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HEMOLYTIC BEHAVIOR OF SHEEP ERYTHROCYTES NON-SPECIFICALLY COUPLED WITH HUMAN C1a

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SUMMARY Sheep erythrocytes non-specifically coupled with human Cla by $CrCl_3$ could be lysed by sequential addition of the remaining components of complement without any antigen-antibody reactions. In marked contrast with EACla, no dissociation of Cla occurred on EDTA treatment of ECla formed with chromium. Hemolytic intermediates, i.e., ECla,4 and ECla,4,2a, were prepared from ECla by methods similar to those used for preparing intermediates of immune hemolysis. Moreover, it was found that the mode of decay of SCla,4,2a sites on ECla,4,2a closely resembled that of those on EACla,4,2a. These findings suggest that the sequence of reactions of complement components initiated by ECla is the same as that of EACla.

INTRODUCTION

We have previously reported that sheep erythrocytes, to which aggregated human IgG is non-specifically coupled through chromium ion, undergo lysis in the presence of guinea pig complement, without any antigen-antibody reactions (Inai and Tsuyuguchi, 1968). The participation of complement (C) in this hemolytic reaction was confirmed by observation of inhibition of hemolysis by EDTA and formation of intermediate cells. It was also reported by Cowan (1954) and Dalmasso and Müller-Eberhard (1964) that non-sensitized sheep erythrocytes could be lysed by complement in the presence of polyethylene glycol which presumably acts as a non-specific sensitizer. Yachnin (1965) and Hinz (1966), too, in their studies on paroxysmal nocturnal hemoglobinuria have suggested that not only erythrocytes from patients but also normal erythrocytes can be lysed by complement under certain circumstances without antibody if the concentration of complement is sufficiently high and the pH is lowered to 6.9. From these findings the question arises as to whether immunoglobulin molecules play any essential role other than that of evoking components of complement to act on the surface of erythrocytes. If immunoglobulin molecules act merely as mediators, then hemolysis should be induced by components of complement alone when they are fixed directly on to the surface of erythrocytes.

In the present paper, the hemolytic behavior of sheep erythrocytes coupled with human Cla was examined by the sequential addition of the remaining complement components. The Cla coupled cells (ECla) not only underwent lysis but also behaved similarly to specifically sensitized sheep erythrocytes carrying Cla (EACla) in their mode of reaction with the remaining complement components.

This work was presented at the US-Japan Complement Seminar in November, 1968 at Tokyo, Japan.

MATERIALS AND METHODS

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

Sheep erythrocytes in Alsever's solution were washed three times with physiological saline and adjusted to a concentration of 1×10^9 cells per ml. Rabbit antibody against boiled sheep erythrocyte stromata was prepared as described by Mayer (1961). Sensitization of sheep erythrocytes with rabbit antibody was performed by the methods of Nelson et al. (1966).

2. *Diluent*

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 method of Mayer (1961). An isotonic sucrose solution in veronal-buffered saline of low ionic strength, 0.09, was prepared as described in the previous report (Inai et al., 1964). The EDTA-veronal buffer employed here contained 0.03 M EDTA in the above isotonic veronal-buffered saline but no Mg^{++} or Ca^{++}.

3. *Components of complement*

1) First component of human complement (Cla)

Partially purified first component of human complement was isolated from the human euglobulin fraction by a method similar to that used for isolation of the first component of guinea pig complement (Borsos et al., 1963). The protein concentration of Cla preparation was 0.03%. This preparation gave a hemolytic activity of 2.25×10^{12} effective molecules per ml. when estimated using EA instead of EAC4.

2) Second component of guinea pig complement (C2)

Partially purified second component of guinea pig complement was prepared by the method of Borsos et al. (1961).

3) Fourth component of human complement (C4)

The fourth component of complement was purified as previously reported (Inai et al., 1967). This purified C4 gave a single precipitin line against horse anti-human serum (from the Research Foundation for Microbial Diseases of Osaka University) on immunoelectrophoresis and contained a negligible amount of Cl inactivator.

4) EDTA-treated guinea pig serum (C-EDTA)

Guinea pig serum was diluted to 1:12.5 with 0.03 M EDTA VB-saline and used as a source of C3, C5, C6, C7, C8 and C9.

4. *Coupling of Cla to sheep erythrocytes*

Coupling of Cla was carried out as follows. To one volume of a suspension of sheep erythrocytes in saline (1×10^9 cells per ml) were added successively one volume of 0.15 mM $CrCl_3$ in saline and one tenth volume of Cla in saline. The reaction mixture was incubated for 1 hour at 30 C with occasional shaking. The erythrocytes were washed twice with saline and once with VB-saline containing Ca^{++}, and resuspended in the same VB-saline at a concentration of 1.5×10^8 cells per ml. These cells carrying Cla sites were designated as ECla.

5. *Preparation of sensitized sheep erythrocytes carrying Cla, C4 sites (EACla,4)*

To EA (1.5×10^8 cells per ml) suspended in VB-saline containing Ca^{++} were added 300 effective molecules per cell of Cla. The mixture was incubated at 30 C for 15 min, washed once and resuspended in the original volume of the same solvent (EACla). Purified C4 was added to EACla to supply 300 effective molecules per cell, and the mixture was incubated at 30 C for 20 min, washed once and resuspended in VB-saline at a concentration of 1.5×10^8 cells per ml.

6. *Examination of the hemolytic behavior of ECla and its hemolytic intermediates*

The hemolytic behavior of ECla was examined as follows. One ml of diluted C4 was added to 0.5 ml of ECla containing 1.5×10^8 cells per ml and incubated at 30 C for 15 min. One ml of C2 was then added at a ratio of 300 effective molecules per cell and the mixture was incubated further at 30 C for 20 min. Finally, 0.5 ml of 1:12.5 C-EDTA was added and the reaction mixture was immediately incubated at 37 C for 60 min with constant mechanical shaking. Then it was mixed with 4.5 ml of cold saline, and centrifuged. The oxyhemoglobin concentration in the supernatant fluid was measured as the optical densities at 414 mμ in a Hitachi spectrophotometer using a cuvette of 1 cm light path.

ECla,4 was prepared as follows: Purified C4 was added to ECla to supply 300 effective molecules per cell. The mixture was incubated at 30 C for 20 min, washed once with VB-saline containing Ca^{++} and resuspended at a concentration of 1.5×10^8 cells per ml. The hemolytic behavior of ECla,4 was

estimated by a method similar to that used for ECla, except that 1.0 ml of VB-saline was used instead of C4.

RESULTS

1. *Hemolysis of sheep erythrocytes coupled with varying amounts of Cla in the presence of excess C4, C2 and C-EDTA*

The amount of Cla required for coupling sheep erythrocytes was measured as follows. To a series of tubes containing 1.0 ml of sheep erythrocytes (1×10^9 cells per ml), 1.0 ml of 150 μM $CrCl_3$ and varying amounts of Cla were quickly added. The cell suspensions were incubated at 37 C for 60 min with occasional shaking. The cells were then washed twice with saline and once with VB-saline containing Ca^{++} and resuspended in the same VB-saline at a concentration of 1.5×10^8 cells per ml. The hemolytic behavior in each tube was estimated by the sequential addition of sufficient amounts of purified human C4, guinea pig C2 and C-EDTA. The percentage hemolysis of ECla was plotted against the amount of Cla used for coupling of erythrocytes.

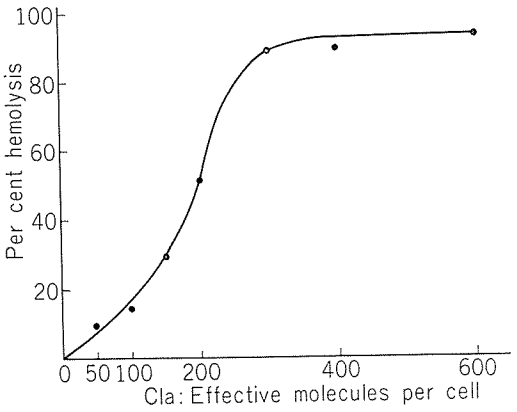

FIGURE 1. *Hemolysis of sheep erythrocytes coupled with varying amounts of Cla in the presence of excess C4, C2 and C-EDTA.*

As shown in Fig. 1, the degree of lysis of various preparations of ECla depended on the amount of Cla used for coupling. The maximum lysis was obtained at coupling ratios higher than 400 effective molecules of Cla per cell. Therefore, in subsequent experiments unless otherwise stated, 400 effective molecules per cell of Cla were used for preparing ECla.

2. *Failure to dissociate Cla from ECla by EDTA treatment*

It is well known that when Cla is fixed on normal sensitized erythrocytes, it can easily be dissociated by EDTA treatment. As reported in the previous paper, Cla was also dissociated from cells coupled with aggregated human IgG with Cla,4 sites by EDTA treatment (Inai and Tsuyuguchi, 1968). Thus, attempts were made to dissociate Cla from ECla by EDTA treatment.

For this experiment a preparation of sheep erythrocytes coupled with Cla at a ratio of 250 effective molecules per cell was chosen. The suspension of ECla cells was divided into two portions and these were centrifuged at 3000 r.p.m. for 10 min. One preparation of cells was resuspended in 0.03 M EDTA and the other in VB-saline as a control, and both were incubated at 37 C for 5 min. The EDTA treated ECla cells were washed once with 0.03 M EDTA and twice with VB-saline. The control cells were washed three times with VB-saline. Finally, both were suspended at a concentration of 1.5×10^8 cells per ml in VB-saline containing Ca^{++}.

The hemolytic behaviors of these two types of ECla cells were compared by the sequential addition of varying amounts of C4, and sufficient amounts of C2 and C-EDTA. The percentage hemolysis of each sample was plotted against the number of effective C4 molecules per cell.

As shown in Fig. 2, no significant difference was observed between EDTA-treated ECla and control ECla, indicating Cla not dissociated from ECla by EDTA-treatment.

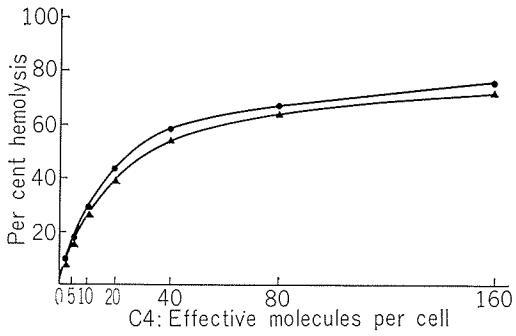

FIGURE 2. *Failure to dissociate Cla from ECla by EDTA treatment.*
●——● EDTA treated ECla
▲——▲ control ECla

3. *Interaction between ECla and purified human C4*

The interaction between erythrocytes coupled with a sufficient amount of Cla and purified human C4 was examined. Erythrocytes coupled at a ratio of 400 effective Cla molecules per cell were treated with varying numbers of effective C4 molecules per cell and then hemolysis was effected by the addition of excess C2 and C-EDTA. In Fig. 3 the percent hemolysis of ECla was plotted against the number of effective C4 molecules per cell.

It was found that the degree of lysis of ECla was a function of the amount of C4 added, but about 5 effective C4 molecules per cell were necessary to obtain 63% lysis of these cells.

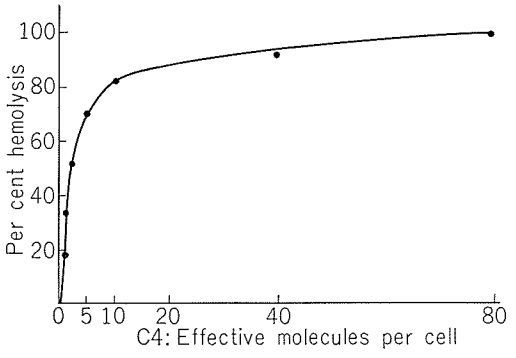

FIGURE 3. *Hemolysis of ECla with varying amounts of C4 in the presence of excess C2 and C-EDTA.*

4. *Interaction between ECla,4 and C2*

The next step of the sequential interaction of components, i.e., the interaction between ECla,4 and C2 was ascertained by the following experiment. Purified human C4 was added to ECla at a ratio of 300 effective molecules per cell, and the mixture was incubated at 30 C for 20 min. Then cells were washed twice and suspended in VB-saline containing Ca^{++}. These cells were found to be hemolyzed by C2 and C-EDTA and were designated as ECla,4.

The Tmax of ECla,4 was estimated at 35 min. This was longer than that for EACla,4 cells, indicating that fewer Cla,4 sites were formed than with ordinary EACla,4. In this experiment, varying numbers of effective molecules per cell of partially purified guinea pig C2 were added to ECla,4, and the mixtures were incubated at 30 C for 35 min and then lysed by addition of excess C-EDTA.

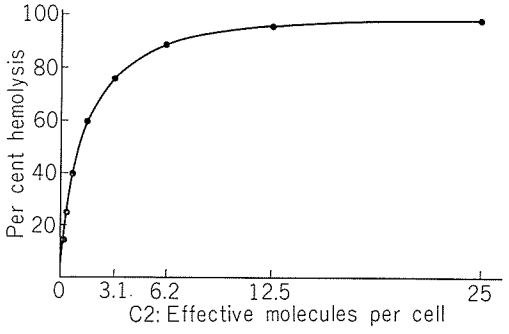

FIGURE 4. *Hemolysis of ECla,4 with varying amounts of C2 in the presence of excess C-EDTA.*

In Fig. 4, the per cent hemolysis of ECla,4 is plotted against the number of effective C2 molecules per cell. The degree of lysis of ECla,4 depended on the amount of C2, and 63% lysis of these cells was obtained at about 2 effective C2 molecules per cell.

5. *Decay of Cla,4,2a sites on ECla,4,2a*

To compare the rate constant of decay (K) of ECla,4,2a with that of EACla,4,2a, the

kinetics of decay of each intermediate were analysed.

To 10 ml of either ECla,4 (1.5×10^8 cells per ml) or EACla,4 (1.5×10^8 cells per ml) prewarmed at 30 C, the same volume of prewarmed C2 was added. An appropriate dilution of C2 was chosen for each intermediate so as to generate about one SCla,4,2a site per cell at Tmax at 30 C.

The mixtures were kept at 30 C until the Tmax and then chilled, washed twice with ice cold VB-saline and resuspended in 50 ml of ice cold VB-saline. Then, 2.5 ml portions of the ECla,4,2a or EACla,4,2a cell suspension were pipetted into a series of test tubes in an ice bath. All of the tubes were then transferred to a water bath at 30 C and zero time was taken as the time when the temperature of the contents of the tubes reached 30 C.

At suitable intervals, 0.5 ml portions of C-EDTA prewarmed at 30 C were added and tubes were then incubated in a water bath at 37 C for 60 min. Then the number of Cla,4,2a sites per cell, i.e., $z=-1n\ (1-y)$, was calculated from the degree of lysis, y, in each tube and log z was plotted against the incubation time at 30 C.

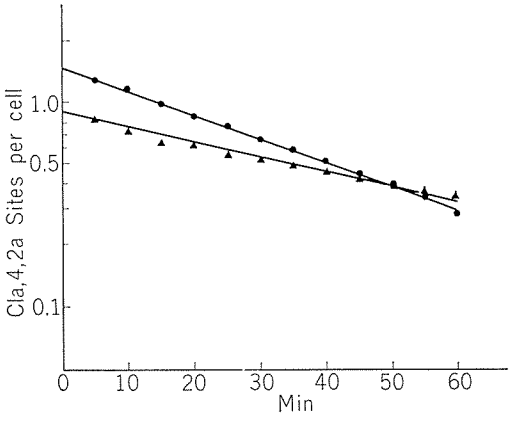

FIGURE 5. *Decay of SACla,4,2a and SCla,4,2a at 30 C.*

● — — ● EACla,4,2a

▲ ——— ▲ ECla,4,2a

As shown in Fig. 5, a straight line was obtained for each cell type and the number of Cla,4,2a sites per cell to these two intermediates decreased gradually indicating the decay of these sites to Cla,4 sites. The rate constant of decay, K, for ECla,4,2a was 0.017, which is the same magnitude as that for EACla,4,2a, namely 0.026. The Thalf for ECla,4,2a was 40 min and that for EACla,4,2a was 26 min at 30 C.

These results indicate that the sequence of reactions of complement components initiated by ECla is the same as that for EACla.

DISCUSSION

The concentration of chromium ion used (150 μM) for coupling of Cla to erythrocytes is sufficient to cause extensive agglutination or lysis of cells unless a certain amount of protein is present. The protein concentration of the Cla preparation used for coupling was approximately 0.03% and under these conditions not much undesirable agglutination and lysis was induced by chromium ion. After washing and dispersing few agglutinated cells by gentle shaking, the coupled cells were stable and no further lysis or agglutination developed. The possible effect of chromium ion on the structure of Cla and especially on the esterase activity has to be considered. Although prior treatment of erythrocytes with chromium ion followed by addition of Cla in the absence of chromium ion was attempted, it did not give satisfactory results, mainly for the reasons stated above.

Non-sensitized erythrocytes to which human Cla molecules were directly coupled in this manner underwent lysis on the addition of the remaining components of complement just as ordinary EACla cells did.

With reference to the previous finding that aggregated IgG could sensitize erythrocytes to complement dependent lysis in a non-specific manner when coupled to the cells through chromium ion (Inai and Tsuyuguchi, 1968),

it must be noted that the present preparation of Cla did not contain any detectable IgG as judged by immunoelectrophoresis. Furthermore it is quite unlikely that the complement induced lysis of ECla in the present work is due to the attachment of a few IgG molecules to the cell because the protein concentration to 0.03% in the coupling reaction was too low induce sensitization even if it represented protein in a pure preparation of IgG (Tsuyuguchi and Inai, 1968).

ECla formed with chromium closely resembled EACla in their biological functions as intermediate cells. ECla were converted to ECla,4 or ECla,4,2a by sequential addition of appropriate purified components and the mode of decay of SCla,4,2a too was similar to that of SACla,4,2a. These results give direct support for the view proposed previously that components of complement can interact directly with normal erythrocytes without antibody, probably by the same sequence of reactions as that in authodox immune hemolysis (Hinz, 1966).

In contrast to usual EACla or the chromium coupled EagGCla used in the previous work (Inai and Tsuyuguchi, 1968), there was no dissociation of Cla from ECla in the presence of EDTA. The failure of ECla to liberate Cla may be ascribed to the strong binding of Cla to the cell surface through chromium ion. The mechanism of EDTA mediated dissociation of Cla from EACla is not understood, but calcium ion may have some effect on the binding of Cla molecules to antibody or the binding of Cl subcomponents together. It also seems that EDTA does not inactivate the Cla site, nor does it disintegrate Cla complex, since the Cla sites on ECla survive EDTA treatment. This can be attributed to the stabilizing effect of chromium ion. It was thus found that the lysis of ECla was not at all affected by the presence or absence of Ca^{++} during the reaction of the remaining complement components.

The similar behavior of ECla to that of EACla in the hemolytic system indicates that at least some of the bound Cl molecules of ECla are properly situated on the surface of erythrocytes for the series of the actions of the remaining complement components, in spite of the absence of mediator antibody. However, as described in the results, 63% lysis of ECla in the presence of excess C4, C2 and C-EDTA required more than 200 effective molecules of Cla for coupling, and the Tmax for ECla,4 cells was very long, namely 35 min. These findings indicate that only some of the Cla molecules fixed on the cell surface are in an active state. This can be explained in many ways as for instance:

1) There may be areas on the erythrocyte surface which are not effected by complement even though they combine with complement through chromium ion.

2) The orientations of active sites of Cla molecules on the cell surface may not always be suitable for subsequent reactions.

3) The activity of Cla molecules may be partially damaged by chromium treatment. Further investigations are needed on the phenomenon.

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REFERENCES

Borsos, T., H. J. Rapp and C. T. Cook 1961. Studies on the second component of complement. III. Separation of the second component from guinea pig serum by chromatography on cellulose derivatives. J. Immunol. 87. 330–336.

Borsos, T. and H. J. Rapp. 1963. Chromatographic

separation of the first component of complement and its assay on a molecular basis. J. Immunol. 91: 851–858.

COWAN, K. M. 1954. Cited in Experimental Immunochemistry. 133–240, 1961. Springfield, Illinois, Charles C. Thomas, 2nd ed.

DALMASSO, A. P. and H. J. MÜLLER-EBERHARD. 1964. Interaction of autologous complement with red cells in the absence of antibody. Proc. Soc. Exp. Biol. Med. 117: 643–650.

HINZ, C. F. 1966. The hemolytic reaction in paroxysmal nocturnal hemoglobinuria. Prog. Hematol. 5: 60–82.

INAI, S., K. FUJIKAWA, H. TAKAHASHI and K. NAGAKI. 1964. Studies on the fourth component of complement. I. Titration of the fourth component of complement in human serum. Biken J. 6: 237–251.

INAI, S., S. HIRAMATSU, and K. NAGAKI. 1967. Separation of C'4 from C'1 inactivator and purification of both substances. Biken J. 10: 155–174.

INAI, S. and I. TSUYUGUCHI. 1968. Complement dependent hemolysis of sheep erythrocytes coupled with non-antibody human IgG. Biken J. 11: 261–267.

MAYER, M. M. 1961. Experimental immunochemistry. Springfield, Illinois, Charles C. Thomas, 2nd ed.

NELSON, R. A., J. JENSEN, I. GIGLI and N. TAMURA. 1966. Method for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. Immunochem. 3: 111–135.

TSUYUGUCHI, I. and S. INAI. 1968. Unpublished data.

YACHNIN, S. 1965. The hemolysis of red cells from patients with paroxysmal nocturnal hemoglobinuria by partially purified subcomponents of the third complement component. J. Clin. Invest. 44: 1534–1546.