Evidence That Prolonged Histamine Suffusions Produce Transient Increases in Vascular Permeability Subsequent to the Formation of Venular Macromolecular Leakage Sites

Proof of the Majno-Palade Hypothesis

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The aim of this study was to determine whether histamine-stimulated increases in macromolecular efflux are dependent on the formation of specific vascular leakage sites, or whether other mechanisms need to be invoked to explain the increase in macromolecular efflux produced by this inflammatory mediator. Intravital light microscopy was used to localize and quantitate vascular macromolecular leakage sites in the noneverted hamster cheek pouch. Fluorimetric measurements of plasma and suffusate tracer (FITC-D 70,000 mol wt) concentrations were utilized to quantitate changes in macromolecular efflux. In some experiments, the FITC-D was injected intravenously either at the start of or after the start of a prolonged histamine suffusion for estimation of the duration of the vascular FITC-D leakage response. In saline control cheek pouches there were few, if any, visible FITC-D vascular leakage sites and only small increases in the $[FITC\text{-}D]_s$. The arteriolar vasodilators papaverine (1×10^{-5} M) and isoproterenol (1×10^{-5} M) failed to increase the formation of vascular FITC-D leakage sites, and the magnitude of the increase in $[FITC\text{-}D]_s$ produced by these agents was similar to that observed in saline controls. Histamine (1×10^{-5} M) suffused for either 15, 60, or 120 minutes produced marked increases in $[FITC\text{-}D]_s$ and in the number of venular FITC-D leakage sites. The venular FITC-D leakage sites began to fade after 10–20 minutes, eventually disappearing altogether. In contrast, the $[FITC\text{-}D]_s$ was markedly increased throughout the 120-minute observation period. Treatment with papaverine prior to and during the 60-minute histamine suffusion failed to prevent the mediator-stimulated vascular leakage response. In contrast, similar treatment with isoproterenol inhibited the histamine-stimulated increases in $[FITC\text{-}D]_s$ and the formation of venular FITC-D leakage sites. When the tracer was injected intravenously at the start of the 60-minute histamine suffusion (1×10^{-5} M), the $[FITC\text{-}D]_s$ and the number of vascular leakage sites were markedly increased. However, when the tracer was injected intravenously 30 minutes after the start of the 60-minute histamine suffusion, there were only minimal increases in $[FITC\text{-}D]_s$ and the formation of venular leakage sites. These findings suggest that prolonged suffusions of histamine produce transient increases in macromolecular efflux which are dependent on the formation of discrete venular macromolecular leakage sites. If venular leakage was pharmacologically inhibited or if the macromolecular tracer was injected intravenously after the start of a prolonged histamine suffusion following the dissipation of the vascular leakage sites, then histamine failed to promote increases in plasma to suffusate FITC-D efflux. (Am J Pathol 1986, 123:570–576)

INTRAVITAL and electron microscopy have been widely employed for visualizing, localizing, and quantitating leakage of macromolecules along the vascular tree.[1–5] Histamine-type inflammatory mediators are reported to produce transient vascular leakage from postcapillary venules lasting as little as 10–20 minutes despite continued exposure to the agent.[2,6–8] The venular macromolecular leakage sites result from endothelial

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cell separation creating large junctional gaps, which permit a virtually unrestricted passage of macromolecules from plasma to interstitium.[2,6] In contrast, prolonged local infusions of mediators into forelimbs[6,8,9] or hindlimbs[10,11] produce sustained increases in the lymph/plasma total protein ratio (L/P protein ratio). This apparent discrepancy has led some investigators to conclude that inflammatory mediators produce sustained increases in protein efflux, thus dismissing the microscopic observations and leading to the suggestion that mediators may produce sustained increases in macromolecular efflux via stimulation of a vesicular transport mechanism.[12] Others have argued that inflammatory mediators produce marked but transient vascular leakage via the formation of venular gaps and a slow but sustained increase in macromolecular efflux via the stimulation of vesicular transport.[13] Although microscopic techniques have yielded valuable information concerning morphologic aspects of vascular leakage, these methods do not yield quantitative data on the impact of the vascular leaks on the plasma to interstitial fluxes of macromolecules, and may not detect the transport of substances via other mechanisms.

Our understanding of the regulation of vascular permeability and edema formation in inflammation is dependent, in part, on our understanding of the mechanism of inflammatory mediator-stimulated increases in vascular permeability. The controversy over the mechanism and duration of mediator-stimulated increases in vascular permeability is attributable to the failure of previous investigators to simultaneously determine effects on plasma to suffusate tracer efflux and on the formation of vascular leakage sites, a limitation of the vascular labeling techniques. A sustained increase in the L/P protein ratio could reflect either a sustained increase in protein transport or simply a slow drainage of the interstitium, a problem not readily resolved by lymph analysis. These considerations prompted the present study to determine the relationship between mediator-stimulated increases in macromolecular efflux and the formation of vascular leakage sites, the duration of the mediator-stimulated vascular macromolecular leakage response, and whether mediators stimulate increases in macromolecular efflux in the absence of vascular leakage site formation. For this study, an intravital microscopic technique was employed which permitted not only the visualization and localization of sites of vascular leakage but also the quantitation of plasma to suffusate fluxes of macromolecules. The use of this modified cheek pouch preparation permitted the correlation of increases in macromolecular efflux with the formation of specific vascular leakage sites, thus providing an opportunity to test the Majno–Palade hypothesis.[2,7] Histamine was studied as a prototype inflammatory mediator because its effects on vascular leakage and L/P protein ratios have been the most thoroughly investigated and because this mediator has been reported to stimulate vesicular protein transport.[12,13]

Materials and Methods

Male golden hamsters weighing 70–120 g were used in these studies. The animals were anesthetized with pentobarbital sodium (6 mg/100 g body wt) intraperitoneally. Body temperature was maintained at 37 C with the use of a heating pad controlled by a rectal thermistor. After making an incision in the cheek skin (2 cm), we inserted a flat plexiglas spatula into the oral cavity and through the skin incision to exteriorize the cheek pouch. The thick avascular layer was removed, and a chamber (2 sq cm) was placed over the cheek pouch and secured to the spatula. The cheek pouch was continuously bathed with a Tris-buffered solution (suffusion solution or suffusate) which was bubbled with nitrogen to provide a pO_2 in the pool of 15–20 mm Hg. The pH of the suffusate was then adjusted to 7.35 at 37 C. The suffusion system was made of borosilicate glass except for the short plastic tubing (5 cm length, PE 20) connecting the suffusion system to the cheek pouch chamber. The flow rate of the suffusate was kept constant at a rate of 1 ml/min throughout each experiment with the use of a Pharmacia peristaltic P-3 pump. After an equilibration period of 30 minutes, the hamsters were given intravenous injections of FITC–dextran (FITC-D) at a dose of 25 mg/100 g body wt. The molecular weight of the fluorescein dextran was 70,000.

A Wild M5-A stereomicroscope fitted for epifluorescence (incidental light) microscopy was used for intravital examination of the cheek pouch vasculature. The pouch was epiilluminated by a 100-watt mercury lamp. Discrete fluorescent-dextran leakage sites could be visually identified when they approached a certain minimal area (about 100 μ in diameter) and fluorescent intensity, which made it possible to visualize and localize sites of macromolecular extravasation along the vascular tree. The number of leakage sites per 2 sq cm was determined at 5-minute intervals throughout the experiment. The effluent suffusate was continuously collected for determination of the FITC-D concentration, which was measured every 5 minutes with an Aminco-Bowman spectrophotofluorometer. Standard curves were developed from fluorimetric measurements of buffer solutions containing known amounts of FITC-D. All samples were read at an excitation wavelength of 490 nm and emitted light at 520 nm after adjustment of the pH to an optimal range between 7.4 and 8.0 with a suitable buffer. The analysis will accurately detect concentrations as low as 1 ng/ml. The concentration of

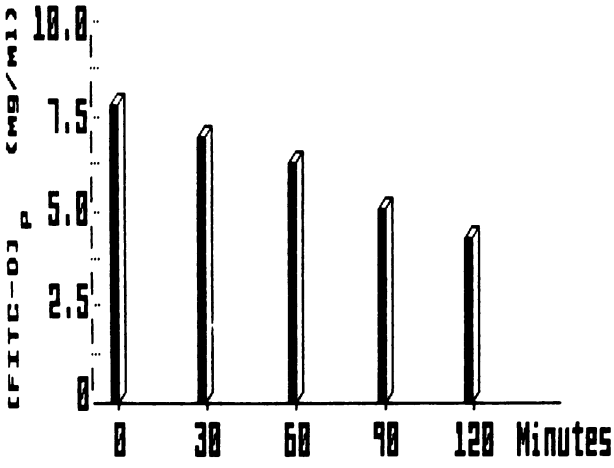

Figure 1—Changes in $[FITC\text{-}D]_P$ after the intravenous injection of FITC-D in control and drug-treated hamsters.

FITC-D in the suffusate ($[FITC\text{-}D]_S$, ng/ml) and in plasma ($[FITC\text{-}D]_P$, mg/ml) was periodically determined throughout each experiment.

Thirty minutes after the intravenous injection of the FITC-D tracer, we suffused the cheek pouch with histamine for either 15 (n = 6), 60 (n = 6), or 120 (n = 6) minutes to produce a histamine concentration of 1×10^{-5} M, and the effects on FITC-D efflux and vascular leakage were determined. Responses to 60-minute suffusions of isoproterenol (1×10^{-5} M, n = 6) and papaverine (1×10^{-5} M, n = 6) were studied in other groups of animals to serve as vasodilator controls for histamine. In another group of animals, the cheek pouch was treated with either isoproterenol (1×10^{-5} M, n = 6) or papaverine (1×10^{-5} M, n = 6) prior to and during the 60-minute suffusion of histamine (1×10^{-5} M). The isoproterenol or papaverine suffusion was started 10 minutes before histamine, and the FITC-D was injected intravenously at the start of the histamine suffusion period. In control animals, the FITC-D was injected intravenously at the start of (n = 6) a 60-minute saline suffusion. In another set of experiments, the FITC-D was injected intravenously either at the start of (n = 6) or 30 minutes after the start of (n = 6) a 60-minute histamine suffusion, which produced a histamine concentration of 1×10^{-5} M.

Results

Effects of Histamine, Isoproterenol, Papaverine, and Saline on $[FITC\text{-}D]_S$ and the Formation of Vascular Leakage Sites

The intravenous injection of the fluorescent dextran tracer produced marked increases in the plasma FITC-D concentration, which then steadily decreased

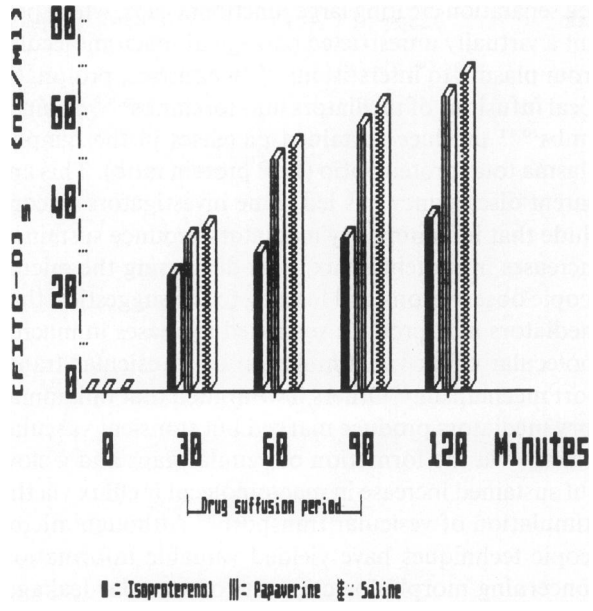

Figure 2—Changes in $[FITC\text{-}D]_S$ in isoproterenol, papaverine, and saline-suffused hamster cheek pouches.

throughout the control and experimental time periods (Figure 1). The suffusate FITC-D concentration increased slightly, essentially approaching a plateau during the 90-minute observation period (Figure 2). There were few, if any, FITC-D vascular leakage sites during the control period, and the increase in $[FITC\text{-}D]_S$ clearly did not correlate with the formation of visible

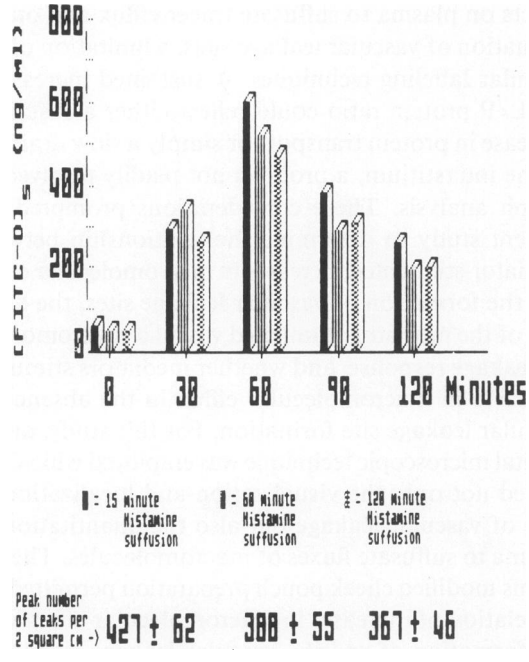

Figure 3—Effects of 15-, 60-, and 120-minute suffusions of histamine on the formation of venular FITC-D leakage sites and $[FITC\text{-}D]_S$ in the hamster cheek pouch.

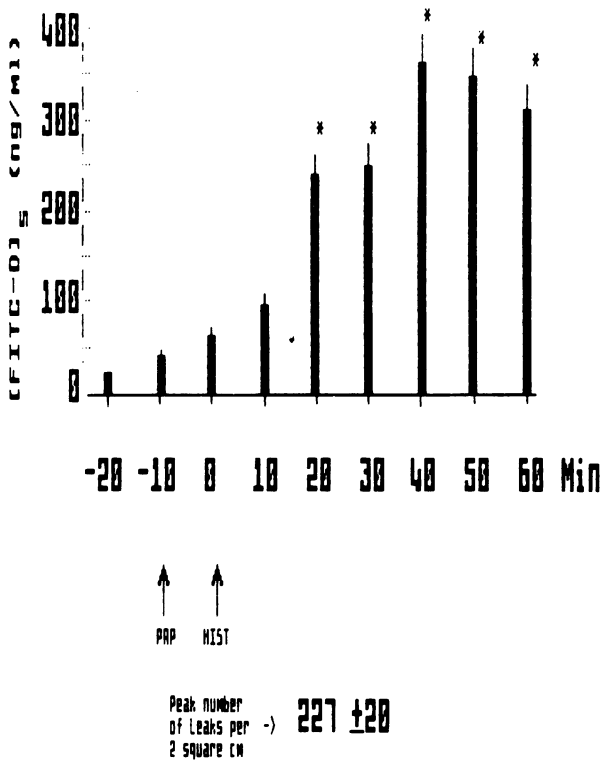

Figure 4—Effects of papaverine on histamine-stimulated increases in the formation of venular FITC-D leakage sites and $[FITC\text{-}D]_s$ in the hamster cheek pouch.

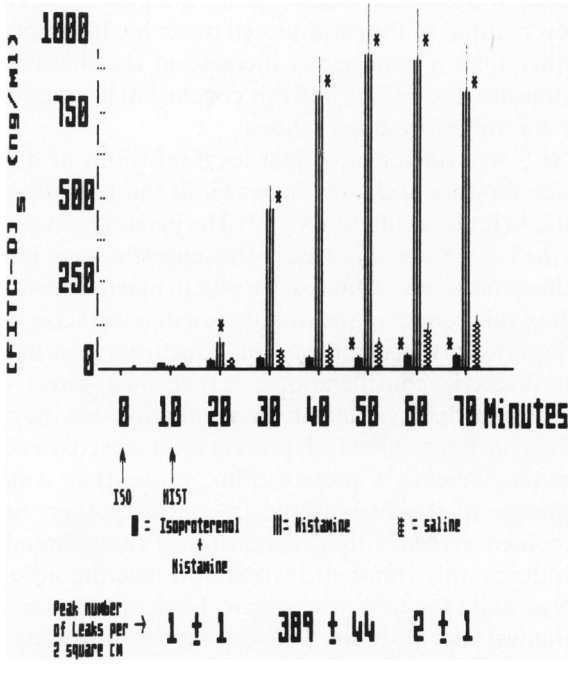

Figure 5—Effects of isoproterenol and histamine, histamine, and saline on the formation of venular FITC-D leakage sites and $[FITC\text{-}D]_s$ in the hamster cheek pouch.

spontaneous FITC-D vascular leakage sites under these conditions.

Fifteen-, 60-, or 120-minute suffusions of histamine (1×10^{-5} M) produced comparable, marked increases in the formation of FITC-D vascular leakage sites and $[FITC\text{-}D]_s$ (Figure 3). The vascular leaks were evident before an increase in $[FITC\text{-}D]_s$ could be detected, and initially appeared as discrete, intensely bright fluorescent spots, permitting the localization of the vascular leakage along the vascular tree. The leakage was restricted to postcapillary venules and was not observed in capillaries or arterioles. After 10–20 minutes, the vascular leaks began to dim and become more diffuse, eventually disappearing altogether. The $[FITC\text{-}D]_s$ increased to peak levels within 30–40 minutes and then slowly decreased with time but remained well above control levels throughout the observation period.

Suffusion of the cheek pouch for 60 minutes with either isoproterenol (1×10^{-5} M, n = 6) or papaverine (1×10^{-5} M, n = 6) failed to promote increases in the formation of vascular FITC-D leakage sites or $[FITC\text{-}D]_s$ relative to that produced in saline controls (n = 12) (Figure 2).

Effect of Isoproterenol and Papaverine on Histamine-Stimulated Increases in Vascular FITC-D Leakage

Treatment of the cheek pouch with papaverine (1×10^{-5} M) prior to and during a 60-minute suffusion of histamine (1×10^{-5} M) failed to prevent the histamine response (Figure 4). In contrast, similar treatment with isoproterenol (1×10^{-5} M) completely inhibited the formation of vascular leakage sites and increase in $[FITC\text{-}D]_s$ produced by histamine (Figure 5). In fact, the peak $[FITC\text{-}D]_s$ during the isoproterenol–histamine suffusion was less than that in saline-treated control cheek pouches.

Effect of Time of Intravenous Tracer Injection on FITC-D Efflux and Vascular Leakage During Prolonged Histamine Suffusions

The intravenous injection of the FITC-D at the start of the 60-minute suffusion of histamine (1×10^{-5} M) revealed in the formation of many vascular FITC-D leakage sites and marked increases in $[FITC\text{-}D]_s$ (Figure 6). In contrast, the intravenous injection of the tracer 30 minutes after the start of the 60-minute suffusion of histamine (1×10^{-5} M) revealed few FITC-D vascular leakage sites and minimal increases in $[FITC\text{-}D]_s$, a response not very different from that in saline-treated control cheek pouches. The FITC-D vascular leaks appeared dim and short-lived, compared with those

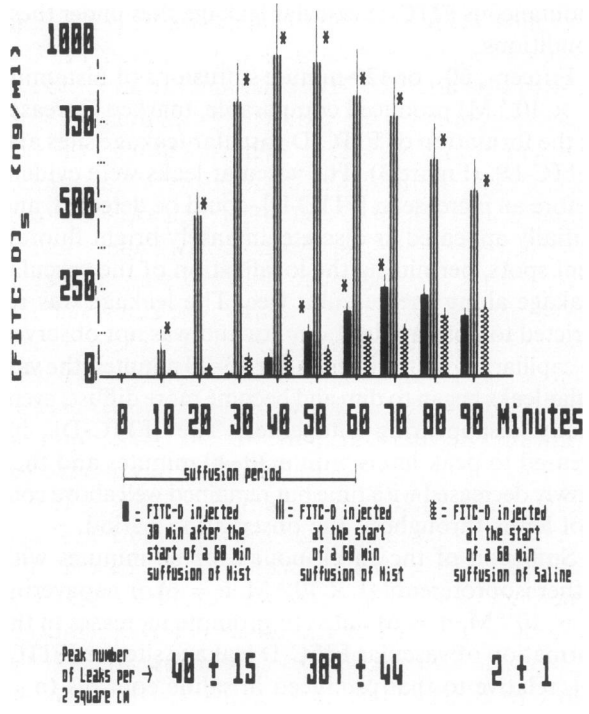

Figure 6—Effects of the intravenous injection of FITC-D at the start of or 30 minutes after the start of a prolonged suffusion of histamine.

produced by the injection of the tracer at the start of the histamine suffusion.

Discussion

In this study a dose of histamine was used which produced marked but submaximal increases in the formation of vascular macromolecular leakage sites in the hamster cheek pouch. Suffusions of histamine (1×10^{-5} M) for either 15, 60, or 120 minutes produced marked, essentially comparable increases in the formation of vascular leakage sites and $[FITC\text{-}D]_s$ (Figure 3). Vascular leakage was observed in postcapillary venules, but not in either arterioles or capillaries. The plasma FITC-D concentration decreased (Figure 1) during the time the suffusate FITC-D increased, and an increased FITC-D suffusate to plasma concentration ratio resulted, evidence for an increased permeability to macromolecules. The vascular leakage sites began to fade and disappear after 10–20 minutes in all animals despite the continued suffusion of histamine. Thus, vascular leakage was transient and independent of the duration of the histamine suffusion. The venular FITC-D leakage sites were evident before an increase in $[FITC\text{-}D]_s$ was detected, which suggests that the increase in FITC-D efflux was dependent on the formation of the vascular leakage sites. The increase in $[FITC\text{-}D]_s$ was largely sustained despite the fact that the vascular leakage sites were rapidly disappearing. These findings suggest two likely interpretations: first, that histamine produces increases in macromolecular efflux, in part, via a second mechanism which is not dependent on the formation of discrete venular leakage sites; second, that histamine-stimulated increases in FITC-D efflux are transient and that a sustained increase in $[FITC\text{-}D]_s$ simply reflects a slow washout of the interstitium.

In order to more precisely determine the period of time that the FITC-D traversed the vascular endothelium, the tracer was injected intravenously either at the start of or 30 minutes after the start of a prolonged histamine suffusion. If the venular leaks were largely closed by this time and if the increase in FITC-D efflux was dependent on the formation of the vascular leaks, then the injection of the tracer 30 minutes after the start of the histamine suffusion would reveal few vascular leaks and minimal increases in $[FITC\text{-}D]_s$. In contrast, the injection of the FITC-D at the start of the histamine suffusion should reveal the formation of venular FITC-D leakage sites and increases in $[FITC\text{-}D]_s$. As illustrated in Figure 6, the intravenous injection of the FITC-D at the start of the histamine suffusion revealed venular FITC-D leaks and increases in $[FITC\text{-}D]_s$. In contrast, the intravenous injection of the FITC-D 30 minutes after the start of the histamine suffusion revealed few venular FITC-D leaks and only minimal increases in $[FITC\text{-}D]_s$, a response not very different from that in saline controls. These data demonstrate that the sustained increase in $[FITC\text{-}D]_s$ produced by prolonged suffusions of histamine reflects the slow removal of the extravasated tracer by the suffusate rather than a continuous increase in the plasma to suffusate tracer efflux, and this conclusion is supported by the following observations.

It is well documented that local infusions of histamine produce sustained increases in the L/P protein ratio in limb vasculature.[6,9,10,12] The persistent increase in the L/P protein ratio led to the suggestion that histamine produces a sustained increase in macromolecular efflux subsequent to the stimulation of a vesicular protein transport mechanism. This conclusion was based on theoretic considerations, rather than direct experimental findings, and on the assumption that the sustained increase in the L/P protein ratio reflected a continuous increase in protein efflux, rather than a slow drainage of the interstitium. In contrast, others have provided evidence that demonstrates that histamine produces only transient increases in macromolecular permeability in limb vasculature. First, short-term (30 minutes) and prolonged (240 minutes) infusions of histamine, an agent which is rapidly metabolized, produce marked, sustained increases in the L/P protein ratio.[6,8] Second, prolonged infusions of histamine pro-

duce only transient increases in capillary filtration coefficient (CFC)[14,15] in maximally vasodilated vascular beds, evidence of a transient increase in vascular permeability. Third, if an independent macromolecular tracer (FITC-D, 70,000 or 150,000 mol wt) is injected intravenously at the start of a histamine infusion, the L/P protein and FITC-D ratios are both markedly increased. In contrast, if the FITC-D is injected intravenously 30 minutes after the start of a prolonged histamine infusion,[6] then only the L/P protein ratio is increased by histamine. The FITC-D L/P ratio is unchanged relative to that in saline-treated control animals under these conditions. These observations are in agreement with the findings from the microscopic studies described in this article and, considered together, support the hypothesis that histamine produces transient increases in macromolecular permeability. The sustained increase in the L/P protein ratio must, therefore, reflect the slow drainage of the swollen interstitium by the lymphatics.

Majno and Palade[2] first demonstrated mediator-stimulated endothelial cell separation, which they attributed to endothelial cell contraction,[7,16] and this conclusion is supported by the findings of others.[17,18] The hypothesis was predicated on the concept that endothelial cells contained contractile elements[19,20] that modulate the dimensions of the junctional gaps of the postcapillary venules. In venular endothelial cells, the contractile elements are localized at cell junctions.[20] Heltianu et al[21] found that histamine receptors are most dense in venular endothelial cells and are strategically localized near endothelial cell junctions in close proximity to contractile elements. Receptors for many mediators and antipermeability agents, including the β-adrenergic stimulants,[22,23] are found in endothelial cells. It is now evident that the increase in vascular permeability produced by inflammatory mediators and their inhibition by antiinflammatory or antipermeability agents are receptor-mediated events.[4,6,8,11,24-28] More recent findings also demonstrate a calcium-dependent link in mediator-stimulated increases in vascular permeability.[24,28-30] However, the studies of Majno and Palade and others do not eliminate the possibility that mediators also produce increases in macromolecular transport via other mechanisms.

The findings from the present study support the Majno–Palade hypothesis. The increase in $[FITC\text{-}D]_s$ was found to be dependent on the formation of specific leakage sites in the postcapillary venules. If the formation of vascular leakage sites was inhibited by treatment with isoproterenol, then histamine failed to produce increases in $[FITC\text{-}D]_s$. If, during a prolonged histamine suffusion, the macromolecular tracer was injected intravenously after the closure of the leakage sites, then histamine failed to produce increases in $[FITC\text{-}D]_s$. Histamine-stimulated formation of vascular FITC-D leakage sites and FITC-D efflux, and the inhibition of these responses by the β-adrenergic stimulants were found to be attributable to a direct action on the vascular endothelium. Other vasodilators with hemodynamic profiles similar to that of histamine failed to produce increases in vascular permeability. Papaverine, an agent with a hemodynamic profile similar to that of isoproterenol, failed to inhibit histamine-stimulated formation of vascular leakage sites and increases in macromolecular efflux. A vesicular transfer mechanism need not be invoked to explain histamine-stimulated increases in macromolecular transport. Vesicles are ubiquitous in vascular endothelial cells. If FITC-D was transported across the vascular endothelium via vesicles, then the extravasation pattern of the tracer would be diffuse, rather than localized, and discrete vascular leakage sites would not be seen. However, an increase in plasma to suffusate FITC-D efflux via vesicles or any other mechanism would be detectable by fluorimetric analysis. We failed to observe increases in $[FITC\text{-}D]_s$ when the FITC-D was injected intravenously 30 minutes after the start of a prolonged histamine suffusion or after treatment with isoproterenol (Figures 5 and 6). We have not, however, ruled out the possibility that vesicles contribute to protein transfer under basal conditions.

The findings of this study also correlate well with findings of studies based on measurements of filtration rates and L/P protein ratios. In the limb vasculature, pretreatment with isoproterenol[6,8,9] prevents the increase in the L/P protein ratio and the formation of edema produced by local infusions of histamine. Histamine produces transient vascular leakage in both limb and cheek pouch vasculature, and the vascular leakage response is subject to inhibition by the same endothelial cell stabilizers in both vascular beds. Moreover, agents that fail to inhibit the histamine-stimulated vascular leakage response in the cheek pouch also fail to prevent the mediator-stimulated increases in the L/P protein ratio in limb vasculature.[6,9] Because the only documented action of the endothelial cell stabilizers affecting mediator-stimulated increases in macromolecular transport is the inhibition of vascular leakage site formation, it is suggested that histamine-stimulated increases in macromolecular efflux in the limb vasculature are attributable to the formation of venular junctional gaps. This markedly increases protein efflux, essentially eliminating the transmural colloid osmotic pressure gradient and thus promoting protein-rich edema formation.[9]

In summary, physiologic, pharmacologic, and morphologic findings are consistent with the hypothesis that the porosity of the vascular endothelium is subject to

direct regulation at the level of the venular endothelial cell, and provide proof of the Majno–Palade hypothesis. The regulation involves receptor activation, calcium, and negative feedback inhibition, which results in a reversible modulation of the dimensions of junctional gaps. Because venular large junctional gap formation is a characteristic feature of the inflammatory process,[31] these findings suggest the possibility of developing a novel class of antiinflammatory agents that act directly on the venular endothelial cell to suppress increases in vascular permeability. The endothelial cell stabilizers inhibit the vascular leakage produced by inflammatory mediators of widely varying chemical composition and may, therefore, have advantages over substances that inhibit synthesis, release, and/or the receptor of a single inflammatory mediator. It remains to be determined whether these mechanisms are operant under normal conditions. However, it has been shown that plasma factors other than proteins and electrolytes are necessary to maintain macromolecular permeability at the normal level.[32,33]

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