Effect of antiproteolytic drugs: epsilon-aminocaproic acid (EACA) and aprotinin on experimental anti-GBM nephritis

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Abstract

Background. Given the evidence accrued by other authors on beneficial effect of protease inhibitors on experimental immune nephritis, and following our preliminary report on abrogation of immune glomerulopathy in the rat by an antifibrinolytic and antiproteolytic drug, epsilon-aminocaproic acid (EACA), we investigated the effect of this drug on the rat autologous anti-GBM nephritis. Along with the EACA we evaluated another protease inhibitor, aprotinin, an antagonist of serine proteases.

Methods. EACA (0.3 g/kg) or aprotinin (5000 kallikrein inhibition units, KlU/kg) was administered intraperitoneally (t.i.d.) from day 0 (preventive protocol) or day 3 (therapeutic protocol) of autologous anti-GBM nephritis induced in Wistar rats. Proteinuria, creatinine clearance and renal histopathology were assessed as markers of disease activity, while glomerular fibrin deposits (immunoperoxidase staining) and standard parameters of coagulation/fibrinolysis of peripheral blood enabled insight into local and systemic haemostatic mechanisms. Glomerular binding of anti-GBM antibodies (immunofluorescence) and serum titres of autologous nephrotoxic antibodies (haemagglutination assay) represented conditions of immune induction of glomerulopathy.

Results. Our experiments indicate that the EACA, and to a lesser extent also aprotinin, are capable of preventing proteinuria (EACA, reduction by 57.6%; aprotinin, reduction by 26.8%, compared to untreated nephritic rats, day 3 post-induction) and glomerular histopathological changes, without affecting endogenous creatinine clearance, otherwise depressed in this model of glomerulonephritis. More importantly, both drugs significantly ameliorated glomerular lesions and proteinuria, even when the treatment was initiated on day 3 post-induction, after the injury has begun (EACA reduced proteinuria by 32.0%, and aprotinin reduced it by 20.9%, day 7). Administration of EACA and aprotinin at doses reducing glomerular injury did not cause any appreciable fibrin deposition in glomeruli of nephritic rats, nor did it modify parameters of systemic coagulation and fibrinolysis in these animals. EACA and aprotinin did not interfere with serum titres of nephrotoxic antibody, nor with the intensity of its binding to the glomerular basement membrane in vivo.

Conclusions. Antiproteolytic drugs utilized in our studies exert their beneficial effect on autologous anti-GBM nephritis through interference with inflammatory phase of the disease, while sparing its immune induction and mechanisms of coagulation/fibrinolysis.

Key words: aprotinin; coagulation; EACA; fibrinolysis; immune glomerulonephritis; proteinuria

Introduction

Growing interest in pathogenesis of immune glomerulonephritis has resulted in the past two decades in description of numerous soluble factors mediating inflammatory glomerular lesions (reviewed in [1]). Given the complexity of interactions between multiple pathways involved in renal inflammation, it is somewhat surprising that experiments aimed at documenting significance of individual mediators by selectively depleting them or blocking their activity, have succeeded in causing a remarkable amelioration of the disease as a whole. This approach has been applied in various experimental models of glomerulonephritis to study a wide spectrum of humoral mediators such as transforming growth factor B in anti-Thy-1 nephritis [2], tumour necrosis factor in autologous anti-GBM disease [3], complement C5b-9 complex in Heymann nephritis [4], and serum proteases in immune complex glomerulopathy [5], to name only a few examples.

The evidence on importance of proteases in pathogenesis of glomerular injury derived from the studies with protease inhibitors [3,5,6] is supported by the in vivo experiments demonstrating induction of proteinuria by infusing active elastase into renal artery of normal rats [7] and a failure to generate proteinuria in mice with neutrophils deficient in neutral proteases [8].
Antiproteolytic drugs in immune glomerulonephritis

We have previously reported on a potential of the EACA, an antifibrinolytic and antiproteolytic drug, to prevent development of toxic immune nephropathy in BN rats [9]. In the present study we assessed the ability of the EACA and another protease inhibitor, aprotinin, both to prevent development of the autologous anti-GBM nephritis in the rat, and to reduce activity of already established glomerular injury in this model system.

Subjects and methods

Animals

Inbred male Wistar rats (weight 250 g) and male rabbits (weight 5 kg) were purchased from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Experimental model

Autologous anti-GBM glomerulonephritis was induced in Wistar rats essentially as previously described [3]. Briefly, subnephritogenic dose of sheep anti-GBM antibody (20 μg/g bodyweight) was administered intravenously to rats preimmunized intraperitoneally 5 days earlier with sheep globulin (12 μg/g in Complete Freund’s Adjuvant—CFA, Sigma, St Louis, USA). This protocol results in development of a proliferative, mainly endocapillary glomerulonephritis with focal necrosis and disruption of the GBM. Glomerular crescents are virtually absent (up to 10% of glomeruli). Proteinuria appears as early as 24 h post-induction, concurrently with neutrophil accumulation, within the next day replaced by abundant macrophage influx. Urinary protein excretion reaches peak on days 3/4 and gradually decreases thereafter with usual disappearance by days 10/12.

Proteinuria

Urinary protein excretion was quantitated in samples collected during 24 h in metabolic cages. Measurements were based on a turbidity method [10].

Creatinine clearance

Creatinine concentrations in plasma and urine were measured with an automatic analyser (Beckman Instruments) and creatinine clearance values (ml/min) were calculated in the usual way.

Histology and immunofluorescence

Samples of renal tissue were fixed in buffered 3.7% formalin and stained with haematoxylin and eosin, and periodic acid-Schiff reagent for light-microscopy. Slides were examined in a blinded fashion by a pathologist (JR). Assessment of glomerular hypercellularity was done by counting cell nuclei in equatorial sections of glomeruli (at least 40 glomeruli per rat) and estimating percentage of tufts with necrosis (at least 100 glomeruli on separated sections).

Frozen tissue samples were subjected to direct immunofluorescence studies with fluorescein-labelled goat anti-rat IgG antibodies (Hyland, UK; diluted 1:32) to determine glomerular binding of autologous nephrotoxic antibodies.

Circulating rat anti-sheep globulin antibody

Titres of autologous rat antibodies, representing host reactivity to sheep anti-GBM GBM antiserum were determined with passive haemagglutination assay utilizing human erythrocytes (group O) coated with sheep globulin [10].

Fibrin deposition in glomeruli

Indirect immunoperoxidase technique on renal tissue sections obtained from paraffin-embedded samples was used. A self-raised polyclonal rabbit anti-rat fibrinogen immunoglobulin (diluted 1:20) served as a primary antibody. It was generated by repeated biweekly subcutaneous inoculations of rabbits with 50 mg of rat fibrinogen (Sigma), firstly emulsified in CFA, secondly in incomplete FA, and then without FA. The specificity of anti-fibrinogen antibodies was ascertained by immunoprecipitation assay using radiiodinated fibrinogen as previously described for immunization against TNF [3].

Peroxidase-conjugated goat anti-rabbit IgG (Dako, Denmark) diluted 1:100 was utilized as secondary antibody. Sections of the rat interstitioma of the testis, characterized by high degree of fibrin deposition [11] served as positive control.

Parameters of haemostasis

Blood samples were obtained by canulating rat carotid artery exposed through a small skin incision under anaesthesia with 15% urethane ethyl (7 ml/kg, 2/3 administered intraperitoneally, 1/3 intramuscularly). Two-milliilitre aliquots were exsanguinated to 3.8% trisodium citrate solution used as anticoagulant. The activity of coagulation/fibrinolysis was assessed in specimens of plasma by determining standard parameters of haemostasis: concentrations of prothrombin and aprotinin with a one-stage Quick method [12], fibrinogen with a tyrosine method [13] and plasminogen with a caseinolytic method [14] utilizing urokinase (Sigma). Moreover, partial thromboplastin time (PTT) and euglobin lysis time reflecting fibrinolytic activity were assessed [14].

Design of the study

Epsilon-aminocaproic acid, 0.3 g/kg bodyweight, or aprotinin 5000 KIU per kg bodyweight (both supplied by Polfa, Poland) were administered by the intraperitoneal route three times a day in 0.9% sodium chloride solution. The dosage of EACA was estimated from previously reported biologically active concentration of the drug preventing skin allograft rejection [15] and being at this dose range free from significant side-effects in the rat [16]. The aprotinin dose employed in our experiments was also reported as active and non-toxic even when applied repeatedly in the rat [17]. Nephritic control animals received sodium chloride solution alone. The average experimental and control group consisted of six rats. Urine collections for assessment of proteinuria were carried out before induction (day –2), and on days 1, 3 and 7. The experiment was terminated on day 8, when animals were exsanguinated through the abdominal aorta under ether anaesthesia, and serum samples stored at –20°C for determination of autologous IgG and creatinine levels. In some experiments animals were exsanguinated on day 4 to assess the above serum parameters. After usual sacrifice on day 8, kidneys were excised and specimens of renal cortex fixed in formalin for light-microscopy or snap frozen in liquid nitrogen for immunofluorescence of GBM-bound autologous
antibodies. Immunoperoxidase studies assessing glomerular fibrin deposition were performed on paraffin-embedded tissue samples harvested on day 8. In some experiments carried out according to the preventive protocol, rats were bled from carotid arteries as described above, on day 8, to determine parameters of haemostasis.

**Preventive protocol**

EACA or aprotinin was given to rats preimmunized with sheep globulin from the day of glomerulonephritis induction, 6 h before the nephritogenic sheep anti-rat GBM antibody and continued t.i.d. until day 8, when the experiment was terminated.

**Therapeutic protocol**

Nephritic rats were given EACA or aprotinin from day 3 post-induction; the frequency and magnitude of dosage being the same as in the preventive protocol.

**Dose-dependence of the effect of EACA or aprotinin on proteinuria**

Three groups of nephritic rats, receiving 0.15 g/kg, 0.3 g/kg and 0.45 g/kg respectively, t.i.d. from day 0 and a control nephritic group were enrolled in this study to determine the effect of graded doses of EACA on proteinuria on days 4 and 8 post-induction. Similar experiments were performed to assess the influence of increasing doses of aprotinin (2500 KIU/kg, 5000 KIU/kg and 7500 KIU/kg t.i.d.) on this nephritis model.

**Statistical method**

Significance of differences between control and experimental groups was calculated utilizing Student’s *t* test.

**Results**

The effects of EACA and aprotinin on proteinuria and creatinine clearance

The EACA (0.3 g/kg t.i.d.) administered from day 0 of the autologous anti-GBM glomerulonephritis (preventive protocol) caused a remarkable reduction in proteinuria otherwise noted in control nephritic rats. In one representative experiment (Table 1) proteinuria in the control nephritic group reached on day 3 the mean value of 151.0 mg/day (SD = 29.1 mg, *n* = 6, pre-experiment proteinuria: 10.3 ± 8.9 mg/24 h), while nephritic rats treated with EACA excreted 76.1 ± 19.3 mg of protein per 24 h (*n* = 8) and the difference is highly significant (*t* = 5.51, *P* < 0.0005). On day 7 the difference was less evident, though still statistically significant (control group, 141.5 ± 30.5 mg, *n* = 6; EACA group, 87.3 ± 40.1 mg, *n* = 8; *t* = 2.87, *P* < 0.01). Nephritic rats treated in parallel with aprotinin were more proteinuric than those in the EACA group (aprotinin-treated, day 3, 112.9 ± 19.6 mg, *n* = 7, *t* = 3.64, *P* < 0.025; day 7, 116.0 ± 12.7 mg, *n* = 7, *t* = 1.91, *P* < 0.05), although significantly less than the control nephritic group (day 3, *t* = 2.72, *P* < 0.01; day 7, *t* = 1.91, *P* < 0.05). The percentage of proteinuria reduction in treated versus untreated nephritic rats assessed from pooled results of three experiments (Figure 1) averaged 57.6% ± 21.7% (*n* = 19) on day 3 in the EACA group and 26.8% ± 13.6% (*n* = 18) in the aprotinin group (*t* = 5.22, *P* < 0.0005). The respective values for day 7 were 44.2% ± 19.3% (*n* = 18) and 24.1% ± 13.0% (*n* = 17) and the difference was also highly significant (*t* = 3.65, *P* < 0.0005).

Treatment with EACA or aprotinin did not result in any large changes in GFR assessed by creatinine clearance (Figure 1), otherwise markedly reduced in this model (pre-experiment value, 1.59 ± 0.26 ml/min, *n* = 6; control nephritic group, day 3, 0.87 ± 0.11 ml/min, *n* = 6, *t* = 6.55, *P* < 0.0005; day 7, 0.91 ± 0.23 ml/min, *n* = 6, *t* = 4.85, *P* < 0.0005). Creatinine clearance in the EACA-treated nephritic group averaged on day 3, 0.96 ± 0.18 ml/min and on day 7, 0.91 ± 0.23 ml/min and was not significantly different from control nephritic group (day 3, *t* = 1.04, *P* < 0.2; day 7, *t* = 0.07, *P* > 0.48). Accordingly, similar values, not significantly different from those seen in control nephritic rats were noted in the aprotinin-treated nephritic group (day 3, 0.94 ± 0.27 ml/min, *n* = 6, *t* = 0.58, *P* < 0.3; day 7, 0.81 ± 0.2 ml/min, *n* = 6, *t* = 0.69, *P* < 0.25).

**Table 1. Proteinuria (mg/24 h) in nephritic rats treated with EACA (0.3 g/kg, t.i.d.) or aprotinin (5000 KIU/kg, t.i.d.) from day 0, compared with the nephritic control group**

<table>
<thead>
<tr>
<th>Nephritic control</th>
<th>Nephritic EACA-treated</th>
<th>Nephritic aprotinin-treated</th>
</tr>
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<tbody>
<tr>
<td>day 3</td>
<td>day 7</td>
<td>day 3</td>
</tr>
<tr>
<td>145</td>
<td>135</td>
<td>111</td>
</tr>
<tr>
<td>118</td>
<td>115</td>
<td>73</td>
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<tr>
<td>175</td>
<td>169</td>
<td>63</td>
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<tr>
<td>158</td>
<td>147</td>
<td>51</td>
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<tr>
<td>190</td>
<td>181</td>
<td>72</td>
</tr>
<tr>
<td>120</td>
<td>102</td>
<td>88</td>
</tr>
<tr>
<td>151.0 ± 29.1</td>
<td>141.5 ± 30.5</td>
<td>76.1 ± 19.3</td>
</tr>
<tr>
<td><em>P</em> &lt; 0.0005</td>
<td><em>P</em> &lt; 0.01</td>
<td><em>P</em> &lt; 0.01</td>
</tr>
<tr>
<td>112.9 ± 19.6</td>
<td>1160 ± 12.7</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
</tbody>
</table>

Statistical significances pertain to the differences with respect nephritic control groups.
Antiproteolytic drugs in immune glomerulonephritis

Results of another experiment aimed at determining the effect of graded doses of EACA or aprotinin on proteinuria in experimental glomerulopathy under study are illustrated in Figure 2. Clearly, the dose-dependence has become evident in 0–0.3 g/kg dose range in case of EACA and from 2500 KIU/kg to 5000 KIU/kg in case of aprotinin. EACA was effective already at 0.15 g/kg regarding nephritic control value (n=6, t=3.92, P<0.0025) and the dose of 0.3 g/kg was significantly more effective than that of 0.15 g/kg (n=6, t=1.98, P<0.05). Increase of aprotinin dose between 2500 KIU/kg and 5000 KIU/kg yielded a significant antiproteinuric result (n=6, t=6.40, P<0.0005). Further increases in dosage of both drugs (EACA: 0.45 g/kg and aprotinin: 7500 KIU/kg) did not result in enhancement of antiproteinuric effect.

Delayed onset of treatment (therapeutic protocol) also generated results indicating that both drugs are capable of ameliorating proteinuria in autologous anti-GBM disease. Results of the studies assessing the effect of EACA and aprotinin administered from day 3 are demonstrated in Figure 1. Already by the first day of treatment both drugs caused a significant antiproteinuric effect, and EACA was more active in this regard (results pooled from two experiments, n=12: day 3–4, nephritic controls: 163.5±20.7 mg; EACA 123.9±17.9 mg, t=5.01, P<0.0005; proteinuria reduced by 24.2%±11.0%; aprotinin 137.8±12.5 mg, t=3.67, P<0.0025, proteinuria reduced by 15.7%±7.7%, EACA vs aprotinin, r=2.18, P<0.025), this influence being more pronounced on day 7–8 (nephritic controls, 168.7±22.3 mg; EACA, 114.3±19.7 mg, t=6.26, P<0.0005; proteinuria reduced by 32.0%±11.7%; aprotinin, 133.0±19.2 mg, t=4.13, P<0.0005, proteinuria reduced by 20.9%±11.4%, EACA vs aprotinin, t=2.36, P<0.0125). The attempt of starting treatment later on (day 5) was unsuccessful: proteinuria in experimental groups was non-significantly different from control nephritic values on days 5, 8 and 11 (data not shown).

Similarly to results obtained with the preventive protocol, administration of the drugs started later in the course of disease did not significantly modify creatinine clearance of nephritic rats (EACA-treated group: day 3, 0.85±0.16 ml/min, n=6, t=0.25, P<0.45; day 7, 0.96±0.21 ml/min, n=6, t=0.39, P<0.35; aprotinin-treated group, day 3, 1.02±0.31 ml/min, n=6, t=1.15, P<0.15; day 7, 0.93±0.17 ml/min, n=6, t=0.17, P<0.45).

Results of an experiment comparing antiproteinuric effects of preventive and therapeutic protocols are summarized in Figure 2 (lower panel).
Glomerular histopathology and binding of autologous nephrotoxic antibodies

Administration of EACA or aprotinin resulted in amelioration of glomerular histological abnormalities seen on day 8 of this model of anti-GBM nephritis (Table 2). Hypercellularity was significantly reduced in rats injected with EACA or aprotinin according to the preventive protocol when compared to control nephritic animals (EACA vs control, \( t = 5.89, P < 0.0005 \); aprotinin vs control, \( t = 4.42, P < 0.001 \)). Therapeutic protocol also provided a notable decrease in glomerular hypercellularity, although in case of aprotinin it did not reach statistical significance (EACA vs control, \( t = 3.54, P < 0.01 \); aprotinin vs control, \( t = 1.42, P < 0.2 \)). Necrosis of glomerular capillaries, not particularly prominent in this nephritis model, was similarly although to a lesser degree affected by antiproteolytic drugs (preventive protocol, EACA, \( t = 3.26, P < 0.005 \); aprotinin, \( t = 1.75, P < 0.0125 \); therapeutic protocol, both not significant, EACA, \( t = 1.43, P < 0.1 \); aprotinin, \( t = 0.38, P < 0.4 \)).

Fluorescein-labelled anti-rat IgG antibodies disclosed linear distribution of GBM deposits typical for this model of glomerulonephritis in control nephritic rats. Glomeruli in preparations obtained from animals treated with EACA or aprotinin did not demonstrate any appreciable differences either in distribution or intensity of staining with regard to controls.

Titres of autologous rat anti-sheep globulin antibodies

Comparison of nephrotoxic autologous antibody titres in nephritic control and experimental rats disclosed no significant differences (day 3, nephritic control, \( 6.5 \pm 1.2 \log_2, n = 6 \); EACA-treated, \( 6.8 \pm 0.7 \log_2, n = 6, t = 0.53, P < 0.35 \); aprotinin-treated: \( 7.0 \pm 1.1 \log_2, n = 6, t = 0.76, P < 0.25 \); day 7, control, \( 8.1 \pm 0.9 \log_2, n = 6 \); EACA-treated, \( 7.9 \pm 0.8 \log_2, n = 6, t = 0.4, P < 0.3 \); aprotinin-treated, \( 8.5 \pm 0.8 \log_2, n = 6, t = 0.81, P < 0.25 \)).

Glomerular fibrin deposits

Immunoperoxidase staining with anti-rat fibrinogen antiserum of kidney tissue sections from rats sacrificed on day 8 of autologous anti-GBM disease failed to reveal any appreciable fibrin deposition in glomeruli of nephritic control rats. Similarly, negative results were obtained with kidneys of rats treated with EACA or aprotinin from day 0 of experimental glomerulonephritis.

Activity of coagulation/fibrinolysis in peripheral blood

Parameters of haemostasis assessed on day 8 of glomerulonephritis have indicated an enhanced clotting tendency in nephritic rats with regard to healthy controls: shortened PTT (nephritic \( 61.5 \pm 9.6 \) s, \( n = 11 \); healthy \( 70.3 \pm 8.4 \) s, \( n = 12, t = 2.32, P < 0.025 \)) and prolonged fibrinolysis time (nephritic \( 197.0 \pm 55.8 \) s, \( n = 10 \); healthy \( 117.1 \pm 43.6 \) s, \( n = 12, t = 3.68, P < 0.0025 \)).

Nevertheless, administration of EACA or aprotinin did not produce any appreciable alterations in the activity of coagulation/fibrinolysis in nephritic rats. Specifically, time of fibrinolysis and plasminogen levels were not significantly different between nephritic control and EACA- or aprotinin-treated animals, indicating lack of notable modification of plasmin activity under these experimental conditions. It is of note that in healthy control rats both protease inhibitors caused a significant prolongation of fibrinolysis time (untreated control group, \( 117.1 \pm 43.6 \) s, \( n = 12 \); EACA-treated, \( 187.3 \pm 48.1 \) s, \( n = 11, t = 3.66, P < 0.0025 \); aprotinin-treated, \( 170.8 \pm 48.5 \) s, \( n = 6, t = 2.29, P < 0.025 \)), reflecting their potential to interfere with plasminogen activation. Elevation of plasminogen levels in aprotinin-treated healthy rats with respect to untreated healthy controls (control group \( 5.92 \pm 1.16 \) units, \( n = 10 \); aprotinin group \( 7.37 \pm 0.95 \) units, \( n = 5; t = 2.25, P < 0.0125 \)) could also be implicated in an anti-plasmin activity of this protease inhibitor.

Discussion

We were initially surprised with the results of pilot experiments, discovering antinephritic potential of the EACA [9], a drug traditionally regarded as pronephritic due to its interference with degradation of fibrin. Moreover, EACA has been demonstrated to potentiate experimental glomerular lesions caused by intravascular coagulation [18]. Nonetheless we embarked on the studies assessing the effect of this drug in experimental rat glomerulonephritis prompted by incidental obser-

Table 2. Glomerular hypercellularity and segmental necrosis in renal sections of nephritic rats treated with EACA or aprotinin

<table>
<thead>
<tr>
<th>Group of rats</th>
<th>Glomerular hypercellularity (cells/gglomerulus)</th>
<th>Glomeruli with segmental necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=6)</td>
<td>40.3 ± 3.2</td>
<td>0</td>
</tr>
<tr>
<td>Control nephritic (n=8)</td>
<td>67.5 ± 4.3</td>
<td>9.0 ± 3.0</td>
</tr>
<tr>
<td>EACA from day 0 (n=8)</td>
<td>51.1 ± 6.6</td>
<td>4.3 ± 2.8</td>
</tr>
<tr>
<td>Aprotinin from day 0 (n=7)</td>
<td>56.8 ± 5.0</td>
<td>5.8 ± 1.8</td>
</tr>
<tr>
<td>EACA from day 3 (n=8)</td>
<td>56.2 ± 8.4</td>
<td>7.0 ± 2.6</td>
</tr>
<tr>
<td>Aprotinin from day 3 (n=6)</td>
<td>62.8 ± 7.2</td>
<td>8.4 ± 3.5</td>
</tr>
</tbody>
</table>

Dosage of the drugs, EACA 0.3 g/kg, t.i.d., aprotinin 5000 KIU/kg t.i.d.
glomerular histopathology with reduction of comple-
delayed onset of treatment is particularly worth
capable of mediating tissue injury directly and through
critical data. Ikehara [23] achieved amelioration of
ectal nephritis in mice with an amidine inhibitor of
protease-dependent path-
dated glomerular hypercellularity and, to a lesser extent,
necrosis in immune
zymes, although have not reported renal histopatholog-
but neutrophils in particular, are
of classical mechanisms than the preventive use of the drug, since delayed onset of inhibiting protease-dependent pathway of inflammatory glomerular injury could have resulted in formation of unrecoverable lesions to renal tufts. Such a possibility was highly probable in this nephritis model, characterized by early infiltration of neutrophils (day 1), shortly replaced by monocytes (day 3), principal cellular mediators of glomerulopathy. Both cell types, but neutrophils in particular, are known to be abundant sources of proteolytic enzymes, capable of mediating tissue injury directly and through activation of other inflammatory mechanisms. Therefore, significant amelioration of the disease with delayed onset of treatment is particularly worth emphasizing and it has not been reported by other authors using protease inhibitors to reduce glomerular injury [5,6]. Specifically, Jennette et al [5] reported on a notable decrease of glomerular necrosis in immune complex nephritis in mice with an amidine inhibitor of trypsin-like proteases, however, without demonstrating its effect on proteinuria, while Baricos et al [6] succeeded in reducing protein excretion in rat anti-GBM glomerulopathy using an inhibitor of cysteine proteases, although have not reported renal histopathological data. Ikehara et al [23] achieved amelioration of severe proteinuria, improvement in renal function and glomerular histopathology with reduction of comple-ment C3 deposits in mouse lupus nephritis, using a synthetic protease inhibitor, PUF-175 (nafamostat), known to block activation of complement and serine proteases. Our accelerated autologous model of glomerular injury is complement-independent, as demonstrated by Schreiner et al [24], who have shown that decompensation has no effect on monocyte infiltration and proteinuria in this nephropathy. Although creatinine clearance is not a precise indicator of the actual glomerular filtration rate, in the settings of this study it has served the purpose of assessing differences between experimental and control groups, indicating that EACA did not affect reduced renal function.

In order to compare the effect of EACA used as a protease inhibitor in nephrotic serum nephritis with another drug of established antiproteolytic activity, we chose aprotinin, a serine protease inhibitor. This drug, a potent antagonist of such enzymes as trypsin, kallikrein, elastase, and plasmin has been found useful in treatment of shock syndrome, pancreatic necrosis and edematous states resulting from increased vascular permeability [17], applications similar to those of EACA. Moreover, aprotinin is also capable of countering fibrinolysis through interference with activation of plasminogen and plasmin [17] and its ability to modify coagulation has recently been utilized in reducing blood losses during major surgical procedures [25]. Interestingly, a successful attempt of its use in treating human nephrotic syndrome has been reported [26], although it concerned only a small group of patients. In our hands, aprotinin was effective against proteinuria and renal histopathological changes in experimental anti-GBM nephritis in a manner comparable to that of EACA, although its effects were clearly less pronounced. In some aspects of its use, such as glomerular hypercellularity and necrosis under therapeutic protocol, the differences with the nephritic control group were not statistically significant. The significance of difference in antinephritic potential of both drugs is unclear, given the similarity of their biological activities; it could certainly become more evident after elucidation of the exact pathogenic mechanisms involved.

Since EACA and other protease inhibitors have been shown to affect mechanisms of immune response adversely [27,28], we sought to assess influence of the EACA and aprotinin on conditions of immune induction of this nephritis model. Results of our experiments indicate that neither serum titres of nephrotic antibody, nor the intensity of its deposition on the glomerular basement membrane was modified by administration of EACA or aprotinin. Because humoral response is the principal determinant of immunopathogenesis of this experimental glomerulopathy [29], we have inferred from the above data that protease inhibitors employed in our studies had not affected immune induction of the disease, while interfering with the effector inflammatory phase.

As mentioned above, the procoagulatory potential of both drugs, especially of EACA, was our major concern when considering their application in experi-
mental glomerulopathy. Nonetheless, it appears from results of our immunoperoxidase studies with anti-rat fibrinogen antibodies that EACA and aprotinin did not cause any appreciable deposition of fibrin in glomeruli of nephritic rats, at least at the doses employed. Apparently clotting is not pathogenetically important in this model system, since fibrin deposits were virtually absent also from glomeruli of untreated nephritic rats. Therefore our data indicate that, while the administration of EACA at the dose range promoting fibrinogenesis may aggravate glomerular injury initiated by local clot formation, as reported by other authors [18], this drug may safely be used in experimental conditions with no demonstrable fibrin deposition before starting the treatment. The activity of systemic coagulation and fibrinolysis in nephritic rats was also not significantly affected by the treatment with EACA or aprotinin. It is noteworthy that the plasminogen levels and times of fibrinolysis in the EACA/aprotinin-treated nephritic groups were not appreciably different from the untreated nephritic controls. Modification of these parameters was expected in animals treated with drugs inhibiting activation of plasminogen to plasmin, and was indeed noted in healthy rats given the drugs with regard to healthy controls (elevation of plasminogen levels and prolongation of fibrinolysis time), but not in nephritic animals and their EACA/aprotinin-treated counterparts. This latter finding not only supports the idea that the fibrinolytic effect of the drugs was probably not related to the inhibition of plasmin.

Serine protease could be responsible for many proinflammatory mechanisms potentially accounting for development of glomerular lesions, such as direct degradation of GBM components [30] or activation of GBM-degrading metalloproteinases [31]. Our results certainly do not rule out the possibility that the local activity of plasmin could have been reduced in the EACA/aprotinin-treated rats and the activity of other proinflammatory proteolytic enzymes, especially serine proteases, could have been affected by these drugs. Since our study was aimed at characterizing conditions of the observed amelioration of glomerular injury and not at identifying mechanisms of this effect, the involvement of particular mediator pathways is unknown. This issue is presently being investigated in our laboratory. Thus far, no firm conclusions could be made as to the nature of glomerular histopathological changes affected by protease inhibitors in our study. Nevertheless it is conceivable that since EACA and aprotinin only partially prevented/reversed glomerular hypercellularity, their effect on recruitment of monocytes to renal tufts or proliferation of intrinsic glomerular cells could have been bypassed by other pathogenic mechanisms, or our intervention only partially affected protease-dependent pathways. Necrosis of capillary loops is not particularly prominent in this model, involving approximately 10% of glomeruli, although preventive protocol of treatment with EACA has demonstrated higher efficacy than the therapeutic one. This observation further emphasizes importance of early inhibition of protease-dependent pathway of glomerular injury, also evident in other parameters assessed in this study.

Beneficial effects of the EACA and aprotinin in experimental rat glomerulonephritis, particularly their ability to reduce glomerular injury when the treatment was started after the appearance of symptoms of the disease, indicate therapeutic potential of protease inhibitors in human glomerulopathies. Indeed, some successful attempts, although in uncontrolled trials, have already been made in this regard in Japan with the above-mentioned nafamostat [32] and in Italy with aprotinin [26]. It is conceivable, that the indications for application of protease inhibitors in individual cases would have to be carefully considered in relation to clinical features and, more importantly, to the renal biopsy findings. Administration of drugs inhibiting glomerular proteases, including plasmin, may even aggravate renal injury in chronically progressive glomerulopathies, characterized by excessive accumulation of mesangial matrix and subsequent sclerosis [33]. Furthermore, tissue plasminogen activator has been successfully used in treatment of experimental crescentic nephritis with prominent glomerular fibrin deposits [34], contrary to our studies employing antagonists of plasminogen in therapy of glomerular lesions without evidence of local coagulation. It therefore appears that while management of glomerulonephritis in the future may indeed utilize selective inhibition of individual inflammatory mediators, this approach would challenge nephrologists to adopt much more stringent criteria for selection and monitoring of treatment than with the presently used, highly non-specific corticosteroid/cytostatic therapy.

Clearly, many issues concerning use of protease inhibitors in treatment of glomerular diseases remain to be solved in experimental settings before this approach could be recommended for clinical applications and we believe that our data, along with results of other authors cited above, merit future efforts in this field.

References
Antiproteolytic drugs in immune glomerulonephritis


Received for publication: 13.2.95
Accepted in revised form: 4.9.95