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C3 Nephritic Factor Stabilization of the Classic C3 Convertase: A Role for C2 in C3 Nephritic Factor Activity (40553)[1]

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The C3 Nephritic Factor (C3Nef) is detectable in a minority of patients with mesangiocapillary glomerulonephritis (1–3) but in 60–70% of patients with partial lipodystrophy (4, 5). C3Nef has some characteristics of normal serum IgG; it is polyclonal and has multiple isoelectric points. C3Nef appears to function by prolonging alternative pathway convertase C3bBb by attachment to this convertase (6–9).

In studies of partially purified C3Nef from two patients with partial lipodystrophy, we find that C2 augments the C3-inactivating function of C3Nef, and that this effect may result from stabilization of the classic C pathway convertase C142 by C3Nef.

Methods. Isolation of C3 Nephritic Factor. C3Nef from two patients (L.C. and C.G.) with partial lipodystrophy was partially isolated by passage of 10 ml of the patient's serum, predialyzed against 0.002 *M* phosphate buffer, pH 7.0, over the anion exchanger triethylamineothyl cellulose (Cellex, T, Bio-Rad Laboratories, Richmond, Calif.) (3). Eluate fractions were concentrated by positive pressure and one aliquot was dialyzed against phosphate-buffered saline with magnesium (5×10^{-4}), pH 7.4, and a second aliquot dialyzed against Veronal-buffered saline with magnesium (5×10^{-4}), pH 7.4. One normal human serum was treated exactly the same for use as a control in the following experiments.

Assay of C3 Nephritic Factor. C3 inactivation by C3Nef (1 part test solution: 1 part normal serum at 37° for 20 min) was determined by one or more of the following procedures: (i) single-direction immunoelectrophoresis of these mixtures and examination for conversion products of C3, (ii) cross gel immunoelectrophoresis for C3 conversion products, (iii) hemolytic titration of residual specific C3 or C3–C9 activity with results being expressed as follows:

$$1 - \frac{\text{hemolytic C3 activity with C3Nef}}{\text{hemolytic C3 activity with buffer}} \times 100.$$

Human C2. Highly purified human C2 preparation (0.143 μg/ml in Veronal-buffered saline with 5×10^{-3} *M* Mg^{2+}, 1.5×10^{-4} *M* Ca^{2+}) was kindly supplied by Dr. R. Stroud (University of Alabama, Birmingham, Ala.). No Factor B was detectable in this preparation.

Hemolytic complement component assays. Buffered saline with metals ($GGVB^{2+}$) were prepared as previously described (10, 11). Methods for the measurement of C1, C4, and C2 have been previously published (10–12).

For specific titration of C3, $EAC\overline{14}$ were prepared and functionally pure guinea pig C2 and human C5, C6, C7, C8, and C9 (Cordis, Miami, Fla.) were used according to recommendations of the supplier. Functional C3 (C3–C9) was also measured by the potassium bromide (KBr) method (13).

T-Max and decay of EAC142. T-Max determinations with guinea pig C2 were performed at 30° according to previously described methods (11).

To determine the effect of partially purified C3Nef (dialyzed against $GGVB^{2+}$) on the decay of EAC142gp, EAC14 cells (1×10^9) at 4° were first premixed at +4° with C3Nef and $GGVB^{2+}$. An equal volume of a 1/500 dilution of functionally pure guinea pig C2 (in $GGVB^{2+}$) was prewarmed at 30°, combined with the cell mixtures, and incubated at 30°. Controls included cell blank control and complete lysis. Final concentration of cells was 5×10^7/ml. At predetermined times,

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duplicate aliquots of this mixture were removed, 0.3 ml of guinea pig complement in 0.04 *M* disodium ethylenediaminetetracetate (C-EDTA) were added and the tubes placed in 37° water bath. Following the removal of the final tube, all tubes were incubated for another 90 min at 37°. One milliliter of normal saline was then added, the tubes were centrifuged, and the optical density was measured at 412 nm.

For the determination of T-Max when using human C2, all incubations were performed at 26° rather than at 30° because of the more rapid formation and decay of EAC142(hu).

Using the average number of sites per cell (*Z*) as our endpoint, we determined the effect of C3Nef on the time required for decay of 50% of the EAC142 activity when using either functionally pure guinea pig C2 or pure human C2.

Immunoelectrophoresis. Immunoelectrophoresis of serum for C3 and Factor B was performed as previously described (10, 14).

Cross gel immunoelectrophoresis was performed with the Laurell apparatus (Medical Research Apparatus Corp., Boston, Mass.) (15).

Complement deficient sera and normal sera. Normal sera used for C3Nef assays were drawn on the day of use or from once-frozen serum (−70°).

Sera from four C2-deficient patients were available. One C2-deficient patient with lupus erythematosus has been extensively reported (16). All C-complement determinations of two patients discovered in this laboratory are normal except for absent C2 protein and C2 hemolytic function. Two other sera from C2-deficient patients have kindly been provided by Dr. V. Agnello (Tufts Medical School, Boston, Mass.).

Sucrose density gradient. The determination of the S-rate of C3Nef in a 10–40% sucrose gradient was performed in collaboration with Dr. Jane Chapitis (Department of Medicine, University of Connecticut Health Center) utilizing a L2-65B Model Ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif.).

Isoelectric focusing. Isoelectric focusing on a cooled platform (LKB Multiphor, Model 2117, LKB Produkter, AB, Sweden) of C3Nef was performed in freshly made 12.5 x 26-cm acrylamide gel slabs (pH 3.5–10, 2% ampholine). The acrylamide gel was sliced into 1 x 1-cm slabs, eluted overnight into 0.4-m GVB^+ (Mg^{2+} 1×10^{-3}) 0.5 *M* EGTA. Equal volumes of eluates and normal serum were incubated for assessment of C3Nef activity by immunoelectrophoretic criteria.

Results. Characterization of C3Nef. On a 10–40% sucrose gradient, serum from L.C. showed C3-inactivating properties at 7.5 S; both C3Nef preparations were present only in the pseudoglobulin (0.005 *M* phosphate, pH 7.0). Both partially purified C3Nef preparations were heat stable (56° for 30 min). The isoelectric point of 3Nef-C.G. was between pH 8.57 and 9.0. No C3 inactivation occurred in Factor B-deficient serum although active in simultaneously tested normal human serum; a dose–response was demonstrable for Factor B-dependent C3 inactivation by both C3Nef preparations (unpublished). Neither C3Nef preparation produced C3 activation in the absence of magnesium.

Effect of Dilution of C3Nef. To examine the relative strength of the C3Nef preparations, 50 μl of dilutions of partially purified C3Nef from each patient were incubated with 50 μl of normal serum and the amount of C3 inactivation measured. In this experiment, a 1/8 dilution of C3Nef-C.G. was as active as undiluted C3Nef-L.C. (54 vs 50% C3 inactivation, respectively) demonstrating greater activity of the former preparation.

The role of C2 in C3Nef activity. In four separate congenitally C2-deficient human sera (C2-DS), C3Nef-L.C. produced only 0–15% inactivation while 62% inactivation of C3 occurred in a simultaneously tested normal serum.

Undiluted C3Nef-C.G. inactivated 56–82% C3 in these same C2-deficient sera. However, at a dilution of 1/8, C3Nef-C.G. had no C3-inactivating properties in C2-deficient serum despite close to maximal C3 inactivation by this 1/8 diluted C3Nef preparation in a simultaneously tested normal serum (Fig. 1). This indicated that at certain concentrations, C3Nef-C.G. was also C2 dependent as had been found for C3Nef-L.C. To study this observation further, pure human C2 was used to reconstitute C2-DS and incubated with diluted C3Nef-C.G. C2 dependency of C3Nef activity was found which was dose dependent (Fig. 2).

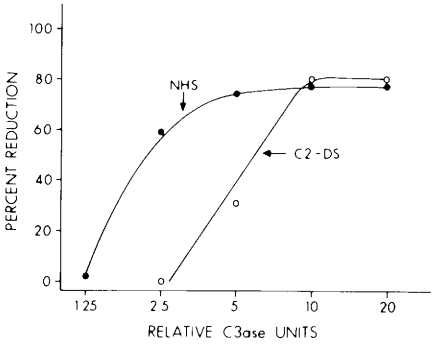

FIG. 1. Comparison of serially diluted C3Nef on normal human serum (NHS) and C2-deficient serum (C2-DS). At a relative C3Nef concentration of 2.5 (1/8 dilution), 58% inactivation of C3 was present in NHS but no detectable C3 inactivation in C2-DS was seen.

Effect of C3Nef on the decay of EAC$\overline{142}$. Undiluted C3Nef-C.G. caused a greater than twofold prolongation of the half-life of EAC$\overline{142}$gp cells using functionally pure guinea pig C2 (Fig. 3). This stabilization of the EAC$\overline{142}$gp cells by C3Nef was related to the C3Nef concentration; the effect varied from a 2.1-fold increase of the half-life of EAC$\overline{142}$gp with neat C3Nef to a 1.2-fold increase with a 1/4 dilution of C3Nef (Fig. 4). Normal serum (C3Nef-NHS) had no effect on the decay of EAC$\overline{142}$gp (39 min C3Nef-NHS, 40 min with buffer).

The possible role of contaminating guinea pig Factor B or C3 in this functionally pure guinea pig C2 used to prepare EAC$\overline{142}$gp

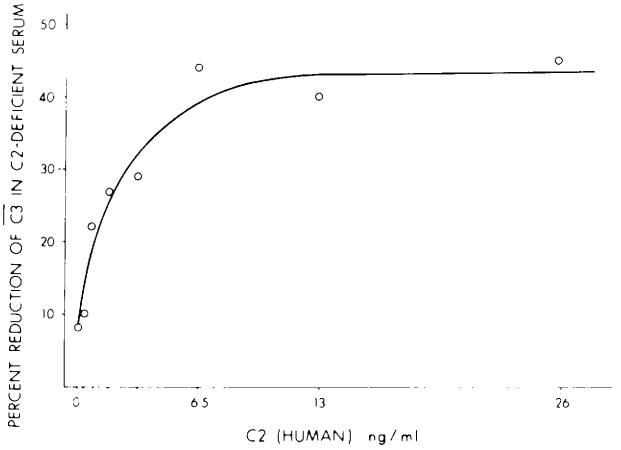

FIG. 2. The effect of C3Nef-C.G. on C2-reconstituted C2-deficient serum. In the absence of added C2, insignificant C3 inactivation (8%) was detected. With greater than 6.5 ng of added pure human C2, maximal C3 inactivation occurred. C2 concentration was expressed as the final concentration of C2 in the reaction mixture.

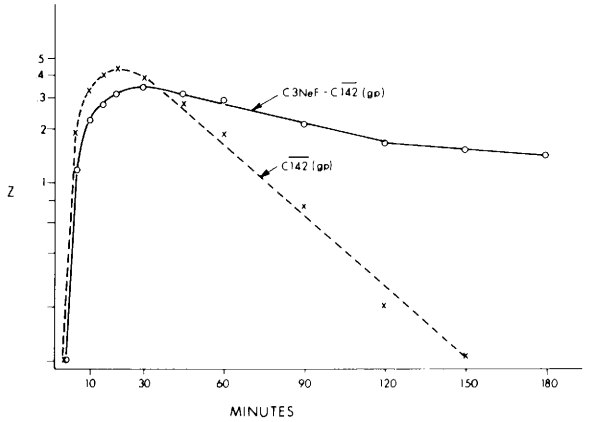

FIG. 3. The effect of C3Nef on the half-life of the classic C3 convertase using guinea pig C2. The half-life of the C3 convertase ($\overline{C142}$gp) was prolonged more than twofold (from 90 to 180 min) when formed in the presence of C3Nef-C.G. Time in minutes on abscissa, the average number of active sites per cell (Z) on ordinate.

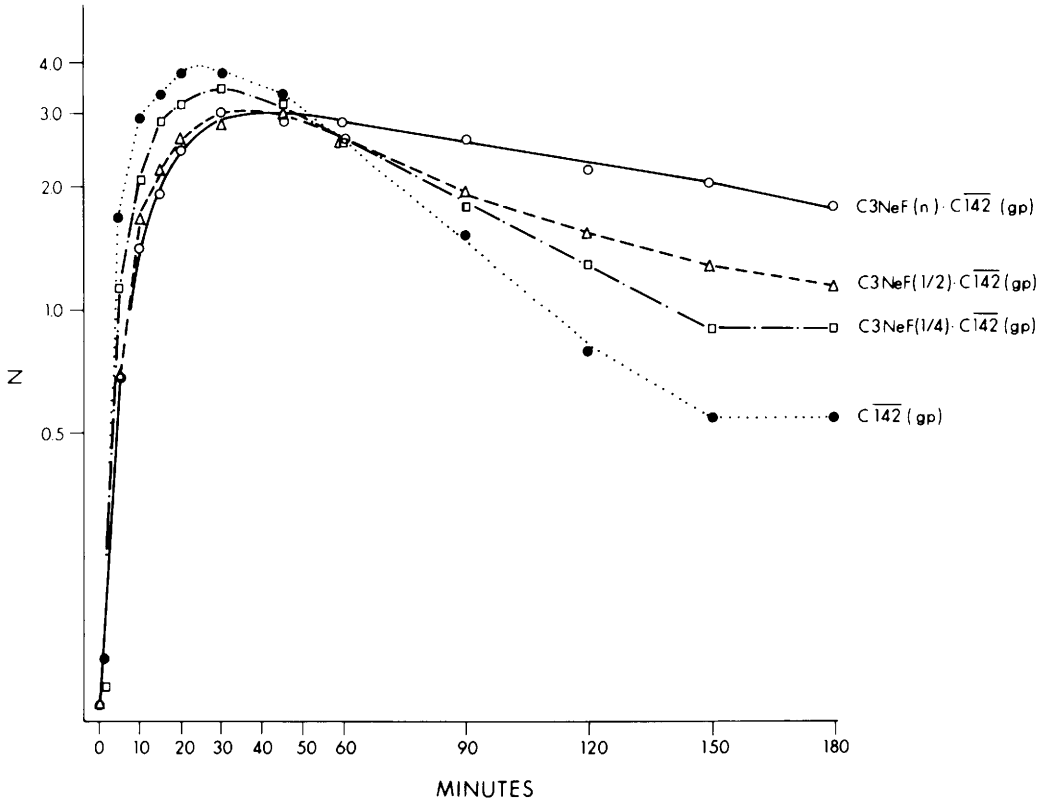

FIG. 4. The effect of decreasing concentrations of C3Nef-C.G. on the prolongation of the decay of the classic C3 convertase. Note that the greater the dilution of C3Nef, the more rapid is the decay of EAC$\overline{142}$gp. Ordinate is time in minutes, the average number of active sites per cell (Z) on ordinate.

cells was evaluated. Following incubation of EAC$\overline{14}$ cells with guinea pig C2 to T-max, the cells were cooled to +4° and washed twice with $GGVB^{2+}$. The washed EAC$\overline{142}$gp cells were resuspended to their previous volume, and the C3Nef preparations were then added for determination of the decay of T-max. Decay of C$\overline{142}$gp-C3Nef-C.G. was prolonged 1.55-fold when compared to EAC$\overline{142}$gp-C3Nef-NHS; this prolongation was most apparent between 60 and 120 min after T-max.

A 1.4-fold prolongation of the decay of EAC$\overline{142}$(hu) was found using purified human C2 instead of guinea pig C2.

Effect of C3Nef on classic complement components. Inactivation of classic C components by C3Nef preparations which were predialyzed against phosphate-buffered saline with magnesium or against VBS^{2+} (Table I) demonstrated inactivation of C4 and C2 and consistently high C3 inactivation.

TABLE I. INACTIVATION OF CLASSIC C COMPONENTS BY C3Nef PREPARATIONS FROM TWO PATIENTS (L.C. AND C.G.)[a]

	Percentage inactivation[b]			
	C1	C4	C2	C3
C3Nef-L.C. + NHS	4.4	33.2	58	78
C3Nef-C.G. + NHS	+3.8	50	79	94
C3Nef-NHS[c]	+8	12	41	7

[a] C component inactivation calculated by:

$100 \times (1 - \text{C concentration with C3Nef/C concentration with buffer})$.

[b] Mixture of 200 μl NHS (using VBS^{2+} as a buffer).

[c] C3Nef-NHS is the IgG fraction isolated from a normal (C3Nef-negative) serum by the same procedure used in the isolation of C3Nef from C3Nef-positive sera.

Discussion. The fluid-phase inactivation of C3 in serum by C3Nef isolated from the sera of two patients with partial lipodystrophy was related to the concentration of C2. This role of C2 was detected utilizing two partially

purified C3Nef preparations but could be demonstrated with one preparation of C3Nef only following dilution of the C3Nef preparation. The specificity and dose dependence of C2 was demonstrated following reconstitution of C2-DS with either human or guinea pig C2.

Since we found that C3Nef was able to stabilize EAC142(hu) and EAC142(gp), the latter in a dose-dependent fashion, we propose that the augmentation of C3Nef activity by C2 may be due, in part, to C3Nef-mediated stabilization of the classic C3 convertase in a manner analogous to C3Nef stabilization of the alternative pathway C2 convertase (C3bBb) (6).

In view of the slightly less maximal Z values with C3Nef, we cannot rule out a delay in the formation of C142-C3Nef sites. Furthermore, we have not excluded the presence of a factor which is separate from C3Nef but which has similar immunoconglutinin-like activity against the classic C3 convertase rather than the alternative pathway C3 convertase.

Four different C2-DS, including one completely asymptomatic individual, were utilized in this study. Therefore, the absent C3 inactivation by C3Nef was not a characteristic of a single serum. It is possible that C2-deficient sera lack another factor which would result in decreased C3Nef activity; Factor B is known to be slightly reduced in C2-DS (16). However, this explanation seems unlikely since reconstitution by human C2 restored C3-inactivating activity by C3Nef in a dose-dependent manner and Factor B was normal in the test sera—98% and 88% (normal 69–130%).

Further evidence in support of participation of early classic C components with C3Nef activity was provided by the depletion of C4 and C2 activity following the incubation of C3Nef in normal serum. Although depletion of C4 and C2 was variable, the mean value for all experiments was considerable and was more than that seen with the control C3Nef preparations. C1 activation was never observed in our experiments in the fluid phase whereas others (17) have reported 70.1% C1 cleavage and C1q binding with C3Nef on a cellular intermediate (EC3bBNef), perhaps reflecting greater efficiency of activation by solid-phase intermediates. (Activation of C4 or C2 was not reported by these investigators.) Activation of the classic C system by C3Nef is demonstrated by these experiments and may account in part for the participation of C2 in C3Nef activity. The stabilization of preformed C142 by semipurified C3Nef is an additional and previously undetected function of C3Nef.

Immune complexes or aggregated proteins in our C3Nef preparations may have produced some early classic C-component inactivation (particularly by the normal C3Nef preparation which was not ultracentrifuged before use). However, the presence of immune complexes in these C3Nef-positive preparations was unlikely since both C3Nef preparations were fully active in calcium-free reaction volumes and C1 consumption was never detected. C1r and C1s were not detected by Ouchterlony analysis in these C3Nef preparations (courtesy of Dr. R. Stroud). Since ultracentrifugation (105,000g) of the C3Nef-positive preparations did not influence C3Nef activity, we feel that neither aggregated proteins nor immune complexes were the cause of C consumption, but we cannot entirely eliminate this possibility.

The participation of classic C components in the activation of the alternative C pathway has been noted by other investigators. Brade demonstrated a 50-fold acceleration of the formation of the alternative C-pathway convertase on zymosan when using normal serum rather than C4-deficient guinea pig serum (18). Also with zymosan as the activating particle, Gigli noted decreased C3 inactivation in C2-DS, an effect which was brought out by dilution of C2-DS (19). Gigli *et al.* attributed the role of C2 to the provision of more C3b caused by "natural antibody" activation of the classic C pathway on zymosan. In another study, decreased early phagocytosis of yeast in C2-DS was correctable by adding C2; this abnormality was interpreted as evidence of decreased efficiency of the alternative C pathway in C2-deficient serum (20).

C2-Dependent activation of C3 could only be seen when comparing dilutions of C3Nef-C.G. in normal and C2-DS. Previous reports of C3Nef activity in C2-DS do not mention

the effect of dilution of C3Nef preparations on the amount of C3 inactivation. Our observation of the stabilization of the classic C3 convertase by C3Nef is an additional explanation of the role of classic C components in the activation of the alternative pathway.

Summary. The C3 Nephritic Factor (C3Nef) from two patients with partial lipodystrophy was partially isolated in order to study the role of early classic C-complement components in C3Nef activity. Both C3Nef preparations were C2 dependent for C3 inactivation, although one C3Nef was C2 dependent only following dilution of the C3Nef preparation. Reconstitution of C2-deficient sera with either human or guinea pig C2 restored C3Nef activity in C2-deficient sera. C3Nef prolonged the decay of the classic complement C3 convertase (EAC142gp) in a dose-dependent manner, and this function of C3Nef may explain, in part, the role of C2 in C3Nef activity.

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