# Sulfated Polysaccharides and Resistance to Russell's Viper Venom.* (27446)

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The importance of connective tissue in resistance to injury is undoubtedly dependent on the functions of the various cells and substances which characterize this tissue. Considerable attention has been given to factors influencing the permeability of connective tissue and interest has been focused largely on hyaluronic acid and its relationship to the viscosity of the ground substance(1). However, other connective tissue mucopolysaccharides possessing different activities may also participate in the over-all protective functions of this tissue. Heparin, for example, is a highly sulfated mucopolysaccharide and has the potentiality to interact with a variety of biologically-active substances and alter their effectiveness(2). This macromolecule is stored in relatively high concentrations in the mast cells of the connective tissue and can be readily secreted from these cells in response to various kinds of injurious stimuli (3). The participation of heparin in local mechanisms of resistance in the tissues has been suggested by the observations that noxious cationic substances such as compound 48/80, polymyxin B, etc., stimulate the secretion of heparin from mast cells and that heparin, in turn, can neutralize the lethal effects of these injurious agents(4).

It has also been observed that local injection of small amounts of Russell's Viper venom can stimulate a rapid degranulation of tissue mast cells(4) and that heparin can inhibit the lethal effects of this noxious material(4,5). This venom is a naturally occurring toxic material which characteristically induces marked changes in the clotting system of the blood in the envenomed individual. These changes can range from a hemorrhagic condition resulting from fibrinoginopenia and vascular injury to an acute generalized thrombosis and death(5). The lethal potency of this venom, however, is largely dependent on the route by which it is administered. For example, mice can tolerate approximately 25 times more venom by the subcutaneous route than by the intravenous route(6). Inasmuch as injection of venom by the latter route involves direct interaction of this noxious material with connective tissue, it is possible that the injurious effects of the venom are modified by heparin and other endogenous mucopolysaccharides of this tissue.

The present study has sought to determine the relative effectiveness of heparin and other connective tissue mucopolysaccharides with regard to their respective abilities to protect mice against the lethal effects of this venom. It was found that this protective activity could be readily demonstrated with some sulfated mucopolysaccharides such as heparin, heparin monosulfate or chondroitin sulfate B, but not with others such as chondroitin sulfates A or C (shark cartilage) or with the non-sulfated mucopolysaccharide, hyaluronic acid. Sulfated polysaccharides of an exogenous origin such as fucoidin, dextran sulfate and polyglucose sulfate also proved to be active. Heparin was found to be effective in very small amounts and was the most active substance tested.

*Materials and methods.* Male mice of the Fairfield-Webster strain (Euers Farm, Austin, Texas) weighing 19 to 21 g were used throughout this study. *Challenge and treatment*: All injections were administered by the intravenous route (except where noted) and were contained in a volume of 0.25 ml sterile, pyrogen-free normal saline. Drugs used were: Russell's Viper venom (Stypven, Burroughs Wellcome & Co.), heparin (Na salt, 129 u/mg, Upjohn Co.), heparin monosulfate (polysaccharide monosulfate, lot No. 817-LLC-250, Upjohn Co.), chondroitin sulfate A (General Biochemicals, Inc.), chondroitin

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sulfate B (beta heparin, Hoffmann-La Roche, Inc.), chondroitin sulfate C (shark cartilage, Kakenyaku Kako Co., Ltd.), hyaluronic acid (Nutritional Biochemicals Corp.), dextran sulfate (10,000 mol. wt., Commercial Solvents Corp.), fucoidin(7) and polyglucose sulfate (8). Data were collected on the basis of numbers of animals surviving for 24 hours after venom challenge. A minimum of 5 animals was used for each dose level tested in the various titrations of venom or polysaccharide activities. Each experiment was repeated at least 2 times and results accumulated. Each result thus represents data obtained from a minimum of 15 animals for each dose level of the various agents tested. The 50% doses were calculated by the method of Miller and Tainter(9).

*Results.* The acute lethal effects of Russell's Viper venom in mice were manifested within 5 to 15 minutes after intravenous challenge. Symptoms of envenomation such as excitement with running and incoordination generally preceded a short phase of convulsive activity which rapidly terminated in death of the challenged animal. Gross and microscopic examination of the organs from animals dying from venom revealed a marked congestion of the liver, spleen, kidney and lungs. In many of the envenomed animals, clotting of the blood was detectable in the inferior vena cava and portal vein almost immediately after death and small well-formed clots were occasionally noted in the right ventricle. A very small percentage of the animals died with a profuse hemorrhage in the lungs. This latter condition became apparent about 30 minutes after challenge and occurred only in mice that had apparently recovered from the initial toxic effects of the venom. It is of interest that this condition never occurred in animals treated with heparin. The majority of all animals surviving the acute toxic phase of envenomation showed rapid recovery and their mortality over a 30-day period was not greater than that of saline-treated controls.

The $LD_{50}$ of the venom preparation used in this study was found to be 3.0 $\pm$ 0.4 $\mu$g/mouse. The dose of venom used for challenge was 5.0 $\mu$g/mouse and was equivalent to an approximate $LD_{95}$. The protective effect of the various polysaccharides was determined by administering one of a series of graded doses of the test material in mixture with the challenge dose of venom. Accumulated results of heparin titrations are shown in Fig. 1. Each point in the figure represents results obtained with 15 animals. It should be noted that there is a straight line relationship between the log of the heparin dose and the probit of the percent survival of the treated animals challenged with the venom. The protective effect of heparin was demonstrated within a dose-response range of 5 to 50 $\mu$g of heparin with a 50% protective dose ($PD_{50}$) of 11.0 $\mu$g/mouse. The results indicate that adequate amounts of heparin can protect animals against this dose of venom and it should be emphasized that this protective effect represents an increase in survivorship rather than merely an increase in survival time.

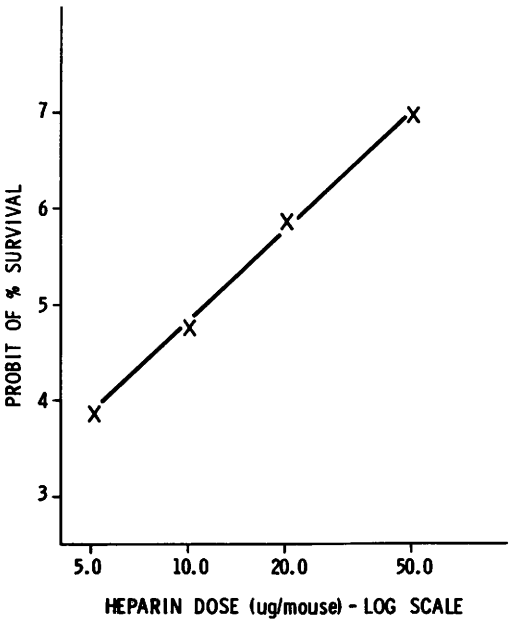


FIG. 1. Dose-response protective effect of heparin in mice inj. intrav. with one of a series of graded amounts of heparin in mixture with a lethal dose of venom.

The protective activity exhibited by heparin was also demonstrated with other polysaccharides of the connective tissues. Of the glucosamine containing mucopolysaccharides, heparin monosulfate was approximately one-seventh as active as heparin and hyaluronic

TABLE I. Comparison of Protective Activities of Various Sulfated Polysaccharides (I.V. Route) *vs* Lethal Dose of Venom.

| | 50% protective doses of test agents ($\mu$g/mouse) | | | | |
|---|---|---|---|---|---|
| Heparin | 11.0 ± 1.96 | Chondroitin sulfate A | >500 | Fucoidin | 260 ±77.8 |
| Heparin monosulfate | 80.0 ±12.9 | Chondroitin sulfate B | 134 ±10.1 | Dextran sulfate | 88 ± 9.3 |
| Hyaluronic acid | >500 | Chondroitin sulfate C | >500 | Polyglucose sulfate | 210 ±55.4 |

acid was inactive (Table I). Inasmuch as heparin is more strongly sulfated than heparin monosulfate and hyaluronic acid is nonsulfated, this limited comparison would appear to suggest that the relative potencies of these substances are associated with degree of sulfation of the respective macromolecules. However, the galactosamine containing mucopolysaccharides, chondroitin sulfates A, B, and C are sulfated to approximately the same degree as heparin monosulfate, yet differ in their activities. Other molecular characteristics must therefore also be of importance. For example, chondroitin sulfate B contains iduronic acid in contrast to the glucuronic acid moiety characteristic of chondroitin sulfates A and C and the activity of chondroitin sulfate B was found to be approximately one-twelfth of that of heparin whereas chondroitin sulfates A and C were inactive.

Exogenous polysaccharides of diverse sources and sulfated to almost the same degree as heparin were also tested. Protective effects could be readily obtained with these "heparinoid" substances, but they were markedly less active than heparin (Fig. 1). Fucoidin, a naturally-occurring sulfated plant polysaccharide, was obtained from Dr. George F. Springer who has reported that it possesses a potent anticoagulant activity(7). The protective activity of this polysaccharide however, was equivalent to only 1/24 of that of heparin. Dextran sulfate, a chemically sulfated bacterial polysaccharide, was somewhat more active with a potency similar to that of heparin monosulfate. Polyglucose sulfate, a chemically sulfated synthetic polymer which was obtained from Dr. P. T. Mora(8), was also active, but to a lesser degree. Despite the considerable differences among the various effective polysaccharides as to source, structure and potency, their dose-response curves were roughly parallel to that of heparin. This would suggest that similar mechanisms of activity may be involved. Quite obviously, however, the greater potency of heparin is a reflection of the unique molecular characteristics of this substance (or mixture of substances(9)) and is not solely dependent on its degree of sulfation.

The protective effects of the connective tissue mucopolysaccharides could also be demonstrated if these substances were administered separately and previous to the challenge dose of venom. Groups of mice were pretreated with one of a series of graded doses of the respective test substances by the intraperitoneal route 30 minutes prior to challenge with the venom by the intravenous route. Heparin was found to be almost 3 times as active as heparin monosulfate and approximately 19 times as potent as chondroitin sulfate B (Table II) whereas chondroitin sulfate A and hyaluronic acid were inactive. Chondroitin sulfate C was not tested. When the $PD_{50}$ results are compared to those obtained in the previous experiment, it is apparent from their "relative activities" that the active mucopolysaccharides are far more effective if allowed to interact directly with the venom prior to injection than if administered previous to and separately from the venom. Pretreatment by the intraperitoneal route is obviously a less sensitive means for comparing heparin to other polysaccharides and it is quite probable that results may be influenced by factors unrelated to the intrinsic protective activities of the respective test

TABLE II. Comparison of Protective Effects of Tissue Mucopolysaccharides (I.P. Route) *vs* Lethal Dose of Venom.

| Test mucopolysaccharides | I.P. $PD_{50}$ ($\mu$g/mouse) | I.V./I.P. ratio |
|---|---|---|
| 1. Heparin | 77 ± 18.6 | .143 |
| 2. Heparin monosulfate | 220 ± 48.2 | .363 |
| 3. Chondroitin sulfate B | 1400 ± 653 | .096 |

substances. For example, heparin monosulfate is a much smaller molecule than either heparin or chondroitin sulfate B and hence may be able to diffuse through the tissues more rapidly than either of these larger macromolecules. This may explain the observation that the "relative activity" of heparin monosulfate is approximately 3 times greater than that of heparin or of chondroitin sulfate B.

*Discussion.* Our results support and extend the previous observations of Ahuja(5) that heparin can protect animals against the lethal effects of Russell's Viper venom. However, the means by which heparin and other sulfated polysaccharides produce their protective effects has not been established.

If the lethal effect of the venom is primarily due to its powerful coagulant action on the blood(5), then the observed protective effect of heparin may be mediated through its potent anticoagulant action on the active proteins of the blood. On the other hand, heparin could produce similar effects by direct neutralization of the toxic constituents of the venom. These two possible mechanisms of protection need not be mutually exclusive and it may very well be that this mucopolysaccharide also possesses a potent antivenom activity in addition to its recognized anticoagulant activity. It is noteworthy in this regard that heparin was found to be approximately 10 times more effective when given simultaneously in mixture with the venom than when given separately and previous to the challenge. Moreover, results in current studies have indicated that heparin can also inhibit a local vasonecrotic effect resulting from local injection of this venom and heparin may therefore have an extravascular protective action. It has also been observed that small amounts of heparin can visibly precipitate this toxic material *in vitro*. This observation would support the idea that direct neutralization of the venom may be an important aspect of the observed protective effects of this mucopolysaccharide.

In view of the observations that heparin can protect mice against the lethal effects of this venom and that heparin is readily secreted from mast cells in response to a local injection of this toxic material(4), one may speculate that heparin can play an important role in natural resistance to this agent. Inasmuch as the noted secretion of heparin from mast cells is a local reaction to this venom (4) and occurs within or in proximity to the initial site of injury, this response of mast cells could be a highly effective and unique connective tissue mechanism for localizing and neutralizing this injurious substance.

*Summary.* Results of this study indicate that heparin can inhibit the lethal effects of Russell's Viper venom in a dose-response manner. This protective activity was also demonstrated with other sulfated mucopolysaccharides of the connective tissue and with sulfated polysaccharides of exogenous origin. Heparin, however, proved to be the most potent substance tested.

The possibility that the secretion of heparin from mast cells is an important factor in natural resistance to this venom has been discussed.

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