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Active immunization against renin in normotensive marmoset

(primates/blood pressure/autoimmune disease/kidney/Freund's adjuvant)

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ABSTRACT **Primate renins (human and monkey) are very similar. We used pure human renin to immunize marmosets (*Callithrix jacchus*) and thereby produce a chronic blockade of the renin–angiotensinogen reaction. After a control period of 2 months, five male marmosets, on their usual sodium-poor diet, were immunized against pure human renin by three subcutaneous injections of 30 μg each, with complete and then incomplete Freund's adjuvant. Three marmosets were injected with adjuvant only and served as controls. Blood sampling and blood pressure measurements were performed weekly. After the third injection, the five marmosets immunized against renin developed a high titer of renin antibodies (50% binding of ^{125}I-labeled human renin at a dilution of ≥1:10,000). The antibodies inhibited the enzymatic activity of both marmoset and human renins. At the same time, systolic blood pressure decreased significantly from 125 ± 13 mm Hg to 87 ± 8 mm Hg (mean ± SD; 1 mm Hg = 133 Pa). Plasma renin enzyme activity was undetectable in three animals. Plasma aldosterone decreased significantly. After 1–4 months with low blood pressure, a normal urinary output, and a normal plasma creatinine, the five marmosets became sick and died within one month. At autopsy an immunological renal disease, characterized by the presence of immunoglobulin and macrophage infiltration colocalized with renin, was found. Granulomatous formations, probably due to Freund's adjuvant, could be seen in the lungs and in the kidney. No immunoglobulin was detectable in extrarenal vessels or in other organs. These experiments demonstrate that, in this primate, a chronic blockade of the renin–angiotensin system can be achieved by active immunization against homologous renin, but this blockade is associated with the development of an autoimmune disease localized in the kidney.**

The inhibition of the renin–angiotensin system at its first and rate-limiting step, the action of renin on angiotensinogen, can be achieved by the use of synthetic renin inhibitors (1) and by immunological tools. Indeed, the early studies on renin inhibition deal with passive or active immunization against renin (2–4). However, interpretation of such results is limited by the fact that renin was not completely purified. Thus, heterologous renin was used for immunization protocol, even though it is now known that renin has a very strong immunological specificity. Finally, only blood pressure was monitored in the earlier experiments, and no plasma renin activity or aldosterone assays were available. The renin antibody titer also could not be directly measured.

The acute passive immunization of renovascular hypertensive dogs (5) and normotensive marmosets (6) with specific homologous renin antibodies clearly showed that the level of blood pressure depended on renin. From this latter study (6) it was concluded that renin regulated blood pressure in the normotensive marmoset ingesting its usual amount of sodium. The present study was designed to examine the effects of active immunization against renin in marmosets and to observe the effects of chronic blockade of the renin substrate reaction.

MATERIALS AND METHODS

Biological Model. Eight male marmosets (*Callithrix jacchus jacchus*), mean (±SD) body weight 350 ± 31 g, fed their usual diet (grains and fruits), were used throughout this study (Na^+ excretion, 0.15 meq per animal per day; K^+ excretion, 1.5 meq per animal per day). Blood samples were collected weekly from the unanesthetized trained marmoset by direct puncture of the femoral vein. Systolic blood pressure (SBP) was also recorded once a week in unanesthetized animals by the tail-cuff method (BP recorder 8005, W+W Electronic, Appelex, Bagneux, France). The validation of the tail-cuff method for blood pressure measurement has been reported in normotensive (7) and hypertensive marmosets (8). Five marmosets were immunized by three repeated subcutaneous injections of 30 μg of highly purified human renin (specific activity, 680 Goldblatt units per mg of protein). Purification was performed by immunoadsorption on a monoclonal antibody from a juxtaglomerular cell tumor as described by Galen *et al.* (9). The first injections were performed in complete Freund's adjuvant (0.5 ml), the two others in incomplete Freund's adjuvant (0.5 ml). Three control animals received only the adjuvant. Injections were repeated at intervals of 3 weeks.

***In Vitro* Experiments.** The presence of renin antibodies was determined by the ability of marmoset plasma to bind pure human iodinated renin. The titer was defined as the antiserum dilution able to bind 50% of the renin tracer. Plasma renin concentration was measured by radioimmunoassay (RIA) of angiotensin I generated by 1 hr of incubation of 1:50-diluted marmoset plasma in the presence of an excess (635 pmol/200 μl) of human angiotensinogen (6). When the plasma renin concentration of the immunized marmoset remained high despite a high titer of renin antibody, the blockade of renin enzymatic activity was tested by incubating undiluted or 1:2-diluted plasma for 1–3 hr at pH 7.5 without addition of exogenous substrate. The ability of marmoset anti-renin serum to inhibit the enzymatic activity of exogenous marmoset and human renin was tested by the incubation of human and control marmoset plasma at 4°C for 24 hr with successive dilutions of antirenin immune serum in 0.01 M sodium phosphate buffer, pH 7.5. Human angiotensinogen was then added and incubations were carried out for 1 hr. Plasma angiotensinogen was measured by the RIA of angiotensin I generated by the incubation of a 1:100 dilution of marmoset plasma in the presence of a large excess of human

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Abbreviations: SBP, systolic blood pressure; PAP, peroxidase–anti-peroxidase.

renin (7.5 ng). Plasma aldosterone was measured by direct RIA (10). Plasma creatinine was measured by a creatinine analyzer (Beckman).

Morphological Studies. After natural death or sacrifice, a complete postmortem examination was conducted on four of five immunized animals and on one control. Kidneys, aorta, lungs, heart, liver, spleen, inguinal lymph nodes, adrenal glands, testis, brain, and cutaneous fragments were removed for standard histological examination. Kidney was fixed in Duboscq Brazil fixative, other organs in buffered 10% Formalin. Tissues were embedded in paraffin after dehydration and were cut at 5–6 μm. Kidney sections were stained with Masson's trichrome, hematoxylin/eosin, and methenamine/silver. Immunomorphology was investigated in the organs of each animal as follows. Fixed sections of kidney with paraffin removed were immunostained with polyclonal rabbit antibody to human renin (11) by the peroxidase–antiperoxidase technique (PAP, Kit Biolyon, Lyon, France). Kidney, aorta, and lung samples were snap-frozen in liquid nitrogen. Cryostat sections were cut at 6 μm from this material and allowed to react with polyclonal rabbit antibody to human renin (11) in indirect immunofluorescence measurements and in direct immunofluorescence measurements with fluoresceinated polyclonal rabbit antibodies to human immunoglobulin, human IgG, human complement component C_3, human complement component C1q, and human fibrinogen. Anti-human immunoglobulin is known to cross-react with marmoset immunoglobulins (12). Slides were examined with a Leitz fluorescence microscope.

Statistical Methods. Results are expressed as mean ± SD. One-way analysis of variance was used for comparison of blood pressure, body weight, and plasma parameters of controls and immunized animals.

RESULTS

Effects of Renin Immunization on Blood Pressure and Hormonal Parameters. SBP, followed for a period of 8 weeks before immunization, was stable (Fig. 1). After the first administration of complete Freund's adjuvant, a nonspecific fall in blood pressure was observed in immunized and control animals. The SBP subsquently returned to previous values in the controls (127 ± 7 mm Hg before, versus 121 ± 8 mm Hg after injections), but after active immunization there was a significant decrease in SBP from 128 ± 7 mm Hg before to 85 ± 5 mm Hg ($P < 0.01$). Before immunization, body weights in the experimental animals were similar to those of controls (350 ± 10 g). After immunization, body weight decreased gradually in immunized animals (286 ± 7 g) but not in controls (334 ± 11 g).

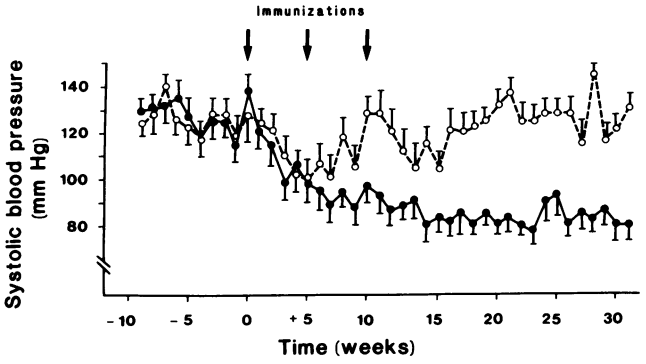

FIG. 1. Effect of active renin immunization on blood pressure in marmosets (1 mm Hg = 133 Pa). ●, Controls ($n = 3$); ○, immunized ($n = 5$). Arrows indicate the time of immunizations.

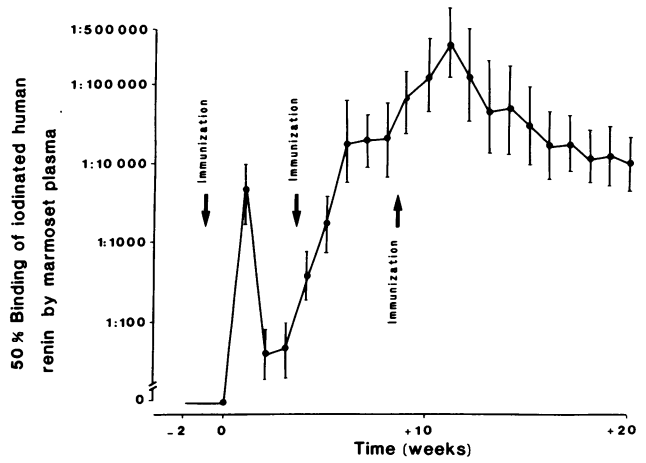

FIG. 2. Appearance of anti-human renin antibodies in marmoset plasma during and after immunization ($n = 5$).

In the five immunized marmosets, after the third injection, the titer of renin antibodies was greater than or equal to 1:10,000 (Fig. 2). No binding of ^{125}I-labeled renin was detected in controls and in preimmune plasma. Plasmas of immunized marmosets blocked exogenously added human and marmoset renin, in parallel dose–response curves (Fig. 3). In three of five marmosets plasma renin concentration decreased to almost zero after immunization (Fig. 4). In two others, despite an antibody titer higher than 1:10,000 and despite a significant decrease in blood pressure, there was no significant decrease in plasma renin concentration. However, pooled plasma samples from these marmosets inhibited the renin angiotensinogen reaction; at pH 7.5, with 1:2 to 1:1 plasma dilution, no angiotensin I was generated in 1 or 3 hr (controls: 1.06 ng of angiotensin I per ml in 1 hr and 2.83 ng of angiotensin I per ml in 3 hr).

Plasma angiotensinogen did not change after immunization (3988 ± 180 pmol/ml before, versus 3343 ± 194 pmol/ml after and 4046 ± 136 pmol/ml in controls). Plasma aldosterone was 979 ± 161 pg/ml in controls and did not vary with time, but plasma aldosterone of immunized marmosets was 1444 ± 150 pg/ml before immunization and decreased significantly ($P < 0.01$) to 416 ± 44 pg/ml after immunization. Plasma creatinine concentration did not change with time in control marmosets (29 ± 12 μM). In immunized marmosets, plasma creatinine did not increase significantly after immunization (33 ± 12 μM before versus 36 ± 17 μM after immunization).

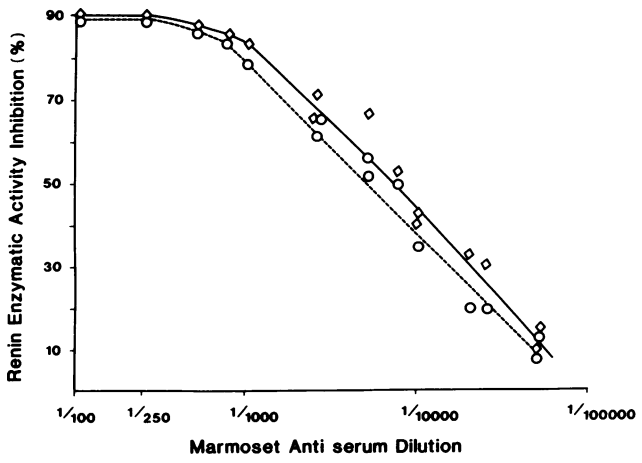

FIG. 3. Inhibition of human (◇) and control marmoset (○) plasma renin enzymatic activity by a terminal serum of one immunized marmoset.

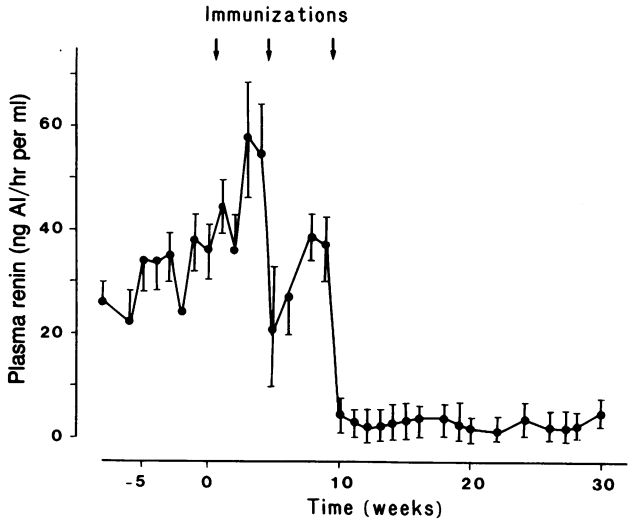

FIG. 4. Disappearance of plasma renin enzymatic activity after immunization. Plasma was diluted 1:50 at pH 6.5 in the assay, and substrate was in excess. Results are mean ± SD for three animals. AI, angiotensin I.

Noticeably, the complete disappearance in PRC was not associated with significant changes in plasma creatinine.

From 2 to 6 months (mean: 25 ± 9 weeks) after immunization, the marmosets became sick, with clinical signs of a progressive disease: very low blood pressure (≤70 mm Hg) and decrease in hematocrit (<25%) and body weight. Plasma creatinine increased just before death (99 ± 13 μM). In these animals hemoglobin was very low (7 ± 0.5 g/100 ml) and platelet count was higher than in controls (10^6/mm^3 versus 0.6×10^6/mm^3). Leukocyte count was low (5×10^3/mm^3 in immunized animals versus 7.7×10^3/mm^3 in controls), with the following differential: neutrophil polynuclears 70%, lymphocytes 25%, and monocytes 5%. The consumption of complement was measured in serum of one immunized animal, one control animal, and a normal human. There was no abnormal complement consumption in the serum of the immunized marmoset (50% hemolytic unit of complement, CH 50 = 297%) as compared to the control marmoset (CH 50 = 322%) and to the normal human serum (CH 50 = 100%). In the last month of life, a renal tubulopathy appeared in these animals, associating major diuresis (30 ml/12 hr versus 5 ml/12 hr in controls), high Na^+ and K^+ output (Na^+ 0.05 meq/hr, K^+ = 0.07 meq/hr in immunized animal versus Na^+ = 0.008 meq/hr, K^+ = 0.03 meq/hr in controls). This sodium diuresis was not reversed by deoxycorticosterone acetate administered subcutaneously at a dose of 0.5 mg over 3 days. Two of the immunized marmosets died spontaneously and three were sacrificed just before death. In contrast, all control marmosets remained healthy.

Morphological Results in Controls. Standard microscopic examination of control marmoset kidneys showed they were optically normal. Fixed sections of control marmoset kidney with paraffin removed and stained with renin antibody by the PAP technique showed a few positive cells in the juxtaglomerular apparatus in approximately 10% of glomeruli. In frozen sections, the juxtaglomerular apparatus and vascular regions were negative. Anti-immunoglobulin, anti IgG, anti-renin, and anti-fibrinogen staining were negative. Only anti-C_3 antibody gave positive granular staining in mesangial cells associated with segmental staining of Bowman's capsules and of tubular basement membranes.

Morphological Results in Immunized Animals. All kidneys from the immunized animals showed microscopic lesions, which were somewhat heterogeneous from one animal to another. The more characteristic features were as follows: (*i*) Glomerular ischemia, attested by retraction of glomerular tufts within the urinary space, which was observed in all animals and ranged from mild to severe; this was associated with hyperplasia of the extraglomerular mesangium (Fig. 5). (*ii*) Inflammatory lesions of arteriolar and arterial vessels, which were prominent in the kidneys of two marmosets. In one marmoset, a granulomatous type of vasculitis occurred, consisting of cellular infiltration of histocytic epithelioid cells and giant multinucleated cells continuously encircling the intrarenal arterial tree from glomerular arterioles to interlobular arteries (Fig. 6). Larger intrarenal arteries were free of lesions. In other marmosets, vascular lesions were of a necrotic type and were restricted to arterioles. The fibrinoid necrosis was associated with few perivascular mononucleated cells and some arterial sections had patchy calcifying necrosis. (*iii*) In two marmosets, there were numerous interstitial nodular granulomas of 300 to 400 μm, which developed in the interstitial connective tissue (independently of the glomerular structure and sometimes pushed into the tubular lumen). They consisted of mononucleated macrophages and multinucleated giant cells. In some giant cells, concentric lamellar Schauman's bodies could be found. Thickening of tubular basement membranes around cortical

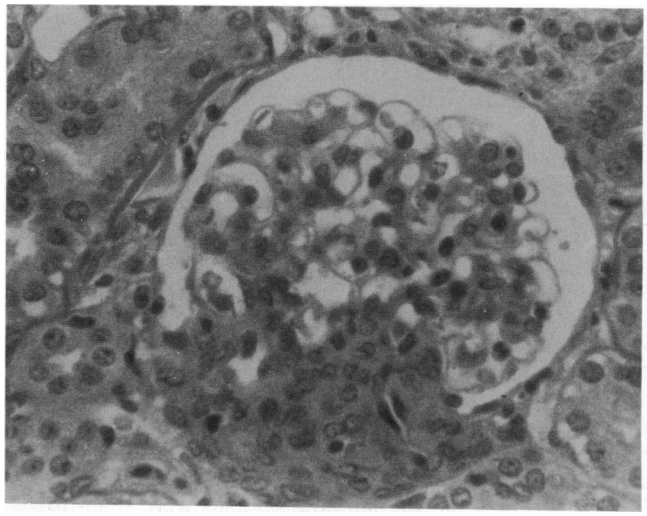

FIG. 5. Hyperplasia of the extraglomerular mesangium in kidney of an immunized marmoset. (×40.)

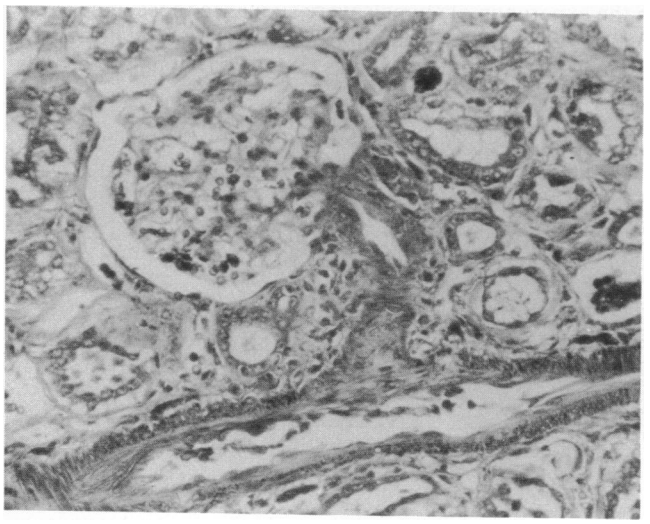

FIG. 6. Perivascular cellular inflammatory process in kidney of an immunized marmoset. (×20.)

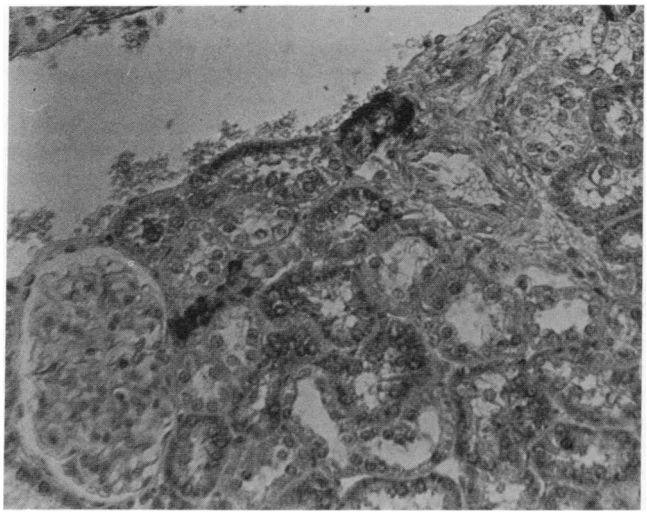

FIG. 7. Immunostaining (PAP technique) of renin on a fixed kidney section of an immunized marmoset. (×20.)

and medullary tubules was observed in two marmosets; on hematoxylin/eosin sections the lesions appeared purple, indicating calcified material.

Immunomorphology. Results were homogeneous from one marmoset to another. Immunostaining of fixed kidney sections after removal of paraffin showed hyperplasia of renin-positive cells (which were present) in nearly all juxtaglomerular apparati, extending down the afferent arteriole and often all the way to the intralobular artery; these recruited cells were localized in the external part of the arterial media (Fig. 7). Immunofluorescence of frozen sections with anti-renin, anti-human immunoglobulin, and anti-human IgG antibodies gave the same patterns of immunostaining, consisting of granular extracellular positive staining in the external part of arteriolar and arterial walls from the juxtaglomerular apparatus to the interlobular arteries (Fig. 8). Interstitial granulomatous lesions and tubular basement membranes were remarkably negative in immunofluorescence with these three antibodies. Anti-human C_3 and C1q gave diffusely positive staining of vascular tissue in exact superposition with anti-renin and anti-immunoglobulin staining. No glomerular staining was observed. Anti-fibrinogen antibody was always negative. Immunostaining of aortic fixed and frozen sections with anti-renin and anti-immunoglobulin antibodies was negative. Contrary to controls, all immunized animals had interstitial granulomatous pneumonia. Granulomas localized around bronchi or in alveolar walls were composed of giant multinucleated cells very rich in calcified Schauman's bodies and associated with few epithelioid cells. In one marmoset, giant cells were spread diffusely through the alveolar walls. These lesions were negative in immunofluorescence studies with anti-renin, anti-immunoglobulin, and anti-IgG antibodies applied to frozen sections. No other significant positive staining was found in all other tissues studied with anti-renin antibody.

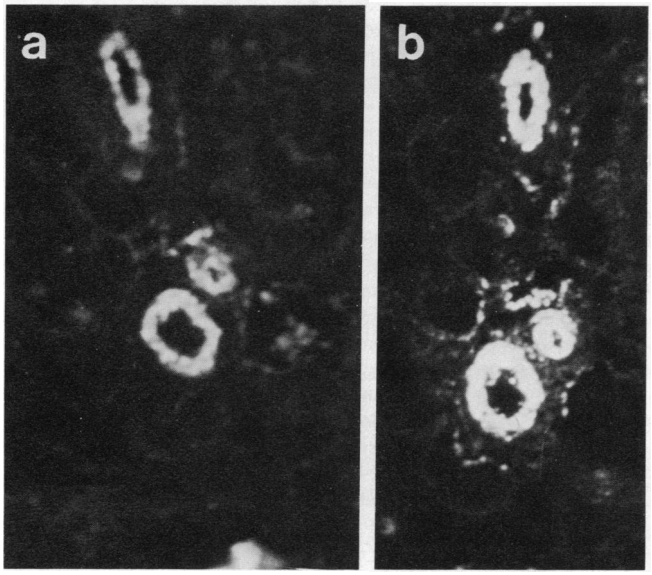

FIG. 8. Colocalization of renin (*a*) and immunoglobulin (*b*) on a frozen kidney section of an immunized marmoset. (×25.)

DISCUSSION

Several factors provide the basis for active immunization of the marmoset with human renin. The biochemical, enzymatic, and immunological properties of marmoset renin are very similar to those of human renin; marmoset renin completely cross-reacts with human renin antibodies, and passive administration of human renin antibodies lowers blood pressure in marmosets (6). Conversely, the absence or insufficiency of interspecies renin homology might explain the failure to obtain a blood pressure lowering or a short-lived hypotensive effect during active immunization (13–18) when heterologous renin is used (13–18). The choice of the normotensive marmoset has several other advantages: on a regular diet (i.e., rich in potassium and poor in sodium), the high plasma renin and aldosterone values indicate a chronic stimulation of the renin–angiotensin–aldosterone system. Indeed, these normotensive animals respond by a significant blood pressure decrease to the passive (6) or active immunization against renin and are a model of choice for testing the acute effect of renin inhibitors (19). Finally, marmosets are known to be good responders to immunogen, as shown by the production of antibodies to human luteinizing hormone-releasing hormone (20). In the present study all five marmosets developed high renin antibody titers. This facility to break down the tolerance to a self protein (i.e., renin) could be due to the low amounts of circulating renin (in the order of the fmol/liter) (21).

The renin antibodies generated in marmosets were able to inhibit *in vitro* the enzymatic activity of exogenous marmoset and human renin. The absence of detectable plasma renin in three marmosets could be attributed to the blockade of circulating renin. An alternative explanation is the absence of renin production by the kidney, but the renin content was high within the kidney in these animals as revealed by immunofluorescence. In the two other animals, plasma renin was not suppressed. However the plasma renin assay involves several factors that could provoke the dissociation of the renin–antibody complex formed *in vivo*, including 1:50 dilution of plasma, exposure to pH 6.5, and an excess of substrate. Indeed, when the generation of angiotensin was tested in conditions of minimal plasma dilution, at neutral pH, the plasmas of these two marmosets did not generate angiotensin I. It is therefore likely that the antibodies produced in these two animals had lower affinities for marmoset renin, explaining the *in vitro* dissociation of the renin–antibody complex under the conditions of the plasma renin assay.

The effect of chronic active renin immunization on blood pressure was quite dramatic in the normotensive marmosets, since the mean decrement in SBP was 45 mm Hg. This decrease occurred approximately 1 month after immunization and persisted throughout the 30 weeks of the experiment. This contrasted with the acute fall in SBP observed after acute passive immunization of approximately 15 mm Hg and a similar lowering effect of renin-inhibitor peptides adminis-

tered acutely (19). The high decrement in SBP observed after anti-renin active immunization could be partly explained by a complete and chronic blockade of the renin enzymatic activity in these animals in which the renin–angiotensin system is especially activated. Because of the autoimmune reaction to the renin immunization, it is also possible that some of the lowering of SBP was not specific for the renin blockade but occurred secondary to the diffuse autoimmune disease.

After renin immunization, plasma aldosterone decreased significantly but remained detectable. These results confirm early experiments (22, 23) involving acute immunization against renin in dogs. In marmosets, the blockade of the renin-angiotensinogen reaction inhibited the secondary hyperaldosteronism but preserved the basal rate of aldosterone secretion. In the kidney, renin immunofluorescence staining was increased in immunized animals. These results extend previous studies using a bioassay for renal renin (24, 25) or the granule and immunofluorescence index (26) to evaluate renin production and storage within the kidney. Renin could be localized by immunofluorescence within the myoepithelioid cells of arterioles on fixed kidney sections but also in the interstitial space on sections of frozen kidney. This increase in renal biosynthesis and storage could be due to the removal of the negative feedback of suppressing angiotensin II, as occurs during converting enzyme inhibition.

During the first months after immunization, the animals were healthy as judged by their body weight, alertness, plasma creatinine, and hematocrit. However, 2–6 months later, they rapidly developed a clinical syndrome characterized by a low body weight, hair loss, passivity, a fall in SBP, and increase in plasma creatinine, which could be attributed to a general disease in these animals. This disease observed in immunized animals 2–6 months after the initial immunization was not observed in control animals and could be attributed to an autoimmune disease. In the immunized marmosets granulomatous formation in the lung and in the kidney could probably be attributed to the Freund's complete adjuvant and more specifically to its mycobacteria (27–29). In the kidney, however, all the lesions could not be attributed to Freund's adjuvant. The colocalization of renin and immunoglobulin within the interstitial space of the immunized kidney indicated the existence of a kidney autoimmune disease directly related to renin. The characteristic distribution of macrophages around the juxtaglomerular apparatus and the arterioles, and the medial necrosis of some arteriolar walls within the kidney, were also in favor of a specific renin autoimmune disease. Finally, the predominant lesions within the kidney, including the presence of immunoglobulin, the existence of a tubulopathy, and a terminal interstitial nephritis showed that active renin immunization induced a kidney-specific autoimmune disease. It can be considered unique in that it predominates at the site of renin production and release, within the afferent arterioles, the interlobular arteries, and the renal interstitium.

Similar observations have been made in autoimmune thyroiditis induced by immunization against thyroglobulin (for review see ref. 21). In these experimental models of thyroiditis, thyroglobulin antibodies are present in plasma and within the thyroid; the humoral autoimmune reaction is associated with a cellular infiltration of the thyroid, particularly with macrophages (30). No lesions could be seen in organs other than the thyroid. As in experimental autoimmune thyroiditis, the present renin autoimmune disease was characterized by autoantibodies and cellular infiltration, which were organ specific since no immunoglobulin or specific cellular infiltration in organs other than kidneys was detected. In early studies of active immunization against hog renin in dog or in rabbit, no evidence of autoimmune disease was shown in the kidney (15–17). In these studies, active immunization was achieved without the utilization of Freund's adjuvant. This raises the question of the role of Freund's adjuvant in the development of this autoimmune tissue disease.

In summary, active immunization against human renin is thus possible in another primate. Before the appearance of the autoimmune disease, renin immunization lowered blood pressure in normotensive animals, decreased plasma aldosterone, and did not induce acute renal insufficiency. It would be worthwhile to conduct experiments with other immunomodulators or with synthetic renin epitopes (31) to see whether it is possible to chronically block renin activity by active immunization without inducing an autoimmune disease.

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