Low Doses of Low Molecular Weight Heparin *In vivo* Do Not Inhibit Delayed-type Hypersensitivity

Sir,

In 1994 Ingber et al. (1) showed that a low dose of low molecular weight heparin (Enoxaparin, Clexane) to a certain degree inhibited the elicitation of a positive patch test in 11 patients. A significant improvement was observed in 3 of 11 patients suffering from chronic allergic contact dermatitis. Since then low dose heparin has been applied in the treatment of severe recalcitrant contact dermatitis with some effect (personal communication). In support of this a study by Lider et al. showed that low dose heparin inhibited lymphocyte traffic as well as delayed-type hypersensitivity in mice (2). The aim of the present study was to evaluate the effect of low dose low molecular weight heparin on the delayed hypersensitivity reaction in rabbits. This model has earlier been used as a valuable model to study mechanisms of delayed-type hypersensitivity (3).

MATERIAL AND METHODS

Delayed-type hypersensitivity reaction

Twenty male New Zealand white rabbits were sensitised to *Mycobacterium tuberculosum*, as previously described (3). Five weeks after the sensitisation the rabbits were randomly allocated to one of three groups (A, B and C). The rabbits in group A ($n=7$) were injected subcutaneously with 50 µg/kg of low molecular heparin – Tinzaparin, Logiparin® (Novo Nordisk, Denmark), while the rabbits in group B ($n=7$) were injected with 50 µg/kg of low molecular heparin – Enoxaparin, Clexane® (Rhone Poulenc, France). The doses of low molecular heparin were chosen in accordance with the protocol of Ingber et al. (1). Group C ($n=6$) were injected with an equivalent amount of sterile saline, 0.9% serving as control group. The rabbits were shaved at an area of 8×20 cm on their backs. After 24 h the rabbits were injected with 10 U of tuberculin (tuberculin-purified protein derivative RT-23, 100 U/ml; Statens Seruminstitut) at two symmetrical sites on their backs. The identity of the rabbits was blinded to the observers performing the determination of erythema, skin thickness, reaction site area and histology.

Clinical examination of the tuberculin skin reaction

At 24 h after the tuberculin injection, clinical examination of the skin was performed by measuring the thickness of the skin on both sites of the tuberculin injection as well as normal skin by using a Digit Outside Micrometer, Series 193 (Mitutoyo, Japan). All values were measured three times each. The increase in dermal thickness is the difference between the thickness of the skin at the site of tuberculin injection and normal, untreated skin. The procedure was repeated at 48 h at the left site and at normal skin. In addition at 24 h and 48 h after the tuberculin injection the erythema of the skin was measured using a portable reflectance spectrophotometer (DermaSpectrometer; Cortex Technology, Denmark). The erythema was expressed as an erythema index between tuberculin injection site and normal skin. The diameter of the infiltrated area was measured at 24 h and 48 h. The average of three individual measurements was used for erythema, skin thickness and diameter.

Histological examination of the tuberculin skin reaction

Biopsies were obtained at 24 h (right site) and at 48 h (left site) after the tuberculin injection. Following local anaesthesia using lidocaine

Table I. *Effect of low dose low molecular heparin on a delayed hypersensitivity reaction*

Mean increase in erythema, skin thickness, mean diameter, oedema, and cell numbers in groups 1 (50 µg/kg of low molecular heparin – Tinzaparin, Logiparin®), 2 (50 µg/kg of low molecular heparin – Enoxaparin, Clexane®) and 3 (sterile saline 0.9%).

Mean ± SD	Group 1 $n=7$	Group 2 $n=7$	Group 3 $n=6$	χ^2	P	P adjusted
24 hours:						
Erythema (left)	10.9 ± 6.0	7.9 ± 3.3	6.1 ± 3.9	3.0	ns	ns
Erythema (right)	6.0 ± 2.5	2.8 ± 3.5	0.7 ± 3.7	6.5	*	ns
Skin thickness (left)	0.3 ± 0.3	0.3 ± 0.2	0.6 ± 0.4	1.9	ns	ns
Skin thickness (right)	0.5 ± 0.5	0.2 ± 0.3	0.5 ± 0.4	2.5	ns	ns
Diameter (left)	14.3 ± 1.4	17.1 ± 2.5	16.0 ± 3.9	0.3	ns	ns
Diameter (right)	4.9 ± 2.0	14.9 ± 1.5	15.7 ± 2.7	5.7	ns	ns
Oedema	1.4 ± 0.5	1.9 ± 0.4	1.2 ± 0.4	5.1	*	ns
Infiltration	1.4 ± 0.5	1.4 ± 1.0	1.7 ± 0.8	0.5	ns	ns
Monocytes	0.6 ± 0.5	0.3 ± 0.5	0.5 ± 0.5	1.2	ns	ns
Lymphocytes	0.4 ± 0.5	0.7 ± 0.8	1.0 ± 0.6	2.5	ns	ns
Neutrophils	1.4 ± 1.0	1.4 ± 1.1	1.0 ± 1.1	0.6	ns	ns
48 hours:						
Erythema (left)	14.5 ± 3.7	15.5 ± 3.7	12.9 ± 4.0	0.6	ns	ns
Skin thickness (left)	1.0 ± 0.5	1.0 ± 0.7	1.0 ± 0.6	0.1	ns	ns
Diameter (left)	17.0 ± 2.2	19.0 ± 6.6	19.2 ± 4.2	0.7	ns	ns
Oedema	1.4 ± 0.5	1.4 ± 0.5	1.3 ± 0.5	0.2	ns	ns
Infiltration	1.6 ± 0.5	2.0 ± 0.8	1.8 ± 1.0	1.4	ns	ns
Monocytes	1.1 ± 0.4	1.1 ± 0.9	0.7 ± 0.5	2.3	ns	ns
Lymphocytes	1.3 ± 0.8	1.0 ± 0.6	1.0 ± 0.6	0.9	ns	ns
Neutrophils	0.1 ± 0.4	0.4 ± 0.5	0.5 ± 0.5	2.0	ns	ns

* $p<0.05$ (two-sided) (Kruskal-Wallis test) significance levels adjusted for multiple comparison using the modified Bonferroni method.

with adrenaline, a 2-mm punch skin biopsy was obtained from the test area at the right site on the back at 24 h and from the left site at 48 h. Additional punch biopsies from normal-looking skin were obtained as controls. The biopsies were fixed and stained with hematoxylin and eosin, as previously described (3). The degree of cellular infiltration and oedema was scored, using scores from 0 to +++, with 0 indicating the number of infiltrating lymphocytes, monocytes and granulocytes as well as the amount of oedema not differing from normal skin, and +++ as the maximum score. Two dermatopathologists separately examined and scored the degree of cellular infiltration and oedema. The average of their individual scores was used.

Statistics

The data were analysed for normality using the Wilk-Shapiro test. Since the data were not found to be normally distributed, non-parametric statistics were used. The results for the three groups were compared using Kruskal-Wallis one-way ANOVAs. The significance levels were adjusted for multiple comparisons using the modified Bonferroni method.

RESULTS

As seen in Table I no difference was found between low dose low molecular weight heparin and saline for the delayed type hypersensitivity reaction. The variation in erythema index seems to show some variation. This can be explained by the shaving of the animals, which irritates the epidermis.

DISCUSSION

We were unable to replicate the results of Ingber et al. (1). There may be several reasons for this discrepancy. Our study was based on a rabbit model, and although inflammatory reactions and the mechanism of heparin in rabbits are similar to reactions in mice and humans there could be species-related differences. Both delayed hypersensitivity reactions, positive patch tests and contact allergies are dependent on allergen recognition by specific T-lymphocytes. It is therefore unlikely that the contradictory results are due to the different models used. Furthermore, the histological characteristics of the reactions are similar when studied in a time-dependent fashion (4–6). The dose of low molecular heparin used could also be critical. We used an equivalent amount of both drugs as used in the original paper (50 μg/kg). Ingber et al. reported that 38% of the patch tests were negative in the group treated with low dose low molecular heparin, while 7% in the control group were negative. The reason for these results remains to be explained.

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Response to the Letter by Zachariae et al.

The negative results reported in the Letter published above do not negate our published results of the effect of a particular batch of a low molecular weight heparin (LMWH) preparation for several reasons, singly or in total.

1. Only some batches of LMWH preparations are effective, since some batches do not contain active molecules. The effective batches have to be pretested in animal models, mice or rats, to ascertain their suitability. Batches that do not work in mice and rats probably will not work in humans. An in vitro test that correlates with in vivo effectiveness will soon be ready for publication.

2. We have never tried a batch of LMWH in rabbits but expect that a batch ineffective in rabbits will probably be ineffective in rats or mice.

3. The doses of LMWH in mice, rats and humans are not to be computed at a single dose per kg. The dose has to be adjusted for surface area. For example, a given LMWH batch may be effective in humans at 15 μg/kg, in rats at 100 μg/kg and in mice at 200 μg/kg. We have no idea where rabbits fit in. In any case, different batches of LMWH will have different optimal doses, which we must ascertain for each batch by dose-response curves in mice or rats. The optimal dose is critical, since the dose-response curve is bell-shaped: higher than optimal doses can be ineffective. Thus, the LMWH batch used in the rabbit study could have been ineffective because that particular batch lacked the active molecules, or the batch may have been potentially effective but was used at the wrong doses.

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