Molecular-weight-dependent effects of nonanticoagulant heparins on allergic airway responses

CARLOS CAMPO, JUSSARA F. MOLINARI, JAIMÉ UNGO, AND TAHIR AHMED
Division of Pulmonary Diseases, University of Miami School of Medicine, Mount Sinai Medical Center, Miami Beach, Florida 33140

Molecular-weight-dependent effects of nonanticoagulant heparins on allergic airway responses. J. Appl. Physiol. 86(2): 549–557, 1999.—We have hypothesized that antiallergic activity of inhaled heparin is molecular-weight dependent and mediated by “nonanticoagulant fractions” (NAF-heparin). Therefore, we studied comparative effects of high-, medium-, and ultralow-molecular-weight (HMW, MMW, and ULMM, respectively) NAF-heparins on acute bronchoconstrictor response (ABR) and airway hyperresponsiveness (AHR) in allergic sheep. Specific lung resistance was measured in 23 allergic sheep, before and immediately after challenge with Ascaris suum antigen, and after pretreatment with inhaled NAF-heparins. Airway responsiveness was estimated before and 2 h postantigen, as the cumulative provocative dose of carbachol in breath units, which increased specific lung resistance by 400%. NAF-heparins attenuated ABR and AHR in a molecular-weight-dependent fashion. HMW NAF-heparin (n = 8) was the least effective agent: it attenuated ABR [inhibitory dose causing 50% protection (ID50) = 4 mg/kg] but had no effect on AHR. MMW NAF-heparin (n = 8) showed intermediate efficacy (ABR ID50 = 0.8 mg/kg, AHR ID50 = 1.4 mg/kg), whereas ULMM NAF-heparin (n = 7) was the most effective agent (ABR ID50 = 0.4 mg/kg, AHR ID50 = 0.2 mg/kg). ULMM NAF-heparin was 3.5 times more potent in attenuating antigen-induced AHR when administered “after” antigen challenge and failed to inhibit the bronchoconstrictor response to carbachol and histamine. In 15 additional sheep, segmental antigen challenge caused a marked increase in histamine in bronchoalveolar lavage fluid that was not prevented by any of the inhaled NAF-heparins. These data indicate that antiallergic activity of inhaled heparin is independent of its anticoagulant action and resides in the <2,500 ULMM chains. The antiallergic activity of NAF-heparins is mediated by an unknown biological action and may have therapeutic potential.

asthma; airway hyperresponsiveness; antigen-induced bronchoconstriction

HEPARIN HAS BEEN USED clinically as an anticoagulant for >50 years (36). Heparin, a glycosaminoglycan, is a complex, highly sulfated, linear polysaccharide comprised of repeating 1 → 4-linked uronic acid and glucosamine residues (21, 26, 29). The structural variability and polydispersity are often the basis of a wide variety of domain structures, with a number of important biological activities ascribed to heparin. These activities result from the ability of heparin to interact with clusters of basic amino acids on numerous proteins, allowing heparin to bind many enzymes and modulate various biological processes (26, 29). The well-known anticoagulant activity, for example, is related to a specific antithrombin III-binding pentasaccharide sequence in the heparin molecule (27, 28). In addition to its anticoagulant activity, the heparin molecule has multiple “nonanticoagulant” properties that include stabilization and activation of various growth factors, regulation of cell growth, angiogenesis, and atherosclerosis (12, 17, 19). The heparin molecule has also been shown to modulate various proteases (41, 42) and to possess anti-inflammatory and immunoregulatory properties (15, 25, 33, 43, 46).

Although the precise role of endogenous mast cell heparin is not known, it has been demonstrated that unfractionated (UF) and fractionated inhaled heparins possess significant antiallergic activity (2, 6, 10, 14, 20, 32). It was hypothesized that the antiallergic action of heparin may be related to its nonanticoagulant properties (2, 3, 6, 20). In a preliminary study a high-molecular-weight (HMW) “nonanticoagulant fraction” of heparin (NAF-heparin) attenuated the antigen-induced bronchoconstriction; however, it failed to modify the postantigen airway hyperresponsiveness (3). Because many biological actions of heparin are molecular weight dependent (13, 32), it is possible that differential effects of NAF-heparin on allergic bronchoconstriction vs. airway hyperresponsiveness may be molecular weight dependent, and a low-molecular-weight (LMW) NAF-heparin may possess more potent antiallergic activity. Therefore, we studied the comparative effects of HMW and medium (MMW)- and ultralow-molecular-weight (ULMM) NAF-heparins on antigen-induced acute bronchoconstrictor response (ABR), airway hyperresponsiveness (AHR), and bronchoalveolar lavage (BAL) histamine release in allergic sheep.

MATERIAL AND METHODS

Animal Preparation

Thirty-eight adult sheep (mean weight 30 kg, range 25–33 kg) were included in the study. All sheep were allergic to Ascaris suum antigen and had been shown to develop only “acute bronchoconstriction” (acute responders) after inhalation challenge with the antigen. This study was conducted in “acute responder” sheep, in which allergic bronchoconstrictor response is inhibited by inhaled heparin (4).

Measurement of Airway Mechanics

Measurement of airway mechanics has been described previously (4, 5). The unsedated sheep were restrained in a cart in the prone position with their heads immobilized. After topical anesthesia of the nasal passages with 2% lidocaine...
solution, a balloon catheter was advanced through one nostril into the lower esophagus. The animals were intubated with auffed endotracheal tube through the other nostril by using a flexible fiber-optic bronchoscope as a guide. Pleural pressure was estimated with the esophageal balloon catheter (filled with 1 ml of air), which was positioned 5–10 cm from the gastroesophageal junction. In this position the end-expiratory pleural pressure is between −2 and −5 cmH₂O. Lateral pressure in the trachea was measured with a side-hole catheter (2.5 mm ID) advanced through and positioned distal to the tip of the endotracheal tube. This has been validated previously (45), and additional measurements of airway resistance were comparable whether obtained with an end-on or a side-hole catheter. Transpulmonary pressure, the difference between tracheal and pleural pressure, was measured with a differential pressure transducer-catheter system, which showed no phase shift between pressure and flow up to a frequency of 9 Hz (model MP45, Validyne, Northridge, CA). For the measurement of pulmonary resistance (Rlung), the proximal end of the endotracheal tube was connected to a pneumotachograph (Fleisch no. 1, Dyna Sciences, Blue Bell, PA). The signals of flow and transpulmonary pressure were recorded on a multichannel recorder, which was linked to an 80,386 DOS personal computer for on-line calculation of Rlung from transpulmonary pressure and flow at isovolume points (respiratory volume was obtained by digital integration). Analysis of at least seven breaths (free from swallowing artifact) was used for the determination of Rlung. Data were expressed as specific Rlung (sRlung) defined as Rlung × thoracic gas volume.

Thoracic gas volume. Thoracic gas volume was measured by a body plethysmographic technique (4, 5). The endotracheal tube was connected to a solenoid valve that could be activated from outside the plethysmograph. The plethysmographic pressure and lateral mouth pressure, measured between the proximal end of the endotracheal tube and the solenoid valve, were measured with a differential gauge (model MP45, Validyne) and a strain gauge (Statham Instruments, Hato Rey, PR), respectively, and displayed on an X-Y oscilloscope provided with a template. The plethysmographic pressure was calibrated manually with a 30-ml syringe at a rate similar to the sheep's spontaneous breathing frequency. After the animal had been enclosed in the plethysmograph, 1–2 min were allowed for stabilization of the plethysmographic pressure. The solenoid valve was activated at expiration, and the slope of the first respiratory cycle against the closed airway was taken for the determination of thoracic gas volume, because subsequent efforts usually produce unsatisfactory slopes caused by the animal straining against the occluded airway. The mean of three measurements was recorded.

Aerosol Delivery System

Aerosols were generated using a disposable medical nebulizer (Raindrop, Puritan Bennett, Lenexa, KS), which produces an aerosol with a mass median aerodynamic diameter of 3.2 μm (geometric standard deviation 1.9) as determined by a seven-stage Andersen cascade impactor. The output from the nebulizer was directed into a plastic T piece, which was interconnected between the Harvard animal respirator and the endotracheal tube. To control the aerosol delivery a dosimeter system, consisting of a solenoid valve and a source of compressed air (20 psi), which was activated for 1 s at the beginning of the inspiratory cycle of the Harvard respirator system, was used. All aerosols were delivered at a tidal volume of 500 ml and a rate of 20 breaths/min.

BAL

The distal tip of a specially designed 80-cm fiber-optic bronchoscope was wedged into a randomly selected subsegmental bronchus. The BAL was performed by an infusion and gentle aspiration of 30-ml aliquots of PBS (pH 7.4) at 30°C by use of 30-ml syringes attached to the working channel of the bronchoscope. The effluent was filtered through a single layer of gauze and placed immediately on ice. The volume of the effluent collected from the BAL was measured and centrifuged at 420 g for 15 min at 4°C. The supernatant was decanted and centrifuged again at 1,000 g at 4°C for 15 min. The supernatant was frozen at −80°C for subsequent histamine analysis (4).

Histamine RIA

Duplicate aliquots from each BAL sample were used for histamine RIA by using a commercial kit from Immunotech International (AMAC, Westerbrook, ME). The sensitivity of the assay is 0.05–2.0 nM, and coefficient of variation is <10%. There is <0.01% cross-reactivity with histidine, serotonin, or t-methyl histamine (4, 16).

Purification and Characterization of NAF-Heparins

The NAF-heparins were derived from porcine intestinal mucosa and are not commercially available. For purification of HMW NAF-heparin (ABL), standard commercial USP heparin was used as the starting material. Heparin (4.5 g) was dissolved in 0.15 mol/l sodium chloride and 5 mmol/l sodium phosphate buffer, pH 7.4, to a concentration of 45 mg/ml. After filtration the sample was applied to 2.3 liters of antithrombin-Sepharose gel packed into a column (model P140×500, Amicon). The high-affinity fraction of heparin was absorbed to the gel; the low-affinity part (NAF-heparin) passed the column and was collected. The NAF-heparin was further purified on 2 liters of Q-Sepharose ff gel packed in a BPG100 column equilibrated in 0.15 mol/l sodium chloride and 10 mmol/l sodium acetate, pH 4.0. Elution was performed with 1.5 mol/l sodium acetate. The material was dialyzed against water for injection and concentrated by reverse-osmosis filtration before it was freeze dried (44). The 10,500-Da product had activated partial thromboplastin time (APTT) activity of 30 IU/mg and antifactor Xa activity of 1 IU/mg (3, 44).

The MMW NAF-heparin (SR-80258) was prepared by periodate degradation of standard heparin. Porcine heparin was solubilized in water and cleaned with periodate oxidation at pH 5.0 at a concentration of heparin and sodium periodate of 2%. The solution was then dialyzed, and β-elimination was conducted in basic conditions (0.2 N NaOH). This was followed by reduction in sodium borohydride and ethanol fractionation (11, 39). The 6,500-Da product had antithrombin activity of 6 IU/mg and antifactor Xa activity of 2 IU/mg.

The ULMW NAF-heparin (KABI-2226) was prepared as sodium salt by nitrous acid depolymerization of porcine heparin followed by various fractionation steps involving ethanol precipitation (38). This was followed by reduction in excess sodium borohydride to convert anhydromannose groups to more stable anhydromannitol (8, 38). The 2,400-Da product had APTT activity of 2 IU/mg and antifactor Xa activity of 36 IU/mg. KABI-2226 has a somewhat lower degree of sulfation than heparin.

Depending on the average molecular weight, various fractionated heparins have been arbitrarily grouped into MMW (>10,000), MMW (>5,000 to <10,000), LMW (>2,500 to <5,000), and ULMW (<2,500) fractions. The HMW, LMW, and ULMW anticoagulant heparins show marked differences
in their biochemical and pharmacological characteristics (18, 40).

Agents. A. suum extract (Greer Diagnostics, Lenoir, NC) was diluted with buffered saline to a final concentration of 82,000 protein nitrogen units/ml and delivered as an aerosol over 20 min (400 breaths). The dose of antigen delivered was kept constant for all animals in all antigen experiments. Carbachol and histamine (Sigma Chemical, St. Louis, MO) were dissolved in PBS for nebulization. HMW NAF-heparin (ABL4) and ULMW NAF-heparin (KABI-2226) were produced by Dr. L. O. Andersson (Pharmacia, Stockholm, Sweden); MMW NAF-heparin (SR-80258) was obtained from Sanofi Pharma (Gentilly, Cedex, France). The NAF-heparins were dissolved in 3 ml of bacteriostatic injection water and administered as an aerosol over 15–20 min.

Experimental Protocol

Antigen studies. For every protocol, each animal was studied on three different experimental days. On experimental day 1, baseline bronchial reactivity to carbachol was determined; on experimental days 2 and 3, bronchial reactivity to carbachol was redetermined 2 h after the antigen challenge, without or after pretreatment with different doses of NAF-heparins in a randomized fashion. This was a parallel, three-group study designed for multiple-dose comparison of three NAF-heparins. Group I (n = 8) was pretreated with aerosolized HMW NAF-heparin (2.5 and 5 mg/kg), group II (n = 8) with aerosolized MMW NAF-heparin (0.62, 1.25, 2.5, and 5 mg/kg), and group III (n = 7) with aerosolized ULMW NAF-heparin (0.15, 0.31, and 0.62 mg/kg). In each group, each animal served as its own control.

To assess baseline airway responsiveness, cumulative dose-response curves to inhaled carbachol were performed on experimental day 1 by measuring sRL before and immediately after inhalation of buffered saline and after each administration of 10 breaths of increasing concentrations of carbachol (0.25, 0.5, 1.0, 2.0, 3.0, and 4.0% wt/vol solution). The bronchoprovocation was discontinued when sRL increased to 400% above the baseline. The cumulative provocative dose of carbachol (in breath units) that increased sRL to 400% above the baseline (PD400) was calculated (1 breath unit = 1 breath of a 1% carbachol solution). Baseline dose-response curves to carbachol were performed in all sheep ≥2 wk after their last exposure to antigen.

For each dose of HMW NAF-heparin (ABL4), the experiments (n = 8) were conducted on experimental days 2 and 3, ≥2 wk apart. For the control antigen experiments, after baseline measurements of sRL, the sheep were challenged with aerosolized A. suum antigen and measurements of sRL were repeated within 5 min after challenge. At 2 h after challenge, when sRL had returned to the baseline, a carbachol dose-response curve was performed to determine the postantigen PD400 as an index of AHR. To evaluate the effect of ABL4 on antigen-induced bronchoconstriction and AHR, the above protocol was repeated after the sheep were pretreated with aerosolized ABL4 (2.5 mg/kg (n = 6) and 5 mg/kg (n = 8)) dissolved in 3 ml of bacteriostatic injection water and administered as an aerosol 30 min before the antigen challenge.

For each dose of MMW NAF-heparin (SR-80258), these experiments (n = 8) were conducted on experimental days 2 and 3, ≥2 wk apart. On each day a standardized antigen challenge was performed, and PD400 of carbachol was determined 2 h postantigen, without or after pretreatment with aerosolized SR-80258 (0.62 mg/kg (n = 5), 1.25 mg/kg (n = 8), 2.5 mg/kg (n = 7), and 5 mg/kg (n = 8)). SR-80258 was dissolved in 3 ml of bacteriostatic injection water and administered as an aerosol 30 min before the antigen challenge.
Table 1. Effect of pretreatment with NAF-heparin on antigen-induced bronchoconstriction

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>n</th>
<th>Antigen Control</th>
<th>HMW NAF-heparin (ABL4)</th>
<th>Baseline</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>8</td>
<td>1.03 ± 0.01</td>
<td>3.00 ± 0.40*</td>
<td>1.03 ± 0.02</td>
<td>2.56 ± 0.29*</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>1.03 ± 0.03</td>
<td>2.92 ± 0.13*</td>
<td>1.04 ± 0.03</td>
<td>1.71 ± 0.24†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>n</th>
<th>Antigen Control</th>
<th>MMW NAF-heparin (SR-80258)</th>
<th>Baseline</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.62</td>
<td>5</td>
<td>1.06 ± 0.06</td>
<td>3.25 ± 0.27*</td>
<td>1.00 ± 0.04</td>
<td>2.33 ± 0.23*</td>
</tr>
<tr>
<td>1.25</td>
<td>8</td>
<td>1.09 ± 0.05</td>
<td>3.21 ± 0.19*</td>
<td>1.08 ± 0.05</td>
<td>1.39 ± 0.05†</td>
</tr>
<tr>
<td>2.5</td>
<td>7</td>
<td>1.10 ± 0.05</td>
<td>3.23 ± 0.22*</td>
<td>1.00 ± 0.04</td>
<td>1.46 ± 0.12†</td>
</tr>
<tr>
<td>5.0</td>
<td>8</td>
<td>1.09 ± 0.05</td>
<td>3.21 ± 0.19*</td>
<td>1.05 ± 0.03</td>
<td>1.52 ± 0.08†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>n</th>
<th>Antigen Control</th>
<th>ULMW NAF-heparin (KABI-2226)</th>
<th>Baseline</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>7</td>
<td>1.06 ± 0.04</td>
<td>3.17 ± 0.37*</td>
<td>1.04 ± 0.02</td>
<td>2.58 ± 0.21*</td>
</tr>
<tr>
<td>0.31</td>
<td>7</td>
<td>1.06 ± 0.04</td>
<td>3.17 ± 0.37*</td>
<td>1.02 ± 0.02</td>
<td>2.17 ± 0.30*</td>
</tr>
<tr>
<td>0.62</td>
<td>7</td>
<td>1.06 ± 0.04</td>
<td>3.17 ± 0.37*</td>
<td>1.00 ± 0.02</td>
<td>1.66 ± 0.08†</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as specific lung resistance (sRL) in cm H2O/s for baseline and postantigen, without and after pretreatment with inhaled "nonanticoagulant fractions" of heparin (NAF-heparin). HMW, high molecular weight; MMW, medium molecular weight; ULMW, ultralow molecular weight. *Significantly different from baseline (P < 0.05); †significantly different from antigen control (P < 0.05).

Table 2. Effect of pretreatment with NAF-heparin on antigen-induced airway hyperresponsiveness

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>n</th>
<th>Baseline</th>
<th>Postantigen</th>
<th>NAF-heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>8</td>
<td>18.0 ± 1.0&lt;br&gt;18.7 ± 1.9</td>
<td>7.0 ± 1.0*</td>
<td>7.0 ± 1.0*</td>
</tr>
<tr>
<td>5.0</td>
<td>6</td>
<td>18.8 ± 2.3&lt;br&gt;18.8 ± 2.3</td>
<td>9.3 ± 1.2*</td>
<td>12.2 ± 1.9*</td>
</tr>
<tr>
<td>0.62</td>
<td>5</td>
<td>16.4 ± 2.3&lt;br&gt;16.4 ± 2.3</td>
<td>7.6 ± 1.5*</td>
<td>11.0 ± 1.8*</td>
</tr>
<tr>
<td>1.25</td>
<td>8</td>
<td>18.8 ± 2.3&lt;br&gt;18.8 ± 2.3</td>
<td>8.8 ± 1.9*</td>
<td>12.9 ± 2.3*</td>
</tr>
<tr>
<td>2.5</td>
<td>7</td>
<td>17.6 ± 2.2&lt;br&gt;17.6 ± 2.2</td>
<td>9.4 ± 2.1*</td>
<td>16.4 ± 1.8†</td>
</tr>
<tr>
<td>5.0</td>
<td>8</td>
<td>18.8 ± 2.3&lt;br&gt;18.8 ± 2.3</td>
<td>8.8 ± 1.9*</td>
<td>16.7 ± 1.1†</td>
</tr>
<tr>
<td>0.15</td>
<td>7</td>
<td>20.5 ± 2.0&lt;br&gt;20.5 ± 2.0</td>
<td>9.8 ± 1.9*</td>
<td>10.5 ± 1.3*</td>
</tr>
<tr>
<td>0.31</td>
<td>7</td>
<td>20.5 ± 2.0&lt;br&gt;20.5 ± 2.0</td>
<td>9.8 ± 1.9*</td>
<td>19.6 ± 2.2†</td>
</tr>
<tr>
<td>0.62</td>
<td>7</td>
<td>20.5 ± 2.0&lt;br&gt;20.5 ± 2.0</td>
<td>9.8 ± 1.9*</td>
<td>24.9 ± 2.9†</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as cumulative provocative dose of carbachol that increased sRL to 400% above baseline (PD400) in breath units (1 breath unit = 1 breath of 1 mg/ml carbachol) for baseline and postantigen, without and after pretreatment with various NAF-heparins. One breath unit = 1 breath of 1 mg/ml carbachol. *Significantly different from baseline (P < 0.05); †significantly different from postantigen (P < 0.05).
were 378 ± 4 and 353 ± 3% without and after pretreatment with KABI-2226 (P = NS); respective values of \( \Delta SR_L \) for carbachol were 333 ± 21 and 338 ± 46% (P = NS), where \( \Delta \) is change in.

Histamine Release in BAL

Baseline concentrations of histamine were comparable in different groups at 0.1–2.9 nM. Segmental antigen challenge caused a marked increase in BAL histamine concentration, which was not inhibited by NAF-heparins (Fig. 5): HMW NAF-heparin (96 ± 20 vs. 84 ± 18 nM, n = 8), MMW NAF-heparin (74 ± 15 vs. 78 ± 8 nM, n = 6), and ULMW NAF-heparin (44 ± 17 vs. 36 ± 17 nM, n = 7).

DISCUSSION

Inhaled heparin has been shown to attenuate antigen-induced acute bronchoconstriction in allergic sheep and to prevent the bronchoconstrictor response to exercise.
and antigen in subjects with asthma (2, 6, 10, 14, 20). Because inhaled heparin did not prolong the partial thromboplastin time, it has been suggested that the antiallergic activity of inhaled heparin observed in previous studies was probably related to its nonanticoagulant properties (2, 6, 20). The results of this study further extend our previous observations and indeed demonstrate that 1) nonanticoagulant fractions mediate the antiallergic activity of inhaled heparin, 2) the antiallergic activity of NAF-heparins is molecular weight dependent, and 3) a ULMW fraction (<2,500) is the most effective NAF-heparin.

The NAF-heparins used in the present study were prepared by different methods, have markedly reduced antifactor Xa and APTT activities, and lacked significant in vivo anticoagulant activity. The HMW NAF-heparin (ABL4) was obtained from commercial heparin by affinity chromatography on antithrombin III sepharose gel and is on average 10,500. It is a potent inhibitor of vascular smooth cell proliferation in vitro, and toxicity studies in vivo did not demonstrate any significant cumulative anticoagulant activity over a 1-mo treatment period (44). The MMW NAF-heparin was prepared by periodate degradation of standard heparin and is on average 6,500. It has also been shown to possess potent antiproliferative activity on vascular smooth muscle cells in experimental mesangio proliferative glomerulonephritis (11, 39). The ULMW NAF-heparin was prepared by nitrous acid depolymerization of porcine heparin followed by ethanol precipitation and fractionation; it is on average 2,400 (38).

It has been suggested that the antiallergic activity of inhaled fractionated heparins is molecular weight dependent (32). An inverse relationship between the antiallergic activity and molecular weight of fractionated heparins was observed (32). The ULMW heparin was the most effective fraction, with ID50 against ABR of 0.5 mg/kg; ID50 of LMW and MMW heparin were 1.25 and 1.8 mg/kg, respectively (32). However, the previous studies were conducted with fractionated LMW heparins with significant anticoagulant activities, as shown by increased anti-Xa activity. The results of the present study are consistent with the previous data and demonstrate that inhibition of antigen-induced ABR by nonanticoagulant heparins is also molecular weight dependent. The HMW NAF-heparin is the least effective fraction, with IC50 against ABR of 4 mg/kg; MMW and ULMW fractions are 5 and 10 times more potent than the HMW fraction, with IC50 of 0.8 and 0.4 mg/kg, respectively (Fig. 6).

Inhaled UF and fractionated LMW heparins have been shown to inhibit the antigen-induced AHR (7, 32).
The present study demonstrates that inhibition of postantigen AHR by UF and fractionated heparins is also related to the nonanticoagulant properties of heparin, inasmuch as NAF-heparins inhibited the postantigen AHR. The inhibition of postantigen AHR by NAF-heparins showed even greater dose and molecular weight dependency than did the inhibition of ABR. Although the HMW NAF-heparin attenuated the antigen-induced ABR, it had no significant effect on postantigen AHR. In contrast, the MMW and ULMW NAF-heparins inhibited the ABR and AHR in a dose-dependent fashion. The MMW NAF-heparin showed intermediate efficacy against AHR, with IC₅₀ of 1.4 mg/kg; the ULMW NAF-heparin was seven times more potent, with IC₅₀ of 0.2 mg/kg (Fig. 6).

Although previous studies have shown that anticoagulant ULMW heparin (CY-222) is the most potent fraction (32), the present observations suggest that potent antiallergic activity of ULMW heparin resides in the NAFs. Although pretreatment with UF and fractionated LMW heparins prevented the antigen-induced AHR, only ULMW heparin attenuated the AHR when administered after the antigen challenge (34). The present data with ULMW NAF-heparin are consistent with this concept and demonstrate its effectiveness in inhibiting AHR whether administered “before” or after the antigen challenge. The ULMW NAF-heparin was more potent in attenuating postantigen AHR when administered after the antigen challenge, as demonstrated by a 3.5-fold lower dose (ID₅₀ = 0.06 vs. 0.2 mg/kg) than that required for prevention of AHR.

The results of the present study also indicate that there are quantitative and qualitative differences between the antiallergic activity of various NAF-heparins, and a molecular-weight-dependent spectrum of activity was observed. The HMW fraction was the least effective, whereas the ULW fraction was the most potent, NAF-heparin. The reason for these differences is not clear. In addition to molecular weight, the differences in the antiallergic properties of various NAF-heparins may also be related to the saccharide chain length and/or variations in the chemical structure. The in vivo pharmacokinetic and pharmacodynamic properties of various inhaled NAF-heparins may also be different. LMW heparins generally have a lower binding affinity to endothelial cells (9) and reduced nonrenal cellular mechanisms of clearance, resulting in increased bioavailability and prolonged in vivo biological activity (37). It is possible that ULMW NAF-heparin may have better tissue penetration and bioavailability at potential sites of action, thus attenuating ABR and AHR, whereas the HMW fraction with limited bioavailability inhibits only ABR at very high doses. Alternatively, the potential sites and mechanisms of action of ULMW NAF-heparins may be different. The HMW fraction by acting only on mast cells may possess antiallergic activity (3), whereas the ULMW fraction, in addition, may also possess mast cell-independent anti-inflammatory properties, thus inhibiting ABR and AHR at very low doses.

The reason for the greater potency and the mechanism of selective inhibition of postantigen AHR by ULMW heparin when administered after the antigen challenge is not known. The glycosaminoglycan heparins have been shown to possess anti-inflammatory properties, including anticomplement action (46) and modulation of T lymphocytes (25), as well as inhibition of neutrophil chemotaxis (33), eosinophil influx (43), and free radical generation (23). Recent evidence shows that heparin strongly binds various cytokines, including tumor necrosis factor and interleukins-4 and -8 (22, 24), and decreases the blood clearance of interferon-γ (30). It has also been suggested that UF heparin and heparin oligosaccharides are effective L- and P-selectin inhibitors and demonstrate anti-inflammatory activity in vivo (35). Although molecular weight dependence of the anti-inflammatory properties of heparin fractions has not been studied, our data suggest that selective inhibition of postantigen AHR by ULMW fractions may be related to its potent anti-inflammatory activities.

The mechanism of antiallergic activity of NAF-heparins is not clear. The most potent NAF-heparin (KABI-2226) had no direct effect on airway smooth muscle, and it failed to inhibit the antigen-induced histamine release in BAL. The in vivo and in vitro studies have demonstrated that UF heparin prevents antigen-induced bronchoconstriction and AHR by inhibition of mast cell mediator release rather than by a direct effect on airway smooth muscle (1, 2, 7, 31). The antiallergic action of UF heparin was further supported by inhibition of antigen-induced histamine release in the BAL (34), as well as by prevention of degranulation and histamine release from isolated mast cells in vitro (7, 31). It has been suggested that the inhibitory effects of UF and fractionated heparins on antigen-induced histamine release in the BAL are also molecular weight dependent (34). Prevention of ABR and AHR by UF and LMW heparin was associated with marked inhibition of antigen-induced histamine release in the BAL, whereas ULMW heparin prevented the ABR and postantigen AHR without inhibiting the histamine release (4, 32, 34). These findings indicate that the structural domain of the heparin molecule responsible for attenuation of ABR and AHR resides in the glycosaminoglycan <2,500-Da chain length, whereas the histamine-release inhibitory domain is located in the >2,500-Da chain length.

The antiallergic activity of NAF-heparins is qualitatively different from UF and fractionated LMW heparins. Whereas a good correlation between the prevention of ABR and inhibition of antigen-induced histamine release in the BAL by UF and LMW heparins has been observed (4, 32, 34), nonanticoagulant heparins attenuated the ABR without inhibiting the histamine release in BAL. This suggests that the histamine release-inhibitory domain of heparin may overlap with anti-thrombin III-binding sites, since all nonanticoagulant heparins (i.e., HMW, MWH, and ULMW) failed to inhibit the antigen-induced histamine release in the BAL. The biological activities of heparin are primarily mediated through its binding of enzymes and various
other proteins (29), but only the antithrombin III-binding site involved in the anticoagulant action has been elucidated as a distinct pentasaccharide sequence (27, 28). The present study provides clear evidence that the antithrombin III-binding site and the domain responsible for the antiallergic activity are distinctly different. The presence of antiallergic activity in the nonanticoagulant fraction is of clinical importance, inasmuch as it may prevent potential bleeding complications that may be associated with long-term use of ULMW heparins.

In summary, the results of this study in conjunction with our previous data suggest that the antiallergic actions of heparin are independent of its anticoagulant activity and the structural domain possessing the antiallergic activity resides in <2,500 ULMW chains. The mechanism of action of ULMW NAF-heparins is independent of histamine release and mediated by an unknown antiallergic action.

Address for reprint requests: T. Ahmed, Div. of Pulmonary Diseases, Mount Sinai Medical Center, 4300 Alton Rd., Miami Beach, FL 33140.

Received 18 March 1998; accepted in final form 21 October 1998.