

MECHANISM OF INJURY OF VIRUS-INFECTED CELLS  
BY ANTIVIRAL ANTIBODY AND COMPLEMENT:  
PARTICIPATION OF IgG,  
F(ab')<sub>2</sub>, AND THE ALTERNATIVE COMPLEMENT PATHWAY\*

By LUC H. PERRIN,† BARRY S. JOSEPH,§ NEIL R. COOPER, AND MICHAEL B. A.  
OLDSTONE

*(From the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla,  
California 92037)*

Virus infection in man may lead to acute, subacute, or chronic illness. Although the source of entry, type of virus, preferred tissue of infection, and mechanism of replication of the virus are important, the manner in which the host interacts with the virus-infected cell determines, in large part, the infection's outcome and the type of disease which results. Host defenses against virus and virus-infected cells operate at humoral [antiviral antibody and complement (C)] (1-3), cellular (sensitized T cells, macrophages) (4, 5), and combined humoral-cellular (K cells, macrophages) (6) levels. Although these various pathways have been described in some detail, little is known of the cellular receptors involved, the quantitative aspects of the reaction, or the biochemical events which lead to lysis of virus-infected cells. While these mechanisms and probably others function simultaneously *in vivo*, we believe that the host's defenses against virus infection will be better understood only after each type of reaction has been characterized in detail.

We have focused initially on humoral defense mechanisms and the interaction of antibody and/or C with various viruses (7, 8) and with virus-infected cells (9). The latter studies indicated that  $1 \times 10^6$  HeLa cells acutely or persistently infected with measles virus consistently can bind from 1 to 7  $\mu$ g of immune IgG at their surface (9, 10). Such cells are lysed by antibody and C, but only when an average of more than  $1 \times 10^6$  molecules of antiviral antibody became bound to each infected cell (9). Furthermore, we found that antibody-initiated C-dependent lysis of several different cell types expressing surface measles virus antigens was mediated exclusively by the alternative C pathway (9).

Now we report antibody-mediated lysis of cells expressing mumps virus, herpes simplex Type I virus, influenza A° virus, or measles virus antigens. Lysis of cells infected with these RNA and DNA budding viruses was dependent on

---

\* This is Publication No. 1074 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92037. This research was supported by U. S. Public Health Service Grants NS-12428, AI 07007, AI 09484, and CA 14692.

† Supported by a postdoctoral grant from the Swiss Medical Research Council.

§ Special postdoctoral fellow of the National Multiple Sclerosis Society.

the presence of specific antibody and proceeded through the alternative C pathway. Lysis was initiated by human IgG antibody, or its  $F(ab')_2$ , but not  $Fab'$  or Fc fragments.

### Materials and Methods

*Viruses and Cell Lines.* Mumps virus, herpes simplex type 1 virus, and measles virus (Edmonston strain) were obtained from the American Type Culture Collection, Rockwell, Md. Influenza A<sup>o</sup>/WSN was a gift of Martin Haspel, Scripps Clinic and Research Foundation, La Jolla, Calif. as was Clone 7-5c-4 of the Wang Kilbourne variant of the Chang conjunctiva cell line. HeLa and Vero cells were obtained from Flow Laboratories Inc., Rockville, Md. After passage at low multiplicities of infection (MOI) (0.1-0.4) in Vero cells for mumps virus, in HeLa cells for herpes simplex type 1 virus and measles virus, and in Chang conjunctiva cell lines for influenza A<sup>o</sup> virus, pools of the respective infectious viruses were collected, separated from cellular debris by centrifugation (5 min, 1,000 g), divided into aliquots, and stored at -70°C until use. The various virus pools were titrated as described (9).

*Cultivating, Infecting, and Handling of Cells.* Vero and HeLa cell lines were cultivated in Eagle's minimal essential media (MEM)<sup>1</sup> with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics; Chang conjunctiva cells were grown in MEM containing 0.2% bovine serum albumin, glutamine, and antibiotics at the time of infection. During the late log phase of their growth in 75 CM<sup>3</sup> Falcon flasks (Falcon Plastics, Oxnard, Calif.), the cells were infected with the different virus preparations at an average MOI of 3. After 2 h of absorption, during which the cells and viruses were gently mixed on a Bellco rocker platform (Bellco Glass Inc., Vineland, N. J.) at 37°C in 5% CO<sub>2</sub>, the cultures were supplemented with 20 ml of appropriate growth media. Cells infected with mumps virus or measles virus were harvested 3-4 days after infection, whereas cells infected with herpes simplex virus or influenza virus were used 1-2 days after infection. Such cells were obtained after vigorously shaking each flask, then removing and discarding the loosely adherent cells. Afterward we treated the adherent cells with trypsin-EDTA as previously reported (9). Preliminary studies indicated that 99% of cells harvested in this method showed viral antigens on their surfaces and greater than 95% viability as judged by trypan blue dye exclusion tests.

*Source of Antiviral Antibody and C.* Human sera were obtained from 13 healthy laboratory workers, two children without known exposure to mumps virus, one 30-yr-old male before and after mumps vaccination, one 12-mo-old infant before vaccination against measles virus, and three women with recurrent herpes simplex type 1 virus infections. Each bleeding was collected in a glass tube and left standing 2 h at room temperature. The sera were centrifuged and used directly or after storage at -70°C as sources of both antiviral antibody and C.

*IgG Fragments.* The IgG fraction from serum of a patient with subacute sclerosing panencephalitis (SSPE) was kindly provided by Peter Lachmann, Royal Postgraduate Hospital, London. Approximately 12% of this IgG preparation contained specific antibodies for measles virus antigen (10). Less than 1% of IgG prepared from the sera of two subjects having antibodies against mumps virus and measles virus contained specific antibodies to measles or mumps virus. After ammonium sulfate precipitation and DEAE cellulose chromatography, both IgG preparations were concentrated to 30 mg protein/ml by negative pressure, dialyzed against Veronal-buffered saline containing Mg<sup>++</sup> and Ca<sup>++</sup> (VBS<sup>++</sup>), centrifuged 10 min at 10,000 g, and shown to be pure by Ouchterlony analysis.  $F(ab')_2$  and  $Fab'$  were made as previously reported (11).  $F(ab')_2$  was reduced with 0.02 M dithiothreitol for 2 h at 20°C and alkylated by addition of 0.05 M recrystallized iodoacetamide to obtain  $Fab'$  that was subsequently chromatographed on Sephadex G100.  $F(ab')_2$  and  $Fab'$  preparations were concentrated to 20 mg/ml, dialyzed against VBS<sup>++</sup>, and, when tested by Ouchterlony analysis for specific antiserum directed against human Fc or human  $Fab'$  fragments (kindly provided by Dr. H. Spiegelberg, Scripps Clinic and Research Foundation, La Jolla, Calif.), showed a precipitin line only with antibody directed toward human  $Fab'$ , but not with antibody against human Fc.

<sup>1</sup> Abbreviations used in this paper: MEM, Eagle's minimal essential media; SSPE, subacute sclerosing panencephalitis; VBS<sup>++</sup>, Veronal-buffered saline containing Mg<sup>++</sup> and Ca<sup>++</sup>.

*Components and Antisera.* Factor B and C2 were purified from human sera as published (12, 13). Factor B was immunochemically pure as determined by Ouchterlony analysis at a concentration of 1.5 mg/ml. C2 was functionally pure and immunochemically free of Factor B.

Human sera containing antibodies to measles virus were depleted of either C4 or factor B by the following procedures. 4 ml of sera containing 0.015 M EDTA and 0.4 M NaCl per ml were run through columns of Sepharose 4B to which IgG fraction of antisera to C4 or Factor B had been coupled (14); effluents were collected and concentrated by negative pressure to the original starting volume and finally dialyzed against VBS++. All manipulations were carried out at 4°C. Some sera were depleted of factor B by heating at 50°C for 25 min. Depleted sera were used directly or stored in small aliquots at -70°C until use. Sera depleted of factor B retained over 75% of the original hemolytic C activity against sheep red blood cells sensitized with antibody (greater than 50 CH<sub>50</sub> U) in each preparation, but sera depleted of C4 lost more than 99.9% of their hemolytic activity. By hemolytic plaque assay (15) sera depleted of factor B had no reactivity, whereas addition of 250 µg purified factor B per ml of depleted sera gave ring size that was equivalent to untreated normal sera. C3 consumption after the addition of inulin was also measured in factor B-depleted and repleted sera. These tests showed loss of ability to sustain C3 consumption on addition of inulin and full reconstitution of this ability by isolated factor B. C4-deficient serum was fully able to mediate inulin-induced C3 consumption (12). Factor B- and C4-depleted sera were free of the respective protein when tested by Ouchterlony analysis with the specific antisera.

Antisera against human properdin were raised in rabbits and antisera against factor B and C3 in goats. All these antisera were free of contaminants, as judged by Ouchterlony analysis, except the rabbit antiproperdin which contained trace amounts of antibodies to human IgG. The contaminating anti-IgG was completely absorbed out by using human IgG insolubilized with ethyl chloroformate (16). IgG was isolated from antifactor B and anti-C4 antisera, and 50 mg of each was coupled to 15 ml of Sepharose 4B (14) to make various affinity columns. Rabbit antibody to HeLa cells was made and characterized as reported (9).

Goat antihuman C4 and antihuman factor B were mixed at equivalence with normal human serum in the presence of 0.01 M EDTA. The precipitate that formed overnight at 4°C was washed with phosphate-buffered saline, dissolved in glycine HCl, and digested with pepsin according to Lachmann (17). After centrifugation at 3,000 g for 5 min, the supernate was collected, dialyzed against 0.05 M Tris buffer pH 8.2, reduced with DDT, and alkylated with iodoacetamide. After chromatography on Sephadex G100 the Fab' fragments were dialyzed against VBS++ and adjusted to a protein concentration of 6 mg/ml. The preparations were free of any Fc contamination by Ouchterlony analysis.

To examine the ability of Fab antisera to C components to block the C system, 100 µl of Fab' antibody to C4 or factor B were mixed with 100 µl of fresh human serum containing antibody to measles virus, mumps virus, influenza virus, or herpes viruses for 10 min at room temperature. A 100-µl aliquot was removed, and two-fold serial dilutions were made with the fresh serum until a final dilution of Fab reagents were 1:16. Various dilutions of these mixtures were tested for their ability to lyse virus-infected target cells by using the microcytotoxicity assay (see below).

*Immunofluorescence.* The IgG fractions of sera containing monospecific antibodies to C4, C3, factor B, and properdin were conjugated to fluorescein isothiocyanate as previously reported (18). 2 vol of fluoresceinated antisera were absorbed with 1 vol of uninfected target cells at 4°C for 3 h to remove natural antibodies against cell surface determinants. The fluoresceinated antisera did not stain the surfaces of uninfected or virus-infected cells grown in growth medium after absorption.

We also used immunofluorescence (direct) to test for measles virus antigens on the surfaces of infected cells and (indirect) for the presence of mumps virus, influenza A<sup>o</sup> virus, and herpes simplex type 1 viruses.

*Cytotoxicity.* To measure cell lysis we used the eosin micromethod described by Joseph et al. (9) with the following modifications: (a) for reconstituting C components after depletion, 1-2 µl of purified C components were added; (b) 8 µl of immune or nonimmune sera depleted of various C components were used, and (c) control sera were supplemented with 1-2 µl of VBS++. The results were expressed either as a cytotoxicity index corrected by removing spontaneously dead cells or as the percentage of dead cells. Nonspecific background lysis never exceeded 10% with a mean of 5% ± SD of 5%.

## Results

*Specificity of Antibody-Initiated C-Dependent Lysis of Cells Infected with Mumps, Herpes Simplex, Influenza A, or Measles Viruses.* Vero cells were susceptible to infection with mumps virus as evidenced by the appearance of viral antigens in the cytoplasm and on the cells' surfaces 24–48 h after addition of virus to the cells. Results were similar when herpes simplex virus and measles virus were added to HeLa cells, and influenza virus was placed on Chang conjunctiva cells. These different virus-infected cells were only used at the time when more than 95% of them were positive by direct or indirect fluorescence. When fresh sera from over 15 persons containing antibodies to these various viruses were added to cells infected with the corresponding virus 48 h or more after initiating infection, cell lysis occurred. Prior heating of these sera at 56°C for 30 min eliminated the lytic effect. However, heated sera could initiate lysis when replenished with a source of C devoid of specific antiviral antibodies.

The specificity of the lytic process was shown in several ways (Tables I and II): (a) Virus-infected cells were lysed only in the presence of IgG (presumably antiviral antibody) that could be detected by immunofluorescence. Neither IgG binding nor specific cell lysis occurred with uninfected cells. (b) Sera having antibody to only one virus and capable of mediating lysis of cells infected with that virus were unable to lyse cells infected with other viruses (Table I). (c) Sera that did not contain immune IgG to a specific virus and that were incapable of lysing cells infected with that virus acquired lytic ability when we added IgG from sera that contained antibody to that virus. An example is shown for mumps virus-infected cells in Table II. In a few instances, primarily with cells infected with herpes simplex Type I virus, IgG bound to the infected cells despite the absence of lysis. This may have been caused by Fc receptors which appeared on cells after infection with this virus (19) or to an inadequate concentration of antiviral antibody.

Further evidence for specificity was observed when we used serum from an individual without a history of mumps virus infection and no evidence of antibodies to mumps virus to lyse Vero cells expressing mumps viral antigens on their surface before and after specific vaccination. As seen in Fig. 1, the prevaccination serum and serum obtained 3 days after vaccination were unable to lyse cells infected with mumps virus. Lysis of mumps virus-infected cells by serum from this individual first occurred 6 days after vaccination and a serum sample obtained 10 days after vaccination was able to kill over 95% of mumps virus-infected Vero cells. Similarly, using a serum devoid of antibodies against mumps virus and adding various amounts of mumps virus-immune IgG obtained from two different subjects, it was shown that the percent of cells lysed depended on the amount of immune IgG added (Fig. 2). At no time were the sera used able to injure uninfected Vero cells. The presence of IgG able to bind to infected cells correlated well with the lytic capacity of the serum. In this as all other studies, IgG did not bind to uninfected Vero cells, and infected cells were not lysed after heating the serum for 30 min at 56°C.

*Pathway of C Activation in Antibody-Initiated Injury of Virus-Infected Cells.* The cytolytic activity of fresh serum containing antibody for cells ex-

TABLE I  
Specificity of Antibody-Initiated Complement-Dependent Lysis of Cells Expressing Viral Antigens on Their Surfaces\*

Fresh serum source	Cells expressing antigens of the following viruses:											
	Mumps virus			Herpes simplex I virus			Influenza A virus			Measles virus		
	Bind- ing of IgG	Percent lysis with		Bind- ing of IgG	Percent lysis with		Bind- ing of IgG	Percent lysis with		Bind- ing of IgG	Percent lysis with	
		Fresh serum	Δ56 serum		Fresh serum	Δ56 serum		Fresh serum	Δ56 serum		Fresh serum	Δ56 serum
LP	0	7	7	+	95	5	+	95	10	ND	ND	ND
BJ	+	86	6	+	99	5	+	87	10	+	10	10
JM	+	76	5	+	95	5	ND	ND	ND	+	96	10
JH	+	91	6	+	84	5	ND	ND	ND	+	9	8
ZB	ND	ND	ND	+	87	5	+	79	10	+	83	9
BB	0	5	5	0	5	5	0	7	7	+	8	7

\* 0, negative; +, positive; ND, not done.

TABLE II  
IgG Dependence of Complement-Mediated Lysis of Cells Expressing Mumps Viral Antigens on Their Surface\*

Immune reagents	Binding of IgG	Percent lysis with	
		Fresh se- rum	Serum Δ56°C‡
IP serum	0	7	7
BB serum	0	5	5
LP serum	0	7	7
BJ serum	+	88	6
BJ IgG	+	0	0
BJ IgG + JP serum§	ND	82	6
BJ IgG + BB serum§	ND	84	7
BJ IgG + LP serum§	ND	83	7

\* 0, negative; +, positive; ND, not done.

‡ These sera were heated at 56°C for 30 min.

§ 2 μl of BJ IgG (30 mg/ml) were added to 8 μl of JP, BB, and LP sera, respectively.

pressing either mumps virus, herpes simplex virus, influenza A° virus, or measles virus antigens on their surfaces was sensitive to dilution. Over 85% of the cytolytic activity disappeared at a 1:4 dilution for each of these viruses, even when the fresh sera were diluted in the same heated sera (56°C, 30 min) to make the antibody concentration constant. Moreover, at this dilution, significant binding of IgG to infected cells occurred in several sera, indicating that the factor limiting lysis was the C system and not the IgG. In contrast, the same C source diluted 1:50 retained over 50% of its original cytolytic titer for sheep red blood cells sensitized with antibody or uninfected HeLa cells coated with rabbit anti-HeLa cell antibodies.

Other experiments (Table III) showed that antibody-initiated C-dependent lysis of virus-infected cells required magnesium but not calcium. In these studies, antibody containing fresh sera chelated with 11 mM EDTA and supple-

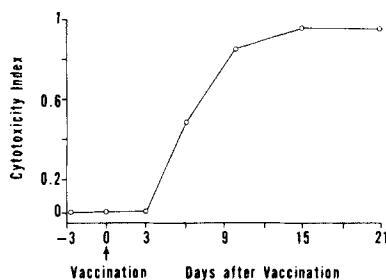


FIG. 1. Ability of serum from an individual previously not exposed to mumps virus to lyse acutely infected vero cells after vaccination. Serum was collected before, at, and after specific mumps vaccination and added to Vero cells expressing mumps virus antigens on its surface. Lysis was measured by microcytotoxicity as described in Materials and Methods.

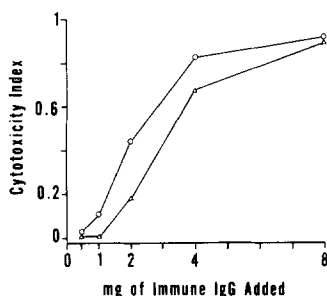


FIG. 2. Ability of varying amounts of IgG antibody to mumps virus in the presence of a human C source devoid of antimumps virus antibodies in lysing Vero cells. Vero cells were acutely infected with mumps virus and were expressing viral antigens on their surface at the time of the test. Antibodies were obtained from a convalescent serum ( $\Delta$ — $\Delta$ ) and from a serum after vaccination ( $\circ$ — $\circ$ ).

mented with 1 mM  $Mg^{++}$  were unable to mediate lysis of cells expressing virus antigens on their surfaces. In contrast, sera chelated with 11 mM EGTA and supplemented with 1 mM  $Mg^{++}$  were fully active (Table III).

The sensitivity to dilution and the lack of calcium dependence strongly suggested primary involvement of the alternative C pathway in the lytic process. In accordance with this interpretation, the cytolytic potential of serum containing antibodies to measles virus for HeLa cells infected with measles virus was not reduced after depletion of C4 by affinity chromatography, although the ability of the C4-depleted sera to lyse sensitized sheep red blood cells was completely abolished by this procedure (over 95% of measles virus-infected HeLa cells were lysed by sera from two individuals either intact or depleted of C4). To better assess the role of the alternative C pathway in antibody-initiated lysis of virus-infected cells, sera were depleted of factor B either by heating at 50°C for 25 min or by affinity chromatography (Table III). Both procedures led to complete loss of ability to lyse mumps virus, herpes simplex virus, influenza A° virus, and measles virus-infected cells. The lytic ability of the sera were fully reconstituted by physiological amounts of highly purified factor B (Table III). Moreover, isolated C2, in excess, was unable to reconstitute the lytic activity in sera depleted of factor B. Similarly, the lytic activity for mumps virus-infected

TABLE III  
*Role of the Alternative C Pathway in Antibody-Initiated Lysis of Virus-Infected Cells\**

C source treatment	Percent lysis after addition of human serum to cells expressing antigens of the following viruses†			
	Mumps virus	Herpes simplex I virus	Influenza virus	Measles virus
None§	78 ± 10	87 ± 7	81 ± 7	90 ± 8
Heated 50°C 20 min	5 ± 10	10 ± 5	10 ± 5	5 ± 10
Heated 50°C 20 min + factor B (250 µg/ml)	84 ± 3	87 ± 8	86 ± 10	88 ± 6
Fab antifactor B	5 ± 5	10 ± 5	10 ± 5	5 ± 5
Fab anti-C4	74 ± 11	82 ± 5	78 ± 8	87 ± 9
Mg EGTA	75 ± 5	83 ± 6	79 ± 7	85 ± 3
Mg EDTA	5 ± 5	10 ± 5	10 ± 5	5 ± 5

\* Spontaneous lysis of infected cells was 5 ± 10% for mumps and measles and 10 ± 5% for herpes simplex I and influenza viruses.

† Values indicate the mean ± SD for at least five different immune sera for cells infected with each virus.

§ Untreated fresh immune serum.

cells of serum lacking mumps virus antibody and reconstituted with immune IgG (8 mg/ml) was lost after heating of the serum at 50°C for 25 min and reconstituted by addition of 250 µg/ml of factor B. Another approach was employed to assess the relative roles of the alternative and classical C pathways in lysis of virus-infected cells in these studies. This is based on our finding that Fab' antibody to factor B and Fab' antibody to C4 specifically block the alternative and classical pathways, respectively. As shown in Fig. 3 the addition of Fab' antifactor B completely blocked cytolysis mediated by the alternative C pathway, while Fab' anti-C4 had no effect. Conversely, Fab' antibody to C4 blocked lysis of HeLa cells coated with rabbit antibody to HeLa cells, a lytic reaction mediated by the classical pathway, whereas Fab' antibody to factor B did not. Lysis was blocked to a dilution of the Fab' anti-C4 of 1:8. We tested 8 different immune sera to mumps virus, 5 immune sera to herpes simplex virus, 11 immune sera to influenza virus, and 10 sera to measles virus with these reagents. In all cases the addition of Fab' antibody to factor B to the serum completely abrogated the cytolytic potential of these sera against cells infected with the respective virus (Table III). In contrast the addition of Fab' antibody to C4 had no significant effect on the ability of the sera to lyse cells infected with mumps virus, herpes simplex virus, influenza A° virus, or measles viruses.

*Binding of Various C Components to Surfaces of Virus-Infected Cells.* We next studied the binding of properdin, factor B, C3, and C4 to the plasma membranes of HeLa cells infected with measles after incubation of these cells with human immune sera (Fig. 4). 10<sup>5</sup> infected HeLa cells expressing measles virus antigens on their surfaces were incubated for 30 min with either growth medium, human serum containing antibodies to measles virus with the same serum depleted of C4, factor B, or properdin. Then these cells were washed and mixed with fluorescein-conjugated antiserum to properdin, factor B, C3, or C4. Virus-infected cells incubated with growth media alone failed to show any

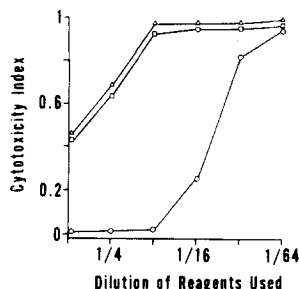


FIG. 3. Inhibition of the ability of measles virus-specific antibodies and C to lyse HeLa cells acutely infected with measles virus upon the addition of goat Fab' antihuman Factor B (○—○). In contrast lysis was not inhibited upon addition of either goat Fab' antihuman C4 (□—□) or VBS++ (△—△).

staining on their plasma membranes. In contrast, greater than 95% of infected cells mixed with immune serum bound C3. Infected cells mixed with C4-depleted sera bound both properdin and factor B (Fig. 4). In all instances observed, factor B binding was significantly less intense than the binding of other C components. When factor B-depleted serum was used, we observed binding of both properdin and C4 to the cells' surfaces. In other experiments both heated serum (56°C for 30 min) and serum from a child devoid of antibodies against measles virus failed to induce binding of C components to the surfaces of infected cells.

*Portion of the IgG Molecule Which Initiates C Lysis of Virus-Infected Cells.* IgG and F(ab')<sub>2</sub> (15 mg/ml) antibody to mumps virus or measles virus in the presence of a C source devoid of antiviral antibodies lysed cells expressing mumps or measles virus antigens on their surfaces, respectively (Table IV). In contrast, Fab' (15 mg/ml) from either antiviral preparation was not able to initiate lysis. Comparable studies with <sup>125</sup>I-labeled F(ab')<sub>2</sub> and <sup>125</sup>I-Fab' showed binding of approximately 5 × 10<sup>6</sup> and 8 × 10<sup>6</sup> molecules of either fragment per cell under the prevailing experimental conditions, indicating that the inability of Fab' to initiate lysis was not due to the failure to bind. Also, unlabeled Fab' inhibited binding of <sup>125</sup>I-IgG to infected cells. Similar results were obtained with IgG, F(ab')<sub>2</sub> (4 mg/ml), and Fab' (4 mg/ml) isolated from the serum of a patient with chronic measles virus infection (SSPE).

Heating the nonantibody-containing C source at 50°C for 25 min prevented IgG- or F(ab')<sub>2</sub>-initiated lysis of infected cells. Lytic ability was completely restored by the addition of physiological amounts of highly purified factor B (250 μg/ml) to the heated C source (Table IV).

*Relationship of Capping of Virus Antigens to Antibody-Mediated Lysis of Virus-Infected Cells.* Immune lysis of cells expressing virus antigens on their surfaces proceeded independently of redistribution and capping of viral antigens. Thus, when HeLa cells expressing measles virus antigens on their surfaces were incubated with various concentrations of cytochalasin B and tested for their ability to be lysed by antibody and C or to redistribute surface viral antigens (Table V), we found that 5 μg/ml of cytochalasin B inhibited capping 36%, while not abrogating immune lysis at all. Similarly, at 20 μg/ml capping



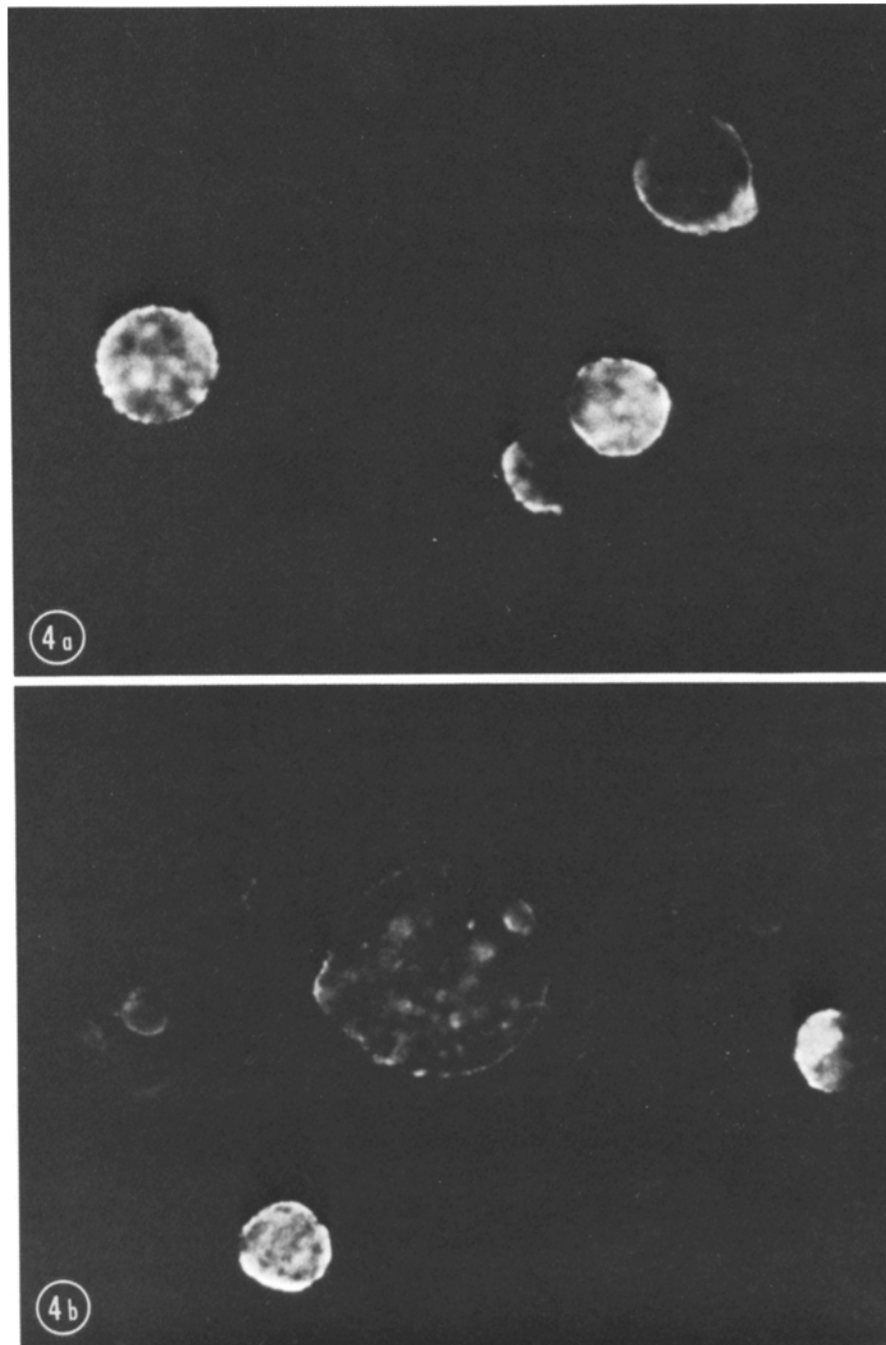


FIG. 4. Presence of human C3 (*a*) and human properdin (*b*) on the surface of HeLa cells acutely infected with measles virus. Living infected cells were incubated with fresh human serum containing antibodies against measles virus before staining with a fluoresceinated goat monospecific antibody to human C3 or human properdin.

was inhibited over 95%, whereas in contrast, lysis was not impeded. Comparable results occurred with concentrations of sodium azide which inhibited capping without abrogating immune cytolysis. Background lysis of virus-infected cells was not appreciably increased after the application of either inhibitor.

### Discussion

Cells infected with mumps virus, herpes simplex virus, influenza A° virus, and measles virus were lysed upon the addition of fresh serum containing both antibody specific for their respective viruses and a functional C source. Only sera containing IgG able to bind to cells expressing the specific viral antigens caused injury. Study of cells infected with mumps,<sup>2</sup> herpes simplex,<sup>2</sup> or measles virus revealed that more than  $1 \times 10^6$  molecules of IgG must bind to infected cells to initiate C-dependent lysis. Absolute specificity was observed in that fresh sera lacking antibody to a given virus were unable to lyse cells infected with that virus, although they acquired this ability after the addition of human IgG containing specific antiviral antibody (Fig. 2, Table II). Further evidence for specificity of this lytic process resulted from sequential studies of the ability of a serum from an individual with no evidence of immune responsiveness to mumps virus to lyse Vero cells expressing mumps viral antigens before and after specific vaccination. Whereas no cytolytic activity was present in sera taken before or 3 days after specific vaccination, cytolysis began 6 days after vaccination and reached its peak by the 10th day after vaccination. The appearance of IgG that bound to infected cells paralleled the acquisition of cytolytic ability.

The involvement of the C system in antibody-initiated lysis of cells infected with measles, mumps, herpes simplex type I, and influenza A° viruses was analyzed in detail. Earlier studies with measles virus (9) had shown that the cytolytic system was markedly sensitive to dilution and required magnesium but not calcium. These findings implied alternative pathway mediation of the lytic event, an impression substantiated by the full cytolytic ability of C2-deficient human serum and an absolute requirement for factor B for lysis. The alternative pathway route for lysis of measles virus-infected cells was confirmed in the present studies, since lysis of HeLa cells infected with measles by fresh human serum containing antibody was sensitive to dilution, independent of calcium, and dependent on factor B. Human serum immunochemically depleted of C4 could still produce lysis, yet serum immunochemically depleted of factor B, but containing antibody, could not lyse infected cells unless reconstituted with physiological concentrations of factor B. In addition, human antibody-initiated lysis of mumps, herpes simplex Type I, and influenza A° virus-infected cells also depended absolutely on the presence of factor B and also on magnesium, but not calcium. Although serum heated at 50°C for 25 min has a C2 concentration which is 30 to 60% of normal, this partial C2 deficiency does not explain the inability of heated serum to lyse virus-infected cells, since isolated C2, free of factor B, failed to reconstitute the lytic activity of such serum, although factor B free of C2 was fully active in this regard. Similarly, serum immunochemically

---

<sup>2</sup> Perrin, L. H. and M. B. A. Oldstone. Unpublished observations.

TABLE IV  
*Site within the IgG Molecule Involved in Initiating C-Dependent Lysis of Cells Expressing Viral Antigens on Their Surfaces*

Reagent	Cells expressing antigens of the following viruses					
	Mumps virus*			Measles virus*		
	Fresh serum	Serum 50°C, 25 min	Serum 50°C, 25 min + factor B‡	Fresh serum	Serum 50°C, 25 min	Serum 50°C, 25 min + factor B
IgG	95	<10	95	99	<10	99
F(ab') <sub>2</sub>	95	<10	95	99	<10	99
Fab'	<10	<10	<10	<10	<10	<10
Immune fresh serum	95	<10	95	99	<10	99

\* Spontaneous lysis of cells infected with each virus was  $5 \pm 5\%$ .

‡ 250  $\mu$ g of factor B per ml of serum.

TABLE V  
*Effect of Cytochalasin B and Capping of Viral Antigens on Antibody-Mediated Dependent Lysis of HeLa Cells Acutely Infected with Measles Virus*

Cytochalasin B	Cells capping	Immune specific lysis	
		Fresh serum	Heated 56°C/30 min
$\mu$ g/ml	%	%	%
30	3	>95	12
20	3	>95	10
10	18	>95	7
5	42	>95	7
0	65	>95	5

depleted of factor B functioned normally at the classical pathway level as shown by its normal  $CH_{50}$  value, although such serum was completely unable to lyse measles virus-infected cells.

Further studies were performed to define which alternative pathway components bound to cells in this system. Fluorescence techniques showed the presence of IgG, properdin, factor B, C3, and C4 on the surfaces of HeLa cells infected with measles virus after incubation with human serum containing antibody to the virus. Factor B binding, although weak, was consistent. None of the components were observed bound to virus-infected cells not incubated with serum or mixed with serum heated to 56°C or originating from a child without measles virus antibodies. IgG, properdin, factor B, and C3 were also bound on use of C4-depleted serum, findings entirely consistent with alternative pathway mediation. The finding of C3b and properdin on the surfaces of infected cells incubated with factor B-depleted serum containing antibody to measles virus may reflect binding of C3b with some classical pathway activation or a direct

attack on C3 by cellular enzymes. Properdin binding is explicable in this context as it binds to C3b (20).

Serum in which C4 was blocked by monospecific Fab' antibody to C4 was fully functional in lysing virus-infected cells, whereas serum depleted of factor B by Fab antibody to factor B was totally unable to lyse virus-infected cells. In our experience the use of Fab' antibody to C4 and Fab antibody to factor B is an effective and specific means of segregating the effects of the classical from the alternative C pathway. Other advantages of the Fab' antibody to C4 and antibody to factor B assay system are that the test is simple, rapid, and avoids inadvertent depletion of other serum components. Furthermore, this test system excludes reactions from C-fixing heteroantibodies, whose diverse influences may explain the discrepancies in reports describing both the presence and absence of a prozonal effect with antibody-initiated C lysis (21, 22).

We observed that  $F(ab')_2$ , but not Fab' fragments, of IgG-initiated C-dependent lysis of mumps virus and measles virus-infected cells. Hence, activation of C took place in the absence of the Fc portion of the molecule. These studies represent the first demonstration of  $F(ab')_2$  activation of alternative C pathway in man and indicate that killing of mumps and measles virus-infected cells could occur without fixation of C1 and other members of the classical C pathway, since the Fc portion of the molecule is required for these activities. Additional evidence for activation of alternative C pathway by  $F(ab')_2$  was provided by experiments in which virus-infected cells coated with immune  $F(ab')_2$  were not lysed by serum devoid of antiviral antibodies and heated at 50°C for 25 min. Complete restoration of immune lysis occurred after addition of the C source with physiological amounts of factor B.

The alternative C pathway has also been reported to be activated in guinea pig serum by aggregated  $\gamma$ -<sup>3</sup> or  $F(ab')_2$  complexed with specific antigen in the absence of the Fc portion of the molecule (23). Similarly, cell bound rabbit IgG can also, at high concentrations, activate the alternative pathway (24). Yet, aggregated human IgG myelomas did not lead to activation of the alternative C pathway in normal (25) and C2-deficient human serum<sup>3</sup>. The discrepancy of the latter findings with ours and with early studies cited above may be explained by hypothesizing that IgG and  $F(ab')_2$  binding to viral antigens on the surfaces of infected cells creates a unique spatial configuration which then can trigger the alternative C pathway or the release of a C1 inhibitor by infected cells.

Even though our results show that antibody initiated lysis of virus-infected cells proceeded via the alternative C pathway and occurred with a C source deficient in C4, C4 was detected on the surfaces of virus-infected cells incubated with immune human serum. This indicates that there is some triggering of the classical pathway which, however, is insufficient to mediate lysis of virus-infected cells. Ohanian and Borsos found large numbers of C4 molecules bound to surfaces of tumor cells incubated with antibody and C in the absence of lysis (26). Their experimental conditions were not appropriate for analysis of alternative C pathway function.

In our studies C4 was not required for lysis of cells expressing herpes simplex

<sup>3</sup> O. Götze. Personal communications.

virus or other viruses on their surfaces. In contrast, Daniels et al. (27) showed that addition of C1 and C4 in excess to herpes simplex virus sensitized with antibody led to enhanced neutralization of the virus. Similarly, we earlier observed activation of the classical C pathway by purified measles virus in the presence of specific antiviral antibody (9). However, measles virus-infected cells were lysed exclusively by the alternative C pathway after incubation with the same antibody source. These observations, coupled with our finding with  $F(ab')_2$ , strongly suggest that the viral antigen configuration on the plasma membrane is responsible for the changes in the bound IgG molecules which permit alternative C pathway activation. It is perhaps relevant that the only other reported example of Ig-initiated activation of the alternative C pathway involves human C and human IgG reacting with cell membrane antigens (28).

For IgG antibody-initiated C-dependent lysis of cells infected with several kinds of viruses, a large number of antibody molecules must bind to each infected cell (9, 10). Theoretically redistribution and concentration of viral antigens (capping) covered with antiviral antibody might facilitate activation of the alternative C pathway. To evaluate this possibility we studied the ability of antibody and C to lyse virus-infected cells exhibiting different degrees of capping. Utilizing cytochalasin and sodium azide as inhibitors of capping, we distinctly segregated lysis from capping, indicating that immune lysis could occur without capping.

Finally, in view of our findings that large numbers of IgG molecules must bind to initiate alternative C pathway activation and immune lysis with three different budding RNA viruses and one DNA virus, probably this pathway is common for other viruses budding from membranes. Although the biological reasons for the requirement of the alternative C pathway are still obscure, nevertheless, our data indicate the value of following the activity of the alternative C pathway during acute viral infections and considering the possible impairment or deficiency in this pathway in tissue sites and in patients having recurrent or persistent virus infections.

### Summary

Antibody-mediated C-dependent lysis of cell lines infected with herpes simplex type 1 virus, influenza A° virus, measles virus, and mumps virus occurred by the alternative C pathway with the participation of IgG antibodies. Lysis occurred only with immune human sera,  $Mg^{++}$  EGTA immune sera, and immune sera depleted of C4 or treated with Fab anti-C4. Lysis did not occur with nonimmune sera,  $Mg^{++}$  EDTA immune sera, and immune sera heated 50°C for 25 min, depleted of factor B or treated with Fab antifactor B. Lysis was restored to heated and factor B immunodepleted immune sera by addition of factor B, but not by addition of an excess of C2. Further studies showed that lysis of HeLa cells infected with measles virus was induced by both immune IgG and  $F(ab')_2$  but not Fab' in the presence of a nonantibody-containing human C source. Lysis of measles virus-infected cells was also independent of movement of viral antigens on the surface of the infected cells, as inhibition of viral antigen capping by cytochalasin B or sodium azide was not associated with abrogation of immune lysis.

The authors thank Professor Peter Lachmann for helpful discussions and Ms. Minick and Ms. Hendrix for assistance in preparation of this manuscript.

Received for publication 20 January 1976.

### References

1. Wiktor, T. J., E. Kuwert, and H. Koprowski. 1968. Immune lysis of rabies virus-infected cells. *J. Immunol.* 101:1271.
2. Dulbecco, R., M. Vogt, and A. G. R. Strickland. 1956. A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus. *Virology.* 2:162.
3. Oldstone, M. B. A. 1975. Virus neutralization and virus induced immune complex disease. *In Progress in Medical Virology.* J. L. Melnick, editor. Karger, Basel. 19:84.
4. Woodruff, J. F., and J. F. Woodruff. 1975. T lymphocytes interaction with viruses and virus infected tissues. *In Progress in Medical Virology.* J. L. Melnick, editor. Karger, Basel, Switzerland. 9:120.
5. Lodmell, D. L., A. Niwa, K. Hayashi, and A. L. Notkins. 1973. Prevention of cell-to-cell spread of herpes simplex virus by leukocytes. *J. Exp. Med.* 137:706.
6. Harfast, B., T. Andersson, and P. Perlmann. 1975. Human lymphocyte cytotoxicity against mumps virus-infected target cells: requirement for non-T cells. *J. Immunol.* 114:1820.
7. Oldstone, M. B. A., N. R. Cooper, and D. L. Larson. 1974. Formation and biologic role of polyoma virus-antibody complexes: a critical role for complement. *J. Exp. Med.* 140:549.
8. Oldstone, M. B. A., and F. J. Dixon. 1971. Acute viral infection: tissue injury mediated by anti-viral antibody through a complement effector system. *J. Immunol.* 107:1274.
9. Joseph, B. S., N. R. Cooper, and M. B. A. Oldstone. 1975. Immunologic injury of cultured cells infected with measles virus. I. Role of IgG antibody and the alternative complement pathway. *J. Exp. Med.* 141:761.
10. Joseph, B. J., L. H. Perrin, and M. B. A. Oldstone. 1976. Quantitation of virus antigens by radioimmune assay on the surfaces of HeLa cells persistently infected with wild type and vaccine strain of measles virus. *J. Gen. Virol.* In press.
11. Spiegelberg, H. L., and W. O. Weigle. 1965. The catabolism of homologous and heterologous 7S gamma globulin fragments. *J. Exp. Med.* 121:323.
12. Götze, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternate pathway of complement activation. *J. Exp. Med.* 134:90s.
13. Cooper, N. R., M. J. Polley, and H. J. Müller-Eberhard. 1970. The second component of human complement (C2): quantitative molecular analysis of its reactions in immune hemolysis. *Immunochemistry.* 7:341.
14. Cuatrecasas, P. 1971. Affinity chromatography. *Annu. Rev. Biochem.* 40:259.
15. Martin, A., P. J. Lachmann, L. Halbwegs, and M. J. Hobart. 1975. Haemolytic diffusion plate assays for factor B and D of the alternative pathway of complement activation. *Immunochemistry.* In press.
16. Avrameas, S., and T. Ternynk. 1967. Biologically active water insoluble protein polymers. I. Their use for isolation of antigens and antibodies. *J. Biol. Chem.* 242:1651.
17. Lachmann, P. J. 1971. The purification of specific antibody as F(ab')<sub>2</sub> by the pepsin digestion of antigen-antibody precipitates, and its application to immunoglobulin and complement antigens. *Immunochemistry.* 8:81.
18. Goldman, M. 1968. *Fluorescent Antibody Methods.* Academic Press, Inc., New York.

19. Westmoreland, D., and J. F. Watkins. 1974. The IgG receptor induced by herpes simplex virus: studies using radioiodinated IgG. *J. Gen. Virol.* 24:167.
20. Schreiber, R. D., R. G. Medicus, O. Götze, and H. J. Müller-Eberhard. 1975. Properdin- and nephritic factor-dependent C3 convertases: requirement of native C3 for enzyme formation and the function of bound C3b as properdin receptor. *J. Exp. Med.* 142:760.
21. Minagawa, T., and M. Yamada. 1971. Studies on the persistent infection with measles virus in HeLa cells. III. Immunolysis of cells in carrier state by antimeasles sera. *Jap. J. Microbiol.* 15:341.
22. Ehrnst, A. C. 1975. Characterization of measles virus-specific cytotoxic antibodies by use of a chronically infected cell line. *J. Immunol.* 114:1077.
23. Sandberg, A. L., B. Oliveira, and A. G. Osler. 1971. Two complement interaction sites in guinea pig immunoglobulins. *J. Immunol.* 106:282.
24. Reid, K. B. M. 1971. Complement fixation by the F(ab')<sub>2</sub> fragment of pepsin-treated rabbit antibody. *Immunology.* 20:649.
25. Spiegelberg, H. L., and O. Götze. 1972. Conversion of C3 proactivator and activation of the alternate pathway of complement activation by different classes and subclasses of human immunoglobulins. *Fed. Proc.* 31:655.
26. Ohanian, S. H., and T. Borsos. 1975. Lysis of tumor cells by antibody and complement. II. Lack of correlation between amount of C4 and C3 fixed and cell lysis. *J. Immunol.* 114:1292.
27. Daniels, C. A., T. Borsos, H. J. Rapp, R. Snyderman, and A. L. Notkins. 1970. Neutralization of sensitized virus by purified components of complement. *Proc. Natl. Acad. Sci. U.S.A.* 65:528.
28. Ferrone, S., N. R. Cooper, M. A. Pellegrino, and R. A. Reisfeld. 1973. Activation of human complement by human lymphoid cells sensitized with histocompatibility alloantisera. *Proc. Natl. Acad. Sci. U.S.A.* 70:3665.