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Microangiographic evaluation of the effects of heparin on progressive Masugi nephritis

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Microangiographic evaluation of the effects of heparin on progressive Masugi nephritis. Unilateral progressive Masugi nephritis was produced in rabbits and studied by microangiography as well as light and immunofluorescence microscopy. The following five groups were studied: *Group 1*. Heparin was started simultaneously with nephrotoxic serum (NTS) and was given for one week. The animals were then sacrificed along with the untreated controls. *Group 2*. This was the same protocol as in group 1 but with three weeks' heparin. *Group 3*. Heparin was started one day after NTS and was given for three weeks. *Group 4*. Heparin was started one to two weeks after NTS and was given for three weeks. *Group 5*: late effect group. Heparin was started simultaneously with NTS, was given for four weeks, and the animals then were sacrificed 10 to 13 weeks later. Heparin dose was 5,000 U, s.c., per day in all treated groups. The number of glomeruli seen per unit of cortex by microangiography was significantly increased in the first through the third groups, as compared to the controls. Group 1 did not show this increase but there was some decrease of immunofluorescent fibrinogen. The late effect group (group 5) showed no modification by the treatment, suggesting that an initial improvement may have been negated by persistent immunologic insults after heparin withdrawal.

Evaluation microangiographique des effets de l'héparine sur la néphrite progressive de Masugi. Une néphrite unilatérale de Masugi a été produite chez des lapins et étudiée par microangiographie et par microscopie photonique et immunofluorescence. Les cinq groupes suivants ont été étudiés: *1)* groupe simultané une semaine, l'héparine est commencée en même temps que le sérum néphrotoxique (NTS) et administrée pendant une semaine, *2)* groupe simultané trois semaines, semblable à groupe 1, mais l'administration d'héparine dure trois semaines, *3)* groupe un jour, l'héparine est commencée un jour après NTS, et administrée pendant trois semaines, *4)* groupe une semaine, l'héparine est commencée une à deux semaines après NTS et administrée pendant trois semaines, *5)* groupe des effets tardifs, l'héparine est commencée en même temps que NTS, donnée pendant 4 semaines, les animaux sont sacrifiés 10 à 13 semaines plus tard. La dose d'héparine est de 5,000 U, s.c., par jour dans tous les groupes traités. Le nombre de glomérules vus par unité de cortex en microangiographie est significativement augmenté dans le premier et le troisième groupe par comparaison avec les contrôles. Le groupe une semaine n'a pas une telle augmentation, mais on observe une diminution de la fluorescence du fibrinogène. Le groupe des effets tardifs n'est pas modifié par le traitement, ce qui suggère que l'amélioration initiale peut avoir été effacée par la persistance du processus immunologique après l'arrêt de l'héparine.

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The beneficial effects of anticoagulants in human as well as experimental glomerulonephritis have been reported [1-4], and it has been suggested that intraglomerular coagulation plays an important role in the progression of various glomerular lesions. However, two recent reports by Bone et al [5] and Border, Wilson, and Dixon [6] have questioned the effects of anticoagulants in experimental glomerulonephritis. There is a need for more basic information on mechanisms and possible limitations of anticoagulants before accepting them as an established treatment.

In the present study, we quantitatively evaluated both the prophylactic and therapeutic effects of heparin in unilateral progressive Masugi nephritis, by the use of microangiography as well as light and immunofluorescent microscopy.

Methods

Experimental model. Albino rabbits weighing 2.0 to 2.5 kg were used. Unilateral progressive Masugi nephritis was produced according to the method of Sarre and Wirtz [7] and modified by Nagasawa [8], which has been described in a previous report [9]. Briefly, after ligation of the right renal artery with fine silk, duck anti-rabbit kidney serum (nephrotoxic serum or NTS) was injected into the ear vein (2.5 ml/kg of body wt). Twenty minutes later, the ligature was released. In this experimental model, the non-ligated kidney is known to progress to a contracted kidney over a period of three or more months. We were able to confirm this phenomenon (Fig. 1).

Experimental groups: Group 1 (simultaneous NTS+H – one week group). Simultaneously with the

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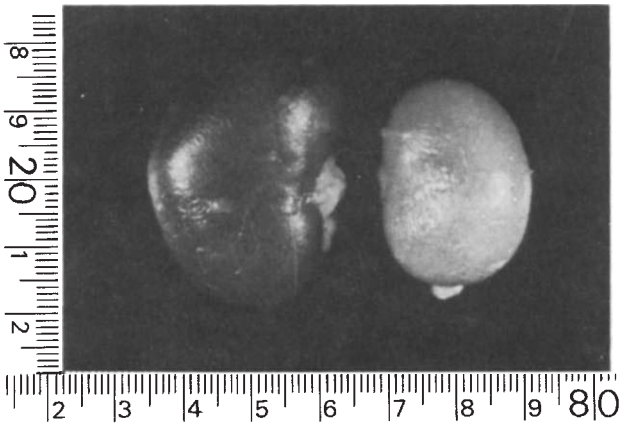

Fig. 1. *Experimental model: The non-ligated kidney progressed to a contracted kidney, while the ligated kidney became hypertrophic.*

NTS injection, 500 U of heparin (H) was infused into the opposite ear vein, and 2,000 U of heparin was administered s.c. around the hip. Then, 2,500 U of heparin was given s.c. every 12 hr (5,000 U per day). In this group, heparin was continued for one week, and then the animals were sacrificed. This heparin dose was the same in all treated groups. In a pilot study, clotting time, by the Lee-White method, in 12 normal rabbits given this heparin dose was prolonged to about twice the normal value at one hour before the scheduled administration of heparin.

Group 2 (simultaneous NTS+H – three weeks group). Heparin was started simultaneously with NTS, as above, and was continued for three weeks. The animals then were sacrificed.

Group 3 (one day group). Heparin was started one day after NTS administration, and was continued for three weeks, as above.

Group 4 (one week group). Heparin was started one (nine rabbits) to two weeks (seven rabbits) after NTS, and was continued for three weeks, as above.

Group 5 (late-effect group). Heparin was started simultaneously with NTS and was continued for four weeks. Thereafter, the rabbits were kept for 10 to 13 weeks after heparin withdrawal, and then were sacrificed.

Groups 1 and 5 had untreated controls with matched surviving periods. The 2nd through 4th groups shared an untreated control group, which was sacrificed three (four rabbits) to five (four rabbits) weeks after NTS. The first two groups were designed to examine the early prophylactic effects of heparin, the third and fourth groups for the therapeutic effects, thus simulating human situations, and the last group for late, or remote effects of prophylactic heparin.

Evaluation of glomerular lesions. After heparinization to prevent thrombus formation, all animals were sacrificed by exsanguination from the femoral artery while under anesthesia. A catheter was inserted into the left main renal artery via its aortic orifice, and one of the first branches of the main renal artery was tightly ligated. A small amount of normal saline was gently infused through the catheter in order to demarcate the area supplied by the ligated branch. In the middle of the demarcated area, a thin slice of the cortex (about 10 × 5 × 1 mm) was obtained. This cortical piece was divided for light, immunofluorescence, and electron microscopy (not included in this report). The remaining kidney was processed for microangiography.

Tissue for light microscopy was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned 2- to 3-μ thick. The sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). One hundred glomeruli were counted each time and evaluated without any knowledge of whether the case was treated or untreated. At one week after NTS injection, microthrombi of fibrin and fibrinoid material in the glomerular tuft were evaluated; at three to five weeks after NTS, crescent formation, and at a later stage, glomerular sclerosis were evaluated, respectively.

Another tissue sample for immunofluorescence microscopy was frozen at −70°C in *N*-hexane, precooled in dry ice and acetone bath, and was cut at 4- to 5-μ thick in cryostat at −20°C. The sections were stained with fluorescein-isothiocyanate-labeled (FITC) goat anti-rabbit γ-globulin serum (Behringwerke, West Germany) and FITC-guinea pig anti-rabbit fibrinogen serum. The intensity of immunofluorescence was graded as −, ±, +, or ++. Rabbit fibrinogen was isolated by Laki's method [10]. Anti-rabbit fibrinogen antibody was raised in guinea pigs, with which FITC was conjugated according to the method of Kawamura [11]. The FITC-labeled anti-fibrinogen serum was made monospecific by appropriate absorption procedures and had an F/P ratio of 1.27.

The processing of microangiography of the kidney was almost the same as described previously [9], according to the method of Ljungqvist [12]. After irrigating the kidney with enough normal saline to wash out the blood, a 7.5% aqueous suspension of fine-grain barium sulfate (Micropaque) was perfused at a hydrostatic pressure of 100 cm of water, then at a pressure of 180 cm of water for 90 min at room temperature. The kidney was fixed for at least 96 hr in 10% neutral buffered formalin and was cut along

its short axis at 750 μ in thickness with a freezing microtome. Angiography was performed at 28 KV and 20 mA on a fine grain photographic emulsion (Fuji FG-15), using a fine focus X-ray machine. The exposure time was 4 min. The microangiogram thus produced was enlarged three times in size on Neo-Pan film (Fuji), using Macro-Apparatus (multiphoto, Nippon opticals). Then, this film was further enlarged 4.5 times to be printed on a photograph, in which the number of glomeruli depicted was calculated over a measured area of the cortex extending from the capsule of the kidney to deep medulla. This area of the cortex was 6.0 × 6.0 mm in original size. One microangiogram in which the radioactive opaque dye distributed evenly from the cortex to medulla was examined in each case in the same manner as the light microscopic section.

Statistical analysis was performed by the *t* test, and the data were expressed as mean ± SEM.

Results

Mortality rate. The death rate before the time of sacrifice is shown in Table 1. There was no significant difference between the treated and control groups. The dead animals were excluded from the present study.

Early prophylactic effects of heparin. Group 1 (simultaneous NTS + H – one week group) and its control, which were sacrificed one week after NTS injection, represented early autologous phase of Masugi nephritis, judged from the presence of rabbit γ-globulin deposited in a linear fashion along glomerular basement membrane (GBM). The most prominent glomerular change was deposition of fibrin-fibrinoid materials in the capillary lumen and occasionally in Bowman's space, i.e., exudative lesions. Although we could not see any significant difference in the incidence of exudative lesions in the treated and control groups by light microscopy, microangiographic examination revealed a definite increase in numbers of the glomeruli visualized in the heparin-treated group as compared to the control ($P < 0.01$) (Table 2, Figs. 2 and 3).

Intrarenal blood vessels appeared to be relatively well-preserved, even in the controls at this stage of the disease.

In group 2 (simultaneous NTS + H – three weeks group) and its control, the pervasive glomerular change was cellular crescent formation. In the group treated with heparin for three weeks, there was a significant decrease in crescent formation and an increase in the glomeruli visualized on microangiography ($P < 0.01$, respectively) as seen in Table 2 and Figure 4. Figure 5 shows examples of both the treated and control groups. In the controls who had survived for three to five weeks after NTS infusion, poor visualization of the smaller vessels as well as the glomeruli was quite marked. On the other hand, the treated group preserved its intrarenal blood supply, as judged from the microangiographs.

Therapeutic effects of heparin. The findings in group 3 (one day group) and group 4 (one week group) are shown in Table 2 and Figure 6, together with those in group 2 (simultaneous NTS + H –

Table 1. Mortality rate in each group

Groups[a]	No. of animals	No. of deaths
1) Simultaneous NTS+H – one week	9	1 (11%)
Control	5	0 (0)
2) Simultaneous NTS+H – three weeks	20	5 (25)
3) One day group	11	3 (27)
4) One week group	22	6 (27)
Control	10	2 (20)
5) Late effect group	20	4 (20)
Control	23	4 (17)

[a] See Method's section for a description of the groups.

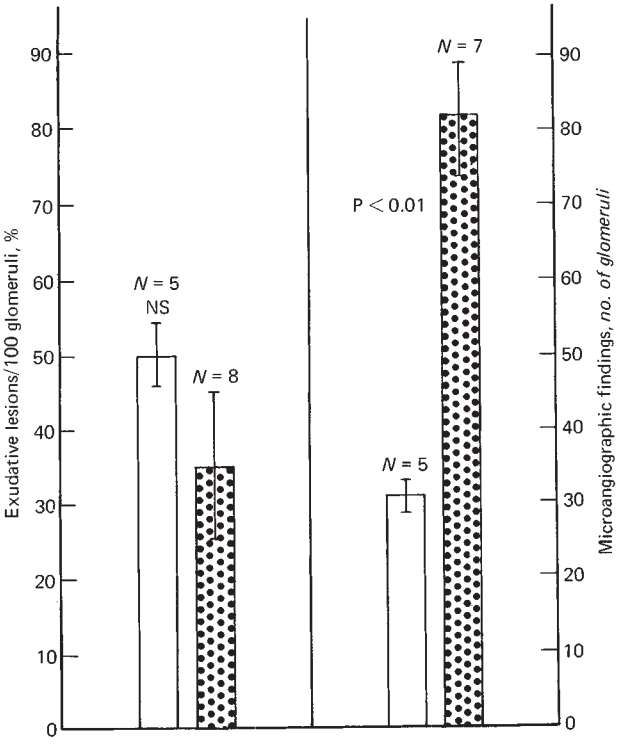

Fig. 2. *Group 1 (simultaneous NTS + H – one week group) and its control: Light microscopic and microangiographic findings. Open bars* represent the control group; *dotted bars,* the heparin group; and —, the mean ± SEM.

Table 2. Light microscopic and microangiographic findings in all experimental groups

Groups[a]	No. of animals	Glomerular changes, *per 100 glomeruli*[b] Exudative lesion	Crescent formation	Hyalinization	No. of animals	Microangiographic findings[b] *no. of glomeruli*
1) Simultaneous NTS+H−one week	8	35.5 ± 9.8	—	—	7	81.9 ± 8.1[c]
Control	5	49.6 ± 2.2	—	—	5	29.8 ± 2.4
2) Simultaneous NTS+H−three weeks	15	—	20.7 ± 7.2[c]	—	14	83.1 ± 7.5[c]
3) One day group	8	—	24.8 ± 10.1[c]	—	6	53.2 ± 12.0[d]
4) One week group	16	—	63.5 ± 7.9	—	13	38.1 ± 3.1
Control	8	—	63.8 ± 8.6	—	7	27.6 ± 5.7
5) Late effect group	16	—		57.1 ± 9.8	16	50.3 ± 6.2
Control	19	—		36.6 ± 8.9	6	66.3 ± 8.5

[a] See Method's section for description of groups.
[b] Values represent the mean ± SEM.
[c] $P < 0.01$ vs. control.
[d] $P < 0.05$ vs. control.

three weeks group). The one day group (group 3), in which heparin was started in the early heterologous phase of Masugi nephritis, behaved like group 2 (simultaneous NTS + H – three weeks group). Although the glomerular filling rate seen by microangiography was a little less than in the latter, it was significant enough to be separated from the control ($P < 0.05$).

Group 4 (one week group), in which heparin was begun in the autologous phase with established glomerular changes, however, showed no histological improvement with treatment. Immunofluorescent staining for fibrinogen in the glomeruli became negative or very faint (±) in about 40% of the treated group, while the control group showed moderate (+)

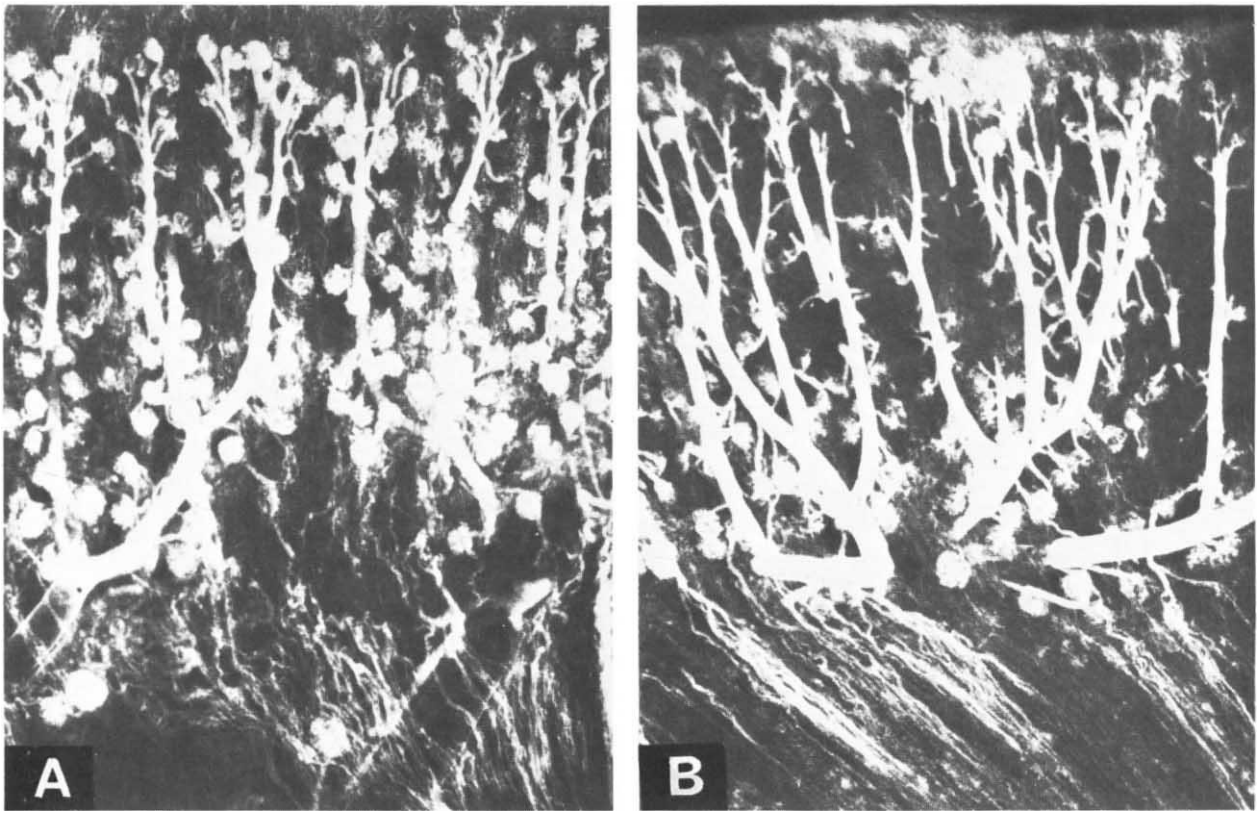

Fig. 3 A. *Group 1 (simultaneous NTS + H – one week group).* An abundant filling of the glomeruli is seen, in a degree comparable to the normal which was shown in the previous report [9]. **B.** *The control group.* In contrast to A, there was a marked decrease of visualized glomeruli, but with relatively preserved intrarenal vessels. (Magnification, × 25.)

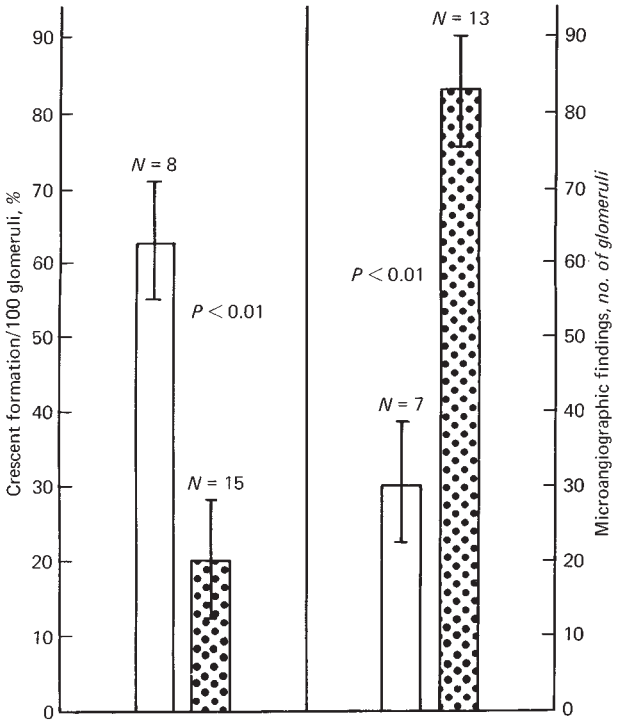

Fig. 4. *Group 2 (simultaneous NTS + H – three weeks group) and its control. Open bars* represent the control group; *dotted bars,* the heparin group; and ⊢⊣, the mean ± SEM.

to heavy (++) staining (Fig. 7). There was no essential difference in histological and immunofluorescent findings between the control rabbits sacrificed three and five weeks after NTS infusion.

Late effect of prophylactic heparin. Data on the late effect group (group 5) and its control are presented in Table 2 and Figure 8. In contrast to groups 1 and 2 (simultaneous NTH+H – one and three weeks groups), the late effect group (group 5) who survived 10 to 13 weeks after cessation of heparin showed increased hyalinization and poor filling of the glomeruli with markedly tortuous intrarenal vessels (Fig. 9). There was no statistically significant difference, however, between the treated and control groups. Immunohistological findings in the treated group were also consistent with the failure of heparin treatment. Except for the completely hyalinized glomeruli, the remaining glomeruli still retained the γ-globulin in a linear fashion along the capillary wall (Fig. 10) and also a considerable amount of fibrinogen-reactive materials deposited in the glomerular tuft (Fig. 11) to the same degree as in the controls.

Discussion

Following the pioneer work on the protective effect of heparin in experimental glomerulonephritis by Silfverskjöld [13] and Kleinerman [14], Vissalli et al [1, 15] have clearly demonstrated that intravascular or intraglomerular coagulation plays an important role in the pathogenesis of glomerular lesions. Glomerular lesions resulting from intravascular coagulation cover the whole range of morphological reactions seen in glomerulonephritis, except for that of epimembranous glomerulonephritis [15, 16]. On the basis of these experimental studies, anticoagulant therapy has been applied to human cases with some beneficial effects [4].

The present results of early prophylactic effects of heparin are consistent with those of Halpern et al [2] and Borrero et al [3], in addition to the investigations cited above [1, 13, 14]. Our report, however, adds the following two points: the first is that anticoagulant effects were evaluated by microangiography. The beneficial effects of early prophylactic heparin were well-correlated with a better blood supply to the small vessels and glomeruli in the renal cortex. Especially in group 1 (the simultaneous NTS + H– one week group), the microangiographs showed the heparin effect more clearly than the routine histologic study. Thus, one of the mechanisms of heparin is probably to prevent microthrombus formation and thus protect the renal cortex from ischemia. Ljungqvist, Sallström, and Biberfeld [17] observed that when the glomerular lesion in Masugi nephritis is sufficiently severe and extensive, the intrarenal vasculature seen by microangiography resulted in alterations implying a relative cortical ischemia. Furthermore, the present result is supported by the experiments of Naish et al [18], who used the defibrinating agent ancrod, which suggested that the protective effect of anticoagulants was related to a reduction in glomerular fibrin deposition.

The second point to be mentioned is that the present study used progressive and severe Masugi nephritis as an experimental model. Conventional Masugi nephritis produced by one shot infusion of NTS into animals with two intact kidneys is usually mild and has a tendency to spontaneous healing [1] For example, in a series by Borrero et al [3], who used the conventional Masugi nephritis, the untreated control showed crescent formation in 34% (mean) of the glomeruli examined, whereas it was 63.8 ± 8.6% in our studies.

Recently, Bone et al [5] and Border, Wilson, and Dixon [6] have reported that they were unable to obtain any beneficial heparin effects on experimental glomerulonephritis, supporting the previous report by Briggs, Kwaan, and Potter [19]. Thomson, Simpson, and Peters [20] have pointed out that a heparin dose of 2,000 U/kg/day was required before there was significant reduction in fibrin deposition and

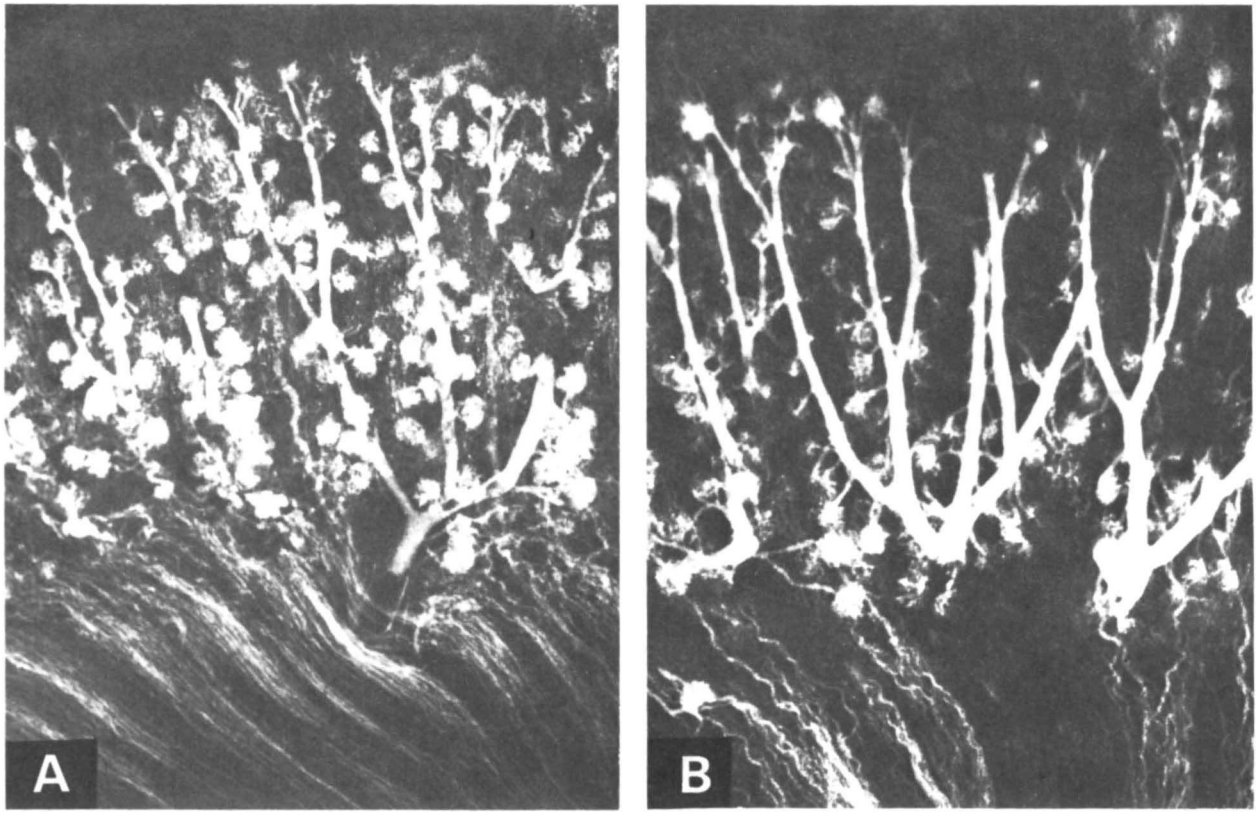

Fig. 5 A. *Group 2 (simultaneous NTS + H–3 weeks group).* Ample glomerular filling rate was maintained, though visualization of the superficial glomeruli in the cortex was slightly less than in Figure 3A. B. *The Control group.* Scanty filling of the glomeruli and damaged intrarenal vessel system are seen. (Magnification, × 25.)

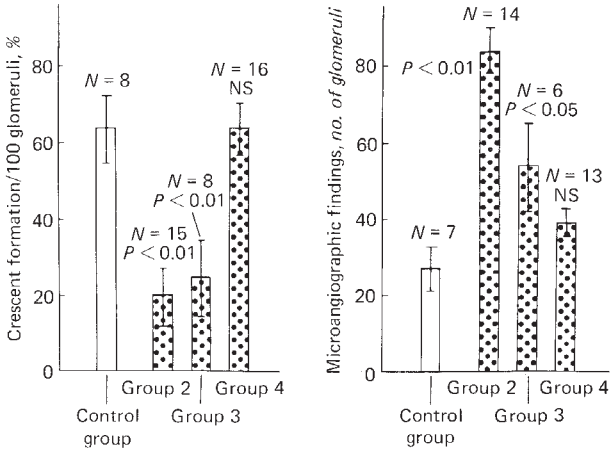

Fig. 6. *Results of group 3 (one day group)* and *group 4 (one week group) in a comparison with group 2 (simultaneous NTS + H–three weeks group). Open bars* represent the control group; *dotted bars,* the heparin group; and ⊢⊣, the mean ± SEM.

extracapillary cell proliferation. But, heparin dosages in these three reports as well as in the present study were more than that. In the studies by Bone et al [5], 57% of the untreated rats with progressive anti-GBM nephritis, produced by the serial administration of heterologous antiserum in two stages, had epithelial crescents in most glomeruli. Although Border et al [6] have examined heparin effects on both immune complex and anti-GBM glomerulonephritis, only the latter will be discussed here. According to their description, the immediate type of anti-GBM glomerulonephritis ran a progressive course leading to death within seven to ten days, with virtually all of the glomeruli involved by cellular proliferation, necrosis, and crescent formation. The severity of glomerular injuries in the report by Briggs et al was not described clearly enough to be compared. The glomerular injuries in those two reports by Bone et al [5] and Border et al [6] appear to be equal to or somewhat more severe than in our series, though an exact comparison is difficult. It is, therefore, conceivable that an extremely severe glomerular injury may be beyond the reach of heparin effects and that the lesser severity of glomerular lesions in the present study may have been more amenable to modulation by heparin.

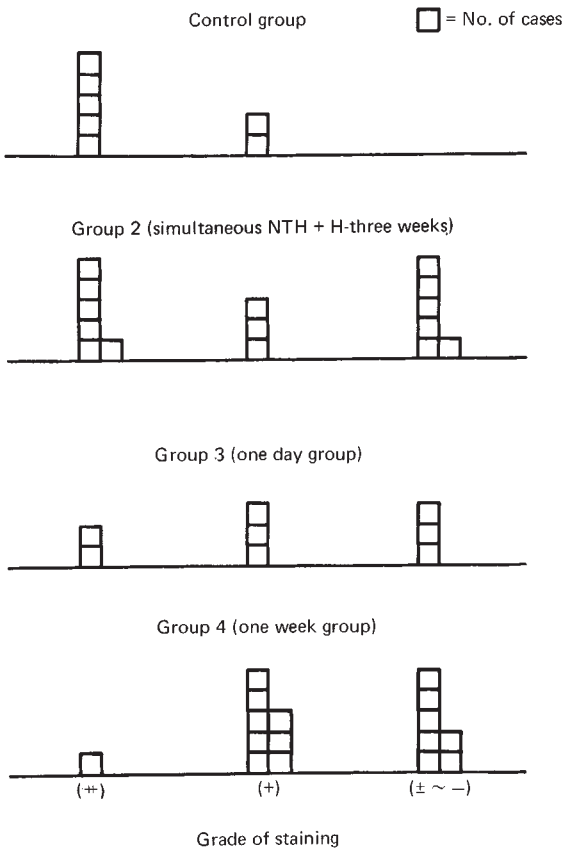

Fig. 7. *Immunofluorescent staining for fibrinogen-reactive materials deposited in the glomeruli.* See Methods section for grading scale.

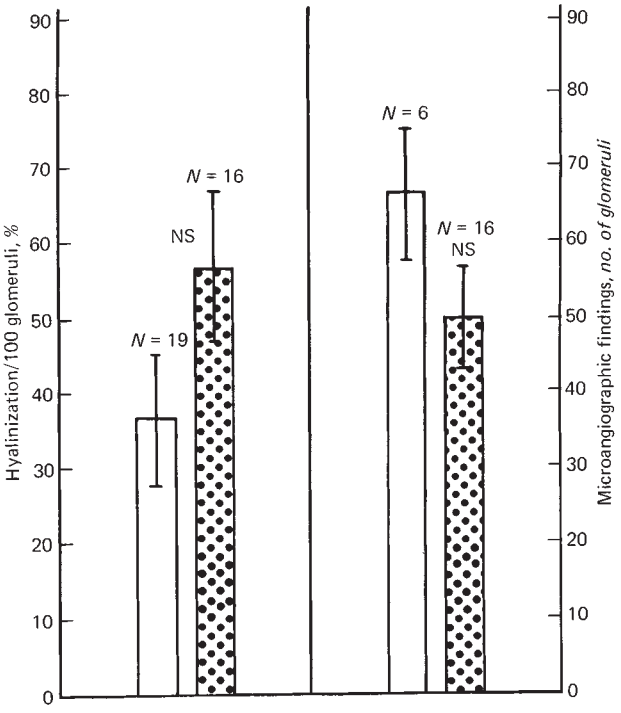

Fig. 8. *Group 5 (late effect group) and its control. Open bars* represent the control group; *dotted bars,* the heparin group; and ⊢⊣, the mean ± SEM.

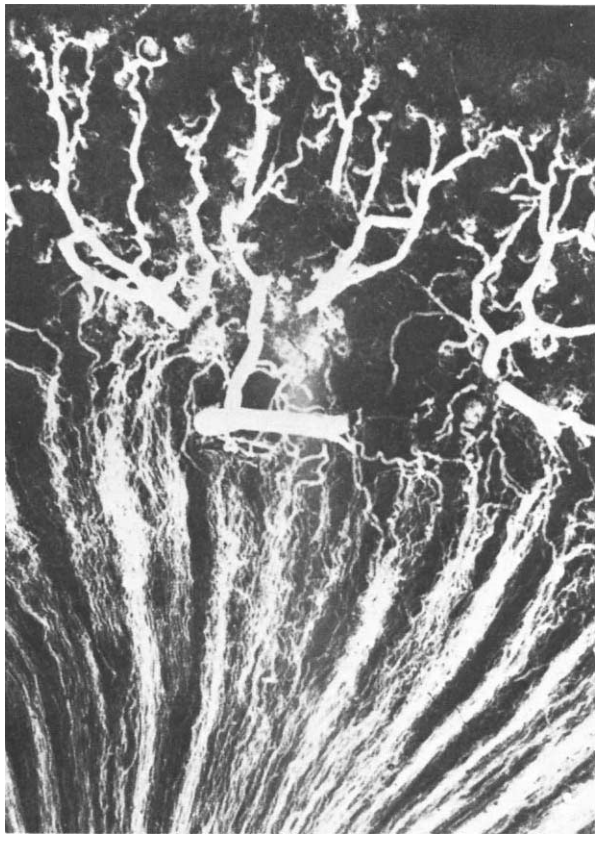

Fig. 9. *A microangiograph from group 5 (late effect group).* A devastated cortical vessel system with marked tortuosity means that it is approaching a contracted state. (Magnification, × 10.)

A therapeutic trial of heparin, which was started one to two weeks after NTS injection, yielded no improvement as judged by both light microscopy and microangiography. The immunofluorescent study, however, disclosed negative or faint staining of fibrinogen-reactive materials in the glomeruli of occasional treated cases. Borrero et al [3] have reported almost the same experience. When they started Warfarin® 14 days after NTS injection, they could see no reduction in crescent formation but some decrease of fresh fibrinous exudate in the glomeruli as compared to the control.

The late effects of prophylactic heparin are of interest. As far as we know, no experimental study has been performed in which the late or remote effect of heparin was evaluated. We found no significant modification by the treatment. It is likely that the initial improvement attained by the early prophylactic heparin regimen had disappeared after stopping heparin. In individual glomeruli from the treated group, definite amounts of fibrinogen as well as γ-globulin were found along relatively well-preserved

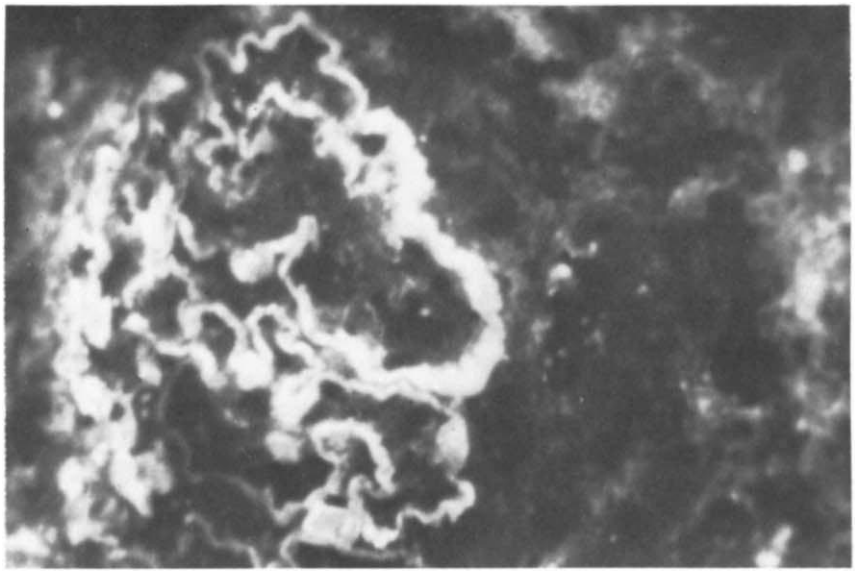

Fig. 10. *The persistent presence of γ-globulin along the glomerular capillary loops which were relatively preserved but compressed by an old fibrinous crescent, from group 5 (late effect group).* (Magnification, ×600.)

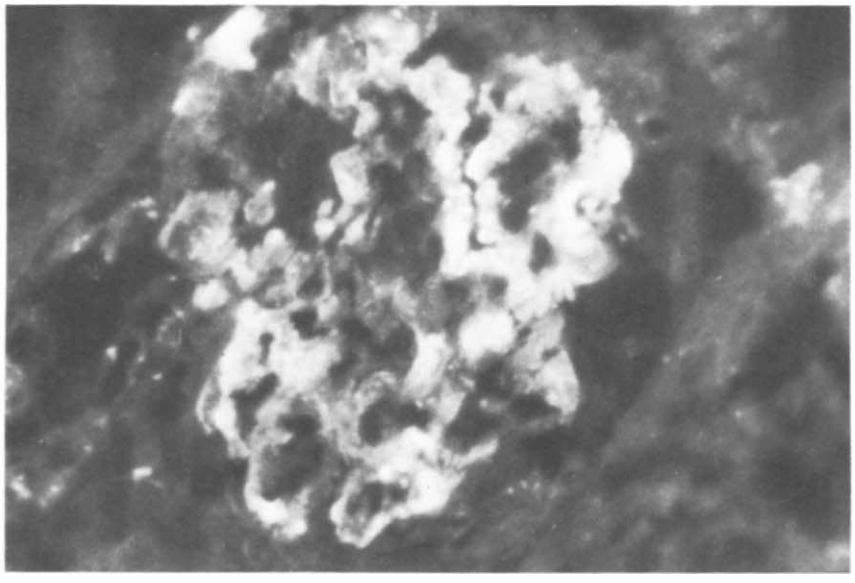

Fig. 11. *Lumpy deposits of fibrinogen-reactive materials by immunofluorescence in a relatively preserved glomerulus, from (group 5) (late effect group).* (Magnification, ×600.)

capillary loops. Two immunological mechanisms, i.e., circulating immune complexes and anti-GBM antibody have been elucidated in the pathogenesis of glomerulonephritis [21]. Since it is known that glomerular deposition of immune complexes and heterologous anti-GBM antibody are not affected by anticoagulant treatment [1, 6], persistent immunologic mechanisms appear to have provoked again the coagulation processes after withdrawal of heparin treatment. These results may represent a limitation of anticoagulant therapy. We suggest that anticoagulants may have to be reinforced by corticosteroid hormone and/or immunosuppressive measures in order to weaken the activity of immunologic mechanisms associated with glomerulonephritis.

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