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A QUANTITATIVE EVALUATION OF ANTICOAGULANTS IN EXPERIMENTAL NEPHROTOXIC NEPHRITIS

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SUMMARY

The protective effects of anticoagulants in nephrotoxic nephritis in rabbits have been studied, using various doses of heparin and defibrination with ancrod. Massive doses of heparin (2000 units/kg/day) were required before significant reduction in glomerular fibrin deposition, extracapillary cell proliferation and urea retention occurred. Doses of 300 and 1000 units/kg/day were insufficient to modify fibrin deposition and cell proliferation. Defibrination with ancrod provided protection, judged by histological and functional criteria, comparable to 2000 units of heparin/kg/day; but fibrin could still be demonstrated in the glomeruli of animals treated with 2000 units of heparin/kg/day, contrasting with the virtual absence of fibrin in animals given ancrod.

INTRODUCTION

Reduction in the severity of the renal lesion in the autologous phase of nephrotoxic nephritis by the use of anticoagulants has been reported by a number of authors (Kleinerman, 1954; Vassalli & McCluskey, 1964; Halpern *et al.*, 1965). The experiments of Naish *et al.* (1972), using the defibrinating agent ancrod ('Arvin') suggested that the protective effect of anticoagulants was specifically related to reduction in glomerular fibrin deposition. Such findings, together with the demonstration that fibrin deposition is associated with crescent formation in human rapidly progressive ('crescentic') nephritis, have led to the clinical use of anticoagulants in this condition (Kincaid-Smith, Saker & Fairley, 1968; Arieff & Pinggera, 1972). However, because no properly controlled trials have been undertaken, there is still no clear evidence that their use is beneficial, nor is there any clear indication from the studies in experimental nephritis whether clinically acceptable doses of anticoagulants are likely to provide protection. Halpern *et al.* (1965) and Kleinerman (1954), in their studies of nephrotoxic nephritis, used massive doses of heparin (3000–6000 units/kg/day) sufficient to

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prolong whole blood clotting times up to thirty times normal. Vassalli & McCluskey (1964), using intravenous warfarin, observed considerable reduction in fibrin deposition and extracapillary cell proliferation; however, such large doses were required that twenty-six of their thirty-four rabbits died from haemorrhage. We therefore thought it important to re-examine the use of heparin in experimental nephritis in rabbits, first to determine the doses required to prevent glomerular fibrin deposition and secondly to compare heparin with ancrod.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits, weighing between 2 and 2·5 kg were used. The animals were fed on a normal water and pellet diet and kept in metabolic cages.

Preparation, characterization and administration of the sheep anti-rabbit glomerular basement membrane serum (nephrotoxic serum)

A high titre of antibody to rabbit glomerular basement membrane (GBM) was prepared in a sheep by injection of particulate rabbit GBM in FCA at 2-weekly intervals. The nephrotoxic serum was decomplemented by heating at 56°C for 30 min and absorbed with normal rabbit erythrocytes and normal rabbit serum. Pilot experiments established that intravenous injection of 0·5–1·5 ml of nephrotoxic serum induced a severe proliferative nephritis with most glomeruli showing some degree of crescent formation by 12 days. Further experiments, using a salt-fractionated gamma-globulin preparation of this serum labelled with ^{125}I and a similar fraction of sheep gamma-globulin labelled with ^{131}I, established that a dose of 1 ml/kg body weight of nephrotoxic serum, given intravenously to ten rabbits, resulted in the deposition of a mean of 180 μg (range 160–220 μg) of nephrotoxic antibody per kidney.

Serum and urine samples

Serum urea and creatinine concentrations were determined by the auto-analyser method, serum C3 concentrations by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) and urinary protein excretion by the Biuret method.

Assessment of anticoagulation

Anticoagulation in the heparin-treated animals was assessed by whole blood clotting times and thrombin clotting times (Douglas, 1962) and defibrination by the clot quality test (Reid, Chan & Thean, 1963).

Histological and immunofluorescent preparations

Portions of kidneys were fixed in 10% buffered formalin, sectioned at 4 μm and stained with Haematoxylin and Eosin and PAS. Specimens were also snap-frozen in liquid nitrogen, sectioned on a cryostat at 4 μm and stained with fluorescein-conjugated antisera to sheep and rabbit IgG and rabbit C3 and fibrin. All antisera were prepared in our laboratory (Fothergill, 1969).

Assessment of the histological and immunofluorescent preparations

Sections were coded and the analysis based on independent 'blind' assessment. Extra-

capillary cell proliferation was assessed by light microscopy, each glomerulus being scored from 0–3 as follows: a score of 0 represented no crescent formation; scores of 1, 2 and 3 represented the involvement of $<1/3$, $1/3$–$2/3$ and $>2/3$ of the circumference of the glomerulus respectively by proliferating extracapillary cells. Fibrin deposition (on immunofluorescent examination) was also scored from 0–3 for each glomerulus, a score of 0 representing no fibrin and scores of 1, 2 and 3 representing fibrin deposition visually assessed to be involving $<1/3$, $1/3$–$2/3$ and $>2/3$ respectively of the surface area of glomerular tuft and crescent. At least fifty glomeruli per animal were assessed by light microscopy and thirty glomeruli by immunofluorescence. Mean scores for extracapillary cellular proliferation and fibrin deposition were then determined for each animal.

Experimental protocol

Forty-two rabbits were used in this study and all were injected intravenously with 1 ml/kg of nephrotoxic serum (day 0). Blood was sampled on alternate days and urine continuously collected for protein estimation. Twenty rabbits received heparin, four ancrod and eighteen control animals received saline.

Heparin group

In sixteen animals sodium heparin (Paines and Byrne) was commenced on day 4 and given subcutaneously twice daily until the animals were killed on day 12. Three dosage regimes were used: five rabbits were given 300 u/kg/day; six were given 1000 u/kg/day; and five were given 2000 u/kg/day. In a further four animals 2000 u/kg/day were again given, but the heparin was commenced on day 1.

Ancrod group

Ancrod (Arvin, Twyford Pharmaceuticals) was commenced on day 1. A dose of 0·5 u/kg was injected intravenously, followed an hour later by 1 u/kg. Defibrination was maintained by twice daily injections of 1 u/kg.

Control group

Commencing on day 4, 1 ml of saline was injected subcutaneously twice daily.

All animals were killed on day 12 and the kidneys removed for histological and immunofluorescent examination.

RESULTS

Deaths

One animal in the control group died from uraemia on day 11. No deaths occurred in treated animals.

Anticoagulation

(1) Animals given 300 u of heparin/kg/day. At 1, 4, and 8 hr after each dose of heparin the ranges of whole blood clotting times were respectively 2·1–3·2 (mean 2·6), 2·5–3·5 (mean 3·1), 2·2–3·2 (mean 2·5) times control. Clotting times had fallen to a range 1·5–2·0 (mean 1·8) times control just prior to the next dose of heparin (i.e. approximately 12 hr).

(2) Animals given 1000 u of heparin/kg/day maintained whole blood clotting times in the

range 4·1–6·2 (mean 5·1) times control at all times. Thrombin clotting times were prolonged to between 30 and 70 sec (control 9–13 sec).

(3) Animals receiving 2000 u of heparin/kg/day had virtually unclottable blood, with thrombin clotting times varying between 50 and 500 sec and whole blood clotting times in the range 15–20 (mean 17·3) times normal at all times.

(4) Ancrod. The clot quality test showed adequate defibrination in all animals given ancrod.

Renal function (Table 1, Fig. 1)

The serum urea and creatinine concentrations remained within the normal range in all animals until day 5, then rose rapidly and progressively in untreated animals and in the groups given 300 and 1000 u of heparin/kg/day. Animals given 2000 u of heparin/kg/day

TABLE 1. Comparison of the serum urea, serum creatinine, fibrin score and crescent score on day 12, between control animals and those treated with heparin and ancrod

Group		Number of animals	Serum urea day 12 (mg/100 ml)	Serum creatinine day 12 (mg/100 ml)	Fibrin score	Crescent score
Saline control	Mean	18	361	7·2	1·81	1·99
	Range		(124–738)	(2·5–14·0)	(0·95–2·4)	(0·62–2·7)
Heparin (300 u)	Mean	5	351	8·7	2·20	2·03
	Range		(150–540)	(4·5–12·5)	(1·4–2·7)	(1·06–2·7)
Heparin (1000 u)	Mean	6	460	9·1	1·80	2·06
	Range		(120–735)	(2·5–13·5)	(0·7–2·6)	(0·48–2·78)
Heparin (2000 u) (day 1)	Mean	4	147	3·5	1·03	1·15
	Range		(120–176)	(2·9–3·9)	(0·85–1·25)	(0·95–1·30)
Heparin (2000 u) (day 4)	Mean	5	154	3·2	0·97	1·20
	Range		(128–180)	(2·5–3·6)	(0·4–1·4)	(0·65–1·5)
Ancrod	Mean	4	68	1·8	0·11	0·49
	Range		(42–142)	(1·2–3·5)	(0·05–0·2)	(0·2–1·0)

and those given ancrod, although showing a rise in the serum urea and creatinine from days 5–9, thereafter showed a progressive fall in these variables (Fig. 1). There was no significant difference ($P > 0·1$)* in the serum urea and creatinine concentrations on day 12 between the control animals and animals given either 300 or 1000 u of heparin/kg/day (Table 1). However, the serum urea and creatinine concentrations on day 12 in the animals given 2000 u of heparin/kg/day were significantly less than the concentrations in control animals and animals given 300 and 1000 u of heparin/kg/day ($P < 0·01$). Similarly the concentrations on day 12 in animals given ancrod were significantly less than in controls and animals given 300 and 1000 u of heparin/kg/day ($P < 0·01$), but were not significantly less than in animals given

* Comparison of the results obtained in the groups of animals was made using the Wilcoxon rank sum test for two samples (Wilcoxon, 1945).

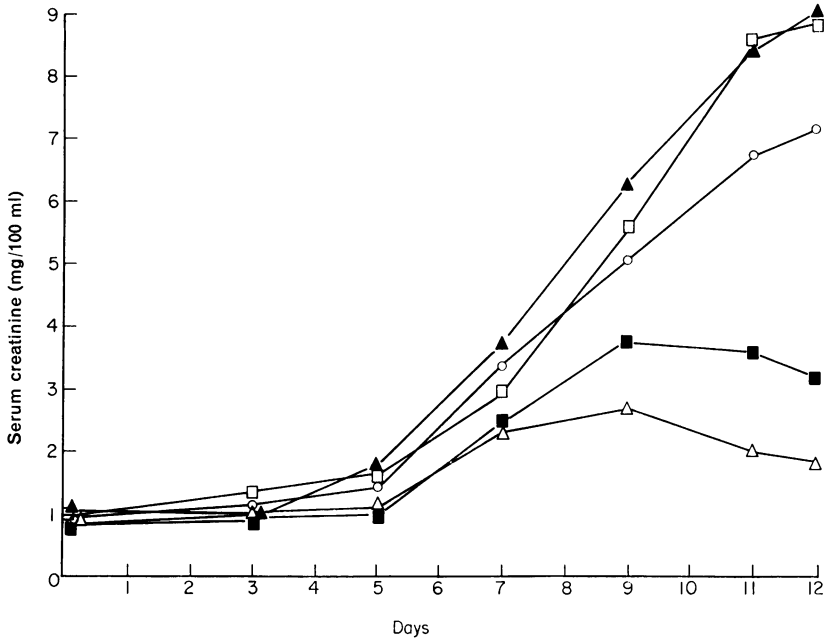

FIG. 1. Mean serum creatinine concentrations for control animals (○), animals given 300 u of heparin (□), 1000 u of heparin (▲), 2000 u of heparin (■) and ancrod (△) on various days after injection with nephrotoxic serum.

2000 u of heparin/kg/day ($P>0{\cdot}1$). There was no difference at any time in the urea and creatinine concentrations, between animals given 2000 u of heparin/kg/day commencing on day 1 and animals given the same dose commencing on day 4.

Serum C3 concentrations

In all animals there was a fall in the serum C3 to a mean value of 52% (range 35–74%) at 24 hr and a subsequent return to normal within 2–3 days. A second persistent reduction in C3 occurred from the 5th or 6th day. On day 12 the mean C3 concentrations for each group were: 40·4% (range 15–71%) control; 47% (range 32–80%); 43% (range 16–70%); and 42% (range 25–55%) in the three groups given 300, 1000 and 2000 u of heparin/kg/day respectively, and 47% (range 36–50%) in the ancrod group. No significant difference exists between the results of any of these groups.

Immunofluorescence results

Sheep IgG, rabbit IgG and rabbit C3 were deposited in a linear fashion along the glomerular basement membrane; no differences could be detected between the various groups of animals. For fibrin deposition (Table 1) no statistically significant difference ($P>0{\cdot}1$) in the mean score per glomerulus was observed between controls and groups given 300 and 1000 u of heparin/kg/day. However, fibrin deposition in animals given 2000 u of heparin/kg/day was significantly lower than in controls or animals given 300 and 1000 u/kg/day ($P<0{\cdot}01$). In the defibrinated animals the fibrin score was not only significantly lower than

for controls but also lower than for animals treated with heparin in the doses 300, 1000 and 2000 u/kg/day ($P<0{\cdot}01$ for each group). No differences were observed between the groups in which heparin was started on day 1 or day 4.

Histopathology results

No significant difference was observed in the degree of extracapillary cell proliferation between controls and animals given 300 and 1000 u of heparin/kg/day ($P>0{\cdot}1$; Table 1). However the score was significantly lower in animals given 2000 u of heparin/kg/day ($P<0{\cdot}01$). Animals treated with ancrod also scored significantly lower than controls and animals given 300 and 1000 u of heparin/kg/day ($P<0{\cdot}01$) but not significantly lower than the group given 2000 u of heparin/kg/day ($P>0{\cdot}1$). Again, commencing heparin on day 1 resulted in the same degree of extracapillary cell proliferation as commencing on day 4.

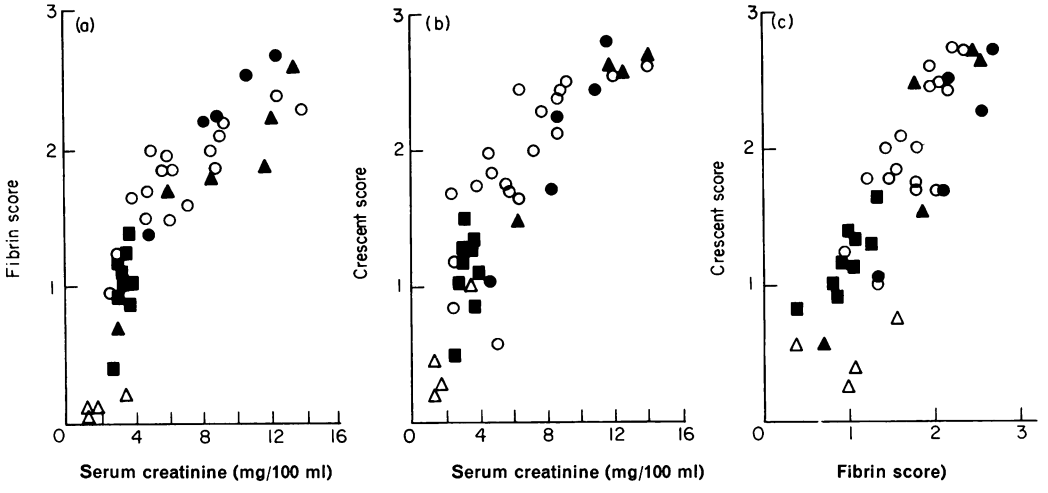

FIG. 2. The relationship between: (a) glomerular fibrin deposition and serum creatinine; (b) extracapillary cell proliferation and serum creatinine; (c) extracapillary cell proliferation and glomerular fibrin deposition, on day 12, for control animals (○), animals given 300 u of heparin (●), 1000 u of heparin (▲), 2000 u of heparin (■) and ancrod (△).

Correlation between the fibrin deposition, extracapillary cell proliferation and serum creatinine

Good correlations existed between the degree of fibrin deposition and serum creatinine, crescent formation and serum creatinine and between fibrin deposition and crescent formation (Fig. 2).

DISCUSSION

These studies of an experimental model of severe 'rapidly progressive' or 'crescentic' nephritis showed that very large doses of heparin were required to provide protection: only large doses of 2000 units/kg/day resulted in a reduction in glomerular fibrin deposition, cellular proliferation and significant preservation of renal function. Such doses caused clotting times to be prolonged fifteen to twenty times normal (far greater than those thera-

peutically practicable in the treatment of rapidly progressive glomerulonephritis in man). Yet even with these doses, glomerular fibrin deposition, though less than in controls, still occurred, contrasting with the almost total absence of fibrin in the glomeruli of animals treated with ancrod. These observations suggest that despite profound anticoagulation by heparin, pathogenetic mechanisms occurring locally within the glomeruli are capable of inducing local coagulation, overriding the effects of circulating anticoagulants. This could possibly result from a failure of heparin, a small but highly charged molecule, to penetrate the mesangium and Bowman's space. However, in normal subjects on high doses of heparin free heparin can be found in the urine (Douglas, 1972).

In other experiments (Naish, Evans, Peters, unpublished observations) sequential renal biopsies in animals given similar doses of nephrotoxic antibody have shown that fibrin deposition is a feature of the autologous phase and cannot be detected until day 5. It is therefore not surprising that no difference was observed between heparinization starting on day 1 and heparinization starting on day 4, nor is it surprising that Naish *et al.* (1972) found that ancrod provided similar protection when started at day -1 and day $+7$.

Our observations raise the possibility that ancrod might be more valuable than heparin in the treatment of rapidly progressive glomerulonephritis in man. However its use is limited to a period of 3–4 weeks, owing to the development of resistance. It is nevertheless possible that in patients with such explosive disease, treatment with ancrod, if given at a sufficiently early stage, may be of benefit.

Like Kleinerman (1954) and Vassalli & McCluskey (1964), but unlike Halpern *et al.* (1965), we were unable to show a reduction in proteinuria with either heparin or ancrod, nor were C3 concentrations modified by any anticoagulant regime. These, and other experiments (Naish *et al.*, 1972, unpublished data) suggest that fibrin deposition plays no part in the primary allergic events causing capillary damage and proteinuria.

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