

EXPERIMENTAL CHRONIC GLOMERULITIS*

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PLATE 92

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The laboratory model which most closely mimicks the spectrum of human glomerulonephritis results from long-term immunization of rabbits with heterologous serum proteins (1-3). The membranous and/or proliferative glomerular lesions in this model resemble those seen in chronic glomerulonephritis or the renal lesions of systemic lupus erythematosus (SLE). In previous studies of the experimental glomerulonephritis, it was concluded that a critical factor determining whether or not a rabbit would develop chronic disease was the *quantity* of antibody formed (1). When the host responded with the production of large amounts of precipitating antibodies, acute, self-limited glomerulonephritis was common but chronic lesions were absent. Animals making moderate amounts of precipitating antibody—amounts sufficient to approximately neutralize the daily antigen dose—developed chronic disease. In an analogy to the quantitative precipitin reaction, animals in this latter group were designated as being in a state of “equivalence.” Animals in “antibody excess” and “antigen excess” were those making large and small (or no detectable) amounts of antibody, respectively. It was suggested that animals in approximate equivalence or slight antigen excess (4) formed more soluble immune complexes than the other two groups and that the chronic lesions were mediated by the deposition of complexes in glomerular capillaries. Immunohistologic studies supported such a role for complexes (1, 2).

In the present study, there is confirmation of the presence of immune complexes in sera of animals with glomerulonephritis. These animals were found to have moderate to large amounts of nonprecipitating antibodies. The nonprecipitating antibodies formed complexes with the antigen employed (bovine serum albumin) which were removed slowly from the circulation, in contrast to almost immediately eliminated complexes formed in animals which did not develop nephritis. It is suggested that the lesser avidity of the smaller complexes formed by nonprecipitating antibodies for the reticuloendothelial system is the main factor determining their slower clearance and their nephrotoxicity. From

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these observations, it is concluded that the *quality*, as well as the *quantity*, of antibody determines whether or not chronic nephritis develops.

Materials and Methods

Mixed breed white rabbits weighing 4-5 lb. were injected 7 days/wk intravenously with crystalline bovine serum albumin (BSA), obtained from Pentex, Inc., Kankakee, Ill. (Since there was initial interest in studying anti- γ -globulin factors that developed, the early phases of immunization used BSA additionally purified by zone electrophoresis (Pevikon, barbital buffer, pH 8.6 (5) to remove trace globulin components.) Later immunization utilized whole crystalline BSA. The daily dose of BSA was 10 mg for several weeks. The doses were increased subsequently to levels as high as 50 mg/day. A few of the animals were subjected to acute experiments (superimposed on chronic immunization) in which as much as 25 mg were given every 2 hr for 24 hr.

Sera were separated from clotted blood obtained by ear artery or cardiac puncture and stored at -60°C in a mechanical freezer. All serological procedures utilized sera obtained 24 hr after administration of BSA unless otherwise stated.

Serum complement (C') levels were determined on samples stored less than 1 wk at -60°C using the method of Kent et al. (6). Normal rabbit sera contained 60-100 50% units (C' 50%).

BSA-tanned cell hemagglutination tests were performed on sera which had been heat-inactivated (56°C for 30 min) and absorbed with washed packed sheep erythrocytes. 2% tanned cells were incubated with an equal volume of 0.01% BSA in pH 7.4 phosphate-buffered saline (PBS) for 45 min, washed three times with PBS, and suspended as a 0.25% suspension in PBS. 0.5 ml of the BSA-coated cells were added to 0.5-ml volumes of serially diluted test sera and agglutination read by settling patterns after overnight refrigeration.

Quantitative precipitin studies were performed in 8-ml calibrated conical centrifuge tubes. Precipitates were washed three times at $0-5^{\circ}\text{C}$. Protein estimations were made by the Folin-Ciocalteu technique using standards of rabbit γ -globulin (7). In some studies, serum C' was absorbed by adding 1.0 mg of washed preformed immune precipitate (egg albumin-rabbit anti-egg albumin) per milliliter of serum. (In studies to be described, C' removal did not influence the results.)

Rabbit γ -globulin concentrations of sera were estimated by a ring diffusion technique employing agar which contained specific duck anti-rabbit γ -globulin. The ring diameters of standards of known concentrations of rabbit Cohn fraction II in semilogarithmic plots permitted estimations of unknowns.

Labeling of BSA (supplied by the authors) with ^{131}I was performed by Mr. B. J. Green of Abbott Laboratories, North Chicago, Ill. The specific activity was 2.05 mc/g. A Packard Gamma Counter served for counting. Concentrations of BSA were calculated from standard curves for each experiment and corrections made on all samples for nonprotein ^{131}I by counting supernatants after precipitation with 20% trichloroacetic acid. Rabbits, that were subjected to *in vivo* studies with ^{131}I BSA, received Lugol's solution (1:5000) in their drinking water for 1 wk before and throughout the experimental period. Globulin-bound ^{131}I BSA was measured by the method of Farr, counting the supernatants after precipitation with 50% saturated ammonium sulfate (8).

Zone centrifugation studies were performed in gradients of sucrose (40-10%) in a SW39 Spinco rotor. After 15-16 hr at 35,000 rpm in a Spinco model L centrifuge, 0.2 or 0.5 ml fractions were collected by drops from the bottoms of tubes.

Tissues were fixed in neutral formalin and prepared for histological studies by routine methods.

Tissues for immunofluorescent studies were frozen in airtight tubes by immersion in dry ice-acetone and stored at -60°C . Cryostat sections ($5\ \mu$) were stained with fluorescein-labeled globulins prepared from duck anti-rabbit γ -globulin and rabbit anti-BSA antisera.

RESULTS

Table I summarizes the data obtained with 22 rabbits injected daily with BSA. Acute proteinuria (between 9 and 14 days) occurred in six animals. 16 rabbits were immunized for periods in excess of 10 wk. Three rabbits developed

TABLE I
Summary of Course of Immunization and Development of Proteinuria in 22 Rabbits

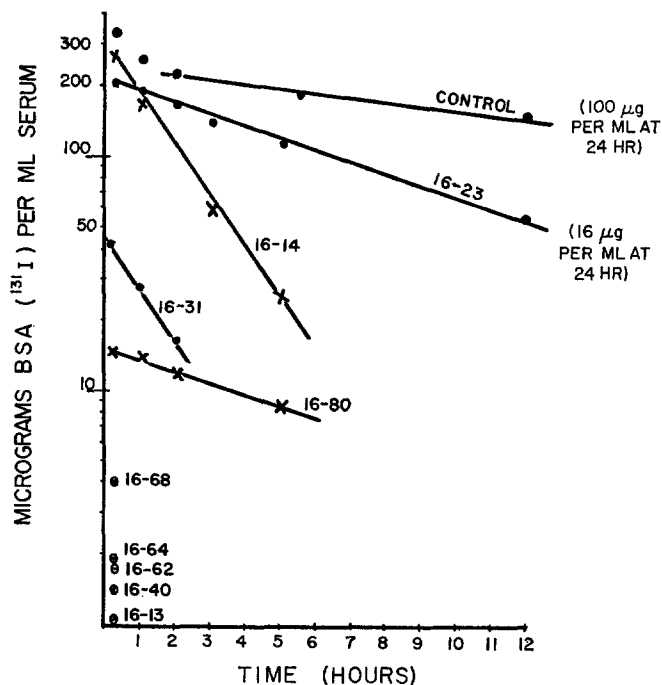
Rabbits	Maxi- mum daily dose BSA	Dura- tion of immuni- zation	Proteinuria		Comments
			Acute	Chronic*	
16-14	30‡	22	0	1+ (18 wk)	Sacrificed 22 wk, soluble complexes present
16-23	30‡	22	0	4+ (18 wk)	" 22 " " " "
16-12	30‡	14	0	0	Died at the end of an acute study at 14 wk in which 300 mg BSA was administered over a 24 hr period. Histologic picture of acute glomerulonephritis
16-13	30	22	0	0	Sacrificed 22 wk
16-40	30	9	4+	0	" 9 "
16-62	30	11	0	0	" 11 "
16-64	30	11	0	0	" 11 "
16-80	30	11	1+	0	" 11 ". No chronic proteinuria but small amount circulating soluble complexes
16-31	50	18	0	3+ (14 wk)	Sacrificed 18 wk, soluble complexes present
16-16	10‡	8	0	0	Died at end of acute experiment at 8 wk
16-18	10‡	10	0	0	" " " " " " " " 10 "
16-19	10	2	3+	0	" during renal biopsy at 2 wk. Histologic picture of acute glomerulitis
16-20	10‡	27	0	0	Sacrificed 27 wk
16-21	10‡	5	0	0	" 5 "
16-26	10	27	0	0	Study terminated at 27 wk
16-32	10	20	0	0	" " " " 20 "
16-34	10	20	0	0	" " " " 20 "
16-38	10	20	3+	0	" " " " 20 "
16-41	10	2	2+	0	Died at 2 wk—acute glomerulonephritis
16-61	10	11	3+	0	Study terminated at 11 wk
16-68	10	11	0	0	Sacrificed 11 wk
16-69	10	11	0	0	Study terminated at 11 wk

* Figures in parentheses indicate the onset of chronic proteinuria. The three rabbits with chronic proteinuria manifested persistent proteinuria until the time of sacrifice.

‡ Seven animals were subjected to acute experiments (1-2 days) after 8-14 wk of immunization, during which time larger doses of antigen were administered. The initial dose for all animals was 10 mg/day.

chronic proteinuria; these rabbits were investigated in the studies to be reported below, along with the other immune animals and nonimmune animals.

Clearance of Antigen in Immune Animals with and without Proteinuria.—Initial studies were done to determine clearance rates of BSA, over a 24 hr period, in animals receiving daily immunization. Text-fig. 1 illustrates such a study in nine immune rabbits and one control rabbit. Each animal was injected with 30



TEXT-FIG. 1. Disappearance rates of ^{131}I -labeled BSA from serum after injection of 30 mg BSA into 10 rabbits. (The control animal had not been immunized.)

mg of BSA and bled at 15 min, 1, 2, 5, 12, and 24 hr intervals. The control animal showed the slowest clearance rate compatible with compartment equilibration and protein catabolism (9). Five of the animals showed very low levels of circulating antigen (less than $4 \mu\text{g}/\text{ml}$) 15 min after injection. Four of nine immune animals had, at 15 min, circulating antigen at levels between 15 and $280 \mu\text{g}/\text{ml}$. The antigen elimination rates were variable. Three of the nine animals exhibited chronic proteinuria at the time of the clearance study (rabbits 16-14, 16-23, and 16-31)—those with the greatest amount of circulating antigen.

Table II summarizes other data from the above experiment. Capillary precipitin tests (using an anti-BSA antiserum) confirmed the results illustrated

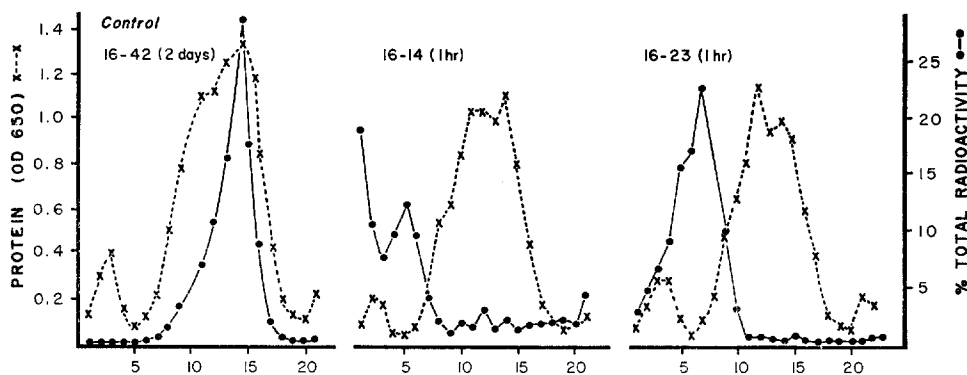
TABLE II
Serological Events in Nine Immune Rabbits Subsequent to Intravenous Injection of 30 mg BSA

Rabbits	Chronic proteinuria	γ-globulin concentration	Serum C' levels (C'50% units) at intervals after injection of BSA (30 mg)						Anti-BSA hemagglutination titers (reciprocal of dilution in thousands) at intervals after BSA injection						Presence of BSA in sera (capillary precipitate with precipitating rabbit anti-BSA antiserum)								
			0	15 min	1 hr	2 hr	5 hr	10-12 hr	24 hr	0	15 min	1 hr	2 hr	5 hr	10-12 hr	0	15 min	1 hr	2 hr	5 hr	10-12 hr		
16-14	1+	3.2	<10	<10	<10	13	35	45	54	64	4	4	8	32	64	64	0	4+	3+	2+	1+	1+	0
16-23	4+	3.7	104	102	111	88	86	86	102	16	4	4	16	8	4	16	0	4+	4+	3+	4+	3+	2+
16-31	3+	2.5	<10	<10	<10	<10	44	47	33	128	32	64	64	64	128	128	0	2+	1+	Tr	0	0	0
16-80	0	3.2	<10	<10	<10	<10	66	47	50	—	—	—	—	—	—	—	0	1+	Tr	0	0	0	
16-13	0	6.1	<10	<10	14	17	30	53	69	256	256	256	256	256	256	256	0	0	0	0	0	0	0
16-40	0	3.2	<10	<10	12	22	46	49	72	64	16	16	32	32	64	64	0	0	0	0	0	0	0
16-62	0	2.3	<10	<10	<10	<10	42	42	36	—	—	—	—	—	—	—	0	0	0	0	0	0	0
16-64	0	4.9	<10	<10	<10	<10	45	71	61	—	—	—	—	—	—	—	0	0	0	0	0	0	0
16-68	0	4.6	<10	<10	<10	<10	47	50	48	—	—	—	—	—	—	—	0	0	0	0	0	0	0

Tr, trace.

in Text-fig. 1. The amounts of circulating BSA were highest in the three animals with chronic proteinuria using isotopic and immunological techniques. Parallel studies of serum complement (C') and hemagglutinating anti-BSA antibody are also summarized in Table II. Eight of the nine immunized animals showed a precipitous fall in serum C', to undetectable levels, 15 min after immunization, with a gradual return to preimmunization levels. The C' levels were stable in rabbit 16-23 and the nonimmune animal. It is noteworthy that rabbit 16-23, which developed proteinuria, had the slowest elimination rate of antigen from circulation, a rate over 12 hr that was almost as slow as the control (Text-fig. 1).

Anti-BSA antibodies (hemagglutination) were detectable in *all* rabbits at *all* intervals, although a fall in titer occurred immediately after injection of antigen

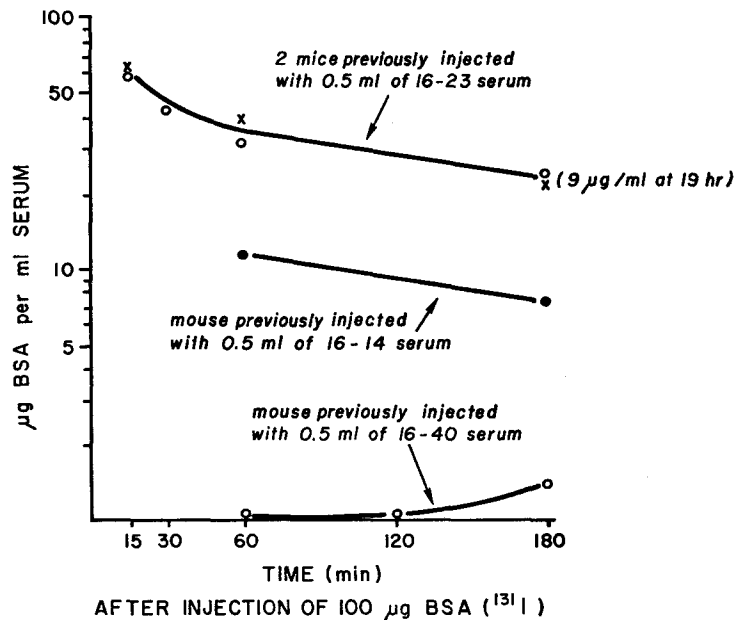


TEXT-FIG. 2. Sucrose density gradient patterns of protein and BSA of three rabbit sera after injection (intravenous) of 30 mg of ^{131}I -labeled BSA. Fractions (from the bottom) were 0.2 ml.

with a return to preimmunization levels over 24 hr. Thus, with the technique of hemagglutination, all animals remained in antibody excess following the intravenous administration of BSA (30 mg)—even though the animals with chronic proteinuria had, concurrently, significant amounts of circulating antigen.

Density Gradient Studies of Sera Containing Labeled Antigen.—The presence of circulating antigen in the three rabbits which developed chronic proteinuria and the absence in all but one of the other rabbits supports the concept that circulating immune complexes induce glomerular injury. It was necessary to determine whether the antigen in such animals was in free or complex form. Density gradient centrifugation studies of sera containing labeled BSA are illustrated in Text-fig. 2. The distribution of free antigen in the gradient is indicated by the study of a control rabbit (16-42) injected 2 days previously with 30 mg of labeled BSA. Sera obtained from two rabbits with chronic proteinuria

(16-14 and 16-23), 1 hr after injection with 30 mg of BSA, had virtually all of the radioactivity in fractions heavier than the 7S γ -globulin (the peak concentration of γ -globulin was in fraction 12). In one animal (16-23), the label appeared maximally in an intermediate zone between 19S and 7S peaks—in a fraction with the lowest protein concentration. In rabbit 16-14, the labeled antigen was distributed at the bottom of the gradient (heavier than the 19S peak) and in the intermediate zone. These data clearly indicated that the

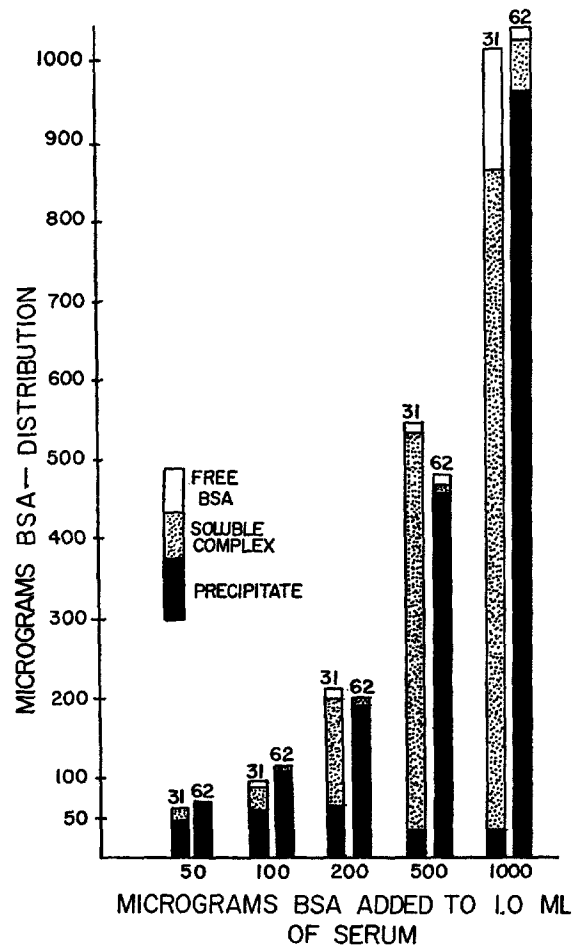


TEXT-FIG. 3. Disappearance rates of ^{131}I -labeled BSA in mice passively immunized with sera of three different rabbits, (16-14 and 16-23 with proteinuria).

circulating antigen in animals with chronic proteinuria existed in the form of complexes and that undetectable quantities of free antigen were present.

Passive Immunization of Mice.—Were the observed differences in antigen clearance rates dependent on variability of the host's clearing mechanisms, or did they reflect unique qualities of the immune response to chronic immunization? To answer this question, mice were passively immunized with 0.5 ml of serum (intravenously) from individual rabbits. 1 hr later 100 μg of ^{131}I BSA was injected intravenously and clearance rates of labeled antigen were determined. Text-fig. 3 illustrates the data obtained. The clearance rates in the mice were quite comparable with those in the rabbits from which the sera derived. Rabbit 16-23 exhibited the slowest clearance, rabbit 16-24 a more rapid clear-

ance, and rabbit 16-40 had almost no detectable antigen 15 min after injection (Text-fig. 1). These data implied that the varied clearance rates in chronically immunized rabbits reflected properties intrinsic to the immune response, rather than differences in clearing mechanisms.



TEXT-FIG. 4. Distribution of ^{131}I -labeled BSA (Farr technique) in two rabbit sera. (Rabbit 16-31 with proteinuria.)

In Vitro Studies of Immune Complex Formation.—Sera of individual rabbits were studied with the Farr technique, using varying quantities of ^{131}I BSA. The distribution of antigen in immune precipitate, in soluble complex (precipitated by 50% saturated $(\text{NH}_4)_2\text{SO}_4$), and in free form (soluble in 50% saturated $(\text{NH}_4)_2\text{SO}_4$), was determined. Data obtained from the study of two sera (16-31)

and 16-62) is illustrated in Text-fig. 4. Sera from rabbit 16-31, which exhibited chronic proteinuria, formed almost entirely soluble complexes. Serum from rabbit 16-62 (without proteinuria) formed precipitating immune complexes. Over a wide range of antigen concentrations, there were negligible quantities of free antigen. The findings, when still larger amounts of antigen (5–10 mg) were added to 1 ml of serum 16-62, did not resemble the pattern observed with serum 16-31. Large excesses of free antigen were required to produce significant quantities of soluble complex. Table III summarizes data obtained from the study of six rabbits using variable amounts of antigen.

Studies of Antibodies Produced in Animals Before Nephritis Appeared.—Did the animals with proteinuria, that possessed largely nonprecipitating antibody,

TABLE III
In Vitro Distribution of ¹³¹I-Labeled BSA (Farr Technique) in Six Rabbit Sera

Animals	Chronic proteinuria	Distribution of antigen (varying amounts) added in vitro to 1 ml of rabbit sera														
		100 μ g			200 μ g			500 μ g			1000 μ g			2000 μ g		
		Ppt.	Complex	Free	Ppt.	Complex	Free	Ppt.	Complex	Free	Ppt.	Complex	Free	Ppt.	Complex	Free
16-14	1+						78	444	6							
16-23	4+				1*	280*	1*									
16-31	3+	58	30	7	64	150	11	35	507	12	33	833	155	11	1280	827
16-80	0	100	9	3	130	76	16	43	438	18	53	629	408	52	950	1130
16-62	0	110	6	0	190	10	0	457	2	12	963	72	12	637	1050	470
16-64	0	110	9	0	205	0	11	490	8	4	987	23	11	765	1015	260

Ppt., precipitate.

* 250 μ g BSA added.

develop this type of antibody late in the course of their chronic immunization, or did they manifest this type of immune response from the beginning? Data relevant to these questions is presented in Table IV. Early in the course of immunization and before the onset of proteinuria, the bulk of antibody produced by these three rabbits lacked precipitating properties.

Pathological Findings.—All rabbits immunized chronically with BSA had mild proliferative glomerulitis. Animals manifesting chronic proteinuria exhibited more severe lesions. The histopathological features were varied, as Fig. 1 illustrates. Of the three animals with chronic proteinuria, rabbit 16-23 which formed immune complex with a sedimentation constant of approximately 10 and which did not experience a significant drop in serum C' (see Text-fig. 2 and Table II), had the mildest lesions. The glomerular alteration was predominately membranous. Rabbits 16-14 and 16-31 showed more severe diffuse proliferative glomerulitis; and, especially in 16-31, there were necrotic foci and hyaline thrombi in glomeruli. Immunofluorescent studies demonstrated BSA in the

glomeruli of animals with chronic proteinuria (Fig. 1). The distribution of BSA was "lumpy." Fluorescein-labeled duck anti-rabbit γ -globulin which stained glomeruli faintly in rabbits 16-14 and 16-23, showed bright fluorescence with rabbit 16-31. Immunofluorescent studies of kidney sections from rabbits without proteinuria were negative. Tissues other than kidney were not subjected to immunofluorescent studies.

TABLE IV
Distribution of 125 I-Labeled BSA (Farr Technique) at Three Different Intervals after Initiation of Immunization of Two Rabbits

Rabbit	Duration of immunization	Proteinuria	BSA added to 0.5 ml serum	Distribution of BSA		
				Ppt.	Complex	Free
			μg	μg	μg	μg
16-14	52 days	0	500	40	450	30
16-14	150 "	0	500	78	444	6
16-14	177 "	1+	250	<1	280	1
16-23	52 days	0	125	<1	140	<1
16-23	150 "	0	250	<1	280	<1
16-23	177 "	4+		Not studied		

Ppt., precipitate.

DISCUSSION

The evidence presented is entirely consistent with the thesis that circulating immune complexes mediate the chronic glomerulitis resulting from daily administration of a heterologous serum protein. The data suggest that the *quality* of the immune response may be as important as the *quantity* of antibody produced in determining whether or not an individual rabbit will develop chronic glomerulitis. Clearly, animals that responded with the production of large amounts of antibody (as determined by precipitin studies) did not develop chronic proteinuria. This confirmed previously reported data (1-3). However, in the present studies, animals developing chronic glomerulitis exhibited appreciable quantities of antibody which was *not* demonstrable by precipitin analysis but which was detected by hemagglutination and the Farr globulin precipitation technique. It appeared that this type of immune response, i.e. production of nonprecipitating antibody, correlated closely with the development of chronic glomerulitis and that the slower reticuloendothelial clearance of complexes formed with such antibodies would favor glomerular deposition.

The passive immunization studies in mice, followed by observations of the clearance of labeled antigen, offered evidence that the phenomena observed were dependent on intrinsic properties of the antibodies; and this was confirmed, *in vitro*, in studies using the Farr technique.

Data, which are summarized in Table III, indicate that the development of nonprecipitating antibody was *not* a late development in the course of chronic immunization—rather, it was evident in the early immune response. The detection of this type of response early may have predictive value in determining which animals will develop chronic disease. (Such studies are in progress.) In previous studies, and in the present one, a minority of animals subjected to chronic immunization have developed chronic glomerular lesions—usually 10–20%.

Why did a minority of animals respond to chronic immunization by the production of predominately nonprecipitating antibody? There are two main possibilities: (*a*) a qualitatively unique antibody is formed which, because of intrinsic properties or lesser avidity for antigen, is unable to form insoluble immune aggregates, or (*b*) the immune response, in such animals, is directed at a limited number of antigenic determinates on the complex protein antigen—a number too small to permit lattice formation and precipitation of the immune reactants. The evidence, which so far favors the latter explanation, will be reported separately.¹

If the above thesis is correct, i.e. that the production of nonprecipitating antibody favors the development of chronic serum sickness, it must be admitted that other factors are likely important. One is the duration of exposure to circulating soluble complexes. The two animals studied in detail possessed largely nonprecipitating antibodies for weeks before chronic proteinuria was observed. Another factor may relate to the varied degree of complexity of soluble complexes formed. There is abundant evidence relating biological properties of immune complexes to their molecular size (10–12). It was of interest that one animal (16-23), that had predominately a low molecular weight complex *in vivo*, exhibited minimal inflammatory lesions—yet had chronic proteinuria with mainly membranous changes. One can calculate from the sedimentation property of the labeled antigen in rabbit 16-23 (Text-fig. 2) that the complex formed was comprised of no more than 2 moles of antibody. The sera of the other animals studied (16-14 and 16-31) formed, *in vivo* and *in vitro*, complexes with antigen that were heavier than 19S components of serum. These more complex soluble immune aggregates would be expected to have greater biological activities; i.e. C' inactivation and the induction of inflammation. The animals with the heavier complexes, unlike rabbit 16-23, exhibited an abrupt decrease in C' upon administration of antigen and manifested more inflammatory glomerular lesions.

Whether or not the experimental studies herein reported have relevance to human disease is unknown. The similarities between the model of experimental chronic serum sickness and SLE are numerous. It is attractive to postulate

¹ Christian, C. L., T. Pincus, and R. C. Haberkern. Manuscript in preparation.

that SLE is immune complex disease in which the antigen is not known.² In the experimental model, if the inducing antigen were not known and available for isotopic labeling, the chances are remote that the offending antigen could have been identified from studies of serum or the intact animal. Even in the animal (16-23) with the largest quantity of circulating antigen (280 $\mu\text{g}/\text{ml}$), the complexes would not have been detected without the isotopic label (Text-fig. 2). If the suggested basis for development of nonprecipitating antibody is correct (i.e. the immune response recognizes a limited number of antigenic determinates on a complex antigen), it may be that the patient with SLE nephritis or post-streptococcal glomerulonephritis responds to an immunologic stimulus with the production of antibodies that have a limited specificity for determinates on a complex environmental or autologous antigen. Theoretically, "autologous" antigens would be more likely inducers of this kind of immune response than exogenous antigens.

SUMMARY

Three of 16 rabbits injected (intravenously) daily with crystalline bovine serum albumin (BSA) for periods in excess of 10 wk developed chronic glomerulonephritis. In vivo, animals with chronic proteinuria formed variable quantities of soluble complex after injection of antigen while animals without proteinuria exhibited rapid removal of the injected BSA. In vitro studies demonstrated that a major part of the antibodies produced by rabbits with chronic nephritis lacked precipitating properties. Interpretations of these observations were presented in the discussion.

It is suggested that, in addition to quantity, quality of antibody plays an important role in the development of chronic serum sickness. Complexes formed with nonprecipitating antibody, which are less rapidly removed from circulation, would have a greater opportunity to deposit in glomeruli and induce inflammation.

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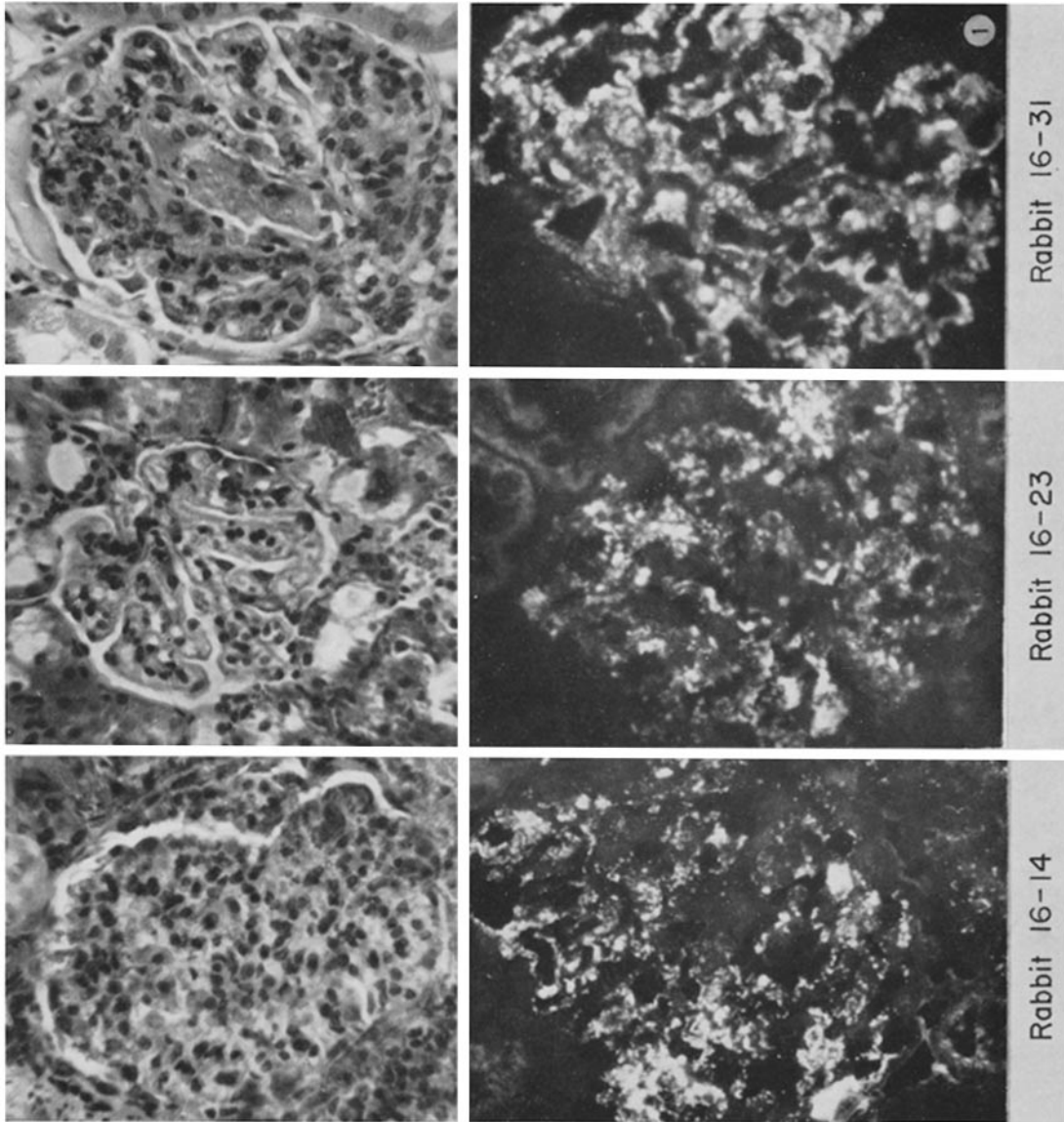
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² In SLE nephritis, recent data make a strong case for nuclear materials being the antigen(s) in immune complexes (13).

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EXPLANATION OF PLATE 92

FIG. 1. Photomicrographs of hematoxylin-eosin-stained kidney sections (upper) and immunofluorescent preparations (lower) stained with fluorescein-labeled rabbit anti-BSA globulin (1:20 dilution). $\times 330$.



(Pincus et al.: Experimental chronic glomerulitis)