STUDIES ON THE ANTIGENIC PROPERTIES OF COMPLEMENT

II. Analysis of Specific Agglutinins against Certain Components of Guinea Pig Complement Fixed on Sensitized Sheep Erythrocytes*

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Evidence has been presented in a companion paper (1) to show that immune sera against guinea pig complement can be produced in rabbits by injection of guinea pig globulin or sensitized erythrocyte stromata exposed to guinea pig complement (SAC′). Sensitized erythrocytes exposed to complement (EAC′) are agglutinated by these immune sera after absorption of the sera with sensitized erythrocytes; and when mixed with the immune serum conjugated with fluorescein they exhibit specific fluorescence on microscopic examination with ultraviolet light. These specific immune reactions have been demonstrated to be unrelated to immunoconglutinins.

The purpose of this paper is to describe the role of each of the fixable components of guinea pig complement in the agglutination of EAC′ by the immune sera prepared against guinea pig complement. Since the fixation of complement to sensitized erythrocytes occurs in a series of reaction steps, it is possible to produce individual complexes, each comprising sensitized erythrocytes with one or more of the components of complement. The agglutinogenic capacity of each of these components in complex with sensitized erythrocytes can then be tested in parallel with examination of the hemolytic function of each component. In a preceding paper (2) a method was described for producing by ether treatment of guinea pig serum a stable complex of sensitized erythrocytes in combination with the first component of complement (EAC′1). This permits analysis of the immune sera for antibodies against the first component of complement. And since this EAC′1 is highly reactive with the fourth component of complement (C′4), an EAC′1,4 can be produced to analyze the immune sera for antibodies against the fourth component. This stepwise procedure can be continued for analysis of other components of complement.

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Materials and Methods

The preparation and use of saline, sensitized cells, and complement reagents, the procedure for determining the hemolytic reactivity of persensitized cells to complement reagents, and the hemagglutination tests have been described elsewhere (1). The immune sera referred to in this paper are identical with those that have been described earlier (1). Three preparations of rabbit anti-guinea pig serum globulin (sera 23, 40, and 41) and two preparations of rabbit anti-SAC′ (sera 26 and 27) were used. Anti-SAC′ was obtained by injecting rabbits with a mixture of Freund's adjuvant and sensitized stromata exposed to guinea pig complement. For control purposes rabbit anti-egg albumin serum was used (serum 29). All immune sera were absorbed with non-sensitized and sensitized erythrocytes.

Sensitization of Erythrocytes.—Erythrocytes sensitized with 8 amboceptor units (EA) were used throughout this investigation for the agglutination tests. This corresponded to one-quarter of the minimal amount of amboceptor that caused agglutination. All cell suspensions were adjusted to a density of 0.5×10^9 cells/ml. For absorption of the immune sera cells sensitized with 16 units of amboceptor were used.

Preparation of EAC′1.—A suitable amount of an EA suspension was chilled and mixed with an equal volume of a chilled dilution of 1:50 of ether-inactivated complement (C′-ether). The mixture was kept for 30 minutes in ice water. The cells were then washed three times with ice cold buffered saline and resuspended in buffered saline (2). The same technique was applied when R4 (hydrazine-inactivated complement) was used as source of C′1. The cells obtained by both procedures showed a high reactivity against R1 and none against R4 or chelated complement, indicating that an EAC′1 preparation had been formed.

Preparation of EAC′1,4.—(*a*) A chilled suspension of EAC′1 was mixed with an equal volume of a chilled dilution of 1:40 of heat-inactivated complement (H) and kept for ½ hour in ice water. The cells were then washed three times with ice-cold buffered saline and resuspended. These cells showed a high reactivity to R1 and to R4 and no reactivity to chelated complement, indicating that an EAC′1,4 preparation had been formed. The incubation in the cold is essential. When the mixture is incubated at 37° the cells show a high reactivity against R4 but their reactivity to R1 is feeble.

(*b*) A suspension of EAC′1 was chilled and mixed with an equal amount of a chilled 1:10 dilution of complement that had previously been chelated by adding EDTA in an amount to give a concentration in the final mixture of 0.01 M EDTA. The mixture was kept in ice water for 5 minutes. The cells were then washed three times with an ice-cold buffer which had been deprived of its bivalent cations by resin passage. Finally the cells were resuspended in normal buffered saline. These cells showed a high reactivity to R4, a moderate or feeble one to R1, and none to chelated complement, indicating that an EAC′1,4 preparation had been formed with some loss of the C′1 activity.

Preparation of EAC′1,4,2.—(*a*) A suspension of EAC′1,4 prepared according to the procedure given in paragraph (*a*) of the section above, was mixed with an equal volume of an endpiece 1:50 derived from R4 (R4-endpiece). The mixture was kept for 5 minutes at 37° and the cells were then washed three times with ice-cold buffered saline, resuspended, and kept in an ice-water bath. The preparation of R4-endpiece was carried out as follows: to 4 ml. of guinea pig serum 1 ml. of a 0.16 M solution of hydrazine was added. The mixture was incubated for 3 hours at 37°. The pH was then adjusted to 7.2 by adding 0.2 M HCl and the mixture was brought up to a final volume of 6 ml. with buffered saline and then chilled. A volume of 45 ml. of chilled HCl 1/250 N was then added dropwise and the volume was made up finally to 60 ml. by adding ice-cold distilled water. The mixture was kept in ice water for 30 minutes and the precipitated euglobulins were spun down. The supernatant was brought up to isotonicity with 10 per cent NaCl solution and to a pH of 7.2 with 0.15 M dibasic sodium phosphate. Finally the volume was brought with saline to a dilution of 1:50 with reference to the original

guinea pig serum. This preparation showed no detectable amount of C′3, since the prolonged incubation with hydrazine inactivates most of it and the rest is precipitated with the euglobulins. The C′2 titer of R4-endpiece is high, usually between 1600 and 3200 units/ml., whereas no trace of C′4 and of C′1 can be detected. When EAC′1,4 was incubated with R4-endpiece the resulting cells showed a high reactivity against R1, R4, and chelated complement, indicating that C′2 had been added without any loss of C′1 or C′4 activity. No formation of E* was detectable.

(*b*) An EA suspension was mixed with equal parts of a dilution of 1:20 of an R3, the latter having been obtained by the cobra venom method (2). The mixture was incubated for 5 minutes at 37° and the cells were washed and resuspended in ice-cold buffered saline. No formation of E* could be detected. The cells reacted strongly with R1, R4, and chelated complement, again indicating that an EAC′1,4,2 preparation had been made.

(*c*) An EA suspension was chilled and mixed with equal amounts of a chilled dilution of 1:50 of active complement (C′). The mixture was kept in the cold for 1 hour and the cells were washed with ice-cold buffered saline. The washed cells were kept for 4 hours in ice water. Approximately 30 per cent of the cells were lost by the formation of E* and the rest of the cells were resuspended in ice-cold buffered saline. The cells showed a strong reactivity with R1, R4, and chelated complement, once again indicating that an EAC′1,4,2 preparation had resulted.

Removal of C′4 from Heat-Inactivated Complement.—EAC′1 was prepared with ether-treated complement as indicated above. The absorption of C′4 from heat-inactivated complement (H) was performed by mixing a sediment of 50 × 10^9 of packed EAC′1 cells in 10 ml. of a 1:10 dilution of the heat-inactivated complement (H) for 30 minutes at 37°. The cells were then spun down and the supernatant, designated as Ha (H minus C′4) was used. On testing, this preparation showed no detectable C′4. Its C′3 titer was in the same range as that of non-treated H. As a control H was treated in the same fashion with EA. No decrease in C′4 or in C′3 was detectable. This control preparation was designated as Ho.

Decomplementation of Guinea Pig Serum.—Guinea pig serum was decomplemented by treating it twice with sensitized stromata as described elsewhere (2).

Absorption of the Immune Sera with EAC′1,4.—Erythrocytes sensitized with 16 units of amboceptor were converted to EAC′1,4 by using the method described in paragraph (*b*) in the section on the preparation of EAC′1,4. The immune sera were absorbed by mixing the sediment of 50 × 10^9 EAC′1,4 cells with 20 ml. of a 1:20 dilution of the respective immune serum. This suspension was incubated for one-half hour at 37°. The cells were then spun down and the supernatant was subjected to the same treatment once more. This treatment exhausted the agglutinating power of the sera against EAC′1,4.

Inactivation of Fixed C′1 by Citrate.—A suspension of EAC′1 was mixed with an equal amount of 0.15 M sodium citrate solution and the mixture was kept at 37° for 30 minutes. The cells were then washed twice and resuspended in buffered saline. This treatment completely destroyed the reactivity of EAC′1 with R1. Citrate should be used for this procedure rather than EDTA because it was observed that prolonged exposure to EDTA in the absence of protein causes a slight spontaneous agglutination of the cells. As a control a portion of EAC′1 was exposed under the same conditions to buffered saline instead of citrate.

EXPERIMENTAL

Samples of a suspension of EA were exposed in sequence to various complement reagents as indicated in Table I. The cells were then washed, resuspended, and subjected to agglutination tests with the anti-guinea pig globulin sera 23, 40, and 41, with the anti-SAC′ sera 26 and 27, and with the anti-egg albumin serum 29. The exposure of EA to the complement reagents was

carried out at 3°. All immune sera were tested against each of the cell preparations in twofold dilution ranging from 1:20 to 1:5120. The results are summarized in Table I.

It can be seen that the preparation EAC'1 showed no agglutination with the immune sera regardless of whether hydrazine-inactivated complement or ether-inactivated complement was used as a source of fixable C'1. When, however, in a second step EAC'1 was exposed to heat-inactivated complement it

TABLE I

Hemolytic Reactivity and Agglutinability of Cells Persensitized with Various Complement Components

EA exposed in sequence to		Formula of hemolytic reactivity	Hemolytic reactivity with C' reagents*				Agglutination, intensity, and endpoint dilution with immune sera					
1st step	2nd step		R1	R4	C'+EDTA	Saline	23	26	27	40	41	29
C'-ether	Saline	EAC'1	xxx	0	0	0	0	0	0	0	0	0
R4	Saline	EAC'1	xxx	0	0	0	0	0	0	0	0	0
C'-ether	H	EAC'1,4	xxx	xxx	0	0	+ 1:160	+ 1:640	+ 1:640	+ 1:640	+ 1:640	0
R4	H	EAC'1,4	xxx	xxx	0	0	+ 1:160	+ 1:640	+ 1:640	+ 1:640	+ 1:640	0
C'	Saline	EAC'1,4,2	xxx	xxx	xxx	0	+++ 1:320	+++ 1:640	+++ 1:640	+++ 1:640	+++ 1:640	0
Saline	Saline	EA	0	0	0	0	0	0	0	0	0	0

* In Tables I to III, the symbols xxx, xx, and x mean complete hemolysis in less than 5, 15, and 30 minutes respectively, $\underline{x}$ means incomplete hemolysis after 30 minutes of incubation at 37°C.

was converted into EAC'1,4 and this preparation showed a marked agglutination with all immune sera except the anti-egg albumin (serum 29). The cells exposed to active complement corresponded to the formula EAC'1,4,2 and showed an agglutination that appeared far stronger than that obtained by using EAC'1,4 cells. Finally the control EA showed no agglutination with any of the sera. The conclusion can be made, therefore, that fixed C'1 itself shows no agglutinogenic properties. On the other hand, fixed C'4 seems to act as an agglutinogen since the cells are rendered agglutinable simultaneously with the fixation of C'4. The question arises as to whether the fixation of this agglutinogen to the cells would parallel the fixation of C'4 under different experimental circumstances. This point was investigated.

A suspension of EA was exposed in sequence to various complement reagents as indicated in Table II. The cell preparations were tested for their hemolytic reactivity and for their agglutination with serum 40 (anti-guinea pig globulin), serum 27 (anti-SAC′, absorbed with E and EA), and serum 29 (anti-egg albumin); and for each type of serum twofold serial dilutions were made from 1:10 to 1:5120. Table II summarizes the results.

TABLE II

Agglutinability of EAC′1 and of EAC′1,4 with Anti-Complement

EA exposed in sequence to			Formula of hemolytic reactivity	Hemolytic reactivity with C′ reagents				Agglutination, intensity, and endpoint dilution with immune sera		
1st step	2nd step	3rd step		R1	R4	C′ + EDTA	Saline	40	27	29
C′-ether	Saline	Saline	EAC′1	xxx	0	0	0	0	0	0
C′-ether	Citrate	H	EA	0	0	0	0	0	0	0
C′-ether	Saline	H	EAC′1,4	xxx	xx	0	0	+ 1:640	+ 1:640	0
H	Saline	Saline	EA	0	0	0	0	0	0	0
C′-ether	Ha	Saline	EAC′1	xxx	0	0	0	0	0	0
C′-ether	Ho	Saline	EAC′1,4	xxx	xx	0	0	+ 1:640	+ 1:320	0
C′-ether	H + EDTA	Saline	EAC′1,4	x	x	0	0	+ 1:320	+ 1:160	0
C′-ether	C′ + EDTA	Saline	EAC′1,4	x	xxx	0	0	++ 1:320	++ 1:320	0
C′-ether + EDTA	H	Saline	EA	0	0	0	0	0	0	0
Saline	Saline	Saline	EA	0	0	0	0	0	0	0

It can be seen that the fixation or failure of fixation of C′4 and of the agglutinogen to sensitized cells occurs in parallel under several well defined circumstances as follows: (*a*) No C′4 and no agglutinogen can be fixed when EA interacts with heat-inactivated complement. (*b*) The fixation of C′4 as well as the fixation of the agglutinogen requires the presence of hemolytically active C′1 on the cells. An EAC′1 exposed to citrate at 37° loses its hemolytic reactivity to R1 as well as its capacity to fix C′4 and the agglutinogen. An EA exposed to ether-inactivated complement in the presence of EDTA showed no lysis with R1 and is unable to fix either C′4 or the agglutinogen. (*c*) By ab-

sorbing heat-inactivated complement with EAC'1 the capacity of the resulting Ha to transfer hemolytically active C'4 or the agglutinogen onto EAC'1 is exhausted. The titration of Ha for C'4 also shows complete absence of C'4. (*d*) The transfer of both C'4 and the agglutinogen to EAC'1 takes place even in the presence of EDTA. Therefore, the transfer of both is independent of calcium and magnesium ions.

The conclusion can be made from this experiment that during the interaction of EAC'1 with heat-inactivated or with chelated complement an agglutinogen indistinguishable from hemolytically active C'4 is transferred to the cells.

An experiment was undertaken to examine the role of fixed C'2 in the agglutination. The cell preparations tested in Table III were exposed to immune sera and to the same immune sera that had previously been absorbed with EAC'1,4. The non-absorbed sera are designated as Nos. 23, 26, 27, 40, and 41, whereas the absorbed sera are designated as Nos. 23*a*, 26*a*, 27*a*, 40*a*, and 41*a*. The absorbed sera were tested in twofold dilutions between 1:20 and 1:5120 against each cell preparation. The non-absorbed sera were used in a dilution of 1:40.

The results summarized in Table III show that it is possible to remove the agglutinins directed against fixed C'4 by absorbing the immune sera with EAC'1,4. Accordingly EAC'1,4 is agglutinated only by the non-absorbed sera whereas it fails to be agglutinated by the absorbed sera. An EAC'1,4,2, produced by attaching C'1, C'4, and C'2 to EA in sequence, as described under (*a*) in the section on preparation of EAC'1,4,2, shows no agglutination with the absorbed sera, whereas it is markedly agglutinated by the non-absorbed sera. The same behavior is shown by an EAC'1,4,2 preparation obtained by exposing EA to complement inactivated by cobra venom. Therefore the fixation of C'2 alone to the complex EAC'1,4 does not transfer any additional agglutinogen distinct from C'4. When, however, EA interacts with active complement the resulting compound (EAC'), corresponding to the hemolytic formula EAC'1,4,2, appears to have acquired an additional agglutinogen apart from C'4, since it shows a strong agglutination with the absorbed sera. It must be noted that during the inactivation of EA with active complement some of the cells are converted to E* which means that a limited reaction had taken place between the resulting EAC'1,4,2 and C'3. It is conceivable, then, that during the interaction of EA with active complement traces of C'3 are fixed even to those cells that are not converted to E*, and that this minute amount of fixed C'3 might act as an agglutinogen. To examine this possibility the following experiment was performed.

An EAC'1,4,2 was obtained by incubating EA with complement that had been treated with cobra venom. Samples of the EAC'1,4,2 suspensions were mixed with equal parts of (*a*) decomplemented guinea pig serum 1:300, (*b*) hydrazine-treated guinea pig serum 1:60, and (*c*) active guinea pig serum 1:600. The incubations were performed at 37° for 10 minutes and resulted in incomplete lysis of the suspensions in all three instances. The remaining cells were then spun down and washed three times with ice-cold saline. The cells were stored in ice

TABLE III

Agglutination of Cells Persensitized with Various C′ Reagents Using Immune Sera Previously Absorbed with EAC′ 1,4

EA exposed in sequence to . . .			Formula of hemolytic reactivity	Hemolytic reactivity with C′ reagents				Agglutination with absorbed sera, intensity, and endpoint dilution					Agglutination with non-absorbed sera 1:40					
1st step	2nd step	3rd step		R1	R4	C′ + EDTA	Saline	23a	26a	27a	40a	41a	23	26	27	40	41	29
C′-ether	Saline	Saline	EAC′1	xxx	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C′-ether	H	Saline	EAC′1,4	xxx	xxx	0	0	0	0	0	0	0	+	+	+	+	+	0
C′-ether	H	R4-end-piece	EAC′1,4,2	xxx	xxx	xxx	0	0	0	0	0	0	+	+	+	++	+	0
C′	Saline	Saline	EAC′1,4,2	xxx	xxx	xxx	0	+ 1:160	+++ 1:640	+++ 1:640	+++ 1:640	+++ 1:640	+	+++	+++	+++	+++	0
C′ + EDTA	Saline	Saline	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R3 (cobra venom)	Saline	Saline	EAC′1,4,2	xxx	xxx	xxx	0	0	0	0	0	0	+	+	+	+	+	0
Saline	Saline	Saline	EA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

water for 4 hours. The loss of cells by lysis was estimated and the remaining cells adjusted to the original concentration. They were then examined for agglutination with the immune sera (Nos. 26, 27, 40, and 41) and with the absorbed immune sera that were deprived of their agglutinins against C'4 (Nos. 26*a*, 27*a*, 40*a*, and 41*a*).

The results summarized in Table IV show that the EAC'1,4,2 cells, obtained by interacting EA with R3, were agglutinated by immune sera but not by the absorbed immune sera. The exposure of the EAC'1,4,2 cells to active complement caused 10 per cent lysis, indicating that a limited reaction with C'3 had taken place at the same time that the remaining cells were rendered agglutinable by the absorbed sera. When, however, the EAC'1,4,2 cells were treated with

TABLE IV

Agglutination of a Persensitized, Partially Lysed EA

EA exposed in sequence to		Approximate loss of cells during the 2nd step	Agglutination with non-absorbed immune sera 1:80					Agglutination with absorbed immune sera 1:80			
1st step	2nd step		26	27	40	41	29	26*a*	27*a*	40*a*	41*a*
		per cent									
R3 (cobra venom)	Saline	<3	++	++	++	++	0	0	0	0	0
R3 (cobra venom)	Decomplemented guinea pig serum	50	+	+	++	++	0	0	0	0	0
R3 (cobra venom)	R4	30	+	+	++	++	0	0	0	0	0
R3 (cobra venom)	C'	10	++	+	++	++	0	+	+	+	0
Saline	Saline	<3	0	0	0	0	0	0	0	0	0

R4 or with decomplemented guinea pig serum the lysis proved to be greater than when active complement was used; and yet in these cases the cells were not rendered agglutinable with the absorbed sera. Therefore, among three reagents that are able to partially lyse EAC'1,4,2 only active complement transmits an additional agglutinogen to the cells, whereas the other two reagents (R4 and decomplemented complement) fail to do so. This experiment indicates that the transfer of an additional agglutinogen to EAC'1,4,2 shows no direct relation to the degree of hemolysis; *i.e.*, the extent to which the EAC'1,4,2 cells have reacted with C'3 to form E*. On this basis the possibility already considered that a sublytic amount of fixed C'3 is identical with the additionally transferred agglutinogen is unlikely.

Attempts were made to further characterize the fixability of the additional agglutinogen to EAC'1,4,2 using different sources of complement reagents.

An EAC'1,4,2 suspension was prepared with complement inactivated by cobra venom as outlined above. Chilled specimens of this EAC'1,4,2 suspension were mixed with equal volumes of several complement reagents indicated in Table V. The mixtures were kept in ice

water for 1 hour, centrifuged, and then the cells washed with ice-cold saline. The cells exposed to chelated complement were washed twice with citrated saline and once with buffered saline. The cells were then exposed to non-absorbed immune sera and to absorbed immune sera that were deprived of their anti-C'4 agglutinins.

Table V shows that an additional agglutinogen different from C'4 can be transferred to EAC'1,4,2 by exposing the cell preparation to active complement or to heat-inactivated complement. This transfer takes place even in the presence of EDTA, and is thus independent of bivalent cations. It is noteworthy that the transfer of this agglutinogen from heat-inactivated complement to EAC'1,4,2 takes place even when the former has been deprived of its C'4 (Ha) by previous exposure to EAC'1. This finding represents additional evi-

TABLE V

Role of Various Complement Reagents as Donors of the Additional Agglutinogen

EA exposed first to R3 (cobra venom) and subsequently to	Agglutination with non-absorbed immune sera 1:80					Agglutination with absorbed immune sera 1:80			
	26	27	40	41	29	26*a*	27*a*	40*a*	41*a*
Saline	+	+	+	+	0	0	0	0	0
C' 1:50	+++	+++	+++	+++	0	+	++	+++	+++
C' 1:50 + EDTA	+++	+++	+++	+++	0	++	+++	+++	+++
H 1:20	++	+++	+++	+++	0	+	+++	++	+
Ha 1:20	++	+++	+++	+++	0	+	+++	++	++
R4 1:10	+	+	+	+	0	0	0	0	0
R3 (zymosan) 1:30	++	++	+++	++	0	0	0	0	0

dence for the conclusion that C'4 is distinct from the additionally transferred agglutinogen. R3 prepared by zymosan as well as R4 prepared by hydrazine both failed to transfer the additional agglutinogen.

DISCUSSION

The experiments reported in this paper show that a clear and very close relationship exists between the fixation of hemolytically active C'4 and the fixation of an agglutinogen to sensitized sheep cells from guinea pig serum. Indeed, it appears that C'4 is truly fixed and acts as an agglutinogen. Neither the fixation of C'1 nor that of C'2 appears to contribute to the agglutinability of the cells with respect to "anti-complement." Furthermore, an additional agglutinogen distinct from C'4 is acquired during the interaction of EA and active complement. The identity of this additional agglutinogen with hemolytically active C'3 is unlikely.

There are several alternatives to explain the fact that neither C'1 nor C'2 is involved in the agglutination. First, one must consider the possibility that both

C'1 and C'2 interact with the cell without being truly "fixed"; *i.e.*, without the attachment of new material to the cell. As far as C'2 is concerned this hypothesis is in accordance with the theory suggested by Lepow and coworkers (3). According to Lepow human C'4 and C'2 may be inactivated by fixed C'1 without being attached to the antigen-antibody aggregate. However, the failure of C'1 to act as an agglutinogen cannot be explained by assuming that this component interacts with EA without being truly fixed, since according to Heidelberger and coworkers (4) the bulk of fixed complement material belongs to the component C'1. If one accepts this latter view, only two explanations can be offered for our findings, namely that C'1 though antigenic (5) is not agglutinogenic, or that it has no antigenic properties at all. If one took into consideration only C'1 and C'2, the data presented here would appear to be consistent with the hypothesis of Lepow referred to above. But with respect to the role of fixed C'4 in agglutination with "anti-complement," our observations cannot be easily reconciled with Lepow's hypothesis, because they indicate that the interaction of the cells with hemolytic C'4 is invariably accompanied by the attachment of an agglutinogen. Since this agglutinogen exhibits all the traits characteristic of C'4 and since no property can be found to distinguish it from C'4, the assumption seems justified that this agglutinogen and C'4 are identical. This would then imply that the interaction between EAC'1 and C'4 leads to a true fixation of the latter.

On the basis of the findings presented in this report it is impossible to determine whether C'3 is antigenic or not. The agglutination technique with persensitized cells is not suitable for an investigation of this problem since hemolysis prevents experiments with more than sublytic amounts of fixed C'3. The additional agglutinogen that we have found to be distinct from C'4 exhibits properties like those characteristic for C'3: it can be fixed from heat-inactivated complement but not from guinea pig serum inactivated by either zymosan or cobra venom; it can be attached only to EAC'1,4,2 and not to EAC'1,4; its fixation is independent of bivalent cations. On the other hand, it is unlike C'3 in that it is not fixable from R4; moreover, it is possible to expose EAC'1,4,2 cells to an amount of C'3 from decomplemented guinea pig serum and from R4 that produces partial lysis without attaching the agglutinogen to the surviving cells. Further studies are required to establish whether this additional agglutinogen bears any relation at all to the process of immune hemolysis. The possibility must be considered that the complement components which are demonstrable in hemolysis may represent only a part of the fixable complement material.

SUMMARY

Sheep erythrocytes sensitized with amboceptor and persensitized thereafter with guinea pig complement are agglutinated by rabbit anti-guinea pig globulin and by immune sera obtained by injection of rabbits with fixed complement.

In this agglutination neither C'1 nor C'2 takes part. Fixed C'4 acts as an agglutinogen. An additional agglutinogen, distinct from C'4, was found on persensitized cells. This additional agglutinogen appears to be distinct from hemolytically active C'3.

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