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Heparin and heparinoids prevent the binding of immune complexes containing nucleosomal antigens to the GBM and delay nephritis in MRL/*lpr* mice

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Heparin and heparinoids prevent the binding of immune complexes containing nucleosomal antigens to the GBM and delay nephritis in MRL/*lpr* mice. Monoclonal anti-nucleosome antibodies (mAbs) complexed to nucleosomal antigens can bind to DNA and to heparan sulfate (HS) in ELISA and to the GBM *in vivo* in a rat renal perfusion system, whereas non-complexed mAbs do not bind [1]. In this study, we analyzed whether heparin (HEP) or N-desulfated/acetylated heparins (DSA-HEP), structurally and functionally strongly related to HS, are able to prevent the binding of these complexed mAbs to DNA and to HS *in vitro* and to rat GBM *in vivo*. In ELISA the binding of nucleosome complexed anti-nucleosome antibodies to DNA and HS was inhibited dose-dependently by HEP, DSA-HEP and low molecular weight (LMW) DSA-HEP. Intravenous injection of nucleosome/anti-nucleosome immune complexes without heparin/heparinoids in BALB/c mice led to GBM binding, while simultaneous injection of heparin/heparinoids with complexed antibodies or pretreatment with heparin subcutaneously prior to injection of complexes prevented this binding. Subsequently, we tested the preventive effect of HEP, DSA-HEP and LMW-DSA-HEP on progression of renal disease in MRL/*lpr* mice. Treatment was started at an age of eight weeks in a dose of 50 μg daily. With all three drugs albuminuria was significantly delayed compared to PBS treated controls (cumulative incidence of proteinuria at 20 weeks in controls 60% vs. 13%, 14% and 6% respectively for HEP, DSA-HEP and LMW-DSA-HEP; $P < 0.05$). At week 21 the glomerulonephritis was histologically less severe in heparin/heparinoid treated animals ($P = 0.02$). In immunofluorescence the amount of immunoglobulin and C3 deposits in the glomerular capillary wall tended to be less in heparin/heparinoid treated mice compared to PBS treated controls ($P = 0.07$). Furthermore, at 20 weeks anti-HS levels in plasma of heparin/heparinoid treated mice were significantly lower ($P < 0.05$). We conclude that interaction of heparin or heparin analogs with HS reactive immune complexes containing nucleosomal antigens prevents the binding of these immune complexes to the GBM and delays nephritis in MRL/*lpr* mice.

Systemic lupus erythematosis (SLE) is an autoimmune disease characterized by the occurrence of numerous autoantibodies, primarily directed against nuclear antigens. Previously we have shown that these anti-nuclear antibodies complexed to nucleosomal antigens are able to bind to the glomerular basement membrane (GBM) *in vivo* [1, 2]. In these nucleosome containing immune complexes the positively charged histones interact with the negatively charged HS in the GBM [1]. The presence of these nucleosomes in the complexes is also responsible for the binding to heparan sulfate (HS) and DNA *in vitro*, since non-complexed antibodies do not bind [1, 3]. In the present study, we investigated whether heparin or heparin analogs, which have structurally and functionally strong similarities with HS, could interfere with the binding of these nucleosome complexed anti-nucleosome antibodies to HS and DNA *in vitro* and to the GBM *in vivo*. Heparin is a drug with many biological activities [4] and the therapeutic effects of heparin in various experimental models of kidney diseases have been investigated. In the renal ablation model heparin reduced systemic blood pressure, proteinuria and the extent of glomerular sclerosis [5–8]. Also in lupus mice heparin reduced the glomerular damage [9]. In the puromycine aminonucleoside-induced nephrotic syndrome, heparin treatment prevented glomerular sclerosis, mesangial proliferation and reduction of the glomerular filtration rate [10]. The development of hypertension and fibrinoid vascular lesions was prevented by heparin treatment in spontaneously hypertensive rats [11, 12]. In most studies a similar protective effect was also found for non-anticoagulant heparinoids [7, 10]. These results indicate that the beneficial effects can be obtained without the risks of anti-coagulation. Because of the renal protective properties and the functional and structural similarities with HS, we analyzed the effects of heparin (HEP) and the non-anticoagulant derivatives,

N-desulfated/acetylated heparin (DSA-HEP) and low molecular weight N-desulfated/acetylated heparin (LMW-DSA-HEP) on manifestations of glomerulonephritis in a spontaneous model of SLE, the MRL/lpr mouse

We assessed the effects of heparin and of heparinoids on autoantibody formation, proteinuria and glomerular Ig and complement deposition in MRL/*lpr* mice both prophylactically as well as therapeutically. In addition, we studied the effects of these

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drugs on the binding of complexed anti-nucleosomal autoantibodies in ELISA to DNA and HS, and *in vivo* to the GBM.

Methods

Animals

MRL *lpr/lpr* mice were bred from stock originally obtained via the Scripps Clinic and Research Foundation (La Jolla, CA, USA) from the Jackson Laboratory (Bar Harbor, ME, USA). All strains were maintained by brother/sister matings at our animal laboratory. For the *in vivo* inhibition studies, the *in vivo* coagulation studies and the *in vivo* assessment of immune modulation by the drugs, BALB/c mice were used, also originating from the Jackson Laboratory.

Drugs

Heparin (HEP) was commercially obtained from Organon Technica BV (Boxtel, The Netherlands). As non-coagulant heparin derivatives DSA-HEP (KF-E4282) and LMW-DSA-HEP (KF-E4567) were used, which were both produced and generously provided by Diosynth BV (Oss, The Netherlands). The N-desulfation of heparin was carried out as described by Inoue and Nagasawa [13]. Briefly, heparin was dissolved in water and passed through a cation-column (Dowex 50W × 12) in the H^+-form. The heparinic acid in the eluate was neutralized with pyridine (approximately 2.5 ml per gram of heparin) to pH 6.5. After freeze-drying, the pyridine salt of heparin was dissolved in 95% dimethylsulphoxide and 5% water and incubated for 30 minutes at 50°C. The desulfated heparin was obtained by adsorption on an anion exchanger (Bayer MP500A), elution with a 20% NaCl solution and precipitation with 75% methanol. Acetylation was carried out as described by Hirano and Ohashi [14]. The N-desulfated material was dissolved in a 10:1 water-methanol mixture and 1/20 of the total volume of acetic anhydride (1 ml per gram of heparin) was added. The pH was kept constant by addition of a Dowex 1 × 8 ion exchanger in the carbonate form. DSA-HEP was obtained by adsorption on an anion exchanger, elution, desalting on a G-10 column and freeze drying. LMW-DSA-HEP was obtained by enzymatic (heparin lyase I, EC.4.2.2.7) digestion of HEP, which then was desulfated and acetylated as described above. The sulfate content was determined by conductometric titration as described by Casu and Gennaro [15]. The N-acetyl content was determined by proton-NMR as described by Casu [16]. For control experiments a neutral polysaccharide Dextran (Pharmacia, Upsalla, Sweden) was used in concentrations identical to those of heparin/heparinoids.

Anticoagulation studies

The anticoagulant properties of DSA-HEP and LMW-DSA-HEP were analyzed and compared with standard HEP *in vitro* and *in vivo*. The assay for anti-factor Xa activity was carried out as described by Teien et al [17, 18] with minor modifications for use in microtiter plates. In addition the effect of DSA-HEP, LMW-DSA-HEP or HEP on the activated partial thromboplastin time (aPTT) was determined by addition of the drugs to normal mouse plasma in a concentration range of respectively 0 to 50 and 0 to 10 μg/ml. For the *in vivo* effect groups of BALB/c and MRL/*lpr* mice were treated for three days with daily subcutaneous injections of 0.1 ml saline or 50 μg of either HEP, DSA-HEP or LMW-DSA-HEP. Two hours after the last injection 0.9 ml blood was obtained by cardiac puncture in a 1 ml syringe containing 0.1 ml of CTAD solution (containing 0.089 M trisodium citrate, 0.021 M citic acid, 15 μM theophyllin, 3.7 μM adenosine and 0.198 μM dipyridamol). The plasma was collected after centrifugation and aPTT and heparin levels were determined, according to Fischer [19]. Briefly, in this assay 10 μl CTAD plasma or standard sample were mixed with 50 μl normal pooled plasma as a source of antithrombin III and 150 μl Tris-imidazole buffer (30 mM in 0.25 M NaCl, pH 8.4) and incubated for five minutes at 37°C. Thereafter 20 μl thrombin (6 U/ml) were added and the mixture was incubated for exactly 30 seconds. To measure the remaining free thrombin 20 μl (2 mM) of the chromogenic substrate S 2238 (KabiVitrium) was added and two minutes later the reaction was stopped with 50 μl 50% acetic acid. The extinction at 450 nm was measured in a EAR 400 microplate reader (SLT, Gröding, Austria). The heparin/AT III-complex mediated anti-thrombin activity (= heparin level) was calculated from a standard curve obtained with heparin. Treatment of BALB/c and MRL/*lpr* mice with HEP resulted in heparin levels of 6.0 ± 3.0 and 3.1 ± 1.5 mg/liter, respectively (controls 0.3 ± 0.2 mg/liter). The aPTT was measured by mixing 100 μl of citrated plasma with 100 μl PTT reagent of Stago (Asnières, France) containing Kieselguractivator and cephalin. After incubation for three minutes at 37°C, 100 μl 25 mM $CaCl_2$ was added and the clotting time was recorded with a KC 10 coagulometer (Amelung, Germany).

Preparation of nucleosome complexed and purified anti-nucleosome mAb

Anti-nucleosome antibodies are directed against nucleosome or subnucleosomal complexes consisting of the histone octamer and DNA. Purified non-complexed anti-nucleosome antibodies do not bind to individual histones or DNA. However, complexed to nucleosomal antigens these antibodies bind to DNA and HS in ELISA and to the GBM *in vivo* [1]. Complexed antibodies of an anti-nucleosomal antibody, mAb #34, were obtained by purification of culture supernatant under physiological conditions as described [1]. Noncomplexed mAb #34 was obtained by purification under high salt conditions preceded by a DNase treatment as previously described [1].

Antigen reactivity of purified and complexed anti-nucleosome mAb and of plasma samples

Anti-DNA and anti-HS reactivities were determined in ELISA as described previously [1, 20]. All ELISAs were titrated and the reciprocal of the dilution giving an absorption of 1.0 at 450 nm was used as titer.

Interaction of heparin or heparinoids with complexed anti-nucleosome mAb

The *in vitro* effects of heparin/heparinoids were analyzed in an inhibition ELISA to HS or DNA using a concentration range of heparin, heparinoids or dextran. Fifty microliters of the different inhibitors were added to the complexed anti-nucleosome mAb #34. After incubation for 45 minutes at room temperature the reactivity of the mAb to HS or DNA was measured in ELISA as described before. In these inhibition experiments the mAb was used in a concentration that gave 50% of the maximal absorption in the respective direct ELISAs.

The *in vivo* effects of heparin/heparinoids on GBM binding of complexed mAb #34 were evaluated in two different ways: (1) by renal perfusion studies in the rat of complexed mAb #34 with or without heparin; (2) by i.v. injection in BALB/c mice of complexed mAb #34 with or without pretreatment of the mice with heparin. For the renal perfusion studies male Wistar rats (150 g) were anesthetized by intraperitoneal administration of 0.15 ml (9 mg) sodium pentobarbital (Narcovet; Appharma, Arnhem, the Netherlands). The control renal perfusion was performed as described before [1]. For inhibition experiments 150 μg of complexed mAb #34 was mixed with 150 μg of heparin/heparinoids for 30 minutes at room temperature before renal perfusion. After perfusion the perfused kidney was taken out and immediately snap frozen in liquid N_2 for evaluation of IgG binding by immunofluorescence (IF).

The glomerular binding after i.v. injection of complexed mAb was evaluated in BALB/c mice injected with 150 μg complexed mAb #34 after pretreatment for three days with either daily subcutaneous injections of 0.1 ml PBS or 50 μg HEP. Two hours after the last injection, the complexed mAb #34 was injected i.v. After five minutes the kidneys were taken out for IF.

*Treatment protocols in MRL/*lpr *mice*

For the first set of experiments to test the preventive effect, eight-week-old MRL/*lpr* mice were treated once daily with a subcutaneous (s.c.) injection of 50 μg of either HEP, DSA-HEP or LMW-DSA-HEP in 0.1 ml PBS. Control animals received 0.1 ml PBS s.c. daily. Each group consisted of 15 animals (8 males and 7 females). Mice were treated from week 8 until week 21. At the start and after 12 weeks of treatment blood was drawn for the determination of the anti-DNA and anti-HS titer in plasma. Albuminuria was screened every week by urine collection in metabolic cages for 18 hours. After 13 weeks of treatment (at the age of 21 weeks) the animals were sacrificed and the kidneys were removed for immunofluorescence.

In a second set of experiments, the therapeutic effect on established albuminuria (albuminuria period before treatment < 7 days) was assessed. MRL/*lpr* mice between the ages of 20 to 30 weeks with an albuminuria of at least 1000 μg/18 hr were randomized for magnitude of albuminuria, age and sex to treatment with either 50 μg HEP, 50 μg LMW-DSA-HEP or 0.1 ml PBS once daily subcutaneously (each group $N = 12$). After the start of the treatment the albuminuria was analyzed every two weeks and the effect on survival was evaluated. At several time points during treatment plasma was collected for determination of anti-DNA and anti-HS titers.

To investigate whether treatment with heparinoids could form a useful addition to immunosuppressive treatment, we performed a pilot experiment in which we tested whether the combination of LMW-DSA-HEP and cyclophosphamide (CY) was more effective than CY treatment alone. MRL/*lpr* mice between the age of 20 to 25 weeks with an albuminuria of at least 300 μg/18 hr and a duration of albuminuria of less than seven days, were randomized for magnitude of albuminuria, age and sex. Mice were treated once daily s.c. with either PBS (group A and group B) or with 50 μg LMW-DSA-HEP (group C). In addition mice in groups B and C received a weekly i.p. injection of CY (Endoxan^R-Asta; Dagra Pharma B.V., Diemen, The Netherlands) in a dose of 20 mg/kg body wt while control mice in group A received an equivalent volume of PBS. Each group consisted of 5 animals. The effect of this treatment regimen was evaluated for albuminuria and survival.

Disease activity assessment

Anti-DNA and anti-HS reactivities were determined in ELISA as described above. The HS ELISA was used to detect nucleosome complexed autoantibodies in the circulation [3]. After collection of urine for 18 hours in metabolic cages, albuminuria was analyzed with radial immunodiffusion with rabbit anti-mouse albumin antibodies (Cappel, West Chester, PA, USA) as described before [21, 22]. Mouse albumin 5 mg/ml (Sigma Chemical Company, St. Louis, MO, USA) was used as a standard. To study the glomerular deposition of mouse Ig and C3 and staining of GBM HS, indirect immunofluorescence was performed on 2 μm cryostat sections of kidney tissue, according to techniques described previously [22]. The coded sections of the kidneys were analyzed by 3 different observers and scored semiquantitatively on a scale from 0 to 4+ (in at least 20 glomeruli per mouse kidney). For staining of Ig and C3 both the amount and intensity of staining was scored. No staining was scored 0; mild staining was scored 1; moderate staining was scored 3 and severe staining was scored 4. For HS staining in the capillary loops, no staining at all was scored 0, staining of 25% off all capillary loops and/or 75% decrease of staining intensity (as compared with a normal BALB/c glomerulus) was scored 1; staining of 50% off all capillary loops and/or 50% decrease of staining intensity was scored 2; staining of 75% of all capillary loops and/or 25% decrease of staining intensity was scored 3, and normal staining of all capillary loops was scored 4. For light microscopy tissue fragments fixed in Bouin's solution were dehydrated and embedded in Paraplast (Amstelstad B.V., Amsterdam, The Netherlands). Four-micrometer sections were stained with periodic acid-Schiff and silver methenamine [21]. Coded sections were analyzed and scored on a scale 0 to 3+. The 0 to 3+ scale was defined as follows: no glomerulonephritis was scored 0; mild/moderate glomerulonephritis was scored 1+ to 2+; and severe glomerulonephritis was scored 2+ to 3+.

In vivo *assessment of immune modulating properties of heparin/heparinoids*

The immunosuppressive properties of HEP, DSA-HEP and LMW-DSA-HEP were studied in normal BALB/c mice by measuring their influence on delayed type hypersensitivity (DTH) and primary antibody response towards bovine serum albumin (BSA) according to methods described previously [23]. In brief, DTH reactivity was determined in 4 groups of 10 BALB/c mice which were treated daily with 0.1 ml PBS s.c. or 50 μg of either HEP, DSA-HEP or LMW-DSA-HEP. Treatment was started at day −2. At day 0 all mice received 0.1 mg BSA in 0.1 ml complete Freund's adjuvant s.c. At day 13 a rechallenge was given of 5 μl BSA s.c. (2 mg/ml) into the pinna of the right ear. As control 5 μl PBS was injected in the pinna of the left ear. At 4, 24 and 48 hours after the rechallenge duplicate measurements of the ear thickness ($\times 10^{-3}$) were made with an engineer's micrometer. The DTH reactivity is expressed as the ratio between the swelling of the antigen injected site (R) and the saline injected site (L) as measured after 48 hours. At day 15 blood was collected from the same animals for the determination of the anti-BSA response. Anti-BSA titers were measured in ELISA [23]. During the whole

Table 1. Biochemical characteristics and anti-coagulant properties of heparinoids and dextran used as a control non-charged polysaccharide

Drug	Abbreviation	Number of sulfate groups per disaccharide	% Of glucosamine residues containing a N-acetyl group	Molecular wt	Anti-Xa activity *U/mg*	aPTT *in vivo* *sec*[a]
Heparin	HEP	3.0	25	15 kDa	200	39 ± 12
N-desulfated/ acetylated heparin	DSA-HEP	2.3	100	14 kDa	<2.4	28 ± 8
Low molecular weight N-desulfated/acetylated heparin	LMW-DSA-HEP	2.3	100	7 kDa	<2.4	25 ± 5
Dextran	Dextran	0	ND	14 kDa	<2.4	ND

Values are given as means ± SD. The aPTT in PBS treated animals was 30 ± 6 sec. The values were statistically not different between drug and PBS treated animals.

[a] The aPTT in mice treated once daily with a subcutaneous injection of 50 μg of either HEP, DSA-HEP or LMW-DSA-HEP compared to mice treated with PBS ($N = 5$ per group)

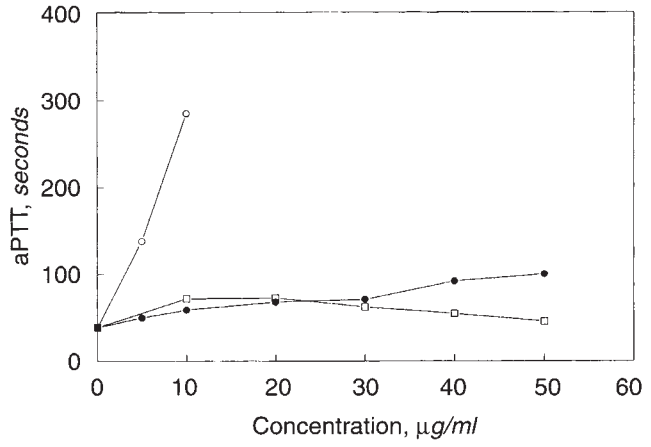

Fig. 1. *Effect of heparin (HEP; ○), N-desulfated/acetylated heparin (DSA-HEP; ●) or low molecular weight N-desulfated/acetylated heparin (LMW-DSA-HEP; □) on aPTT* in vitro.

experimental period the daily administration of the drugs was continued.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U*-test. A two-sided $P < 0.05$ was considered to be statistically significant. Spearman's correlation coefficient was used for linear regression analysis.

Results

Characteristics and anti-coagulant properties

The biochemical characteristics of HEP, DSA-HEP, LMW-DSA-HEP and dextran are listed in Table 1. As can be seen only HEP had an anti-factor Xa activity *in vitro* while the other preparations were devoided of any anti-factor Xa activity. A similar pattern was observed analyzing the effect on the activated partial thromboplastin time (aPTT) *in vitro*. Even addition of large amounts of DSA-HEP and LMW-DSA-HEP gave hardly any prolongation of the aPTT (Fig. 1). To prolong the aPTT to 100 seconds 25 times more DSA-HEP than HEP was needed while addition of LMW-DSA-HEP had no effect. The effect on coagulation *in vivo* using 50 μg of the various drugs is also shown in Table 1. In all drug treated animals the aPTT was not significantly different from control animals receiving PBS.

Inhibition of the binding of complexed anti-nucleosome mAb to HS or DNA by heparin or heparinoids in ELISA

In pure non-complexed form mAb 34 reacted only with nucleosomes and had no reactivity with DNA or HS. Complexed with nucleosomes, however, this mAb bound not only to nucleosomes but also to DNA and HS as described before [1]. The binding of complexed mAb #34 to HS and DNA could be inhibited by HEP, DSA-HEP and LMW-DSA-HEP in a dose-dependent manner as shown in Figure 2. More DSA-HEP and LMW-DSA-HEP were required to achieve a similar degree of inhibition as with heparin, which in all probability is due to the partial desulfation of the former reagents. In contrast to heparin and the heparinoids, dextran was not able to inhibit the binding of complexed mAb #34 in these competitive inhibition ELISAs. This inhibition was not unique for mAb #34 since HEP, DSA-HEP and LMW-DSA-HEP could also inhibit to a similar degree the binding of other previously described [1] complexed anti-nucleosome mAbs (#2 and #32) to HS and DNA (data not shown)

Inhibition of glomerular binding of complexed anti-nucleosome mAb by heparin and heparinoids

Previously, we have shown that after renal perfusion of complexed anti-nucleosome mAb #34 glomerular binding occurs, while in purified form the mAb does not show any binding [1]. In this study, we tested the effect of heparin/heparinoids on this GBM binding by renal perfusion (in Wistar rats) of complexed mAb #34 with or without heparin/heparinoids ($N = 3$ per group). When, prior to perfusion, complexed mAb #34 was mixed with PBS clear binding of the complex to the glomerulus was seen. After perfusion of complexed anti-nucleosome mAb #34 mixed with dextran a comparable binding was observed (Fig. 3A). However, the addition of heparin, DSA-HEP or LMW-DSA-HEP to the complexed mAb #34 could completely prevent the glomerular binding (Fig. 3B). Similar results were obtained after pretreatment of BALB/c mice with either PBS or HEP. BALB/c mice were pretreated for three days with either daily s.c. injections of PBS or HEP ($N = 4$ per group). Two hours after the last injection the complexed mAb #34 was injected i.v. Pretreatment of the mice with HEP resulted in detectable heparin levels (6.0 ± 3.0 mg/liter; controls, 0.3 ± 0.2 mg/liter) in the plasma at the time of injection of the compexed anti-nucleosome mAb #34. When complexed mAb #34 was injected i.v. after pretreatment with a daily subcutaneous injection of PBS glomerular binding was observed identical to that shown in Figure 3A. In contrast to this,

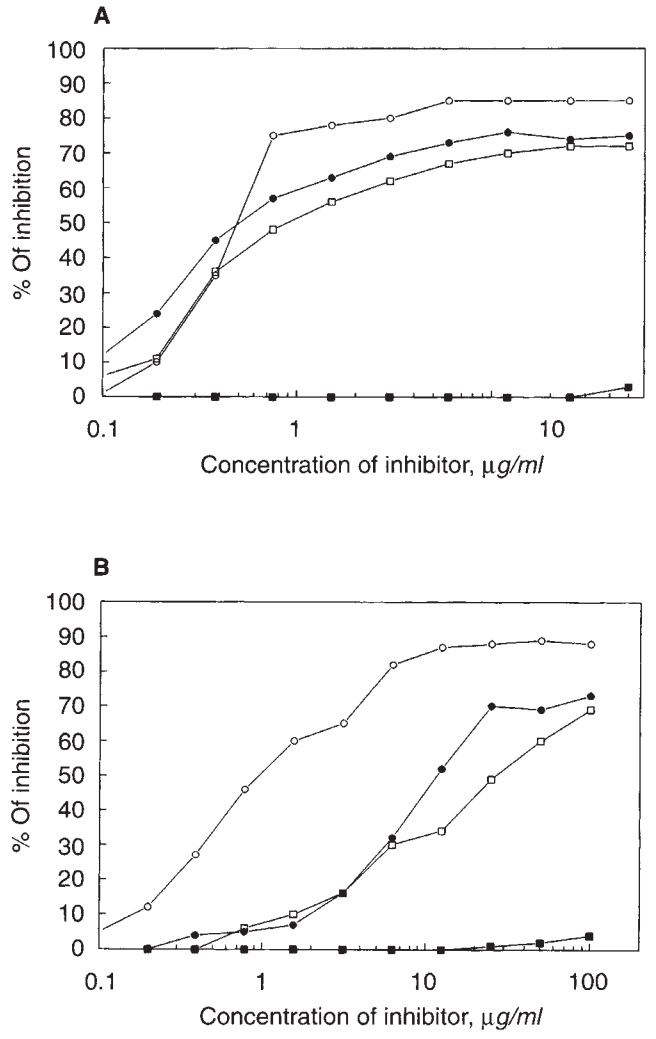

Fig. 2. *Inhibition of the binding of the complexed anti-nucleosome mAb #34 to HS* (**A**) *and DNA* (**B**) *in the inhibition ELISA.* Binding of complexed #34 was inhibited dose dependently by HEP (○), DSA-HEP (□) and LMW-DSA-HEP (●). Dextran (■) could not inhibit the binding. For abbreviations see legend Figure 1.

i.v. injection of complexed mAb #34 in heparin pretreated mice did not lead to any GBM binding.

*Effects on glomerular disease in MRL/*lpr *mice*

In the preventive study a daily dose 50 μg of HEP, DSA-HEP or LMW-DSA-HEP significantly delayed the onset of albuminuria in MRL/*lpr* mice compared to PBS treated controls ($P < 0.05$; Fig. 4). At 20 weeks the cumulative incidence of albuminuria in PBS treated animals was 60%, while in the drug treated groups this was respectively 13%, 14% and 6% for HEP, DSA-HEP and LMW-DSA-HEP. Furthermore, the administration of HEP or heparinoids prevented the development of the histological hallmarks of glomerulonephritis. At week 21, 10 out of 15 PBS treated control MRL/*lpr* mice had glomerulonephritis. In contrast, mice treated with HEP, DSA-HEP and LMW-DSA-HEP, respectively, showed a significant lower prevalence of glomerulonephritis (3/15, 3/14 and 2/15 of the mice, respectively, $P = 0.03$, $P = 0.03$, $P = 0.008$; Fisher Exact test; Fig. 5). In direct immunofluorescence of kidney sections of drug-treated animals we found compared with PBS treated controls, that there was a decrease in the amount of immunoglobulin deposits in the glomerular capillary wall ($P = 0.07$), but not in the mesangium ($P = 0.90$; Figs. 6 and 7 A, B). The pattern of C3 deposition along the glomerular capillary wall was identical to that of the Ig deposits. Less C3 deposits were observed in heparin/heparinoid treated groups (Fig. 7 C, D). Previously, we have shown that the staining of HS in the GBM of MRL/*lpr* mice is significantly decreased and correlated with the extent of IgG deposits [22]. In the present study we observed the same phenomenon, since HS staining was markedly decreased in the GBM of PBS treated mice compared with PBS treated controls. However, in heparin/heparinoid treated mice this decrease in HS staining was much less as compared to PBS treated controls (Fig. 7F). As we have described before [22] and also found in this study, there was an inverse correlation between HS staining and albuminuria ($r_s = -0.65$, $P < 0.0001$) and GBM Ig deposits ($r_s = -0.54$, $P < 0.0001$). At the start of the treatment the anti-DNA and anti-HS reactivities in plasma were similar in all groups and rose with age (data not shown). After 12 weeks of treatment the anti-HS levels were significantly lower in HEP, DSA-HEP or LMW-DSA-HEP treated groups ($P < 0.05$, 0.03 and 0.05, respectively) compared to PBS treated controls (Fig. 8). At that time there were no differences in anti-DNA antibody levels between the groups. To establish whether HEP, DSA-HEP and LMW-DSA-HEP could also exert an antiproteinuric effect in MRL/*lpr* mice with an established albuminuria (range: 1000 to 9000 μg/18 hr) animals were treated daily with 50 μg HEP or LMW-DSA-HEP. In the PBS treated group there was an increase of albuminuria in 90% of the mice. HEP and LMW-DSA-HEP treated groups showed an increase of albuminuria in 55% and 60% of the mice, respectively. This difference was not statistically significant. Both drugs also had no effect on survival, anti-DNA or anti-HS titers (data not shown).

In the pilot experiment, in which the additive effect of heparin to immunosuppressive treatment was tested, MRL/*lpr* mice with an established albuminuria were treated with PBS/CY or LMW-DSA-HEP/CY. Control mice were treated with PBS only. This experiment revealed that there was a significant beneficial effect on survival for the LMW-DSA-HEP/CY treated mice as compared to PBS/CY treated mice ($P < 0.05$) and that the LMW-DSA-HEP/CY treatment halted the progression of the proteinuria.

Effects on immune reactivity

Treatment with 50 μg of either HEP, DSA-HEP or LMW-DSA-HEP had no effect on DTH reactivity or on the primary antibody response towards BSA (Table 2).

Discussion

This study shows that heparin and non-anticoagulant heparin derivatives are able to inhibit the binding of nucleosome complexed antibodies to DNA and HS. Also the *in vivo* GBM binding of these complexes after renal perfusion was prevented. Furthermore, these drugs prevented the development of glomerular lesions in MRL/*lpr* mice. In addition, we found that heparin/heparinoid treatment can retard the onset of albuminuria and significantly reduces anti-HS reactivity in plasma. Because heparin has many biological actions in inflammation and tissue repair this beneficial effect could be due to number of actions. It has

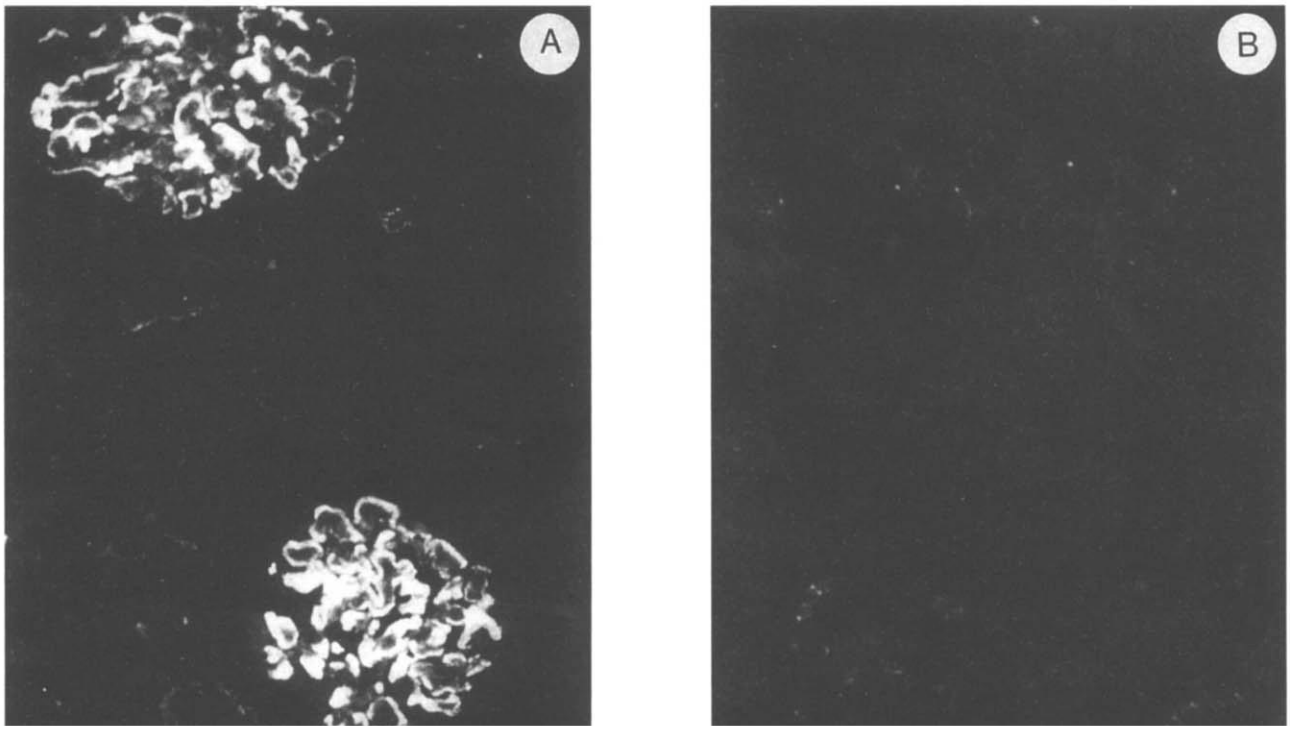

Fig. 3. *Direct immunofluorescence of glomerulus after perfusion of complexed anti-nucleosome mAb #34 mixed with dextran (A) or DSA-HEP (B).* After perfusion of complexed mAb #34 mixed with dextran clear binding of the complex to the glomerulus was observed (A). However, the addition of DSA-HEP to the complexed mAb #34 could completely prevent this binding (B).

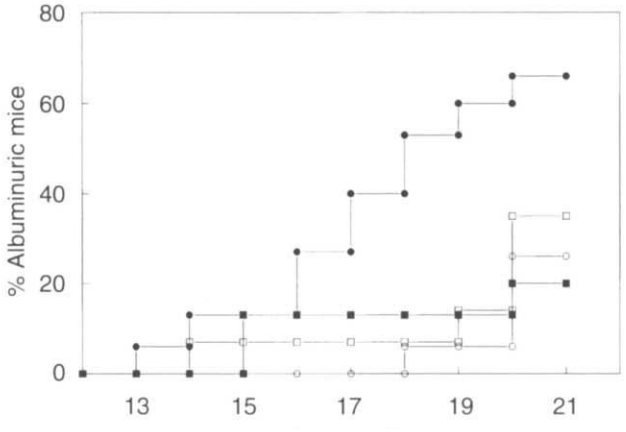

Fig. 4. *Cumulative incidence of albuminuria in the various groups of MRL/*lpr *mice.* Starting at the age of eight weeks MRL/*lpr* mice were treated once daily with either 50 μg HEP, DSA-HEP or LMW-DSA-HEP and compared with PBS treated controls. Each group consisted of 15 animals. Albuminuria was considered to be present if the urinary albumin excretion exceeded 300 μg/18 hr (upper limit of albuminuria (mean + 2 × SD) in non-SLE normal control mice: 100 μg/18 hr). PBS (●), HEP (■), DSA-HEP (□) and LMW-DSA-HEP (○), $P < 0.05$.

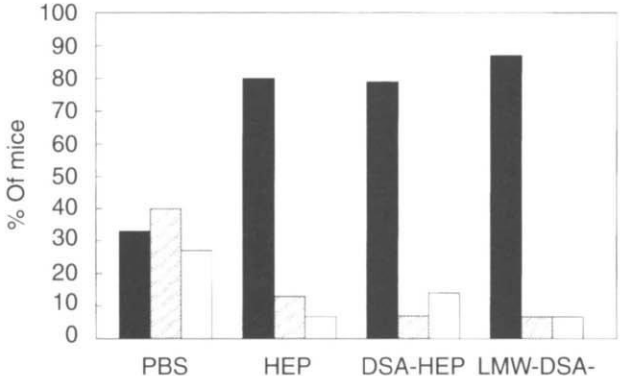

Fig. 5. *Severity of glomerular lesions in MRL/*lpr *mice treated with PBS, HEP, DSA-HEP or LMW-DSA-HEP.* No glomerulonephritis (score 0, ■), mild glomerulonephritis (score <2+, ▨), severe glomerulonephritis (score >2+, □).

been shown that heparin inhibits mesangial cell proliferation [24–26], modulates proteoglycan synthesis *in vitro* [13, 26, 27] as well as *in vivo* [10, 28, 29], exerts an anti-mitogenic effect on glomerular epithelial cells [30] and inhibits the binding of immune complexes to mesangial cells at least *in vitro* [31, 32]. From our data we cannot draw conclusions whether these properties contributed to the anti-proteinuric observed effect, but there were no differences between the control and drug treated mice in the extent of mesangial matrix expansion and mesangial immunoglobulin deposition. The beneficial effect of heparin could also be due to its immunosuppressive properties [33–36]. However, it is unlikely that this immunosuppressive effect is responsible for the diminution of the glomerulonephritis, since no effect on primary immune reactions like DTH and antibody response was evident. Also, the fact that the levels of anti-DNA antibodies were

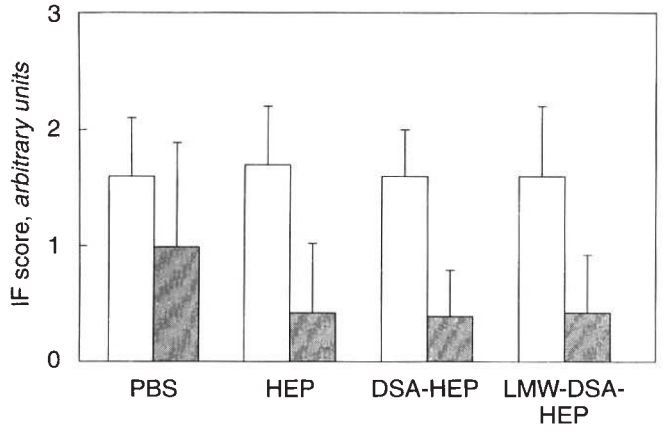

Fig. 6. *The amount of Ig deposition in glomerular capillary loops (▨) and mesangium (□) in glomeruli of mice treated with PBS, HEP, DSA-HEP or LMW-DSA-HEP.* Deposition of Ig in the mesangium is similar in all groups. A smaller amount of Ig deposition in the GBM was observed in heparin/heparinoid treated mice compared to PBS treated controls.

unaltered does not provide support for an immunosuppressive effect. Therefore, an alternative explanation for the renoprotective effect seems more likely. Several years ago we found that anti-DNA antibodies could cross react with HS [3]. Later it became apparent that this binding was not a direct antibody binding but due to nucleosomes complexed to the antibody [2]. The histone part of the nucleosome was responsible for the binding to HS. Based on these *in vitro* results we could show that anti-nuclear antibodies complexed to nucleosomal antigens are potential nephritogenic immune complexes via their interaction with HS and other anionic sites in the GBM [1]. These complexes can be identified in the circulation by using an anti-HS-ELISA [3]. This anti-HS reactivity correlates with the onset and exacerbation of renal manifestations of SLE [37–39]. In this study we show that heparin and heparinoids are able to prevent the binding of these complexed anti-nucleosome antibodies to DNA and HS in ELISA. In our view this inhibition is due to the binding of heparin/heparinoids to cationic charged nucleosomal antigens within the complex. This explanation is different from that offered by others showing inhibition of binding of SLE auto-antibodies to HS or DNA in ELISA by heparin [9, 40]. In these latter studies inhibition of "direct" binding was suggested. That inhibition of binding of nucleosome complexed anti-nuclear antibodies takes place also *in vivo* is suggested by our finding that the plasma anti-HS reactivity in the heparin/heparinoid treated mice was significantly reduced. The inhibitory effect of heparin/heparinoids is not restricted to serum-derived complexed autoantibodies but also to antibodies eluted from glomeruli in SLE nephritis. In glomerular eluates from albuminuric MRL/*lpr* mice, besides strong anti-DNA reactivity, we also found anti-HS reactivity. Both reactivities could be inhibited by heparin and heparinoids (van Bruggen et al, unpublished observations). Similar results have been published not only for MRL/*lpr* mice but also for human SLE nephritis [9]. These data support the suggestion that the protective effect of heparin and heparinoids can be explained by their ability to prevent the binding of nucleosome containing immune complexes to HS in the GBM.

In line with the *in vitro* findings, we found in the acute GBM binding studies that heparin/heparinoids were able to prevent GBM binding both after renal perfusion and after i.v. injection of nucleosome complexed antibodies. More importantly, a similar mechanism seems to occur in the chronic model of lupus nephritis in MRL/*lpr* mice. The onset of albuminuria was significantly postponed by heparin and the used heparinoids. This was also reflected by the decrease in histological severity of the glomerulonephritis in the drug treated animals. Furthermore, the amount of immunoglobulin deposits in the glomerular capillary wall tended to be less in heparin/heparinoid treated MRL/*lpr* mice compared to PBS treated controls. This decrease in IgG deposits is relevant for the degree of proteinuria, since we found a strong correlation between GBM-Ig deposits and albuminuria. Complement activation is an important contribution to tissue damage in diseases mediated by immune complexes like lupus nephritis. The pattern of C3 deposition along the capillary wall was identical to the Ig deposition. This suggests that the reduced deposition of C3 in drug treated animals is secondary to the decrease of IgG deposition. However, we cannot exclude that heparin and the heparinoids had a direct effect on complement, since inhibition of complement activation *in vitro* and *in vivo* has been described [41, 42].

In this study we could confirm in the control group our earlier observation that in MRL/*lpr* mice the staining of GBM-HS is strongly reduced and related to proteinuria and GBM-Ig deposition [22]. Heparin/heparinoids were able to prevent this decrease of HS staining significantly. As outlined above, this seems to be due to the reduction of deposition of nucleosome complexed autoantibodies in the GBM. A similar mechanism has been demonstrated for other cationic molecules like human platelet-derived cationic molecules [43] and platelet factor 4 [44], which binding to the glomerular polyanion was inhibited by heparin. However, in recent years data has emerged that points to additional mechanisms which could explain the observed beneficial effect of heparin. It was found that glycosaminoglycans (GAGs) like heparin could prevent the progressive loss of HS-associated anionic charges in the GBM in adriamycin nephropathy [45] and in streptozotocin-induced diabetic nephropathy [46, 47]. The significance of these observations is supported by our findings that GBM-HS expression in adriamycin nephropathy [48] and diabetic nephropathy [49] is significantly correlated to proteinuria. Indeed, in patients with insulin-dependent diabetes microalbuminuria was substantially reduced by heparin treatment [50]. These protective effects were not related to the anticoagulant properties of the agents. It is therefore conceivable that exogenous administration of GAGs can interfere with the functional decrease of the glomerular polyanion, that is, HS. A similar mechanism might have contributed to the attenuation of the decreased expression of HS in the GBM by heparin/heparinoids as observed in this study. However, once albuminuria was established, heparin or heparinoid treatment did not reduce it, suggesting that the loss of HS was not corrected anymore. We therefore assume that immune complexes, once they are bound to the GBM, are not resolved by heparin/heparinoids.

Our preliminary data on the combination treatment of LMW-DSA-HEP and CY suggest that addition of non-anticoagulant heparinoids to immunosuppressive drugs in the initial phases of treatment of lupus nephritis could be of clinical importance. After initiation of the immunosuppressive treatment for disease flares the reduction of the autoantibody production is not immediate.

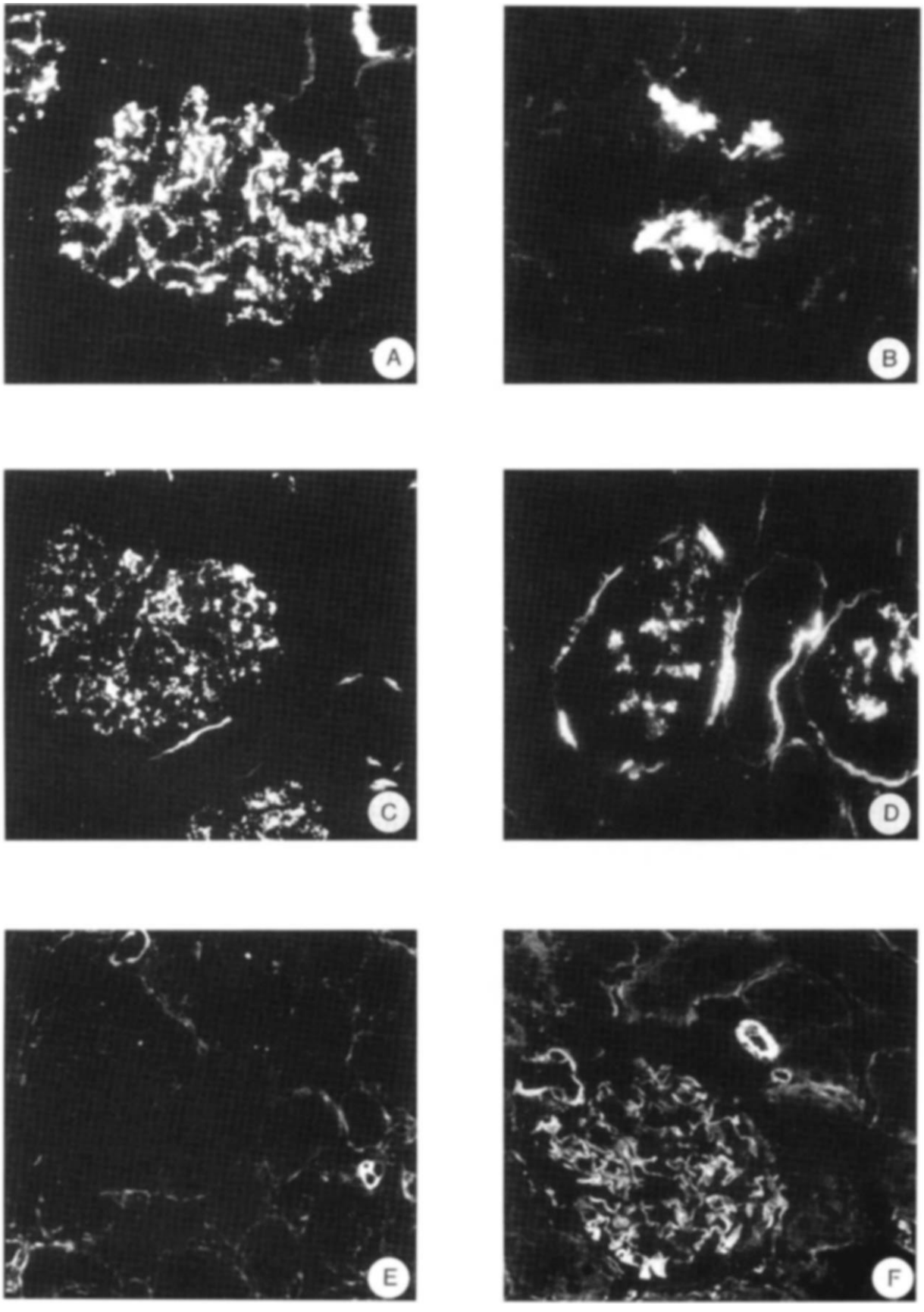

Fig. 7. *Representative examples of the immunofluorescence findings of glomeruli of MRL/*lpr *mice showing deposition of Ig after PBS treatment (***A***) or HEP treatment (***B***); staining for C3 in PBS treated mice (***C***) and HEP treated mice (***D***); staining for HS in PBS treated mice (***E***) and HEP treated mice (***F***).* The few animals that developed albuminuria in the treated heparin/heparinoid treated groups showed a staining pattern similar to that of albuminuric PBS treated controls.

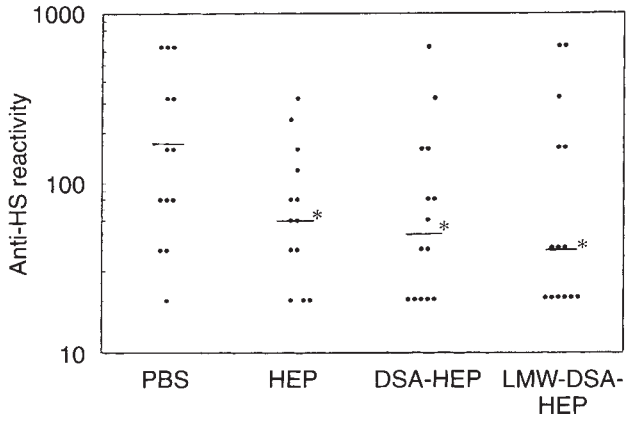

Fig. 8. *The anti-HS reactivity at the age of 21 weeks in plasma samples of MRL/*lpr *mice treated with PBS, HEP, DSA-HEP or LMW-DSA-HEP.* Individual titers are given plus the median value for each group. $^*P < 0.05$.

Table 2. Influence of 50 μg daily of either HEP, DSA-HEP or LMW-DSA-HEP on delayed type hypersensitivity (DTH) and primary antibody response after immunization with BSA

Treatment	DTH R/L ratio[a]	Primary antibody response[b]
PBS	1.5 ± 0.2	100 (20–480)
HEP	1.4 ± 0.2	80 (40–320)
DSA-HEP	1.3 ± 0.1	280 (10–640)
LMW-DSA-HEP	1.3 ± 0.2	320 (10–1900)

Control animals were treated daily with PBS. Results are expressed as means ± SD or medians (range).

[a] ratio between the antigen (R) and PBS (L) injected site

[b] titer

During this period, temporary treatment with heparinoids could prevent further glomerular damage by reducing the deposition of nephritogenic complexes. Further studies are needed to see whether this promising option is realistic.

In conclusion, our study indicates that heparin, and more importantly non-coagulant heparin derivatives, are able to bind to nephritogenic complexes consisting of nucleosomes and antinuclear antibodies. This binding of heparin/heparinoids prevents the subsequent deposition of these nephritogenic immune complexes in the GBM. By this diminution of IgG deposition the ensuing glomerular inflammation and albuminuria is reduced.

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