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Role of the plasma contact system in the pathogenesis of experimental anti-GBM glomerulonephritis

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SUMMARY

To study the participation of the Hageman factor-related contact system of plasma in the pathogenesis of glomerulonephritis (GN), an anti-BM GN was induced in a group of 10 normal Brown Norway rats and another of seven Brown Norway BN/Mai Pfd f rats. The latter strain is characterized by a congenital deficiency of plasma prekallikrein and of high-molecular weight kininogen, with lengthening of the activated partial thromboplastin time. In the deficient group, one animal developed crescents in < 25% of glomeruli, five in 25–50% and one in 50–75%. In the group of normal rats, extracapillary proliferation was of greater severity: one animal showed crescents in less than 25% of glomeruli, two in 50–75% and five in more than 75% of glomeruli. Although in both groups intense glomerular fibrin deposition was documented, the intensity of these deposits was less severe in the deficient animals. These data suggest, in the first place, that functional integrity of the contact system is not a necessary requirement for glomerular fibrinogenesis, other mechanisms being implicated in this phenomenon. On the other hand, this functional deficit has exerted a protective effect on crescent formation, which suggests that the contact system can play a role as a mediator of injury in glomerulonephritis, perhaps through the release of contact system-dependent mediators of inflammation.

Keywords contact system anti-GBM glomerulonephritis plasma prekallicrein deficient rats HMW-kininogen deficient rats

INTRODUCTION

It has been suggested that the Hageman factor-related contact system of plasma (CS) could play a role in the pathogenesis of glomerulonephritis (GN) as a mediator of injury, on the basis of its inflammatory potential (Cochrane, 1978). Such participation could take various forms, as has been recently reviewed (Cochrane & Griffin, 1982; Colman, 1984), of which the most significant are the activation of the classical pathways of coagulation and the release of phlogistic substances. In fact, there already exist some papers which indirectly support this hypothesis. Studies *in vitro* have shown that collagen and glomerular basement membrane (GBM), acting as negatively charged surfaces, are capable of activating the CS (Niewiarowski *et al.*, 1965; Cochrane *et al.*, 1972). An immunologically mediated lesion at the endothelial level could expose GBM and lead to activation of the CS. Thus, Hageman factor (HF) and plasma prekallikrein (PPK) deposits have been detected by immunofluorescence techniques in the glomerular capillary wall in patients with different types of GN (Berger & Yaneva, 1982; Yamabe *et al.*, 1984). On the other hand, glomerular fibrinogenesis is a feature of certain types of GN, although the mechanisms by which it is produced are poorly understood. Glomerular fibrin is sometimes associated with crescent formation and irreversible renal damage. Hypothetically it is easy to relate glomerular fibrinogenesis with a possible activation of the CS.

This study is to test the possible role of the CS in the development of experimental GN, and specially its hypothetical implication in glomerular fibrinogenesis and in the pathogenesis of determined glomerular lesions such as extracapillary proliferation with crescent formation. We have therefore developed an experimental model of anti-GBM GN (aGBM GN) in a strain of Brown Norway rats characterized by a congenital deficit of two CS proteins: PPK and high molecular weight kininogen (HMWK).

Both proteins are essential for an adequate functioning of this biological system, and their lack determines the absence of CS activation and of the physiopathological pathways which depend upon it. This strain of rats was the first described by Damas & Adam (1980), their findings being confirmed later by Oh-Ishi *et al.* (1982), who further characterized the quantitative deficit of these proteins.

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MATERIAL AND METHODS

Animals

Females of two different strains of rats were used: normal Brown Norway rats and BN/Mai Pfd f Brown Norway rats, carriers of a partial congenital deficit of PPK and a total congenital deficit of HMWK (Oh-Ishi *et al.*, 1982). The weight of the animals at the beginning of the experiment was between 80 and 120 g.

Anti-GBM serum

aGBM serum was obtained through repeated immunization of rabbits with rat glomerular antigen emulsified with Freund's complete adjuvant, and was decomplemented by heating at 56°C for 30 min and then adsorbed with rat plasma and rat red cells.

Induction of aGBM GN

GN was induced by a single intravenous injection of 2 ml of aGBM serum. In order to accelerate the autologous phase, all animals were pre-immunized with two 1 mg doses of rabbit IgG in complete Freund's adjuvant, 30 and 15 days before induction.

Experimental groups

This protocol was applied in two experimental groups: the BNn group, consisting of 10 normal Brown Norway rats, and the BNd group, consisting of seven BN/Mai Pfd f rats.

Experimental animals were killed 28 days after induction, or beforehand if they displayed oliguria, oedemas and tachypnea. Three samples of renal tissue were obtained. One was fixed in 10% formalin in PBS for light microscopic study. The other two were frozen in liquid nitrogen and stored at −70°C for immunofluorescence and to study cortical fibrinolytic activity (CFA). A 24-h urine collection was obtained weekly in order to determine proteinuria and urinary fibrin/fibrinogen degradation products (FDP).

The control group included six normal and six deficient rats, matched for sex and weight, untreated with aGBM serum and not subjected to manipulation. For evaluation of PPK concentration and activated partial thromboplastin time (APTT), blood samples were taken by open heart puncture, under ether anaesthesia, using plastic tubes containing 1/10 volume of 3·8% sodium citrate. The plasma samples were stored at −70°C.

PPK concentration and APTT

APTT was studied according to the method of Proctor & Rapaport (1961). PPK concentration was determined as described by Stormorten *et al.* (1978), using chromogenic substrate S-23-2 and a PPK activation containing ellagic acid and phospholipid, and rich in HMWK and HF (AB Kabi diagnostic, Stockholm, Sweden). PPK concentration is expressed as a percentage with respect to a pool of plasma from six Brown Norway normal rats (100%).

Proteinuria and urine FDP

Proteinuria (mg/24 h) was estimated by the trichloroacetic acid method (Henry, Sobel & Segalove, 1956). Urinary FDP (mg/24 h) was detected according to a variant of the method of Merskey, Kleiner & Johnson (1966), based on inhibition of haemagglutination of sheep red blood cells sensitized with rat plasma, using rabbit anti-rat fibrinogen serum. The sensitivity of our method was 2·4 μg/ml.

Assessment of the histological preparations

The material obtained for light microscopy studies were stained with haematoxylin–eosin, PAS and Masson's trichrome stain. Crescents were defined as the proliferation of at least four adjacent epithelial cells. According to the percentage of glomeruli with such lesions, the following scale was established: 1+, less than 25%; 2+, 25 to 50%; 3+, 50 to 75%; 4+, more than 75% of glomeruli with crescents.

Assessment of immunofluorescence preparations

Direct immunofluorescence was performed by routine technique using commercial fluorescein isothiocyanate labelled (FITC) anti-sera against rat IgG and rabbit IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands). Anti-rat fibrinogen FITC serum was prepared in our laboratory by injecting rabbits with purified rat fibrinogen. The intensity of rabbit IgG and rat IgG deposits was graded from 0 to 3+. Evaluation of fibrin-related antigen (FRA) deposits was performed in two complementary ways: using a subjective scale of intensity from 0 to 4+ and by counting the percentage of glomeruli with extracapillary FRA deposition over a minimum of 50 glomeruli per section.

Cortical fibrinolytic activity

CFA was demonstrated using the fibrin slide technique, as reported by Sánchez Ibarrola, Quazzaz & Naish (1981). For each experimental animal minimum grading is 0 and maximum is 12 points. This is a semiquantitative method which determines the presence of the tissue activator of Plasminogen.

Statistics

The results of the quantitative parameters studied are expressed as the mean ± standard deviation. The statistical analysis was done using Student's test and the correlation index (r).

RESULTS

Control group

General data. No significant differences between the animals of the two different strains were observed. Thus, the results are discussed as one homogenous group. The mean proteinuria was insignificant (2·85 ± 0·91 mg/24 h). In no case were urinary FDP detected. The average CFA was 5·476 ± 0·97 points. Direct immunofluorescence showed the absence of pathological deposits of rabbit IgG, rat IgG and FRA. In the same way, no worthwhile anomalies were found in light microscopic study.

Assessment of APTT and PPK concentration. See Table 1. These two parameters have been analysed in the plasma of animals from the control group. The mean PPK concentration in the normal Brown Norway rats was 100·83 ± 12·78%, whilst in the deficient rats the result was 33·33 ± 19·36%, the difference being highly significant ($2P < 0·001$). The APTT in the lot of normal rats varied between 29 and 32 s (mean 31 ± 0·6 s), whilst in the deficient animals it fluctuated between 35 and 53 s (mean 42·66 ± 6·47 s), the difference between the means being highly significant ($2P < 0·001$).

Table 1. Plasma prekallikrein concentration and activated partial thromboplastin time in normal and deficient rats from the control group

	% of plasma prekallikrein	APTT (seconds)
Control BNn-1	90·8	32
Control BNn-2	103	32
Control BNn-3	119·4	29
Control BNn-4	82·6	31
Control BNn-5	106·2	32
Control BNn-6	103	31
$\bar{x}$	100·83	31·16
s.d.	12·78	1·17
Control BNd-1	16·5	45
Control BNd-2	59·1	37
Control BNd-3	43	38
Control BNd-4	15·4	53
Control BNd-5	17	46
Control BNd-6	49	37
$\bar{x}$	33·33	42·66
s.d.	19·36	6·47
	$2P<0·001$	$2P<0·001$

BNn group

Of the 10 animals in the group, two died before the experiment was completed, it not being possible to gather material for study. Another four rats were killed (three on the 14th day and one on the 21st day), as they showed oliguria, oedemas and tachypnea. Significant proteinuria was found from the 7th day, reaching a maximum on the 14th day ($251 \pm 30·05$ mg/24 h) and slightly decreasing until day 28 ($180·8 \pm 32·8$ mg/24 h). Likewise, by day 7, a quantifiable elimination of urine FDP was observed ($1·32 \pm 0·74$ mg/24 h), which increased, reaching a maximum value of $4·55 \pm 2·57$ mg/24 h on day 28 (Tables 2 and 3).

Direct immunofluorescence showed intense linear deposits (3+) of rabbit IgG and rat IgG in all animals. Likewise, in all sections studied, intense glomerular ARF deposits were detected, predominantly in extracapillary localization, affecting an average of $94 \pm 11·9$% of glomeruli with an intensity of 4+ in six cases and of 3+ in the remaining two cases (Table 4). It must be emphasized that the intensity of the glomerular ARF deposits in the animals killed before the end of the experiment was similar to that of those killed on day 28.

In all the animals of this group a marked increase in CFA (average grading $11·62 \pm 0·99$ points) was shown with respect to the control group ($2P<0·001$). The four rats killed beforehand had the maximum score (Table 4).

BNn group has uniformly developed a severe extracapillary proliferative GN with a great number of crescents: 4+ in five cases and 3+ in another two. Only BNn rat 5 showed lesions of minor intensity (1+). In the same way, the animals killed beforehand had glomerular lesions of similar severity to the rest of the group (Table 4).

Table 2. Weekly proteinuria in experimental groups (mg/24 h)

	Days			
	7	14	21	28
BNn				
n	10	8	5	4
$\bar{x}$	200·54	251·87	198·29	180·8
s.d.	33·41	30·05	33·54	32·8
BNd				
n	5	7	7	7
$\bar{x}$	305·76	217·63	192·85	185·17
s.d.	48·24	40·55	22·74	40·34
t	4·979	2·958	0·336	0·184
	$2P<0·001$	$2P<0·01$	NS	NS

Table 3. Weekly urine-FDP in experimental groups (mg/24 h)

	Days			
	7	14	21	28
BNn				
n	10	8	5	4
$\bar{x}$	1·32	4·25	3·2	4·55
s.d.	0·74	2·21	0·95	2·57
BNd				
n	5	7	7	7
$\bar{x}$	17·74	13·05	15·79	6
s.d.	8·13	6·61	5·27	3·63
t	6·667	2·286	5·209	0·697
	$2P<0·001$	$2P<0·05$	$2P<0·001$	NS

BNd group

In this group, proteinuria reached its maximum on day 7 ($305 \pm 48·4$ mg/24 h), decreasing slightly until day 28

Table 4. BNn group: immunofluorescence, cortical fibrinolytic activity and percentage of crescents on light microscopy

	Immunofluorescence					
			FRA			
	Rabbit IgG	Rat IgG	SI	%ExFRA	CFA	LM
BNn-1*	3+	3+	4+	100	12	4+
BNn-2†	3+	3+	4+	100	9	4+
BNn-4	3+	3+	4+	100	12	4+
BNn-5	3+	3+	3+	68	12	1+
BNn-6	3+	3+	4+	100	12	3+
BNn-7	3+	3+	4+	100	12	4+
BNn-9*	3+	3+	4+	100	12	3+
BNn-10*	3+	3+	3+	84	12	4+
			$\bar{x}$	94	11·62	
			s.d.	11·9	0·99	

SI, subjective intensity of FRA deposits; %ExFRA, percentage of glomeruli with extracapillary FRA; LM, light microscopy.

* Animals killed on day 14.

† Animal killed on day 21.

(185·77 ± 40·34 mg/24 h). In summary (Table 2), both groups presented an intense proteinuria throughout the experiment. On day 7 the mean proteinuria of BNd group was significantly greater than that of the BNn group ($2P < 0.001$), whilst on the 14th day it was the BNn group which showed the greater elimination ($2P < 0.01$). The urinary elimination of FDP was notably high in the BNd group, reaching a maximum of 17·74 ± 8·13 mg/24 h on day 7 and decreasing slightly until day 28. It was significantly greater than in the BNn group on day 7 ($2P < 0.001$), day 14 ($2P < 0.05$) and day 21 ($2P < 0.001$) (Table 3).

Direct immunofluorescence studies showed intense linear deposits (3+) of rabbit IgG and rat IgG in all animals, in the same way as in group BNn. Likewise, in all sections studied intense glomerular deposits of FRA were detected, predominantly in extracapillary location. They involved a mean of 91·4 ± 3·43% glomeruli with an intensity of 4+ in two cases and of 3+ in five animals.

All the animals in the BNd group have shown an important increase in CFA (11·62 ± 0·53 point) with respect to the control group ($2P < 0.001$). There was no statistical difference within both experimental groups.

The animals in the BNd group also showed extracapillary proliferative changes with crescent formation: 1+ in one rat, 2+ in six and 3+ in one (Table 5). In summary, although the two groups developed a diffuse extracapillary proliferative GN, the degree of crescent formation was considerably more significant in the BNn group.

Linear correlation betwen the proteinuria and urine FDP

In neither of the groups was found a statistically significant linear correlation between the two parameters observed throughout the whole experiment.

DISCUSSION

The use of the BN/Mai Pfd f strain to study the possible role of the activation of the CS in aGBM GN looks adequate because of the well-documented deficiency of these animals in some of the components of this biological system essential for its activation. We have been able to show the low concentration of PPK in the animals used in this work, as has been previously reported in the literature (Oh-Ishi *et al.*, 1982). Besides, our rats showed an increase in APTT compared with normal Brown Norway control rats. This parameter had not been checked before in this strain of rats.

Table 5. BNd group: immunofluorescence, cortical fibrinolytic activity and percentage of crescents on light microscopy

	Immunofluorescence					
			FRA			
	Rabbit IgG	Rat IgG	SI	%ExFRA	CFA	LM
BNd-1	3+	3+	3+	92	12	2+
BNd-2	3+	3+	3+	84	11	2+
BNd-3	3+	3+	3+	94	11	2+
BNd-4	3+	3+	4+	94	11	3+
BNd-5	3+	3+	4+	90	12	2+
BNd-6	3+	3+	4+	92	11	2+
BNd-7	3+	3+	4+	92	12	2+
			$\bar{x}$	91·14	11·62	
			s.d.	3·43	0·53	

SI, subjective intensity of FRA deposits; %ExFRA, percentage of glomeruli with extracapillary FRA; LM, light microscopy.

Our aim was to focus on the problem of glomerular fibrin deposition and extracapillary proliferation, because of the many unsolved problems in this field. The interrelationship between several biological systems (contact system, coagulation, fibrinolysis, kinins, etc; Cochrane, 1978; Cochrane & Griffin, 1982) could play a role in the mechanisms leading to glomerular fibrin deposition. Besides these interactions could be responsible for the glomerular fibrinolytic response and be significant in the modulation of the inflammatory process.

Up to now, several pieces of indirect evidence have considered the participation of the CS in the pathogenesis of glomerulonephritis. Berger & Yaneva (1982) detected glomerular deposits of HF and PPK in patients with membranous GN, suggesting the implication of the CS in the alteration of glomerular permeability. Yamabe *et al.* (1984), showed deposition of HF both in the capillary wall and the glomerular mesangium in patients with IgA nephropathy. In an experimental model of aGBM GN in dogs (Nakamura, Kazama & Be, 1979) a decrease of PPK concentration with elongation of APTT has been demonstrated. These authors implicated the CS in the pathogenesis of GN.

In this paper, we have induced an accelerated model of severe glomerulonephritis characterized by the presence of extracapillary proliferation and the deposition of glomerular fibrin. These two features are present in the animals deficient in proteins of the CS, and this fact becomes a new argument against the implication of an unique thrombin-dependent mechanism in the generation of glomerular fibrin (Bone *et al.*, 1975; Border, Wilson & Dixon, 1975).

However, when the deficient animals are compared with normal BN rats, some striking differences appear. The animals with an impaired contact system develop a less severe GN, with a minor degree of extracapillary proliferation and glomerular fibrin deposition. We think these data can be taken as evidence for a role of the contact system in the pathogenesis of this experimental model because of the protective effect afforded by the deficiency of components of such biological system shown in this paper. Such protection could be exerted through the lack of contact system dependent mediators of inflammation in the BN deficient rats.

Considering protein excretion in these animals the picture looks different. The protection afforded in the CS deficient animals when we look at the histological glomerular damage is not present when the alteration of glomerular permeability is considered. It is worth emphasizing that structural damage and protein excretion are not related parameters as demonstrated in human glomerulonephritis. However the correlation between glomerular structural damage and the best index of glomerular function, i.e. glomerular filtration, seems to be present in our study. In this sense we can speak of protection in the BNd rats bearing in mind that several of the animals in the BNn group died before the end of the experiment, probably of renal failure, although we have no biochemical data to confirm this possibility. The different evolution of proteinuria between the normal

and the deficient groups of rats cannot be explained with the data which are available in this study. Perhaps minor histological damage from the beginning could allow a higher protein excretion as occurred in day 7 in the BNd animals.

These deficient animals showed an increased tissue fibrinolytic response as well as a higher excretion of urinary FDP and a lesser deposition of glomerular fibrin when compared with the normal BNn group. Perhaps minor inflammatory damage at the endothelial level in the deficient group, because of the lack of mediators, could be responsible for a better fibrinolytic response and, thereby for more efficient fibrin clearance and higher excretion of FDP in urine. The method used here to assess the tissue fibrinolytic response, being semi-quantitiative, is not sufficient to demonstrate this possibility.

The lack of correlation between the elimination of proteins and FDP in urine speaks clearly in favour of fibrinolytic mechanisms in the clearance of glomerular fibrin, at least in this experimental model.

In summary, the data presented in this paper, together with other information previously reported support a pathogenetic role for the contact system in the development of immunological glomerulonephritis.

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