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The Hamster Cheek Pouch Preparation as a Model for Studies of Macromolecular Permeability of the Microvasculature

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ABSTRACT

A method for intravital microscopy studies of macromolecular permeability is described. The cheek pouch of an anaesthetized hamster was everted and a single layer preparation was mounted on a microscope stage and dissected during observation with a stereomicroscope at 10–16 times magnification. The cheek pouch preparation was superfused with a buffered electrolyte solution with pH=7.35 at 37°C and with a PO_2<4kPa (<30 mmHg). Fluorescein labelled dextran $\bar{M}_w$=145 000 (FITC-dextran 145) was used as a tracer for permeability.

Sites of microvascular permeability to macromolecules were indicated by extravasation of FITC-dextran 145. Leakage of this tracer from the microcirculation was found to occur at the postcapillary venules only. The number of FITC-dextran leakage sites was used for quantitation of the permeability during intravital observations in untreated control and indomethacin-treated hamsters. The number of leakage sites was significantly increased at 120 min in control hamsters but not until 300 min in the indomethacin-treated hamsters. There was a significant difference in permeability at 120 min in control hamsters as compared to indomethacin-treated. Analysis of PGE_2-activity in pooled samples of exposed cheek pouches at 5 hours showed 20 times higher activity in these pouches when compared to the unexposed pouches. Indomethacin was found to inhibit PGE_2-synthesis and to reduce the number of leaking postcapillary venules. The model allows studies on the dynamics of macromolecular transport in the microvasculature and its relation to other vascular parameters such as vessel diameter and blood flow. It should also be useful for studies of inflammatory and immunological responses in the microcirculation and the pharmacology of small vessel permeability to macromolecules.

INTRODUCTION

Permeability of microvessels has often been evaluated from observations of the extravasation of intravascularly injected dyes such as Patent Blue V (19), Evans Blue (T-1824), pontamine sky blue (17), trypan blue, sodium fluorescein (12). These dyes become bound to circulating plasma proteins, particularly the albumins and are therefore assumed to trace the movement of macromolecules across the vascular wall. Since the degree of binding is not known precisely, the reliability of these studies has been questioned (15). For the purposes of studies of macromolecular permeability it is therefore a prerequisite to have a tracer with well defined molecular weight and with a label of high chemical stability. Fluorescein labelled dextrans (FITC-dextrans) are according to de Belder & Granath (6) stable and possess biological properties similar to ordinary dextrans (20, 22).

The use of FITC-dextrans for direct observations under fluorescent microscopy in a transparent tissue such as the rabbit ear chamber and rabbit mesentery has already been described by Jonsson et al. (14) and Arfors et al. (1) as well as in preliminary reports for the cheek pouch (24, 25). The results obtained have shown that FITC-dextran is a suitable tracer for permeability studies.

The purpose of this study was to assess an experimental model in which quantitative studies of macromolecular permeability could be made by direct intravital observations of extravasated FITC-dextrans. The advantage of such a model would be that simultaneous light and fluorescent light microscopic observations could be performed with instantaneous correlation between the site of FITC-dextran extravasation and the vessel morphology. Such a correlation will be of relevance for a better understanding of the mechanisms and of the morphological structure involved in the transport of solute from blood to the tissue.

Rous et al. (19) described what they called a "gradient of permeability along the capillaries" suggesting higher permeability on the venous side of the capillary. This was later confirmed by Zweifach

(29). Majno & Palade (16), using small particles as tracers, showed a gradient of permeability in electron microscopic studies. The greatest permeability was found in venules with a diameter of 20–30 μm. However, the precise mechanism by which these molecules leave the circulating blood is still not clear.

This paper describes a method for in vivo study of macromolecular permeability in which the number of FITC-dextran leakage sites in postcapillary venules is used for quantitation. The paper also describes how indomethacin inhibition of the endogenous synthesis of prostaglandins affects the leakage of FITC-dextran 145 from the circulating blood to the interstitium of the cheek pouch.

MATERIALS AND METHODS

FITC-dextran 145, $\bar{M}_w$=145 000 (Pharmacia Fine Chemicals, Uppsala, Sweden) with a degree of substitution of 2 FITC-molecules per 1 000 glucose molecules in the polysaccharide chain was used in this study. FITC-dextran 145 was given in a dose of 50 mg per 100 g b.w. as an intravenous injection of a 5% solution in 0.9% saline over a period of 20–30 min, at the end of the preparation procedure. Indomethacin (Merck, Sharp & Dohme, Rahway, N.J., USA) was used for the inhibition of prostaglandin synthesis in some hamsters. 20 mg of indomethacin was dissolved in 0.5 ml of ethanol and 1.5 ml of a sodium phosphate buffer, 0.154 M, pH=7.4, and given as an intravenous injection in a dose of 2 mg per 100 g b.w.

Male golden hamsters of one breed weighing 70–120 g were used. Pentobarbital (Nembutal, 60 mg per ml, Abbott, USA) was given in a dose of 6 mg per 100 g b.w. intraperitoneally and anaesthesia supplemented with intravenous injections of pentobarbital (30 mg/ml) through a femoral vein catheter (P.E. 10). A tracheal cannula (P.E. 160) was inserted. Body temperature was maintained at 37°C by a heating pad controlled by a rectal thermistor. The hamster was placed on a microscopic stage similar to that of Duling (9) with minor modifications. The cheek pouch was gently everted and mounted with 4–5 needles pinned into a circular well filled with silicone rubber to give a plane bottom layer, avoiding stretching of the tissue but preventing shrinkage. In this position the pouch was submerged in a superfusion solution which continuously flushed the pool of the microscope stage. Before the pouch was pinned, large arterioles and venules were located with the aid of a Zeiss binocular stereomicroscope.

Fashioning of single layer preparation started with incision of the upper layer to swing a triangular flap to one side. The exposed area was dissected at 10–16 times magnification under the stereomicroscope, the fibrous almost avascular connective tissue covering the vessels being removed with ophthalmic surgical instruments. Mounting and dissecting the pouch took 20–30 min. The dissected part of the pouch was 125–150 μm thick. Dissected pouches with petechial formation or those without blood flow in all vessels were discarded.

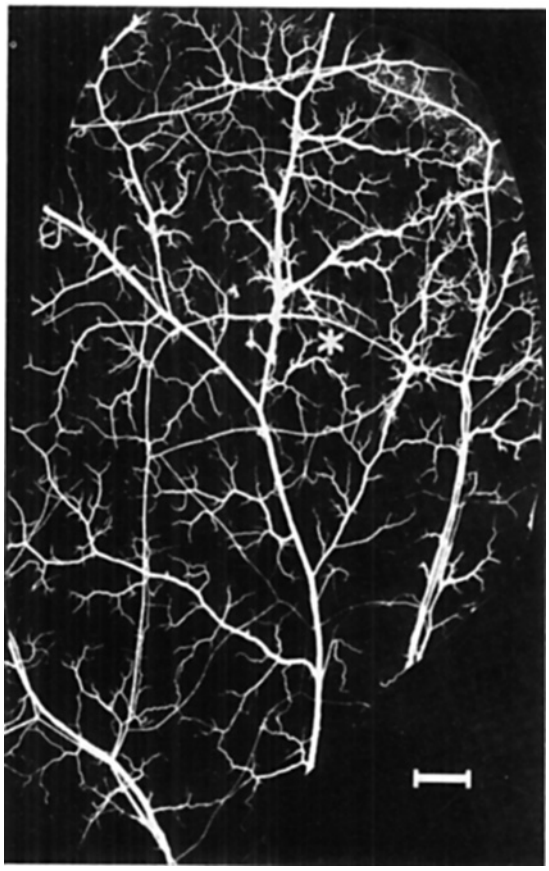

Fig. 1. Overview of hamster cheek pouch preparation made from 70 single photographs as shown in Fig. 2 which were mounted and reproduced in reduced scale. One mm is indicated in the figure. Area of Fig. 2 is outlined.

The superfusion solution was a Tris-buffer solution as described by Duling (9) 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 superfusion system was all made of borosilicate glass except a 5 cm length of plastic tubing connecting the superfusion system with the cannula supplying the pool on the stage. Superfusion solutions were prepared freshly each day by diluting sterile concentrated stock solutions with sterile, pyrogen-free distilled water. The temperature of the superfusion solution was controlled from a thermistor in the pool close to the cheek pouch and kept at 37°C.

Observations were made with a Leitz Ortholux Microscope with 3.5× and 12× (UM 20) long distance objectives or a 23× water immersion objective, and with 10× oculars. The light source was a 100 W Hg D.C. lamp (Irem Model El XH5 P/L). The specific light filters used for observations in fluorescent light (Leitz B.G. 12, B.G. 38, G.G. 455, and K.P. 490) were positioned between the light source and the condensor to give a light for optimal

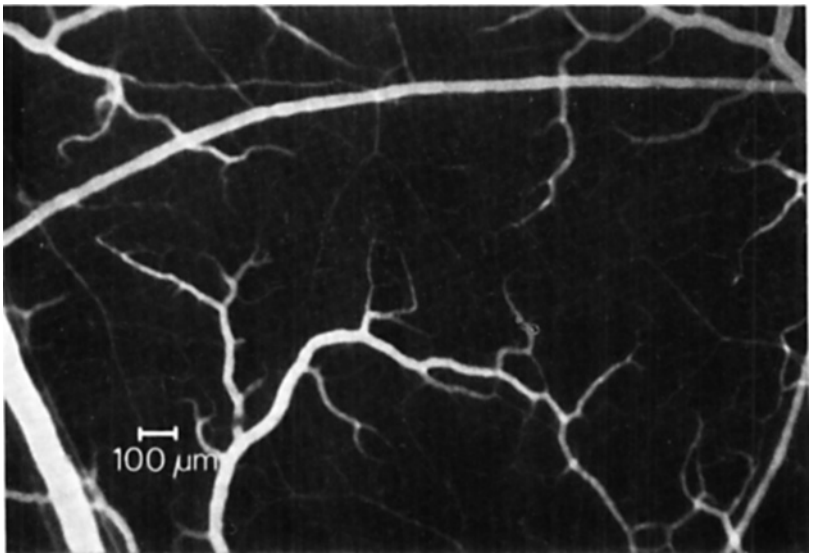

Fig. 2. Detail of Fig. 1 taken at 35× magnification. 100 µm is indicated.

excitation at 490 nm of the FITC-dextran. A barrier filter (K 530) was placed between the objective and the eye pieces. A Leitz Orthomat automatic camera and Kodak Panestar 2484 were used for photographic recording. Exposure times at 35× magnification were 40–60 sec for fluorescent light photographs.

The total observed area of the part of the pouch was roughly circular, and its area was estimated from the mean of two diameters—proximal to distal and left to right side—measured with a calibrated scale at 35× magnification. Another part of the pouch, the central area representing approximately 10% of the total observation area, was chosen for comparison with the total observation area. The size of this central area was estimated using a graticule in the eye piece of the microscope. Observations on the number of leakage sites were made by scanning the total observation area twice at 35× magnification at half-hourly intervals. The fluorescent spots formed at leakage sites could be traced when they reached a certain minimal size and fluorescent intensity. Each was classified as a leakage site when its diameter was larger than 100 µm. A leakage site is shown in Fig. 3*c*.

Fifteen male hamsters were anaesthetized and prepared for microscopic observations as described above and observed for 5 hours. Ten hamsters were used as controls and 5 hamsters were given an intravenous injection of indomethacin (Merck, Sharp & Dohme, Rahway, N.J., USA) 2 mg per 100 g b.w.

At the end of the 5-hour observation period the observed and non-observed non-muscular parts of the pouches were separated from the body by rapid transection and immediately frozen in an ethanol-dry ice mixture and stored at −60°C until extracts were prepared. Prostaglandin activity was assayed by a technique similar to that one used for assay of the acidic lipid extracts of burn blister fluid (5). Extraction was made from pooled samples of 8 control and 5 indomethacin-treated hamsters. Smooth muscle activity of these extracts was determined on rat fundus strips using PGE_2 as a reference.

RESULTS

In all the cheek pouch preparations, FITC-dextran appeared in the microcirculation within 30 sec of the start of injection, indicating adequate perfusion of the cheek pouch. In all hamsters FITC-dextran leakage sites appeared as fluorescent spots in the interstitium surrounding the vessels at some time during the observation period of 5 hours. All leakage sites in controls as well as indomethacin-treated animals, were at postcapillary venules. A typical leakage site is shown in ordinary light in Fig. 3*a* and in fluorescent light before the leakage has been recognized in Fig. 3*b*. In Fig. 3*c* extravasated FITC-dextran can be seen.

One additional indomethacin-treated hamster was used to map the whole observation area following injection of FITC-dextran. Altogether 70 photographs were taken, mounted and reproduced in reduced scale as shown in Fig. 1. One of the 70 prints from the central part of the area is shown in Fig. 2. The total exposure time for all these photographs was more than one hour and therefore such a procedure cannot be used for studying rapid changes of permeability.

48 photos with the same magnification as in Fig. 2 from 7 hamsters in another study (27) were used for an estimation of the number of postcapillary venules (in the range 8–20 µm) per unit area of the cheek pouch preparation. There were from 480 to 830 postcapillary venules per cm^2 with a mean value of 680±180 (S.D.) (Table I).

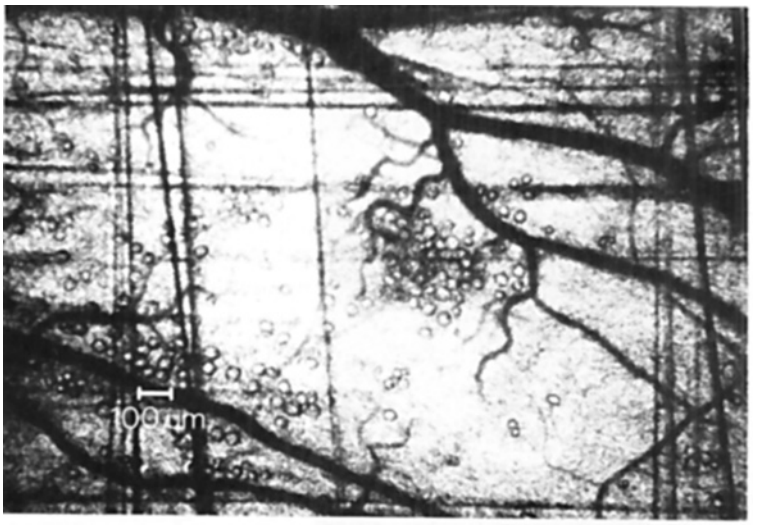

Fig. 3 a. Photograph in ordinary light at 35× magnification showing the normal character of the vasculature and a group of fat cells.

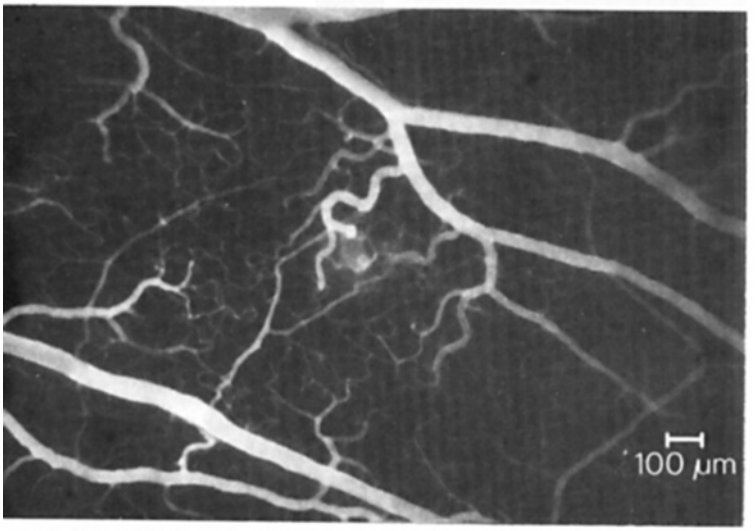

Fig. 3 b. Same area as in Fig. 3*a* in fluorescent light showing microvasculature in more detail than in ordinary light. No extravasation of FITC-dextran.

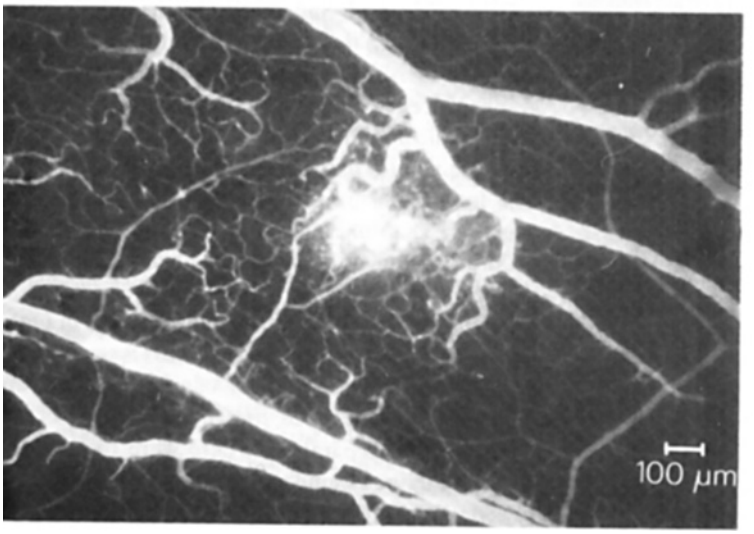

Fig. 3 c. Same area as in Fig. 3*b* 75 min later showing extravasation of FITC-dextran (=leakage site).

Table I. *Number of postcapillary venules with diameters in the range 8–20 μm as estimated from 48 photographs in 7 hamsters*

Hamsters	Number of photographs	Mean value and S.D. of number of postcapillary venules per cm²
1	4	480±120
2	6	770±170
3	6	580± 90
4	4	610±140
5	9	670± 90
6	9	680±140
7	10	830±220
	48	680±180

Fig. 4 shows the results of counts of the numbers of leakage sites at half hourly intervals for 5 hours in the control and indomethacin-treated hamsters. All control hamsters showed an increase in the number of leakage sites during the experiment, although this increase appeared late in two hamsters. Single leakage sites were identified up to 240 min after injection of FITC-dextran when in some occasions they started to fuse. This confluence of fluorescent spots of FITC-dextran was even more apparent at 270 min so mean values of observations are given only up to 210 min. The increase in the mean numbers of leakage sites from 90 min (5.0±4.3 per cm²) to 120 min (12.1±8.8 per cm²) in control hamsters was significant as shown by a *t*-test for paired observations ($t=2.358$, D.F.=8 and $P<0.05$).

In the indomethacin-treated group a significant difference was not seen between two consecutive mean values until 300 min of observation. A *t*-test for the difference between paired observations at 270 and 300 min gave a *t*-value of 3.146 (D.F.=4 and $P<0.05$). There was no confluence of leakage sites in the indomethacin-treated hamsters and it was possible to count all leakage sites at every half hourly interval. The S.D. of mean results was smaller compared to the control group.

Mean values of the number of leakage sites for the two groups have been compared with a *t*-test in Table II. There was a significant difference between the two groups at 120 min which became more pronounced by the time up to 210 min.

A comparison of the results from counting FITC-dextran leakage sites in the total observation area and the central area showed no significant difference in either of the two series so only total observation area data have been described.

It was a common finding that those leakage sites which appeared with the most intensive fluorescence were those localised to groups of fat cells, as shown in Fig. 3*c*. Fig. 3*a* shows a group of fat cells in ordinary light and Fig. 3*b* the same area in fluorescent light. Figs. 3*a* and *b* were taken at 75 min following injection of FITC-dextran when no leakage was observed and Fig. 3*c* at 150 min when it can be observed.

Table III shows the smooth muscle stimulating activity expressed as ng PGE_2 per g wet tissue of dissected left pouches and undissected right pouches. This activity was about 20 times higher in the pooled samples of the pouches observed in microscope for 5 hours as compared to the contralateral pouches which were untouched until the time of sampling. There was no detectable activity in any of the pooled samples of indomethacin-treated hamsters.

DISCUSSION

The aim was to study whether the hamster cheek pouch preparation could be used as a model for detailed permeability experiments such as localization of macromolecular leakage sites, definition of the type and size of leaking vessels, investigation of the effects of repeated applications of mediators

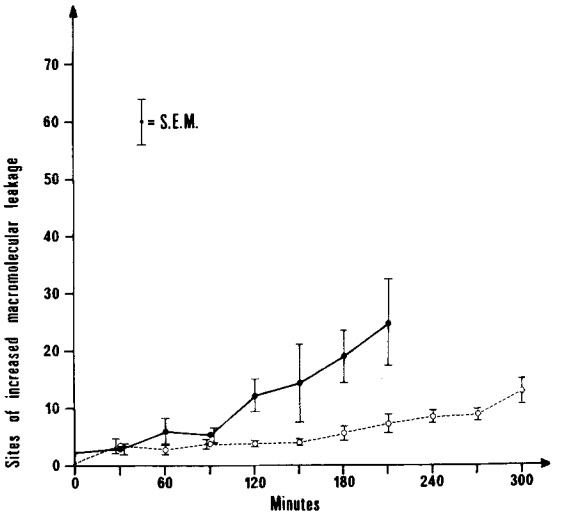

Fig. 4. Mean number of sites per cm² (±S.E.M.) with leakage of FITC-dextran 145 in cheek pouch preparations. The results from 10 control hamsters are indicated by the solid line. The dashed line represents the results from 5 indomethacin-treated hamsters.

Table II. *t-test for difference between mean number of sites of increased leakage of FITC-dextran 145 per cm² in whole observation area of cheek pouch preparation in untreated (controls) and indomethacin-treated hamsters*

	Minutes of observation							
	0	30	60	90	120	150	180	210
Control								
n	10	10	10	10	10	10	10	10
$\bar{x}$	2.2	2.8	5.8	5.0	12.1	14.1	18.9	24.8
S.D.	3.0	2.2	6.8	4.3	8.8	13.6	14.6	24.0
Indomethacin								
n	5	5	5	5	5	5	5	5
$\bar{x}$	0.4	3.3	2.6	3.6	3.6	3.9	5.4	7.1
S.D.	0.8	2.0	1.5	1.7	0.6	1.2	3.1	4.1
t	1.760	0.389	1.385	0.900	3.017	2.351	2.793	2.274
P	0.104	0.703	0.193	0.383	0.014	0.043	0.019	0.046

and inhibitors of increased vascular permeability (vasoactive amines, kinins and prostaglandins), and the effects of stasis to the microvasculature described in preliminary reports (24, 25, 26).

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 other plasma proteins. Levick & Michel (15) found that capillaries, when perfused with solutions containing Evans Blue-albumin-complex with no unbound dye, appeared less permeable when compared to perfusion with Evans Blue in an unbound form. We have chosen FITC-dextran as a tracer for several reasons; it is available with varying molecular weights; it can be detected at lower concentrations in the microscope than other vital dyes; properties of dextrans as permeability tracers are known (3, 10, 11); the binding of the fluorescent chromophore to the dextran molecule is stable (6); FITC-dextrans seem to have the same properties as unlabelled dextrans (22); there is no plasma protein binding of FITC-dextran (20).

The constant flux of superfusion solution will continuously remove water soluble compounds such as FITC-dextran from tissues, but we were still able to localize extravasation from areas of the microcirculation. These macromolecular leakage sites were at postcapillary venules. We have used the number of such leaking sites per unit area as a measure of macromolecular permeability although we are aware that the human eye cannot recognize all leakage sites and FITC-dextran may be removed before its concentration has reached levels high enough for recognition.

In pilot experiments prior to the present study of 15 hamsters we occasionally observed a rapid increase in the number of FITC-dextran leakage sites all over the preparation. This increase did not appear to be correlated to the physiological conditions of the preparations as judged from factors such as deterioration of blood flow or leucocyte margination. However, it was found that by using sterile superfusion solution and by careful cleaning of the superfusion system and all surgical instruments we managed to avoid these rapid increases although we cannot offer an explanation. Even with these precautions we observed in pilot experiments that the number of FITC-dextran leakage sites increased

Table III. *Smooth muscle stimulating activity in the cheek pouches on untreated control and indomethacin treated hamsters*

Pooled samples of dissected left pouches and undissected right pouches after 5 hours of microscopic observation of left pouches

No. of hamsters	Wet weight of pooled samples in g	Smooth muscle stimulating activity (ng PGE_2/g tissue)
8 control, right	1.62	1.3
8 control, left	1.44	25.7
5 indomethacin, right	0.69	0
5 indomethacin, left	1.10	0

with time although few such sites were seen initially following the injection of FITC-dextran when the preparation was ready for microscopic observations. We, therefore, interpreted these leakages as manifestations of a delayed inflammatory response to the dissection and superfusion of the cheek pouch. Whether as mediators or modulators prostaglandins appear to play a central role in this response, they may provide balance in the inflammatory reaction in that they appear able to both mediate and suppress the inflammatory response (28). To minimize the influence of endogenous prostaglandins we used a dose of indomethacin which has been found to inhibit synthesis of prostaglandins in burn injured tissue of dogs (4) and in the rabbit mesentery (1).

Our studies have revealed a clear difference between the untreated control and the indomethacin-treated group in the mean number of leakage sites and also in standard deviation which was smaller in the latter group.

The difference in permeability suggested that inhibition of prostaglandin synthesis reduced the effects of the inflammatory stimulus most probably provided by the continuous superfusion resulting in a change of tissue composition. This suggestion is supported by our findings in the experiments in which paraffin oil was used instead of electrolyte buffer superfusion demonstrating insignificant difference in FITC-dextran 145 permeability between the untreated and the indomethacin-treated group and no such difference in PGE_2-activity between observed and non-observed cheek pouch samples as found in the present study (27). Although the increase in the number of leakage sites became significant at 120 min in the control and at 300 min in the other group of the present study the magnitude of this increase was small as compared to the number of available or potential leakage sites, e.g. postcapillary venules. The estimated number ranged between 500 to 800 per cm^2 which means that less than 5% of such postcapillary venules were actually observed to leak at maximal increase in permeability. This should not be interpreted as FITC-dextran 145 only permeated the vessel wall at sites which were possible to identify in the microscope as we have described that FITC-dextran in the interstitium can be detected with a photomultiplier on the same microscope before it was actually observed (21).

Leakage sites were, without exception, localised around postcapillary venules. It was a frequent observation that the most intense fluorescence from a leakage site during the first hours was seen at congregation of fat cells as shown in Fig. 3*c*. This was more obvious in the indomethacin group where almost all leakage sites were found at groups of fat cells. Already Rous et al. (19) found a gradient of capillary permeability and this was later confirmed by Zweifach (29). Majno & Palade (16) found by electron microscopy that histamine induced extravasation of carbon particles appeared in the postcapillary venules with a diameter of 20–30 μm. More recently Simionescu et al. (23) have found that the endothelial cell junctions in the postcapillary venules have such different properties that it might explain a higher permeability in this area of the vascular tree. The morphology of leakage sites by using simultaneous fluorescence and electron microscopy has been described in preliminary reports (13, 18).

Bioassay of pooled samples of cheek pouches showed that indomethacin 2 mg per 100 g b.w. totally inhibited PGE_2-activity. In untreated control animals the PGE_2-activity of the untouched cheek pouches was low (1.3 ng/g tissue) but in dissected and superfused pouches it was 20 times higher (25.7 ng/g tissue). The method for bioassay of PGE_2-activity was not sensitive enough to measure the activity of single cheek pouch samples. For this reason several samples were pooled to constitute sufficient tissue for analysis. It was, thus, impossible to correlate individual permeability changes with PGE_2-activity but we suggest that higher activity in the superfused pouches indicated an inflammatory process causing increased permeability. These results are supported by other studies of FITC-dextran 150 permeability in the rabbit mesentery (1) in which it was also found that indomethacin counteracted FITC-dextran leakage significantly. It should also be noted that even in the indomethacin-treated group there was a significant increase in permeability at the end of the observation period although there was no detectable level of PGE_2-activity.

An intravenous injection of dextran—depending on the dose and speed of injection—increases plasma volume and results in effects on the coagulation and fibrinolytic systems including a reduction in platelet activity as reviewed by Bygdeman (8). Berman & Fulton (7) showed an antithrombotic effect of dextran in the hamster, a finding which was

later confirmed by Arfors et al. (2) in the cheek pouch in ADP-induced thrombus formation. The dose of FITC-dextran in our study was adjusted to give a good optical resolution in fluorescent light without exceeding the plasma level of dextran at which such effects become significant.

In conclusion: We have described a model for studies of microvascular permeability to macromolecules by intravital microscopy which enables the correlation between macromolecular leakage sites and vessel morphology and other vascular events. Indomethacin 2 mg per 100 g b.w. was found to reduce the number of leaking postcapillary venules as compared with an untreated control group of hamsters. The model allows studies of macromolecular transport and its relation to other vascular parameters, vessel diameter and blood flow and it should be useful for studies of the pharmacology of small vessel permeability to macromolecules.

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