PROTEOLYTIC ENZYME TREATMENT REDUCES GLOMERULAR IMMUNE DEPOSITS AND PROTEINURIA IN PASSIVE HEYMANN NEPHRITIS

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Immunopathologic studies (1) in humans and experimental animals have established that deposition and/or in situ formation of immune complexes mediate most forms of glomerular injury. In spite of numerous reports of treatments of idiopathic immune complex glomerulonephritis in humans and experimental glomerulonephritis in animals, results with the more traditional approaches have generally been unsatisfactory. Recently, dissociation of immune deposits from glomeruli by administration of excess antigen (2–5) or anti-Ig (6) has been more successful. A role for complement in redistribution and clearance of immune complexes from glomeruli has also been explored (7), with results suggesting that the deposits are not static once formed; this knowledge has not yet been exploited therapeutically.

As a generalized, nonspecific modality, we hypothesized that exogenous proteases should be capable of removing glomerular immune deposits. To test this idea, we chose to study passive Heymann nephritis (PHN),[1] a widely used experimental model in rats that closely resembles human membranous nephropathy. Insight into the pathogenesis of Heymann nephritis has been greatly advanced in recent years, and direct binding of antibody to fixed glomerular antigen is now considered to be the principal mechanism of subepithelial immune deposit formation (8, 9). The responsible antigen appears to be a 330-kD glycoprotein of proximal tubular epithelial brush border membranes which is also present on glomerular epithelial podocytes (10, 11). Tissue injury is thought to result from activation of complement without involving inflammatory cells (12).

PHN has other features that make it an attractive model system. Injection of heteroantibody specific for a glomerular antigen leads to accumulation in glomeruli, and after 3–6 d the deposited antibody may induce transient proteinuria. Since the culprit Ig is from a different animal species, this proteinuria is referred

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[1] *Abbreviations used in this paper:* Fx1A, fraction 1A of renal cortical brush border membranes; PHN, passive Heymann nephritis.

to as the heterologous phase of the disease. By 7–10 d, the recipient animal usually develops an immune response to the heterologous Ig. The resulting autologous antibody binds to the heteroantibody still present in the glomeruli, thereby inciting a new phase of glomerulonephritis, termed autologous because it is caused by host antibody. The autologous phase is typically more pronounced and persistent than the heterologus phase. This biphasic character of glomerular immune complex formation makes PHN particularly well adapted for study at multiple time points. The effects of removal of heteroantibody before onset of the autologous phase and the effects of treatment during or after development of the autologous phase can be studied independently simply by intervening at different times after injection of the heteroantibody.

In the present study, we treated PHN with proteolytic enzymes at different phases of the disease and examined the effects on glomerular immune complexes and urinary protein excretion. The results show that exogenous enzymes can prevent or remove glomerular immune complex deposits and decrease proteinuria.

Materials and Methods

Animals. Male Sprague Dawley rats weighing 250 g were obtained from Zivic Miller (Allison Park, PA).

Induction of PHN. Fx1A tubular antigen was prepared from Sprague Dawley renal cortices (13). New Zealand white rabbits were immunized with 10 mg of fraction 1A of renal cortical brush border membranes (Fx1A) in CFA (Difco Laboratories Inc., Detroit, MI) in the rear footpads. 2 wk later, a booster immunization with the same preparation was given in multiple subcutaneous sites and repeated biweekly. Rabbits were periodically bled and we assessed antibody against tubular antigen by indirect immunofluorescence. Antiserum against Fx1A was heat inactivated (56°C, 30 min) and absorbed with 0.1 ml normal rat serum, and subsequently, 1 ml packed rat blood cells per ml antiserum. The IgG fraction of the rabbit antiserum was prepared by precipitation with half-saturated $(NH_4)_2SO_4$ followed by DEAE-cellulose column chromatography in 0.0175 M potassium phosphate buffer, pH 6.30. This preparation is referred to as Heymann antibody; its specificity was confirmed by indirect immunofluorescence. To induce PHN, it was given by tail vein.

Experimental Design. Six groups of animals were studied so that doses of Heymann antibody could be varied and protease treatment could be instituted at different phases of the disease (Table I).

Group A, rats were randomly divided into two subgroups after receiving 5 mg Heymann antibody on day 1. Rats in subgroup 1 were treated with 2.5 ml of a mixture of chymopapain (1 mg/ml) and subtilisin BPN′ (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO) in PBS, pH 7.4, given intraperitoneally twice daily from days 1–7; control rats (subgroup 2) were given corresponding injections of 2.5 ml normal saline. Animals were killed on day 12.

Group B, rats received 10 mg Heymann antibody intravenously on day 1, and were randomly subdivided. Subgroup 1 rats were treated with chymopapain and subtilisin twice daily on days 1–5 as above, and control rats (subgroup 2) were treated with saline instead of protease. Animals were killed on day 5.

Group C, rats received 10 mg Heymann antibody. Protease (subgroup 1) or saline (subgroup 2) injections were begun on day 6 and continued until they were killed on day 12.

Group D, after 60 mg of Heymann antibody was given as three 20-mg injections at 1-h intervals, rats were allocated randomly to four subgroups. Subgroup 1 rats were given protease on days 1–7, subgroup 2 rats on days 6–12, and subgroup 3 rats on days 6–18. Control rats (subgroup 4) did not receive any treatment. Rats were killed on day 18.

TABLE I
Experimental Design for Induction and Treatment of Passive Heymann Nephritis

Group	*n**	Heymann antibody (mg)†	Treatment	Days of treatment	Day killed
A1	16	5	Protease§	1–7	12
2	19	5	Saline	1–7	12
B1	12	10	Protease	1–5	5
2	12	10	Saline	1–5	5
C1	12	10	Protease	6–12	12
2	13	10	Saline	6–12	12
D1	7	60	Protease	1–7	18
2	8	60	Protease	6–12	18
3	6	60	Protease	6–18	18
4	11	60	—	—	18
E1	10	40	Protease	15–25	25
2	8	40	—	—	25
F1	6	0	Protease	1–5	5
2	6	0	Protease	1–12	12
3	6	0	Saline	1–5	5
4	6	0	Saline	1–12	12
5	6	0	—	—	5
6	6	0	—	—	12

* Number of animals.
† IgG fraction of rabbit anti-Fx1A antiserum given on day 1.
§ Chymopapain plus subtilisin.

Group E, 15 d after administration of 40 mg Heymann antibody given as two 20-mg injections 1 h apart, rats were randomly divided into protease-treated and control groups. Enzyme treatment was started on day 15 and continued through day 25, when all animals were killed.

Group F, to assess possible toxicity of protease treatment in normal rats, animals were randomly divided into six subgroups. Subgroup 1 rats received enzymes intraperitoneally as above on days 1–5, and were killed on day 5; rats in subgroup 2 received enzymes intraperitoneally on days 1–12, and were killed on day 12; subgroup 3 received saline intraperitoneally on days 1–5, and were killed on day 5; subgroup 4 rats received saline intraperitoneally on days 1–12, and were killed on day 12; rats in subgroups 5 and 6 did not receive any treatment and were killed on days 5 and 12, respectively.

Morphology. Renal tissue obtained at sacrifice was processed for light, immunofluorescence, and electron microscopy (14). For light microscopy, after fixation in 10% neutral formalin, sections of paraffin-embedded tissue cut at 2 μm were stained with H and E and periodic acid-silver methenamine.

For immunofluorescence microscopy, blocks of renal cortex were snap-frozen in chilled 2-methylbutane suspended in liquid nitrogen and stored at −70°C. Cryostat sections, 4 μm, were fixed in acetone for 1 min, washed in PBS, incubated in a moist chamber with antiserum, washed in PBS, and mounted in glycerol-PBS. Triplicate sections were reacted with fluorescein-conjugated goat antibody against rabbit IgG (Miles Laboratories Inc., Naperville, IL), rhodamine-conjugated goat antibody to rat IgG (United States Biochemical Corp., Cleveland, OH) and fluorescein-conjugated goat anti-rat C3 (United States Biochemical Corp.); all antisera were diluted 1:10. Coded slides were examined in a fluorescence microscope (E. Leitz, Inc., Wetzlar, Federal Republic of Germany) independently by two observers, and the intensity of staining was scored as − to 2+. In the

TABLE II

Immune Deposits in Glomeruli of Rats Injected with 5 or 10 mg Heymann Antibody and Treated with Protease

Group*	n†	Heymann antibody injected (mg)	Grade of glomerular immunofluorescence§							
			Rabbit IgG			Rat IgG			Rat C3	
			−	1+	2+	−	1+	2+	−	+
A1	16	5	10	6	0	11	5	0	16	0
2	19	5	2	17	0	2	17	0	17	2
B1	12	10	8	4	0	7	5	0	12	0
2	12	10	3	9	0	7	5	0	12	0
C1	12	10	4	2	6	5	3	4	12	0
2	13	10	0	5	8	0	6	7	13	0

* Subgroup 1 rats received protease; subgroup 2 rats received saline.
† Number of animals.
§ Data are number of rats positive for each immune reactant at the intensity indicated.

occasional instance when the scores from the two observers differed, the section was reexamined by both observers and a score was agreed upon before decoding.

For electron microscopy, 0.5-mm^3 blocks were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, postfixed in 1% OsO_4, stained en-bloc in uranyl acetate, dehydrated, and embedded in Spurr's epoxy. Sections from three randomly selected rats from each subgroup were evaluated in a 201C electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ). The number and size of electron dense deposits in subendothelial, intramembranous, subepithelial, and mesangial glomerular sites were determined by planimetry with a computerized morphometry system (Bioquant, R & M Biometrics, Inc., Nashville, TN). The data were normalized to the length of basement membrane studied for each specimen.

Urine Studies. 24-h urine collections were done on individual rats in metabolic cages without food but with free access to water. Urine was collected under 0.5 ml mineral oil and centrifuged at 2,000 rpm for 20 min at 4°C. Supernatants were stored at −20°C until tested. Protein content was determined by precipitation in 3% sulfosalicylic acid (15).

Antibody Response to Heterologous Protein. Before sacrifice, blood was drawn from the aorta, and serum was obtained and frozen at −70°C until tested. For measurement of antibody levels against rabbit IgG, we used ELISA (16). 96-well polystyrene flat-bottomed microtiter plates (Flow Laboratories, Inc., McLean, VA) were coated with 100 μl/well of normal rabbit IgG (1 μg/ml in 0.1 M sodium carbonate, pH 9.4) at 37°C for 16 h. After coating and between all subsequent steps, wells were washed three times with PBS. The coated plates were blocked by incubation with 1% BSA (Sigma Chemical Co.) in PBS at 37°C for 1 h. Serum samples, in serial twofold dilutions, were added (100 μl/well) and incubated for 1 h at room temperature. The plates were then incubated for 1 h at room temperature with alkaline phosphatase–conjugated goat anti-rat IgG (Cappel Laboratories, Cochranville, PA) diluted 1:100 in PBS. The chromogenic substrate *p*-nitrophenyl disodium phosphate (Sigma Chemical Co.) was added at 4 mg/ml in glycine/$MgCl_2$ buffer, pH 10.5. After 180 min, optical densities were measured at 410 nm in a microplate reader (Dynatech Laboratories, Inc., Alexandria, VA).

Serum Creatinine. Serum creatinine was measured by the picric acid method (17).

Results

Morphological Studies. Table II gives the immunofluorescence results for rabbit IgG (the injected heterologous Heymann antibody), rat IgG (autologous antibody to the injected rabbit IgG), and rat C3 in groups A, B, and C (see Table

I). Granular deposits of both rabbit and rat IgG along the glomerular capillary loops, characteristic of PHN, were seen in 17 of 19 rats in the control animals of group A2. On the other hand, only 6 of 16 rats treated with enzymes on days 1–7 (group A1) were positive for rabbit IgG ($\chi^2 = 10.4$, $p < 0.01$), and only 5 of 16 were positive for rat IgG ($\chi^2 = 12.6$, $p < 0.001$) at the time of sacrifice. In rats given 10 mg of Heymann antibody, treated on days 1–5, and killed in the heterologous phase on day 5 (group B), only 4 of 12 protease-treated rats were positive for rabbit IgG compared with 9 of 12 saline-treated rats ($\chi^2 = 4.2$, $p < 0.05$); there was no difference in frequency of positivity for rat IgG. However, after the same 10-mg dose of Heymann antibody, when we began protease treatment later (on day 6) and continued until day 12, significantly fewer rats in the enzyme-treated group had deposits of rabbit and rat IgG; 4 of 12 protease treated rats (group C1) had no rabbit IgG compared with 0 of 13 controls (group C2) ($\chi^2 = 5.16$, $p < 0.025$), and 5 of 12 protease treated rats had no deposits of rat IgG compared with 0 of 13 controls ($\chi^2 = 6.77$, $p < 0.01$) (Fig. 1). There were no deposits of C3 in rats given 5 or 10 mg of Heymann antibody (groups A, B, and C), except for two rats in group A.

When larger (40–60 mg) doses of Heymann antibody (Table I, groups D and E) sufficient to induce proteinuria, i. e., "nephritogenic" doses, were injected, all animals were positive for both rabbit and rat IgG; the intensity of immunofluorescence in control rats, while somewhat greater than in enzyme-treated rats, was not significantly different (data not shown). Positivity for rat C3 was 8 of 21 (38%) in protease-treated vs. 8 of 11 (73%) in control rats, which approaches significance ($\chi^2 = 3.5$, $p < 0.1$). In the group F1 and F2 controls given protease but not Heymann antibody (Table I), none showed immunofluorescence for rabbit IgG, rat IgG, or rat C3.

By electron microscopy, all rats given Heymann antibody had electron dense deposits in the subepithelial layer of the capillary basement membrane; intramembranous and paramesangial deposits were seen rarely, <1% of all deposits; we did not see any subendothelial and mesangial deposits. As shown in Table III, in all groups studied, rats treated with protease had significantly fewer deposits per unit basement membrane length than the corresponding controls given saline (all groups, $p < 0.01$) (see also Fig. 2). Among rats given subnephritogenic doses of Heymann antibody (5 or 10 mg), the deposits were small and scattered, and we made no attempt to measure their area. However, in group D rats given 60 mg of Heymann antibody, a dose resulting in proteinuria (see below), the deposits were larger and their areas were quantified. Sizes of the deposits were variable, and there was no significant difference in mean size in protease-treated (0.050 ± 0.051) and saline-treated (0.036 ± 0.045) rats. Total cross-sectional area of the deposits per unit basement membrane length, which is dependent on both size and number of deposits, was significantly less in protease-treated rats (15.7 ± 1.87 nm) than in controls (28.6 ± 1.68 nm, $p < 0.001$). The deposits remaining in protease-treated rats were subjectively less dense.

By routine light microscopy, group D and E rats (Table I) given 60 and 40 mg of Heymann antibody had rare epimembranous spikes and deposits, indicative of a membranous nephropathy. There were no differences between the protease-

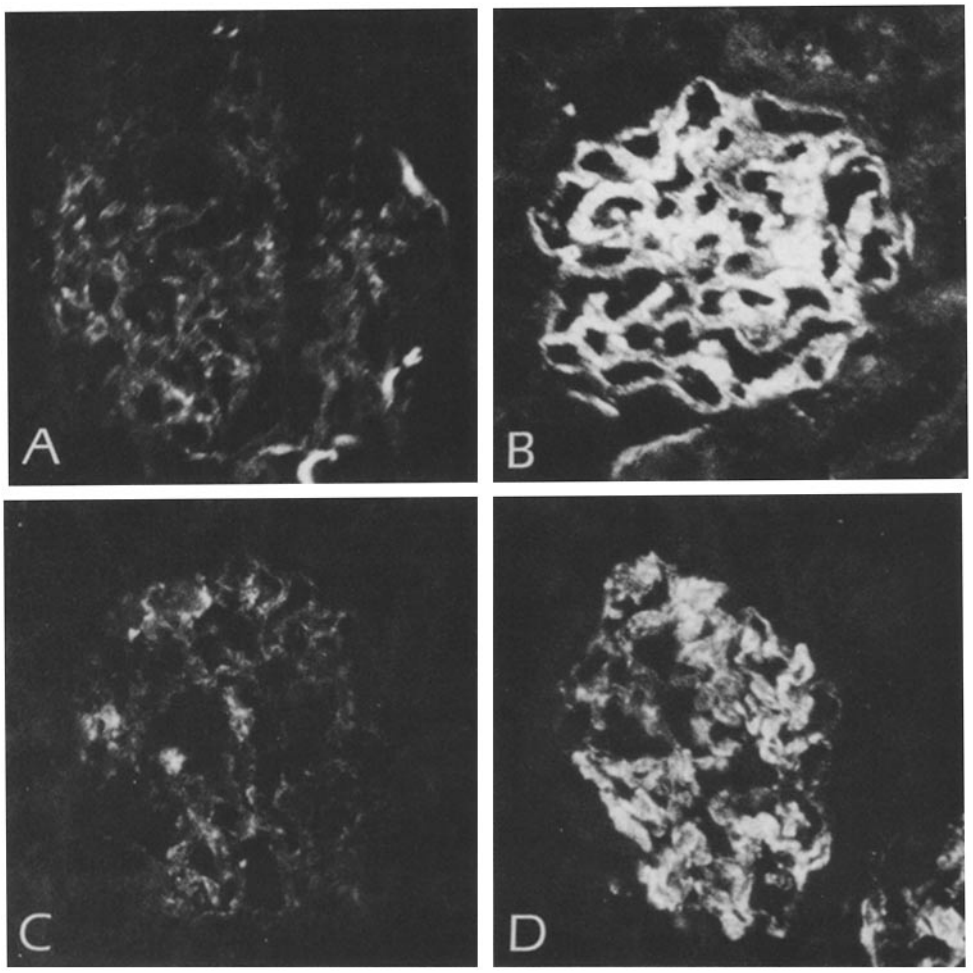

FIGURE 1. Immunofluorescence micrographs of glomeruli from group C rats given 10 mg Heymann antibody on day 1, treated with protease (*A* and *C*) or saline (*B* and *D*) on days 6–12, and killed on day 12. Sections from protease-treated rats stained for heterologous rabbit IgG showed weaker fluorescence (*A*) than did untreated controls (*B*). Sections from protease-treated rats stained for autologous rat IgG likewise revealed weaker fluorescence (*C*) than controls (*D*). The *A* and *B*, and *C* and *D* pairs were exposed for the identical times. × 430.

treated rats and controls in either group. Light microscopy showed no abnormalities in the other groups, which received less Heymann antibody.

Urine Studies. No rat in groups A, B, and C, given low doses of Heymann antibody, developed proteinuria in either the heterologous phase (day 4–5) or the autologous phase (day 11–12) of PHN. 24-h urine protein excretions in rats given 60 mg Heymann antibody (group D) are shown in Fig. 3. Normal male Sprague Dawley rats weighing 250 g excreted <6 mg protein in 24 h. During the heterologous phase of PHN (day 4–5), no subgroup (D1–D4) had significant proteinuria. Control rats (group D4) given Heymann antibody but not treated with enzymes developed significant proteinuria by day 11–12, i. e., in the autologous phase, which increased with time. The mean urine protein excretions

TABLE III

Morphometric Analysis of Electron Microscopic Glomerular Deposits in Rats Given Different Doses of Heymann Antibody and Treated with Protease

Animal group	Heymann antibody (mg)	Treatment	Duration in days	Length of glomerular basement membrane examined (μm)	Number of electron dense deposits	Number of deposits per micrometer of basement membrane
A1	5	Enzyme	1–7	477	122	0.256*
2	5	Saline	1–7	234	156	0.667
B1	10	Enzyme	1–5	984	71	0.072*
2	10	Saline	1–5	1,079	222	0.206
C1	10	Enzyme	6–12	1,673	279	0.167*
2	10	Saline	6–12	1,510	510	0.338
D3	60	Enzyme	6–18	1,185	377	0.318*
4	60	—	—	883	720	0.815

* Significantly less than corresponding control group, $p < 0.01$.

in group D1 (treated during the heterologous phase) on day 11–12, day 15–16, and days 17–18 were significantly less at all three times (4.5 ± 0.8, 9.8 ± 3.1, and 5.6 ± 0.8 mg/24 h) than in the group D4 controls (17.6 ± 6.5, 41.5 ± 14.2, and 37.6 ± 13.8 mg/24 h; all p values <0.05). Group D3 rats, treated during the entire period of the autologous phase, also excreted significantly less protein on day 11–12 (5.0 ± 0.7 mg/24 h), day 15–16 (6.8 ± 1.3 mg/24 h), and day 17–18 (7.4 ± 2.1 mg/24 h) than group D4 control rats (all p values <0.05). Group D2 rats, given protease only during the first part of the autologous phase, had less proteinuria than control rats, but the differences did not attain statistical significance.

To learn whether protease therapy could reverse proteinuria, we gave treated rats in group E, which received 40 mg Heymann antibody, protease from day 15, when all rats had significant proteinuria, until day 25 (Fig. 4). Before the onset of treatment, the rats excreted 13.0 ± 4.1 mg/24 h on day 14–15. A significant reduction of proteinuria occurred by 7 d of treatment, at which time protease therapy had reduced proteinuria to almost normal values; treated rats excreted 7.5 ± 1.6 mg/24 h on day 21–22 and 9.4 ± 1.2 mg/24 h on day 24–25; on the other hand, the untreated controls excreted 12.8 ± 2.1 mg/24 h ($p < 0.05$) and 17.1 ± 3.3 mg/24 h ($p < 0.05$) at these times.

Antibody Response to Heterologous Protein. The host serum antibody response to rabbit IgG after injection of Heymann antibody was measured by ELISA (Table IV). There were no significant differences between protease-treated and control animals.

Serum Creatinines. Among rats given 5, 10, and 60 mg of Heymann antibody, the serum creatinine ranged between 0.61 and 0.82 mg/dl, with no significant differences among subgroups or from age-matched normal rats by analysis of variance ($F = 0.832$).

Possible Adverse Effects of Protease Treatment. Study by light and electron microscopy in group F animals (Table I) revealed no differences between protease-treated subgroups 1 and 2 vs. control groups 3–6. Therefore, if protease

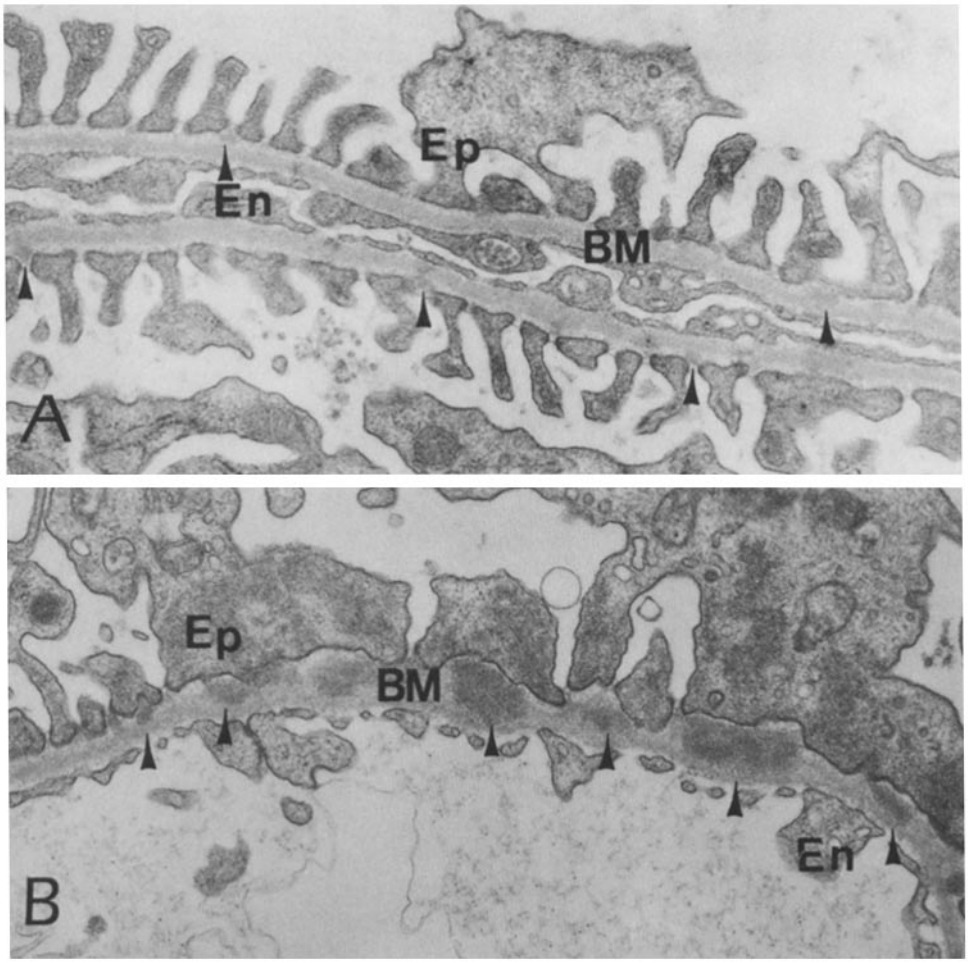

FIGURE 2. Electron micrographs from group *D* rats given 60 mg Heymann antibody on day 1, and killed on day 18. In rats treated with protease on days 6–18 (*A*), significantly fewer electron dense deposits (*arrows*) are seen in the glomerular basement membrane compared with untreated controls (*B*). *BM*, basement membrane; *Ep*, epithelial podocyte; *En*, endothelium. × 21,000.

altered glomerular structure, any effects on cellular morphology or organization of the basement membrane were not discernible by microscopy.

We also examined possible induction of proteinuria by administration of protease to normal rats. Enzyme-treated subgroups F1 and F2 did not manifest significant proteinuria on day 4–5 or day 11–12 compared with saline-treated rats (subgroups 3 and 4) and untreated rats (subgroups 5 and 6) (data not shown).

Protease treatment also had no significant effect on renal function, as reflected in serum creatinine levels. Among rats in group F, the mean serum creatinines within the subgroups ranged between 0.19 and 0.55 mg/dl on day 12; the least significant difference for $p < 0.05$, computed from analysis of variance, is 0.75.

No systemic signs of illness were apparent in protease-treated rats, although we saw 2–3 ml of blood in the intraperitoneal fluid of one-fourth of the killed

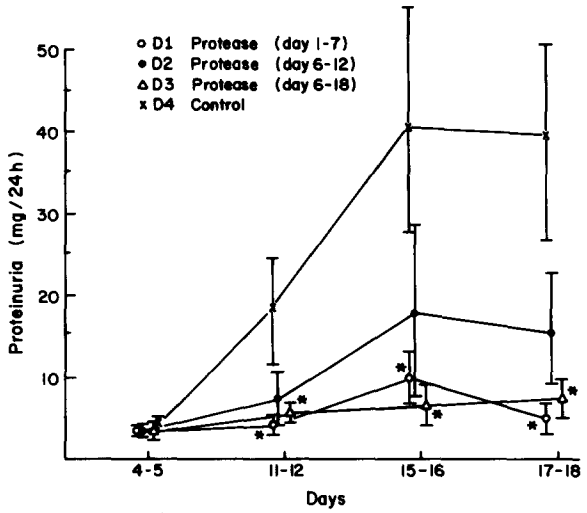

FIGURE 3. 24-h protein excretions by group D rats given 60 mg Heymann antibody on day 1 and killed on day 18 are shown over the 18-d course of study. All protease-treated rats showed numerically less proteinuria than untreated controls (x) at all time points after day 4–5. These differences were statistically significant (asterisk, $p < 0.05$) in subgroup D1 rats (O), treated only during the heterologous phase (day 1–7), and in subgroup D3 rats (Δ) treated throughout the autologous phase (day 6–18).

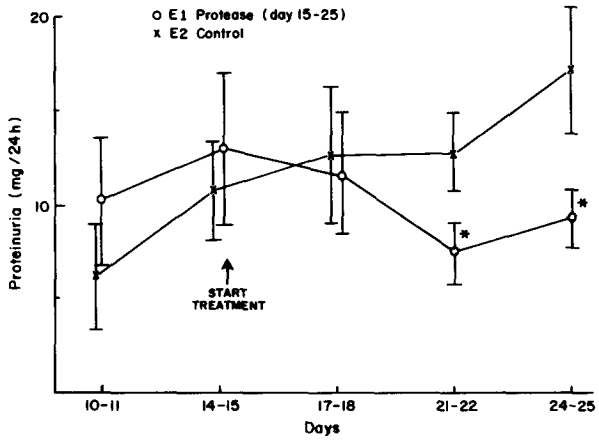

FIGURE 4. 24-h protein excretions by group E rats given 40 mg Heymann antibody on day 1 and killed on day 25 are shown over the course of study. Protease-treated rats (O) excreted significantly less protein than controls (x) after a week of treatment (day 21–22), a difference that was maintained until sacrifice (asterisk, $p < 0.05$).

animals. None of the control animals given enzymes, but not Heymann antibody, died or appeared ill during the course of the experiments.

Discussion

Membranous nephropathy in humans has a characteristic histopathology in which granular deposits of IgG and complement are localized along the glomer-

TABLE IV
Serum Antibody Levels Against Rabbit IgG in Rats Injected with Heymann Antibody

Group	*n**	Day of assay	ELISA OD ± SE
A1	14	12	0.114 ± 0.018
2	16	12	0.104 ± 0.012
B1	12	5	0.194 ± 0.028
2	12	5	0.256 ± 0.066
C1	11	12	0.140 ± 0.021
2	12	12	0.174 ± 0.047
F†	36	5 or 12	0.081 ± 0.011

* Number of animals.
† Group F includes all subgroups; there were no significant differences among the subgroups of these nonimmunized rats by analysis of variance ($F = 0.907$).

ular capillary wall, as determined by immunofluorescence, and exclusively in the subepithelial space, seen by electron microscopy (18, 19). Autologous immune complex nephritis and PHN are experimental glomerulonephritides in rats, initiated when antibody binds to an antigen in the glomerulus. Both models closely resemble human membranous nephropathy. Autologous immune complex nephritis is induced by immunization with an antigen, Fx1A, derived from the brush border of renal proximal tubular epithelial cells; PHN is induced by heterologous antibody against Fx1A.

Many studies have dealt with the treatment of membranous nephropathy in humans and experimental models; however, the results have been unsatisfactory and in many instances controversial. Treatment of the human disease is currently generally limited to corticosteroids. While proteinuria and nephrotic symptoms are reduced, the effect of steroids on the ultimate course of the disease remains unknown. Moreover, the risks of immunosuppression and the other effects of longterm steroid therapy are well known.

With respect to therapy in the experimental models, while some regimens can prevent disease, treatment of already established disease has not succeeded (20–27). Treatment of autologous immune complex nephritis dates from the use of cortisone, adrenal corticotrophic hormone, and nitrogen mustard by Heymann et al. in 1962 (20). Corticosteroids and cytotoxic drugs can reduce protein excretion only if the animals are treated at or before the time of immunization (21–23). Vasoactive amine antagonists with potent antiserotonin and antihistamine activity have either been ineffective or useful only if given before nephritis (24–27).

In recent years, PHN has been studied more widely than autologous immune complex nephritis because of its more rapid induction. Feenstra et al. (28) found that azathioprine was effective in preventing ultrastructural changes and in reducing protein excretion, again only when started before or simultaneously with the administration of Heymann antibody. Salant et al. (29) used corticosteroids and vasoactive amine blockade; however, neither immune deposits nor protein excretion were reduced, although they studied only the heterologous phase. The efficacy of these therapeutic approaches to both autologous immune complex nephritis and PHN thus appears to lie in suppression of the autologous

immune response and/or diminished delivery of immune complexes to glomeruli by virtue of hemodynamic effects, rather than in modulation of effector mechanisms or removal of immune deposits. Because such strategies are only prophylactic, they cannot be applied to the therapy of natural glomerulonephritis in humans or animals.

Lotan et al. (30) reported a significant reduction in proteinuria and glomerular C3 in PHN after treatment with DMSO, even when treatment began after the onset of proteinuria. They suggested that DMSO alters the configuration of the immune deposits and inhibits deposition of C3.

Since proteinuria occurs in PHN, the efficacy of treatment can be monitored by measuring urinary protein as well as by assessing glomerular immune deposits morphologically. Glomerular immune complexes, in sufficient quantity and of appropriate character, can induce alterations in function, often manifest as proteinuria; complexes are then said to be nephritogenic. In such cases, deposits may be so pronounced that morphologic criteria like intensity of immunofluorescence may be insensitive to significant changes in actual amounts of immune reactants. On the other hand, with lesser, subnephritogenic quantities of immune reactants, glomerular deposits may still be evident morphologically and yet allow quantitative differences to be readily appreciated. Since the presence or absence of glomerular dysfunction appears to reflect the quantity of immune reactants deposited, morphological evaluation and nephritis measured by altered glomerular performance (e. g., proteinuria) are both useful parameters, but at different doses of Heymann antibody.

We considered that proteases might be efficacious in PHN by directly digesting the glomerular immune complexes. In the present work, PHN was induced by intravenous injection of Heymann antibody. First, subnephritogenic doses below the threshold for proteinuria were given. Among rats given 5 mg Heymann antibody, the minimum dose leading to autologous glomerular immune deposits observable by immunofluorescence on day 12 (group A), protease treatment was successful in removing glomerular immune deposits. 10 mg of Heymann antibody, the minimum dose permitting identification of rabbit IgG on day 5 in the heterologous phase, was given in two different experiments (groups B and C). In group B, animals were treated with enzymes during the entire heterologous phase (days 1–5), while in group C they were treated during the autologous phase (days 6–12). We saw significant differences in both intensity and frequency of heterologous rabbit antibody, as detected by immunofluorescence, between treated and control rats in group B at the end of the heterologous phase on day 5. When treatment commenced on day 6 (group C), a time when maximal deposition of rabbit IgG has already occurred (31), immune deposits of both rabbit and autologous rat IgG were significantly less on day 12. In addition to results observable by immunofluorescence, the small subepithelial electron dense deposits typically seen in rats given these amounts of Heymann antibody were also significantly reduced in number by protease treatment. Together, these results show that protease treatment can remove immune deposits from glomeruli in PHN. Since protease treatment did not inhibit the antibody response of the host to the heterologous rabbit IgG, depressed antibody synthesis can be excluded as the explanation for the reduction in immune deposits.

60 mg Heymann antibody, a large nephritogenic dose that can induce proteinuria, was administered to rats in group D. By immunofluorescence, deposits were readily noted, and there were no significant differences in rabbit IgG, rat IgG, or rat C3 between treated and control rats. However, with the larger and more numerous electron dense deposits that develop at this dose, there were conspicuous and statistically significant reductions after protease treatment. In addition, treatment during the heterologous phase ceasing on day 7 essentially prevented proteinuria well into the autologous phase. Treatment begun after the heterologous phase and continued for 6 d reduced but did not eliminate proteinuria; however, the marked increase in proteinuria observable on days 15–16 and 17–18 in untreated control rats was completely prevented by continuing enzyme therapy to day 18. These results suggest that protease is effective not only when started at the time disease is initiated (heterologous phase), but even when delayed until the usual time of onset of the autologous phase.

The group E experiment, using 40 mg of Heymann antibody, aimed at determining whether proteinuria could be reversed by protease therapy. 40 mg was purposely chosen to produce mild but consistent proteinuria in the autologous phase. The results showed that enzyme therapy was indeed effective, even when begun as late as day 15.

We favor the interpretation that protease treatment directly removes glomerular immune deposits and thereby ameliorates proteinuria. The prevention or reversal of proteinuria by such treatment is not simply due to digestion of circulating Heymann antibody since immunofluorescence for rabbit IgG was bright in all kidneys after large doses of Heymann antibody, and levels of host serum antibody against rabbit IgG were not different in treated and control rats. We cannot exclude several other less likely possibilities contributing to the diminished proteinuria. Among these, protease could conceivably change the conformation of immune deposits, interfere with actions of the membrane attack complex of complement, or act on glomerular cell receptors that are important in coupling immunologic stimuli to pathophysiological responses. However, none of these alternative hypotheses can account for the differences in intensity of immune reactants observed by immunofluorescence or the decreased number and size of deposits seen by electron microscopy.

Overall, the experiments suggest some general potential for the therapeutic use of proteolytic enzymes in glomerular immune complex disease. We did not see overt toxicity, and renal function was not compromised. Doubtless, the regimen can be improved and other enzymes deserve consideration. Additional studies will be required to investigate the effects of proteases on various mediator systems and inflammatory cells relevant to glomerulonephritis.

Summary

We investigated the effect of proteolytic enzyme treatment on the course of passive Heymann nephritis (PHN). PHN was induced by intravenous injection of Heymann antibody into Sprague Dawley rats. Protease-treated rats received intraperitoneal chymopapain and subtilisin. In rats given subnephritogenic doses of Heymann antibody (5 or 10 mg, insufficient to cause proteinuria), glomerular immune deposits were assessed by immunofluorescence and electron microscopy.

In rats given 5 mg Heymann antibody and treated with protease in the heterologous phase of the disease (days 1–7), fewer animals were positive for rabbit IgG and rat IgG, as determined by immunofluorescence on day 12, compared with controls ($p < 0.01$). Rats given 10 mg Heymann antibody and treated on days 1–5 were less frequently positive for rabbit IgG on day 5 than controls ($p < 0.05$). When treatment was given on days 6–12 (autologous phase), fewer rats had glomerular rabbit and rat IgG compared with controls ($p < 0.025$). Protease treatment of rats given nephritogenic doses of Heymann antibody (≥40 mg, causing proteinuria) did not result in significant differences in immunofluorescence deposits. However, protease treatment significantly reduced the number of electron dense deposits at all doses of antibody ($p < 0.01$). Furthermore, rats given 60 mg Heymann antibody followed by enzyme treatment in the heterologous phase (days 1–7) or throughout the autologous phase (days 6–18) had significantly reduced protein excretion during the autologous phase compared with control rats ($p < 0.05$). After onset of significant proteinuria on day 15 in rats given 40 mg Heymann antibody and treated from day 15 until day 25, there was significantly less ($p < 0.05$) proteinuria on days 21–22 and 24–25 than in control rats; thus, enzymes could reverse proteinuria. In normal rats, administration of proteases did not have significant effects on urinary protein excretion, serum creatinine, or renal morphology, nor did protease affect anti-rabbit IgG antibody production in rats injected with Heymann antibody. The overall results indicate that proteolytic enzyme treatment can prevent or remove glomerular immune deposits and can prevent or reverse proteinuria.

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