

LYSIS OF MEASLES VIRUS-INFECTED CELLS
BY THE PURIFIED CYTOLYTIC ALTERNATIVE
COMPLEMENT PATHWAY AND ANTIBODY*

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Lysis of virus-infected cells by human serum requires both IgG antibody to the virus and an intact alternative complement pathway. It has been shown that in the presence of antibody, serum either immunochemically depleted of C4 or genetically deficient in C2 can lyse virus-infected cells, whereas factor-B-depleted serum cannot. In these studies either anti-viral IgG or its F(ab')₂ fragment induced lysis in serum, whereas its Fab' fragment did not (1, 2). These observations were made with a variety of RNA- and DNA-budding viruses, and a range of human sera containing antibody to virus, suggesting that this might be a mechanism of wide biological applicability and relevance for clearing virus-infected cells.

It has recently been shown that an intact alternative pathway of complement activation can be assembled from six isolated plasma proteins: C3, factors B and D, β1H, C3b inactivator (C3bINA),¹ and properdin (P) (3). Combination of the above six proteins and the five proteins of the membrane-attack pathway of complement (C5, C6, C7, C8, and C9) at their respective physiological concentrations, led to the formation of a cytolytically active alternative pathway whose function was qualitatively and quantitatively identical to the alternative pathway in serum. The isolated cytolytic alternative pathway was capable of lysing rabbit erythrocytes (4) and, upon addition of lysozyme, certain strains of *Escherichia coli* (5) with an efficiency virtually identical to C4-depleted human serum.

The availability of the isolated cytolytic alternative pathway allowed us to definitively examine the complement-dependent lysis of antibody-coated measles virus-

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¹ Abbreviations used in this paper: C3bINA, C3b inactivator; IAP, isolated alternative pathway; ICAP, isolated cytolytic alternative pathway; MEM, minimal essential Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics; P, properdin; SDS, sodium dodecyl sulfate; VBS, Veronal-buffered saline; VBS⁺⁺ OVA, Veronal-buffered saline with 1.2 mM MgCl₂, 0.15 mM CaCl₂, and 0.1% ovalbumin.

infected HeLa cells. In this communication we report that by using the isolated cytolytic alternative pathway we have been able to document the absolute role of the alternative pathway and IgG antibody in the mediation of this lysis. This study also demonstrates that although properdin is not essential for alternative-pathway activation by these antibody-coated cells, it is absolutely required for their lysis.

Materials and Methods

Virus and Cells. HeLa cells were acutely infected with measles virus (Edmonston strain) at a multiplicity of infection of one plaque-forming unit per cell, and then maintained in suspension culture in minimal essential Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics (MEM) for 40 h; 100% of cells then showed surface staining with fluorescein-conjugated measles antibody (1) and cell viability was >90%.

Cytotoxicity Assay. In initial studies, lysis was assessed by the eosin microcytotoxicity assay as in previous studies (1, 2). A small-scale ^{51}Cr -release assay was developed to facilitate quantitation of lysis while still enabling the use of small volumes of reagents. Infected cells were washed in MEM and labeled with ^{51}Cr (200 $\mu\text{Ci}/3 \times 10^6$ cells in 500 μl MEM for 45 min at 37°C). 2×10^6 cells in 100 μl MEM were then incubated with varying amounts of IgG or its fragments for 30 min at 4°C, washed once, and suspended at 1×10^6 cells/ml in serum-free MEM with 0.01 M Hepes and 0.1% ovalbumin (Ovalbumin grade V, Sigma Chemical Co., St. Louis, Mo.). 5- μl fractions containing 5×10^3 cells were dispensed in wells of a U microtiter plate (Cooke Engineering Co., Alexandria, Va.) and the complement source was added in amounts ranging from 0 to 30 μl diluted in Veronal-buffered saline (VBS), containing 1.2 mM MgCl_2 , 0.15 mM CaCl_2 , and 0.1% ovalbumin (VBS⁺⁺ OVA). After incubation for 90 min at 37°C, 200 μl of phosphate-buffered saline were added to each well, the plate was centrifuged at 1,000 rpm for 5 min and 150 μl of supernate from each well was counted. Maximum release was obtained with 1% Nonidet P-40 (Shell Chemical Co., New York) and spontaneous release in MEM alone was 10% or less of the maximum. Specific release was calculated from the formula: the percentage of specific release = (sample - spontaneous release)/(maximum - spontaneous release).

Preparation of Immunochemically Depleted Sera. Human sera immunochemically depleted of specific complement proteins were prepared as described elsewhere (6) by adsorption with the IgG fraction of monospecific antiserum covalently coupled to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). Sera depleted of complement proteins but with intact C1 activity were prepared by passage through such affinity columns in VBS containing 1.0 M NaCl and 2 mM EDTA. These sera depleted of alternative pathway proteins exhibited total hemolytic titers against antibody-coated sheep erythrocytes of >90% of the control normal serum. C4-depleted serum prepared in this manner could also be reconstituted with regard to control serum upon addition of physiological concentrations of purified C4.

Purified Complement Components. C3 (7), factor B (8), factor D (9), C3bINA (10), β 1H (10), P (11), C5 (7), C6 (12), C7 (12), C8 (13), and C9 (14) were prepared as described. Purity of the isolated proteins was assessed, in part, by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Representative gel patterns have been published previously (4). In this study, at least two different preparations of each protein were used. Proteins were radioiodinated using the lactoperoxidase method of David and Reisfeld (15).

Preparation of the Isolated Cytolytic Alternative Pathway. The mixture of purified components at their physiological concentrations was prepared as described (4). Possible C3b contamination of C3 preparations was eliminated by incubation of C3 with appropriate amounts of β 1H and C3b inactivator just before the addition of the other eight isolated proteins. Final component concentrations were: 1,200 $\mu\text{g}/\text{ml}$ C3, 200 $\mu\text{g}/\text{ml}$ factor B, 2 $\mu\text{g}/\text{ml}$ factor D, 470 $\mu\text{g}/\text{ml}$ β 1H, 34 $\mu\text{g}/\text{ml}$ C3bINA, 20 $\mu\text{g}/\text{ml}$ P, 72 $\mu\text{g}/\text{ml}$ C5, 64 $\mu\text{g}/\text{ml}$ C6, 54 $\mu\text{g}/\text{ml}$ C7, 54 $\mu\text{g}/\text{ml}$ C8, and 58 $\mu\text{g}/\text{ml}$ C9. Before use in the cytotoxicity assays, the isolated component mixture was tested for its ability to lyse rabbit erythrocytes as described previously (4) and compared with C4-depleted serum.

Antibody. The IgG fraction of serum from patients with subacute sclerosing panencephalitis was isolated by anion exchange chromatography and concentrated to 24 mg/ml. F(ab')₂

TABLE I
Role of Alternative Complement Pathway in IgG and Complement-dependent Lysis of Measles Virus-infected HeLa Cells by Serum

Component depleted	Depletion experiments		Reconstitution experiments: percentage of lysis on addition of depleted component
	Percentage of lysis in depleted serum		
		%	%
None (normal serum)		95	—
Factor B		5	95
Factor D		7	95
P		20	95
C4		95	—
C2		95	—

Human sera containing IgG antibody to measles virus were immunochemically depleted of various complement components by affinity chromatography (as described in Methods), except for C2 which was obtained from a patient with genetic deficiency of C2. Depleted sera were reconstituted with a physiological concentration of the purified depleted component. Lysis was assessed by the eosin microcytotoxicity assay (1). Data represent the results of three experiments. Spontaneous lysis of infected cells in MEM was $5 \pm 10\%$; lysis of uninfected HeLa cells in reagents was $5 \pm 5\%$ (mean \pm 2 SD).

fragments were prepared by digestion of IgG with 3% wt/wt pepsin, and Fab' were obtained by reduction of F(ab')₂ with dithiothreitol and alkylation with iodoacetamide (16). There was no evidence of IgG in the F(ab')₂ or Fab' in the Fab' preparations on overloaded 7% SDS polyacrylamide gel electrophoresis under nonreducing conditions. IgG and its fragments were radiolabeled with ¹²⁵I by the lactoperoxidase method using Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.).

Antibody-binding Assays. Infected cells were incubated with ¹²⁵I-labeled IgG, F(ab')₂ or Fab' under the same conditions as were used to precoat cells with antibody for the cytotoxicity assay. Cells were then layered on 300 μ l of 20% sucrose in a microfuge tube, pelleted in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 1.5 min, the tip of the tube was cut off, and radioactivity in pellet and supernate was determined.

Kinetics of C3 Uptake. 2×10^7 cells in 200 μ l VBS⁺⁺ OVA were incubated in 200 μ l of the mixture of the six isolated alternative pathway proteins containing [¹²⁵I]C3, at 37°C. Aliquots of 2×10^6 cells were removed at timed points and the cells pelleted through 20% sucrose in VBS⁺⁺ OVA as above; [¹²⁵I]C3b uptake was calculated from the number of counts in the pellet. [¹²⁵I]C3b uptake from the same component mixture containing 0.01 M EDTA was simultaneously determined at each time point, and served as a control.

Results

Lysis of Measles Virus-infected Cells by Sera Depleted of Specific Complement Components. The ability of serum containing antibodies to measles virus to lyse measles virus-infected HeLa cells was abolished by immunochemical depletion of individual components of the alternative complement pathway. As shown in Table I, immunochemical depletion of factor B, factor D, or P, significantly abrogated lysis. Reconstitution of the depleted serum with physiologic amounts of the depleted component totally restored the lytic activity. In contrast, immunochemical depletion of C4 or genetic deficiency of C2 did not abrogate lysis, in confirmation of previous results.

Comparative studies showed that results given by the small-scale ⁵¹Cr-release assay correlated well with those obtained in the eosin dye microcytotoxicity assay used in

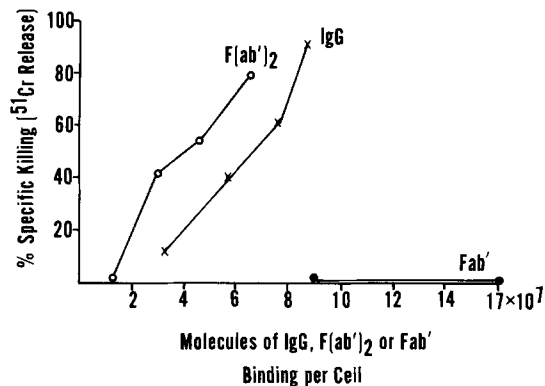


FIG. 1. Requirements for IgG, F(ab')₂ and Fab' for lysis of measles virus-infected cells via the isolated cytolytic alternative pathway (ICAP). 2×10^5 measles virus-infected HeLa cells were preincubated with increasing amounts of IgG (X) or its F(ab')₂ (O) and Fab' (●) fragments as described in Methods. 5×10^3 cells were then incubated with 20 μ l of the ICAP and lysis assessed by ⁵¹Cr release as described. Data for lysis are plotted against the number of molecules of IgG, F(ab')₂ or Fab' binding per measles virus-infected cell, as determined in a parallel binding assay using ¹²⁵I-labeled antibody or fragments.

the above experiments and in previous studies (1, 2); maximum ⁵¹Cr release correlated with morphological cell death. Kinetic studies showed that lysis of infected cells coated with IgG began at 30 min and was maximal by 60–90 min.

Lytic Activity of the Isolated Cytolytic Alternative Pathway. Measles virus-infected HeLa cells coated with IgG (at the maximal dose used in the IgG dose-related lysis curve in Fig. 1) were incubated in C4-depleted human serum, whole human serum without antibody, or the mixture of 11 proteins which constitute the isolated cytolytic alternative pathway. The dose-related lysis curves (Fig. 2) showed that the isolated cytolytic alternative pathway and C4-depleted serum were functionally equivalent in their capacity to lyse virus-infected cells; 50% lysis required 5 μ l of the isolated cytolytic alternative pathway or 14 μ l of C4-depleted serum. Lysis of infected cells without antibody and of uninfected cells, incubated in the isolated cytolytic alternative pathway or C4-depleted serum, was not greater than in the MEM control. Heating the isolated cytolytic alternative pathway at 50°C for 20 min completely abrogated lysis of IgG-coated cells.

Requirements for IgG, F(ab')₂, and Fab'. The amounts of IgG, F(ab')₂, or Fab' required to induce lysis of measles virus-infected cells in the isolated cytolytic alternative pathway were determined. Fig. 1 shows the dose-related lysis produced by preincubating the cells with increasing quantities of IgG, F(ab')₂, or Fab'. The results from the cytotoxicity assay are plotted against the number of IgG, F(ab')₂, or Fab' molecules binding per cell, as determined from a binding assay using these same proteins after radiolabeling and performed under conditions identical to those used to precoat cells for the lysis assay. Equivalent amounts of IgG, F(ab')₂, or Fab' bound per cell for a given amount of protein offered (e.g., twice as many Fab' as F(ab')₂ molecules bound for an equal amount of offered protein). The dose-related lysis curves for IgG and F(ab')₂ are very similar, but comparable amounts of Fab' did not induce lysis. Binding of 4×10^7 molecules of F(ab')₂ or 6.5×10^7 molecules of IgG antibody per cell was required for lysis of 50% of the measles virus-infected cells by the isolated cytolytic alternative pathway. In contrast, binding of $>1 \times 10^8$ molecules per cell of Fab' did

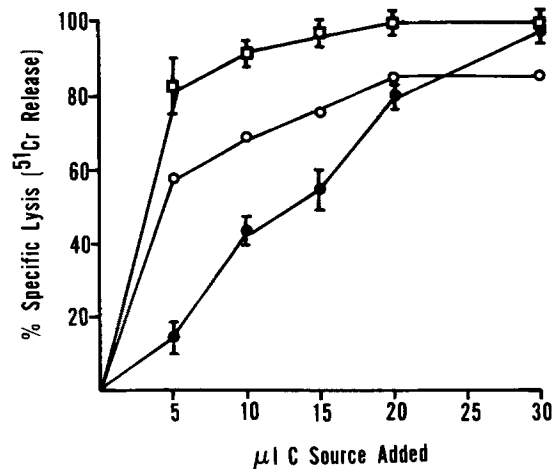


FIG. 2. Dose-related lysis of measles virus-infected HeLa cells coated with IgG antibody to measles virus by the following sources of complement (C) whole human serum lacking antibody to measles (□), human serum immunochemically depleted of C4 (●), and the ICAP (○). Lysis was assessed by ^{51}Cr release as in Methods. Each point represents the mean of duplicate assays \pm 1 SE. There was no lysis of uninfected HeLa cells or infected cells without antibody.

not induce any lysis by the isolated cytolytic alternative pathway, despite the fact that this was equivalent to the amount of the divalent $\text{F}(\text{ab}')_2$ inducing maximal lysis.

Requirement for P for Cell Lysis. P has been previously shown to play a nonessential but enhancing role in the alternative-pathway-mediated deposition of C3b onto activating particles (3), and for the lysis of rabbit erythrocytes (4) and certain gram-negative bacteria (5). In contrast to the aforementioned activators, the lysis of measles virus-infected cells is dependent upon antibody and the target is a nucleated cell presumably capable of effecting membrane repair. We therefore examined the role of P in the lysis of these cells. As shown in Fig. 3, deletion of P from the isolated cytolytic alternative pathway totally abrogated the lysis of IgG-coated virus-infected cells. In parallel experiments, the same reaction mixtures containing or lacking P were tested for their ability to lyse rabbit erythrocytes. Results obtained were similar to those previously reported (4) which showed that deletion of P only reduced lysis of rabbit erythrocytes by a factor of 2.6.

Enhancement of Pathway Activation by P. To determine the role P plays in the activation of the alternative pathway by these cells, the kinetics of C3b deposition onto antibody-coated measles virus-infected cells were studied using the isolated alternative pathway either containing or lacking P. As shown in Fig. 4, P was not absolutely required for C3b deposition onto the cell surface but did enhance the kinetics of pathway activation. In the presence of P, C3b was rapidly deposited onto the IgG-coated measles virus-infected cells during the 1st 10–20 min of the reaction. At 60 min, 15×10^5 C3b molecules were bound per cell. Deletion of P from the reaction mixture significantly decreased the rate of C3b deposition but had less effect on the number of C3b molecules ultimately bound per cell. After incubation of the cells for 60 min with the isolated protein mixture lacking P, C3b deposition was approaching that which was seen in the presence of P.

The deposition of C3b onto antibody-coated virus-infected cells from the isolated alternative pathway containing 0.01 M EDTA has been subtracted at each time

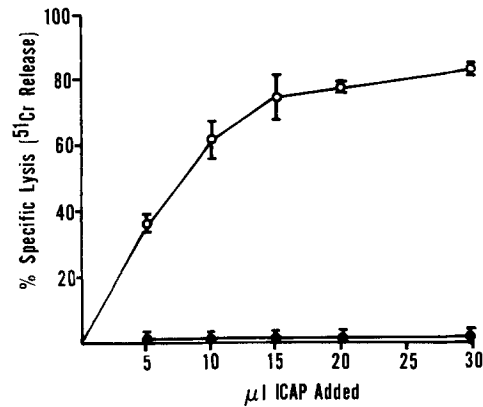


FIG. 3. Requirement for P for lysis of IgG-coated measles virus-infected HeLa cells by the ICAP. Lysis of 5×10^9 IgG-coated infected cells by increasing doses of the ICAP (○) and the ICAP with P omitted (●).

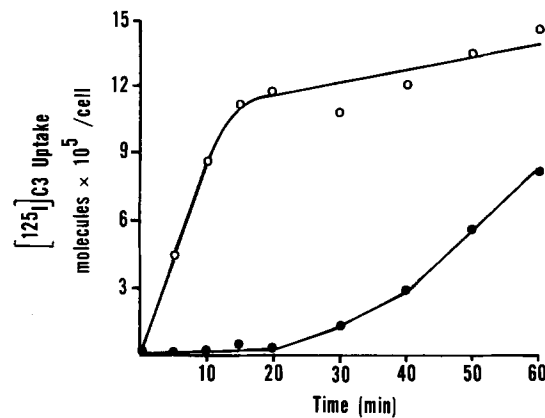


FIG. 4. Effect of P on the kinetics of [^{125}I]C3 uptake from the isolated alternative pathway (IAP) by IgG coated measles virus-infected HeLa cells. [^{125}I]C3 uptake, measured as described in Methods, is shown from the IAP containing P (○) and with P omitted (●). [^{125}I]C3 uptake from the IAP containing 0.01 M EDTA has been subtracted at each time point.

point; this deposition in the presence of EDTA averaged 3×10^5 C3b molecules per cell and showed no increase with time.

Discussion

This study makes the following points. First, measles virus-infected cells are lysed by IgG antibody to measles virus, or its $\text{F}(\text{ab}')_2$ fragment, and the components of the isolated cytolytic alternative pathway, a system composed solely of the 11 highly purified proteins of the alternative pathway of activation and membrane attack pathway of complement. IgG and $\text{F}(\text{ab}')_2$ induce equivalent dose-related lysis by the isolated cytolytic alternative complement pathway, whereas comparable or larger doses of Fab' are ineffective. Second, this lysis of measles virus-infected HeLa cells exhibits an absolute requirement for P. Third, P is not essential for C3b uptake onto the measles virus-infected cell in the presence of antibody but does enhance the rate at which deposition occurs.

These observations provide conclusive evidence for the role of IgG antibody and the alternative complement pathway in the lysis of measles virus-infected cells. They thus confirm earlier findings from this laboratory that lysis of virus-infected cells by whole serum is dependent on the presence of IgG and an intact alternative pathway (1, 2). Furthermore, the use of the 11 highly purified cytolytic alternative pathway components rules out the participation of any other serum factors in this process. These results also demonstrate that the proteins of the isolated cytolytic alternative pathway are sufficient for activation and cell lysis in a system where lysis is dependent on IgG, and are functionally equivalent in this respect to the pathway in whole serum.

The fact that both IgG and F(ab')₂ fragments of measles antibody-induced lysis extends previous findings (2) by showing that IgG and F(ab')₂ are equally effective on a molecular basis in inducing lysis over a range of doses. Moreover, an equivalent amount of Fab' bound to the infected cell was totally ineffective in inducing lysis. This clear requirement for divalent IgG may indicate that the role of IgG is to group or patch viral antigen on the surface of infected cells or alternatively, that a site other than the combining site, present on F(ab')₂ but lost on reduction to Fab', is required for IgG to induce lysis.

We found an absolute requirement for P for the lysis of virus-infected HeLa cells. The fact that deletion of factor B or D abrogates lysis (Table I) would be expected as both are required, with C3, for generation of the alternative pathway C3 convertase C3bB. P, however, is a regulatory protein, whose demonstrated function is that of delaying the rapid intrinsic decay of C3bB. Moreover, P has been shown not to be essential for the lysis of rabbit erythrocytes (4) or *E. coli* (5) by the isolated cytolytic alternative pathway. The contrasting requirement shown here may reflect the fact that lysis of nucleated cells, which have the capacity for membrane repair, probably requires extensive C3 deposition and generation of C5b-C9 complexes on the plasma membrane, both of these events would be enhanced by P. The accelerated C3 uptake shown here in the presence of P is consistent with its established function of stabilizing C3bB.

Other workers have also reported that measles virus-infected cells are lysed by antibody and the alternative pathway in serum, although they sometimes used heterologous sera as a source of complement (17, 18). Hicks et al. (17) reported that both classical and alternative pathways combined were considerably more effective than either alone. In contrast, as shown here, the isolated cytolytic alternative pathway is of comparable efficiency to whole serum in dose-related lysis of virus-infected cells; this suggests a minor or negligible contribution of the classical pathway to lysis, despite the fact that the IgG bound to the infected cell is capable of causing C3 uptake by activation of the classical pathway. It should be emphasized that by employing a cell line of human origin and human antibody and complement sources, our experiments avoid problems inherent in the use of heterologous sera.

The mechanism by which IgG induces lysis via the alternative pathway is unknown. The production of lysis by the isolated cytolytic alternative pathway conclusively rules out any possibility that C1 could be activating the alternative pathway, as described for the lysis of sheep erythrocytes heavily sensitized with IgG antibody by C4-deficient guinea pig serum (19). This mechanism was viewed as unlikely on the basis of the previously described lack of requirement for calcium ions for lysis of virus-infected cells, which can be mediated by magnesium EGTA serum (1, 2).

Current evidence indicates that solid-phase initiation of the alternative pathway depends on distinctive surface properties of the known particulate activators, such that C3b deposited on them from the fluid phase is protected from its serum inactivators (20, 21, 3). Thus, it has been shown that access of $\beta 1H$ to C3b deposited on activating particles is restricted (22, 23), the C3b is protected from the concerted inactivating effects of $\beta 1H$ and C3bINA, and the C3 convertase, C3bB, can be generated with subsequent amplification of C3 turnover and surface deposition, binding of P, and cleavage of C5 (3, 24). There is no information available to suggest how IgG might play a role in such a scheme of activation. Our own studies, to be presented elsewhere, indicate that measles virus-infected cells can activate the alternative pathway in the absence of IgG and that, although IgG is essential for lysis, it does not itself initiate activation.

Thus, the dominant role of the alternative pathway in the antibody-dependent and-complement-dependent lysis of virus-infected cells is now amply established; the precise role of IgG or its F(ab')₂ fragment in inducing such lysis remains to be uncovered.

Summary

The dependence of antibody-and-complement-mediated lysis of virus-infected cells on the alternative pathway was examined utilizing the isolated cytolytic alternative pathway—a system consisting of the six purified proteins of the alternative pathway of activation (C3, factors B and D, $\beta 1H$, C3b inactivator and properdin), and the five proteins of the membrane attack pathway (C5–9) of complement. HeLa cells acutely infected with measles virus were lysed by anti-viral IgG and the isolated cytolytic alternative pathway with an efficiency comparable to whole human serum. IgG and its F(ab')₂ fragment were equally effective in inducing lysis by the isolated cytolytic alternative pathway, binding of $\cong 5 \times 10^7$ molecules per cell being required for 50% lysis; in contrast, no lysis occurred when equivalent or greater amounts of Fab' were bound to the virus-infected cell. Properdin was required for lysis. No lysis occurred if properdin was deleted from the isolated cytolytic alternative pathway, and lysis was diminished by 80% in properdin-depleted serum. Uptake of [¹²⁵I]C3b from the isolated alternative pathway onto measles virus-infected cells occurred in the absence of properdin, but was accelerated in the presence of properdin. The 11 proteins of the isolated cytolytic alternative pathway are thus sufficient for lysis of measles virus-infected cells bearing anti-viral IgG or F(ab')₂ without any other serum protein.

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