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REACTIVITY AND COMPATIBILITY BETWEEN THE SECOND COMPONENT OF COMPLEMENT AND EAC'1, 4 FROM DIFFERENT COMPONENT SOURCES[1]

KAZUYOSHI NAGAKI, KATSUMI FUJIKAWA and SHINYA INAI
The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka
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SUMMARY Human and guinea pig C'2 in whole serum and in a partially purified form were measured with EAC'1 hu, 4 hu, EAC'1 hu, 4 gp, EAC'1 gp, 4 hu and EAC'1 gp, 4 gp. The Tmax[2] of these cellular intermediates differed, and the decay rate constants of EAC'1, 4 with C'2 hu and of EAC'1, 4 with C'2 gp were 0.106 to 0.117 and 0.022 to 0.031, respectively. Very low reactivity of partially purified C'2 hu was elicited with EAC'1 hu, 4 gp and EAC'1 gp, 4 gp, but the reactivity of C'2 in whole human serum was more. There was little difference in the reactivities of guinea pig C'2 with four preparations of EAC'1, 4. The highest C'2 titers of human and guinea pig sera were obtained when measured with EAC'1 gp, 4 hu.

INTRODUCTION

Recent studies have shown that guinea pig complement is composed of at least nine components (INOUE *et al.*, 1965, 1966) and human complement of at least eight (HODDING *et al.*, 1966), but there is little information available about the interchangeability of components of human and guinea pig complement. BIER *et al.* (1945) showed that four components of human and guinea pig complement were mutually interchangeable. But these results were obtained with classical R-reagents, which have since been found to have certain limitations.

Since a method for titration of guinea pig C'2 using a cellular intermediate was first reported (BORSOS *et al.*, 1961a), methods for titration of the components of human and guinea pig complement using cellular intermediates have been developed by several investigators (BORSOS *et al.*, 1961a, HOFFMANN, 1960, INAI *et al.*, 1964). Recently, AUSTEN and BEER (1964) applied the principles of the method of BORSOS *et al.* to the measurement of C'2 in human complement and reported that it could be measured in the serum by its specific interaction with a cellular intermediate prepared with sensitized sheep erythrocytes and components of guinea pig complement.

To investigate the interchangeability of

1 Part of this paper was presented at the Third Symposium on Complement in July 1966 at Hakone, Japan.

2 The time at which the number of SAC' 1, 4, 2 sites reached a maximum.

human and guinea pig C'2, the specific interaction between human and guinea pig C'2 and various cellular intermediates prepared from various combinations of human and guinea pig components of complement should be studied. Recently, purification of the fourth component of human complement which contains almost negligible " C'1 destroyer " has been achieved (NAGAKI *et al.*, 1966), and using this the cellular intermediate, EAC'1,4 was successfully prepared. This cellular intermediate bound fair amounts of the first and fourth components of human complement and was suitable for use in titration of the second component of human complement.

This paper is on the kinetics and titration of the second component of human and guinea pig complements with four cellular intermediates prepared by various combinations of the first and fourth components of human and guinea pig complements.

MATERIALS

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

Sheep erythrocytes were stored at 4°C with an equal volume of Alsever's solution. Before use, erythrocytes were washed twice with physiological saline and once with buffer, and were adjusted to a concentration of 1×10^9 cells per ml.

Antibody against boiled sheep erythrocyte stromata was prepared by the method of KABAT and MAYER (1961).

Standardized sheep erythrocyte suspension was allowed to react with an equal volume of 1 : 300 antibody for 10 minutes at 37°C.

2. *Diluents*

Isotonic saline veronal gelatin (to 0.1%) buffers of 0.15 ionic strength containing Mg^{2+}(to 5.0×10^{-4}M) and/or Ca^{2+} (to 1.5×10^{-4}M) are referred to here as So‡ and So±. Isotonic saline sucrose veronal gelatin (to 0.1%) buffers of 0.09 ionic strength containing Mg^{2+} (to 5.0×10^{-4}M) and/or Ca^{2+}(to 1.5×10^{-4}M) are referred to as S_4‡ and S_4±. EDTA veronal buffer was prepared by diluting 0.30 M EDTA ten fold with isotonic saline veronal gelatin (to 0.1%) buffer without added Ca^{2+} or Mg^{2+}.

3. *Human and guinea pig sera*

Blood for human serum specimens was obtained from healthy donros. Guinea pig blood was obtained by cardiac puncture. Both bloods were allowed to clot at room temperature for about one hour and then centrifuged at 4°C. The sera were removed and were used on the same day or divided into portions and stored at −70°C.

4. *The first component of human complement* (C'1 hu)

The first component of human complement was isolated from the human euglobulin fraction by the same method used for isolation of the first component of guinea pig complement. This sample of C'1 hu had a hemolytic activity of 7.5×10^{11} effective molecules per ml, when titrated by the method of BORSOS *et al.*

5. *The second component of human complement* (C'2 hu)

Two columns (each 6×60 cm) were packed with 80 gm of DEAE cellulose (Brown Comp.) and equilibrated at pH 7.4 with 0.09 M NaCl in 0.01 M Tris-HCl buffer. A further column (3×60 cm) was packed with 6 gm of CM-Sephadex C-50 (Pharmacea Comp.), and equilibrated at pH 6.0 with 0.1 M NaCl in 0.01 M phosphate buffer containing 1 mM EDTA. About 700 ml of pooled human serum were adjusted to pH 5.8 with 0.15 N HCl, dialyzed against 20 *l* of cold distilled water overnight and centrifuged, and the supernatant was adjusted to pH 7.4 with 0.09 N NaOH and the ionic strength to 0.09 with 3 M NaCl. The supernatant was divided into two portions, which were applied to the two DEAE cellulose columns, and were eluted with 0.09 M NaCl in 0.01 M Tris-HCl buffer. The first 350 ml of eluate were discarded and the next 550 ml were collected and pooled. Then EDTA was added at a final concentration of 1 mM. This material was then concentrated to about 200 ml by ultrafiltration and adjusted to pH 6.0 with 0.1 N HCl. It was applied to the CM-Sephadex C-50 column, which was then thoroughly washed with about 1300 ml of 0.10 M NaCl in 0.01 M phosphate buffer of pH 6.0 containing 1 mM EDTA and then with 0.20 M NaCl in the same buffer. The active fractions were pooled, neutralized to pH 7.4 with

0.01 M Na_2HPO_4 and stored at −20°C. This partially purified C'2 hu had no C'1 or C'4 activity.

6. *The fourth component of human complement* (C'4 hu)

The fourth component of human complement was isolated from the human pseudoeuglobulin fraction employing two DEAE cellulose columns at pH 7.4, a CM-Sephadex C-50 column at pH 5.5 and Pevikon C-870 block zone electrophoresis at pH 8.6. This purified C'4 hu gave a single line on immunoelectrophoresis against potent antihuman serum horse serum obtained from the Osaka Microbial Diseases Research Foundation and contained almost negligible "C'1 destroyer" activity. One mg of this C'4 hu protein had a hemolytic activity of 2.2×10^{13} effective molecules.

Details of the method for purification of C'4 hu and its nature will be published in another paper.

7. *The first component of guinea pig complement* (C'1 gp)

The first component of guinea pig complement was obtained by the method of Borsos *et al.* (1963), and contained no C'2 or C'4 activity. Various lots of C'1 gp had hemolytic activities of 7.5×10^{11} to 13.5×10^{11} effective molecules per ml when titrated by the method of Borsos *et al.* (1963).

8. *The second component of guinea pig complement* (C'2 gp)

The second component of guinea pig complement was separated by the method of Borsos *et al.* (1961 b). Various lots of C'2 gp had hemolytic activities of 1.2×10^{11} to 11.3×10^{11} effective molecules per ml and contained no C'1 or C'4 activity.

9. *R-reagents of human and guinea pig sera*

R1 and R4 of human and guinea pig sera were prepared by the method of Kabat and Mayer. R1, 4 of human and guinea pig sera were prepared from R4 by dialysis overnight against cold distilled water.

10. *EDTA treated guinea pig complement* (C'-EDTA)

Guinea pig complement was diluted to 1 : 12.5 with 0.03 M EDTA veronal buffer and used as a source of the complex of the third component of complement for titration of C'2.

To 0.5 ml of 1 : 12.5 C'-EDTA was added 2.5 ml of EAC'1, 4, 2 containing 7.5×10^7 cells.

METHODS AND RESULTS

1. *Kinetic analysis of the formation of* EAC'1, 4, 2

(1) *Preparation of* EAC'1, 4

Various samples of EAC'1, 4 were prepared as described below and used for kinetic analyse of the formation and decay of EAC'1, 4, 2 and for measurement of C'2.

a) EAC'1 hu, 4 hu

To EA suspended in $So^{\pm}$ were added 300 effective molecules per cell of C'1 hu. The mixture was incubated at 30°C for 15 minutes, washed once and resuspended in the original volume of $So^{\pm}$ (EAC'1 hu). Purified C'4 hu was added to EAC'1 hu so as to supply 300 effective molecules per cell, sensitized at 37°C for 20 minutes, washed once and resuspended in $So^{\ddagger}$ at a concentration of 5×10^7 cells per ml.

b) EAC'1 hu, 4 gp

EAC'4 gp, prepared from EAC'1 gp, 4 gp by treatment with EDTA veronal buffer, was suspended in $S_4^{\pm}$ and mixed with 300 effective molecules per cell of C'1 hu. The mixture was incubated at 30°C for 15 minutes, washed once and resuspended in $So^{\ddagger}$ at a concentration of 5×10^7 cells per ml.

c) EAC'1 gp, 4 hu

To EA suspended in $S_4^{\pm}$ were added 300 effective molecules per cell of C'1 gp. The mixture was incubated at 30°C for 15 minutes, washed once and resuspended in $S_4^{\pm}$ (EAC'1 gp). This EAC'1 gp was mixed with 300 effective molecules per cell of purified C'4 hu. The mixture was incubated at 37°C for 20 minutes, washed once and suspended in $So^{\ddagger}$ at a concentration of 5×10^7 cells per ml.

d) EAC'1 gp, 4 gp

For preparation of EAC'1 gp, 4 gp, the method of Nishioka *et al.* (1963) was employed, but $S_4^{\pm}$ was used as diluent throughout instead of $So^{\pm}$. The final concentration was adjusted to 5×10^7 cells per ml in $So^{\ddagger}$.

(2) *Formation of* EAC'1, 4, 2

The kinetics of EAC'1, 4, 2 formation were analyzed using one of four preparations of EAC'1, 4 described above and C'2 hu or C'2 gp. The optimal dilutions of C'2 hu and C'2 gp were determined by preliminary tests on each cellular intermediate.

The procedures used for analysis of the kinetics of formation of EAC'1, 4, 2 were as follows : at zero time 27 ml of EAC'1, 4 in $So^{\ddagger}$ (5×10^7 cells per ml), were prewarmed to 30°C and then mixed 18 ml of C'2 in $So^{\ddagger}$, also at 30°C. After suitable intervals, 2.5 ml samples were withdrawn and introduced into

0.5 ml of 1 : 12.5 C′-EDTA. These mixtures were immediately transferred to a water bath at 37°C and incubated for 60 minutes with constant mechanical rocking. Then 4.5 ml of cold 0.15 M NaCl were added to each tube and the contents were mixed and centrifuged. The oxyhemoglobin contents of the supernatant fluids were measured by the optical density at 414 mμ in a Hidachi spectrophotometer using a cuvette of 1 cm light path.

As shown in Figs. 1, 2, 3 and 4, use of EAC′1, 4 and C′2 from different sources resulted in marked differences in EAC′1, 4, 2 formation. These experiments were repeated, and the data obtained are summarized in Table 1.

Using four preparations of EAC′1, 4, composed of different combinations of C′1 and C′4, but C′2 from a single source, different values of Tmax were obtained. Moreover, when a sample of EAC′1, 4 composed of a fixed combination of C′1 and C′4 was allowed to react with C′2 from different sources, different values of Tmax were observed.

When C′2 hu was added to EAC′1 gp, 4 gp, the value of Tmax was longer than that with C′2 gp, but when C′2 hu was added to three other preparations of EAC′1, 4, the values of Tmax were shorter than that with C′2 gp.

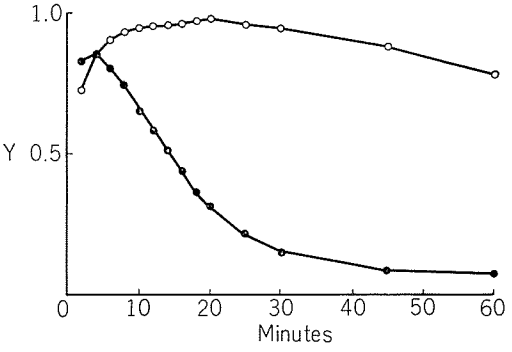

FIGURE 1 EAC′1, 4, 2 formation at 30°C.
Sources of Components
C′1 : Human
C′4 : Human
○———○ Guinea Pig C′2
●———● Human C′2

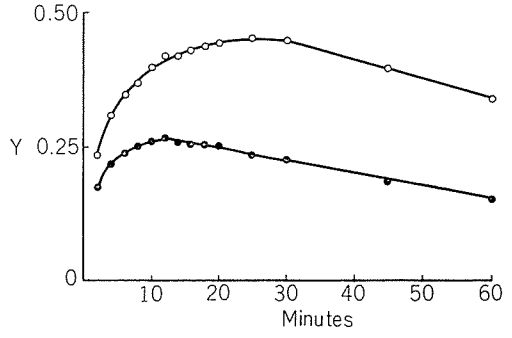

FIGURE 2 EAC′1, 4, 2 formation at 30°C.
Sources of Components
C′1 : Human
C′4 : Guinea Pig
○———○ Guinea Pig C′2
●———● Human C′2

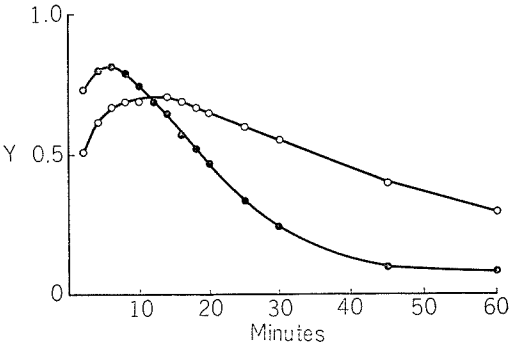

FIGURE 3 EAC′1, 4, 2 formation at 30°C.
Sources of Components
C′1 : Guinea Pig
C′4 : Human
○———○ Guinea Pig C′2
●———● Human C′2

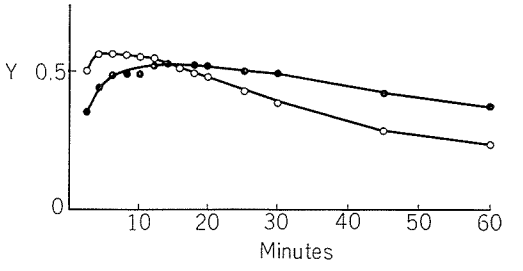

FIGURE 4 EAC′1, 4, 2 formation at 30°C.
Sources of Components
C′1 : Guinea Pig
C′4 : Guinea Pig
○———○ Guinea Pig C′2
●———● Human C′2

TABLE 1 *Tmax of EAC'1,4 Cells* (minutes)

Sources of Components of EAC'1,4		Source of C'2	
C'1	C'4	Human	Guinea Pig
Human	Human	4-6	18-20
Human	Guinea Pig	10-12	25-30
Guinea Pig	Human	6-12	14-16
Guinea Pig	Guinea Pig	12-20	4-12

2. *Kinetic analysis of the decay of* EAC'1, 4, 2

In titration of C'2, the magnitude of the rate constant of decay (K) of EAC'1, 4, 2 must be known.

To measure K, 15 ml of prewarmed EAC'1, 4 (10^8 cells per ml) were mixed with appropriate dilutions of prewarmed C'2, so as to generate between one and two SAC'1, 4, 2 per cell at Tmax, at 30°C. The mixtures were kept at 30°C for the period required to reach Tmax and then chilled, washed twice with ice cold So‡ at 0°C and resuspended in 50 ml of ice cold So‡. Then 2.5 ml portions of EAC'1, 4,2 were pipetted into a series of test tubes in an ice bath. All 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 prewarmed C'-EDTA (1 : 12.5) were added to the tubes which were then transferred to a water bath at 37°C for a further 60 minutes. Then 4.5 ml of cold 0.15 M NaCl was added to each tube and the contents were mixed and centrifuged. The oxyhemoglobin contents of the supernatants were measured at 414 mμ. Then the number of SAC'1, 4, 2 per cell, i.e., $Z = -ln(1-y)$, was calculated from the degree of lysis, y, and log Z was plotted against the time of incubation of EAC'1, 4, 2 at 30°C. The results gave a straight line.

As seen in Figs. 5, 6, 7 and 8 and in Table 2, the rate constant of decay, K, of EAC'1, 4, 2 gp ranged from 0.022 to 0.031, whereas the K value of EAC'1, 4, 2 hu was between 0.106 and 0.117. The T half of EAC'1, 4, 2 gp, that is the time required to reduce the number of SAC'1, 4, 2 per cell by one half due

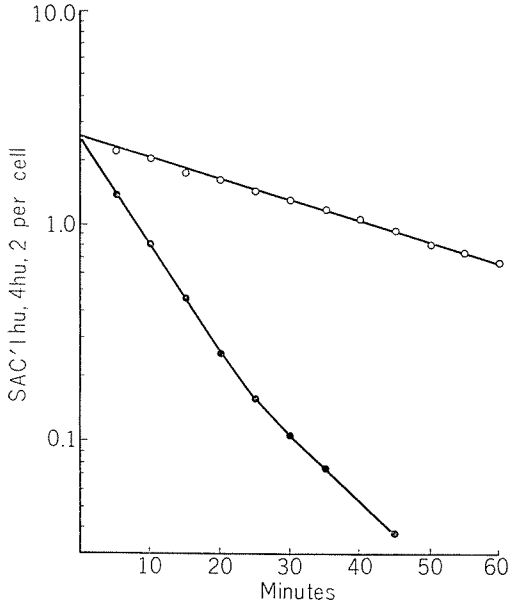

FIGURE 5 Decay of SAC',1 4, 2 sites at 30°C.
Sources of Components
C'1 : Human
C'4 : Human
○——○ Guinea Pig C'2
●——● Human C'2

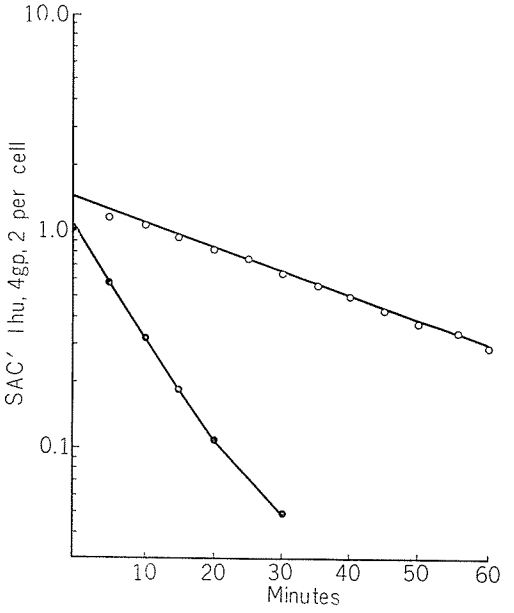

FIGURE 6 Decay of SAC'1, 4, 2 sites at 30°C.
Sources of Components
C'1 : Human
C'4 : Guinea Pig
○——○ Guinea Pig C'2
●——● Human C'2

to their decay to SAC'1, 4, was also much longer than that of EAC'1, 4, 2 hu.

From these results, it is concluded that the rate constant of decay and the half life of SAC'1, 4, 2 are independent on the sources of C'1 and C'4, but dependent on the source of C'2.

3. *Titration of the second component of complement in human and guinea pig sera and of partially purified second component of complement obtained from human and guinea pig sera*

C'2 was titrated as follows: one milliliter aliquots of serial two-fold dilutions of sample were preincubated in a water bath at 30°C, and then mixed with 1.5 ml of prewarmed EAC'1, 4 (5×10^7 cells per ml). The mixtures were incubated for the period required to reach Tmax with constant shaking. At the end of this period, 0.5 ml of C'-EDTA (1 : 12.5) was added to each tube and tubes were then incubated for a further 60 minutes at 37°C with constant shaking. Then 4.5 ml of cold 0.15 M NaCl was added to each tube with thorough mixing and tubes were centrifuged. The oxyhemoglobin contents of the supernatants were measured at 414 mμ. When the proportion of the cells lysed was defined as y, and $-ln(1-y)$ were plotted against the reciprocal of the dilution of sample, a straight line was obtained up to about 80% lysis. The value of $-ln(1-y)$ was termed the Z value (BORSOS *et al.*, 1961 a), and in this titration refers to the number of SAC'1, 4, 2 per cell. The reciprocal of dilution of the sample which produces one SAC'1, 4, 2 per cell was arbitarily defined as the C'2 units per ml of sample.

As shown in Table 3, there was marked difference in the reactivities of partially purified C'2 hu with four kinds of EAC'1, 4. Partially purified C'2 hu was most reactive with EAC'1 gp, 4 hu, and least reactive with EAC'1 hu, 4 gp and EAC'1 gp, 4 gp. In contrast, there was little difference in the reactivities of partially purified C'2 gp with four kinds of EAC'1, 4, though the titers measured with EAC'1 gp, 4 hu and EAC'1 gp, 4 gp were slightly higher

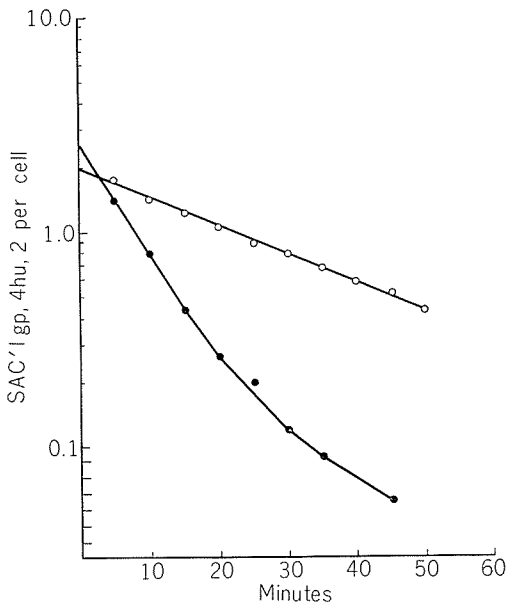

FIGURE 7 Decay of SAC'1, 4, 2 sites at 30°C.
Sources of Components
C'1 : Guinea Pig
C'4 : Human
○——○ Guinea Pig C'2
●——● Human C'2

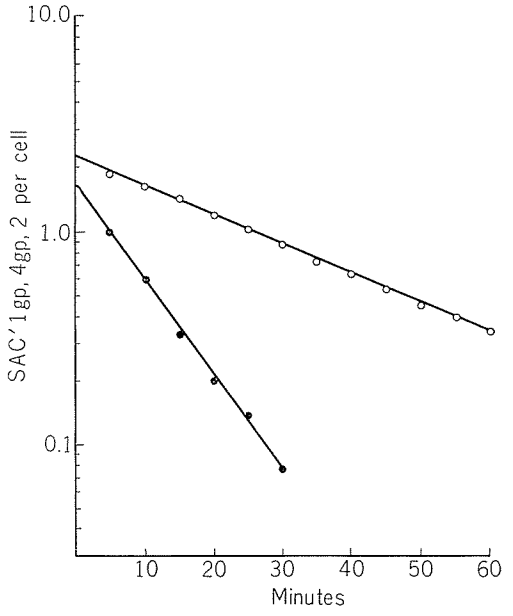

FIGURE 8 Decay of SAC'1, 4, 2 sites at 30°C.
Sources of Components
C'1 : Guinea Pig
C'4 : Guinea Pig
○——○ Guinea Pig C'2
●——● Human C'2

TABLE 2 *Rate Constant of Decay (K) and Thalf of EAC'1,4,2 Cells*

Sources of Components of EA'1,4,2			K (min.$^{-1}$)	Thalf (min.)
C'1	C'4	C'2		
Human	Human	Human	0.106	6.5
		Guinea Pig	0.022	31.1
Human	Guinea Pig	Human	0.110	6.5
		Guinea Pig	0.024	28.5
Guinea Pig	Human	Human	0.117	6.0
		Guinea Pig	0.031	22.0
Guinea Pig	Guinea Pig	Human	0.106	6.5
		Guinea Pig	0.030	23.0

TABLE 3 *Titers of Partially Purified C'2 with Four Kinds of EAC'1,4 Cells*

Sources of Components of EAC'1,4		Partially Purified Human C'2		Partially Purified Guinea Pig C'2	
C'1	C'4	Lot A	Lot B	Lot A	Lot B
Human	Human	215	470	425	650
Human	Guinea Pig	9.5	1.5	340	770
Guinea Pig	Human	520	865	1,430	1,550
Guinea Pig	Guinea Pig	21	15.5	2,850	3,000

TABLE 4 *Titers of C'2 in Pooled Sera with Four Kinds of EAC'1,4 Cells*

Sources of Components of EAC'1,4		C'2 in Pooled Human Serum		C'2 in Pooled Guinea Pig Serum	
C'1	C'4	Lot A	Lot B	Lot A	Lot B
Human	Human	890	930	14,000	16,800
Human	Guinea Pig	280	490	18,500	20,500
Guinea Pig	Human	1,550	960	41,000	26,500
Guinea Pig	Guinea Pig	385	285	32,000	42,000

than those measured with EAC'1 hu, 4 hu and EAC'1 hu, 4 gp.

However, as shown in Table 4, when samples of C'2 in pooled human and pooled guinea pig sera were used, there was not so marked a difference in the reactivities to four kinds of EAC'1, 4 as in the case with partially purified C'2 hu, although, slightly lower titers were obtained for EAC'1 hu, 4 gp or EAC'1 gp, 4 gp with C'2 in human serum and for EAC'1 hu, 4 hu or EAC'1 hu, 4 gp with C'2 in guinea pig serum.

From these results, the following conclusions are drawn.

(1) On titration of partially purified C'2, the sources of C'1 and C'4 used to form EAC'1, 4 affect the results. Partially purified C'2 hu showed high titers when measured with EAC'1, 4 to which C'4 hu was bound, and partially purified C'2 gp showed high titers when measured with EAC'1, 4 to which C'1 gp was bound.

(2) There was no marked difference in the titer when C'2 in human serum was measured with

four kinds of EAC'1, 4 unlike the case with partially purified C'2 hu. There were slight differences between the titers of C'2 in guinea pig serum when measured with four kinds of EAC'1, 4, as in the case with partially purified C'2 gp. The titer of C'2 in guinea pig serum is about half to those of C'1 and C'4, but the titer of C'2 in human serum is about a hundredth to those of C'1 and C'4. Thus, at a dilution of human serum suitable for titration of C'2, there are still high concentrations of C'1 and C'4, which may form new SAC'1 hu, 4 hu on EAC'1 hu, 4 gp and EAC'1 gp, 4 gp as active sites for C'2 hu.

The above conclusions were tested by further experiments.

4. *Titration of the second component of complement in human and guinea pig R-reagents*

C'2 in human and guinea pig R1, R4 and R1, 4 were titrated by the same method as that described in section 3. At the same time the value of C'2 in the original serum was measured.

As shown in Tables 5 and 6, C'2 in human and guinea pig R1, R4 and R1, 4 showed almost the same reactivities with four kinds of EAC'1, 4 as those of partially purified C'2 hu and C'2 gp, although the C'2 titers varied with each R-reagent.

These results did not explain the difference in reactivity of purified C'2 with four kinds of EAC'1, 4. However, the conclusion that C'1 and C'4 in human serum may participate in forming new active sites on EAC'1, 4 for C'2 in human serum (SAC'1 hu, 4 hu) was confirmed, because the reactivities of C'2 in serum lacking C'1 and/or C'4 with four kinds of EAC'1, 4 showed the same pattern as those of partially purified C'2.

TABLE 5 *Titers of C2, C1 and C4 in Human R1, R4, R1,4 and Their Original Serum*

Sources of Components of EAC'1,4		C'2 Titers in Human Serum			
C'1	C'4	Original Serum	R1	R4	R1,4
Human	Human	930	550	535	300
Human	Guinea Pig	490	8.9	42	1.2
Guinea Pig	Human	960	1,230	555	520
Guinea Pig	Guinea Pig	285	120	31.5	10.0
Titers of C'1 and C'4					
	C'1	116,000	0	35,000	44
	C'4	88,000	122,000	0	0

TABLE 6 *Titers of C'2, C'1 and C'4 in Guinea Pig R1, R4, R1,4 and Their Original Serum*

Sources of Components of EAC'1,4		C'2 Titers in Guinea Pig Serum			
C'1	C'4	Original Serum	R1	R4	R1,4
Human	Human	16,800	10,200	14,000	8,900
Human	Guinea Pig	20,500	7,000	17,800	17,000
Guinea Pig	Human	26,500	21,000	24,500	22,000
Guinea Pig	Guinea Pig	42,000	22,500	40,500	36,000
Titers of C'1 and C'4					
	C'1	63,500	0	26,000	14.3
	C'4	72,000	39,000	0	0

DISCUSSION

In 1945, BIER *et al.* showed that each component of lhuman complement might be replaced by the corresponding component of guinea pig complement and *vice versa* in immune hemolysis, and other workers have confirmed these results. But these studies were made with classical R-reagents, which have since been shown to have certain limitations.

In 1964, the fourth component of human complement was titrated with the cellular intermediates, EAC'1 gp, C'2 gp and guinea pig C'-EDTA (INAI *et al.*, 1964). And, the first component of human complement was measured with EAC'4 gp, C'2 gp and guinea pig C'-EDTA. C'2 in human serum was measured by its interaction with EAC'1 gp, 4 gp by AUSTEN and BEER (1964). But, these authors employed only one cellular intermediate for titration of C'2 in human serum. Experiments on the decay of EAC'1 gp, 4 gp, 2 have been made using EAC'1 gp, 4 gp and a mixture of diluted human serum and partially purified C'2 gp. However, the results cannot be regarded as giving a true decay curve of EAC'1 gp, 4 gp, 2 hu.

It is very difficult to prepare EAC'1 hu, 4 hu containing sufficient amounts of bound C'1 hu and C'4 hu, because in human as well as in guinea pig serum, the existence of a substance which inactivates C'1 has been reported. This substance was named C'1 destroyer (NAGAKI *et al.*, 1965) and C'1 inactivator (NELSON *et al.*, 1966) in human and guinea pig sera, respectively. This substance was suggested to be identical with C'1 esterase inhibitor. It was reported that partially purified guinea pig C'4 was contaminated with C'1 inactivator (NELSON *et al.*, 1966). MÜLLER-EBERHARD *et al.* did not refer to contamination of their purified preparation of human C'4 with C'1 destroyer (1963). Efforts have been made to remove C'1 destroyer from purified preparations of human C'4 and successful results have been obtained (NAGAKI *et al.*, 1966). With this purified C'4 hu and partially purified C'1 hu, EAC'1 hu, 4 hu which had a Tmax of 6 minutes was prepared. Thus, four cellular intermediates, EAC'1 hu, 4 hu, EAC'1 hu, 4 gp, EAC'1 gp, 4 hu and EAC'1 gp, 4 gp were prepared and their reactivities with C'2 hu and C'2 gp were compared.

A marked difference was seen in the formation of EAC'1,4,2 cell. According to the theoretical analysis reported by BORSOS *et al.* (1961a), Tmax depends upon the average number of SAC'1,4, the specific rate constant of formation of SAC'1,4,2 and the decay constant of SAC'1,4,2. In the present experiments, Tmax of each EAC'1,4 preparation varied with the source of C'2. The average number of SAC'1,4 of each EAC'1,4 must be fixed, and so the difference in the Tmax of EAC'1,4,2 hu and EAC'1,4,2 gp must be due to a difference in the formation constant and or decay constant of SAC'1,4,2.

No experiments were made on the rate constant of formation of SAC'1,4,2.

From experiments on the rate constant of decay of SAC'1,4,2, it was concluded that this constant varied with the source of C'2 but not with the source of SAC'1,4. AUSTEN and BEER employed a mixture of partially purified C'2 gp and diluted human serum for experiments on decay because they could not use diluted human serum as a source of C'2. These experiments led them to conclude that the rate constant of decay of SAC'1 gp, 4 gp, 2 gp/hu was identical with that of SAC'1 gp, 4 gp, 2 gp. However, it was clear that the rate constant of decay of SAC'1,4,2 hu must be much greater than that of SAC'1,4,2 gp and the half life of SAC'1,4,2 hu must be much shorter than that of SAC'1,4,2 gp, when partially purified C'2 hu was employed.

There were marked differences in the reactivities of partially purified C'2, and especially C'2 hu, with four kinds of EAC'1,4. The titers of partially purified C'2 hu measured with EAC'1 hu, 4 gp and EAC'1 gp, 4 gp were much lower than those with EAC'1 hu, 4 hu and EAC'1 gp, 4 hu. However, when C'2 in whole serum were measured, there was

no marked difference in the reactivities of C'2 hu and C'2 gp. Moreover, when C'2 in R-reagents, R1, R4 and R1,4, were measured, their C'2 reactivities were similar to that of partially purified C'2. The following conclusions are drawn from these results. At the optimal dilution of human serum for titration of C'2, there are high concentrations of C'1 and C'4 which form SAC'1 hu, 4 hu sites on EAC'1,4 and react with C'2. However, at the optimal dilution of guinea pig serum for titration of C'2, there are only one to two molecules of C'1 and C'4. Hence, the reactivities of partially purified C'2 hu with four kinds of EAC'1,4 differ from those of C'2 in human serum, while the reactivities of partially purified C'2 gp are similar to those of C'2 in guinea pig serum.

Human C'2, and especially purified C'2 hu, gave very low titers with EAC'1 hu, 4 gp and EAC'1 gp, 4 gp, and guinea pig C'2 gave relatively low titers with EAC'1 hu, 4 hu and EAC'1 hu, 4 gp. These phenomena might be due to the incompatibility of human C'2 with C'4 gp and of guinea pig C'2 with C'1 hu, but this possibility requires investigation.

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