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Purification of the fourth, second and fifth components of mouse complement

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Summary. The fourth, second and fifth components of mouse complement were purified by a combination of polyethylene glycol precipitation, ion exchange chromatography and gel filtration. The final products were homogeneous on SDS-PAGE, and the activity yields were 8·5% for C4, 32% for C2 and 40% for C5. C4 was composed of three polypeptide chains with mol. wts of 90,000, 78,000 and 32,000. C2 was composed of a single polypeptide chain with a mol. wt. of 115,000 and cleaved by C1s into two fragments with mol. wts of 80,000 and 35,000. The half life of C4b2a was 7 min and was not prolonged by the iodination of C2. C2 activity could not be measured using EAC14^{hu} or EAC14^{gp} cells, but measurement was possible with the use of EAC14^{mo} cells with purified C5 components of mouse complement. C5 was composed of two polypeptide chains with mol. wts of 135,000 and 84,000.

This is the first report on the purification of functionally active mouse complement components C4, C2 and C5 from plasma.

INTRODUCTION

Most information about the complement system has been obtained from studies of guinea-pig and human complements. The lack of a sensitive haemolytic assay method for mouse complement components has been a major difficulty for characterization of the mouse complement system. We have already reported a functional assay method for components C1, C4, C2, C3 in mouse sera (Fukuoka *et al.*, 1982). Recently, some other laboratories have described haemolytic assays for mouse C4 (Ferreira, Nussenzweig & Gigli, 1978; Atkinson, Mcginnis & Shreffler, 1980) and mouse C2 (Gorman *et al.*, 1980).

It was shown that structural genes of mouse C4 were linked to the H-2 complex which was examined by the measurement of C4 antigen (Shreffler, 1964) and that of C4 haemolytic activity (Atkinson *et al.*, 1980). The linkage of mouse C2 activity to the H-2 complex was also shown by Gorman *et al.* (1980). However, during the investigation of mouse C4 in our laboratory, we observed that there were differences in the functional and molecular states of C4 depending on whether they existed in serum or in plasma (Fukuoka *et al.*, 1983). So it is necessary for an examination of linkage of C2 to measure the amount of C2 protein. Therefore, in this paper, we report on the purification of mouse C2, the preparation of specific antibodies and the examination of the functional and biochemical properties. Furthermore, though the structural properties of mouse C4 (Ss) and C5 (MuBl) protein have been relatively well characterized because antibodies to C4 and C5 could be easily prepared, functionally active C4 and C5 have not been purified as yet. In this paper, we show a method of purification for C4, C2 and C5 components of mouse complement.

Abbreviations: PEG, polyethylene glycol; EACA, ε-amino-n-caproic acid; PMSF, phenylmethylsulphonyl-fluoride; PBS, phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulphate.

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MATERIALS AND METHODS

Materials

ICR male mice ($Ss^{h}Slp^{-}$) were obtained from the Funabashi Farm, Chiba, and reared in our laboratory until 12–15 weeks of age. Mice were bled by cardiac puncture into EDTA to a final concentration of 10 mM. After centrifugation, plasma was stored at −70° until use.

GVB^{++}, G-GVB^{++} and EDTA-GVB were prepared as previously described (Fukuoka *et al.*, 1982). QAE-Sephadex A-50, CM-Sephadex C-50, SP-Sephadex C-50, Sephadex G-200 were purchased from Pharmacia Chemicals. DEAE-BioGel-A was purchased from Bio-Rad Laboratories. The PEG 4000, EACA, benzamidine hydrocloride and PMSF used were products of Wako Chem, Tokyo Kasei, Tokyo Kasei and Sigma, respectively.

Haemolytic assay

C4 activity was measured as previously described but with some modification (Fukuoka *et al.*, 1982). Briefly, 25 μl of $EAC1^{gp}$ (1×10^{8} cells/ml) and $^{oxy}C2^{hu}$ (100 unit/cell) were added to the test samples (25 μl) serially diluted in G-GVB^{++} in the wells of a microtitre plate. After incubation at 37° for 10 min, 25 μl of purified mouse C5 (30 unit/cell) and 1:20 diluted guinea-pig serum in 0·04 M EDTA-GVB (guinea-pig C-EDTA) were added to the wells, then further incubated for 60 min at 37°. The microtitre plate was centrifuged at 1000 r.p.m. for 5 min; the reciprocal dilution giving 50% haemolysis was determined by visual inspection.

C2 assay was carried out with some modification of our previously reported method (Fukuoka *et al.*, 1982). EAC14 was prepared by using $EAC1^{gp}$ and functionally pure mouse C4 (100 units/cell). Twenty-five microlitres of diluted sample and $EAC14^{mo}$ (1×10^{8} cells/ml) were incubated at 37° for 5 min. After 25 μl of purified mouse C5 (30 unit/cell) and guinea pig C-EDTA were added, the mixture was incubated at 37° for 60 min. For kinetical analysis, 1·5 × 10 cm glass tubes were used. A limited amount of mouse C2 (2 unit/cell) was mixed with equal volumes of $EAC14^{mo}$ (1×10^{8} cells/ml) and purified mouse C5 (30 unit/cell), and then incubated at 30°. At time intervals, 0·75 ml samples were removed and 0·75 ml of guinea-pig C-EDTA was added. After incubation at 37° for 60 min, 2·25 ml of cold 0·15 M NaCl solution was added, and the mixture was centrifuged at 2000 r.p.m. for 10 min. The degree of lysis was measured by absorption at 414 nm.

C5 activity was measured as follows: EAC142 was generated by incubating $EAC14^{hu}$ (1×10^{8} cells/ml) with an equal volume of $^{oxy}C2^{hu}$ (100 unit/cell) at 37° for 10 min and washed once with cold G-GVB^{++}. Serially diluted test samples in GVB^{++} in microtitre wells were mixed with an equal volume of functionally pure guinea-pig C3 (100 unit/cell) and 1:30 diluted guinea-pig C6–9 reagent prepared by the method of Cooper & Muller-Eberhard (1970). Then the mixture was incubated at 37° for 60 min.

Polyacrylamide gel electrophoresis

This was carried out by the method of Weber & Osborn (1969), with 5% and 7·5% polyacrylamide gels. Samples were treated by adding an equal volume of 0·2 M phosphate buffer, pH 7·2 containing 2% SDS and 5 M urea, and incubating at 60°C for 30 min. Protein was detected by staining with Coomassie Brilliant Blue and glycoprotein by the Schiff reagent as described by Zacharius *et al.* (1969).

RESULTS

Purification of C4, C2 and C5 components

C4 After addition of PMSF to 60 ml of ICR EDTA-plasma for a final concentration of 0·5 mM, 50% PEG 4000 in PBS containing 0·5 mM PMSF was added for a final concentration of 5% in an ice bath. After stirring for 30 min at 4°, the mixture was centrifuged at 6000 r.p.m. for 20 min. The 5% PEG precipitates contained almost all the C1 and C4-bp activities. As the 5% supernatant fraction contained a trace amount of C1, protease inhibitor was added to the buffer to diminish the loss of C4 activity. The 5% PEG supernatant fraction was dialysed against 0·02 M phosphate buffer (PB) pH 7·5/10 mM EACA/10 mM benzamidine/2 mM EDTA/0·03 M NaCl, and applied to a QAE-Sephadex A-50 column equilibrated with the same buffer. C4 activity was eluted at a higher NaCl concentration than any other components detected in our assay system. The salt concentration of the eluting buffer was raised linearly, and C4 fractions eluted at 0·28 M NaCl were dialysed against 0·02 M acetate buffer pH 5·6/10 mM EACA/10 mM benzamidine/2 mM EDTA/0·03 M NaCl and chromatographed on SP-Sephadex C-50 equilibrated with the same buffer. C4 fractions eluted at 0·14 M NaCl were

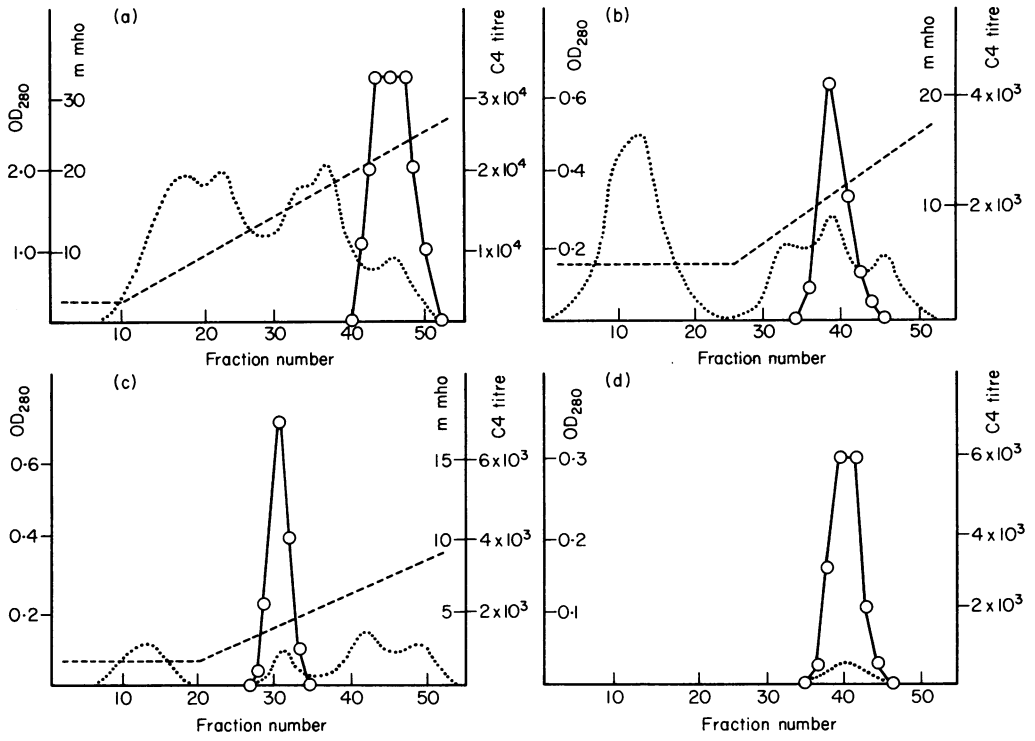

Figure 1. Chromatography of component C4. (a) QAE-Sephadex A-50 chromatography (2·5 × 40 cm) of the 5% PEG supernatant fraction of ICR EDTA-plasma. (b) SP-Sephadex C-50 chromatography (1·5 × 30 cm) of the post QAE-Sephadex pool of component C4. (c) DEAE-BioGel A chromatography (1·5 × 30 cm) of the post SP-Sephadex pool of component C4. (d) Sephadex G-200 gel filtration (2·5 × 60 cm) of the post DEAE-Bio-Gel chromatography of component C4. (· · · ·) OD_{280}; (○) C4 haemolytic activity; (– – –) specific conductivity at 4° (m mho/cm).

dialysed against 0·01 M PB pH 8·0/5 mM benzamidine/2 mM EDTA and applied to a column of DEAE-BioGel-A equilibrated with the same buffer. DEAE-BiolGel-A which was used by Bolotin *et al.* (1977) for purification of human C4 was also effective for purification of mouse C4. A purification of about 10-fold could be attained and most of the contaminated protein including inactive C4 could be removed at this step (Fig. 1). C4 fractions eluted at 0·05 M NaCl were concentrated by the negative pressure ultrafiltration and finally passed through a column of Sephadex G-200 with PBS containing 2 mM EDTA. A

Table 1. Summary of C4 purification

Step	Total OD_{280}	Total C4	Yield	Specific activity	Fold purification
EDTA-plasma	2900	376,000	100	130	1
5% PEG supernatant	2534	376,000	100	148	1·1
QAE-Sephadex A-50	33·4	103,000	27	3080	24
SP-Sephadex C-50	5·6	41,400	11	7390	57
DEAE-Bio Gel-A	0·56	36,300	9·7	64,820	499
Sephadex G-200	0·34	31,900	8·5	93,820	722

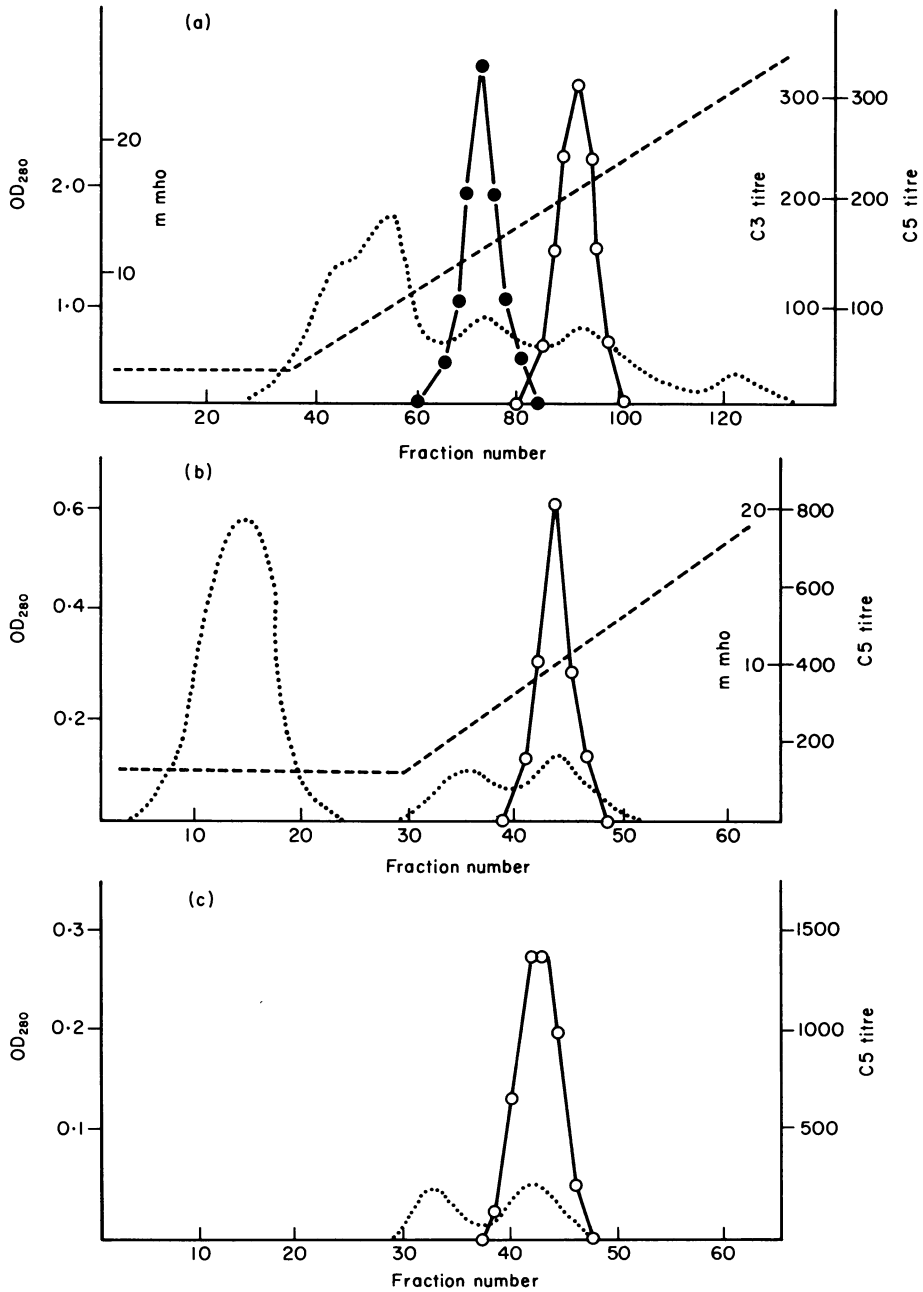

Figure 2. Chromatography of component C5. (a) QAE-Sephadex A-50 chromatography (2·5 × 40 cm) of the 5–12% PEG precipitate fraction of ICR EDTA-plasma. (b) CM-Sephadex C-50 chromatography (1·5 × 30 cm) of the post QAE-Sephadex pool of component C5. (c) Sephadex G-200 gel filtration (2·5 × 60 cm) of the post CM-Sephadex pool of component C5. (· · · ·) OD_{280}; (●) C3 IA activity; (○) C5 haemolytic activity; (– – –) specific conductivity at 4°C (m mho/cm).

trace amount of IgG was found in some lots of purified C4 and removed by Sepharose 4B column coupled with rabbit anti-mouse IgG. A summary of the C4 purification is depicted in Table 1.

C5. To the 5% PEG supernatants, 50% PEG in PBS containing 0·5 mM PMSF was further added up to a final concentration of 12%. After 30 min at 4°, the precipitates were recovered by centrifugation at 6000 r.p.m. for 20 min, and dissolved in 0·02 M PB pH 7·8/10 mM EACA/2 mM EDTA/0·02 M NaCl. The 5–12% PEG precipitates contained almost all of the C3 and C5, about 50% of factor B and about 10% of C2. Though the C4 protein (Ss) was recovered in this fraction, its haemolytic activity was lost. The 5–12% PEG precipitates were applied to a column of QAE-Sephadex A-50 equilibrated with the same buffer. C3 and C5 were eluted at 0·18 M NaCl and 0·22 M NaCl, respectively (Fig. 2). In the case of the purification of human complement components, separation of C3 and C5 was very difficult. However, mouse C3 was almost completely separated from mouse C5 fraction by QAE-Sephadex A-50 chromatography. Factor B and C4 protein was also separated from C3 and C5 fractions on this column. The C5 fraction was rechomatographed on the same column and dialysed against 0·02 M acetate buffer pH 6·0/10 mM EACA/2 mM EDTA/0·08 M NaCl, and applied to a column of CM-Sephadex C-50 equilibrated with the same buffer. The C5 fraction eluted at 0·16 M NaCl was concentrated and finally passed through a column of Sephadex G-200 with PBS containing 2 mM EDTA. The purification of C5 is summarized in Table 2. C5 was purified about 2000-fold from plasma.

C2. The 12% PEG supernatant fraction is a good source of C2 purification containing about 90% of C2 and 50% of factor B activities in plasma but no C1, C4, C3 and C5 activities. The 12% PEG supernatant was dialysed against 0·02 M PB pH 7·8/10 mM EACA/10 mM benzamidine/2 mM EDTA/0·03 M NaCl, then mixed in batches with QAE-Sephadex A-50 equilibrated with the same buffer. The gel was packed into the column and washed with the same buffer. C2 and factor B were eluted at the same fraction (0·12 M NaCl). Next, these fractions were dialysed against 0·02 M acetate buffer pH 6·0/10 mM EACA/10 mM benzamidine/2 mM EDTA/0·05 M NaCl and applied to a column of CM-Sephadex C-50 equilibrated with the same buffer. Factor B and C2 were eluted at 0·2 M NaCl and 0·26 M NaCl, respectively (Fig. 3). This chromatography was very effective for C2 purification; more than 99% of other proteins were removed and 500 fold purification was attained. Human factor B was shown to have properties similar to human C2, and was often contaminated by C2 fraction. However, mouse factor B could be easily separated in this step. The C3bINA (I) activity, which was measured by inactivation of a limited number of C3b on EAC^{hu} (IA inhibition), was also separated from the C2 fraction. Pooled C2 was homogeneous on SDS-PAGE (Fig. 4). A summary of the C2 purification is depicted in Table 3. C2 was purified about 16,000-fold from plasma.

Characterization of C4, C2 and C5 components

Purified C4 was composed of three polypeptide chains, α, β, and γ with mol. wts of 90,000, 78,000 and 32,000, respectively, on SDS-PAGE under a reduced condition (Fig. 4). Furthermore, when each chain stained with Shiff reagent, the α and β chains stained but the γ chain did not stain (data not shown). These results were in agreement with the report of Roos, Kornfeld & Shreffler (1980), which was based on results obtained from the immunoprecipitation of synthesized C4 by cultured peripheral macrophage with anti-Ss serum.

Table 2. Summary of C5 purification

Step	Total OD_{280}	Total C5	(%) Yield	Specific activity	Fold purification
EDTA-plasma	5000	28,000	100	5·6	1
5–12% PEG fraction	620	28,000	100	45	8
QAE-Sephadex A-50	72	27,000	96	375	67
QAE-Sephadex A-50	33	26,000	92	780	140
CM-Sephadex C-50	1·7	11,000	40	6500	1160
Sephadex G-200	0·9	11,000	40	12,000	2180

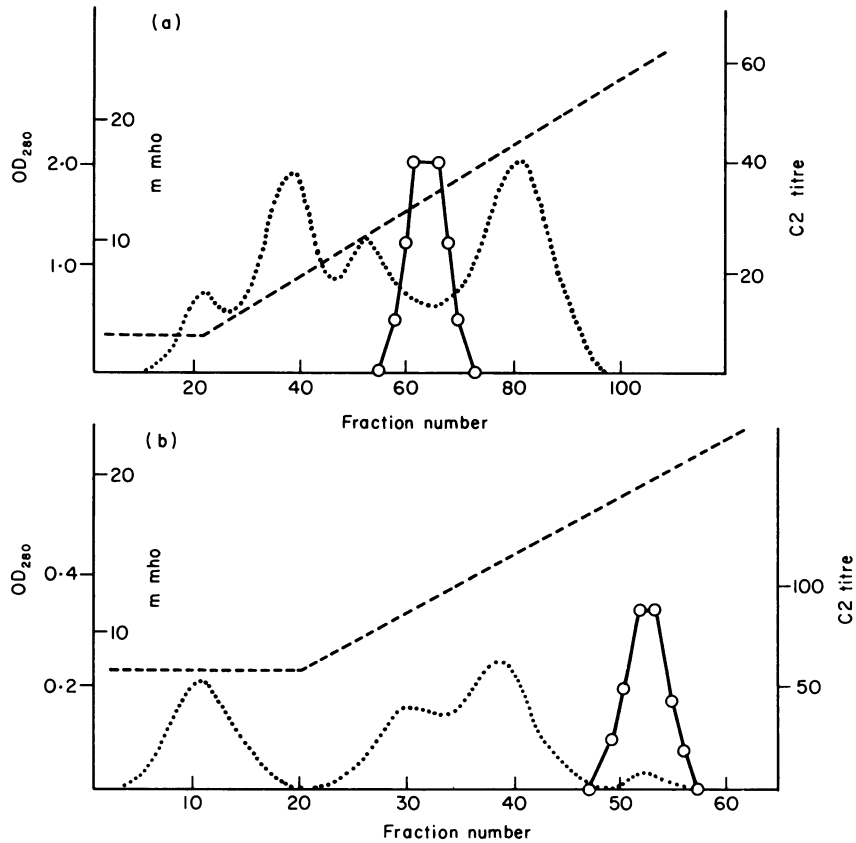

Figure 3. Chromatography of component C2. (a) QAE-Sephadex A-50 chromatography (2·5 × 40 cm) of the 12% supernatant PEG fraction of ICR-EDTA-plasma. (b) CM-Sephadex C-50 chromatography (1·5 × 30 cm) of the post QAE-Sephadex pool of component C2. (· · · ·) OD_{280}; (○) C2 haemolytic activity; (– – –) specific conductivity at 4°C (m mho/cm).

Table 3. Summary of C2 purification

Step	Total OD_{280}	Total C2	Yield (%)	Specific activity	Fold purification
EDTA-plasma	5000	8000	100	1·6	1
12% PEG supernatant	3500	7400	92	2·1	1·3
QAE-Sephadex A-50	80	4500	56	55·6	35
CM-Sephadex C50	0·1	2600	32	26,000	16,250

After the treatment of C4 with C1s at 37° for 1 hr, a fragment with a mol. wt of 82,000 corresponding to α′ was detected on SDS-PAGE (data not shown). Though the mol. wt of the α chain was smaller than those reported by other laboratories (Roos *et al.*, 1980; Ferreira *et al.*, 1978), a decrease of 8000 for the α chain caused by C1s treatment agreed with results of Ferreira *et al.* (1978). C4 treated with C1s migrated more anodally than native C4 on immunoelectrophoresis which was consistent with human C4 (Bolotin *et al.*, 1977) (Fig. 5).

Purified C2 was composed of a single polypeptide

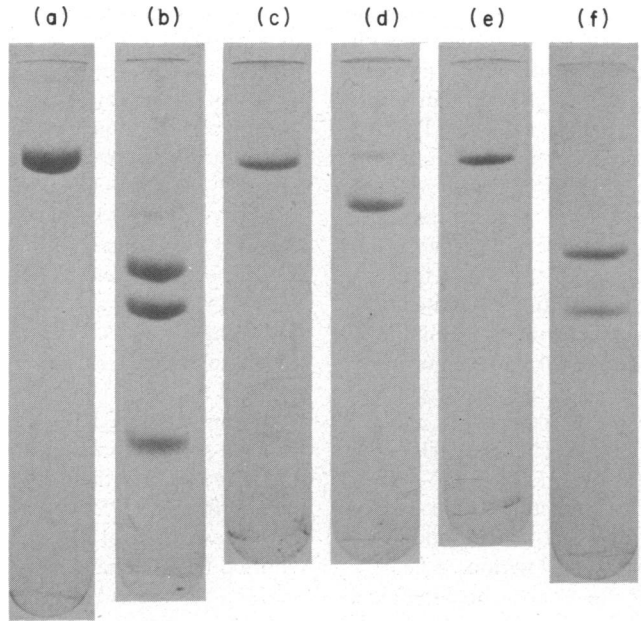

Figure 4. SDS-polyacrylamide gel electrophoresis of C4, C2 and C5. Electrophoresis on 5% polyacrylamide gel of C4 and C5, and on 7·5% polyacrylamide gel of C2 in the presence of SDS. (a) Purified C4; (b) purified C4 (reduced); (c) purified C2; (d) purified C2 after treatment with $C1s^{hu}$; (e) purified C5; (f) purified C5 (reduced).

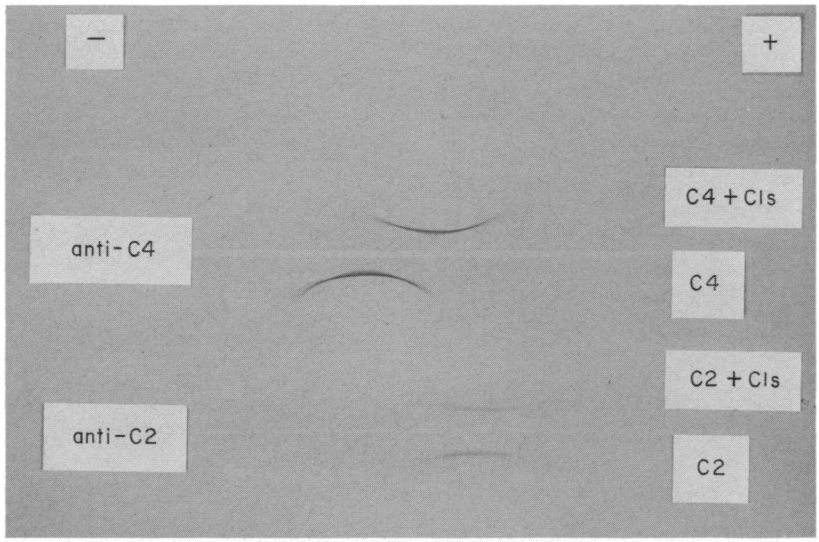

Figure 5. Immunoelectrophoretic analyses of C4 and C2.

chain with a mol. wt of 115,000 on SDS-PAGE whether reduced or not. When C2 was treated with C1s at 37° for 1 hr, C2 was cleaved and fragments with mol. wts of 80,000 and 35,000 could be identified on SDS-PAGE (Fig. 4). The C2 was glycoprotein which stained with Shiff reagent (data not shown). Purified C2 was emulsified with Freund's complete adjuvant and subcutaneously injected into a rabbit to produce antiserum. The rabbit was given a booster injection 2 weeks later and bled after 1 more week. This antiserum proved to be monospecific anti-C2 serum as follows. (i) The $F(ab')_2$ fragment of this anti-C2 inhibited only C2 activity; C4, C3, C5 activities were not inhibited. (ii) Partially purified C2 was subjected to 6% polyacrylamide gel electrophoresis. When half of the gel was put on the agarose gel and developed against anti-C2 serum, a single line could be seen. The other half of the gel was sectioned and eluted with EDTA-PBS. When the eluates were examined for C2 activity, such activity was identified in the sectioned gels corresponding to the precipitin line against anti-C2 serum. When C2 was treated with C1s at 37° for 1 hr, the precipitin line migrated more anodally than native C2 on immunoelectrophoresis (Fig. 5). Compared to the observation of human C2 reported by Nagasawa & Stroud (1977), the fragment which migrated anodally might be C2a.

Iodination of human C2 enhances C2 activity and retards the decay of C2 from EAC142 (Polly & Muller-Eberhard, 1967). Human and mouse C2 were iodinated by the method of Polly & Muller-Eberhard (1967). Iodinated human C2 showed twice as much activity as untreated human C2, and retarded the decay of C2 from EAC142. But the iodination of mouse C2 by various concentrations of I_2 showed no enhancement of C2 activity and showed the same decay profile as untreated samples. Purified C2 was stable for at least 1 year when stored at −70° and also relatively stable even when thawed and refrozen several times.

Purified C5 was composed of two polypeptide chains with mol. wts of 135,000 (α) and 84,000 (β) on SDS-PAGE under a reduced condition (Fig. 4). Phan & Ward (1979) reported that the α chain of human C5 stained with Shiff reagent, while the β chain scarcely stained if at all. However, when mouse C5 stained with Shiff reagent on SDS-PAGE, both α and β chains stained to the same degree (data not shown).

DISCUSSION

We succeeded in separating some mouse complement components by using PEG fractionation. However, it was essential that the separation should be done by adding EDTA and various protease inhibitors to the buffer and all procedures were done at low temperatures. Good recovery of each activity was possible and fractionated components were stable at least a week. The elution patterns of mouse C4, C2, C3, C5 and factor B on QAE-Sephadex chromatography were similar to those of human complement components when 5% PEG supernatant was applied. That is, both C2 and factor B were eluted at low NaCl concentrations. Next, C3, C5 and C4 were eluted in the order of measuring NaCl concentration. Anion exchange chromatography was not so effective for the separation of human C3 and C5, but was very effective in separating mouse C3 and C5. C4 activity was lost by the PEG precipitation even if various protease inhibitors were added at the PEG fractionation. So C4 was purified from 5% PEG supernatant fraction.

The low yield of C4 activity on DE-52 column reported by Goldman, Bangalore & Goldman (1978) seems to be the results of inactivation by C1s because of direct application of plasma to the column. However, another reason for the low yield could be the necessity of mouse C5 for the detection of mouse C4 activity as we showed in a separate paper (Fukuoka *et al.*, 1983). That is, incomplete separation of C4 and C5 on the column led to the detection of C4 activity which overlapped the C5 fraction. Similarly, the coincidence of the C4 active fraction and the C2 fraction which was reported by Goldman *et al.* (1978) seems to have been caused by the overlapping of C4, C2 and C5 fractions. Because mouse C5 was also necessary for the measurement of mouse C2 with $EAC14^{mo}$. It seems improbable that early classical components functioned as a unit as suggested by Goldman *et al.* (1978).

Mouse C2 was composed of a single polypeptide chain which was similar to human C2 (Keer & Porter, 1978) and guinea-pig C2 (Keer & Gagnon, 1982). However, molecular weights of C2 and fragments C2a and C2b were larger than those of human and guinea-pig C2. Iodination of mouse C2 showed no stabilization of C3 convertase. Gorman *et al.* (1980) also reported no effect of iodination of mouse serum on C2 activity.

Mouse C4 was well characterized because rabbit anti-Ss (C4) serum could be easily prepared by the immunization of partially purified Ss protein and absorption of the antiserum with Ss^1 strain serum. Immunoprecipitated C4 was prepared from serum or

culture supernatant of macrophage. However, purification of haemolytically active mouse C4 has not been reported. Our results of characterization were in good agreement with those of previous reports. That is, purified C4 was composed of three polypeptide chains and the γ chain did not stain with Shiff reagent (Roos, *et al.*, 1980), and C1s treatment of C4 resulted in the appearance of α' fragment (Ferreira *et al.*, 1978).

Anti-C5 serum also could be easily prepared by injecting C5-sufficient mouse serum into C5-deficient mice. Though the characterization of mouse C5 was done by using immunoprecipitated C5, purification of haemolytically active mouse C5 also has not been reported. Finally, the effect of trypsin digestion on C4 and C5 was examined to obtain preliminary results. In the case of C4, the α chain was rapidly digested, but the β and γ chains were not susceptible under the conditions employed. In the case of C5, the α chain was susceptible but the β chain was resistant. These properties were consistent with the reported results regarding human C5 (Wetsel & Kolb, 1982) and guinea-pig C5 (Kinoshita *et al.*, 1981).

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