Published in final edited form as:

Vaccine. 2009 January 7; 27(2): 319. doi:10.1016/j.vaccine.2008.09.092.

In vitro evidence that commercial influenza vaccines are not similar in their ability to activate human T cell responses¹

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Abstract

We evaluated three commercial trivalent inactivated vaccines (TIVs) from the 2007–2008 season in terms of their ability to elicit in vitro T cell responses. T cell-mediated immunity may offer a more cross-reactive vaccine approach for the prevention of pandemic or epidemic influenza. Human cytotoxic T cell lines demonstrated differences in matrix protein 1 and nucleocapsid protein recognition of autologous target cells. Peripheral blood mononuclear cells stimulated with each of the TIVs showed statistically significant differences between the vaccines in the numbers of IFN γ producing cells activated. These data suggest that TIV vaccines are not similar in their ability to activate human T cell responses.

Keywords

T cells; influenza; vaccine

Introduction

Human influenza is a highly contagious acute respiratory illness that is responsible for significant morbidity and excess mortality in the elderly and the very young worldwide. Although effective antiviral medications targeting the neuraminidase (NA) glycoprotein are available, prevention of influenza morbidity and mortality is primarily through the immunization of target groups at high risk for mortality or hospitalization [1]. The World Health Organization (WHO) makes annual recommendations on the composition of the annual influenza vaccine on the basis of surveillance of circulating strains worldwide. Commercial influenza vaccines are evaluated for potency by the content of the surface antigen hemagglutinin (HA) detected by a single radiation diffusion method with 15 μ g of HA required for each component. Inactivated influenza vaccine production differs by manufacturer and the composition of some components in these vaccines has been shown to differ [2].

¹Financial support: This work was supported by Funding from the National Institute of Allergy and Infectious Diseases/National Institutes of Health Grant U19 AI-057319

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Current vaccine approaches depend primarily on the induction of antibodies to the viral surface protein HA that neutralizes the infectivity of the virus and secondarily to the NA protein that interferes with the release of newly replicated virus from the host cell [3,4]. However, murine model studies have demonstrated the importance of CD8⁺ T cells in reducing viral titers and decreasing morbidity [5–7] and the contribution of CD4⁺ T cells in the generation of CD8⁺ memory T cells [8]. Studies to date on the cytotoxic T lymphocyte (CTL) repertoire to influenza A viruses in humans indicate that influenza memory T cell responses are directed to a number of epitopes mostly on internal proteins including the nucleocapsid protein (NP), the nonstructural protein 1 (NS1), and the matrix protein 1 (M1)[9,10]. Most of these highly conserved CD8⁺ T cell cross reactive epitopes have been found to be conserved in H5N1 viruses from recent outbreaks [11]. These murine studies suggest that cell-mediated immunity appears to be important in both restricting influenza A virus replication and reducing disease severity, and potentially may offer a more cross-reactive vaccine approach for the prevention of pandemic or epidemic influenza.

In this study, we evaluated three different commercial TIVs from the 2007–2008 season in terms of their ability to elicit in vitro T cell responses. We performed CTL assays with defined human CD8⁺ and CD4⁺ T cell lines to quantitate specific lysis of autologous vaccine pulsed BLCLs (B lymphoblastoid cell-lines), quantitated the number of IFN γ (interferon gamma) producing cells by enzyme-linked immunosorbent spot (ELISPOT) and quantitated specific cytokine producing cells by intracellular cytokine staining (ICS) assays. These assays were performed to address the following questions related to vaccine induced in vitro T cell immune responses. 1) Do all vaccines elicit similar levels of T cell effector function such as cytolytic activity and numbers of IFN γ cells? 2) Can differences in these T cell functions be explained by the protein content of these vaccines? 3) What are the functional characteristics of the T cells that are responding to these vaccines?

Methods

PBMC donors

Prevaccination blood samples were obtained under an Institutional Review Board (IRB) protocol from 30 healthy individuals (5 males and 25 females) who subsequently received the licensed 2005–2006 trivalent inactivated vaccine manufactured by Sanofi Pasteur comprised of H1N1 A/New Caledonia/20/99 and H3N2 A/California/07/2004 strains. The mean/median age of these 30 PBMC donors was 44.6 years and 44.5 years, respectively, with a range from 26–58 years of age.

PBMC were also obtained approximately 1–2 months post recovery from three individuals (NFLU 001–003) who were diagnosed with clinical influenza A infection during the 2006–2007 influenza season for use in ICS assays. The mean/median age of these PBMC donors was 55.3 years and 57 years respectively. In terms of immunization status, only NFLU003 received the TIV vaccine during the influenza season prior to illness.

PBMC Cryopreservation and Thawing procedure

PBMC are separated from whole blood using Ficoll-Paque medium and cryopreserved using freezing medium consisting of 90% fetal bovine serum (FBS), 10% DiMethylSulfOxide (DMSO). Vials are placed in a Nalgene Cryo 1° Freezing Container (Nalgene) in the bottom of a $\leq -70^{\circ}\text{C}$ mechanical freezer and left undisturbed for a minimum of 4 hours. The frozen vials are removed from the container, placed on dry ice and transferred to a permanent, long-term storage liquid nitrogen tank. To thaw cells, cryogenic vials are placed into a rack in a 37°C water bath. Once the cells are thawed, the outside of the vial is rinsed with 70% ethanol, allowed to dry and cells are transferred immediately to a 15mL tube containing 5 – 10mLs of

pre-warmed RPMI-10 with 20µg/mL DNase. Cells are centrifuged at $400 \times g$ (1400 rpm) for ≥ 5 minutes and the supernatant is aspirated. Cells are re-suspended in 5 – 10mLs of RPMI-10/tube. For all experiments performed, cryopreserved cells were used. Viability of cells after thawing was above 90%.

Vaccines

The 2007–2008 vaccines from three licensed US manufacturers of trivalent inactivated influenza vaccines (TIV) were obtained from a vaccine supplier (Flu Shot Center, Fox River Grove, Illinois) and used in ELISPOT, ICS, and CTL assays. The vaccine manufacturers are designated as Vaccine 1 (Fluzone manufactured by Sanofi Pasteur, Swiftwater, PA), Vaccine 2 (Flulaval manufactured by ID Biomedical Corporation of Quebec, Quebec, Canada and distributed by Glaxo Smith Kline, Belgium) and Vaccine 3 (Fluvirin manufactured by Novartis, United Kingdom) in the text. The virus strains that were used to produce these inactivated vaccines were an A/Solomon Islands/3/2006 (H1N1)-like virus; an A/Wisconsin/67/2005 (H3N2)-like virus; and a B/Malaysia/2506/2004-like virus.

CTL lines

Several previously described influenza epitope specific CTL lines: CD4+ M1₁₇₋₃₁ specific T cell line 1–3 restricted by HLA-DR1, CD8+ NP₃₈₃₋₃₉₁ specific T cell line 1-1 restricted by HLA-B27, CD8+ M1₅₈₋₆₆ specific T cell line 1–7K restricted by HLA-A2 [10] and a newly defined CD4+ HA₂₆₇₋₂₈₃ specific T cell line 3E-2 (Babon et al. manuscript submitted for publication) were established by limiting dilution techniques as previously described [12] and used as effector cells in cytotoxicity assays.

⁵¹Cr release assay

Autologous BLCL were established as previously described [13] for use as target cells in cytotoxicity assays. Target cells were first labeled with 0.25 mCi of ⁵¹Cr for 60 min at 37°C. Following labeling, the cells were washed three times and resuspended at 1.5×10^4 /ml in RPMI containing 10% FBS. Effector cells were added to 1.5×10^3 ⁵¹Cr-labeled target cells at various effector/target cell (E/T) ratios. For peptide pulsed targets, ⁵¹Cr-labeled target cells were incubated with the indicated amount of peptide in 96-well round bottom plates for 30 min at 37°C before the addition of effector cells. The peptides remained in the wells for the duration of the assay. For vaccine pulsed targets, 5µl of the influenza vaccine in 1 ml of RPMI-10 was added to 0.5×10^6 BLCL and incubated at 37°C for 16h, after which the effector cells were added. If a CD8 T cell line was to be used, 5µl of the influenza vaccine was also mixed with 100 µg of ISCOMATRIX™ (ISCOM) in 1 ml of PBS containing 0.1% of BSA, incubated at 4°C for 24 h, and added to 0.5×10^6 BLCL for 1 h [14]. The BLCL was washed and incubated at 37°C for 16h, after which the effector cells were added. Plates were centrifuged at $200 \times g$ for 5 min and incubated for 4 h at 37°C. Supernatants were harvested (Supernatant Collection System, Skatron Instruments) and counted in a Hewlett–Packard counter. Specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. All assays were performed in triplicate. Negative controls included uninfected or unpulsed target cells. Background levels of spontaneous lysis were between 15–32%.

Enzyme-linked immunospot assay to quantitate the frequency of IFN γ producing cells (ELISPOT)

On Day 0, 96-well filtration plates (Millipore, Bedford, MA) were pre-wet with 35% ethanol, washed three times with PBS and coated with 5µg/ml of mouse anti-human IFN γ monoclonal antibody 3420-3-1000 (Mabtech, Cincinnati, Ohio) and incubated overnight at 4°C. On Day 1, plates are washed with PBS three times and PBS containing 10% FBS was added at 100

µl/well for 2 hr at 37°C to block non-specific binding. Prevacination PBMC from each of the 30 donors were thawed and resuspended in RPMI containing 10% FBS supplemented with penicillin-streptomycin, glutamine, and HEPES at 2×10^5 cells/well. Cells were incubated in the plates at 37°C for 20 hours with a final 1:256 concentration (based on the specified 15 µg/ml amount of HA content) of each of the three commercial vaccines. As a positive control, cells were incubated at 37°C for 15 hours with live influenza A/Wisconsin/67/2005X-161B (H3N2) virus, which was kindly provided by Dr. Michel De Wilde and Dr. Robert Ryall from Sanofi Pasteur, at a final dilution of 1:16. The optimal concentrations of the H3N2 virus and the vaccines were determined in preliminary experiments using PBMC from an individual with substantial T cell responses to this influenza strain. Medium was used as a negative control and Phytohemagglutinin (PHA), (final concentration in assay = 20µg/ml) (Sigma, St Louis, MO) and CEF peptide pool 3651-1 (Mabtech, Cincinnati, Ohio) was used as positive controls. On Day 2, the plates were washed and then incubated with biotinylated murine anti-human IFN-γ Antibody 3420-6-1000 (Mabtech, Cincinnati, Ohio). Spots were developed using fresh substrate buffer (NovaRed SK-4800 (Vector Labs, Burlingame, CA). The plates were read by an ImmunoSpot® S4 pro Analyzer and analyzed using ImmunoSpot® 4.0 software (CTL Analyzers LLC, Cleveland, OH). The frequency of peptide-specific IFN-γ-producing cells was calculated as (average number of spots in the virus wells – average number of spots in medium wells/number of cells/well) and converted to the number of IFN-γ-producing cells per 10^6 PBMC. The number of spots in the negative control wells (medium alone) ranged from 0 to 10. Experiments were performed in duplicate.

Intracellular cytokine staining (ICS)

PBMC from three naturally infected individuals with laboratory confirmed influenza were thawed and washed with 5–10ml of prewarmed RPMI 1640 with 10% heat-inactivated human AB serum and 20µg/ml of DNase. Cells were then centrifuged at 1400 rpm for 5 minutes, resuspended in the same medium and then counted. The number of cells per tube was 1×10^6 cells. Vaccine was added at a final dilution 1:250 dilution (determined in preliminary studies using the PBMC of a donor with a detectable response) and incubated for 11–13 hours at 37°C. The following day, phorbol myristate acetate (PMA)-ionomycin was added to the positive control tube and incubated for 15 minutes at 37°C prior to the addition of .7 µl of Golgi Stop and 1 µl of Golgi Plug (BD Pharmingen) to all tubes. Cells were incubated for an additional 5 h at 37°C and then washed with 1ml phosphate buffered saline (PBS) and centrifuged at 1200 rpm for 8 min.

After decanting, 1 µl of Live Dead Aqua (Invitrogen) viability stain/tube was added for 20 minutes at room temperature. Cells were then washed with 1 ml of PBS at 1200 rpm for 8 minutes. After decantation, cells were resuspended in 100 µl of surface stain cocktail that included CD3-PerCPCy5.5 (BD Biosciences), CD8- PE Cy7 (BD Biosciences), and CD4-PAC BLUE (BD Biosciences), CD56-PE (BD Biosciences) and incubated for 30 minutes in the dark. FACS buffer (2% fetal bovine serum, 0.1% sodium azide in PBS) was added and cells were then centrifuged at 1200 rpm for 8 minutes. Cells were incubated with 250 µl of Cytofix/Cytoperm (BD Pharmingen) for 20 min at 4°C in the dark and washed with 2–3 ml of PermWash (BD Pharmingen) and stained with an ICS antibody cocktail that included interleukin 2 (IL2)- APC (BD Biosciences) and IFNγ – Alexa 700 (BD Biosciences) in the dark for 30 min at 4°C. Cells were washed with 2–3 ml of PermWash and then resuspended in 0.15 ml of Cytofix and sent for flow cytometry analysis on FACS ARIA machine. Data was analyzed using FlowJo software (version 7.1; Tree Star). For CD4 and CD8 T cell subsets, live cells were gated using Live- Dead stain and then the lymphocyte gate was drawn using forward and side scatter populations. Further gating was done on either the CD3+/CD4+ or CD3/CD8 + cells, CD3–/CD56+ cells, CD3+/CD56+ cells for either interferon gamma (IFNγ) or IL2

cytokine positive cells. Single experiments with no replicates were performed for NFLU 001 and NFLU003. An additional experiment was performed for NFLU 002 with replicates.

Western blotting

The 2007/2008 TIVs were separated in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a Immuno-Blot™ PVDF membrane (Bio-Rad, Hercules CA) in a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) as previously described [15]. Influenza A/New Caledonia/20/99 IVR-166 and A/Wisconsin/67/2005X-161B were used as positive controls and allantoic fluid was used as a negative control. Non-specific binding was blocked by incubating the membrane in 5% instant nonfat dry milk (Carnation, Vevey, Switzerland) in phosphate buffered saline-Tween 20 solution (PBS-T). Primary and secondary antibodies were used as follows: (1) goat polyclonal anti-influenza A M1 antibodies, vF-20 and vN-20, (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution and donkey anti-goat IgG conjugated with horse radish peroxidase (HRP) at 1:2000 dilution (Santa Cruz Biotechnology); (2) rabbit polyclonal anti-NP antibody (IMGEX, San Diego, CA) at 2 µg/ml and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) at 1:5000 dilution; (3) mouse monoclonal anti-HA (H1) antibody (C102) (Santa Cruz Biotechnology) at 1:200 dilution and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) at 1:2000 dilution; and (4) mouse monoclonal anti-HA (H3) antibody (12CA5) (Roche Applied Science, Indianapolis, IN) at 0.1 µg/ml and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) at 1:2000 dilution. All dilutions were made with 1% bovine serum albumin in PBS-T. Bound antibodies were visualized by a Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and the Kodak BioMax XAR film (Eastman Kodak, Rochester, NY) was exposed to the membrane. The intensity of the bands on the film was quantitated using Quantity One® software (Version 4.5.1 (Bio-Rad)).

Proteomic Analyses

Influenza virus proteins present in the vaccines were identified and quantified by capillary UPLC-MS using MS^E analysis methods described by Silva et al [16]. A 300 µl aliquot of each vaccine was mixed with 16.5 µl of 2% PPS acid cleavable detergent (Protein Discovery, Inc.) and 5 µl of dithiothreitol (321 mM), both in 50 mM ammonium bicarbonate, and incubated for 30 min at 60°C to reduce disulfides. The proteins were then alkylated by the addition of 5 µl of 1 M iodoacetamide (Sigma Chemical Co.) in 50 mM ammonium bicarbonate for 30 min at room temperature. Trypsin digestion was then performed by the addition of 2 µl of sequencing grade modified porcine trypsin (100 ng/µl; Princeton Separations Inc.) followed by heating at 30°C overnight. Following digestion, the samples were acidified by the addition of 7.1 µl of concentrated (11.7 M) hydrochloric acid to destroy the detergent and then centrifuged. The supernatant was transferred to a clean tube and stored at -80°C until analysis. Triplicate UPLC-MS^E analysis of the tryptic digests was performed using a Waters Q-TOF Premier mass spectrometer equipped with a Waters NanoAcquity capillary UPLC system. The peptides were injected onto a 180 µm × 22 mm Symmetry C18 trapping column (Waters, Milford, MA) at 5 µl/min and then separated on a Waters BEH130C18 column (1.7 µm particle diameter, 100 µm × 100 mm) using a 30 min solvent gradient from 5 to 45% acetonitrile in 0.1% formic acid at 600 nl/min. Low collision energy MS and high collision energy MS^E spectra were alternately acquired and the charge state reduced. The isotope was then deconvoluted and database searched against the complete SwissProt protein database (July 5, 2007 download) using Waters Identity^E software to identify all proteins present. All MS^E spectra used to identify peptides were exported in the *.pkl format for further Mascot and X!Tandem database searches against a database compiled in-house containing the gallus gallus, and the influenza B and influenza A (H1N1 and N3N2) NIH-NCBI protein database (May 16, 2008 download, 77,029 entries). For all peptide identifications, the peptides were required to be identified in 2 out of the 3 replicates. For all database searches, 2 or more peptides were required for protein

identification. Results from these analyses were quantified by the peptide counting protein abundance index (PAI) method [17] and compared to the absolute quantification results calculated by the Identity^E method using the peptide precursor ion plot peak areas from the 3 most intense peptides for each protein[16].

Statistical Analysis

For the ELISPOT assays, the number of IFN γ producing cells in the prevaccination PBMC samples from the 30 subjects were analyzed using a generalized estimating equation method, clustering by patient to account for within subject correlation. A statistically significant result was defined as $p < .05$. Analysis using a non-parametric test (Friedman's) yielded similar results. The percentages of cytokine producing cells (Ifn γ or IL2) produced by the CD4, CD8, NK subsets in NFLU 002 were compared using the Friedman's non parametric test with a statistically significant result defined as $p < .05$.

Results

Commercial vaccines from three licensed manufacturers elicit different in vitro CTL responses

We tested four influenza specific CD4 and CD8 T cell lines in CTL assays to assess their recognition of BLCLs pulsed with each of the three commercially available TIVs. Using a previously defined CD8+ NP specific CTL clone 1-1, significant specific lysis (17 %) was seen only with BLCL pulsed with Vaccine 1 and ISCOM (Figure 1A). ISCOM has been described as a cage like vehicle made up of glycosides, cholesterol, and phospholipids which aids in the presentation and processing of viral proteins to specific CD8+ T cells [14, 18]. There was high specific lysis (74%) of peptide pulsed BLCL used as a positive control and minimal lysis of unpulsed target cells or target cells pulsed with ISCOM alone (Data not shown). For the M1-specific CD8 T cell line 1-7K, high specific lysis was seen with target cells pulsed with Vaccine 1 and ISCOM (20–38%) over a 10 fold concentration and with Vaccine 2 and ISCOM (25.4%) at the highest concentration (5 μ l) (Figure 1B). In a preliminary experiment with this CD8 M1 T cell line without ISCOM, specific lysis of target cells pulsed with each of the TIV (5 μ l) ranged from 0–6% [19]. For the HA (H3)-specific CD4+ T cell line 3E-2, there were high levels of specific lysis of BLCL pulsed with either Vaccine 1, Vaccine 2, or Vaccine 3 (43–58%) which were seen over a 10 fold dilution of vaccine (5-.5 ul) (Figure 1C). For the M1-specific CD4 T cell line 1-3, high specific lysis was seen with Vaccine 1 (46–70%) over a 100 fold dilution (5-.05 μ l) while specific lysis of Vaccine 2 and 3 (32–65%) pulsed target cells was seen over a 10 fold concentration (5-.5 μ l) (Figure 1D). In summary, we demonstrated differences in CTL activity with targets pulsed with each of the TIVs. The higher levels of specific lysis of targets pulsed with Vaccine 1 by the NP and M1 specific cell lines suggest differences in the protein content of these vaccines that manifested as differences in the induction of T cell effector function, in this case, cytolytic activity.

Commercial vaccines elicit significantly different numbers of IFN γ producing cells

We used the IFN γ ELISPOT assay to determine whether differences in cytolytic activity seen with commercial vaccines would also be detected by testing for another T cell function, IFN γ production. Prevaccination PBMC from 30 healthy subjects were stimulated with each of the TIVs at a final dilution of 1:256 which was determined in preliminary experiments using PBMC of a known responder (Figure 2). The influenza A/Wisconsin/67/2005 (H3N2) virus was used as a positive control (Data not shown). Prevaccination PBMC were used to minimize the complicating factors of either recent influenza infection or vaccination. For these 30 donors, high prevaccination frequencies of IFN γ producing cells were seen to the H3N2 virus (Avg. SFU/ 10^6 = 1209.347) (Data not shown) and to each of the three TIVs (415.3 to 990.0 SFU/ 10^6) (Figure 2). The differences in IFN γ ELISPOT responses between live virus and the

different vaccines is likely due to the fact that live virus stimulates CD4+ and CD8+ T cells while the vaccines which are killed viruses may only stimulate CD4+ T cells. The wide range of values may be due to differences in individual levels of exposure to influenza virus either naturally or through yearly vaccination. Using Vaccine 1 as the comparison group (mean 804.3, median 595.8), Vaccine 2 elicited a significantly higher average number of IFN γ producing cells (mean = 989.9, median = 839.40) (Friedman's test $p < .05$) and Vaccine 3 had a significantly lower average number of IFN γ producing cells (mean = 415.3, median = 346.7) (Friedman's test $p < .05$).

Intracellular cytokine staining identifies vaccine differences in cytokine production by CD4, CD8 T and NK cells

We performed preliminary ICS experiments using limited available PBMC from three individuals (NFLU 001–003) with laboratory confirmed influenza to determine the potential contributions of CD4 and CD8 T cells to the production of IFN γ seen in the ELISPOT results. After stimulation of PBMC with each of the TIVs, we measured both IFN γ and IL2 production in these cell subsets. CD4+ T cells produced the majority of the CD3+ IFN γ + cells (40–93% of CD3+ lymphocytes) though CD8+ T cells did produce IFN γ (1–30% of CD3+ lymphocytes) (Data not shown). The majority of IFN γ -producing cells were CD4 or CD8 single positive cells and the contribution of the double positive CD4/CD8 T cell subset was minimal. As a whole, a lower percentage of both CD4 and CD8 T cells produced IL2 (range 0–.09 %) as compared to IFN γ (range 0 – .86 %) (Data not shown). Other than in donor NFLU 002, the highest responses were seen in the CD4+ IFN γ producing subset (.13–.22%) with Vaccines 1 and 2 (Data not shown).

For NFLU 002 PBMC, Vaccine 1 and Vaccine 2 induced similar CD4+ IFN γ producing T cell responses (.13–.29%) that were higher than what was seen with Vaccine 3 (.05–.11%) (Table 1). In this donor, the highest IFN γ responses were seen in the CD8 subset with Vaccine 1 and 2 inducing similar responses than either Vaccine 3 (.86 % vs. .83% vs. .22%) (Figure 3 and Table 1). Using Friedman's non parametric test, statistical analysis suggested differences between the three vaccines in terms of CD4 IFN γ and CD8 IFN γ production between all three TIVs ($p > .05$ but $p < .01$). Though limited, this data from an individual recovered from natural influenza A infection suggests vaccine related differences between TIV vaccines in the induction of T cell in vitro effector functions that deserve further study.

Western blotting reveals differences in internal protein content of commercial vaccines

Western blotting was performed to compare the amount of M1 and NP in the TIVs (Figure 4). We confirmed that all three vaccines had a similar amount of HA proteins (H1 and H3) and the amount of NP in all three vaccines was also similar. M1 protein was detected in similar levels in Vaccines 1 and 2, but was not detected in Vaccine 3 by an antibody recognizing the N-terminus of M1 (vN-20). The same result was obtained by an antibody raised against an internal region of influenza A M1 (vF-20) (data not shown).

Mass spectrometry confirmation of Western Blot results

Mass spectrometry analysis was performed on the three TIV vaccines. Scaffold protein identification software analysis (Waters, Milford MA) using Swiss Prot databank demonstrated differences in protein expression between the vaccines. There was no detection of matrix protein 1 peptides using SWISS PROTEIN influenza A or B virus strains in Vaccine 3 while influenza A HA and NP peptide sequences were detected in all vaccines tested. In spectral counting analysis, which reflects the relative abundance of proteins in the samples, M1 protein was not detected in Vaccine 3. Mass spectrometry peptide identification data revealed the presence of the NP sequence SRYWAIR which contains 7 of the 9 amino acids of

the ₃₈₃SRYWAIRTR₃₉₁ T cell epitope, which our NP specific CTL line 1-1 recognizes, in only Vaccine 1.

Discussion

The 1976 swine influenza outbreak prompted the institution of the National Immunization Program which required national multi-center vaccine trials to establish the safety and immunogenicity of the then commercially available inactivated influenza A/New Jersey/76 virus, A/Victoria/75 and B/Hong Kong/72 vaccines [20–22]. These vaccine trials evaluated the effects of age, multiplicity of dose, and manufacturing process (split vs. whole vaccines) on the antigenicity of the vaccines as measured by hemagglutination antibody inhibition (HAI) titers. In the unprimed population, whole virus vaccines, as opposed to split vaccines, were more reactogenic and antigenic, suggesting that the manufacturing process can affect vaccine immunogenicity. Data from these cooperative trials solidified the use of HAI titers as the primary measure of immunogenicity of future vaccines. Today, additional scientific evaluation of vaccine immunogenicity has broadened to not only include humoral immune response but also cellular immune responses measured by quantitating the number of IFN γ and other cytokine producing T-cells, cytotoxicity, tetramer staining and T-cell proliferation responses [23,24].

Inactivated vaccines induce specific short term protection in healthy adults due to the frequent antigenic variation of the HA and NA proteins in the influenza virion [25]. Cytotoxic T cells are likely to play a role in reducing viral titers [5–7,26–29] and while CD4 T cells may not be important in the primary response to influenza, they are important in the generation of memory CD8 T cells. Our study examined the pattern of cellular responses to each of three licensed TIVs from the 2007–2008 season using influenza specific T cell lines in CTL assays, PBMC from healthy donors to quantitate the number of influenza specific IFN γ producing T cells, and PBMC from individuals acutely recovered from influenza A virus infection.

It has been reported that in addition to the surface proteins HA and NA, NP and M1 influenza proteins have been found in TIVs [30,31]. A recent paper by Garcia-Canas used two dimensional high performance liquid chromatography to characterize TIVs from different manufacturers [32]. HA content was relatively consistent but differences in the content of the NP protein and M1 proteins between the tested vaccines in a single season were found. In our study, western blot results showed similar amounts of H1 HA and H3 HA which can explain the similar levels of lysis (43–58%) of autologous target cells pulsed with each of the three vaccines at the highest concentration tested (5 μ g) seen with the HA CD4 T cell line. The absence of detectable M1 protein by Western blot for Vaccine 3 may partially explain the significantly lower number of IFN γ producing cells in ELISPOT assays and the lower specific lysis against targets pulsed with this vaccine seen with the CD4 M1 1–3 and CD8 M1 1–7 cell lines. Western blot results also suggested that Vaccine 3 had a lower amount of NP than vaccines 1 and 2, but the difference was not significant enough (about 20%) to explain the difference in CD8 CTL activity seen between vaccine 3 (1.2 %) and vaccine 1 (17%). However, mass spectrometry peptide identification data revealed the presence, in Vaccine 1 but not the other vaccines, of the NP sequence SRYWAIR, which contains 7 of the 9 amino acids of the NP ₃₈₃SRYWAIRTR₃₉₁ T cell epitope, which may help to explain the higher magnitude of CTL activity seen with Vaccine 1. Given the fact that several published escape mutants, including SGYWAIRTR, are associated with this peptide sequence, we might have suspected that this sequence would be detected in either Vaccine 2 or 3 [33,34] but it was not. Since the mass spectrometry analysis was designed to compare the relative abundance of proteins, this analysis may not have had the sensitivity to detect these mutated sequences.

We used prevaccination PBMC to quantitate the number of IFN γ producing cells in ELISPOT assays to avoid the confounding factors of recent natural infection or vaccination. We found a wide range of prevaccination immunity detected in response to influenza vaccines in these PBMC indicative of prior influenza virus exposure. As shown in our previous study, prior antigenic experience is likely to be an important factor in the response to any vaccine [35]. As a group, Vaccine 2 induced statistically significant higher number of IFN γ T cells from PBMC in vitro than did either Vaccine 1 or 3 ($p < .05$).

Taking advantage of an expected higher frequency of influenza specific T cells in individuals recently recovered from natural influenza A infection, we performed limited ICS assays examining which T cell subsets were responding to the vaccine. We found variability in the pattern of responses in this small subset of individuals. With TIV stimulation, we were able to detect CD4 IFN γ responses at a frequency of $> .1\%$ in all donors for at least one vaccine with CD4 IL2 responses detected in only two of the donors (Data not shown).

Inactivated influenza vaccine would not be expected to be efficiently presented to CD8 T cells but we were able to detect CD8 IFN γ responses in both NFLU 002 (.22–.86%) and NFLU 003 (.12–.17%). The detection of higher CD8 T cell responses in NFLU002 may be related to several factors including advanced age (75 years old), a more severe illness requiring hospitalization, and no TIV immunization during the influenza season. In our recent publication examining immune responses in individuals who received inactivated influenza vaccine, we showed that CD8 CD45RA+ cells increased significantly after immunization [35]. Data using HLA matched dendritic cells suggest that these inactivated vaccines can be presented to CD8 T cells (unpublished data), similar to the ISCOM effect, which can explain the induction of CD8 T cell responses that we observed post inactivated influenza vaccination.

There are no definitive immune correlates of protection related to T cells for viral infections such as influenza. There is growing evidence that the frequency and magnitude of a T cell response may not reflect the full functional potential of a T cell [36]. Mean fluorescence intensity (MFI) of the staining for a particular cytokine can be used as an estimate of the amount of cytokine produced by that particular T cell. For NFLU 002, the geometric MFI of the IFN γ and IL2 cytokines produced by CD4 T cells was higher than that seen for CD8 (Data not shown and Figure 3) suggesting that these CD4 T cells produced more IFN γ than CD8 T cells (Figure 3). The interpretation of our ICS results is limited given the number of subjects. Despite this, results from NFLU002 are suggestive that differences in TIV protein content can result in functional differences.

The 2007–2008 TIVs were mismatched for the predominant circulating H3N2 and B strains of the influenza season [37]. The majority of the H3N2 viruses isolated were of the A/Brisbane/10/2007-like strain that is a “drifted” variant from the vaccine A/Wisconsin-like strain whereas the majority of circulating influenza B viruses (94%) were B/Florida/04/2006-like viruses, from a different B lineage than what was included in the vaccine. This year’s influenza season was more severe than the previous three seasons in terms of peak outpatient visits for influenza-like illness and percentage of deaths related to pneumonia/influenza [37]. However, preliminary results showed an overall vaccine effectiveness of 44%, suggesting that vaccination provided a degree of protection against influenza-illness and influenza related complications even with an antigenic mismatch [38–40]. Protection may be in part modulated by prior cellular immune responses induced by natural exposure or boosted by vaccination.

Increasing attention should be paid to vaccines based on cellular immune responses especially to the highly conserved internal proteins of the influenza virus that might induce more cross subtype and within subtype protection against HA variation. These responses may be important in subpopulations, such as the elderly, in which the TIV offers less than optimal protection.

Despite a lower level of protection (50%) in the elderly, vaccination has been found to reduce the risk of hospitalization for pneumonia or influenza and the risk of death in this population (25–50%) which may in part be related to the induction of T cell responses[39,41]. In addition, a recent paper suggested that ex vivo measures of cellular immune responses (IFN γ /IL10 ratio and granzyme B response) were better correlates of protection in immunized elderly individuals compared with antibody responses [23].

Our study demonstrates in vitro differences in the T cell responses to three commercially available inactivated influenza vaccines. Our results suggest that cell-mediated immune responses to influenza vaccination may vary depending on the internal protein content of vaccines from different manufacturers. Future studies will need to address whether or not the observed in vitro differences in T cell immune responses observed translate into in vivo differences that are clinically relevant and impact on protection.

Acknowledgments

We thank Alan L. Rothman for discussion and Dr David Burt and Pasteur-Merieux for providing ISOMATRIXTM. We also wish to thank Dr. Jeffrey Kennedy, Karen Longtine, Jaclyn Longtine, and Melissa O'Neill for their help in acquiring the blood samples. This work was supported by the National Institutes of Health (NIH)/the National Institute of Allergy and Infectious Diseases (NIAID) grant U19 AI-057319. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH/NIAID

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Figure 1.

Detection of M1, NP, and M1 proteins in TIV by cytotoxic CD8⁺ and CD4⁺ T cell lines. **A.** The CD8⁺ T cell line, 1-1 specific to the NP₃₈₃₋₃₉₁ epitope was tested for recognition of autologous target cells infected with three TIV vaccines at concentrations from 5-.05 μg/ml at an E/T ratio =10. ISCOMATRIX was first incubated with vaccine for 24 hours, added to autologous BLCL for 1 hour, washed and then incubated overnight at 37 °C. **B.** The CD8⁺ T cell line, 1-7K, specific to M1₅₈₋₆₆ epitope was tested for recognition of autologous target cells infected with three TIV vaccines at concentrations from 5-.05 μg/ml at an E/T ratio =10. ISCOMATRIX was first incubated with vaccine for 24 hours, added to autologous BLCL for 1 hour, washed and then incubated overnight at 37 °C. **C.** The CD4⁺ T cell line, 3E-2 specific to HA₂₆₇₋₂₈₃ epitope was tested for recognition of autologous target cells infected with three TIV vaccines at concentrations from 5-.05 μg/ml at an E/T ratio =10. **D.** The CD4⁺ T cell line, 1-3, specific to M1₁₇₋₃₁ epitope was tested for recognition of autologous target cells infected with three TIV vaccines at concentrations from 5-.05 μg/ml at an E/T ratio =10.

Figure 2.

ELISPOT responses to three commercial TIV vaccines using prevaccination PBMC from 30 donors. Prevaccination PBMC from 30 healthy donors were tested against each of the TIV vaccines for the frequency of IFN γ producing cells using IFN γ ELISPOT assay. Responses with media background were subtracted from each value. All values were expressed as SFU/ 10^6 . The A/Wisconsin/67/2005X-161B (H3N2) virus was tested in each subject as a positive control with average SFU/ 10^6 = 1209 ± 816 (Data not shown).

Figure 3.

CD4 and CD8 T cell IFN γ responses to three commercial inactivated influenza vaccines in Donor NFLU002. Intracellular cytokine staining assay was performed on PBMC obtained from a naturally infected individual NFLU 002. For CD4 and CD8 T cell subsets, dead cells were gated out using Live- Dead stain viability marker and then the lymphocyte gate was drawn using forward and side scatter populations and further gating was done on the CD3+/CD4+ or CD3/CD8+ cells for either IFN γ cytokine positive cells. Shown in the first row are the flow cytometry plots depicting % of IFN γ -producing CD3+/CD8+ cells after stimulation with each of the three vaccines. The second row shows the flow cytometry plots depicting % of IFN γ producing CD3+/CD4+ T cells. Medium and PMA controls are also shown for both CD4 and CD8 populations. Geometric mean fluorescence intensity for each population is represented as MFI.

Figure 4.

Comparison of the amount of internal proteins in the vaccines. **A.** M1. 45 μ l of three TIVs were separated in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a goat polyclonal antibody recognizing the N-terminus of influenza A M1 was used. NC (H1N1) and W (H3N2) are A/New Caledonia/20/99 IVR-166 (H1N1) and A/Wisconsin/67/2005X-161B (H3N2) used as positive controls, and allantoic fluid was used as a negative control. Relative intensity of the band is shown. **B.** NP. A rabbit polyclonal antibody specific to influenza A NP was used to detect the NP. **C.** HA (H1) as a control, HA (H1) was detected by a mouse monoclonal antibody specific to influenza A H1 HA. **D.** HA (H3) as a control, HA (H3) was detected by a mouse monoclonal antibody specific to influenza A H3 HA.

Table 1

Percentage of CD4 and CD8 cytokine staining in NFLU 002

Percentage of cytokine staining cells in NFLU 002				
		Vaccine 1 ^a	Vaccine 2 ^a	Vaccine 3 ^a
CD8				
	IFN	.86 (.42–1.3)	.83(.46–1.2)	.22 (.04–.32)
	IL2	.03 (0–.11)	0 (0)	.01 (0–.02)
CD4				
	IFN	.22(.13–.29)	.20(.19–.22)	.08(.05–.11)
	IL2	.09(.04–.14)	.06(.03–.10)	.05(.02–.11)

^a Average cytokine production in PBMC after stimulation with each of the TIVs in intracellular cytokine staining assay. Background has been subtracted. Experiment was performed twice with the conditions tested three times. Numbers in parenthesis represent the range of values obtained.