DEFICIENCY OF THE SIXTH COMPONENT OF COMPLEMENT IN RABBITS WITH AN INHERITED COMPLEMENT DEFECT*, ‡, §

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In 1961 a strain of rabbits was described which was characterized by an inherited trend for a deficiency in serum complement activity. The serum of these animals was found to lack the activity of the classical third component of complement (1). In recent years it was possible to demonstrate that the classical third component of guinea pig (2) and of human (3 *a* to 3 *c*) complement consists of at least six distinct components, which are now being referred to as C'3, C'5, C'6, C'7, C'8, and C'9 (4). On the basis of this information and with the aid of purified components from human and rabbit serum, it was possible to define the inherited complement defect in rabbits. It is the purpose of this paper (*a*) to show that the deficient component is identical with the sixth component (C'6) of normal rabbit complement, and (*b*) to describe a method for the purification of rabbit C'6.

Materials and Methods

Serum and Serum Fractions.—Pooled normal rabbit serum (raC') was obtained from bleedings of 12 rabbits. It was stored at −70°C until it was used. Complement-deficient serum (C'_{def}) was obtained from at least 6 deficient rabbits and the individual sera were pooled before storage at −70°C. The pseudo- and euglobulin fractions of serum pools were prepared by dialysis against phosphate buffer pH 5.2, ionic strength 0.01, and separated by centrifugation at 1000 *g* in the cold.

Preparation of Purified Complement Components.—The functionally pure second component of complement (C'2) of guinea pig serum (5) was kindly supplied by Dr. Manfred Mayer. It was used in the titration of C'3 by immune adherence. The purified sixth component (C'6) of human serum was prepared according to a previously published method (6).

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Antisera.—Antiserum to the third component of rabbit complement (C'3) was prepared in ducks by the injection of zymosan particles which had been treated with rabbit complement, as described earlier (7). For the immunochemical detection of the fifth component of complement (C'5), a mouse antiserum against mouse MuB1 (8) was employed. It was prepared by the injection of C57BL/J strain mouse serum into A/HeJ strain mice. The antiserum is able to react with the MuB1 analogue of certain other species, including rabbits. MuB1 was shown to represent C'5 of mouse serum (9). Sheep anti-rabbit γ-globulin serum was kindly supplied by Dr. Charles Cochrane. Antiserum to rabbit C'6 was prepared by the injection of purified rabbit C'6 into C'6-deficient rabbits. Four injections were administered intravenously in intervals of 2 wk.

Chromatography.—In the first step of the purification procedure of rabbit C'6, euglobulin was separated chromatographically on triethylaminoethyl-cellulose (TEAE-cellulose) by stepwise elution. A 5 × 60 cm column containing approximately 900 ml packed TEAE was equilibrated with phosphate buffer pH 7.2, ionic strength 0.05 (starting buffer). After the euglobulin, which was obtained from 300 ml serum, was applied, the column was washed with 3000 ml of starting buffer. C'6 activity was then eluted with 1500 ml of starting buffer containing 4.0 g of NaCl per liter. The conductivity of the eluting buffer was 7 millimhos.

Preparative Electrophoresis.—Column fractions containing C'6 activity were pooled and the NaCl concentration in the pool was raised to 1 M. This measure was found to preserve C'6 activity during the subsequently performed concentration of the protein. The pool was concentrated by ultrafiltration in collodion sacks to approximately 5 ml. The material was then applied to a Pevikon block for electrophoresis in barbital buffer pH 8.6, ionic strength 0.05, at 4°C (10). A potential gradient of 3.5 v per cm was applied for 42 hr.

Starch Gel Electrophoresis.—The method of Smithies (11) was employed using the discontinuous buffer system described by Poulik (12). The material was applied at a protein concentration of approximately 10 mg per ml by filter paper carrier. After 3 hr at a potential gradient of 10 v per cm, the gel was sliced into two halves, one of which was stained for protein. The other half served for the localization of C'6 activity. Small segments were cut out of the gel, mechanically disintegrated and eluted with veronal buffer pH 7.4. C'6 activity was determined on the supernatants after removal of the starch gel particles by centrifugation.

Density Ultracentrifugation.—This was performed according to Kunkel (13) using a 10 to 40% sucrose density gradient in phosphate buffer pH 7.0, ionic strength 0.1, and a Spinco SW 39 swinging bucket rotor. Experiments were carried out for 20 hr at 35,000 RPM.

Labeling of C'6 Preparations with Radioactive Iodine.—Iodine labeling was performed with carrier free iodine^{125} using the chloramine T method, as described in detail by McConahey and Dixon (14). The specific radioactivity was approximately 30,000 CPM per μg protein.

C'6 Assay.—For the detection and quantitation of C'6 activity sensitized sheep erythrocytes (EA) were used which had reacted with complement-deficient rabbit serum (C'_{def}). These cells are, in the following, referred to as EAC'_{def}. 5 ml EA were incubated at 32°C with 5 ml C'_{def} diluted 1 to 12 in veronal buffer. After 10 min the cell suspension was poured into 100 ml of ice cold veronal buffer containing Na_3H EDTA in a final concentration of 0.005 M. After centrifugation in the cold, the cells were resuspended in 5 ml of EDTA containing veronal buffer. 0.5 ml of EAC'_{def} were incubated at 32°C for 60 min with 0.4 ml C'_{def} diluted 1 to 12 together with a 0.1 ml aliquot of the test solution.

The immune adherence assay and the titration of C'3 in rabbit serum was performed according to Nishioka and Linscott (15).

RESULTS

Absence of an Inhibitor in the Serum from Rabbits with Inherited Complement Deficiency.—Initially, the possibility existed that lack of hemolytic activity in

complement-deficient serum was due to the presence of a powerful complement inhibitor interfering with an otherwise unimpaired complement system. This possibility was virtually eliminated by the finding that addition of C'_{def} serum did not dimimish the activity of normal rabbit serum (1). To exclude further that normal complement activity is obscured by an inhibitor, C'_{def} serum was subjected to electrophoretic fractionation, thus possibly effecting

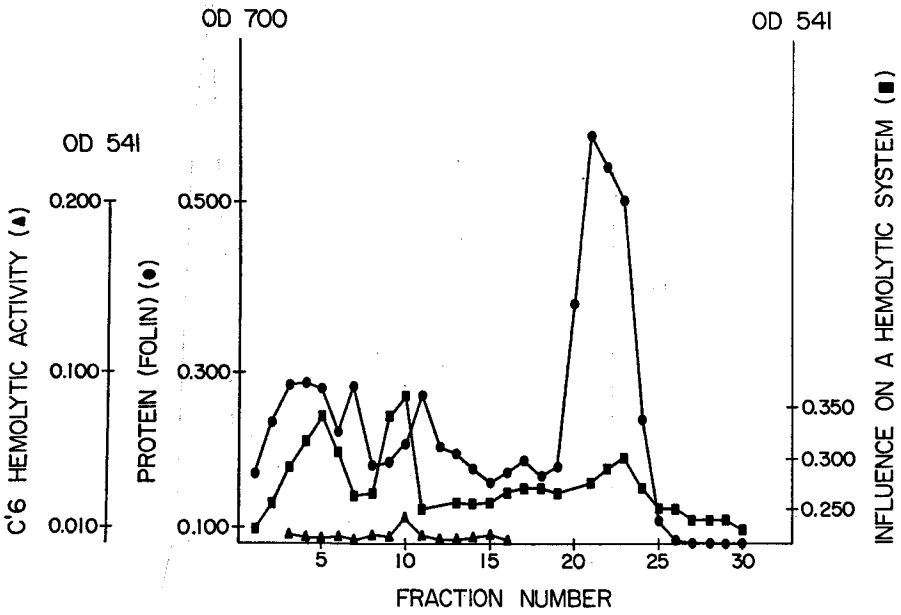

FIG. 1. Demonstration of absence of complement inhibitor in fractions of complement-deficient rabbit serum separated by Pevikon block electrophoresis. Fractions were incubated with a standardized hemolytic system which, per se, yielded a degree of lysis equivalent to $OD_{541} = 0.250$. C'6 activity was not demonstrable.

the separation of hemolytic from inhibitory activity. As demonstrated in Fig. 1, none of the fractions of the electrophoretically separated deficient serum contained either C'6 activity or marked anticomplementary activity.

Localization of C'6 in Electrophoretic Fractions of Normal Rabbit Serum.—In contrast to C'_{def} serum, C'6 activity could readily be demonstrated in fractions of normal rabbit serum, as illustrated in Fig. 2. The peak activity was localized in the inter-β-γ-region. The activity was detected by utilizing the complement-deficient serum as a reagent. The deficiency of this serum was overcome only by addition of the inter-β-γ-material which showed a very restricted distribution of its electrophoretic mobility.

Definition of the Missing Activity as C'6.—The electrophoretic mobility of the

normal serum constituent that reconstituted deficient serum suggested its identity with C'6, C'7, or C'8, since these activities have been found in human serum to behave as slowly migrating β-globulins (3 *b*, 6). To test this hypothesis, varying amounts of purified human C'6 were added to the defective serum and the hemolytic activity of the mixtures was measured. As listed in

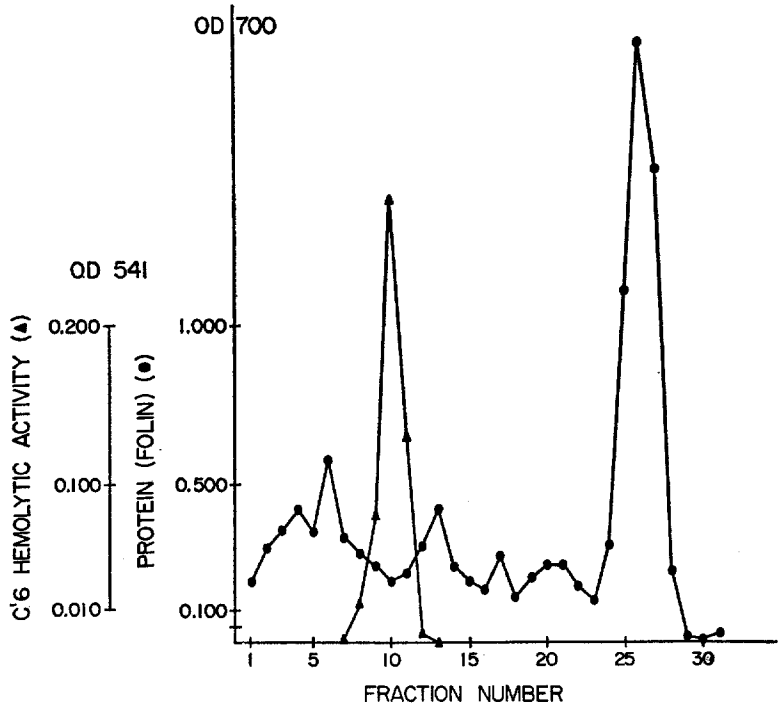

FIG. 2. Localization of C'6 activity in fractions of normal rabbit serum separated by Pevikon block electrophoresis. The activity is distributed as a well defined peak in the inter β-γ-region.

Table I, increasing amounts of C'6 led to increasing hemolytic activity. In contrast, purified human C'7 and purified human C'8 lacked the capacity to reconstitute rabbit $C'_{def.}$

To make certain that the reconstituting factor was identical with C'6, a preparation of human C'6 was subjected to starch gel electrophoresis. Eluates of segments of the gel were then tested for reconstituting activity using rabbit C'_{def}, and for human C'6 activity utilizing the conversion of the thermolabile intermediate complex EAC'1a,4,2a to the thermostable complex EAC'1a,4,2a,3,5,6,7 (3 *b*, 6). Fig. 3 shows the identical distribution of both activities in the starch gel. Conforming to the nomenclature currently in use for the human complement system (3 *a* to 3 *c*, 4), the rabbit component reconstituting the

TABLE I

Reconstitution of Complement-Deficient Rabbit Serum by Purified Human C'6

Reciprocal of C'6 dilution	Hemolysis (OD 541)
20	0.329
40	0.280
80	0.262
160	0.197
320	0.117
640	0.079
1280	0.041
Buffer control	0.001

Conditions: 0.2 ml of human C'6; 0.2 ml of rabbit C'_{def} diluted 1:10; and 0.2 ml of EA.

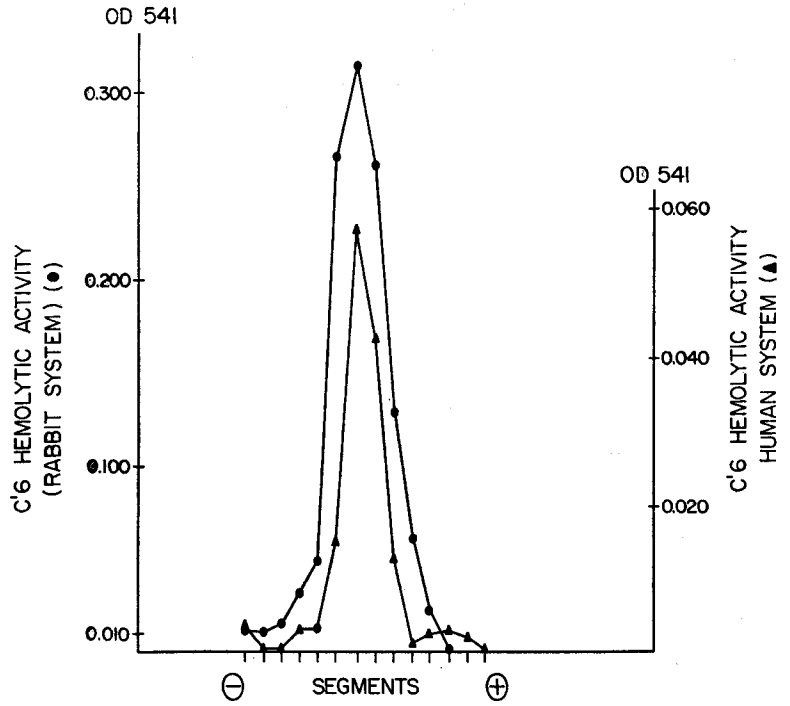

FIG. 3. Starch gel electrophoresis of purified human C'6. Comparison of the distribution of the activity which reconstitutes complement-deficient rabbit serum and of human C'6 activity.

hemolytic activity of serum from complement-deficient rabbits was therefore designated C'6.

Purification of C'6 from Normal Rabbit Serum.—Although C'6 activity is present in both the euglobulin and pseudoglobulin fraction of serum, the eu-

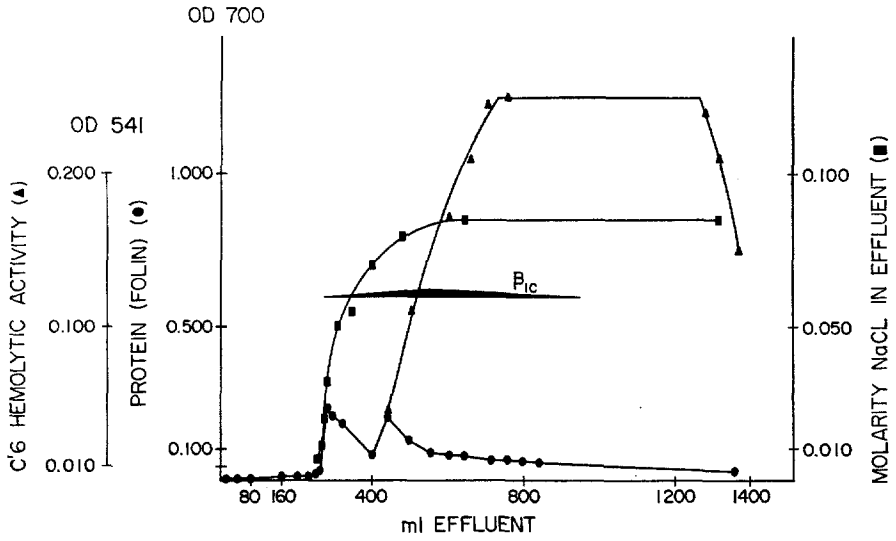

FIG. 4. Chromatography of the euglobulin fraction of normal rabbit serum on TEAE-cellulose. Fractions were obtained by step-wise elution. The first 800 ml of effluent which contained rabbit C'3 (β_{1C}) were discarded, the next 400 ml containing the major portion of C'6 activity were pooled and concentrated for application to Pevikon block electrophoresis.

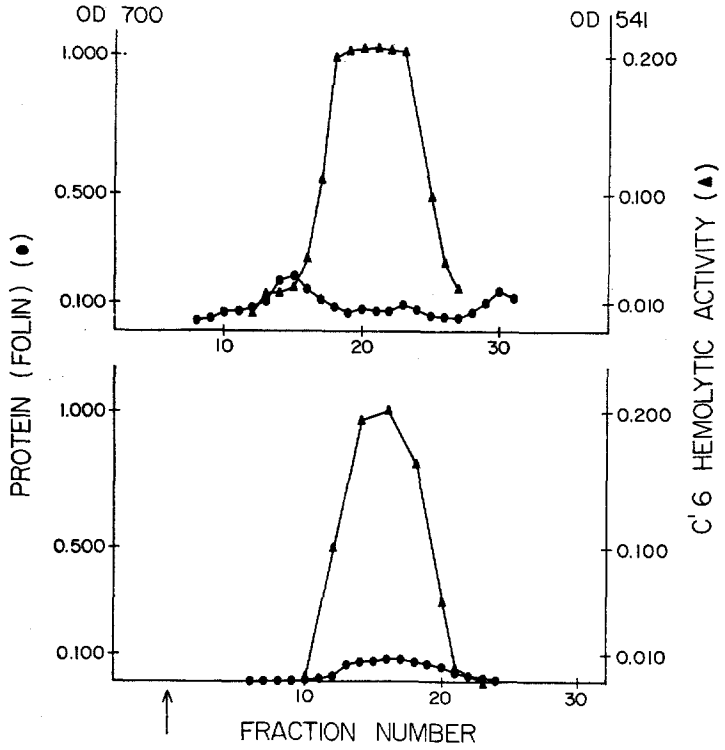

Fig. 5. Zone electrophoresis on Pevikon of C'6 containing fraction obtained from euglobulins by TEAE chromatography. C'6 containing block fractions (upper pattern) were pooled, concentrated and once again subjected to block electrophoresis (lower pattern). Anode was at the right.

globulins were used as starting material because this afforded a greater degree of initial purification. The euglobulins were first separated by chromatography on TEAE-cellulose (Fig. 4). Fractions containing C'6 activity were concentrated and the material was then subjected to preparative electrophoresis

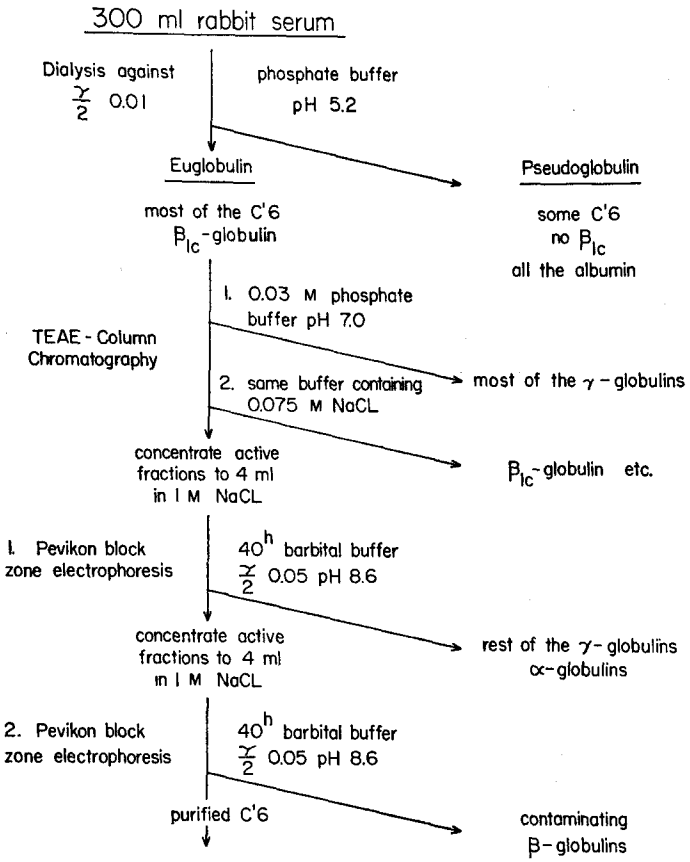

Fig. 6. Procedure of purification of C'6 from normal rabbit serum.

(Fig. 5). In those cases where it was obvious that contaminating proteins overlapped with the activity, the active fractions were pooled, concentrated, and once again subjected to electrophoresis under identical conditions (Fig. 5). The final material was concentrated to approximately 10 mg protein per ml. The purification procedure is summarized in Fig. 6.

Characterization of Purified Rabbit C'6.—Concentrated purified rabbit C'6 yielded one relatively broad protein zone on starch gel electrophoresis. No additional bands could be discovered. Comparison of the distribution of the protein with the distribution of the activity revealed lack of complete cor-

relation. The activity peak coincided with the anodal edge of the protein zone. The cathodal part was devoid of detectable activity. This suggested that most of the protein present in this preparation was not associated with active C'6.

Analysis with anti-C'3 and anti-C'5 sera in Ouchterlony plates showed absence of those proteins in the C'6 preparations. However, analysis with an anti-rabbit γ-globulin serum revealed the presence of γ-globulin in the final C'6 preparations. Quantitative precipitin analysis of I^{125}-labeled C'6 preparations established that at least 80% of the total protein represented rabbit γ-globulin. Using an antiserum to rabbit C'6 raised in C'6 deficient animals (see below), approximately 5% of the radioactively labeled material was precipitated in antibody excess.

Ultracentrifugal analysis in a sucrose density gradient showed the C'6 activity to sediment somewhat less rapidly than 7S γ-globulin. The sedimentation rate was calculated to be 6S. Although considerable purification was achieved, these results indicated that C'6 is contaminated with γ-globulin of electrophoretic and chromatographic properties closely resembling those of C'6. Identity with 7S γ-globulin is precluded by the distinctly different sedimentation behavior of the two entities. Rabbit C'6 in whole serum or in the purified form resisted heating to 56°C.

Characterization of the Complex Formed by the Reaction of Deficient Serum with Sensitized Erythrocytes.—In a previous paper (1) it was shown that the reaction of EA with C'_{def} leads to the formation of EAC'1a,4,2a. Since it was not possible at that time to decide whether later reacting complement components also participated in the reaction, the EAC'_{def} complex was subjected to further analysis.

To find out whether C'3 was attached, the cells were tested with an antiserum to rabbit C'3. Strong agglutination was observed and allowed the conclusion that the EAC'_{def} complex was at least in the EAC'1a, 4,2a,3 state. This was further corroborated by the observation that these cells yielded a strongly positive immune adherence phenomenon. Immune adherence has previously been shown by Nishioka and Linscott (15) to be caused by cell-bound C'3.

These results were in agreement with the finding that C'3 was present in the deficient serum in approximately the same concentration as in normal serum, which was determined by the use of Ouchterlony plates and a duck antiserum to rabbit C'3. When the reactivity of C'3 in deficient and in normal rabbit sera were examined by immune adherence, comparable titers were found for both types of sera.

To determine whether C'5 was attached to the EAC'_{def} complex, cells were set up for agglutination with an antiserum to C'5. No detectable agglutination occurred, although C'5 is definitely present in defective serum, as evidenced by positive Ouchterlony tests with specific antiserum to C'5

In another set of experiments, the ability of the EAC'_{def} complex to react with purified C'6 was investigated. A mixture of EAC'_{def} and C'6 was incubated

at 32°C and samples were withdrawn at certain intervals of time. After centrifugation the supernatants were removed and the cells were resuspended in a dilution of defective serum in the presence of EDTA. Although these cells contained on their surface the first, second, third, and fourth components, no evidence was obtained for an interaction with C'6. It was concluded that in the absence of C'6, C'5 cannot effectively react with EAC'1a,4, 2a,3 sites. The EAC'_{def} complex may therefore be defined as being in the EAC'1a,4,2a,3 state.

TABLE II

Inhibition of Rabbit C'6 by Anti-C'6 Prepared in a C'6-Deficient Rabbit

Tube No.	Reciprocal of antiserum dilution*	Remaining activity of C'6 in whole serum‡
1	Undiluted	0.002
2	2	0.003
3	4	0.029
4	8	0.080
5	16	0.111
6	32	0.128
7	Buffer control	0.140
		Remaining activity of purified C'6
8	2	0.041
9	4	0.039
10	8	0.159
11	16	0.199
12	Buffer control	0.151
	Tube No. 9 reincubated with:	
13	Buffer	0.034
14	C'6 (60', 62°C)	0.141
15	Fraction from C'_{def}§	0.022

* Antiserum was diluted in serum from the same animal obtained prior to immunization. The reaction mixture contained 0.003 M EDTA.

‡ OD 541.

§ Prepared exactly according to the method used for purification of C'6 from normal rabbit serum.

Formation of an Antibody to C'6 in Deficient Rabbits.—Several deficient animals were injected with purified C'6 material. The animals responded with the formation of an antibody which functionally blocks C'6 activity. When purified C'6 or normal rabbit serum were incubated in the presence of EDTA with increasing amounts of antiserum, increasing inactivation of C'6 was observed (Table II). The inhibition could be partially reversed by the addition of heat

inactivated C'6 (60 mins, 62°C) to the reaction mixture. A similar reversal of inhibition could not be achieved by addition of protein from defective serum which chromatographically and electrophoretically corresponded exactly to C'6 of normal serum. Inhibition of hemolysis was also observed when the antibody was added to EA simultaneously with normal rabbit complement. Sera collected from the same animals prior to immunization with C'6 were free of inhibitory activity. The data indicate that C'6- deficient serum lacks an antigen which is present in normal rabbit serum and which is intimately associated with C'6. It is not possible, however, on the basis of these experiments to decide whether C'6 is totally absent from deficient serum or whether it is present in an immunochemically modified and inactive form.

DISCUSSION

Inherited defects of the hemolytic serum complement system have been observed to date in guinea pigs (16), mice (8, 17), and rabbits (1). In all these instances, the lesion involved the classical third component of complement. While the guinea pig defect cannot be defined in terms of modern concepts because the deficient strain was lost many years ago, the mouse defect has been shown to involve the fifth component of complement (9). In the present communication, evidence is set forth indicating that complement-deficient rabbits lack the sixth component of complement

The evidence is based on the observation that functionally pure human C'6 reconstitutes completely the hemolytic capacity of deficient rabbit serum. In contrast, C'7 and C'8, both possessing physicochemical characteristics similar to those of C'6 (18), failed to render rabbit C'_{def} hemolytically active. Reconstitution could also be accomplished by a factor purified from normal rabbit serum. This normal rabbit serum component was shown to be the rabbit analogue to human C'6. The accumulated evidence fully supports the previously advanced concept that this complement abnormality is due to a true deficiency of a complement component rather than a suppression of activity by an inhibitor.

Rabbit C'6 has an electrophoretic mobility of an inter-β-γ-globulin and a sedimentation rate of 6S. Although substantial purification of C'6 from normal rabbit serum has been possible, the final preparation contained approximately 90% of a contaminating protein. Almost all of this represents γ-globulin of an electrophoretic and chromatographic behavior similar to that of C'6. C'6 is distinguished from γ-globulin by a lower sedimentation rate. Final purification might be achieved by recycling on Sephadex G-200, the usefulness of which was suggested by a slight but definite mobility difference between γ-globulin and C'6 upon starch gel electrophoresis.

Of considerable interest is the finding that the erythrocyte-antibody-complement complex prepared with C'_{def} was in the state EAC'1a,4,2a,3. Theoretically, one would expect that C'6-deficient serum would lead to the formation

of EAC'1a,4,2a,3,5 cells. The observed formation of EAC'1a,4,2a,3 suggests that C'5 cannot exert a lasting effect on the cells in the absence of C'6. This observation is in agreement with observations made on the human complement system which indicate that C'5, C'6, and C'7 are functionally interdependent (3 *b*).

Attempts to produce an antibody to C'6 in complement-deficient animals were undertaken in order to find out whether absence of C'6 activity is accompanied by lack of the serum protein which normally is endowed with this activity. Prior to immunization, serum from deficient animals was found to be devoid of anticomplementary activity. After several injections of partially purified rabbit C'6, the serum of the animals acquired the capacity to inhibit hemolytic rabbit complement. An analysis of the inhibition effect showed it to be specific for C'6. While inhibition could be overcome with heat-inactivated rabbit C'6, it was unaffected by a serum fraction which was prepared from deficient rabbit serum and which in electrophoretic and chromatographic properties was identical with partially purified C'6 from normal rabbit serum. Whereas these results do not definitely show that deficient serum lacks the physicochemical correlate to C'6 activity, they do demonstrate that it lacks an antigenic quality intimately associated with the active principle.

The absence of C'6 from the deficient animals explains fully a number of biologic phenomena that have been observed in experiments with these animals and their serum. Thus, deficient serum lacks the capacity to kill Gram-negative bacteria even after their sensitization with antibody (19), since this capacity requires all the components of complement. It also lacks the ability to generate leukocyte chemotactic activity (20) which has recently been found to depend on the activated C'5, C'6, and C'7 complex (21). Furthermore, an impairment of the passive Arthus reactivity was noticed with these animals (22) which is most probably related to the strikingly reduced chemotactic activity of their serum.

Serologic reactions known to involve only the first four complement components were found to be unimpaired in defective animals or in their serum. These are immune adherence, erythrophagocytosis by polymorphonuclear leukocytes in vitro and enhanced immune clearance of *Salmonella typhi* in vivo (23).

By contrast, certain other phenomena are presently not sufficiently understood to relate them clearly to the C'6 defect. Thus, C'6-deficient rabbits, unlike their normal litter mates, develop only markedly suppressed delayed hypersensitivity to tuberculin (24) and with a certain frequency fail to reject skin homografts (24, 25). On the other hand, they do develop nephrotoxic nephritis apparently in a normal fashion (26), although this experimental disease has been shown to be complement dependent (27). It appears that C'6 is not an essential factor in the mechanism of this disease.

It is hoped that the definition of the nature of the complement defect will

render these animals more useful in future immunological and immunopathological studies.

SUMMARY

A strain of rabbits with an inherited complement deficiency was shown to lack the sixth component of the hemolytic complement system. A method was elaborated for the partial purification of this component from normal rabbit serum. Upon injection of partially purified rabbit C'6 into C'6-deficient animals, an antibody was obtained which specifically inhibited the hemolytic activity of C'6. The data suggest that C'6-deficient serum either lacks the C'6 molecule or contains it in a chemically modified and inactive form.

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