

Suppression of Experimental Autoimmune Diseases and Prolongation of Allograft Survival by Treatment of Animals with Low Doses of Heparins

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Abstract

The ability of activated T lymphocytes to penetrate the extracellular matrix and migrate to target tissues was found to be related to expression of a heparanase enzyme (Naparstek, Y., I. R. Cohen, Z. Fuks, and I. Vlodavsky. 1984. *Nature (Lond.)* 310:241-243; Savion, N., Z. Fuks, and I. Vlodavsky. 1984. *J. Cell. Physiol.* 118:169-176; Fridman, R., O. Lider, Y. Naparstek, Z. Fuks, I. Vlodavsky, and I. R. Cohen. 1987. *J. Cell. Physiol.* 130:85-92; Lider, O., J. Mekori, I. Vlodavsky, E. Baharav, Y. Naparstek, and I. R. Cohen, manuscript submitted for publication). We found previously that heparin molecules inhibited expression of T lymphocyte heparanase activity in vitro and in vivo, and administration of a low dose of heparin in mice inhibited lymphocyte traffic and delayed-type hypersensitivity reactions (Lider, O., J. Mekori, I. Vlodavsky, E. Baharav, Y. Naparstek, and I. R. Cohen, manuscript submitted for publication). We now report that treatment with commercial or chemically modified heparins at relatively low doses once daily (5 µg for mice and 20 µg for rats) led to inhibition of allograft rejection and the experimental autoimmune diseases adjuvant arthritis and experimental autoimmune encephalomyelitis. Higher doses of the heparins were less effective. The ability of chemically modified heparins to inhibit these immune reactions was associated with their ability to inhibit expression of T lymphocyte heparanase. There was no relationship to anticoagulant activity. Thus heparins devoid of anticoagulant activity can be effective in regulating immune reactions when used at appropriate doses.

Introduction

Activated T lymphocytes express an endoglycosidase that specifically degrades the heparan sulfate moiety of the subendothelial extracellular matrix (ECM),¹ which seals the vascular compartment (1-3). This heparanase was found to be asso-

ciated with the ability of lines of T lymphocytes reactive to myelin basic protein (BP) to penetrate the blood-brain barrier, to accumulate in the central nervous system, and to cause experimental autoimmune encephalomyelitis (EAE) (1). This paper provides more direct evidence that heparanase is critical to the ability of T lymphocytes to negotiate through vascular barriers; inhibition of expression of endogenous heparanase by administration of a suitable dose of heparin deranged lymphocyte traffic and functionally incapacitated T lymphocytes adoptively mediating delayed-type hypersensitivity.² The studies constituting this paper were done to investigate the effects of heparin on cell-mediated immunological reactions of clinical interest: autoimmune disease and allograft rejection. We now report that relatively low doses, but not high doses of heparin were markedly effective in preventing or inducing remissions of EAE and adjuvant arthritis (AA) in rats. Low-dose heparin also prolonged the survival of fully allogeneic skin grafts on mice. These effects were produced also by heparin molecules devoid of anticoagulant activity.

Methods

Animals

Rats. Inbred Lewis rats were obtained from the Animal Breeding Center of the Weizmann Institute of Science, Rehovot, Israel. Rats used were 2-3 mo old and were matched for age and sex in each experiment.

Mice. Inbred mice of strains C57BL/6 (H-2^b), BALB/c (H-2^d), and SJL (H-2^e) were purchased from Jackson Laboratories (Bar Harbor, ME). 1.5-2-mo-old females were used.

Active induction of AA

To induce active AA, rats were inoculated intradermally at the base of the tail with 0.1 ml CFA containing 10 mg/ml *Mycobacterium tuberculosis* H37Ra (MT) in incomplete Freund's adjuvant (DIFCO Laboratories, St. Louis, MO) (4). The system described by Trentham et al. was used to assess severity of arthritis (5). Briefly, each paw was graded clinically based on erythema, swelling, and deformity of the joint. To avoid unconscious bias in judging the clinical score, arthritis was graded by a colleague ignorant of the experimental protocol and the identity of the group. For the sake of uniformity with the clinical scoring of EAE, the range of AA clinical scores was computed to be 0-100 instead of 0-16 as described by Trentham et al. The clinical diagnosis of AA was confirmed by histologic examination of the joints of selected rats (6).

EAE adoptively transferred by lymph node cells

Adoptive EAE was produced as described by Panitch and McFarlin (7). Donor rats were sensitized in the hind footpads with guinea-pig BP

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1. Abbreviations used in this paper: AA, adjuvant arthritis; BP, basic protein of myelin; EAE, experimental autoimmune encephalomyelitis; EAT, experimental autoimmune thyroiditis; ECM, extracellular matrix; MT, *Mycobacterium tuberculosis*.

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dissolved in PBS and emulsified with an equal volume of incomplete Freund's adjuvant containing added MT. Each rat received subcutaneously a total of 0.1 ml containing 50 μ g BP and 400 μ g MT. The draining lymph nodes were removed 9 d later and their cells were washed and seeded in DME containing 10 μ g/ml BP, 1% fresh autologous serum, and additives as described (8). After 4 d of incubation (37°C, 7.5% CO₂ in humidified air) the cells were collected, washed twice in PBS, counted, and injected intravenously into naive syngeneic recipient rats at a concentration of 1.5 \times 10⁷ cells/ml per rat. Rats were observed daily for signs of EAE, which were rated according to the method described by McFarlin et al. (9), except that the scores were adjusted to a scale of 0–100 instead of 0–4. The individual judging the clinical scores was ignorant of the experimental design.

Skin allografting

Full-thickness skin grafting was done as described (10). Briefly, the dorsal skin of recipient mice was shaved, washed with PBS, and dried. A circular piece of skin \sim 1 cm in diameter was removed and replaced by donor skin, which was cleaned of fat and connective tissue. The attachment of the skin graft to the recipient was achieved using an acrylic plastic spray (Nobecutane spray; Astra, Södertälje, Sweden).

Degradation of ECM heparan sulfate by T lymphocyte heparanase

Lymphocytes (2 \times 10⁷/ml) suspended in 0.5 ml RPMI 1640 medium containing 5% FCS, antibiotics and 1.5 μ g/ml Con A were seeded on ³⁵S-labeled ECM tissue culture wells, pH 6.5, and incubated at 37°C, in 10% CO₂ as described (3).² 5 μ g/ml heparins were added to the cell cultures. After 48 h, the medium was collected, centrifuged at 10,000 g for 3 min and filtered through Sepharose 6B columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Low molecular weight labeled material eluted in peak II (K_{av} , \diamond 0.70, fractions 30–40) contained heparanase-mediated degradation products of heparan sulfate side chains, as confirmed by their sensitivity to deamination with nitrous acid (1–3).

Treatment with heparins

Modified heparins that were prepared by Institut Choay (Paris, France), were donated by Prof. A. Eldor (Department of Oncology, Hadassah Medical Center, Jerusalem), and included: total desulfated heparin; N-desulfated, N-acetylated heparin; total desulfated, N-acetylated heparin; and total desulfated, N-resulfated heparin. All these heparins exhibited < 5% the anticoagulant activity of unmodified commercial heparin. Unmodified commercial heparin was obtained from Leo Pharmaceutical Products (Ballerup, Denmark). The heparins were diluted or dissolved in PBS and the designated amounts were injected subcutaneously in a volume of 0.1 ml daily into treated mice or rats. Control animals were sham injected with PBS.

Results

Low-dose heparin induces remission of AA. AA is a disease inducible in certain strains of rats by immunization to antigens of MT (11). The disease appears \sim 11–12 d after immunization and is characterized by mononuclear cell infiltration of the synovia, most prominent in the small joints of the extremities, with pannus formation. The process may progress for months resulting in destruction of bone and ankylosis of joints. AA appears to be caused by T lymphocytes that recognize an antigen of MT cross-reactive with the proteoglycan of joint cartilage (12). AA is thus an autoimmune disease induced by molecular mimicry (13).

To test the effects of treatment with heparin, we induced AA in Lewis rats by immunization to MT and then 8 d later, a week before the onset of overt clinical arthritis, we treated the rats with a single dose of commercial heparin of 1, 5, 10, 20, or 40 μ g administered subcutaneously daily for 7 d. The progres-

sion of AA was observed for 90 d. Fig. 1 shows the results in the groups receiving 1, 20, or 40 μ g. It can be seen that administration of 20 μ g of heparin led to a marked decrease in both severity and duration of AA. In contrast, administration of either 1 or 40 μ g had no effect. Doses of 5 and 10 μ g produced a partial inhibition of disease (not shown). Therefore, similar to suppression of T lymphocyte migration and delayed type hypersensitivity as shown in another investigation,² a relatively low dose of heparin produced a marked suppression of T lymphocyte reactivity, in this case that associated with AA. Likewise, increasing the dose of heparin led to a decrease in its inhibitory effect on T lymphocyte reactions. Histologic examination of the joints by light and electron microscopy at 90 d showed marked pannus formation and destruction of articular cartilage in the untreated rats and minimal lesions in the clinically well, treated rats (Stanescu, R., et al., manuscript in preparation).

Fig. 2 shows that heparin was effective in aborting AA even when treatment was begun on day 21, after the clinical onset of arthritis. Signs of AA did not recur after cessation of treatment at day 50. Thus, suppression of arthritis for part of its course by administration of heparin caused a permanent remission of disease.

Inhibition of AA produced by chemically modified heparin. To investigate the nature of the heparin molecules responsible for enzyme inhibition, disease suppression, and anticoagulation, we tested the effects of several chemically modified heparins.

Fig. 3 shows the results of an experiment in which, instead of commercial heparin, Lewis rats were treated with a chemically modified, N-desulfated, N-acetylated heparin devoid of anticoagulant activity. Here it can be seen that a daily dose of 20 μ g was more effective than higher doses (200 or 2,000 μ g). Similar to unmodified commercial heparin, the modified heparin thus was more effective when administered at a relatively low dose than at much higher doses. Similar to unmodified heparin, doses of 1 μ g or less daily had no effect on arthritis (not shown).

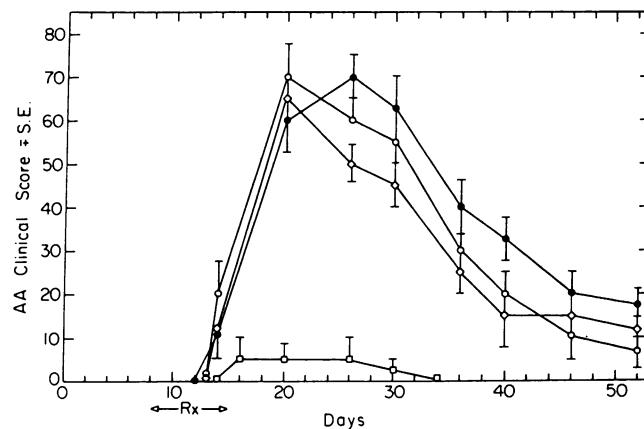


Figure 1. Prevention of AA using commercial heparin. Lewis rats were immunized with MT for the induction of AA. 8 d later, the rats were divided into four groups of five to six rats each of which was treated (□, \diamond , ○) for 7 d with commercial heparin or remained untreated (●). Heparin was administered subcutaneously once daily at various doses: 1 (○); 20 (□) and 40 μ g (\diamond). Rats were examined daily and scored for arthritis.

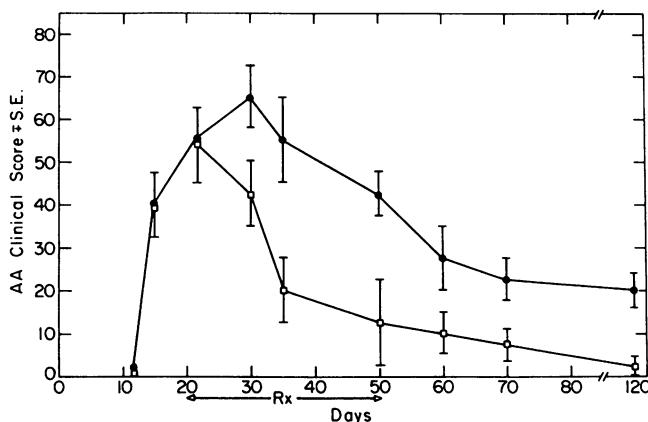


Figure 2. Treatment of established AA with commercial heparin. Groups of 10 rats were inoculated with MT to induce AA. One group remained untreated (●), and the other was treated with commercial heparin, 20 μ g/d from day 20 until day 50 after immunization (□). AA was observed daily until day 120. From day 35 on, the severity of AA of the two groups was significantly different ($P < 0.05$).

Table I summarizes our experience with commercial heparin and four heparins modified by desulfatation and acetylation in which we compared their effects on coagulation, on heparanase degradation of ECM, and on AA.

It can be seen that anticoagulation and heparanase inhibition were unrelated. The four modifications led to a significant loss of anticoagulant activity, while two of the modified heparins depleted of anticoagulant activity retained their activity as heparanase inhibitors: totally desulfated, partially N-resulfated, and N-desulfated and N-acetylated.

The modified heparins were tested for their ability to inhibit AA when administered between days 8 and 14. Table I shows the percent inhibition of arthritis on day 21 as a measure of the effect of the heparin given earlier. It can be seen that the N-desulfated, N-acetylated heparin was about as effective as unmodified heparin. The desulfated partially N-resulfated heparin was relatively less effective in inhibiting AA, although it produced a clear decrease in the severity of disease. The two

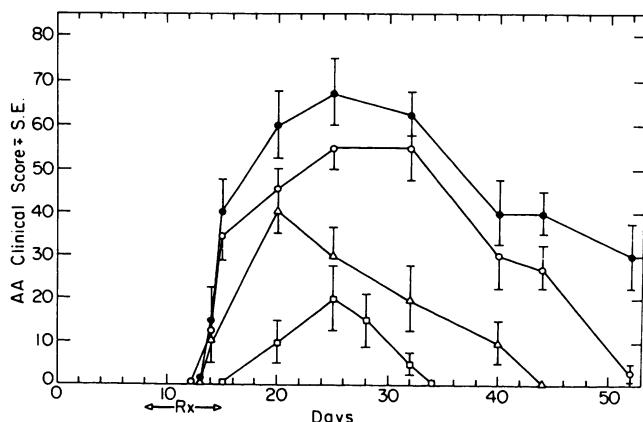


Figure 3. Prevention of AA using modified heparin. Groups of 10 rats were immunized with MT to induce AA, and treated in the same manner as described in the legend to Fig. 1 with N-desulfated, N-acetylated heparin, or remained untreated (●). The daily doses were: 20 (□), 200 (△), and 2,000 μ g (○).

Table I. Prevention of AA and Inhibition of Heparanase Activity by Heparins Are Correlated

Heparin	Anticoagulant activity U/mg	% Inhibition of heparanase activity	
		% Inhibition of heparanase activity	% Inhibition of AA
Commercial	150	100	80
N-desulfated	11.5	99	75
N-acetylated	0	0	<5
Total desulfated	14.5	3	<5
Total desulfated	11.5	96	50
N-resulfated			

Heparins were assayed for anticoagulant activity by their capacity to inhibit partial thrombin time test as described (20). Inhibition of heparanase activity was tested by the effect of the test heparin on the degradation of 35 S-labeled ECM by heparanase secreted by the ESB subline of a DBA/2 mouse T lymphoma line as described (20). The ability of heparin to inhibit AA was measured by the percent reduction in AA clinical score observed on day 21 in rats treated with 20 μ g of the heparin daily on days 8–14 after induction of AA by immunization with MT.

other modified heparins that were not inhibitors of heparanase in vitro had no significant effect on AA. Thus there appeared to be an association between the ability of a heparinoid to inhibit heparanase in vitro and its ability to influence AA in vivo. In contrast, there was no correlation of these phenomena with anticoagulant activity. With regard to the effects of the chemical modifications on heparanase and AA, it appears that sulfatation of the heparins was critical; totally desulfated heparins, with or without N-acetylation, were ineffective while N-resulfation restored both activities. N-desulfated, N-acetylated heparin, of which the O-sulfate groups were still intact, was also effective in inhibiting both heparanase and AA.

Low-dose heparin inhibits T lymphocyte-mediated EAE. The effects of low-dose commercial and modified heparins on adoptively transferred EAE were also studied. Fig. 4 shows the effect of treating Lewis rats with a subcutaneous inoculation of 20 μ g of commercial or N-desulfated, N-acetylated heparin daily. Treatment was begun 1 d before rats received a single intravenous inoculation of 1.5×10^7 activated cells of anti-BP primed lymph node cells. It can be seen that the treated rats had a marked reduction in the severity of adoptive EAE. Similar to the effects of heparinoids on AA, a higher dose (50 μ g) or a lower dose (1 μ g) of heparinoid had a markedly decreased effect on EAE (not shown).

Heparins inhibit rejection of skin allografts. Primary rejection of skin allografts is accompanied by invasion of host T lymphocytes into the graft (14). If expression of T lymphocyte heparanase is critical to this invasion then treatment of graft recipients with heparin might impede penetration of the graft by host T lymphocytes thus prolonging survival of the graft. Fig. 5 B illustrates that a daily dose of 5 μ g of heparin markedly extended the 50% survival time of fully allogeneic skin grafts from day 10 to day 20. Fig. 5 A shows that N-desulfated, N-acetylated heparin also prolonged graft survival. Higher doses (20–25 μ g) and lower doses (1 μ g) of heparinoids were

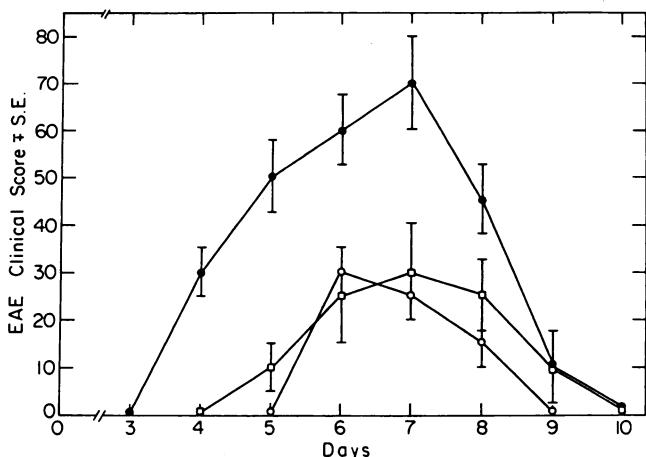


Figure 4. Prevention of adoptively transferred EAE using commercial or modified heparins. Groups of seven rats were inoculated intravenously with 1.5×10^7 activated anti-BP lymph node cells. The rats received 20- μ g daily doses of commercial heparin (□) or N-acetylated, N-desulfated heparin (○) beginning 1 d before inoculation of the lymph node cells until 10 d later. One group of recipient rats received no heparin (●). Rats were examined daily for EAE.

much less effective in prolonging allogeneic skin grafts (not shown).

Discussion

Heparin has been shown previously by others to act as an antiinflammatory agent effective in modulating EAE (15) or preventing delayed-type hypersensitivity (16). It was also shown to influence the traffic of lymphocytes (17). Our discovery that commercial heparin or certain chemically modified heparins at surprisingly low doses have a specific inhibitory effect on T lymphocyte heparanase expression and cell traffic provides a rationale and guide to the use of heparinoids as immunomodulators.² A key observation was that these heparanase inhibitors were less effective at higher doses than they were at lower doses both *in vivo* and *in vitro*. As shown in this report, we were able to inhibit the experimental autoimmune diseases EAE and AA in rats, and allograft rejection in mice by using suitable doses of heparins.

Heparins can inhibit the activity of free heparanase enzyme, probably by competing with the heparan sulfate sub-

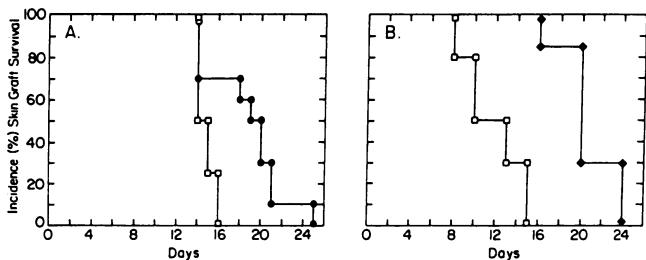


Figure 5. Heparins inhibit rejection of skin allografts. (A) Donor skin originating from C57BL/6 mice was grafted to BALB/c recipients that were (●) or were not (□) treated daily with 5 μ g of N-desulfated, N-acetylated heparin beginning 1 d before grafting and continuing until rejection. (B) Donor skin originating from SJL mice that were (●) or were not (□) treated with 5 μ g commercial heparin daily as described for A.

strate which they resemble. However, competitive inhibition is unlikely to be the mechanism by which heparins inhibit T cell-mediated reactions, because inhibition of free enzyme *in vitro* does not appear to decrease at higher concentrations of heparins (manuscript in preparation). Indeed, preliminary evidence suggests that the inhibitory effect of heparins may result from a direct interaction with T lymphocytes, leading to a decrease in the expression of heparanase by the cells.² This cellular effect of heparins appears to be most efficient at an optimal concentration, thereby accounting for the peculiar dose-response characteristics of inhibition of T cell-mediated reactivity.

Although coagulation probably has a role in cell mediated immune reactions (18), it is clear that the effects of heparins described here and in another investigation² were unrelated to any effects on coagulation; the doses of heparin were a fraction of those required for anticoagulation and modified heparins depleted of anticoagulation properties were effective. In contrast, there appeared to be an association between the chemical identity of heparinoids active as inhibitors of heparanase expression and their effects on T lymphocyte functions. Heparin is a crude mixture of ill-defined molecules only some of which are inhibitors of heparanase. It is thus difficult at present to isolate the effect of heparanase inhibition from the many other biological activities associated with whole heparin (19). Nevertheless, these results suggest that even crude commercial heparin used at relatively low doses may have a therapeutic potential in autoimmune and other conditions in which it would be desirable to selectively inhibit lymphocyte traffic.

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