Renal-Clip Hypertension in Rabbits Immunized Against Angiotensin II

By Graham J. Macdonald, M.B., B.Sc, William J. Louis, M.D. (Melb.), Vincenzo Renzini, M.D. (Perugia), Graham W. Boyd, M.D., Ph.D., and W. Stanley Peart, M.D.

ABSTRACT

The rabbit immunized against angiotensin II was shown to be a valid model for the study of renal-clip hypertension. In particular, there was a specific and complete blockade of the pressor effect of high doses of intravenous renin and angiotensin in vivo, even at angiotensin II production rates which far exceeded those associated with renal-clip hypertension. Despite this, four immunized rabbits developed hypertension after renal-artery clipping with contralateral nephrectomy, and in three of these the hypertension was severe. In four other rabbits, there was no evidence of modification of an established hypertension after immunization against angiotensin II. In both groups, the specific absence of pressor response to high doses of renin and angiotensin II after immunization was confirmed. These studies provide strong evidence that angiotensin is not the sole or even the major factor in either the initiation or maintenance of this form of hypertension.

ADDITIONAL KEY WORDS

renin plasma angiotensin radioimmunoassay angiotensin II production rate noradrenaline metabolic clearance rate antibody-bound angiotensin II

- Although there is often a good correlation between plasma levels of hormones and their biological effects, this is perhaps not always the case. For example, levels of angiotensin may give no indication of production rates, metabolic clearance rates and changes in sensitivity. Even when all these measurements can be made it is still not certain that they will give unequivocal data on the role of a hormone such as angiotensin in initiating and maintaining disorders like hypertension. Specific blocking agents would obviously be extremely useful in this regard.

In the case of angiotensin, attempts have been made to overcome this lack of a specific blocking agent. For example, Drury and his associates (1, 2) studied rabbits with renal hypertension in which tachyphylaxis to renin was induced with large doses of heterologous renin. They found that this procedure caused no notable reduction in the degree of hypertension. However, these were complicated experiments and have not received widespread recognition, perhaps because of a reluctance to apply results obtained acutely to a chronic disease such as hypertension. Other approaches to this problem include immunization against crude renal extracts (3-6) and the chronic administration of phospholipid compound which Sen et al. (7) have suggested is a specific inhibitor of renin in vitro and in vivo.

The recent development of specific antibodies to angiotensin II (8-12) has suggested that chronic immunization could be used to study the action of angiotensin specifically in various situations, including hypertension (12-14), and we have applied this approach to the study of renal-clip hypertension in the rabbit. Previously Eide and Aars (13) have reported
that angiotensin II immunization in the rabbit did not affect the development of hypertension produced by wrapping the kidneys in silk soaked in turpentine, but it is by no means certain the mechanism of this form of hypertension is the same as that associated with a renal-artery clip. In an investigation of renal-clip hypertension in rats, Christlieb et al. (14) suggested that angiotensin II immunization may reduce the elevated blood pressure, but interpretation of their studies is difficult since antiangiotensin titers were very low and poorly correlated with the variable falls in blood pressure.

Before attempting to apply this approach to a study of renal-artery clip hypertension in the rabbit, it seemed to us essential to first demonstrate that the angiotensin-immune rabbit provided a valid experimental model for this purpose. This was established, and the experimental model was studied in relation to renal-clip hypertension. It was shown that prior immunization against angiotensin II in the rabbit does not prevent the development of renal-clip hypertension, and that immunization of rabbits already hypertensive does not lower blood pressure. A preliminary account of this work has been reported previously (15).

**Materials and Methods**

Studies were performed in conscious unrestrained cross-bred rabbits weighing 2 to 3 kg and fed a standard laboratory pelleted diet. Hypertension was induced by carrying out a right nephrectomy and placing a silver clip 6 mm long and 5 mm wide with a gap of 0.55 mm on the left renal artery. The operation was performed under thiopentone-sodium pentobarbital anesthesia (4).

**Blood Pressure Measurement.**—Blood pressure was measured daily using an ear capsule (18). Readings were made in a warmed room (58 to 30°C) by two observers independently and without reference to previous measurements. On each occasion, multiple readings were taken until five consecutive identical values were obtained. With this technique the day-to-day variability in 48 normotensive rabbits was 4.2 mm Hg (SD) or 5.1% (coefficient of variation); that of rabbits with established hypertension was 4.8 mm Hg (SD) or 4.0% (coefficient of variation).

During all intravenous infusions and injections (given via an ear-vein cannula inserted under local anesthesia), blood pressure responses were recorded directly from a polyethylene cannula passed down the central ear artery under local anesthesia and connected to a Devices strain-gauge-recorder system.

**Blood Sampling.**—Arterial samples were made from a two-way tap in the arterial pressure-recording line. Venous blood samples were taken directly from the marginal ear vein after dilatation of vessels locally with topical xylene, and application of silicone grease to prevent hemolysis.

**Immunization.**—Rabbits were immunized against valyl-5-angiotensin II amide adsorbed onto carbon, as reported previously (12).

**Plasma Angiotensin Immunoassay.**—The radioimmunoassay for determining plasma levels of angiotensin II has been described previously (17). In the present studies, angiotensin assays were performed on smaller quantities of blood using proportionately less 0.2M 2, 3 dimercaprol (BAL) and 0.3M EDTA (pH 7) for angiotensinase inhibition. Plasma samples were made up to 10 ml with sterile 0.9% NaCL and extracted with Fuller's earth exactly as described previously for angiotensin I in human plasma (18). Recovery of added angiotensin II (0.1 to 1 ng/ml) from blood was 75% (n = 5) and from plasma 85 to 94% (n = 4). Replicate estimations were made in 7 experiments in which the plasma angiotensin II level ranged from 10 to 200 pg/ml; the coefficient of variation was 6.8%.

Measurement of angiotensin II bound to circulating antibody was made by acidifying plasma to pH 2 with 1N HCl prior to extraction as to dissociate the antigen-antibody complex. In three experiments, recovery of 5 ng angiotensin II added to immune plasma and extracted in this way ranged from 70 to 85%. In seven experiments in which bound endogenous plasma angiotensin II ranged from 1 to 10 ng/ml, analysis of replicate estimates showed a coefficient of variation of 4.4%.

Angiotensin II immunosassays were performed with 125I-valyl-5-angiotensin II amide and standardized against valyl-5-angiotensin II amide. Although angiotensin antibodies were produced against valyl-5-angiotensin II, the particular one used in the assay showed complete cross reaction with isoleucyl-5-angiotensin II.

Antibody titer—In-vitro titers of antibody were assessed by both immunoassay and bioassay. On immunoassay, antibody titer was expressed as the dilution of plasma at which 0.25 ml bound 80% of a 100 pg 125I-angiotensin II label after 18 hours at 4°C. The bioassay measurement described previously (15) was expressed as the amount of valyl-5-angiotensin II pressor activity.
neutralized per milliliter of plasma after incubation at 0°C for 1 minute, as assessed in the anesthetized ganglion-blocked rat. Blood samples were taken into BAL-EDTA, which inhibited angiotensinase and prevented it from stripping the antibody-angiotensin II complex and hence giving falsely high results of "free" antibody titer. With some antibodies, EDTA has been shown to unbind the antigen-antibody complex (17) but in the present study this effect was overcome by adding calcium (0.02M) to the immunoassay buffer.

In-vivo antibody activity was assessed by measuring pressor responses to rabbit renin, valyl-5-angiotensin II amide, and isoleucyl-5-angiotensin II in comparison with norepinephrine.

Metabolic Clearance Rates of Angiotensin II from Plasma.—Constant infusions of isoleucyl-5-angiotensin II, 15 to 85 ng/min, were used to determine the angiotensin II metabolic clearance rates (M.C.R.) in conscious rabbits. By infusing angiotensin II at the standard rate (r) until a constant plasma level (x) was achieved, the metabolic clearance rate of angiotensin II from plasma could be calculated from the formula: M.C.R. = r/x (19, 20).

Having determined the metabolic clearance rate of angiotensin II in any given situation, the endogenous production rate (R) of angiotensin II in vivo could be calculated from a knowledge of the circulating basal plasma angiotensin II level (Xb), using a rearrangement of the same formula: R = M.C.R. * Xb.

Drugs used were angiotensin (valyl-5-angiotensin II amide: Hypertensin (Ciba); L-noradrenaline bitartrate B.P. (Koch-Light) and rabbit renin (MRC 66/133) obtained from the Division of Biological Standards, National Institute for Medical Research, Mill Hill, London. The rabbit renin unit used was the same as that described elsewhere for pig renin (21).

Results

PART 1. ANGIOTENSIN II-IMMUNE RABBIT AS A VALID EXPERIMENTAL MODEL

Before the animal actively immunized against angiotensin II could be accepted as a valid model for the study of renal-clip hypertension, it was essential to demonstrate (a) that the antibody produced by immunization specifically binds the naturally occurring angiotensin II of the species concerned; (b) that levels of antibody and the rate of antigen-antibody interaction are such that complete neutralization of the action of angiotensin II occurs in vivo as well as in vitro; (c) that the antigen-antibody complex is biologically inactive; (d) that immunization does not produce a nonspecific change in the animal, either in the basal state or in its response to other agents; (e) that the antibody capacity is not saturated in vivo by any increase in endogenous angiotensin II production which might occur either as a compensatory response to the development of antibodies, or as a result of the experimental condition under study, viz., in this case, hypertension.

Immunological Characterization of the Type of Angiotensin II Present in the Rabbit.—Angiotensin II was generated in vivo rather than in vitro for this investigation, since it seemed likely from the work of Ng and Vane (22) that there would be very little limitation to the conversion of angiotensin in vivo, and so all angiotensin II necessary for accurate bioassay, rabbit renin equivalent to

![Figure 1](http://circres.ahajournals.org/)

**FIGURE 1** Characterization of the type of angiotensin II present in the rabbit. Rabbit angiotensin, prepared as described in the text, was immunoassayed using an antibody that differentiated between valyl-5-angiotensin II amide (Val^5^AII Amide) and isoleucyl-5-angiotensin II (Ile^5^AII). The curves were obtained by incubating constant amounts of ^125^I-valyl-5-angiotensin II amide (100 pg) and antibody (final dilution, 1:2400) with varying amounts of each angiotensin for 18 hours at 4°C. It is clear that rabbit angiotensin behaved immunologically as isoleucyl-5-angiotensin II, and not as valyl-5-angiotensin II.
2,000 pig renin units was injected intravenously as a single dose into a rabbit actively immunized against valyl-5-angiotensin II amide. Ten minutes later, 8 ml of blood was drawn into BAL and EDTA and centrifuged at 4°C. The resulting plasma was acidified to pH 2 to dissociate the endogenously formed angiotensin from the antibody, and the freed angiotensin extracted with Fuller's earth in the usual way (see Methods). The dried angiotensin was then dissolved in immunoassay buffer and its potency (ng/ml) determined by comparison with valyl-5-angiotensin II using either bioassay (23) or immunoassay with an antibody which did not discriminate between valyl-5- and isoleucyl-5-angiotensin II. A close correlation (within 5%) was seen between these two methods of assay, which makes it unlikely that inert peptide fragments were interfering with the immunoassay in these circumstances. Having established the number of nanograms per milliliter of rabbit angiotensin present, the unknown solution was again subjected to immunoassay, but this time with an antibody which did distinguish between valyl-5- and isoleucyl-5-angiotensin II in that about four times as much isoleucyl-5-angiotensin II was necessary to produce any given degree of inhibition on the standard curve. The result is shown in Figure 1, where it is clear that rabbit angiotensin II behaves immunologically as isoleucyl-5-angiotensin II and not as the valyl-5 compound. Therefore, provided that there is not a third form of the hormone which behaves immunologically as isoleucyl-5-angiotensin II it seems probable that rabbit angiotensin is the isoleucyl-5 form, as in porcine, equine and human angiotensin (24-26) rather than the valyl-5 form which occurs in the ox (27, 28).

Antibody Binding In Vitro and In Vivo.—In vitro, the immunoassay titer and bioassay capacity measurements gave reliable results and there was a good correlation between the two (Fig. 2). It is of interest that once developed, bioassay titers of 500 ng angiotensin II/ml and more were maintained indefinitely, provided rabbits were given boosting injections once every 4 to 6 weeks. The high specificity of this type of antibody, as tested with various fragments and analogues of angiotensin II has been reported previously (29).

In-vivo binding of antibody was also measured, but in this and all other in-vivo studies, only rabbits with in-vitro bioassay titers over 700 ng/ml were used. These studies showed a specific blockade of intravenous renin in immunized rabbits. 200 units of rabbit renin increased the level of endogenous angiotensin II bound to antibody by approximately 2.0 ng/ml, but although this is extremely high in comparison with the usual level of circulating free angiotensin II, it represented only 0.36% of the total circulating antibody capacity and was not associated with any rise in blood pressure.

The effect of immunization on the blood pressure response to intravenous renin is
summarized in Figure 3. It is apparent that rabbit renin in the dosage used produced large rises in blood pressure in nonimmune rabbits and that hypertensive rabbits may have shown a somewhat increased sensitivity. By contrast, none of the immunized animals showed any response to 400 and 800 units of renin intravenously, and four out of five showed no response to 1600 units as well. The blood pressure rise in the one rabbit which did respond to 1600 units was both small and transient. Blood pressure responses to norepinephrine are also shown in Figure 3.

Figure 4 shows that dose-response curves for angiotensin II injections in normal and hypertensive rabbits are parallel, but it is clear that valyl-5-angiotensin II amide had no effect in the immunized rabbit until doses greater than 0.8 μg were given, and even then, amounts some 200 times greater than normal were needed to raise the blood pressure by, say, 20 mm Hg. A similar result was obtained when angiotensin II was infused into immune rabbits (Fig. 4). It is also interesting to note with increasing doses of angiotensin in the immunized rabbit.

Biologic Inactivity of the Antigen-Antibody Complex.—This was demonstrated during the in-vivo experiments above, in which it was shown that high levels of antibody-bound angiotensin II were not associated with any increase in blood pressure. When as much as 2000 units of rabbit renin were injected intravenously in an immunized rabbit, levels of antibody-bound angiotensin II rose by up to 30 ng/ml without any increase in blood pressure.

Lack of Nonspecific Alteration of Pressor Responsiveness in the Immunized Rabbit.—This was evident from the normal or slightly increased blood pressure response to norepinephrine in the immunized animal in comparison with nonimmune controls (Fig. 5).

Quantification of the Plasma Levels and Production Rates of Angiotensin II after Immunization and Development of Hypertension.—In the use of the actively immunized animal as an experimental model for the study of hypertension, it was important that the
INJECTION OF ANGIIOTENSI I I (FIGURE 4

Dose-response curves for injections and infusions of valyl-S-angiotensin II amide in normotensive (•—•) and hypertensive nonimmune (o o) rabbits and in immune rabbits (• •).

antibody capacity in vivo was not saturated by any increase in endogenous angiotensin II production which might occur either as a compensatory response to the development of neutralizing antibodies or as a result of the hypertension itself.

In Figure 5 are shown the levels of plasma venous angiotensin II (and blood urea) in normal rabbits and in rabbits with a left renal-artery clip and right nephrectomy. The mean angiotensin II level in 17 normal rabbits was 40 ± 5.1 (se) pg/ml. Significant elevation did not occur in 18 rabbits with mild hypertension (systolic blood pressure elevated less than 25 mm Hg), the mean value for angiotensin II being 47 ± 5.4 (se) pg/ml (P > 0.3). In 16 severely hypertensive rabbits the mean angiotensin II level of 64 ± 10.4 (se) pg/ml was higher than normal (P < 0.05), although it is evident that there was a wide scatter of the individual results.

Mean blood ureas were significantly elevated in hypertensive rabbits (P < 0.01). The individual values were: normals, 39 ± 1.8 (se) mg/100 ml; mild hypertensives, 45 ± 2.9 mg/100 ml; severe hypertensives, 57 ± 3.1 mg/100 ml.

Table 1 summarizes the results of studies on metabolic clearance rates and secretion rates of angiotensin II in normotensive and hypertensive nonimmune rabbits and in immune rabbits. All angiotensin II levels are in arterial blood. Prior to infusion, basal levels of plasma angiotensin II were low in nonimmune rabbits (9 to 28 pg/ml). By contrast, in immune rabbits the plasma level of antibody-bound angiotensin II (recovered by dissociation at pH 2) was high (760 to 2768 pg/ml).

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

Basal levels of plasma venous angiotensin II (•) and blood urea (o) in normotensive rabbits and in rabbits with mild and severe renal-clip hypertension. Mean ± se is indicated.
Metabolic Clearance Rates and Secretion Rates of Angiotensin II Derived from Arterial Levels of Angiotensin II during Infusions of Isoleucyl-5-Angiotensin II in Normotensive and Hypertensive Non-immune Rabbits and Immune Rabbits

<table>
<thead>
<tr>
<th>Type of rabbit</th>
<th>Basal</th>
<th>15-30 ng/min infusion</th>
<th>45-50 ng/min infusion</th>
<th>M.C.R. (ml/min)</th>
<th>Secretion rate (ng/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive nonimmune</td>
<td>541</td>
<td>19</td>
<td>80</td>
<td>117</td>
<td>322-332</td>
</tr>
<tr>
<td>Hypertensive nonimmune</td>
<td>872</td>
<td>18</td>
<td>80</td>
<td>90</td>
<td>292</td>
</tr>
<tr>
<td>Immune*</td>
<td>701</td>
<td>9</td>
<td>63</td>
<td>64</td>
<td>261</td>
</tr>
<tr>
<td>Immune*</td>
<td>800</td>
<td>269</td>
<td>5725</td>
<td>11750</td>
<td>6-7-4</td>
</tr>
</tbody>
</table>

*The angiotensin II level in immune plasma refers to antibody-bound angiotensin recoverable after acidification to pH 2.

However, these values still represented less than 0.3% of the total antibody capacity to bind angiotensin II as calculated in vitro by bioassay (see Methods).

This absence of saturation of antibody in the immune rabbit was confirmed in studies of metabolic clearance rates of angiotensin II from plasma during angiotensin infusions (Table 1). Firstly, clearance values obtained in immunized rabbits (5.9 to 9.3 ml/min) were very much lower than those of normal rabbits, either hypertensive or normotensive (199 to 383 ml/min) and since these data were calculated from the total plasma angiotensin II level (free, antibody-bound, or both) this reflects a high degree of protection of infused angiotensin II from clearance, due to the presence of circulating antibody. Corresponding with this, it was observed that when isoleucyl-5-angiotensin II was infused at rates of up to 85 ng/min, total plasma angiotensin II levels in immunized rabbits rose some 30 times higher than in nonimmune rabbits (Table 1). Secondly, a threefold increase of angiotensin II infusion rate in the immunized animal trebled the amount of angiotensin recovered from circulating plasma antibody and hence the metabolic clearance rate of angiotensin II bound to antibody in arterial plasma during angiotensin infusions in an immune rabbit. The infusion rates used were 13 and 40 ng/min. A plateau was reached after about 40 min, and these equilibrium values were used to calculate metabolic clearance rate.
Production of hypertension in angiotensin-immunized rabbits (251, 790, 794) by left renal artery constriction and contralateral nephrectomy. Each point represents the mean weekly systolic pressure. The shaded area represents 1 SD above and below mean systolic blood pressure in 36 nonimmune rabbits with left renal artery constriction and contralateral nephrectomy.

Angiotensin II from plasma remained low and constant (Fig. 6). Such a quantitative relationship would be expected to hold only if the increment of infused angiotensin were totally bound to antibody. Thirdly, there was no increase in blood pressure in response to the angiotensin II infusions in the immunized animal.

Endogenous angiotensin II production rates were also calculated (see Methods) in normotensive and hypertensive nonimmune rabbits and in immune rabbits (Table 1). The values obtained in three normal rabbits ranged from 6 to 8 ng/min. There was no evidence of any increased angiotensin II production in the two hypertensive rabbits studied; in fact, the actual levels recorded were somewhat below this normal range. In the three immunized rabbits (790, 791, and 800), the calculated secretion rates varied from 6.3 ng/min to 21.9 ng/min.

It should be stressed that it was not intended that these studies of metabolic clearance rates and secretion rates of angiotensin II in hypertensive rabbits should be definitive in their own right but merely that they should provide an indication of the amounts of angiotensin II which would have to be neutralized in a study of renal-clip hypertension. Thus the highest level of angiotensin II seen in hypertensive nonimmune rabbits was 140 pg/ml (Fig. 5) and the mean metabolic clearance rate in these rabbits was 263 ml/min. This gave a calculated maximum angiotensin II production rate in the hypertensive immune rabbit of approximately 37 ng/min. Allowing for the low metabolic clearance rate of antibody-bound angiotensin in the immune rabbit, it was calculated that the maximum level of bound angiotensin II in the hypertensive immune rabbit would be 5 ng/ml, which is still less than 1% of the total antibody-binding capacity at the levels of immunization studied.
PART 2. STUDY OF RENAL-CLIP HYPERTENSION IN THE ANGIOTENSIN II-IMMUNE RABBIT

Assessment of Hypertension.—The average systolic blood pressure in 48 normal rabbits, determined by the Grant-Rothschild ear capsule, was 83 ± 5 (so) mm Hg. Hypertension was considered to be present when the systolic blood pressure consistently exceeded 95 mm Hg. In most studies confirmation of the presence or absence of hypertension was obtained by direct arterial pressure recording (e.g., Table 2).

Effect of Immunization on Basal Blood Pressure.—Chronic immunization by itself had no significant effect on blood pressure, the average systolic pressure of seven undipped rabbits which developed antibody titers on immunoassay greater than 1:500 being 82 ± 7 (SD) mm Hg (P = 0.6).

Development of Renal-Clip Hypertension in the Angiotensin II-Immune Rabbit.—Figure 7 summarizes systolic blood pressure levels in three immunized rabbits before and after nephrectomy and left renal artery cupping. It is apparent that in rabbits 251 and 794 there was a rapid rise in blood pressure to clearly hypertensive levels. Rabbit 790 showed a slower but definite and sustained rise. The presence of hypertension was confirmed in all three animals by direct arterial pressure recording (Table 2). The two rabbits with severe hypertension died of hypertensive complications—no. 251 at 3 weeks and no. 794 at 4 weeks after renal-artery clipping. Two days before death, blood urea concentrations in these two rabbits were 69 and 37 mg/100 ml, respectively. At autopsy, both had gross cardiomegaly, pleural effusions and pulmonary edema.

In-vitro assessment of the degree of immunization was made prior to renal-artery clipping and again when hypertension had developed. In the bioassay angiotensin II neutralization test, 1 ml of plasma from the immune rabbits bound at least 750 ng angiotensin II amide on bioassay, and titers on radiomunoassay exceeded 1:2500 (see Methods). In vivo studies carried out when hypertension was established showed the complete absence of a pressor response to
Intravenous injections of up to 800-1600 units of rabbit renin and 0.8 μg angiotensin II amide, doses which caused a marked elevation of blood pressure in normal and hypertensive nonimmune rabbits (Table 2). Infusions of valyl-5-angiotensin II amide at 0.2 μg/kg/min also had no pressor effect. If huge doses of angiotensin II (51 μg iv) were given, definite pressor responses could be obtained which were similar to those obtained with 0.8 μg angiotensin in hypertensive nonimmune rabbits. Responses to intravenous injections of noradrenaline were, in contrast, slightly augmented in the hypertensive immune rabbit (Table 2).

**Effect of Angiotensin II Immunization on Established Renal-Clip Hypertension.**—Figure 8 shows the effects of angiotensin II immunization in four rabbits with established hypertension. Rabbits 748, 767 and 791 all had elevated blood pressures of 120 mm Hg or greater. Five weeks after immunization, rabbits 748 and 767 had immunosassay titers of 1:50 and 1:150, respectively. Both rabbits died from complications of hypertension before the effect of intravenous injections of renin, angiotensin and norepinephrine could be investigated. Rabbit 791 developed a immunosassay titer of 1:800 six weeks after immunization, and bioassay at this stage showed that 1 ml of plasma neutralized 1.2 μg of angiotensin II amide. In-vivo studies in this animal then showed that neither the intravenous injection of 1600 units of rabbit renin nor of 0.8 μg of angiotensin II amide caused any increase of blood pressure. Angiotensin II infused at 0.2 μg/kg/min for 10 minutes also had no pressor effect, but the pressor response to norepinephrine was normal (Table 2).

Despite this specific insensitivity to exogenous and endogenous angiotensin II, systolic blood pressure remained very high (Fig. 8). At this point the clip was removed from the left renal artery of rabbit 791 and blood pressure fell to normal levels in 8 hours (Fig. 9).

The effect of chronic immunization on a rabbit with a milder degree of hypertension (no. 710) is also seen in Figure 8. Blood pressure rose to a stable level of approximately 105 mm Hg before immunization, and this was maintained despite the development of high antibody titers both on immunoassay (1:3000) and bioassay (neutralization of 1.4 μg angiotensin II/1 ml of plasma). These titers were maintained over 6 months without any change in the level of blood pressure. In-vivo studies again showed the complete absence of a pressor response to large doses of renin and angiotensin II amide but a normal or augmented response to norepinephrine (Table 2).

Figure 10 shows a further experiment.
Rise in systolic blood pressure after left renal artery constriction and contralateral nephrectomy in an angiotensin-immune rabbit (791) and in normal control (817). Once systolic pressures reached 130 mm Hg, the clips were removed under general anesthesia, with a subsequent steep fall in blood pressure to normotensive levels in both animals.

Effect of angiotensin immunization on systolic blood pressure in a rabbit with established renal-clip hypertension. Antibody titers are shown either as a dilution on immunoassay or, in parentheses, on bioassay. After the application of the first clip the early fall in blood pressure rose and then fell before the development of detectable antibody titer. Severe hypertension resulted from the application of a second clip, despite the presence at this stage of high titer of antibody.

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(rabbit 800) in which immunization was commenced after the development of moderate hypertension due to the usual procedure of left renal-artery clipping with contralateral nephrectomy. In this case the blood pressure fell soon after the primary immunization procedure, but since no antiangiotensin could be detected on immun assay at this stage, it was possible that, as occurs from time to time, a spontaneous fall in blood pressure had occurred. To investigate this, a second clip (0.55-mm gap) was placed on the left renal artery after boosting injections had produced substantial titers of antibody. Subsequently, severe hypertension developed despite increasing levels of antiangiotensin. At this stage the animal was unresponsive to large doses of renin but had a normal response to norepinephrine (Table 2).

In all of these studies, the definitive criterion of effective immunization against angiotensin II was the complete absence of a pressor response to both intravenous angiotensin II and rabbit renin. Nonetheless, it was of interest to examine the extent to which antibody capacity was saturated in vivo by endogenous angiotensin II production in the hypertensive immune animal. To do this, bound angiotensin II in plasma was dissociated from antibody by acidification to pH 2 prior to extraction (see Methods). As in the normotensive immune rabbit (see Part 1), this study showed that although the levels of angiotensin II bound to antibody in five hypertensive immune animals were as high as 2 ng/ml, this still represented less than 0.5% of the total antibody capacity for angiotensin II, as determined by the in-vitro bioassay neutralization test.

Figure 9 illustrates the relatively slow rise in blood pressure which may occur after renal-artery clipping. On removal of the clip, however, blood pressure may fall rapidly to normotensive levels within 24 hours. Of the two rabbits shown, one (791) was immune at the time of removal of the clip and the other (817) nonimmune.

Discussion
It is evident from our studies that the rabbit immunized against angiotensin II amide adsorbed on carbon (12) provided a valid model for the study of the role of angiotensin in renal-clip hypertension. Firstly, in-vitro bioassay and immun assay measurements in the immunized animal demonstrated the presence of high titers of antibody, almost all of which was in a form uncomplexed with angiotensin. However, despite this, it could not be assumed that all endogenous angiotensin II would be neutralized by antibody in vivo, where competition between antibody and receptor for angiotensin II would occur, and for this reason, in-vivo neutralization studies were performed as well. Even here, it was not considered adequate merely to infuse angiotensin II intravenously since angiotensin II production in vivo probably occurs at a point much closer to the receptor, viz., during passage of blood through the lung (22), and this would be associated with a less favorable balance in the competition between antibody and receptor for angiotensin. Therefore, the definitive investigation was based on the intravenous injection of rabbit renin, and it was crucial to validation of the model that the immunized rabbit showed no pressor response to doses of renin which caused sharp rises in blood pressure in normal rabbits. These injections were associated with substantial increases in the plasma level of angiotensin II bound to antibody without causing any significant degree of saturation of antibody capacity (<0.5%).

The specificity of angiotensin inhibition is shown by the finding of an intact response to norepinephrine in the immunized animal, and by the observation that large injections of angiotensin II could exceed antibody capacity and produce rises in blood pressure (Fig. 4). This would not be the case if the lack of pressor responsiveness were due to a general pressor refractoriness or to a specific tachyphylaxis to angiotensin (30, 31). The high degree of specificity of the antibody as tested in vitro with various fragments and analogues.
of angiotensin II has been reported previously (29).

Having established the validity of using the angiotensin II-immune rabbit as an experimental model, the results of Part 2 of this study make it extremely difficult to sustain any major role for circulating renin or angiotensin in either the initiation or maintenance of renovascular hypertension in the rabbit, as induced by the usual technique of unilateral renal-artery clipping with contralateral nephrectomy. Hypertension developed despite prior effective immunization in all four animals of the present study, and in two of these the hypertension was severe, with eventual death from hypertensive complications.

The studies in rabbits with established hypertension (Fig. 7) were made difficult by the high mortality before development of detectable antibody titers. However, in those rabbits which did develop such titers there was no evidence of modification of the hypertensive process. In the two hypertensive immune rabbits given intravenous injections of renin (791 and 710), levels of angiotensin II bound to antibody increased up to 40 times without any increase in blood pressure, indicating an enormous excess of circulating antibody. Rabbits 748 and 767 died prior to the test infusion of renin but both had readily detectable titers of antiangiotensin II in immunoassay, and since with blood taken into BAL and EDTA this test is a measure of circulating free antibody in vivo (see Methods) it seems likely that these rabbits too were effectively immunized against angiotensin II in vivo.

The specificity of the blockade to angiotensin in the immunized animal has been discussed previously (Part 1). Of particular importance is the normal or even slightly enhanced pressor sensitivity to nonadrenaline in hypertensive immune rabbits.

It could be argued that, since hypertension is a chronic disease, the duration of immunization in hypertensive animals was not long enough to allow a return of blood pressure to normal levels. However, this could not explain the development of hypertension in previously immunized animals or the rapid fall in blood pressure immediately after unclipping the renal artery. Also, the possibility that hypertension in our experiments could have been produced or maintained artificially by chronic boosting injections of angiotensin II adsorbed on carbon has been excluded by the absence of hypertension at any stage during immunization in the unclipped rabbit. These results were similar to those reported recently by Eide and Aan (13) in rabbits made hypertensive by wrapping kidneys in silk soaked in turpentine. These rabbits developed titers similar to those reported here, and similar criteria were used to assess the degree of immunization. In contrast, Christlieb et al. (14) found that in the rat with renal-clip hypertension, angiotensin II immunization may be associated with a reduced blood pressure. However, antibody titers in these latter experiments were extremely low and not effective in causing complete neutralization of the pressor effect of exogenous angiotensin. Nor was there any consistent relationship between the degree of blood pressure reduction and antibody titer. Thus it is difficult to relate any reduction in blood pressure purely to a specific blockade of angiotensin. Also, Hedwall (32) has carried out a study with passive angiotensin immunization in rats with renal-clip hypertension and reached precisely the opposite conclusion.

We consider that the evidence from our experiments is strongly against any major involvement of circulating angiotensin II in the pathogenesis of renal-clip hypertension in the rabbit. It does not, however, preclude the theoretical possibility that angiotensin II could still be involved if it were produced by the action of renin on renin substrate locally in arterioles at sites inaccessible to antibody, although it must be said that such a likelihood appears remote. The fall of blood pressure which occurred after unclipping the renal artery in the hypertensive immune rabbit is of considerable interest and is similar to that found in the hypertensive nonimmune rabbit, both by ourselves and by other workers (33). Although interpretation of the early fall could
be complicated by the anesthesia and surgery, the fact that this blood pressure reduction persisted during the period of rapid recovery and afterwards strongly suggests that it was due to the acute removal or re-institution of a renal mechanism which is not the renin-angiotensin system.

References
ANGIOTENSIN AND RENAL-CLIP HYPERTENSION


Renal-Clip Hypertension in Rabbits Immunized Against Angiotensin II
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