American Journal of Pathology, Vol. 127, No. 3, June 1987

Amelioration of Immune Complex-Mediated Glomerulonephritis by Synthetic Protease Inhibitors

J. CHARLES JENNETTE, MD,
RICHARD R. TIDWELL, PhD,
J. DIETER GERATZ, MD,
DAVID H. BING, PhD, and
RONALD J. FALK, MD

From the Departments of Pathology and Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, and B R Laboratories, Inc., Boston, Massachusetts

Proteases are involved in the pathogenesis of inflammatory diseases by participating in the activation of mediator systems and by producing proteolytic tissue injury. Homeostatic control of inflammation is accomplished in part by physiologic protease inhibitors. The authors investigated the effectiveness of a number of synthetic protease inhibitors in ameliorating the glomerular injury induced by immune complex-mediated glomerulonephritis in mice. Two amidine-type protease inhibitors, bis (5-amidino-2-benzimidazolyl)methane and 1,2-bis (5-amidino-2-benzimidazolyl)ethane, had the greatest effects. They caused a marked reduction in glomerular necrosis ($P < 0.001$) but did not affect the amount or site of immune complex localization or leukocyte influx. The inhibition constants of the protease inhibitors against nine purified physiologic proteases were determined. These results were discussed in relation to the effectiveness of the protease inhibitors in reducing glomerular injury. This investigation indicates that the administration of synthetic protease inhibitors can have a beneficial effect on immune-mediated inflammatory injury. (Am J Pathol 1987, 127:499–506)

PROTEOLYTIC enzymes influence or control a large variety of humoral and cellular reactions of the body. They are prominently involved in the activation of inflammatory mediator systems by participating in such multistep pathways as complement activation, Hageman factor-dependent kinin generation, coagulation, and fibrinolysis.[1,2] Upon release from leukocytes, proteases can also be direct effectors of tissue injury.

Recognition of this importance of proteolysis in many pathophysiologic processes has prompted interest in exogenous and endogenous protease inhibitors as anti-inflammatory agents. Physiologic (endogenous) protease inhibitors, such as α_1-proteinase inhibitor (α_1-antitrypsin) and α_2-macroglobulin, play a homeostatic role in counteracting proteolytic processes, including inflammation. At sites of inflammation, mediator and effector proteolytic events are dominant over antiproteolytic control mechanisms. It might be possible to regain anti-inflammatory control by the administration of supplementary exogenous antiproteases, such as synthetic protease inhibitors. To test this hypothesis, we chose to study the effects of four synthetic protease inhibitors on murine immune complex-induced glomerulonephritis.[3] We selected three synthetic amidino compounds with a wide inhibitory spectrum against trypsinlike proteases and a rather specific inhibitor of complement.

Materials and Methods

Experimental Animals and Protease Inhibitor Doses

One hundred twenty-one 16–18-week-old male BALB/c mice (Charles River Breeding Laboratories) were divided among 9 different experimental groups (Table 1). Ninety mice (Groups I–VI) received daily intraperitoneal injections of 4 mg of horse apoferritin (HAF) (Sigma Chemical Co.) for 12 days. On Days 7–12, Group I (30 mice) received intraperitoneal in-

Supported by PHS Grant HL 19171-09 and NIH Grant DK 34855-02.

Accepted for publication January 27, 1987.

Address reprint requests to J. Charles Jennette, MD, 713 Brinkhous-Bullitt Bldg. Department of Pathology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.

Table 1—Experimental Groups

Group	Number of mice	HAF (mg/day)	Days to sacrifice	Protease inhibitor*
I	30	4	13	none
II	20	4	13	BABIM (20 mg/kg/day)
III	10	4	13	BABIM (5 mg/kg/day)
IV	10	4	13	BABIE
V	10	4	13	K-76 COONa
VI	10	4	13	ABI
VII	12	0	13	BABIM
VIII	9	4	33	BABIM
IX	10	4	33	None

*BABIM, bis (5-amidino-2-benzimidazolyl)methane; BABIE, 1,2-bis(5-amidino-2-benzimidazolyl)ethane; K-76 COONa, 6,7-dihydroxy-2,5,5,8a-tetramethyl,1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene-1-spiro-2′-(7′carboxyl-6′-formyl-4′-hydroxy-2′,3′-dihydrobenzofuran); ABI, 1-(4-amidinobenzyl)indole.

jections of 0.1 ml phosphate-buffered saline (PBS), and Groups II–VI received daily intraperitoneal injections of synthetic protease inhibitors in 0.1 ml PBS. The protease inhibitors used were bis(5-amidino-2-benzimidazolyl)methane (BABIM); 1,2-bis(5-amidino-2-benzimidazolyl)ethane (BABIE); 1-(4-amidinobenzyl)indole (ABI); and 6,7-dihydroxy-2,5,5,8a-tetramethyl,1,1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene-1-spiro-2′-(7′carboxyl-6′-formyl-4′-hydroxy-2′,3′-dihydrobenzofuran) (K-76 COONa). The three amidines were synthesized according to previously described methods.[4,5] K-76 COONa was obtained from W. Miyazaki and Otsuka Pharmaceutical Co. (Rockville, Md).[6,7]

The protease inhibitor daily dose was 20 mg/kg in all groups except Group III, which received 5 mg/kg. Group VII contained 12 mice, which received 13 daily injection of PBS but no HAF. On Days 7–12, half of this group received 5 mg/kg/day BABIM and half 20 mg/kg/day BABIM.

On Day 13, all mice in Groups I–VII were anesthetized with ether, blood was obtained from the retroorbital venous plexus, and the animals were killed by cervical dislocation. Kidney tissue was processed for light, immunofluorescence, and transmission electron microscopy by previously reported methods.[3] For direct immunofluorescence microscopy, we immunostained 4-μ frozen sections of kidney with fluoresceinated goat antibodies to mouse IgG, IgM, and C3 and rhodaminated goat anti-HAF (Cappel Laboratories, Inc., Cochranville, Pa).

To test the effect of BABIM on survival, 19 mice (Groups VIII and IX) received 4 mg/kg/day intraperitoneal HAF for 32 days, or until death. On Days 7 through 32, Group VIII (9 mice) received 20 mg/kg BABIM in 0.1 ml PBS, and Group IX (10 mice) received PBS alone. On Day 33, mice that had not expired earlier were sacrificed and sampled as described above.

Pathologic Analysis of Renal Lesions

Kidney tissue from all but 2 of the 121 mice was evaluated by light microscopy. Two Group IX animals that died prior to sacrifice were unsuitable for microscopic study. The overall severity of light-microscopic abnormalities was subjectively quantified on a scale of 0 to 4+. No immunofluorescence was designated by 0, 0.5+ indicated trace focal segmental staining, and 1+ to 4+ indicated progressively more intense and widespread staining from slight (1+) to maximum (4+). The location of immunostaining, that is, mesangial and/or capillary wall, was also recorded.

Immunofluorescence microscopy was carried out on all animals except those in the survival study (Groups VIII and IX). Mesangial and capillary wall immunostaining of nonnecrotic glomeruli were each quantified on a scale of 0 to 4+ (0, none; 4+, maximal). In animals that received HAF, nonnecrotic glomeruli had well-defined granular mesangial and capillary wall immune deposits that outlined the normal structure. In necrotic segments, the normal structure was disrupted, which resulted in the localization of immunoglobulins, complement, and HAF in a smudged distribution. This corresponded to foci of necrosis that by light microscopy had replacement of the normal architecture by amorphous acidophilic material, karyolysis, and karyorrhexis. For each animal, except those in Groups VIII and IX, the percentage of globally necrotic glomeruli was determined by immunofluorescence microscopy. Because immunofluorescence microscopy was not performed on renal tissue from Groups VIII and IX, the percentage of globally necrotic glomeruli in those mice was determined by light microscopy. The P values for glomerular necrosis in the various experimental groups were calculated using the Statistical Analysis software package from Human Systems Dynamics on an Apple IIe computer.

Transmission electron microscopy was carried out on a minimum of 2 animals per group. Particular attention was paid to the extent and location of glomerular electron-dense deposits and the characteristics of glomerular inflammation.

Assay of Plasma Creatinine and Anti-HAF Antibodies

By means of a standard autoanalyzer method,[8] creatinine was quantitated in plasma from survivors in

Groups VIII and IX and from 10 animals in Group I, 10 animals in Group II, and all animals of Group IV.

Plasma anti-HAF antibody activity was measured in all animals by means of an enzyme immunoassay.[9] The wells of 96-well microtiter plates (Costar, Cambridge, Mass) were filled with 0.1 mg/ml HAF in PBS. The plates were left for 1 hour at room temperature, and then overnight at 4 C. After three washes with 0.5% Tween-20 (Sigma) in PBS, the wells were filled with 5% bovine serum albumin (Sigma) for 1 hour and then washed with PBS. Mouse plasma samples diluted in PBS were incubated in wells for 1 hour at room temperature. After washing, wells were incubated for 20 minutes with peroxidase-labeled goat antibodies specific for mouse IgG, IgM, and light chains (Tago, Inc. Burlingame, Calif), then washed. A solution of 0.04% ortho-phenylendiamine dihydrochloride (Eastman Kodak, Rochester, NY) in pH 5.0 citrate phosphate buffer with 0.012% hydrogen peroxide was added. After 20 minutes, the optical density at 450 nm was determined by means of a Titertek Multiscan (Flow Laboratories, Helsinki, Finland). All samples were run in duplicate. Test samples were compared with standard pools of plasma from control mice and hyperimmunized BALB/c mice. Results are expressed as a percentage of the value obtained for the concurrently analyzed standard hyperimmune pool.

Determination of Inhibition Constants (K_i Values) of Protease

The potency of the inhibitors against various purified proteases was determined from amidase or esterase assays employing sensitive synthetic substrates. The inhibition constants (K_i values) were obtained graphically according to Dixon[10] by plotting the reciprocals of the initial reaction velocities at two different substrate concentrations against different inhibitor concentrations.

Incubations were carried out at 37 C with the majority of the enzymes, the only exceptions being the C1r and C1s assays, which were run at 23 and 25 C, respectively. The pH of the tests was 8.1 with trypsin, thrombin, urokinase, plasma kallikrein, and pancreatic kallikrein. It was 8.3 with factor Xa, 7.6 with plasmin, 7.5 with $C1\bar{r}$, and 8.0 with $C1\bar{s}$. Details of the assays for trypsin, factor Xa, thrombin, urokinase, plasmin, and pancreatic kallikrein were reported previously.[11,12] The substrates used in those assays were the following: for trypsin, thrombin, plasmin and pancreatic kallikrein N-benzoyl-DL-arginine-*p*-nitroanilide HCl (Sigma); for factor Xa N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide HCl (S-2222, Kabi Vitrum, Stockholm, Sweden), and for urokinase L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide HCl (S-2444, Kabi Vitrum).

Plasma kallikrein assays contained 1.3×10^{-3} plasma equivalence units of the enzyme per milliliter of 0.05 M Tris-HCl buffer. Hydrolysis of the substrate H-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide HCl (S-2302, Kabi Vitrum)[13] was followed colorimetrically at 410 nm, as with all other nitroanilides.

For the evaluation of $C1\bar{s}$, the protease (1.1 nM) was incubated in 0.038 M Tris-HCl buffer containing 150 μM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent),[14] 1 mM NaCl, 10 μM EDTA, 1% (vol/vol) DMF, 100 μM KH_2PO_4, 400 μM Na_2HPO_4, and the substrate thiobenzyl benzyloxycarbonyl-L-lysinate (Peninsula Laboratories).[15] The reaction was monitored by the color development at 412 nm which results from the chromogenic attack on DTNB by the mercaptan released.

The assay for $C1\bar{r}$ was based on the same principles as the one for $C1\bar{s}$, ie, on hydrolysis of a thioester. The reaction mixture contained 25.2 nM $C1\bar{s}$, 0.05 M Tris-HCl, 650 μM DTNB, 750 μM NaCl, 5 μM EDTA, 0.5% (vol/vol) DMF, 50 μM KH_2PO_2, 200 μM Na_2HPO_4, and the substrate benzyloxycarbonyl-glycyl-L-arginine thioisobutyl ester HCl (Enzyme Products).[16]

Trypsin (bovine, crystallized) was obtained from Worthington Biochemical Corporation. Human Factor Xa was a product of Diagnostic Reagents, Thame, Oxon, England. Thrombin (bovine, topical) was purchased from Parke, Davis and Company. High-molecular-weight human urokinase was contributed as Winkinase by Winthrop Laboratories. Human plasmin and human plasma kallikrein were bought from Kabi Vitrum. Porcine pancreatic kallikrein was a gift of Farbenfabriken Bayer AG, Wuppertal-Elberfeld, West Germany. Highly purified preparations of C1r and C1s were obtained by a previously published procedure.[17]

Complement Hemolytic Assay

The total hemolytic complement activity of serum from mice given K-76 COONa (Group V) and ABI (Group VI) was determined by means of a modified standard CH_{50} assay. Sheep red blood cells were sensitized (EA) with a 1 : 75 dilution of anti-sheep hemolysin (Difco Laboratory, Detroit, Mich), and washed in cold gelatin veronal buffered saline. Mouse blood was harvested from the retroorbital plexus and clotted at room temperature. After separation of serum from clot, the serum was immediately placed on ice and

evaluated by CH_{50} during the same day. Serum was added to EA 30 minutes after hemolysin had been added. Hemolysis was measured spectrophotometrically.

Results

All animals that received HAF developed diffuse proliferative glomerulonephritis. This glomerulonephritis had the pathologic features previously described by us in BALB/c mice given HAF.[3] Of the 119 mice examined by light microscopy, all had severe glomerular injury except for the 12 mice in Group VII, which had no lesions. In mice that received HAF, glomeruli were either markedly hypercellular (Figure 1) or had segmental to global necrosis (Figure 2). In nonnecrotic glomeruli, the degree of glomerular hypercellularity was not significantly different between the experimental groups. The degree of glomerular necrosis, however, did vary among the experimental groups. Glomerular necrosis was very apparent by immunofluorescence microscopy, producing a smudged appearance in the glomeruli (Figure 3), compared with the discrete granular immune deposits seen in nonnecrotic glomeruli (Figure 4). There

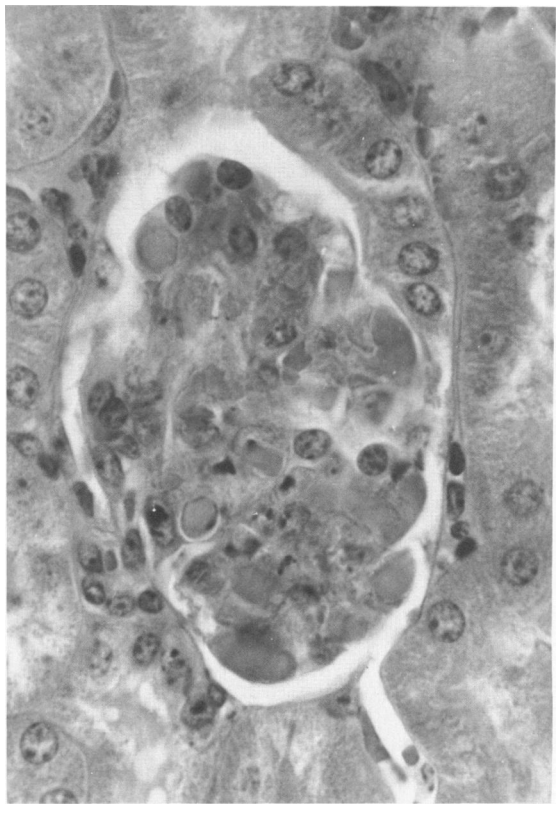

Figure 2—Extensive necrosis with loss of nuclei in a glomerulus from a Group I mouse that received HAF for 12 days and no protease inhibitor. (H&E, ×900)

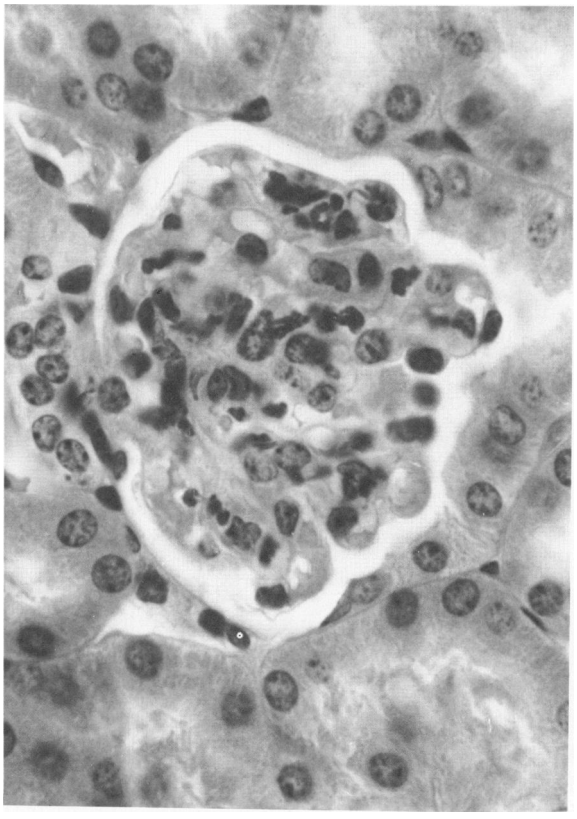

Figure 1—Marked hypercellularity in a glomerulus from a Group II mouse that received HAF for 12 days and 20 mg/kg BABIM for 6 days. (H&E, ×900)

Figure 3—Smudged staining shown by immunofluorscence microscopy in two glomeruli from a Group I mouse that received HAF for 12 days and no protease inhibitor. (Anti-mouse IgG, ×600)

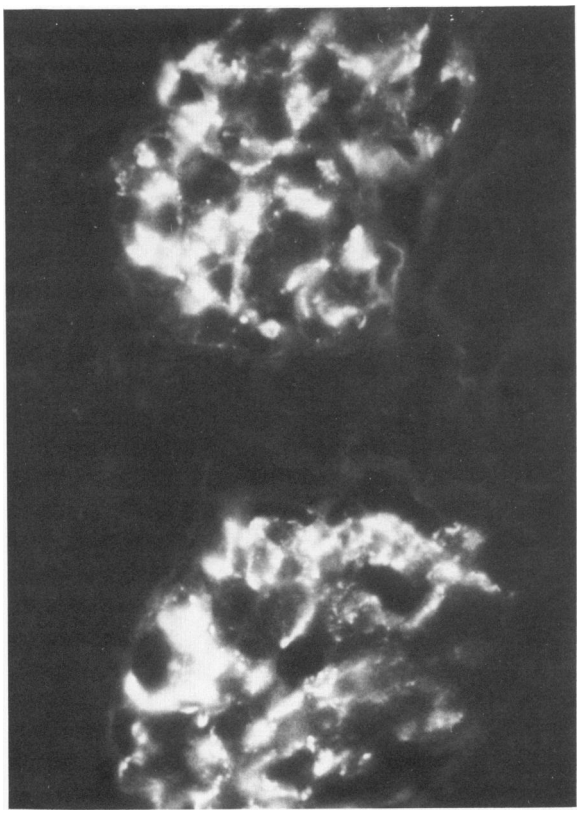

Figure 4—Granular, predominantly mesangial staining by immunofluorescence microscopy in two glomeruli from a Group II mouse that received HAF for 12 days and 20 mg/kg BABIM for 6 days. (Anti-mouse IgG, ×600)

were no discernable ultrastructural differences in nonnecrotic glomeruli between control mice given HAF and no protease inhibitor and mice given HAF and an inhibitor. All nonnecrotic glomeruli had large global mesangial electron-dense deposits and scattered small subepithelial and subendothelial dense deposits. All glomeruli had hypercellularity with mononuclear and polymorphonuclear leukocytes. Necrotic glomeruli had large masses of amorphous moderately electron-dense material, cellular debris, and reduced intact cells.

Short-Term (13-Day) Experiment

As shown in Table 2, of the mice that received 12 days of HAF (Groups I–VI), those that received no protease inhibitor had the greatest percentage of globally necrotic glomeruli (47.5%). There was a striking and statistically highly significant reduction in necrosis in mice that had been given 20 mg/kg/day BABIM, 5 mg/kg/day BABIM, or 5 mg/kg/day BABIE. The other two inhibitors studied (K-76 COONa and ABI) were much less effective than either BABIM or BABIE in reducing necrosis, and the differences with respect to the inhibitor-free controls were not statistically significant.

Table 2—Protease Inhibitor Effects on Glomerular Necrosis, Glomerular Immune Complex Localization, and Plasma Anti-HAF Antibody Levels in Mice That Received HAF for 12 Days

Protease inhibitor	% Necrotic glomeruli	Glomerular immunostaining*			Plasma anti-HAF
		IgG	HAF	C3	
None	47.5	3.2	3.5	3.0	51.0
BABIM (20 mg/kg/day)	14.8 (p = 0.001)	3.1	3.5	3.1	48.9
BABIM (5 mg/kg/day)	5.1 (p = 0.001)	3.4	3.2	3.7	43.6
BABIE	8.0 (p = 0.002)	3.6	3.6	3.8	58.0
K-76 COONa	23.7 (p = 0.06)	2.6	3.4	2.8	48.9
ABI	28.7 (p = 0.17)	3.0	3.9	3.0	56.6

*Mean intensity of mesangial immunofluorescence in nonnecrotic glomeruli.

By immunofluorescence microscopy, there was no difference among Groups I–VI mice with respect to the distribution or intensity of immunostaining for IgG, IgM, C3, or HAF in nonnecrotic glomeruli. Glomerular immunostaining was most intense in mesangial regions, but almost all glomeruli had a minor degree of capillary wall staining for IgG, C3, and HAF (Figure 4). Plasma levels of anti-HAF antibodies did not differ among the groups of mice that received HAF.

Plasma creatinine was measured in 10 mice from Groups I (no protease inhibitor), II (BABIM), and IV (BABIE). The mean creatinine was 30.1 μmol/l in Group I, 23.9 μmol/l in Group II, and 27.4 μmol/l in Group IV. Only the difference between Groups I and II was statistically significant ($P = 0.005$).

Group VII mice that received BABIM but no HAF had no glomerular lesions by light or electron microscopy, only low intensity glomerular immunoglobulin immunostaining, no glomerular HAF immunostaining, and a plasma anti-HAF antibody assay activity of only 13.4% (± 5.5 SD). Age-matched normal BALB/c mice had a mean anti-HAF activity of 9.5% (± 1.9 SD).

Long-Term (33-Day) Survival Experiment

In mice that received HAF for 32 days or until death caused by renal failure, 7 of 10 that did not receive BABIM died within 33 days, whereas only 3 of 9 died that had received 20 mg/kg/day BABIM. Of the 19 mice in groups VIII and IX, 17 were examined by light microscopy. All of these mice had proliferative glomerulonephritis. There was no difference in the

degree of hypercellularity in non-necrotic glomeruli between mice that received BABIM and those that did not.

There was, however, a marked difference in the extent of necrosis. Mice that received BABIM (Group VIII) had on average 8.4% globally necrotic glomeruli, compared with 33.1% in mice not receiving BABIM (Group IX). In either group, the amount of necrosis was different between those mice that died prior to 33 days, compared with those sacrificed at 33 days. In Group VIII (BABIM-treated), mice dying prior to 33 days had 16.7% necrotic glomeruli and those sacrificed at 33 days had 4.3%. In Group IX, those dying early had 44.0% necrotic glomeruli, compared with 15.0% in sacrificed mice.

Plasma creatinine level and anti-HAF activity were measured in the 6 Group VIII and 3 Group IX mice that survived for 33 days. There was no difference in anti-HAF antibody activity between the two groups. Plasma creatinine was not significantly different; however, the groups of mice evaluated were small. Group VIII mice had a mean serum creatinine level of 32.7 μmol/l, and Group IX had 36.2 μmol/l.

Complement Hemolytic Activity

The dose of K-76 COONa administered to each mouse was based on two factors. First the concentration of K-76 COONa was determined that was required to inhibit *in vitro* 95% of the lysis of sensitized sheep red blood cell (SRBCs) by mouse serum. This concentration was then used to calculate the *in vivo* dose. Second, the administered dose was equivalent to that reported as effective in other systems.[7] Total hemolytic activity of serum from experimental animals that received K-76 COONa (Group V), however, was no different from that of control mouse serum.

The total hemolytic activity of serum taken from mice that received ABI (Group VI) also was no different from that of control serum. The serum concentration of ABI used in Group VI inhibited *in vitro* 38% of SRBC lysis, compared with control serum. When the serum concentration of ABI in mice was increased to be equivalent to 95% inhibition of SRBC lysis *in vitro*, all mice died within 24 hours.

Inhibition Constants of Protease Inhibitors

Table 3 gives the inhibition constants of BABIM, BABIE, and ABI with nine trypsinlike proteases. Not included in the table is the only non-amidino compound studied, K-76 COONa. At concentrations of up to 1×14^{-4} M it did not produce any inhibition of the proteases tested.

Table 3—Inhibition Constants (K_i Values) of BABIM, BABIE, and ABI for Nine Trypsinlike Proteases

Protease	Ki (μM*) BABIM	BABIE	ABI
Trypsin	0.017 ± 0.006†	4.68 ± 0.33†	4.59 ± 0.43‖
Factor Xa	10 (irreg.)‡	2.68 ± 0.11‡	25.6 ± 1.83
Thrombin	4.15 ± 0.65†	11.6 ± 1.1†	13.1 ± 1.5§
Urokinase	2.33 ± 0.36§	21.5 ± 7.9	25.6 ± 3§
Plasmin	2.65 ± 0.47§	39.2 ± 3.67	111.0 ± 14§
Plasma Kallikrein	0.35 (irreg.)	2.53 ± 1.12	2.28 ± 0.83
Pancreatic Kallikrein	17.3 ± 1.4†	36.5 ± 6.6†	18.0 ± 4.47
C1r̄	524		16.3
C1s̄	>100.0 (irregular)	166.0 ± 90.5	31.4 ± 5.44

*Values are means ± SD (n = 3, except in the case of C1r̄, where values are the mean of only two assays).
†From reference 4.
‡From reference 18.
§From reference 12.
‖From reference 5.

Discussion

These studies indicate that the systemic administration of certain protease inhibitors can alter the morphologic characteristics of immune complex-mediated glomerulonephritis in mice. Some of the synthetic protease inhibitors tested effected a striking reduction in the extent of glomerular necrosis while not changing at all the glomerular influx of leukocytes, site or amount of glomerular immune complex localization, or magnitude of anti-HAF antibody production.

The protease inhibitors having the greatest effects on glomerular injury, BABIM and BABIE, produced a 69–89% reduction in the number of glomeruli with global necrosis. Because glomerular complement localization and leukocyte influx were not reduced, it is most likely that the protease inhibitors produced their effects by inactivating or preventing the release of leukocyte lytic proteases.

The glomerular filtration rate, as evidenced by plasma creatinine levels, was only modestly improved in mice given BABIM. This is not surprising in view of the inability of the protease inhibitors used in these experiments to prevent components of glomerular inflammation other than necrosis. This also is most likely the basis for the more impressive reduction in necrosis than in mortality. The reduction of glomerular necrosis produced by protease inhibitors, however, did correlate with improved survival. In mice given HAF for 32 days or until death caused by renal failure, 70% not given BABIM died, compared with 33% given BABIM. In each treatment group, mice

that died had more necrosis than mice that survived. In mice that did not receive BABIM, those that died had an average of 44% globally necrotic glomeruli, compared with 15% in those that survived. In mice given BABIM, those that died had 17% necrosis and those that survived had 4% necrosis.

The most plausible explanation for the beneficial influence of the amidine-type inhibitors is that they interfered with the damaging action of proteases in the glomeruli. The specificity requirements of the inhibitors allow us to narrow the search for the protease(s) involved to those with activity against arginyl or lysyl bonds, ie, with trypsinlike characteristics. Even with those restrictions, however, there still remains a long list of proteases that have to be considered. It would include intracellular as well as humoral enzymes. To begin, factor XIIa and plasma kallikrein may both be implicated. They are normally present in plasma in the zymogen form, but they can be activated at the site of endothelial cell injury in the glomeruli and may, in turn, stimulate degranulation of neutrophils.[19,20] Among others, elastase, collagenase, and plasminogen activator would be secreted with the granules[20–24] and would be in a position to promote tissue necrosis. Additional amounts of the three proteases could be recruited from stimulated macrophages accumulating in the glomeruli.[25–27] Freshly released collagenase is probably initially present as the zymogen but can be fully activated by plasma kallikrein or plasmin.[28,29] Factor XIIa, kallikrein, plasminogen activator, and plasmin would all be susceptible to inhibition by amidines. Once active, however, neither collagenase nor elastase would be affected. It should be noted that all proteases in the clotting cascade except factor XIIa are trypsinlike and can be expected to be blocked by amidino compounds. The same holds true for the early components of the classical pathway of complement activation, ie, C1r and C1s.

In an attempt to correlate the necrosis-preventing activity of the three amidines selected with their ability to inhibit one or more well-defined proteases, the inhibition constants (K_i values) were obtained for nine likely target enzymes (Table 3). In addition to trypsin, two clotting factors (Factor Xa and thrombin), two fibrinolytic agents (plasmin and urokinase), two members of the kinin generating system (pancreatic and plasma kallikrein), and two components of the complement system ($C1\bar{r}$ and $C1\bar{s}$) were examined. As BABIM and BABIE were much more effective than ABI in the prevention of glomerular necrosis, any protease considered a candidate for the mediator of necrosis would have to show significantly lower K_i values with BABIM and BABIE than with ABI. From the data in Table 3 it is evident that no protease fulfilled these conditions, except possibly Factor Xa. One might speculate that prevention of thrombosis in glomerular capillaries by the blockage of Factor Xa could have reduced the glomerular injury.

ABI is an inhibitor of $C1\bar{r}$ and $C1\bar{s}$ (Table 3), and K-76 COONa is an inhibitor of C5.[30] At the doses used in this investigation, however, no reduction in blood hemolytic complement activity was produced. Therefore, the ABI and K-76 COONa data neither support nor rule out a role for complement inhibition in reducing necrosis.

Complement inhibition at any step would not explain the antinecrosis activity of BABIM. Besides being only a very weak inhibitor of purified C1r and C1s (Table 3), BABIM had no effect on guinea pig complement-induced hemolysis, even at a concentration of 1×10^{-4} M (data not shown).

The amelioration of experimental immune complex-mediated glomerulonephritis by amidines is further evidence for the role of proteases in the induction of immune-mediated inflammation. This anti-inflammatory role for protease inhibitors also is supported by the reports of patients with a congenital deficiency of α_1-antitrypsin who develop glomerulonephritis and necrotizing arteritis.[31,32] The usefulness of synthetic protease inhibitors in the treatment of another type of experimental glomerulonephritis has been reported by Ikehara et al.[33] They showed that the immune complex-mediated "lupus" glomerulonephritis that develops spontaneously in NZB/NZW F_1 mice can be reduced in severity by the administration of FUT-175 (6-amidino-2-naphthyl *p*-guanidinobenzoate dimethanesulphonate). As in our study, they observed no major change in immune deposits. There was, however, a reduction in glomerular injury and an improvement in renal function.

Protease inhibitors are of potential value in studying pathogenic mechanisms of inflammation and as antiinflammatory pharmacologic agents.

References

1. Barrett AJ: The many forms and functions of cellular proteinases. Fed Proc 1980, 39:9–14
2. Wintroub BU: Inflammation and mediators. Int J Dermatol 1980, 19:436–442
3. Iskandar SS, Jennette JC, Wilkman AS, Becker RL: Interstrain variations in nephritogenicity of heterologous protein in mice. Lab Invest 1982, 46:344–351
4. Tidwell RR, Gerazt JD, Dann O, Volz G, Zeh D, Loewe H: Diarylamidine derivatives with one or both of the aryl moieties consisting of an indole or indole-like ring: Inhibitors of arginine-specific esteroproteases. J Med Chem 1978, 21:613–623

5. Tidwell RR, Geratz JD, Dubovi EJ: Aromatic amidines: Comparison of their ability to block respiratory syncytial virus induced cell function and to inhibit plasmin, urokinase, thrombin, and trypsin. J Med Chem 1983, 26:294–298
6. Miyazaki W, Tamaoka H, Shinohara M, Kaise H, Izawa T, Nakano Y, Kinoshita T, Hong K, Inoue Kozo: A complement inhibitor produced by Stachybotrys complementi, nov. sp. K-76, a new species of fungi imperfecti. Microbiol Immunol 1980, 24:1091–1108
7. Konno S, Tsurufuji: Induction of zymosan-air-pouch inflammation in rats and its characterization with reference to the effects of anticomplementary and anti-inflammatory agents. Br J Pharmac 1983, 80:269–277
8. Schirmeister JH, Willmann H, Kiefer H: Endogenes kreatinine in serum und harn. Klin Wochenschr 1963, 44:878–882
9. Falk RJ, Jennette JC: Immune complex induced glomerular lesions in C5 sufficient and deficient mice. Kidney Int 1986, 30:678–686
10. Dixon M: The determination of enzyme inhibitor constants. Biochem J 1953, 55:170–171
11. Geratz JD, Stevens FM, Polakoski KL, Parrish RF, Tidwell RR: Amidino-substituted aromatic heterocycles as probes of the specificity pocket of trypsin-like proteases. Arch Biochem Biophys 1979, 197:551–559
12. Geratz JD, Shaver SR, Tidwell RR: Inhibitory effect of amidino-substituted heterocyclic compounds on the amidase activity of plasmin and of high and low molecular weight urokinase and on urokinase-induced plasminogen activation. Thromb Res 1981, 24:73–83
13. Glaeson G, Aurell L, Friberger P, Gustavsson S, Karlson G: Designing of peptide substrates: Different approaches exemplified by new chromogenic substrates for kallikreins and urokinase. Haemostasis 1978, 7:62–68
14. Ellman GL: Tissue sulfhydryl groups. Arch Biochem Biophys 1959, 82:70–77
15. Green GDJ, Shaw E: Thiobenzyl benzyloxycarbonyl-L-lysinate, substrate for a sensitive colorimetric assay for trypsin-like enzymes. Anal Biochem 1979, 93:223–226
16. McRae BJ, Lin TY, Powers JC: Mapping the substrate binding site of human C1r and C1s with peptide thioesters: Development of new sensitive substrates. J Biol Chem 1981, 256:12362–12366
17. Bing DH, Andrews JM, Morris KM, Cole E, Irish V: Purification of subcomponents C1q and C1r and C1s of the first component of complement from the Cohn fraction I by affinity chromatography. Prep Biochem 1980, 10:269–275
18. Tidwell RR, Webster WP, Shaver SR, Geratz JD: Strategies for anticoagulation with synthetic protease inhibitors: Xa inhibitors versus thrombin inhibitors. Thromb Res 1980, 19:339–349
19. Wachtfogel YT, Pixley RA, Kucich U, Abrams W, Weinbaum G, Schapira M, Colman RW: Purified plasma factor XIIa aggretates human neutrophils and causes degranulation. Blood 1986, 67:1731–1737
20. Wachtfogel YT, Kucich U, James HL, Scott CF, Schapira M, Zimmerman M, Cohen AB, Colman RW: Human plasma kallikrein releases neutrophil elastase during blood coagulation. J Clin Invest 1983, 72:1672–1677
21. Janoff A: Human granulocyte elastase. Further delineation of its role in connective tissue damage. Am J Pathol 1972, 68:579–591
22. Henson PM: Pathologic mechanisms in neutrophil-mediated injury. Am J Pathol 1972, 68:593–612
23. Kopitar M, Lebez D: Intracellular distribution of neutral proteinases and inhibitors in pig leucocytes: Isolation of two inhibitors of neutral proteinases. Europ J Biochem 1975, 56:571–581
24. Kopitar M, Stegnar M, Accetto B, Lebez D: Isolation and characterization of plasminogen activator from pig leucocytes. Thromb Diath Haemorrh 1974, 31:72–85
25. Banda MJ, Werb Z: Mouse macrophage elastase: Purification and characterization as a metalloproteinase. Biochem J 1981, 193:589–605
26. Mainardi CL, Seyer JM, Kang AH: Type-specific collagenolysis: A type V collagen-degrading enzyme from macrophages. Biochem Biophys Res Commun 1980, 97:1108–1115
27. Unkeless JC, Gordon S, Reich E: Secretion of plasminogen activator by stimulated macrophages. J Exp Med 1974, 139:834–850
28. Eeckhout Y, Vaes T: Further studies on the activation of procollagenase, the latent precursor of bone collagenase: Effects of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. Biochem J 1977, 166:21–31
29. Nagase H, Cawston TE, DeSilva M, Barrett AJ: Identification of plasma kallikrein as an activator of latent collagenase in rheumatoid synovial fluid. Biochim Biophys Acta 1982, 702:133–142
30. Hong K, Kinoshita T, Miyazaki W, Izawa T, Inoue K: An anticomplementary agent, K-76 monocarboxylic acid: Its site and mechanism of inhibition of the complement activation cascade. J Immunol 1979, 122:2418–2423
31. Moroz SP, Cutz E, Williamson Balfe J, Sass-Kortsak A: Membranoproliferative glomerulonephritis in childhood cirrhosis associated with alpha$_1$-antitrypsin deficiency. Pediatrics 1976, 57:232–238
32. Lewis M, Kallenbach J, Zaltzman M, Levy H, Lurie D, Baynes R, King P, Meyers A: Severe deficiency of alpha$_1$-antitrypsin associated with cutaneous vasculitis, rapidly progressive glomerulonephritis and colitis. Am J Med 1985, 79:489–494
33. Ikehara S, Shimamura K, Aoyama T, Fujii S, Hamashima Y: Effect of FUT-175, a new synthetic protease inhibitor, on the development of lupus nephritis in (NZB × NZW) F$_1$ mice. Immunol 1985, 55:494–600