

## Stabilization of the classical pathway C3 convertase C4 $\bar{2}$ , by a factor F-4 $\bar{2}$ , isolated from serum of patients with systemic lupus erythematosus

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**Summary.** Sera from sixteen patients with SLE were investigated for the presence of a factor which would conserve convertase activity on preformed EAC1<sup>EP</sup> 4<sup>hu2hu</sup> for 30 min at 30° in EDTA. Although such a factor could not be detected readily in the sera, chromatography on DE-52 cellulose yielded fractions appearing as three peaks in one patient and as two peaks in a second patient. These peaks were capable of conserving C4 $\bar{2}$  activity and were designated as F-4 $\bar{2}$ . Purification of F-4 $\bar{2}$  from the second peak eluting between 4 and 7 mS on DE-52 was obtained by SP-C50, S-300 and QAE-A50 chromatography. F-4 $\bar{2}$  exhibited charge heterogeneity upon SP-C50 chromatography. On polyacrylamide gel electrophoresis the final material migrated as one band, which coincided with the position of F-4 $\bar{2}$  activity upon elution from a parallel gel. F-4 $\bar{2}$  had an apparent molecular weight of 150,000 and reacted with anti-IgG in Ouchterlony analysis. Sepharose-bound anti-IgG was capable of neutralizing F-4 $\bar{2}$  activity. The purified material was shown to prolong the half-life ( $T_{1/2}$ ) of performed cell-bound C4 $\bar{2}$  in GVB-EDTA at 30° from 5 to 80 min.

### INTRODUCTION

The complement system is comprised of at least eight-

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een plasma proteins and consists of four functional divisions: two pathways for activation, the classical and the alternative; a single amplification mechanism which is used by both activating pathways; and a final common effector pathway towards which the activating and amplifying sequences are directed (Müller-Eberhard, 1975; Fearon & Austen, 1976). Antigen-antibody complexes may be involved in the activation of the classical pathway, resulting in the formation of the classical pathway convertase C4b2b, a (Nagasawa & Stroud, 1977) which is responsible for initial C3 cleavage. Activation of the classical pathway has been shown to occur in patients with SLE (Lewis, Carpenter & Schur, 1971). In addition these patients may develop autoantibodies (Holman, 1971; Miescher, Rothfield & Miescher, 1976). Since it is known that antibodies may be found in sera of patients with membranoproliferative glomerulonephritis and/or partial lipodystrophy (Davis, Ziegler, Gelfand, Rosen & Alper, 1977; Daha, Austen & Fearon, 1978; Scott, Amos, Sissons, Lachmann & Peters, 1978) with specificity for the amplification convertase, C3b,Bb, and which are capable of stabilizing C3b, Bb convertase activity, we searched for a factor in sera of SLE patients which would sustain and conserve classical C3 convertase activity.

### MATERIALS AND METHODS

#### *Sera*

Blood samples from sixteen patients with SLE were

obtained from the Department of Rheumatology, allowed to clot for 2 h at 0°, centrifuged for 10 min at 2000 *g* and frozen in aliquots at -80° until use. The diagnosis of SLE was based on the presence of at least four of the preliminary criteria (Cohen, Reynolds, Franklin, Kullea, Ropes, Shielman & Wallace, 1971). Blood samples from ten healthy individuals were also obtained and treated in a similar fashion.

#### Materials

XM-50 Diaflo ultrafiltration membranes (Amicon Corp., Lexington Mass), Sepharose 4B, Sephacryl-S300, Sephadex G-150, Quaternary aminoethyl-A50 Sephadex (QAE-A50), Sulphopropoxyl-C50 Sephadex (SP-C50) (Pharmacia Fine Chemicals, The Hague, The Netherlands), DE52-cellulose (Whatman Biochemicals, Kent), and LiBr (Merck, Amsterdam) were obtained as indicated.

#### Preparation of complement components

C1<sup>8p</sup> and C2<sup>8p</sup> (Nelson, Jensen, Gigli & Tamura, 1966) were functionally purified and C5, C6, C7, C8 and C9 were obtained from Cordis Corp., Miami, Florida. C3<sup>hu</sup> was purified by polyethylene glycol precipitation as described (Tack & Prahl, 1976). C2<sup>hu</sup> was purified to homogeneity from 1000 ml of fresh frozen normal human serum by chromatography of the fraction of serum precipitating between 2 and 3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> on Biorex-70 in 0.01 M phosphate buffer at pH 6.0 and further purified to homogeneity as described (Nagasawa & Stroud, 1977). The final material contained 1.5 mg protein as determined by Folin analysis (Lowry, Rosebrough, Farr & Randall, 1951), with human serum albumin as a standard, and had a functional activity of 560 units/ $\mu$ g protein.

Anti-C3 and anti-IgG were obtained by immunization of rabbits with purified C3 and IgG. IgG was prepared as described (Daha *et al.*, 1978). Anti-IgG was rendered specific for its heavy chain by absorption with Sepharose-bound  $\kappa$  and  $\lambda$  chains. Sepharose-bound anti-C3 and anti- $\gamma$  were prepared according to Cuatrecasas (1970) by coupling the IgG fraction of these antisera to Sepharose 4B.

#### Assays

Isotonic Veronal-buffered saline (VBS), pH 7.5, containing  $5 \times 10^{-4}$  M magnesium,  $1.5 \times 10^{-4}$  M calcium (VBS<sup>2+</sup>) and 0.1% gelatin (GVB<sup>2+</sup>), half-isotonic GVB<sup>2+</sup> with 2.5% dextrose (DGVB<sup>2+</sup>) (Nelson *et al.*, 1966) and GVB containing 0.01 M EDTA (0.04 M GVB-EDTA) or 0.04 M EDTA (0.04 M GVB-EDTA)

were used as diluents for the haemolytic assays. EAC4<sup>hu</sup> was prepared as described (Rapp & Borsos, 1970). EAC1<sup>8p4hu</sup> was prepared by interaction of EAC4<sup>hu</sup> with 200 effective molecules of C1<sup>8p</sup> per intermediate in DGVB<sup>2+</sup> for 30 min at 30°, followed by further incubation for 90 min at 0°. EAC1<sup>8p4hu2hu</sup> was prepared by incubation of washed EAC1<sup>8p4hu</sup> for 5 min at 30° in DGVB<sup>2+</sup> with the appropriate amount of purified C2<sup>hu</sup> such that eight calculated effective haemolytic sites were formed per intermediate. C2 (Rapp & Borsos, 1970) and C3 (Ruddy & Austen, 1969) haemolytic activity were measured as described.

To assay for the factor (F-4 $\bar{2}$ ) which conserves classical pathway C3 convertase activity, 0.1 ml dilutions of the fractions containing F-4 $\bar{2}$  in 0.01 M GVB-EDTA were incubated with 0.1 ml EAC1<sup>8p4hu2hu</sup> bearing eight haemolytic sites per intermediate for 30 min at 30°; residual haemolytic sites were then developed by the addition of 0.3 ml guinea-pig serum (GPS) diluted 1:30 in 0.04 M GVB-EDTA for 60 min at 37°. After addition of 1.5 ml isotonic saline, the extent of haemolysis was determined and the average number of haemolytic sites per cell (*Z*) was calculated, with a reagent mixture without F-4 $\bar{2}$  or containing buffer alone as the reagent blank. PAGE analysis and SDS-PAGE analysis were performed as described (Daha, Stuffers-Heiman, Kijlstra & Van Es, 1979).

## RESULTS

#### Isolation of F-4 $\bar{2}$

Twenty-two millilitres of serum from patient 1 (Table 1) were dialysed against 1000 ml of 0.01 M Tris-HCl, pH 8.0, containing 0.002 M EDTA for 8 h at 4° and the pseudoglobulin fraction was applied on a 2.5  $\times$  30 cm DE-52 column equilibrated with dialysis buffer. After collection of sixty fractions of 2.5 ml a linear NaCl gradient of 800 ml was applied to the column. F-4 $\bar{2}$  and protein content were assessed in every fifth fraction together with the conductivity in every tenth fraction (Fig. 1). F-4 $\bar{2}$ , measured in 1:5 dilutions of the fractions, was found in the effluent, between 4 and 7 mS and between 9 and 11 mS. The third peak of activity at least was caused by C3, and was also found in all normal sera investigated. Although the distribution of C3 haemolytic activity in a regular C3 assay of 1:1000 dilutions of the fractions was found between fractions 140-185, with peak activity in fraction 150, the distribution in the F-4 $\bar{2}$  assay was different, presumably because of inhibition of activity by an unde-

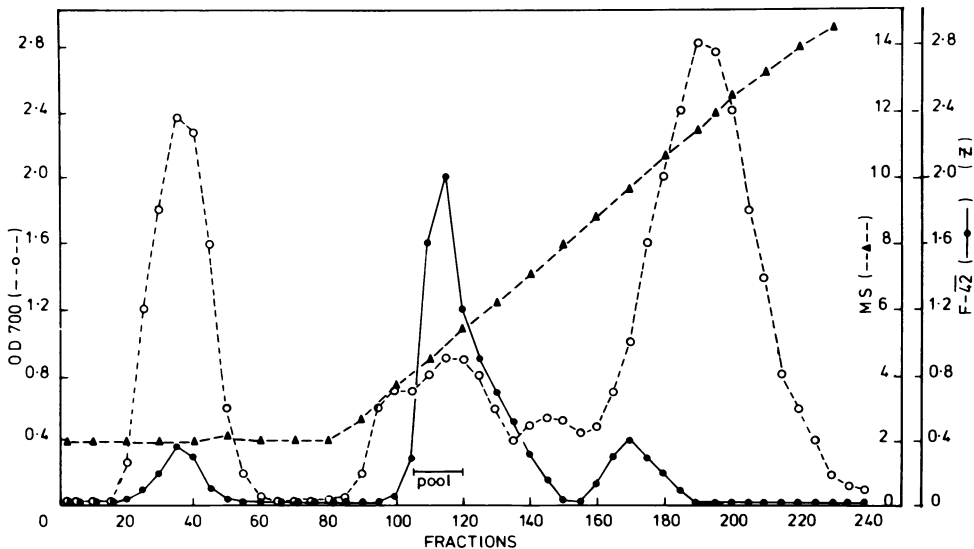
**Table 1.** Detection of F-42 activity after DE-52 chromatography in sera of patients with SLE

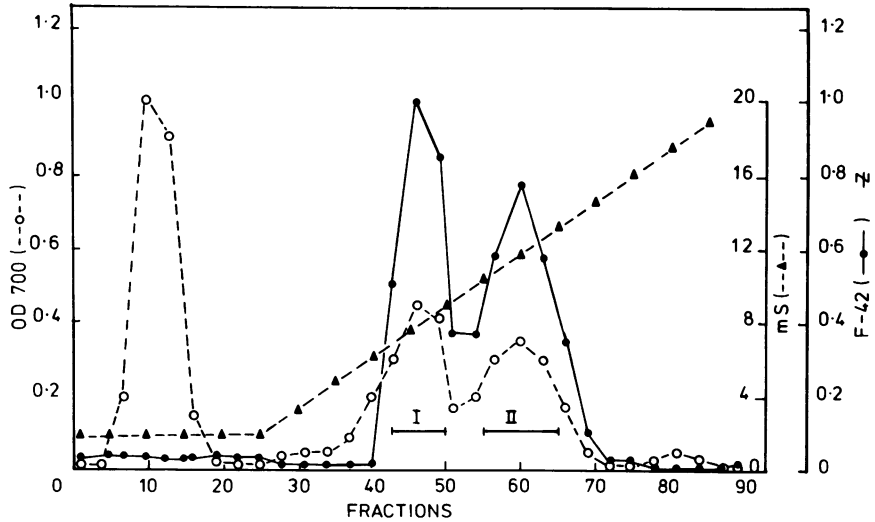
Patient	F-42		
	Effluent	4-7 mS	8-11 mS
1	+	+	+
2	-	+	+
14 other SLE patients	-	-	+
Normals	-	-	+

finer inhibitor. Since fractions 105-120 exhibited the greatest activity and were free of C3, these fractions were studied further. They were pooled, dialysed against 2 l of 0.01 M sodium acetate buffer, pH 6.0, containing 0.002 M EDTA and applied to a 1.5 x 30 cm column containing SP-C50; the column was equilibrated with dialysis buffer. After collection of fifteen fractions of 2.5 ml each, a linear NaCl gradient in 300 ml starting buffer was applied to the column. F-42 activity assessed in 1:5 dilutions of the fractions exhibited charge heterogeneity and eluted as two peaks with peak activities at 8 and 12 mS (Fig. 2), respectively. Fractions 43-50 and 55-65 were pooled separately, concentrated and subsequently subjected to gel

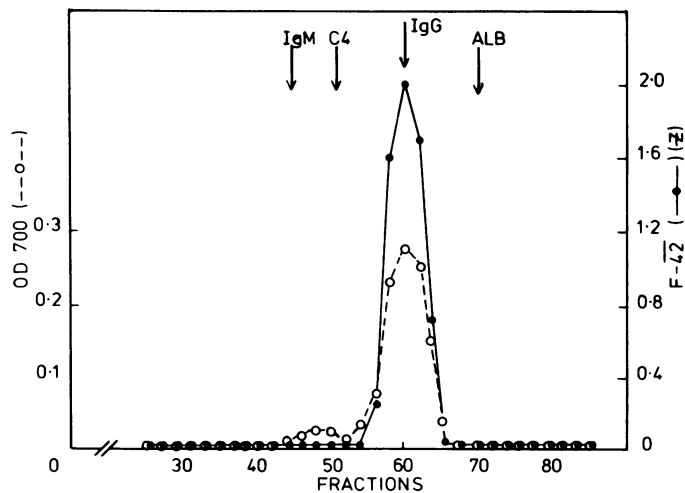
filtration on a 2.5 x 90 cm Sephacryl S-300 column, which had previously been calibrated with human IgM, C4, IgG and albumin as molecular weight markers. Fractions of 4.6 ml were collected and assayed for protein content. F-42 activity assessed in 1:5 dilutions of the fractions, as depicted for pool I in Fig. 3, filtered from the column with an apparent molecular weight of 150,000 and coincided with the major protein peak. The molecular weight of F-42 from pool II was identical.

Further purification of F-42 was obtained by chromatography of fractions 58-65, after dialysis against 0.01 M Tris-HCl, pH 8.0, containing 0.02 M EDTA on a 0.9 x 15 cm QAE-A50 column. After collection of fourteen fractions of 1 ml each, a linear NaCl gradient of 100 ml was applied to the column. F-42, assayed in 1:5 dilutions of the fractions, coincided with the only protein peak and eluted at 5 mS (Fig. 4). In a similar way, the fractions containing F-42 from the S-300 step of pool II were fractionated by QAE-A50 chromatography. This F-42 activity eluted with peak activity at 4.7 mS. The QAE pool I and pool II of F-42 were pooled and concentrated to 0.4 ml each. Sixteen SLE sera and ten normal sera were fractionated on DE52. F-42 activity was found in the effluent of one patient's serum (patient 1); the sera of two patients exhibited activity between 4 and 7 mS (Table 1, patient 1,2), and all sixteen sera exhibited F-42 activity which could be

**Figure 1.** Anion exchange chromatography of the pseudoglobulin pool of serum from patient 1 (Table 1) on DE-52 cellulose. Protein content (○), conductivity (▲) and F-42 activity (●) in a 1:5 dilution of the fractions are shown.



**Figure 2.** Cation exchange chromatography of the second DE-52 pool (fractions 105–120) containing F-42 activity on SP-C50. Protein content (○), conductivity (▲) and F-42 activity (●), in a 1:5 dilution of the fractions are shown.



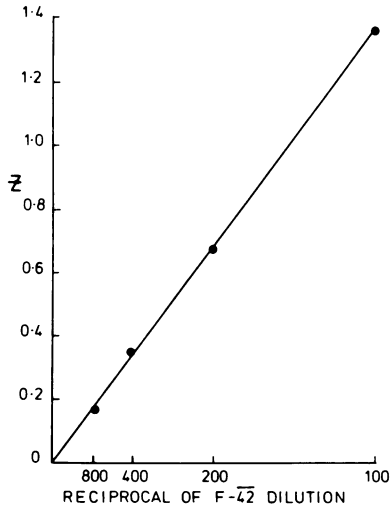
**Figure 3.** Sephacryl-S300 gel filtration of pool I from the SP-C50 step containing F-42. The position of the markers IgM, C4, IgG and albumin, which were previously determined, is shown at the top. Protein content (○) and F-42 activity (●), in a 1:5 dilution of the fractions are depicted.

inhibited only partially with anti-C3, whereas normal sera exhibited F-42 activity between 9 and 11 mS which could be inhibited completely by anti-C3.

#### Functional characterization

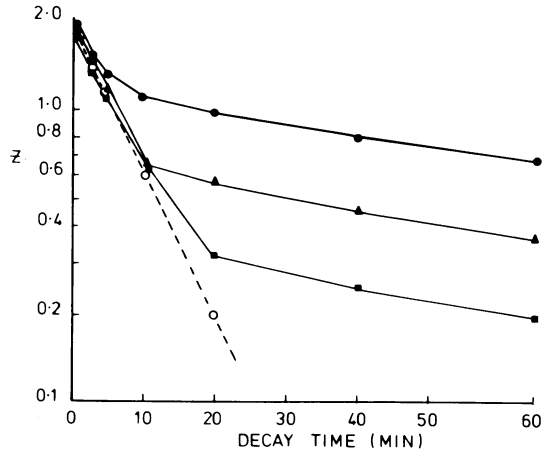
To obtain information on the activity of the final

material isolated from pool I (Fig. 2), serial two-fold dilutions were assayed for F-42 activity. As may be seen from Fig. 4 a linear dose-response of F-42 activity was obtained. One unit of F-42 was defined as that amount of F-42 which induced one site per cell ( $Z = 1$ ). When functionally purified C3-C9 was used to develop residual EAC:142 activity, a linear dose response was



**Figure 4.** Dose-response of F-42 activity assessed by incubation of F-42 dilutions with EAC1<sup>sp4hu2hu</sup>, bearing eight haemolytic sites per cell, for 30 min at 30° and subsequent development of residual convertase sites with GPS-EDTA for 60 min at 37°.

also seen. In order to find out what the mechanism of F-42 action is, EAC1<sup>sp4hu2hu</sup> bearing approximately two effective sites per intermediate was prepared and washed with ice cold 0.01 M GVB-EDTA;  $1 \times 10^8$  intermediates were resuspended at 30° in 0.01 M GVB-EDTA or in 0.01 M GVB-EDTA containing 1:50, 1:100 and 1:200 dilutions of F-42. In a kinetic experiment, 0.1 ml aliquots were removed at timed intervals and added to 0.3 ml GPS-EDTA for 60 min at 37° to develop residual convertase sites. The number of residual haemolytic sites was determined using EAC1<sup>sp4hu</sup> as a reagent blank. As depicted in Fig. 5, EAC1<sup>sp4hu2hu</sup> decayed with a half-life ( $T_{1/2}$ ) of 5 min at 30°, while intermediates exposed to F-42 exhibited a biphasic decay: namely a first phase of decay compatible with a  $T_{1/2}$  of 5 min and thus similar to that of EAC1<sup>sp4hu2hu</sup>, and a second phase of decay with a  $T_{1/2}$  of approximately 75 min. To determine whether stabilization of EAC1<sup>sp4hu2hu</sup> by F-42 persisted after washing, EAC1<sup>sp4hu2hu</sup> was prepared and added to three different dilutions of F-42 in 0.1 M GVB-EDTA for 30 min at 30° or kept at 0° without F-42. Subsequently all four batches of intermediates were washed twice with ice-cold 0.01 M GVB-EDTA, and finally resuspended at a concentration of  $1 \times 10^8$  intermediates/ml in 0.01 M GVB-EDTA at 30°. The rate of decay was determined as described in the previous experiment. The interme-

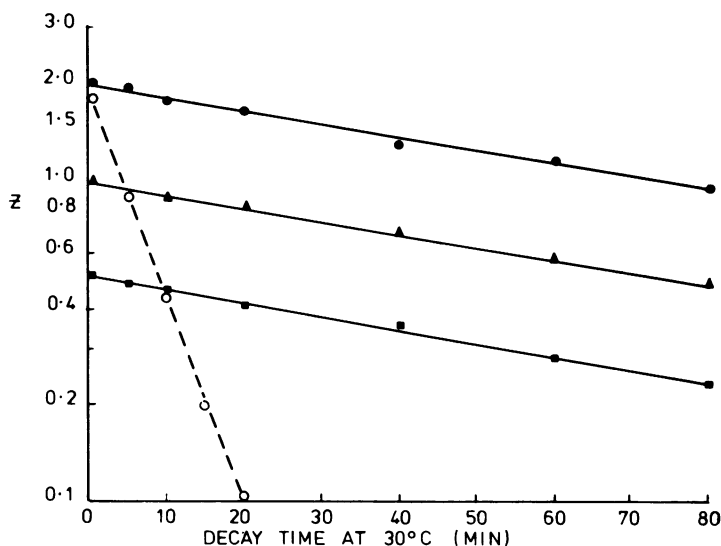


**Figure 5.** Kinetics of decay at 30° in 0.01 M GVB-EDTA of cell-bound C4<sup>hu2hu</sup> alone (o) or in 1:50 (●), 1:100 (▲) and 1:200 (■) dilutions of F-42.

diates exposed to 1:50, 1:100 and 1:200 dilutions of F-42 had 2.05, 1.09 and 0.49 effective sites at time zero, respectively, and decayed with a  $T_{1/2}$  of 80 min at 30° (Fig. 6). Cell-bound C42 in GVB-EDTA decayed with a  $T_{1/2}$  of 5 min. To determine whether binding was essential for stabilization, 0.2 ml two-fold dilutions of F-42 were incubated with  $5 \times 10^8$  performed EAC1<sup>sp4hu2hu</sup> bearing 200 effective sites per cell or with the same number of EAC14 or EA for 30 min at 30°. Following centrifugation, residual F-42 activity was assessed in the supernatant with EAC1<sup>sp4hu2hu</sup> bearing eight effective sites per cell. As shown in Table 2, EAC1<sup>sp4hu2hu</sup> was capable of binding 0.41, 0.35 and 0.23 units of F-42 in inputs of 1.41, 0.69 and 0.36 units, respectively. No detectable binding to EA and EAC14 occurred. Binding of F-42 to EAC1<sup>sp4hu2hu</sup> bearing 200 sites per cell was investigated at 2°, 20° and 30°. Compared with experiments at 30°, binding decreased by 10% and 20% at 20°, respectively.

### Immunochemical characterization

The final material from pool I (Fig. 2), after QAE-A50 chromatography, exhibited one band after application of 50 µg of protein to 5% polyacrylamide gels and subsequent electrophoresis. F-42 activity eluted from a parallel gel coincided with the stained protein band. SDS-PAGE analysis also revealed only one band with a molecular weight of 152,000. Upon Ouchterlony analysis against anti- $\mu$ ,  $\alpha$ ,  $\gamma$ -C3, -C4, -C5, - $\beta_1$ H, -C3bINA and properdin it only exhibited a precipi-



**Figure 6.** Kinetics of decay at 30° of cell-bound C4<sup>hu2hu</sup> (o) and of C4<sup>hu2hu</sup> pretreated with 1:50 (●), 1:100 (▲) and 1:200 (■) dilutions of F-42 in 0.01 M GVB-EDTA. Prior to decay intermediates were washed with ice-cold GVB-EDTA and subsequently resuspended in GVB-EDTA at 30°.

tation line with anti- $\gamma$  and showed identity with normal human IgG. One precipitation line was also observed with anti-whole human serum. Immunoelectrophoresis of the material and development of precipitation lines with anti-IgG and anti-whole human serum indicated its migration as a  $\gamma$  globulin.

To determine whether anti-IgG would also remove F-42 activity from the F-42 preparation, 0.3 ml dilutions of F-42 in 0.01 M GVB-EDTA were incubated with either 0.15 ml buffer alone or with 0.15 ml Sepharose-bound anti-C3 or anti- $\gamma$  for 30 min at 30° and 30 min at 0°. The reaction mixtures were centrifuged and residual F-42 activity in the supernatant was determined. As depicted in Fig. 7, anti- $\gamma$  was capable of

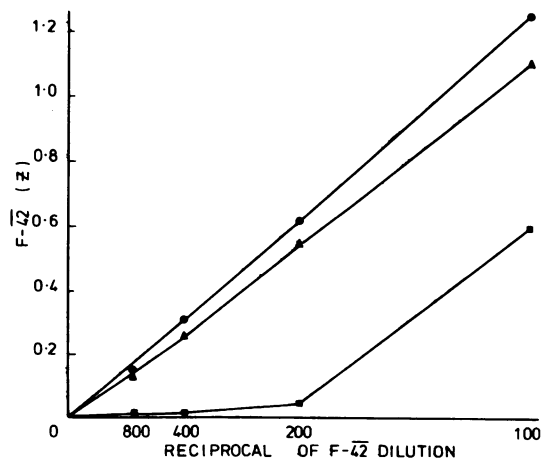
removing F-42 activity fully from 1:100, 1:200 and 1:400 dilutions of F-42, while anti-C3 had no significant effect. Efforts to elute F-42 activity with 3 M LiBr or with 0.3 M glycine, HCl, pH 2.8, were only partially successful. Although it was possible to recover 80% of the bound protein, less than 10% functional F-42 activity could be retrieved.

**Table 2.** Residual F-42 activity after incubation of three dilutions of F-42 with various cellular intermediates or buffer for 30 min at 30°

Intermediate	1.41*	0.69*	0.36*
EA	1.35 (96)	0.65 (94)	0.33 (92)
EAC1 <sup>SP4hu</sup>	1.34 (95)	0.66 (96)	0.32 (89)
EAC1 <sup>SP4hu2hu</sup>	0.93 (66)	0.32 (46)	0.11 (30)

\* Input of F-42 (100%) in GVB-EDTA.

The percentages of residual F-42 activity after absorption are given in parentheses.



**Figure 7.** Inhibition of F-42 activity by Sepharose-bound anti- $\gamma$  (■). Incubation with anti-C3 (▲) and buffer (●) served as controls.

## DISCUSSION

Sera of sixteen patients with systemic lupus erythematosus were examined for a factor capable of conserving the activity of the cell-bound classical C3 convertase, C4 $\bar{2}$  in the presence of EDTA for 30 min at 30°. Such a factor was found in two patients. Stabilization of C4 $\bar{2}$  by F-4 $\bar{2}$  exhibited a biphasic decay which could be explained by the fact that F-4 $\bar{2}$  acts by binding to C4 $\bar{2}$ . Since the degree of binding was directly correlated with the degree of stabilization it may be concluded that the action of F-4 $\bar{2}$  is non-enzymatic.

It was reported recently that the serum of a patient with acute glomerulonephritis (AGN) also contained an IgG immunoglobulin which was capable of binding to C4 $\bar{2}$  and to stabilize this convertase (Halbwachs, Leveille & Leibowitch, 1980). Also it was shown by Gorski & Müller-Eberhard (1980), that an antibody prepared in rabbits against the  $\beta$ -chain of C4 is capable of stabilizing C4 $\bar{2}$ . Whether the antibody found in the sera of patients with SLE (Daha, Hazevoet, Van Es and Cats, 1980) or in the patient with AGN is directed against C4, C2 or the complex of C4 and C2, is not clear. However, the experiment described in Table 2 suggests that the complex of C4 and C2 is capable of binding F-4 $\bar{2}$ . F-4 $\bar{2}$  purified to homogeneity will allow further investigation.

Recently it was reported (McLean & Nilson, 1979) that partially purified C3NeF is capable of inducing a two-fold stabilization of EAC1<sup>EP4hu2hu</sup> and to a lesser extent of EAC142<sup>hu</sup>. It is known that highly purified C3NeF can combine with C3b,Bb, the amplification convertase of complement, in a 1:1 molar ratio (Daha, Austen & Fearon, 1977) and that more than 90% of the highly purified and radiolabelled C3NeF may bind to EAC4b,3b,Bb (Daha *et al.*, 1977). Because partially purified C3NeF was used in the paper mentioned above (McLean & Nilson, 1979), it is not clear whether C3NeF directed to C3b,Bb or to a different population of proteins induces stabilization of the classical convertase. The factor described in this paper, F-4 $\bar{2}$ , has many characteristics in common with C3NeF. It stabilizes C4 $\bar{2}$  much like C3NeF stabilizes C3b,Bb (Schreiber, Medicus, Götze & Müller-Eberhard, 1975; Daha, Fearon & Austen, 1976); it seems to bind to C4 $\bar{2}$  like C3NeF binds to C3b,Bb (Schreiber, Götze & Müller-Eberhard, 1976; Daha *et al.*, 1977), and it has a similar molecular weight and electrophoretic mobility.

The experiments described here suggest that F-4 $\bar{2}$  may be found in patients with SLE and that it is presumably an antibody directed against C4 $\bar{2}$ .

Autoantibodies to various body constituents are known to occur in SLE (Holman, 1971; Miescher *et al.*, 1976), and this study suggests that C4 $\bar{2}$  may contain neoantigens which may induce antibody production. Whether formation of F-4 $\bar{2}$  occurs in all SLE patients and whether fluctuation in F-4 $\bar{2}$  levels during disease occurs requires further investigation. Currently, however, it is still difficult to quantify the levels of F-4 $\bar{2}$ , since factors such as C3 or inhibitors which have not yet been defined may influence the assay system.

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