PRODUCTION OF ANTIRENIN TO HOMOLOGOUS RENIN AND ITS EFFECT ON EXPERIMENTAL RENAL HYPERTENSION*

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The most direct evidence for the participation of the renin-angiotensin pressor mechanism in experimental renal hypertension has been the lowering of blood pressure in these animals by antirenin (1–3). In human essential hypertension similar evidence is lacking, since antirenin produced in man by the administration of heterologous (hog) renin, while it can inactivate the renin of other animals, is ineffective against human renin (4). This observation probably explains the failure of the blood pressure to fall in hypertensive patients with high titers of antirenin to hog renin (5). Since angiotensin II, the active pressor octapeptide of the humoral mechanism of renal hypertension, does not exhibit this species specificity, attempts were made in this laboratory to produce antiangiotensin, by using an angiotensin II–protein complex as an antigen (6, 7). We were successful in producing antiangiotensin, but the titer of the antibody was too low and the antigen-antibody interaction was too slow to effect a lowering of the blood pressure of hypertensive dogs and rabbits.[1] During the present studies, we investigated the possibility that homologous renin might become antigenic as a result of appropriate chemical alteration of the renin molecule. The long-term objective of this investigation was to make human renin antigenic, for man, and to determine the effect of antirenin to human renin on human essential hypertension.

Materials and Methods

Renin.—Rat and rabbit renins were prepared according to the procedures previously described (8). Large scale preparations of dog renin were carried out in the following manner.

Preparation of Dog Renin.—

10 kg of dog kidneys, stored at −20°C, was thawed, decapsulated, and put through a meat

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grinder. The process of freezing and thawing of the kidneys, of normotensive as well as of hypertensive dogs, increases the yield of renin more than twofold (9). By the slow addition of 7500 ml of distilled water, at 60°C, the ground tissue was warmed to 25°C, and the suspension was stirred mechanically for 15 minutes at room temperature. For the extraction of renin, the suspension was thoroughly mixed with a filter aid (1.4 kg of Johns Manville Celite No. 503), and the extract was separated from the insoluble residue by means of a hydraulic filter press. This resulted in the recovery of 12,000 ml of fluid. The dry residue was broken up into small pieces, reextracted at room temperature with 7500 ml of water, and the second extract (9000 ml) was obtained by means of the filter press. The two extracts, containing the renin, were combined to give a total volume of 21,000 ml. The solution was cooled to 0°C, and 408 ml of 5 N H_2SO_4 was added, dropwise, with constant stirring, to lower the pH to 2.5. After 10 minutes, at 0°C, the pH was readjusted to 6.5, by the slow addition of 520 ml of 5 N KOH, which resulted in precipitation of denatured, inert proteins. Centrifugation yielded 16,000 ml of supernatant. The precipitate (6100 ml) was reextracted, by stirring for 15 minutes, at room temperature, with 13,000 ml of water, and centrifugation yielded a supernatant which, combined with the previous one, measured 30,600 ml. This solution was cooled to 0°C, adjusted to pH 4.5 with approximately 60 ml of 5 N H_2SO_4, and 11,600 gm of $(NH_4)_2SO_4$ was added slowly, with stirring, to a concentration of 2.4 M. The precipitate (approximately 2000 ml) containing the renin, was obtained by permitting sedimentation overnight, followed by centrifugation. It was resuspended in 500 ml of water and brought into solution by dialysis for 16 hours against cold, distilled water. The solution (2400 ml) was adjusted to pH 7.5, by the addition of approximately 9 ml of 5 N KOH, and clarified by centrifugation. It contained approximately 10 units (u) of renin and 37 mg protein per ml (specific activity 0.27u/mg). The final yield of dog renin, by this procedure, was 2.3 units per gm of kidney, on the average, with a range of 1.8 to 2.7 units/gm in 10 different preparations. For the purpose of storage, the renin solution was adjusted to 1 per cent NaCl and kept at −20°C at pH 7.5. Under these conditions, there was no loss of activity due to prolonged storage. In contrast to hog renin, however, dog renin was less stable in acid solution. At pH 4.6, in 1 per cent NaCl, and −20°C, for example, there was a loss of 50 per cent of activity after storage for 7 weeks.

Bioassay: The assay of renin was carried out according to the procedure previously described (10). The sample of renin was assayed by intravenous injection (external or internal saphenous vein) into a normal, trained, unanesthetized dog. The rise of the direct, mean, femoral arterial blood pressure was determined, and pressor activity was then expressed in terms of units. The quantity of extract required to raise the pressure by 30 mm Hg in at least two dogs, was regarded as containing a unit of renin. The assay of antirenin was carried out by a procedure previously described (4, 11). One unit of antirenin is defined as the minimum amount of antiserum which completely inactivates one unit of renin during incubation at room temperature, *in vitro*, for 2 minutes. The antirenin titer of a serum is then expressed as the number of units of renin inactivated by 1 ml of the serum.

Acetylation of Renin.—

This was carried out according to the general procedure of Fraenkel-Conrat *et al.* (12, 13). It has been reported that, under these conditions, the acetylation is confined primarily to the free amino groups.

Acetylation of rat renin: To 90 ml of rat renin (513 units; specific activity 0.32 unit/mg) 120 ml of half saturated sodium acetate solution and 3.3 ml of acetic anhydride was added dropwise with constant stirring. The solution was stirred for 1 hour, at 0°C, and then dialyzed against cold, distilled water and lyophilized. The average specific activity of the acetylated rat renin, in six different preparations, was 35 per cent of that of the untreated renin (range 28 to 42 per cent).

Acetylation of rabbit renin: To 20 ml of rabbit renin (3000 units; specific activity 2.0 units per mg) 133 ml water and 204 ml of half saturated sodium acetate was added. The solution

was cooled to 0°C, and 5.4 ml of acetic anhydride was added, as described previously. The pH of the final solution was 5.4–5.5. The solution was stirred for 1 hour, at the same temperature, and then dialyzed against distilled water, at 0°C, for 16 to 18 hours. The dialyzed solution was lyophilized, and redissolved in normal saline. The average specific activity of eight different preparations of acetylated rabbit renin was 46 per cent of that of the untreated rabbit renin (range 40 to 53 per cent).

Acetylation of dog renin: To 320 ml of dog renin (3000 units; specific activity 0.27 unit per mg), kept at 0°C, 41 gm of anhydrous sodium acetate was added. This was followed by the addition, dropwise, of 7.3 ml of acetic anhydride, with constant stirring. The pH of this solution was 5.4–5.5. The molar ratio of acetic anhydride to protein was estimated to be 500:1. After the solution was stirred for 1 hour, it was dialyzed for 17 hours against cold (0°C) distilled water. To the dialyzed solution (610 ml) 6.7 ml of 2.5 N KOH and 600 mg NaCl was added, to bring the pH to 6.8 and the salt concentration to 0.1 per cent. The solution was then lyophilized, made up to 60 ml with water (pH 6.8; 1 per cent NaCl) and stored at −20°C. Acetylation brought about a loss of pressor activity, 50 per cent on the average, with a range of 28 to 76 per cent in 18 separate experiments.

Immunization with Acetylated and with Untreated Renin.—

Immunization of rats: Sprague-Dawley female rats weighing between 200 and 250 gm were immunized in the following manner (11). On days 1, 8, and 15, a mixture of 0.5 ml of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit) and an equal volume of acetylated, or untreated, rat renin of appropriate concentration (Table I) was injected intraperitoneally. On all other days (5 days a week for 3 to 5 weeks), the same amount of acetylated, or untreated, rat renin alone was injected intraperitoneally. Three days after the last injection all rats were bled from the abdominal aorta, under ether anesthesia, and the sera were stored at −20°C.

Immunization of rabbits: New Zealand white rabbits (Albino Farms, Red Bank, New Jersey) weighing 5 to 6 pounds were immunized with renin, according to the following general schedule. On days 1 and 15, the rabbits were given an intraperitoneal injection of a mixture of 2.0 ml of complete Freund's adjuvant and an equal volume of acetylated, or untreated, rabbit renin of appropriate concentration (Table II). On all other days (5 days a week for 5 weeks) the same amount of acetylated, or untreated, rabbit renin but without Freund's adjuvant, was injected intraperitoneally. Three days after the last injection, the rabbits were bled by cardiac puncture and the sera were stored at −20°C.

Immunization of dogs: In the dog, the subcutaneous administration of heterologous renin is known to be more effective in producing antirenin than the intraperitoneal administration. Subcutaneous injections of complete Freund's adjuvant, however, induce a marked acute and chronic granulomatous reaction, with ulceration, of skin and subcutaneous tissue. In most of the experiments, therefore, the immunization was carried out without adjuvant, as follows (Table III): The injections were given subcutaneously (occasionally intramuscularly) three times a week, until an adequate titer of antirenin was attained. At frequent intervals, a small sample of blood was obtained from the femoral artery and the serum was tested for antirenin.

In two of the dogs, Freund's incomplete adjuvant was used (Table III, dogs 2-7 and 5-4), and the following schedule of immunization was employed: On the 1st day of the week, 1 ml of the untreated, or of the acetylated, dog renin was mixed briefly with 0.5 ml of adjuvant and injected subcutaneously. On the following 4 days of each week (for 8 weeks), the same amount of renin was injected without adjuvant.

In all cases, the dose of antigen given during the different immunization procedures is described in terms of pressor units. In the case of the acetylated renins, therefore, the degree of inactivation following acetylation has to be considered in determining the total units administered. Thus a weekly dose of 300 pressor units of acetylated dog renin was equivalent to 600 original units, since the acetylated dog renin had a specific pressor activity of only half that of the untreated renin.

RESULTS AND DISCUSSION

The results of the immunization of the rat, rabbit, and dog are summarized in Tables I to III.

TABLE I

Immunization of Rats With Acetylated or Untreated Rat Renin

No. of rats	Treatment of rat renin	Immunization procedure	Titer of antirenin to rat renin, units/ml of serum
2	None	2 units/day, 5 days/wk., for 5 wks., without adjuvant	0
2	None	Same, but with adjuvant	0
2	Acetylation	0.7 unit/day, 5 days/wk., for 5 wks. without adjuvant	0.85
2	Acetylation	Same, but with adjuvant	1.7
2	Acetylation	10 units/day, 5 days/wk. for 3 wks., with adjuvant	3.5

TABLE II

Immunization of Rabbits With Acetylated or Untreated Rabbit Renin

No. of rabbits	Treatment of rabbit renin	Immunization procedure	Titer of antirenin to rabbit renin, units/ml of serum
2	None	10 units/day, 5 days/wk., without adjuvant	0
2	None	Same, but with adjuvant	0
1	None	20 units/day, 5 days/wk., without adjuvant	0
2	Acetylation	5 units/day, 5 days/wk., without adjuvant	2.5
2	Acetylation	Same, but with adjuvant	2.8
3	Acetylation	10 units/day, 5 days/wk., with adjuvant	3.2
2	Acetylation	25 units/day, 5 days/wk., with adjuvant	5.3

It is clear from the results in Tables I to III that homologous renins were not antigenic in the rabbit, rat, and dog, even after administration with Freund's adjuvant, but that they became antigenic and elicited the formation of antirenin, after acetylation. The optimum conditions for the formation of antirenin in this manner were not fully established, but it appears from the results in Tables I to III that, in almost all cases, an increase in the dose of acetylated renin administered resulted in an increase of antirenin formation.

In the dog, the serum levels of antirenin attained in different animals varied from 0.5 unit/ml in dog 5–7, to 8.3 units/ml in dog 8 (Table III).

Such wide variations in individual animals have been observed previously also in the response of dogs to immunization with heterologous (hog) renin (4).

Cross-Reactivity of Antirenin with Untreated and with Acetylated Renin.— The antirenin produced to homologous, acetylated renin in the rat was effective, with approximately equal efficiency, in the inhibition of the pressor activity of untreated and of acetylated rat renin. The same result was found in similar studies in the rabbit.

TABLE III

Immunization of Dogs With Acetylated or Untreated Dog Renin

Dog	Treatment of renin	Weekly dose of renin, units	Immunization period	Titer of antirenin to dog renin, units/ml of serum
			wks.	
2-7*	None	48	8	0
5-4*	Acetylation	48	8	2.1
8	None	300	8	0
8	Acetylation	150	6	1.4
	Acetylation	300	4	8.3
2	Acetylation	300	4	3.4
2-8	Acetylation	150	5	1.4
	Acetylation	300	6	5.4
1-5	Acetylation	300	9	0.8
5-7	Acetylation	300	13	0.5

* Incomplete Freund's adjuvant used for immunization.

The titer of the antirenin produced by the immunization of the dog with acetylated dog renin was first determined for untreated dog renin and then evaluated for its efficiency in the inhibition of acetylated dog renin. In the case of 5 separate preparations of acetylated dog renin, considerable individual variations in the susceptibility to the effect of the antirenin were found. For example, in one preparation, 1 unit of acetylated dog renin was inactivated by 0.5 unit of antirenin. In contrast, in the case of 4 other preparations of acetylated dog renin the amount of antirenin required varied from 0.7 to 6.0 units. It was also found that the greater the degree of inactivation of a preparation of renin during acetylation the larger was the amount of antirenin required for neutralization.

Acetylation lowered the specific activity of rat, rabbit, and dog renin to 35, 46, and 50 per cent on the average, respectively, of the original value. This could be interpreted as an indication of the formation of an altered molecular

configuration of renin, characterized, furthermore, by its capacity to induce the formation of antirenin in the homologous animal. The higher activity of such antirenin against acetylated than against the corresponding untreated renin, which was observed in a few of the experiments with dog renin, lends further support to the concept of an alteration of the molecular configuration of renin by acetylation.

In previous studies, by several investigators, the antibody to acetylated, homologous, rabbit serum albumin (14) and to altered, autologous rabbit

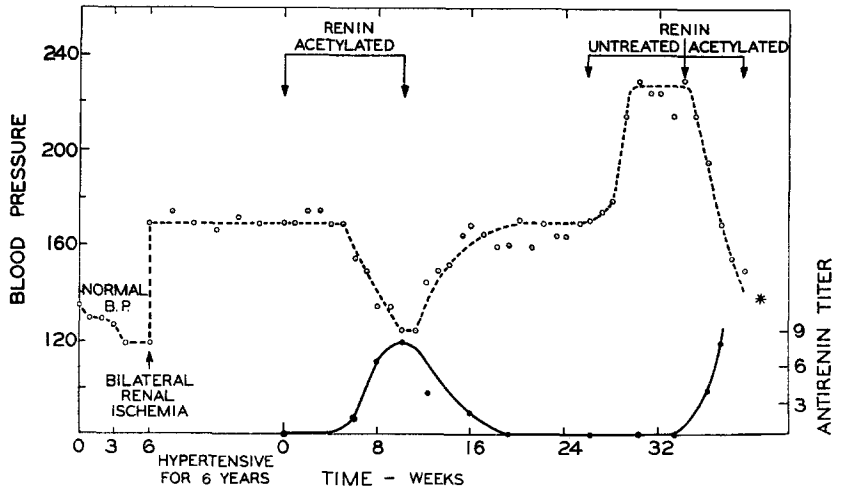

FIG. 1. Immunization of a chronic hypertensive dog with acetylated and with untreated dog renin (Dog 8). Mean femoral artery blood pressure, mm Hg; Antirenin, units per ml of serum.

* Two months after discontinuation of the immunization, blood pressure had risen again to 190 mm Hg.

serum gamma globulin (15, 16) was found to be active against only the treated but not against the native antigen.

Recently, however, Williams and Kunkel (17) have shown that the development of antibody to native rabbit gamma globulin can be demonstrated, by special means, in antiserum produced by the injection of native or treated autologous rabbit gamma globulin.

In our study, only the acetylated, but not the untreated, homologous renin was antigenic, and the antirenin produced neutralized the pressor action of both the untreated and the acetylated renin.

Production of Antirenin to Acetylated Dog Renin in a Hypertensive Dog.— The pressor action of dog renin was abolished by its incubation, *in vitro*, with antirenin which had been produced (Table III) by immunization of the dog with acetylated dog renin. In order to test the efficiency of this antirenin,

in vivo, a chronic renal hypertensive dog (No. 8), was immunized with acetylated dog renin (Table III). This animal had hypertension for more than 6 years, produced by constriction of both main renal arteries. Subcutaneous injections of acetylated dog renin were given on Monday, Wednesday, and Friday throughout the period of the experiment. Mean femoral arterial blood pressure was determined weekly, on Monday morning, before the injection of the renin, and antirenin titers of the blood serum were determined at frequent intervals. The results of the first course of immunization, which lasted 10 weeks, are plotted in Fig. 1.

Starting at about 5 weeks after the beginning of immunization with acetylated dog renin, there was a progressive rise in the antirenin titer of the serum, and the blood pressure fell progressively, until it reached the normotensive level, in about 10 weeks. After the immunization was discontinued, the blood pressure began to rise, gradually reaching the previous hypertensive level, in about 10 weeks, at which time the antirenin titer had dropped to a point below the detectable level.

As a control, untreated dog renin was then administered, for 8 weeks, in the same dog, in the same manner, and in the same weekly amount (300 units) as for the acetylated dog renin (Table III). There was no fall of blood pressure; there was even a significant rise, which remains unexplained. Throughout this period the titer of antirenin remained below the detectable point.

In the 33rd week, the administration of untreated dog renin was discontinued, and a second course of injections of acetylated dog renin was started. This was now followed by a more rapid fall of mean arterial blood pressure and a more rapid rise of the titer of the antirenin than during the first course of immunization with acetylated dog renin. This is characteristic of an anamnestic response.

The results of this experiment are consistent with the concept that the renin-angiotensin pressor system plays a part of primary importance in the maintenance of the blood pressure at an elevated level, even in the chronic phase of experimental hypertension due to renal ischemia, and that this type of hypertension is reversible, even in the chronic phase.

SUMMARY

1. Procedures are described for the extraction and partial purification of dog renin, on a large scale, as well as for the acetylation of rat, rabbit, and dog renin.

2. Untreated homologous renin was not antigenic in rat, rabbit, or dog, but the acetylation of homologous renin made it antigenic.

3. Immunization of rats, rabbits, and dogs, with acetylated rat, rabbit, and dog renin, respectively, resulted in each case in the development of antirenin to the homologous, untreated, as well as to the acetylated renin.

4. The progressive development of antirenin as a result of repeated, sub-

cutaneous injections of acetylated dog renin, in a dog with experimental renal hypertension for more than 6 years, was accompanied by a correspondingly progressive fall of the mean arterial blood pressure to the prehypertensive level. This points up the important part played by the renin-angiotensin mechanism in the maintenance of the hypertension, even in the chronic phase of experimental renal hypertension.

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