STUDIES ON THE ANTIGENIC PROPERTIES OF COMPLEMENT

I. Demonstration of Agglutinating Antibodies against Guinea Pig Complement Fixed on Sensitized Sheep Erythrocytes*

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(Received for publication, August 20, 1959)

The knowledge that exists of the role of complement in immune reactions has been gained from investigations limited almost entirely to studies *in vitro*. Very little is known of the role of complement in immune reactions *in vivo*. It has not been demonstrated that complement fixation occurs in animal tissues *in vivo* in some way analogous to the fixation of complement to antigen-antibody aggregates *in vitro*. That some part of complement can actually become fixed to immune aggregates is indicated by the work of Heidelberger and associates who demonstrated the uptake of organic nitrogen by immune precipitates exposed to complement (1). However, such determination of the actual fixation of complement is applicable only to highly purified immune precipitates. Further elucidation of the fixation of complement by more complex immune aggregates, such as sensitized erythrocytes, could result if specific agglutinins against fixed complement could be experimentally produced and then used in an agglutination test with sensitized erythrocytes exposed to complement. The conjugation of such agglutinating antibodies with fluorescein could then also be employed to attempt visual demonstration of complement on sensitized erythrocytes. If this attempt were successful, it would appear reasonable to expect that complement fixed to sensitized cells in animal tissues might also be detected with the use of fluorescein-conjugated antibodies against complement.

Over the past 60 years many attempts have been made to demonstrate that antibodies can be produced against complement. Two principal methods have generally been employed to produce immune sera against complement. One method consisted in immunizing animals with fresh active serum (2–7); the other in immunizing animals with serum complement in its fixed form; *i.e.*, in antigen-antibody-complement aggregates (8–11). Evaluation of the immune sera produced by these various methods was made in several ways. Some investigators estimated the anticomplementary activity of the immune sera by mixing the latter with the complement used for the immunization

* This work was supported by research grant H-1803 of the National Heart Institute of the National Institutes of Health and by the Helen HayWhitney Foundation.

(2–4, 10). Substantial objections have been raised against the conclusions derived from this type of evaluation (12–14). In attempt to obviate some of the difficulties inherent in this problem another investigator determined the anticomplementary activity of the immune serum by interacting the latter with EAC'1,4 (EA exposed to midpiece) and observing the decay of the hemolytic reactivity of the EAC'1,4 with respect to endpiece (7). The decay of the fixed complement was interpreted as being effected by specific antibodies against complement (anti-complement).

Finally, other investigators evaluated the immune sera against complement by studying the agglutination reaction of these immune sera with "alexinized" red cells (sensitized cells exposed to complement) (6–9). This reaction was at first considered to be the result of specific antibodies against complement. It was possible, however, to demonstrate that the immune serum against the complement of animals of one species agglutinated red cells persensitized with complement not only of that species but of other species in addition. Furthermore, alexinized cells were in some instances agglutinated by immune sera from animals immunized with bacterial antigens alone (15). The latter agglutination occurred in many cases despite previous exhaustive absorption of the immune sera with the bacterial antigen used for the immunization. Moreover, this agglutinating capacity could not be removed by exposing the immune serum to fresh non-fixed complement, but could be abolished by absorbing the immune serum with alexinized cells; *i.e.*, with complement in its fixed state (15, 16). This particular agglutination phenomenon was considered to be due to the presence of immunoconglutinins in the immune sera (16). Immunoconglutinins were conceived as antibodies of the auto- or hetero- type against a serological determinant characteristic only of fixed complement. It was proposed that this determinant is common only to the fixed complement of many animal species and not to non-fixed complement (16).

Recently it has been shown that the globulin fraction of an anti-guinea pig globulin serum, labelled with fluorescein, reacts with a rickettsial antigen-antibody complex that was previously exposed to guinea pig complement (17). This indicated that not only were there antibodies present in the anti-guinea pig globulin serum against guinea pig complement but also that complement fixed to an antigen-antibody aggregate other than sensitized erythrocytes could be detected.

With respect to the formation of antibodies against complement much of the data quoted above is inconclusive and in some cases confusing. This is especially true with regard to the analysis of immunoconglutinins. It is conceivable that some immune sera, produced by injection of animals with fixed or non-fixed complement, may contain not only true antibodies against free or fixed complement but may also contain immunoconglutinins. It is clear that any simple evaluation of the agglutinating capacity of such immune sera would give equivocal results.

The present paper is concerned with the production and evaluation of antibodies against complement in the absence of immunoconglutinins. This report is followed by a companion paper that analyzes the role of the individual components of complement as agglutinogens (18).

Materials and Methods

Incubations were performed in a water bath at 37°C. The hemagglutination tests were performed in an air incubator at 37°. All centrifugations were carried out in the cold. The procedures for the preparation and storage of buffered saline, citrated saline, sensitized sheep erythrocytes (EA), sensitized stromata (SA), guinea pig complement (C′), and complement reagents have been described in a previous communication (19). Chelation was performed by adding EDTA (ethylenediaminetetraacetate) to the respective reagents to give a final concentration of 0.01 M.

Rabbit anti-sheep hemolysin (amboceptor) was purchased[1] and the hemolytic titer was determined according to the method of Kabat and Mayer (20). The amboceptor showed a hemolytic activity of 1600 units/ml. Optimal sensitization for complement titrations was obtained by sensitizing sheep cells with 4 hemolytic units. The agglutination titer of the amboceptor was determined as follows: 0.2 ml. of each dilution in a series of amboceptor dilution was placed into Kahn tubes and 0.2 ml. of a suspension of 10^9 red cells/ml. was added. The mixtures were then incubated for 30 minutes at 37°. After an additional incubation of half an hour at room temperature the endpoint was read with the naked eye. With this technique the amboceptor showed a barely visible agglutination at a dilution of 1:50 which we refer to as 1 agglutination unit. For the sensitization of sheep cells an amboceptor concentration was used which never exceeded 8 hemolytic units or respectively ¼ of an agglutination unit.

Guinea pig serum as well as decomplemented guinea pig serum were obtained, prepared, and kept as described previously (19). The agglutination titer of heat-inactivated guinea pig serum was determined as described for the amboceptor; and the hemolysin titer of guinea pig serum was evaluated analogously, starting with undiluted fresh guinea pig serum and using unsensitized cells. No complement pool was used that showed an agglutination of red cells in undiluted guinea pig serum or a hemolysin titer of more than 1 unit/ml. Additional controls were performed for each pool of complement in the following way: 5 ml. of a chelated suspension of sensitized cells (8 hemolytic units, density 0.5×10^9/ml.) was mixed with 5 ml. of a chelated dilution of guinea pig serum (1:10) and incubated for one-half hour at 37°. The cells were then washed twice with citrated saline and once with buffered saline, resuspended, restandardized, and finally tested for agglutination against three different anti-guinea pig globulin sera as indicated below. This test was always negative, indicating that apart from complement no detectable amount of guinea pig globulin was attached to the cells when guinea pig serum at a dilution of 1:10 or higher was allowed to interact with sensitized cells. The same control procedure performed with non-sensitized cells gave similar results.

In testing the hemolytic reactivity of cell-fixed complement the persensitized cells were exposed to the complement reagents R1, R4, and chelated C′ as described in the previous publication (19). The time required for 100 per cent hemolysis to occur is symbolized by xxx, xx, and x, indicating total hemolysis after less than 5, 15, and 30 minutes respectively. Incomplete hemolysis after 30 minutes incubation is indicated by the symbol x̲.

Immunizations.—Three rabbits were immunized with guinea pig globulin and two with sensitized stromata exposed to guinea pig complement (SAC′). For control purposes an immune serum was produced against sensitized stromata (SA), and against egg albumin. Immunizing injections were given to each animal both intracutaneously and intramuscularly, using Freund's adjuvant. Each animal received one dose of antigen per week. Three doses were given and 1 week after the last injection the animals were bled. Rabbit anti-human globulin and sheep anti-rabbit globulin were purchased.[2]

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[1] Carworth Laboratory Inc., New City, New York.

[2] Sylvana Chemical Co., Orange, New Jersey.

Preparation of Guinea Pig Globulin.—Precipitation of 40 ml. of guinea pig serum by half saturation with ammonium sulfate was performed in the cold. The precipitate was washed three times with half-saturated ammonium sulfate and then dialyzed in the cold for 12 hours against buffered isotonic saline of pH 6.0 with constant stirring and repeated changing of the dialyzing fluid. The globulin was brought to the original volume of the guinea pig serum. During the immunization period each animal received a total of 12 ml. of this antigen. Titrations of the complement components were performed on the guinea pig globulin. Accordingly, each animal received in total 19,200 units of C′1, 9,600 units of C′2, 4,800 units of C′3, and 19,200 units of C′4.

Fixation of Complement on Stromata.—Stromata, sensitized with 40 units of amboceptor per 10×10^9 stromata (SA), were obtained by the glycerin method previously described (19). Thirty ml. of a suspension of 10×10^9 SA/ml. was brought with buffered saline to a final volume of 270 ml. To this suspension 30 ml. of fresh guinea pig serum was added and the suspension incubated at 37°. The supernatant was kept for the titration of the remaining complement components and the sediment (SAC′) was washed seven times with ice-cold buffered saline and finally resuspended in a volume of 30 ml. Two rabbits were immunized with this SAC′ preparation according to the procedure mentioned above. A single dose of antigen consisted of 4 ml. of the suspension of SAC′; *i.e.*, an amount of SAC′ that corresponded to 40×10^9 persensitized cells. Titrations of the complement components were carried out on an untreated specimen of complement and on the supernatant from the mixture of complement with SA. A comparison of the respective titers revealed that during the immunization period each animal theoretically received a total of approximately 32,000 units of fixed C′1, 15,000 units of fixed C′2, 15,000 units of fixed C′3, and 32,000 units of fixed C′4. One of the control animals was injected with a total of 120×10^9 sensitized stromata that were not exposed to complement (SA). A second control animal received a total dose of 12 ml. of a 5 per cent solution of egg albumin.

Immune Sera.—The immune sera were heat-inactivated and kept in the ice box with merthiolate added to give a concentration of 1:10,000. The titer of precipitins in the immune sera was estimated by using the ring test and the antigen dilution method; and in addition the titer of agglutinins against non-sensitized sheep erythrocytes was determined. The general characteristics of each serum are given in Table I.

Prior to their use for the purposes of this investigation the immune sera were absorbed with non-sensitized and with sensitized sheep erythrocytes to the point of complete exhaustion of the agglutinating power in a dilution of 1:5.

Agglutination Technique.—Agglutination tests with the absorbed immune sera were performed in polyethylene hemagglutination plates with hemispherical chambers.[3] Each chamber received 0.2 ml. of the immune serum dilution and 0.05 ml. of the cell suspension (density 0.5×10^9/ml.). The plate was shaken gently, wrapped in cellophane, and placed for 30 minutes in an incubator at 37°. The plate was then shaken gently again and kept at room temperature for another 30 minutes before reading the results. The agglutinations were evaluated by placing the plate on a horizontal piece of ground glass illuminated from beneath. Degrees of agglutination were estimated visually and recorded as +, ++, +++, and ±.

Labelling of the Immune Sera.—A solution of the immune globulin of 10 mg./ml. was labelled with fluorescein isocyanate according to Goldman and Carver (21).

Preparation of Complement Persensitized Stromata on the Slide.—For purposes of demonstrating optically the reaction between fixed complement and labelled anti-complement smears of persensitized red cells were made. After being dried at room temperature the smears were dipped into distilled water saturated with CO_2 and then rinsed in buffered saline. The labelled

[3] Prestware Ltd., London.

immune globulin was placed on the wet surface of the slide; the slide was then incubated at 37° in a wet chamber for 30 minutes, rinsed with saline, embedded in alkaline glycerin with a coverslip, and examined with a dark-field condenser in ultraviolet light (Zeiss fluorescence microscopy equipment). Another series of experiments was performed utilizing smears of sensitized cells exposed after lysis in CO_2 water to either complement, heated complement, or chelated complement. The smears treated with chelated complement were rinsed first in citrated saline and then in buffered saline. The labelled globulin was then applied as described above.

TABLE I

Precipitin and Agglutinin Titers of the Immune Sera

Immune serum No.	Type of antigen injected	Precipitin titer against guinea pig serum (40, 41, 23, 26, 27, 28) and egg albumin (29)	Agglutinin titer against sheep erythrocytes
40	Guinea pig globulin	16,000	1
41	Guinea pig globulin	8,000	2
23	Guinea pig globulin	8,000	1
26	Sensitized stromata persensitized with guinea pig complement (SAC′)	2,000	25
27	Sensitized stromata persensitized with guinea pig complement (SAC′)	4,000	50
28	Sensitized stromata (SA)	0	50
29	Egg albumin	8,000	2

EXPERIMENTAL

Samples of red cells, two of them non-sensitized, and the rest sensitized with 8 amboceptor units (density 0.5×10^9/ml.), were chilled and each mixed with an equal amount of a chilled dilution of 1:40 of the complement reagents as shown in Table II. The mixtures were kept in ice water for 1 hour and the cells were then washed three times in ice cold buffered saline[4] and kept in the cold for 4 hours to accomplish lysis of E* (22). The cells were then washed once more, resuspended at the original density, and examined for their hemolytic reactivity and agglutinability.

Table II shows that the preparation EAC′, *i.e.* EA which had been exposed to C′ in the cold, corresponded to the formula EAC′1,4,2 and was strongly agglutinated by an anti-guinea pig globulin (serum 23) as well as by an anti-SAC′ deprived of its Forssman agglutinins (serum 27). The absence of agglutination in all controls shows that this agglutination was specific. No agglutination occurred with either the sera taken from the animals before immunization (sera 23_0, 28_0, 27_0, 29_0) or with immune sera heterologous to guinea pig serum (sera 29 and 28). The controls show that the sera which were able to agglutinate the complex EAC′ were unable to agglutinate EA which had been exposed to heat-

[4] The cells exposed to chelated complement were washed twice in citrated saline and then in buffered saline.

inactivated, decomplemented, or chelated guinea pig serum. Furthermore, no agglutination with any immune serum was obtained when non-sensitized cells were exposed to active complement. Finally, the controls showed no trace of spontaneous agglutination with either non-sensitized or sensitized cells.

TABLE II

Hemolytic and Agglutination Reactions of Persensitized Erythrocytes

Cell preparation	Hemolysis with				Agglutination‡ with normal and immune sera§ diluted 1:20							
	R1	R4	C′ and EDTA	Saline	23_0	23	27_0	27	29_0	29	28_0	28
EA exposed to active complement	xxx	xxx	xxx	0	0*	+++*	0*	+++*	0*	0*	0	0
EA exposed to heat-inactivated complement	0	0	0	0	0	0*	0	0*	0	0	0	0
EA exposed to complement chelated with EDTA	0	0	0	0	0	0*	0	0*	0	0	0	0
EA exposed to decomplemented complement	0	0	0	0	0	0*	0	0*	0	0	0	0
E (non-sensitized cells) exposed to active complement	0	0	0	0	0	0*	0	0*	0	0	0	0
E (non-sensitized cells)	0	0	0	0	0	0*	0	0*	0	0	0	0
EA	0	0	0	0	0	0*	0	0*	0	0	0	0

‡ The symbol * indicates that for the respective combination of cells and serum all dilutions between 1:10 and 1:2560 were tested in addition to the dilution 1:20.

§ The symbol 23_0 means a serum taken from the animal *before* immunization; 23 means serum taken *after* the immunization. This applies also to the other sera.

The agglutinin titers of the immune sera with respect to the complex EAC′ (the latter prepared as indicated in the previous section) were determined. Before the agglutination test the sera were absorbed with non-sensitized and with sensitized sheep cells; any further reference to immune sera implies that this procedure had been carried out. The agglutination endpoint was reached at a serum dilution of 1:1280 with the sera 26, 27, 40, and 41, and at 1:640 with serum 23.

Attempts were made to determine the minimal amount of sensitizing amboceptor necessary to enable sheep cells to fix a sufficient amount of complement that would be detectable by agglutination with the immune sera 23 and 27. It was found that this minimal amount corresponded to 1 hemolytic unit. A parallel experiment revealed that this amount of amboceptor also represented the minimum necessary to render the cells agglutinable by sheep anti-rabbit globulin serum.

Experiments were performed to see what minimal amount of complement would be required to render suitably sensitized cells agglutinable with the immune sera 23 and 27.

Cells sensitized with 8 amboceptor units were exposed to equal volumes of serially diluted complement. The persensitization was carried out at 3° (3 hours) and at 37° (30 minutes). The complement had a lytic activity of 500 units/ml.

It was found that fixable complement can be detected by agglutination in an amount of one-eighth of a full unit when the persensitization was performed at 3°. The respective amount for persensitization at 37° was one-sixteenth of a unit.

The sera 23 and 27 were absorbed by using the complex SAC′ (complement-persensitized stromata) as an absorbent.

An amount of 30×10^9 of packed SAC′ was taken up in 3 ml. of a 1:10 dilution of serum 23. The same procedure was applied to serum 27. The suspensions were stirred and incubated for 45 minutes at 37° with occasional stirring. The absorbent was then spun down and the sera examined in serial dilution for agglutinins against the complex EAC′. The sera were also tested for precipitins to guinea pig serum in serial dilutions. Non-absorbed batches of both sera 23 and 27 were examined in the same way and served as controls.

This experiment revealed that the agglutinating power of both sera against the complex EAC′ was completely exhausted by absorption with SAC′. Controls showed that absorption of the immune sera with non-persensitized SA did not influence the agglutinating power. The precipitating potency of serum 27 (anti-SAC′) against guinea pig serum was exhausted simultaneously with the disappearance of the agglutinating power, whereas in the case of anti-guinea pig globulin (serum 23) precipitins persisted at the same titer, even when the agglutinating potency was exhausted by absorption with SAC′.

In another experiment samples of sera 23, 26, 27, 40, and 41 were each mixed with a double volume of guinea pig serum and the mixtures incubated for 30 minutes at 37°. The precipitates were then spun down and the supernatant mixtures heated for 30 minutes at 62° to destroy the component C′3; and as a control non-precipitated samples of both immune sera were heated in the same way. All of these sera were then centrifuged and tested in dilution series (starting with a dilution of 1:20) for their agglutination strength against EAC′ and for their capacity to precipitate with guinea pig serum diluted 1:10.

The results of this experiment showed that both the agglutinating and the precipitating properties of the immune sera disappeared completely when the sera had been previously mixed with an excess of antigen (guinea pig serum). In contrast the results with the control (non-precipitated) immune sera showed that these sera retained their agglutinating and precipitating properties.

The sera 23 and 27 were precipitated with guinea pig serum that had been previously decomplemented with SA. The procedure was performed as indicated

above. In both cases the precipitation of the two immune sera with decomplemented complement resulted in exhaustion of their agglutinating power against EAC′. Therefore, despite the removal of the fixable complement there still remained in this apparently decomplemented guinea pig serum material capable of abolishing the agglutinating power of the immune sera against EAC′. The results of experiments with the sera 23 and 27 are summarized in Table III.

In another series of experiments serum 27 (anti-SAC′) was examined in a precipitin test against a dilution series of fresh guinea pig serum and of decomplemented guinea pig serum. No difference was found in the positive precipitin

TABLE III

Precipitation and Agglutination Reactions of Absorbed Sera 23 and 27

Immune sera	Absorbed or precipitated with	Reaction in a subsequent test with	
		Guinea pig serum (precipitation)	EAC′ (agglutination)
Anti-SAC′ (No. 27)	—	Positive	Positive
Anti-guinea pig globulin (No. 23)	—	Positive	Positive
Anti-SAC′ (No. 27)	SA	Positive	Positive
Anti-guinea pig globulin (No. 23)	SA	Positive	Positive
Anti-SAC′ (No. 27)	SAC′	Negative	Negative
Anti-guinea pig globulin (No. 23)	SAC′	Positive	Negative
Anti-SAC′ (No. 27)	Guinea pig serum	Negative	Negative
Anti-guinea pig globulin (No. 23)	Guinea pig serum	Negative	Negative
Anti-SAC′ (No. 27)	Decomplemented guinea pig serum	Not tested	Negative
Anti-guinea pig globulin (No. 23)	Decomplemented guinea pig serum	Not tested	Negative

reactions against both the active and the decomplemented guinea pig serum (antigen dilution technique).

These results indicate that some altered complement components may be rendered unfixable and therefore unremovable by the process of decomplementation with SA and therefore may persist in decomplemented complement and react with the immune serum. An alternative explanation is that complement may be antigenically related to some non-complement material of serum.

Cross-Reactions with Human Complement.—

Sera 23 and 27 were tested for agglutination against a complex EA which had been persensitized with human complement. At the same time a rabbit anti-human globulin was tested for agglutination against a complex EA persensitized with guinea pig complement (EAC′). Persensitization with guinea pig serum was carried out in the cold using a dilution of 1:80. The same dilution was used in the case of human complement but the mixture was incubated at 37° for 30 minutes, since previous experience has indicated that the persensitization with

human complement in the cold yields unsatisfactory results with regard to hemolytic reactivity as well as to agglutinability. Controls were made exposing samples of EA to both human and guinea pig heat-inactivated and chelated complement. Table IV summarizes the results.

This experiment shows that cell-fixed guinea pig complement reacts not only with anti-guinea pig complement (sera 23 and 27) but also with an anti-human globulin. On the other hand fixed human complement reacts with anti-human globulin as well as with anti–guinea pig complement. Since no agglutinogen is taken up from either heat-inactivated or chelated human complement, the

TABLE IV

Cross-Reactions of Immune Sera

EA exposed to	Hemolysis with guinea pig C′ reagents				Agglutination with immune sera, intensity and endpoint dilution			
	R1	R4	C′ + EDTA	Saline	Serum 23	Serum 27	Rabbit anti-human globulin	Serum 29 (anti-egg albumin)
Human C′	x	x	0	0	+++ 1:320	+++ 1:160	+++ 1:320	0
Heat inactivated human C′	0	0	0	0	0	0	0	0
Chelated human C′ (EDTA)	0	0	0	0	0	0	0	0
Guinea pig C′	xxx	xxx	xxx	0	+++ 1:1280	+++ 1:1280	+++ 1:80	0
Heat inactivated guinea pig C′	0	0	0	0	0	0	0	0
Chelated guinea pig C′ (EDTA)	0	0	0	0	0	0	0	0

fixed agglutinogen can be justifiably regarded as representing complement material. It is noteworthy that in the cross-reaction with human C′ the heterologous cross-reacting serum shows considerably lower agglutinin titers than the homologous serum. Additional experiments showed that EA persensitized with horse complement or with rat complement were agglutinated by the sera 23, 26, 27, 40, and 41. Agglutination of the cells persensitized with rat or horse complement did not occur when the immune sera were absorbed with the respective rat and horse serum.

Another technique in addition to the precipitation and agglutination reactions was utilized to further demonstrate the nature of the reactions involved. The globulin fraction of serum 23 was labelled with fluorescein isocyanate and its effect upon sensitized and complement-persensitized stromata was studied on microslides.

Smears of the sediments of sensitized cells (8 units) and non-sensitized cells were made and lysed as described in the section on methods. The slides were then exposed to guinea pig serum, the latter having previously been absorbed by the complex EA in the presence of EDTA to remove any traces of Forssman antibody and antibodies reacting with fixed rabbit amboceptor.

The EDTA was subsequently removed after absorption by dialysis against buffered saline. One portion of this complement was then divided into two parts that were heat-inactivated for 30 and 60 minutes respectively, another portion was chelated with EDTA, and still another decomplemented by absorption with the complex SA. Each of these preparations of complement were finally diluted 1:10 and applied to the surface of smears of both sensitized and non-sensitized cells. The slides were washed in buffered saline and then exposed to the labelled rabbit anti-guinea pig globulin (No. 23) for 30 minutes at 37°. The results are represented in Table V.

It can be seen that a marked fluorescence occurred only when stromata of sensitized cells had been exposed to active complement. When stromata of sensitized cells were exposed to guinea pig serum that had previousy been chelated, heat-inactivated, or exposed to an antigen-antibody complex (decom-

TABLE V

Demonstration of Cell-Fixed C′ with Fluorescent Antisera

Smear material	Smear exposure to	Fluorescence after treatment with labelled immune globulin from serum 23
Non-sensitized stromata	C′	0
Sensitized stromata (SA)	C′	+++
SA	Heat-inactivated C′ (30 min.)	± (traces)
SA	Heat-inactivated C′ (60 min.)	± (traces)
SA	Chelated C′ (EDTA)	0
SA	Decomplemented C′ (treatment with SA)	0
SA	Saline	0

plementation), no fluorescence occurred after exposure to the labelled anti-complement. Furthermore, stromata of non-sensitized cells that had been exposed to guinea pig serum likewise showed no fluorescence after exposure to the labelled anti-complement. Traces of fluorescence occurred when the sensitized stromata had been persensitized with heat-inactivated complement; and this was anticipated, for it is well known that immune precipitates exposed to heat-inactivated complement take up a small quantity of nitrogenous material. Furthermore, more than traces of fluorescence, but strikingly less than marked fluorescence occurred when stromata of heavily sensitized (30 units) cells had been persensitized with heat-inactivated complement. This increase to more than traces of fluorescence was reduced to traces, however, when the complement used for the persensitization of heavily sensitized stromata was first heat-inactivated for 60 minutes instead of 30 minutes.

In addition it was possible to demonstrate by another experiment that smears of the complex EAC′ (the latter prepared in the cold according to the description above) showed strong fluorescence after exposure to the labelled

globulin 23; the controls consisting of smears of EA, that had been exposed in the test tube to heat-inactivated or to chelated complement, showed no traces of fluorescence under the same conditions.

Additional controls were performed to demonstrate that the uptake of fluorescent material from labelled globulin 23 could be blocked by treating the complex EAC′ first with non-labelled immune globulin 23 before applying the labelled immune globulin. In this control the uptake of fluorescent material was markedly reduced when the complex EAC′ had first been treated with globulin from non-labelled serum 23, whereas no blocking occurred when the complex EAC′ had been first treated with normal rabbit serum. In addition, absorption of the labelled globulin 23 with heavy amounts of SAC′ abolished completely the capacity of that serum to stain stromata derived from the complex EAC′. On the other hand absorption of the labelled globulin 23 with the complex SA failed to diminish the staining capacity with respect to the complex EAC′.

Experiments to be reported in a future communication have been carried out with anti-rat complement immune globulin conjugated with fluorescein to test for the presence of complement that might have been fixed *in vivo* in kidneys of rats that were injected with rabbit anti-rat kidney serum. The results of these experiments show that rat complement, not detectable in the kidneys prior to injection of nephrotoxic serum, is present in the walls of glomerular capillaries after the injection of nephrotoxic serum. Furthermore, it was demonstrated with anti-guinea pig complement immune globulin conjugated with fluorescein that these same sites in sections of these very kidneys can additionally fix guinea pig complement (23).

DISCUSSION

The experiments reported in this paper show that the injection of rabbits with guinea pig globulin or of stromata persensitized with guinea pig complement, each in conjunction with Freund's adjuvant, renders the sera of these animals capable of reacting specifically with guinea pig serum material fixed to sensitized red cells. Under suitable conditions this reaction manifests itself in agglutination of these persensitized red cells. The agglutinogenic substance fixable from fresh guinea pig serum is not fixable from guinea pig serum inactivated by heat, by chelation, or by exposure to an antigen-antibody aggregate. In parallel with the fixation of the agglutinogenic substance the cells also acquire hemolytic reactivity against the complement reagents R1, R4, and chelated complement, thus indicating that these cells have reacted with the complement components C′1, C′4, and C′2. The agglutinogenic material fixed on the cells is, therefore, indistinguishable from hemolytic complement. These experiments, of course, cannot be offered as evidence in proof of the fact that the fixable agglutinogen is simply identical with the hemolytically active complement

components. It is conceivable that during complement fixation a material that is not hemolytically active may be taken up in addition to the hemolytically active components. In other words, the fixable material may contain complement components that are hemolytically "mute."

It is noteworthy that the capacity of the immune sera to agglutinate complement persensitized cells was acquired during immunization and proved to be exhaustible by absorption of the immune sera with fixed complement as well as by precipitation with non-fixed complement, *i.e.* with guinea pig serum. No agglutinating capacity could be found in two control sera obtained by immunizing rabbits with egg albumin or SA and none could be found in the serum samples taken from all rabbits prior to immunization. The agglutinating capacity of the immune sera can, therefore, be attributed to antibodies homologous to fixable complement material. The cross-reactions between human and guinea pig complement indicate that the fixable material from human and guinea pig complement are antigenically related.

Before ascribing the reaction between persensitized cells and immune sera to a specific agglutination, one must consider the alternative possibility that this may be a non-specific reaction involving conglutinins or immunoconglutinins. First, this alternative would imply that rabbit serum may contain a heat-stable conglutinin that would act upon cells persensitized with guinea pig complement. However, all the controls show that sera from rabbits prior to immunization as well as immune rabbit sera heterologous to complement are not able to agglutinate cells persensitized with guinea pig or human complement. Yet the term "conglutinin" implies that there are present in normal serum elements reactive with heterologous complement.

Secondly, this alternative would imply that the immune sera examined in our experiments might contain a substance acquired during the immunization which is able to react only with fixed complement but not with free, non-fixed complement. This possibility can be ruled out, however, because the agglutinating power of all our immune sera was removed by mixing the immune sera with guinea pig serum. In the case of the anti-SAC′ serum (serum 27) the agglutinating power was removed completely by absorption with SAC′; *i.e.*, by alexinized stromata; simultaneously the precipitating potency of the serum with respect to guinea pig serum was also exhausted. In the case of serum 23 (anti-guinea pig globulin) precipitins against guinea pig serum persisted even when the agglutinating capacity of the serum was exhausted by absorption with SAC′. This indicates that within the fraction of guinea pig globulin at least two different serological entities can be distinguished: one which includes fixable complement and another which is entirely unrelated to fixable complement.

It is noteworthy that a decomplemented guinea pig serum still reacts with an anti-SAC′ serum and deprives it of its agglutinating power with respect to

alexinized cells. The meaning of this result is obscure. One might assume that one or several factors belonging to the fixable complement are not fully absorbed during the decomplementation and escape the detection in the hemolysis test. It is also conceivable that complement may be related antigenically to other non-complement substances in serum. Finally, one might assume that complement may become altered in such a way that it loses its capacity to be fixed without losing its antigenic properties.

SUMMARY

Evidence is presented to show that guinea pig complement fixed on sensitized sheep red cells acts as a specific agglutinogen. Agglutinating antibodies that react with cell-fixed complement can be produced by immunizing rabbits with a complex of stromata-amboceptor-complement or with guinea pig serum globulin. These agglutinins can be removed by precipitation with guinea pig serum. They are, therefore, distinct from immunoconglutinins.

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