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Enhancement of Anti-Influenza A Virus Cytotoxicity following Influenza A Virus Vaccination in Older, Chronically Ill Adults

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We studied anti-influenza cytotoxicity by bulk peripheral blood mononuclear leukocyte (PBL) cultures derived from older, chronically ill volunteers undergoing vaccination. Vaccinees received either cold-recombinant, live-attenuated influenza A/Korea/1/82 (H3N2) virus intranasally or inactivated monovalent influenza A/Taiwan/1/86 (H1N1) subvirion vaccine intramuscularly. PBL were collected pre- and postvaccination and in vitro stimulated by autologous PBL infected with influenza A virus homologous and heterosubtypic to the respective vaccine strain. Cytotoxicity was measured against influenza A virus-infected autologous and human leukocyte antigen (HLA)-mismatched PBL targets infected with influenza A virus homologous or heterosubtypic to the vaccine virus strain. Vaccinees infected with the live-attenuated virus developed significant rises in mean anti-influenza, HLA-restricted cytotoxicity that was cross-reactive against influenza A viruses homologous and heterosubtypic to the vaccine virus. The enhanced cross-reactive cytotoxicity was inducible postvaccination by in vitro stimulation with autologous PBL infected with the homologous influenza A (H3N2) virus and with influenza A (H1N1) virus. In contrast, after vaccination with inactivated monovalent subvirion vaccine, volunteers developed significant increases in mean anti-influenza, HLA-restricted cytotoxicity only against autologous PBL infected with homologous influenza A (H1N1) virus. Increased cytotoxicity occurred only after in vitro stimulation with autologous cells infected with homologous influenza A (H1N1) virus. Mean gamma interferon levels in supernatant fluids of influenza A virus-stimulated effector PBL did not increase postvaccination, despite increased levels of anti-influenza cytotoxicity displayed by the effector cells. We conclude that the live-attenuated influenza A virus infection induced a broader range of enhanced anti-influenza cytotoxicity than did the inactivated subvirion vaccine.

Development of more effective influenza A virus vaccines which will gain wider acceptance with populations at risk for influenza A virus-related complications than the currently licensed inactivated virus vaccine is a desirable goal. During recent influenza epidemics in the United States, influenza resulted in an average of 20,000 excess deaths per year (35). However, in a 1987 population-based survey of influenza immunization levels among U.S. adults in 32 states, only 32.2% of adults aged ≥65 years had received standard influenza vaccination in the previous 12 months and other data suggest the rate of vaccination to be lower (8). Administration of a live-attenuated influenza A virus vaccine intranasally offers the advantages of a preferred route of administration and induction of nasal secretory antibody. Murine studies suggest that live-attenuated virus vaccines induce an influenza A virus-specific cytotoxic T-cell response which is cross-protective and cross-reactive among influenza A virus subtypes, but a lack of cross-reactivity and lower levels of cytotoxic T-cell activity result from inactivated virus vaccine injection (34).

Evidence that cell-mediated immunity plays a role in the reduction of severity of illness and recovery from influenza A virus infection is based on murine and human studies. Adoptive transfer of influenza A virus-specific murine cytotoxic T cells and of influenza-immune T cells to syngeneic mice limit influenza A virus replication in lung tissue of the recipient mice and reduce mortality (21, 31, 38). Preexisting influenza A virus-specific memory cytotoxic T-cell activity correlates with accelerated viral clearance after wild-type influenza A virus challenge of young adults (23).

In this study, we immunized older, chronically ill volunteers with either live-attenuated influenza A virus or with monovalent inactivated influenza A subvirion vaccine to determine which preparation induced the better anti-influenza cytotoxicity in vitro postvaccination. We hypothesized that the live-attenuated influenza A virus infection would induce a higher level of cytotoxicity and a response cross-reactive among influenza A virus subtypes compared with that of the inactivated virus vaccine.

MATERIALS AND METHODS

Vaccines. The cold-recombinant, live-attenuated influenza A/Korea/1/82 (H3N2) virus vaccine was derived from cold-adapted influenza A/Ann Arbor/6/60 virus by H. F. Maassab using methods previously described (9). The resulting cold-recombinant virus (CR-59, lot numbers E-204 and E-223) contained six genes that code for internal proteins from the donor cold-adapted strain and genes that code for hemagglutinin (HA) and neuraminidase derived from influenza A/Korea/1/82 (H3N2) virus. In addition to being cold adapted, the vaccine virus was temperature sensitive (restrictive temperature, 39°C). The inocula of H3N2, CR-59 vaccine virus were $10^{7.2}$ for lot E-204 and $10^{7.5}$ for lot E-223 (undiluted as provided by the National Institute of Allergy and Infectious Diseases, Bethesda, Md.) PFU/0.5-ml intranasal dose.

The inactivated monovalent influenza A/Taiwan/1/86 (H1N1) subvirion vaccine (Connaught Laboratories, Inc., Swiftwater, Pa.; lot number 4624) contained 15 μg of H1 hemagglutinin (HA) per dose and included a mixture of other

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influenza A/Taiwan/1/86 (H1N1) proteins, including neuraminidase and internal viral proteins. A monovalent subvirion vaccine of an H3N2 subtype would have been the preferable preparation but was not available to us from the manufacturer.

Clinical studies and selection of patients. Volunteers were recruited from a Veterans Affairs population of older, chronically ill adults who were ambulatory and not institutionalized. Inclusionary criteria were the presence of significant chronic obstructive pulmonary disease (COPD), other chronic medical illness, and age over 55 years. Volunteers were not excluded if there was preexisting antibody to H1 and H3 HA or a history of parenteral trivalent inactivated influenza virus vaccination in a previous influenza virus season at least 9 months prior to entry into our study. None had previously been vaccinated with a live-attenuated, cold-recombinant influenza A virus vaccine.

The protocol for clinical studies was approved by the Human Subjects Committee of the Huntington Veterans Affairs Medical Center, Huntington, W.Va., where the study was conducted. The study was conducted in the summer and fall seasons prior to the influenza season. The first group of volunteers was intranasally inoculated with live-attenuated influenza A/Korea (H3N2) CR-59 virus vaccine as previously described (14). Subsequently, monovalent inactivated influenza A/Taiwan/1/86 (H1N1) virus subvirion vaccine was administered intramuscularly in the deltoid muscle to a second group of volunteers recruited and enrolled into the study after the first group. Volunteers gave written informed consent.

Standard spirometry was performed in the clinical pulmonary function testing laboratory before and 10 to 14 days after vaccination with both vaccines in the individuals with a history of lung disease with a computerized series 2500 Pulmonary Function Laboratory (Gould, Dayton, Ohio) using previously described methods (14). The forced expiratory volume (FEV_1) and forced vital capacity (FVC) were determined (1, 2), and the degree of obstruction to airflow was categorized on the basis of the FEV_1/FVC (percent).

Nasal wash specimens were obtained before and daily for 4 days after vaccination with CR virus vaccine and were inoculated onto tissue culture cells as previously described (4, 14).

Serological tests. Nasal wash specimens (20 ml of L-15 [Leibovitz] medium [Whittaker Bioproducts, Inc., Walkersville, Md.]) were obtained before and at 14 and 28 days after administration of CR-59 (H3N2) virus vaccine. Serum specimens were obtained from each vaccinee before and 28 days after administration of the CR-59 and the inactivated Taiwan/H1N1 virus vaccines.

Hemagglutination inhibition (HAI) antibodies in serum were measured by using whole virus homologous to the vaccine strains in a standard microtiter assay (36).

The CR-59 vaccine virus was grown in the allantoic cavities of eggs, and the HA was extracted and purified by previously described methods (28). The Taiwan/H1N1 virus HA was provided to us by Connaught Laboratories, Inc. (gift of C. Susan Brown). A one-step enzyme-linked immunosorbent assay (ELISA) was used to determine the anti-influenza virus HA serum and nasal wash immunoglobulin titer and immunoglobulin isotype as previously described using the homologous HA for the respective vaccinee group (14, 15, 25, 26). In addition, the nasal wash immunoglobulin anti-HA titers were corrected to a total secretory immunoglobulin A (IgA) concentration of 100 μg/ml. The total secretory IgA concentration in each concentrated nasal wash specimen was determined by ELISA. The sequence of reagents from solid phase up consisted of the following: (i) rabbit anti-human immunoglobulins IgG, IgA, and IgM (Cappel, West Chester, Pa.), (ii) nasal wash specimen, (iii) goat anti-human IgA conjugated with alkaline phosphatase (Cappel), and (iv) *p*-nitrophenyl phosphate disodium substrate. The standard curves of known secretory IgA concentration were determined by using serial dilutions of human secretory IgA (Jackson ImmunoResearch Laboratories, Inc., Avondale, Pa.) instead of nasal wash specimen in the ELISA.

Preparation of PBL. Heparinized peripheral venous blood was obtained from each volunteer before and at 14 and at 28 days after administration of the vaccines. A sample of heparinized peripheral venous blood from each volunteer was delivered to the Tri-State American Red Cross, Huntington, W.Va., for HLA class I type determination by complement lysis. For subsequent cytotoxicity studies, PBL were separated by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) density gradient centrifugation (7) and adjusted to a final cell concentration of 10^6 cells per ml in RPMI 1640 medium supplemented with 10% pooled human AB serum (Flow Laboratories, McLean, Va.), penicillin G (250 U/ml), and gentamicin sulfate (50 μg/ml). All pooled human AB serum added to cell culture medium in this and subsequent steps was heat inactivated at 56°C for 30 min before use.

Preparation of influenza A virus pools for infection of PBL. Influenza A/Chile/1/83 (H1N1), Taiwan/1/86 (H1N1), and Korea/1/82 (H3N2) CR-59 recombinant clone 19 were propagated in the allantoic cavities of embryonated hen eggs and were found to have 50% tissue culture infective doses ($TCID_{50}$s) equal to 10^7, 10^9, and 10^7/ml, respectively, in rhesus monkey kidney cell monolayers and hemagglutination titers of 1:512, 1:512, and 1:128, respectively, using guinea pig erythrocytes.

Anti-influenza cytotoxicity assay. (i) Target cell preparation. Fresh PBL (10^6 cells per ml) derived from the peripheral venous blood samples from volunteers were incubated in RPMI 1640 with 10% human AB serum for 4 days after which time phytohemagglutinin-P (PHA) at a concentration of 8 μg/ml was added to the cell culture for the following 3 days at 37°C in 5% CO_2. On the day of the cytotoxicity assay, the cells were washed in medium with no serum, samples were infected with influenza A viruses homologous and heterosubtypic to the vaccine virus strain by incubation for 1.5 h with virus (influenza A/Chile [H1N1], $10^{6.5}$ $TCID_{50}$/10^6 PBL; influenza A/Taiwan [H1N1], $10^{8.5}$ $TCID_{50}$/10^6 PBL; and influenza A/Korea [H3N2] CR-59, $10^{6.5}$ $TCID_{50}$/10^6 PBL) in medium with no serum, washed, and incubated for 60 min in medium containing 10% heat-inactivated fetal bovine serum (FBS). Control uninfected PBL were incubated with allantoic fluid not containing virus and then treated the same as target cells infected with influenza virus. The target cells were then pelleted, suspended, and incubated for 60 min in 0.3 ml of medium with 75 μCi of sodium [^{51}Cr]chromate (New England Nuclear, Boston, Mass.) per 10^6 cells. The labeled targets were then washed, the concentration was adjusted to 10^5 cells per ml, and 0.1 ml was added to each well of round-bottom 96-well microtiter plates. We have previously documented by flow cytometric indirect immunofluorescent antibody analysis the expression of influenza A virus on the cell surface of infected PBL incubated with influenza A virus in vitro (16). However, in the current study we did not determine whether there were individual differences in infectivity of PBL with influenza A virus based on, for instance, differences in HLA type.

In vitro-stimulated memory cytotoxic effector cells. Fresh PBL derived from the peripheral venous blood samples from volunteers were suspended at 10^6 cells per ml in RPMI 1640 with 10% human AB serum (responder cells). Other samples of autologous cells were incubated with live influenza A viruses homologous and heterosubtypic to the vaccine virus strain for 1.5 h in medium without serum (stimulator cells) at the same multiplicity of infection used to infect the target PBL. The responder cells were then incubated with autologous stimulator cells at a ratio of 9:1 at 37°C in 5% CO_2 for 7 days after which the assay for cytotoxic activity was performed.

Assay of cytotoxic activity. A standard ^{51}Cr release assay was used (22, 37). On the day of the assay, the effector cells were pelleted, samples of cell supernatant fluid were collected and frozen at −70°C, the cells were suspended in medium with 10% FBS, and added in 100-μl volume to target cells in 96-well microtiter plates at effector/target ratios of 50:1 and 25:1. Effector cells stimulated by autologous PBL infected with homologous and heterosubtypic influenza A viruses (see Cytotoxic Effector Cells section above) were added to target cells to detect cytotoxicity against autologous target cells infected with homologous and heterosubtypic viruses, uninfected target cells, and influenza A virus infected HLA-mismatched targets. The plate was incubated for 6 h after which the cell supernatant fluids were harvested, using a supernatant collection system (Skatron, Inc., Sterling, Va.), and the radioactivity was counted in a gamma counter as counts per minute. Percent specific lysis was calculated as [(experimental release − minimum release)/(maximum release − minimum release)] × 100. Maximum release was measured by incubating target cells in 10% Triton X-100, and minimum release was measured by incubation in medium with 10% FBS. In the results, the time point of percent specific lysis determination is reported as the time of venipuncture pre- or postvaccination from which the fresh PBL used in the assay were derived.

For all determinations in the study, the means of the maximum release of ^{51}Cr from CR-59 (H3N2)-infected target cells, Chile/H1N1-infected target cells, Taiwan/H1N1-infected target cells, and uninfected target cells incubated with allantoic fluid were 4,443 ± 350 cpm (range, 540 to 13,455 cpm), 3,741 ± 397 cpm (range, 665 to 11,886 cpm), 4,881 ± 640 cpm (range, 1,411 to 15,761 cpm), and 4,925 ± 438 cpm (range, 837 to 15,664 cpm), respectively. For all determinations in the study, the means of the minimum release of ^{51}Cr from CR-59 (H3N2)-infected target cells, Chile/H1N1-infected target cells, Taiwan/H1N1-infected target cells, and uninfected target cells incubated with allantoic fluid were 1,291 ± 97 cpm (range, 339 to 3,743 cpm, 29.1% of mean maximum release), 1,235 ± 101 cpm (range, 297 to 2,894 cpm, 33.0% of mean maximum release), 1,385 ± 189 cpm (range, 481 to 4,393 cpm, 28.4% of mean maximum release), and 1,174 ± 105 cpm (range, 261 to 3,861 cpm, 23.8% of mean maximum release), respectively.

IFN-γ ELISA. Levels of gamma interferon (IFN-γ) in supernatant fluids of the responder/stimulator PBL cell cultures (10, 16, 37) were measured using an IFN-γ ELISA similar to that described by Van der Meide et al. (33). The sequence of reagents from solid phase up in this ELISA was as follows: (i) murine monoclonal antibody to IFN-γ (Interferon Sciences, Inc., New Brunswick, N.J.) in 0.05 M carbonate buffer (pH = 9.6) was dispensed into wells of polystyrene microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.), and the plates were incubated at 4°C for 20 h; (ii) the wells were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma Chemicals, St. Louis, Mo.); (iii) 4% bovine serum albumin in PBS was then added to the wells, the plates were incubated at room temperature for 30 min and aspirated; (iv) test samples containing IFN-γ were added, and the plates were incubated for 1 h at 37°C; (v) the plates were washed five times with PBS containing 0.05% Tween 20; (vi) polyclonal rabbit anti-human gamma leukocyte IFN antibody (Chemicon International, El Segundo, Calif.) in PBS containing 1% bovine serum albumin and 0.05% Tween 20 was added, and the plates were incubated for 1 h at 37°C; (vii) the wells were washed five times with PBS containing 0.05% Tween 20; (viii) goat anti-rabbit gamma globulin alkaline phosphatase conjugate (Bio-Rad Laboratories, Richmond, Calif.) was added, and the plates were incubated at ambient temperature for 2 h; (ix) the wells were washed five times with PBS containing 0.5% Tween 20; and (x) *p*-nitrophenyl phosphate disodium (Sigma) diluted in diethanolamine buffer was added, the plates were incubated at 37°C for 2 h and the optical density (OD) of *p*-nitrophenol yellow chromogen was measured at 405 nm using a multichannel spectrophotometer (Dynatech). Background OD was measured for each test IFN sample by elimination of the initial step (murine monoclonal antibody to IFN-γ), and the value was subtracted from the OD obtained when all the steps were included. Serial twofold dilutions of human reference IFN-γ (Gg 23-901-530, 4,000 IU/ml; National Institutes of Health, Bethesda, Md.) were included in each experiment and a standard curve of $\log_{10}$ (IFN-γ [international units per milliliter]) versus $\log_{10}$ (OD) was plotted (the correlation coefficient for the linear regression was always >0.990). The IFN-γ level (in international units per milliliter) in each cell supernatant fluid was then determined by comparison of the OD for the sample and the corresponding point on the standard curve.

Statistical methods. Statistical tests included chi-square analysis with Yates' correction and two-tailed Fisher exact test for two-by-two comparisons. Differences in prevaccination and postvaccination mean percent specific lysis and mean log (IFN-γ level), and logarithmically transformed prevaccination serum HAI and IgG reciprocal antibody titers were compared by using the Wilcoxon signed ranks test for paired data and Mann-Whitney U test for independent data samples. Arithmetic means are reported with the standard error of the mean.

RESULTS

Patient population. Fifteen chronically ill volunteers (mean age, 64.3 ± 1.1 years; range, 57 to 71 years) were evaluated for in vitro PBL anti-influenza cytotoxicity, in vitro PBL IFN-γ production, serum and nasal wash anti-HA antibodies, and nasal wash shedding of vaccine virus, before and after intranasal inoculation of CR-59 (H3N2) virus. Seven had severe COPD ($FEV_1/FVC < 45\%$), three had moderate COPD ($FEV_1/FVC = 45$ to 59%), three had mild COPD ($FEV_1/FVC = 60$ to 69%), one had small airways disease, and one had normal spirometry. Three volunteers had heart disease requiring chronic chemotherapy, two had neurologic disease (cerebrovascular accident and seizure disorder), two had hypertension, and one each had lung cancer, breast cancer, anemia, diabetes mellitus, and chronic alcohol abuse. Fourteen (93.3%) volunteers were infected by the vaccine virus by criteria including a fourfold rise in serum antibody titer to H3 HA (by ELISA, 9 exhibited rises in measured levels of IgG and IgA to H3 HA, and/or HAI

antibody), a fourfold rise in nasal wash IgA antibody titer to H3 HA (11 volunteers), and/or nasal wash shedding of vaccine virus (5 volunteers). These volunteers are referred to as those who were infected by the vaccine virus. Nine of the infected volunteers had received trivalent inactivated influenza virus vaccine 9 to 24 months previously, and five had never been vaccinated against influenza virus. Of the nine (60%) volunteers (mean age, 64.0 ± 1.5 years) with a fourfold serum antibody titer rise, two had a fourfold rise in HAI antibody titer, nine had a fourfold rise in IgG antibody titer to H3 HA, and six had a fourfold rise in IgA antibody titer to H3 HA. The prevaccination geometric mean serum HAI, IgG, and IgA reciprocal antibody titers of those volunteers with a subsequent fourfold serum antibody rise were lower than those of the six volunteers without a fourfold serum antibody rise (HAI, 12.7 versus 32.0; IgG, 201.4 versus 805.4, IgA, 274.2 versus 1,016), although these differences did not achieve statistical significance. Three of five patients who shed CR-59 virus postvaccination developed a fourfold serum antibody rise, and the other two developed only a fourfold nasal wash antibody rise without a serum antibody response. There was no difference in prevaccination geometric mean reciprocal serum HAI and IgG antibody titers to H3 HA when volunteers who subsequently shed vaccine virus postvaccination were compared with those who did not. Four of the five volunteers who shed vaccine virus had received trivalent inactivated virus vaccine 9 to 24 months previously. The only uninfected volunteer was 65 years old and had severe COPD, no history of influenza A virus vaccination, a prevaccination serum HAI reciprocal antibody titer of 16, and prevaccination IgG and IgA reciprocal antibody titers of 80. The five volunteers (mean age, 64.8 ± 2.1 years) with a fourfold rise in nasal wash antibody titer and no serum antibody response had prevaccination geometric mean HAI, IgA, and IgG reciprocal serum antibody titers higher than the corresponding values for the volunteers with a fourfold serum antibody rise (serum HAI, 36.8 versus 12.7 [not significant]; serum IgG, 1,279 versus 201.4 [$P < 0.05$]; serum IgA, 1,687 versus 274.2 [$P < 0.05$]).

Seven chronically ill volunteers (mean age, 66.6 ± 1.3 years; range, 63 to 73 years) were evaluated for anti-influenza cytotoxicity, IFN-γ, and serum antibody responses before and after parenteral inactivated influenza A/Taiwan/1/86 (H1N1) virus vaccine administration. Three volunteers had COPD (one had severe COPD, and two were unable to cooperate with pulmonary function testing), three had diabetes mellitus, three had coronary artery disease, two had hypertension, and two had neurologic diseases (Parkinson's disease and a history of cerebrovascular accidents). Five (71%) volunteers (mean age, 67.8 ± 1.4 years) had a fourfold rise in serum antibody titer to H1 HA (five by HAI antibody, three by IgG antibody, and two by IgA antibody). Two of these five had received trivalent inactivated influenza virus vaccine 12 months previously, and three had never received influenza virus vaccine. The prevaccination geometric mean reciprocal serum antibody titers to H1 HA of the volunteers who experienced a fourfold antibody titer rise were similar to the corresponding values of the two vaccinees who did not experience a fourfold serum antibody rise (HAI, 21.1 versus 22.6; IgG, 2,228 versus 2,559; IgA, 242.7 versus 320.0). Of the two volunteers who did not have a fourfold antibody rise, one had COPD, Parkinson's disease, coronary artery disease, and a history of chronic alcohol abuse, and the other had coronary artery disease and a history of a cerebrovascular accident. Both had received trivalent inactivated influenza virus vaccine 12 months previously.

Anti-influenza cytotoxicity of PBL from CR-59 virus vaccine recipients. Effector PBL were in vitro stimulated with influenza A/Korea (H3N2) CR-59 virus-infected stimulator cells. The mean percent specific lysis by PBL from volunteers infected with CR-59 vaccine virus significantly increased at 14 days postvaccination against influenza A/Korea (H3N2)-infected target autologous PBL and at 14 and 28 days postvaccination against influenza A/Chile (H1N1)-infected target autologous PBL (Table 1). There was no significant change in mean percent specific lysis of control uninfected target cells which were incubated in vitro with uninfected allantoic fluid rather than with allantoic fluid containing live influenza A virus. Significant rises in mean percent specific lysis of the H3N2 and H1N1 influenza A virus-infected target cells occurred in the cases of volunteers exhibiting a fourfold serum antibody rise and in those with a fourfold nasal wash antibody rise (Table 1).

Prevaccination and 28 day postvaccination percent specific lysis of H3N2 infected targets minus percent specific lysis of control uninfected targets did not correlate with concomitant reciprocal serum antibody titers to H3 HA by linear regression and the Kendall Tau test.

Effector PBL were in vitro stimulated with influenza A/Chile (H1N1) virus-infected stimulator PBL. The mean percent specific lysis by these PBL from volunteers infected with CR-59 virus significantly increased 14 days postvaccination against both H3N2 and H1N1 virus-infected target autologous PBL (Table 2). There was no significant change in mean percent specific lysis of control uninfected target cells. Significant rises in mean percent specific lysis of influenza A virus-infected target cells occurred in the case of volunteers exhibiting a fourfold serum antibody rise (Table 2).

Prevaccination mean percent specific lysis of H3N2- and H1N1-infected autologous target cells by PBL in vitro stimulated by H3N2 virus was low. No difference was detectable in mean percent specific lysis of target cells when those who subsequently shed vaccine virus were compared with those who did not [(H3N2 virus-infected targets) − (control uninfected targets), 4.1% ± 4.2% versus 4.6% ± 2.3% (not significant); (H1N1 virus-infected targets) − (control uninfected targets), 2.0% ± 1.0% versus 7.3% ± 3.0% (not significant)].

For all CR-59 vaccinees, the values for prevaccination and postvaccination mean minimum release of radiolabel as a percentage of mean maximum release were similar for each target cell population (H3N2 infected, prevaccination, 33.0%, 14 and 28 days postvaccination, 27.6 and 29.6%; H1N1 infected, prevaccination, 35.9%, 14 and 28 days postvaccination, 29.5 and 32.9%; uninfected, prevaccination, 27.5%, 14 and 28 days postvaccination, 23.2 and 22.4%). Thus, it is unlikely that the observed changes in percent specific lysis postvaccination were due to variability in the assay caused by differences in minimum (spontaneous) ^{51}Cr release as a proportion of maximum release.

Anti-influenza cytotoxicity of PBL from influenza A/Taiwan (H1N1) virus vaccine recipients. Effector PBL were in vitro stimulated with influenza A/Taiwan (H1N1) virus-infected stimulator PBL. The mean percent specific lysis by these PBL from volunteers with a fourfold rise in serum antibody titer significantly increased 14 days postvaccination against only the H1N1-infected target autologous PBL (Table 3). There were no significant increases in mean percent specific lysis against the H3N2-virus infected or the control unin-

TABLE 1. Lysis of autologous target PBL by PBL effectors stimulated in vitro by influenza A/Korea/1/82(H3N2) CR-59 virus measured before and after intranasal inoculation of volunteers with CR-59 virus (effector/target ratio, 50:1)

CR-59 vaccinee group	No. of vaccinees	Time before or after vaccination	Specific lysis (%) of target PBL (mean ± SEM)		
			H3N2-infected targets[a]	H1N1-infected targets[b]	Control targets[c]
Infected with CR-59 virus	14	Before	10.8 ± 3.2	10.3 ± 3.2	6.6 ± 1.7
		14 days after	26.5 ± 5.9[d]	31.4 ± 5.6[e]	8.7 ± 2.2
		28 days after	16.9 ± 2.9	28.3 ± 5.6[d]	7.1 ± 1.8
Fourfold serum antibody rise to CR-59 virus	9	Before	14.6 ± 4.3	13.6 ± 4.5	8.9 ± 1.9
		14 days after	24.4 ± 7.8	31.6 ± 6.6[d]	9.1 ± 2.8
		28 days after	17.5 ± 3.2	28.5 ± 6.1	7.6 ± 2.5
Fourfold nasal wash antibody rise to CR-59 virus	11	Before	7.7 ± 3.2	9.5 ± 4.0	6.9 ± 2.9
		14 days after	30.4 ± 7.1[d]	35.2 ± 6.1[e]	9.4 ± 2.6
		28 days after	17.8 ± 3.7	30.3 ± 7.0[d]	7.7 ± 2.3
Fourfold nasal wash antibody rise to CR-59 virus without serum antibody rise	5	Before	3.8 ± 2.5	4.3 ± 2.1	2.6 ± 2.6
		14 days after	30.3 ± 9.5[d]	31.1 ± 12.0	8.1 ± 3.8
		28 days after	15.7 ± 6.4	27.8 ± 12.2	6.2 ± 2.5
Uninfected	1	Before	3.2	11.0	1.2
		14 days after	5.4	5.3	6.0
		28 days after	1.7	0.0	3.3

[a] Target PBL infected with influenza A/Korea (H3N2) CR-59 virus.
[b] Target PBL infected with influenza A/Chile (H1N1) virus.
[c] Target PBL incubated with allantoic fluid and uninfected.
[d] Postvaccination value higher than prevaccination value in the respective vaccinee group and category ($P < 0.05$).
[e] Postvaccination value higher than prevaccination value in the respective vaccinee group and category ($P < 0.01$).

fected target autologous cells. Prevaccination and postvaccination (at 28 days) percent specific lysis of H1N1-infected targets minus percent specific lysis of control uninfected targets did not correlate with concomitant reciprocal serum antibody titers to H1 HA by linear regression and the Kendall Tau test.

Mean percent specific lysis of H3N2 and H1N1 virus-infected autologous target cells did not increase postvaccination when effector PBL were in vitro stimulated with influenza A/Korea (H3N2) virus (Table 4). Thus, increased levels of crossreactive memory anti-influenza cytotoxicity were not induced by the inactivated virus vaccine.

TABLE 2. Specific lysis of autologous target PBL by PBL effectors stimulated in vitro by influenza A/Chile (H1N1) virus measured before and after intranasal inoculation of volunteers with influenza A/Korea/1/82 (H3N2) CR-59 virus (effector/target ratio, 50:1)

CR-59 vaccinee group	No. of vaccinees[a]	Times before or after vaccination	Specific lysis (%) of target PBL (mean ± SEM)		
			H3N2-infected targets[b]	H1N1-infected targets[c]	Control targets[d]
Infected with CR-59 virus	11	Before	4.8 ± 1.6	10.6 ± 3.9	3.7 ± 1.3
		14 days after	22.7 ± 5.4[e]	32.8 ± 7.2[e]	4.9 ± 1.8
		28 days after	16.1 ± 4.7	25.7 ± 7.4	7.6 ± 1.4
Fourfold serum antibody rise to CR-59 virus	9	Before	6.1 ± 2.0	11.6 ± 4.8	4.3 ± 1.4
		14 days after	25.8 ± 6.8[e]	31.6 ± 9.1	4.0 ± 1.8
		28 days after	14.4 ± 5.1	24.7 ± 8.3	7.4 ± 2.0
Fourfold nasal wash antibody rise to CR-59 virus	9	Before	6.2 ± 2.0	11.7 ± 5.1	2.3 ± 1.3
		14 days after	17.3 ± 6.1	31.3 ± 9.4	4.3 ± 2.1
		28 days after	16.4 ± 6.1	23.1 ± 8.1	8.9 ± 1.6
Fourfold nasal wash antibody rise to CR-59 virus without serum antibody rise	3	Before	1.1 ± 1.2	7.6 ± 7.6	
		14 days after	14.4 ± 7.2	37.9 ± 1.7	
		28 days after	20.6 ± 12.5	28.3 ± 18.4	8.1 ± 0.8
Uninfected	1	Before	0.0	2.1	3.5
		14 days after	0.0	2.0	3.2
		28 days after	0.0	0.0	0.0

[a] Data available for 12 of the 15 CR-59 virus vaccinees.
[b] Target PBL infected with influenza A/Korea (H3N2) CR-59 virus.
[c] Target PBL infected with influenza A/Chile (H1N1) virus.
[d] Target PBL incubated with allantoic fluid and uninfected.
[e] Postvaccination value higher than prevaccination value in the respective vaccinee group and category ($P < 0.05$).

TABLE 3. Specific lysis of autologous target PBL by PBL effectors stimulated in vitro by influenza A/Taiwan/1/86 (H1N1) virus measured before and after injection of monovalent influenza A/Taiwan (H1N1) inactivated subvirion vaccine (effector/target ratio, 50:1)

Vaccinee group	No. of vaccinees	Times before or after vaccination	Specific lysis (%) of target PBL (mean ± SEM)		
			H3N2-infected targets[a]	H1N1-infected targets[b]	Control targets[c]
All Taiwan/H1N1 vaccinees	7	Before	17.5 ± 5.2	17.6 ± 5.5	7.9 ± 2.3
		14 days after	23.8 ± 7.9	37.0 ± 7.1[d]	7.2 ± 4.0
		28 days after	9.1 ± 4.5	22.5 ± 5.5	5.1 ± 1.9
Fourfold serum antibody rise to Taiwan/H1N1	5	Before	15.8 ± 7.2	10.7 ± 3.8	8.7 ± 3.0
		14 days after	19.3 ± 10.2	29.0 ± 6.5[e]	2.8 ± 1.9
		28 days after	7.5 ± 5.5	17.3 ± 6.1	3.5 ± 1.6
No fourfold serum antibody rise to Taiwan/H1N1	2	Before	22.0 ± 3.0	34.9 ± 5.7	6.3 ± 3.2
		14 days after	32.9 ± 9.6	53.0 ± 7.7	16.1 ± 7.0
		28 days after	13.3 ± 6.9	35.7 ± 2.6	9.0 ± 3.6

[a] Target PBL infected with influenza A/Korea (H3N2) CR-59 virus.
[b] Target PBL infected with influenza A/Taiwan (H1N1) virus.
[c] Target PBL incubated with allantoic fluid and uninfected.
[d] Postvaccination value higher than prevaccination value in the respective vaccinee group and category ($P = 0.06$).
[e] Postvaccination value higher than prevaccination value in the respective vaccinee group and category ($P < 0.05$).

For all influenza A/Taiwan (H1N1) vaccinees, the prevaccination and postvaccination mean minimum release of radiolabel as a percentage of mean maximum release were similar for each target cell population (H3N2 infected, prevaccination, 27.7%, 14 and 28 days postvaccination, 28.5 and 24.9%; H1N1 infected, prevaccination, 29.7%, 14 and 28 days postvaccination, 28.5 and 27.3%; uninfected, prevaccination, 19.6%, 14 and 28 days postvaccination, 24.5 and 23.2%). Thus, it is unlikely that the observed changes in percent specific lysis postvaccination were due to variability in the assay caused by differences in minimum (spontaneous) ^{51}Cr release as a proportion of maximum release.

Changes in the proportion of vaccinees with specific levels of anti-influenza cytotoxicity postvaccination. Of the 14 volunteers infected with CR-59 virus, percent specific lysis of H3N2 virus- and H1N1-virus infected autologous targets by H3N2 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 10% against both target populations measured prevaccination in 3 (21%) volunteers each and was greater than 10% against both target cell populations when measured 14 or 28 days postvaccination in 12 (86%) volunteers each, ($P < 0.001$, comparing postvaccination with prevaccination proportions). Percent specific lysis of H3N2 virus- and H1N1 virus-infected autologous targets by H3N2 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 20% measured prevaccination in 2 (14%) and 1 (7%) volunteers, respectively, and was greater than 20% when measured at 14 or 28 days postvaccination in 8 (57%) and 9 (64%) volunteers, respectively ($P < 0.05$ and $P < 0.01$, comparing postvaccination with prevaccination proportions).

Of the 11 volunteers with a fourfold nasal wash antibody titer rise to H3 HA after CR-59 virus inoculation, percent specific lysis of H3N2 virus- and H1N1 virus-infected autologous targets by H3N2 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 10% measured prevaccination in 1 (9%) and 3 (27%) volunteers, respectively, and was greater than 10% when measured 14 days and/or 28 days postvaccination in 10 (91%) and 10 (91%) volunteers, respectively ($P < 0.01$ and $P < 0.01$, comparing postvaccination proportions with prevaccination). Percent specific lysis of H3N2 virus- and H1N1

TABLE 4. Specific lysis of autologous target PBL by PBL effectors stimulated in vitro by influenza A/Korea/1/82 (H3N2) CR-59 virus measured before and after injection of monovalent influenza A/Taiwan/1/86 (H1N1) inactivated virus vaccine (effector/target ratio, 50:1)

Vaccinee group	No. of vaccinees	Time before or after vaccination	Specific lysis (%) of target PBL (mean ± SEM)		
			H3N2-infected targets[a]	H1N1-infected targets[b]	Control targets[c]
All Taiwan/H1N1 vaccinees	7	Before	15.6 ± 4.7	18.8 ± 6.4	6.3 ± 2.3
		14 days after	8.3 ± 2.0	21.1 ± 5.8	6.9 ± 3.6
		28 days after	3.1 ± 1.1[d]	11.1 ± 2.9	3.1 ± 1.9
Fourfold serum antibody rise to Taiwan/H1N1	5	Before	16.8 ± 7.4	16.5 ± 9.7	5.5 ± 3.4
		14 days after	6.8 ± 2.6	16.4 ± 6.8	4.6 ± 2.0
		28 days after	2.2 ± 1.4[d]	12.7 ± 3.7	1.7 ± 1.1
No fourfold serum antibody rise to Taiwan/H1N1	2	Before	13.3 ± 1.1	23.4 ± 3.4	7.5 ± 2.7
		14 days after	11.2 ± 1.7	30.5 ± 7.1	11.6 ± 8.2
		28 days after	5.3 ± 0.85	7.2 ± 2.6	6.9 ± 4.8

[a] Target PBL infected with influenza A/Korea (H3N2) CR-59 virus.
[b] Target PBL infected with influenza A/Taiwan (H1N1) virus.
[c] Target PBL incubated with allantoic fluid and uninfected.
[d] Prevaccination value higher than 28 day postvaccination value in the respective vaccinee group and category ($P < 0.05$).

TABLE 5. HLA class I major histocompatibility complex restriction of anti-influenza cytotoxicity in vaccinees without shared HLA class I loci[a]

Vaccinee group	Class I HLA type						Influenza A virus subtype		% Specific lysis of mismatched target cells	% Specific lysis of autologous target cells[b]
	Effector cells and autologous target cells			Mismatched target cells			Stimulating effector cells in vitro	Infecting target cells in vitro		
	A	B	C	A	B	C				
CR-59	1, 3	45, 49	w6	2, 24	5, 14	w5	H1N1	H1N1	39.3	60.4
	3, 28	7, w53	w4, w7	2, 23	44	–[c]	H3N2	H3N2	13.8	11.9
	3, 28	7, w53	w4, w7	2, 23	44	–[c]	H3N2	H1N1	11.1	11.4
	2, 23	44	–[c]	3, 28	7, w53	w4, w7	H3N2	H3N2	10.5	12.9
	2, 23	44	–[c]	3, 28	7, w53	w4, w7	H3N2	H1N1	3.7	12.7
Taiwan/H1N1	2, 28	37, 44	w6	1, 30	8, w53	w4, w7	H1N1	H1N1	10.5	45.2
	2, 28	37, 44	w6	1, 30	8, w53	w4, w7	H1N1	H3N2	7.1	47.0
	2, 28	37, 44	w6	1, 30	8, w53	w4, w7	H3N2	H3N2	7.7	12.1
	2, 28	37, 44	w6	1, 30	8, w53	w4, w7	H3N2	H1N1	8.1	19.5

[a] Effector cells were collected 14 days postvaccination in each case.
[b] Target cells autologous to the effector cells.
[c] Nonreactive at C locus.

virus-infected autologous targets by H3N2 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 20% against both target cell populations measured prevaccination in one (9%) volunteer each and was greater than 20% against both target cell populations when measured at 14 or 28 days postvaccination in eight (73%) volunteers each ($P < 0.01$, comparing postvaccination proportions with prevaccination).

Of the seven volunteers who received the inactivated influenza A virus vaccine, percent specific lysis of H1N1 virus- and H3N2 virus-infected autologous targets by H1N1 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 10% measured prevaccination in two (29%) and three (43%) volunteers, respectively, and was greater than 10% when measured at 14 or 28 days postvaccination in six (86%) and four (57%), respectively. Percent specific lysis of H1N1 virus- and H3N2 virus-infected autologous targets by H1N1 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 20% measured prevaccination in one (14%) and 2 (29%) volunteers, respectively, and was greater than 20% when measured at 14 or 28 days postvaccination in four (57%) and three (43%) volunteers, respectively. Of the five inactivated virus vaccinees with a fourfold serum antibody titer rise to H1 HA postvaccination, percent specific lysis of H1N1 virus- and H3N2 virus-infected autologous targets by H1N1 virus-stimulated PBL minus percent specific lysis of control uninfected targets was greater than 10% measured prevaccination in zero (0%) and two (40%) volunteers, respectively, and was greater than 10% when measured at 14 or 28 days postvaccination in four (80%) and two (40%) volunteers, respectively ($P < 0.05$ and not significant, comparing postvaccination with prevaccination proportions).

Thus, the proportion of CR-59 virus vaccinees with a higher level of percent specific lysis against autologous targets infected with virus homologous and heterosubtypic to CR-59 virus minus percent specific lysis of control uninfected targets increased significantly postvaccination. The proportion of inactivated virus vaccinees with a higher level of percent specific lysis against autologous targets infected with virus homologous to vaccine virus minus control uninfected target lysis increased postvaccination; however, there was no increase against autologous targets infected with virus heterosubtypic to the subvirion vaccine virus minus control uninfected target lysis.

HLA class I restriction of cytotoxicity. HLA restriction was studied utilizing PBL effectors from 11 vaccinees, seven of whom received the CR-59 virus vaccine and four the inactivated influenza A/Taiwan (H1N1) subvirion vaccine. These studies were done whenever adequate numbers of effector cells were available to measure cytotoxicity against HLA class I mismatched in addition to autologous target PBL. In general, when percent specific lysis of autologous target cells by effector cells obtained both pre- and postvaccination was greater than approximately 12%, the percent specific lysis of the HLA class I mismatched target cells was lower (Tables 5, 6, and 7). This was also generally true whether the effector cells were in vitro induced with H3N2 or H1N1 virus-infected stimulator cells. When the percent specific lysis of autologous target PBL was less than 12%, the lysis of HLA-mismatched target cells was similar in magnitude. This suggests that the underlying background nonspecific target cell lysis was between 0 and 12%. This is consistent with the level of mean percent specific lysis of uninfected autologous target cells, which was less than 10% (Tables 1, 2, 3, and 4). The number of effector cells available did not allow measurement of lysis of HLA-mismatched, uninfected target cells by heterologous effector cells. Therefore, these control values could not be included in Tables 5, 6, and 7.

Effector PBL from vaccinees sharing one locus at HLA-A2, HLA-A28, or HLA-B8 with target PBL from other vaccinees did not lyse the mismatched target cells to the same degree as autologous targets (Table 6). The percent reduction in percent specific lysis of mismatched targets sharing one of these three HLA class I loci compared with lysis of autologous targets ranged from 34 to 100% (Table 6). Cytotoxicities of PBL from vaccinees sharing three HLA loci (HLA-B8, 38, and Cw7) differed with respect to HLA restriction (Table 7). Effector PBL from the vaccinee with HLA-A1,30 lysed autologous target cells more efficiently than the heterologous targets from the vaccinee with HLA-A24,28; however, effector PBL from the HLA-A24,28 vaccinee lysed the HLA-A1,30 target cells to the same degree as autologous cells (Table 7). This does not appear to be due to significant non-CTL activity since the HLA-A24,28 effectors

TABLE 6. HLA class I major histocompatibility complex restriction of anti-influenza cytotoxicity in vaccinees with one shared HLA class I locus

Vaccinee group	Class I HLA type[a]						Influenza A virus subtype		% Specific lysis of mismatched target cells	% Specific lysis of autologous target cells[b]	Time (days) pre- or post-vaccination tested
	Effector cells and autologous target cells			Mismatched target cells			Stimulating effector cells in vitro	Infecting target cells in vitro			
	A	B	C	A	B	C					
CR-59	1, **28**	44, w53	w4	3, **28**	w22, w62	w3	H3N2	H1N1	0	21.2	14
	3, **28**	w22, w62	w3	1, **28**	44, w53	w4	H1N1	H1N1	0	16.7	14
	1, **2**	7, 8	w7	**2**	13, w62	w3, w6	H3N2	H3N2	7.9	21.3	14
Taiwan/H1N1	1, 30	**8**, 38	w7	2	**8**, 44	w5	H1N1	H1N1	11.7	42.9	Pre[c]
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H1N1	H1N1	18.7	63.9	14
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H1N1	H1N1	11.9	32.0	28
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H1N1	H3N2	0	26.3	Pre
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H1N1	H3N2	8.5	46.4	14
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H3N2	H1N1	13.6	28.2	Pre
	1, 30	**8**, 38	w7	2	**8**, 44	w5	H3N2	H1N1	6.4	40.6	14
	24, 28	**8**, 38	w7	2	**8**, 44	w5	H1N1	H3N2	0	17.7	Pre
	24, 28	**8**, 38	w7	2	**8**, 44	w5	H1N1	H3N2	11.0	19.4	14
	24, 28	**8**, 38	w7	2	**8**, 44	w5	H1N1	H1N1	13.6	26.8	Pre
	24, 28	**8**, 38	w7	2	**8**, 44	w5	H1N1	H1N1	33.4	42.1	14
	24, 28	**8**, 38	w7	2	**8**, 44	w5	H3N2	H1N1	9.3	18.5	Pre
	2	**8**, 44	w5	1, 30	**8**, 38	w7	H1N1	H1N1	6.1	25.2	14
	2	**8**, 44	w5	1, 30	**8**, 38	w7	H3N2	H1N1	10.3	15.5	28
	2	**8**, 44	w5	1, 30	**8**, 38	w7	H1N1	H3N2	11.8	22.8	Pre
	2	**8**, 44	w5	1, 30	**8**, 38	w7	H1N1	H3N2	0.1	28.4	28
	2	**8**, 44	w5	1, 30	**8**, 38	w7	H3N2	H3N2	0	9.1	14
	2	**8**, 44	w5	24, 28	**8**, 38	w7	H1N1	H3N2	0	22.8	Pre
	2	**8**, 44	w5	24, 28	**8**, 38	w7	H1N1	H3N2	10.7	28.4	28
	2	**8**, 44	w5	24, 28	**8**, 38	w7	H3N2	H3N2	0	9.1	14
	2	**8**, 44	w5	24, 28	**8**, 38	w7	H3N2	H1N1	2.1	15.5	28

[a] Loci in boldface type were shared by effector and mismatched target cells.
[b] Target cells autologous to the effector cells.
[c] Pre, Prevaccination.

did not lyse the targets from another vaccinee sharing only one class I locus (HLA-B8 [Table 6]). Also, it is not due to differences in mean percent minimum (spontaneous) release of radiolabel from the target cells (HLA-A1,30 targets, CR-59 [H3N2] infected, 18.3% and Taiwan/H1N1-infected, 22.6%; HLA-A24,28 targets, CR-59 [H3N2] infected, 26.8%, and Taiwan/H1N1 infected, 29.7%; HLA-A2, B8,44, Cw5 targets, CR-59 [H3N2] infected, 27.5%, and Taiwan/H1N1 infected, 20.4%). The possibility of significant HLA class II-restricted cytotoxicity in these bulk PBL cultures was not investigated. In certain cases, this type of activity may have contributed to the less marked reduction in percent specific lysis of targets which were mismatched to various degrees by class I HLA loci but may not have been mismatched for class II HLA loci, which were not considered in this study. Also, we did not exclude the possibility that target cells of different HLA types may have been infected to various extents by influenza A virus. If there were resultant significant differences in expression of influenza A virus proteins, this could have contributed to differences in percent specific lysis of autologous and HLA-mismatched target cells.

IFN-γ production. Five vaccinees infected with CR-59 virus and one of the inactivated virus vaccinees with a fourfold serum antibody rise had a fourfold postvaccination rise in in vitro IFN-γ level in response to influenza A/H3N2 or H1N1 virus stimulation. The geometric mean IFN-γ levels measured in supernatant fluids of PBL stimulated in vitro with homologous and heterosubtypic influenza A virus-infected autologous PBL and those stimulated with PHA did not change significantly after vaccination with either the CR-59 virus or inactivated virus vaccines (Table 8). In the group which subsequently shed CR-59 virus, the prevaccination geometric mean IFN-γ level in supernatant fluids of PBL stimulated in vitro with CR-59 virus-infected autologous PBL was lower than the level in the volunteers who did not shed virus (17.6 versus 69.2 IU/ml [$P < 0.05$]). There were no differences in geometric mean IFN-γ levels between volunteers who subsequently did and did not shed CR-59 virus when the PBL were stimulated in vitro with influenza A/H1N1 virus-infected autologous PBL or with PHA. Also, the prevaccination geometric mean IFN-γ level to CR-59 virus in the patients who developed a fourfold nasal wash reciprocal antibody titer rise to CR-59 virus without a concomitant fourfold rise in serum antibody was higher than in those volunteers with a fourfold serum antibody rise postvaccination with CR-59 virus (96.2 versus 30.3 IU/ml [not significant]). Although a causal mechanism is not proven and is unlikely, a higher prevaccination in vitro-induced IFN-γ level was associated with a less severe vaccine virus infection manifested by lack of virus shedding or lack of systemic antibody response. There was no significant correlation by linear regression and the Kendall Tau test between IFN-γ levels and concomitant percent specific lysis by the corresponding effector PBL against autologous target cells infected with influenza A virus homologous to the virus used to stimulate the effector PBL.

TABLE 7. HLA class I major histocompatibility complex restriction of anti-influenza cytotoxicity in two vaccinees with three shared HLA class I loci and in one vaccinee whose effector cells were tested against mismatched target cells not HLA typed

Vaccinee group	Class I HLA type[a]						Influenza A virus subtype		% Specific lysis of mismatched target cells	% Specific lysis of autologous target cells[b]	Time (days) pre- or post-vaccination tested
	Effector cells and autologous target cells			Mismatched target cells			Stimulating effector cells in vitro	Infecting target cells in vitro			
	A	B	C	A	B	C					
CR-59	1, 28	44	w6		ND[c]		H3N2	H3N2	2.4	34.8	Pre[d]
	1, 28	44	w6		ND		H1N1	H1N1	22.3	16.0	Pre
	1, 28	44	w6		ND		H1N1	H3N2	7.0	17.5	Pre
Taiwan/H1N1	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H1N1	H1N1	24.2	42.9	Pre
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H1N1	H1N1	21.3	63.9	14
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H1N1	H1N1	24.6	32.0	28
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H1N1	H3N2	15.1	26.3	Pre
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H1N1	H3N2	12.1	46.4	14
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H3N2	H1N1	12.5	28.2	Pre
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H3N2	H1N1	4.1	40.6	14
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H3N2	H3N2	4.8	14.8	Pre
	1, 30	**8, 38**	w7	24, 28	**8, 38**	w7	H3N2	H3N2	4.1	13.6	14
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H1N1	H1N1	34.5	26.8	Pre
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H1N1	H1N1	51.7	42.1	14
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H1N1	H3N2	8.9	17.7	Pre
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H1N1	H3N2	29.3	19.4	14
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H3N2	H1N1	33.5	18.5	Pre
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H3N2	H1N1	32.8	20.4	14
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H3N2	H3N2	5.0	11.8	Pre
	24, 28	**8, 38**	w7	1, 30	**8, 38**	w7	H3N2	H3N2	12.4	8.8	14

[a] Loci in boldface type were shared by effector and mismatched target cells.
[b] Target cells autologous to the effector cells.
[c] HLA class I typing not available for these cells from one vaccinee.
[d] Pre, Prevaccination.

DISCUSSION

This study is important because of the older, chronically ill patients enrolled and demonstration of cross-reactive anti-influenza cytotoxicity induced by live-attenuated influenza A virus infection but not by the inactivated virus vaccine injection. These patients would be expected to have had experience with both H1N1 and H3N2 virus subtypes due to either previous wild-type virus infection or immunization. Despite the likely prior exposure to influenza A virus, prevaccination levels of in vitro-stimulated influenza A virus cytotoxicity were relatively low, close to the magnitude of background killing of uninfected autologous target cells. This low level of preexisting immunity may have contributed to the susceptibility of most recipients to the live-attenuated vaccine virus infection. The prevaccination mean percent specific lysis of influenza A virus-infected targets was low in both the persons with and without documented nasal shedding of live-attenuated virus, so we could not correlate reduced virus shedding with preexisting level of cytotoxicity.

Prevaccination mean serum anti-influenza virus antibody titers were higher in those CR-59 vaccinees who subsequently manifested vaccine virus infection by only a nasal wash antibody titer rise than among those who developed a fourfold serum antibody rise to vaccine virus. A higher prevaccination mean serum antibody titer was also found in those without a fourfold antibody titer rise to vaccine virus compared with those who experienced a fourfold serum antibody titer rise. Thus, preexisting serum antibody to HA was associated with prevention of systemic, serum antibody response as was the prevaccination IFN-γ level. There was no statistical difference in prevaccination serum antibody

TABLE 8. IFN-γ levels in supernatant fluids of PBL stimulated with influenza A viruses and PHA[a]

Vaccinee group	No. of vaccinees	Inducing agent	Geometric mean IFN-γ level (IU/ml)		
			Prevaccination	14 days postvaccination	28 days postvaccination
CR-59 virus vaccine					
Infected	14	H3N2	45.8	48.4	65.5
		H1N1	16.7	36.7	49.8
		PHA	219	189	375
Fourfold serum antibody rise	9	H3N2	30.3	30.7	38.5
		H1N1	9.3	20.0	28.5
		PHA	242	181	474
Fourfold nasal wash antibody rise without serum antibody rise	5	H3N2	96.2	110	170
		H1N1	96.4	173	265
		PHA	176	214	220
Uninfected	1	H3N2	23.7	38.2	37.4
		H1N1	41.5	30.9	7.6
		PHA	355	NT[b]	390.2
Inactivated virus vaccine					
Fourfold serum antibody rise	5	H3N2	130	80.9	130
		H1N1	167	221	282
		PHA	598	417	630
No serum antibody rise	2	H3N2	31.2	25.4	56.9
		H1N1	41.1	26.5	68.7
		PHA	189	163	184

[a] PBL collected from recipients of live-attenuated influenza A/Korea/1/82 (H3N2) CR-59 virus or inactivated influenza A/Taiwan/1/86 (H1N1) virus vaccine.
[b] NT, Not tested.

titers to vaccine virus among patients who subsequently did or did not shed vaccine virus in nasal secretions.

The enhanced mean percent specific lysis of influenza A virus-infected autologous target cells following live-attenuated influenza virus infection was more extensive than that after the inactivated virus injection. The CR-59 virus vaccine infection was associated with an in vitro inducible cytotoxicity which was cross-reactive against autologous target cells infected with virus both homologous and heterosubtypic to the vaccine virus. This enhanced cross-reactive cytotoxicity postvaccination was inducible in vitro with both the vaccine virus and with heterosubtypic influenza A virus-infected autologous stimulator cells. This may have been due to stimulation of memory cytotoxic T-cell populations with specificity for internal virus proteins shared by the H3 and H1 viruses, such as matrix protein, nucleoprotein, and viral polymerase, and for possible shared T-cell epitopes on H3 and H1 hemagglutinin molecules. Cross-reactive $CD8^+$ HLA class I-restricted clones have been characterized to have specificity for viral nucleoprotein (31), and in general, cross-reactive cytotoxic T-cell activity is due to $CD8^+$ cells and is class I restricted (5, 18, 20, 22). $CD4^+$ HLA class II major histocompatibility complex-restricted clones with specificity for viral M protein and nucleoprotein with cross-reactivity against heterosubtypic influenza A viruses have also been described previously (13). Perhaps the recent CR-59 virus infection resulted in the presence of $CD4^+$-inducible cytotoxic T cells in the in vitro bulk PBL cultures in some cases, unlike what has been reported in bulk cultures of PBL obtained from individuals without recent in vivo influenza virus infection or vaccination (6).

The rise in percent specific lysis seen postvaccination was most likely not due to natural killer cell activity, because prevaccination in vitro stimulation with influenza A virus resulted in relatively low levels of influenza virus-infected target lysis, no postvaccination rise in lysis of target cells infected with heterosubtypic virus by PBL from inactivated virus vaccinees occurred, and the cytotoxicity demonstrated HLA restriction in general. The presence of significant levels of antibody-dependent cell-mediated cytotoxicity is also unlikely for the same reasons, and also because the cells were washed with medium containing FBS prior to culturing the effector and target PBL populations together in the anti-influenza cytotoxicity assay plates and subsequent incubation during the cytotoxicity assay was in medium supplemented with FBS. In vitro production of antibody to influenza A virus which could result in antibody-dependent cell-mediated cytotoxicity has been reported by Greenberg et al. (17) but was seen after overnight incubation. The shorter duration of incubation of effector cells with target cells in our assay also makes antibody-dependent lysis less likely.

A rise in mean percent specific lysis of influenza A virus-infected autologous target cells was seen postinfection with live-attenuated virus both in vaccinees who had only a fourfold nasal wash antibody titer rise and in those with a serum antibody titer rise. This suggests that only mild viral infection in the nasal mucosa resulted in an increase in circulating PBL with in vitro inducible cytotoxicity against influenza A virus. This is encouraging with respect to the ability of the limited, subclinical live-attenuated influenza A virus infection observed in this population to induce a wide ranging immunologic response which could be protective. Ennis et al. (12) reported a rise in memory cytotoxic T-cell activity following live virus vaccination in some persons whose serum anti-influenza antibody titer did not rise. Nasal wash antibody titers and viral shedding were not reported, but one can speculate that these may have been indicative of virus infection if they had been measured.

The inactivated virus vaccine also resulted in enhanced in vitro inducible cytotoxicity against autologous target cells infected with influenza A virus homologous to the vaccine virus strain, but not against target cells infected with heterosubtypic virus. The postvaccinal enhancement in cytotoxicity occurred after in vitro induction with virus homologous to the inactivated virus vaccine strain, but not after induction with heterosubtypic virus-infected stimulator PBL. Thus, the anti-influenza cytotoxicity stimulated by the inactivated subvirion vaccine was more limited compared with that following live-attenuated virus infection. Possible explanations for the differences observed are as follows: (i) influenza A virus of the H3N2 subtype was better able to stimulate cross-reactive responses than the H1N1 subtype or (ii) intranasal infection with live-attenuated virus was better able to stimulate cross-reactive responses than subvirion inactivated virus vaccine administered intramuscularly (different presentation of vaccine virus antigen in vivo). We were unable to determine from our data which is the most likely explanation.

There are several concerns which should be included in the assessment of the design, implementation, and biological significance of this study. The results of cytotoxicity assays are affected by a number of variables. Our two vaccine groups were studied sequentially, not in parallel, and fresh PBL were used in all of the assays. We did not exclude the possibility that PBL of different HLA type had various degrees of susceptibility to infection with influenza A virus. The level of minimum release of radiolabel from target cells which we considered acceptable in this study was higher than upper limits set in other investigations, although some human studies have not commented on this technical aspect at all (11, 12, 23). The major effect of higher minimum release is to reduce the sensitivity of detection of target cell lysis if present and reduce the calculated percent specific lysis value reported. The ranges of both minimum and maximum release were wide, but the mean percent minimum release was similar before and after vaccination for each influenza A virus-infected target cell population and similar between target cell populations for all determinations in the study.

Not excluded was the possibility that the influenza A virus-infected stimulator cells added to the effector cell cultures later interfered with the cytotoxicity assay by serving as cold, unlabeled targets. Our method of in vitro stimulation of effector cells was the same as that reported in the literature (11, 12, 22, 23). In addition, it is known that influenza A virus infection of monocytes and lymphocytes is abortive without the production of new infectious virions so that no more than the initially infected stimulator cells could have been infected in the effector cell cultures (24, 30). If any of these stimulator cells were still present after 7 days of incubation and if they interfered with effector cell recognition and lysis of radiolabeled target cells, this could have reduced the sensitivity of the assay, but it was a constant factor throughout the study.

The different responses to the live-attenuated and inactivated virus vaccines suggest a clinical advantage for the live-attenuated virus if these differences were not just simply due to the different viral subtypes in the two vaccines. A broader, cross-reactive cytotoxic T-cell response has also been reported following live virus infection compared with inactivated virus injection in the murine model (3, 27, 34). A murine cytotoxic T-cell clone with antigen specificity for a

shared epitope on the hemagglutinin molecule lysed target cells infected with H1 and H2 virus strains and significantly reduced virus titers in the lungs of mice infected with the H1 and H2 virus strains, but not those infected with an H3 subtype in vivo (19). This provides evidence for the possible clinical importance of cross-reactive cytotoxic T cells and the desirability of their induction by an influenza A virus vaccine.

Human influenza A virus-specific, HLA-restricted cytotoxic T-cell activity is stimulated by in vivo administration of live-attenuated influenza A virus, wild-type influenza A virus, and inactivated influenza A virus in young adults (11, 29). In young adults, in vitro inducible cytotoxic T-cell-mediated percent specific lysis of autologous target cells infected with influenza A H1N1 virus of at least 10% correlated with protection from experimental wild-type influenza A H1N1 virus infection manifested by reduced viral shedding but not reduced symptoms of viral infection (23). Postvaccination levels of percent specific lysis by H3N2 and H1N1 virus-stimulated effector cells from CR-59 (H3N2) vaccinees against targets infected with H3N2 and H1N1 virus and by H1N1 virus-stimulated effector cells from inactivated virus vaccinees against targets infected with H1N1 virus were well above 10% in our study after subtracting percent specific lysis of control uninfected targets. Our methods were similar to those of McMichael et al. (23), but whether a specific level of percent specific lysis in their study is directly comparable to the same numerical level in our study with respect to correlation with protection from challenge virus was not determined. Also, it is not well established what constitutes a significant rise in percent specific lysis in the individual patient in response to vaccination.

Ennis et al. (11) reported that peak in vitro inducible cytotoxicity occurred in young healthy adults 14 days after vaccination with live-attenuated and inactivated influenza A virus vaccines. However, they did not report whether there were differences in the induction of cross-reactive cytotoxicity against heterosubtypic influenza A virus-infected autologous target PBL with one mode of vaccination compared with the other. Virus-infected target cells showing certain HLA class I antigens (A1, A9, and B8) were less susceptible to lysis by HLA-matched cytotoxic T cells than target cells which matched effectors at other HLA loci.

The live-attenuated, cold-adapted, temperature-sensitive reassortant viruses retain their internal genes (encoding PA, M protein, PB2, and PB1) from the cold-adapted parent influenza A virus and acquire the RNA segments which encode the HA and NA glycoproteins from the wild-type virus (32). There may be antigenic differences in internal proteins among wild-type influenza A viruses and the donor cold-adapted influenza A parent virus. Since cross-reactive cytotoxic T cells are often directed against internal proteins, the live-attenuated virus may not always induce a cytotoxic T-cell response against the contemporary wild-type influenza A virus strains.

Mean IFN-γ levels in supernatant fluids of effector PBL cultures did not increase significantly postvaccination in either of our vaccinee groups. IFN-γ levels did not correlate with the concomitant effector cell killing of infected autologous target cells. A higher prevaccination IFN-γ level, however, was associated with a less severe CR-59 vaccine virus infection manifested by lack of viral shedding and lack of a serum anti-influenza antibody titer rise. This cannot be considered a causal mechanism, since IFN-γ was measured after 7 days of in vitro culture. The ability of PBL to produce IFN-γ in response to in vitro stimulation may, however, be a marker for an immunologic mechanism which resulted in the less severe vaccine virus infection. Yamada et al. (37), using methods similar to ours, reported IFN-γ levels correlated with cytotoxic T-cell activity by in vitro-stimulated PBL collected at one time point from 12 normal healthy adults apparently not recently vaccinated or infected with influenza A virus. The older, chronically ill population we studied was different from theirs and may have contributed to the difference in results between the two studies with respect to correlation between IFN-γ production and anti-influenza cytotoxicity. Addition of anti-IFN-γ antibody to effector cell cultures by Yamada et al. (37) did not inhibit the development of in vitro cytotoxic T-cell activity, suggesting that the generation of cytotoxic T-cell activity was not IFN-γ dependent.

In summary, we have detected significant enhancement of in vitro-inducible, HLA-restricted anti-influenza A virus cytotoxicity after vaccination with live-attenuated influenza A virus and monovalent inactivated influenza A virus vaccines in patients at risk for severe influenza A virus infection and influenza A virus-related morbidity. The enhancement of cytotoxicity cross-reactive against heterosubtypic influenza A viruses after the live-attenuated CR-59 virus infection would be an advantage of this vaccination approach over that of inactivated monovalent virus vaccine injection, if it is long-lasting, protective, and actually due to type of vaccine and mode of administration.

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