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Ancrod improves survival in murine systemic lupus erythematosus

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Ancrod improves survival in murine systemic lupus erythematosus. The effect of ancrod, a defibrinating agent, on murine lupus glomerulonephritis in the male BXSB mouse was studied to determine the relationship between macrophage procoagulant activity (PCA), fibrin deposition and glomerulonephritis. Marked renal disease and fibrin deposition were noted by three months of age in control mice, whereas little or no disease was seen in ancrod treated mice until five months of age. Similar high titers of anti-DNA antibodies and renal deposition of IgG were seen in both groups of mice. PCA rose with age in both ancrod treated and untreated mice, although it was significantly higher in control animals than in the ancrod treated group. Furthermore, ancrod therapy resulted in a decrease in plasma PCA inducing activity (PIF) and a decrease in the effectiveness of PIF to induce PCA in peritoneal macrophages in vitro. No mortality was observed in the 20 ancrod treated mice, whereas 10 of 20 control animals died. We conclude that defibrination with ancrod delays the development of renal fibrin deposition and glomerulonephritis and improves survival in BXSB mice. This was associated with a decrease in plasma PCA inducing activity and with an inhibitory effect on PCA induction. These results suggest that PCA contributes to injury in murine lupus glomerulonephritis by promoting fibrin deposition.

A number of investigators have shown that injury in glomerulonephritis (GN) is dependent upon activation of the coagulation system. Defibrination reduced the incidence of glomerular microthrombi [1], decreased fibrinogen deposition in the glomeruli with a reduction in crescent formation [2], and protected against experimental nephrotoxic serum nephritis [3]. Pollak et al found that treatment with ancrod improved renal function and reduced thrombi in patients with lupus GN [4]. Our previous studies have shown that induction of monocyte/macrophage (MO/MA) procoagulant activity (PCA) occurs in patients with active lupus nephritis, and also in the male BXSB mouse as GN develops with age [5, 6]. Studies by Holdsworth and Wiggins et al have also implicated PCA as being important in the induction of injury in other experimental models of GN [7–9].

The synthesis and secretion of PCA by MO/MA is now known to be an integral component of the host's immune response [10, 11]. PCA can be induced by a number of immunostimulatory molecules in collaboration with T lymphocytes, and this process is tightly controlled by both helper and suppressor cells as well as soluble mediators [12, 13]. The exact means by which PCA leads to the development of injury in GN is unknown. Theoretically, the molecule could be relevant by directly promoting coagulation with formation of fibrin, or, PCA might be chemotactic resulting in the recruitment of inflammatory cells [14].

Ancrod is a proteolytic enzyme derived from the venom of the Malayan Pit viper, Agkistrodo Rhodostoma, which causes hypofibrinogenemia, hypoplasminogenemia, and an elevation in fibrin degradation products [15, 16]. This molecule cleaves fibrinopeptide A from fibrinogen without cleavage of fibrinopeptide B, thereby preventing normal polymerization of fibrin. In addition, ancrod activates the fibrinolytic system [16, 17]. These present studies were performed to assess the effect of ancrod on the BXSB male mouse, which predictably develops an age-related lupus-like GN [18], and specifically to determine the relationship between PCA induction and fibrin deposition in this model of GN.

Methods

Mice

BXSB male mice, four weeks of age, were obtained from Jackson Laboratories, Bar Harbour, Maine, USA, and were housed in the Department of Laboratory and Animal Services at the University of Toronto. Blood urea nitrogen (BUN) was determined using Azostix (Miles Laboratories, Rexdale, Ontario, Canada).

Treatment protocols

Ancrod (Arvin) was obtained from Connaught Laboratories Limited, Willowdale, Ontario, Canada. Ancrod was provided in isotonic saline and 0.0025 M phosphate and 0.005% wt/vol chlorbutol. Ancrod was diluted in RPMI-1640 (Flow Laboratories, Mississauga, Ontario, Canada), and each mouse received 100 μl of medium containing five units of ancrod by subcutaneous injection every Monday, Wednesday and Friday starting at five weeks of age. This dosage had previously been determined to reduce circulating fibrinogen to less than 30% of normal values. As shown below, fibrinogen levels were subsequently followed in all animals. At each time point, at least seven mice from both ancrod treated and control groups were sacrificed

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and sera, kidney tissue and splenic mononuclear cells assessed. Of necessity, mice sacrificed were those surviving up to that point in time. Independently, survival was determined in two cohorts (N = 20 in each group) of mice.

Fibrinogen levels

Two hundred μl of blood was collected from the retroorbital plexus into heparinized tubes containing 10 μl of 0.5 M sodium citrate, pH 5, just prior to ancrod administration. Blood was centrifuged for five minutes at 8,800 × g; plasma was then removed, 50 μl were added to 50 μl of a 1/30 dilution of 30 mg/ml protamine sulphate (Eli Lilly Canada Inc., Toronto, Canada), and then combined with 450 μl of 25 mM $CaCl_2$ pH 7.2. The reaction was started by adding 50 μl of thrombin, (1000 μ/ml; Parke Davis Ltd., Toronto, Canada), and fibrinogen was measured by a change in optical density (Lancer Fibrinogen Analyzer, Sherwood Medical Industries, Cooksville, Ontario, Canada), as compared to a fibrinogen standard. Fibrinogen levels were measured in each mouse twice weekly for the first week and once weekly thereafter and were reported in g/liter. This assay measures clottable protein. Fibrin degradation products (FDP) have been shown not to interfere with the assay and are not detected by this assay.

Cell isolation

Spleen cells. Spleens were removed aseptically from BXSB mice, and cells were resuspended in RPMI 1640 medium (Flow Laboratories, Mississauga, Ontario, Canada) as previously described. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (density 1.034; Pharmacia, Montreal, P.Q., Canada) at 1400 × g at 22°C for 12 minutes [6]. The cells at the interface were removed and adjusted to 1×10^6 splenic mononuclear cells per ml. The viability of these cells was greater than 95% as demonstrated by trypan blue exclusion. Since our previous studies have identified MO/MA as the source of PCA within peripheral blood mononuclear cells/spleen cells, assays for PCA were done on this total population without further separation [6]. Studies of splenic cell populations showed no differences in the ratio of macrophages to lymphocytes in the different treatment groups or time points. The percentage of macrophages was quantified by nonspecific esterase uptake, and was consistently 10 to 12% of total mononuclear cells as previously described [6].

Peritoneal macrophages. Peritoneal macrophages were isolated from four- to six-week old BXSB male mice three days after intraperitoneal thioglycollate injection as previously described [6]. Approximately 10^7 macrophages were isolated from each animal.

Procoagulant activity

Samples of frozen-thawed and sonicated cells (total content PCA) were assayed for the capacity to shorten recalcification time of citrated normal pooled human plasma in a one-stage clotting assay, as described previously [6]. Purified splenic mononuclear cells at 1×10^6/ml in 12 × 75 mm polypropylene tubes (Falcon Plastics, Oxnard, California, USA) were subjected to three cycles of freeze-thaw and sonication. An aliquot of the cellular homogenate (0.1 ml) was added to 0.1 ml of citrated normal human platelet poor plasma (Helena Laboratories, Beaumont, Texas, USA) at 37°C and 0.1 ml of 25 mM $CaCl_2$ was added to start the reaction. The time in seconds for the appearance of a fibrin gel was recorded. To establish units, a rabbit brain thromboplastin standard (Dade Division, American Hospital Supply, Miami, Florida, USA) at 36 mg dry mass per ml was assigned a value of 100,000 mU. Log dilutions of this standard were used to derive a standard curve as previously described. Similarly, log dilutions of macrophages expressing PCA gave a linear curve parallel to the thromboplastin standard when plotted on a log-log scale. Media with or without 10% fetal calf serum and buffers were all without activity [6].

Plasma procoagulant activity inducing factor

At the time of sacrifice, blood was collected from the axillary artery of control and ancrod treated mice into heparinized tubes. To assess procoagulant inducing factor (PIF), 20 μl of plasma from control or ancrod treated mice were added to 1×10^6 peritoneal macrophages from six-week-old male BXSB mice which were supplemented with 6×10^6 splenic cells suspended in 1 ml of RPMI 1640 (Flow Laboratories, Mississauga, Ontario, Canada). The suspension was incubated overnight, the medium was removed and the cells resuspended in 1 ml of RPMI 1640. Cells (1×10^6) were subjected to three cycles of freeze-thawing and sonication and assayed for total content PCA as above.

Effect of ancrod on procoagulant activity inducing factor (PIF)

In order to determine whether ancrod was able directly to inhibit the ability of PIF to induce PCA, peritoneal macrophages were stimulated with 20 μl of positive plasma from six-month-old BXSB mice, or 10 μg of lipopolysaccharide (LPS; *E. coli* 0111:B4) or 20 μg of human IgG aggregated by heating to 63°C for 20 minutes in the presence or absence of increasing concentrations of ancrod up to 5 U/ml.

Endotoxin contamination

All media and buffers were assayed for endotoxin contamination by a standard limulus assay (E. Toxate; Sigma Chemical Co., St. Louis, Missouri, USA) and contained less than 0.1 ng/ml of endotoxin, the lower limits of the assay.

Assay for anti-DNA antibodies

Single stranded DNA (ssDNA) was prepared by placing calf thymus DNA type I (Sigma Chemical Co.; diluted to 1 mg/ml in distilled water), in a boiling water bath for 15 minutes followed by rapid cooling in an ice bath for 15 minutes. One hundred microliters of the denatured DNA diluted to 2.5 μg/ml in 0.9% saline, was placed into the wells of a 96-well polystyrene plate (NUNC, Gibco Scientific, Mississauga, Ontario, Canada) which in turn was incubated overnight at 4°C. After washing in distilled water containing 0.05% Tween 20, the plates were blocked with 3% bovine serum albumin, washed and then air-dried before use in the assay.

Sera in serial twofold dilutions were added to the coated DNA plates along with appropriate positive (sera from NZB and MRL/1 mice) and negative (sera from DBA/2 and CBA/J) controls. After a 60 minute incubation at room temperature, the plates were washed as above, following which 100 μl of diluted peroxidase-conjugated, affinity purified, goat anti-mouse IgM or

Table 1. Effect of ancrod on plasma fibrinogen, blood urea nitrogen and survival

Age of mice *months*	Plasma fibrinogen[a] *g/liter*		Blood urea nitrogen[b] *mg/100 ml*		Survival *% of total*	
	Control	Ancrod	Control	Ancrod	Control	Ancrod
1.25	2.46 ± 0.8	1.95 ± 0.75	—	—	100	100
2.00	1.95 ± 0.65	0.45 ± 0.18[c]	10 ± 2	10 ± 2	80	100
3.00	1.25 ± 0.3	0.47 ± 0.17[c]	15 ± 1.9	15.1 ± 1.5	70	100
4.00	1.4 ± 0.2	0.49 ± 0.2[c]	30 ± 5	15 ± 4[c]	60	100
5.00	1.35 ± 0.11	0.35 ± 0.05[c]	40.5 ± 5	23 ± 6[c]	55	100
6.00	1.45 ± 0.22	0.67 ± 0.3[c]	49.5 ± 5.5	10 ± 1[c]	50	100

Data are expressed as the mean ± standard error of the mean.
[a] 4 animals/group/time point (normal fibrinogen = 1 to 3 g/liter)
[b] 7 mice per group
[c] $P < 0.01$
— not done

IgG antibody (Jackson Immunoresearch, Avondale, Pennsylvania, USA) was added to each well. The plates were incubated at room temperature for 60 minutes. After washing, 100 μl of 0.015% hydrogen peroxide substrate containing 0.2% o-phenylene diamine was added and incubated for 30 minutes at RT. The optical density at 450 nm of each well was determined using an MR-600 plate reader (Dynatech Industries, McLean, Virginia, USA). The results were expressed as optical density units where absorbance values 2 standard deviations above the mean of normal controls (0.200 O.D.) were deemed to be positive.

The assay for anti-double stranded DNA (anti-dsDNA) was performed identically to the anti-ssDNA antibody assay omitting the denaturation step.

Renal pathology

Whole kidneys were removed from sacrificed animals. Specimens for histologic study were embedded in paraffin. Thereafter, 4 micron sections were stained with hematoxylin and eosin, (H & E) and were reviewed by a pathologist (JS) in a double blind fashion. Biopsies were assessed by a scoring system similar to that used by Balow and colleagues in the assessment of activity in human lupus nephritis [19], and points were assigned as follows: glomerular cellular proliferation-3, leukocyte exudation-3, karryorrhexis or fibrinoid necrosis-6, cellular crescents-6, hyaline deposits-3, interstitial inflammation-3.

Immunofluorescence

Portions of kidneys were embedded in Tissue-Tech (Miles Laboratories, Napleville, Illinois, USA) and snap-frozen in liquid nitrogen. Thereafter, the samples were kept at −70°C prior to further assessment. Three micron sections were cut and to determine fibrinogen deposition were stained initially with rabbit anti-mouse fibrinogen (Dr. E. Plow, Scripps Clinic and Research Foundation, La Jolla, California, USA), and thereafter with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pennsylvania, USA). The goat anti-rabbit IgG was absorbed with normal mouse serum prior to use. To determine IgG deposition, sections were stained with fluorescein isothiocyanate labelled goat anti-mouse IgG (Cappel Laboratories). The sections were subsequently assessed by JS in a double blind manner.

Statistical analysis

Data from both ancrod treated and control mice were compared by means of the Wilcoxon Rank Sum Test, giving a comparison of two independent variables at various time points.

Results

Fibrinogen levels

These data are shown in Table 1. As can be seen, up to five months of age, plasma fibrinogen was maintained at less than 30% of control values. However, at six months of age, despite continued therapy, plasma fibrinogen rose to 52% of control values (0.77 g/liter vs. 1.47 mg/liter).

Blood urea nitrogen

Table 1 compares BUN in ancrod treated and control mice. The values are significantly higher ($P < 0.01$) in the controls at four, five and six months.

Anti-DNA antibodies

DNA binding assessed by all parameters (IgG, IgM, single and double stranded DNA) varied from 1:100 to 1:3200. There were no differences in any parameter at any time point between control and ancrod treated animals (data not shown).

Mortality

A striking difference was observed in mortality between those animals treated with ancrod and control mice. Over a six month period, none of 20 animals in the ancrod treated group died, whereas 10 out of 20 control animals (50%) died. These data are shown in Table 1.

Procoagulant activity

Spontaneous PCA was assessed in both control and ancrod treated mice as shown in Figure 1. An increase in PCA was observed in splenic cells from two-month-old BXSB mice, rose markedly by three months and persisted for the length of the study. In contrast, a lesser rise in PCA was observed in ancrod treated mice at three months and a significant difference in PCA values between control and treated mice was seen from two months of age on.

Procoagulant activity inducing factor (PIF)

PIF was compared in ancrod treated and control mice. The latter group had PIF values significantly higher than ancrod treated mice ($P < 0.01$) from two months of age on. However, a modest increase in PIF also occurred in the treated animals

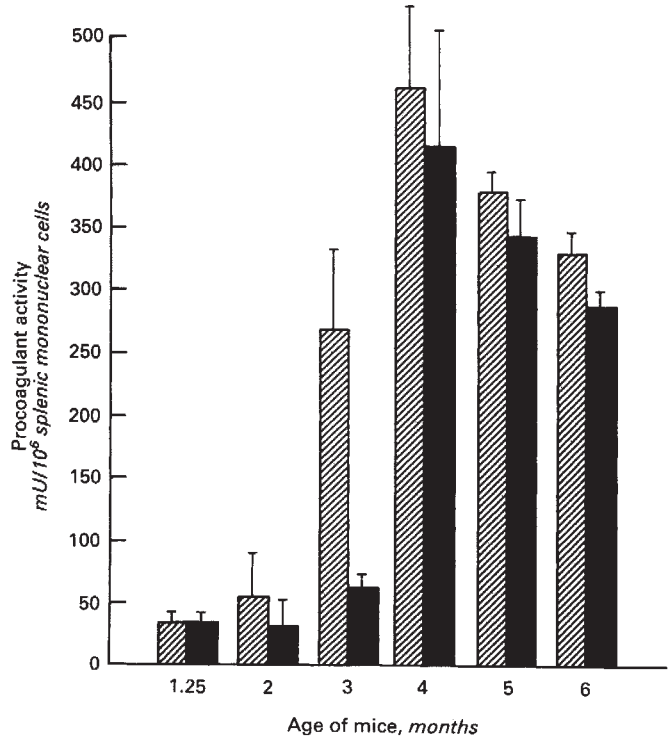

Fig. 1. *Spontaneous procoagulant activity in control (▨) and ancrod treated (■) BXSB mice.* Values represent the means ± 1 standard error of the mean of mice/group. The differences between ancrod treated and control animals were significant ($P < 0.01$) at 2 and 3 months of age, and significant ($P < 0.5$) at 4, 5 and 6 months of age.

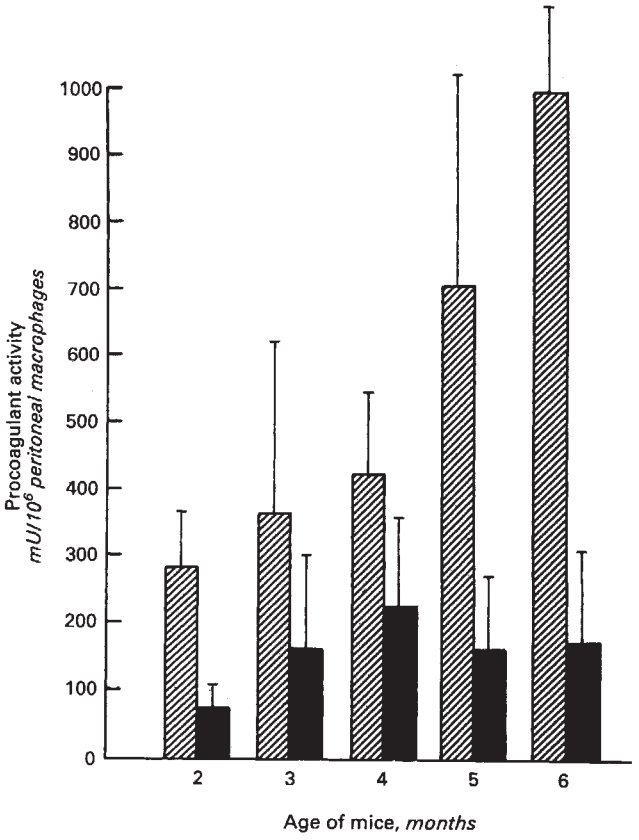

Fig. 2. *A comparison of plasma procoagulant-inducing factor in control (▨) and ancrod treated (■) mice.* Values represent the mean ± standard error of the mean of 5 assays done in duplicate. The values at 2 to 6 months of age were significantly different between the two groups ($P < 0.01$).

starting at three months of age (Fig. 2). No PIF was found in plasma from four-week-old BXSB mice or two- and four-month old A/J or Balb/cJ mice (data not shown).

Effect of ancrod on PIF

Ancrod had no effect on the ability of lipopolysaccharide (LPS) to induce procoagulant activity in peritoneal macrophages, whereas it was markedly inhibitory to the stimulatory effects of aggregated mouse IgG and PIF. Forty-two percent inhibition of PIF induced PCA was observed at a concentration of 1.25 U/ml ancrod, and 68% inhibition at 5 U/ml. One hundred percent inhibition of aggregated IgG induced PCA was observed at all concentrations of ancrod tested (Table 2).

Histology

The activity index was assessed for all mice. Mean activity scores are presented in Figure 3 and show that control mice had significantly more active glomerulonephritis (Fig. 4A and B) at three, four and five months of age ($P < 0.01$). At five and six months of age severe histologic disease was seen in both treated and untreated mice, although at five months of age, five of seven ancrod treated mice had normal kidneys (Activity index = 0).

Immunofluorescence

Immunofluorescence was graded from negative to 3+. Control animals had increasing amounts of glomerular fibrin from two months of age, which was significantly greater than that present in treated mice at all time points ($P < 0.01$). At four months of age, when disease was well established, glomeruli from control mice were strongly positive (2 to 3+) for fibrinogen (Fig. 4C), whereas ancrod treated mice were negative or had only trace amounts (Fig. 4D).

Both control and ancrod treated mice had marked and

Table 2. Effect of ancrod on procoagulant activity inducing factor

Ancrod U/ml	Stimulus			
	Control	LPS	Aggregated IgG	Plasma
	PCA *mU/10⁶ peritoneal macrophages*			
—	174 ± 54	1750 ± 25	439 ± 71	3296 ± 243
0.3125	—	1489 ± 154	142 ± 16[a]	3845 ± 327
0.625	—	1500 ± 143	—	—
1.25	—	1309 ± 193	136 ± 10[a]	1900 ± 182[a]
2.5	—	1472 ± 188	—	—
5.0	194 ± 39	1533 ± 169	146 ± 10[a]	1064 ± 118[a]

LPS (*E. coli* 0111:B4) was added at a final concentration of 10 μg/ml. Ancrod (Arvin) concentration is expressed as units/ml.

Aggregated IgG (Agg IgG) was mouse IgG at a final concentration of 0.9 mg/ml. Positive plasma was isolated from 5-month-old BXSB mice and 20 μl was added to each culture. PCA is total content procoagulant activity in mU/10⁶ peritoneal macrophages.

[a] $P < 0.01$ as compared to PCA without ancrod

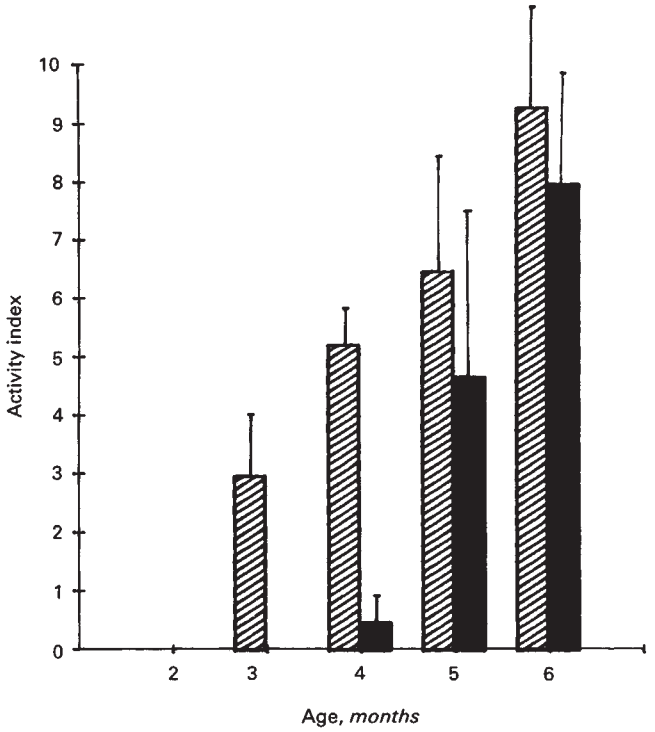

Fig. 3. *Renal activity index in BXSB mice.* Symbols are: (▨) control; (■) ancrod treated mice. Values are derived as described in **Methods** and are the mean ± 1 standard error of the mean. There was no activity in either group of mice at 2 months of age and in ancrod treated mice at 3 months of age. There was a significant difference between the 2 groups of mice at 3, 4 and 5 months of age.

equivalent deposition of IgG in renal glomeruli from three months of age on. In three-month-old mice, deposition was primarily within the mesangium, while older mice generally had prominent capillary loop deposition as well.

Discussion

Fibrin deposition is a prominent feature of many forms of GN, although the mechanism is uncertain [2–4]. We have recently demonstrated that monocytes (human) and splenic macrophages (mouse) contain large amounts of PCA in both patients and mice with active lupus GN [5, 6]. Wiggins, Glatfeller and Brukman [7] and Holdsworth and Tipping [8] have demonstrated induction of PCA in macrophages isolated from glomeruli of animals with antiglomerular basement membrane antibody mediated glomerulonephritis. Rickles and colleagues, using a monoclonal antibody against macrophage activation antigen, have shown surface expression of PCA in patients with active GN [20]. These data suggest participation of macrophage PCA in glomerular injury. Induction of macrophage PCA preceded fibrin deposition and may be responsible for its presence in active disease [9]. This does not prove that fibrin is formed as a consequence of PCA induction, but it is highly suggestive.

Our previous data and that of others have shown a 50% mortality at five months in the BXSB male mouse [6, 21]. These present studies demonstrate that ancrod, given intermittently to BXSB mice in doses sufficient to reduce fibrinogen, significantly improved mouse survival. While we were unable to perform detailed studies on animals that died, we attributed their death, as emphasized by others, to renal failure secondary to GN [21]. This was associated with a decrease in glomerular fibrin deposition, improved renal function and a delay in the appearance of active GN. However, circulating anti-DNA antibody levels and renal IgG deposition were unaffected, indicating no effect of ancrod on autoantibody production or renal immune complex (IC) deposition. The documented delay in renal disease is even more remarkable if one considers that control animals studied were survivors and thus had less severe disease than control mice that died of disease and could not be studied. The onset of more severe glomerular disease and fibrin accumulation in five- to six-month ancrod-treated mice, associated with an increase in plasma fibrinogen levels, may be related to resistance to ancrod as has been described. The mechanism for this resistance is unknown but could be related to the development of antibodies to ancrod [22].

These data support the concept that fibrin deposition is crucial to the development of serious glomerular injury in this mouse strain. Fibrin likely contributes to injury by reducing blood flow through glomerular capillaries, and by recruitment of inflammatory cells through the creation of a meshlike network [23].

The amelioration of GN seen in association with fibrin depletion was accompanied by an unexpected sustained reduction in PCA and PIF, although the major effect was seen at months two and three. The fact that both renal function and survival were so profoundly affected despite no effect on formation and deposition of IC emphasizes the requirement for subsequent activation of mediator systems in order for inflammation and disease to occur. The mechanism by which ancrod reduced PCA induction is uncertain. Our previous work has demonstrated that a circulating plasma factor (PIF), which develops with age, is responsible for inducing PCA in BXSB mice [6]. Studies in our laboratory have shown no inhibitory effect of ancrod on the PCA assay itself. However, ancrod markedly inhibited the ability of aggregated IgG and plasma from five-month-old BXSB mice to induce PCA in vitro in macrophages from six-week-old mice, whereas it had no inhibitory effect on LPS induction of PCA (Table 2). This data suggests that ancrod interferes with the interaction of PIF and mononuclear cells resulting in reduced levels of PCA. The nature of PIF in this model is unknown; however, recent studies in our laboratory have shown it to have a molecular weight of 400,000 to 900,000 daltons (manuscript in preparation). Studies in human lupus using endothelial cells have suggested the stimulatory factor to be IC [24]. The similarity of inhibition by ancrod of plasma and aggregated IgG PCA induction is consistent with this hypothesis. Although ancrod did inhibit PCA induction, the beneficial effect of the drug is probably related to the creation of abnormal fibrinogen preventing the formation of fibrin.

These data are in agreement with those of Tipping and Holdsworth, who showed that accumulation of glomerular macrophages preceded that of fibrin, and that the latter paralleled the development of glomerular crescents and impairment of renal function in an experimental model of antiglomerular basement membrane antibody-induced disease in the rabbit [9]. They have also shown benefit of both fibrinolysis and defibrina-

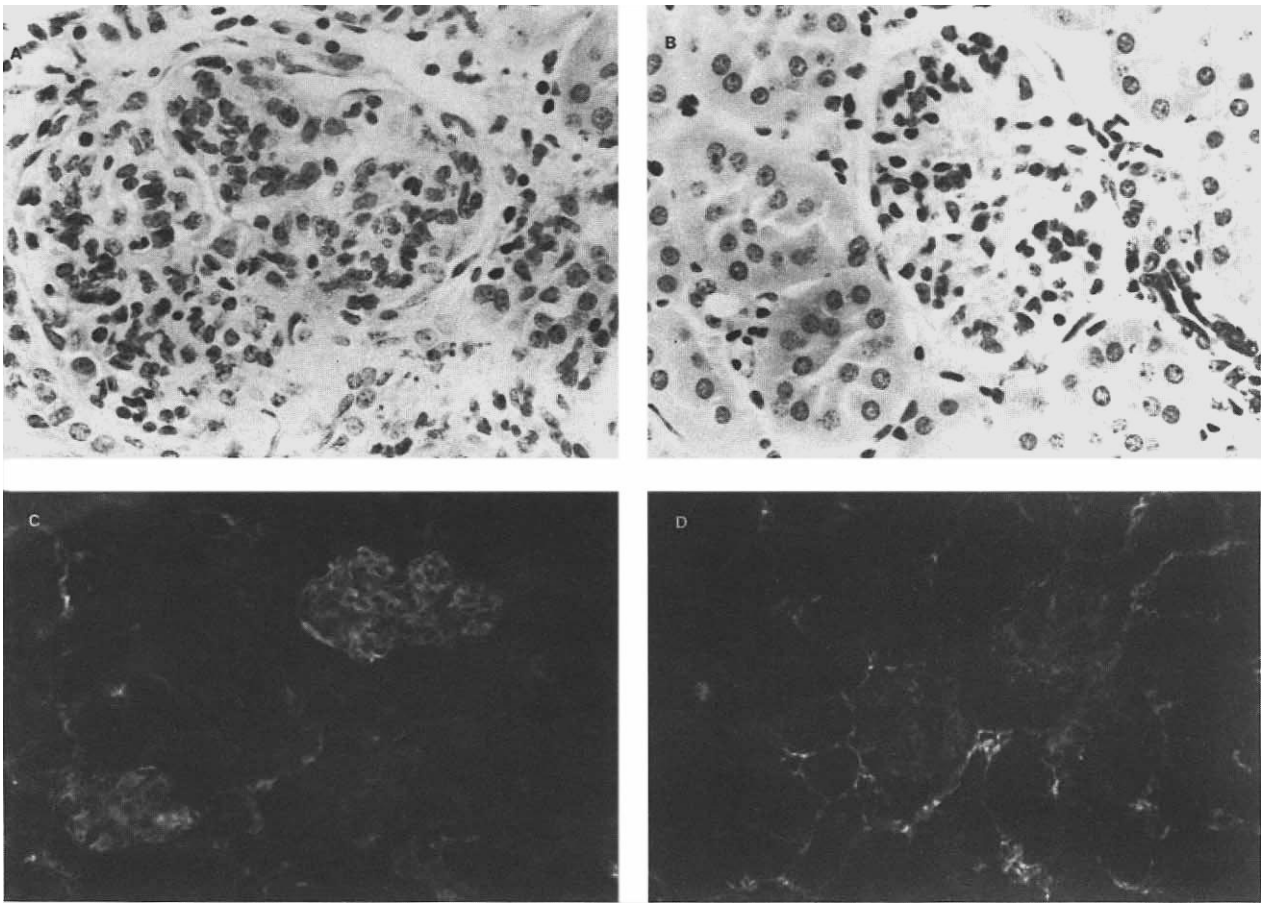

Fig. 4. *Renal histology and immunofluorescence in BXSB mice.* **(A)** Severe proliferative glomerulonephritis in control mice at 4 months. (H & E × 200). **(B)** Normal glomerulus from ancrod treated mouse at 4 months of age. (H & E × 200). Immunofluorescence using a rabbit anti-mouse fibrinogen demonstrating: **(C)** fibrin deposition in glomeruli of control animal at 4 months of age, whereas **(D)** glomeruli from ancrod treated mice had only trace amounts of fibrin (× 20).

tion in this disease but did not assess PCA [25]. These studies are also consistent with data of Cohen et al showing that anticoagulants interfere with delayed hypersensitivity reactions, not by interfering with the process of sensitization, but by acting at a critical step in the formation of the inflammatory infiltrate [26].

Unlike certain other anticoagulants, the effect of ancrod is specific, in that it has no effect on coagulation, other than directly converting fibrinogen to a soluble form of fibrin which is rapidly removed by the reticuloendothelial system [27]. Pollak and colleagues have shown that the resulting production of fibrin does not alter IC clearance by the reticuloendothelial system, and would not be expected to affect the course of GN by that mechanism [28]. Nevertheless, it has also been noted that FDP which accumulate in some patients on ancrod therapy suppress lymphocyte immune responsiveness in vitro [29]. Since autoantibody production and renal IC deposition were unaffected by ancrod, depressed lymphocyte responsiveness was probably not responsible for the amelioration of lupus GN seen in this study.

The profound effect of ancrod on mortality in this study, along with the reports by Pollak and Holdsworth, suggest that this agent may be clinically useful in the therapy of lupus nephritis, although it does not appear to alter the formation or deposition of IC [4, 26]. Future experiments will be directed towards further definition of the role of PCA in GN and autoimmune disease and characterization of PIF.

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Appendix. Abbreviations

GN	glomerulonephritis
PCA	procoagulant activity
MO/MA	monocyte/macrophages

H&E	haematoxylin and eosin
FDP	fibrin degradation products
BUN	blood urea nitrogen
PIF	procoagulant inducing factor
LPS	lipopolysaccharide
IC	immune complexes

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