ABSTRACT
Evidence is presented that treatment with a highly sulfated low-molecular-weight heparin fraction, LU 51198, protects the ex vivo perfused rabbit heart from human complement-mediated injury. Hearts from male New Zealand White rabbits were perfused under constant flow in the Langendorff mode. After equilibration, normal human plasma was added to the perfusate as a source of complement. Either control (n = 8) or LU 51198 (0.6 mg/ml; n = 7) was added to the perfusion medium 10 min before the addition of human plasma. Hemodynamic variables were obtained for both groups before treatment of human plasma. Hemodynamic variables were obtained for both groups before treatment (baseline), 10 min after treatment (zero) and after the addition of human plasma. Compared to control-treated hearts, variables recorded during perfusion with human plasma, including coronary perfusion pressure, left ventricular developed pressure, and left ventricular end-diastolic pressure, along with a reduction of creatine kinase and potassium efflux, were significantly improved in hearts treated with LU 51198 (P < .05). ELISA assays were used to analyze lyphatic effluent for the presence of iC3b, Bb and SC5b-9 proteins derived from the activation of human complement. The increased presence of the Bb fragment in the effluent obtained from LU 51198-perfused hearts suggests an accelerated dissociation of the convertases responsible for complement amplification, an observation that coincided with protection from complement-mediated damage in the presence of the glycosaminoglycan. The lysis of rabbit red blood cells upon exposure to human plasma was inhibited by LU 51198, which is evidence of the drug’s ability to modulate complement reactivity. The results of this study indicate that a highly sulfated heparin fraction, LU 51198, can reduce tissue injury and preserve discordant organ function that otherwise would be compromised during activation of the human complement cascade.

The ability of the glycosaminoglycan heparin to interfere with the complement cascade was demonstrated initially by Ecker and Gross (1929), who reported that guinea pig plasma failed to hemolyze sheep erythrocytes in the presence of heparin sulfate. Other investigators have shown that glycosaminoglycans, including heparin-related compounds that have sulfated structural domains, are able to regulate one or more of the serine proteases in the complement cascade (Raepple et al., 1976; Weiler et al., 1978; Sharath et al., 1985; Meri and Pangburn, 1990). Recent studies demonstrating that the glycosaminoglycans inhibit complement activation in vivo, independently of an action on the coagulation system, provide a compelling argument for the implementation of this class of compounds in experimental models of cellular injury that directly involve the complement cascade (Weiler et al., 1992; Black et al., 1995; Gralinski et al., 1996c). The rationale for the use of human plasma for induction of complement-mediated tissue injury derives from the studies of Platts-Mills and Ishizaka (1974) demonstrating that human serum caused the hemolysis of rabbit red blood cells as a result of activation of the alternative pathway of complement. The ability of rabbit lung tissue to activate the human complement system was demonstrated by Seeger et al. (1989).

We examined the potential anticomplement properties of a LMWH derivative with structural modifications that altered the sulfation characteristics present in the glycosaminoglycan pentasaccharide. The degree of glycosaminoglycan sulfation has been associated with complement inhibition when examined in vitro with the use of purified complement com-
components (Sharath et al., 1985; Mailet et al., 1988). However, studies detailing the effects of glycosaminoglycan structural modification on complement inhibition in a model of cellular injury dependent on human complement activation are lacking. The objective of the current study was to examine whether LU 51198, a highly sulfated LMWH derivative, could reduce myocardial injury resulting from activation of the human complement system. We used the rabbit isolated heart perfused in the presence of NHP. The experimental model involves a direct interaction between the human alternative complement pathway and the endothelial cell surface of the perfused rabbit heart (Gralinski et al., 1996b; Homeister et al., 1992). The formation of complement neoantigens was assessed to determine a mechanism for the observed anticomplement activity in the presence of LU 51198.

Materials and Methods

Guidelines for human and animal research. The procedures used in this study are in accordance with the guidelines of the Internal Review Board of the University of Michigan, with the regulations of the United States Department of Health and Human Services for the Protection of Human Research Subjects (part 46 of title 45 of the Code of Federal Regulations, as amended) and with the guidelines of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Health Care, and the animal care and use program conforms to the standards in “The Guide for the Care and Use of Laboratory Animals,” DHEW Publ. No. (NIH) 86–23.

Langendorff perfused rabbit heart. Male New Zealand White rabbits (2.2–2.4 kg) were euthanized by cervical dislocation. The hearts were removed rapidly and attached to a cannula for perfusion through the aorta. The final perfusion medium (250 ml) consisted of a recirculating modified Krebs-Henseleit buffer (pH 7.4, 37°C) delivered at a constant rate of 20 to 25 ml/min that established an initial mean coronary artery perfusion pressure of 60 to 65 mmHg. The composition of the buffer medium, in millimoles per liter, was as follows: NaCl, 117; KCl, 1.41; CaCl2·H2O, 2.4; MgCl2·6H2O, 1.2; NaHCO3, 25; KH2PO4·H2O, 1.1; glucose, 5.0; monosodium L-glutamate, 5.0; sodium pyruvate, 2.0; bovine serum albumin, 0.25% w/v. The Krebs-Henseleit buffer passed through a gas porous “lung” consisting of Silastic Medical Grade Tubing (Dow Corning, Midland, MI) 5.49 m in length, with I.D. 1.47 mm and O.D. 1.96 mm. The membrane “lung” was exposed continuously to a mixture of 95% O2/5% CO2 to obtain an oxygen partial pressure within the perfusion medium equal to 500 mmHg. The hearts were paced throughout the protocol via electrodes attached to the right atrium. Pacing stimuli, 3 Hz, 4-msec duration, 4-msec delay, were delivered from a laboratory square-wave generator (Grass SD-5, Quinca, MA).

The pulmonary artery was cannulated with polyethylene tubing to facilitate collection of the pulmonary artery effluent representing the coronary venous return to the coronary sinus. The superior and inferior vena cava and the pulmonary veins were ligated to prevent loss of perfusate from the otherwise severed vessels. A left ventricular drain, thermostor probe and latex balloon were inserted via the left atrium and positioned in the left ventricle. The fluid-filled latex balloon was connected with rigid tubing to a pressure transducer to permit measurement of left ventricular developed pressure. The intraventricular balloon was expanded with distilled water to achieve an initial BL LVEDP of 5 mm Hg. Coronary perfusion pressure was measured with a pressure transducer connected to a sidearm of the aortic cannula. Left ventricular developed pressure represents the difference between peak systolic left ventricular pressure and LVEDP. Because the rate of coronary artery perfusion was maintained constant, alterations in the coronary artery perfusion pressure served as an indicator of changes in coronary arterial resistance. All hemodynamic variables were monitored continuously using a multichannel recorder (Grass Polygraph 79D, Quincy, MA).

Hearts were maintained at 37°C throughout the experimental period by enclosing the heart in a temperature-regulated double lumen glass chamber and passing the perfusion medium through a heated reservoir and delivery system.

Preparation of NHP. After receiving informed consent, we obtained human blood by venipuncture from donors who were fasted for 12 hr and who had not ingested any form of medication in the past 7 days. The blood was placed into tubes containing heparin (14 IU/ml final concentration) and mixed. The blood was centrifuged at 2000 g for 10 min, and the plasma was collected. NHP was stored at −70°C until used (not more than 72 hr). Immediately before use, the plasma was thawed, centrifuged (40 min at 27,200 × g, 4°C) and filtered through a C-18 cartridge (Millipore).

Assessment of complement inhibition using NHP and rabbit erythrocyte lysis. Complement-mediated RBC hemolysis was assessed by a modified turbidimetric technique similar to that described previously (Polhill and Pruitt, 1978; Friedrichs et al., 1994). Rabbit blood (2 ml) was collected and centrifuged at 2000 × g for 10 min at room temperature. The plasma layer was discarded, and RBC were washed three times with Hanks balanced salt solution (pH = 7.4). A solution of erythrocytes was prepared in Hanks’ balanced salt solution to a final cell concentration of 2.5 × 109 cells/ml. The assay for detection of hemolysis was performed with a FAP-4 platelet aggregometer (Bio/Data Corp., Hatbore, PA) by using microvolume cuvettes and a stir speed of 1200 rpm. One hundred percent light transmission was defined by lysing 2.5 × 109 cells/ml (100 μL) with 4% NHP (final concentration). Plasma (180 μl) and vehicle control (saline) or LU 51198 (20 μl) were added to the cuvette and warmed to 37°C. The assay was initiated by addition of 20 μl of 6.7% NHP, achieving a final concentration of 4% NHP in the cuvette. RBC lysis resulted in an increase in light transmittance that was monitored for 10 min. LU 51198 was tested in this assay at final concentrations ranging from 0.006 to 2.0 mg/ml. The assay was repeated three times using red cells from different rabbits exposed to the same batch of NHP (n = 3).

Drugs. LU 51198 was provided by Knoll AG, Ludwigshafen, Germany. The compound was synthesized by randomly O-sulfating LMWH fragments with a pyridine-SO3 complex. LMWH was produced by selective deaminative cleavage of unfractionated heparin with nitrous acid. The mean molecular mass was 3711 daltons with antifactor Xa activity of 4.6 IU/mg (personal communication).

Experimental protocol. Two treatment groups were used to determine the ability of a highly sulfated glycosaminoglycan to inhibit the effects of complement activation in the rabbit isolated heart (fig. 1): Group 1: Control, consisted of hearts perfused with 4% NHP (n = 8); Group 2: Treatment group, consisted of hearts perfused with
4% NHP in the presence of 0.6 mg/ml LU 51198 (n = 7). The concentration of LU 51198 was based on a previous study from our laboratory that utilized heparin sulfate and N-acetylated heparin as anticomplement compounds (Friedrichs et al., 1994) and was calculated on the basis of its ability to inhibit RBC lysis using a unit/mass conversion comparison with heparin sulfate. All hearts underwent 10 min of equilibration on the Langendorff apparatus before the addition of control (Kreb-Henseleit buffer) or LU 51198 to the perfusion medium. Ten minutes after the addition of vehicle or LU 51198, NHP (4% v/v) was added to the perfusion medium. Hemodynamic variables including coronary perfusion pressure, left ventricular developed pressure and LVEDP were recorded before the addition of vehicle or LU 51198 (BL), before the addition of 4% NHP (zero, 0), and every 5 min thereafter. The data was stored on disk using a MacLab data acquisition system (MacLab, Milford, MA) and software interfaced with a Macintosh Power Book 140 and disk drive (Apple Computer, Cupertino, CA) with final storage on an UltraDrive120MO optical disk (GCC Technologies, Waltham, MA).

**Measurement of activated complement components SC5b-9, Bb fragment and iC3b.** The appearance of the following complement neoantigens in the lymphatic fluid collected from the heart was monitored using enzyme-linked immunosassays commercially available from Quidel (San Diego, CA): soluble C5b-9 (s-MAC), Bb fragment and iC3b as previously described (Homeister et al., 1992; Gralinski et al., 1996a; Yasuda et al., 1990). The assays used antibodies directed against human complement components, thus allowing for assessment of activation of the human complement added to the perfusion medium. Supplied standards and control sera were used to determine the amount of neoantigen present in the effluent samples using derived standard curves. Lymphatic flow rates were determined by monitoring the time to collect 1 ml of lymphatic fluid. Because damaged hearts generated lymphatic effluent at a greater rate than normal hearts, complement neoantigen concentrations were normalized to lymphatic flow (neoantigen concentration multiplied by lymphatic flow rate).

**Statistical analysis.** The data presented in the text, figures and tables are expressed as mean ± S.E.M. for the indicated number of determinations. Two-way ANOVA was used to examine the influence of time on all repeated measurements. If significance was determined, t test was used as a post-hoc analysis to determine statistical differences between groups. All t test comparisons performed were two-tailed. A P value less than .05 was considered significant. All statistical evaluations were performed using a Macintosh 6100 computer (Apple Computer, Cupertino, CA) and Statview 4.01 software (Abacus Concepts Inc., Berkeley, CA).

**Results**

**Inhibition of human complement activation—NHP and Rabbit RBC Lysis.** LU 51198 inhibited rabbit erythrocyte lysis after exposure of 4% NHP (final concentration) in a concentration-dependent manner (fig. 2). In the absence of LU 51198 (vehicle control-treated), 96% ± 2% rabbit erythrocyte lysis occurred. This is not significantly different from the results with lower concentrations of LU 51198 tested in this assay. The ability of LU 51198 to inhibit complement-mediated hemolysis was evident at a concentration of 0.6 mg/ml. One hundred percent inhibition of plasma-mediated hemolysis was achieved with 2.0 mg/ml. An IC<sub>50</sub> for LU 51198 in this cell lysis assay was estimated at 0.7 mg/ml.

**Left ventricular developed pressure.** The addition of 4% NHP to the perfusion medium produced a significant alteration in the measured hemodynamic variables of the Langendorff perfused rabbit heart. Exposure of the control hearts to human plasma resulted in an increase in coronary perfusion pressure, an increase in LVEDP and a decrease in left ventricular developed pressure. The addition of LU 51198 alone to the perfusion medium did not produce any significant changes in the recorded functional parameters. The percentage change from BL in left ventricular developed pressure for each of the experimental groups during the periods before and after the addition of 4% NHP plasma to the perfusion medium is shown in figure 3. After the addition of NHP to the perfusion medium, the developed pressure increments throughout the perfusion with 4% NHP. BL data were obtained immediately before the addition of vehicle or LU 51198. The graph demonstrates the protective effect of the highly sulfated LMWH derivative with respect to functional preservation during complement-mediated myocardial injury. Control: C (n = 8); LU51198 (0.6 mg/ml): ■ (n = 7). Data are mean ± S.E.M. * P < .05 vs. vehicle.
recorded from hearts perfused with LU 51198 was impaired to a much lesser extent compared with hearts in the control-treated group. The maintenance of left ventricular function in the LU 51198-treated hearts became more apparent as the time of exposure to NHP increased (fig. 4). Beginning at 15 min after exposure to human plasma and continuing throughout the remainder of the experimental protocol, hearts perfused in the absence of LU 51198 exhibited a progressive decrease in left ventricular developed pressure, which plateaued at 30 min.

**LVEDP.** The mean LVEDP between the two experimental groups of hearts did not differ before addition of NHP to the perfusion medium, which suggests that LU 51198 did not influence this hemodynamic variable directly. When exposed to human plasma, the LVEDP of control-treated hearts increased significantly compared with values recorded from hearts perfused in the presence of LU 51198 (P < .05). As shown in figure 5, a rapid increase in the LVEDP with a subsequent plateau was observed in the control-treated hearts as the duration of exposure to the human plasma increased. Approximately 30 min after exposure of the control-treated hearts to human plasma, LVEDP increased maximally (fig. 5). Pretreatment of the perfused hearts with LU 51198 provided significant protection against the increase in LVEDP (P < .05).

**Coronary perfusion pressure.** Coronary perfusion pressure during the periods before and after addition of NHP is shown in figure 6. Coronary artery perfusion pressure increased progressively after the addition of human plasma to the perfusion medium, which indicates an increase in coronary vascular resistance. Compared with results obtained in the control-treated group, hearts perfused with LU 51198 exhibited significant protection against the progressive increase in coronary perfusion pressure throughout the experimental protocol (P < .05).

**Creatine kinase and potassium release.** The coronary venous drainage was collected from the pulmonary artery for the determination of creatine kinase activity and potassium ion release before and after the addition of NHP to the perfusion medium. The data are summarized in figures 7 and 8 for each of the experimental groups. Creatine kinase activity and the potassium ion concentration increased progressively in the venous effluent of the control hearts after addition of NHP to the perfusion medium, which indicates myocardial cellular injury. Compared with results obtained from the control group, hearts perfused with LU 51198 exhibited significant protection against the progressive cytosolic release of creatine kinase and potassium ion throughout the experimental protocol (P < .05).

As determined by the recorded changes in myocardial contractile function, coronary vascular resistance, creatine kinase release and potassium ion efflux, perfusion with the highly sulfated LMWH derivative LU 51198 protects the rabbit heart against the damaging effects resulting from exposure to NHP, presumably by preventing activation of the human complement cascade.
Determination of complement components iC3b, Bb fragment and SC5b-9. The results from the ELISA assays are shown in figure 9, A-C. A decrease in iC3b formation was measured in the lymphatic fluid from LU 51198-treated hearts perfused with 4% NHP compared with hearts perfused in the absence of the glycosaminoglycan (fig. 9A). Lymphatic effluents collected from hearts perfused with LU 51198 displayed an increase in the immunoreactive Bb fragment, which suggests an accelerated dissociation of the alternative pathway C3 convertase in the presence of the drug (fig. 9B). The lymphatic fluid effluent from hearts perfused in the presence of human plasma showed a progressive increase in immunoreactive SC5b-9, which indicates formation of the MAC. The presence of LU 51198 in the perfusion medium completely suppressed the formation of SC5b-9 throughout the period of human plasma perfusion, a result that coincided with protection of the heart from complement-mediated damage when hemodynamic variables were used as the arbiter of myocellular injury (fig. 9C).

Discussion

The objective of this study was to determine the potential for LU 51198, a highly sulfated LMWH derivative, to inhibit activation of human complement. Complement activation plays a major role in many cardiovascular pathophysologies, including vasculitis (Smiley and Moore, 1989), myocardial ischemia and reperfusion injury (Weisman et al., 1990), cardiac transplant rejection (Baldwin et al., 1995) and atherosclerosis (Seifert and Kazatchkine, 1988). Pharmacologic interventions such as LU 51198 or other heparin analogs that inhibit complement activation could potentially alleviate tissue injury directly mediated by the formation of MAC. Likewise, LU 51198 could limit neutrophil-mediated damage by inhibiting the generation of the anaphylatoxin C5a in complement-related pathophysologies.

In the present study, complement activation was determined with the use of a well-characterized in vitro bioassay in which an organ from a discordant species is perfused in the presence of NHP (Platts-Mills and Ishizaka, 1974; Seeger et al., 1989; Homeister et al., 1992). The results indicate that LU 51198 can provide protection against the direct cytotoxic effects resulting from activation of the complement cascade. The ability of LU 51198 to protect rabbit erythrocytes from complement-mediated hemolysis demonstrated its inhibition of human complement activation (Polhill and Pruitt, 1978). Rabbit hearts, representative of a discordant tissue, when perfused in the presence of NHP exhibited deleterious alterations in LVEDP, left ventricular developed pressure and coronary vascular resistance. The changes in cardiac function were accompanied by a loss of cytosolic creatine kinase and intracellular potassium. Perfusion of the discordant tissue with NHP was associated with activation of the human complement system, as evidenced by the appearance of immunoreactive complement components in the lymphatic fluid effluent from the perfused heart. LU 51198 added to the perfusion medium prevented or reduced the alterations in cardiac function and the loss of cytosolic creatine kinase and intracellular potassium ion. The observed absence of immunoreactive SC5b-9 and the reduction in iC3b in the lymphatic fluid effluent of hearts perfused in the presence of LU 51198...
is in accordance with the concept that LU 51198 was able to prevent and/or modulate activation of the complement cascade.

Glycosaminoglycans such as LU 51198 may function as an anticomplement intervention by accelerating the dissociation of an enzymatically active complex designated the C3 convertase, C3bBb (Maillet et al., 1988). C3 convertase cleaves serum C3 to C3b, forming a positive amplification feedback pathway leading to formation of the cytotytic MAC, C5b-9. One of two outcomes can be expected, depending on the extent of the MAC deposition on the target cell. The presence of cell-associated and humoral regulators of complement activation can limit the number of cytotytic complexxes on the target tissue so that myocardial cells may be subjected to reversible alterations in membrane integrity. On the other hand, multiple complex formation may overwhelm the cellular protective mechanisms, thereby resulting in an alteration of the intracellular environment through an increase in membrane permeability, with subsequent swelling of the affected cells, resulting in irreversible cellular injury. These irreversible changes would be reflected in this model of complement-mediated myocardial injury through myocardial dysfunction (changes in contractility and coronary arterial resistance), as illustrated in figure 4. In the erythrocyte lysis assay, 0.6 mg/ml LU 51198 inhibited approximately 40% of the complement-mediated erythrocyte hemolysis. This same concentration of LU 51198 completely protected the plasma-perfused isolated rabbit heart from complement-mediated dysfunction.

Polysulfated glycosaminoglycans appear to inhibit the classic and alternative complement pathways, which suggests a possible effect on complement components common to both pathways [Rashmir-Raven et al., 1992]. An important event in the activation of both the classic and the alternative pathways of complement on nucleated cell surfaces is the covalent binding of the C3 activation product C3b to the target-cell membrane and the ultimate proteolytic conversion of C3b to C3bBb. The latter has an enzymatic activity and functions as a C3 convertase. C3bBb is unstable, and factor B is readily displaced by a fluid-phase component, factor H. Factor H, a serum protein, controls the formation of the alternative complement pathway C3 and C5 convertases in vivo. The dissociation of the C3 and C5 convertases by factor H and other regulatory proteins results in the generation of the Bb fragment. The complex formed between factor H and C3b is susceptible to attack by the C3b inactivator, factor I, to yield the inactive iC3b. Thus, under normal conditions, factor H serves the role of modulating the potentially explosive complement cascade.

Highly sulfated glycosaminoglycans inhibit the enzymatic function of the C3bBb complex by potentiating the endogenous regulatory molecule associated with the complex (Meri and Pangburn, 1994; Boackle et al., 1983; Pangburn et al., 1991; Koistinen, 1993). Glycosaminoglycans augment factor H activity in the fluid phase and in the presence of C3b attached to an alternative pathway activating surface [Koistinen, 1993], such as the endothelial cells of the perfused rabbit heart. The outcome of treatment with a glycosaminoglycan would be to limit the formation and tissue deposition of the terminal cytotytic MAC, thereby protecting the target organ from irreversible cellular injury.

We examined the formation of the neoantigens associated with both the mechanistic sites involved in the factor H-
complement interaction. The generation of iC3b was reduced in the presence of LU 51198, which suggests that the glycosaminoglycan does not potentiate the activity of factor H at the site in the complement cascade. An increased formation of the Bb fragment was observed in hearts perfused in the presence of LU 51198, a finding in accordance with the concept that the activity of factor H is enhanced by the glycosaminoglycan at the level of the alternative pathway C3 and C5 convertase formation. The reduced formation of the C3 and C5 convertases would result in a decreased formation of iC3b and SC5b-9. The suggested mechanism for the protective action of LU 51198 is consistent with the experimental observations obtained in the present study as well as with those of Meri and Pangburn (1994). The latter investigators demonstrated that the interaction of the polyion binding site on factor H with glycosaminoglycans depends on the number, orientation and polymeric arrangement of sulfate groups. They suggest that most, but not all, sulfated glycosaminoglycans participate in the protection of host tissue from complement damage by promoting inactivation of tissue-bound C3b. The observed increase in immunoreactive Bb fragment when hearts are perfused with human plasma in the presence of LU 51198 suggests the accelerated dissociation of the C3bBb convertase, possibly because of the glycosaminoglycan-related augmentation of factor H activity.

The results of this investigation present support for the concept that a highly sulfated glycosaminoglycan derivative, LU 51198, can modulate the activation of the complement cascade as evidenced by its ability to confer protection on the rabbit heart perfused with human plasma containing the intact complement system. Compared with control-treated hearts, hemodynamic variables recorded during perfusion with NHP, including coronary perfusion pressure, left ventricular developed pressure and LVEDP, were improved in hearts perfused in the presence of LU 51198. In addition, hearts perfused with LU 51198 in the presence of NHP generated an increased amount of the human complement Bb fragment. The increased generation of the Bb fragment may be associated with a reduction in the amount of functional C3 convertase, a result that could explain the observed anticomplement action of LU 51198 as has been proposed for other glycosaminoglycans (Meri and Pangburn, 1994). The present study demonstrates the ability of a highly sulfated glycosaminoglycan derivative to modulate complement activation in an experimental model in which a discordant tissue, serving as a target organ, is perfused with human plasma. The in vitro model provides a convenient means of assessing pharmacologic interventions directed toward inhibition of the human complement system. LU 51198 and related glycosaminoglycans, with and without anticoagulant activity (Black et al., 1995; Friedrichs et al., 1994), may provide a useful therapeutic approach for limiting tissue injury related to activation of the complement cascade.

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