

# AUTOANTIBODIES IN HUMAN GLOMERULONEPHRITIS<sup>1</sup>

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It has often been suggested that the picture and course of human glomerulonephritis could be best explained by an antigen-antibody reaction. There are many clinical facts which support such a hypothesis. The interval of 10 to 20 days between the primary streptococcal infection and the first appearance of clinical signs of acute glomerulonephritis, the persistent absence of direct evidence of streptococcal invasion of the kidney itself with evidence of severe general involvement of the glomerular apparatus and the often irreversible progress of the disease seem strongly to suggest such a possibility. In our studies on the nephrotic syndrome (1) during the last few years using the fluorescein and other methods for determining capillary permeability, we had the impression that during this stage of chronic glomerulonephritis we are dealing with a diffuse damage of the entire capillary network throughout the body, possibly produced by an "allergic" reaction.

Good evidence has been presented that by an antigen-antibody reaction a picture very similar to human glomerulonephritis and its different stages can be produced in experimental animals. Masugi (2) has shown that a chronic glomerulonephritis can be produced by the injection of a heterologous immune antikidney serum. Sarre and Wirtz (3) were able to demonstrate that such antibodies are rapidly and selectively adsorbed by the kidney and thus disappear out of the circulation, giving rise to specific changes in the kidney. They were also able to show that other organs bind these specific antibodies to a much lesser extent. Swift and Smadel (4) demonstrated the specificity of such antikidney serum by the fact that an injection of kidney extract immediately preceding the injection of the antikidney serum prevented kidney damage. Injection of liver extract did not afford this protection. Burky (5) in turn showed that

lens material of rabbits when incubated with staphylococcus toxin and injected into rabbits produced antibodies to lens which in turn attacked the lens of the same animal species. Hecht, Sulzberger and Weil (6), by the utilization of the synergistic action of staphylococcus toxin and of homologous skin antigen, succeeded in the production of specific antiskin antibodies.

Cavelti (7-9) was able to produce, in a high percentage of rabbits and rats, a picture similar to human glomerulonephritis and rheumatic heart lesions by the injection of homologous kidney and heart extracts, respectively, incubated with streptococci or staphylococci. He also succeeded in showing the appearance of specific antibodies in the blood of such animals with the modified colloid technique of Cannon and Marshall (10). Schwentker and Comploier (11) also demonstrated that in rabbits antibodies to kidney can be produced by injecting a rabbit kidney extract previously incubated with staphylococcus or streptococcus extract. They also showed that in the majority of recent cases of scarlet fever antibodies to kidney can be found by complement fixation one to two weeks after the acute phase but that similar antibodies can be demonstrated only in a small percentage of normal individuals.

Stimulated by these experiments and search for a possible "allergic" mechanism which would explain the increased capillary permeability in acute nephritis and the nephrotic stage we investigated the bloods of 23 patients with different stages of glomerulonephritis and of 68 controls in whom there were no signs of glomerulonephritis by history or clinical examination. Since we deviated in some major points from the colloid method as suggested by Cannon and Marshall (10) and by Cavelti (12) a full description of the method used is essential to enable reproducibility and to avoid if possible pitfalls in the extremely delicate and somewhat unpredictable method.

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## METHOD

*I. Preparation of collodion particles*

All water used in the preparation of collodion particles is triply distilled in glass and all apparatus used is completely made of glass. One-half pound of collodion (non-flexible Merck or Mallinckrodt) is very slowly poured into 2 liters of water with constant stirring. The mass so formed is then washed with four changes of just enough water to cover it. The mass is then dried after excess water has been pressed out with clean hands. The mass is now left to dry for three days in a beaker at room temperature. Thereafter a 5% stock solution is prepared by adding acetone to the mass in a water bath at 40° under constant stirring. This stock solution may be used for three months if kept in a dark bottle.

For a test of 400 tubes 75 cc. of stock solution are needed to obtain the necessary particles. A motor-driven stirring rod is suspended close to the bottom of a beaker containing the stock solution. This is done in a water bath at 40° C. While stirring, a mixture of three parts water to one part acetone is added in a fine stream until a gelatinous mass separates and the supernatant fluid becomes cloudy. This supernate is then discarded and the mass redissolved in a somewhat smaller volume of acetone. This procedure is repeated twice, each time discarding the supernate. The fourth and succeeding supernates are collected in a flask containing 300 cc. cold water. This procedure is repeated until the gelatinous mass fails to form. Compressed air is then bubbled through the suspension until all acetone has evaporated. This requires two days. The suspension is then filtered through glass wool. Subsequently, the suspension is washed by the following procedure: 1) In an angle centrifuge the suspension is spun at 3000 r.p.m. for five minutes. The largest particles settle down and are discarded. 2) The supernate is recentrifuged at 2500 r.p.m. for one hour. The supernate is now discarded and the precipitate resuspended by gentle agitation in 50 to 100 cc. of water to give a milky suspension. 3) Again this suspension is centrifuged at 2500 r.p.m. for one hour. The supernate is discarded and the precipitate resuspended and centrifuged as before. 4) At the end of this third one-hour period the particles are resuspended in the smallest volume of water that will permit complete resuspension and centrifuged at 3000 r.p.m. for five minutes discarding the precipitate. The supernate is used as stock suspension and should not be kept for more than a week. It is recommended that the collodion suspension be examined under the Fisher-Kahn viewer before use for the absence of self-agglutination. If clumps are visible the suspension must be discarded.

*II. Preparation of the antigen*

The kidneys obtained from autopsies performed within a few hours after death are cut up, washed in several changes of saline until free of blood and ground in a Waring Blendor. Enough 1.1% saline solution made with triple distilled water is added during the grinding

to make a 20% emulsion. Care should be taken that the solution does not overheat during the grinding process. The suspension is then centrifuged at high speed until the supernate is absolutely clear. This takes between two and three hours at 3000 r.p.m. If the solution does not clear at this speed further centrifugation at 5000 r.p.m. should be used. The clear supernate is stored at approximately -20° C. The effectiveness of antigens seems to decrease after 10 days. The antigens differ widely in antigenic properties, a question which requires further study.

*III. Coating of collodion particles*

After studying many methods of coating the following was found most satisfactory. The final collodion suspension described above is tested for the dilution which is necessary to make it match tube No. 3 of McFarland's scale (13). Small amounts of undiluted collodion suspension and undiluted antigen solution are then mixed in such a way that when the proper amount of 1.1% saline is added to make the collodion suspension match McFarland's tube No. 3 the antigen is in a dilution of 1:60. Various antigen titrations with different antigens and different test sera indicated that an antigen dilution of 1:60 is the most effective dilution although variations with different antigens are considerable. The final mixture of collodion and antigen is permitted to stand at room temperature for one hour before use.

*The sera*

Bloods to be tested should be collected on the day before use with dry syringes, allowed to clot firmly and the sera taken off. They are kept overnight in an ordinary refrigerator. Prolonged storage of sera shows increasing non-specificity in tests. The last 46 tests were run with the sera inactivated for one hour at 56° C. It is the authors' impression that this procedure eliminates non-specificity but also depresses somewhat the specific titres obtained. It has been accepted at present as part of the routine procedure.

*The test*

Serial dilutions of the sera by the doubling method and employing 1.1% saline are set up in serological test tubes so that each tube contains 0.5 ml. of the diluted serum. The first tube contains a dilution of 1:5. Then 0.2 ml. collodion-antigen mixture is added to each tube. Controls are made up of each serum (1:5 to 1:40) with 0.2 ml. uncoated collodion in such a dilution as to match McFarland's tube No. 3. Additional controls are made of 0.5 ml. saline and 0.2 ml. of coated collodion particles but no serum. The tubes are stoppered and allowed to stand at room temperature for one hour. Thereafter they are spun in an angle centrifuge for three minutes at 1400 r.p.m. Each rack of tubes is then inverted sharply three times to resuspend the particles and the tubes are then read in a Fisher-Kahn viewer. The positive tubes will show discrete small particles with a clear

surrounding fluid, while the negative tubes show a milky suspension. Numerous modifications of this last method were tested but the method as described above was found to be the most reliable. The use of kidneys of young rabbits as antigens was studied but the tests were too few to be conclusive.

The method is very delicate and cumbersome. Occasionally, entire runs will be negative or positive, without any known deviation in technique. Such runs have to be excluded.

The particular antigen used in our tests seems to be of great importance. It was found that adult human kidneys are much less satisfactory than kidneys of infants or stillbirths. This fact has been demonstrated by us repeatedly and seems rather important not only for the performance of the test but also for the general aspect of the physiologic mechanism. But even kidneys from young individuals vary considerably in antigenic quality for unknown reasons. Fractionation and concentration of the antigen may eventually aid in the explanation of this observation. In order to secure a uniform statistical basis each test was run in such a way that approximately the same number of control and nephritic bloods were used in each run. In 11 tests the sera were absorbed with human liver extracts. The results did not differ from those obtained without absorption. To exclude the possibility of blood group factors influencing the results 11 tests were carried out with absorption of the sera with mixed red cells. Again the results obtained with or without absorption did not deviate, indicating that these factors do not influence the specificity of the results obtained.

#### RESULTS

Twenty-three cases of glomerulonephritis were examined in 122 individual determinations. The cases were arbitrarily classified as early or late nephritis on the basis of duration of signs or symptoms, a duration of less than one year being considered early and longer than one year being considered late. The 12 cases of early nephritis included four cases of acute glomerulonephritis, four which started with a nephrotic syndrome and four cases of chronic glomerulonephritis of less than

one year's duration. The 11 cases of late nephritis were all chronic glomerulonephritis ranging in duration from one and one-half to 12 years. Six of the late cases had marked reduction of renal function with inulin clearances of less than 18 ml. per minute and chemical retention. In all, 44 determinations were done in the early and 78 in the late cases. The results are given in Table I.

The group of nephritics showed titres of 1:10 or above in 75% of the determinations. The average titre in this group of positive reactions was 1:623. Of the determinations in nephritics, 18% showed a titre of 0, and 7% a titre between 0 and 1:10. The highest titres obtained in this group (1:80,000) were obtained on two occasions from different specimens. Since these values were exceptionally high they were not used as such in the statistics but reduced arbitrarily to the next high value of 1:5000 in order not to raise the average titre disproportionately. Of 23 cases in all stages of nephritis 18, or 78.3% were positive more than 60% of the times tested.

Splitting the group of nephritics up between early and late nephritics the following results were obtained. Of the 12 cases considered as early nephritis with 44 determinations 30, or 68% showed titres of or above 1:10. The average titre was 1:918. A titre of 0 was obtained in 11, or 25% of the determinations; 7% had titres between 0 and 1:10. The 11 cases of late nephritis with 78 determinations showed a titre of 1:10 or above in 61 tests, or 78% of the determinations. The average titre was 1:337. A titre of 0 was present in 11, or 14% of the determinations and a titre below 1:10 but above 0 in seven tests, or 9%.

Of all nephritics examined there was not a single case where on repeated determinations the titre

TABLE I  
*Antibody determinations in nephritic and control groups*

	No. of cases	No. of determinations	Positive determinations		Aver. titre of positive determinations	Cases consistently positive*	
			no.	per cent		no.	per cent
Total nephritis	23	122	91	75	1:623	18	78
Early nephritis	12	44	30	68	1:918	7	58
Late nephritis	11	78	61	78	1:337	11	100
Controls	68	126	24	19	1:78	4	6

\* Nephritics 60% or above. Controls 50% or above.

was persistently below 1:10. The age distribution of the nephritics as well as the control cases is given in Table II.

Sera of 68 control cases with various ailments or normals were subjected to 126 determinations (Table I). This group included the following diagnoses: normal young adults, 33; active pulmonary tuberculosis, 11; diabetes, 11; essential hypertension with demonstrable renal involvement, two; essential hypertension without demonstrable renal involvement, three; acute upper respiratory infection, two; Laennec's cirrhosis, one; secondary anemia of unknown etiology, one; syringomyelia, one; inactive rheumatic fever, one; advanced arteriosclerosis, two. The titres in 24

ther study with the view of purification. If, in the majority of cases of chronic glomerulonephritis, antibodies to normal kidney tissue are constantly or commonly circulating in the blood the progressive course of the disease would be well explained. Whether the wide variations in titre in an individual case and the occasional complete absence of a positive titre are due to technical imperfections of the delicate method or whether they really represent variations in antibody formation remains to be seen. One case with the nephrotic stage of chronic glomerulonephritis showed a titre of zero during an attack of measles. Subsequently, the nephrotic phase disappeared as is often observed. Two weeks after the measles the titre went up to 1:40. If it is true that as a result of measles the antibody level in general is severely depressed it would possibly explain the temporary clearing of certain phases of chronic glomerulonephritis subsequent to rubeola. The fact that none of the cases of glomerulonephritis showed a consistently negative titre seem to confirm the impression that the mechanism of the disease is related to an antigen-antibody reaction. The presence of high anti-streptolysin titres and their increase subsequent to reinfections seem to indicate that the streptolysins may possibly be the factor which split up certain otherwise nonantigenic kidney proteins and combine in a hapten linkage so that they become antigenic. The antibodies in turn are then no longer restricted to the hapten-kidney linkage but attack kidney substance directly in the form of a direct precipitin reaction, as demonstrated *in vitro* by the precipitin reaction with the kidney antigen made visible by the collodion agglutination technique. The high specificity obtained by using infants' kidneys and the marked nonspecificity obtained from adult material may explain the high incidence of glomerulonephritis in young individuals. Further studies to correlate antistreptolysin titres with autoantibodies to kidney are in progress. Rantz and his collaborators (14) have shown that during the first two years of life antistreptolysin titres are very low. This may explain the relatively low incidence of glomerulonephritis in infancy in spite of the high antigenicity of infant kidney tissue. Cavelti's original report that a high percentage of cases of rheumatic fever show antibodies to heart in their sera has recently been modified (15) since tests with a modified antigen

TABLE II

Age distribution of nephritic and control groups

Ages yrs.	Early nephritis no.	Late nephritis no.	Control group no.
0-5	5		6
5-10	1		3
10-20	2	4	21
20-30	2	1	30
30-50	2	6	6
over 50			2

tests, or 19.0% of the control determinations were 1:10 or above. The average titre in this positive group was 1:78. Seventy-six determinations, or 60% of the control tests showed a titre of 0, while 26, or 21.0% showed titres above 0 but below 1:10. Of the 68 control cases four, or 6% were positive more than 50% of the times tested. On each one of these four only one determination was done. It should be stated that of the 68 control cases 26 were tested more than once. In these, 84 determinations were done, of which 20, or 23.8% were positive (1:10 or above). None of these 26 cases were positive in more than 50% of the tests (Table I).

DISCUSSION

From the data described it will appear that in a high percentage of nephritics of all stages of the disease the occurrence of antibodies to normal kidney can be demonstrated. The results described may still be improved in the future since many of the tests were technically imperfect in the beginning and the antigen certainly requires much fur-

did not show the same degree of specificity. Here again the decisive role of the antigen has been demonstrated, and it is possible that infant's heart may yield better and more consistent results. The low incidence of positive titres in normal controls makes the results more significant.

At present the technical difficulties of the method described in this paper and its occasional complete failure do not make it applicable as a routine clinical test. It is to be hoped that with further knowledge of the antigen new and simpler methods can be worked out for more general use. Our attempts with the complement-fixation method as suggested by Schwentker have failed. The methods as suggested by Hecht, Sulzberger and Weil (6) should be investigated.

One cannot avoid speculating on the possibility of neutralizing these ant kidney antibodies as a therapeutic approach if they should prove to be a link in the pathogenesis of glomerulonephritis.

The fact that all authors were forced to employ many injections of their "nephrotoxins" to produce persisting lesions suggests that human nephritis does not develop and progress from a single insult but rather from the constant presence and occasional stimulation of antibodies to renal tissue. This lends support to the thought that a maintained neutralization of the antibodies may arrest or interfere with the progress of the disease.

#### SUMMARY

1. The clinical and experimental evidence supporting the concept that glomerulonephritis is due to a continuous organ-specific antigen-antibody reaction is reviewed.

2. A modified collodion particle technique for detecting antibodies to kidney tissue is described.

3. In 23 cases of glomerulonephritis of all stages antibodies to human renal tissue were demonstrated in 75% of the 122 tests done (average titre 1:623) and 18, or 78.3% of the cases were positive in more than 60% of the tests.

4. In 12 cases considered as early nephritis 68% of the 44 determinations done were positive (average titre 1:918).

5. In 11 cases considered as late nephritis 78% of the 78 determinations done were positive (average titre 1:337).

6. In 68 control subjects antibodies to human renal tissue were demonstrated in 19% of the 126 determinations (average titre 1:78). Four, or 6% of the 68 cases were positive in more than 50% of the tests.

7. Renal antigens obtained from infants or stillbirths show a greater specificity and higher titres than antigens from adult renal tissue.

8. The importance of the continuous presence of antibodies to kidney in a high percentage of cases of nephritis is discussed in relation to the clinical course of the disease and a possible therapeutic approach is suggested.

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