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IMMUNOLOGIC STUDIES IN HYPERTENSION INDUCED BY RENAL ARTERY STENOSIS IN THE RAT

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USAMA T. SAMHURI

A thesis presented in partial fulfillment of the requirements for the degree of Master of Science

Department of Bacteriology and Virology
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August, 1974

AMERICAN UNIVERSITY OF BEIRUT

Thesis Title:

IMMUNOLOGIC STUDIES IN HYPERTENSION INDUCED BY RENAL ARTERY STENOSIS IN THE RAT

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ABSTRACT

Immunization of rats against angiotensin-II conjugated to bovine gamma globulin prevented the development of hypertension induced by unilateral constriction of the renal artery. The immunity produced after giving one booster injection afforded better protection against hypertension than that produced after giving two or three booster injections.

The immune sera produced after giving one and three booster shots were compared by neutralization bioassay tests in nephrectomized rats. Sera from the animals given one booster injection blocked the pressor activity of angiotensin-II more effectively than sera from the animals given three booster injections.

These results suggest that the renin-angiotensin system may play a role in the pathogenesis of hypertension due to renal artery stenosis with the contralateral kidney kept intact.

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INTRODUCTION

Clinical renovascular hypertension in man can be simulated in experimental animals by constriction of the renal artery with the contralateral kedney kept intact.

The decreased blood flow in the kidney with the constricted artery stimulates the release of certain humoral factors which are thought to be implicated in the pathogenesis of this form of hypertension. In the plasma of animals with renal artery stenosis (RAS) and of patients with renovascular hypertension, there is a high concentration of the enzyme renin, which is produced by the juxtaglomerular cells in the kidney. Renin initiates a chain of reactions leading to the production of a highly vasoactive actapeptide called angiotensin-II (AN-II). These observations suggest that the stimulation of the renin-angiotensin system following RAS in experimental animals, or renal artery constriction in clinical cases, may be responsible for the observed rise in blood pressure and the state of hypertension (Brunner et al., 1971). Although this hypothesis was suggested about half a century ago, intensive experimental work to verify its validity is still pursued and the problem has remained unsettled.

With the progress in immunologic methods, the most direct approach to testing the above mentioned hypothesis is to eliminate specifically the effect of renin and angiotensin-II by antibodies acquired either passively or actively by immunization. During the

last 15 to 20 years, the immunologic approach has been widely employed in research in experimental hypertension.

The results of the immunologic studies are contradictory. Some workers succeeded in ameliorating the state of hypertension induced in animals by RAS following immunization against AN-II (Christlieb et al., 1969). Others showed that prolonged immunization regimens failed to protect the animals against induced hypertension although the antibody titers obtained were high (Fide, 1972; Oates et al., 1974).

The present work is an attempt to clarify the problem by showing that (1) immunization against angiotensin-II can protect rats against hypertension induced by renal artery stenosis, (2) duration of the immunization schedule is an important factor for this protection to take place, (3) immune sera can neutralize the in vivo pressor effect of synthetic exegenous angiotensin-II injected intravenously into normal rats, and finally (4) the effective immune state is probably maintained for a very short duration.

REVIEW OF THE LITERATURE

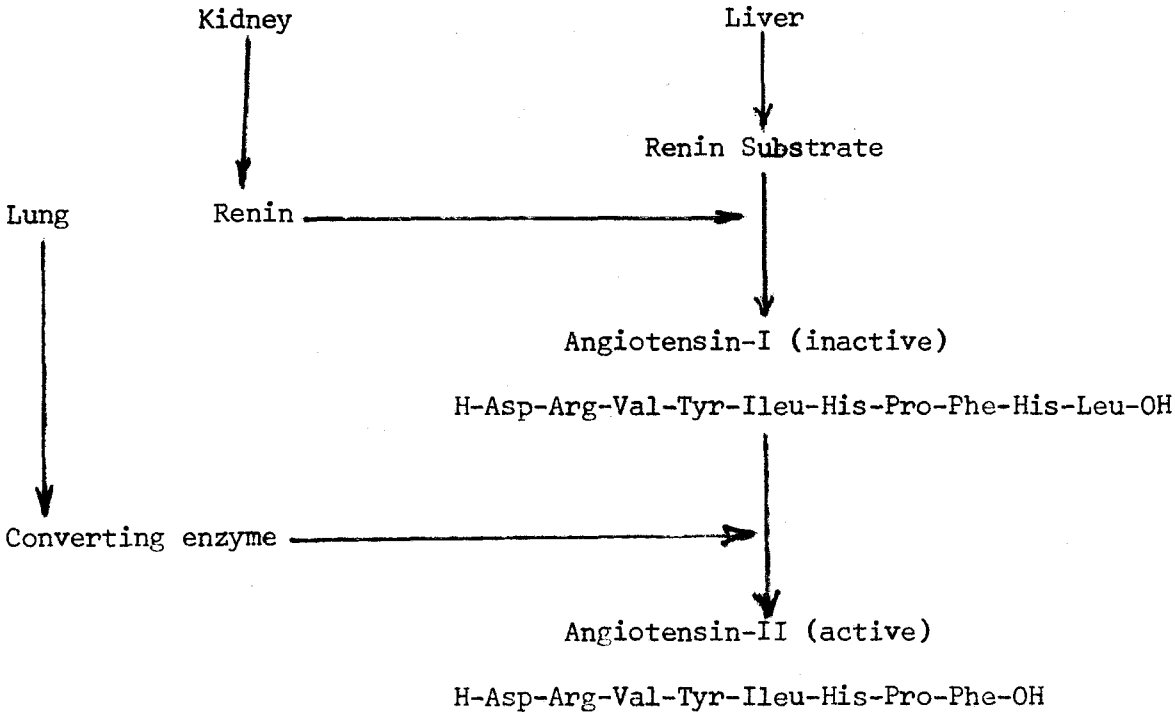
The link between renal hypertension and the renin-angiotensin system is based on two historical experiments. Goldblatt et al. (1934) found that partial constriction of one renal artery and removal of the other kidney in the dog resulted in persistent hypertension. Removal of the clamp from the constricted artery resulted in a drop in the blood pressure to normal. This finding attracted attention to experimental hypertension, and the search to understand the mechanisms underlying the rise in blood pressure began.

The search was guided by another historical observation in 1898: Tigerstedt and Bergman demonstrated that the kidneys contain a highly pressor substance by injecting crude extracts of kidneys into normal rabbits. This pressor substance was identified later as the enzyme renin (Page and McCubbin, 1968; Goodman and Gillman, 1970; Gross, 1971; Berman and Vertes, 1973). Thus, with Goldblatt's finding in mind, the powerful pressor system initiated by renin started to be linked with clinical and experimental hypertension of renal origin.

Renin is a proteolytic enzyme released by cells of the juxtaglomerular apparatus. Once released, it acts upon a substrate attached to an alpha-2-globulin (renin substrate) produced in the liver and a decapeptide molecule called angiotensin-1 (AN-I) is formed. This substance is converted

to an octapeptide, angiotensin-II (AN-II), by the action of the pulmonary converting enzyme. AN-II is a powerful pressor substance and causes vasoconstriction and immediate rise in blood pressure. However, its half-life in the circulation is very short, 1-2 minutes, due to destruction by a group of enzymes known as the angiotensinases and blood pressure drops down to normal (Goodman and Gillman, 1970).

The diagram below summarizes these reactions:



To establish the role of the renin-angiotensin system in renal hypertension, the need arose to measure both renin and AN-II in plasma of patients or experimental animals. It was shown that

the level of plasma renin increases in clinical (Catt et al., 1971) and experimental (Schaechtelin et al., 1963) renovascular hypertension. Yet an elevated level of renin and AN-II in plasma does not necessarily implicate them in the pathogenesis of hypertension. It is important to show that their elimination will result in partial or complete disappearance of the hypertensive state. This led to the suggestion that immunization against renin or AN-II may result in protection against hypertension (Helmer, 1958; Wakerlin, 1958; Deodhar et al., 1964; Weiser et al., 1969). The work on anti-renin antibodies has not been completely reliable due to the impurity of renin extracts and the difficulty of obtaining pure renin (Macdonald et al., 1972). AN-II, on the other hand, has a well known chemical structure (Elliot and Peart, 1957) and hence can be synthesized and obtained in pure form.

Angiotensin-II is a low molecular weight substance, hence alone may fail to induce an immune response; it should be conjugated to a high molecular weight protein carrier before its use as an immunogen. Using a water soluble carbodiimide as a link, AN-II was successfully conjugated to rabbit serum albumin (Goodfriend et al., 1964). The conjugate was highly immunogenic and stimulated the production of specific antibodies. Other carriers such as porcine gamma globulin and poly-L-lysine or adsorption on microparticles of carbon have also been employed to enhance the immunogenicity of AN-II (Waite, 1972).

Once it became possible to induce antibodies against AN-II, immunization proved the most reliable method of producing specific

blockers (antibodies) of the pressor action of this substance. This served as the basis for testing the role of AN-II in the pathogenesis of experimental hypertension. Antibodies can be either introduced passively or induced by active immunization.

Passive immunization of rats with anti-AN-II serum prepared in rabbits was attempted by many investigators. Hedwall (1968) showed that rabbit immune serum failed to lower the blood pressure in rats made hypertensive by renal artery stenosis with the contralateral kidney kept intact. However, the antibody titers in the sera he used were low and probably were insufficient to block completely the physiologic effect of angiotensin-II (Macdonald et al., 1972). With similar experiments, Brunner and coworkers (1971) showed that there was a significant drop in the blood pressure of the rats. They also showed that normal rats injected with the immune serum did not respond to the pressor activity of angiotensin-II.

Similar contradictory findings were reported with active immunization. While Christlieb et al. (1969) reported a drop in blood pressure of rats made hypertensive by renal artery stenosis then actively immunized against angiotensin-II, Johnston et al. (1970), Eide (1972), and Oates et al. (1974) failed to make similar observations.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (bred in the animal house of the School of Medicine, American University of Beirut) weighing 250-350 grams each were used. They were maintained on a standard diet with free access to water.

Preparation of Antigen for Immunization

Val-5 hypertensin II-asp-β-amide (AN-II) (Hypertensin-CIBA) was conjugated to bovine gamma globulin (BGG) using the method of Goodfriend et al. (1964) as modified by Christlieb et al. (1969). The carbodiimide used was 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide metho-p-toluenesulfonate (K & K Laboratories, Inc.), which will be reffered to as CDI.

Five mg of BGG and 10 mg of AN-II were dissolved in 0.25 ml of distilled water. The mixture was added to 0.125 ml of distilled water containing 75 mg of CDI. A similar mixture was prepared to serve as mock-antigen by mixing the same amounts and reagents except AN-II. Both solutions were left for 40 minutes at room temperature with continuous mild magnetic stirring. At the end of 40 minutes, a white turbid colloidal suspension was obtained in the flask containing AN-II and a thick, heavy precipitate in the mock-antigen flask. The contents of both flasks were dialyzed against distilled water at $4^{o}C$ for 14-18 hours, and the dialized colloidal preparations were lyophilized and kept at $4^{o}C$ until use.

Immunization of Rats

Phosphate-buffered saline (PBS), pH 7.3, was used as solvent for the antigen. PBS was prepared as follows:

NaCl	8.00 g
K_2HPO_4	1.21 g
KH_2PO_4	0.34 g
Distilled water	1 liter

The solution was autoclaved for 15 minutes at 121°C and 15 lb per inch square pressure and stored at 4°C.

Five groups of rats, each group consisting of 8-10 animals, were immunized or mock-immunized according to the following schedule:

Group I : primary immunization only

Group II : primary immunization only

Group III : primary immunization and one booster

Group IV : primary immunization and two boosters

Group V : primary immunization and three boosters

The first booster injection in each animal of groups III, IV, and V was given 21 days after primary immunization. Subsequent boosters were given at one week intervals.

In each group, 4-5 of the animals were immunized with AN-II-BGG (experimental) while the other 4-5 rats were mock-immunized

with CDI-BGG (control). For primary immunization, 40 mg of the appropriate conjugate were dissolved in 1 ml of PBS. The solution was emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco), thus giving a final concentration of 20 mg per ml. Each animal received 0.2 ml of the emulsion, 0.05 ml subcutaneously from each foot pad, and hence a total of 4 mg of the appropriate conjugate.

All booster injections were given without adjuvant, each consisted of a total of 4 mg of the appropriate conjugate dissolved in 1 ml of PBS. Half a ml was given intraperitoneally and the other 0.5 ml intramuscularly from hind limbs. All injections were done with the rats under mild ether anesthesia.

Renal Artery Stenosis

Renal artery stenosis was performed one week following the last booster injection in each of the rats in groups III, IV and V. Rats in groups I and II were stenosed 10 and 21 days after primary immunization, respectively.

Stenosis of the renal artery was done under ether anesthesia. A rectangular copper piece, 2 mm X 6 mm in dimensions, was bent from one side to make a clip into which the artery was inserted and partially constricted. The contralateral kidney was left intact.

Measurement of Blood Pressure

The systolic blood pressures of all rats were determined once every two days, one week before starting and throughout the immunization schedule. After stenosis, blood pressure was measured daily for three weeks.

The rat was heated for 5-7 minutes under a 250 watt infra-red lamp fixed 25 cm above. It was then kept in a special holder warmed by a temperature control unit (Physiography MK IV, E & M Instrument Co., Inc., Houston, Texas) with the tail exposed out. A cuff was inserted around the proximal end of the tail, while the distal end was attached to a pneumatic pulse transducer. Both the cuff and the transducer were connected to an electro-sphegmograph with an output leading to the amplifier unit of a physiograph pen recorder (all from E & M Instrument Co., Inc.). The arterial pulse and cuff pressures were recorded simultaneously, and the mean of 5-7 successive readings was taken as the actual systolic pressure.

Preparation of the Immune Serum

Two groups of rats, each consisting of ten animals, were immunized exactly as described previously for groups III and V. Five of the rats in each group were mock-immunized to use their sera as control.

One week after receiving the last booster, all rats were bled from the carotid artery under sodium pentobarbital anesthesia (Abott Laboratories, North Chicago, Illinois, 60064, USA; 2.5 mg per 100 g body weight, given intraperitoneally). Eight to 10 ml of blood were collected from each rat and left standing for 20 - 30 minutes at room temperature. The clot was then broken gently with a wooden applicator and the samples centrifuged for 15 minutes at 1,500 rpm. Serum was collected in 1 ml volumes in small glass tubes and kept at -20°C until use.

Bioassay of the Immune Sera

The different sera were tested in vivo (within one week of their collection) for their ability to neutralize the pressor activity of angiotensin-II.

Rats bilaterally nephrectomized 12-24 hours earlier were anesthetized with sodium pentobarbital and bilaterally vagotomized. Blood pressure was recorded directly by cannulating the carotid artery with a polyethylene catheter connected to a Grass Recorder (Model 7B Polygraph, Grass Instruments Co., Quency, Massachussetts, USA) via a statham transducer (Model P23Gb, Statham Laboratories, Inc., Hato Rey, Puerto Rico). Infusions were given into the left jugular vein through a polyethylene catheter.

The neutralizing effect of each serum was assessed by mixing

0.1 ml of the serum with 0.1 ml of each of different 0.9% saline solutions containing 1000, 500, 250 or 100 ng of angiotensin-II per ml. The mixture was incubated with mild shaking for one minute in an ice-cold water bath. The volume of each injection was such that a total of 10 ng of angiotensin-II (whether bound to antibody or free) from the incubated mixture was administered to the animal. Thus volumes of 20, 40, 80 and 200 ul were injected from mixtures of angiotensin-II containing sera with concentrations of 1000, 500, 250 and 100 ng per ml, respectively.

Both the mock-immune and immune sera were tested. The sensitivity of the rat was tested at the start of and during every experiment by infusing 10 ng of angiotensin-II alone. Serum or saline alone were also injected to assess the effect of volume on increase in blood pressure. The mean of 2-4 determinations was taken as the actual rise in blood pressure above the baseline.

RESULTS

Effect of Immunization on the Development of Hypertension

The mean systolic blood pressure of all rats was 116±1.6 SE before immunization. After immunization the mean did not change significantly for both the mock-immunized (113 ± 0.6, $P > 0.3$) and the immunized (120 ± 1.3, $P > 0.2$) rats. Figures (1 - 5) and tables (I - V) show the blood pressures of all rats before and after immunization and following renal artery stenosis.

Groups I and II

Primary immunization failed to protect rats in these two groups against hypertension. In group I, the mean systolic pressures during the first week after stenosis were 135 ± 3.9 and 136 ± 2.4 ($P > 0.4$) for the mock-immunized and immunized rats, respectively. In group II, the means were 143 ± 3.5 and 139 ± 5.7 ($P > 0.3$) (tables I, II, VI and Figures 1, 2).

Groups III, IV and V

Rats in these groups received one, two and three booster injections, respectively, after primary immunization. There was no significant rise in blood pressure during the first week after stenosis in all groups. During the second week, however, blood pressure remained within normal range only in group III (126 ± 2.0) and rose significantly in groups IV (141 ± 2.3) and V (138 ± 0.7) (tables III, IV, V and Figures 4, 5).

Bioassay of the Immune and Control Sera

Each serum was tested against different concentrations of angiotensin-II. The results are shown in tables (VII, VIII). Antibodies in the different immune sera neutralized best the pressor activity of 50 and 25 ng of angiotensin-II. Table (IX) summarizes the results of the bioassay experiments. Immune sera from the one-booster rats were more effective than immune sera from the three-booster rats in neutralizing the pressor activity of angiotensin-II.

TABLE I

SYSTOLIC BLOOD PRESSURE OF RATS GIVEN PRIMARY IMMUNIZATION (GROUP I) AND OPERATED 10 DAYS LATER

	Blood Pressure (mm Hg)										
	Experimental Rats[1]						Control Rats[2]				
Day	1	2	3	4	5	Mean (±SE)	Day	1	2	3	4	Mean (±SE)
-14	125	107	118	112	115	115 ± 3.0	-14	125	115	125	120	121 ± 2.4
-12	120	120	118	122	125	121 ± 1.2	-12	117	122	122	105	117 ± 4.0
-10	Primary Immunization						-10	Primary Immunization				
- 8	115	120	110	128	112	117 ± 3.2	- 8	115	110	105	105	109 ± 2.4
- 6	115	125	125	118	122	121 ± 1.9	- 6	115	124	125	110	119 ± 3.6
- 2	105	125	120	115	128	118 ± 4.1	- 2	115	125	115	110	116 ± 3.1
0	Stenosis						0	Stenosis				
+ 1	125	130	125	125	150	131 ± 4.8	+ 1	144	110	105	115	119 ± 8.7
2	115	140	130	145	140	134 ± 5.3	2	140	130	110	125	126 ± 6.3
3	105	135	115	135	145	127 ± 7.3	3	145	135	130	115	131 ± 6.3
4	115	145	130	150	170	142 ± 9.3	4	170	140	130	120	140 ±10.8
5	120	145	115	140	155	135 ± 7.6	5	180	140	135	135	148 ±10.9

TABLE I (Cont 'd)

Blood Pressure (mm Hg)

Day	Experimental Rats[1] 1	2	3	4	5	Mean (±SE)	Day	Control Rats[2] 1	2	3	4	Mean (±SE)
6	130	140	125	135	160	138 ± 6.0	6	170	137	140	130	144 ± 8.8
7	125	155	122	140	185	146 ±11.5	7	160	135	135	115	138 ± 8.5
8	125	180	140	180	150	155 ± 10.9	8	165	135	135	115	138 ±10.3
9	125	180	140	180	160	157 ± 10.9	9	155	130	130	115	133 ± 8.3
10	130	170	140	170	155	153 ± 8.0	10	160	145	145	120	143 ± 8.3
11	132	175	145	165	155	154 ± 7.5	11	165	150	150	118	148 ± 9.9
12	135	170	145	175	160	157 ± 7.5	12	170	150	150	125	149 ± 9.2
13	140	165	145	170	160	156 ± 5.8	13	175	155	155	130	154 ± 9.2
14	140	170	150	175	175	162 ± 7.2	14	180	160	160	135	159 ± 9.2
15	138	170	145	180	175	162 ± 8.4	15	185	165	165	135	163 ±10.3
16	Bleeding			170	170	170 ± 0.0	16	Bleeding		170	140	155 ±15.0
17	-	-	-	180	180	180 ± 0.0	17	-	-	175	140	158 ±17.5
18	-	-	-	185	175	180 ± 5.0	18	-	-	170	145	158 ±12.5
19	-	-	-	175	180	178 ± 2.5	19	-	-	170	150	160 ±10.0
20	-	-	-	180	175	178 ± 2.5	20	-	-	170	155	163 ± 7.5
21				Bleeding			21.				Bleeding	

1. Rats immunized with AN-II-BGG and Freund's complete adjuvant.
2. Rats mock-immunized with CDI-BGG and Freund's complete adjuvant.

TABLE II

SYSTOLIC BLOOD PRESSURE OF RATS GIVEN PRIMARY IMMUNIZATION (GROUP II) AND OPERATED 21 DAYS LATER

Blood Pressure (mm Hg)

Experimental Rats[1]						Control Rats[2]					
Day	6	7	8	9	Mean (±SE)	Day	5	6	7	8	Mean (±SE)
-24	110	102	110	115	109 ± 2.7	-24	111	100	105	112	107 ± 2.8
-22	100	108	115	110	108 ± 3.1	-22	100	113	130	100	108 ± 4.9
-21	Primary Immunization					-21	Primary Immunization				
-20	110	122	116	110	115 ± 2.9	-20	110	113	115	102	110 ± 2.9
-18	115	106	125	110	114 ± 4.1	-18	108	108	112	118	112 ± 2.4
-16	110	105	120	108	112 ± 3.3	-16	108	112	105	110	109 ± 1.5
-14	100	115	126	100	110 ± 6.2	-14	110	105	108	122	111 ± 3.7
-12	115	105	112	105	109 ± 2.5	-12	100	115	105	126	112 ± 5.8
-10	114	118	115	100	112 ± 4.0	-10	100	118	115	125	115 ± 5.3
- 8	119	115	120	108	116 ± 2.7	- 8	115	104	117	106	111 ± 3.2
- 6	118	120	120	120	120 ± 0.5	- 6	110	122	106	110	112 ± 3.5
- 4	120	125	110	118	118 ± 3.1	- 4	122	105	118	102	112 ± 4.9
- 2	118	124	126	120	122 ± 1.8	- 2	115	120	120	121	119 ± 1.4
0		Stenosis				0		Stenosis			
+ 1	140	160	160	120	145 ± 9.6	+ 1	100	110	112	120	111 ± 4.1
2	130	160	160	122	143 ± 9.9	2	135	132	134	138	135 ± 1.3
3	130	138	145	122	134 ± 4.9	3	144	145	140	150	145 ± 2.1
4	135	165	140	120	140 ± 9.4	4	140	135	138	140	138 ± 1.9

TABLE II (Cont "d)

Blood Pressure (mm Hg)

Day	Experimental Rats[1] 6	7	8	9	Mean (±SE)	Day	Control Rats[2] 5	6	7	8	Mean (±SE)
5	140	132	140	125	13" ± 3.6	5	142	135	142	138	140 ± 1.2
6	170	140	146	120	144 ±1^.3	6	160	145	166	155	157 ± 4.4
7	160	170	195	120	161 ±15.6	7	155	138	180	138	153 ± 9.9
8	165	160	170	126	15F ± 9.9	8	142	130	142	150	141 ± 4.1
9	180	170	170	132	163 ±10.6	9	137	135	165	155	148 ± 7.2
10	190	140	145	126	150 ±13.8	10	145	144	165	140	149 ± 5.6
11	170	170	150	120	15? ±11.^	11	144	136	148	160	147 ± 5.0
12	170	145	160	130	151 ± 8.8	12	140	134	170	150	149 ± 7.9
13	170	145	155	145	154 ± 5.9	13	140	144	185	170	160 ±10.7
14	Bleeding		160	144	152 ± 8.0	14	Bleeding		175	180	178 ± 2.5
15	-	-	140	145	143 ± 2.5	15	-	-	180	165	173 ± 7.5
16	-	-	150	140	145 ± 5.0	16	-	-	-	-	-
17	-	-	160	135	1'8 ±12.5	17	-	-	160	145	153 ± 7.5
18	-	-	160	130	145 ±17.5	18		-	150	172	161 ±11.0
19	-	-	-	-	-	19	-	-	165	180	173 ± 7.5
20	-	-	-	-	-	20	-	-	180	185	183 ± 2.5
21			Bleeding			21			Bleeding		

1. Rats immunized with AN-II-BGG and Freund's complete adjuvant.
2. Rats mock-immunized with CDI-BGG and Freund's complete adjuvant.

TABLE III

SYSTOLIC BLOOD PRESSURE OF RATS GIVEN SECONDARY IMMUNIZATION (GROUP III) AND OPERATED ONE WEEK AFTER THE BOOSTER SHOT

Blood Pressure (mm Hg)

Experimental Rats[1]						Control Rats[2]						
Day	10	11	12	13	Mean (±SE)	Day	9	10	11	12	13	Mean (±SE)
-32	132	130	124	130	129 ± 1.7	-32	110	110	105	110	115	110 ± 1.6
-30	130	130	126	126	130 ± 1.5	-30	110	120	130	100	125	117 ± 5.4
-28		Primary	Immunization			-28		Primary	Immunization			
-26	126	118	130	122	124 ± 2.9	-26	112	100	120	110	120	112 ± 3.7
-24	124	122	128	126	125 ± 1.3	-24	110	110	116	108	115	113 ± 2.5
-22	126	123	124	126	125 ± 0.8	-22	108	120	104	112	117	112 ± 2.9
-20	125	118	121	122	122 ± 1.4	-20	110	105	108	115	122	112 ± 2.9
-18	120	122	120	120	121 ± 0.5	-18	110	105	114	110	124	113 ± 3.2
-16	123	124	121	121	122 ± 0.8	-16	124	110	110	125	126	121 ± 3.7
-14	124	119	122	120	124 ± 1.1	-14	122	116	105	100	102	109 ± 4.3
-12	120	121	122	122	121 ± 0.5	-12	110	110	120	106	116	112 ± 2.5
-10	127	121	122	120	123 ± 1.6	-10	124	115	106	116	100	112 ± 4.2
- 8	118	120	120	122	120 ± 0.8	- 8	100	110	106	120	110	109 ± 3.3
- 7		Booster				- 7			Booster			
- 6	116	114	118	116	116 ± 0.8	- 6	120	120	120	125	122	121 ± 0.9
- 4	-	-	-	-	- -	- 4	120	110	102	120	118	114 ± 3.5

TABLE III (Cont 'd)

Blood Pressure (mm Hg)

Day	Experimental Rats[1] 10	11	12	13	Mean (±SE)
-2	-	-	-	-	-
0		Stenosis			
+1	125	115	102	115	114 ± 4.7
2	100	100	105	110	104 ± 2.4
3	100	122	100	120	111 ± 5.4
4	127	110	110	120	117 ± 4.2
5	120	122	100	118	115 ± 5.1
6	100	105	117	118	110 ± 4.6
7	105	100	-	115	107 ± 4.4
8	120	150	120	120	128 ± 7.5
9	135	155	119	122	133 ± 8.2
10	130	Bleeding	110	120	120 ± 5.8
11	145	-	130	110	128 ±10.1
12	140	-	122	125	129 ± 5.6
13	-	-	-	-	-
14	Bleeding	-	124	120	122 ± 2.0

Day	Control Rats[2] 9	10	11	12	13	Mean (±SE)
-2	112	108	105	125	124	114 ± 3.5
0			Stenosis			
+1	120	118	138	148	150	135 ± 6.8
2	108	140	125	158	133	133 ± 8.3
3	135	134	124	140	150	137 ± 4.2
4	125	138	150	143	142	140 ± 4.1
5	140	144	142	-	132	140 ± 2.6
6	135	147	145	150	137	143 ± 2.9
7	130	140	135	160	142	141 ± 5.1
8	137	160	146	175	158	155 ± 6.5
9	-	-	-	-	-	-
10	134	160	152	-	136	146 ± 6.3
11	135	140	155	-	160	148 ± 5.9
12	140	144	160	-	140	146 ± 4.8
13	136	136	155	-	140	142 ± 4.5
14	Bleeding		145	-	148	147 ± 1.5

TABLE III (Cont 'd)

Blood Pressure (mm Hg)

Experimental Rats[1]						Control Rats[2]						
Day	10	11	12	13	Mean (±SE)	Day	9	10	11	12	13	Mean (±SE)
15	-	-	105	110	108 ± 2.5	15	-	-	158	-	152	155 ± 3.0
16	-	-	106	117	112 ± 5.5	16	-	-	150	-	140	145 ± 5.0
17	-	-	130	125	128 ± 2.5	17	-	-	155	-	142	149 ± 6.5
18	-	-	130	105	118 ±12.5	18	-	-	141	-	135	138 ± 3.0
19	-	-	122	125	124 ± 1.5	19	-	-	142	-	140	141 ± 1.0
20	-	-	104	136	120 ±16.0	20	-	-	155	-	140	152 ± 3.5
21	-		Bleeding			21				Bleeding		

1. Rats immunized with An-II-BGG, booster shot given 3 weeks after primary immunization.
2. Rats mock-immunized with CDI-BGG, booster shot given 3 weeks after primary immunization.

TABLE IV

SYSTOLIC BLOOD PRESSURE OF RATS GIVEN TERTIARY IMMUNIZATION (GROUP IV) AND OPERATED ONE WEEK AFTER THE LAST BOOSTER SHOT

Blood Pressure (mm Hg)

	Experimental Rats[1]					Control Rats[2]				
Day	14	15	16	17	Mean (±SE)	14	15	16	17	Mean (±SE)
-38	112	114	120	122	117 ± 2.4	104	108	113	100	106 ± 2.8
-36	124	120	120	124	122 ± 1.2	120	110	110	121	115 ± 3.0
-35		Primary Immunization					Primary Immunization			
-34	126	126	120	122	124 ± 1.5	110	100	112	110	108 ± 2.7
-32	127	126	127	125	126 ± 0.5	118	105	104	120	112 ± 4.2
-30	128	124	125	125	126 ± 0.9	102	104	115	108	107 ± 2.9
-28	120	122	121	122	121 ± 0.5	106	110	108	110	109 ± 0.9
-26	122	124	115	118	120 ± 2.0	105	104	95	110	104 ± 3.1
-24	122	123	122	120	122 ± 0.6	105	95	110	100	103 ± 3.2
-22	121	123	118	119	120 ± 1.1	102	115	106	104	107 ± 2.9
-20	126	124	120	122	123 ± 1.3	120	115	120	124	120 ± 1.8
-18	123	124	121	122	123 ± 0.6	125	122	120	120	122 ± 1.2
-16	122	124	121	122	122 ± 0.6	118	116	120	112	117 ± 1.7
-14		Booster					Booster			
-12	-	-	-	-	-	120	115	115	120	118 ± 1.4

TABLE IV (Cont 'd)

Blood Pressure (mm Hg)

Day	Experimental Rats[1] 14	15	16	17	Mean (±SE)	Day	Control Rats[2] 14	15	16	17	Mean (±SE)
-10	-	-	-	-	-	-10	117	120	112	120	117 ± 1.9
- 8	-	-	-	-	-	- 8	120	100	125	110	114 ± 5.5
- 7		Booster				- 7		Booster			
- 6	120	97	100	90	102 ± 6.4	- 6	125	124	105	115	117 ± 4.7
- 4	118	110	97	110	109 ± 4.3	- 4	126	118	120	107	118 ± 3.9
- 2	118	112	105	108	111 ± 2.8	- 2	126	112	120	119	119 ± 2.9
0		Stenosis				0		Stenosis			
+ 1	110	120	120	97	112 ± 5.5	+ 1	115	122	132	160	132 ± 9.9
2	120	130	130	120	125 ± 2.9	2	145	140	150	160	149 ± 4.3
3	120	130	140	135	131 ± 4.3	3	144	143	140	160	147 ± 4.5
4	119	115	110	130	119 ± 4.2	4	142	140	145	162	147 ± 5.0
5	142	115	120	142	130 ± 7.1	5	140	145	150	162	150 ± 4.7
6	140	92	140	145	129 ±12.5	6	150	150	137	160	149 ± 4.7
7	142	117	145	165	142 ± 9.8	7	145	148	140	162	149.± 4.7
8	140	100	140	145	131 ±10.4	8	142	-	140	138	139 ± 1.0
9	142	100	150	145	134 ±11.5	9	160	-	150	162	156 ± 6.0
10	145	136	140	165	147 ± 6.4	10	165	-	144	150	147 ± 3.0

TABLE IV (Cont 'd)

Blood Pressure (mm Hg)

Experimental Rats[1]						Control Rats[2]					
Day	14	15	16	17	Mean (±SE)	Day	14	15	16	17	Mean (±SE)
11	144	130	140	170	146 ± 8.5	11	145	-	142	158	150 ± 8.0
12	140	132	140	160	143 ± 5.9	12	145	-	150	165	158 ± 7.5
13	140	140	138	155	143 ± 3.9	13	140	-	140	140	140 ± 0.0
14	Bleeding		135	150	143 ± 7.5	14	Bleeding	-	Bleeding	144	144 ± 0.0
15	-	-	138	150	144 ± 6.0	15	-	-	-	155	155 ± 0.0
16	-	-	130	132	131 ± 1.0	16	-	-	-	140	140 ± 0.0
17	-	-	105	134	120 ±14.5	17	-	-	-	145	145 ± 0.0
18	-	-	110	130	120 ±10.0	18	-	-	-	140	140 ± 0.0
19	-	-	125	135	130 ± 5.0	19	-	-	-	130	130 ± 0.0
20	-	-	116	135	126 ± 9.5	20	-	-	-	142	142 ± 0.0
21			Bleeding							Bleeding	

1. Rats immunized with AN-II-BGG, boosters given 3 weeks after primary immunization and at one week intervals.

2. Rats mock-immunized with CDI-BGG, boosters given 3 weeks after primary immunization and at one week intervals.

TABLE V

SYSTOLIC BLOOD PRESSURE OF RATS GIVEN PRIMARY IMMUNIZATION AND THREE BOOSTERS (GROUP V) AND OPERATED ONE WEEK AFTER THE LAST BOOSTER

Blood Pressure (mm Hg)

	Experimental Rats[1]						Control Rats[2]						
Day	18	19	20	21	Mean (±SE)	Day	18	19	20	21	22	Mean (±SE)
-46	120	118	125	130	123 ± 2.7	-46	120	122	104	105	108	112 ± 3.8
-44	121	122	116	124	121 ± 1.7	-44	119	123	110	106	100	112 ± 4.2
-42		Primary immunization				-42		Primary immunization				
-40	122	120	126	126	124 ± 1.5	-40	110	120	105	109	108	110 ± 2.7
-38	126	126	120	125	124 ± 1.4	-38	112	118	100	102	100	106 ± 3.7
-36	-	120	120	122	121 ± 0.7	-36	120	122	110	110	110	114 ± 2.7
-34	-	115	116	115	115 ± 0.3	-34	115	116	112	104	105	110 ± 2.5
-32	124	120	124	126	124 ± 1.3	-32	115	100	120	100	100	107 ± 4.4
-30	114	120	123	124	120 ± 2.3	-30	100	118	95	106	118	107 ± 4.7
-28	117	120	120	120	119 ± 0.8	-28	105	112	110	100	118	109 ± 3.0
-26	120	120	120	124	121 ± 1.0	-26	120	120	115	110	120	117 ± 2.0
-24	119	119	126	120	121 ± 1.7	-24	115	121	118	118	120	118 ± 1.0
-22	119	121	122	120	121 ± 0.6	-22	122	118	100	109	106	111 ± 4.0
-21		Booster				-21		Booster				
-20	-	-	-	-	-	-20	102	120	106	120	118	113 ± 3.8
-18	-	-	-	-	-	-18	118	125	120	125	125	123 ± 1.5
-16	-	-	-	-	-	-16	107	125	109	120	120	116 ± 3.5

TABLE V (Cont 'd)

Blood Pressure (mm Hg)

Day	Experimental Rats[1] 18	19	20	21	Mean (±SE)	Day	Control Rats[2] 18	19	20	21	22	Mean (±SE)
-14		Booster				-14		Booster				
-12	110	120	115	110	114 ± 2.4	-12	122	112	120	115	120	118 ± 1.9
-10	115	120	110	120	116 ± 2.4	-10	117	114	120	118	110	116 ± 1.7
- 8	115	117	117	100	112 ± 4.1	- 8	120	110	102	115	108	111 ± 3.1
- 7		Booster				- 7		Booster				
- 6	118	122	106	115	115 ± 3.4	- 6	114	100	118	104	110	109 ± 3.3
- 4	126	108	120	120	119 ± 3.8	- 4	126	114	110	120	126	119 ± 3.1
- 2	126	120	115	115	119 ± 2.6	- 2	124	124	120	125	107	120 ± 3.4
0		Stenosis				0		Stenosis				
+ 1	114	124	120	120	120 ± 2.0	+ 1	122	130	126	130	112	124 ± 3.3
2	122	120	100	114	114 ± 4.9	2	135	140	130	150	140	139 ± 3.3
3	118	130	126	114	122 ± 3.7	3	148	165	140	160	180	159 ± 6.9
4	120	130	144	140	134 ± 5.4	4	140	155	150	175	170	158 ± 6.4
5	122	132	140	138	135 ± 4.0	5	165	175	165	185	195	177 ± 5.8
6	120	132	138	148	135 ± 5.9	6	160	180	160	180	175	171 ± 4.6
7	148	126	132	140	137 ± 4.8	7	140	185	155	185	190	171 ± 9.9
8	142	140	135	140	141 ± 2.7	8	140	185	150	175	180	168 ± 9.8
9	140	144	140	126	138 ± 3.9	9	150	190	160	150	165	163 ± 7.3

TABLE V (Cont 'd)

Blood Pressure (mm Hg)

Experimental Rats[1]						Control Rats[2]						
Day	18	19	20	21	Mean (±SE)	Day	18	19	20	21	22	Mean (±SE)
10	145	130	142	137	131 ± 3.3	10	143	167	150	160	190	162 ± 8.1
11	140	132	142	137	138 ± 2.2	11	175	170	140	185	195	173 ± 9.3
12	136	130	140	140	137 ± 2.4	12	144	177	1401	170	180	162 ± 8.4
13	136	148	140	138	141 ± 2.6	13	154	145	142	170	160	154 ± 5.1
14	Bleeding		140	132	136 ± 4.0	14	144	142	Bleeding			143 ± 1.0
15	-	-	144	144	144 ± 0.0	15	142	150	-	-	-	146 ± 4.0
16	-	-	140	130	135 ± 5.0	16	140	160	-	-	-	150 ±10.0
17	-	-	115	120	118 ± 2.5	17	138	160	-	-	-	149 ±11.0
18	-	-	120	110	115 ± 5.0	18	135	180	-	-	-	158 ±22.5
19	-	-	120	130	125 ± 5.0	19	140	160	-	-	-	150 ±20.0
20	-	-	140	140	140 ± 0.0	20	144	165	-	-	-	155 ±10.5
21			Bleeding			21	Bleeding					

1. Rats immunized with AN-II-BGG, boosters given 3 weeks after primary immunization and at one week intervals.
2. Rats mock-immunized with CDI-BGG, boosters given 3 weeks after primary immunization and at one week intervals.

TABLE VI

COMPARISON OF THE MEAN SYSTOLIC BLOOD PRESSURES OF RATS WITH DIFFERENT IMMUNIZATION REGIMENS ONE AND TWO WEEKS AFTER STENOSIS

	Systolic Blood Pressure (mm Hg)									
	Group									
	I[1]		II[2]		III[3]		IV[4]		V[5]	
	E[6]	C[7]	E	C	E	C	E	C	E	C
Base line[8]	119±1.1	116±2.0	114±1.3	112±0.9	123±1.0	113±0.9	119±1.8	113±1.4	119±0.9	113±1.0
First week[9]	136±2.4	135±3.9	143±3.5	139±5.7	111±1.7	138±1.3	126±3.6	146±2.4	127±3.4	157±7.3
P[10]	> 0.4		> 0.3		< 0.0005		< 0.0005		< 0.002	
Second week[11]	156±1.1	146±3.4	154±3.4	154±4.7	126±2.0	147±1.7	141±2.3	147±2.8	138±0.7	160±3.7
P	< 0.01		> 0.4		< 0.0005		< 0.05		< 0.0005	

1. Rats with primary immunization and stenosed 10 days later.
2. Rats with primary immunization and stenosed 21 days later.
3. Rats with primary immunization and one booster shot, stenosed one week after the booster.
4. Rats with primary immunization and two booster shots, stenosed one week after the last booster.
5. Rats with primary immunization and three booster shots, stenosed one week after the last booster.
6. Rats immunized with AN-II-BCG (experimental)
7. Rats mock-immunized with CDI-IGG (control).
8. Mean (±SE) of the daily systolic pressures before stenosis.
9. Mean (±SE) of the daily systolic pressures during the first week after stenosis.
10. Probability calculated by the Student t-test.
11. Mean (±SE) of the daily systolic pressures during the second week after stenosis.

TABLE VII

BIOASSAY OF SERA OBTAINED FROM RATS WITH SECONDARY IMMUNIZATION (GROUP III)

	Rise in Blood Pressure (mm Hg) Angiotensin-II (ng)					Rise in Blood Pressure (mm Hg) Angiotensin - II (ng)			
IS*	100	50	25	10	CS**	100	50	25	10
1	10.6	11.3	7.3	15.0	1	20.6	19.3	20.6	27.3
2	25.6	9.0	11.6	12.5	2	25.6	23.0	23.0	24.0
3	23.3	10.0	10.0	17.0	3	25.3	26.6	25.3	21.0
4	12.0	10.0	6.6	17.0	4	23.3	21.3	19.3	24.0
5	20.6	14.6	14.6	19.0	5	19.3	22.0	16.6	27.0
Mean (±SE)	18.4±3.0	10.9±0.9	10.0±1.5	16.1±1.1	Mean(±SE)	22.8±1.3	22.4±1.2	20.9±1.5	26.3±0.9
P	>0.1	<0.0005	<0.0005	<0.0005					

* Immune serum from rats immunized with AN-II-BGG, obtained on the 8th day after giving the last booster shot

** Control serum from rats mock-immunized with CDI-BGG, obtained on the 8th day after giving the last booster shot

TABLE VIII

BIOASSAY OF SERA OBTAINED FROM RATS WITH PRIMARY IMMUNIZATION AND THREE BOOSTERS (GROUP V)

	Rise in Blood Pressure (mm Hg) Angiotensin-II (ng)					Rise in Blood Pressure (mm Hg) Angiotensin-II (ng)			
IS*	100	50	25	10	CS**	100	50	25	10
1	12.5	12.5	5.0	12.5	1	10.5	11.5	13.5	16.0
2	9.0	11.0	13.0	17.0	2	20.0	19.0	19.0	23.0
3	24.0	19.0	17.0	19.0	3	24.0	27.0	29.0	29.0
4	17.0	13.0	20.0	21.0	4	21.0	25.0	25.0	29.0
5	13.0	16.0	17.0	20.0	5	21.0	25.0	27.0	30.0
Mean (±SE)	15.1±2.6	12.3±2.8	14.4±2.6	17.9±1.5	Mean (±SE)	19.3±2.3	21.5±2.8	22.7±2.8	25.4±2.7
P	> 0.1	< 0.02	< 0.05	< 0.02					

* Immune serum from rats immunized with AN-II-BGG, obtained on the 8th day after giving the last booster shot.

** Control serum from rats mock-immunized with CDI-BGG, obtained on the 8th day after giving the last booster shot.

TABLE IX

SUMMARY OF THE BIOASSAYS OF SERA OBTAINED FROM RATS WITH SECONDARY IMMUNIZATION (GROUP III) AND THOSE WITH PRIMARY IMMUNIZATION AND THREE BOOSTERS (GROUP V).

Mean (±SE) rise in blood pressure (mm Hg)

		Angiotensin -II (ng)			
		100	50	25	10
Group III	IS[a]	18.1 ± 3.0 [c]	10.9 ± 0.9	10.0 ± 1.5	18.1 ± 1.1
	CS[b]	22.8 ± 1.3	22.4 ± 1.2	20.0 ± 1.5	26.3 ± 0.9
	P [d]	> 0.1	< 0.0005	< 0.0005	< 0.0005
Group V	IS	15.1 ± 2.6	12.3 ± 2.8	14.4 ± 2.6	17.9 ± 1.5
	CS	19.3 ± 2.3	21.5 ± 2.8	22.7 ± 2.8	25.4 ± 2.7
	P	> 0.1	<0.02	<0.05	<0.02

a. Immune sera from rats immunized with AN-II-BGG.
b. Mock-immune sera from rats mock-immunized with CDI-BGG
c. The mean (±SE) of rise in blood pressure (mm Hg) obtained with 5 different sera
d. Probability calculated using the Student t-test

Fig. 1. Systolic blood pressure of rats given primary immunization (group I) and operated 10 days later.

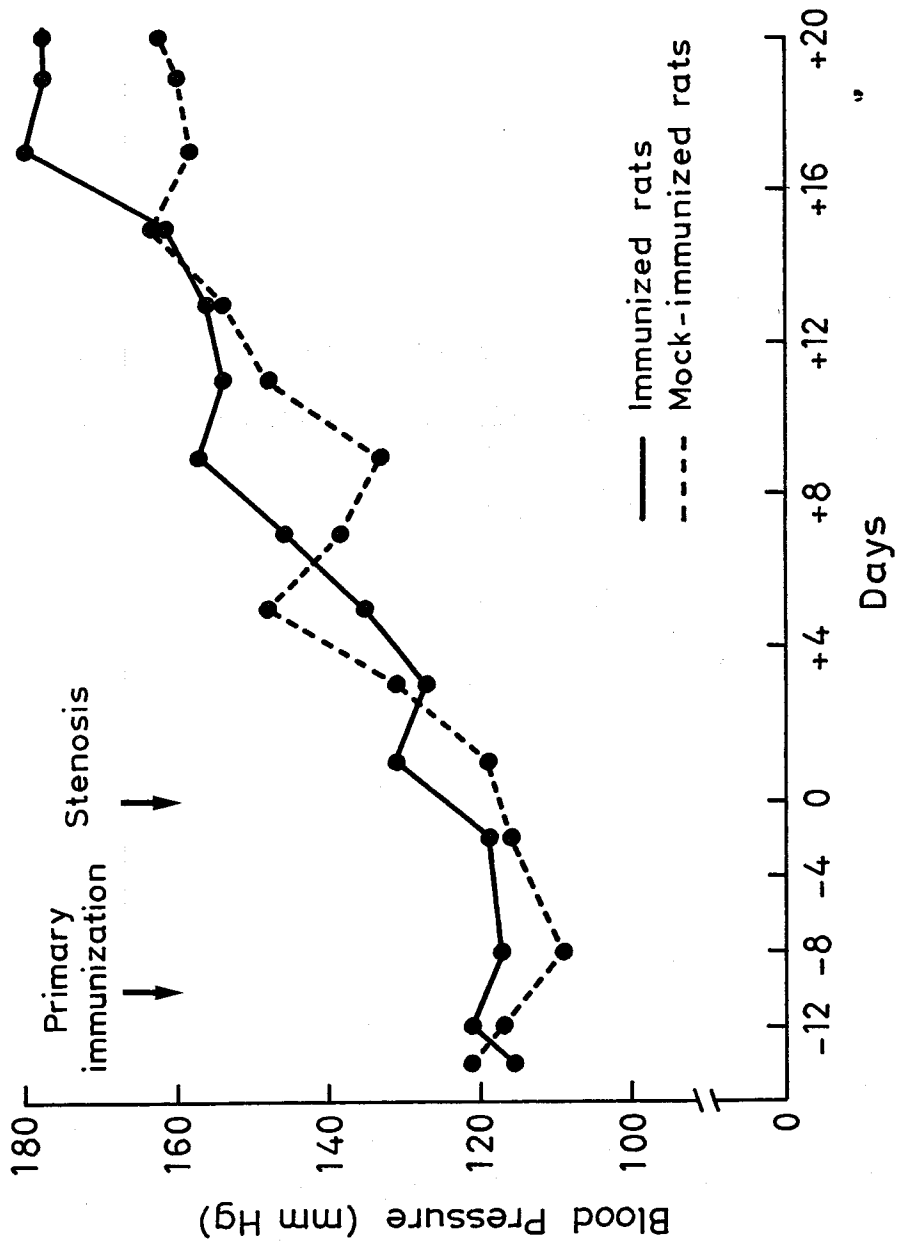

Blood Pressure (mm Hg)
180
160
140
120
100
0
-12 -8 -4 0 +4 +8 +12 +16 +20
Days
Primary immunization
Stenosis
Immunized rats
Mock-immunized rats

Fig. 2 Systolic blood pressure of rats given primary immunization (group II) and operated 21 days later.

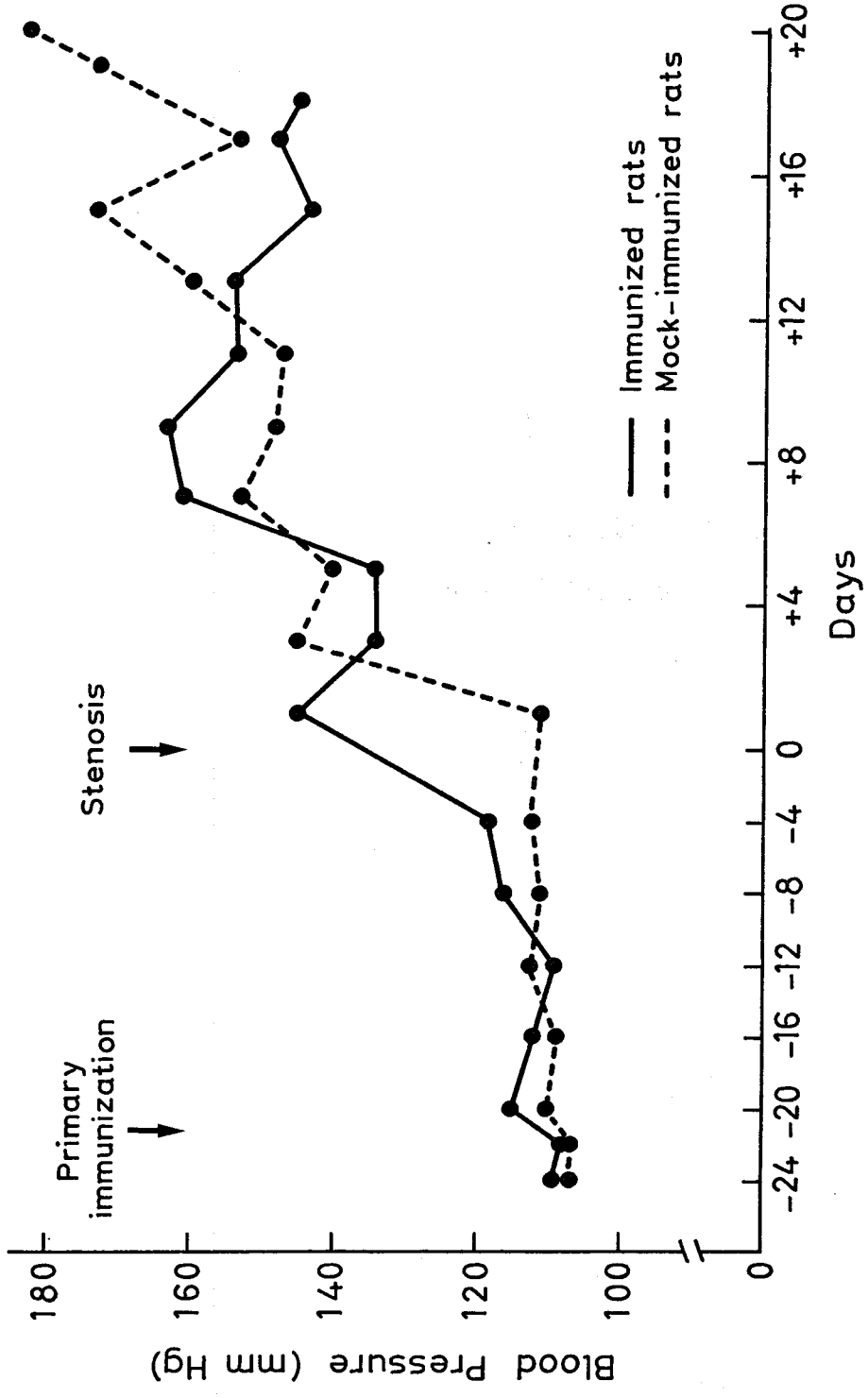

Blood Pressure (mm Hg)
180
160
140
120
100
0
Primary immunization
Stenosis
-24 -20 -16 -12 -8 -4 0 +4 +8 +12 +16 +20
Days
Immunized rats
Mock-immunized rats

Fig. 3 Systolic blood pressure of rats given secondary immunization (group III) and operated one week after the last booster shot.

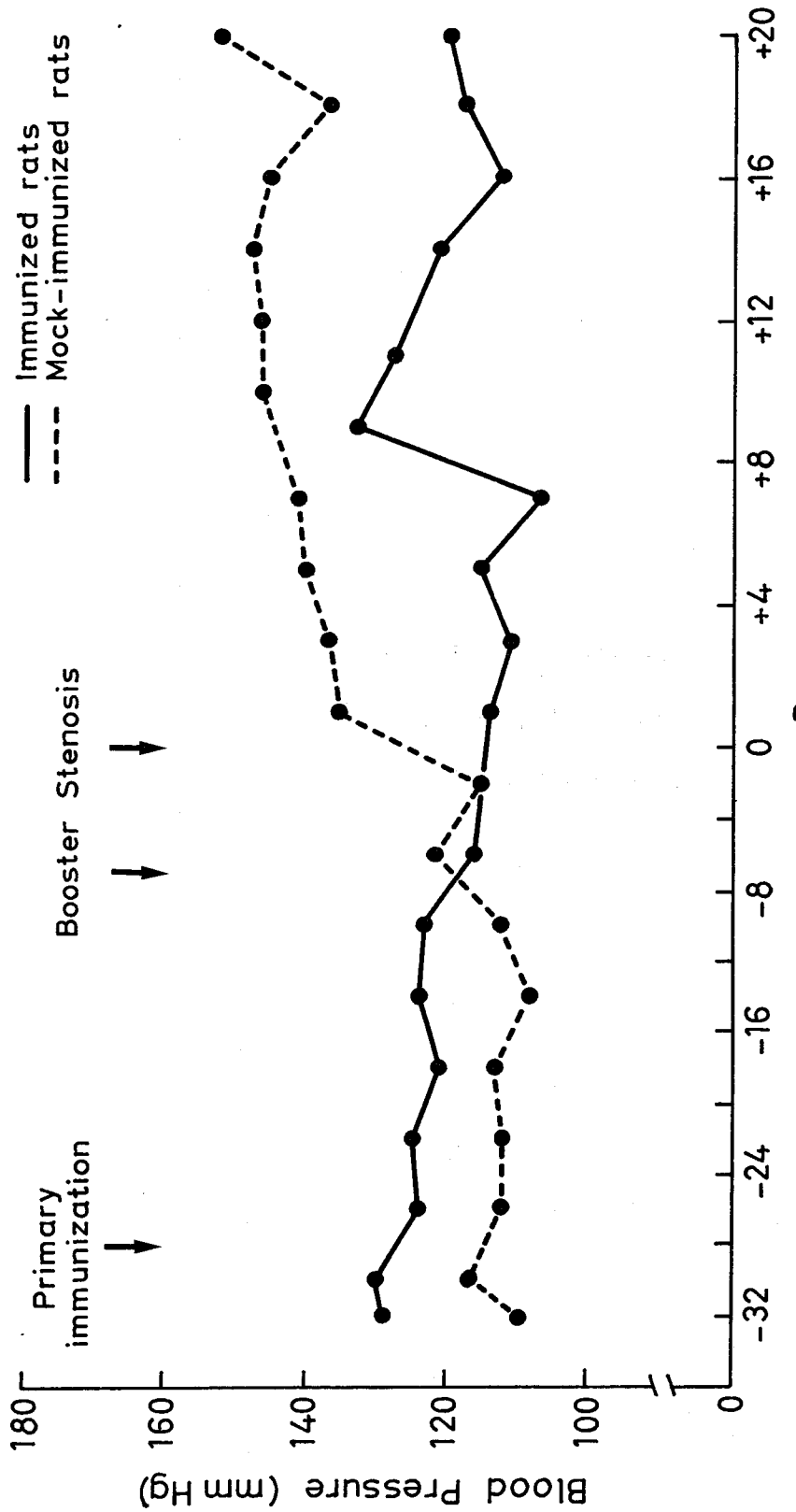

Blood Pressure (mm Hg)
180
160
140
120
100
0
-32
-24
-16
-8
0
+4
+8
+12
+16
+20
Days
Primary immunization
Booster
Stenosis
Immunized rats
Mock-immunized rats

Fig. 4 Systolic blood pressure of rats given tertiary immunization (group IV) and operated one week after the last booster shot.

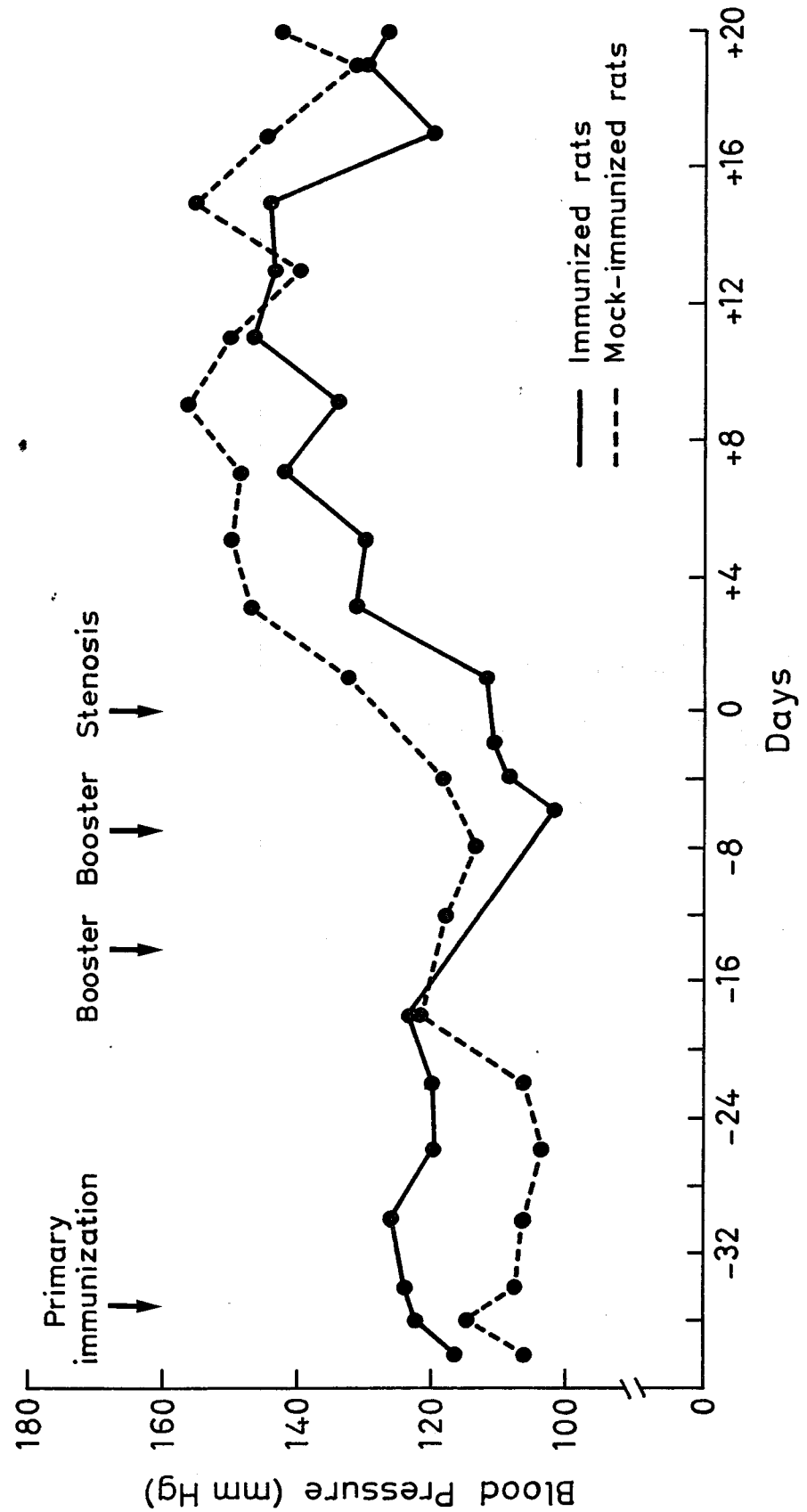
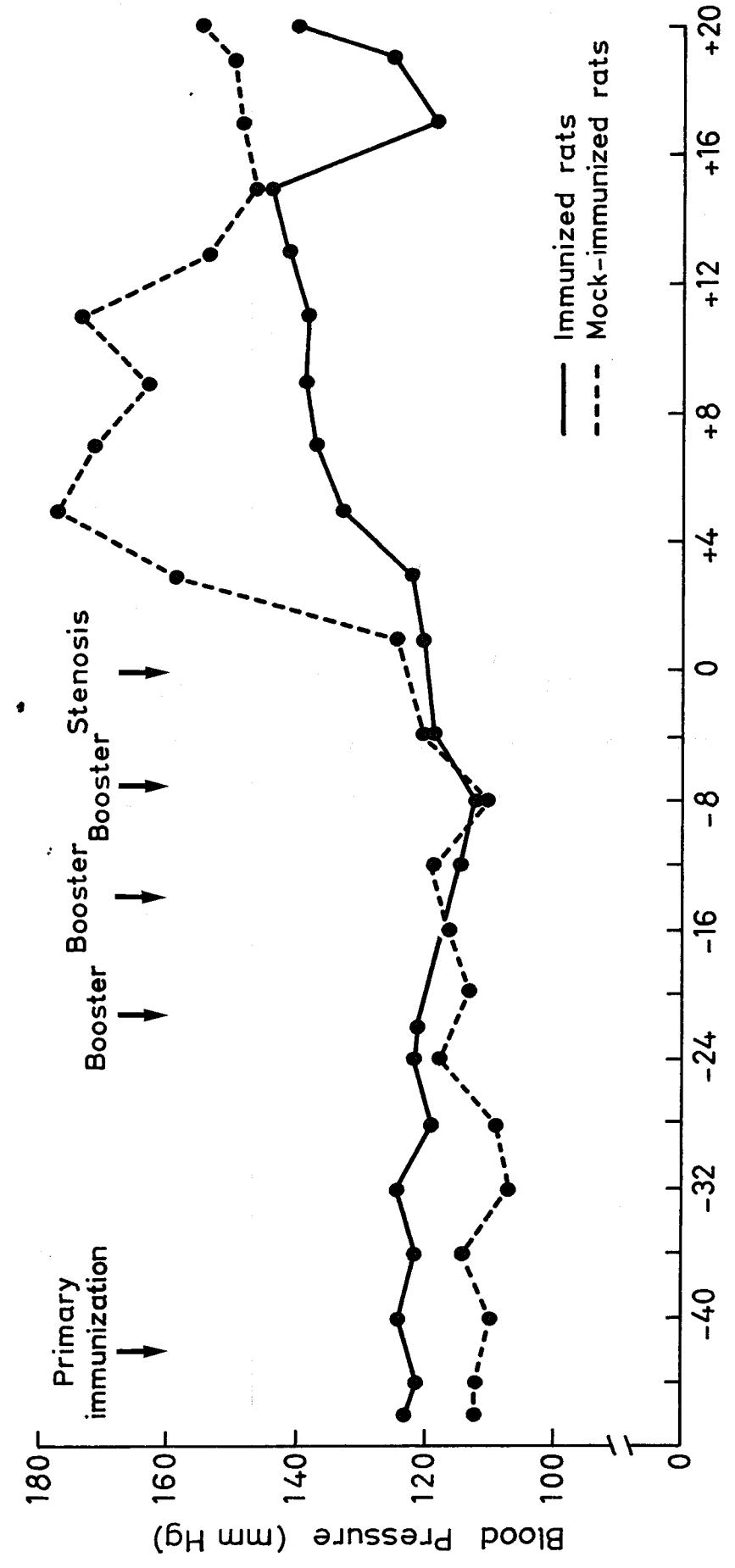

Blood Pressure (mm Hg)
180
160
140
120
100
0
Primary immunization
Booster
Booster
Booster
Stenosis
-32
-24
-16
-8
0
+4
+8
+12
+16
+20
Days
Immunized rats
Mock-immunized rats

Fig. 5 Systolic blood pressure of rats given primary immunization and three booster shots (group V) and operated one week after the last booster.


Blood Pressure (mm Hg)
180
160
140
120
100
0
-40
-32
-24
-16
-8
0
+4
+8
+12
+16
+20
Days
Primary immunization
Booster
Booster
Booster
Stenosis
Immunized rats
Mock-immunized rats

DISCUSSION

The results obtained in this work suggest that: (1) protection of rats against hypertension induced by renal artery stenosis can be afforded by immunization only when booster injections are given following primary immunization and (2) giving one booster injection results in a more effective protection than giving two or three boosters (tables I - V and figures 1 - 5).

These findings can probably be explained by Burnet's clonal selection theory concerning the dynamics of the immune response. According to this theory, an antigen selects certain precomitted lymphoid cells to start the process of antibody formation. Cells with antibody receptors of relatively high affinity for the particular antigen will preferentially capture the antigen and be stimulated (Steiner and Eisen, 1967). Since each cell makes antibody molecules of a single specificity, the heterogeneity of the binding affinity of antibodies reflects a corresponding heterogeneity of antibody forming cells (Gershon et al., 1968). With low antigen doses, the cells with higher affinity will be selected. Larger doses progressively exhaust the high affinity cells and this eventually results in the stimulation of lower-affinity lymphocytes. This was shown by studies of the affinity of rabbit antibodies against 2,4-dinitrophenyl (DNP) conjugated to bovine gammaglobulin (BGG) (Siskind, 1970).

The fact that rats which received one booster were better protected against angiotensin-II than those which received two or three boosters may suggest that higher affinity antibodies were produced in the animals with one booster. Further boosters introduced more antigen and probably resulted in the stimulation of lower affinity antibody forming cells and the secretion of late antibodies with less antigen-binding capacity.

Complete evidence to support the above conclusions may be obtained by isolating the presumably high affinity antibodies present in the sera of rats with reduced immunization regimens and subjecting these antibodies to further chemical and physical characterization. These antibodies should also be compared to low-affinity antibodies as to their avidity to synthetic and natural rat angiotensin-II employing equilibrium dialysis and similar studies.

In light of these observations, it seems that the failure of immunization against angiotensin-II to protect rabbits (Johnston et al., 1970; Macdonald et al., 1970; Louis et al., 1970) and rats (Johnston et al., 1970; Eide, 1972; Oates et al., 1974) against hypertension induced by renal artery stenosis can be attributed to prolonged immunization schedules, which probably resulted in the formation of antibodies of low affinity to the antigen.

These findings support the view that the renin-angiotensin system may play a role in the pathogenesis of hypertension induced by renal artery stenosis in the rat, at least during the first one or two weeks after stenosis.

Another possible explanation for the findings observed is based on the fact that the antigen used for boosting could have formed complexes with already present antibody and as such neutralized the protective effects of this antibody. To provide proof for this possibility, one requires a study of the fate of the antigen, its clearance, and the demonstration of antigen-antibody complexes in circulation.

It is also worthwhile to keep in mind that antigenic competition might explain the findings in this work, whereby the carrier (BGG) might compete with antibody formation to the hapten (AN-II). One might demonstrate this phenomenon by determining antibody levels to BGG at various times after immunization, or by using rat serum albumin as carrier; since no such competition would take place with this carrier.

Finally, it would be of interest to study the effects of antigen-antibody complex formation on the secretion of renin substrate and renin enzyme from the liver and the kidney, respectively. No information is available concerning this possibility, but preliminary reports indicate that renin activity decreases in the course of immunization (Birbari, 1974), suggesting that blocking of renin release could result from antigen-antibody complexes.

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