Variance in the Activity of the Fourth Component of Mouse Complement

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FUKUOKA, Y., OKUDA, T. and TACHIBANA, T. Variance in the Activity of the Fourth Component of Mouse Complement. Tohoku J. exp. Med., 1983, 141 (2), 225-235 — There are two methods for hemolytic titration of 04. One is a method using EAC\textsuperscript{EP}, oxyC\textsubscript{2hu} and EDTA-C\textsuperscript{EP} (E-C method) and the other using EAC\textsuperscript{EP}, oxyC\textsubscript{2hu} and C4D\textsuperscript{EP} (C4D method). The 04 titers obtained by these two methods differed depending on whether the 04 exists in plasma or in serum. In the case of Ssh strain, serum 04 activity assayed by the E-C method was higher than that by the C4D method, while plasma 04 activity assayed by the E-C method was lower than that by the C4D method. It was hypothesized that these results would occur from the difference in the state of 04 molecule present in serum or plasma. From chromatographic analysis, it was apparent that the 04 molecule in serum existed in at least four different molecular states; free 04 molecule with low Ss antigenicity, hemolytically inactive Ss protein, active 04 with high molecular weight and low Ss antigenicity, and inactive Ss protein with high molecular weight. Their expression of Ss antigen and the hemolytic 04 activity were different, and each type of 04 molecule behaved differently in the assay of the two methods. Furthermore an important role of mouse C5 in the hemolytic assay of mouse 04 is discussed.

The genetically defined mouse is frequently used as an experimental animal in immunological research. However, most of information on the complement system has thus far been obtained from human and guinea pig complement, so the characterization of the mouse complement system has become important. Since the report of Démant et al. (1973), the similarity between the Ss protein which is controlled in the S region of the H-2 complex and mouse 04 has been shown (Meo et al. 1975; Ferreira et al. 1978; Ross et al. 1978; Parker et al. 1980). The authors reported in a previous paper (Fukuoka et al. 1982) that 04 hemolytic activity could be measured using EDTA treated guinea pig serum and the level of

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Abbreviations used in this paper: H-2, major histocompatibility in mouse; Ss, serum serological substance; Ss-bp, Ss binding protein; C4D, C4 deficient; SRBC, sheep red blood cell; EA, SRBC sensitized with anti-SRBC antiserum; VBS, isotonic veronal-buffered saline; GVB\textsuperscript{++}, VBS containing 0.1% gelatin, 0.5 mM MgCl\textsubscript{2} and 0.15 mM CaCl\textsubscript{2}; glu-GVB\textsuperscript{++}, low ionic strength VBS containing 2.5% glucose, 0.1% gelatin, 0.5 mM MgCl\textsubscript{2} and 0.15 mM CaCl\textsubscript{2}; EDTA-GVB, VBS containing 0.1% gelatin and 0.04 M EDTA; VBS\textsuperscript{++}, VBS containing 1 mM MgCl\textsubscript{2} and 0.15 mM CaCl\textsubscript{2}; EDTA-VBS, VBS containing 2 mM EDTA; EACA, ε-amino caproic acid; SRID, single radial immunodiffusion.
C4 activity did not always correlate with the estimated amount of Ss antigen. A different method for the titration of mouse C4 activity was reported by Ferreira et al. (1978), who showed a correlation between the levels of C4 activity and Ss protein. In the current paper, it is shown that there are differences in the functional and molecular states of C4 molecule present in serum and plasma.

**MATERIALS AND METHODS**

**Mice**

Specific pathogen free BALB/c mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, and ICR, C3H/He mice from the Funabashi Farm, Chiba. DDI mice were supplied from the Mouse Breeding House, Tohoku University, and DDD, DDK, B10.D2/6, A/J, AKR and CBA mice from the Institute of Medical Science, Tokyo University. All mice used were reared in our laboratory until 12-15 weeks of age.

**Serum and plasma**

After anesthetized mice were bled by cardiac puncture, the blood was allowed to clot for 15 min at room temperature, kept on ice for 60 min and centrifuged to separate serum. For plasma 1/10 volume of 0.1 M EDTA was taken in a syringe and each mouse was bled by cardiac puncture. The blood was kept on ice and centrifuged as soon as possible. Serum and plasma were stored at -70°C until used.

**Buffers and cellular intermediates**

GVB+, glu-GVB++ and EDTA-GVB were prepared as previously described (Fukuoka et al. 1982). EAC1 and EAC142 were prepared by using guinea pig C1, human C4 and oxidized human C2. EACmo was made by reacting one volume of 1:30 diluted BALB/c male serum with an equal volume of EA (1 x 10⁹ cells/ml) at 37°C for 15 min in GVB++. Then the cells were incubated in 0.01 M EDTA-GVB at 37°C for 60 min. After washing, the cells were suspended in GVB++ (2 x 10⁸ cells/ml).

**Antisera**

Anti-Ss serum was prepared according to the method of Passmore and Biesel (1977), and checked with the standard antiserum kindly provided by Dr. V. Nussenzweig. Anti-Ss-bp serum was kindly supplied from Dr. M. Takahashi. Mouse antiserum to mouse C5 (MuB1) was prepared by the method of Cinader et al. (1964).

**Quantitation of the Ss, Ss-bp and MuB1 protein**

The concentration of Ss protein was estimated by a single radial immunodiffusion technique (Mancini et al. 1965). The Ss protein in the column fractions was estimated by hemagglutination inhibition of EACmo with diluted anti-Ss serum on microtiter plate. The levels of the Ss-bp and C5 protein were measured by rocket immunoelectrophoresis according to the method of Laurell (1972), and expressed as relative levels to pooled serum of normal BALB/c male mice.

**Purification of mouse C5 from plasma**

This process will be described in detail in a separate publication. Briefly, after addition of phenylmethyl-sulfonyl-fluoride (1 mM) to mouse EDTA plasma, the 4-12% polyethylene glycol 4000 precipitate was dissolved in 0.02 M phosphate buffer (pH 7.4) with 2 mM EDTA and 10 mM EACA, and chromatographed on QAE-Sephadex with the same buffer. The C5 fraction eluted with 0.1 M NaCl was concentrated and passed through a Sephadex G-200 and finally applied to a column of Sepharose 4B conjugated with IgG fraction of anti-mouse C5 deficient serum. The recovery of C5 activity was 10%.

**Complement titration**

Two different methods were used for titration of C4 hemolytic activity. One is described in a previous paper (Fukuoka et al. 1982). Briefly, equal volumes of sample, EAC1 exp (1 x 10⁸ cells/ml) and oX Y C2ha were incubated at 37°C for 10 min. After addition of
guinea pig serum at 1:20 dilution in 0.04 M EDTA-GVB, further incubation at 37°C for 60 min was done, then the reciprocal of sample dilution giving 50% hemolysis was determined by visual inspection. This method is termed the E-C method. The other is a minor modification of the method of Ferreira et al. (1978). Serial dilutions of test samples were incubated with an equal volume of EAC18P (1 × 10⁸ cells/ml) at 37°C for 10 min, and oxYC2hu and 1:100 diluted C4D guinea pig serum were added. This method is termed the C4D method. The R4 reagent was prepared according to Lachmann et al. (1973), and used for some experiments instead of C4D serum.

Hemolytic assay for C5 was performed by using EAC14oxY2hu (1 × 10⁸ cells/ml) and 1:15 diluted B10.D₂/o male plasma with GVB++.

**RESULTS**

*Correlation between the level of C4 hemolytic activity and Ss protein levels in plasma*

In a previous paper, the authors showed that the C4 titer in serum obtained by the E-C method does not always correlate with the level of Ss antigen (Fukuoka et al. 1982). Those results did not coincide with the observation of Ferreira et al. (1978) in which C4 titer in plasma was determined by the C4D method. To examine this disparity, titration of C4 activity in plasma was carried out using the E-C and C4D methods. As shown in Fig. 1, the C4 titer in plasma obtained by both E-C and C4D methods increased with age in males and slightly increased with age in females. The level of Ss antigen also increased with age in a manner similar to that of the C4 titer (Fig. 2). Therefore in the case of plasma, good correlation between C4 titer and Ss antigen level could be seen.

![Fig. 1. The age dependent changes of C4 titer in plasma from BALB/c mice. Each point represents the mean C4 titer of five male (●—●, △—△) and female (○—○, ◊—◊) mice by the E-C and C4D methods, respectively, and bars indicate the range of maximum and minimum C4 titers.](image)

**Differences of C4 activity in serum and plasma of various inbred strains**

The results of C4 titration using various strains which differ in the content of Ss protein and C5 protein are shown in Table 1. In the case of Ss₅C₅⁺ strain, C4 titer of plasma was the same by either method, while C4 titer of serum assayed by
the C4D method was lower than that by the E-C method. Though the Ss\textsuperscript{4}C5\textsuperscript{+} strain showed the same C4 titer as Ss\textsuperscript{b} strain in serum by the E-C method, low titers were obtained in plasma by either method. Moreover, Ss\textsuperscript{b}C5\textsuperscript{-} strain showed no C4 activity in plasma by either method, and C4 activity in serum only by the E-C method. Ss\textsuperscript{4}C5\textsuperscript{-} strain showed results similar to those obtained in Ss\textsuperscript{b}C5\textsuperscript{-} strain. As described above, it is evident that these two methods of C4 titration give quite different results for various strains irrespective of the amount of Ss protein.

**Elution profile of C4 activity on Sephadex G-200 by the E-C and C4D methods**

Ferreira et al. (1977) described a new protein in mouse serum which forms complexes with the Ss protein, which appeared in the first protein peak on Sephadex G-200, and which they labeled Ss binding protein (Ss-bp). Ss-bp is not
associated with the Ss protein in plasma collected with EDTA. To examine whether the difference in C4 titers of serum and plasma depends on the presence of either free or complex form of Ss protein, C4 activity of serum and plasma on Sephadex G-200 were measured by both the E-C and C4D methods.

When DDI (Ss\textsuperscript{b}C5\textsuperscript{+}) plasma was eluted with EDTA-VBS (Fig. 3), C4 activity could be detected at the ascending limb of the second protein peak (middle protein peak) by either method with yields of 90%. The Ss protein also appeared in the same position. When DDI serum was eluted with VBS\textsuperscript{++} (Fig. 4), however, C4 activity was detected by the E-C method at the first and the middle protein peak with a recovery of more than 90%, and the Ss protein was also found at the same position. C4 activity measurable by the C4D method was found only in the middle protein peak.

When the ICR (Ss\textsuperscript{b}C5\textsuperscript{+}) serum was eluted with EDTA-VBS, C4 activity

![Figure 3](image-url)  
**Fig. 3.** Sephadex G-200 gel filtration of DDI plasma with EDTA-VBS containing 10 mM EACA. Similar results were obtained with ICR and BALB/c plasma.

![Figure 4](image-url)  
**Fig. 4.** Sephadex G-200 gel filtration of DDI serum with VBS\textsuperscript{++} containing 10 mM EACA and 10 mM benzamidine. Similar results were obtained with ICR and BALB/c serum.
was found only in the middle protein peak by either method with a recovery of about 20%, showing the same elution pattern of Ss protein as VBS++ (data not shown). In the case of the Ss<sup>C5</sup> strain serum eluted with VBS++, C4 activity could be detected only at the first protein peak by the E-C method. Moreover, a similar result was obtained when Ss<sup>C5</sup>- strain serum was eluted with VBS++ (data not shown).

### Further characterization of C4 molecule in serum of Ss<sup>b</sup> strain

When the C4 fraction of the first protein peak on Sephadex G-200 in Fig. 4 was further chromatographed on QAE-Sephadex, most of the C4 activity could be separated from the Ss antigen fraction with a recovery of 60% (Fig. 5). The Ss-bp was detected as two peaks coinciding with the C4 activity peak and the Ss antigen peak. The middle protein peak in Fig. 4 was chromatographed on QAE-Sephadex, and the C4 activity could be also separated from the Ss antigen (data not shown). The results suggest that there are two types of Ss-bp complexes in the first protein peak, that is, C4/Ss-bp complex and Ss/Ss-bp complex.

These two complex fractions were separately applied on Sephadex G-200 with high salt concentration buffer. When C4/Ss-bp complex was applied, C4 activity was detected at the position corresponding to the first protein peak (Fig. 6A). When Ss/Ss-bp complex was applied, however, Ss antigen was detected at the position corresponding to the middle protein peak (Fig. 6B). When the Ss-bp fraction and Ss fraction in Fig. 6B were mixed and eluted on Sephadex G-200 with VBS++, most of the Ss antigen could be detected at the first protein peak (Fig. 6C). It is therefore clear that the association of the inactive Ss protein and Ss-bp was reversible depending on both divalent cation and ionic strength of the buffer.

![Fig. 5. QAE-Sephadex chromatography of ICR serum C4 fraction derived from the first protein peak on Sephadex G-200 eluted with VBS++ containing 10 mM EACA and 10 mM benzamidine. The column (2 x 6 cm) was equilibrated with 0.01 M Tris-HCl, pH 7.4, containing 0.15 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM EACA and 10 mM benzamidine. Elution was carried out with a linear gradient of 0.05~0.45 M NaCl.](image-url)
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Effect of anti-Ss on the activity of each type of C4 molecule

The constant amount of C4 activity from various fractions was treated with varying dilution of F(ab')2 fragments of rabbit anti-Ss, and inhibition of C4 activity was measured. As shown in Fig. 7, F(ab')2 anti-Ss blocked the C4 activity of the second protein fraction of serum eluted through Sephadex G-200 and the C4 activity of the partially purified C4 fraction from plasma. The C4 activity of the QAE-Sephadex C4 fraction derived from the first protein fraction of serum on Sephadex G-200, however, was not affected by F(ab')2 anti-Ss or by F(ab')2 of normal IgG.

Fig. 7. Effect of anti-Ss on C4 activity. Three units of each C4 fraction were pretreated with serial 2-fold dilution of 1 mg/ml F(ab')2 of rabbit anti-Ss for 10 min at 37°C. Then C4 activity was measured by the E-C method. As a control, F(ab')2 of normal rabbit IgG was used. △-△, C4 fraction of the QAE-Sephadex derived from the first protein fraction on Sephadex G-200 with serum; ○-○, C4 fraction of the QAE-Sephadex derived from the second protein fraction on Sephadex G-200 with serum; •-•, partially purified C4 fraction from plasma.
Effect of mouse C5 on the detection of C4 activity

Since C4 activity in plasma of C5− strain could not be detected by either method, a possible effect of mouse C5 on measurement of C4 activity was examined. IgG fraction of mouse anti-MuB1 was covalently bound to CNBr-activated Sepharose 4B, then packed into the column and washed with EDTA-PBS. Partially purified mouse C4 fraction from plasma was applied to the column to deplete C5. As shown in Table 2, complete removal of C4 activity from the sample was documented by both C4D and E-C methods. When purified mouse C5 was added to this C5 depleted sample, C4 activity was recovered.

Table 2. C4 activity after immunoadsorption of mouse C5

<table>
<thead>
<tr>
<th>Immunoadsorption</th>
<th>Buffer</th>
<th>+Mouse C5</th>
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<tbody>
<tr>
<td>A/MuB1-Sepharose</td>
<td>0</td>
<td>240</td>
</tr>
<tr>
<td>NMS-Sepharose</td>
<td>200</td>
<td>240</td>
</tr>
</tbody>
</table>

DISCUSSION

Though the C4D method was approximately twice as sensitive as the E-C method in the measurement of C4 activity in plasma, in some strains the C4D method was too low to measure the C4 activity in serum. Other laboratories have reported the failure to measure the C4 activity in mouse serum using C4D guinea pig serum (Carroll and Capra 1978; Goldman et al. 1978). In the case of three Ss+C5+ strains, C4 activities in serum were the same in both sexes by the E-C method, but in plasma, C4 activity was higher in male than in female mice. This fact suggests that the discrepancy is closely associated with the state of C4 whether it exists in plasma or serum.

When mouse serum was filtered on Sephadex G-200 with VBS++, C4 activity could be detected in both the first and the middle protein peak by the E-C method. Ferreira et al. (1978) reported that when serum or plasma was eluted with EDTA-VBS, C4 hemolytic activity was found only in the middle protein peak by using the C4D method. We found that this discrepancy comes from the different C4 assay method and elution buffer. That is, C4 activity of the first protein peak cannot be detected by the C4D method, and when serum was eluted with EDTA-VBS, C4 activity of the first protein peak was entirely inactivated by EDTA.

To investigate the possible participation of any particular factors in C4D guinea pig serum which lead to different C4 titers by the two methods, the normal guinea pig serum artifically devoid of C4 (R4) was compared with C4D serum. The R4 serum functioned in the C4D method in the same manner as did the C4D serum. At present, there is no definite answer whether or not other factors were involved in the difference of the two methods.

Following the view of Ferreira et al. (1978), B10.D2/0 or A/J serum which is Ss+C5− strain would show the same elution pattern of C4 activity as BALB/c serum.
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(Ss\textsuperscript{h}C5\textsuperscript{+}). But C4 activity could be detected only at the first protein peak, though the elution pattern of Ss protein was the same as that of Ss\textsuperscript{h}C5\textsuperscript{+} serum. Moreover, when A/J plasma was eluted with EDTA-VBS, the elution pattern of Ss protein was the same as that of Ss\textsuperscript{h}C5\textsuperscript{+} serum, but C4 activity was not detected in any fraction (data not shown). Since mouse C5 is always detected in the middle protein peak, it is considered that the presence of mouse C5 is necessary to detect the C4 activity of the middle protein peak by either method, while the C4 activity of the first protein peak does not need mouse C5 to detect it. Mouse C5 was then highly purified from plasma for the first time, as described in materials and methods (Fig. 8). When this purified mouse C5 was added to the middle protein peak fraction of A/J plasma or B10.D\textsubscript{2}/o serum, C4 activity could be detected by E-C and C4D methods. Furthermore, when partially purified C4 fraction from plasma was treated with anti-MuB1-Sepharose 4B column, C4 activity was depleted and also recovered by adding highly purified mouse C5. Therefore, it is considered that the C4 molecule at the first protein peak would form a convertase acting on guinea pig C5, while C5 convertase generated from the C4 molecule at the middle protein peak would only activate mouse C5.

The possibility is outlined in this paper that two kinds of Ss-bp complexes exist in mouse serum. Though the presence of Ss-bp in human and guinea pig serum has been reported (Scharfstein et al. 1978; Burge 1980), we could not find any hemolytically active C4/Ss-bp complex in these sera (data not shown). Then the presence of C4/Ss-bp complex was unique to the mouse complement system at present. Since the middle protein peak fraction was also separated in active C4 and inactive Ss protein fractions (data not shown), it is supposed that the C4 molecule would be present in at least four forms in Ss\textsuperscript{h} serum, as shown in Fig. 9. When the Ss antigenic expression was compared with the C4 activity measured by the E-C method, the Ss antigenic expression of C4/Ss-bp was lower than that of

Fig. 8. SDS-PAGE of purified mouse C5. SDS-PAGE was performed in 5% gel containing 1% SDS and 4 M urea. Electrophoresis was carried out according to Weber and Osborn (1969). (1) Unreduced mouse C5. (2) Reduced mouse C5.
free active C4. Moreover, though hemolytic activity of free C4 was inhibited by F(ab')2 anti-Ss, that of C4/Ss-bp complex was not. These results indicate that the Ss antigen of C4/Ss-bp complex does not play an important role for expression of C4 activity. It is therefore conceivable that the binding of C4 molecule to Ss-bp would cause functional and conformational changes of the C4 molecule, which is one of the unique characters of the mouse complement system.

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References


