Localization of Renin in Juxtaglomerular Cells of Rabbit and Dog Through the Use of the Fluorescent-Antibody Technique

By ROBERT EDELMAN, A.B., AND PHYLLIS M. HARTROFT, PH.D.

RENAL juxtaglomerular cells (JG cells) are located in afferent arterioles, usually just before they enter the glomeruli. By both light and electron microscopy, their cytological characteristics strongly suggest a secretory function.

Nearly a quarter of a century ago, Goormaghtigh proposed the theory that JG cells are the site of elaboration of the pressor substance, renin. Marshall and Wakerlin reported that the solubility characteristics of canine renin and JG granules of the dog correspond. Bing and Kazimierczak, using microdissection techniques, localized renin to the area of the renal corpuscle including the vas afferens and juxtaglomerular apparatus, but they found none in the glomerular tuft itself. Evidence obtained in this laboratory showed a direct correlation between the bio-assay of renin and the degree of granulation of JG cells in sodium-deficient and control animals. Tobian et al. demonstrated a similar correlation in hypertensive rats and in rats fed excess dietary salt (7 per cent).

Johnson and Wakerlin first prepared antibodies to renin by injecting hog renin into dogs. Lamfrom, Haas, and Goldblatt showed the ability of similarly prepared antirenin to neutralize not only renin from the hog, but also that from rabbit, dog, and several other species as well. Nairn and co-workers, using the indirect "sandwich" variation of the fluorescent-antibody technique, reported the localization of renin in the glomerular tuft of hog kidneys, but not in the "juxtaglomerular areas." However, in Nairn’s report, JG cells were not demonstrated by conventional tinctorial methods in control sections from areas similar to those used for fluorescence microscopy.

In the present study, antirenin preparations were coupled with fluorescein dyes by the procedures outlined by Coons and Kaplan to provide further evidence for the site of elaboration of renin in the kidney.

Methods

Antirenin

Antirenin is an antibody that will not precipitate, agglutinate, nor fix complement with antigen. Therefore, it is necessary to depend upon bio-assay for estimation of its titer. A unit of antirenin is the amount that will completely neutralize the pressor activity of one canine unit of renin. Antirenin was assayed in the dog according to the method of Lamfrom, Haas and Goldblatt.

Antirenin was prepared by parenteral injection (subcutaneous or intramuscular) of rabbit renin (specific activity: 2 units per mg.) into dogs. The dog serum contained 2 units of antirenin per cc. serum.

Antirenin to hog renin was also prepared. Hog renin with a specific activity of 10 units per mg. protein, stored frozen in small concentrated batches, was thawed and diluted just before use. Two littermate Basenji dogs (female, nine months of age, 10 Kg.) were injected with 50 units of this hog renin (0.5 to 1 cc.) subcutaneously every other day. One of the dogs received, in addition, Freund's adjuvant without Mycobacterium tuberculosis (0.5 to 1 cc. Bayol F and 2 to 3 drops Arlacel A), emulsified with the renin for five minutes immediately before each injection. In this dog, at the end of one month, the amount of antirenin in its...
serum had risen to almost 200 units per cc.; in the other dog, the titer was 50 units per cc. At this time, serum was obtained from both dogs for conjugation with fluorescein dyes (fluorescein isocyanate and fluorescein isothiocyanate). In addition, nonimmune serum was obtained from a noninjected littermate dog for control, and was similarly conjugated.

**Anticanine Globulin**

Anticanine globulin was prepared by injecting an adult, female, albino rabbit with normal canine globulin. Eighty mg. of canine globulin in 2 cc. saline (plus adjuvant) were injected subcutaneously once a week for four weeks. At the end of this period, rabbit-immune globulin was obtained from the anticanine serum by precipitation with an equal volume of saturated ammonium sulfate and subsequent dialysis (against 0.01 M phosphate buffer, pH 7.0 to 7.4). This antiglobulin, which gave a positive flocculation ring test with the canine globulin, was used in the indirect method of staining (vide infra).

**Conjugation of Antisera with Fluorescein Dyes**

The procedures of coupling (labeling) the immune globulin and serum to fluorescein dyes were essentially the method of Coons and Kaplan. The antisera (either whole serum or the globulin fraction) were coupled with fluorescein isocyanate-prepared in the laboratory. In addition, a commercial preparation of fluorescein isothiocyanate* was coupled with serum containing antirenin to hog renin, giving equally bright or even brighter staining. Using a Dowex 1X4 anion exchange resin to remove excess dye, three days were saved in dialysis with this batch of conjugated serum. Because of its greater stability during storage, use of the commercially available dye proved of definite advantage, but it was found that different lots of the same dye varied markedly in staining intensity. After conjugation, the titer was not significantly reduced during the process of conjugation of antisera with the dye. The conjugated sera were stored in a deep freeze (—12°C). The conjugated sera were frozen (one to three seconds) in a beaker of liquid Freon cooled on dry ice (—40 to —60°C) which was then surrounded by liquid nitrogen.

**Tissue Adsorption of Conjugated Antirenin**

Adsorption with both liver and bone-marrow powder was found necessary to remove non specific fluorescence of labeled antirenin preparations. The use of either of these powders alone resulted in excessive nonspecific background staining. The powders were prepared in the following manner. Fresh rabbit liver was homogenized without diluent in a Waring blender for several minutes. The homogenate was then placed in excess acetone; the acetone was changed three to four times during a 24-hour period, and the tissue was dried by filtration under vacuum. Essentially the same procedure was used with rabbit bone marrow, except that it was not necessary to homogenize it before placing it in acetone.

Liver powder, 100 mg., was added to 2 cc. of conjugated antisera, the mixture stirred intermittently for one-half to one hour at room temperature, centrifuged at 3,000 r.p.m. for 20 minutes, and the conjugate poured off. Then, bone-marrow powder, 100 mg., was added to the partly adsorbed sera, centrifuged, and the supernatant saved, ready to be put on kidney sections. Adsorptions, as described, were performed only once with each powder rather than twice or more with just one, as is frequently done. This procedure resulted in a loss in volume of 0.5 cc. of the original 2.0 to 2.5 cc., but did not reduce the antirenin titer significantly. The amount of tissue powder used was critical. Doubling to tripling the amount of powder (i.e., 200 to 300 mg./2 cc.) greatly reduced, and even sometimes abolished, all specific staining.

**Animals and Preparation of Tissue**

Male, weanling, albino rabbits, in groups of two, were fed a diet deficient in sodium12 for periods of 2, 3 1/2, 6, 8, and 10 weeks. At each time period, a control rabbit fed ad libitum the same basal diet supplemented with 0.6 per cent sodium chloride was also sacrificed. In addition, renal tissue was obtained from weanling rats which had been maintained on a sodium-deficient diet since the age of two months. Sodium-deficient animals were used in order to take advantage of the prominent, hypergranulated JG cells which result.13

In all cases, renal tissue was obtained while the animal was under pentobarbital anesthesia. Thin slices (i.e., 2 mm. square) were loosely wrapped in aluminum foil and swiftly frozen (one to three seconds) in a beaker of liquid Freon cooled on dry ice (—40 to —60°C) which was then surrounded by liquid nitrogen. Procedures of freeze-drying, embedding in ethylene glycol distearate, and sectioning with a Servall microtome at 1 to 2 μ were carried out according to the description given by Lacy and Davies.14 Once embedded, the tissue blocks could be stored indefinitely at room temperature and used repeatedly.

At the same time that tissue was taken for freeze-drying, an adjacent portion of each kidney was also fixed in Helly's fluid, embedded in paraffin, and stained by the Bowie technique in order to determine the degree of granulation of JG cells (JGI).15 The remainder of each rabbit kidney was put in a deep freeze and was later homogenized.

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*Sylvania Chemical Co., Orange, New Jersey, lot no. 1001.

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with saline; the resulting crude renin extracts were assayed by the method of Gross and Lichtlen, in which the nephrectomized rat is used to test pressor activity of the extracts. Blood pressure was measured by direct cannulation of the femoral artery. After assays in the rat were completed, the crude extracts were stored in a deep freeze for several months and were then thawed and partially purified for assay in the dog. The advantage of the latter method is that the actual content of renin (dog units/Gm.) can be determined.

Fluorescent Staining

The frozen-dried sections were immersed in xylol for five minutes to remove the ethylene glycol distearate and then air-dried at room temperature. In the direct technique, one to two drops of labeled, adsorbed antirenin (whole serum or the globulin fraction only) were left in contact with the tissue section, with precautions being taken to avoid evaporation and to prevent the drop from spreading out over the glass slide. The length of time necessary for this staining procedure depended upon the titer of that antirenin used. In the case of antirabbit renin (2 units/cc), 14 to 18 hours at 4 C. were required, whereas with the antihog renin (50 to 200 units/cc), only 20 minutes at room temperature were necessary. An increase in neither speed nor intensity of staining was achieved with incubation at 37 C.

By the indirect technique (“sandwich” method), sections were incubated first with unlabeled antirabbit renin for 12 hours at 4 C. After a 15-minute wash in isotonic buffered saline (pH 6.5 to 7.8), the sections were treated with adsorbed, labeled (with both fluorescein isocyanate and rhodamine B isothiocyanate) anticanine globulin for 12 hours at 4 C.

Before microscopy, all slides were washed with two to three changes of fresh, buffered, isotonic saline (0.01 M phosphate, pH 7.0 to 7.8). Sections have been stored (refrigerated) in this buffer solution for as long as one and one-half months without loss of staining. After a minimum of 10 minutes, sections were mounted in buffered glycerin under a cover glass and examined with the ultraviolet microscope. When necessary, the cover slip was removed and sections were rewashed until nonspecific background staining no longer interfered.

Fluorescence Microscopy and Photography

A standard Leitz microscope was fitted with a cardioid darkfield condenser, a rhodium-coated mirror, and a 2B Wratten protective filter in the eyepiece. The light source was a Zeiss mercury vapor arc lamp, model HBO200, with a Corning ultraviolet filter, no. 5840.

For photography, the microscope was fitted with a 35 mm. Exacta camera. Daylight color film (Kodak Ectachrome, type E and type EH high speed) was used. Use of the high-speed film, which required a much shorter exposure (one and one-half minutes with type EH and five minutes with type E), completely compensated for the fading of specific fluorescence which occurred after four to five minutes' exposure to ultraviolet light.

Results

Staining with “High Titer” Labeled Antihog Renin

Staining with fluorescein-labeled anti-hog renin uniformly revealed an intensely brilliant, yellow-green fluorescence of cytoplasmic granules in JG cells of sodium-deficient rabbits (fig. 1). The nuclei of JG cells were dark and unstained amidst these brightly fluorescing granules. The specific yellow-green fluorescence precisely localized to granules contrasted strikingly with the diffusely distributed light-blue autofluorescent tubules and vascular elastic laminae. The glomeruli appeared almost completely dark except for any red cells which appeared a relatively light, tawny magenta. Similar results were obtained in renal tissue from the sodium-deficient dog, except that the granules, although equally bright (fig. 2, C and D), were typically smaller than those in the sodium-deficient rabbit. In sodium-deficient rats, on the other hand, fluorescent staining of JG granules was faint or nonexistent, although many granules could be demonstrated in the JG cells visualized in Bowie-stained paraffin sections from the same kidneys. The significance of this negative result will be discussed subsequently.

Staining with “Low Titer” Antirabbit Renin

Granules in JG cells of sections of rabbit kidneys treated with fluorescein-labeled homologous antirenin (2 units/cc.) reacted positively just as those treated with “high titer” antihog renin, but, as mentioned, staining time had be be lengthened to 12 to 18 hours.

Relation of Fluorescent Staining of JGI and Renin Activity

It was found that the rabbit tissues giving the best fluorescent staining had the highest JGI (degree of granulation of JG cells de-
(A) Photomicrograph of kidney from rabbit fed a sodium-deficient diet for three and a half weeks. (Arrows) indicate abnormally great numbers of dark-staining granules in the juxtaglomerular cells located in the preglomerular afferent arteriole. (B) Field similar to that in (A) of a section stained with antihog renin labeled with fluorescein isothiocyanate. The JG granules (arrows) fluoresce a bright yellow-green (white in this photograph). Glomerulus containing autofluorescent red blood cells is to the left. The surrounding tubules (T) show pale, diffuse (bluish-white) autofluorescence. JGI for this animal was 73; renin content 13 units/Gm. Frozen-dried section viewed by ultraviolet microscope. (C) Extreme hypergranulation of JG cells in a rabbit fed a sodium-deficient diet for eight weeks. Afferent arteriole cut longitudinally, demonstrating prominent granulation of JG cells in its wall (glomerulus out of field). Paraffin section, Bowie stain. (D) Field similar to that in (C) of a section stained with fluorescein-labeled antihog renin. Arteriole, cut longitudinally, is seen entering a glomerulus (dark area to the left). Note brilliant fluorescent staining of individual JG granules surrounding dark, nonfluorescent nuclei. JGI for this animal was 168. Frozen-dried section viewed by ultraviolet microscope.

determined in Bowie-stained sections) and highest renin content (table 1, figs. 1 and 2). For example, rabbit no. 2, with a JGI of 73 and a renin content of 13 units per Gm., revealed a great deal more fluorescent staining than a control rabbit (no. 6) with a JGI of 16 and a renin content of 5. Most kidneys of control animals showed specific fluorescence, although

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Figure 2

(A) Kidney of rabbit fed control diet for three and a half weeks. (Arrows) indicate JG cells, not as prominent as in sodium-deficient rabbits in figure 1. Paraffin section, Bowie stain. (B) Field similar to that seen in (A) of a section stained with fluorescein-labeled antihog renin. Glomerulus occupies most of field. (Arrow) points to two small JG cells with brightly fluorescent granules in their cytoplasm. Bright spots to the left are autofluorescent red cells in the glomerulus. JGI for this animal was 29; renin content 7 units/Gm. Frozen-dried section viewed by ultraviolet microscope. (C) Section from kidney of dog maintained on sodium-deficient diet for two years. Glomerulus is to the upper right and the macula densa (MD) to the lower left. Note that the JG granules seen here at the vascular pole (arrows) are typically smaller and more dispersed than in sodium-deficient rabbits (see fig. 1). Paraffin section, Bowie stain. (D) Similar field from same kidney as in (C) in a section stained with antihog renin labeled with fluorescein isothiocyanate. Note that, although the JG-cell granules (arrows) are not so concentrated as in rabbit kidneys (see fig. 1), they do show equally bright specific fluorescence. Glomerulus containing bright autofluorescent red blood cells is to the upper left. (T)—surrounding autofluorescent tubules. Frozen-dried section viewed by ultraviolet microscope.

the staining intensity was not so great as in the sodium-deficient animals because of the fewer number of granules per JG cell (fig. 2, A and B). The JGI of one control animal (no. 7) in which even a diligent search failed to reveal staining in any of many sections was
Table 1

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Diet</th>
<th>Time on diet, weeks</th>
<th>Rat assay, mm. Hg</th>
<th>Dog assay, units/Gm.</th>
<th>JGI</th>
<th>Fluorescence microscopy</th>
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</thead>
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<tr>
<td>1</td>
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<td>8</td>
<td>-§</td>
<td></td>
<td>108</td>
<td>++++</td>
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<td>2</td>
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<td>13</td>
<td>78</td>
<td>+++++</td>
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<td>16</td>
<td>+</td>
</tr>
<tr>
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<td>+25</td>
<td>7</td>
<td>29</td>
<td>+</td>
</tr>
<tr>
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<td>2</td>
<td>+18</td>
<td>5</td>
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<tr>
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<td>6</td>
<td>+ 2</td>
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<td>18</td>
<td>0</td>
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</table>

*Renin content is expressed as pressor activity in the case of the rat assay (pressure rise in mm. Hg with injection of 0.1 cc. of 1:50 dilution) and as units per gram of kidney in the case of the dog assay (1 unit = rise in blood pressure of 30 mm. Hg).

†JGI is an index of degree of granulation of JG cells.

§Fluorescence microscopy expressed as 0 to 4 plus indicates the frequency and prominence of JG cells showing specific fluorescent staining.

In order to rule out the possibility that the staining was the result of an antigen-antibody complex nonspecific for renin and antirenin, the following tests for specificity of staining were undertaken and the results are given below: (1) Treatment of other rabbit tissues (heart, lung, and liver) with labeled antirabbit renin and labeled antienzyme globulin on rabbit tissue, showed specific fluorescence of JG cells. In addition, glomerular elements were very slightly stained, particularly in thick sections (3 to 4 μ) or in those in which JG cells were poorly granulated. Results obtained by using orange-red-staining rhodamine B isothiocyanate were inferior to those secured by using fluorescein derivatives because of poor color differentiation between background and specific staining.

Tests for Specificity of the Above Staining for Renin

In order to rule out the possibility that the staining was the result of an antigen-antibody complex nonspecific for renin and antirenin, the following tests for specificity of staining were undertaken and the results are given below: (1) Treatment of other rabbit tissues (heart, lung, and liver) with labeled antirabbit and antihog renin resulted in no staining. (2) Preincubation of two units of rabbit renin with two units of homologous labeled antisera resulted in no staining of JG cells in rabbit kidney through use of the direct method of staining. Normal staining was obtained with the same amount of labeled antisera (two units) preincubated with saline as a dilution control. The procedure was repeated with labeled antihog renin and its homologous antigen with the same result. On staining by the “sandwich” method, neutralization of antirenin with renin prior to the application of the antibody to the tissue abolished all JG-cell staining (fig. 3, A and B). However, it is of considerable importance that this procedure did not abolish glomerular staining when present. (3) Conjugated serum from a normal, nonimmunized, littermate dog failed to stain. (4) Following incubation with either heterogenous labeled antisera to beef insulin or to human gamma globulin, specific staining in rabbit kidneys was absent. (5) Preincubation of sections (rabbit kidney) with unlabeled antirabbit renin produced only faint staining.

Discussion

There can be little doubt that the fluorescent staining of JG cells was specific as a result of the formation of an antigen-antibody complex. The staining was limited to granules of these cells which were positively identified as juxtaglomerular; the intensity of staining was impervious to repeated washings; and all the criteria for specificity, represented by the
tests described herein, were completely satisfied.

On the other hand, there may still be doubt that this antigen-antibody complex was actually that of renin-antirenin. Because the specific activity (purity) of the renin available to us in the present investigation was only 10 units/mg., although highly purified hog renin (780 units/mg.) has been prepared, the possibility remains that the specific staining may have been the result of an unknown protein (and its antiprotein) from JG cells present in the renin extracts. However, evidence presented below strictly supports the concept that renin is being demonstrated by our method.

Previously published studies from our own and other laboratories support the concept that the JG cell is the source of renin. Furthermore, in the present study, the amount of extractable renin, determined by bio-assay, paralleled the intensity of fluorescent staining in frozen-dried sections from the same kidney. Also of significance was the fact that the species affinity of the antirenin used was identical by both the fluorescent-antibody technique and by bio-assay of antirenin. Thus, antirenin produced in the dog with injections of hog renin will neutralize the pressor effect of both canine renin and rabbit renin, but has little neutralizing effect on rat renin. Similarly, in this study, it was found that fluorescein-labeled antirenin (dog to hog) stained JG cells of the rabbit and dog, but not those of the rat. The results with “low titer” and “high titer” antirenin can also be cited as additional evidence. Using conjugated antirabbit renin with a titer of only 2 units per cc., prolonged incubation (12 to 18 hours) was necessary to achieve suitable fluorescent staining of JG cells, whereas the antirenin containing 200 units per cc. required only a few minutes.

The results of this study provide very strong evidence, after all these years, to support Goormaghtigh’s theory that the site of renin elaboration is the JG cell. Procedures are now under way in an attempt to produce immunologically pure renin for use in the blocking test for specificity, with the objective of completing the evidence for the existence of
only a single antigen-antibody system and, thereby, eliminating the possibility of cross reaction with others.

It must be emphasized that in the use of the direct fluorescent-antibody technique, glomerular staining was absent. In contrast, Nairn et al.° reported localization of renin in the glomerulus by using the indirect or "sandwich" fluorescent-antibody technique employing labeled antiglobulin and unlabeled antirenin, rather than the direct procedure with labeled antirenin alone. In our hands, any slight glomerular staining that was demonstrated by the indirect technique was not abolished by previous treatment with unlabeled renin, although Nairn reported the opposite result. Further studies in this and other laboratories will be necessary to resolve this apparent difference.

Summary

Canine antisera to rabbit and hog renin were coupled to several fluorescein dyes. The antiserum was immunologically adsorbed once each with rabbit-liver and with bone-marrow powder, thereby removing excess dye and eliminating nonspecific staining. Mounted frozen-dried sections of kidneys from sodium-deficient rabbits (in which hyperplasia and hypergranulation of juxtaglomerular [JG] cells were present), from control rabbits, from a sodium-deficient dog, and from sodium-deficient rats were treated with the adsorbed labeled antiserum. Ultraviolet microscopy (direct technique) revealed an intense yellow-green fluorescence sharply limited to the granules of the juxtaglomerular cells in all sections studied from kidneys of rabbit and dog. JG granules in rats did not fluoresce, an observation in accord with the species specificity of renin.

The indirect ("sandwich") method was also employed (with adsorbed, labeled, rabbit antiserum to canine globulin), and although slight staining of glomerular epithelium resulted, that in the JG granules was far more intense. In our hands, the faint glomerular staining was not blocked by prior treatment with unlabeled renin. Staining of JG granules in any kidney paralleled the intensity of JG granulation by light microscopy and the amount of extractable renin in the same kidney. JG-granule staining (direct and indirect techniques) was blocked by neutralization of the antirenin with renin. Heterogenous antisera (to insulin; to human gamma globulin) failed to stain. Other rabbit tissues (heart, lung, liver) similarly treated with labeled antirenin never stained.

If this work can be repeated with immunologically pure renin, the evidence presented here, together with previously published studies from this and other laboratories, will establish beyond any reasonable doubt that the source of renin in the kidney is the juxtaglomerular cell, as postulated first by Goormaghtigh nearly a quarter of a century ago.

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