Brief Definitive Report

ASSEMBLY OF THE CYTOLYTIC ALTERNATIVE PATHWAY OF COMPLEMENT FROM 11 ISOLATED PLASMA PROTEINS*

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It has been shown recently that the alternative pathway of complement activation can be assembled from six isolated plasma proteins including C3, Factors B and D, β1H, C3b inactivator, and properdin (1). The functional activity of this mixture has been shown to be comparable to that of the alternative pathway in serum with respect to initiation and amplification. The cytolytic membrane attack pathway is known to be composed of five plasma proteins, C5, C6, C7, C8, and C9 (2). Cells capable of initiating the alternative pathway in serum have been demonstrated to undergo lysis (3–6). The question arose, therefore, as to whether the composite mixture of the isolated alternative pathway proteins and the isolated membrane attack pathway proteins is capable of causing lysis of cells such as rabbit erythrocytes which activate the alternative pathway. It is the purpose of this communication to report that the combination of the 11 isolated proteins in physiological concentrations constitutes on intact alternative pathway with cytolytic activity quantitatively comparable to that of serum.

Materials and Methods

Purified Components. C3 (7), Factor B (8), Factor D (9), C3b Inactivator (10), β1H (10), native properdin (11), C5 (7), C6 (12), C7 (12), C8 (13), and C9 (14) were prepared as described elsewhere. The mixture of purified components at physiological concentrations was prepared as follows: C3 (4,500 μg/ml) was incubated with β1H (1,500 μg/ml) and C3bINA (102 μg/ml) for 30 min at 37° so as to inactivate any C3b contaminant in the C3 preparation. After cooling at 0°C, the remaining eight components were added such that the final protein concentrations were: 1,200 μg/ml C3; 200 μg/ml Factor B; 2 μg/ml Factor D; 470 μg/ml β1H; 34 μg/ml C3bINA; 20 μg/ml nP; 72 μg/ml C5; 64 μg/ml C6; 54 μg/ml C7, 54 μg/ml C8, and 58 μg/ml C9.

Neuraminidase-Treated Sheep Erythrocytes (E_s-NA). E_s-NA were prepared as described by Pangburn and Müller-Eberhard (5). Sheep erythrocytes suspended in 0.5 M sodium acetate buffer pH 5.5 containing 0.9% NaCl and 0.1% $CaCl_2$ were incubated for 60 min, at 37°C with 10 U/ml of *Vibrio cholerae* neuraminidase (Behring Diagnostics, Somerville, N. J.) and then washed and stored in DGVB.

C4-Depleted Human Serum. C4-depleted human serum was prepared by immuneadsorption on columns of Sepharose 4B (Pharmacia Fine Chemicals) to which the IgG fraction of monospecific goat anti C4 serum had been coupled as described previously (4).

Buffers. VB: isotonic veronal buffered saline pH 7.4; Mg-GVB: VB containing 0.1% gelatin and 1.2 mM $MgCl_2$; DGVB: one-half physiological ionic strength VB containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 2.5% Dextrose.

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Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in 7% polyacrylamide gels using the method of Weber and Osborn (15).

Lysis of Erythrocytes by the Alternative Pathway. This process was performed as described elsewhere (4). Total reaction mixtures of 160 μl contained varying amounts of either C4-depleted serum or purified components, 1.2 mM $MgSO_4$, Mg-GVB, and 10^7 erythrocytes suspended in Mg-GVB. After incubation for 10 min at 37°C, lysis was stopped by addition of 1 ml cold Mg-GVB and centrifugation. Hemolysis was determined spectrophotometrically at 412 nm.

Chemotaxis Assay for Human Polymorphonuclear Leukocytes. This process was performed using the chemotaxis under agarose method of Chenoweth et al. (16). 1 ED_{50} U is defined as that amount of material which elicits one-half of the maximal chemotactic response.

Results

Demonstration of the 11 Isolated Proteins. Fig. 1 shows the eleven isolated proteins as they appear after electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels. All proteins were analyzed at a concentration of ≅ 1 mg/ml. Excluding C3, this concentration is higher than the concentration of these proteins in serum. In the case of Factor D, it is 500 times the physiological concentration (17). All proteins except C8 appear as single bands under nonreducing conditions. Factors B (18) and D (18), β1H (19), C6 (12), C7 (12), and C9 (20) are known to be single chain proteins. Properdin consists of four identical noncovalently bonded polypeptide chains (11, 21). C3 (22, 23), C3bINA (10) and C5 (22) are composed of two disulfide-linked nonidentical chains. C8 consists of two SDS dissociable subunits, the heavier representing the disulfide linked α- and γ-chains and the lighter constituting the β-chain (13). Inspection of the stained gels indicate 95–99% purity of the isolated proteins.

The Cytolytic Activity of the 11 Isolated Proteins. The 11 proteins were admixed at serum concentration: C3, 1,200 μg/ml; Factor B, 200 μg/ml; Factor D, 2 μg/ml; properdin, 20 μg/ml; C3bINA, 34 μg/ml; β1H, 470 μg/ml; C5, 72 μg/ml; C6, 64 μg/ml; C7, 54 μg/ml; C8, 54 μg/ml; and C9 58 μg/ml. The solvent was veronal-buffered physiological saline pH 7.4 containing 1.2 mM $MgSO_4$. Cells used were rabbit erythrocytes (E_R), sheep erythrocytes (E_S), and neuraminidase-treated sheep erythrocytes (E_S-NA). Fig. 2 shows the dose-related lysis of E_R and E_S-NA but not of E_S by the isolated component mixture. 50% lysis of E_R was obtained with 13.8 μl and of E_S-NA with 35.5 μl of the component mixture. No lysis was observed when in control experiments either the six alternative pathway proteins or the five membrane attack pathway proteins were omitted, or when individual proteins, such as Factor B, C3, or C7 were deleted from the complete mixture. In the absence of activating cells, the component mixture was stable at 37°C with respect to consumption of Factor B, C3, and C5. Also shown in Fig. 2, is the lytic capacity of C4 depleted human serum. The dose-response curves are virtually identical to those obtained with the component mixture, 50% lysis of E_R was caused by 13 μl and of E_S-NA by 33 μl of the serum.

The Nonessential, Regulatory Role of Properdin in the Lysis of Rabbit Erythrocytes. Previously reported experiments with properdin-depleted human serum indicated that properdin enhances rabbit erythrocyte lysis but is not essential (4). We have also shown that properdin is not essential for the initiation of the alternative pathway assembled from isolated proteins (1). As shown in Fig. 3, lysis of rabbit erythrocytes by the isolated component mixture proceeds in absence of properdin. 50% lysis was effected by 37 μl of mixture without properdin and by 16 μl of mixture with properdin.

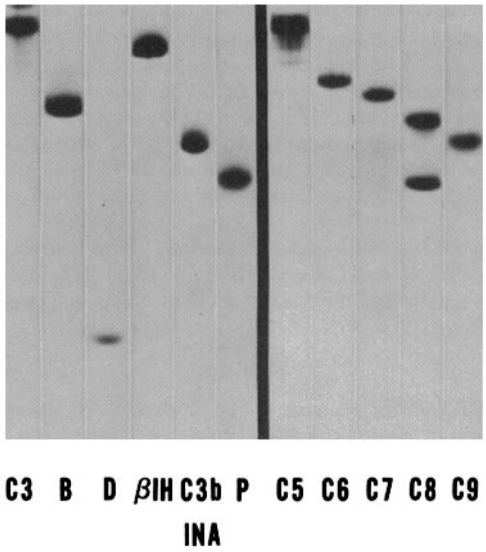

FIG. 1. Proteins of the cytolytic alternative pathway. 20-μg samples of each protein were subjected to SDS-polyacrylamide gel electrophoresis in 7% gels under nonreducing conditions. Gels were stained with Coomassie Blue dye R250. Proteins C3 through properdin (P) constitute the components of the alternative pathway of activation. Proteins C5 through C9 constitute the components of the membrane attack pathway.

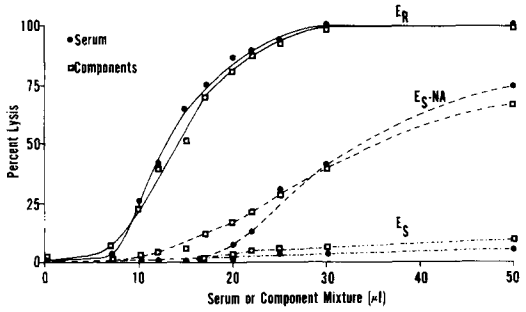

FIG. 2. Assembly of an intact cytolytic alternative pathway from 11 purified proteins. 160 μl reaction mixtures containing 10^7 erythrocytes, Mg-GVB, and varying amounts of either C4-depleted serum (●) or the mixture of 11 purified components (□) (see Materials and Methods) were incubated for 10 min at 37°C. Reactions were stopped by addition of 1 ml cold Mg-GVB and centrifugation. Lysis was determined spectrophotometrically at 412 nm. Shown are the dose-response curves for lysis of rabbit erythrocytes (——) neuraminidase-treated sheep erythrocytes (-----), and untreated sheep erythrocytes (-·-·-).

Generation of chemotactic activity in the isolated system. When the mixture of isolated components was incubated with 5 mg/ml zymosan for 45 min at 37°C chemotactic activity for polymorphonuclear leukocytes could subsequently be detected (Table I). C4-depleted serum after treatment with zymosan also contained chemotactic activity but fourfold less than the mixture.

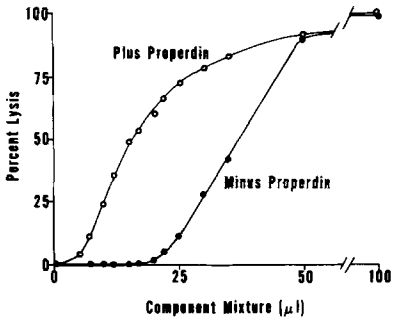

FIG. 3. The nonessential role of properdin in the alternative pathway. Dose-response curves for rabbit erythrocyte lysis using the isolated component mixture either containing (○) or lacking (●) native properdin were generated as described in Fig. 2.

TABLE 1

Zymosan-Induced Generation of Chemotactic Activity in the Purified Component Mixture or in C4-Depleted Human Serum

System	Chemotactic Activity (ED_{50}/ml)	
	+ Zymosan	+ Buffer
Purified components	2,128	<100
C4-depleted human serum	588	<100

Discussion

This is the first demonstration of the generation of cytolytic activity of the alternative pathway assembled entirely from isolated proteins. That the alternative pathway of complement activation can be assembled from C3, Factors B and D, and the three known regulatory factors has been documented (1). Whether the interaction of this activation system with the membrane attack system requires additional factors had not been critically evaluated heretofore. The results of this study indicate that no factors other than the 11 isolated proteins are required for the lysis of the alternative pathway activators: rabbit erythrocytes and neuraminidase-treated sheep erythrocytes. The quantitative similarity of the dose-response curves obtained with the component mixture and with C4-depleted serum further support our conclusion. No immunoglobulin requirement was found for the two cytolytic systems studied. We do not wish to imply on the basis of these results, that other cytolytic systems involving the alternative pathway may not need additional factors. It is known, for instance, that the cytolytic function of the pathway with regard to measles virus-infected cells is dependent on IgG with anti-measles virus specificity (24). And the complement-dependent lysis of gram-negative bacteria appears to involve lysozyme and perhaps other factors in addition to the membranolytic complex of complement (25, 26). Whether the results of this study have general validity for alternative pathway-dependent cytolysis necessitates a systematic investigation using the isolated component system described here and various cellular activators of the pathway.

Summary

The known cytolytic function of the alternative pathway in serum was quantitatively reproduced by combining 11 isolated plasma proteins at their respective serum

concentrations. These proteins are: C3, Factor B, Factor D, C3b inactivator, β1H, native properdin, C5, C6, C7, C8, and C9. In absence of activators of the alternative pathway, this mixture was stable at 37°C as evidenced by lack of consumption of Factor B, C3, and C5. Upon addition of either rabbit erythrocytes or neuraminidase-treated sheep erythrocytes, cell lysis ensued and the extent of lysis was dependent on dose of the component mixture. The dose-response curves obtained with the isolated component mixture and with C4-depleted serum were virtually indistinguishable. Nonactivator erythrocytes (untreated sheep erythrocytes) were not lysed by the component mixture. Deletion of properdin resulted only in a twofold diminution of the hemolytic activity of the component mixture. No immunoglobulin requirement was apparent. These results indicate that the cytolytic systems studied are internally sufficient and capable of coupling the initiation and amplification sequence with the cytolytic membrane attack sequence.

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