Cytotoxic Lymphocytes and Antibody-Dependent Complement-Mediated Cytotoxicity Induced by Administration of Influenza Vaccine

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Recently defined aspects of cellular and humoral antiviral immunity were evaluated in 10 young adults given influenza vaccines containing A/USSR/77 (H1N1) antigens. Cytotoxic lymphocytes were measured by using cryopreserved lymphocytes as effector cells and syngeneic, virus-infected lymphocytes as target cells. An assay previously developed in this laboratory was adapted for the measurement of antibody-dependent, complement-mediated cytotoxicity. Antiviral cytotoxic lymphocyte responses were detected in 5 of 10 volunteers between 3 and 10 days after the initial vaccination. These responses were found both in individuals who were previously primed and in individuals who were not primed to influenza A/USSR/77 antigens. The complement-mediated lysis assay was found to be more sensitive than the hemagglutination inhibition test and probably detected antibodies to both subtype-specific and cross-reactive antigenic determinants. These responses to influenza antigens are similar to those obtained in studies of murine influenza which indicate that cytotoxic lymphocytes and antibody-dependent, complement-mediated cytotoxicity have a role in the early response to acute infection.

Immune lysis of infected cells is probably an important host defense mechanism against many viral infections. Since viral antigens are often expressed on the surface of infected cells, immune recognition and cell lysis may occur early in the infectious cycle, before virus assembly has occurred (25). Several mechanisms exist which may produce immune lysis, including antibody-dependent, complement-mediated cytotoxicity, antibody-dependent, cell-mediated cytotoxicity, and cytotoxic T lymphocytes (CTL).

Studies of influenza vaccine-related immunity are important because of the large number of people vaccinated annually and because responses to vaccination are likely to be similar in some respects to those induced by natural infection. Some cytotoxic immune responses have been examined after infection and vaccination of mice. The development of antibody which can sensitize cells to complement-mediated lysis was recently described by work performed in this laboratory (28). CTL have also been studied

extensively (4-7), and we have recently reported evidence that they have an important role in murine influenza pneumonia (8). Immune responses in humans to influenza infection and vaccination have been less well characterized. Assays of humoral immunity have not yet included complement-mediated cytotoxicity, and studies of cell-mediated immunity have been mostly restricted to lymphocyte transformation assays (3). Studies of antibody-dependent lymphocyte cytotoxicity for xenogeneic target cells have been reported by Greenberg and co-workers (11).

In 1977 China, Russia, and Europe experienced widespread outbreaks of infection by influenza A/USSR/77, an H1N1 virus (12). Limited outbreaks followed in the United States in early 1978 (12). In anticipation of additional disease in the 1978-79 influenza season, vaccines were developed containing antigens of this virus. Since H1N1 strains of influenza have not been prevalent since 1953, most individuals 25 years of age or less lacked antibody to these antigens (12). We therefore chose to study young adults, some of whom would not be immunologically primed to H1N1 antigens, and to examine the development of complement- and lymphocyte-

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mediated cytotoxicity in these individuals after the administration of influenza (H1N1) vaccines.

MATERIALS AND METHODS

Volunteers and vaccines. Ten students, male and female, aged 22 to 33 were studied. Two of these had previously received influenza A/New Jersey/76 vaccine; all were selected according to the criteria used for the 1978 national influenza vaccine clinical trials. Experimental lots of monovalent A/USSR/77 (approximately 60 μ g of hemagglutinin) and trivalent A/USSR/77, A/Texas/77, and B/Hong Kong (approximately 20 μ g of hemagglutinin each) whole-virus vaccines (Connaught Laboratories, Swiftwater, Pa.) were given intramuscularly, 4 weeks apart. There were no significant side effects produced by the vaccines.

Specimen collection. Blood specimens were collected on the day of the first vaccination and at intervals thereafter. Serum removed from coagulated blood was stored at -20°C . Lymphocytes were separated from specimens treated with sterile, preservative-free sodium heparin (Hynson, Wiscoll & Dunning, Baltimore, Md.) by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) gradient centrifugation. Specimens treated with acid citrate dextrose solution A (Media Production Unit, National Institutes of Health, Bethesda, Md.) were defibrinated by using 3-mm glass beads (VWR Scientific, Baltimore, Md.), calcium chloride (Fisher Scientific Co., Fairlawn, N.J.), and standard thrombin (Bureau of Biologics, Bethesda, Md.), and the buffy coat was separated by the use of Plasmagel (HTI Corp., Buffalo, N.Y.). Non-adherent lymphocytes were then obtained by passage of the buffy-coat suspension through a nylon wool column (16) (Fenwall, Deerfield, Ill.). Erythrocytes were lysed by treatment with tris(hydroxymethyl)aminomethane-ammonium chloride buffer (22).

Cryopreservation of lymphocytes. Ficoll-Hypaque-purified (FH) or nylon wool-purified (NW) lymphocytes were suspended at a concentration of 5×10^6 to 10×10^6 cells per ml in RPMI 1640 media containing penicillin, streptomycin, and 10 mM HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Suspensions were chilled in an ice bath; then, working in a 4°C cold room, an equal volume of 15% dimethyl sulfoxide in the same medium was added slowly with gentle mixing. One-milliliter portions were immediately placed in 2-ml polyethylene freezer vials and transferred to a precooled, programmed biological freezer. The temperature was lowered at $1^{\circ}\text{C}/\text{min}$ to -35°C , after which the vials were transferred to the vapor phase of a liquid nitrogen tank for storage. At the time of assay, cells were thawed quickly by gentle agitation in a 37°C water bath and immediately diluted in an equal volume of medium containing 20% human AB serum (Flow Laboratories, McLean, Va.). After equilibration for 5 min, the cells were slowly diluted with an additional 10 ml of medium, centrifuged at 1,000 rpm for 10 min, and resuspended in medium for use. Lymphocyte suspensions prepared in this fashion were 80 to 95% viable, as demonstrated by trypan blue exclusion. Lymphocyte suspensions which had been passed through nylon wool columns contained 0 to 2% neutrophils and 0.2% macrophages.

Characterization of lymphocyte suspensions. E-rosetting cells were quantitated by using 2-aminoethyl isothiourea uronium bromide hydrobromide (Sigma Chemical Co., St. Louis, Mo.) according to the method described by Kaplan and Clark (17). Cells bearing Fc receptors were separated from lymphocyte suspensions by adherence to EA monolayers composed of type O, Rh⁺ erythrocytes and human anti-D serum (Ortho Diagnostics, Raritan, N.J.) according to the method of Van Oers et al. (27).

Viruses. Virus pools were prepared as mouse-adapted influenza A/Port Chalmers/73 (H3N2) (33) or in embryonated hen eggs from stocks of the prototype influenza viruses A/USSR/90/77 (H1N1), A/Japan/305/57 (H2N2), and B/Hong Kong/73 or from the high-yielding recombinant of an A/Texas/77-like H3N2 virus designated X49 (supplied by E. D. Kilbourne). Equal portions of virus pools in allantoic fluid were stored at -70°C .

Antibody determinations. Hemagglutination-inhibiting (HI) antibody was measured as described by Tauraso et al. (26), using the virus pools described above. Sera were pretreated with receptor-destroying enzyme (Microbiological Associates, Bethesda, Md.). Neutralizing antibody was assayed by the method of Appleyard and Maber (1). Briefly, 100 50% tissue culture infectious doses of serum-treated virus were inoculated into tube cultures of rhesus monkey kidney cells; after 4 days the monolayers were tested for hemadsorption of 0.1% washed guinea pig erythrocytes.

Antibody was also assayed by a modification of the method of complement-mediated lysis previously described by this laboratory (28). Target cells were prepared from primary BALB/c mouse embryo cells infected with influenza A/USSR/77 virus or A/Port Chalmers/73 at a multiplicity sufficient to produce infection of $\geq 90\%$ of the cells after 18 h of incubation. Antibody to H3N2 virus was assayed with A/Port Chalmers/73 virus, since this virus produced more efficient infection of mouse cells than did the A/Texas/77 virus. The cells were then harvested by trypsinization and radiolabeled by incubation for 30 min at 37°C in minimal essential medium with 10% fetal bovine serum containing 100 μCi of [^{51}Cr]sodium chromate per ml (New England Nuclear Corp., Boston, Mass.). They were then washed twice and suspended for use at 5×10^5 cells per ml. Portions (0.1 ml) of target cell suspensions were placed in tissue culture tubes (16 by 125 mm; Falcon Plastics, Oxnard, Calif.). To these tubes were added 0.2 ml of test serum and 0.1 ml of an appropriate dilution (1:4 or 1:8, depending on the lot used) of guinea pig complement (Flow Laboratories) for test lysis, 0.2 ml of medium plus 0.1 ml of complement for control lysis, and 0.3 ml of a 10% Brij 35 solution for maximal lysis. The tubes were incubated for 2 h at 36°C , the supernatants were carefully aspirated, and the cell sediments and supernatants were counted separately on a gamma counter (model 1185, G. D. Searle & Co., Des Plaines, Ill.). The percentage of chromium release for individual tubes was calculated and the percentage of specific immune lysis (SIL) was determined from the equation:

$$\% \text{ SIL} = \frac{(\% \text{ lysis by antibody} + \text{complement}) - (\% \text{ lysis by complement only})}{(\% \text{ maximal lysis}) - (\% \text{ lysis by complement only})}$$

Serum antibody titers were determined by calculating the 50% SIL endpoint for serial serum dilutions.

Lymphocyte transformation assays. Methods for lymphocyte transformation assays have been previously described (2, 8). Fresh or cryopreserved FH lymphocytes were suspended in RPMI 1640 containing 10% fetal bovine serum, penicillin, streptomycin, and 2×10^{-4} M 2-mercaptoethanol. Volumes of 0.2 ml of cell suspension were placed in wells of sterile, flat-bottomed 96-well plastic plates (Microtest II; Falcon). To quadruplicate wells was added 10 μ l of antigen, concanavalin A (ConA), or medium. Antigen was prepared from virus pools by inactivation with ultraviolet irradiation and used at an appropriate dilution to produce optimal response. The plates were incubated for 68 h at 37°C in a 5% CO₂ atmosphere; at that time 0.1 ml of medium containing 1 μ Ci of [³H]thymidine (New England Nuclear Corp.) was added to each well, and the plates were incubated for an additional 4 h. Cells were then collected with a MASH II harvester (Microbiological Associates), and the incorporation of thymidine was determined by scintillation counting. The transformation index was calculated by dividing the mean counts per minute incorporated into stimulated cultures by the mean counts per minute incorporated into control cultures.

Lymphocyte cytotoxicity assay. A chromium release microcytotoxicity assay was used, similar to that described by McMichael et al. (19, 20) and Galama et al. (10). Effector cells were cryopreserved NW lymphocytes, prevaccination for control lysis and post-vaccination for test lysis. Target cells were prepared from syngeneic, cryopreserved NW lymphocytes; 10⁶ cells were suspended in 5 ml of RPMI 1640 supplemented with 20% human AB serum and phytohemagglutinin M (Difco Laboratories, Detroit, Mich.) in a 1:100 final dilution. After 72 h of incubation at 37°C in a 5% CO₂ atmosphere, the cells were washed and suspended in 1 ml of medium containing 200 hemagglutination units of influenza A/USSR/77 virus and 100 μ Ci of [⁵¹Cr]sodium chromate per ml for 90 min at 37°C. Cells were then suspended in 5 ml of medium with 10% AB serum, incubated for 4 h at 37°C in a 5% CO₂ atmosphere, washed twice, and suspended in medium at a concentration of 10⁵ cells per ml. Portions (0.1 ml) of target cells were placed in wells of round-bottom, 96-well microtiter plates; to triplicate or quadruplicate wells of target cells was added 0.1 ml of medium containing 10⁶ control or test lymphocytes or 10% Brij 35 detergent, the latter for maximal lysis. Cultures were incubated at 37°C in a 5% CO₂ atmosphere for 18 h, the supernatant was harvested with a Titertek supernatant collection system (Flow Laboratories), and the released chromium was measured on a gamma counter (model 1185; G. D. Searle & Co.). Test lysis and control lysis were compared by the Student *t* test. Percentage of specific immune lysis (SIL) was calculated by the equation:

$$\% \text{ SIL} = \frac{\text{test lysis} - \text{control lysis}}{\text{maximal lysis} - \text{control lysis}} \times 100$$

RESULTS

Antibody determinations. The results of titrations of antibody to influenza A/USSR/77 in sera before vaccination are shown in Table 1. Two volunteers had antibody by the HI test, five by the complement-mediated lysis assay, and seven by the neutralization test. Only two volunteers, 26 and 33 years of age, had antibodies by all three assays. This finding was expected since individuals less than 26 years of age would not be likely to have had previous exposure to H1N1 influenza viruses. Prior vaccination with A/New Jersey/76 vaccine did not appear to have had an effect on base-line A/USSR/77 antibody titers, since one of the two who had received the vaccine was antibody positive and the other was negative by all three assays. After the first and second vaccinations the respective frequencies of antibody positivity were 10/10 and 10/10 by neutralization, 9/10 and 10/10 by complement-mediated lysis, and 7/10 and 10/10 by HI assay.

In Fig. 1 are compared the geometric mean titers (GMT) of antibody to A/USSR/77 obtained by the three assays to each time point. Since the lowest serum dilution tested in the HI assay was 1:10, a negative result at this dilution was assigned a value of 1:5. Although sera were tested at lower dilutions in the other assays (1:2 by neutralization, 1:4 by complement-mediated lysis) to maintain uniformity in performing calculations of GMT, all sera with titers of $\leq 1:5$ were assigned a titer of 1:5. Consistently higher

TABLE 1. *Prevaccination anti-influenza A/USSR/90/77 (H1N1) antibody titers*

Volunteer no.	Age (yr)	Antibody titers ^a		
		HI	Complement-mediated lysis	Neutralization
1	22	<10	<4	4
2	23	<10	<4	4
3	23	<10	4.6	2
4	24	<10	<4	<2
5	23	<10	<4	<2
6	25	<10	14.2	2
7	25	<10	<4	<2
8	24	<10	7.7	8
9	33	20	29	8
10	26	160	158	64

^a Expressed as reciprocals of highest dilution producing positive results for HI and neutralization tests and as reciprocal of 50% SIL endpoint dilution for complement-mediated lysis assay.

FIG. 1. Geometric mean serum antibody titers after influenza vaccine. Values for HI (▲) and neutralization (■) are expressed as reciprocals of the highest serum dilution giving positive results; values for the complement-mediated lysis assay (●) are expressed as the reciprocal of the 50% SIL endpoint dilution. Monovalent influenza A/USSR/77 vaccine was given on day 0, and trivalent A/USSR/77, A/Texas/77, B/Hong Kong/73 vaccine was given on day 28 of the study.

titers were obtained by the complement-mediated lysis assay than by the HI test. This difference was significant when titers obtained on all sera tested were compared by the paired *t* test ($\text{GMT} \pm \text{standard deviation} = 19.1 \pm 3.2$ versus 13.2 ± 3.2 ; $P < 0.001$). Neutralization antibody titers exceeded those obtained by the other assays after the second but not the first vaccination. The neutralization test was not significantly more sensitive than the complement-mediated lysis assay when results obtained from all sera tested were compared by the paired *t* test.

The specificity of the complement-mediated lysis assay was examined by comparing lysis of target cells infected with A/USSR/77 and A/Port Chalmers/73 viruses. The A/Port Chalmers/73 virus was used in preference to A/Texas/77 to assay for antibodies to H3N2 antigens since the mouse-adapted virus produced more uniform infection of target cells. Results of comparative testing of specimens from three volunteers are presented in Table 2. After the first vaccination, all three volunteers had increases in serum antibody to A/USSR/77 virus as measured in both the HI and complement-mediated lysis assays, but none developed increases in antibodies to H3N2 antigens. After the second vaccination there were increases in antibodies to H3N2 and H1N1 antigens demonstrated by both assays. These results indicate that the complement-mediated lysis assay de-

fects subtype-specific antibodies after vaccination.

Lymphocyte transformation. Peripheral blood lymphocytes from all volunteers tested before vaccination proliferated in response to the mitogen ConA and to the influenza antigens A/USSR/77 (H1N1), A/Port Chalmers/73 (H3N2), and A/Japan/57 (H2N2); the latter antigen is also a nonspecific B- and T-lymphocyte mitogen (2). No differences were observed between the responses of the antibody-negative and -positive vaccinees. The changes in lymphocyte responsiveness to these agents over time are depicted in Fig. 2. In all volunteers, between 3 and 7 days postvaccination the incorporation of thymidine into cultures stimulated with influenza A/USSR/77 (H1N1), A/Port Chalmers/73 (H3N2), or ConA was depressed. By 10 days after vaccination there was a sharp rise in lymphocyte responsiveness to all agents followed by a decline to base-line levels in approximately 28 days. Responses to A/Texas/77 (H3N2) were first tested on day 7 and thereafter showed similar sequential changes. In contrast, lymphocyte responsiveness to A/Japan/57 did not show an initial depression preceding the period of increased responsiveness. The effect of secondary vaccination on day 28 appeared to be qualitatively different. Seven days after the vaccination, transformation indices to all of the influenza antigens tested and to ConA were in-

TABLE 2. Subtype specificity of complement-mediated lysis assay

Volunteer no.	Days after first vaccination ^b	Serum antibody titer ^a			
		H3N2		H1N1	
		Complement-mediated lysis	HI	Complement-mediated lysis	HI
2	0	<8	NT ^c	<4	<10
	28	<8	20	47.4	20
	42	245	64	68.2	32
6	0	<8	NT	14.2	<10
	28	<8	10	33.8	10
	42	348	64	76.5	32
8	0	<8	NT	7.7	<10
	28	<8	<10	126	40
	42	176	64	164	128

^a Expressed as reciprocals of 50% endpoint dilutions for complement-mediated lysis assay or of highest dilutions producing positive test results for neutralization and HI assays.

^b Monovalent A/USSR/77 vaccine was given on day 0 and trivalent A/USSR/77, A/Texas/77, B/Hong Kong/73 vaccine was given on day 28.

^c NT, not tested.

creased, not depressed. Since it was not possible to test the volunteers on day 3 after the second vaccination, it is not known whether depressed responsiveness occurred at this time. These se-

FIG. 2. Transformation of peripheral blood lymphocytes by influenza antigens and ConA (Δ) after vaccination on day 0 with monovalent influenza A/USSR/77 vaccine and on day 28 with trivalent influenza A/USSR/77, A/Texas/77, B/Hong Kong/73 vaccine. Influenza antigens used for stimulation were: $\bullet$, A/USSR/77; $\blacksquare$, A/Texas/77; $\blacktriangle$, A/Japan/300/57; $\circ$, A/Port Chalmers/1/73.

quential changes in lymphocyte responsiveness were similar in all volunteers.

Functional viability characterization of cryopreserved lymphocytes. FH and NW cryopreserved lymphocytes were tested by trypan blue exclusion and found to be 80 to 90% viable. By staining with Wright-Giemsa stain, the NW cells were shown to be 98 to 99% lymphocytes and 0.2% macrophages. E-rosetting cells comprised 89 to 92% of the NW lymphocytes and 40 to 70% of FH lymphocytes; sequential changes in the frequencies of E-rosetting cells occurred in some volunteers, but these variations did not correlate with transformation indices, antibody responses, or lymphocyte cytotoxicity. The percentage of NW lymphocytes which were adherent to EA monolayers, that is, that possessed Fc' receptors, was 15 to 20%. The functional capacity of the cryopreserved cells was tested in lymphocyte transformation assays; transformation indices of four cryopreserved FH lymphocyte suspensions stimulated with influenza A/USSR/77 (H1N1) antigen or ConA were 50 to 100% of those obtained testing the same lymphocytes on the day of collection.

Cell-mediated cytotoxicity. The results of chromium release assays are presented in Table 3. Significant specific immune lysis of influenza-infected target cells was produced by lymphocytes from 5 of 10 volunteers (no. 2, 3, 6, 8 and 10). The amount of chromium released in the presence of lymphocytes obtained postvaccination from other volunteers was not significantly greater than the amount released in the presence of control lymphocytes (Student *t* test for independent variables). There was no lysis of uninfected target cells. Cytotoxicity was generally detected on only one of the assay days, on day 3 in three and day 7 in one volunteer. However,

TABLE 3. Lymphocyte cytotoxicity for influenza A/USSR/77 (H1N1)-infected syngeneic target cells

Volunteer no.	% SIL on given day after vaccination ^a				
	3	7	10	28	35
1	≤ 0.0	1.6	< 0.0	≤ 0.0	≤ 0.0
2	48.9 (< 0.001) ^b	46.9 (< 0.001)	42.6 (< 0.001)	NT ^c	≤ 0.0
3	13.5 (< 0.05)	2.1	3.7	≤ 0.0	≤ 0.0
4	≤ 0.0	2.7	3.9	≤ 0.0	≤ 0.0
5	6.7	7.5	8.4	1.7	≤ 0.0
6	33.7 (< 0.001)	8.6	≤ 0.0	NT	≤ 0.0
7	≤ 0.0	7.2	≤ 0.0	6.8	≤ 0.0
8	≤ 0.0	11.4 (< 0.05)	≤ 0.0	3.0	≤ 0.0
9	≤ 0.0	7.2	≤ 0.0	≤ 0.0	≤ 0.0
10	10.6 (< 0.05)	≤ 0.0	4.2	4.7	NT

^a Volunteers were given monovalent A/USSR/77 vaccine on day 0 and trivalent A/USSR/77, A/Texas/77, B/Hong Kong/73 vaccine on day 28.

^b Numbers in parentheses indicate probability by the Student *t* test expressing significant differences between test and control counts per minute. All other values were nonsignificant.

^c NT, not tested.

high levels of specific immune lysis were produced by lymphocytes from volunteer no. 2 on days 3, 7, and 10 postvaccination. The occurrence of a cytotoxic lymphocyte response did not correlate with serological status before vaccination or with the degree of enhancement of lymphocyte transformation which occurred subsequently. Because of the limited number of lymphocytes available, it was not possible to characterize the effector cells other than by the assays mentioned above. No cytotoxic lymphocyte responses were found on the 7th day after the second vaccine administration.

DISCUSSION

Immune responses to influenza vaccines are usually demonstrated by measurement of HI antibody, although antibodies to neuraminidase (9) and neutralizing antibody can also be detected (1). The efficacy of influenza vaccines has been demonstrated in studies which indicate that protective immunity correlates, in general, with a certain minimum titer of HI antibody (14). In addition, individuals with no prior exposure to closely related antigens often require more than one vaccine inoculation to achieve adequate responses. Thus, the HI antibody results obtained in this study were similar to those found in other studies.

Rises in antibody titers to A/USSR/77 antigens were detected in all volunteers by all three assays used in this study. The assay for neutralizing antibody was found to be more sensitive than that for HI antibody, since more prevaccination serum specimens contained antibody detected by the former type of assay than the latter. Similar types of comparative results have been obtained by others (18). The reasons for this difference in sensitivity are probably that (i) serum cannot be tested for HI antibody at very low dilutions because of the need for pretreatment to remove inhibitors and (ii) much smaller amounts of virus are used in the neutralization assay. The complement-mediated lysis assay was also more sensitive than that for HI antibody, in terms of both GMT obtained on comparative serum testing and sensitivity for detecting antibody in low-titered sera. This difference may be related to the same limitations of the HI test mentioned above. However, some of the findings of this study suggest that the difference may be due, in part, to detection of antibodies to cross-reactive antigens by the complement-mediated lysis assay. Three volunteers who were less than 26 years of age had low levels of antibody to A/USSR/77 antigens detected before vaccination by the complement-mediated lysis assay. Since these three volunteers had

probably not encountered H1N1 antigens previously, the results suggest that the test can detect cross-reactive antigens.

The specificity of the complement-mediated lysis assay was examined further by comparative testing of sera for antibodies to H3N2 and H1N1 viruses. Rises in antibody to H3N2 antigens occurred after administration of vaccine containing A/Texas/77 antigens but not after administration of monovalent A/USSR/77 vaccines. These results indicated that the assay was capable of detecting subtype-specific antibody, despite the indication discussed above that antibodies to cross-reactive determinants were also detected. These results are consistent with earlier findings demonstrating the presence of both specific and cross-reactive determinants on the hemagglutinin molecule (23). Moreover, antigens expressed on the surface of influenza-infected cells have been found to render them susceptible to both subtype-specific and cross-reactive cell-mediated lysis (9). Both types of antibody-dependent cytotoxicity are of potential biological importance. Antibody to cross-reactive determinants may be of protective value to an individual infected with a virus of a subtype they have not previously encountered, whereas subtype-specific complement-fixing antibody may be involved in immunity to homologous virus.

Cellular immune function is probably an important part of host defense against influenza infection, as indicated by the histological finding of lymphocytic infiltration during influenza pneumonia (13). Lymphocyte transformation *in vitro*, although not a measure of effector cell function, has been used as a measure of cell-mediated immune responsiveness. The proliferative response to influenza antigens is often cross-reactive. The responding cells are generally T lymphocytes, except in the case of the H2N2 viruses which have been shown to be mitogenic for murine B cells (2). The results of lymphocyte transformation assays presented here are consistent with earlier findings (3, 15). However, some of the data are unique. Within 3 to 7 days of initial vaccination there was depression of lymphocyte responses to most of the influenza antigens and ConA. The only exceptions were the responses measured to influenza A/Japan/57, an H2N2 strain which is mitogenic for murine B cells. These results raise the possibility that H2H2 antigens are also mitogenic for human B cells and suggest that vaccination may produce a selective suppression of T-lymphocyte responsiveness. This suppression did not correlate with the numbers of T cells, indicating that it results from a change in cell func-

tion rather than a change in the number of available responding cells. The suppression might have been due to the direct effect of viral antigens on the available responding cells, to the activation of a population of suppressor cells, or to some other immune regulatory mechanism. After the period of depression and after secondary vaccination there was a rise in transformation indices to values greater than those obtained before vaccination; the rise after secondary vaccination came earlier, and either it was not preceded by a period of depression or the depression occurred before the time when measurements were made. Unfortunately, it was not possible to study the volunteers at as many time points after the second vaccination as after the first. Results similar to these were obtained by Dolin et al. (3) and Jennings et al. (15) in studies of volunteers infected with influenza, but they did not observe depressed responsiveness in recipients of inactivated vaccines. This disparity may relate to differences in virus strains used or in the amount of viral antigen inoculated. The findings of this study indicate that the results of lymphocyte transformation assays must be interpreted in relationship to both past and recent immunization history. Transformation measured *in vitro* may be subject to immunoregulatory mechanisms induced by vaccination.

Cytotoxic lymphocytes which lysed syngeneic, virus-infected target cells were found to be present within the first 10 days postvaccination in 5 of 10 volunteers. Although the lymphocyte suspensions used in the cytotoxicity assays had been enriched for T cells by passage through nylon wool columns, insufficient numbers of lymphocytes were available to permit full definition of the cells mediating the cytotoxicity by further fractionation procedures. There is limited evidence that human CTL are HLA restricted, that is, that they are capable of lysing only target cells with which they share HLA antigens (19, 20, 24). Extensive evidence indicates that a similar phenomenon governs the function of murine CTL (4, 7). Since the target cells used in our assays were syngeneic, the method should be satisfactory for the measurement of HLA-restricted CTL. In this respect, the assay system described here includes an important innovation over that described by Greenberg et al. (11), which involved the use of xenogeneic target cells.

The temporal characteristics of the cell-mediated cytotoxicity response are of interest. The actual role of CTL as a host defense mechanism has been examined in mice infected with influenza (8) and cytomegalovirus (21). The responses generally coincide with the acute phase of infection, during which time the cytotoxic

cells circulate in the peripheral blood and are distributed to infected tissues. CTL can be found in lungs of mice suffering from influenza pneumonia (8). These findings suggest that this cell type performs an important function early in the course of infection. The demonstration of an early cytotoxic cell response in this study is consistent with the possibility that the functions performed by human and murine cytotoxic lymphocytes are similar.

The significance of immune responses to influenza antigens relates to great numbers of individuals vaccinated and infected annually. Through the studies described here we have demonstrated that vaccination can effect a variety of changes in cellular immune function and can induce the capacity for both cell-mediated and antibody-dependent antiviral cytotoxicity. Lysis of virus-infected cells by these mechanisms may function *in vivo* to limit the extent of virus replication early in the course of infection. The outcome of influenza infection may well be partially determined by the capacity of individuals to develop these immune functions.

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