mM), isoproterenol plus MIX, or control buffer and then tested for histamine (10^{-4} M)-mediated permeability. Each bar represents the mean and standard deviation of at least triplicate determinations. Ordinate: increase in permeability expressed as a percentage of control. * $P < 0.05$. ** $P < 0.01$.

was combined with MIX. Recent studies, however, have shown that MIX may cause effects on endothelial cells by mechanisms other than PDE inhibition[39]; and Hong[40] reported an apparent inhibition of endothelial-cell phospholipase A_2 and phospholipase C by MIX. These results have important implications in the development of pharmacologic controls of vasopermeability.

Other *in vitro* models of vascular permeability have been reported. Taylor et al[41] cultivated endothelial cells on gelatin-coated micropore filters, and Furie et al[12] modified this system by using amnion membrane instead of filters. Shasby and associates have used the filter technique to show that endothelial cell monolayers become reversibly permeable to albumin in the presence of cytochalasin B or D[42] or a calcium chelator.[43] Using endothelial cell-coated microcarriers packed in columns, Boiadjieva et al[7] measured dye extraction from buffer by the microcarriers in a method based on the same principles as described here and demonstrated the long-term, irreversible destruction of endothelial monolayers by homocysteine and dicloxacillin. An important advantage of the present model is that it combines the ability to do sensitive assay-type experiments as found with the models of Taylor et al,[41] Furie et al,[12] and Shasby et al[42] with the ability to use portions of a single microcarrier cell culture as found with the model of Boiadjieva et al.[7] Another feature of this model is the relative ease with which reproducible assay-type data can be generated.

The $AgNO_3$-staining characteristics of both Cytodex- and Gelibead-cultured endothelial cells is another important factor distinguishing these endothelia from those cultured in plastic dishes. This staining pattern is similar to that found with endothelium *in vivo* and to that found by Furie et al,[12] who cultured bovine endothelial cells cloned from bovine adrenal cortex microvessels on human amnionic membrane. The reason for the different staining patterns of microcarrier versus plastic dish-cultured cells in unknown. This characteristic allowed us, however, to demonstrate the morphologic changes at the cell junctions induced by histamine and thrombin. While the increased microcarrier dye-staining following thrombin treatment was associated with a shape change of the cells and, in many cases, a severe disruption of cell junctions, histamine caused relatively minor discontinuities of the cell junctions, and if any shape change occurred, it was hardly evident in semithin sections through the beads and cells. This relative potency of thrombin with respect to histamine was also reflected in permeability studies where these agents were used at optimal concentrations in the same assay runs.

Using human umbilical vein endothelial cells cultured on microcarriers, we have been able to mimic many but not all of the *in vivo* effects of some "direct-action" mediators of permeability. The use of thrombin, the effects of which have been known since 1973,[44,45] was important in the initial development of this model. It is proposed that further characterization of the effects of thrombin, histamine, and bradykinin, as well as further elucidation of the control mechanisms of endothelial cells on vasopermeability, can be made through the incorporation of this *in vitro* model with its *in vivo* counterparts.

Currently we are investigating the nature of the gap formation between adjacent endothelial cells by scanning and transmission electron microscopy. Using intima sections stripped from the wall of bovine pulmonary artery, Meyrick and Brigham[46] demonstrated histamine-induced focal dilatations in the endothelial intercellular junctions, although increased diffusion of ^{125}I-albumin across the intima was not detected. It remains to be ascertained whether the changes we observe *in vitro* are similar to those observed earlier *in vivo* in acute inflammation induced with mechanical,[47] physical, and chemical stimuli,[48] which were similar to those first observed when vasoactive agents were injected.[17]

References

1. Movat HZ: The Inflammatory Reaction. Amsterdam, Elsevier Biomedical Publishers, 1985, pp 1–383
2. Wedmore CV, Williams TJ: Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature (Lond) 1981, 289:646–650

3. Majno G: Mechanisms of abnormal vascular permeability in acute inflammation, Injury, Inflammation and Immunity. Edited by L Thomas, JW Uhr, L Grant. Baltimore, Williams and Wilkins Co., 1964, pp 58-63
4. Hugli TE: The structural basis for anaphylatoxin and chemotactic functions of C3a, C4a and C5a. CRC Crit Rev Immunol 1982, 1:321-366
5. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Exp Biol Med 1970, 133:1384-1387
6. Killackey JJF, Johnston MG, Movat HZ: An *in vitro* system for the study of vascular permeability using endothelial cells cultured on collagen coated microcarriers (Abstr). Fed Proc 1984, 43:971
7. Boiadjieva S, Hallberg C, Hogstrom M, Busch C: Exclusion of trypan blue from microcarriers by endothelial cells: An in vitro barrier function test. Lab Invest 1984, 50:239-246
8. Laposata M, Dovnarsky DK, Shin HS: Thrombin-induced gap formation in confluent endothelial cell monolayers in vitro. Blood 1983, 62:549-556
9. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. J Clin Invest 1973, 52:2745-2756
10. Johnston MG, Walker MA: Lymphatic endothelial and smooth muscle cells in tissue culture. In Vitro 1984, 20:566-572
11. Mano Y, Sawasaki K, Takahashi K, Goto T: Cultivation of arterial endothelial cells from human umbilical cord. Experientia 1983, 39:1144-1146
12. Furie MB, Cramer EB, Naprstek BL, Silverstein SC: Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electric current. J Cell Biol 1984, 98:1033-1041
13. Rubin B, Laffan RJ, Kotler DG, O'Keefe E, Dermio D, Goldberg ME: SQ 14225 (d-3-mercapto-2-methylpropanoyl, L-proline), a novel orally active inhibitor of angiotensin I converting enzyme. J Pharmacol Exp Ther 1978, 204:271-280
14. Hopkins NK, Gorman RR: Regulation of endothelial cell cyclic nucleotide metabolism by prostacyclin. J Clin Invest 1981, 67:540-546
15. Grega GJ, Kline RL, Dobbins DE, Haddy FJ: Mechanisms of edema formation by histamine administered locally into canine forelimbs. Am J Physiol 1972, 223: 1165-1177
16. Kline RL, Scott JB, Haddy FJ: Mechanisms of edema in canine forelimbs by locally administered bradykinin. Am J Physiol 1973, 225:1051-1059
17. Majno G, Palade GE: Studies on inflammation: I. The effect of histamine and serotonin on vascular permeability: An electron microscopic study. J Biophys Biochem Cytol 1961, 11:571-606
18. Majno G, Shea SM, Leventhal M: Endothelial contraction induced by histamine-type mediators: An electron microscopic study. J Cell Biol 1969, 42:647-672
19. Hammersen F: Endothelial contractility—does it exist? Adv Microcirc 1980, 9:95-134
20. Simionescu N, Heltianu C, Antohe F, Simionescu M: Endothelial cell receptors for histamine. Ann NY Acad Sci 1982, 401:132-149
21. Mustard JF, Packham MA: The reaction of the blood to injury, Inflammation, Immunity and Hypersensitivity. 2nd edition. Edited by HZ Movat. Hagerstown, Md, Harper & Row, 1979, pp 557-664
22. Camussi G, Aglietta M, Malavasi F, Tetta C, Piacibello W, Sanavio F, Bussolino F: The release of platelet-activating factor from human endothelial cells in culture. J Immunol 1983, 131:2397-2403
23. Prescott SM, Zimmerman GA, McIntyre TM: Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) when stimulated with thrombin. Proc Natl Acad Sci USA 1984, 81:3534-3538
24. Humphrey DM, McManus LM, Satouchi K, Hanahan DJ, Pinckard RN: Vasoactive properties of acetyl glyceryl ether phosphorylcholine and analogues. Lab Invest 1982, 46:422-427
25. Humphrey DM, McManus LM, Hanahan DJ, Pinckard RN: Morphologic basis of increased vascular permeability induced by acetyl glyceryl ether phosphorylcholine. Lab Invest 1984, 50:16-25
26. Morley J, Page CP, Paul W: Inflammatory actions of platelet activating factor (PAF-acether) in guinea pig skin. Br J Pharamacol 1983, 80:503-509
27. Harlan JM, Harker LA, Reidy MA, Gajdusek CM, Schwartz SM, Striker GE: Lipopolysaccharide-mediated bovine endothelial cell injury in vitro. Lab Invest 1983, 48:269-274
28. Gimbrone MA: Culture of vascular endothelium. Prog Hemost Thromb 1976, 3:1-28
29. Evensen SA: Injury to cultured endothelial cells. The role of lipoproteins and thrombin-active agents. Haemostasis 1979, 8:203-210
30. Lerner RG, Cheong LC, Nelson JC: Thrombin-induced endothelial cell retraction (Abstr). Thromb Haemost 1979, 42:244
31. Galdal KS, Evensen SA, Nilsen E: Thrombin-induced shape changes of cultured endothelial cells: Metabolic and functional observations. Thromb Res 1983, 32:57-66
32. Pearson JD, Carlton JS, Hutchings A: Prostacyclin release stimulated by thrombin or bradykinin in porcine endothelial cells cultured from aorta and umbilical vein. Thromb Res 1983, 29:115-124
33. Hong SL: Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. Thromb Res 1980, 18:787-795
34. Johnsson AR, Erdös EG: Release of histamine from mast cells by vasoactive peptides. Proc Soc Exp Biol Med 1973, 142:1252-1256
35. Prsad CM, Adamski SW, Svensjo E, Grega GJ: Pharmacological modification of edema produced by combined infusions of prostaglandin E_1 and bradykinin in canine forelimbs. J Pharmacol Exp Ther 1980, 220:293-298
36. Marciniak DL, Dobbins DE, Maciejko JJ, Scott JB, Haddy FJ, Grega Gj: Antagonism of histamine edema formation by catecholamines. Am J Physiol 1978, 234:H180-H185
37. Persson CGA, Erjefalt I, Grega GJ, Svensjo E: The role of β-receptor agonists in the inhibition of pulmonary edema. Ann NY Acad Sci 1982, 384:544-557
38. Brotherton AFA, Hoak JC: Role of Ca^{2+} and cyclic-AMP in the regulation of the production of prostacyclin by the vascular endothelium. Proc Natl Acad Sci USA 1982, 79:495-499
39. Brotherton AFA, MacFarlane DE, Hoak JC: Prostacyclin biosynthesis in vascular endothelium is not inhibited by cyclic-AMP. Studies with 3-isobutyl-l-methylxanthine and forskolin. Thromb Res 1982, 28:637-647
40. Hong SL: Inhibition of prostacyclin synthesis in endothelial cells by methylisobutylxanthine is not mediated through elevated cAMP levels. Biochim Biophys Acta 1983, 754:258-263
41. Taylor RF, Price TH, Schwartz SM: Neutrophil-endothelial cell interactions on endothelial monolayers grown on micropore filters. J Clin Invest 1981, 67:584-587

42. Shasby DM, Shasby SS, Sullivan JM, Peach MJ: Role of endothelial permeability. Circ Res 1982, 51:657–661
43. Shasby DM, Shasby SS, Welsh MJ: Reversible changes in pulmonary endothelial cell cytoskeleton in the control of endothelial permeability: Role of extracellular calcium (Abstr). Fed Proc 1984, 43:513
44. McDonald RI, Shepro, D, Rosenthal M, Booyse FM: Properties of cultured endothelial cells. Ser Haematol 1973, 6:469–478
45. Rafelson ME Jr, Hoveke TP, Booyse FM: The molecular biology of platelet-platelet and platelet-endothelial interactions. Ser Haematol 1973, 6:367–381
46. Meyrick B, Brigham KL: Increased permeability associated with dilatation of endothelial cell junctions caused by histamine in intimal explants from bovine pulmonary artery. Exp Lung Res 1984, 6:11–25
47. Marchesi VT: The passage of colloidal carbon through inflamed endothelium. Proc R Soc Lond [Biol] 1962, 156:550–552
48. Movat HZ, Fernando NVP: Acute inflammation: I. The earliest fine structural changes at the blood-tissue barrier. Lab Invest 1963, 12:895–910

Acknowledgments

The authors wish to thank Dr. O. Casulo and the following staff of Doctor's Hospital, Toronto, for supplying human umbilical cords: L. Sabanal, C. Attard, M. Hodge, N. Lavoie, B. Brabin, M. Quon, C. Bhella, E. Mitchell, E. Comanescu, L. Norona, and M. Piecha; Dr. Herenda, Grace Packers, Toronto for supplying bovine tissue; Mr. M. Walker for technical assistance; Mrs. O. Freitag for preparing the figures, the photographs, and semithin sections; Dr. B. A. Killackey for assistance in preparing this manuscript, and Ms. M. Michael for word processing.