Activation of C3 in human serum by the alternative pathway may be induced by a variety of substances including microbial polysaccharides such as zymosan (1) and gram-negative bacterial lipopolysaccharides (2), and by certain mammalian cells, such as rabbit erythrocytes (Er) and lymphocytes (3), and some human lymphoblastoid cell lines (4). Effective C3 cleavage by the alternative pathway requires that the reaction advance to a positive feedback mechanism in which C3b (5), the major cleavage fragment of C3, interacts with B and D (6, 7) to form an amplification C3 convertase (C3b,Bb) (8-10). The activity of C3b,Bb is enhanced by the binding of properdin (P) (11-13), a 223,000-mol wt \( \gamma \)-globulin, which retards decay of the otherwise labile convertase (11). Endogenous regulation of this amplification convertase occurs at three points: intrinsic decay of the C3b,Bb complex by irreversible dissociation of Bb, which becomes inactive Bi (8, 11); extrinsic decay-dissociation of the convertase, even when stabilized with P, by displacement of Bb from C3b with \( \beta \)1H, a 150,000 mol wt \( \beta \)-globulin (14, 15); and, after a removal of protective Bb, inactivation of C3b by C3b inactivator (C3bINA), (16-18), a 95,000 mol wt \( \beta \)-globulin, to prevent regeneration of the convertase at that site (19).

The presence of active D in whole plasma and serum suggests that amplified C3 cleavage by the alternative pathway involves either activation of an additional protein or merely circumvention of the normal regulatory mechanisms by the reactants already present. Amplification by deregulation does occur when serum lacks a control protein, such as C3bINA (19, 20); contains the pathobiologic protein, C3 nephritic factor (21), which stabilizes and protects C3b,Bb from decay-dissociation by \( \beta \)1H (14); or is incubated with cobra venom factor (22), a...
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functional analog of C3b that is resistant to C3bINA (23). The concept of a continuously operative, low grade fluid phase C3b-generating system is supported by studies of the interaction of purified proteins in the absence of the regulatory proteins, C3bINA and β1H. Thus, native C3 and B interact with D to commence B and C3 cleavage (24), and the efficiency of C3 cleavage is greatly augmented by the presence of P (25). The introduction of C3bINA and β1H to such reaction mixtures prevents their transition to amplified C3 and B inactivation and has made possible the demonstration of a mechanism of activation of the alternative pathway by E r. C3b deposited on the surface of E r by the low grade fluid phase reaction and the P-stabilized amplification C3 convertase (P,C3b,Bb) formed at that site are relatively protected from the action of the regulatory proteins, thereby causing membrane-associated amplification of C3 cleavage.

Materials and Methods

Preparation of Alternative Pathway Factors. B (6), C3 (24, 26), β1H (14), D (11), P (27), and C3bINA (27) were purified to homogeneity and quantitated as described. Purified P did not contain functionally detectable P; P did not induce C3 and B cleavage upon addition to normal serum (28) and did not remain bound to an erythrocyte antibody complement 4b,3b intermediate (EAC4b,3b) during washing in isotonic buffer (29). 300 μg purified C3bINA emulsified in complete Freund’s adjuvant yielded a monospecific rabbit antiserum that recognized no antigens in human serum genetically deficient in C3bINA (19). C3 was trace-labeled with 125I by insolubilized lactoperoxidase (30) (Worthington Biochemicals Corp., Freehold, N. J.). 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 (31), after which the gels were stained with Coomassie blue. Each protein preparation was homogeneous by densiometric scanning and exhibited the appropriate molecular weight for its polypeptide chains. B (32), D (32), P (33), and β1H (15) each demonstrated a single stained band corresponding to mol wt of 100,000, 25,000, 58,000, and 150,000, respectively. In the case of P, the molecular weight represents four apparently identical noncovalently linked subunits (33). C3 (34, 35) and C3bINA (27), which consists of two nonidentical covalently linked polypeptide chains, revealed two bands which were 125,000 and 80,000 mol wt for C3, and 55,000 and 42,000 mol wt for C3bINA.

Assays. Veronal-buffered saline, pH 7.5, containing 0.1% gelatin (GVB), 1.5 × 10^-4 M calcium, and 5 × 10^-4 M magnesium (GVB++), half-isotonic GVB++ with 2.5% dextrose (DGVB++), and GVB containing 0.04 M ethylenediaminetetraacetate (EDTA) (GVB-EDTA) were used as diluents (36). Erythrocytes from sheep (E s) and New Zealand albino rabbits (E r) were collected into Alsever’s solution, washed in saline, and stored in GVB-EDTA. The quantitative relationship between the number of molecules of C3b bound to erythrocytes and the number of hemolytically active convertase sites generated upon interaction with P, B, and D was examined with E rC3b and E rC3b formed with increasing amounts of 125I-C3. Replicate samples of 4 × 10⁹ E s and E r containing 500 μg B and 1.0 μg D were incubated with 800, 400, 200 μg, or no 125I-C3 in 2.0 ml DGVB++ for 60 min at 30°C. The cells were washed twice with GVB-EDTA, incubated for an additional 120 min at 37°C in GVB-EDTA, and washed in DGVB++. The number of C3b molecules/erythrocyte was calculated from the 125I bound in the presence of B and D after subtracting 125I nonspecifically bound in the absence of B and D. C3b hemolytic activity was assayed by incubating 1 × 10⁹ E sC3b and E rC3b with 0.2 μg P, 10 ng D, and 10 ng B in 0.2 ml DGVB++ for 30 min at 30°C. After addition of 0.3 ml rat serum diluted 1:20 in GVB-EDTA and further incubation of the cells for 60 min at 37°C, the percent hemolysis was determined and the average number of hemolytic sites/cell (Z) was calculated, with reaction mixtures containing the E s and E r previously incubated with B and D alone serving as reagent blanks. Uptake of 125I-C3b at the three inputs of 125I-C3 was 0.14, 0.14, and 0.13%, respectively, for E s and 0.22, 0.19, and 0.18%, respectively, for E r. Lysis of E sC3b and E rC3b was linearly related to the number of C3b molecules/cell, and E sC3b was 3.5 times more susceptible to complement-mediated lysis than E rC3b at equivalent amounts of bound C3b (Fig. 1). In further experiments, E sC3b and E rC3b were formed with a C3 input of 800 μg/4 × 10⁹ E; and C3b
Resistance of C3b on E' to Inactivation by C3bINA. The relative susceptibility of C3b on E'C3b and E'C3b to inactivation by C3bINA was compared by incubating 1 × 10^6 of each intermediate in 0.1 ml GVB++ for 60 min at 37°C during which replicate samples received 0.1 μg C3bINA in 0.1 ml GVB++. Each reaction was stopped by addition of 2 ml ice-cold GVB++, sedimentation, and washing of the intermediates at 4°C. Loss of C3b activity from E'C3b and E'C3b proceeded as first order reactions, but the rates of inactivation differed markedly (Fig. 2). C3b on E'C3b exhibited a half-life ($t_\frac{1}{2}$) of 41 min in the presence of C3bINA with 64% loss of activity in 60 min, while C3b on E'C3b lost only 8% activity and had an estimated $t_\frac{1}{2}$ of 480 min. Since $\beta$1H has been observed to augment inactivation of C3b by C3bINA (15), the experiment was repeated with 0.05 μg C3bINA and 0.5 μg $\beta$1H. C3b on E'C3b again demonstrated relative resistance to inactivation when compared to E'C3b, with the former intermediate having a $t_\frac{1}{2}$ of 220 min and the latter a $t_\frac{1}{2}$ of 16 min. To establish that the resistance of C3b on E'C3b to the regulatory proteins existed under natural circumstances, the experiment was repeated with 0.1 ml of normal human serum diluted 1:100 with GVB-EDTA instead of purified C3bINA and $\beta$1H. E'C3b again demonstrated relative resistance to inactivation, with C3b function declining 14% in 60 min, yielding a $t_\frac{1}{2}$ of 280 min; while E'C3b was 86% inactivated in the same interval with a $t_\frac{1}{2}$ of 22 min.

Resistance of P.C3b,Bb, on E' to Decay-Dissociation by $\beta$1H. The susceptibility of the P-stabilized amplification convertase on E' and E' to $\beta$1H-mediated decay-dissociation was examined by comparing the dose-response effects of $\beta$1H on the decay of the cell-bound convertases. E'C3b and E'C3b, 4 × 10^6,
were incubated with 8 \( \mu g \) P, 0.4 \( \mu g \) D, and 0.2 \( \mu g \) B for E'C3b and 8 \( \mu g \) P, 0.4 \( \mu g \) D, and 1 \( \mu g \) B for E'C3b in 6 ml DGVB ++ for 30 min at 30°C to generate the intermediates bearing P,C3b,Bb. Intermediates lacking P also were formed by incubating \( 1 \times 10^8 \) E'C3b with 0.1 \( \mu g \) D and 1 \( \mu g \) B, and \( 1 \times 10^8 \) E'C3b with 0.1 \( \mu g \) D and 5 \( \mu g \) B. The intermediates bearing the P-stabilized amplification convertase were washed twice with ice-cold GVB-EDTA diluted 1:1 with 5% dextrose, and \( 1 \times 10^8 \) of each intermediate were re suspended at 30°C in 2 ml of the same buffer alone or containing 0.22, 0.66, or 2.0 \( \mu g \) \( \beta 1H \). The intermediates formed in the absence of P were washed and resuspended in buffer alone. Incubation was continued at 30°C, and 0.2-ml samples were removed at varying intervals and added to rat serum diluted 1:20 in GVB-EDTA to develop the residual convertase sites. The \( t_1 \)'s of E'C3b,Bb and E'C3b,Bb were 4 min, and P effected similar degrees of stabilization, extending the \( t_1 \)'s to 28 and 29 min, respectively (Fig. 3). The susceptibility of E'C3b,Bb,P to \( \beta 1H \)-mediated decay-dissociation was such that 0.22 \( \mu g \) \( \beta 1H \) reduced the \( t_1 \) to 11 min and 2.0 \( \mu g \) \( \beta 1H \) reduced the \( t_1 \) to less than that of the unstabilized convertase. In contrast, 2.0 \( \mu g \) \( \beta 1H \) reduced the \( t_1 \) of E'C3b,Bb,P only to 12 min. Thus, E' did not change the requirement for P-stabilization or alter the intrinsic decay of the amplification convertase, but did protect the P-stabilized convertase from extrinsic decay-dissociation by \( \beta 1H \).

Interaction of E' with the Alternative Pathway Reconstructed with Purified
Proteins. The alternative complement pathway was reconstructed with \( \frac{1}{10} \)th serum levels of purified C3, B, D, P, C3bINA, and \( \beta \)1H. Two identical reaction mixtures containing 70 \( \mu \)g C3, 12.5 \( \mu \)g B, 0.05 \( \mu \)g D, 1.25 \( \mu \)g P, 2.5 \( \mu \)g C3bINA, and 25 \( \mu \)g \( \beta \)1H in 0.5 ml GVB++ were prewarmed to 37°C and added to 1 \( \times \) 10^6 E\(^s\) and E\(^r\), respectively. During further incubation at 37°C, two samples of 25 \( \mu \)l were removed at each timed interval from each reaction mixture. One sample was added to 2.5 ml ice-cold DGVB++ and centrifuged, and the supernate was assayed for hemolytically active C3 and B. The other sample was added to 1 ml rat serum diluted 1:50 in GVB-EDTA and incubated for 60 min at 37°C, after which the percent hemolysis of E\(^s\) and E\(^r\) was measured. In the presence of E\(^s\) only low grade inactivation of C3 and B occurred, reaching 10% in 60 min. In contrast, E\(^r\) induced about 50% inactivation of C3 and B by 10 min which reached 76 and 93% inactivation of C3 and B, respectively, at 30 min (Fig. 4). E\(^r\) was not converted to an intermediate that could be lysed by the source of C3-C9, while lysis of E\(^s\) increased from 0 to 40% in the first 20 min, declining thereafter to 8% by 60 min. Thus, E\(^r\) had no effect on the regulated mixture of alternative pathway proteins, while E\(^s\) markedly amplified C3 and B inactivation and was converted to an intermediate susceptible to lysis by C3-C9. The \( T_{\text{max}} \) kinetics observed for the lysis of the E\(^r\) intermediate reflect reexpression of endogenous control mechanisms after fluid phase C3 and B have been largely consumed.

To assess directly the capacity of the alternative pathway reaction with purified proteins to deposit C3b on the activating target cells, the reaction was repeated with \(^{131}\)I-C3 in the presence and absence of \( \beta \)1H. Three reaction mixtures, each containing \(^{131}\)I-C3, B, D, P, C3bINA, and \( \beta \)1H in 0.5 ml GVB++ in
FIG. 4. Interaction of $E^r$ and $E^s$ with the alternative complement pathway reconstructed with purified C3, B, D, P, C3bINA, and $\beta_1$H. Lower panel: time-course of inactivation of C3 ($\bullet$) and B ($\circ$) by $E^r$ and of C3 ($\Delta$) and B ($\triangle$) by $E^s$. Upper panel: kinetics of generation of hemolytically active sites on $E^r$ ($\bullet$) but not on $E^s$ ($\Delta$) taken from the reaction mixtures depicted in the lower panel at the times indicated.

The concentrations noted above, and three reaction mixtures of the same proteins except for $\beta_1$H were incubated at 37°C alone, with $2 \times 10^9 E^s$ and with $2 \times 10^9 E^r$, respectively. 100-μl samples were removed at timed intervals and added to 5 ml ice-cold DGVB++, the cells were sedimented and the supernates removed for hemolytic assay of C3. The cells were washed four times in GVB-EDTA and assessed for bound $^{125}$I-C3b. Control reaction mixtures containing the proteins and cells in 0.5 ml GVB-EDTA were treated identically and established that nonspecific binding of $^{125}$I-C3 was less than 0.2% for both cell types. In the presence of $\beta_1$H, $E^r$ induced 82% inactivation of C3 in 30 min; this inactivation was accompanied by net binding of $^{125}$I-C3b that was equivalent to 12.4% of the C3 molecules cleaved (Table I). In the absence of $\beta_1$H, 100% of the C3 was inactivated within 15 min and net binding of $^{125}$I-C3b was reduced to 0.14% of the C3 input. Minimal inactivation and negligible binding of $^{125}$I-C3 occurred with $E^s$ in the complete reaction mixture. The omission of $\beta_1$H resulted in rapid C3 inactivation with net $^{125}$I-C3b uptake that was 0.16% of the initial C3 input. C3 inactivation was also complete by 15 min in the $\beta_1$H-deficient reaction mixture incubated without erythrocytes, indicating that the absence of this regulatory protein permitted amplified cleavage of C3 to occur in the fluid phase. Thus, deregulation of endogenous controls at the surface of $E^r$ but not $E^s$ results in
local deposition of C3b by formation and function of surface-bound amplification convertase. In the absence of the regulatory protein, β1H, the fluid phase reaction spontaneously advances to amplification, consuming C3 and B, so that specific deregulation at the surface of E with concomitant accumulation of C3b cannot occur.

Discussion

Cleavage of C3 by the alternative complement pathway occurs in at least two distinct phases: continuous generation of C3b by low grade interaction of native C3, B, D, and P, and subsequent amplified cleavage of C3 by the interaction of C3b, B, D, and P. Transition to C3b-dependent amplification of C3 cleavage is normally prevented by C3bINA (19, 20) and β1H (14, 15), which limit formation and function of C3b,Bb. That the interaction of B and D with C3b (5) was much more efficient than with C3 was recognized in earlier studies (24, 25), but the concept that regulatory proteins restricted the reaction to the initial phase could not be validated with isolated proteins until the recent characterization of the function of β1H. Lachmann and Halbwachs (37) had suggested that the intense C3 and B cleavage observed in serum genetically deficient in (19) or immunochemically depleted of C3bINA (20) was due to initial generation of C3b by noncomplement-dependent proteolytic cleavage of C3 followed by unregulated amplification. The subsequent finding (24) that interaction of native C3, B, and D led to slow and then rapid inactivation of C3 and B in the absence of any demonstrable contamination of the starting materials with C3b, and even in the presence of C3bINA, indicated that the alternative pathway proteins themselves could generate the initial C3b. The presence of P in such reaction mixtures increased the efficiency of initial C3 cleavage as evidenced by reduced requirements for B and D and a shortening of the lag phase before amplified C3 and B inactivation (25). The introduction of both C3bINA and β1H into reaction mixtures of C3, B, D, and P limits the reaction to low grade C3 cleavage without progression to the amplification phase. This circumstance has permitted demonstration that activators of the alternative pathway, such as zymosan (27) and E,

| Time (min) | Rabbit erythrocytes | Sheep erythrocytes |
|-----------|---------------------|-------------------|
|           | with β1H | without β1H | with β1H | without β1H |
| 15        | 11,120  | 82%       | 130  | 100%   | 10    | 140  | 100%   |
| 30        | 9,200   | 83%       | 130  | 100%   | 0     | 90   | 100%   |
| 45        | 7,840   | 82%       | 120  | 100%   | 30    | 90   | 100%   |
| 60        | 9,980   | 64%       | 150  | 100%   | 20    | 170  | 100%   |
provide privileged sites that protect C3b deposited by the low grade fluid phase reaction and the P,C3b,Bb formed at that site from the action of regulatory proteins, resulting in membrane-associated amplified C3 cleavage.

The initial experiments demonstrating that E, as compared to E', afforded C3b a protected location contrasted the effects of inactivating principles on bound C3b. Generation of E,C3b and E',C3b by incubation of the cells with purified 125I-C3,B, and D resulted in slightly more binding of 125I-C3b to E than to E'. The number of hemolytic sites/cell was linearly related to the number of molecules of 125I-C3b/cell, permitting the precise measurement of cell-bound C3b activity (Fig. 1). When these intermediates were treated with C3bINA alone, with a combination of C3bINA and β1H, or with dilute whole human serum, inactivation of C3b bound to E at 60 min was 64, 93, and 86%, respectively (Fig. 2). In contrast, these same treatments had little effect on C3b bound to E', and the inactivation at 60 min ranged from 8 to 19%. Thus, C3b molecules bound to E relative to those on E' were protected from the action of C3bINA alone or in combination with β1H.

Furthermore, once the P-stabilized amplification convertase had been formed on the bound C3b, the surface of E' manifested an additional point of deregulation. As shown in Fig. 3, intrinsic decay and the magnitude of P-stabilization of C3b,Bb was the same on E' and E. However, 10-fold more β1H was required for comparable decay-dissociation of P,C3b,Bb on E' as on E'. Thus, the E' membrane, by offering a protected microenvironment for C3b, facilitates rapid formation of bound amplification convertase which, in turn, is relatively resistant to the action of β1H and deposits additional C3b by cleavage of C3, thereby shifting the alternative pathway to its amplification phase.

The hypothesis that the protective surface of E' was responsible for the transition from fluid phase low grade to membrane-associated amplified C3 cleavage was supported by such an occurrence in a reaction mixture constituted only of the purified proteins of the alternative pathway. D was utilized in these reaction mixtures since it was invariably present in plasma and serum. Partially purified C3bINA had been reported to be contaminated with initiating factor, a β-globulin consisting of two covalently linked polypeptide chains of 85,000 mol wt (38, 39). The C3bINA utilized in these reconstitution experiments was purified by a different procedure (27) and revealed only two disulfide linked polypeptide chains of 55,000 and 42,000 mol wt when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. As shown in Fig. 4, incubation of P, C3, B, D, C3bINA, and β1H at 10% of their serum concentrations was associated only with low grade C3 and B inactivation, indicating that P, lacking P, did not advance the fluid phase reaction to amplification. The addition of E' to the mixture of these purified proteins resulted in inactivation of B and C3 in association with formation of membrane-bound amplification convertase on E', as indicated by lysis of the E' intermediate upon interaction with C3-C9. E' did not advance the low grade C3 and B inactivation, which reached approximately 10% at 60 min, and was not converted to an intermediate susceptible to lysis by subsequent exposure to a source of C3-C9.

To demonstrate that increased C3b fixation by E' resulted from deposition by particle-bound amplification C3 convertase rather than by fluid phase conver-
tase, binding of C3b was quantitated during reactions in the presence and absence of β1H. As shown in Table I, interaction of E⁺ with ¹²⁵I-C3, B, D, P, C3bINA, and β1H induced 82% inactivation of C3 in 30 min and was accompanied by a net binding of 11,120 molecules of ¹²⁵I-C3b/E⁺, which is equivalent to 12.4% of the C3 cleaved at that time point. In contrast, when β1H was omitted from the reaction mixture there was complete inactivation of C3 within 15 min in the unregulated fluid phase reaction and the net uptake was only 150 molecules of ¹²⁵I-C3b/E, representing 0.14% of the C3 cleaved. Thus, the presence of a regulated fluid phase reaction is essential for efficient fixation of C3b on the activating principle. As E⁺ did not advance the complete reaction to amplification, there was no binding of C3b, and the omission of β1H again led to complete inactivation of C3 that produced less than 0.2% net binding of ¹²⁵I-C3b.

The facilitating role in alternative pathway activation of specific antibody and classical complement components noted by others (40-42) probably pertains to the phase of initial deposition of C3b. Once C3b is bound to the protecting surface of the activator, the reaction readily shifts to the amplification phase because of the increased rate of C3b generation and efficiency of C3b binding induced by the particle-bound C3b-dependent convertase.

The capacity of a microbial surface, by analogy to zymosan and E⁺, to protect and thereby accumulate increasing amounts of C3b by local circumvention of the regulatory proteins is compatible with a unique role for the alternative pathway in host defense. The specificity of the pathway resides in the capacity of certain surfaces to induce transition from constant low grade fluid phase C3 cleavage to localized amplification because of the selective inability of C3bINA and β1H to deal with their substrates when deposited on these surfaces. The implication of these surface effects would hold even if initial C3 cleavage and binding was facilitated by specific antibody and classical complement or by involvement of additional principles.

**Summary**

Cleavage of C3 by the alternative complement pathway occurs in at least two distinct phases: continuous low grade generation of C3b by the interaction of native C3, B, D, and P, and subsequent amplified cleavage of C3 by the interaction of C3b, B, D, and P which forms the amplification convertase, P,C3b,Bb. Transition to C3b-dependent amplification is necessary to achieve substantial C3 cleavage and is normally limited by the combined action of C3b inactivator (C3bINA) and β1H. An activator of the alternative pathway, such as rabbit erythrocytes (E⁺), provides sites that protect bound C3b and P,C3b,Bb from the action of these regulatory proteins and permits C3b deposited by the low grade fluid phase reaction to assemble a membrane-associated amplification convertase which can deposit additional protected C3b.

Under conditions in which the control proteins, C3bINA and β1H, almost completely inactivated C3b bound to sheep erythrocytes (E⁺), which does not activate the alternative pathway, the function of C3b bound to E⁺ was diminished by less than one-fifth. Further, the P-stabilized amplification convertase on E⁺ was 10-fold less sensitive to β1H-mediated decay-dissociation than the convertase on E⁺. The addition of E⁺ to a regulated mixture of purified C3, B, D, P, C3bINA, and
\( \beta_1 \text{H} \) resulted in amplified inactivation of C3 and B by formation of the amplification convertase on \( E^r \) as indicated by its lysis with subsequent exposure to C3-C9. In contrast, \( E^s \) did not advance the low grade fluid phase inactivation of C3 and B to amplified inactivation and the cell was not converted to an intermediate susceptible to lysis by C3-C9. Since \( E^r \) and \( E^s \) did not differ in their inefficient fixation of C3b generated during an unregulated fluid phase reaction, the activating capacity of \( E^r \) must reside in its protection of bound C3b and P, C3b, Bb from the regulatory proteins rather than in enhanced capacity to bind C3b from the fluid phase. When the reaction is limited to low grade fluid phase turnover, introduction of \( E^r \) but not \( E^s \) results in a 100-fold increase in the deposition of C3b, indicating that surface-dependent activation of the alternative pathway is characterized by efficient deposition of C3b on the initiating surface. Thus, the activating surfaces advance the interaction of the alternative pathway proteins to the amplification phase because of the selective inability of the regulatory proteins to deal with their substrates when deposited on these surfaces and results in a specificity that is not necessarily dependent on adaptive immunity.

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