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Comparison of *In Vitro* Immunostimulatory Potential of Live and Inactivated Influenza Viruses

Vesna Blazevic, C. Mac Trubey, and Gene M. Shearer

ABSTRACT: Live influenza viruses, heat-inactivated virus, and a trivalent formalin-inactivated influenza vaccine were analyzed for their *in vitro* stimulatory properties on immune cells from healthy donors. Lymphocyte proliferation induced by each influenza antigen was comparable. Influenza vaccine stimulated significantly lower production of interferon-γ (IFN-γ) compared with live and heat inactivated viruses, whereas both vaccine and heat-inactivated influenza induced lower levels of IFN-α compared with live virus. Furthermore, only live virus generated influenza-specific cytotoxic T lymphocyte (CTL) activity. A significant increase in monocyte expression of CD80, CD86, CD40, and human leukocyte antigen-DR (HLA-DR) was also induced by live influenza virus. Our results suggest that immunization with live influenza vaccines might induce immune responses that would not be induced by conventional inactivated vaccines, including CTL generation, antiviral IFN-γ and IFN-α cytokine production, and increased antigen presentation and costimulatory capacity on antigen presenting cells (APC). *Human Immunology 61, 845–849 (2000).* © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: influenza; vaccine; interferon-γ; interferon-α; costimulatory molecules

ABBREVIATIONS

IFN	interferon
CTL	cytotoxic T lymphocytes
HLA	human leukocyte antigen
APC	antigen presenting cells
PBMC	peripheral blood mononuclear cells

INTRODUCTION

Immunization is the most effective method for preventing influenza infections. Vaccination of elderly individuals and individuals who are at risk for developing complications of influenza are strongly recommended. Current influenza vaccines are trivalent, formalin-inactivated vaccines with effectiveness from 70%–90% in young, healthy individuals [1] and up to 50% in elderly people [2]. During the past two decades live, attenuated, cold-adapted influenza vaccines have been developed to replace or complement current vaccine formulations [3]. Live influenza vaccine has been shown to be more effective than the inactivated vaccine in the prevention of influenza infection and clinical illness in children [4], healthy adults [5], and in elderly people [6]. The ease of intranasal administration, safety and high efficacy make live influenza vaccine a good candidate for use in diverse human populations.

We have compared the *in vitro* immunostimulatory potential of live influenza A viruses, heat-inactivated influenza, and a trivalent formalin-inactivated influenza vaccine. Lymphocyte proliferation, cytokine production, and CTL activity stimulated by different influenza preparations were analyzed. Our results suggest that live influenza virus has greater potential for inducing T cell and APC activation in healthy individuals than non-replicative heat-inactivated influenza or vaccine preparation.

From the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health (V.B., G.M.S.), Bethesda, Maryland, USA, and Intramural Research Support Program, SAIC Frederick, NCI-FCRDC (C.M.T.), Frederick, Maryland, USA.

Address reprint requests to: Gene M. Shearer, Ph.D., Experimental Immunology Branch, NCI, NIH, Bldg. 10, Rm. 4B36, Bethesda, MD 20892, USA; Tel: (301) 402-3246; Fax: (301) 496-0887; E-Mail: sheareg@exchange.nih.gov.

Received May 15, 2000; revised June 18, 2000; accepted June 23, 2000.

0198-8859/00/$–see front matter
PII S0198-8859(00)00170-1

MATERIALS AND METHODS

Viruses

The influenza type A viruses, A/PR/8 [A/Puerto Rico/8/34(H1N1)] and A/Bangkok [A/Bangkok/RX73(H3N2)] were grown in embryonated hen eggs and used as infectious allantoic fluids (8.5–9.3 EID50/ml). The 1998–99 influenza vaccine is an egg-derived, formalin-inactivated trivalent subunit formulation that contains 30 μg/ml of the hemagglutinin antigens of influenza A/Beijing/262/95 (H1N1), A/Sydney/5/97 (H3N2), and B/Harbin/7/94 strains (Wyeth-Ayerest Laboratories Inc., Marietta, PA, USA).

Lymphocyte Proliferation and Cytokine Production

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy blood bank donors (Blood Transfusion Department, National Institutes of Health, Bethesda, MD, USA). The PBMC were cultured for 6 days at 1.5×10^6 cells/ml culture media (RPMI 1640 medium [Gibco, Grand Island, NY, USA] supplemented with antibiotics, L-glutamine and 5% human AB serum [Sigma, St. Louis, MO, USA]) with and without the following antigens: influenza A/PR/8 (1:800), influenza A/Bangkok (1:800), heat-inactivated (56°C) influenza A/PR/8 (A/PR/8-inact; 1:800) and formalin-inactivated influenza vaccine (1:5000). The optimal concentrations of influenza preparations used were determined in preliminary experiments as described below, through six tenfold dilutions of virus and vaccine. Triplicate proliferation assay cultures were set up in 96-well flat-bottomed plates (Costar, Cambridge, MA, USA) and cytokine production cultures were set up in 24-well plates (Costar). After the 5-day incubation period, cells were pulsed with 1 μCi of [^{3}H]thymidine overnight and radioactivity measured. Results are expressed as a stimulation index (cpm in the presence of influenza antigen/cpm in medium only). After 6 days of influenza stimulation, culture supernatants were assayed for cytokine production using commercial ELISA kits: IFN-γ (R&D, Cambridge, MA, USA) and IFN-α (Biosource International, Camarillo, CA, USA).

Immunofluorescence Staining

PBMC (1.5×10^6) were incubated with or without influenza A/PR/8 (1:800) for 24 h in ultra low attachment plates (Costar) to avoid monocyte adherence to plastic. After incubation, cells were washed and stained with: anti-CD80-FITC, anti-CD86-PE, anti-CD40-FITC, anti-HLA-DR-PE, or isotype control antibodies (all from Pharmingen, San Diego, CA, USA). Analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA, USA). Monocytes were gated based on their forward and side angle scatter characteristics and results are expressed as mean fluorescence intensity.

Cytotoxic T-Lymphocyte Assay

Influenza-specific CTL were generated by stimulating PBMC (1.5×10^6/ml culture media) with live influenza A/PR/8 (1:800) or influenza vaccine (1:5000) for 6 days. Autologous 3-day phytohemagglutinin (Life Technologies, Grand Island, NY, USA) stimulated T-cell blasts were used as target cells. The blasts were infected with influenza A/PR/8 (1:4 dilution) for 1 h and incubated for 2 h additional in medium containing 200 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA, USA). After washing, target cells were mixed with effector cells at different ratios in a 4 h chromium release assay. Control target cells were uninfected T-cell blasts. Results are expressed as mean percent lysis of triplicate determinations and are calculated by the formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. Spontaneous release of ^{51}Cr from the targets did not exceed 25% of maximum detergent release.

Statistical Analysis

Two-tailed Student's *t*-tests for paired and unpaired data were used for statistical analysis and p values ≤ 0.05 were considered significant.

RESULTS

Proliferation, IFN-γ, and IFN-α Production Induced by Live Influenza Virus, Heat-Inactivated Influenza and Influenza Vaccine

PBMC from eight donors were stimulated with the two live influenza virus strains, as well as with heat-inactivated A/PR/8 and influenza vaccine. Lymphocyte proliferation (Figure 1A) induced by all four different influenza antigen preparations was comparable. We also tested the ability of the different influenza preparations to induce the production of the cytokines IFN-γ and IFN-α by PBMC (Figures 1B and 1C). Stimulation with live influenza viruses (A/PR/8 and A/Bangkok, respectively) induced comparable amounts of both cytokines (518.4 ± 165.9 compared with 526.8 ± 267.3 pg/ml for IFN-γ, and 1367.8 ± 480.1 compared with 1178.0 ± 349.2 pg/ml for IFN-α). Heat-inactivated A/PR/8 stimulated production of IFN-γ similar to stimulation with live virus (451.2 ± 113.2 pg/ml). In contrast, the influenza vaccine induced production of IFN-γ was reduced compared with live viruses (190.7 ± 71.2 pg/ml, $p = 0.04$). Furthermore, IFN-α production induced by heat-inactivated virus (266.6 ± 134.8 pg/ml, $p = 0.031$) was reduced compared with live viruses, and the vaccine did not stimulate influenza-specific IFN-α (39.5 ± 18.7

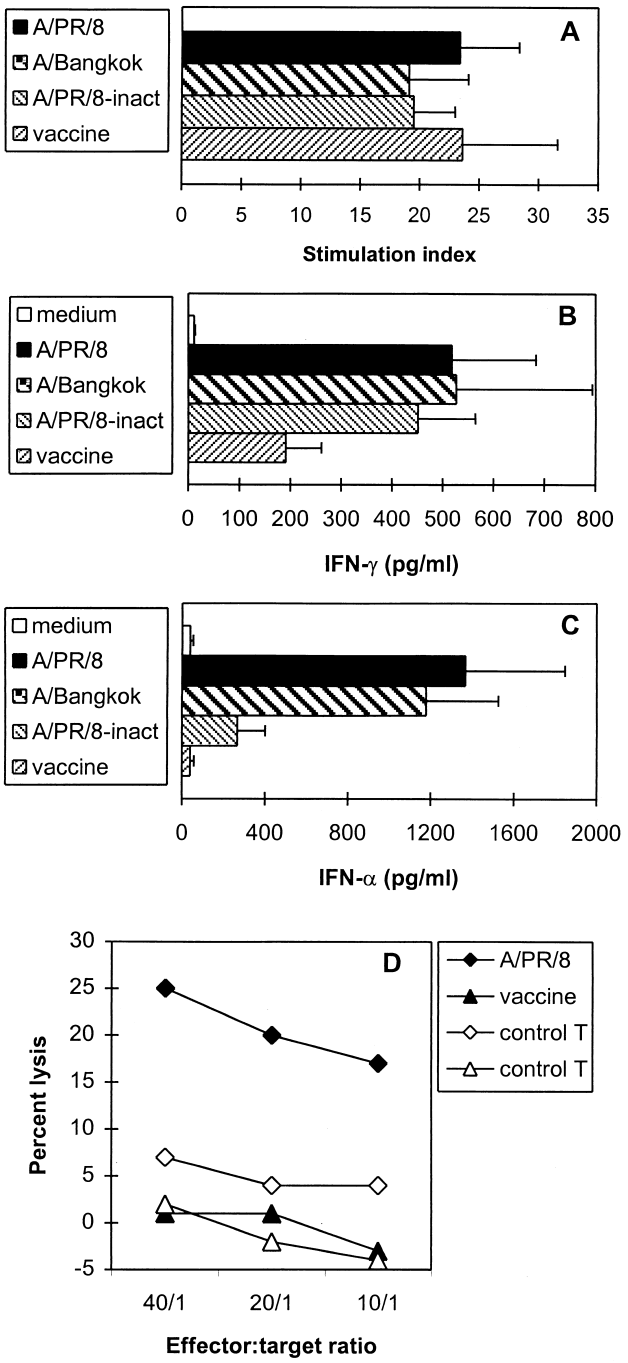

FIGURE 1 Lymphocyte proliferation (A), IFN-γ (B), IFN-α (C) production, and CTL activity (D) induced by live influenza (A/PR/8 and A/Bangkok), heat-inactivated virus (A/PR/8-inact) and influenza vaccine. Mean values ± SEM of 8 (A), 11 (B), and 5 (C) individual experiments are shown. Effector cells were generated either by influenza A/PR/8 or vaccine stimulation of PBMC for 6 days. Cytotoxicity was assayed on influenza-infected (filled symbols) and uninfected, control target cells (empty symbols). One representative experiment of three performed is shown.

pg/ml, $p = 0.024$) above the culture media background (38.2 ± 14.5 pg/ml).

Induction of CTL Responses by Live Influenza Virus but not by Influenza Vaccine

We also tested generation of influenza-specific CTL responses by live influenza A/PR/8 and influenza vaccine (Figure 1D). Effector cells that were stimulated by live virus lysed influenza-infected target cells, whereas vaccine stimulated effector cells did not.

Expression of Costimulatory Molecules on Monocytes by Live Influenza Virus Stimulation

It was previously demonstrated that only live influenza viruses induce expression of costimulatory molecules on human alveolar macrophages and dendritic cells [7, 8]. Therefore, we analyzed the expression of costimulatory molecules on peripheral blood monocytes stimulated for 24 h with live influenza A/PR/8. As shown in Table 1, influenza virus induced a significant upregulation of CD80, CD86, CD40, and HLA class II molecules on human blood monocytes.

DISCUSSION

New influenza vaccine formulations to complement or replace current vaccine strategies are under investigation. One of the candidates is live, cold-adapted trivalent influenza vaccine that has proven safe and effective in fighting infection and clinical illness [1, 9]. This study shows that infectivity and replicative capacity of the virus are important in inducing influenza-specific cellular immune responses, as a loss in immunostimulatory capacity was apparent with virus inactivation.

The cytokines IFN-γ and IFN-α have been shown to be crucial for controlling several viral infections, including influenza [5, 10]. Therefore, we tested the effect of the different influenza preparations on the production of these cytokines. Influenza vaccine induced significantly lower production of IFN-γ compared with live and heat-inactivated influenza. Furthermore, IFN-α production induced by both heat-inactivated influenza and the vac-

TABLE 1 Expression of costimulatory molecules on peripheral blood monocytes

	CD80	CD86	CD40	HLA-DR
Culture medium	4.6 ± 1.7	472 ± 96	34 ± 9.4	465 ± 130
Influenza A/PR/8	79 ± 13.6	858 ± 118	84 ± 16	908 ± 148
p value[a]	0.002	0.013	0.007	0.03

Mean fluorescence intensity ± SEM of seven experiments for CD80 and CD86, and four experiments for CD40 and HLA-DR are shown.

[a] A p value determined by paired, two-tailed Student's t-test.

cine was defective. These results indicate that IFN-γ production is only affected by formalin inactivation of the virus and not by heat inactivation, suggesting that the immunostimulatory capacity of influenza is dependent on the method of inactivation. Furthermore, high IFN-α production was induced only by infective and fully replicative influenza. These observations are of potential importance not only because of the direct antiviral effect of these cytokines, but also because these cytokines are important for the generation of cell-mediated immunity. IFN-γ has been shown to non-specifically induce antigen presentation of APC [11], and IFN-α has been demonstrated to prime type 1 T-helper responses in humans [8].

Although inactivated and subunit influenza vaccines have been shown to induce strong antibody responses, they lack the ability to generate influenza-specific CTL [6] and mucosal (IgA antibody) [2] immune responses. It is believed that these responses are important in protection of the host during the early stages of influenza infection and, thus, contribute to the reduction of complications from infection in risk populations [12]. We demonstrate that live replicating virus is essential for inducing influenza-specific CTL activity *in vitro*. PBMC stimulated with live influenza but not influenza vaccine were able to lyse infected targets. An *in vivo* murine model has suggested that live influenza immunization can induce heterosubtypic immunity after live virus challenge through generation of cross-reactive CTL, while inactivated influenza failed [13]. Furthermore, mice immunized with live influenza generate Th1 type response in contrast to Th2 response generated by inactivated virus immunization [13].

We further show that incubation of peripheral blood monocytes with live influenza induces a significant increase in expression of costimulatory molecules. It is possible that influenza virus by inducing expression of CD80, CD86, CD40, and HLA class II molecules on APC lowers the threshold for activation of T cells, making them more sensitive to antigenic stimulation [14]. Also, a significant increase in HLA class II by influenza virus could allow for increased antigen presentation. Upregulation of HLA and costimulatory molecules on influenza infected human alveolar macrophages [7] and dendritic cells [8] has also been demonstrated.

Our data show that live influenza, in contrast to inactivated virus, induces three complementing effects to control influenza infection: (1) production of cytokines with direct antiviral effect; (2) generation of influenza-specific CTL; and (3) local induction of cytokines and increased costimulatory molecule expression that could augment APC function and T-cell activation. Induction of influenza-specific CTL responses to conserved viral proteins could be effective against emerging influenza strains, for which antibodies would not be effective. In conclusion, our findings may provide understanding for generating more effective cell-mediated immune responses following immunization with different formulations of influenza vaccines, and, therefore, may have impact on the design of new influenza vaccination strategies.

ACKNOWLEDGMENTS

This project has been funded in part with Federal Funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-56000. We thank Drs. Jonathan Yewdell and Jack Bennink (NIAID, NIH) for providing influenza viruses, and Dr. Matthew Dolan for providing influenza vaccine. We also thank Dr. Ligia Pinto (NCI, NIH) for helpful suggestions and Dr. William Biddison (NINDS, NIH) for critical review of the manuscript. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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