BIKEN JOURNAL Vol. 11 261-267 1968

COMPLEMENT DEPENDENT HEMOLYSIS OF SHEEP ERYTHROCYTES COUPLED WITH NON-ANTIBODY HUMAN IgG[1]

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(Received September 26, 1968)

SUMMARY Sheep erythrocytes, non-specifically coupled with aggregated human IgG by $CrCl_3$, underwent lysis in the presence of diluted guinea pig serum without any antigen-antibody reaction. Participation of complement in this hemolytic reaction was confirmed by the inhibition of the hemolysis by EDTA and the formation of intermediate cells. Hemolysis of IgM-coupled erythrocytes by complement was also evident, whereas IgA-coupled erythrocytes were not lysed even at much higher concentrations of complement.

INTRODUCTION

Immune hemolysis involves three consecutive events which take place on the very surface of erythrocytes; a specific antigen-antibody interaction, binding of complement (C′) by the antibody molecule(s), and the sequential actions of complement components. It is also known that erythrocytes coupled with antigens such as bacterial polysaccharides or synthetic haptens can also be lysed by complement, if antibodies to these antigens are present. On the other hand, it has been shown that complement can also be bound by aggregates of either non-specific IgG (ISHIZAKA & ISHIZAKA, 1959) or the Fc fragment which is devoid of any antibody combining site (AMIRAIAN and LEIKHIM, 1961, ISHIZAKA *et al.*, 1962). The question therefore arises as to whether the specific binding of antibody to erythrocytes is essential for the completion of the lysis by complement.

To answer this question, aggregated human non-specific IgG was directly coupled to sheep erythrocytes using chromium ion and the reaction of the cells with guinea pig complement was followed. The coupled cells not only underwent lysis but also showed a striking resemblance to specifically sensitized cells in their mode of reaction with complement.

MATERIALS AND METHODS

1. *Sheep erythrocytes (E), rabbit hemolysin (A) and sensitized sheep erythrocytes (EA)*

These were prepared and standardized as described in the previous report (NAGAKI *et al.*, 1967).

2. *Diluents*

Isotonic veronal-buffered saline (VB-saline) containing 0.1% gelatin, 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$ was prepared according to the description of MAYER (1961). Isotonic veronal-buffered solution of low ionic strength, 0.09, was prepared as described previously (INAI *et al.*, 1967a). The EDTA-veronal

1 Parts of this work were presented at the fifth Symposium on Complement in July, 1968 at Tokyo.

buffer used contained 0.03 M EDTA in isotonic veronal-buffered saline but no Mg^{++} or Ca^{++}.

3. *Guinea pig serum*

Normal hemolysin was eliminated from guinea pig sera by repeated absorption with packed sheep erythrocytes at 0°C.

4. *Components of complement*

1) First component of guinea pig complement (C'1 gp)

This was obtained by the method of BORSOS *et al.* (1963).

2) Second component of guinea pig complement (C'2 gp)

This was isolated by the methods of BORSOS *et al.* (1961).

3) Fourth component of human complement (C'4 hu)

This was isolated as described in the previous report (INAI *et al.*, 1967b).

4) EDTA treated complement (C'-EDTA)

Guinea pig serum was diluted to 1 : 12.5 with 0.03 M EDTA VB-saline, and used as a source of C'3, C'5, C'6, C'7, C'8 and C'9.

5. *Human immunoglobulins*

1) IgG

Commercial Cohn Fraction II (Nutritional Biochemicals Corporation) was used. Aggregated IgG was prepared by heating at 63°C for 15 min. and by precipitation with Na_2SO_4 as described by CHRISTIAN (1958), and by ISHIZAKA and ISHIZAKA (1959). The fraction obtained at 0.36 M of Na_2SO_4 was used as aggregated IgG.

2) IgA and IgM

IgA and IgM were prepared from serum of patients with IgA myeloma and macroglobulinemia, respectively. These were kindly provided by Dr. TADAMITSU KISHIMOTO[2] and Dr. HIROMICHI HARA[2]. Heated IgA and IgM were also used in this experiment.

6. *Coupling of proteins to sheep erythrocytes*

This was carried out by the methods described by KISHIMOTO *et al.* (1968). To one volume of an erythrocyte suspension containing 1×10^9 cells per ml in saline were added successively one volume of 0.15 mM $CrCl_3$ in saline and one tenth volume of 2% protein solution in saline. The reaction mixture was incubated for one hour at 30°C with occasional shaking. The erythrocytes were washed three times with saline and resuspended in veronal-buffered saline at a concentration of 5×10^8 cells per ml.

7. *Hemolysis of protein-coupled erythrocytes with gruinea pig serum*

Hemolysis of protein-coupled erythrocytes with diluted guinea pig serum was followed using the 1/2.5 scale of Mayer's C'H50 titration method (NAGAKI *et al.*, 1965). A sample of 0.4 ml of 5×10^8 cells per ml protein-coupled erythrocytes and varying amounts of diluted guinea pig serum were mixed in a final volume of 3.0 ml, and incubated at 37°C for 60 minutes with constant mechanical shaking. The reaction mixtures were centrifuged and the optical densities of the supernatants were measured at 541 mμ in a Hitachi spectrophotometer using a cuvette of 1 cm light path.

8. *Quantitative C' fixation analysis*

The C' fixation properties of immunoglobulins were examined by the methods described by MAYER (1961) with slight modification. Incubation of test samples and guinea pig C' was performed at 37°C for 90 minutes.

RESULTS

1. *Hemolysis of sheep erythrocytes coupled with aggregated IgG (EagG) by diluted guinea pig serum*

EagG was capable of hemolysis in the presence of guinea pig serum. Absorbed guinea pig serum diluted to 1/500 was used throughout. The serum had a C'H50 titre of 670 (1/n= 0.200) as assayed with EA (series A, Table 1), while it gave no detectable lysis with untreated cells, E, alone (series C). Table 1 shows clearly that EagG could be lysed by absorbed guinea pig serum even at the low concentrations used (series B). For comparison the apparent C'H50 of the same serum was also calculated from the data with EagG giving a value of 430 (1/n=0.365), which is quite close to that obtained with EA.

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TABLE 1 *Hemolysis of EA, EagG and E by diluted guinea pig serum.*

Guinea pig serum (×500) (ml)	2.0	1.2	1.0	0.8	0.6	0.5	0.4	—
VB-saline (ml)	0.6	1.4	1.6	1.8	2.0	2.1	2.2	2.6[a]
Erythrocytes (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
(A) EA								
O.D. 541		0.629	0.528	0.380	0.171	0.118	0.073	0.640
Per cent hemolysis		98.0	82.7	59.5	26.8	18.5	11.4	
(B) EagG								
O.D. 541	0.608	0.308	0.227	0.146	0.078	0.056	0.049	0.645
Per cent hemolysis	94.2	47.8	35.2	22.6	12.1	8.6	7.6	
(C) E								
O.D. 541		0.022	0.021	0.020	0.020	0.020	0.024	0.630

a; H_2O

Erythrocytes incubated with IgG only could not be lysed by absorbed guinea pig serum, indicating that there was no normal antibody in IgG to sheep erythrocytes.

In Fig. 1 the percentage hemolysis of EA and that of EagG are plotted against the volume of the diluted serum. The typical sigmoid curve for EagG closely resembles that for EA.

2. *Kinetics of hemolysis of EagG by guinea pig serum*

The kinetics of hemolysis of EagG by guinea pig serum was examined as follows. Three flasks each containing 4.8 ml of suspension containing 5×10^8 cells per ml EagG and 19.2 ml of VB-saline were cooled in an ice bath and 12.0 ml of 1/160, 1/250 or 1/500 diluted guinea pig serum were added to them. The contents of the flasks were thoroughly mixed and incubated at 37°C with constant mechanical shaking. After suitable intervals the oxyhemoglobin contents of the supernatant fluids were estimated from the optical density. As

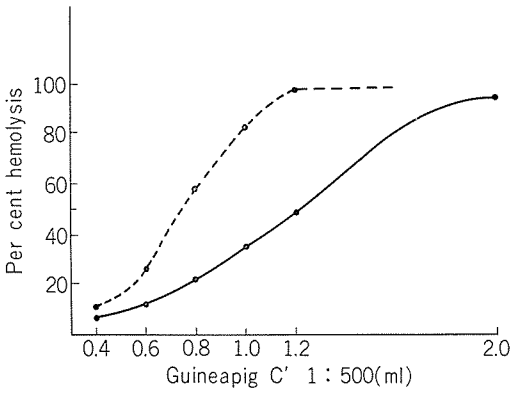


FIGURE 1. Hemolysis of EA and EagG by diluted guinea pig serum.
●——● EagG
●------● EA

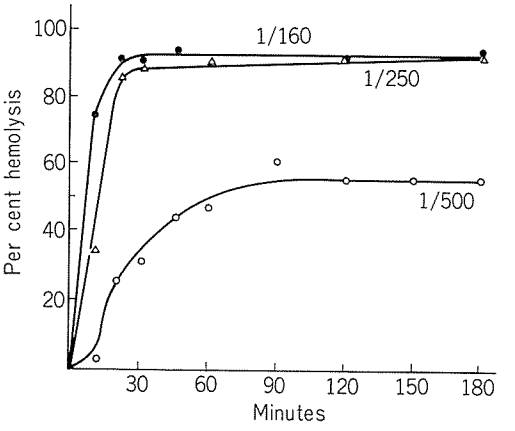


FIGURE 2. Kinetic hemolysis curves of EagG with varying dilutions of guinea pig serum.
●——● 1/160 dilution of guinea pig serum
△——△ 1/250 ,, ,,
○——○ 1/500 ,, ,,

shown in Fig. 2, these curves closely resemble those obtained from optimally sensitized sheep erythrocytes with homologous antibody in a limited complement system.

3. *The effect of EDTA on the hemolysis of EagG by guinea pig serum*

The results described above suggested that the hemolysis of EagG by guinea pig serum is due to complement action. Hence the inhibitory effect of EDTA on hemolysis of EagG was examined. Seven flasks (A, B, C, D, E, F and G) each containing 4.0 ml of EagG (5×10^8 cells per ml) and 15.75 ml of VB-saline were placed in an ice bath. Each flask received 10.0 ml of 1/175 diluted guinea pig serum at zero time. Flask A received 0.25 ml of 0.3M EDTA to chelate Ca^{++} and Mg^{++} in the reaction mixture 5 minutes prior to the addition of guinea pig serum. At time O, flask B received the same amount of EDTA as A, and G, the control, received VB-saline alone instead of EDTA. All flasks were immediately transferred to a water bath and incubated at 37°C with mechanical shaking. EDTA was added to C, D, E and F after 2.0, 4.0, 8.0 and 13.0

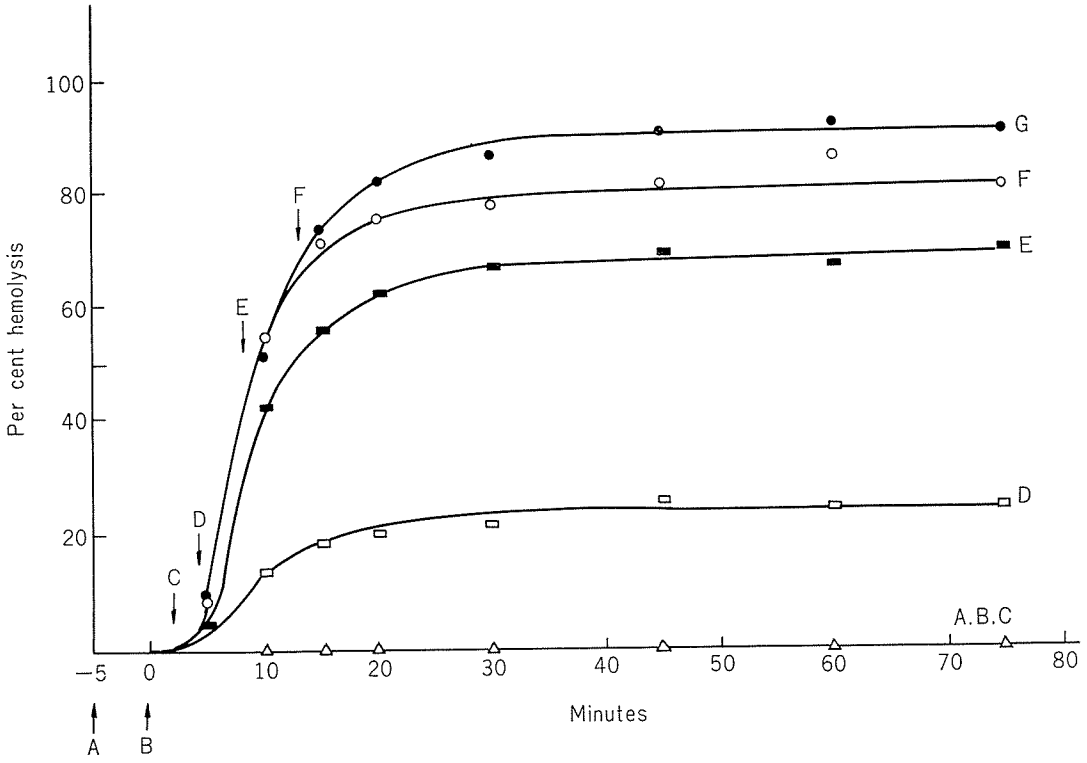


FIGURE 3. The effect of EDTA on the hemolysis of EagG by guinea pig serum. EDTA was added to A, B, C, D, E, and F at −5, 0, 2, 4, 8 and 13 minutes after addition of guinea pig serum, respectively, as indicated by arrows. Curve G represents the course of hemolysis in a control reaction mixture which did not receive EDTA.

△——△ A, B and C
□——□ D
■——■ E
○——○ F
●——● G (control)

minutes, respectively. At suitable intervals 3.0 ml aliquots were removed from each flask and the hemolysis in each reaction mixture was measured. As shown in Fig. 3, the addition of EDTA did not stop hemolysis unless it was added during the first few minutes of the reaction.

The mode of inhibition by EDTA of hemolysis of EagG seems to be identical to that of hemolysis of EA. The requirements for Ca^{++} and Mg^{++} in the early steps of hemolysis suggest that a sequence of reactions of complement components analogous to that in immune hemolysis is involved in this hemolytic system.

4. *Formation of intermediate cells*

1) Formation of EagGC'la and EagG C'la, 4

These intemediate cells were prepared by sequential addition of purified components of complement to EagG. EagG was suspended in VB-saline containing only Ca^{++}, and 300 effective molecules per cell of C'1gp were added. The mixture was incubated at 30°C for 15 minutes, washed twice and resuspended in the same buffer at a concentration of 1.5×10^8 cells per ml. These cells could be hemolysed by sequential addition of C'4, C'2 and C'-EDTA. Therefore, these cells had C'la sites like EAC'la and were designated as EagG C'la. Purified C'4hu was added to EagG C'la at a ratio of 300 effective molecules per cell, incubated at 30°C for 20 minutes, washed twice and suspended in VB-saline containing only Ca^{++}. These cells could be hemolysed by adding C'2 and C'-EDTA and were designated as EagGC'la,4. The Tmax of EagG C'la, 4 was 14 minutes. The reaction between EagGC'la and varying amounts of C'4 in the presence of sufficient C'2 and C'-EDTA was examined by the same methods as those used for titration of C'4 (INAI *et al.*, 1964).

The per cent hemolysis of EagGC'la is plotted against the amount of C'4 in Fig. 4. It was calculated from these results that about 10 effective molecules of C'4 per cell were needed to obtain 63% lysis of these cells.

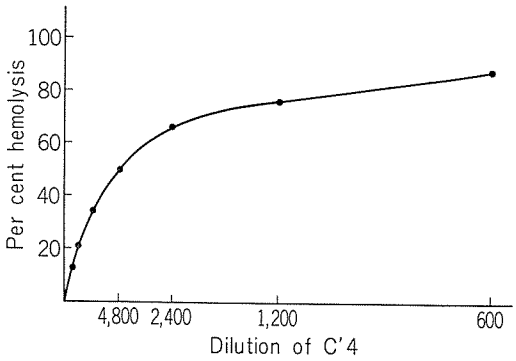


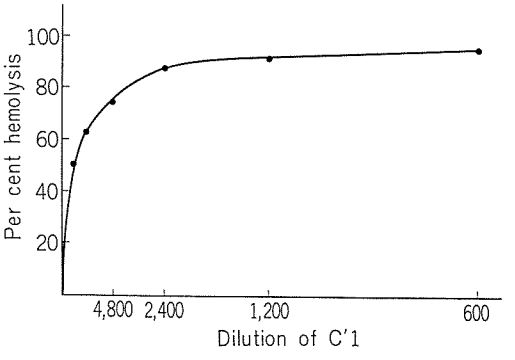
FIGURE 4. Hemolysis of EagGC'la by varying amounts of C'4 in the presence of sufficient amounts of C'2 and C'-EDTA.

EagGC'la,4 also reacted with C'2 and formed EagGC'la,4,2a which was lysed by the addition of C'-EDTA.

2) Formation of EagGC'4

EagGC'4 could be obtained from EagG C'la,4 using EDTA by a similar method to that used for preparation of EAC'4 from EAC'la,4. As shown in Fig. 5, where the per cent hemolysis, of EagGC'4 is plotted against the amount of C'1 in the presence of excess C'2 and C'-EDTA, about 20 effective molecules of C'1 per cell were needed for 63% lysis of EagGC'4. Regeneration of EagG C'la,4 from EagGC'4 by C'1 is evident.

From these results it was confirmed that various intermediate cells such as EagGC'la,



FIGHURE 5. Hemolysis of EagGC'4 by varying amounts of C'1, in the presence of excess C'2 and C'-EDTA.

EagGC'la,4 and EagGC'4 could be prepared using components of complement.

5. *Hemolysis of erythrocytes coupled with IgA or IgM by guinea pig complement*

Purified preparations of IgA and IgM were used for coupling and IgA-coupled (E-A) and IgM-coupled cells (E-M) were prepared by the same procedures as those used for EagG. The effect of absorbed guinea pig serum on the hemolysis of these cells was examined similarly. As shown in Fig. 6 the E-A cells gave no detectable hemolysis even with high concentrations of guinea pig serum, whereas significant lysis was obtained with the E-M cells though to a lesser extent than that with EagG cells. From the positive agglutination reaction of each cell preparation with the respective rabbit antiserum, it was evident that both IgA and IgM were fixed on the surface of sheep erythrocytes.

The effects of C′ on the hemolysis of erythrocytes coupled with heated IgA or IgM were also examined. Erythrocytes coupled with heated IgM showed the same degree of hemolysis as that of E-M. But erythrocytes coupled with heated IgA could not be lysed even with a high concentration of C′ as observed with E-A.

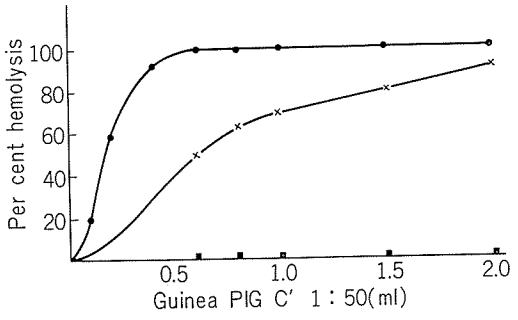


FIGURE 6. Hemolysis of erythrocytes coupled with various immunoglobulins by guinea pig serum.
●——● EagG
×——× E-M
■——■ E-A
The quantities of each immunoglobulin, used in this experiment, required to fix 50 C'H50 were as follows: aggregated IgG; 1.83 μg N, IgA; 850 μg N, IgM; 125 μg N.

DISCUSSION

In the present experiments the coupling of aggregated human IgG to erythrocytes by $CrCl_3$ rendered the cells susceptible to the action of guinea pig serum without participation of any antigen-antibody reaction. The findings of the inhibitory effect of EDTA on this type of hemolysis and the formation of intermediates indicated clearly that the hemolysis of EagG is actually due to the interaction between IgG molecules and complement on the surface of the erythrocytes.

In the present study IgM-coupled erythrocytes were also hemolysed by complement, while IgA-cells were not. The results are consistent with the facts that IgM as well as IgG is a good complement fixer but IgA lacks this capacity (ISHIZAKA *et al.*, 1966).

It is uncertain whether some changes in the conformation of the molecule occur during the course of coupling by $CrCl_3$ which may result in an enhancement of complement binding, or whether there is any damage to the cell which increases the susceptibility to lysis. However, the effect of chromium ion, if any, is thought to be very small, and the protein coupled cells, as far as tested, did not show any increased lability under physiological conditions. In any event, the present results support the previous suggestion by ISHIZAKA and ISHIZAKA (1959) that the mechanisms of complement fixation by aggregated IgG in the presence and absence of specific antigen-antibody complexes are probably analogous, if not identical, and it may safely be concluded that hemolysis by complement can take place only if proteins which are capable of binding complement are simply adsorbed on the surface of erythrocytes.

The significance of such a non-specific sensitization of erythrocytes deserves attentions with regard to hemolytic diseases of unknown etiology.

AKNOWLEDGEMENTS

The authors are indebted to Dr. Kozo Inoue and Dr.

Sayaka Utsumi, Department of Bacteriology, Osaka University, Medical School for criticism and advice.

They also wish to thank Miss Michiko Ueno and Miss Noriko Akagi for their technical assistance.

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