MECHANISM OF ACTION OF THE C4 NEPHRITIC FACTOR

Deregulation of the Classical Pathway C3 Convertase*

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Cleavage of the third component of complement (C3) and subsequent assembly of the attack mechanism (C5-C9) occurs as a function of at least two distinct enzymes generated during the activation of the classical (1) and the alternative (2–4) pathways. The classical pathway C3 convertase is formed in the fluid phase (5), or is bound to particles (6) by a sequential reaction involving the reversible binding of C2 to C4b in the presence of Mg^{2} to form the C4b2 complex, and by subsequent cleavage of the bound C2 by $C\bar{1}s$ to generate C4b2a (7). The latter decays by dissociation of C2a, becoming functionally inactive C2i (8). C4b and $C\bar{1}s$ remain in their active state and are able to regenerate the convertase with additional C2 (8). These reactions are remarkably similar to those occurring during the formation of the alternative pathway C3 convertase (3, 4).

Although a great deal of knowledge has been accumulated on the modulation of the alternative pathway (9, 10), the same information relating to the classical pathway enzyme is less complete, except for the knowledge that the C4b2a, formed either on particles or in the fluid phase, is thermolabile (5, 6). At physiologic temperatures, the C2a fragment is released from the complex with a half-life of 7 min, thus rendering the enzyme inactive. The recent demonstration of a serum protein, designated C4b binding protein (C4-bp),[1] which exhibits a marked affinity for C4b (11), suggested that this protein may also regulate the function of the C4b2a enzyme. It has been shown that C4-bp accelerates the decay rate of C4b2a (12) by dissociating C2a from C4b (13). Therefore, similarly to β1H in the alternative pathway (14, 15), C4-bp provides the means for an extrinsic decay of C4b2a. C4-bp may also regulate the generation of C4b2a enzyme by impairing the uptake of C2 by C4b (12). The capacity of C4b to form the classical pathway C3 convertase is limited by the proteolytic action of the serum enzyme C4b/C3b inactivator (C4b/C3bINA). This reaction results in the cleavage of the α' polypeptide chain of C4b yielding the C4d (α2) fragment of the α' chain, and C4c, which contains the two remaining fragments of the α' chain (α3 and α4) and the intact β and γ chains (12). Cleavage of C4b in the fluid phase by

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[1] *Abbreviations used in this paper:* C3Nef, C3 nephritic factor; C4-bp, C4b binding protein; C4b/C3bINA, C4b/C3b inactivator; $DGVB^{++}$, $GVB^{=}$ mixed with 5% dextrose in water containing 0.005 M $MgCl_2$ and 0.00015 M $CaCl_2$; GVB^{++}, veronal-buffered saline containing 0.1% gelatin, 0.005 M $MgCl_2$, and 0.00015 M $CaCl_2$; NFc, nephritic factor of the classical pathway; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus.

C4b/C3bINA absolutely requires the presence of C4-bp (16, 17), whereas the surface-bound C4b proceeds independently of these proteins (12).

In contrast to these reactions, which dampen the formation and function of C4b2a, a plasma protein that is functionally characterized by its capacity to stabilize the classical pathway C3 convertase has recently been described (18, 19). This protein, a gammaglobulin, has been isolated from the plasma of one patient suffering from acute post-infectious glomerulonephritis (18) and from some patients with systemic lupus erythematosus (SLE) (19). Because of the physicochemical and functional similarities with C3 nephritic factor (C3Nef; 20), it has been postulated that this protein may represent an antibody to the C4b2a enzyme. It has been termed the nephritic factor of the classical pathway (NFc; 18).

In this paper, we describe the mechanism of action of NFc and its relationship to the control proteins C4-bp and C4b/C3bINA. We demonstrate that it prevents the intrinsic decay of C4b2a, as well as the extrinsic decay mediated by the effect of C4-bp. In addition, we show that the C4b in the stable C4b2a enzyme is protected from the proteolytic action of the C4b/C3bINA.

Materials and Methods

Reagents. Agarose was obtained from Behring Diagnostics, American Hoechst Corp., Sommerville, N. J.; *N,N'*-methylene-bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulphate, and sodium dodecyl sulphate were obtained from Bio-Rad, Richmond, Calif.; diisopropylfluorophosphate was purchased from Calbiochem, San Diego, Calif.

Buffers. Phosphate-buffered saline (Dulbecco's PBS) was obtained from Grand Island Biological Co., Grand Island, N. Y. Other buffers used were PBS containing 2 mM Na_3H EDTA, pH 7.55; barbital buffer, pH 8.6, containing 0.023 M sodium barbital, 0.0037 M barbituric acid; 0.002 M barbital, with NaCl added to achieve a conductivity of 7.4 mS at 0°C, and 0.002 M Na_3H EDTA (VBS-EDTA); veronal-buffered saline containing 0.1% gelatin, 0.005 M $MgCl_2$, and 0.00015 M $CaCl_2$; $GVB^{=}$ mixed with an equal volume of 5% dextrose in water containing 0.005 M $MgCl_2$ and 0.00015 M $CaCl_2$ ($DGVB^{++}$).

Purified Complement Components. Guinea pig and human $C\bar{1}$ (21) and $C\bar{1}s$ (22), human C4 (23), C2 (24), C4-bp (11), C3, C5 (25), and C4b/C3bINA (26) were purified by published techniques. C4 was radiolabeled with ^{125}I as described (27); the specific activity was $\simeq 4 \times 10^5$ cpm/μg of protein. These proteins were quantitated functionally by hemolytic techniques and their protein concentration was measured by the method of Lowry et al. (28), or by their absorbance at 280 nm, assuming that an absorption of 1.0 equals 1 mg of protein/ml.

Cellular Intermediates and Assays. EA and $EAC\bar{1}$ were prepared from sheep erythrocytes, rabbit anti-sheep erythrocytes, and human $C\bar{1}$, and were converted to $EAC\bar{1}4b$ by using highly purified C4 at a concentration of 6 μg/1 $\times 10^8$ cells (12). The C4 deposited on the cellular intermediate $EAC\bar{1}4b$ represented 10% of the C4 input, as assessed by an uptake of ^{125}I-labeled C4. C4, C2, and C3 were measured by hemolytic titrations (29). Rat serum diluted 1:20 in EDTA-$GVB^{=}$ was used as a source of C3-C9. The C6, C7, C8, and C9 used in C3 and C5 titrations were purchased from Cordis Laboratories (Miami, Fla.).

Isolation and Purification of the NFc. The identification of this factor was originally reported on serum and EDTA plasma from a patient (R. A. M.) with postinfectious acute glomerulonephritis (18). The purification, as previously described (18), consisted of separation of the euglobulin fraction of the plasma, or serum, obtained by dialysis against 5 mM PO_4, pH 5.9, containing 2.5 mM EDTA. The supernate was then applied to a carboxymethyl cellulose column equilibrated with the same buffer and eluted with a salt gradient. The samples with NFc activity eluted between 7 and 14 ms. They were pooled, concentrated, and applied to a Sephadex G-200 column in PBS. The stabilizing activity was found superimposed with the ascending portion of the IgG peak; its physicochemical characteristics have been previously described (18).

Structural Analysis of Cell-Bound ^{125}I-labeled C4. Sodium dodecyl sulphate-polyacrylamide gel

electrophoresis (SDS-PAGE) was performed as previously described (30). In all cases, a 3% stacking gel and 5–15% gradient running gels were used. Approximate molecular weight determinations under reducing conditions were calculated by using the purified C4 as standard.

Cellular intermediates, carrying ^{125}I-labeled C4, were lysed in 50 mM Tris-HCl, pH 6.8, containing 2% $NaDodSO_4$, 6 M urea, 10% glycerol, and 0.1 M dithiothreitol. The samples were heated for 3 min in boiling water and applied to polyacrylamide gels. After electrophoresis, the gels were stained and dried. Radioautography was carried out by exposing the dried gels to an X-omat R film XR-S at −70°C for 12–72 h. Supernatant fluids were analyzed in the same manner after the proteins were precipitated using trichloroacetic acid.

Results

EAC$\overline{1}$4b2a: Formation and Functional Stabilization in the Presence of NFc. To demonstrate the effect of NFc on the formation of the classical pathway convertase, four samples of EAC$\overline{1}$4b cells, prewarmed at 30°C, were incubated with an equal volume of a limited concentration of C2 diluted with $DGVB^{++}$, or with increasing concentrations of NFc. Samples were removed at zero time, and at intervals thereafter, and the C4b2a activity generated was developed with rat complement in EDTA. As shown in Fig. 1, the presence of NFc during the generation of C4b2a resulted in the detection of more C3 convertase sites. This was evidenced by increased lysis of the EAC4b2a by the rat complement in EDTA that was used as a source of C3 to C9. Once the maximal reactivity of the cells was reached, the hemolytic potential of the cells remained unchanged throughout the 60 min of incubation, as compared with the EAC$\overline{1}$4b2a generated either in the presence of low concentrations of NFc or in $DGVB^{++}$. Thus, it appears that NFc augments C3 deposition and the assembly of the attack mechanism, resulting in increased lysis.

That EAC$\overline{1}$4b2a, prepared in the presence of NFc, augmented C3 cleavage and the initiation of the assembly of the membrane attack mechanism, was investigated by measuring the amount of C2, C3, and C5 consumed by these EAC$\overline{1}$4b2a prepared in the presence of NFc. Two samples of EAC$\overline{1}$4b2a were generated with excess C2, diluted either in buffer or in buffer containing NFc. The cells were separated by centrifugation and the supernate was kept for measurement of residual C2 activity. The cells were then washed with ice cold buffer, resuspended in buffer containing

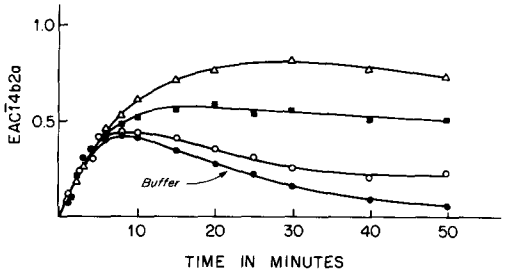

FIG. 1. Formation of cell-bound C4b2a. Four samples of EAC$\overline{1}$4b (1×10^8 ml) were prewarmed at 30°C and incubated with an equal volume of C2 (0.5 functionally effective molecules 1×10^8 cells) diluted in $DGVB^{++}$ (●), or 20 μg/ml NFc (△); 10 μg/ml (■); 5 μg/ml (○). At zero time and intervals thereafter, 0.5 ml was removed and added to 0.75 ml of rat complement diluted 1:20 in 0.04 M EDTA in $GVB^{=}$. After 1 h at 37°C, 2.5 ml of saline was added, the unlysed erythrocytes were sedimented by centrifugation, and the hemoglobin in the supernate was read in a spectrophotometer at OD_{414}. The percent lysis as compared with a sample of cells 100% lysed was calculated and expressed as $Z = -\ln\left(\frac{1}{\%\ \text{lysis}}\right)$.

excess C3, and further incubated at 37°C for 1 h. The cells were separated and the supernate were measured for residual C3 hemolytic activity and compared with a C3 sample that was incubated in buffer alone. A sample of $EAC\overline{1}4b3b$ cells, prepared in the absence of NFc after prolonged incubation to assure complete decay of C2, was divided into four equal aliquots that received either C2, C2 and NFc, NFc alone, or buffer. After 15 min incubation at 30°C, the cells were washed and their capacity to use C5 was measured by incubating each cell preparation with purified C5. The results of these experiments are shown in Fig. 2. $EAC\overline{1}4b$ incubated with C2 in the presence of NFc consumed less C2. However, the convertase generated was capable of consuming more C3 (44%), as compared with the C3 convertase formed in the absence of NFc (14%). A greater use of C5 by the C5 convertase prepared in the presence of NFc was also shown (48 and 18%, respectively). It should be emphasized that this increase in C5 use was not due to a larger number of C3b present on the erythrocytes because the C3b was originally deposited using $EAC\overline{1}4b2a$ prepared in the absence of NFc. These experiments demonstrate that the enhanced hemolytic activity observed in the presence of NFc was not due to the generation of a larger number of C4b2a. It is significant that the results also show that in addition to the C4b2a convertase, NFc affects the function of the C5 convertase (C4b2a3b).

Next, we investigated the effect of NFc on the decay of cell-bound C4b2a. $EAC\overline{1}4b2a$ cells were prepared with a limited concentration of C2. The cells were washed in $DGVB^{++}$ and divided into four equal samples. These samples were prewarmed at 30°C and at zero time each was mixed with an equal volume of

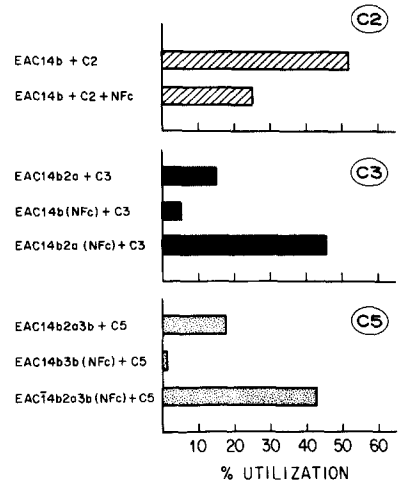

FIG. 2. C2, C3, and C5 use by $EAC\overline{1}4b$ and C2 in buffer or in buffer with 40 μg of IgG containing NFc activity. $EAC\overline{1}4b2a$ were prepared with excess C2 diluted in buffer or in buffer containing 40 μg NFc. After 15 min incubation at 30°C, the supernate was separated and measured for residual C2 activity. The cells were washed and resuspended in buffer containing C3. A sample of $EAC\overline{1}4b$ preincubated with NFc without C2 was included as control. After 60 min incubation at 37°C, the supernatant fluid was measured for residual C3 activity. The $EAC\overline{1}4b3b$ were generated by incubated $EAC\overline{1}4b2a$ with C3 in the absence of NFc, followed by decay of C2. These cells were converted to $EAC\overline{1}4b2aC3b$ or $EAC\overline{1}4b2a3b$ (NFc) by incubation with C2 in buffer or C2 in buffer containing NFc. After 15 min of incubation at 30°C, the cells were washed in ice cold buffer and mixed with buffer containing purified C5. The mixtures were incubated 60 min at 37°C and the residual C5 was measured in the supernatant fluids.

prewarmed $DGVB^{++}$ containing increased amounts of NFc or $DGVB^{++}$ alone. 0.5-ml samples were removed at intervals and the C4b2a sites/cells were assessed by adding rat complement-EDTA. The results (Fig. 3) demonstrate that C3 convertase on $EAC\bar{1}4b2a$ incubated with buffer decayed with a half-life of 7.5 min, whereas in the presence of increasing concentrations of NFc, the decay was abolished. The initial decay observed at low NFc concentration may represent unstabilized C4b2a sites. In a separate experiment, it was shown that IgG or IgM isolated from normal serum failed to alter the decay of the C3 convertase, and that the stabilized convertase remained fully active after 5 h of incubation at 30°C.

Protection of $EAC\bar{1}4b2a$ (NFc) from Accelerated Decay by C4-bp. The capacity of C4-bp to accelerate the intrinsic decay of the stabilized C3 convertase (C4b2a) was investigated in the following experiment. $EAC\bar{1}4b2a$ and $EAC\bar{1}4b2a$ (NFc) were generated by incubating equal samples of $EAC\bar{1}4b$ with C2, diluted in $DGVB^{++}$ or in $DGVB^{++}$ containing NFc. Each sample was washed in ice cold $DGVB^{++}$, resuspended in $DGVB^{++}$, and divided into two equal aliquots. One sample of each received 9.5 ml of $DGVB^{++}$ and the other received 9.5 ml of $DGVB^{++}$ containing C4-bp. At the time of mixing, and at intervals thereafter, 0.5-ml samples were removed from each mixture and the number of remaining C4b2a sites was measured hemolytically. The half-life of the C4b2a enzyme (Fig. 4) when incubated in buffer alone was 7.5 min, but it was shortened to 2.5 min in the presence of C4-bp. In contrast, C4-bp failed to alter the hemolytic activity of the stabilized convertase. A dose response of the effect of C4-bp demonstrated that the stabilized enzyme was totally resistant to the effect of C4-bp even at doses 30 times greater than that sufficient to accelerate the decay of the nonstabilized convertase by >50%.

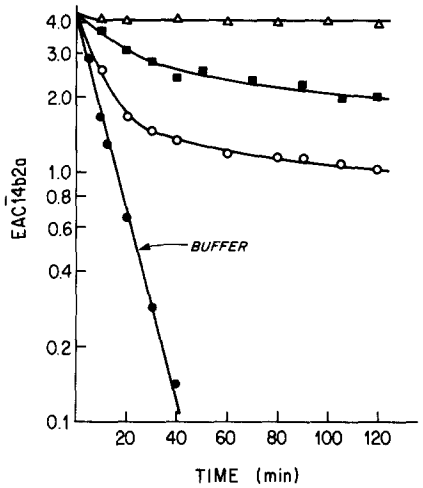

FIG. 3. Decay of $EAC\bar{1}4b2a$ and $EAC\bar{1}4b2a$ (NFc). $EAC\bar{1}4b2a$ were prepared by incubating for 5 min. $EAC\bar{1}4b$ (1×10^8 cells/ml) were prewarmed at 30°C with C2 diluted in $DGVB^{++}$ to contain 5 functionally effective U/1 $\times 10^8$ cells. The cells were washed in cold $DGVB^{++}$ and resuspended in the same buffer to a concentration of 5×10^8/ml. 1-ml samples were added: one to $DGVB^{++}$ (●), and one each to IgG containing NFc activity diluted in $DGVB^{++}$ to final concentrations of 40 μg (△), 20 μg (■), and 5 μg (○). The final cell concentration was 5×10^7/ml. At the time of mixing and at intervals thereafter, 0.5 ml was removed from each reaction mixture and added to 0.75 ml of rat complement diluted in 0.04 M EDTA-$GVB^{=}$, and the hemoglobin released was measured as described after 1 h incubation at 37°C.

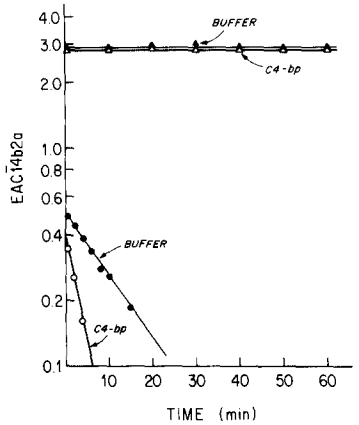

FIG. 4. Effect of C4-bp on the decay of EAC$\overline{1}$4b2a and EAC$\overline{1}$4b2a (NFc). EAC$\overline{1}$4b2a and EAC$\overline{1}$4b2a (NFc) were generated by incubating for 5 min at 30°C two 15-ml samples of EAC$\overline{1}$4b (1×10^8/ml) with an equal volume of C2 containing 5 effective U/1×10^8 cells diluted in DGVB^{++} alone, or in DGVB^{++} containing 20 μg NFc. The cells were washed and resuspended in 1.5 ml DGVB^{++}, and two 0.5-ml aliquots were removed from each sample. One aliquot of each received 9.5 ml DGVB^{++}: EAC$\overline{1}$4b2a (●) and EAC$\overline{1}$4b2a (NFc) (▲), and the other two received 9.5 ml C4-bp (10 μg/1×10^8 cells), EAC$\overline{1}$4b2a (○), EAC$\overline{1}$4b2a (NFc) (△), both prewarmed at 30°C. At the time of mixing and at intervals thereafter, 0.5-ml samples were removed from each reaction mixture and the number of C4b2a sites were developed by the addition of 0.75 ml of rat complement-EDTA. After 60 min incubation at 37°C, the amount of hemoglobin released by each sample was measured. The ordinate depicts the hemolytic activity of each sample during the decay time, which is represented by the abcissa.

TABLE I

Release of Radioactivity from EAC$\overline{1}$4b2a and EAC$\overline{1}$4b2a (NFc) by the Regulatory Proteins C4-bp and C4b/C3bINA

	EAC$\overline{1}$4b2a (NFc)				EAC$\overline{1}$4b2a			
	C4-bp C4b/ C3bINA	C4-bp	C4b/ C3bINA	Buffer	C4-bp C4b/ C3bINA	C4-bp	C4b/ C3bINA	Buffer
Percent counts released	9	3	6	1	53	7	42	6
Percent counts on cells	89	96	91	95	43	93	56	92

Cells were prepared with ^{125}I-C4. Total counts in each reaction mixture, 17,500 ± 2%.

Protection of C4b in EAC$\overline{1}$4b2a (NFc) from Proteolytic Cleavage by C4b/C3bINA. The susceptibility of C4b2a (NFc) to the proteolytic action of the C4b/C3bINA was investigated structurally and functionally. Two samples of EAC$\overline{1}$4b cells, prepared with ^{125}I-labeled C4, were incubated with excess C2 and either buffer or NFc. After 30 min of incubation at 30°C, the cells were centrifuged, washed with ice cold DGVB^{++}, and resuspended in the same buffer. Each sample was divided into four aliquots and incubated with either DGVB^{++}, C4-bp, C4b/C3bINA, or with both C4-bp and C4b/C3bINA. After 1 h at 37°C, DGVB^{++} was added to each sample, the supernatant fluids were removed and the cells were carefully washed with cold DGVB^{++}. Subsequently, the radioactivity of the cells and supernates was measured (Table I). The cells were then lysed, the membranes were solubilized in SDS-urea, and both the membranes and the supernates were subjected to SDS-PAGE, and an

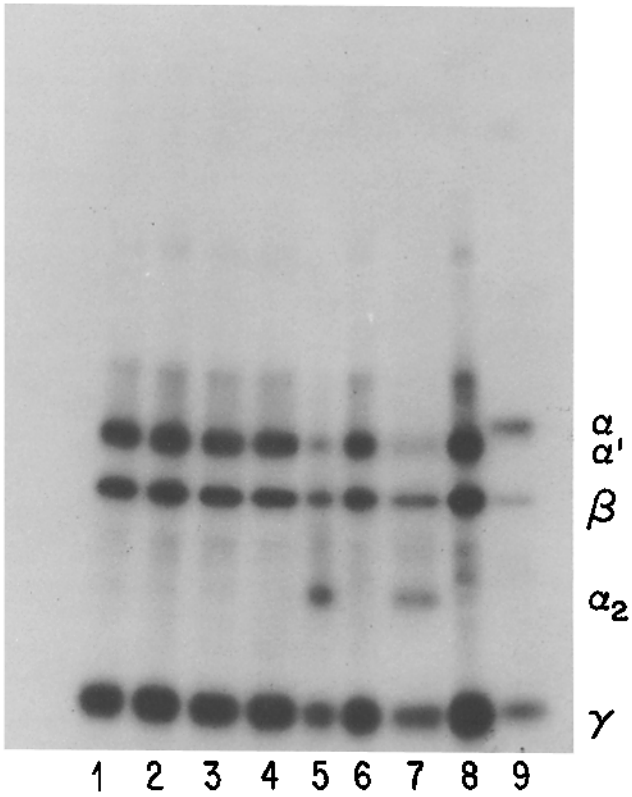

FIG. 5. Cleavage of C4b in EAC$\overline{1}$4b2a and EAC$\overline{1}$4b2a (NFc) by C4-bp and C4b/C3bINA. EAC$\overline{1}$4b prepared with ^{125}I-C4 were converted to EAC$\overline{1}$4b2a with C2 diluted either in buffer containing NFc or alone. The EAC$\overline{1}$4b2a (NFc) and EAC$\overline{1}$4b2a generated were each divided into four equal samples; one sample of each received either C4-bp and C4b/C3bINA, C4-bp, C4b/C3bINA, or GVB^{++}. After 1 h at 37°C, the cells were separated, washed until the supernates were free of radioactivity. The cells were lysed and the membranes were dissolved in dithiotreitol-SDS. These samples and a sample of reduced ^{125}I-C4 were applied to a 5–15% polyacrylamide gradient slab gel and electrophoresis was performed. EAC$\overline{1}$4b2a (NFc) was treated with: C4-bp + C4b/C3bINA (track 1), C4-bp (track 2), C4b/C3bINA (track 3), and buffer (track 4). EAC$\overline{1}$4b2a was treated with: C4-bp + C4b/C3bINA (track 5), C4-bp (track 6), C4b/C3bINA (track 7), and buffer (track 8). Track 9 depicts ^{125}I-C4 used to prepare the cellular intermediates.

autoradiograph was performed. The results show that the EAC$\overline{1}$4b2a (NFc) were completely protected from the action of the control proteins. This was evidenced by the lack of radioactivity released into the supernate (Table I) and the intactness of the α' chain of C4b following treatment with C4b/C3bINA alone or in addition to C4-bp (Fig. 5). In contrast, the unstabilized EAC$\overline{1}$4b2a released 50% of the radioactivity in the supernates after treatment with C4b/C3bINA alone or with C4-bp. SDS-PAGE analysis of these supernates revealed C4c (α3, α4, β, and γ), whereas C4d (α2) could be recognized as being attached to the treated cells (Fig. 5). Incubation of EAC$\overline{1}$4b with NFc failed to protect C4b, which indicates that the effect of NFc is dependent upon the presence of the C2a, which must be bound to C4b (C4b2a).

The functional activity of EAC$\overline{1}$4b2a (NFc) and EAC$\overline{1}$4b2a after exposure to the control proteins, was investigated by measuring the capacity of the cellular intermediates to cleave C3. Although the C4b/C3bINA alone, or in the presence of C4-bp,

abolished >50% of the C3 cleaving activity of the nonstabilized cell-bound convertase (Table II), the stabilized enzyme completely circumvented the effect of the control proteins. Identical C3 utilization was afforded by EAC$\overline{1}$4b2a (NFc), which was pretreated with buffer or with the control proteins. Moreover, the stabilized convertase, after incubation at 37°C, used the same number of C3 units without the addition of C2, whereas the nonstabilized convertase required, as was expected, additional C2.

Discussion

Regulation of the classical pathway C3 convertase is critical in determining whether the initial activation of the complement system leads to the formation of C3b, to the initiation of the amplification step mediated by C3b, and to the assembly of the protein complex involved in the membrane attack. On erythrocyte-bound C4b2a, this regulation is exerted through at least three different natural mechanisms: (*a*) the intrinsic decay of the enzyme, which is the consequence of the temperature-dependent instability of the convertase (8), leading to the dissociation of C2a; (*b*) an accelerated decay-dissociation of C2a from the C4b2a complex, which is mediated by the effect of the normal serum protein, C4-bp (12); and (*c*) the subsequent inactivation of the uncovered C4b by the serum enzyme C4b/C3bINA (12). Proteolysis of the α' chain of C4b into C4c and C4d (12) by the C4b/C3bINA prevents the formation of the classical pathway C3 convertase (12).

Recently, a factor that is functionally characterized by its capacity to stabilize the classical pathway C3 convertase, was isolated from the plasma of a patient with acute postinfectious glomerulonephritis (18). Similar material was also noted in some patients with SLE (19). This factor was found to be associated with a serum protein that, according to its physicochemical and antigenic properties, could not be distinguished from IgG (18). The protein binds to EAC$\overline{1}$4b2a, but not to EAC$\overline{1}$4b or EAC3bBb, and its biologic activity is associated with a portion of the molecule that behaves as the antigen-combining site of IgG. These findings are compatible with the concept that this IgG or "IgG-like" molecule is acting as an autoantibody (18), similar

TABLE II

Effect of the Control Proteins C4-bp and C4b/C3bINA on C3 Consumption by EAC$\overline{1}$4b2a and EAC$\overline{1}$4b2a (NFc)

Reaction mixture*	Percent C3 used	
	C3 added	C2 + C3 added
EAC$\overline{1}$4b2a, buffer	14	50
EAC$\overline{1}$4b2a, C4-bp	4	46
EAC$\overline{1}$4b2a, C4b/C3bINA	4	24
EAC$\overline{1}$4b2a, C4-bp, C4b/C3bINA	2	20
EAC$\overline{1}$4b2a (NFc), buffer	76	76
EAC$\overline{1}$4b2a (NFc), C4-bp	75	76
EAC$\overline{1}$4b2a (NFc), C3bINA	76	76
EAC$\overline{1}$4b2a (NFc), C4-bp, C4b/C3bINA	74	76

* Cellular intermediates were washed after 60 min incubation with control proteins or buffer. The cells were resuspended in buffer containing either C3 alone, or C3 and C2. After 60 min of incubation, the cells were removed by centrifugation and the C3 in the supernate was measured hemolytically.

to the C3Nef of the alternative pathway (31). The stabilization of the classical pathway C3 convertase, recently ascribed to the C3Nef (32) may in fact represent a protein identical to the one described here.

Our investigation has permitted the elucidation of the mechanism(s) by which NFc regulates the classical pathway C3 convertase. The presence of NFc during the formation of cell-bound C4b2a resulted in the generation of a larger number of C4b2a sites (Fig. 1), as evidenced by a greater hemolytic activity of the EAC$\overline{1}$4b2a. This augmented lysis was interpreted as being the consequence of enhanced cleavage of C3 and C5 and the assembly of the proteins of the attack mechanism, which is probably a result of the stabilization of the convertase. The generation of an apparently larger number of C4b2a sites was the result of a marked retardation in the intrinsic decay of C2a from the C4b2a complex, rather than an increased use of C2, leading to the formation of more C4b2a. Two lines of evidence support this view: (*a*) the generation of EAC$\overline{1}$4b2a in the presence of NFc was associated with a lesser use of C2 in spite of the generation of a large number of EAC4b2a (Fig. 2); and (*b*) the prolongation of the half-life of the cell-bound enzyme from 7.5 min in the absence of NFc, to >5 h in its presence (Fig. 3). The effects of NFc were observed when it was added during the generation of the enzyme (Figs. 1 and 4), or during the decay process (Fig. 3). Thus, the increase in the number of generated C4b2a sites represents the formation of a stable convertase, in which the intrinsic decay of C2a is markedly retarded. Further, it appears that NFc can also generate a more stable C5 convertase (Fig. 2). The capacity of the NFc used in these experiments to stabilize the C4b2a enzyme is greater than that isolated by Daha et al. (13) from patients with SLE, probably reflecting a greater affinity of this molecule for the C4b2a complex.

Alone, stabilization of the classical pathway convertase in the presence of the control proteins C4-bp and C4b/C3bINA could not account for the persistence of an active C4b2a complex in serum as was seen in patient R. A. M. This observation prompted studies to examine whether NFc could interfere with the function of the control proteins. 30 times the amount of C4-bp sufficient to reduce the half-life of C4b2a on sheep erythrocytes by 50% was ineffective in altering the decay of the NFc-stabilized convertase (Fig. 4). The resistance of C4b2a (NFc) to the accelerating effect of C4-bp on the decay dissociation of C2a was recently confirmed by the demonstration of the limited ability of C4-bp to dissociate ^{125}I-C2 from the enzyme complex (13). Therefore, a second mechanism by which NFc induces enhanced C3 cleavage in serum can be attributed to the formation of a classical pathway convertase that is resistant to the regulatory action of C4-bp.

The inability of C4-bp to accelerate the decay of C2a from C4b2a (NFc) suggested that the C4b in the complex was resistant to proteolytic cleavage by C4b/C3bINA. Indeed C4b, in the NFc-stabilized convertase, was found to maintain its functional and structural integrity as shown by C3 use (Table II), and the preservation of the structure of α' chain of C4b in the complex (Fig. 5). However, C2a must be complexed to C4b for NFc to exert its protective function, as demonstrated by the inability of this protein to prevent the proteolytic cleavage of C4b on the cellular intermediate in the absence of C2a.

It should be noted that the EAC$\overline{1}$4b2a treated with C4b/C3bINA alone was no longer capable of regenerating the convertase when additional C2 was added (Table II). There is an apparent disagreement of these data with those previously published

(12), which demonstrated that EAC$\overline{1}$4b treated with C4b/C3bINA alone could bind and activate limited C2, whereas in the presence of C4-bp, no activation took place. This can be explained by the presence of uncleaved C4b sites capable of binding C2 in the absence of C4-bp but not in its presence.

The resemblance of C3Nef (33–35) to NFc in the formation, decay, and regeneration of the classical and alternative pathway C3 convertases, demonstrates a closer similarity between these two enzymes. Based on these similarities, it could be expected that a serum protein similar to properdin of the alternative pathway (36–38) could exist in normal human serum to stabilize the classical pathway enzyme.

Summary

Three mechanisms that regulate the formation and function of the classical pathway C3 convertase (C4b2a) have been elucidated: (*a*) an intrinsic decay of the enzyme that is temperature dependent; (*b*) an extrinsic decay mediated by the effect of the serum protein C4b binding protein (C4-bp); and (*c*) inactivation of C4b by the proteolytic action of C4b/C3b inactivator (C4b/C3bINA), which cleaves the α' chain of C4b to yield C4d (α2) and C4c (α3, α4, β, and γ chains). A fourth mechanism described here is based on the observation that the IgG fraction of the serum of certain patients with glomerulonephritis contains a protein termed C4 nephritic factor (NFc), which prevents the intrinsic decay of C4b2a. This protein, which prolongs the half-life of surface-bound C4b2a from 7.5 min to >5 h, increases the use of C3 and C5. It also inhibits the decay produced by C4-bp by preventing the dissociation of C2a from the C4b2a complex. Additionally, the C4b/C3bINA alone, or in the presence of C4-bp, fails to cleave the α' chain of C4b in the surface-bound stabilized C4b2a complex. This protective property of NFc requires the presence of C2a, because C4b was not protected unless it was bound to C2a. Thus in the presence of NFc, the three natural controls of the function of the classical pathway convertase, intrinsic decay, extrinsic decay, and proteolytic cleavage, are bypassed.

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