Brief Definitive Report

HEMAGGLUTININ-SPECIFIC COMPLEMENT-DEPENDENT CYTOLYTIC ANTIBODY RESPONSE TO INFLUENZA INFECTION

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The host defense response to influenza infection is complex. Specific humoral antibodies develop to the strain-specific surface antigens, the hemagglutinin and the neuraminidase, and to the internal antigens (matrix and nucleoprotein) which are common to all influenza A viruses (1). Antibodies to the hemagglutinin, which is the major surface antigen, neutralize viral infectivity (2). In addition to antibodies which have been detected against virion antigens, a cytotoxic T-cell response with specificity against the viral hemagglutinin on influenza-infected target cells (3–5) has been recently described. A more cross-reactive cytotoxic T-cell response has also been observed when a nonpermissively infected target cell is used in cytotoxicity assays (6, 7). The present report describes the development during influenza infection and after vaccination of a cytolytic humoral antibody response which is directed against the hemagglutinin on infected target cells. This antibody-mediated lysis of infected cells is complement dependent, as has been reported with other virus infections (8–11).

Materials and Methods

Viruses. The A Equine-1 Detroit/3/65 virus (Heq1Neq1), and the H3Neq1 and Heq1N2 recombinants of this virus with A/Port Chalmers (H3N2) were provided by Dr. Brian Murphy of the National Institutes of Health, Bethesda, Md. These viruses had not been adapted by mouse passage. Recombinant MRC-9 (H3N2) derived from A/Port Chalmers/1/73 and A/PR/8/34 (H0N1) was provided by Dr. Geoffrey Schild of the National Institute for Biological Standards and Control, Holly Hill, England, and had been adapted for growth in mice by repeated intranasal passage of infected lung tissue. Other influenza A and B strains were obtained from the Bureau of Biologics and have been described earlier (3, 4). The same lots of virus were used to infect mice and target cells.

Animals. 3-wk-old BALB/c mice were obtained from the National Institutes of Health Small Animal Section, Bethesda, Md.

Immunization of Mice. Mice were immunized by either: (*a*) intranasal infection with $10^{3.5}$ egg-infectious doses (EID_{50}) of the A/PR/8/34 (H0N1), A/Scotland/840/74 (H3N2) or A/England/42/72 (H3N2) strains of influenza virus; or (*b*) intraperitoneal inoculation of formalin-inactivated whole virus containing 70 chick-cell agglutinating units/dose and two booster doses of live virus (10^6 EID_{50}) at 3-wk intervals. These were first administered intranasally, and then intravenously for the A/Port Chalmers/1/73 (H3N2), A/Victoria/3/75 (H3N2) and the B/Hong Kong/5/72 virus strains.

Complement and Sera. Mice were bled from the heart, the blood was quickly clotted, and the sera were pooled and immediately frozen in aliquots at −70°C. Sera from nonimmune BALB/c mice served as the source of complement (C′) in these studies because guinea pig and rabbit C′

were tested and showed high levels of nonspecific lysis. Test sera from infected or immunized animals were prepared in a similar manner. Heat inactivation for 30 min at 56°C removed all C′ activity.

Antibody Assays. The titer of hemagglutination-inhibition (HI) antibodies was determined by standard microtiter methods after removal of nonspecific inhibitors (12). Neutralizing antibodies were determined by a plaque assay (13) in canine kidney cells (Flow Laboratories, Inc., Rockville, Md.) after preincubation of serially diluted serum samples with 50 plaque-forming units of virus, and 50% plaque-neutralizing antibody titers were then calculated. Neuraminidase inhibiting antibodies were detected using standard methods (14).

Cytolytic Antibody Assay. A syngeneic target cell system developed for assaying T-cell cytotoxicity was utilized for assaying C′-dependent, antibody-mediated lysis of influenza-infected cells (3). Monolayers of second-passage BALB/c mouse kidney cells were infected with a multiplicity of infection which resulted in ≅90% of the cells showing membrane fluorescence after 18 h of incubation. This was determined by indirect immunofluorescent staining with a specific mouse anti-influenza antisera and fluorescein-conjugated goat anti-mouse immunoglobulin. Infected and control target cells were trypsinized, labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.), washed, and 5×10^4 cells in 0.1 ml of Eagle's minimal essential medium containing 10% fetal calf serum was put into 16 x 125-mm tissue culture tubes (model 3033; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Test sera (0.2 ml) and C′ (0.1 ml of a 1:4 dilution) were then added and duplicate samples were incubated for 2 h at 36°C. Additional tubes contained target cells plus 0.3 ml of a 10% solution of Brij detergent (Sigma Chemical Co., St. Louis, Mo.) to determine the maximum released. Radioactivity in supernates and cell sediments was determined separately in a Searle gamma counter (Searle Radiographics, Inc., Des Plaines, Ill.) and the percent of ^{51}Cr released was calculated. The percent of specific immune lysis (SIL) was derived from the following formula:

$$\% \text{ SIL} = \frac{(\% \text{ lysis by Ab + C}') - \% \text{ lysis by C}' \text{ only}}{\% \text{ maximum lysis} - \% \text{ lysis by C}' \text{ only}}.$$

Background ^{51}Cr released by infected and noninfected cells with medium with C′ was usually 15–30%.

Results

Initially, the ability to detect cytotoxic antibodies was evaluated using several pools of sera which had relatively high titers of HI (1:128–1:512) and virus-neutralizing (1:256–1:1,028) antibodies obtained from mice immunized and boostered with A/Port Chalmers virus. These hyperimmune nonheat-inactivated sera lysed infected, but not control target cells. Heated immune sera or nonimmune, nonheated sera did not lyse infected cells. The addition of fresh nonheated, nonimmune mouse serum to the heat-inactivated immune mouse serum restored the lytic capability of the immune sera. A 1:4 dilution of nonimmune mouse sera was subsequently used as the source of C′ in further testing of these immune sera. The 50% cytolytic antibody titer of four serum pools of similarly prepared hyperimmune sera varied less than twofold in repeated assays. This reproducibility was in the same range as that obtained with neutralization or HI assays performed on the same sera. The 50% cytolytic antibody titers were generally lower than neutralizing antibodies ($P < 0.01$) and the HI titers ($P < 0.05$) of these sera.

After the reproducibility of the assay had been established, an experiment was performed to study the development of cytolytic antibodies during primary infection with a mouse-adapted (MRC-9) A/Port Chalmers virus. Fig. 1 illustrates the rapid replication of virus in the lungs of infected mice. By day 4, pulmonary virus titers reached 10^6 EID_{50}, fell to 10^5 EID_{50} by day 6, and no

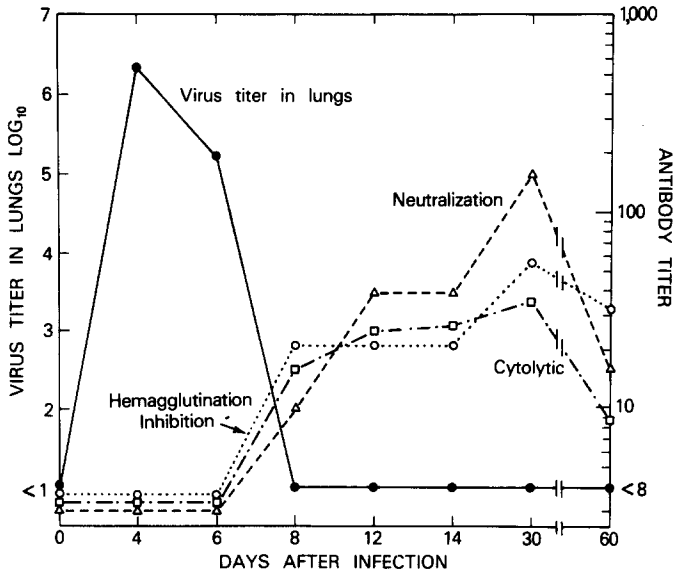

FIG. 1. Antibody production after intranasal infection with 5 mean lethal doses of A/Port Chalmers virus in 3-wk-old BALB/c mice. The reciprocal HI (○), cytolytic (□), and neutralizing (△) antibody titers represent the means of four individual assays performed on serum pools obtained from 10 mice sacrificed on the day indicated after infection. The pulmonary virus titers (●) are indicated as EID_{50}/0.1 ml.

infectious virus could be detected on day 8. No antibodies were detectable until day 8 by virus neutralization, hemagglutination-inhibition, or cytolytic assays. All three antibody titers then began to rise in parallel, reaching peak titers around day 30, and they were lower when tested on day 60.

Mice were also immunized intraperitoneally with formalin-inactivated intact A/Port Chalmers vaccine (MRC-11; Merrell-National Laboratories, Cincinnati, Ohio) and their antibody responses were measured by the same techniques. The cytolytic antibodies were detected as early as day 8 and rose in parallel with the viral-neutralizing and HI antibodies through day 30. Ratios of cytolytic to neutralizing antibody titers appeared to be similar to those obtained in naturally infected mice.

The specificity of the cytolytic C′-dependent antibody response was then evaluated by testing sera of mice after immunization. The upper portion of Table I shows the specific antibody responses to two influenza A viruses of different subtypes (H0N1 and H3N2) and to influenza B virus. The results indicated that the cytolytic antibody response was specific for the virus subtype in a way that was comparable to the HI response in these same mice. Cross-reactivity was observed (lower part of Table I) when mice were infected with one of four different members of the H3N2 subtype isolated between 1972 and 1975. These viruses share the same major hemagglutinin and neuraminidase (H3N2) on their surfaces, but there has been some slight antigenic drift of the hemagglutinin antigen of these strains. The data in the lower portion of Table I show that cross-reactivity was generally observed with immune mouse sera to these closely related viruses by both the cytolytic and the hemagglutination-

TABLE I
Specificity of Cytolytic C'-Dependent Antibodies

Experiment	Immunizing virus	Antibody titer on target cells infected with:					
		B/Hong Kong	A/PR/8	A/Port Chalmers	A/England	A/Scotland	A/Victoria
1	B/Hong Kong/5/72	13 (32)*	<8 (<8)	<8 (<8)			
	A/PR/8/34 (HON1)	<8 (<8)	31 (64)	<8 (<8)			
	A/Port Chalmers/5/73 (H3N2)	<8 (<8)	<8 (<8)	27 (256)			
2	A/Port Chalmers/1/73 (H3N2)			43 (512)	32 (512)	30 (128)	33 (256)
	A/England/42/72 (H3N2)			12 (16)	25 (128)	18 (16)	9 (16)
	A/Scotland/840/74 (H3N2)			17 (16)	23 (64)	24 (64)	23 (32)
	A/Victoria/3/75 (H3N2)			26 (256)	12 (512)	18 (256)	26 (512)

* Reciprocal of cytolytic antibody titer (reciprocal of HI antibody titer).

TABLE II
Hemagglutinin Specificity of Antiserum to A/Port Chalmers (H3N2) Virus

Virus used to infect target cells	Antibody titer	
	Cytolytic*	Hemmagglutination inhibition‡
A/Port Chalmers/1/73 (H3N2)	80.4	512
A/Victoria/3/75 (H3N2)	42.8	128
A/Japan/170/62 (H2N2)	<8	<8
A/PR/8/34 (HON1)	<8	<8
A/Equine-1 Detroit/3/65 (Heq1Neq1)	<8	<8
Recombinant (Heq1N2)	<8	<8
Recombinant (H3Neq1)	23.1	256

* Expressed as the reciprocal of the 50% cytolytic antibody titer.
‡ Reciprocal of the serum HI antibody titer.

inhibition techniques. Thus, the specificity of the cytolytic C'-dependent antibody appears to be similar to that of the HI antibody.

Further evaluation of the specificity of the cytolytic response was performed by assaying an immune A/Port Chalmers (MRC-9) serum against several influenza A virus strains with different hemagglutinin and neuraminidase surface antigens. This serum contained antibodies to both the hemagglutinin (1:512) and the neuraminidase (1:128) of A/Port Chalmers virus as determined by standard HI and neuraminidase-inhibiting assays. Table II shows that the antiserum lysed only cells infected with strains containing the same hemagglutinin (H3) regardless of the neuraminidase content. In contrast, this serum did not lyse cells infected with strains containing the same neuraminidase (N2), but those having a different hemagglutinin. Furthermore, this serum did not lyse cells infected with the A/PR/8/34 (H0N1) used to prepare this recombinant virus, demonstrating that the cytolytic specificity is not determined by common internal antigens shared by type A influenza viruses such as nucleocapsid or matrix proteins.

Discussion

The results reported in this paper indicate that there is a readily detectable cytolytic antibody response to influenza infection. These antibodies are induced by either infection with live virus or by immunization with inactivated vaccine.

Cytolytic antibodies can be detected soon after infection or immunization, and they tend to parallel the production of antibodies detected by neutralization or hemagglutination techniques.

The specificity of cytolytic antibodies appears to be primarily directed against the hemagglutinin of the virus. There was no cross-reactive lysis by immune sera of target cells infected with influenza viruses of another type or subtype; there was, however, cross-reactivity within the H3N2 subtype using viruses with minor differences in hemagglutinin antigens. In experiments with recombinant viruses, it is clear that the hemagglutinin induces a cytolytic antibody response which could be detected on target cells infected with a virus containing the same hemagglutinin, regardless of the neuraminidase antigen present. These results indicate that there is a hemagglutinin-specific cytolytic antibody response to influenza infection. These observations parallel our recent observations on the specificity of the cytotoxic T-lymphocyte response specific to the viral hemagglutinin after influenza infection and vaccination (3–5).

Although the present studies do not address the in vivo roles of these antibodies, their results are consistent with the long-recognized importance of hemagglutinin specificity in understanding the epidemiology of influenza. The present data support the importance of the hemagglutinin antigen by demonstrating a specific antibody response which recognizes viral hemagglutinin on infected cells and binds C′. Antibody with C′ may speed lysis of infected cells or act indirectly by releasing chemotactic factors which attract phagocytic cells during acute infection (15). These immunological reactions may be immunopathological (8) as well as beneficial to the host. The present assay, as well as the recently described assay for the specific cytotoxic cell response, may aid future investigations of the host response and mechanisms of recovery from influenza.

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