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# STUDIES ON THE FOURTH COMPONENT OF COMPLEMENT
# II. THE FOURTH COMPONENT OF COMPLEMENT IN GUINEA PIG AND HUMAN PLATELETS*

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SUMMARY Activity of the fourth component of complement (C'4) was found in extracts of guinea pig and human platelets, but not in leucocytes extracts, when activity was measured with EAC'1 cells.

Further, it was found that the reactivation of hemolytic activity of ammonium-treated guinea pig serum with leucocyte extracts described by MALTANER in 1935, might be due to contamination of the leucocyte suspension from blood with platelets.

From further investigations it was concluded that platelets might both contain and adsorb C'4 activity.

## INTRODUCTION

In the previous publications (INAI *et al.*, 1963, 1964), it was stated that the diminution of serum complement activity found in a patient with chronic myelogenous leukemia might be due to a remarkable reduction in C'4 activity in the serum. It was thought that changes in white cell counts of the blood of this patient might be related to this phenomenon.

As OSLER stated in his review (1961), there is little information available on the complement activities of tissues and body fluids other than serum, especially about the activities of the components of complement. As far as we know, MALTANER's report (1935) on the substance in guinea pig leucocytes obtained from blood which is complementary to the activity of ammonium-treated serum (R4) in the hemolytic system is an only information available on the possible existence of complement or its components in leucocytes. But in 1953, AMANO *et al.* failed to obtain a supplementation of the hemolytic activities of all R-reagents with an extract of leucocytes obtained from guinea pig peritoneal exudate.

These results both seemed very suggestive to us in relation to the observation on the remarkable reduction in C'4 activity in the serum of the patient with chronic myelogenous leukemia, mentioned above.

We have confirmed the findings of these two groups of investigators that extracts of guinea pig leucocytes obtained from blood could supplement the hemolytic activity of R4, but that

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extracts of leucocytes from peritoneal exudate could not. To resolve the discrepancies between these two results the experimental procedures employed previously were carefully examined, and it was found that the leucocyte preparations from peripheral blood were heavily contaminated with platelets while there were very few platelets in the peritoneal exudate.

In this paper a method for separation of leucocytes and platelets in blood samples is described and results on the titration of the C'4 activity of extracts of these cells are reported. These investigations showed that platelets had low C'4 activity while leucocytes had none. It is also suggested that most of the C'4 activity in the platelet preparation might be of intracellular origin.

## MATERIALS AND METHODS

1. *Guinea pig complement (guinea pig C')*

Blood from guinea pigs was obtained aseptically by cardiac puncture. After standing the sample for about 30 minutes at room temperature the serum was separated, centrifuged and either used on the same day or stored at −20°C until use.

2. *Hemolysin*

Pooled rabbit antiserum against boiled sheep red cell stromata prepared according to the method of KABAT and MAYER (1961), was employed throughout these studies.

3. *Diluents*

Sucrose veronal buffer and NaCl veronal buffer were prepared as in the previous paper (INAI *et al.*, 1964) and gelatin (Difco) was added to a final concentration of 0.1 per cent. Diluents with an ionic strength of 0.09 were prepared by mixing 6 parts of NaCl veronal buffer with 4 parts of sucrose veronal buffer, and those diluents containing calcium (to 0.00015 M) with or without magnesium (to 0.0005 M) were designated as $GVB^{++}$(SUC.4)[1] and $GVB^{+-}$(SUC.4)[2], respectively.

These diluents, ionic strength 0.09, were employed in all the studies except for titration of complement, which was done in $GVB^{++}$(SUC.0)[3], having an ionic strength 0.147. For chelating $Ca^{2+}$ and $Mg^{2+}$, 0.03 M EDTA-GVB containing 0.03 M EDTA in $GVB^{--}$(SUC.0)[4] was used.

4. *Erythrocytes*

Sheep blood, drawn aseptically, was preserved at 2–5°C with an equal volume of Alsever's solution. On the day of use erythrocytes were washed twice with 0.15 M NaCl, once with $GVB^{+-}$(SUC.4), resuspended in $GVB^{+-}$(SUC.4) and standardized by reading the optical density at 541 mμ of a water-lysed aliquot in a Coleman Junior type spectrophotometer.

5. *Sensitized sheep erythrocytes* (EA)

Hemolysin diluted to 1 : 300 in $GVB^{+-}$(SUC.4) was added to an equal volume of washed cell suspension containing $1 \times 10^9$ cells per ml, and the suspension was incubated in a water bath at 37°C for 10 minutes for sensitization.

6. *The first component of complement* (C'1)

C'1 isolated from guinea pig serum by the method of BORSOS *et al.* (1963), was used in place of the C'1 preparation used in our previous work, and stored at −20°C. The different samples of C'1 isolated by this method contained $1.65 \times 10^{12}$ to $6.38 \times 10^{12}$ effective molecules of C'1 per ml when titrated by the method of BORSOS (1963) using EAC'4.

7. *The second component of complement* (C'2)

C'2 was obtained from guinea pig serum according to the method of BORSOS *et al.* (1961). The different preparations of C'2 contained $1.29 \times 10^{11}$ to $1.13 \times 10^{12}$ effective molecules of C'2 per ml, when titrated by the method of BORSOS using EAC'1,4 and C'-EDTA (BORSOS *et al.* 1961 a).

8. *EDTA treated complement* (C'-EDTA)

Guinea pig complment was diluted to 1 : 12.5 with 0.03 M EDTA-GVB, used as a source of the C'3

1. $GVB^{++}$(SUC.4): Mixture of 6 parts of saline veronal buffer and 4 parts of sucrose veronal buffer containing $Ca^{2+}$(to 0.00015 M) and $Mg^{2+}$ (0.0005 M).
2. $GVB^{+-}$(SUC.4): Mixture of 6 parts of saline veronal buffer and 4 parts of sucrose veronal buffer containing $Ca^{2+}$(to 0.00015 M) without added $Mg^{2+}$.
3. $GVB^{++}$(SUC.0): Saline veronal buffer containing $Ca^{2+}$(to 0.00015 M) and $Mg^{2+}$(to 0.0005 M).
4. $GVB^{--}$(SUC.0): Saline veronal buffer without added $Ca^{2+}$ and $Mg^{2+}$.

complex for titration of C'1, C'4, and C'2. 0.5 ml of 1 : 12.5 C'-EDTA was added to 2.5 ml of EAC'-1,4,2.

9. *EA with C'1 activity* (EAC'1)

The results given in the previous paper (INAI *et al.*, 1964) on the condition for sensitization of EA with C'1 were confirmed when C'1 prepared by BORSOS' method was used at an ionic strength 0.09. Optimal amounts of C'1 (predetermined) were mixed with EA and incubated for 15 minutes in a water bath at 30°C. Then they were washed twice with $GVB^{+-}$ (SUC.4), resuspended in $GVB^{+-}$(SUC.4) at a concentration of $1.5 \times 10^8$ cells per ml and used on the same day. These cells were shown to contain no C'4 activity.

10. *EA with C'1 and C'4 activities* (EAC'1,4)

To 20 ml of EA ($1 \times 10^9$ cells per ml) in $GVB^{+-}$ (SUC.4) precooled to 0°C was added 0.5 ml of precooled guinea pig C' with thorough mixing, and the mixture was incubated at 0°C for 7.5 minutes (NISHIOKA and LINSCOTT, 1963). The cells were centrifuged and washed twice with cold $GVB^{+-}$ (SUC.4). They were then resuspended in the same buffer and incubated at 37°C for 2 hours. Then they were centrifuged and resuspended in $GVB^{+-}$ (SUC.4) to a concentration of $1 \times 10^9$ cells per ml and stored at 0°C for use within a week. The T max of cells prepared by this method was measured by a modification of BORSOS' method (BORSOS *et al.* 1961 a) using $GVB^{++}$(SUC.4) at a cell concentration of $3.0 \times 10^7$ cells per ml before addition to C'-EDTA, and it was usually between 2 and 4 minutes.

11. *EA with C'4 activity* (EAC'4)

Packed EAC'1,4 cells were suspended in prewarmed 0.03 M EDTA-GVB, and incubated at 37°C for 5 minutes. Then the cells were centrifuged, washed once with 0.03 M EDTA-GVB, three times with $GVB^{+-}$(SUC.4), resuspended in the same buffer at a concentration of $1 \times 10^9$ cells per ml and stored at 0°C. EAC'4 cells prepared by this method had no C'1 activity.

12. *Titration of complement*

To a series of $15 \times 110$ mm test tubes containing varing amounts of test sample were added 0.4 ml of EA ($5 \times 10^8$ cells per ml in $GVB^{++}$(SUC.0)); the total reaction volume was made up to 3.0 ml with $GVB^{++}$(SUC.0). Reaction mixtures were placed in a water bath at 37°C for 60 minutes with constant shaking. At the end of the incubation period, tubes were centrifuged, and the degree of lysis was measured with a Coleman Junior type spectrophotometer at a wave-length of 541 m$\mu$, and then C'H50 was calculated as usual manner.

13. *Titration of C'4*

Titration of C'4 was done as described in the previous paper (INAI *et al.*, 1964) at an ionic strength 0.09.

14. *Titration of C'1*

To a series of $15 \times 110$ mm test tubes containing 1.0 ml of diluted sample was added 0.5 ml of EAC'4 ($1.5 \times 10^8$ cells per ml), and mixtures were placed in a water bath at 30°C for 15 minutes with constant shaking. At the end of this period, 1.0 ml of C'2, diluted in $GVB^{++}$(SUC.4) to supply about 300 effective molecules per cell, was added to each tube. After 30 minutes' incubation at 30°C with constant shaking, 0.5 ml of C'-EDTA was added to each tube which was then quickly transferred to a water bath at 37°C. After 60 minutes' incubation with constant shaking, unlysed cells were centrifuged off and the hemoglobin content of each tube was measured with a Coleman Junior type spectrophotometer at a wave-length of 414 m$\mu$. For calculation of the C'1 titer, $-\ln(1-y)$ was calculated from the per cent lysis, $y$, and the reciprocal value of $-\ln(1-y) =$ unity was multiplied by the number of cells ($7.5 \times 10^7$) to obtain the number of effective C'1 molecules per ml.

15. *Titration of C'2*

A series of $15 \times 110$ mm test tubes containing 1.0 ml of sample, diluted appropriately with $GVB^{++}$ (SUC.4), was placed in a water bath at 30°C. Exactly 1.5 ml of prewarmed EAC'1,4 ($5 \times 10^7$ cells per ml) suspended in $GVB^{++}$ (SUC.4) were added to the series of tubes, staggered at uniform intervals of 1 minute. Then the tubes were incubated for the period of time, T max, and 0.5 ml of C'-EDTA were added to all the tubes at the same intervals used for the EAC'1,4 additions. Immediately after addition of C'-EDTA, the tubes were transferred to a water bath at 37°C. After 60 minutes' incubation with constant shaking, unlysed cells were centrifuged off, and the hemoglobin content of each tube was measured with a Coleman Junior type spectrophotometer at a wave-length of 414 m$\mu$. Effective molecules of C'2 were calculated in the usual manner (BORSOS *et al.*, 1961).

16. *Estimation of the activity of trypsin and of trypsin inhibitor*

The activities of trypsin and of trypsin inhibitor were titrated by the method described by KUNITZ (1947) with a slight modification. Lyophilized crystalline trypsin (MOCHIDA) was used throughout the present experiments and the tryptic units in this sample was $(T.U.)^{case}=4.4\times10^{-3}$.

Activity of soybean trypsin-inhibitor (NBC) was estimated and it was calculated that 1 $\mu$g of this inhibitor inhibited 0.5468 $\mu$g of trypsin. To ensure the complete suppression of trypsin activity, five fold this amount of trypsin-inhibitor was used.

Preliminary experiments showed that 200 $\mu$g per ml of trypsin inhibitor, or 20 $\mu$g per ml of trypsin which was inactivated by 100 $\mu$g per ml of trypsin inhibitor, had no effect on the titration of isolated C'4.

## RESULTS

1. *Preparation of crude leucocyte extract and its C'4 activity*

For isolation of leucocytes from blood, HAMMERSTROM's procedure (1963) was adopted with several modifications. Silicon-coated glasswares were used in each step of separation of the cells. As shown in Fig. 1, guinea pig blood was obtained by heart puncture and Na-citrate was added to a final concentration of 0.5 per cent. Aliquots of 10 ml of blood were put in a 20 $\times$180 mm test tubes, and 2 ml of 7 per cent dextran (mol. wt. $18\times10^4$) in saline solution was added. Tubes were closed with a parafilm sheet and inverted slowly several times avoiding formation of bubbles. Tubes were then placed in a rack at an angle of 45° and allowed to stand for 60 minutes at room temperature. The supernatant was collected and centrifuged at 3,000 r.p.m. for 5 minutes. Precipitated cells were suspended in 2 ml of saline and 6 ml of distilled water were added to them. After stirring the mixture for 30 seconds with a glass rod, 2 ml of 3.5 per cent saline was added and the tubes were centrifuged. Most of the contaminating erythrocytes were lysed by this procedure. Sedimented cells were washed 4 times with 50 ml volumes of saline and were suspended in $GVB^{++}$(SUC.4) at a concentration of $1\times10^7$ to $3\times10^7$ cells per ml. Crude leucocyte extracts were prepared by freezing-thawing these suspensions, followed by centrifugation at 5,000 r.p.m. for 10 minutes at 0°C.

Human blood was obtained from 3 healthy men in this laboratory. Human leucocyte extracts were prepared as described above, except that the period of standing of blood in dextran solution for separation of plasma was reduced to 30 minutes.

The extracts were usually diluted 1: 5 to 1: 80 and C'4 activities were measured.

The C'4 titres of these preparations were between $3\times10^8$ and $5\times10^8$ effective molecules per ml for both human and guinea pig leucocytes. The leucocyte preparations obtained as described above contained several kinds of white blood cells, and it was of interest to see from what kinds of cells the C'4 activity in the leucocyte extracts originated.

To study this the ascitic cells of guinea pigs were collected from the peritoneal cavity. Several guinea pigs were each injected intraperitoneally with 10 ml of aseptic physiological saline. After an appropriate period, the peritoneal cavities were washed with citrate-saline. This washing fluid was centrifuged at 5,000 r.p.m. for 10 minutes at 0°C, and precipitated cells were washed 3 times with 50 ml volumes of saline and differential counts of cells were made. When the peritoneal washing fluid was collected within 15 hours after injection of saline, more than 90 per cent of the cells were polynucleated cells. On the other hand, when the washing fluid was collected 72 hours after injection of saline, more than 80 per cent of the cells were mononucleated cells. The washed cells were extracted using the same procedures as described above. The concentration of cells used for preparing the extracts of leucocytes from the peritoneal cavity was almost the same as that from the peripheral blood. However, C'4 activity of the extracts obtained from ascitic cells was almost zero regardless of the cell type. The discrepancies

between results with peripheral blood leucocytes and ascitic cells suggested the possible role of contaminating platelets in the preparations from the peripheral blood. Indeed, cells obtained by these procedures from peripheral blood were contaminated with $2\times10^8$ to $5\times10^8$ platelets per ml of the leucocyte suspension, but cells from the peritoneal cavity contained only about $1.5\times10^7$ platelets per ml.

2. *Separation of platelets and leucocytes from plasma and their C'4 activities*

The procedures for separation of platelets and leucocytes from guinea pig and human plasma are shown in Fig. 2. Separation of plasma was performed as described in Experiment 1. The plasma was divided into three equal portions. The first portion served for the separation of platelets. It was repeatedly centrifuged at 1,000 r.p.m. for 10 minutes at 0°C until the number of leucocytes in the supernatant was reduced to less than 10 per cmm. In most cases, the number of contaminating leucocytes decreased to this level after three or four centrifugations. Then this leucocyte-free plasma was centrifuged at 5,000 r.p.m. for 15 minutes at 0°C. The sedimented platelets were washed five times with 50 ml volumes of saline, and suspended in $GVB^{+-}$(SUC.4) at a concentration of $1\times10^9$ platelets per ml. This suspension was designated as P.

The second portion of the plasma was employed for separation of leucocytes. It was centrifuged at 1,000 r.p.m. for 10 minutes at 0°C. The sediment was washed with ice cold saline and centrifuged at 1,000 r.p.m. 4 times in order to eliminate contaminating platelets. The final sediment was obtained by centrifuga-

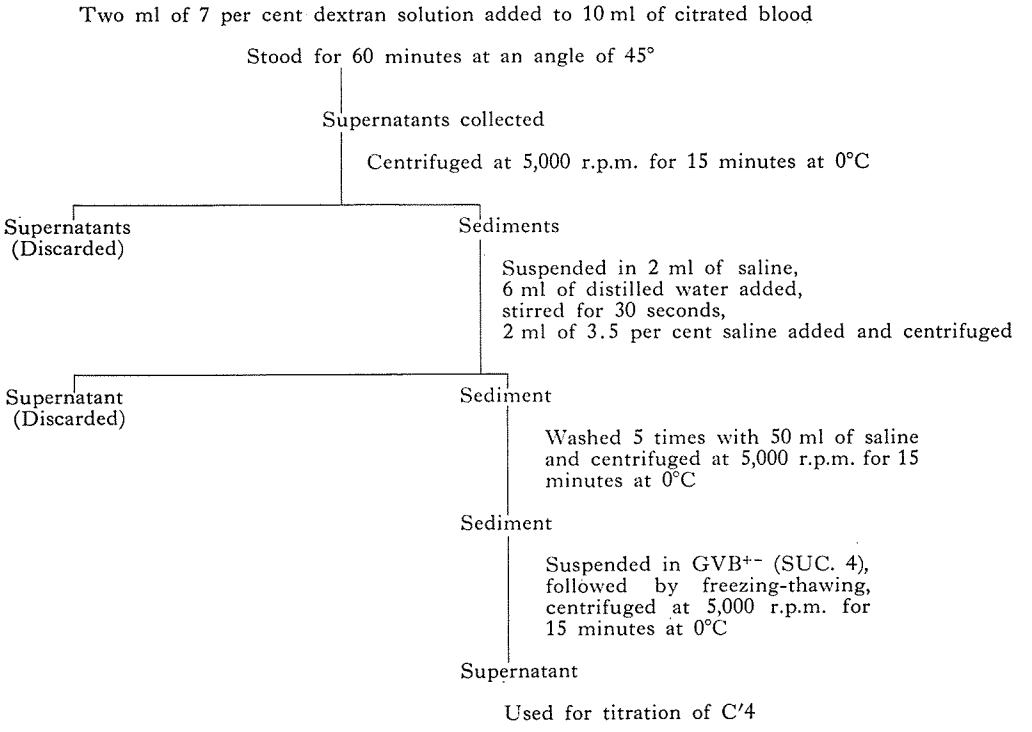


FIGURE 1 Preparation of crude guinea pig leucocyte extract

tion of the last cell suspension at 5,000 r.p.m. for 15 minutes, and the red cells contaminating this sediment were lysed by the method described in Experiment I. The resultant cells were suspended in $GVB^{+-}$(SUC.4). This suspension contained $1.5 \times 10^7$ leucocytes per ml and was designated as L.

As a control, a crude leucocyte suspension was prepared from the third portion of the plasma by the same procedures described in Experiment I and this was designated as LP.

The number of leucocytes and platelets used in these suspensions were twice as many as in the peripheral blood.

These three suspensions were extracted by the procedures described in Experiment I. The protocol for titration of C'4 activity in these three extracts obtained from guinea pig is shown in Table 1.

It can be seen that C'4 activity was detected in P and LP but not in L. As shown in Table 2, C'4 contents in P and LP of human origin were in the same order as those in guinea pig material, but a trace of C'4 was found in human L which was not seen in guinea pig material.

From these results, it was considered that platelets from both guinea pig and human blood may have considerable C'4 activity, whereas

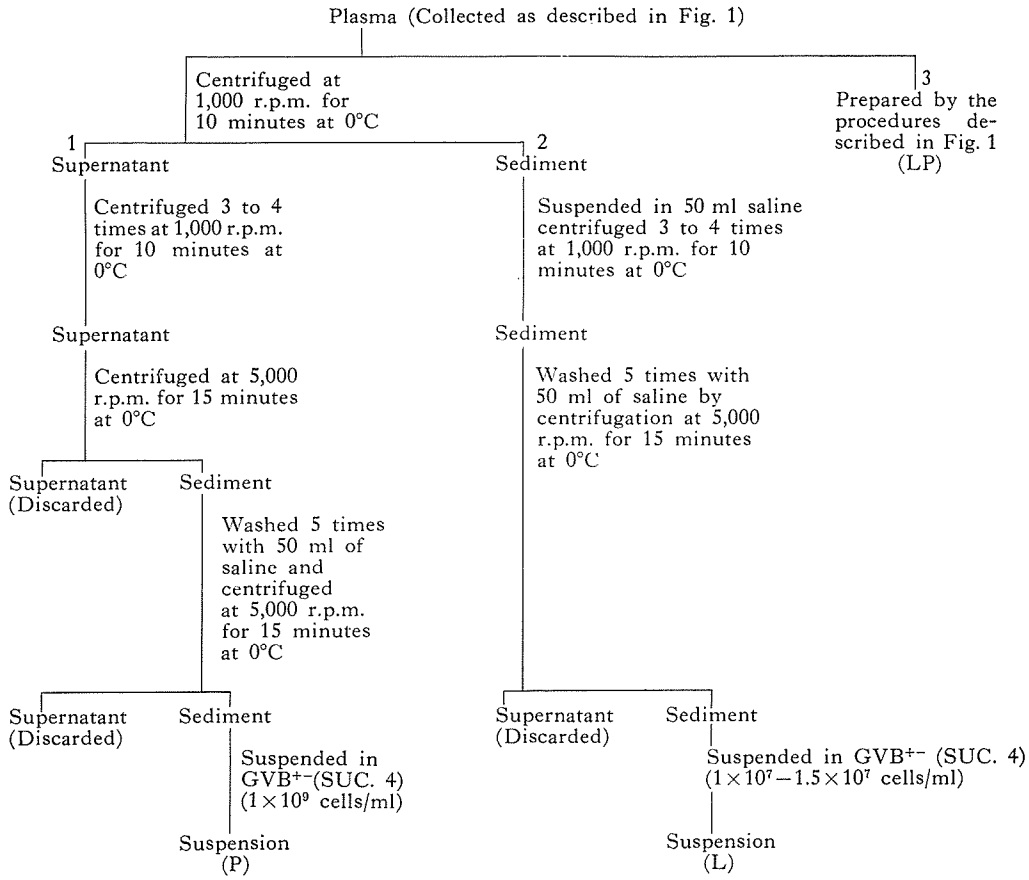


FIGURE 2 Separation of platelets and leucocytes from plasma

leucocytes may have little or no C'4 activity. However, it was necessary to decide whether C'4 activities in human and guinea pig LP were actually derived only from platelets. To study this the experiments similar to that described above were carried out and C'4 activity per platelet was calculated. As shown in Table 3, if it is assumed that C'4 activities in both LP and P originated only from platelets, C'4 activities per guinea pig platelets in LP and P, respectively, could be calculated to be 0.99 and 1.27 effective molecules, and in human platelets in LP and P, respectively, to be 0.45 and 0.44 effective molecules. Therefore, in both

TABLE 1 *Titration of C'4 in extract of guinea pig leucocytes and platelets*

| | | Tube No. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| EAC'1 | ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Extract | 1.0 ml | 1 : 5 | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | — | — | — |
| GVB$^{++}$-SUC.4 | ml | — | — | — | — | — | 1.0 | 2.5 | 2.5* |
| C'2 | ml | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | — | — |
| 1 : 12.5 C'-EDTA | ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | — | — |
| Extract obtained from | | OD 414 mμ | | | | | | | |
| L | | 0.100 | 0.075 | 0.072 | 0.058 | 0.058 | 0.050 | 0.005 | 0.830 |
| P | | 0.810 | 0.700 | 0.525 | 0.370 | 0.240 | | | |
| LP | | 0.635 | 0.545 | 0.430 | 0.305 | 0.205 | | | |

L Not detected
P $12.7\times10^8$ Effective molecules/ml } C'4 in each extract
LP $7.88\times10^8$ Effective molecules/ml

* $H_2O$

TABLE 2 *Titration of C'4 in extract of human leucocytes and platelets*

| | | Tube No. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| EAC'1 | ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Extract | 1.0 ml | 1 : 5 | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | — | — | — |
| GVB$^{++}$-SUC.4 | ml | — | — | — | — | — | 1.0 | 2.5 | 2.5* |
| C'2 | ml | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | — | — |
| 1 : 12.5 C'-EDTA | ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | — | — |
| Extract obtained from | | OD 414 mμ | | | | | | | |
| L | | 0.205 | 0.170 | 0.120 | 0.100 | 0.090 | 0.075 | 0.005 | 0.830 |
| P | | 0.630 | 0.490 | 0.355 | 0.240 | 0.185 | | | |
| LP | | 0.620 | 0.455 | 0.325 | 0.225 | 0.160 | | | |

L $0.075\times10^8$ Effective molecules/ml
P $4.43\times10^8$ Effective molecules/ml } C'4 in each extract
LP $4.05\times10^8$ Effective molecules/ml

* $H_2O$

human and guinea pig material, C'4 activities per platelet in LP and P were almost the same.

On the other hand, assuming C'4 activities in LP and L to be derived only from leucocytes, the effective molecules of C'4 per leucocyte were calculated to be as follows: 52.5 per leucocyte in guinea pig LP, not detected in guinea pig L, 22.5 in human LP and 0.44 in human L. Namely, C'4 activities per leucocyte were quite different in LP and L in both cases. On the basis of these calculations, it seems reasonable to assume that C'4 activity in LP may originate from platelets, and C'4 activity in L may be due to contaminating platelets. Indeed, in practice, it is difficult to remove clotted platelets completely from packed leucocytes by low speed centrifugation.

The following experiment also suggests that the platelets are the source of C'4 activity in preparation P. Guinea pig platelets were separated from blood by the procedures described above, and platelets suspensions of different concentrations were prepared. The C'4 activities of these samples were titrated. As shown in Fig. 3, C'4 activities of the extracts were proportional to the number (concentration) of platelets used in the extractions.

From these results C'4 activity in one guinea pig platelet was calculated to be 1 to 3 effective molecules.

3. *Other components of complement in platelets and leucocytes*

The C'1 and C'2 activities in extracts of

TABLE 3 *Calculated C'4 activity per guinea pig and human platelets*

| | | | Extract obtained from | | |
|---|---|---|---|---|---|
| | | | P | LP | L |
| Guinea pig | Number of cells used for extraction | Leucocytes | — | $1.5\times10^7$ | $1.5\times10^7$ |
| | | Platelets | $10\times10^8$ | $8.0\times10^8$ | — |
| | C'4 eff. mol./ml | | $12.7\times10^8$ | $7.88\times10^8$ | Not detected |
| | Calculated C'4 eff. mol. per platelet | | 1.27 | 0.99 | — |
| Human | Number of cells used for extraction | Leucocytes | — | $1.8\times10^7$ | $1.6\times10^7$ |
| | | Platelets | $10\times10^8$ | $9.0\times10^8$ | — |
| | C'4 eff. mol./ml | | $4.43\times10^8$ | $4.05\times10^8$ | $0.075\times10^8$ |
| | Calculated C'4 eff. mol. per platelet | | 0.44 | 0.45 | — |

TABLE 4 *Activities of some components of complement in human and guinea pig platelets and leucocyte extracts*

| | Platelet extract | | Leucocyte extract | |
|---|---|---|---|---|
| | Human | Guinea pig | Human | Guinea pig |
| Cell number | $1.04\times10^9$/ml | $1.1\times10^9$/ml | $1.6\times10^7$/ml | $1.75\times10^7$/ml |
| C'1 eff. mol./ml | 0 | 0 | 0 | 0 |
| C'2 eff. mol./ml | Not detected | $8.63\times10^8$ | Not detected | $7.88\times10^8$ |
| C'4 eff. mol./ml | $4.43\times10^8$ | $21.4\times10^8$ | $0.075\times10^8$ | $0.495\times10^8$ |

guinea pig and human cells were also tirated. As shown in Table 4, no C'1 activity was found in L or P of either human or guinea pig origin. C'2 activity was observed in guinea pig P and L, being, $8.6\times10^8$ and $7.88\times10^8$ effective molecules per ml, respectively. But in the case of L, C'2 activity might have been derived from contaminating platelets, since C'4 activity in this sample of L was $4.95\times10^7$ effective molecules per ml. No C'2 activity was found in extracts of human cells by this titration method.

4. *The effect of trypsin and hydrazine on the C'4 activities of separated platelets*

It is obvious from the results described above that the platelets separated from peripheral blood have C'4 activity, but it is uncertain whether this activity is inside the platelets or only adsorbed on their surface. To study this the destructive effects of trypsin and hydrazine on C'4 activity of the platelets was examined. HJORT *et al.* (1955) reported that most of the platelet accelerator activity of normal platelets is destroyed by trypsin without altering the appearance of the platelets, so that the platelet accelerator might be adsorbed plasma proaccelerin. Thus, if the C'4 of platelets was derived from plasma C'4 adsorbed on the platelets, it might be inactivated by trypsinization. On the other hand, if the activity was inside the platelets then hydrazine might penetrate into them and destroy the C'4 activity, because it is a small sized molecule.

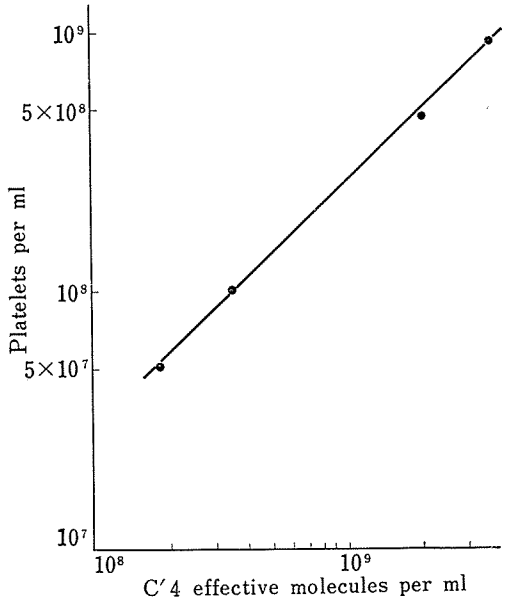


FIGURE 3 Relationship between C'4 activity in platelet extracts and the number of platelets used in the extractions.

1) *The effect of trypsin and hydrazine on isolated C'4*

To determine the amount of trypsin and hydrazine necessary for destruction of the C'4 activity of isolated platelets, the destructive effects of these compounds on isolated C'4 was examined.

The effect of trypsin on isolated C'4 was studied as follows. To tubes containing 1.0 ml of isolated C'4 were added 1.0 ml volumes of serial dilutions from 2 to 80 $\mu$g of trypsin. After incubation at 30°C for 20 minutes 1.0 ml of trypsin inhibitor solution of a concentration sufficient to inhibit the trypsin activity was added to each tube. Then the tubes were reincubated at 30°C for 10 minutes to suppress the trypsin activity. After this 2.0 ml of $GVB^{+-}$(SUC.10) was added to each tube to obtain the optimal ionic strength for titration of C'4. Finally, the residual C'4 activity in each tube was titrated.

The effect of hydrazine on isolated C'4 was estimated as follows. To tubes containing 1.0 ml of isolated C'4 were added 1.0 ml of 0.0015 to 0.020 M hydrazine solution. After incubation at 30°C for 30 minutes each tube received 1.0 ml of saline containing an equimolar amount of HCl and 2.0 ml of $GVB^{+-}$(SUC.10). The residual C'4 activity in each tube was titrated.

The results of these experiments are summarized in Table 5. It was found that 10 $\mu$g of trypsin inactivated $3.78\times10^{10}$ effective molecules of C'4, while 0.006 M of hydrazine destroyed $5.73\times10^{10}$ effective molecules of C'4.

TABLE 5 *Effect of hydrazine and trypsin on isolated C'4*

| | Final concentration | Reaction temperature and time | C'4 Inactivated (eff. mol.) |
|---|---|---|---|
| Hydrazine | 0.006 M | 37°C 30 min. | $573\times10^8$ |
| Trypsin | 10 $\mu$g | 37°C 20 min. | $330\times10^8$ |

2) *Elution of C'4 from isolated platelets*

Preliminary experiments showed that a considerable amount of C'4 activity was eluted from platelets suspended in saline or GVB at room temperature or at 37°C. To confirm this phenomenon, the following experiments were carried out. Isolated platelets ($1\times10^{10}$ cells) were suspended in an appropriate amount of ice cold saline and equal volumes were distributed in 8 tubes and centrifuged at low temperature. The supernatant was discarded. Each sediment was suspended in 1.3 ml of ice cold $GVB^{+-}$(SUC.4) and 6 of the 8 tubes were incubated at 30°C. At appropriate intervals tubes were centrifuged in the cold and C'4 activity of each supernatant was titrated. Each sediment was also resuspended in 1.3 ml of $GVB^{+-}$(SUC.4), and after 5 times freezing-thawing treatment tubes were centrifuged and each supernatant was also titrated. As a control, one tube was centrifuged without incubation and C'4 activities of the supernatant and sediment were titrated. The latter values represented C'4 activities in the supernatant and sediment at zero time, respectively. The last tube was incubated at 30°C for 60 minutes and was then immediately treated by freezing-thawing without centrifugation. The C'4 activity of this tube represented the total C'4 activity of the platelets in the tube after 60 minutes incubation.

As shown in Fig. 4, C'4 activities in the supernatants rapidly increased to a plateau value after 20 minutes which represented about 45 per cent of the total C'4 activity of the platelets, while C'4 activities in sediments decreased rapidly reaching a minimum after 20 minutes. It was

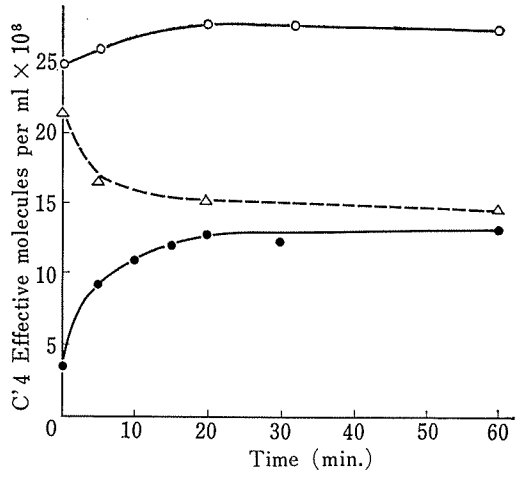


FIGURE 4 Elution of C'4 from guinea pig platelets at 30°C.
○——○ Total
△——△ Sediment
●——● Supernatant

considered that C'4 adsorbed on the surface of the platelets is readily eluted under the present conditions while C'4 in the platelets is not eluted after 20 minutes of incubation. Therefore, trypsinization and hydrazine treatment of platelet suspensions were carried out after preincubation at 30°C for 30 minutes.

3) *The effect of trypsin and hydrazine on the C'4 activity of platelets*

Isolated platelets ($3\times10^9$ cells) were suspended in 3.0 ml of ice cold saline and 1.0 ml portions were distributed in 3 tubes. Tube A was frozen immediately. To elute the adsorbed C'4 from the surface of the platelets and to examine the effect of trypsin on C'4 activity which was not eluted from the platelets, both tubes B and C were incubated at 30°C for 30 minutes. Then, 20 $\mu$g of trypsin in 1.0 ml of saline was added to tube B, and it was reincubated for 20 minutes. Then 100 $\mu$g of trypsin inhibitor in 1.0 ml of saline was added to the reaction mixture and it was reincubated at 30°C for 10 minutes. In place of trypsin and trypsin inhibitor 2.0 ml of saline was added to tube C, and it was incubated at 30°C for 30

minutes. Tubes B and C were then centrifuged and the respective supernatants were separated. Two ml of $GVB^{++}$(SUC.10) were added to these to adjust the ionic strength to 0.09. The sediments of these two tubes were each suspended in 1.0 ml of $GVB^{++}$(SUC. 4). All three tubes were titrated for C'4 activity in the usual manner.

The results, shown in Table 6, indicate that C'4 activity of the starting material (Tube A) was $22.5 \times 10^8$ effective molecules. After trypsinization of the platelets (Tube B), C'4 activity of the supernatant and sediment were respectively zero and $5.6 \times 10^8$ effective molecules. In the control tube (Tube C), C'4 activity of the supernatant was $10.9 \times 10^8$ effective molecules and that of the sediment was $12.4 \times 10^8$ effective molecules.

TABLE 6 *Effect of trypsin on C'4 activity of platelets*

| | | C'4 eff. mol./Tube($\times 10^8$) |
|---|---|---|
| Before trypsinization (Tube A) | | 22.5 |
| After treatment with 20 $\mu$g of trypsin (Tube B) | Supernatant | 0 |
| | Sediment | 5.55 |
| Control After incubation with saline at 30°C for 20 minutes (Tube C) | Supernatant | 10.9 |
| | Sediment | 12.4 |

Each tube contained $1 \times 10^9$ platelets.

TABLE 7 *Effect of hydrazine on C'4 activity of platelets*

| | | C'4 eff. mol./Tube ($\times 10^8$) |
|---|---|---|
| Before treatment | | 14.2 |
| After treatment with 0.006 M of hydrazine | Supernatant | 0 |
| | Sediment | 0 |
| Control After incubation with saline at 30°C for 30 minutes | Supernatant | 4.35 |
| | Sediment | 9.85 |

These results show that the C'4 eluted from the platelets was completely destroyed by trypsin while only about half of C'4 which was not eluted was destroyed.

The effect of hydrazine on C'4 activity of platelets was tested in a similar way using 0.012 M hydrazine and equimolar HCl, instead of trypsin and trypsin inhibitor. As shown in Table 7, C'4 activities of the both supernatant and sediment were zero, and it is obvious from this result that not only the C'4 which was eluted but also the C'4 in the platelets was destroyed by hydrazine.

## DISCUSSION

In 1935, MALTANER reported that an extract of guinea pig blood leucocytes supplmented the hemolytic activity of ammonium treated guinea pig serum (R4). She considered this supplementary activity to be due to C'4. However, recent progress on the components of guinea pig complement shows that ammonium or hydrazine treated guinea pig serum lacks not only C'4 activity, but also C'3c and other activities (NISHIOKA *et al.* 1963). Moreover, the method employed by MALTANER for obtaining a leucocytes suspension from blood is unsatisfactory, for the leucocytes suspension is contaminated with platelets.

In 1953, AMANO *et al.* failed to supplement the hemolytic activities of all R-reagents with extracts of guinea pig leucocytes from peritoneal exudates.

These reports and the observation that there was a remarkable reduction in C'4 activity in the serum of a patient with chronic myelogenous leukemia (INAI *et al.*, 1963) prompted us to reinvestigate MALTANER's report.

In this paper, estimation of C'4 activity was carried out with EAC'1 cells instead of R-reagent, and an effort was made to separate the platelets and leucocytes completely. Unlike MALTANER, we found no appreciable C'4 activity in either guinea pig or human leucocytes. However, guinea pig and human platelet preparations did contain C'4 activity. Thus, it

was calculated that guinea pig and human platelets contained 1 to 3 and 0.44 effective molecules of C'4 activity per cell, respectively. These levels are significant and can explain MALTANER's results if it is assumed that her preparation was contaminated with platelets. However, these C'4 titres of the platelets are too small to influence the level of C'4 activity in the serum, because C'4 titre of normal human sera is between $5\times10^{12}$ and $2\times10^{13}$ effective molecules per ml, and platelet counts in normal human blood are between about $2\times10^{5}$ to $3\times10^{5}$ per cmm. Thus C'4 activity derived from the total platelets in one ml of blood should be only $1\times10^{8}$ to $2\times10^{8}$ effective molecules. In fact, we have encountered two interesting cases of chronic myelogenous leukemia. In one case, the platelet count in the peripheral blood increased to about $2\times10^{6}$ per cmm for about a month, and in another case, the platelet count in the peripheral blood decreased to less than 500 per cmm for about a month, but in both cases, C'4 levels of sera were in almost within the normal range throughout these periods. Details of studies on the C'4 activities of the sera of these patients will be reported elsewhere.

Numerous studies have established the existence of several coagulation factors that either originate in platelets or are specifically adsorbed by platelets from plasma (ADELSON, RHEINGOLD and CROSBY, 1961). Moreover, normal platelets "have" many kinds of proteins almost identical to serum proteins. But the question as to whether the plasma proteins observed in platelets are carried by a simple adsorption was not answered yet (BEZKOROVAINY and RAFELSON, 1964). MALTANER also could not determine whether her preparations of guinea pig leucocytes "contain or convey" the complementary substance. In our experiments, when platelets were washed several times at 0°C, no C'4 activity was found in the supernatant. When the platelet suspension was incubated at 30°C, some C'4 activity was found in the supernatant, and it reached a maximum after 20 minutes incubation. The fact that this C'4 activity is easily eluted suggests that it may be originated from the surface of platelets (that is adsorbed C'4).

HJORT *et al.* (1953) reported that human platelets possessed an accelerator activity equal to about 6 per cent of the proaccelerin activity of normal citrated plasma, and trypsin destroyed 90 per cent or more of the platelet accelerator activity of normal platelets without altering their appearance. These workers concluded that the platelet accelerator was adsorbed plasma proaccelerin. Trypsin and hydrazine were found to destroy free C'4. If all C'4 is adsorbed on the surface of the platelets, all the activity should be destroyed by trypsinization. However, when platelets were trypsinized they still retained a moderate amount of C'4 activity, while hydrazine-treated platelets lost their C'4 activity completely. This suggests that hydrazine may penetrate into the platelets and inactivate the C'4 while trypsin does not because it is a larger molecule. The partial loss of C'4 activity in trypsinized platelets might be due to the invasion of trypsin into partially damaged platelets or to the digestion of some portion of the platelets. In either case, the platelets can be considered to contain C'4.

Thus it is concluded that platelets contain and adsorb C'4.

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