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Lymphocyte Cytotoxicity to Influenza Virus-Infected Cells: Response to Vaccination and Virus Infection

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Peripheral blood leukocytes obtained from volunteers at various times following influenza vaccine or live influenza virus infection were assayed for cytotoxicity against influenza virus-infected cells. Cytotoxicity was highest on days 3 and 7 following vaccination with commercial A/Port Chalmers/1/73 inactivated influenza virus vaccine. Maximal cytotoxicity was found 9 days after infection induced by intranasal inoculation of a strain of A/Scotland/840/74 influenza virus. Individuals naturally infected with A/Victoria/3/75 were also found to have elevated cytotoxicity approximately 1 week after onset of illness. Cytotoxicity levels decreased toward base line approximately 30 days after the virus exposure. The effector mechanism(s) responsible for the early, transient elevation in specific immune release to influenza virus-infected cells may be different from the antibody-dependent cell cytotoxicity demonstrated in the peripheral blood leukocytes from volunteers who had a remote experience with influenza virus.

A cell-mediated immune response that may be important in the control of influenza virus infection is cell-mediated destruction of influenza virus-infected cells (1, 6, 11; B. S. Criswell, R. B. Couch, and S. B. Greenberg, Fed. Proc. **34**:948, 1975). Previous studies from this laboratory have demonstrated that lymphocyte cytotoxicity to xenogeneic cells infected with type A influenza virus is antibody dependent and that the effector cell is probably a nonadherent, nonphagocytic, Fc receptor-bearing cell (8, 9). These results were obtained in volunteers who had no known exposure to either inactivated or live influenza virus antigen for at least 2 months. In these individuals, specific antihemagglutinin antibody (AHAb) was found by a sensitive radioimmunoassay (RIA) to be present on the surface of the lymphocytes. When this antibody was removed by warming the separated lymphocytes to 37°C for 30 min, cytotoxicity was greatly reduced.

To evaluate the development of cell cytotoxicity, volunteers were tested before and early after vaccination with inactivated influenza virus, after intranasal inoculation with live virus, and following occurrence of natural infection with influenza virus. The kinetics of these early cytotoxicity responses and tests for the effector mechanism(s) are presented.

MATERIALS AND METHODS

Volunteers. Volunteers were healthy adults between the ages of 18 and 45 years. Naturally infected individuals were 18 to 62 years of age. Those volunteers challenged with live virus were participating in studies for evaluation of effectiveness of experimental influenza virus vaccines. Written informed consent was obtained for all studies (12).

Vaccinations. A monovalent inactivated influenza virus vaccine, A/Port Chalmers/1/73 (H3N2) (700 chick cell agglutinating [CCA] units, Zonomune, Eli Lilly & Co., Indianapolis, Ind.) was used, except for one volunteer who received trivalent influenza virus vaccine containing two type A components (A/Port Chalmers/1/73, A/Scotland/840/74) and B/Hong Kong/72 influenza virus (1,200 CCA units, Zonomune, Eli Lilly & Co.). Blood specimens were collected before and at various times after vaccination. No untoward reactions were reported in any of the vaccinated volunteers.

Live-virus inoculations. Viruses used in challenge studies were either the fluid harvested from passage 5 in human embryonic kidney (HEK) tissue cultures of a strain of A/Scotand/840/74 or passage 2 in embryonated hen's eggs of A/Victoria/3/75.

Before use in volunteers, the two inocula were processed and safety tested as previously described (12). Volunteers were inoculated by nasal instillation of 0.5 ml of a dilution of virus that contained approximately 1,000 50% tissue culture infectious doses per 0.5 ml of A/Scotland/840/74 and approximately 10,000 50% tissue culture infectious doses per 0.5 ml of A/Victoria/3/75.

After challenge with virus, volunteers were examined daily for illness, and nasal wash specimens were collected for virus isolation. The methods used for isolation and identification of virus isolates have been previously described (14). Blood was collected before and periodically after virus inoculation for cytotoxicity assays and for influenza virus serological tests.

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Cytotoxicity assays. Peripheral blood leukocytes (PBL) were assayed for cytotoxicity to influenza virus-infected target cells by the ^{51}Cr release assay previously described (8). Briefly, heparinized blood was obtained, and the buffy coat was removed after centrifugation. Leukocytes were obtained from the buffy coat by either the Technicon lymphocyte separator (Technicon Corp., Tarrytown, N.J.) or by Ficoll-Isopaque density centrifugation (2). Target cells (approximately 10^7) consisted of baby hamster kidney (BHK-21) cells labeled with 0.1 mCi of ^{51}Cr ($Na_2{}^{51}CrO_4$; CJS-1, Amersham/Searle, Arlington Heights, Ill.) and inoculated at a multiplicity of infection of 1 to 10 with an influenza virus recombinant strain containing the hemagglutinin antigens A/Hong Kong, A/Port Chalmers, A/Scotland, and an equi-1 neuraminidase: H3hkNeq1, H3pcNeq1, or H3scNeq1. Control target cells consisted of uninfected, chromium-labeled cells. After 16 h of incubation, the monolayers were trypsinized and washed, and the cells were counted by trypan blue exclusion. Specific immunofluorescence with anti-influenza virus antisera was employed to demonstrate suitable degree of infection in target cells (>80%). Immunofluorescence was read on fixed cells and demonstrated cytoplasmic and membrane fluorescence.

In most experiments, 5×10^4 target cells in 0.1 ml was added to 5×10^6 PBL. After a 4-h incubation at 37°C, 3 ml of cold tris(hydroxymethyl)aminomethane buffer with 10% fetal calf serum was added, and each tube was centrifuged at $1,000 \times g$ for 10 min. The supernatant fluid and cell sediment from each tube were counted in a gamma counter (Amersham/Searle) to determine the percentage of chromium released.

The percentage of specific immune release (%SIR) was calculated from the following formula:

$$\text{Infected } [(L_v - S_v)/(M_v - S_v)] - \text{Uninfected } [(L_c - S_c)/(M_c - S_c)] \times 100,$$

where L represents the percentage of ^{51}Cr released from tubes containing lymphocytes and target cells, S represents the percentage of ^{51}Cr released from tubes containing only tris(hydroxymethyl)aminomethane buffer and target cells, and M represents maximum ^{51}Cr release after freeze-thaw lysis.

Preparation of eluate. In some experiments, lymphocytes obtained by Ficoll-Isopaque centrifugation were incubated at 37°C for 30 min in serum-free minimal essential medium (10^6 lymphocytes per ml). After the incubation period, lymphocytes were centrifuged for 5 min at $800 \times g$ and the eluate (supernatant) was removed. The lymphocytes were washed twice with minimal essential medium and recounted. In designated experiments, the lymphocytes that were incubated at 37°C for 30 min were tested for cytotoxicity with and without the autologous eluate.

Characterization of lymphocyte subpopulations and monocytes. E-rosette-forming lymphocytes (T cells) were determined by the method of Jondal et al. (10). Surface immunoglobulin-bearing cells (B cells) were determined by an immunofluorescent antibody technique with fluorescein-conjugated anti-human immunoglobulin (polyvalent) antisera (Meloy Laboratories, Springfield, Va.) (4). The percentage of 200 cells constituted the proportion for each cell type. The percentage of lymphocytes not accounted for by the sum of B and T lymphocyte determinations was termed null cells. Total counts of each cell type were determined by calculation using the total leukocyte count and a differential for percentage of lymphocytes. The frequency of monocytes was determined by acridine orange staining (7).

Using these techniques, no significant differences in total T, B, null, or monocyte counts were detected in the volunteers on the test days after vaccination or live influenza virus challenge.

Tests for antibody to influenza virus. Standard viral neutralization tests were performed using A/Hong Kong/68 (H3N2), A/Victoria/75 (H3N2) and antigenic hybrid viruses containing the relevant hemagglutinin and the neuraminidase of an equi-1 virus (H3hkNeq1, H3pcNeq1, H3scNeq1) (14). The hybrid viruses provide a specific measure of antibody to the hemagglutinin glycoprotein. The starting dilution of serum for neutralizing antibody was 1:4. In addition, AHAb titers for serum and lymphocyte eluates were determined using a double-antibody RIA (17). The neutralization test was performed in rhesus monkey tissue culture with 32 50% tissue culture infectious doses of the appropriate recombinant influenza virus, using absence of hemadsorption as an end point of neutralization.

Statistical analysis. Cytotoxicity results were analyzed by the paired-sample t test.

RESULTS

Effect of inactivated influenza vaccine on cell cytotoxicity. Lymphocytes obtained from 17 volunteers before and at various times after vaccination with inactivated A/Port Chalmers/73 influenza virus were tested for cytotoxicity to virus-infected target cells (Fig. 1). Seven demonstrated cytotoxicity before vaccination with a mean %SIR of 19.9 ± 5.5%. The mean %SIR increased to 25.6 ± 6.6% on day 3, although this increase was not statistically significant ($P > 0.1$, $t = 0.83$). No significant differences were noted between cytotoxicity levels for any of the time periods. Cytotoxicity responses to H3hkNeq1-infected target cells exhibited a similar pattern in these seven volunteers (data not shown).

Among the 10 volunteers with no preexisting cytotoxicity to H3pcNeq1, mean %SIR was 12.3 ± 2.0 and 8.0 ± 3.2%, respectively, on day 3 and day 7 after vaccination. The mean %SIR for day 3 and day 7 was significantly higher than the prevaccination level ($P < 0.001$, $t = 6.24$ and $P < 0.005$, $t = 4.07$, respectively). Each of the 10 volunteers demonstrated positive %SIR on day 3 with a range of 3.6 to 23.4%. In the eight volunteers who were available for testing, mean cytotoxicity levels were positive, but decreased by day 14 and were not different from prevaccination levels on day 35. Of these 10 volunteers, 7 were available for testing approximately 70

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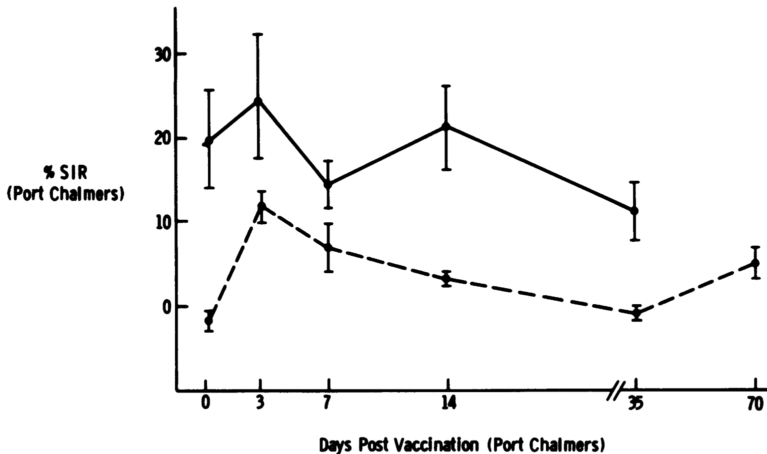

FIG. 1. *Development of cytotoxicity after vaccination with inactivated influenza vaccine. %SIR was measured in volunteers who lacked (----) or demonstrated (—) cytotoxicity to H3pcNeq1-infected cells prior to vaccination. Each point represents the mean ± standard error for the group tested.*

days after vaccination, and low but positive % SIR was demonstrated in all but one. However, the mean %SIR was not significantly higher on day 70 than on day 35 ($0.1 > P > 0.05$, $t = 2.58$). The cytotoxicity response using H3hkNeq1-infected target cells exhibited a similar response pattern in these 10 volunteers (data not shown).

Nine of 10 volunteers with no detectable cytotoxicity to H3pcNeq1 prior to vaccination also lacked detectable neutralizing antibody, and eight of these developed a fourfold or greater rise in titer (Table 1). Six of the seven with prevaccination positive %SIR also had neutralizing antibody, and one of these and the seronegative person developed a fourfold or greater rise in titer after vaccination. The mean neutralizing antibody titer at day 35 among those without prevaccination cytotoxicity was considerably lower than either the prevaccination or postvaccination titer for those who initially exhibited cytotoxicity.

Effect of nasal inoculation with A/Scotland influenza virus on cell cytotoxicity in volunteers. Six volunteers who had no detectable serum neutralizing antibody to H3scNeq1 or H3pcNeq1 were infected with a strain of A/Scotland/840/74 influenza virus. Infection was documented by virus isolation as well as serological response. Cell cytotoxicity before and after inoculation is shown in Fig. 2. Five of the six volunteers had no measurable cytotoxicity prior to inoculation. A pattern of development and persistence of cytotoxicity similar to that seen in volunteers given inactivated vaccine was demonstrated in these volunteers. Cell cytotoxicity was detected in all volunteers on day 9 after inoculation, and the mean level was significantly higher than day 0 values ($P < 0.025$, $t = 3.59$). Mean cytotoxicity values had returned to near base line by day 14, but, in one volunteer, consistently elevated %SIR was measured on days 9, 14, and 28 after inoculation. His value returned to a level near those of other volunteers by day 60.

TABLE 1. *Serum neutralizing antibody response after vaccination with inactivated A/Port Chalmers/1/75 (H3N2) influenza virus vaccine*[a]

Prevaccination %SIR[b]	No. vaccinated	No. with fourfold rise in antibody[c]	Geometric mean antibody titer[d]	
			Day 0	Day 35
Absent	10	8	1.6	17
Present	7	2	84	372

[a] 700 CCA units.
[b] %SIR to H3pcNeq1-infected BHK-21 cells.
[c] Neutralizing antibody titer was determined using H3pcNeq1.
[d] Reciprocal of neutralizing titer.

Effect of eluate on cell cytotoxicity after vaccination with inactivated influenza virus or nasal inoculation with live virus. Three individuals who had serum neutralizing antibody to H3pcNeq1 but had not been vaccinated or exposed to influenza virus for at least 2 months were tested for cytotoxicity. After their PBL were separated, a portion was incubated at 37°C for 30 min and the eluate (supernatant) was removed. Degree of cytotoxicity was measured for the PBL fraction, for the lymphocytes that had been warmed, washed, and mixed with fresh medium, and for the lymphocytes that had been warmed, washed, and mixed with the autologous eluate (Table 2). Cytotoxicity was

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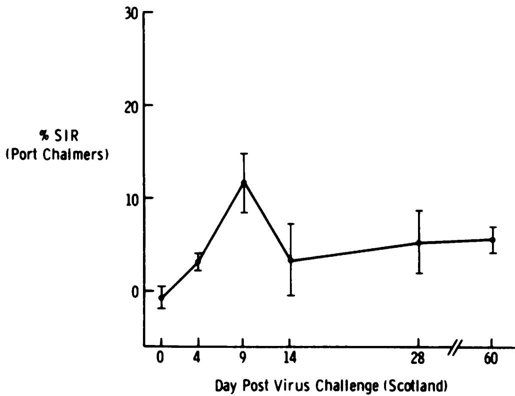

FIG. 2. *Development of cytotoxicity after intranasal inoculation with A/Scotland/840/74 influenza virus. %SIR was measured in volunteers who lacked cytotoxicity to H3pcNeq1-infected cells prior to vaccination. Each point represents the mean ± standard error for the group.*

TABLE 2. *Effect of eluate on cell cytotoxicity after exposure to inactivated or live influenza virus*

Volunteer	Time from vaccination or infection (days)	%SIR[a]		
		PBL[b]	Lymphocytes tested with[c]: Medium alone	Lymphocytes tested with[c]: Eluate
1	>60[d]	64.3	5.9	54.5
2	>60[d]	44.5	2.7	32.0
3	>60[d]	9.7	−0.5	10.8
4	7[e]	8.0	9.2	4.4
5	9[e]	32.8	27.7	23.3
6	9[e]	50.8	43.7	45.6
7	9[e]	19.0	20.8	17.7
8	9[e]	30.2	14.4	25.9
9	9[e]	19.5	12.4	15.0

[a] Determined using H3pcNeq1-infected BHK-21 cells.

[b] Lymphocyte-target cell ratio = 100:1.

[c] After incubation at 37°C for 30 min. %SIR was <2% when these eluates were tested without lymphocytes. All eluates had detectable antibody by RIA except for volunteer no. 4.

[d] Volunteers had no known infection or vaccination with influenza virus during the preceding 60 days.

[e] Volunteer no. 4 was vaccinated with A/Port Chalmers/1/73 influenza virus vaccine (700 CCA units), and his cells were tested on day 7. Volunteers no. 5 to 9 were inoculated intranasally with A/Scotland/74, and their cells were tested on day 9.

markedly reduced in the warmed and washed lymphocytes in the three volunteers. However, autologous eluate restored cytotoxicity to these lymphocytes. All three eluates had detectable AHAb to the Port Chalmers hemagglutinin by RIA.

A single volunteer was identified who had no detectable AHAb or cytotoxicity to H3hkNeq1 or H3pcNeq1 by RIA. He was vaccinated with a commercial influenza virus vaccine containing inactivated A/Port Chalmers/1/73, A/Scotland/840/74, and B/Hong Kong/72 influenza viruses. On day 7 after vaccination, his PBL were separated, and a sample was incubated at 37°C for 30 min and processed as described above. Unlike the three other volunteers tested, there was no loss of cytotoxicity in the warmed and washed lymphocytes compared with that detected in PBL. The lymphocytes to which eluate was added back actually demonstrated lower levels of cytotoxicity. There was no detectable AHAb by RIA in this eluate.

Volunteers infected with A/Victoria/3/75 were similarly tested on day 9 for cytotoxicity to H3pcNeq1-infected cells. Four of five volunteers had small reductions in cytotoxicity detected with their warmed and washed lymphocytes when compared to the PBL fraction. Eluate did not increase the cytotoxicity of the warmed and washed lymphocytes in four of the five tested volunteers. However, AHAb to the Port Chalmers hemagglutinin was found in all five eluates when tested by RIA.

Effect of natural infection with A/Victoria/75 (H3N2) on cell cytotoxicity. Four individuals with naturally acquired influenza infection with A/Victoria influenza virus were tested for cytotoxicity to H3pcNeq1-infected cells from 2 to 5 days after the onset of symptoms (Fig. 3). All were ill with upper res-

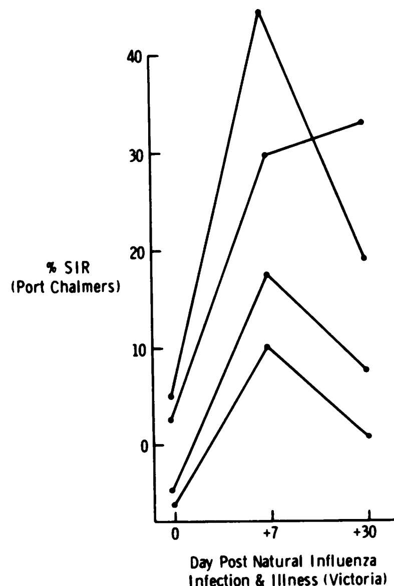

FIG. 3. *Cytotoxicity in four individuals who were tested while clinically ill (day 0) with A/Victoria/3/75 and during convalescence (days 7 and 30).*

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piratory tract symptoms and fever. All had documented influenza virus infections by virus isolation and fourfold rises in serum neutralizing antibody to A/Victoria/75 (H3N2).

PBL obtained 7 days after the acute illness sample, when all volunteers were symptom free, demonstrated a rise in %SIR for all four cases. PBL tested 30 days after the illness sample demonstrated reduced levels of cytotoxicity compared to the day 7 samples in three of the four subjects.

DISCUSSION

Cell cytotoxicity to influenza virus-infected target cells developed in volunteers who had received either inactivated or live antigen stimulation. Maximal cytotoxicity was measured approximately 3 to 7 days after vaccination, 9 days after live virus challenge, and 7 days after natural influenza illness. In most individuals, there was a decline in cytotoxicity to low levels approximately 30 days after the antigenic stimulation regardless of how it was provided, i.e., by vaccine or live virus.

These cytotoxicity responses contrast with our earlier studies in individuals who had no known exposure to influenza virus for at least 2 months. In those volunteers, cytotoxicity to influenza virus-infected cells was shown to be mediated by small quantities of specific antibody that is cell associated and by lymphocytes that have characteristics in common with K cells (9). The small quantities of specific antibody were detected by RIA in eluates from PBL that had been incubated at 37°C for 30 min. A similar role for specific cell-associated antibody could not be shown in the volunteers in the present study who were tested 7 to 9 days after vaccination or after live virus infection. In one volunteer, both the PBL fraction and the lymphocytes that had been incubated at 37°C for 30 min demonstrated equal levels of cytotoxicity on day 7 following vaccination. Eluate that had been removed from the lymphocytes after incubation at 37°C of 30 min failed to increase cytotoxicity of autologous lymphocytes and lacked specific AHAb when measured by RIA. In five volunteers who were tested 9 days after receiving a live virus challenge, the lymphocytes that had been incubated at 37°C for 30 min demonstrated somewhat reduced cytotoxicity. Although the eluates removed from these five individuals had detectable AHAb by RIA, the cytotoxicity demonstrated after cell-associated antibody was removed remained at a high level.

Thus, the effector mechanism responsible for the early transient cytotoxicity responses has not been defined by these experiments, and alternatives to antibody-dependent cell cytotoxicity must be considered. The T cell has been reported to be responsible for cell cytotoxicity to virus-infected cells in many animal studies (3, 5). Although one study in humans reported T-cell cytotoxicity to rubella virus (16), a recent study has failed to document T-cell cytotoxicity in humans vaccinated with vaccinia virus (13). In this latter study, syngeneic as well as allogeneic and xenogeneic target cells were used, and the effector cell was thought to be a K cell. If the K cell is operating in our early cytotoxicity responses, then specific antibody would have to be secreted in vitro during the 4-h incubation period for antibody-dependent cell cytotoxicity to be operative. If the 4-h incubation time is sufficient for active secretion of antibody to develop, the present data suggest that cells with this capability are found in peripheral blood only in the period early after immunization. Studies are in progress in our laboratory to clarify this point.

Since macrophages were not specifically removed from the separated lymphocytes, we cannot exclude these cells as possible nonspecific effectors mediating the early cytotoxic responses. Macrophages may exhibit nonspecific cytotoxicity; in a study with Semliki Forest virus in mice, such cytotoxicity was maximal 7 days after challenge and declined rapidly thereafter (15).

Maximal cytotoxicity responses after live virus infection appeared in most instances during the period of recovery from infection and illness. This suggests that this immune response could play a role in the recovery phase of influenza virus infections. Although an in vivo function for cell cytotoxicity has not been demonstrated, such a mechanism for destroying virus-infected cells could help promote recovery by impairing the full development and release of influenza virus from infected cells.

We have already shown that antibody-dependent cell cytotoxicity to influenza virus-infected cells is present in individuals exposed to influenza virus in the distant past. If the early cytotoxic responses measured within days of exposure to influenza virus are also dependent on specific antibody and Fc receptor-bearing lymphocytes, then antibody-dependent cell cytotoxicity may be important as a host defense against influenza virus infections.

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