FORMATION OF A HEMOLYTICALLY ACTIVE CELLULAR INTERMEDIATE BY THE INTERACTION BETWEEN PROPERDIN FACTORS B AND D AND THE ACTIVATED THIRD COMPONENT OF COMPLEMENT

BY DOUGLAS T. FEARON, K. FRANK AUSTEN, AND SHAUN RUDDY

(From the Departments of Medicine, Harvard Medical School and the Robert B. Brigham Hospital, Boston, Massachusetts 02120)

(Received for publication 7 June 1973)

The availability of hemolytically active cellular intermediates formed with components of the classical complement system has been critical to the determination of the order in which the components interact, their mechanism of interaction, and their quantitation. Although it has been possible to achieve lysis of erythrocytes by the activation of properdin factors with certain complex polysaccharides (1, 2) or cobra venom factor (CoVF) (3), the formation of hemolytically active cellular intermediates with properdin factors has not been previously accomplished.

Activation of the terminal complement sequence (C3-C9) by CoVF involves the participation of two plasma proteins: properdin factor B (4, 5), also termed C3 proactivator (C3PA) (6), and glycine-rich beta glycoprotein (GBG) (7), a 100,000 mol wt pseudoglobulin; and factor D (8), a 25,000 mol wt euglobulin, apparently identical with C3PA convertase (9) and GBGase (10). Initiation of fluid-phase reactions between factors B and D may also be accomplished by the activated form of properdin factor A (9, 11, 12), which is identical with the major cleavage product (C3b) of the third component of complement (C3).

In the present study, cell-bound C3b was shown to interact with factors B and D, generating a cellular intermediate able to activate the terminal complement sequence as assessed by its lysis in the presence of C3-C9.

Materials and Methods

Preparation of Plasma Proteins.—Factors B and D were prepared from pooled fresh frozen citrated human plasma as described (8). The factor B preparation yielded a single line on alkaline disk gel electrophoresis and immunoelectrophoretic analysis with goat antinormal human serum and was quantitated by radial immunodiffusion against monospecific antibody...
The factor D preparation yielded no lines on alkaline disk gel electrophoresis, having only 86 µg protein/ml by Folin assay (14), and had no factor B activity. The activated first component of guinea pig complement (C1 popover) and the human components C4, C2, and C3 were prepared and measured as previously described (15–18). The C3 preparation contained 830 µg C3/ml as assayed by radial immunodiffusion (19), no immunochemically detectable C5, and was free of factors B and D activities. Human C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories, Miami, Fla.

Cellular Intermediates of the Classical Complement System.—Low ionic strength Veronal-buffered saline with dextrose, pH 7.5, µ = 0.075, containing 0.1% gelatin, 1.5 X 10⁻⁵ M Ca⁺⁺, and 5 X 10⁻⁴ M Mg⁺⁺ (DGVB⁺⁺) (15, 20), and Veronal-buffered saline containing 0.04 M ethylenediaminetetraacetate (GVB-EDTA) were used as diluents.

Sheep erythrocytes in the states EA, EAC1 (15), EAC4, and EAC14 (21) were prepared according to described methods. As indicated in the Results section, in one experiment EAC14 generated by reacting EAC1 with purified C4, 100 U C4/10⁸ EAC1 were used in the formation of EAC42. EAC42 were made by incubating EAC14 (10⁸ cells/ml DGVB⁺⁺) with an equal volume of C2 (1000 U/ml) for "Tmax time" at 30°C, followed by immediate washing in ice-cold GVB-EDTA. EAC43 were prepared by incubating EAC42, 10⁸ cells/ml GVB-EDTA, with an equal volume of GVB-EDTA containing 8.3 µg C3/ml for 30 min at 30°C, washing twice, and allowing the C2 to decay during a subsequent 120-min incubation at 37°C in GVB-EDTA. Guinea pig serum diluted 1:19 in GVB-EDTA (C-EDTA) was used as a source of C3-C9; in some experiments functionally pure human C3-C9 were used.

RESULTS

The Conversion of EAC43 by Factors B and D to an Active Intermediate Capable of Being Lysed by C3-C9.—Sheep E, EA, EAC4, and EAC34 were incubated with factors B and D, and C3-C9 in various combinations, and the extent of hemolysis was determined (Table I). Only EAC43 were lysed, and this lysis was dependent on the presence of factors B and D. Although C3b was present on the cells, additional native C3 was required as an ingredient in the terminal complement mixture.

The ability of EAC43 to produce intermediates that, after being exposed to factors B and D, C3 and C5, and then washed, were capable of lysis by incubation with the remaining terminal complement components was examined. Three reaction mixtures were prepared each containing 5 X 10⁸ EAC43 and 0.8 µg of factor D in 1.5 ml of DGVB⁺⁺: to the first was added 10 µg of factor B; to the second, factor B and 50 U of C3; and to the third, factor B, C3, and 50 U of C5. After incubation for 30 min at 30°C, the cells were washed twice with ice-cold DGVB⁺⁺ and resuspended in 2.5 ml of DGVB⁺⁺ containing 50 U each of C3-C9, C5-C9, and C6-C9, respectively, for an additional 60-min incubation at 37°C. 100% lysis occurred within each group of cells, indicating that intermediates were formed at 30°C that maintained the activities of the proteins to which they were exposed, factors B and D, C3 and C5, through two washes. In the absence of factor B hemolytically active intermediates were not formed.

Quantitative Effects of Cell-Bound C3b and Factor B in Formation of Hemolytically Active Sites.—The quantitative relationship of cell-bound C3b and the number of effective hemolytic sites generated during exposure to excess amounts of
TABLE I
Conversion of EAC43 by Factors B and D to an Active Intermediate Capable of Being Lysed by C3-C9*

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Factor B</th>
<th>Factor D</th>
<th>C3-C9</th>
<th>Lysis (Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>EA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>EAC4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.02</td>
</tr>
<tr>
<td>EAC43</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.98</td>
</tr>
<tr>
<td>EAC43</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.03</td>
</tr>
<tr>
<td>EAC43</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>EAC43</td>
<td>+</td>
<td>+</td>
<td>C5-C9</td>
<td>0.02</td>
</tr>
<tr>
<td>EAC43</td>
<td>+</td>
<td>+</td>
<td>C3, C6-C9</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* 0.5 × 10⁸ cells, 0.3 µg of factor B, 0.4 µg of factor D, and 50 U each of C3, C5, C6, and C7 were incubated in 1.5 ml of DGVB⁺⁺ for 30 min at 30°C; 25 U of C8 and C9 were added; and incubation was continued for an additional 60 min at 37°C in a final volume of 2.5 ml of DGVB⁺⁺. After addition of 5 ml of saline, the extent of hemolysis was determined and the average number of effective hemolytic sites per cell was computed (Z).

Factors B and D and C3-9 is shown in Fig. 1. Batches of EAC14 prepared with purified C4 were converted to EAC42 and exposed to variable amounts of C3, ranging from none to 0.064 µg/10⁶ EAC42, and the C2 was allowed to decay. 5 × 10⁷ EAC43 from each batch were treated with 12 µg of factor B and 0.86 µg of factor D in 1.0 ml of DGVB⁺⁺ for 30 min at 30°C, followed by addition of 1.5 ml of C-EDTA and further incubation for 60 min at 37°C. A linear stoichiometric relationship between the amount of C3 used to prepare the EAC43 intermediates and the generation of effective hemolytic sites was observed, with 0.04 µg of C3 being sufficient for generation of one site per 10⁶ cells. Simultaneously, a classical effective molecule titration of this C3 using 2000 U³⁰⁸Cas/Cas/10⁶ EAC14 and 50 U each of C3-C9/10⁶ EAC14 revealed that 0.16 µg was required for generation of one site per 10⁶ cells.

The quantitative requirement for factor B was examined by incubating 5 × 10⁷ EAC43, prepared as described in Materials and Methods, with 0.86 µg of factor D and variable amounts of factor B in 1.0 ml of DGVB⁺⁺ for 30 min at 30°C, followed by development with 1.5 ml of C-EDTA for 60 min at 37°C. Fig. 2 reveals that a linear relationship was obtained between the average number of hemolytic sites per cell and the input of factor B, and that 0.17 µg were capable of producing one site per 10⁶ EAC43.

Kinetics of Formation and Decay of the Factor B-Dependent Hemolytically Active Site.—The generation and maintenance of the hemolytic activity formed
Fig. 1. C3b-dependent hemolytic sites per cell (Z) generated during formation of EAC43 by addition of variable amounts of C3 to EAC42.

Fig. 2. Hemolytic sites per cell (Z) formed by addition of variable amounts of factor B to a mixture of EAC43 and factor D.

by the interaction of EAC43 and factor D with factor B was examined in the experiments carried out at 30° and 37°C (Fig. 3). 5 ml of DGVB++ containing 0.75 μg of factor B and prewarmed to 30° or 37°C were added to 5 ml of DGVB++ containing 5 X 10^8 EAC43 and 4.15 μg of factor D also prewarmed to the corresponding temperature. At varying times, 1.0-ml portions were removed and incubated with 1.5 ml of C-EDTA for 60 min at at 37°C. At both 30° and 37°C,
generation of the hemolytically active enzyme began without detectable lag periods, consistent with first-order kinetics. Maximal activity at 30°C was obtained after approximately 30 min, while peak activity at 37°C occurred by 10 min.

The rate of decay of the factor B-dependent site on the washed intermediate was assessed at 30° and 37°C (Fig. 4). $8 \times 10^8$ EAC43 were incubated with 1.20 μg of factor B and 6.64 μg of factor D in 16 ml of DGVB++ for 30 min at 30°C. The cells were washed twice in 20 ml of ice-cold DGVB++, resuspended in 16 ml of DGVB++, and divided into two equal portions that were equilibrated at 30° and 37°C, respectively. 1-ml samples were removed at varying times and developed with 1.5 ml of C-EDTA for 60 min at 37°C. Decay of the hemolytically active site was more rapid at 37°C with a half-life of 15 min, while at 30°C the half-life was 50 min. In both instances, the kinetics of decay were consistent with a first-order reaction.

**DISCUSSION**

The demonstration that the fluid-phase interaction of C3b (activated factor A), C3Pase (factor D), and C3PA (properdin factor B) generated C3-cleaving activity (9) suggested that C3b participated in the formation of an additional convertase distinct from the classical C3 convertase. When this fluid-phase interaction was studied for its capacity to bring the complement reaction to completion as appreciated by hemolysis, the efficiency was so limited that it was necessary to use erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (1, 2) that are uniquely susceptible to lysis by complement components. The present studies demonstrate that the factor B-dependent C3 convertase can be affixed to an erythrocyte by use of an intermediate bearing C3b, and that this convertase brings the hemolytic reaction to completion with an efficiency comparable to that of classical convertase.

The intermediate EAC43, employed to initiate formation of the factor B-
dependent convertase, was devoid of C1 and C2 as indicated by its failure to lyse upon exposure to either \( \alpha xC2^{th} \) followed by C-EDTA (unpublished observations), or to C3-C9 alone (Table I). The absence of cell-bound C1 and C2 resulted from interacting EAC142 with C3 in the presence of EDTA followed by washing and further incubation in EDTA for 2 h at 37°C. The evidence that the EAC43 intermediate was lysed by a new pathway included requirements for factors B and D and cell-bound C3b. The quantity of C3b required for the EAC43 intermediate to be hemolytically active through the formation of the factor B-dependent convertase was similar to that observed in the formation of the classical enzyme, C423. The linear relationship between the availability of C3b and the extent of lysis when factors B and D and C3-C9 were present in excess clearly distinguishes this pathway from the reaction of classical C3 convertase with the terminal complement sequence (Fig. 1).

The formation of the factor B-dependent convertase through the interaction of EAC43 and factor D with factor B began without a lag period and the number of active sites reached a peak \( (T_{\text{max}}) \) earlier at 37°C than at 30°C (Fig. 3). The factor B-dependent site was stable to washing at 0°C, but exhibited first-order decay kinetics with a half-life of 15–20 min at 37°C and 60 min at 30°C (Fig. 4). The decayed enzyme can be regenerated by the addition of fresh factor B.
DOUGLAS T. FEARON, K. FRANK AUSTEN, SHAUN RUDDY

1311

(unpublished results). Although factor D is essential for the formation of the factor B-dependent convertase and does not appear to decay, it is not yet possible to distinguish whether its action is from the fluid phase or after binding to the intermediate.

The factor B-dependent convertase can form subsequent intermediates with C3, and with C3 and C5, which tolerate washing at 0°C. The recognition of an intermediate that did not require C5 for subsequent lysis revealed that the mechanism of attack by the terminal complement sequence induced by the factor B-dependent convertase is similar to that initiated by the classical convertase in which C3 and C5 can be bound in the absence of C6 and C7. The previously described hemolytic reactions initiated by CoVF and complex polysaccharides are also dependent on factors B and D but appear to be mediated by the hemolytically inefficient reactive lysis mechanism, whereby C5 is bound to the cellular membrane as part of the nascent trimolecular complex, C567, performed in the fluid phase (22, 23).

The linear stoichiometry of the effective molecule titration of C3b (Fig. 1) and factor B (Fig. 2) and the first-order kinetics displayed by the generation (Fig. 3) and decay (Fig. 4) of the factor B-dependent hemolytic site are characteristics consistent with the "one-hit" theory as initially developed for the classical complement system (24). There is also a remarkable similarity of the factor B-dependent convertase to the classical C3 convertase: both are capable of activating the terminal complement sequence; both are formed during the interaction of three plasma proteins; both show a \( T_{\text{max}} \) time of optimal generation of active sites as a consequence of temperature-dependent decay reactions; and after decay, each can be regenerated with the respective addition of factor B and C2. The functional analogy of factor B and C2 has been noted previously in studies of the role of factor B in CoVF (25) and zymosan-dependent (26) activation of the C3-cleaving enzyme.

The use of hemolytically active cellular intermediates to examine the reactions occurring with C3b and factors B and D has allowed extension of the one-hit theory to this molecular sequence, development of effective molecule titrations, recognition of the analogies to the functional characteristics of classical C3 convertase, and discrimination of the probable mechanism of terminal complement activation from reactive lysis. Furthermore, these studies have contributed to the appreciation of the potential role of this pathway for complement-mediated inflammation. Conceptually, it is possible for C3b produced by activation of the classical complement system, the properdin system, or some other enzymatic reaction to initiate intense utilization of the terminal components by formation of the factor B-dependent convertase. This view is consistent with the recent demonstration that lymphocyte tumor cells that had adsorbed trypsin-produced C3b were susceptible to factor B-dependent lysis (27). It is not clear whether the reported CoVF and lipopolysaccharide-induced activation of B lymphocytes (28) is through fixation of factor B-dependent convertase or the later fixation of C567 by reactive lysis.
SUMMARY

The present studies demonstrate that the factor B-dependent C3 convertase can be affixed to an erythrocyte by use of an intermediate bearing C3b and that this convertase brings the hemolytic reaction to completion with an efficiency comparable to that of classical convertase. The evidence that the EAC43 intermediate was lysed by a new pathway includes requirements for factors B and D and cell-bound C3b for subsequent lysis by the terminal components, C3-C9. The linear stoichiometry of the effective molecule titrations of C3b and factor B, and the first-order kinetics displayed by the generation and decay of the factor B-dependent hemolytic site are characteristics consistent with the one-hit theory as initially developed for the classical complement system. The use of hemolytically active cellular intermediates to examine the reactions occurring with C3b and factors B and D has allowed extension of the one-hit theory to this molecular sequence, development of effective molecule titrations, recognition of the analogies to the functional characteristics of the classical C3 convertase, and discrimination of the probable mechanism of terminal complement activation from reactive lysis.

REFERENCES

11. Pensky, J., L. Wurz, L. Pillemer, and I. H. Lepow. 1959. The properdin system and immunity. XII. Assay, properties and partial purification of a hydrazine...
sensitive factor (Factor A) in the properdin system. Z. Immunoforsch. 118:329.


