COMPLEMENT AND ISOHEMAGGLUTININS IN URINARY PROTEINS[1]

By S. SEIFTER and E. E. ECKER

(From the Institute of Pathology, Western Reserve University and the University Hospitals, Cleveland)

(Received for publication May 6, 1946)

It has been recently established that the diminution or extinction of the serum complement titer which sometimes occurs in the course of infectious disease is coincident with a decrease or disappearance of primarily C'4 and secondarily C'2 and C'1 (1). It was also found that prognosis was grave for those patients whose complement titers fell to zero or near-zero. The mechanism of masking, inactivation, or interference with the production of these components, whatever the case may be, has not as yet been fully investigated, nor has the manner in which the dimution of titer may contribute to greater susceptibility to infection.

In cases of kidney disease, in which sometimes a lowering of serum complement has also been observed, the excretion of protein in the urine presents a situation distinct from that generally found in cases of uncomplicated infectious disease. While not always reflecting itself in a decrease of serum complement titer, for reasons discussed below, a loss of complement by means of urinary excretion nevertheless could take place in kidney disease, and could be a contributory factor to decreased resistance to superimposed infection.

The present study is therefore concerned with the analysis, chiefly with respect to the complement components, of a series of urine specimens obtained from normal individuals, from cases of infectious disease, and from patients with kidney disease.

METHODS

1. Preparation of urine specimens. Urine samples, consisting of single voidings, were usually processed within a few hours after collection. Specimens were measured for volume, adjusted to pH of about 6.6 with either 0.1 N NaOH or 0.1 N HCl, filtered or centrifuged to remove insoluble material, and chilled to 1° C. One of 2 methods, both of which were designed to preserve complement activity and both of which gave comparable results, was then used to prepare urine or urinary protein solutions for testing. These methods are as follows.

(*a*) The urine, which had been given preliminary treatment as described above, was dialyzed at 1° C. for 24 hours against 2 changes of medium, each consisting of 4 liters of 0.9 per cent NaCl mixed with 20 ml. of potassium phosphate buffer of pH 6.6 and ionic strength 0.3. This method was used particularly for apparently highly concentrated urines excreted in low volumes.

(*b*) The urine, adjusted to pH 6.6 and filtered, was diluted with ¼ its volume of the potassium phosphate buffer of pH 6.6 and ionic strength of 0.3. Thirteen grams of ammonium sulfate were then added for each 25 ml. of the buffered urine, and the mixture shaken until all of the salt was dissolved. The resulting mixture was then allowed to stand at 1° C. for at least 1 hour, following which it was filtered just to dryness at 1° C. on a fine sintered glass funnel. The collected precipitate was then dissolved in a minimum quantity of the buffered saline prepared as described under Method (*a*), the protein solution enclosed in a cellophane membrane, and dialyzed against repeated changes of the buffered saline at 1° C. until free from sulfate. This latter process required from 24 to 36 hours. The contents of the dialysis sac were then centrifugalized at 1° C., the supernate was poured off, measured for volume, and tested for complement component activity. Occasionally the precipitate which formed upon dialysis was more than scant, but invariably it was insoluble in 18 per cent NaCl or phosphate buffer of pH 7.8. Suspensions of these precipitates, when tested for complement component activity, were always found to be negative.

This method led to a complete separation of the urinary proteins.

2. Testing for complement component activities. Testing complements, previously designated as specifically inactivated complements, were prepared from fresh human serum as described elsewhere (2). The dialyzed urine or the urinary protein solution, in amounts of 0.15, 0.30, and 0.50 ml., was tested alone and in combination with each of the various testing complements used in 3-unit amounts. After incubation for 30 minutes at 37.5° C. with the standard dose of sensitized sheep red cells, the test mixtures were centrifugalized and their supernates read for per cent hemolysis by visual comparison with prepared standards (2).

Since all of the dialyzed urines and the urinary proteins were inactive when tested alone, the degree of hemolysis which they produced in combination with *an excess* of a given testing complement was a measure of the amount of the given component present in the urine solution.

3. Detection of isohemagglutinins. In some cases the

[1] Aided by a grant from the Commonwealth Fund.

dialyzed urine or the urinary protein solution was tested by both slide and test tube methods for isohemagglutinin type against A, B, and O cell suspensions.

4. Electrophoresis. Several samples of treated urines were subjected to electrophoresis using a veronal buffer of pH 7.8 and ionic strength of 0.1.

RESULTS

1. Complementary activity

Table I summarizes the significant data obtained with respect to complement activity of the urinary proteins. In the table a given complement component activity is expressed in the number of units per gram of protein found in, or isolated from, the urine, which caused complete hemolysis of the standard dose of sensitized sheep red cells in the presence of an added excess of the other 3 components. As a basis for comparison, the table includes the number of such units of each of the components which would be found in a normal serum of average complement titer (33.3 units per ml. of serum) and protein content (7.0 per cent). Since the complement components are associated with the globulins, their units could more properly be expressed per gram of *globulin,* but even such designation would be arbitrary, and accordingly is made only in 2 instances.

a. Normal urines. Urine specimens from 3 normal individuals were treated according to Method (*b*). In each case a very minute amount of protein was obtained which showed no complement component activities.

b. Infectious diseases. The urines of 2 patients with meningococcal meningitis, one with scarlet fever, and one with both rheumatic fever and Type XII pneumococcal meningitis, were studied. Although all of the urines contained some protein, no complement component activities were detected in any of them. The data obtained with respect to the rheumatic fever-meningitis patient, because of the relatively high urinary excretion of protein, are included in Table I.

c. Nephrotic syndrome; lipoid nephrosis. Urine specimens from 3 children were treated and tested; complement components were found in the urinary proteins of each case at one time or another

TABLE I

Content of complement components in urinary proteins

Patient	Diagnosis	Date of urine sample	Total protein in urine	Approximate number of units of C′ component isolated from the urine			
				C′1	C′2	C′3	C′4
			grams per cent	*per gram protein*			
D. C.	Rheumatic fever; Type XII pneumococcal meningitis	3-3-45	0.44	0	0	Tr?	Tr?
D. M.	Lipoid nephrosis	2-5-45	0.58	100	440	100	100
D. M.	Lipoid nephrosis	2-13-45	0.32	0	80	80	80
A. S.	Lipoid nephrosis; upper respiratory infection	2-5-45	0.33	0	200	80	80
A. S.	Lipoid nephrosis; upper respiratory infection	2-13-45	0.56	0	40	0	20
J. G.	Lipoid nephrosis	2-5-45	0.42	40	250	200	100
J. Q.	Chronic glomerulonephritis; nephrotic syndrome; upper resp. infect.	4-21-44	0.54	0	425	0	850
L. B.	Acute glomerulonephritis	5-2-44	0.61	0	475	700	650
E. M.	Acute glomerulonephritis; nephrotic syndrome	2-27-45	1.73	0	40	90	80
M. P.	Arteriosclerotic heart disease; diabetes mellitus	2-22-45	0.22	0	250	175	70
			Total globulin in urine	Units C′ component isolated from urine			
			grams per cent	*per gram globulin*			
D. M.	Lipoid nephrosis	2-13-45	0.08	0	275	275	275
E. M.	Acute glomerulonephritis; nephrotic syndrome	2-27-45	0.79	0	90	200	180
Units C′ component per gram of protein in an average normal serum (approximate)				4,275 10,300	475 475	475 700	4,275* 11,200†

* Approximated as described by Ecker, E. E., Seifter, S., and Dozois, T. F., J. Lab. Clin. Med., 1945, **30**, 39.

† Approximated from figures given by Bier, O. G., Leyton, G., Mayer, M., and Heidelberger, M., J. Exper. Med., 1945, **81**, 449.

in the course of the disease, but 2 of the total of 7 urine samples failed to show any component activity whatsoever. As shown in Table I, C'2 and C'3 chiefly were excreted, followed by C'4, and in 2 of the 5 urine samples, by C'1.

All 3 of these patients were edematous.

d. Glomerulonephritis. One urine sample from each of 2 patients, and 2 samples from a third patient, were studied. C'2 and C'4 were present in all of the urines, C'3 was present in 3 of the samples, and C'1 was present in none.

These patients were also edematous.

e. Arteriosclerotic heart disease and diabetes mellitus. Two urine samples obtained from one patient were studied. One of these contained about 1 per cent protein, and showed considerable C'2 and C'3 activities and some C'4. The second sample contained relatively slight traces of protein, and was found to have no complement component.

This patient also was edematous.

f. Arteriolar nephrosclerosis. One urine obtained from a patient was examined and found to contain a very minute amount of protein with questionable traces of C'1, C'3, and C'4 activities.

This patient did not have edema.

2. *Isohemagglutinin activity*

As a beginning in the study of the excretion of antibodies, the isohemagglutinin activities of a number of dialyzed urines and the urinary protein solutions were determined qualitatively. After these tests were completed, the clinical records of the patients were checked for blood types, and in every case in which such a record was available it served to confirm the urinary tests if these were at all positive. In several cases, particularly in which the content of urinary protein was low, no tests were obtained at all. Table II gives a summary of the positive results obtained by test tube titration.

3. *Electrophoresis*

The urinary proteins obtained in one case with nephrotic syndrome and those from one case of acute glomerulonephritis were examined in the Tiselius apparatus. In confirmation of the observations of Luetscher (3) and Blackman and Davis (4), Table III shows that the urine of this patient contained considerably more albumin than did that of the nephritic patient, and that the latter urine contained a large amount of gamma globulin. What is of particular interest, in relation to the present study, is that both of these urinary protein solutions contained complement activity and isohemagglutinins, and that the globulin fractions generally associated with these activities were shown to be present in the electrophoretic diagrams.

TABLE II

The isohemagglutinin activities of urinary proteins

Patient	Date	Clumping by urinary proteins of cells of type			Blood type from urine	Blood type from hospital records
		A	B	O		
D. M.	2-13-45	−	++++	−	A	A
D. M.	2-19-45	−	+++	−	A	A
A. S.	2-13-45	−	±	−	A?	A
A. S.	2-22-45	−	−	−		A
M. P.	2-20-45	+	++++	−	O	O
E. M.	2-27-45	−	++++	−	A	A

TABLE III

Electrophoretic analysis of urinary proteins

Patient	Diagnosis	Date	Concentrations (per cent of total)			
			Albumin	Globulins		
				Alpha	Beta	Gamma
D. M.	Nephrotic syndrome	2-13-45	74.5	2.6	9.8	13.0
E. M.	Acute glomerulonephritis; nephrotic syndrome	2-27-45	55.1	6.7	10.5	27.9

DISCUSSION

In the several samples of urine obtained from normal individuals and from people suffering with uncomplicated infectious disease, no complement component activities were detected. On the other hand, the data in Table I show that in one case or another each of the 4 complement components was identified in the urinary proteins excreted in kidney disease, particularly in those cases in which there was an associated edema. In this respect, the edema probably has no significance other than as an indication of the profound reduction of the serum proteins as evidenced by the following: DM had a serum protein content of 3.3; AS, 4.3; EM, 3.62; JG, 5.6; and JQ, 4.81 per cent.

As seen in Table I, the consistent and high excretion of C'2 is outstanding, the activity figures

often approaching that for the normal serum. In sharp contrast are the infrequency of C'1 excretion, and the relatively low value obtained for this component even when it is present. Since the excretion of a protein through the damaged kidney may be related to its solubility, it must be pointed out that C'2 is extremely soluble even in distilled water, whereas C'1 behaves as a euglobulin, and is easily precipitated. Furthermore, C'1 has an apparent isoelectric point at about pH 6 (5), and may be one of those more insoluble globulins, discussed by Luetscher (3), which are precipitated from dilute solutions of pH 5.5 to 6.5, and aid in the formation of the urinary "casts." Blackman and Davis (4) believe that the hyaline materials which collect in the glomeruli and tubules in patients with "progressing nephrotic nephritis," are probably derived from globulins other than fibrinogen. It may be, then, that C'1 is one of these globulins, and is retained in the process of formation of the hyaline materials.

The excretion of C'3 and C'4, both of which are in themselves relatively soluble, or at least may be attached to soluble proteins, is fairly consistent, though not as striking in amount as is the excretion of C'2.

Though red blood cells were found in some of the urine specimens tested, particularly in 2 of the cases of glomerulonephritis, the occurrence of complement components cannot be attributed in any great measure to the presence of whole blood, for the following reasons: (*a*) In a number of urine specimens in which complement components were present, no red blood cells were seen nor were positive benzidine tests obtained. (*b*) Contrary to what would be expected if whole blood were present, the entire complement complex was not found, but only certain components. (*c*) The amounts of complement components, particularly of C'2 and C'3, found in those urines showing some red cells, were relatively so great, that the urines would have had to consist in large measure of whole blood, a circumstance ruled out by the relatively low red cell and protein contents of the urines. From a physiologic standpoint, the discovery of complement components in the urine is extremely interesting, inasmuch as it demonstrates that normal plasma proteins, and particularly those with specific functions, are excreted in kidney disease. In addition to the complement components, the isohemagglutinins have been demonstrated in the urinary protein excreted in kidney disease. While the authors have not as yet had the opportunity to study the excretion of antibodies to infectious agents, there is no reason to believe that these are not excreted.

The possible immunological significance of this work now becomes apparent. It is well known that a patient with kidney disease is particularly subject to infection, frequently pneumococcal in origin, and expressing itself as a septicemia, peritonitis, or an upper respiratory disease. This general lack of resistance to infection exhibited by individuals with kidney disease may in part be related to excretion of protein, as follows: (*a*) in the sense of Cannon (6), by the depletion of protein reserves, with consequent reduction of matrix necessary for the production of antibodies and complement; (*b*) by the loss to the urine of preformed antibodies, as suggested by Bell (7); (*c*) by the loss over a period of time of complement components, particularly C'2 and C'4, which together with C'1 are necessary for the acceleration of opsonification of invading organisms (8); and (*d*) by the diminution in the serum of any one or more of the 4 components of complement, all of which are necessary for bactericidal action (9).

If sufficient complement is excreted in the urine to predispose the patient, in some degree, to infection, a concomitant reduction of the serum complement should be noted. In some cases of kidney disease a simultaneous lowering of the serum complement titer and serum protein content has been observed; however, this has not been the general rule. The usual test for serum complement titer, being a test for a *dissolved* protein complex and therefore dependent upon *plasma volume,* necessarily fails to reveal the full character of changes occurring in the plasma. Plasma volume variations taking place with edema might tend to offset and mask the decrease of serum protein. Furthermore, the patients studied here have been undergoing treatment, inclusive of transfusions of whole blood and plasma, thus complicating even more the expected relationship between loss of serum protein to the urine and decrease of serum complement titer. These considerations are the basis for the statement made at the beginning of this paper that loss of serum protein may not always be reflected in diminished complement titer.

SUMMARY

1. Complement component activities have not been detected in, or isolated from, the urines of 3 normal individuals, nor in those of 4 people suffering with infectious disease.

2. Complement component activities have been identified in the precipitated urinary proteins of patients showing nephrotic syndrome, and in cases of acute and chronic glomerulonephritis, as well as arteriosclerotic heart disease with a nephrotic component.

3. The excretion of C'2 was most consistent and in largest relative quantity. C'3 and C'4 were excreted in the urines of these cases with good consistency, but C'1 was seldom excreted, and then only in relatively small amount.

4. It is pointed out that C'2 is a very soluble protein, whereas C'1 is a euglobulin; and that the high excretion of the one and the retention of the other is probably related to their solubilities.

5. Isohemagglutinins have been isolated with the urinary proteins in 5 instances. Identification of these was in agreement with the blood types of the patients.

6. The physiological significance of these findings lies in the demonstration that normal plasma proteins, and particularly those with specific functions, may be excreted in the urine by patients with kidney disease.

7. The immunological significance of this study is that it may explain in part the predisposition of patients with kidney disease to infection. The loss of complement substances and antibodies, both necessary for the opsonification and killing of invading organisms, may be contributing factors to the diminished resistance of these cases.

The authors express their thanks to Drs. Max Miller and Walter Heymann of the University Hospitals for their cooperation in supplying clinical materials.

BIBLIOGRAPHY

1. Ecker, E. E., Seifter, S., Dozois, T. F., and Barr, L., Complement in infectious disease in man. J. Clin. Invest., 1946, **25**, 800.
2. Ecker, E. E., Pillemer, L., and Seifter, S., Immunochemical studies on human serum. I. Human complement and its components. J. Immunol., 1943, **47**, 181.
3. Luetscher, J. A., Jr., Electrophoretic analysis of plasma and urinary proteins. J. Clin. Invest., 1940, **19**, 313.
4. Blackman, S. S., Jr., and Davis, B. D., Electrophoretic and Kjeldahl analysis of protein in nephritic urine and the effect of proteinuria on the human kidney. South. M. J., 1943, **36**, 247.
5. Pillemer, L., Seifter, S., San Clemente, C. L., and Ecker, E. E., Immunochemical studies on human serum. III. The preparation and physicochemical characterization of C'1 of human development. J. Immunol., 1943, **47**, 205.
6. Cannon, P. R., Antibodies and the protein reserves. J. Immunol., 1942, **44**, 107.
7. Bell, E. T., The pathogenesis of glomerulonephritis including lipoid nephrosis. Proc. Inst. Med. Chicago, 1939, **12**, 306.
8. Ecker, E. E., Pillemer, L., and Kuehn, A. O., The opsonins of normal and immune sera. II. The opsonins of sera of different species, the role of complement in opsonic activity and the combination of an immune serum and a normal serum as influencing opsonization. J. Immunol., 1942, **43**, 245.
9. Dozois, T. F., Seifter, S., and Ecker, E. E., Immunochemical studies on human serum. IV. The role of human complement in bactericidal phenomena. J. Immunol., 1943, **47**, 215.