Br. J. Pharmacol. (1993), **110**, 1496–1500

Suppression by intradermal administration of heparin of eosinophil accumulation but not oedema formation in inflammatory reactions in guinea-pig skin

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1 Heparin is widely used in the treatment of thrombotic disorders and as an aid in surgery. Anti-inflammatory effects of heparin have also been described. In this study, we have investigated the effects of locally-injected heparin on the oedema formation and eosinophil accumulation induced by various inflammatory stimuli in guinea-pig skin.

2 Heparin dose-dependently suppressed the accumulation of ^{111}In-labelled eosinosphils induced by i.d. injection of zymosan-activated plasma (ZAP). The ^{111}In-eosinophil accumulation induced by other inflammatory stimuli (compound 48/80, platelet activating factor, interleukin-8 and in a passive cutaneous anaphylaxis reaction) was also suppressed by locally-injected heparin.

3 Oedema formation in response to these same stimuli was not altered by the local injection of heparin.

4 Fucoidin, a negatively-charged sulphated algal polymer, had no effect on the ^{111}In-eosinophil accumulation or oedema formation induced by ZAP. Nevertheless, fucoidin significantly suppressed the oedema formation induced by i.d. injection of cationic protein-containing extracts of *Schistosoma mansoni* larvae. Heparin also inhibited oedema induced by the extracts, suggesting that both fucoidin and heparin were effectively neutralizing the cationic protein of the extracts to inhibit their oedema-inducing activity.

5 Thus, heparin significantly inhibited the accumulation of ^{111}In-eosinophils, but not oedema formation, induced by various inflammatory stimuli. This, taken together with the lack of effect of fucoidin, suggests that heparin interferes with the process of eosinophil trafficking by a mechanism that does not depend on neutralisation of the charge of the stimulatory molecules.

Keywords: Heparin; eosinophil; oedema formation; inflammation; fucoidin; passive cutaneous anaphylaxis reaction

Introduction

Heparin is a linear anionic polyelectrolyte drug obtained commercially from beef lung or porcine intestinal mucosa and used mainly in the prevention and treatment of thrombotic disorders (Jaques, 1980; Hirsh, 1991; Freedman, 1992). Anti-inflammatory actions of heparin have also been described. For example, it has been shown to suppress neutrophil function and chemotaxis *in vitro* (Matzner *et al.*, 1984; Bazzoni *et al.*, 1993), reduce neutrophil migration into the inflamed rat paw (McGovern, 1957) and inhibit increases in vascular permeability in rabbit skin (Lecomte & Hugues, 1954; Carr, 1979). Heparin also appears to diminish the bronchoconstrictor response of sheep to allergic and non-allergic stimuli (Ahmed *et al.*, 1992) and the immediate response to antigen in the skin and lungs of allergic human subjects (Bowler *et al.*, 1993).

Heparin is found in many tissues, especially in association with mast cells granules where it binds to histamine and cationic proteins (Jaques, 1980). Mast cells appear to play an important role in allergic diseases such as asthma and rhinitis (Wasserman, 1987; Holgate & Church, 1992). Thus, mast cells are directly responsible for the acute clinical manifestations of allergic diseases (Holgate & Church, 1992) but may also influence the chronic states through the release of cytokines (Plaut *et al.*, 1989; Bradding *et al.*, 1992; Kulmburg *et al.*, 1992). Another feature of allergic inflammation is the presence of eosinophils which are potent effector cells capable of causing tissue destruction and dysfunction (Djukanovic *et al.*, 1990; Kay, 1991). Mast cells are thought to contribute to the recruitment of eosinophils through the release of mediators including phospholipids and chemotactic cytokines (Kulmburg *et al.*, 1992) and this may be relevant to allergic disorders such as asthma.

In preliminary studies, we observed that heparin effectively inhibited experimental inflammation in guinea-pig skin induced by cationic protein-containing extracts of *Schistosoma mansoni* larvae (Teixeira *et al.*, unpublished observations). Interestingly, eosinophil accumulation was inhibited much more effectively than the formation of oedema which suggested an effect other than merely neutralization of cercarial cationic proteins. In the present study, we investigated whether local administration of heparin could also inhibit the accumulation of eosinophils and oedema formation induced by known mediators of inflammation in guinea-pig skin, and whether another highly anionic molecule, the sulphated algal polymer fucoidin, could mimic the effects of heparin.

Methods

Preparation of zymosan-activated plasma

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des-Arg. Guinea-pig heparinized (10 iu ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min at 3000 *g*). The activated plasma was desalted in a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C. The maximum concentration of heparin in ZAP was approximately 7 iu ml^{-1} (i.e. 0.7 iu per skin site).

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of IgG_1-rich sera are given elsewhere (Weg *et al.*, 1991). Briefly, male guinea-pigs (Harlan Porcellus, 350–400 g) were immunized with bovine gamma-

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globulin (BGG) in Freund's complete adjuvant (0.2 mg BGG 0.2 ml^{-1} of adjuvant s.c.). A boost of antigen in Freund's incomplete adjuvant was given on day 21 and the serum prepared on day 30. For the skin assays, recipient animals received an injection of 50 μl of a 1/50 dilution of the antisera i.d., followed 16–20 h later by the i.d. injection of antigen (BGG, 1 μg per site).

Induction, purification and radiolabelling of guinea-pig eosinophils

The method is described in detail elsewhere (Faccioli *et al.*, 1991). Briefly, eosinophils obtained from the peritoneal cavity of horse serum-treated ex-breeder female guinea-pigs (Harlan Porcellus, Oxon) were purified on a discontinuous percoll gradient. The cells obtained were washed twice in HBSS (calcium- and magnesium-free) and labelled with $^{111}InCl_3$ (100 μCi in 10 μl) chelated to 2-mercaptopyridine-N-oxide. The purity of the eosinophils obtained was always greater than 93%. The main contaminating cells were monocytes with a few neutrophils (<3%).

Measurement of local oedema formation and eosinophil accumulation in guinea-pig skin

Radiolabelled eosinophil infiltration and oedema formation were measured simultaneously. ^{125}I-labelled human serum albumin (~5 μCi) was added to the ^{111}In-labelled eosinophils and these were injected intravenously (5×10^6 cells per animal) into recipient guinea-pigs (350–400 g, Harlan Porcellus, Oxon) anaesthetized with Hypnorm (0.15 ml, i.m.). After 5 min inflammatory stimuli or antigen (in the sites pretreated with antiserum) were injected i.d. in 0.1 ml volumes into the dorsal skin of shaved animals with or without heparin. All drugs were mixed before the injection. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response (^{111}In-labelled eosinophil accumulation and oedema formation) was assessed after 2 h. At this time, a blood sample was obtained by cardiac puncture, the animals were killed by an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess of blood and the skin sites punched out with a 17 mm punch. The samples were counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks) and the counts were cross-channel corrected for the two isotopes.

Eosinophil accumulation is expressed as the number of ^{111}In-labelled eosinophils per skin site and oedema formation as μl of plasma obtained by dividing the ^{125}I counts of the skin sample by the ^{125}I counts in 1 μl of plasma.

Materials

Reagents The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): bradykinin, bovine gamma globulin, compound 48/80, zymosan, fucoidin. Hanks solutions, HEPES and horse serum were purchased from Gibco Limited (Paisley, Penfrewshire). Percoll was purchased from Pharmacia (Milton Keynes, Bucks), PAF from Bachem (Saffron Walden, Essex) and porcine intestinal mucosa heparin (Monoparin, 5000 iu ml^{-1}) from C.P. Pharmaceuticals (Wrexham, Wales). $^{111}InCl_3$ and ^{125}I-labelled human serum albumin were purchased from Amersham International (Amersham, Hertfordshire). Cercarial extracts were kindly generated and donated by Dr M.J. Doenhoff and C. McNiece (School of Biological Sciences, Bangor, Wales). Human recombinant interleukin-8 (IL-8) was a gift from Dr I. Lindley (Sandoz, Vienna, Austria).

Statistics

Data are presented as mean ± s.e.mean for the number of experiments indicated and were analyzed by using two-way analysis of variance (ANOVA) on normally distributed data. P values were assigned by the Neuman-Keuls procedure. Values of $P<0.05$ were considered significant.

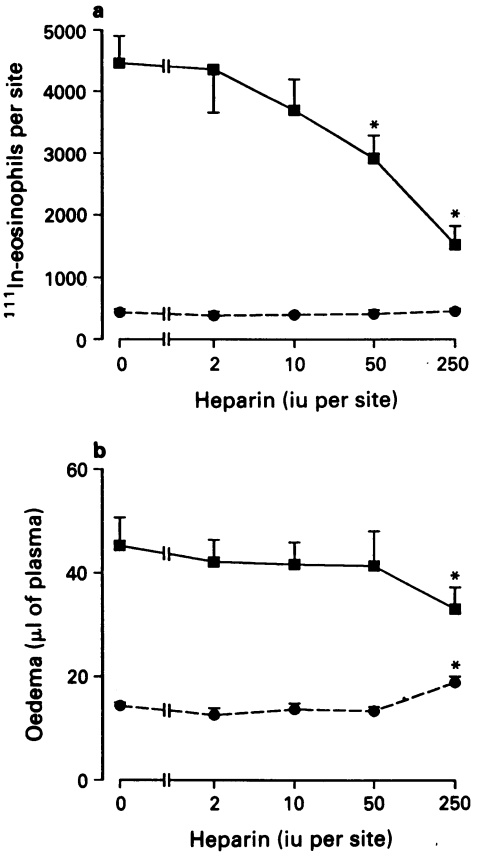

Figure 1 Effect of increasing concentrations of heparin on the accumulation of ^{111}In-labelled eosinophils (a) and oedema formation (b) induced by zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. Heparin was injected alone (●) or mixed with ZAP (30% in saline, ■). Results are mean ± s.e.mean of $n = 4$ guinea-pigs. *$P<0.05$ when compared to values obtained in the absence of heparin.

Results

Two hours after the injection of the ^{111}In-labelled eosinophils, 9.6 ± 1.3% ($n = 22$) of the labelled cells were circulating and over 94% of the total blood ^{111}In was bound to these cells. Although histology was not performed in this study, previous work from our laboratory has shown that the eosinophils do accumulate within 2 h in sites of inflammation (Faccioli *et al.*, 1991; Collins *et al.*, 1993). Autoradiographic data confirmed that radiolabelled cells migrated into the tissue (Weg *et al.*, unpublished observations).

Effect of heparin on ^{111}In-eosinophil accumulation

The effect of increasing doses of heparin on ZAP (10% solution in saline)-induced inflammation in guinea-pig skin is shown in Figure 1. Local administration of heparin dose-dependently inhibited ZAP-induced eosinophil accumulation (Figure 1a). At a dose of 50 iu per site, heparin also effectively inhibited the eosinophil accumulation induced by increasing doses of ZAP (Figure 2a) although the effectiveness of heparin appeared to diminish at higher doses of ZAP. Thus, eosinophil accumulation induced by 10%,

Table 1 Effect of i.d. heparin on eosinophil accumulation and oedema formation in guinea-pig skin

	^{111}*In-eosinophils* *Heparin* (iu/site)			*Oedema* (μl of plasma) *Heparin* (iu/site)		
	Control	10	50	*Control*	10	50
ZAP	4224 ± 357	3170 ± 304	2462 ± 288*	27.8 ± 2.0	27.6 ± 2.6	24.2 ± 2.2
PAF	6748 ± 548	3814 ± 604*	3079 ± 511*	69.2 ± 2.6	62.6 ± 5.0	61.9 ± 5.2
PCA	13067 ± 2723	8619 ± 1908*	6876 ± 2367*	47.5 ± 2.4	41.6 ± 2.5	49.5 ± 4.7
48/80	4170 ± 1027	2028 ± 113*	1166 ± 93*	46.6 ± 4.1	47.8 ± 2.8	39.6 ± 2.0
IL-8	1036 ± 140	902 ± 113	711 ± 100*	14.4 ± 1.7	15.8 ± 1.6	16.2 ± 2.1
Sal	750 ± 56	544 ± 48	570 ± 58	14.2 ± 0.6	14.4 ± 1.1	13.8 ± 0.8

All stimuli and the antigen were mixed with heparin prior to i.d. injection. ^{111}In-eosinophil accumulation and oedema formation were assessed after 2 h. The following stimuli were used: zymosan-activated plasma (ZAP, 10% in saline), PAF (5×10^{-9} mol per site), compound 48/80 (48/80, 10 μg per site), interleukin-8 (IL-8, 10^{-10} mol per site), antigen (PCA reaction, 1 μg of antigen per site) and saline injected sites (Sal). Results are expressed as the mean ± s.e.mean for 4–6 guinea-pigs. *$P<0.05$ when compared to control values.

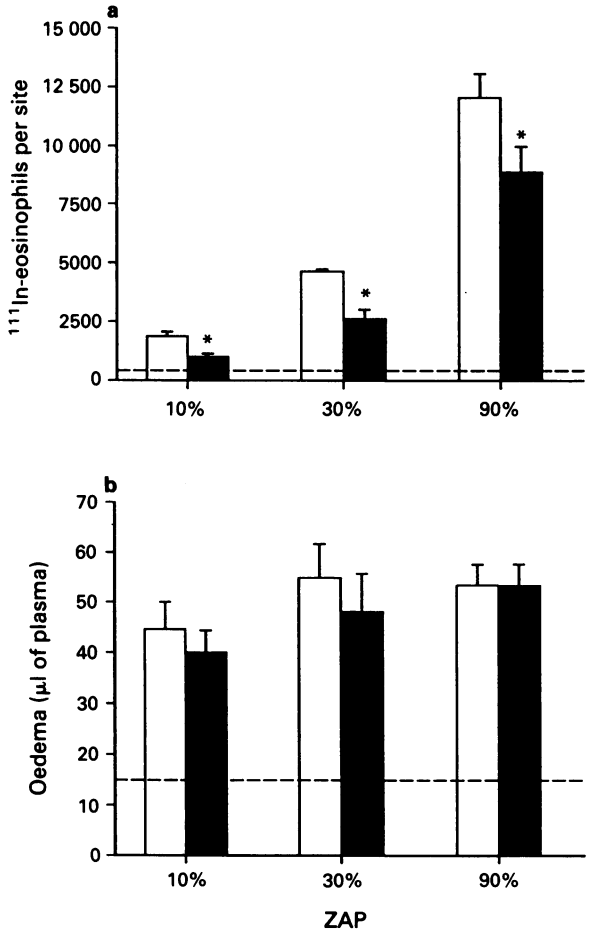

Figure 2 Effect of heparin on the accumulation of ^{111}In-eosinophils (a) and oedema formation (b) induced by increasing concentrations of zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. ZAP was injected alone (open columns) or mixed with heparin (50 iu per site, solid columns). The line across the graph represents background values obtained in response to i.d. injection of saline. Results are mean ± s.e.mean of $n = 4$ guinea-pigs. *$P<0.05$ when compared to values obtained in the absence of heparin.

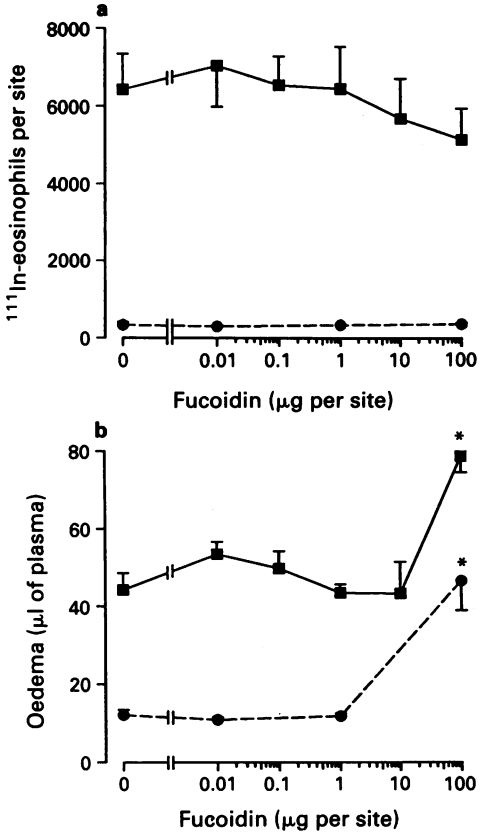

Figure 3 Effect of increasing concentrations of fucoidin on the accumulation of ^{111}In-labelled eosinophils (a) and oedema formation (b) induced by zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. Fucoidin was injected alone (●) or mixed with ZAP (30% in saline, ■). Results are mean ± s.e.mean of $n = 4$ guinea-pigs. *$P<0.05$ when compared to values obtained in the absence of fucoidin.

30% and 90% ZAP was suppressed by 64, 49 and 27%, respectively.

The effects of heparin on the inflammatory response induced by different stimuli is depicted in Table 1. In these experiments, local administration of heparin effectively inhibited the accumulation of eosinophils induced by various stimuli and in a PCA reaction (Table 1). Whereas heparin did not significantly inhibit ZAP-induced eosinophil accumulation at the dose of 10 iu per site (Figure 1a), it was effective against most mediators studied at this dose.

Effect of heparin on oedema formation

Local administration of heparin had no significant effects on local oedema formation induced by all mediators tested and in a PCA reaction (Figure 1b, Figure 2b and Table 1). Oedema formation induced by ZAP (10% in saline) was inhibited only at the top dose of heparin (250 iu per site) but it also induced significant oedema formation when injected alone (Figure 1b). In contrast, as presented above, heparin significantly reduced eosinophil accumulation in the same

Table 2 Effect of i.d. fucoidin and heparin on the oedema formation induced by extracts of *S. mansoni* larvae in guinea-pig skin

	% inhibition
Fucoidin (10 μg per site)	67.3 ± 7.9*
Heparin (50 iu per site)	27.5 ± 5.6*

Cercarial extracts and fucoidin or heparin were mixed prior to the i.d. injections and oedema formation was assessed after 2 h. Intradermal injection of cercarial extract alone (5 μg of protein per site) induced a leakage of 57.0 ± 9.5 μl compared with 13.3 ± 2.1 μl in saline-injected sites. Results are mean ± s.e.mean of $n = 4-6$ guinea pigs. *$P < 0.05$ when compared to values obtained in the absence of heparin or fucoidin.

sites. Bradykinin-induced oedema formation was also not significantly affected by heparin (bradykinin 10^{-10} mol per site, 52.1 ± 5.8 μl; bradykinin + heparin 10 iu per site, 47.5 ± 3.6 μl; bradykinin + heparin 50 iu per site, 44.0 ± 4.2 μl).

Effect of fucoidin on local inflammation in guinea-pig skin

The effects of fucoidin, a fungal polysaccharide with many electronegative charges, is shown in Figure 3. Fucoidin had no effect on ZAP-induced eosinophil accumulation when used up to 100 μg per site (Figure 3a). When injected alone or with ZAP, fucoidin, at the dose of 100 μg per site, caused an increased formation of oedema, but it had no effect when injected in smaller doses (Figure 3b). Nevertheless, fucoidin at a dose of 10 μg per site significantly inhibited the oedema formation induced by cationic protein-containing extracts of *Schistosoma mansoni* larvae (Table 2).

Discussion

The discovery of heparin some 70 years ago opened new and exciting possibilities in the treatment of human disease. With the use of this drug, it has been possible to perform cardiac and orthopaedic surgeries effectively, to anticoagulate properly high risk patients and, more recently, it is being used in the prevention of thrombotic complications following surgery, long term hospitalization and cancer (Hirsh, 1991; Freedman, 1992). Heparin has also received some attention previously as an anti-inflammatory drug but the results of different trials are conflicting (Boyle *et al.*, 1964; Fine *et al.*, 1968). Recent reports have again stressed the anti-inflammatory actions of heparin in man (Page, 1991; Volkl *et al.*, 1991).

We have found that heparin, when injected i.d. with several different known mediators of inflammation, antigen or compound 48/80, effective inhibited the accumulation of ^{111}In-labelled eosinophils. ZAP-induced neutrophil accumulation was assessed in some experiments and it was also found to be inhibited (data not shown). For example, ^{111}In-neutrophil accumulation induced by ZAP (10% in saline) was inhibited by 42% ($n = 5$, $P < 0.05$) by local administration of heparin (50 iu per site). Interestingly, heparin had no significant effect on local oedema formation induced by the same mediators or in a PCA reaction.

Because of its electronegative charge, heparin can bind to many different substances and this may lead to their inactivation or enhancement of their function (Jaques, 1980; Yamashita *et al.*, 1992). For example, heparin may interact with eosinophil cationic protein leading to inactivation of this highly toxic protein (Fredens *et al.*, 1991). If this happens *in vivo*, it may prove to be an important mechanism by which heparin, and possibly mast cells, may control tissue damage by cationic proteins (Fredens *et al.*, 1991). Heparin has also been shown to interact with and inhibit the action of compound 48/80, antigen and bradykinin (Jaques, 1980). We cannot exclude this interaction in our system since the stimuli were mixed with heparin prior to injection. However, the observation that oedema formation induced by the various stimuli was unchanged in the same sites that eosinophil (and neutrophil) accumulation was suppressed suggests that heparin inhibits eosinophil accumulation by a mechanism other than neutralization of the mediators injected or generated endogenously. This suggestion is supported by the results of the experiments carried out with cationic protein-containing extracts of *S. mansoni* larvae. Fucoidin significantly inhibited, by approximately 70%, extract-induced oedema formation, presumably as a result of charge mediated-neutralization. Heparin (50 iu per site) was less effective (approximately 30% inhibition). However, at this same 'charge-neutralizing' dose, fucoidin had no effect on ZAP-induced inflammation while heparin was inhibitory for eosinophil accumulation.

Leucocytes express different adhesion molecules which play an important role in their interaction with endothelial cells and migration into the tissue (reviewed in Williams & Hellewell, 1992). The first step in the leucocyte-endothelial cell adhesion is the loose attachment and rolling which occurs via a selectin-dependent mechanism (Ley *et al.*, 1991b; Williams & Hellwell, 1992). Heparin and also fucoidin, when given systemically, have been shown to inhibit the process of rolling and this appears to be related to the capacity of these drugs to interfere with L-selectin-mediated adhesion (Ley *et al.*, 1991a; 1993; Arfors & Ley, 1993). The observation that local administration of fucoidin did not inhibit eosinophil accumulation in guinea-pig skin suggests that interference by heparin with leucocyte rolling *in vivo* is unlikely to be the mechanism of its inhibitory action in the present study. However, heparin is actively taken up by endothelial cells (Hiebert & Jaques, 1976; Hiebert & Liu, 1991) and this leaves the possibility that it may be re-expressed on the lumenal side of the circulation where it could inhibit rolling of eosinophils. Furthermore, the ability of heparin to displace the binding of chemokines to proteoglycans on the endothelial surface (Rot, 1992; Tanaka *et al.*, 1993) may also represent a further mechanism by which it could interfere with leucocyte adhesion to endothelial cells, at least for IL-8-induced eosinophil accumulation.

Heparin and related polysaccharides may interact with extracellular matrix components and this may be important for the control of local matrix metabolism (Claman, 1969; Yamashita *et al.*, 1992). After attaching to endothelial cells, leucocytes have to interact with the extracellular matrix to move into the tissue (Nathan & Sporn, 1991; Williams & Hellewell, 1992). Heparins are capable of inhibiting tumour metastasis (Saiki *et al.*, 1991), an effect which is related to their ability to modulate the interaction of tumour cells with the extracellular matrix (McCarthy *et al.*, 1990; Asch *et al.*, 1991). Thus, this inhibitory effect of heparin on cell-extracellular matrix interaction may represent an additional mechanism by which heparin could inhibit tissue accumulation of eosinophils.

Eosinophils are potent cells associated with various allergic processes (Djukanovic *et al.*, 1990; Kay, 1991; Thorne & Mazza, 1991). In these diseases, eosinophil numbers and state of activation are related to worsening of symptoms and to the degree of lesion to the target organ (Djukanovic *et al.*, 1990). The safety of heparin (Jaques, 1980) and its capacity to inhibit eosinophil accumulation (this study) and function (Fredens *et al.*, 1991) may represent a potential therapeutic target for the control of diseases in which the oesinophils play an important pathogenetic role, such as asthma. The effects of heparin on the acute symptoms of allergen challenge in man may also be relevant to this situation (Bowler *et al.*, 1993).

In summary, we have shown in this study that local

administration of heparin dose-dependently inhibits the eosinophil accumulation in skin, induced by mediators of inflammation whereas it had little effect on oedema formation. This suggests that an interference with eosinophil traffic rather than just neutralization of the mediators is responsible for the inhibitory effects of heparin. The lack of effect of fucoidin, a highly electronegative molecule, on the accumulation of eosinophils further supports this idea.

M.M.T. was supported by Sandoz, Basel and P.G.H. by The National Asthma Campaign. We thank Dr M.J. Doenhof and Mr C. McNiece for the gift of cercarial extracts.

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(Received May 17, 1993
Revised July 16, 1993
Accepted July 29, 1993)