Renovascular Hypertension in Rats Immunized with Angiotensin II

By Ivor Eide

ABSTRACT

The experimental design which most closely reproduces clinical renovascular hypertension is constriction of one renal artery, with the other renal artery and kidney left intact. To test the role of renin and angiotensin in the pathogenesis of renovascular hypertension, attempts were made to induce such hypertension in rats previously immunized with angiotensin. In 29 highly immunized and 33 control rats, one renal artery was partially constricted and the other kidney and renal artery left intact. Preoperative blood pressures were equal in all rats (means: immunized, 118 ± 0.95; controls, 117 ± 0.70 mm Hg). Both groups developed hypertension during the 13 days following operation (means: immunized, 173 ± 3.42; controls, 169 ± 4.65 mm Hg). The high blood pressures persisted throughout the observation period (56 days). Immune sera completely inactivated large amounts of angiotensin (mean, 1130 ± SD 887 ng/ml antiserum; range 200—4000), and high intravenous doses of renin and angiotensin had no effect on the blood pressure of immunized rats. These data provide strong evidence that the direct pressor effect of circulating angiotensin is not essential for the development of hypertension evoked by constricting one renal artery in the rat.

KEY WORDS
antigen-antibody reactions antibodies immunoassay juxtaglomerular apparatus kidney diseases renal artery obstruction renin

Rabbits immunized with angiotensin develop hypertension after renal encapsulation or constriction of one renal artery when the contralateral kidney is removed (1–5). It may therefore be questioned whether development of renal hypertension is dependent on an increase in circulating angiotensin.

Renal hypertension of this type, however, has no definite clinical counterpart, as most cases of renovascular hypertension are due to unilateral renal artery stenosis, with the contralateral kidney intact. The experimental parallel, i.e., unilateral renal artery constriction with the opposite kidney untouched, produces hypertension in rats, while other experimental animals usually remain normotensive. Christlieb et al. (6) found such hypertension alleviated by immunization with angiotensin, but this could not be confirmed by Johnston et al. (5). Whatever the reason for the difference in results, both groups started immunization subsequent to the development of hypertension; neither group investigated whether antibody titers were sufficient for the complete inactivation of
injected angiotensin. Renal hypertension may convert into the chronic form within a few weeks and later persist independent of the causative agent (7), whereas sufficient immunization takes several months. Accordingly, the most favorable condition for such experiments would be adequate immunization prior to application of the renal clip.

Whether renovascular hypertension could be induced in rats already highly immunized with angiotensin was therefore investigated by constricting one renal artery and leaving the other kidney in situ.

Methods

Wistar rats, weighing 150–200 g and kept on a standard laboratory diet, were immunized with multiple intramuscular injections of angiotensin (1-Asp-5-Val-angiotensin II amide) coupled to human serum albumin according to the method of Goodfriend et al. (8). During ether anesthesia, 0.7–0.8 mg of conjugate was injected together with Freund’s complete adjuvant at intervals of 3–4 weeks for 11 months.

IMMUNOLOGICAL EVALUATION

The following immunological tests have been described in detail in a previous publication (2).

a. Angiotensin Neutralization Test.—In this test, antiserum or a dilution thereof was incubated with angiotensin for 1 minute in vitro, and 0.05–0.10 ml of the incubation mixture were assayed by the pressor response in the anesthetized normal rat. The highest final dilution of antiserum which completely inactivated the pressor effect of a standardized quantity (40 ng in 1 ml of saline) of angiotensin was taken as the antibody titer. The amount of angiotensin inactivated per milliliter of antiserum might then easily be calculated and used as an index of antibody concentration.

b. Measurement of Angiotensin II Bound to Circulating Antibody.—Immune complexes between angiotensin and antibody in the rat plasmas were dissociated according to Macdonald et al. (4). Plasma samples of 0.5 ml to which had been added 0.3x EDTA (pH 7.0) and 0.2x 2,3 dimeracrol were acidified to pH 2.0, made up to 10 ml with 0.9% NaCl, and extracted exactly as described by Boyd et al. (9). The dried extract was dissolved in 1 ml of immunoassay buffer and 2 x 100 µleters used for duplicate radioimmunoassay. In five experiments, the recovery of 1.6–2.4 ng of angiotensin II added to 0.5 ml normal rat plasma varied between 90 and 115% (mean 105%); when added to immune plasma in six experiments, recovery varied between 88 and 96% (mean 92%). The coefficient of variation for the six experiments on immune plasma was 3.5%.

The angiotensin II radioimmunoassay procedure described previously (2) was made with an antiserum against 5-Val-angiotensin II which bound nearly 70% of added 125I-5-Val-angiotensin II (2,000–2,500 count/min) at a final dilution of 1:256,000 and easily permitted the quantification of 10-pg amounts of 5-Val-angiotensin II. There was nearly complete cross reaction with 5-Ile-angiotensin II but no cross reaction with 5-Ile-angiotensin I within the concentrations used.

c. Injection of Angiotensin, Renin, and Norepinephrine.—Angiotensin, hog renin, and norepinephrine were alternately injected intravenously into the immunized and the control rats, and the carotid blood pressure responses measured with a mercury manometer in the anesthetized, ganglion-blocked rats. Each injection was completed within 4 seconds.

INDUCTION OF RENOVASCULAR HYPERTENSION

At least 14 days after the last immunization, the immunized rats were anesthetized with ether and through a left flank incision a silver clip (2.0 mm wide, 0.2 mm i.d.) was placed on the left renal artery. The right kidney and renal artery were left intact. The same operation was performed on the control rats.

BLOOD PRESSURE DETERMINATIONS

The systolic pressure was taken by the photoelectric method on the tail of the conscious rat, preheated for 5 minutes at 40°C, according to the principle described by Weinman et al. (10). A cuff 2 cm wide was placed proximally, and the distal part of the tail transilluminated with two bulbs driven at low voltage (Fig. 1). The arterial pulses were sensed by photo-resistors and amplified with an integrated high gain amplifier. Respiratory or accidental movements of the rat and electrical noise from the power network were filtered off with a band-pass filter. The output of the high gain amplifier was connected to a Sanborn AC-DC preamplifier. Cuff pressures were simultaneously measured with a Statham transducer (P23A) and a Sanborn carrier preamplifier. Both the pulse tracings and cuff pressures were ultimately recorded with a two-channel Sanborn Recorder, and the mean of five successive readings was taken as the actual systolic pressure.

Figure 2 shows the correlation between blood pressure measured in the tail (y) and aortic systolic blood pressure (x) measured through an indwelling polyethylene catheter in four rats with blood pressures varied during graded bleeding or intravenous administration of angiotensin, norepinephrine, and histamine. The mean ratio between
ANTIIANGIOTENSIN ANTIBODIES

y and x was 0.95 ± 0.061, the regression equation \( y = 5.0 + 1.00 \times x \), and the coefficient of correlation 0.98.

**BLOOD SAMPLING**

Blood for radioimmunological testing was collected in tubes containing EDTA by cutting the tail tip under ether anesthesia. Centrifugation was performed immediately after cooling and 2.3 dimercaprol added subsequently. Otherwise the samples were allowed to coagulate on ice bath and were spun down in a refrigerated centrifuge. Each blood sample amounted to 0.5–1.5 ml. The procedure was found not to influence later blood pressure recordings. Immune plasma and serum were kept frozen at \(-20^\circ\text{C}\).

**STATISTICAL METHODS**

Student's *t*-test was used in comparisons between blood pressures. Wilcoxon's signed rank test for paired comparisons was applied in evaluations of the data obtained with the angiotensin neutralization test.

**Results**

Thirty-eight rats were immunized for 10–11 months, each receiving 14–15 injections. Of these, four rats died during application of the renal arterial clip, and one was excluded because of high blood pressure prior to the operation.

**DEVELOPMENT OF HYPERTENSION**

Twenty-nine rats were considered to be highly immunized on the basis of immunological test results described below. Of these, 26 (89%) developed hypertension upon application of the renal arterial clip (> 160 mm Hg). Corresponding figures for the nonimmunized group were 29 hypertensive out of 33 operated (88%).

Blood pressures were measured in all the immunized and control rats for 7 and 5 days,
FIGURE 3

Mean systolic tail blood pressure in 29 immunized rats and in 33 control rats before and after application of clip on left renal artery. Vertical bars indicate SE of mean.

respectively, prior to the operation (Fig. 3). The corresponding mean blood pressures were 118 ± SE 0.95 and 117 ± 0.70 mm Hg, with no significant difference (0.30 > P > 0.20, Student’s t-test). Within 13 days of the application of the clip, both groups had become hypertensive, with similar values of blood pressure (immunized: 173 ± SE 3.42; controls: 169 ± SE 4.56, 0.30 > P > 0.20) and remained so until killed. Blood pressures of all the rats were measured between 12 and 16 days postoperatively. Six rats were selected at random for measurements at 32 days; 12 on days 41 and 42, and 4 on day 51.

Tail blood pressures in the awake rats were compared with carotid blood pressures performed during light pentobarbital anesthesia. The last tail pressure (y) measured in each rat was paralleled with its carotid systolic blood pressure (x) recorded on the day of sacrifice, and there was fairly good correspondence (mean of y/x = 1.03 ± SD 0.08; regression line y = 18 + 0.92 x; coefficient of correlation r = 0.82). These comparisons were undertaken in 31 immunized rats and 18 controls, comprising both normo- and hypertensives.

In two immunized rats (blood pressures 206 and 163 mm Hg) and in two immunized (208 and 180 mm Hg) the clips were removed at different times within the hypertensive course. In 2 days the blood pressures of these rats fell below 140 mm Hg.

IMMUNOLOGICAL TESTING

In the present work, the efficacy of immunization was tested by the angiotensin neutralization test and by injecting the immunized rats intravenously with angiotensin, renin, and norepinephrine. Both tests measured the capacity of the immunized rats for inactivation of the pressor effect of angiotensin, the former in vitro, the latter in vivo. The amount of angiotensin bound to antibody was tested radioimmunologically.

a. Angiotensin Neutralization Test.—The angiotensin neutralization test was performed on the sera from all 38 immunized rats. Most of the antisera were highly potent, completely inactivating large amounts of angiotensin (Fig. 4). In the 29 rats considered as highly immunized, a mean antibody titer corresponding to 1130 ± SD 887 ng inactivated per ml was found (range 200-4000 ng/ml). Thirteen of these rats were also tested 13-26 days postoperatively, and 22 rats 30-56 days postoperatively. Within these groups, 6 randomly chosen rats were tested both 13-16 and 43-55 days postoperatively.

The results (Table 1) showed an excess of free antibody throughout the hypertensive period, indicating concentrations of circulating angiotensin insufficient for saturation of

FIGURE 4

Angiotensin neutralization test. Sixty microliters of antiserum was incubated with 40 ng of angiotensin in 1 ml of saline for 1 minute; 0.15 ml was injected intravenously (AT + AS) into an immunized rat. Note complete inactivation of the pressor effect. AT = identical injections of angiotensin, but without antiserum added. NaCl = saline, 0.15 ml, i.v. AP = carotid artery pressure.
TABLE 1

Antibody Concentrations Measured with Angiotensin Neutralisation Test in Sera from Immunized Rats, before and after Constriction of the Left Renal Artery

<table>
<thead>
<tr>
<th></th>
<th>Preoperative</th>
<th>13-16 days</th>
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<th>30-56 days</th>
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<tr>
<td><strong>Mean ± SD</strong></td>
<td>1130 ± 887</td>
<td>548 ± 257</td>
<td>664 ± 510</td>
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<td><strong>Range</strong></td>
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<tr>
<td><strong>No. of rats</strong></td>
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<td>6</td>
<td>13</td>
<td>22</td>
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<tr>
<td><strong>Difference</strong></td>
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<tr>
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Values are ng of angiotensin inactivated per ml of antiserum.

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Values are ng of angiotensin inactivated per ml of antiserum. *The rats were randomly selected. †Difference between the means of the corresponding pre- and postoperative concentrations. ‡Wilcoxon's signed rank test, paired comparisons.

the antibody capacity in both early and late stages. In all three groups tested with the angiotensin neutralization test, however, there was a decline in immunological activity with time. Mean reductions (359 and 350 ng/ml) of antibody concentrations were statistically significant only in the largest groups (22 rats tested for 30-56 days, and 13 rats tested for 13-26 days postoperatively), although of equal magnitude (300 ng/ml) also in the smaller group (6 rats tested for 13-16 and 43-55 days postoperatively). The antibody titers were consequently reduced during the postoperative period, the reduction probably being caused by suboptimal conditions for antibody production postoperatively.

Sera from unimmunized, hypertensive, and normotensive control rats were invariably free from inactivating capacity, thus excluding any appreciable effects of angiotensinases during the incubation time (1 min at 4°C). Of four rats excluded for immunological reasons, three had antibody concentrations between 100 and 200 ng/ml, the titers of the fourth were uncertain.

b. Endogenous Angiotensin II Complexed with Antibody.—Angiotensin bound to antibody in immune plasma from 12 hypertensive rats at different times postoperatively ranged between 2.50 and 17.60 ng/ml (mean, 8.42 ± 5.21). The antibody titer in these plasmas varied between 580 and 2000 ng/ml (mean, 1120 ± 465 ng/ml). Accordingly, on average only 0.75% of the antibody binding capacity was occupied by endogenously formed angiotensin. It is therefore obvious that the reduction of free antibody titer postoperatively was due to factors other than partial saturation with angiotensin.

c. Injections of Angiotensin, Renin, and Norepinephrine.—Angiotensin, in single doses of 16 ng, always caused pressor responses in 18 hypertensive control rats (mean, 28 ± 10; range, 12-55 mm Hg). A minimum dose of 16 ng was therefore injected in most (N = 29) of the immunized rats. Seven were injected between days 15 and 25, and 22 between days

![Figure 5](http://circres.ahajournals.org/Downloaded from http://circres.ahajournals.org/)

**FIGURE 5**

Carotid pressure (AP) responses to intravenous injections of angiotensin, renin, and norepinephrine. Top: 32, 80, 500, and 1000 ng of angiotensin (AT) and 0.1 Coldblatt units of renin (R) injected into an immunized, hypertensive rat. Bottom: 16 ng angiotensin and 0.1 Coldblatt units of renin (R) injected into a hypertensive control rat. NE = 32 ng; NE* = 16 ng of norepinephrine. Note the complete inactivation of the pressor effect of angiotensin and renin in the immunized rat.
The former period coincided with the final development of hypertension, and the latter with more stabilized blood pressures. The entire hypertensive period was accordingly covered by the injection tests and the angiotensin neutralization test. There were no differences between early and late responses. Thirteen rats gave no responses to 80 ng, 6 rats completely inactivated single doses between 32 and 48 ng, and 5 rats inactivated between 16 and 32 ng of angiotensin (mean of 24 rats, 57 ± 26). One rat gave no response to 1000 ng (Fig. 5). In performing these injections, the doses were not increased in excess of the inactivating capacity for angiotensin. Four rats reacted to 16 ng with elevations of blood pressure and were therefore considered to be insufficiently immunized; these rats also presented low titers with the neutralization test (see above).

From 15 hypertensive, immunized rats chosen at random from the total material, 13 were selected as highly immunized. On increasing injections, faint pressor responses (mean 9.9 ± 5.7 mm Hg) first appeared in these rats at doses averaging 490 ng (range 80–1000), which was therefore taken as the threshold dose of peptide giving measurable pressor responses. The effect of these doses, calculated per ng angiotensin, was on average only 1.1% of that in unimmunized, hypertensive rats. By comparison, the effect of norepinephrine in immunized rats was 0.74 ± 0.29 mm Hg/ng, and in unimmunized rats 0.87 ± 0.24 mm Hg/ng; this difference was not, however, statistically significant (0.20 > P > 0.10). Correspondingly, in the immunized rats the pressor effect of angiotensin averaged 3.5% and in control rats 189% that of norepinephrine. Decreased responses to angiotensin in the immunized rats were not, therefore, caused by a general decline in vascular reactivity.

Intravenous injections of 0.1 Goldblatt units of hog renin invariably caused pressor responses (mean, 29.9 ± 3.57; range, 9.02–51 mm Hg) in unimmunized, hypertensive control rats. The same dose caused no elevations in blood pressure when injected into the hypertensive rats that were considered as highly immunized (Fig. 5). Endogenously formed angiotensin was therefore inactivated by the antiangiotensin antibodies. The four rats that were considered to be insufficiently immunized all responded to this dose with elevations of blood pressure.

d. Comparison between Immunological Tests a and c.—In each of the 15 rats injected with large doses, the pressor effects of angiotensin were divided by that of norepinephrine to correct for differences in pressor reactivity. By plotting these ratios along the ordinate and the antibody concentrations along the abscissa, a nonlinear correlation approaching an exponential course was found, which indicated insufficient inactivating capacity only for the rats with the lowest antibody titers (Fig. 6). Four rats with technically failed injection tests (2 died during anesthesia, and 2 bled to death because of unsuccessful cannulation of the carotid artery), but
Maximal blood pressures in 33 rats (solid circles) with different antiangiotensin antibody titers, comprising rats with high and low titers. Note absence of correlation. Mean of maximal blood pressures in 33 control rats (open circle) was 191 mm Hg ± 2 s, indicated by vertical bars.

Discussion

In 1943, Fell et al. (11) demonstrated that active immunization with histamine protected rabbits against anaphylactic responses to ovalbumin. Endogenously formed histamine could thus be immunologically inactivated. Similarly, the effects of endogenous gastrin (12), insulin (13), and angiotensin (1) were abolished by immunization with the respective substances. Immunization with insulin not only elevated blood glucose concentrations but also effected permanent diabetes (13).

Accordingly, several examples illustrate a pathogenetic role of, or conversely, a prophylactic effect of antibodies against physiologically or pathophysiologically active low-molecular substances. A reasonable hypothesis would therefore be that renal hypertension could be precluded by immunization with angiotensin, if this substance were of prime importance in the pathogenesis.

In the immunized rats of the present study, an excess of free antibody persisted throughout the hypertensive period (from postoperative day 13), with sufficient titers to completely neutralize large amounts of angiotensin, greatly exceeding ordinary pressor doses for

relatively high antibody titers (mean, 640 ng/ml), were therefore judged to be highly immunized. The total material of highly immunized rats accordingly amounted to 29 (25 with successful injection tests, and 4 with high antibody titers as determined by the angiotensin neutralization test).

In 17 unimmunized hypertensive control rats there was a linear correlation between the pressor responses to angiotensin (y) and to norepinephrine (x) \( y = -21 + 2.12 x \); coefficient of correlation 0.76).

Relation between Antibody Titer and Hypertension.—If circulating angiotensin were responsible for renovascular hypertension, higher pressure levels would be expected in rats with low antibody titers than in those with high titers. In Figure 7, the maximal blood pressures (y) of the immunized rats \( n = 33 \) surviving application of the clip, including those with poor titers, were plotted against the concentrations of antibody (x). Blood pressures were found to be virtually uninfluenced by antibodies against angiotensin (regression line \( y = 186 + 0.0025 x \); coefficient of correlation 0.1; Student's t-test on the regression coefficient, 0.60 > P > 0.50).
hypertensive rats. However, a significant decrease in free antibody titers during the postoperative period was observed. This decline was not caused by partial saturation of antibody, since in the hypertensive immunized rats an average of less than 1% of the antibody was complexed with angiotensin. The great majority of antibody binding sites consequently remained free, and it is therefore highly unlikely that hypertension was caused by unbound angiotensin. Similar findings were made by Macdonald et al. (4), who, in addition, increased the concentration of complex in immunized rabbits 40 times, although blood pressure remained unaltered.

In the immunized animal there must always be a breakthrough point at which the pressor effect begins to be seen. The localization of this point would indicate how far the dose response curve had been moved to the right in the immunized versus the unimmunized animals. The rabbits of Macdonald et al. (4) were also examined with large injections of angiotensin, and the threshold dose was 0.8 mg, i.e., about 0.32 µg/kg body weight. In the present work, the corresponding dose was 0.49 µg to immunized rats, i.e., on average 2.45 µg/kg. Consequently, the rats were at least as efficiently immunized as the rabbits. The threshold dose in unimmunized hypertensive rats was 0.004-0.010 µg, which demonstrates the great inactivating capacity of antiangiotensin antibodies.

The conditions for single injections are certainly somewhat different from those existing during continuous infusion. However, supposing a biological half-life of 1–2 minutes for circulating angiotensin (14), injections of 0.49 µg to rats with a blood volume of 10 ml within a few seconds (vein-artery circulation time) would increase the concentration in blood by some 50 ng/ml. This increase is several times the concentration of complexed angiotensin found in the immunized rats of the present study, and would certainly (according to the law of mass action) militate against unbound angiotensin as the direct cause of hypertension. This conclusion is reinforced by the demonstration of the consid-

erably reduced metabolic clearance rates of angiotensin in immunized animals (4).

However, it has not yet been proved that antibodies are capable of inactivating any effect of angiotensin, and hypertension could have been caused by some indirect mechanism. In this context the results of Peach (15) are of special interest. He found that antiangiotensin antibodies abolished the catecholamine-releasing effect of angiotensin II during in situ perfusion of the cat adrenal gland. Although the true significance of adrenal catecholamines in the development of renovascular hypertension is unknown, his data demonstrate another effect of angiotensin which is inactivated by antibodies. A similar example is the sympathetic stimulating effect of angiotensin demonstrated by Aars and Akre (16). This effect is absent in rabbits immunized with angiotensin (17). But neither the stimulating effect on secretion of aldosterone nor the central nervous action demonstrated by Dickinson (18) have yet been examined. The present results indicate that the closest experimental reproduction of hypertension due to unilateral renal artery stenosis in man may develop independent of the direct pressor effect of circulating angiotensin II.

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