

**HEMAGGLUTININ-SPECIFIC CYTOTOXIC T-CELL
RESPONSE DURING INFLUENZA INFECTION**

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Studies on the specificity of the cytotoxic T-lymphocyte response of mice to influenza virus infection are highly relevant to an understanding of the role of effector T cells in influenza immunity. Epidemiological observations have repeatedly indicated that epidemic illness occurs after a significant antigenic change in the hemagglutinin of influenza A virus (1). These observations are consistent with the development in both humans and mice of hemagglutinin-specific neutralizing (2) and cytolytic (3) antibodies in response to influenza infection. Similarly if T-cell-mediated cell lysis plays a major direct role in vivo immunity then cytotoxic T cells would be expected to exhibit hemagglutinin specificity. Recent reports (4, 5) however have revealed a high degree of cross-reactive lysis of target cells infected with various influenza A viruses. The pattern of cross-reactivity was complex and not accountable by known serological relationships between the hemagglutinins of influenza A viruses. In contrast to these studies we have readily detected hemagglutinin-specific cytotoxic T cells in influenza-infected mice. Indeed cytotoxic T cells were capable of discriminating minor antigenic variations among influenza viruses of the same subtype specificity (6). Because of the apparent conflict of these data with the other recently published data we undertook additional experiments on the specificity of cytotoxic T lymphocytes in influenza-infected mice.

Materials and Methods

Mice. BALB/c mice and C3H mice were obtained from the Animal Production Unit of the National Institutes of Health, Bethesda, Md.

Viruses. The A/Georgia/74 isolate of A/Port Chalmers virus (H3N2), the A Equine-1 Detroit/3/65 virus (Hq1Neq1), and the recombinants of these viruses (H3Neq1 and Hq1N2) were provided by Dr. Brian Murphy of the National Institutes of Health. These viruses had not been adapted by mouse passage. A/Port Chalmers/1/73 (H3N2) designated MRC-9 after recombination with A/PR/8/34 by Dr. Geoffrey Schild of the National Institute for Biological Standards and Control, Holly Hill, England, and A/PR/8/34 (HON1) had been adapted for growth in mice by repeated intranasal passage of infected lung tissue extracts. Virus stocks for use in immunization were prepared by inoculating fertile hen's eggs. The allantoic fluid harvests of these eggs were used as the source of virus in these experiments. The identity of the virus pools was confirmed serologically using monospecific chicken anti-influenza typing antisera.

Immunization. 3-wk-old BALB/c and C3H mice were inoculated intranasally with various doses of the above virus strains. Control mice received diluent intranasally. Infection was verified by observing pulmonary pathology, by the development of specific anti-influenza antibodies, or by assay of pulmonary virus titers. Lungs were homogenized and then diluted in phosphate-buffered saline containing 0.1% bovine albumin. Three eggs were inoculated intra-allantoically with each

10-fold dilution. After 48 h incubation at 35°C, the allantoic fluids were tested for the presence of viral hemagglutinin with a 0.25% suspension of chick erythrocytes in phosphate-buffered saline.

Preparation of Spleen Cells. Spleens were removed from infected and control mice at various times after viral inoculation. Erythrocytes were lysed by exposure of the spleen cell suspensions to ammonium chloride buffer (7). After centrifugation the lymphocytes were suspended in minimum essential medium supplemented with 10% fetal calf serum at a concentration of 10^7 /ml.

Preparation of Cervical Lymphocytes. Lymph nodes were removed from the anterior cervical region of control and infected BALB/c mice and were finely teased apart on a Petri dish using a surgical scalpel. After gentle pipetting in minimum essential medium supplemented with 10% fetal calf serum the lymphocytes were washed twice, counted, and resuspended at a concentration of 10^7 /ml.

Preparation of Target Cells. Kidneys were removed from weanling BALB/c and C3H mice and cultures were established. L929 cells (L cells) were obtained from the Bureau of Biologics Cell Culture Section. Confluent monolayers of L cells and of second passage kidney derived cells in 35-mm plastic Petri dishes were inoculated with approximately $10^{6.5}$ EID₅₀ (dose of virus capable of infecting 50% of inoculated eggs) of virus. The dishes were incubated at 36°C for 1 h, washed, and incubated in fresh medium for an additional 18 h. At this time greater than 90% of the kidney-derived cells showed membrane fluorescence when reacted with specific mouse anti-influenza antisera followed by fluorescein-conjugated goat anti-mouse gamma globulin antiserum. The cells were removed from the dishes by trypsinization and, after washing, were incubated with ^{51}Cr (supplied as $\text{Na}_2^{51}\text{CrO}_4$; New England Nuclear, Boston, Mass.) at a concentration of 100 $\mu\text{Ci}/\text{ml}$ for 30 min at 36°C. The cells were washed twice in medium and resuspended to a concentration of 10^5 /ml.

Lymphocyte Cytotoxicity Assay. ^{51}Cr -labeled target cells (10^4 in a 0.1 ml volume of minimal essential medium containing 10% fetal calf serum) were added to the wells of a microtiter plate (Microtest II; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Replicates of eight wells received 10^6 viable spleen or cervical lymph node cells from either infected or uninfected mice, in a vol of 0.1 ml. Additional wells contained target cells plus 0.1 ml of a 10% solution of Brij detergent (Sigma Chemical Co., St. Louis, Mo.) to determine the maximum releasable ^{51}Cr . The microtiter plates were incubated at 36°C for 18 h. The cells were centrifuged at 1,500 rpm for 10 min. Aliquots of 0.1 ml were removed from each well and the amount of radioactivity present was determined. The mean value of the counts released by the spleen cells from infected mice (immune) was compared with both the mean counts released by spleen cells from uninfected mice (control) and the mean counts released by exposure to Brij detergent (max). The percent immune lysis was calculated from the formula:

$$\% \text{ lysis} = \frac{\text{immune} - \text{control}}{\text{max} - \text{control}} \times 100.$$

In each experiment the immune and control values were compared using Student's *t* test.

Results

An experiment was performed to evaluate the induction of a cytotoxic T-cell response during A/Port Chalmers influenza infection in BALB/c mice. Fig. 1 illustrates the cytotoxic T-cell responses detected in the cervical lymph nodes, and the spleens of infected mice. Significant cytotoxicity was detected 4 days after infection. The response persisted in the spleen for a longer period of time than in the cervical lymph nodes. The pulmonary virus titers peaked by day 2 after infection and fell to undetectable levels by day 8. The cytotoxic lymphocytes in these experiments have been demonstrated to be T cells by their nonadherence to nylon wool columns and the loss of cytotoxicity by treatment with anti- θ -serum plus complement (6).

Experiments were performed to determine whether cytotoxic lymphocytes were recognizing the hemagglutinin, the neuraminidase, or internal compo-

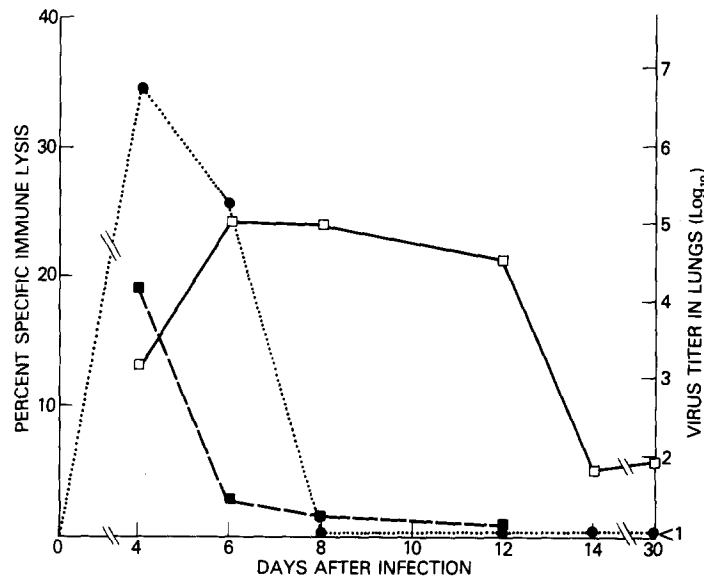


FIG. 1. Cytotoxic T-cell response in BALB/c mice after infection with A/Port Chalmers virus. Percent specific immune lysis is indicated by: (■—■) by cervical lymph node cells and (□—□) by spleen cells. Pulmonary virus titers (EID_{50} \log_{10}) are indicated by (●····●). No specific lysis occurred with spleen or lymph node cells from influenza-infected mice were tested on uninfected target cells.

nents common to influenza A viruses. Spleen cells from BALB/c mice infected with one of two parental strains of virus expressing either the H3N2 or the Heq1Neq1 surface antigens, or with one of two recombinant strains (Heq1N2 and H3Neq1) were tested 9 days later for cytotoxicity against BALB/c target cells infected with the same viruses. Table I indicates that significant cytotoxicity occurred only when the hemagglutinin of the immunizing virus was the same as that of the virus used to infect the target cells. Significant cytotoxicity was not observed when the immunizing virus had the same neuraminidase as the target cell but a different hemagglutinin.

The recently reported experiments (4, 5) which failed to demonstrate strong hemagglutinin specificity of cytotoxic cells in influenza-infected mice used the C3H mouse-derived transformed L929 cell line rather than early passage normal tissue-derived cells. Because of the requirement of *H-2* compatibility between lymphocyte and target cells for lysis to occur we infected C3H mice and tested their lymphocytes on virus-infected syngeneic kidney-derived cells and on virus infected L cells. The viruses used were A/Port Chalmers (H3N2) and A/PR/8 (HON1).

Table II illustrates that virus-specific cytotoxicity was detected on kidney-derived target cells. In contrast considerable cross-reactivity was noted when spleen cells from A/Port Chalmers-infected mice were tested on virus-infected L929 cells. Moreover in this experiment even though anti-A/PR/8 cytotoxic cells were generated in the immunized mice no significant cytotoxicity was demonstrated when infected L cells were used as target cells.

TABLE I
Percent Specific Immune Lysis on Target Cells Infected With
Recombinant Strains of Influenza Virus

Antigens of immunizing virus	Virus used to infect target cells			
	H3N2	Heq1Neq1	H3Neq1	Heq1N2
H3N2	37.8‡	-4.9	17.0‡	-0.2
Heq1Neq1	2.7	22.8‡	-1.4	17.8*
H3Neq1	43.0‡	-1.9	19.3‡	2.0
Heq1N2	-0.2	8.2‡	1.3	31.0‡

The degree of lysis achieved against target cells infected with the same virus or a virus sharing the same hemagglutinin was significant as indicated (* = $P < 0.01$, † = $P < 0.001$). Spleen cells were tested 10 days after infection. Significant lysis was not observed when the target cell was infected with a virus which had the same neuraminidase, but not the same hemagglutinin as the virus used to immunize mice.

TABLE II
Percent Specific Immune Lysis by Spleen Cells from C3H Mice on
Infected Target Cells

Virus used to immunize	C3H kidney cells		L929 cells	
	A/Port Chalmers	A/PR/8/34	A/Port Chalmers	A/PR/8/34
A/Port Chalmers (H3N2)	103.0*	10.7	41.2*	24.2*
A/PR/8/34 (HON1)	13.9	39.5*	8.3	4.9

The degree of lysis achieved on target cells was significant as indicated (* = $P < 0.001$). The absence of (*) indicates significant lysis was not detected. The spleen cells were tested 9 days after intranasal infection.

Discussion

These experiments illustrate the importance of the target cell in determining the specificity of the cytotoxic lymphocyte response of influenza-infected mice. The experiment using recombinant strains clearly indicates a major response to the hemagglutinin antigen. On the other hand the use of L cells as target cells can clearly lead to a false negative result and conversely can indicate a broadly cross-reactive response. L929 cells are transformed and are known to be relatively nonpermissive for influenza virus and this may lead to the expression of greater amounts of internal viral components which are known to express common antigens (8). Since influenza infection in vivo involves a productive lytic infection of epithelial cells it is probable that the use of L cells as target cells may not accurately reflect the in vivo specificity of the major cytotoxic T-cell response. The studies with L929 cells do however indicate that a more cross-reactive response occurs. Similarly, influenza-infected mice are known to produce some antibody directed against determinants common to influenza A viruses. The important point however is that immune responses to these common antigens play a relatively minor role in protective immunity (1). We would therefore suggest that the published data are consistent with the interpretation that there is a specific cytotoxic response to the major surface antigen during influenza infection, which can be readily detected on a *H-2* compatible productively infected target cell. In addition, there is a less specific cytotoxic T-cell response detectable on infected L929 cells.

There is additional evidence for the presence of a cross-reactive population of

T cells in influenza-infected mice. Butchko et al. (9) have reported that the stimulation of protein synthesis by T lymphocytes from influenza-immunized mice shows significant cross-reactivity among influenza A viruses. This stimulation could be detected months after infection, and may well reflect a distinct population of T lymphocytes differing from cytotoxic T cells. At present there is no evidence to suggest that these T cells can be considered as effector T cells in resistance to influenza. The question of whether cytotoxic T cells are protective deserves serious study and as indicated in this paper, their role in protection cannot be dismissed because of the use of a target cell inappropriate to demonstrate specific cytotoxicity.

Summary

Specific cytotoxic thymus-derived (T) lymphocytes were detected in the cervical lymph nodes and spleen during influenza infection of mice. The cytotoxic T cells can distinguish target cells infected with different influenza A subtypes. Infection with parent viruses and their recombinant progeny possessing the hemagglutinin of one parent and the neuraminidase of the other demonstrated that significant cytotoxicity occurred only when the hemagglutinin of the immunizing viruses was the same as that of the virus used to infect the target cell. In addition to this specific cytotoxic response to the major surface antigen, a cross-reactive response could be detected when the relatively nonpermissive L cell was used as the target cell. These results indicate there is a specific cytotoxic T-cell response to the surface hemagglutinin, and a cross-reactive cytotoxic response, not directed to the hemagglutinin, during influenza infection. The cytotoxic T-cell response specific for the hemagglutinin antigen may play an important role in in vivo immunity to influenza.

Received for publication 23 May 1977.

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