A New Mechanism in One-Kidney, One Clip Hypertension

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SUMMARY The renin-angiotensin system does not appear to be involved in the maintenance of elevated blood pressure in experimental one-kidney, one clip hypertension. Paradoxically, direct immunization with purified hog kidney renin lowers the blood pressure of rabbits with this form of hypertension. Antirenin antibodies were removed and the IgG fraction prepared from the plasma of such immunized rabbits. The antibodies thus obtained lowered the blood pressure of other hypertensive rabbits. The same antibodies, detected with a fluorescein-labeled second antibody, stained the cytoplasm of smooth muscle and certain other cells in sections of kidney, aorta, carotid artery, heart, liver, pancreas, adrenal gland, and small intestine from normal and hypertensive rabbits. We suggest that renin is converted into a form that is present most conspicuously in arterial and arteriolar smooth muscle. Its function in this location is unknown but must involve vasoconstriction as its neutralization by specific antibody lowers the blood pressure of one-kidney, one clip hypertensive rabbits. (Hypertension 7: 72-80, 1985)

Key Words • renin • antirenin • blood pressure • immunofluorescence • hybridoma

The mechanism responsible for maintaining the elevated blood pressure in experimental one-kidney, one clip (1K1C) hypertension is not known. The renin-angiotensin system does not appear to be involved as specific blockade of this system does not lower the blood pressure of animals with this form of hypertension. A partial reduction in blood pressure (not to normotensive levels) has been produced by Bengis and colleagues in 1K1C hypertensive rats by long-continued administration of SQ14225. This partial reduction may result from increased bradykinin levels. Watkins and co-workers have shown that administration of SQ14225 does not prevent the development of 1K1C hypertension in dogs.

The blood pressure of 1K1C hypertensive animals can be lowered to normal by immunization with heterologous kidney extracts. This was shown by Wakerlin and co-workers in 1953 and has since been confirmed by other investigators using dogs, rabbits, and monkeys. Initially it was believed that the blood pressure lowering effect was due to the formation of antibodies to the heterologous renin present in the kidney extracts, with resulting neutralization of the endogenous renin in the hypertensive animal. As we now know that renin does not play a role in this form of hypertension, however, the fall in blood pressure could not be due to neutralization of renin. In addition, we have shown that the blood pressure of 1K1C hypertensive rabbits can be lowered with kidney extracts that do not contain renin.

We concluded that hog kidney contains an unknown substance, not renin, that elicits a cross-reacting antibody in hypertensive rabbits. The antibody neutralizes a similar unknown substance in the rabbit and causes a fall in blood pressure. We have designated the unknown substance antigen M and believe that it must play an essential role in maintaining an elevated blood pressure in 1K1C hypertensive rabbits. (To simplify the discussion we have also called the corresponding and probably very similar substance in the rabbit antigen M.)

We therefore attempted to isolate antigen M from hog kidney. During the course of this work, we were able to purify hog kidney renin with immobilized monoclonal antirenin. Immunization of 1K1C hypertensive rabbits with this purified renin lowered blood...
pressure to normal. From these rabbits we have isolated a blood pressure lowering antibody that is not anti-renin. Using immunocytochemical techniques, we have demonstrated localization of this antibody in arterial and arteriolar smooth muscle tissue sections from both normal and hypertensive rabbits.

Materials and Methods

Protein was determined by an automated modification (Technicon Auto Analyzer) of the method of Lowry and co-workers; crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used for standardization.

Renn was measured in terms of the amount of angiotensin I liberated at pH 7.4 from an excess of partially purified, angiotensinase-free hog renin substrate.9 The bioassay of angiotensin I was performed in the anesthetized rat,11 and the results are expressed as picomoles of angiotensin I formed per minute or in terms of Goldblatt units (GU) based on standard hog renin preparation "59" (kindly supplied by Dr. Erwin Haas, 9.1 GU/mg, step 5). A Goldblatt unit of renin is defined as the amount of enzyme that is required to raise the mean blood pressure of a trained, unanesthetized dog 30 mm Hg, and in our assay, 1 GU is equal to 333 pmol angiotensin I formed per minute.

Plasma renin activity (PRA) was determined by the method of Haber and associates; radioimmunoassay was used to quantitate the liberated angiotensin I.

Antirenin was determined by its inhibitory effect on hog renin and is expressed in terms of the amount of renin neutralized. Incubation mixtures included diisopropylfluorophosphate and, in the case of plasma samples, ethylenediaminetetraacetic acid (EDTA) as well. The smallest concentration of antirenin that can be readily detected is 0.01 unit per milliliter. The method has been described in detail.

Preparation of Purified Hog Renin

Hog kidney cortex was extracted with water in the presence of inhibitors. After a brief acid treatment insoluble material was removed and protein in the supernatant solution further purified by ammonium sulfate precipitation and dialysis. It was then purified by affinity chromatography on pepstatin-aminoheptyl Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) followed by chromatography on monoclonal antirenin–Sepharose 4B. The final step was gel filtration on a 1.1 x 118-cm column of Sephacryl S-200 (Pharmacia Fine Chemicals). The final product had a specific activity of 2122 GU/mg protein. Subsequent preparations used in this work all had specific activities of 2000 GU/mg of protein or greater. This is the value found by Inagami and Murakami9 for pure hog renin.

Evidence for the purity of the renin was obtained by disc gel electrophoresis at pH 9.5, flat bed electrofocusing (LKB 2117 Multibhor), sodium dodecyl sulfate (SDS) gel electrophoresis under reducing and nonreducing conditions (Miles Canalco Co.), and SDS gel filtration before and after reaction of the renin with C-labeled diazoacetyl norleucine methyl ester. The method of purification and description of purity have been described in detail.

Preparation and Use of Renin-Sepharose Columns

Purified renin, 1 to 2 mg, was reacted with 2.5 g (dry weight) of cyanogen-bromide-activated Sepharose 4B. The directions provided by the manufacturer were used with the exception that 0.1 M sodium phosphate, 0.5 M sodium chloride, pH 7.5, was used instead of the usual coupling buffer and the final alkaline washes were replaced with 0.1 M sodium borate, 0.5 M sodium chloride buffer, pH 7.5. The renin–Sepharose conjugate received a final wash with phosphate-buffered saline (PBS), pH 7.5, and was used to prepare columns approximately 1 x 15 cm. Potassium thiocyanate, 3 M in PBS, was pumped through the column for 1 to 6 hours after each use. All traces of potassium thiocyanate were then removed by pumping through PBS for an extended period. Several columns were needed as their capacity decreased each time they were used.

Preparation of IgG Fractions for Passive Immunizations

IgG for control passive immunizations was prepared from normal rabbit serum (Pel-Freeze, nonhemolyzed, sterile; Pel-Freeze Biologicals, Rogers, AR). The serum was diluted with an equal volume of PBS and the pH adjusted to 6.0. Ammonium sulfate (0.338 g/ml) was added and after adequate stirring the precipitate was collected, dissolved, and dialyzed overnight against a continuous flow of 0.1 M sodium phosphate buffer, pH 8.1. The solution was applied to a protein A–Sepharose column. Elution was achieved with a gradient from 0.1 M sodium phosphate, pH 8.1, to 0.05 M citric acid. Approximately 35% of the protein applied to the column was recovered as IgG. The eluate was dialyzed for 4 hours against 0.01 M sodium phosphate, 0.145 M sodium chloride, pH 7.4, to remove excess citrate and phosphate. It was then concentrated by ultrafiltration in an Amicon filter (Amicon Corp., Lexington, MA) with a PM10 membrane. The concentrated solution was centrifuged at 48,200 g for 10 minutes and then sterilized with a 0.2 μm filter (Nalgene 160–2020, Nalgene Labware Div., Nailge/Sybron Corp., Rochester, NY). The antibodies were stored at −80 °C. Protein concentrations ranged from 20 to 40 mg/ml.

Immune antibodies for passive immunizations were prepared from plasma taken at weekly intervals from rabbits whose blood pressures had been lowered by direct immunization. Blood samples (up to 30 ml) were drawn into one-tenth volume of 2% EDTA from the central artery of the ear and were chilled in ice as soon as they were obtained. Plasma was separated without delay and was frozen until used. Plasma samples from a single or more rabbits were pooled for further processing and the preparation of the IgG fraction.

Antirenin antibodies, which ranged from less than 0.01 to more than 10 antirenin units/ml, were removed
either before the ammonium sulfate step or immediately after.

In the former case plasma, at pH 7.4, was simply pumped through a renin-Sepharose column, as described previously, followed by sufficient PBS to allow recovery of the protein from the column. Antirenin assay was then performed on the effluent and the process repeated if necessary to remove all of the detectable antirenin. After this had been accomplished the immune IgG antibodies were prepared by the same method that was used to prepare the normal IgG preparations with the single exception that ammonium sulfate was added in two steps to eliminate fibrinogen. The fraction that was collected and used was that precipitating between 0.114 and 0.338 g ammonium sulfate per milliliter.

In those cases in which antirenin was removed after the ammonium sulfate step, the same two-step addition of ammonium sulfate was used. In addition, the ammonium sulfate precipitate was dialyzed against PBS, pH 7.4, to prepare the solution for the renin-Sepharose column. After the antirenin was removed as described previously, a sufficient amount of solid NaH₂PO₄·H₂O was added to bring the phosphate molarity to 0.05. The pH was then adjusted to 8.1 and the solution applied to the protein A-Sepharose column. The rest of the process was carried out exactly as described for the normal IgG preparations.

Precautions Used in the Preparation of IgG Fractions for Passive Immunizations

All solutions used in the operation of the renin-Sepharose and protein A-Sepharose columns as well as all others that were used throughout the procedure were prepared with sterile, pyrogen-free water. After preparation they were resterilized by filtration and were stored in the cold until needed. All of the procedures except for the final sterilization were carried out at 4 °C or less. When a delay in the processing of more than a few hours occurred, the preparations were frozen. Columns were kept in the cold at all times and contained 0.02% sodium azide in the appropriate buffer when not in use. Every effort was made to complete the procedure in the least possible time, thus the entire process from pooling of the protein A-Sepharose column eluate fractions to the freezing of the final sterile preparation was accomplished in 1 working day.

Production of Hypertension and Measurement of Blood Pressure

Hypertension was produced in young, male, white New Zealand rabbits weighing between 2.2 and 2.6 kg by application of a silver clip to the left renal artery and removal of the right kidney in the same operation. Systolic blood pressures were measured 5 days per week in the central artery of the ear of warmed rabbits. The rabbits that were used in experiments had exhibited a sustained form of benign hypertension with pressures of 30 mm Hg or more above their control level for a period of at least 4 weeks. These methods have been described in detail.

Method of Direct Immunization

The purified renin to be used as the antigen was sterilized by filtration (Nalgene 160-2020) and diluted to the required concentration in sterile PBS, pH 6.0. The antigens were stored at -80 °C.

An initial dose of 100 μg of renin in 1 ml of PBS was emulsified with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected intramuscularly into hypertensive rabbits with a satisfactory blood pressure record. Thereafter, the animals were injected subcutaneously 5 days per week with 20 to 50 μg of antigen in 1 ml of PBS, pH 6. This schedule, which is similar to that used by Lamfrom and colleagues in their early work on antirenin in dogs, has been the most effective in lowering blood pressure.

Some of the passive immunizations reported in this paper were done with IgG prepared from antibodies raised with partially purified renin. In this case the purification had been performed through the pepstatin-Sepharose column step, which resulted in a specific activity of approximately 100 GU/mg protein. The immunization schedule with such preparations was the same, although the amount of protein used in each dose was usually greater than in the case of the purified antigen. The specific activity of the purified antigen was 2000 GU/mg protein or greater.

Method of Passive Immunization

The blood pressure of rabbits to be passively immunized was determined several times a day during a control period of 3 days or longer. Antibodies were then given very slowly into the marginal ear vein in two or three divided doses over 2 to 3 hours. Multiple blood pressures were obtained for several days thereafter or until the blood pressure returned to its control level.

The dose of normal IgG used as a control ranged from 97 to 415 mg/kg body weight. In 6 of the 23 experiments the dose approximated 400 mg/kg. The dose of immune IgG that had been obtained with purified renin ranged from 213 to 420 mg/kg. The dose of immune IgG obtained with partially purified renin ranged from 219 to 283 mg/kg.

The composition of the PBS used for controls was 0.01 M sodium phosphate, 0.145 M sodium chloride, pH 7.4. It was given in a dose of 11 ml/kg, which approximated the volume of immune and normal IgG given in the other experiments.

Demonstration of Antigen in Tissues

Portions of heart, lung, liver, spleen, pancreas, small intestine, kidney, adrenal, carotid artery, aorta, and skeletal muscle were taken from normotensive and hypertensive rabbits and snap-frozen by immersion in liquid nitrogen. Frozen tissue was stored at -70 °C and cryostat-sectioned (American Optical Spencer Cryocut) at 4-μm thickness. Slides were air dried at -20 °C for 5 minutes and stored at -70 °C until stained.

Slides to be stained were equilibrated in PBS (in this procedure 0.15 M NaCl, 0.01 M sodium phosphate,
pH 7.4) for 5 minutes, fixed by immersion in acetone for 1 minute, and washed in PBS for 5 minutes.

Each of three 1 mg/ml IgG fractions of rabbit immune serum (187/106, 131B/106, 229A/102) was used to treat replicate slides from each tissue specimen. Rabbit antiovine IgG was used on control slides. After a 30-minute incubation at ambient temperature, all slides were washed in PBS and incubated at ambient temperature with a drop of fluorescein-isothiocyanate-conjugated goat antirabbit IgG (U.S. Biochemicals Corporation, Cleveland, OH) diluted 1:20. After a final wash in PBS for 5 minutes, slides were mounted with 40% glycerol in PBS and examined in a Leitz Ortholux microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, NJ) equipped with a Floempak epifluorescence.

The specificity of the IgG fraction of rabbit immune serum 187/106 that was used in the passive immunization of rabbit 894 (illustrated in Figure 2 and in the immunofluorescence micrographs of Figures 3 and 4) was further characterized by extinction/inhibition studies with cryostat sections of rabbit carotid artery. Replicate sections were incubated with serial dilutions of the immune 187/106 antibody in PBS that ranged in concentration from 3 to 100 μg/ml. For this special purpose the antibody was adsorbed three more times (to make a total of three times) with hog renin-Sepharose 4B. A second set of sections was prepared that duplicated the first set in every way except that the dilutions of immune antibody were made in PBS containing 200 μg/ml of purified hog renin. A third set of sections to be used as controls was incubated with corresponding concentrations of the same antibody diluted in PBS containing renin as well as with those stained with antiovine IgG. This procedure was designed to determine if tissue binding of the immune antibody was inhibited by excess free renin or if the concentration of antibody required for positive staining would be increased.

Double-labeled immunofluorescence studies of rabbit kidney and carotid artery were performed with a monoclonal mouse IgG antirenin antibody and the rabbit immune 187/106 antibody. A mouse monoclonal IgG antibody to complement receptor (44D) was used as a control. Sections were incubated first with a 1 mg/ml solution of either the monoclonal antirenin or monoclonal antibody to complement receptor. After washing, all were then incubated with rhodamine-conjugated goat antimouse IgG (Cappel Laboratories, Cochrunville, PA) at a 1:40 dilution in PBS. The sections were then washed and incubated with the rabbit immune IgG antibody 187/106 at a concentration of 1 mg/ml in PBS. Finally, the sections were washed, incubated with fluorescein-conjugated goat antirabbit IgG, washed, and then mounted. In this fashion localization of renin and antigen M could be simultaneously assessed and a determination made as to whether prior treatment with monoclonal antirenin prevented staining of the tissue by the rabbit immune IgG antibody 187/106.

Results

The effect of direct immunization of rabbit 699 with purified renin is illustrated in Figure 1. The left renal artery was clipped and the right kidney removed on the ninth day. The blood pressure rose in 25 days from a preclipping average level of 70 mm Hg to approximately 100 mm Hg, where it remained for another 112 days. At this time an initial dose of 100 μg of purified renin in Freund's complete adjuvant was given intramuscularly. Thereafter, a 20 μg per day maintenance...
dose of purified rennin was given subcutaneously 5 days per week throughout the 36-day injection period. Sixteen days after the initial injection the blood pressure began to fall. Within 9 days it had fallen to a level of 68 mm Hg. For another 40 days (28 days after the last injection) the pressure remained at approximately 75 mm Hg. Thereafter the pressure rose slowly to a hypertensive level.

Many hundreds of rabbits have been directly immunized in similar fashion, with both purified and impure rennin. Approximately 25% responded with a significant fall in blood pressure. Some did not respond during the first immunization period but required a second period of injections. Nearly all developed antirenin titers ranging from less than 0.01 to greater than 10 units antirenin per milliliter. All rabbits remained active and healthy, did not lose weight, and appeared normal in all other respects.

The effect of passive immunization of rabbit 894 is illustrated in Figure 2. After a 3-day control period, a total of 1449 mg of IgG preparation 187/106 (483 mg/kg) was given in 4 doses over a 2-hour period. This preparation was derived from the plasma of hypertensive rabbits whose blood pressure had been lowered as a result of immunization with purified rennin and from which antirenin antibodies had been subsequently removed. The blood pressure began to fall within 90 minutes of the last injection and reached a minimum of 57 mm Hg approximately 12 hours after the last injection. The blood pressure remained between 60 and 70 mm Hg for the next 48 hours after which it slowly climbed to a hypertensive level over the next 6 days. The rabbit remained active and in good condition throughout the experiment.

A total of 32 such experiments have been done. The results are tabulated in Table 1 and include 7 experi-

![Figure 2](https://example.com/figure2.png)

**TABLE 1** Change in Systolic Blood Pressure of Hypertensive Rabbits Following Passive Immunization

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<tr>
<th>Phosphate-buffered saline mm Hg</th>
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-3.25 ± 2.68(8)*

-1.08 ± 4.58(12)**

-14.2 ± 6.08(5)**

-12.85 ± 7.51(7)**

The change in blood pressure is the difference between the average of the 10 pressures obtained during the 72-hour period before intravenous injection and those obtained between 4 and 48 hours after injection.

*Mean and standard deviation (number of experiments).

**p < 0.005 when compared with the phosphate-buffered saline or normal IgG controls.
ments performed with antibodies taken from hypertensive rabbits whose pressure had been lowered as a result of direct immunization with pure renin, 5 experiments in which impure renin was used as the antigen, 12 control experiments that used normal rabbit IgG, and 8 control experiments that used PBS.

In the seven experiments employing antibody produced with pure renin as the antigen the decrease in blood pressure ranged from 5 to 28 mm Hg with a mean fall of 12.85 ± 7.51 mm Hg (Table 1). The range of fall in blood pressure in the five experiments in which impure renin was used as the antigen was from 4 to 20 mm Hg; the mean change was −14.2 ± 6.08 mm Hg. In 12 control experiments where normal IgG was given the change in pressure ranged from +7 to −8 mm Hg with a mean change of −1.08 ± 4.58 mm Hg. Finally, in eight control experiments employing PBS the change in pressure ranged from 0 to −9 mm Hg, with a mean of −3.25 ± 2.68 mm Hg. There was a highly significant difference when the response to either of the two immune IgG antibodies raised with either pure or impure renin was compared with those obtained with normal IgG or with PBS (p < 0.005; Student’s t test).

The PRA measured in five hypertensive rabbits before injection of immune IgG antibodies ranged from 1.00 to 2.12 ng angiotensin I/ml/hour (mean 1.46 ± 0.41) and from 1.72 to 4.10 ng/ml/hour (mean 3.25 ± 1.13) 24 hours after injection. Similar measurements made in five hypertensive rabbits that received control injections of PBS yielded PRA values ranging from 0.93 to 3.25 ng/ml/hour (mean 2.44 ± 1.04) before injection and 2.08 to 3.99 ng/ml/hour (mean 2.93 ± 0.75) after injection. The relative increase in PRA that occurred in those rabbits receiving the immune IgG was statistically significant when compared with those that received control injections of PBS (p < 0.05).

Three of the IgG antibodies that had been obtained from rabbits immunized with purified renin were used to demonstrate the presence and distribution of antigen in tissues taken from normotensive and hypertensive rabbits. Each of the antibodies (187/106, 131B/106, 229A/102) was used as a primary antibody to stain replicate sections of heart, lung, spleen, pancreas, small intestine, kidney, adrenal gland, carotid artery, aorta, and skeletal muscle. Two of the antibody preparations had been through a renin–Sepharose immuno-affinity column and were free of detectable antirenin; the third, 229A/102, which had not been through the renin–Sepharose column, still contained antirenin (3 units/ml). The results obtained with 229A/102 were not different from those obtained with the two antirenin-free antibodies, which indicates that antirenin antibodies do not interfere with staining by the rabbit immune anti-antigen M antibody. Control sections were produced by using rabbit antirabbit IgG as the primary antibody. All sections were stained with a fluorescein-conjugated goat antirabbit antiserum.

Representative photographs of sections of two of the tissues stained with the identical primary antibody (187/106) that was used in the passive immunization of

Figure 2 are shown in Figures 3 and 4 together with controls. Small, medium, and large arteries that were present in all sections, as well as sections of carotid artery and aorta, revealed bright cytoplasmic staining of the cells of the lamina media. This staining was distinct in appearance from the autofluorescence of the elastic lamellae owing to differences in color. Staining in the large muscular artery (carotid) was more conspicuous, owing to a greater relative amount of medial smooth muscle, than was staining in the large elastic artery (aorta). While the smooth muscle cells in the small intestine (muscularis propria and muscularis mu-
Immunofluorescence micrographs of sections of renal cortex from a hypertensive rabbit A There is intense staining of the lamina media of a cortical arteriole stained with the same immune rabbit IgG (187/106) that was used to lower blood pressure in the passive immunization experiment of Figure 2. Note also the perinuclear staining in tubular epithelial cells B In contrast, there is only weak artifactual staining in the lamina adventitia in the control section stained with rabbit antirabbit IgG (×200)

FIGURE 4 Immunofluorescence micrographs of sections of renal cortex from a hypertensive rabbit. A There is intense staining of the lamina media of a cortical arteriole stained with the same immune rabbit IgG (187/106) that was used to lower blood pressure in the passive immunization experiment of Figure 2. Note also the perinuclear staining in tubular epithelial cells. B In contrast, there is only weak artifactual staining in the lamina adventitia in the control section stained with rabbit antirabbit IgG (×200).

cosae) and bronchus stained brightly, striated muscle from the heart and skeletal muscle revealed staining only in association with the arterial supply. Striated muscle fibers were completely negative. Epithelial cells in renal tubules, liver, bronchus, small intestine, pancreas, and adrenal gland all showed varying degrees of cytoplasmic staining, all in a perinuclear distribution (Figure 4). This staining was most conspicuous in the thin loop of Henle in the kidney and in the adrenal cortex. Cytoplasmic staining of cells lining the vasa recta of the renal medulla also was observed. The only other positive staining was observed in the cytoplasm of reticuloendothelial cells lining liver and spleen sinusoids. In these cells there was staining throughout the cytoplasm, but perinuclear areas appeared more intense.

Extinction/inhibition immunofluorescence studies with rabbit carotid artery as the tissue substrate and the immune rabbit IgG 187/106, which was used in the passive immunization of rabbit 894 shown in Figure 2 and the immunofluorescence micrographs of Figures 3 and 4, revealed that the minimum concentration of antibody needed for cytoplasmic staining of vascular smooth muscle was 50 μg/ml. The fluorescent intensity of the stained tissue increased progressively up to 200 μg/ml, at which point it was comparable to that observed in Figure 3 in which the antibody concentration had been 1 mg/ml. Control sections incubated with rabbit antirabbit IgG were uniformly negative. Neither the threshold of 50 μg/ml needed for positive fluorescence nor the intensity at any given concentration of antibody was affected by the presence of 200 μg/ml of purified hog renin.

In the double-labeled immunofluorescence studies, sections of kidney that had been stained first with mouse monoclonal antirenin and then with the immune rabbit IgG 187/106 revealed a distribution and intensity of staining owing to the latter rabbit antibody that was identical to that found in sections that had been incubated first with the mouse monoclonal anticomplement receptor (44D). In addition, rhodamine fluorescence indicated binding of the mouse monoclonal antirenin antibody to arterioles in close proximity to glomeruli in the kidneys in a pattern similar to that described by others. 20-23 The antirenin antibody did not bind to epithelium or smooth muscle cells in arterioles in the kidney that were remote from glomeruli nor was there staining of the smooth muscle cells of the carotid artery. No staining for mouse IgG was observed in the sections that had been incubated first with the mouse monoclonal anticomplement receptor (44D) that was used as a control. Overlap of staining between the immune rabbit IgG 187/106 and the monoclonal antirenin was confined to the periglomerular arterioles.

Thus the cytoplasmic staining of arterial smooth muscle by the immune rabbit IgG 187/106 was unaffected by three successive absorptions by purified renin-Sepharose 4B and by the presence during staining of excess free purified hog renin. The binding of the immune rabbit IgG 187/106 was not paralleled by the binding of the monoclonal antirenin antibody, nor was the binding of the rabbit IgG influenced by preincubation with monoclonal antirenin.

Discussion

We demonstrated in earlier work 8 that the blood pressure of hypertensive rabbits could be lowered by direct immunization with hog kidney preparations from which all the renin had been removed with antirenin-Sepharose columns. The high-titer antirenin used for removal of renin had been raised in normal rabbits in an impure hog renin preparation (22 GU/mg) as antigen. The same antirenin did not lower the blood pressure of hypertensive rabbits when given intravenously. 3 Therefore it could not have contained antibodies to antigen M.

When we attempted the purification of antigen M from hog kidneys it became obvious that the removal of renin by this means was not possible on a suitable scale. A large variety of methods were unsuccessful in...
removing renin from our preparations and in purifying antigen M. The assay, which employed direct immunization of hypertensive rabbits, was not quantitative and required 4 to 6 weeks to perform. Rapid progress was made after we produced a hybridoma (F32V111C4, American Type Culture Collection, Rockville, MD) that secreted a monoclonal, anticytolytic antirenin. This antibody, when used in immobilized form, allowed us to prepare highly purified hog renin. Although the preparation exhibits heterogeneity both as to size and charge, a critical examination using several methods did not reveal the presence of non-renin impurities.

We were then surprised to discover that direct immunization with our pure renin preparation lowered the blood pressure of hypertensive rabbits. (Failure of more than 25% of the rabbits to respond could be due to a low degree of cross-reaction as well as to the usual wide individual variation in immune response. Moreover, it would be necessary to neutralize an adequate amount of antigen M in the animal before a titr of can become apparent. Partial neutralization of antigen M may stimulate its further production. In this case still higher titers of anti-antigen M antibodies would be needed before the blood pressure could be expected to fall.) This completely unexpected finding forced us to decide between two apparently unlikely alternatives. First, that our renin preparation was not pure and still contained a small amount of antigen M in spite of affinity chromatography with a monoclonal antirenin column and a rigorous examination of its purity. Second, that the renin itself was the source of antigen M. After injection into the rabbit, renin might undergo a conformational change or enzymatic cleavage that would expose new antigenic sites. The resulting molecule might then have some or all of the antigenic sites that were present in the original renin molecule and in addition those new sites peculiar to antigen M. The new molecule when used as an antigen would elicit two different types of antibody molecules—those that react with renin and those that react with antigen M. Under these circumstances the relative concentrations of each antibody that might occur in any one immunized rabbit might vary over a wide range.

The first alternative would imply that antigen M, although unrelated to renin, stubbornly resists separation from renin and that a very small amount of the substance remains as impurity in our final purified renin preparation after chromatography on peptatinaminohexyl-Sepharose, monoclonal antirenin-Sepharose, and S200 Sephacryl. Careful examination of the results of polyacrylamide gel electrophoresis of the purified renin preparation would suggest that the amount of an unrelated antigen M that could be present could not exceed 5% and more likely is less than 1%. Were there to be as much as 5% of antigen M in the renin that we used to directly immunize our hypertensive rabbits, then the immunizations and the consequent reductions in blood pressure (Figure 1) were achieved with an initial dose of 5 µg and daily doses of 1 to 2.5 µg. Such small doses are more likely to induce tolerance rather than immunity in white New Zealand rabbits We therefore accepted the second alternative—that renin itself is in some manner the source of antigen M—as being more probable.

To demonstrate the existence of the antibody to antigen M in the absence of antirenin we removed the latter antibody from the plasma of hypertensive rabbits directly immunized with renin by passage through a column of pure renin immobilized on Sepharose.

At present we have no way of determining the titer of antibodies to antigen M in the plasma of the immunized rabbits. For this reason only those rabbits were bled whose blood pressure had been lowered as a result of the immunization, this being considered the best, although hardly conclusive evidence of the existence of circulating antibodies against antigen M.

The antiserum was purified with protein A-Sepharose chromatography. This method yielded a well-defined IgG preparation.

The removal of the antirenin from the antibody preparations may have resulted in loss of anti-antigen M. Furthermore, in order to use a purified IgG antibody preparation the IgM antibodies were sacrificed. Both of these procedures militated against the success of the experiment. In spite of these potential difficulties, the decrease in blood pressure in the hypertensive rabbits receiving the immune IgG preparations was statistically highly significant (Table 1).

In five of the hypertensive rabbits that were passively immunized with immune IgG antibodies there was a statistically significant increase in PRA as compared with controls. This finding provides additional evidence that the fall in blood pressure in the passively immunized rabbits could not have been due to antirenin. Furthermore, it would appear that the pressor action of the increased PRA might limit the blood pressure lowering action of the anti-antigen M antibodies.

The same antibody preparations that were obtained with pure renin as an antigen and that lowered the blood pressure of hypertensive rabbits by passive immunization after removal of antirenin were used to demonstrate the presence of antigen M in rabbit tissues. Very unexpectedly the antigen was found, when visualized by means of a fluorescein-labeled second antibody, to be present not only in sections of kidney but in sections of aorta, carotid artery, liver, heart, pancreas, adrenal gland, and small intestine from both normal and hypertensive rabbits. In all cases smooth muscle, especially arterial and arteriolar, stained specifically and strongly whereas cardiac and skeletal muscle did not stain. Epithelial cells in several of these tissues including renal tubules and the thin loop of Henle showed varying degrees of cytoplasmic staining in a perinuclear distribution.

The present experiments do not allow us to determine the relative concentrations of antigen M in tissues from normal and hypertensive rabbits. Apparently there is approximately as high a concentration in the normal as in the hypertensive animals. Examination of the renin-angiotensin system would suggest that this
finding should not remove antigen M from consideration as essential to the vasoconstrictive mechanism. For example, angiotensin-converting enzyme is present in excess in both normal and hypertensive animals and angiotensin I is in either case converted to angiotensin II with great speed. Yet inhibition of the converting enzyme is a very effective method of lowering blood pressure in renin-dependent hypertension.

Edelman and Hartroft and others have used immunohistological methods to demonstrate that the presence of renin in the kidney of rabbits and other species is sharply limited to the juxtaglomerular apparatus and associated arterioles. Renin also has been demonstrated in glial and neuronal cells in several locations in the brain by immunohistological methods. Finally, renin has long been known to be present in extracts of blood vessels as demonstrated by radioimmunoassay and other means; however, the concentrations of the enzyme in vascular tissue is exceedingly low and no reports of its immunohistological localization have appeared. Our own experiments were also negative. Our sections of carotid, aorta, kidney, and other tissue treated with monoclonal anti-renin did not show specific staining except for the juxtaglomerular apparatus and closely associated arterioles of the kidney. Careful consideration of the foregoing work demonstrates that it would not be possible to confuse the staining of tissues produced by antigen M with that of renin.

We therefore suggest that hog renin, injected into rabbits as an antigen, must be converted in vivo into antigen M, which elicits anti-antigen M antibodies. Furthermore, endogenous rabbit renin also must be converted to an antigen M substance and is located most conspicuously in arterial and arteriolar smooth muscle cells throughout the animal. Its function in these and other locations is unknown but must involve arterial and arteriolar vasconstriction because its neutralization with antibody lowers the blood pressure of rabbits with chronic 1K1C hypertension.

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