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Dimensions of Postcapillary Venules Sensitive to Bradykinin and Histamine-induced Leakage of Macromolecules

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ABSTRACT

Microvascular leakage of macromolecules was studied in the hamster cheek pouch preparation using fluorescein labelled dextran (FITC-dextran 145 $\bar{M}_w$ = 145,000) as a tracer. When the preparation is superfused with 10^{-5} M histamine or 10^{-7} M bradykinin the permeability to macromolecules increases exclusively at postcapillary venules. Microinjections of 30 - 200 picolitres (pl) of 0.1 M histamine and 10^{-4} M bradykinin close to arterioles and capillaries caused extravasation from several postcapillary venules at a distance from site of injection but not from arterioles or capillaries. The minimal diameter of the postcapillary venules where leakage occurred was (n = 45) 8.6 ± 2.6 (S.D.) µm and the maximal diameter was 14.0 ± 5.3 µm. Histamine and bradykinin caused leakage of macromolecules in postcapillary venules but not in arterioles, capillaries or larger venules even when these were exposed to high local concentrations of these agents.

INTRODUCTION

Early observations on permeability properties of the microcirculation demonstrated that the vessels most permeable to vital dyes injected into the circulation were the postcapillary venules (10). Levick & Michel (4) found that capillaries, when perfused with vital dye-albumin-complex with no unbound dye, appeared less permeable when compared to perfusion with vital dye in an unbound form. For the interpretation of observations on microvascular permeability using vital dyes, it is important to know their molecular sizes and the nature of bonds with macromolecules such as albumin and plasma proteins. We have used FITC-dextran in this study for several reasons: it is available with different molecular weights, it can be detected at lower concentrations in the microscope than other vital dyes, and the binding of the fluorescent chromophore to the dextran molecule is stable (1). Furthermore, FITC-dextrans seem to have the same properties as unlabelled dextrans (13) and

they can be used as tracers in electron microscopy (14) which was confirmed for the hamster cheek pouch by Olson et al. (8). The postcapillary venules represent the points at which injury reaction can be shown in most tissues. In their classical electron microscopical study Majno & Palade (5) showed that postcapillary venules of 20 - 30 µm diameter leaked carbon particles through gaps formed between endothelial cells as a result of histamine stimulation. They suggested that this leakage was due to endothelial cell contraction, a hypothesis which was supported by further studies (6,7). In a preliminary study using intravital microscopy we reported that histamine and ADP caused FITC-dextran leakage in postcapillary venules of the hamster cheek pouch without giving actual dimensions of these venules (16). Similar type of leakage was observed with bradykinin (17,18).

The aim of this study was to determine the dimensions of the postcapillary venules sensitive to the action of histamine and bradykinin as studied by intravital microscopy during virtually non-inflammatory conditions before the application of histamine and bradykinin. This study should then provide the basis for an ultrastructural study of the vessels sensitive to histamine and bradykinin. It was also our intention to study if local concentrations of these mediators considerably higher than those effective on topical application to the entire cheek pouch preparation might induce leakage of macromolecules from seemingly non-reactive vessels, e.g. arterioles, capillaries and large venules.

MATERIALS and METHODS

Eight male hamsters weighing 70 - 100 g were prepared for intravital microscopy of macromolecular permeability with FITC-dextran 145 as described by Svensjö et al. (19). Briefly, hamsters were anaesthetized with pentobarbital and supplemented with intravenous injections through a femoral vein catheter (P.E. 10). Indomethacin (2 mg · 100 g^{-1} b.w.) was given intravenously to inhibit endogenous synthesis of prostaglandins. The cheek pouch was gently everted and mounted on a microscopic stage and continuously superfused with a Tris-buffered electrolyte solution as described by Duling (2) which was bubbled with N_2 to give a PO_2 in the pool of 2 - 3.3 kPa (15 - 25 mmHg). pH of the superfusant was adjusted to 7.35 at 37°C. The flow of the superfusion was 5 - 10 ml per min. A single layer preparation was made and FITC-dextran 145 ($\bar{M}_w$ = 145,000, D.S. = 0.002; Pharmacia Fine Chemicals, Uppsala, Sweden), 50 mg · 100 g^{-1} b.w. was given as an i.v. injection of a 50 g/l solution in 9 g/l saline. Observations were made with a Leitz Ortholux Microscope with 3.5 x and 12 x (UM 12) long distance objectives and with 12.5 x oculars. The cheek pouch preparation was scanned at 44 x magnification for FITC-dextran leakage sites at 5, 15 and 30 min following injection of FITC-dextran. Preparations were accepted for use if total number of leakage sites was less

than 5 at 30 min.

A Leitz Micromanipulator was used to place micropipettes for injection of 30 - 200 pl volumes of histamine and bradykinin close to arterioles, capillaries or venules. Micropipettes were made from borosilicate glass tubes, outer diameter = 1 mm and inner diameter = 0.5 mm. A David Kopff Pipette Puller Model 700 C and a standardized procedure with constant setting of pull strength and heating to achieve pipettes of same shape was used. Once pulled, pipettes were treated with Desicote® and completely filled with water saturated Silicon-paraffin oil. Small volumes of bradykinin and histamine solutions (30 - 200 pl) could then be drawn into the pipettes followed by an even smaller, 0.5 pl of oil which sealed the pipette and protected from fluid leakage.

For calibration of pipettes containing fluid to be injected 10 pipettes were filled with volumes of water in the range 30 - 200 pl. The length of water between oil-water interfaces in the pipette tip was measured with a stereomicroscope. The contents of the pipette were then rapidly emptied into a plastic cup filled with water saturated paraffin oil and with its base replaced by a transparent Teflon film. The droplet of fluid assumed an almost perfect spherical conformation and its diameter was then measured with a stereomicroscope and the droplet volume was calculated. This volume was plotted versus the length of pipette containing the fluid and the relation was used for calculation of bradykinin and histamine volumes in the microinjection experiments. Within the volume range used in this study we found an almost linear correlation between estimated volume and actual volume measured by injection of fluid into paraffin oil. Pipettes containing bradykinin (Bachem AG, Bubendorf, Switzerland) 10^{-4} - 10^{-3} M and histamine acid phosphate (PDH Biochemicals Ltd., Poole, England) 0.1 - 1 M were filled immediately before usage.

The following procedure was used to study the effects of local interstitial injection of histamine or bradykinin. An area of the microcirculation devoid of leakage and not used earlier was placed in the optical axis of the microscope. The pipette was placed with its tip in the optical axis, descended and placed with its tip towards the actual vessel to be studied as close as possible or 2 - 5 µm from the vessel wall during observation at 150 x magnification. The pipette was left in this position for a couple of minutes during which time photos were taken in fluorescent and ordinary light, 44 x magnification, as seen in Figs. 1 - 4. If the placing of the pipette did not cause any effect (like altered blood flow, leucocyte stickiness or FITC-dextran leakage) the histamine or bradykinin solution was injected during observation at 150 x magnification in ordinary light. Following most of the successful injections one or several leakage sites were seen within 30 - 60 sec. Further photos were taken at 44 x and 150 x magnification.

The next interstitial injection was made 10 - 15 min later in another area of the preparation which was at least 1 mm apart from the previous injection site. Kodak Panestar 2484 was used and prints were made in the format 9 x 12 cm. A millimeter scale was placed on the microscope stage and a print of this scale was used for calibration. Diameters and distances were then measured in a Zeiss binocular stereomicroscope with oculars for high accuracy. These measurements were always made on "fluorescent" prints (and in most experiments also in ordinary light prints) because these prints showed the vessels much more clearly, as shown in Figs. 1 b and c. Due to light scattering of the vessel wall the estimated diameters from "fluorescent" prints will be larger. Ratio between fluorescent and ordinary light measurements was therefore calculated from prints of the same area with the same focus setting at the two subsequent exposures. The calculated ratio was then used to convert all "fluorescent" values (inner diameters), when these were not available from measurements in ordinary light. The dimensions of leaky venules were measured at two sites: at the edge of the leakage site proximal to the capillary and at the edge distal to the capillary. When several leakage sites appeared as a result of one injection the smallest value of the proximal measurement and the largest measurement of distal measurement was chosen to describe the range of leakiness.

Statistical analysis was done using Student's t-test, $p < 0.05$ being considered significant.

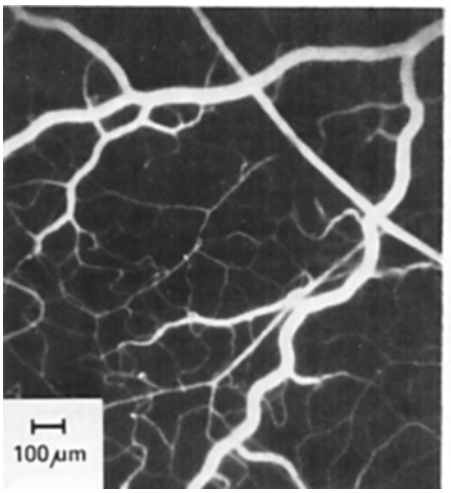
RESULTS

FITC-dextran 145 appeared in the microcirculation within 30 sec after starting the intravenous injection in all cheek pouch preparations and all observable vessels showed fluorescence within 75 sec. The number of spontaneous leakage sites that developed during the first half hour after FITC-dextran 145 injection and before any bradykinin or histamine application were less than 3 per cm^2. These observations were used as qualitative measure to define a stable preparation that was good enough for further experimentation.

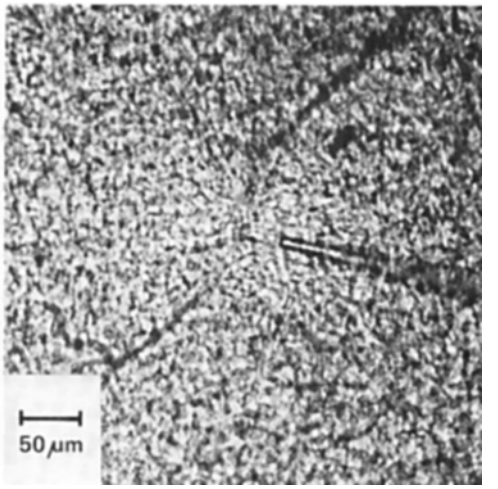
In all, 64 microinjections were made, 30 with histamine and 34 with bradykinin solution in the micropipettes. In 5 of the injections there was no clear indication that fluid came out of pipette. Of the remaining 59, there were 9 successful injections (fluid was seen to come out of pipette) where no leakage was observed. Two of these 9 were at arterioles, 4 at capillaries and 3 at venules with a diameter of 15, 15 and 32 µm. 50 injections resulted in one or several leakage sites at postcapillary venules as shown in Tables 1, 2 and 3 and in Figs. 1 - 4.

A series of 5 - 10 photos were taken of all injections and 45 of these series of prints (~400 prints) of successful injections with subsequent

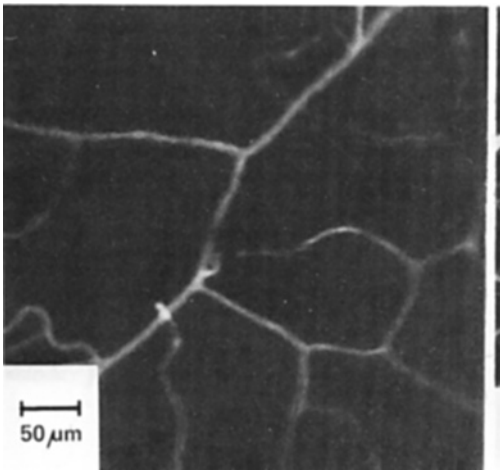
Fig. 1. Injection of 67 pl 0.1 M histamine against arteriolar capillary Ø 4.8 µm.



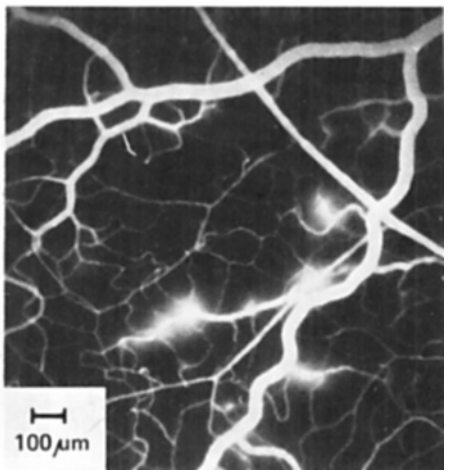
a. Before injection, 55 x, fluorescent light.



b. Before injection. Pipette placed against arteriolar capillary. Ordinary light 190 x.



c. Before injection, same area and focus as b., 190 x fluorescent light.



d. 1 1/2 min after injection, several postcapillary venular leakage sites, 55 x, diameter of leaking vessels 8.8 - 15.2 µm.

leakage were used for the determination of leakage sites and vessel diameters.

A typical series of photos from one microinjection experiment (No. 5) is shown in Fig. 1. Histamine (0.1 M, 67 pl) injection close to an arteriolar capillary produced no leakage at the site of injection but there was leakage in postcapillary venules located 280 - 620 µm away from injection site.

Arteriolar constriction as a result of histamine injection was seen in some experiments as shown in Fig. 2 d. The arteriole feeding the leaking venules was constricted.

Injection of bradykinin close to a postcapillary venule resulted in leakage only around the site of injection (Fig. 3).

When the injection was made against a venule of 17.1 µm, leakage appeared at a postcapillary venule of 12.0 µm diameter and 400 µm apart from the injection site (Fig. 4).

34 photos, 17 in ordinary light and 17 in fluorescent light of the same area with the same focus setting were used to determine the ratio between vessel diameter as measured in fluorescent light and ordinary light (inner diameter). The ratio was 1.347 $\pm$ 0.176 (S.D.).

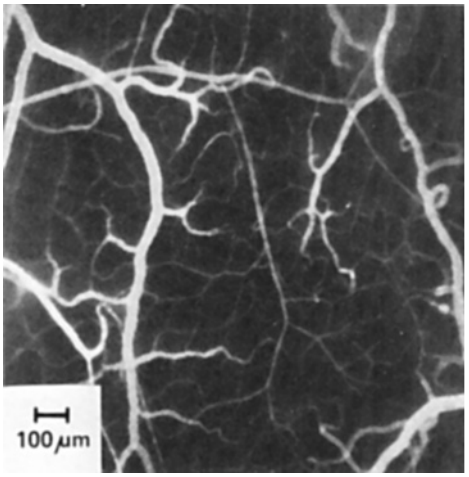
All measurements are given in Tables 1, 2 and 3. Before mean values in these tables were calculated the results of histamine injections were compared with those of bradykinin. This comparison showed that there was no significant difference between the results of these mediators. Therefore all diameter measurements were used for the calculation of mean values regardless of the agent used, histamine or bradykinin.

In 14 out of 18 injections close to venules the leakage was seen at or very close to the pipette. In four injections the diameter at the site of injection was larger than the maximal inner diameter of the leaking venule.

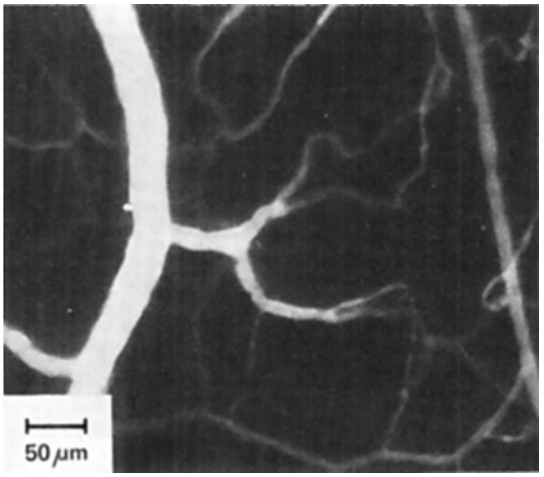
The diameters of leaking venules at the site of leakage and measured proximal to capillaries (minimal leaking diameter) were independent of the site of injection, 8.4 $\pm$ 2.7 (S.D.) µm, 7.9 $\pm$ 2.0 µm and 9.3 $\pm$ 2.8 µm respectively for close to arteriolar, capillary and venular injection as shown in Tables 1, 2 and 3. There was no significant difference between the diameters measured distal to capillaries, 13.1 $\pm$ 3.9 µm, 14.9 $\pm$ 7.8 µm and 13.9 $\pm$ 3.9 µm, respectively.

In order to describe a size range for the postcapillary venules sensitive to histamine and bradykinin all 45 values of minimal and maximal leaking diameter were used. This range was 8.6 ($\pm$ 2.6) - 14.0 ($\pm$ 5.3) µm. The length of the venular area sensitive to histamine and bradykinin varied from 50 to 400 µm.

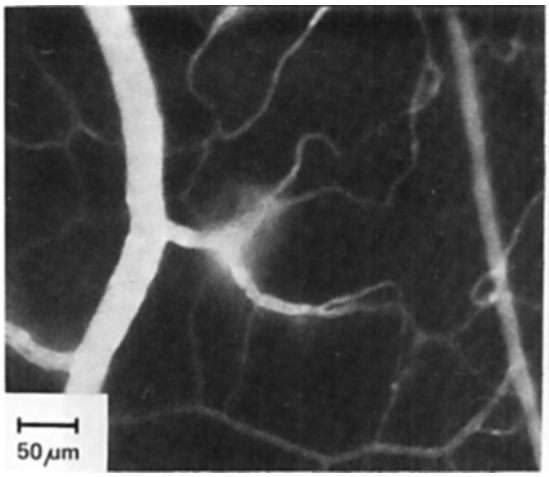
Fig. 2. Injection of 79 pl 0.1 M histamine close to postcapillary venule Ø 9.1 µm.



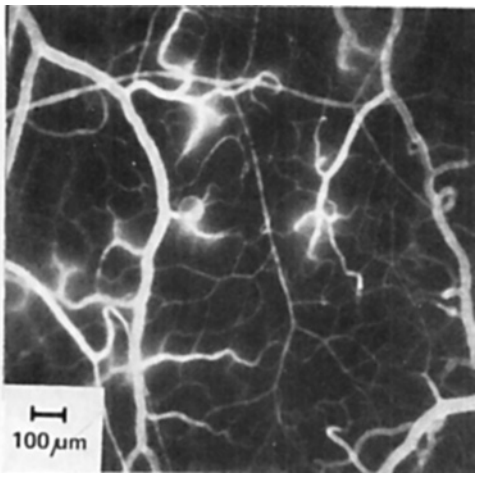
a. Before injection, 55 x. fluorescent light.



b. Before injection, fluorescent light 190 x.

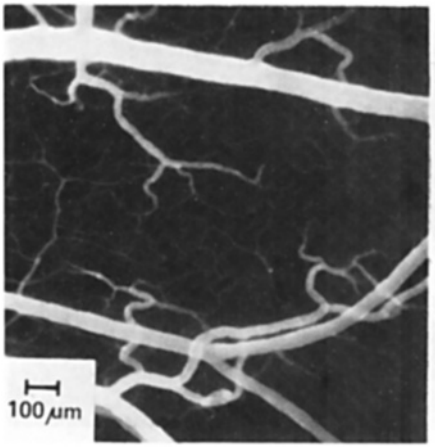


c. 30 - 60 sec after injection 190 x.

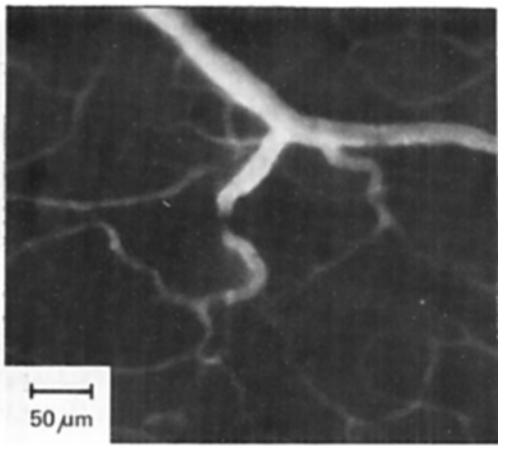


d. 1 1/2 min after injection, 55 x, fluorescent light. Range of leakiness: 6.1 - 10.8 µm.

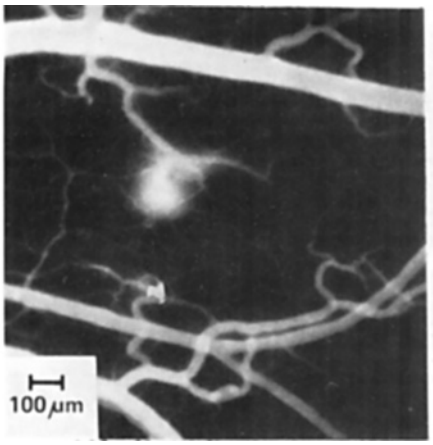
Fig. 3. Injection of 32 pl 10^{-3} M bradykinin against postcapillary venule Ø = 10.2 µm.



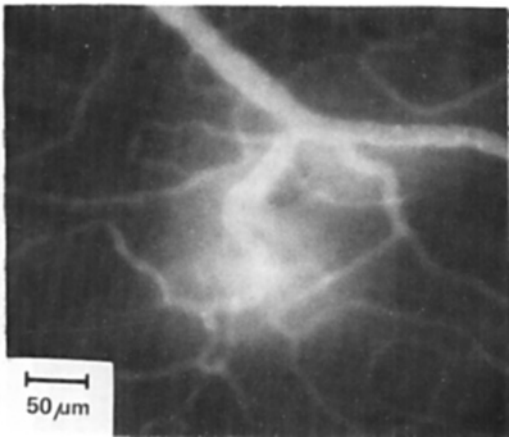
a. Before injection, 55 x, fluorescent light.



b. Before injection, 190 x, fluorescent light, position of pipette indicated.



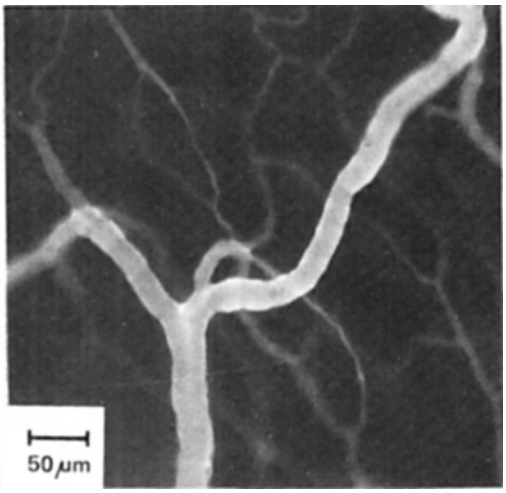
c. 1 min after injection, 55 x, same area as in a. showing leakage at site of injection. Range of leakiness: 10.0 - 14.5 µm.



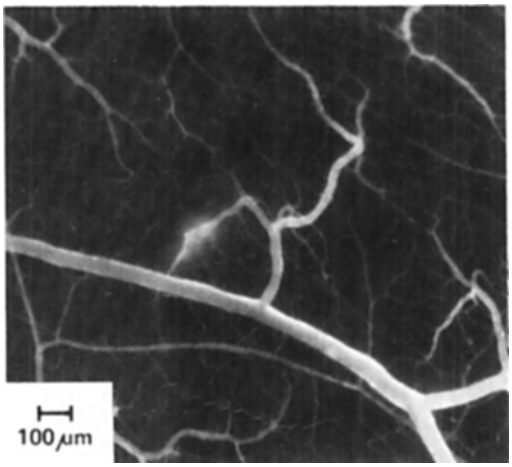
d. 1 45 - 2 30 after injection 190 x, same area as in b.

Fig. 4. Injection of 123 pl 0.1 M histamine against venule Ø = 17.1 µm.

a. Before injection, 190 x, ordinary light, position of pipette indicated by arrow.



b. Before injection, 190 x, fluorescent light, same area and focus as in a.



c. 3 min after injection, 55 x, fluorescent light, leakage at distant (400 µm) post-capillary venule (12.0 µm) but not at site of injection.

TABLE 1. Interstitial injections of bradykinin (Bk) and histamine (H) close to arterioles.

Exp. No.	Mediator and Conc.	Injected volume	Diameter at site of injection	Diameter of leaking venule: Minimal (proximal to capillary)	Diameter of leaking venule: Maximal (distal to capillary)	Distance to leakage site: Closest	Distance to leakage site: Furthermosts	No. of leakage sites
	mol/l	pl	µm	µm	µm	µm	µm	
1	H 1	152	4.9	8.2	14.5	120	1300	8
2	H 1	112	11.1	7.4	12,3	160	160	1
2	H 1	48	10.5	7.1	19,5	60	360	3
2	H 1	180	7.6	6.8	11.4	40	660	10
3	H 1	94	9.4	4.9	11.5	240	360	4
5	H 0.1	118	5.7	13.4	14.3	220	560	4
5	H 0.1	67	4.8	8.8	15.2	280	620	8
5	BK 10^{-4}	63	7.1	3.9	3.9	100	100	1
6	H 0.1	87	6.8	12.5	17.0	160	520	5
7	BK 10^{-3}	97	5.6	10.2	14.5	100	400	3
7	BK 10^{-3}	131	5.4	9.7	15.4	160	300	6
8	BK 10^{-4}	40	5.6	9.6	13.1	90	150	1
8	BK 10^{-4}	90	7.6	6.5	8.3	55	160	2
		n	13	13	13	13	13	13
		$\bar{x}$	7.1	8.4	13.1	137	435	4
		S.D.	2.1	2.7	3.9	75	320	3

TABLE 2. Interstitial injections of bradykinin (Bk) and histamine (H) close to capillaries.

Exp. No.	Mediator and Conc.	Injected volume	Diameter at site of injection	Diameter of leaking venule: Minimal (proximal to capillary)	Diameter of leaking venule: Maximal (distal to capillary)	Distance to leakage site: Closest	Distance to leakage site: Furthermosts	No. of leakage sites
	mol/l	pl	µm	µm	µm	µm	µm	
1	H 1	115	6.5	8.8	39.0	340	1040	4
3	H 0.1	60	–	8.6	9.9	180	200	2
3	H 1	120	7.1	5.5	9.9	80	480	3
3	BK 10^{-4}	134	5.0	5.0	7.6	280	440	5
4	H 0.1	118	3.5	11.1	11.5	260	460	3
4	H 0.1	153	4.3	10.8	14.3	240	500	4
6	H 0.1	44	4.5	7.1	13.5	160	800	8
6	H 0.1	80	3.3	7.0	16.6	140	540	15
6	BK 10^{-4}	44	4.8	–	–	140	140	1
6	BK 10^{-4}	82	4.5	9.5	15.4	220	240	2
6	BK 10^{-4}	92	6.5	5.4	11.1	55	110	2
7	BK 10^{-3}	137	3.9	8.8	16.5	250	290	2
7	BK 10^{-4}	129	5.9	6.1	17.7	290	430	3
8	BK 10^{-4}	77	3.3	8.7	11.3	50	90	1
		n	13	13	13	14	14	14
		$\bar{x}$	4.9	7.9	14.9	192	411	4
		S.D.	1.3	2.0	7.8	91	268	4

TABLE 3 . Interstitial injections of bradykinin (Bk) and histamine (H) close to venules.

Exp. No.	Mediator and Conc.	Injected volume	Diameter at site of injection	Diameter of leaking venule: Minimal (proximal to capillary)	Diameter of leaking venule: Maximal (distal to capillary)	Distance to leakage site: Closest	Distance to leakage site: Furthermosts	No. of leakage sites
	mol/l	pl	µm	µm	µm	µm	µm	
1	H 0.1	169	12.2	9.1	12.2	0	540	2
1	H 0.1	48	8.0	7.8	12.3	0	520	4
2	H 1	103	7.8	7.6	11.5	0	1000	6
3	H 1	190	23.5	10.2	23.5	0	100	2
3	H 0.1	126	31.8	6.2	9.8	200	200	1
3	BK 10^{-4}	87	15.0	15.0	15.0	0	0	1
4	H 0.1	123	17.1	12.0	12.0	400	400	1
4	BK 10^{-4}	140	4.5	4.5	7.2	0	0	1
5	H 0.1	52	11.9	10.5	19.1	0	440	4
5	BK 10^{-4}	77	14.9	7.8	10.8	60	140	2
5	BK 10^{-4}	103	14.7	10.8	14.7	0	160	2
6	H 0.1	60	17.7	8.8	17.0	40	620	5
6	H 0.1	79	9.1	6.1	10.8	0	500	9
6	BK 10^{-4}	112	14.8	13.8	15.0	30	140	2
7	BK 10^{-4}	103	6.2	6.2	10.6	0	0	1
7	BK 10^{-3}	32	10.2	10.0	14.5	100	400	3
7	BK 10^{-3}	70	15.1	7.9	17.7	0	250	4
8	BK 10^{-4}	67	14.8	12.6	16.3	0	90	2
		n	18	18	18	18	18	18
		$\bar{x}$	13.9	9.3	13.9	46	306	3
		S.D.	6.4	2.8	3.9	102	267	2

DISCUSSION

This study shows that the morphological site of macromolecular leakage induced by bradykinin and histamine is quite independent of the site of injection of these agents, e.g. at arterioles, capillaries or venules. All the leakages developed at postcapillary venules within a segment from 8.6 $\pm$ 2.6 µm to 14.0 $\pm$ 5.3 µm inner diameter. Both bradykinin and histamine induced the same type of leakage in venules of the same dimensions but histamine alone caused arteriolar constriction. On a molar basis bradykinin is ~400 times more effective than histamine.

The hamster cheek pouch has virtually no leakage sites immediately after it has been prepared for intravital observations. If the hamster is given indomethacin and the pouch is superfused with a Tris-buffered electrolyte solution there is very little increase in number of leakage sites over several hours (19). Preliminary experiments with iontophoretic application of histamine in micropipettes demonstrated that this technique was unreliable. It was impossible (with the iontophoretic technique) to assess if histamine was actually flowing out of the pipette once it had been inserted in the interstitium. Bradykinin cannot be applied with iontophoresis. We therefore used an injection technique where the mediator solution was "locked" in between two paraffin oil interfaces. In this way it was possible to watch the oil-water-interface in the

pipette under the microscope and to use the oil meniscus as an indicator of the injection. The injected volumes were small (30 - 200 pl) and concentrations could remain high only locally. One hundred pl of 1 M histamine diluted in the superfusion solution covering the pouch would give a final concentration of less than 10^{-10} M, which in our experience is far below permeability increasing concentrations (17, 18). The most distant leakage site was found 1 mm away from the pipette but most leakages were within a shorter distance. Although one cannot be sure that the concentration achieved with agents at the endothelial cell in regions tested is identical, due to the variability in thickness of vessel wall, it was felt that the concentrations used were sufficient in all areas tested to stimulate the endothelial cell.

We think that the major advantages of our technique are: the use of FITC-dextran gives a bright and clear view of the microvasculature; the same preparation can be used for several injections without using one area more than once or an area which might have been exposed to an effective concentration of the tested agent; the spontaneous increase in macromolecular leakage over time is small; the preparation can be continuously watched for any changes in blood flow and leakage.

A successful injection was defined as one in which the pipette was seen to empty. In 50 out of 59 successful interstitial injections of bradykinin and histamine resulted in FITC-dextran leakage. The nine failing attempts (with successful injections) to induce leakage were made at such vessels that may not be reactive. Six injections were close to arterioles or capillaries. Three injections were made close to venules of a size (>15 µm) close to or larger than the maximal mean diameter of leaking venules. Even though leakage in postcapillary venules at distance from capillary or arteriolar injection site was not seen in all experiments, we do not think it interferes with our main conclusion. Only postcapillary venules of a certain size appear to leak. Concentrations of histamine or bradykinin 10^{4}- 10^{5} times higher than those effective in topical applications do not induce observable leakage at arterioles, capillaries or larger venules.

In studies of subdermal microvessels in the rabbit the highest density of pericytes was found around venules of 20 - 30 µm diameter (9). This was also the diameter of the venules found to be most sensitive to histamine (5). Rutili & Hagander (12) studied permeability of subcutaneous tissue in the rabbit with FITC-dextrans of various molecular weights. The evaluation of macromolecular transport indicated that the postcapillary venule has the highest permeability to macromolecules. Studies on the permeability of various FITC-dextrans in the subcutaneous tissue of the rabbit (12) were in good agreement with those performed in the hamster cheek pouch (11). Both studies were concerned with tissue which was not stimulated by any pharmacological agent. The

suggestion could be made that the number of pericytes determines the degree of sensitivity of each segment of the capillary and venular beds. That is, the more numerous the pericytes, the more sensitive is the microvessel to connective tissue released substances like serotonin and histamine as the pericytes may alert the endothelium to the action of these substances (9).

Simionescu et al. (15) have shown that ~25 % of the intercellular junctions of the venular endothelium (pericytic venule) were open and these gaps of 30 - 60 Å appeared more rapidly permeated by the tracer macromolecule, ($\bar{M}_w$ = 1,900, mol.diam. ~20 Å). They described intracytoplasmic fine filaments in the endothelial cells of the pericytic venule. However, the possible contraction of these filaments was not discussed. The size of these pericytic venules was given as inner diameters with a range 8 - 16 µm. This result is consistent to the range of diameters of leaking venules we have found, 8.6 - 14.0 µm. The intercellular junctions of the venular endothelium are known to be susceptible to chemical mediators of inflammation like histamine and serotonin (5) and bradykinin (3). In other studies we have provided indirect evidence for endothelial cell contraction as the mechanism for leakage induced by bradykinin (18). In that study bradykinin was shown to dilate arterioles of the hamster cheek pouch. In the present study we observed arteriolar constriction with histamine and at the same time FITC-dextran leakage. Thus, from our experiments it seemed reasonable to conclude that the leakage we observed had little to do with changes in blood flow or pressure.

It remains to be studied if the physiological patency of the junctions in pericytic venules of mouse diaphragm (15) and our findings of macromolecular leakage in cheek pouch venules is a reflection of a similar morphology of the postcapillary or pericytic venules in these two animal species.

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