British Journal of Pharmacology (1998) 123, 943–951

Histamine-induced biphasic macromolecular leakage in the microcirculation of the conscious hamster: evidence for a delayed nitric oxide-dependent leakage

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1 Late effects (up to 3 h) of intravenously-injected histamine on FITC-dextran extravasation were investigated in the conscious hamster, by use of computer-assisted image analysis of fluorescence distribution in a microscopic window of dorsal skin fold preparations. This analysis allowed measurement of local (skin) and general (all organs) extravasations caused by a bolus injection of histamine (1 mg kg^{-1}, i.v.)

2 Histamine doses higher than 0.01 mg kg^{-1} caused biphasic local and general extravasations. Initial phases developed fully within 15 min (for local) and 60 min (for general) and were followed by late phases beginning 90 min after histamine injection. Although the initial and late phases of histamine-induced extravasations had differential apparent reactivities to the autacoid, all the effects of histamine on the microcirculation (1 mg kg^{-1}) were inhibited by pyrilamine (1 mg kg^{-1}, i.v.) but not by cimetidine (1 mg kg^{-1}, i.v.).

3 Pretreatment with N^G-monomethyl-L-arginine (L-NMMA, 30 mg kg^{-1}, i.v.) or N^G-nitro-L-arginine methyl ester (L-NAME, 100 mg kg^{-1}, i.v.) did not affect the initial phases but did prevent the late phases of local and general extravasations triggered by 1 mg kg^{-1} histamine. The inhibitory effects of L-NAME were reversed by L-arginine (30 mg kg^{-1}) but not by D-arginine (30 mg kg^{-1}) according to the enantioselectivity of nitric oxide synthase (NOS). A late NO-mediated venular dilatation occurred in response to plasma histamine.

4 A low dose of aminoguanidine (1 mg kg^{-1}, i.v.), a selective inhibitor of the inducible isoform of NOS (iNOS), mimicked the inhibitory effects of L-NAME on the late phases of histamine-induced macromolecular extravasations and venular dilatation.

5 Pretreatment with dexamethasone (1 mg kg^{-1}, i.v.) prevented both the initial and late phases of histamine-induced extravasations. Fucoidan (1 or 25 mg kg^{-1}, i.v.) prevented the late phases without affecting initial phases, consistent with a role for leukocytes adhesion in the development of the late NO-mediated effects of histamine.

6 We conclude that intravenous injection of histamine triggers a biphasic inflammatory cascade via initial activation of H_1 receptors which induces a late NO-mediated PMN-dependent extravasation process.

Keywords: N^G-nitro-L-arginine methyl ester; L-arginine; aminoguanidine; permeability

Introduction

Inflammation is characterized by microvascular permeability to plasma protein and recruitment of polymorphonuclear leukocytes (PMN) to the site of injury. Histamine induces both macromolecular extravasation and PMN adhesion. The direct permeabilizing effects of histamine on endothelium barrier function have been studied by use of intravital microscopy and topical suffusion of the autacoid on exteriorized tissues from different animal species (Fox *et al*., 1980; Wu & Baldwin, 1992; Yuan *et al*., 1993; Mayhan, 1994; Paul *et al*., 1994). These studies demonstrate that activation of endothelial histamine receptors induces a transient increase in macromolecular permeability (peak at 3 to 5 min, total recovery 15 to 20 min after the onset of histamine suffusion) through a phospholipase C nitric oxide (NO) cyclic GMP (guanosine 3′:5′-cyclic monophosphate)-dependent mechanism. Such a mechanism would account for the protective effects of nitric oxide synthase (NOS) inhibitors against the oedema that rapidly develops following subcutaneous injection of histamine (Texeira *et al*., 1993; Paul *et al*., 1994). Histamine is also responsible for rapid expression of the P-selectin adhesion molecule at the endothelial surface from preformed P-selectin stored in the Weibel-Palade bodies. This increases the rolling and sticking of leukocytes on cultured endothelial cells as well as in *in vivo* microcirculation systems (Lorant *et al*., 1991; Asako *et al*., 1994; Kubes & Kanwar, 1994; Thorlacius *et al*., 1995; Gaboury *et al*., 1996; Kubes & Gaboury, 1996). Kubes and Gaboury (1996) suggested that histamine induces PMN-dependent extravasation rather than a direct permeabilizing effect. Once stimulated, PMN produce cytokines and growth factors which are known inducers of the *de novo* protein synthesis involved in the self-amplification of the inflammatory cascade. The Ca^{2+}-independent NOS isoform is expressed in macrophages, smooth muscle cells and fibroblasts treated with interleukin-1β (IL-1β) or tumour necrosis factor-α (TNF-α) (Busse & Mulsch, 1990; Hauschildt *et al*., 1990; Werner-Felmayer, 1990). Higher levels of NO synthesis following expression of this iNOS have been implicated in the pathogenesis of chronic inflammation and circulatory shock (Laszlo *et al*., 1995a,b). Thus, it is possible that histamine-induced PMN adhesion/activation promotes delayed NO-dependent macromolecular extravasation.

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The aim of this study was to determine by intravital microscopy whether plasma histamine is able to induce delayed (up to 3 h) NO-mediated macromolecular extravasation in conscious animals. The dorsal skin fold preparation (Endrich *et al*., 1980) allows observation of the skin microcirculation in conscious animals for prolonged periods of time (up to 4 weeks). This eliminates the influence of acute inflammatory reactions elicited by surgical procedure and the effects of anaesthetics on the microcirculation. This preparation combined with a specially adapted computer-assisted image analysis, makes possible the long-term study of the effects of histamine on macromolecular extravasation. The results describe delayed NO-mediated PMN-dependent effects of histamine which could be inhibited by the selective iNOS inhibitor, aminoguanidine.

Methods

Surgical procedure

Male Syrian golden hamsters (6–8 weeks old, 60–80 g, Dépré, Saint Doulchar, France) were used. Titanium window chambers allowing microscopic observation of the dorsal skin microcirculation (Endrich *et al*., 1980) were surgically implanted. Animals were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). The back of the hamster was shaved and a depilatory ointment applied to remove any remaining hair. Two titanium frames were sewn on to the hamster so as to sandwich the extended double layer of dorsal skin. Once the first frame of the chamber was fixed, the opposite skin layer was completely removed in a defined circular area (15 mm diameter). The remaining exposed layer was moistened with 0.9% saline solution and covered by a cover slip fixed on to the second frame allowing observation of the microcirculation through the chamber. A subcutaneous venous catheter was then inserted into the jugular vein, positioned on the dorsal side of the neck and sutured to the frames. The animal was allowed to recover from anaesthesia and surgical procedure for 48 h. There was no apparent sign of discomfort, such as changes in sleeping and feeding habits during this recovery period. Meticulous care was taken, but if major surgical trauma occurred, the preparation rapidly exhibited signs of inflammation or haemorrhage during the recovery period, the animal was immediately killed by intravenous injection of a lethal dose of anaesthetic.

Experimental protocol

Experiments were performed two days after surgery in a dark, quiet and temperature-controlled (21°C) laboratory. A conscious hamster was placed into a specially-designed plexiglass cylinder. This was fixed to the microscope stage with the window of the chamber to be positioned perpendicular to the light beam of a Leitz Ergolux microscope (Leica, France). Use of this cylinder limited animal movements and the skin microcirculation could be observed at a global magnification of ×125 throughout 180 min experiments. The fluorescent macromolecular tracer, FD-150 (150 kD, 63 mg kg^{-1}), was injected (bolus) via the catheter inserted into the jugular vein. There was a 15 min equilibration period, then histamine (from 0.001 to 1 mg kg^{-1}; 1 ml kg^{-1}) was intravenously-injected through the same catheter (time t = 0). Antiselectin compound (fucoidan), NOS inhibitors (L-NAME, L-NMMA and aminoguanidine), NOS substrate (L-arginine), dexamethasone, histamine receptor antagonists (pyrilamine and cimetidine) or appropriate vehicle were intravenously-injected (1 ml kg^{-1}) as required 15 min before, or 1 or 90 min after injection of histamine. The time-course of the histamine-induced macromolecular extravasation was determined by analysing FD-150 fluorescence distribution in the dorsal skin preparation every 15 min throughout the experiment. The preparation was sequentially epi-illuminated (for 1 min at intervals of 15 min) at the optimal excitation wavelength for FITC (450–490 nm) with a 100 W mercury bulb and filters (I_3 block, Leica) positioned between the light source and the condenser. The stability of the excitation light intensity was periodically checked with a luxmeter. A black and white CCD video camera (HPR 610, ICAP, Meylan, France) was connected to the microscope. The CCD video camera was not light-adjusted to avoid compensation between bright and dark zones of the images. Images were sent to a VHS video-recorder (Mitsubishi, BV 2000E, Bioblock, France) for off-line computer-assisted image analysis.

Computer-assisted image analysis of the fluorescence distribution

Fifteen minutes after intravenous injection of 63 mg kg^{-1} FD-150, epi-illumination of the hamster dorsal skin fold preparation allows observation of the arterioles and venules in the small area (0.35 mm^2) in the window. The vascular network accounted for 30–40% of the total image surface, allowing the light intensity in both interstitial and intravascular compartments to be analysed simultaneously by video image processing (Visicap) (Figure 1a). The analogue video image was digitized into x, y arrays of 512 by 512 picture elements (pixel) as previously described (Bekker *et al*., 1989). Each pixel was associated with an 8-bit grey level (a number between 0 (black) and 255 (white)). The digitized data consisted of the x, y positions of each pixel in the image and their corresponding grey value. The grey level histogram was constructed by scanning of the entire image. Gaussian peaks (2 or 3) were fitted to the histograms by use of the Microcal Origin Peak Fitting Module software. Statistical analysis revealed that pixels were normally distributed in two homogeneous populations along the grey level axis. On average, at t = 0, these two gaussian curves were centered at 82 ± 4 (35 ± 3% of the total population of pixels) and 178 ± 7 (53 ± 5% of the total population of pixels) ($n = 130$) of the 256 attributable grey levels (Figure 1b). The right-hand peak represented the brighter venular population of pixels whereas the left-hand peak represented the darker interstitial population of pixels. Arterioles were always darker than venules and were responsible for a third minor peak. The difference between arteriolar and venular grey levels may be due to the three-dimensional nature of the preparation and to the smaller arteriolar diameter. The grey level of an individual pixel is determined by the fluorescence of FD-150 molecules on the corresponding light path, including out of focus molecules.

Histamine-induced fluorescence redistribution

Interstitial and venular peaks remained stable for 3 h after bolus injection of FD-150 in preliminary experiments. Bolus injection of histamine (1 mg kg^{-1}, i.v.), 15 min after injection of the fluorescent tracer, induced time-dependent redistribution of the light intensity in the window (Figure 1c, e and g) which resulted in a progressive rightward shift of the interstitial peak and a concurrent leftward shift of the venular peak (Figure 1d, f and h). The rise in the interstitial mean grey level may reflect histamine-induced FD-150

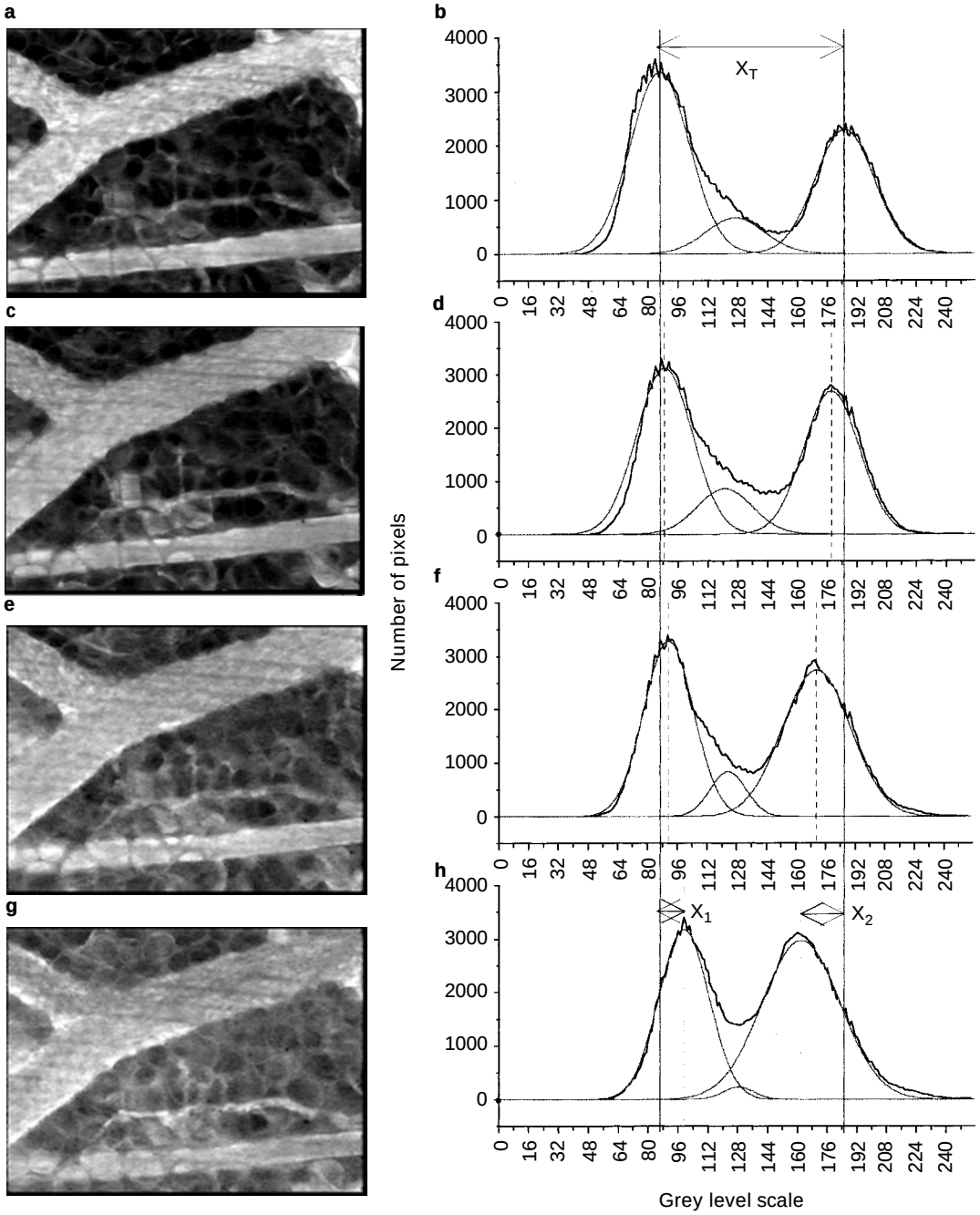

Figure 1 Time-dependent effects of intravenously-injected histamine (1 mg kg^{-1}) on fluorescence distribution in vascular and interstitial spaces. Images (× 125 magnification) are from a representative 180 min experiment at t = 0 (before, a), 60 (c), 120 (e) and 180 min (g) after histamine injection. The corresponding grey level histograms (b, d, f, and h) were derived from computer-assisted image analysis and were fitted by use of Microcal Origin Peak Fitting Module software. The time-dependent increase in interstitial light intensity (left-hand peak) resulted in an absolute rightward shift of the mean grey level of the darker peak (X_1). Intravenular light intensity (right-hand peak) decreased resulting in an absolute leftward shift of the mean grey level of the brighter peak (X_2). X_1 is the local extravasation index and X_2 the general extravasation index. They were normalized with X_T (before injection of histamine). At the end of the experiments only two peaks were detected. The darker peak accounted for $44 \pm 3\%$ of the total population of pixels and the brighter peak for $56 \pm 3\%$ of the pixels. At t = 0, they accounted for $53 \pm 5\%$ (darker) and $35 \pm 3\%$ (brighter) reflecting both histamine-induced venular dilatation and loss of a third minor arteriolar peak which was probably included in the brighter peak. Mean vessel diameters before inflammatory stimulus were 90 ± 5 μm for the observed collecting venules and 52 ± 5 μm for the arterioles.

accumulation in the interstitial fluid of the dorsal skin, as the initially extravasated tracer was not removed. The histamine-induced decrease in intravascular fluorescence may account for the progressive loss of FD-150 by the vasculature in all irrigated organs. In a separate set of experiments, haematocrits measured at the end of 180 min experiments were not significantly different in histamine- and saline solution-treated animals ($50.4 \pm 1.8\%$, $n = 7$, for histamine vs $52.5 \pm 4.8\%$, $n = 7$, for saline solution). This rules out any influence of red blood cells on the decrease in the venular light intensity observed in response to histamine. So, both interstitial rightward and venular leftward peak shifts probably account for changes in the amounts of interstitial and venular FD-150.

Measurements of histamine-induced macromolecular extravasation

For quantification of the amounts of macromolecules in the interstitial and intravascular compartments, it was assumed that a linear relationship exists between pixel grey level and number of FD-150 molecules present along the corresponding light path. Preliminary intravascular calibration experiments showed that the venular mean grey level was proportional to the dose of FD-150 injected (from 20 to 140 mg kg^{-1}). Interstitial calibration was not available in this study, but previous studies with the same image digitization system have shown that, in inflammatory conditions, interstitial grey level is linearly related to the amount of extravasated FITC-dextran (Bekker *et al*., 1989; Armenante *et al*., 1991). Thus, for a given time, the size of the histamine-induced peak shifts (X_1 and X_2 values in Figure 1h) were assumed to be proportional to the number of FD-150 molecules accumulated in the interstitial fluid of the skin (X_1) and to the number of FD-150 molecules lost by the vasculature (X_2). As the number of macromolecules diffusing through the permeabilized endothelium depends on the pre-existing gradient of concentration between intravascular and interstitial spaces, both shift amplitudes were normalized by use of the difference between venular and interstitial mean grey levels before injection of histamine (X_T). Therefore the rightward shift of the interstitial peak was measured every 15 min, called 'local extravasation' and expressed as X_1/X_T. The leftward shift of the venular peak was calculated every 15 min, called 'general extravasation' and expressed as X_2/X_T.

Venular diameter measurements

Systemic application of histamine produces general vasodilatation and hypotension. Haemodynamics influence macromolecular permeability. Unfortunately, we were unable to measure arterial blood pressure in the conscious hamster as we found that clots rapidly collapsed catheters. Measurements of venular diameter have been made in the same conscious animals to determine whether histamine-induced local and general extravasations were associated with the histamine-induced decrease in venular shear rate. Venular diameter measurements (Visicap software) were made by use of a mathematical transformation of the digitized images into bicolor images. A grey level threshold was defined between the mean grey levels of the interstitial and venular pixels. Pixel grey levels above this threshold were changed to black and those below the threshold to white. In the transformed images the vessels were black and the tissues, white. A segment was placed perpendicular to the longitudinal axis of the vessel. Visicap software determined the number of black pixels along the segment and converted it into micrometres. Venular diameter was measured every 15 min and expressed as % of the diameter measured at $t=0$ or $t=90$ min.

Drugs

Fluorescein-isothiocyanate dextran (150,000 mol wt), histamine hydrochloride, cimetidine, pyrilamine, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine, D-arginine, aminoguanidine, water-soluble dexamethasone and fucoidan were dissolved in saline 0.9% NaCl solution. All drugs were intravenously injected. They were purchased from Sigma Chemical (Saint-Quentin Fallavier, France).

Statistical analysis

Results are expressed as mean ± s.e.mean. Analysis of variance (ANOVA, Statistica, Statsoft) was used for statistical comparison and significance was determined with Scheffe's *post hoc* test. P values <0.05 were considered significant.

Results

Effects of histamine

The time-dependent changes in fluorescence distribution illustrated in Figure 1 reflected histamine-induced macromolecular extravasation since in control experiments, saline solution injection 15 min after fluorescent tracer caused no significant change in interstitial or venular light intensities during the 180 min of the experiments (Figure 2a and b). Time-courses of histamine-induced FD-150 accumulation in the interstitial fluid (local extravasation) and decrease in the venular compartment (general extravasation) were biphasic (Figure 2a and b). In both cases, the initial phase developed as soon as the inflammatory mediator was injected and reached steady states at $t=15$ min (for local extravasation) and $t=60$ min (for general extravasation). The later phases of both local and general extravasations began at t = 90 min and accounted for 50–75% of the total changes in interstitial and venular fluorescence measured at the end of the experiments: 17.22 ± 5.53%, $P<0.05$ for local and 22.91 ± 2.19%, $P<0.001$

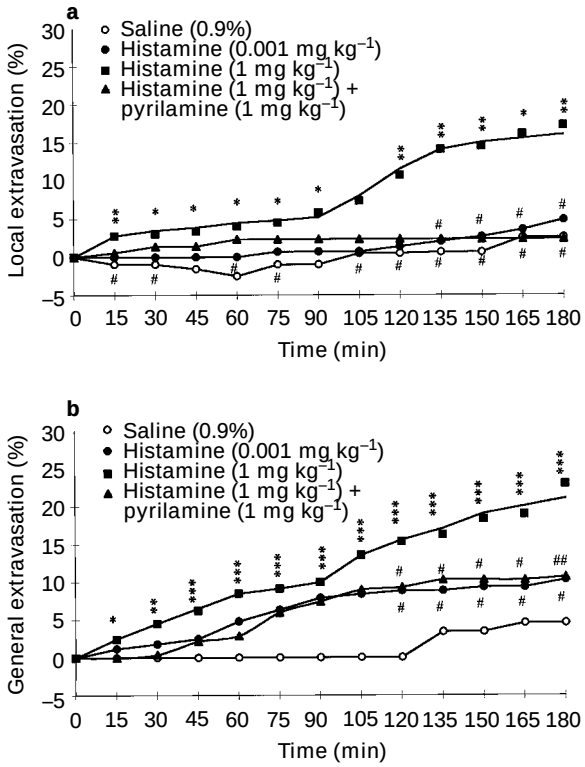

Figure 2 Time-courses of local (a) and general (b) extravasation induced by saline solution (0.9%), by a low dose (0.001 mg kg^{-1}, i.v.) or a large dose (1 mg kg^{-1}, i.v.) of histamine. Effects of an H_1-histamine receptor antagonist were evaluated by a pretreatment with pyrilamine (1 mg kg^{-1}, i.v.), 15 min before histamine (1 mg kg^{-1}, i.v.). *Denotes a significant difference from the values obtained with saline solution injection and #denotes a significant difference from 1 mg kg^{-1} histamine (*,#$P<0.05$; **,##$P<0.01$; ***$P<0.001$) according to Scheffe's *post-hoc* test.

for general extravasations. Histamine induced significant time-dependent venular dilatation in the same animals. Venular diameter was 107.52±2.11% ($P<0.01$) of the initial diameter in histamine-treated animals at t = 180 min whereas in control experiments it was 94.83±2.44%.

Effects of various doses of histamine (from 0.001 to 1 mg kg^{-1}) were measured to determine apparent reactivities of initial and late phases of histamine-induced extravasations. The lowest dose tested (0.001 mg kg^{-1}) did not provoke local extravasation (4.85±3.16% at t = 180 min). With 0.01 and 0.1 mg kg^{-1} histamine, the time courses of the local extravasation were similar to that obtained with 1 mg kg^{-1} (data not shown). Therefore, the two phases of local extravasation would exhibit the same apparent reactivity to intravenously-injected histamine. In the same set of experiments, the late phase of general extravasation, (measured between $t = 90$ min and $t = 180$ min) exhibited a clear dose-dependent profile ($EC_{50} = 0.03$ mg kg^{-1}), whereas the early phase (measured between $t = 0$ and $t = 90$ min) developed fully even with 0.001 mg kg^{-1} histamine (Figure 2b). Using this dose, a delay was observed in the onset of the initial phase of histamine-induced general extravasation. Thus, with regard to general extravasation, the initial phase appeared more sensitive to histamine than the late phase.

Pretreatment with the H_2 receptor antagonist, cimetidine (1 mg kg^{-1}), did not affect the time-courses of local and general extravasations (data not shown). Such a pretreatment with the H_1 receptor antagonist, pyrilamine (1 mg kg^{-1}), prevented local extravasation induced by 1 mg kg^{-1} histamine. In these experiments, the initial phase of histamine-induced general extravasation was delayed whereas the late phase of general extravasation was suppressed (Figure 2b). Injection of cimetidine or pyrilamine alone did not affect fluorescence distribution throughout the 180 min of the experiments (data not shown).

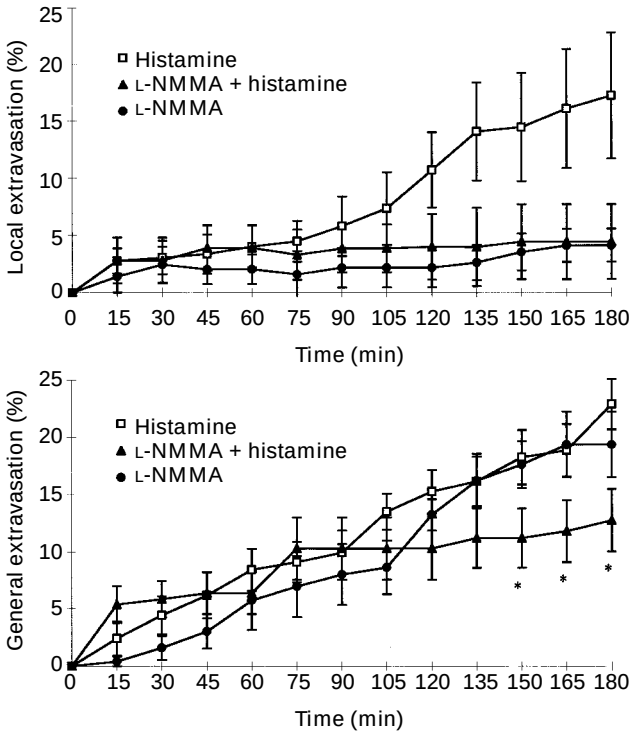

Figure 3 Effect of previous NO synthesis inhibition by L-NMMA (30 mg kg^{-1}, i.v.; $n = 6$) on the time-courses of local (a) and general (b) extravasation induced by intravenously-injected histamine (1 mg kg^{-1}). Control experiments to check the effects of a single injection of L-NMMA on the fluorescence distribution are included (solid circles). A significant difference from the values obtained without NO synthase inhibitor are denoted by $*P<0.05$, according to Scheffe's *post-hoc* test.

Effects of L-NMMA and L-NAME

Preparations were pretreated with the NOS inhibitors, L-NMMA (30 mg kg^{-1}) and L-NAME (100 mg kg^{-1}), to investigate the involvement of NO synthesis in the fluorescence changes caused by 1 mg kg^{-1} histamine. L-NMMA did not affect the early phases but totally prevented the late phases of histamine-induced local and general extravasations (Figure 3a and b). The more potent inhibitor of NOS, L-NAME had the same effects on histamine-induced local and general extravasations (see below). In control experiments without histamine, L-NMMA (30 mg kg^{-1}) caused time-dependent general extravasation but did not provoke local extravasation. In contrast, L-NAME (100 mg kg^{-1}) injected alone induced both local (10.54±3.13%; NS) and general (12.73±2.36%; $P<0.05$) extravasations.

The differential effects of L-NAME (100 mg kg^{-1}) on the initial and late phases of histamine-induced extravasations were evaluated separately by measuring absolute changes in interstitial and venular fluorescence during the first 90 min and the remaining 90 min (Figure 4a and b). The same sequential analysis was performed on histamine-induced venular dilatation (Figure 4c). L-NAME did not affect the initial phase of histamine-induced local and general extravasations but it did significantly prevent venular dilatation during the first 90 min. The effect of L-NAME (100 mg kg^{-1}) on histamine-induced venular dilatation was partially reversed by injection of L-arginine (30 mg kg^{-1}) at $t = 1$ min but not by injection of D-arginine (30 mg kg^{-1}) (Figure 4c).

The late phases of histamine-induced local and general extravasations were prevented by L-NAME pretreatment. L-Arginine, injected 1 min after histamine, did not significantly reverse the inhibitory effects of L-NAME on histamine-induced macromolecular extravasations. However, the late phases of histamine-induced local and general extravasations were totally restored if L-arginine was injected at $t = 90$ min. Injections of D-arginine at $t = 90$ min did not have the same restorative effects as L-arginine (Figure 4a and b). Histamine-induced increase in venular diameter during the late phases of extravasation was significantly reduced by L-NAME pretreatment. Addition of L-arginine at $t = 90$ min completely restored histamine-induced venular dilatation whereas D-arginine was ineffective. In control experiments, L-arginine alone was able to induce rapid local and general extravasations: 10.45±1.98% ($n = 8$, $P<0.05$) and 17.59±3.75% ($n = 8$, $P<0.01$) after 180 min, respectively.

Effects of aminoguanidine

The 90 min required for the histamine-induced NO-dependent extravasations and venular dilatation to be resolved, led us to evaluate the effects of the selective inhibitor of iNOS, aminoguanidine (Hasan *et al*., 1993) on these processes (Figure 5a and b). Injection of a low dose of aminoguanidine (1 mg kg^{-1}), 15 min before histamine, significantly inhibited the late phases of both extravasation and venular dilatation without affecting the initial phases. Aminoguanidine did not cause the NO-dependent venular constriction observed with L-NAME during the first 90 min of the experiment. In control

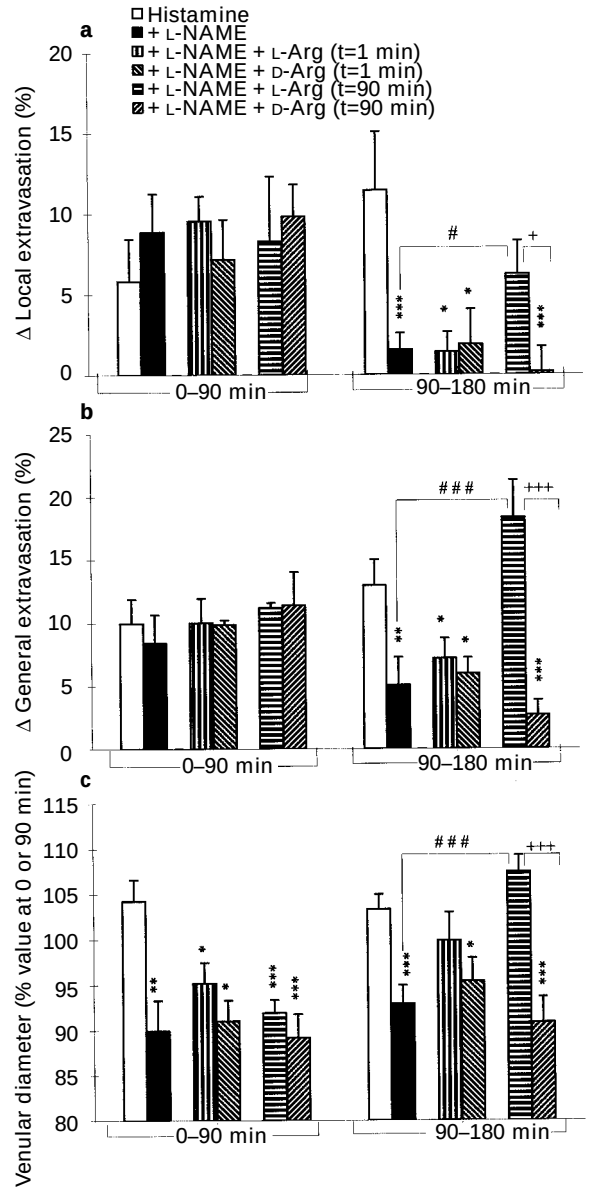

Figure 4 Sequential analysis of the effects of NO-synthesis modulators L-NAME (100 mg kg^{-1}, i.v.; $n=7$), L-arginine and D-arginine (30 mg kg^{-1}, i.v.; $n=5-6$) on local (a) and general (b) extravasation and venular diameter (c) induced by histamine (1 mg kg^{-1}, i.v.) during the first 90 min and the remaining 90 min of the experiments. L- and D-arginine were injected either 1 or 90 min after the injection of histamine, in the presence of L-NAME. Individual effects of drugs are specified in the text. *Denotes a significant difference from the extravasation obtained with histamine alone ($n=9$). $^{\#}$and $^{+}$Denote a significant difference from the appropriate control ($^{*\#+}P<0.05$; $^{**}P<0.01$; $^{***\#\#\#+++}P<0.001$) according to Scheffe's *post-hoc* test.

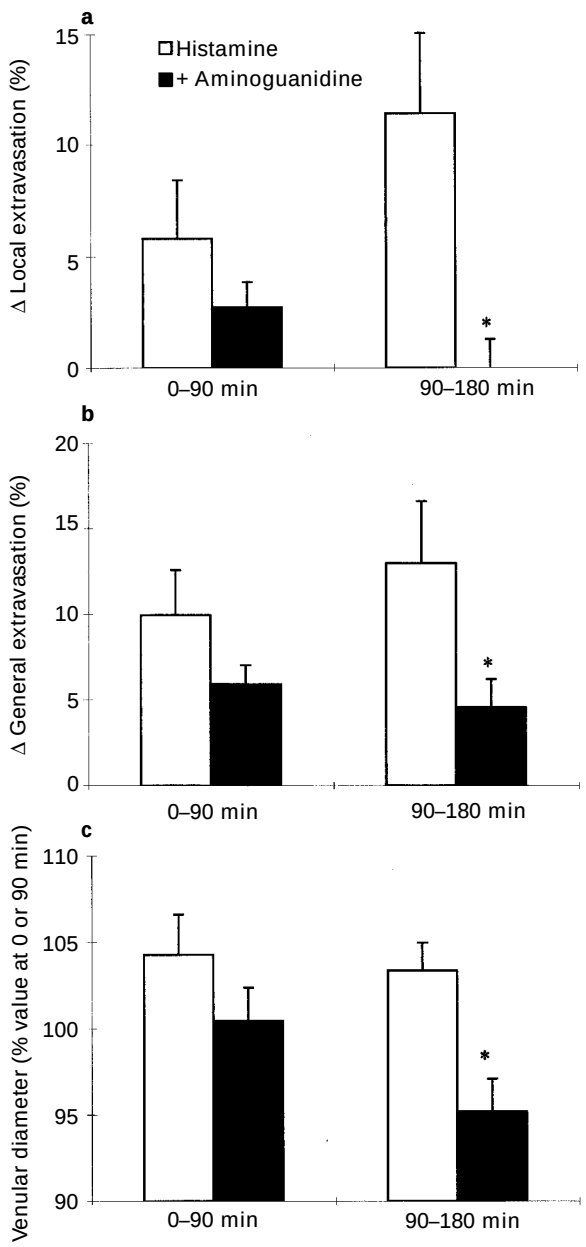

Figure 5 Sequential analysis of the effects of pretreatment with the selective inhibitor of iNOS, aminoguanidine (1 mg kg^{-1}, $n=8$), on histamine-induced local (a) and general (b) extravasation and on venular diameter (c). $^{*}P<0.05$, a significant difference from the effects of histamine ($n=9$) (Scheffe's *post-hoc* test).

180 min experiments, aminoguanidine did not provoke local ($4.61\pm1.12\%$, $n=6$, NS) or general ($8.96\pm2.63\%$, $n=6$, NS) extravasations.

Effects of dexamethasone

We investigated the effects of dexamethasone, which prevents expression of the gene for iNOS, on histamine-induced macromolecular extravasation (Figure 6a and b). Pretreatment with this glucocorticoid totally inhibited the histamine-induced local extravasation (Figure 6a). At the end of the experiments, the local extravasation index was not significantly different from that measured at the end of experiments performed with saline solution alone, ie without histamine and dexamethasone ($-8.54\pm5.59\%$ vs $2.57\pm3.23\%$). Dexamethasone alone did not cause any changes in basal FD-150 distribution throughout 180 min experiments: $1.60\pm4.55\%$ ($n=6$) for local and $5.43\pm1.12\%$ for general index.

Effects of fucoidan

Animals were pretreated with the selectin-binding carbohydrate, fucoidan, to investigate whether PMN adhesion was involved in the histamine-induced local and general extravasations. Fucoidan, 1 or 25 mg kg^{-1}, prevented the late phases of histamine-induced local and general extravasations without affecting their initial phases (Figure 7a and 7b). Fucoidan also significantly prevented the late histamine-induced venular

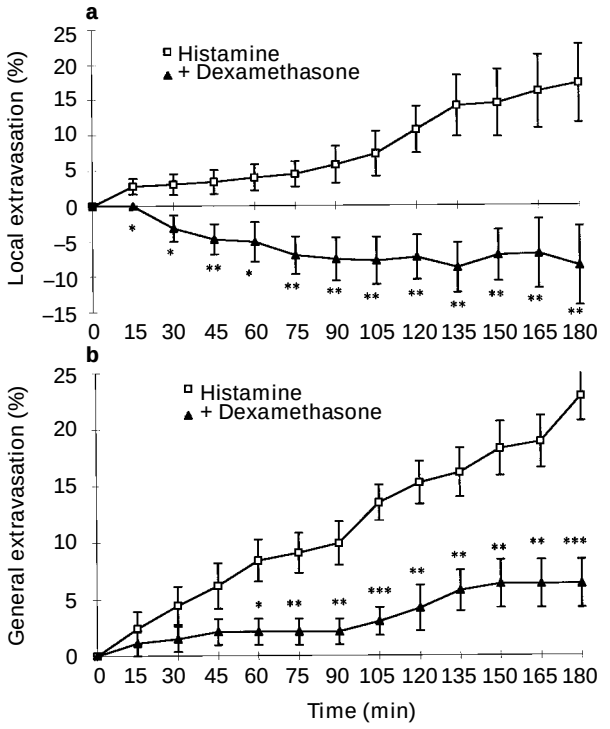

Figure 6 Inhibition by a glucocorticoid, dexamethasone (1 mg kg^{-1}, i.v., $n=7$), of histamine (1 mg kg^{-1}, i.v.)-induced local (a) and general (b) extravasation. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, significance of difference from the effects of histamine according to Scheffe's *post-hoc* statistical test.

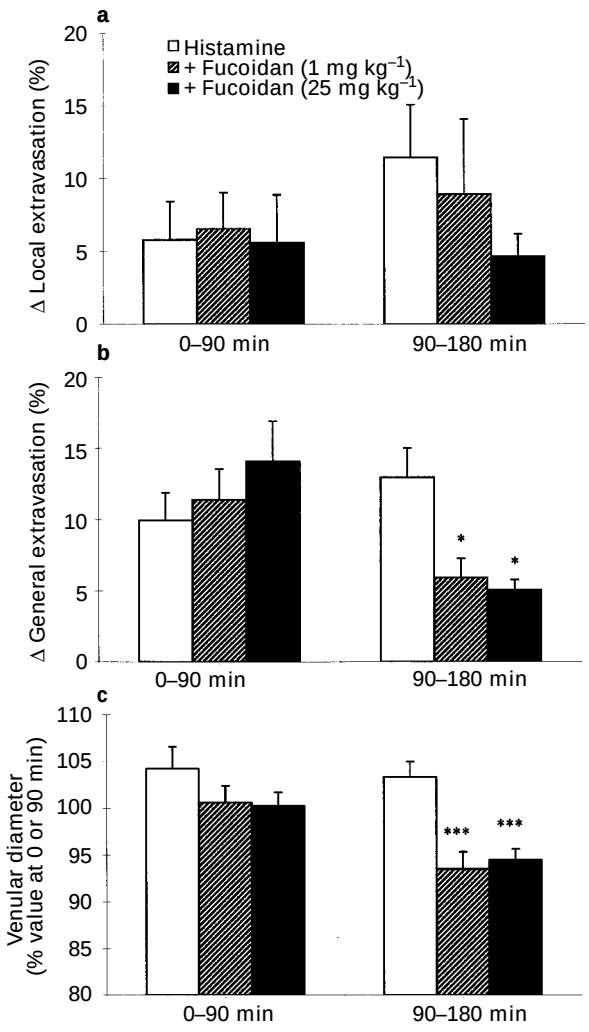

Figure 7 Sequential analysis of the effects of two doses (1 or 25 mg kg^{-1}, i.v., $n=6$ and 5) of the selectin-binding carbohydrate, fucoidan, on local (a) and general (b) extravasation and venular diameter (c) induced by histamine (1 mg kg^{-1}, i.v.) during the first 90 min and the remaining 90 min of the experiments. $^{*}P<0.05$; $^{***}P<0.001$, significance of difference from the value obtained with histamine alone ($n=9$), according to Scheffe's *post-hoc* test.

dilatation at both doses (Figure 7c). In control experiments, fucoidan alone had no effect on local ($3.44\pm1.55\%$, for 1 mg kg^{-1}, $3.65\pm2.68\%$ for 25 mg kg^{-1}) or on general index ($4.58\pm1.16\%$ for 1 mg kg^{-1}, $3.16\pm1.14\%$ for 25 mg kg^{-1}).

Discussion

Bolus injection of histamine caused inflammatory cascades resulting in simultaneous biphasic local and general extravasations over the course of the 180 min experiments. There were discrete differences between these two phenomena: (i) the initial phase of general extravasation developed over 60 min whereas that of local extravasation developed within 15 min. (ii) Local and general extravasations had differential apparent reactivities to histamine. (iii) Dexamethasone prevented local extravasation but only partially inhibited general extravasation. Nevertheless, all the pharmacological interventions that inhibited local extravasation simultaneously prevented general extravasation, suggesting that these two processes are regulated by a qualitatively similar histamine-triggered inflammatory cascade. Although local extravasation is a homogeneous response of the cutaneous tissue, general extravasation results from the responses of all the organs, some being more or less sensitive to the pharmacological interventions tested. This might explain the differences between histamine-induced local and general extravasations.

Immediately after histamine injection, the rate of FD-150 diffusion through the endothelium in response to plasma histamine transiently exceeded the rate of putative FD 150 drainage by the lymphatics, accounting for initial phases of local and general extravasations. Numerous *in vivo* studies performed on exteriorized organs in different animal species have shown that direct activation of endothelial H_1 receptors by topical suffusion of histamine causes the transient formation of gaps and the transient appearance of macromolecular leakage sites in post-capillary venules via a PLC/NO/cyclic GMP-dependent mechanism (Wu & Baldwin, 1992; Yuan *et al*., 1993). In our study, the histamine-induced fluorescence redistribution during the first 60 min of the experiment was not affected by pretreatment with L-NMMA or L-NAME, whereas these molecules did cause a reduction in venular diameter via their known vascular effects on the constitutive endothelial NOS (Mayhan, 1994; Paul *et al*., 1994; Laszlo *et al*., 1995a,b). Thus, the initial phases of local and general extravasations would not be related to the direct NO-mediated permeabilizing effects of histamine on the endothelium and would be independent of the known NO-mediated vasodilatation induced by the autacoid. Histamine-induced P-selectin-dependent adhesion of PMN was not involved in these initial phases, as fucoidan had no effect on these initial phases. The inflammatory processes involved in the early phases of histamine-induced macromolecular extravasation are unknown. One possible explanation would be that plasma

histamine activates leukocytes independently from their adhesion, through the liberation of platelet-activating factor (PAF) (Lorant *et al*., 1991; Watanabe *et al*., 1991). Leukocyte-derived inflammatory mediators may act synergistically to cause immediate NO-independent macromolecular leakage.

The 180 min experiments of this study showed that there were late phases in the histamine-induced local and general extravasations beginning about 90 min after histamine injection. The late phases resulted from initial activation of an H_1 receptor population and were inhibited by the non-selective inhibitors of NO-synthases, L-NMMA and L-NAME. The inhibitory effects of 100 mg kg^{-1} L-NAME were reversed by 30 mg kg^{-1} L-arginine but not by 30 mg kg^{-1} D-arginine according to the stereospecificity of NOS activity. These results demonstrate that a bolus injection of histamine triggers late NO-mediated macromolecular leakage in the hamster microcirculation. As discussed above, NO synthesis inhibition by L-NAME prevents the direct permeabilizing effects of several mediators including histamine (Texeira *et al*., 1993; Yuan *et al*., 1993; Mayhan, 1994; Paul *et al*., 1994; Laszlo *et al*., 1995a,b). Nevertheless, this direct NO-mediated permeabilizing effect of histamine is rapid and transient (Svensjö, 1990; Wu & Baldwin, 1992; Mayhan, 1994), a time-course incompatible with the sustained, late, NO-dependent leakage that we observed. Furthermore, the direct NO-mediated effect of histamine on endothelial barrier function is independent of PMN adhesion, whereas we found that histamine-induced late NO-dependent extravasations were prevented by fucoidan, at doses known to prevent PMN rolling and sticking caused by different inflammatory mediators including histamine (Kubes & Gaboury, 1996; Van Osselaer *et al*., 1996).

Aminoguanidine (1 mg kg^{-1}), totally prevented the late phases of extravasation and vasodilatation triggered by histamine. Aminoguanidine has a 50 to 100 fold selectivity for iNOS relative to the endothelial form (Hasan *et al*., 1993; Laszlo *et al*., 1995a). However, several authors have shown that high doses of aminoguanidine (50 mg kg^{-1}) cause leukocyte adherence and protein leakage in the rat intestinal microvasculature, resulting from inhibition of the constitutive endothelial isoform (Laszlo *et al*., 1995a; Lopez-Belmonte & Whittle, 1995). In our study, injection of 1 mg kg^{-1} aminoguanidine alone did not mimic the pro-inflammatory effects of the non selective inhibitors of NO-synthases, L-NMMA and L-NAME, alone. Furthermore, 1 mg kg^{-1} aminoguanidine prevented the late extravasations and venular dilatation induced by histamine, but failed to reproduce the inhibitory effects of L-NAME on the histamine-induced NO-mediated dilatation during the first 90 min of the experiments. Thus, it is probable that the inhibitory effects of aminoguanidine on the late phases of histamine-induced extravasation resulted from inhibition of iNOS. Consistent with histamine-induced iNOS expression, pretreatment with the glucocorticoid, dexamethasone, prevented late phases of extravasations. However, with regard to a putative initial phase requirement for NO-synthase induction and the inhibitory effects of dexamethasone on these early phases, this set of experiments was not conclusive.

We found that histamine-induced late NO-dependent extravasations were prevented by fucoidan. Thus, late histamine-induced NO-dependent macromolecular extravasation is related to histamine-induced PMN adhesion/activation. This is apparently controversial since NO is known to present anti-adhesive properties (Kubes, 1995). NOS inhibitors have been shown to promote PMN adhesion and associated macromolecular extravasation, through oxidative activation of mast cells and subsequent liberation of potent pro-adhesive mediators including histamine (Kurose *et al*., 1993; Gaboury *et al*., 1996). Such a mechanism might account for the general extravasation that we observed in response to injection of either L-NAME or L-NMMA alone. The lack of significant effect of these compounds on local extravasation might be explained by a lower sensitivity of the cutaneous tissue to NOS inhibition. Although NO-synthesis inhibition promotes PMN adhesion, it does not affect histamine-induced PMN adhesion (Gaboury *et al*., 1996). Thus, in the present study, NO-synthesis inhibition would not increase further histamine-induced PMN adhesion via mast cell degranulation, but would prevent a PMN-dependent process. The direct permeabilizing effect of NO on the endothelium is involved in the leakage observed in response to several mediators including histamine (Texeira *et al*., 1993; Yuan *et al*., 1993; Mayhan, 1994; Paul *et al*., 1994; Laszlo *et al*., 1995a,b). Furthermore, our control experiments showed that injection of L-arginine alone was able to induce rapid macromolecular extravasation, probably through activation of constitutive NO-synthase.

The relationship between PMN adhesion and the late NO-mediated extravasation and venular dilatation remains unclear. Reduction of venular shear rate by NO might favour the interaction of PMN with the endothelium (Kurose *et al*., 1993; Davenpeck *et al*., 1994) and subsequent PMN-dependent extravasation. Histamine might also induce leukocyte adhesion/activation through rapid expression of P-selectin at the endothelial surface and liberation of PAF (Lorant *et al*., 1991; Watanabe *et al*., 1991). Such an early activation of leukocytes would result in the liberation of cytokines and growth factors known to induce *de novo* expression of iNOS in different cells, such as monocytes, macrophages, smooth muscle cells and fibroblasts but also in endothelial cells (Busse & Mulsch, 1990; Hauschildt *et al*., 1990; Werner-Felmayer *et al*., 1990). Such expression of iNOS in the endothelial layer might be of critical importance in the late extravasation process observed in response to plasma histamine.

This study provides new insights into the effects of histamine in the microcirculation of awake hamsters and is consistent with a bolus injection of histamine causing an inflammatory cascade, which in turn induces NO-mediated extravasation through a PMN-dependent mechanism. However, the high pharmacological doses of histamine required to demonstrate the late phase of extravasation would result in plasma concentrations at least 100 to 1000 times higher than the normal concentration in the plasma (Brackett *et al*, 1990). The physiological relevance of this study to the anaphylactic reaction remains to be determined but mast cell degranulation in the vicinity of the vasculature can certainly cause local bursts of histamine in the intravascular compartment.

This study was supported by the ''Association Nationale de la Recherche Technique'' (convention n° 92651). The authors thank Dr Anger-Leroy for helpful discussions and suggestions.

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(Received September 25, 1997
Accepted November 17, 1997)