Proc. Natl. Acad. Sci. USA
Vol. 74, No. 4, pp. 1683–1687, April 1977
Immunology

Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms

(properdin/C3b inactivator/β1H regulatory protein/C3 convertase)

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Contributed by K. Frank Austen, January 10, 1977

ABSTRACT **The surface of zymosan (Zy), by affording a protected microenvironment for C3b and the amplification convertase stabilized by properdin, P,C3b,Bb, shifts the alternative complement pathway from slow fluid phase turnover to the amplification phase of its expression. This mode of activation is in contradistinction to that of the classical pathway, which follows conversion of a proenzyme, C1, to its active form, C1. Under conditions in which the control proteins, C3b inactivator (C3bINA) and β1H, completely inactivated C3b on the sheep erythrocyte intermediate, EAC4b,3b, the activity of C3b bound to Zy, ZyC3b, was diminished by only one-third. Further, when ZyC3b was converted to ZyC3b,Bb,P there was an additional point of deregulation in that the convertase was resistant to β1H-mediated decay-dissociation while P,C3b,Bb on the sheep erythrocyte exhibited the usual susceptibility to β1H.**

That Zy alone could indeed promote rapid C3 cleavage by the alternative pathway through assembly and protection of the amplification convertase on its surface was demonstrated with a mixture of alternative pathway proteins, C3, B, $\overline{D}$, P, C3bINA, and β1H, that had each been purified to homogeneity. Interaction of these proteins at one-tenth their relative serum concentrations with Zy permitted low-grade inactivation of C3 and B to advance to the level of amplification after a 15 min lag period. Because the reaction of the purified proteins proceeded spontaneously when either regulatory protein was deleted, the effect of Zy was attributed to deregulation rather than to conversion of one of the proteins to a specific initiating state. The alternative pathway, through the normal presence of $\overline{D}$, interacts with a microbial surface, such as Zy, to amplify deposition of C3b by circumvention of endogenous regulatory mechanisms, thereby augmenting host defense.

The classical activating pathway of complement (C) is initiated by antigen–antibody complexes that bind and convert C1 to its activated state, $\overline{C1}$ (1), which then cleaves C4 and C2 to form C4b2a (2, 3), the classical C3 convertase. The latter sequentially cleaves C3 and C5 to generate from the effector sequence a variety of biologically active products and the C5b6789 cytolytic complex (4). An alternative pathway was discovered (5) when zymosan (Zy), an insoluble polysaccharide-containing derivative from yeast cell walls, was observed to inactivate C3 in serum without apparent utilization of C1, C4, or C2. This reaction was characterized by participation of a nonclassical complement component, properdin (P), a 223,000 molecular weight (M_r) γ globulin (6, 7), and by the lack of a requirement for specific antibody. Expression of the alternative pathway is dependent upon a positive feedback mechanism in which C3b (8), the major cleavage fragment of C3 that is generated by a C3 convertase, interacts with B and $\overline{D}$ to form the amplification C3 convertase, C3b,Bb (9–11), that is stabilized by P (12). C3b serves as a receptor for B, a 100,000 M_r β globulin (13, 14), in a magnesium-dependent, reversible, binding reaction that exposes a site on B that is susceptible to cleavage by $\overline{D}$, a 25,000 M_r serine protease (15, 16). $\overline{D}$ releases the 20,000 M_r Ba fragment to uncover fully the C3-cleaving site in the 80,000 M_r Bb fragment which remains bound to C3b. Regulation of the amplification convertase occurs at three points: intrinsic decay of the inherently labile C3b,Bb complex (9, 12); extrinsic decay-dissociation of C3b,Bb by displacement of Bb from C3b by β1H, a 150,000 M_r β globulin, an effect that even reverses stabilization of the convertase by P (17, 18); and irreversible inactivation of C3b by C3b inactivator (C3bINA) (19), thereby preventing regeneration of the convertase at that site (20). In view of the presence of $\overline{D}$ in whole serum or plasma, amplification of the alternative pathway could involve either activation of an additional protein or merely circumvention of the normal regulatory mechanisms.

Abbreviations: M_r, molecular weight; P, properdin; C, complement; C3bINA, C3b inactivator; P,C3b,Bb properdin-stabilized amplification C3 convertase; Zy, zymosan; ZyC3b, zymosan particle bearing the major cleavage fragment of C3; E, sheep erythrocytes; EAC4b,3b, sheep erythrocyte sensitized with specific antibody and bearing the major cleavage fragments of C4 and C3; EDTA, ethylenediaminetetraacetate; VBS, Veronal-buffered saline; GVB, VBS containing 0.1% gelatin; GVB^{++}, GVB containing 0.15 mM calcium and 0.5 mM magnesium; $DGVB^{++}$, half-isotonic GVB^{++} with 2.5% dextrose; GVB-EDTA, GVB containing 0.04 M EDTA.

MATERIALS AND METHODS

Preparation of Alternative Pathway Factors. B (15), C3 (21), β1H (17), and $\overline{D}$ (12) were purified to homogeneity and quantitated as described. B and C3 were trace-labeled with ^{125}I by insolubilized lactoperoxidase (Worthington Biochemical Corp., Freehold, NJ) (22) to specific activities of 82,000 cpm/μg and 20,500 cpm/μg, respectively. To isolate P the euglobulin fraction of serum obtained by dialysis against 0.002 M ethylenediaminetetraacetate (EDTA), pH 7.5, was resolubilized in 0.15 M NaCl, dialyzed against 0.15 M Tris·HCl, pH 9.5, and applied to QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the same buffer. The effluent fractions containing P were pooled, dialyzed against 0.025 M Na acetate, 0.05 M NaCl at pH 6.0, and applied to SP-Sephadex C-50 (Pharmacia Fine Chemicals) equilibrated in the same buffer. P was eluted by a linear NaCl gradient, pooled, concentrated by ultrafiltration with a UM-10 Diaflo membrane (Amicon Corp., Lexington, MA), and quantitated by radial immunodiffusion (23).

For purification of C3bINA (24), plasma was dialyzed against 0.01 M Tris, 0.06 M NaCl at pH 7.8, and applied to QAE-Sephadex A-50 equilibrated with the same buffer. C3bINA was eluted with a linear NaCl gradient, and brought to pH 7.0 by addition of 0.1 M HCl and to 60% $(NH_4)_2SO_4$ saturation. The pool was then mixed with moist CM-cellulose CM-52 (Reeve Angel, Clifton, NJ) which had been washed in 0.03 M $NaPO_4$, 60% $(NH_4)SO_4$ at pH 7.0. The suspension was poured into a

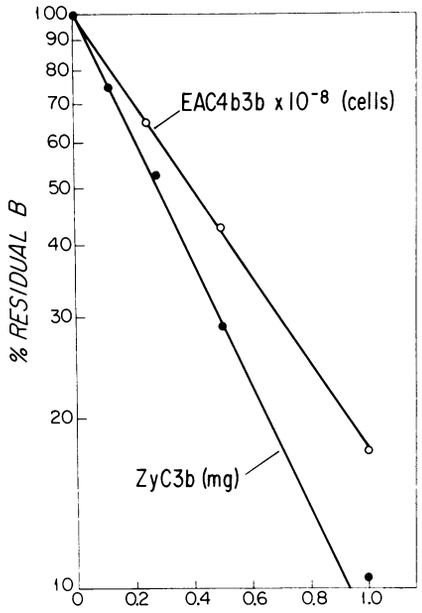

FIG. 1. Inactivation of the hemolytic activity of B by increasing concentrations of particle-bound C3b, EAC4b,3b (○) and ZyC3b (●), in the presence of $\overline{D}$.

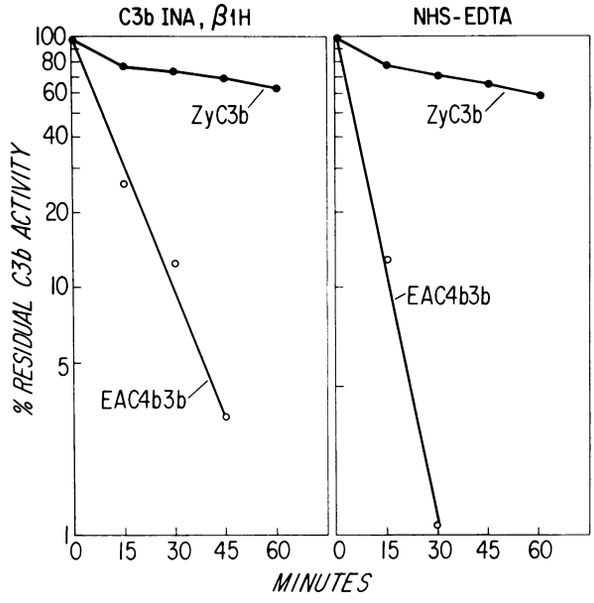

FIG. 2. Time course of inactivation of C3b bound to sheep E (○) and Zy (●) by C3bINA and β1H (*left*) and by diluted normal human serum (NHS) (*right*).

column, the column was packed, and a linear gradient of $(NH_4)SO_4$ from 60 to 10% saturation was applied. Fractions containing C3bINA were pooled and brought to pH 6.0 by addition of 0.1 M HCl, and gradient solubilization was repeated as above except at pH 6.0. The fractions containing C3bINA were concentrated by precipitation in 70% $(NH_4)_2SO_4$, solubilized in H_2O and filtered on Sephadex G-150 Superfine (Pharmacia Fine Chemicals) with 0.05 M Veronal, 0.3 M NaCl at pH 7.5 as the eluant. C3bINA appeared as a distinct protein peak which was concentrated by precipitation in 70% $(NH_4)_2SO_4$ and resolubilized with Veronal-buffered saline (VBS) containing 0.2 M glycine. To establish purity, 25–50 μg of each alternative pathway protein was reduced with 0.1 M dithiothreitol in 8 M urea and subjected to electrophoresis in 7% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (25), after which the gels were stained with Coomassie blue.

Assays. VBS, pH 7.5, containing 0.1% gelatin (GVB), 0.15 mM calcium and 0.5 mM magnesium (GVB^{++}), half-isotonic GVB^{++} with 2.5% dextrose ($DGVB^{++}$), and GVB containing 0.04 M EDTA (GVB-EDTA) were used as diluents (26). Hemolytic assays for C3 (27) and B (28) were carried out as previously described. Sheep erythrocytes (E) sensitized with antibody (A) and bearing C3b, EAC4b,3b, were prepared by incubating 1×10^9 EAC1,4,2 with 150 μg of unlabeled or ^{125}I trace-labeled C3 for 30 min at 30° followed by washing and further incubation in GVB-EDTA for 180 min at 37° (9). Zy bearing C3b, ZyC3b, was prepared by incubating 20 mg of Zy (Schwarz/Mann, lot no. AZ-2290), which had been boiled and washed, with 1 mg of C3, 0.5 mg of B, and 0.05 mg of $\overline{D}$ in 1 ml of $DGVB^{++}$ for 60 min at 30° followed by washing and further incubation in GVB-EDTA for 120 min at 37°. To assess the function of particle-bound C3b, dilutions of EAC4b,3b or ZyC3b were incubated with 0.15 μg of B and 0.05 μg of $\overline{D}$ in 0.2 ml of GVB^{++} for 60 min at 37°, followed by centrifugation and hemolytic assay of residual fluid phase B activity. Inactivation of B was directly related to the input of particle-bound C3b (Fig. 1). Zy treated with C3 in the absence of B and $\overline{D}$ did not have this activity, ruling out nonspecific adsorption of C3 capable of functioning in this assay.

RESULTS

Regulation of the Function of Particle-Bound C3b and P,C3b,Bb by C3bINA and β1H. The relative susceptibility of C3b bound to sheep E and to Zy to inactivation by purified C3bINA and β1H was studied in a kinetic experiment, with amounts of particle-bound C3b that gave approximately 75% B inactivation in the presence of $\overline{D}$. Five samples each of 8×10^7 EAC4b,3b and of 0.5 mg of ZyC3b in 0.1 ml of GVB^{++} were incubated at 37° for 60 min, during which replicates of each received 0.05 μg of C3bINA and 0.5 μg of β1H in 0.1 ml of GVB^{++} at 15 min intervals. All reactions were stopped by addition of 2 ml of ice-cold GVB^{++} and sedimentation of the particles at 4°. The particles were washed three times in 2 ml of GVB^{++} and assayed for residual functional C3b. Loss of C3b function on EAC4b,3b proceeded as a first-order reaction with greater than 95% inactivation after 45 min treatment (Fig. 2 *left*). In contrast, C3b bound to Zy was relatively resistant to inactivation with only 35% loss of function after 60 min of treatment with C3bINA and β1H. Addition of Zy to EAC4b,3b did not alter the rate of inactivation of C3b. C3b was also markedly protected on Zy relative to sheep E when C3bINA was used alone. The experiment was repeated with 0.1 ml of a 1:25 dilution of whole human serum in GVB-EDTA instead of C3bINA and β1H. Loss of C3b function on EAC4b,3b was complete in 30 min, while C3b bound to Zy had lost only 41% activity by 60 min (Fig. 2 *right*).

The capacity of β1H to release radiolabeled Bb from the P-stabilized amplification convertase on sheep E and Zy was also examined. EAC4b,3b, 5×10^8, and 2.5 mg of ZyC3b were each incubated with 0.5 μg of $\overline{D}$, 25 μg of P, and 13 μg of ^{125}I-labeled B (^{125}I-B) in 6 ml of $DGVB^{++}$ for 45 min at 30°, washed three times in ice-cold $DGVB^{++}$, and resuspended to 2.6 ml of $DGVB^{++}$. A 0.5-ml portion from each was assessed at zero time for bound ^{125}I-Bb (Table 1). Four samples each of EAC4b,3b,Bb,P or ZyC3b,Bb,P in 0.5 ml of $DGVB^{++}$ were then incubated for 30 min at 30° with 0.5 ml of $DGVB^{++}$ alone or containing 0.02 μg of β1H, 0.06 μg of β1H, and 0.18 μg of β1H, respectively, in the case of the sheep E intermediates, and 0.18 μg of β1H, 0.54 μg of β1H, and 1.62 μg of β1H, respec-

Table 1. Residual ^{125}I-Bb on erythrocyte and zymosan intermediates after treatment with β1H

Treatment	Bound ^{125}I-Bb, cpm	
	EAC4b,3b,Bb,P	ZyC3b,Bb,P
None (zero time)	2897	2431
Buffer (30 min, 30°)	1246 (41%)	1001 (39%)
β1H, 0.02 μg	666 (21%)	
0.06 μg	342 (9%)	
0.18 μg	176 (4%)	995 (38%)
0.54 μg		952 (36%)
1.62 μg		831 (31%)
EDTA control	75	105

Numbers in parentheses refer to percent binding relative to the zero time samples after correction of each experimental reaction mixture by subtracting counts bound in EDTA.

tively, for the Zy intermediates. The reaction mixtures were then washed three times in ice-cold DGVB^{++}, resuspended in 0.5 ml of DGVB^{++}, and assayed for residual ^{125}I-B. Additional samples of EAC4b,3b and ZyC3b were incubated with ^{125}I-B, $\overline{D}$, and P in the presence of 0.02 M EDTA to prevent convertase formation, and these served as controls for nonspecific binding of ^{125}I-B. Both P-stabilized intermediates released 60% of the specifically bound ^{125}I-Bb during incubation in buffer alone, reflecting comparable rates of intrinsic decay-dissociation. In contrast, 0.18 μg of β1H decay-dissociated an additional 37% of the ^{125}I-Bb from EAC4b,3b,Bb,P, leaving a residual of only 4%; while this concentration of β1H had no effect on ^{125}I-Bb bound to the Zy intermediate. Both intermediates formed without P had net ^{125}I-Bb uptakes of only 4%. Thus, Zy did not change the requirement for P-stabilization and did not alter intrinsic decay but did protect the bound P,C3b,Bb complex from extrinsic decay-dissociation by β1H.

Reconstruction of the Serum Reaction with Purified Alternative Pathway Proteins. To assess the possibility that the role of Zy in activation of C3 by the alternative pathway resided principally in its capacity to circumvent the action of the regulatory proteins, the entire system was reconstructed with purified proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified C3, B, $\overline{D}$, P, C3bINA, and β1H, in their reduced states, revealed that each protein preparation was homogeneous and exhibited the appropriate molecular weight for its polypeptide chains. B, $\overline{D}$, P, and β1H each demonstrated a single stained band, and C3 and C3bINA, which consist of two covalently linked polypeptide chains of unequal size (24, 29), revealed two bands (Fig. 3). Reaction mixtures were constituted to contain approximately one-tenth the respective serum concentrations of each protein. The mixtures containing 2.5 μg of B, 13.5 μg of C3, 0.25 μg of P, 0.01 μg of $\overline{D}$, 0.5 μg of C3bINA, and 5 μg of β1H, or lacking either C3bINA or β1H, were incubated in 0.1 ml of GVB^{++} at 37° in the presence and absence of 0.5 mg of Zy. At timed intervals 0.01-ml samples were removed and assayed for residual hemolytic C3 and B. The complete reaction mixture containing both control proteins exhibited no accelerated inactivation of C3 and B. Introduction of Zy induced a phase of amplified inactivation of C3 and B after 15 min (Fig. 4). The absence of β1H permitted total consumption of both C3 and B by 15 min and thus an effect by Zy could not be recognized. The absence of C3bINA also allowed almost complete inactivation of B in 15 min, but C3 consumption at this time was only 20–25% and did not increase significantly thereafter. An effect by Zy in the absence of C3bINA was limited to slight augmentation of C3 consumption.

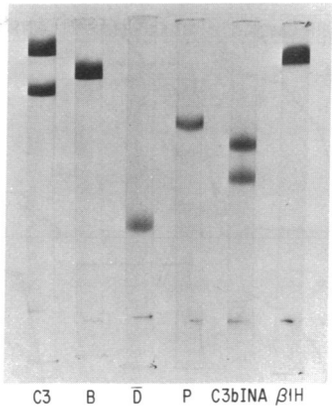

FIG. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified reduced proteins of the alternative complement pathway. The analysis of β1H was carried out on an occasion separate from that on which all the other proteins were analyzed. Electrophoresis is from top to bottom, and the position of the tracking dye is indicated at the bottom.

The incomplete C3 inactivation in the reaction mixture lacking C3bINA probably reflects rapid decay-dissociation of fluid phase P,C3b,Bb by β1H before the convertase could cleave C3. The capacity of Zy to induce accelerated C3 cleavage in a mixture of purified C3, B, $\overline{D}$, and P that was regulated by C3bINA and β1H reveals a mechanism of Zy action by deregulation rather than recruitment of additional plasma proteins.

The importance of the surface supplied to the fluid phase reactants was analyzed by comparing the effect of an EAC4b,3b intermediate with that of Zy on the reconstructed reaction mixtures. Less than 10% inactivation of C3 and B occurred after 60 min in mixtures containing purified proteins alone or with 8×10^7 EAC4b. The presence of Zy resulted in more than 80% inactivation of C3 and B (Fig. 5). In contrast, 8×10^7 EAC4b,3b, carrying 53,000 ^{125}I-C3b molecules per cell, induced only 20% inactivation of C3 and B in 15 min and had no effect thereafter even though this input of cell-bound C3b molecules was equivalent to 10% of the C3 molecules initially available in the fluid phase.

DISCUSSION

The view that the alternative complement pathway is continuously in low grade operation because of the normal presence of $\overline{D}$ and that activation represents deregulation to the point of amplified C3 cleavage is entirely compatible with results obtained when serum lacks C3bINA (20), contains C3 nephritic factor (30), or encounters a complex microbial cell wall surface, such as Zy (5). In each of these instances regulation would be circumvented, albeit by different mechanisms (refs. 17 and 31; Fig. 4), and the alternative pathway would be able to move from low-grade activity to C3b-dependent amplification. Attempts to validate this hypothesis with the purified isolated proteins began with the observation that the interaction of native C3, B, and $\overline{D}$ led to complete C3 and B inactivation in the absence of any demonstrable contamination of the starting materials with C3b (21). The introduction of purified P into such reaction mixtures increased the efficiency of C3 cleavage as evidenced by reduced requirements for $\overline{D}$ and B and a shortening of the lag phase prior to progressive B and C3 cleavage (28). Because these reconstituted fluid phase systems cleaved C3 without a requirement for addition of Zy, two possibilities for endogenous regulation of the alternative pathway seemed likely; that is, the active principle, $\overline{D}$, must be

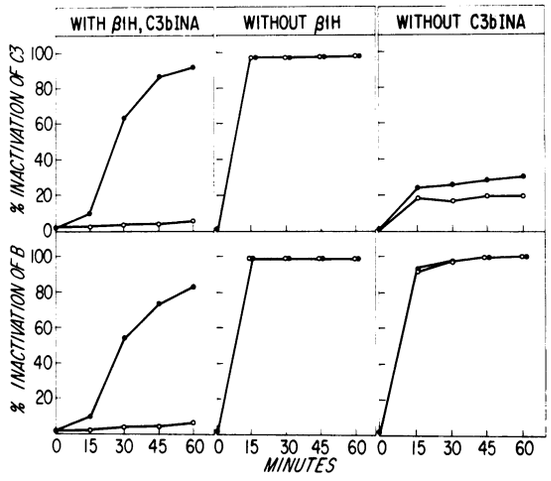

FIG. 4. Time course of inactivation of hemolytic C3 and B by reaction mixtures containing C3, B, $\overline{D}$, P, and the control proteins indicated, without (○) and with (●) the introduction of Zy.

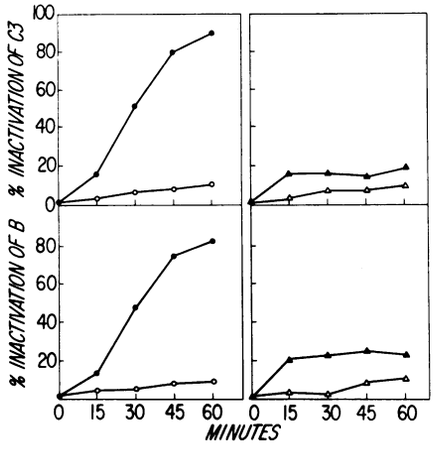

FIG. 5. Time course of inactivation of hemolytic C3 and B by reaction mixtures containing C3, B, $\overline{D}$, P, C3bINA, and β1H alone (○), and with Zy (●) (*left*). Identical reaction mixtures with EAC4b (△) and EAC4b, ^{125}I-C3b (▲) (*right*).

derived from a precursor, D, or the low-grade chronically active system must be held in check by regulatory proteins. Although a trypsin-activatable prototype of $\overline{D}$ was recognized (16), $\overline{D}$ was invariably present in normal serum and plasma as indicated by direct isolation (8, 15, 16). When attention was first directed to the other possibility by introduction of the regulatory protein, C3bINA, into the reaction mixture of B, $\overline{D}$, and C3, the system was not suppressed (21). However, the subsequent finding of a second control protein, β1H (17, 18), has made possible the creation of reaction mixtures of C3, B, $\overline{D}$, P, C3bINA, and β1H reconstituted to their relative serum concentrations that do not spontaneously progress to amplified C3 and B inactivation.

The initial experiments demonstrating that Zy offered C3b a protected location compared the action of the control proteins on C3b carried by Zy, ZyC3b, and by the sheep E intermediate, EAC4b,3b. The concentrations of particle-bound C3b used for these studies were adjusted so that their interaction with the same amounts of B and $\overline{D}$ yielded comparable B cleavage (Fig. 1) and comparable uptake of ^{125}I-Bb (Table 1). Under these circumstances a mixture of purified C3bINA and β1H completely inactivated the C3b on EAC4b,3b in 60 min, while the function of the Zy-bound C3b was diminished by only 35% during the same time period (Fig. 2 *left*). When diluted normal serum was incubated with the particles, there was complete loss of functional C3b from EAC4b,3b in 30 min while that on Zy was reduced by only 41% over a 60 min interval (Fig. 2 *right*). Thus, C3b bound to Zy is protected from the action of the regulatory proteins when compared to its distribution on sheep E. Further, when the P-stabilized amplification convertase, P,C3b,Bb, is formed on Zy, deregulation of an additional control occurs. As shown in Table 1, the formation and intrinsic decay of the amplification convertase on Zy is no different from that of convertase assembled on the sheep E. However, while P,C3b,Bb on sheep E is subject to dose–response extrinsic decay-dissociation by β1H, ^{125}I-Bb in the complex on Zy is relatively resistant to this effect of β1H. The prolongation of the half-life of the amplification convertase on the Zy surface facilitates further deposition of C3b which itself would be protected and available to form additional P,C3b,Bb. Thus, the microbial surface, by offering a protected microenvironment for C3b and P,C3b,Bb, readily shifts the alternative pathway to the C3b-dependent amplification phase of its expression.

In order to establish that Zy could induce C3 cleavage by the alternative pathway through assembly and protection of surface-bound amplification convertase, it was critical to demonstrate this effect with a mixture of the purified proteins of the alternative pathway. C3, B, $\overline{D}$, P, C3bINA, and β1H were purified to homogeneity as defined not only by previous criteria that involved analytical alkaline or acid disc gel electrophoresis or isoelectric focusing (17, 21, 23) but also sodium dodecyl sulfate polyacrylamide gel electrophoresis after reduction (Fig. 3). Partially purified C3bINA has been reported to be contaminated with initiating factor (32), a β globulin consisting of two covalently linked polypeptide chains of 85,000 M_r (32). The gel of C3bINA purified by a different procedure (24) revealed only two chains of approximately 50,000 and 40,000 M_r (Fig. 3). Further, the C3bINA protein elicited in a rabbit a monospecific antiserum that yielded a single precipitin arc with normal serum and no arcs with serum from an individual genetically deficient in C3bINA (20). Finally, incubation of 100 μg of purified C3bINA in 0.2 M glycine·HCl, pH 2.2, for 5 hr at 4° did not generate C3 nephritic-factor-like activity as had been reported for initiating factor (32). Thus, the preparation of C3bINA contained no physically or functionally detectable contaminants.

Interaction of C3, B, $\overline{D}$, and P and the regulatory factors, C3bINA and β1H, at one-tenth their serum concentrations permitted low-grade inactivation of C3 and B which did not accelerate during 60 min at 37°. The introduction of Zy gave a modest increase in C3 and B inactivation during the initial 15 min of interaction, which was followed by accelerated inactivation to more than 80% by 60 min (Fig. 4 *left*). The omission of β1H from the reaction permitted complete inactivation of C3 and B in the fluid phase by 15 min and served to illustrate that C3bINA is not sufficient to prevent the low-grade interaction of the alternative pathway proteins from proceeding to amplification (Fig. 4 *middle*). When C3bINA was omitted from the reaction mixture, B utilization was complete after 15 min, while C3 utilization was limited to 20% with only a slight augmentation in the presence of Zy (Fig. 4 *right*). It seems likely that in the absence of C3bINA, B was rapidly consumed through formation of P,C3b,Bb in the fluid phase, but β1H-mediated decay-dissociation of the convertase was so marked that efficient C3 cleavage was prevented. Thus, the presence of both C3bINA and β1H is necessary to retard fluid phase amplification of C3 cleavage so that the reaction can be directed to the surface of Zy by local circumvention of these regulatory proteins. The failure of the sheep E membrane, even when fortified with C3b, to lead to progressive and complete C3 and

B cleavage in the presence of $\overline{D}$, P, C3bINA, and β1H further indicates that the critical issue is not initial C3b formation but the site of deposition (Fig. 5).

A critical biologic function of C3b is interaction with C3b receptors on host phagocytic cells, a process termed immune adherence, which leads to enhanced phagocytosis of the particle bearing C3b (33). The capacity of a microbial surface, such as Zy, to protect and thereby accumulate increasing amounts of C3b by local circumvention of the host regulatory mechanisms is compatible with a unique role of the alternative pathway in host defense. The specificity of this system, in contrast with the classical pathway which is dependent on the host adaptive immune response, would reside in the selective inability of the regulatory proteins to deal with their substrates when the latter are deposited on the surface of the invading organism.

This work was supported by Grants AI-07722, AM-05577, AI-10356, and RR-05669 from the National Institutes of Health. D.T.F. is a postdoctoral fellow of the Helen Hay Whitney Foundation.

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