FUNCTION OF COMPONENTS OF COMPLEMENT IN IMMUNE HEMOLYSIS*

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Although the activity of complement is exhibited in bactericidal, bacteriolytic, and hemolytic phenomena, the hemolysis of sensitized red blood cells is the most clearly defined property. Nevertheless, the rôle of the various components of complement in immune hemolysis has not been determined under the most favorable conditions.

Liefmann and Cohn (1) assert that the disappearance of complement in immune hemolysis is due to the effect of hemolysis rather than to an initial fixation of complement. On the contrary, Eagle (2, 3) and Ponder (4) state that complement must first be bound by sensitized erythrocytes before hemolysis occurs. These authors made no attempt to identify the components operating in this fixation. Many investigators (5–8) have claimed that the mid-piece is fixed by sensitized red blood cells. Brin (9) states, however, that the end-piece alone is bound by immune aggregates. Still others hold that both end-piece and mid-piece combine (10–12). It has also been asserted that the fourth component is involved in the fixation of complement prior to hemolysis (13–15). Furthermore, Nathan (16) concludes that the third component of complement is not fixed by sensitized sheep red blood cells.

Pillemer, Seifter, and Ecker (17) have shown that C′4, C′2, and varying amounts of C′1[1] are always fixed by antigen-rabbit antibody aggregates. In agreement with Nathan, these authors showed that C′3 is not fixed by sensitized red blood cells.

Fixation of the components of complement in the work last mentioned was determined by measuring the residual component activity in fresh guinea pig serum after fixation. This, however, did not disclose whether the adsorbed components were functionally active, since under the experimental conditions employed no visible changes occurred which could be attributed to the action of combining complement components. However, if the immune aggregate employed in fixation is a substrate which upon resuspension and appropriate treatment shows a visible manifestation, such as hemolysis, the

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[1] The symbols C′1, C′2, C′3, and C′4 refer to mid-piece, end-piece, third component, and fourth component respectively in the older terminology (18). In the present paper the term "mid-piece" is used to indicate the CO_2-insoluble fraction of complement (which may often contain C′1, C′3, and C′4); the term "end-piece" is applied to the CO_2-soluble fraction, which may contain C′2, C′3, and C′4.

nature and rôle of the fixed complement components can then be determined. The present paper presents the results of an investigation in which sensitized sheep red blood cells were used as an immune system to determine the rôle of the complement components in immune hemolysis.

Materials

Complement.—Immediately after withdrawal, the blood of normal guinea pigs which had been maintained on a high vitamin C diet was placed in the ice box (3–4°C.) for about 1 hour. The serum was then separated by centrifugation and used promptly in the experiments. In the experiments reported here the serum was never allowed to stand more than 1 hour before use.

A unit of complement was considered to be the smallest amount of a 10 per cent dilution of fresh serum which caused 100 per cent hemolysis of 1 cc. of a 2.5 per cent suspension of sheep red blood corpuscles containing 5 units of anti-sheep cell rabbit serum per cc.

Sheep Red Blood Cells.—Defibrinated sheep blood was strained through cotton gauze and washed four times with 10 volumes of physiological saline. After the final washing the cells were left in a thick suspension, each cubic centimeter of which contained approximately 10 billion cells as determined by cell count. Care was taken that the bloods employed were obtained daily from the same sheep so as to avoid, as far as possible, errors due to variations in the resistance of red blood cells to the action of complement.

1 cc. of a 2.5 per cent dilution of the thick suspension, or 250,000,000 erythrocytes, was taken as a unit of sheep red blood cells.

Anti-Sheep Cell Rabbit Serum.—The anti-sheep red blood cell rabbit serum contained 10,000 units per cc. It was not anticomplementary in twice the quantities employed. A unit of anti-sheep cell rabbit serum was considered to be the smallest amount of antiserum necessary for the complete hemolysis of 250,000,000 red blood cells in the presence of 1 unit of complement. Nevertheless, 5 units of antiserum was always added to each unit of cells in order to avoid errors due to the possible presence of a natural hemolysin in guinea pig serum.

Methods

Specifically Inactivated Complements.—Methods for the preparation and assay of these have been previously described (17, 26).

In estimating the degree of fixation of complement or its components by sensitized red blood cells, most workers have allowed complement to react with red cell aggregates for a few seconds, since longer periods at the temperatures employed by them (either room temperature or 37°C.) would have resulted in hemolysis, and consequent overshadowing of exact fixation data. The present authors (17) have shown that 60 minutes treatment at 1°C. results in almost complete fixation of the combining components of complement, differing only slightly from fixation at room temperature. In the experiments reported here fixation was carried out at 1°C. for 1 hour, thus achieving fixation without hemolysis.

General Method for the Determination of the Rôle of the Components of Complement

in Immune Hemolysis.—The reactants (complement, red cells, and antiserum) were combined in amounts previously estimated to contain 40 units each of complement and red cell substrate and 200 units of antiserum.

Therefore, to 1 cc. of packed red blood cells (containing 10 billion cells per cc.), 40 units of undiluted complement[2] were added with mixing, and the mixture was chilled to 1°C. 1 cc. of diluted antiserum containing 200 units, previously chilled to 1°C., was then added and the contents thoroughly mixed; the mixture was allowed to stand for 1 hour at 1°C. At the end of this time, it was centrifuged in an angle-centrifuge at 2750 R.P.M. at 1°C. for 10 minutes. The clear supernatants were decanted and tested for their content of complement components by the method previously described (17).

The packed erythrocytes were then resuspended in 10 volumes of 0.9 per cent saline, chilled to 1°C., thoroughly mixed, and again centrifuged. This washing was repeated three times. The saline washings, except for minute traces in the initial washing, contained no complement component activity and were discarded. The washed erythrocytes were made up to a volume of 40 cc. with 0.9 per cent saline, so that each cubic centimeter contained 1 unit of cells, 5 units of anti-sheep cell rabbit serum, and the combining components from 1 unit of complement.

1 cc. of each erythrocyte suspension was then incubated at 37°C. for 30 minutes. If 10 per cent or less hemolysis occurred, a quantity of each specifically inactivated complement or individual component equivalent to the amount present in 1 unit of complement was added to 1 cc. of the red blood cell suspension, and incubated at 37°C. for 30 minutes. The amount of hemolysis produced was estimated by comparison with a series of standard hemoglobin solutions and expressed as percentage of complete hemolysis. The same precautions expressed in previous publications (17) were observed in these experiments in order to obtain comparable and reproducible data. The temperature conditions were rigidly controlled. For the further clarification of the data, the per cent hemolysis resulting from the inter-reactivations of the specifically inactivated complements is included in each protocol.

The Fixation of the Components of Complement by Sensitized Sheep Cells

In this experiment a comparison of the effects of normal complement and of specifically inactivated complements, as well as mid-piece and end-piece, was made by the separate addition of each to a volume of sensitized erythrocytes. The results obtained are summarized in Table I, and reveal the following facts: (1) All of C′4 and C′2 in normal serum was removed or inactivated by the sheep cell aggregates. (2) Apparently little C′1 and no C′3 of normal serum were fixed. (3) The inactivation of C′4 by hydrazine previous to fixation, while it did not markedly influence the combination of C′3

[2] Either untreated complement or specifically inactivated complements equivalent to quantities representing 40 units of fresh complement. All specifically inactivated complements which had been diluted during inactivation or separation were concentrated by lyophilization to their original volumes before employment in fixation experiments.

and C'2, resulted in an increase of the fixation of C'1, since upon the addition of end-piece only 40 per cent hemolysis occurred. (4) The removal of C'3 by the insoluble carbohydrate prepared from yeast resulted in the almost complete fixation of the other components of complement. (5) The inactivation of C'1 and C'2 by heat inhibited the fixation of C'4. (6) When end-

TABLE I

Packed sheep R.B.C. suspension —10 billion per cc.	40 units —0.28 cc.— normal serum or treated serum	Anti-sheep red blood cell rabbit serum (1:20)	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements: Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum treated with insoluble carbohydrate and hydrazine	Serum heated at 56°C. for 30 min.	CO_2-soluble fraction (end-piece)	CO_2-insoluble fraction (mid-piece)
cc.		*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	Normal guinea pig serum	1	5	5	85	15	50	90	0
1	Serum treated with hydrazine	1	0	0	70	0	40	40	0
1	Serum treated with insoluble carbohydrate	1	0	0	10	0	tr	10	0
1	Serum treated with hydrazine and insoluble carbohydrate	1	0	0	10	0	10	10	0
1	Serum heated at 56°C. for 30 min.	1	0	90	90	60	0	0	0
1	End-piece	1	0	80	75	50	0	0	80
1	Mid-piece	1	0	0	50	0	0	tr	0

Specifically inactivated complements	Hemolysis						
	per cent						
Serum treated with hydrazine	0	0	85	0	90	90	0
Serum treated with insoluble carbohydrate	0	85	0	10	90	45	60
Serum treated with hydrazine and insoluble carbohydrate	0	0	10	0	60	25	0
Serum heated at 56° for 30 min	0	90	90	60	0	0	0
End-piece	0	90	45	25	0	0	90
Mid-piece	0	0	60	0	0	90	0

piece which contained C'2, C'4, and a small amount of C'3, was added to sensitized sheep cell suspensions, little or no fixation of C'2 and C'4 occurred. (7) Practically all of C'1 was removed or inactivated in the fixation of mid-piece, which apparently contained no C'2 or C'4. (8) C'4 did not combine in the absence of active C'1.

These results are similar to those reported in an earlier publication (17), and show that the direct and immediate inactivation of whole fresh complement by fixation to sensitized red blood cells is due to a union of almost all of C'2 and C'4 and a part of C'1. As pointed out by Nathan (16) and ourselves (17) C'3 does not combine under these conditions.

The Nature and Rôle of the Fixed Complement Components

In order to learn whether the inactivation of complement during fixation is due to an actual adsorption of C'2-C'4 and C'1 and whether these components are functionally active after fixation, the resuspended cells were

TABLE II

1 cc. of 2.5 per cent suspension of sensitized R.B.C. which had previously been treated with	Hemolysis after incubation at 37°C. (control)	Hemolysis produced after addition of					
		Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum treated with insoluble carbohydrate and hydrazine	Serum heated at 56°C. for 30 min.	CO_2-soluble fraction (end-piece)	CO_2-insoluble fraction (mid-piece)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Normal guinea pig serum	10	95	5	tr.	25	5	30
Serum treated with hydrazine	0	0	0	0	0	0	0
Serum treated with insoluble carbohydrate	0	60	0	0	15	10	10
Serum treated with insoluble carbohydrate and hydrazine	0	0	0	0	0	0	0
Serum heated at 56°C. for 30 min.	0	0	0	0	0	0	0
End-piece	0	0	0	0	0	0	0
Mid-piece	0	0	0	0	0	0	0

treated as outlined in the section of this paper dealing with methods. The results are shown in Table II, and reveal the following:—

1. The resuspended cells which contained the combining components of 1 unit of complement showed only 10 per cent hemolysis when incubated at 37°C. for 30 minutes, indicating that although C'4 and C'2 and a small amount of C'1 had combined, another fraction of complement which did not combine is necessary for hemolysis. The addition of serum deprived of C'4 to the resuspended cells caused 95 per cent hemolysis, while the addition of serum deprived of C'3 yielded a hemolytic titer no greater than that of the resuspended cell control. C'3, therefore, although not fixed, is necessary for the final action of complement, in this case, hemolysis. Apparently, heating of serum to

56°C. and the fractionation of serum by CO_2 diminished the hemolytic activity of C'3. The implications of this finding are being investigated further.

2. The resuspended cells which contained the combining components from complement lacking C'4 showed no hemolysis when incubated at 37° for 30 minutes, and further showed no hemolytic activity when mixed with the various other components of complement. This indicates that C'4 must combine first before the other components exhibit their activities and that unless C'4 combines either previous to or simultaneously with C'2 and C'1, no hemolysis occurs, even though the two thermostable components are fixed to the sensitized cells. This confirms the views of Deissler (13) and disproves those of Ueno (15).

3. The resuspended cells to which the combining components from serum deprived of C'3 had been fixed, yielded results qualitatively similar to those in which the cells had been allowed to fix untreated serum. This indicates that C'3 does not participate in the initial fixation, but plays its rôle after the combining components have been fixed.

4. The resuspended cells which had been allowed to fix either heated serum, serum derprived of both C'3 and C'4, or end-piece, showed no hemolytic activity when added to the various complement components.

5. The resuspended cells which had been allowed to fix mid-piece free of C'4 showed no hemolytic activity, indicating that although C'1 had been completely fixed, it was functionally inactive in producing hemolysis.

The Fixation of C'1 by Sheep Cell Aggregates and the Conditions Necessary for Its Hemolytic Activity

Many investigators have assumed that mid-piece is the combining component of complement. However, it has been shown that combining mid-piece, free of C'4, is functionally inert in so far as producing immune hemolysis is concerned. The following experiments, therefore, were conducted to determine the rôle of C'1 and C'4 in immune hemolysis, and the influence of the order of their combination upon the hemolysis of sensitized red blood cells. Mid-piece, containing no C'4, was added in varying amounts to sensitized sheep cells. Further, mid-piece and different serum fractions known to contain C'4 as well as other components of complement were combined and then added to sheep cell aggregates. The results of this experiment show the following: Whereas, in this instance, the fixation of normal serum resulted in the removal of 60 per cent of mid-piece activity, 95 per cent of C'4 activity, and no significant removal of C'3 activity, the fixation of mid-piece lacking in C'4, resulted in the removal of all of C'1 activity. However, if serum heated to destroy C'1 and C'2 activity, was added to mid-piece and then allowed to fix, this resulted in fixation of about 45 per cent of C'1. Since

C′3 is not fixed, it is apparent that this effect is due to the added C′4. Heated end-piece which contained C′4 did not act similarly, this being as yet unexplained.

In order to determine whether the adsorbed C′1 was functionally active, the resuspended cells which contained the combining components of the variously treated mid-pieces, were added to end-piece and other complement fractions and the hemolytic titers determined.

The results of this experiment revealed the following:—

1. Although C′1 combines with sensitized red cells in the absence of C′4, it is functionally inert in producing hemolysis.

2. The fixation of a combination of mid-piece and serum heated at 56°C. for either 10 or 30 minutes to red blood cells, followed by the addition of complement lacking C′4, resulted in the hemolysis of the resuspended red blood cells. This was not the case upon the addition of serum lacking in C′3 to such resuspended cells. It is, therefore, evident that C′4 and C′1 must both be present for both of these components to be functionally active after fixation and that C′4 must fix before or simultaneously with C′1. Furthermore, it is again seen that C′3, although not a combining component, is necessary for the production of hemolysis.

3. The addition of mid-piece to serum heated at 56°C. for 60 minutes, which treatment destroys most of the activity of C′4, and then fixation of these by sensitized sheep cells, resulted in only slight hemolysis of the suspended red blood cells, while the addition of serum heated at 66° for 15 minutes, which results in total destruction of C′4, gave no subsequent hemolytic activity.

DISCUSSION

Evidence derived from recent data and from the facts disclosed in this paper point to the following explanation of the action and rôle of the components of complement in immune hemolysis.

Anti-sheep cell rabbit serum by itself does not combine with complement or any of its components, or at least does not inactivate them. Nevertheless, when the specific antibody enters into the primary combination with the red cell, the surface pattern of the antiserum molecule or the antiserum-cell aggregate changes and in turn different groups are in contact with the complement complex. This alteration in the surface pattern increases the affinity for C′4, C′2, and C′1. The adsorption of these complement components on the red cell-antibody complex then renders the red cell amenable to the action of the unadsorbed C′3 and hemolysis results. Since C′3 is not fixed by the sensitized cell and is apparently not used up in the process of hemolysis, it appears to have certain enzymic qualities.

Osborn (19) comments on the fact that complement-antibody systems resemble enzymic systems in certain respects, in that small amounts of com-

plement produce changes in large quantities of substrate, in that complement is thermolabile, and in that the reaction is highly specific. He further points out that when the substrate is highly concentrated as compared to complement, the speed of the reaction is independent of the concentration of the unhemolyzed corpuscles. He points out that the one argument which has been advanced against classifying complement as an enzyme is the fact that complement and anti-sheep cell rabbit serum appear to obey the law of definite proportions. While the data presented here contribute little one way or another to the enzymic interpretation of C′1, C′2, and C′4, they appear to warrant the classification of C′3 as a catalyst, since this component is not fixed and is not used up in the process of hemolysis (13, 16, 20, 21).

Although Bessemans (22) asserts that mid-piece and end-piece are mutually supplementary, others claim that optimal hemolysis results when end-piece and mid-piece are present in the same proportions as in normal serum. Parsons (23) states that end-piece must be present in relatively high proportion, while mid-piece may be reduced considerably. Browning and Mackie (12) state that on increasing end-piece, a smaller quantity of mid-piece is needed for complete hemolysis. Since their experiments were reported before the discovery of C′4, the reason for these discordant results is apparent. It is evident from the experiments reported in the present paper that the activity of C′1 after fixation is pre-determined by the fixation of C′4. It is probable that when mid-piece contains C′4 together with C′1, it is to a certain degree supplementary to end-piece. Mid-piece devoid of C′4 is not supplementary and in fact may prove anticomplementary when used in excess, as has been frequently observed. This was clearly indicated in experiments on pure C′1 (24). In these instances, the adsorbed C′1 in the absence of C′4 may occupy all of the "key spots" (25) on the red cell-antibody complex and therefore prevent further combination by any C′4 molecules.

SUMMARY

1. At a temperature of 1°C., C′2, C′4, and C′1 combine with sensitized sheep erythrocytes, while C′3 does not combine.

2. C′1, although combining with sensitized cells in the absence of C′4, is hemolytically inert unless C′4 combines previous to, or simultaneously with it.

3. C′4 does not combine in the absence of C′1.

4. Although C′3 is not fixed by antibody-sheep cell aggregates, it is essential for hemolysis, operating on the sensitized red cell after the fixation of C′4, C′2, and C′1 and behaving as if it were a catalyst.

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