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Research paper

Granzyme B ELISPOT assay to measure influenza-specific cellular immunity

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ABSTRACT

The immunogenicity and efficacy of influenza vaccination are markedly lower in the elderly. Granzyme B (GrzB), quantified in fresh cell lysates, has been suggested to be a marker of cytotoxic T lymphocyte (CTL) response and a predictor of influenza illness among vaccinated older individuals. We have developed an influenza-specific GrzB ELISPOT assay using cryopreserved PBMCs. This method was tested on 106 healthy older subjects (ages 50–74) at baseline (Day 0) and three additional time points post-vaccination (Day 3, Day 28, Day 75) with influenza A/H1N1-containing vaccine. No significant difference was seen in GrzB response between any of the time points, although influenza-specific GrzB response appears to be elevated at all post-vaccination time points. There was no correlation between GrzB response and hemagglutination inhibition (HAI) titers, indicating no relationship between the cytolytic activity and humoral antibody levels in this cohort. Additionally, a significant negative correlation between GrzB response and age was observed. These results reveal a reduction in influenza-specific GrzB response as one ages. In conclusion, we have developed and optimized an influenza-specific ELISPOT assay for use with frozen cells to quantify the CTL-specific serine protease GrzB, as a measure of cellular immunity after influenza vaccination.

1. Introduction

Influenza outbreaks continue to plague all regions of the globe. The 2009 influenza A pandemic was caused by the H1N1 virus, the same subtype responsible for the Spanish flu pandemic that killed between 20 and 100 million people in 1918–1919 (Murray et al., 2006). Influenza often leads to death in patients suffering from underlying illnesses such as congestive heart failure and chronic obstructive pulmonary disease, and can also lead to additional illnesses including pneumonia and bacterial infection, which can result in death (Small et al., 2010). These complications are especially prevalent in the elderly population (adults 65+ years of age) (McElhaney, 2011).

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As recommended by the World Health Organization (WHO), annual vaccination is effective at reducing the global health burden associated with influenza (CDC, 2012). The immune system responds to vaccination by producing influenza-specific antibodies. The hemagglutination inhibition assay (HAI) is used to quantify influenza-specific antibody levels by measuring the highest dilution of serum that prevents hemagglutination (Meijer et al., 2006). In addition to stimulating an adaptive humoral immune response, vaccination also primes the cellular immune response by promoting the formation of memory cytotoxic T lymphocytes (CTLs) that are specific for influenza virus. Therefore, subsequent exposure to influenza elicits a secondary immune response against viral pathogens through cytolytic mechanisms.

Previous research indicates a dampening of this response in the elderly population due to immunosenescence (McElhaney, 2011; McElhaney et al., 2009). This is likely due to the decline in CTL response with replicative senescence as one ages, among other mechanisms (McElhaney, 2011). Because of immunosenescence, questions arise regarding the efficacy of influenza vaccines in the elderly population (McElhaney et al., 2009).

To better understand the immunogenicity of influenza vaccines in the elderly, there must be an explicit, sensitive, quantitative assay to measure influenza-specific cell mediated immunity (CMI) in these individuals in addition to using HAI to measure humoral immunity. Previous research has suggested that Granzyme B (GrzB) levels correlate with the cytolytic activity of CTLs responsible for the reduction and control of cytopathogenic viruses (e.g., influenza) (McElhaney et al., 2009). When activated CTLs recognize virally infected host cells, the apoptotic pathway is induced through the cooperation of perforin and GrzB (Trapani and Smyth, 2002). Perforin, a protein with structural and functional similarities to complement component 9, is polymerized in the presence of calcium, resulting in the formation of channels in the target cell lipid membrane (Shresta et al., 1998). These pores are utilized by GrzB, a serine protease, to pass into the cytoplasm of the target cell where it cleaves death substrates to effectuate cell death. This form of granule-mediated killing can be quantified, making it a candidate as a measure of the cellular immune response. In addition, GrzB (expressed primarily by CTLs, natural killer (NK) cells, and dendritic cells) is reported to have additional immune-related functions, such as degradation of viral proteins, receptor cleavage, cytokine-like functions, and immunosuppressive function (Jahrsdorfer et al., 2010).

There are significant variations in normal GrzB activity and levels between individuals. A sensitive and accurate assay is needed to quantify levels of GrzB as a measure of cellular immune response to vaccination (Karulin et al., 2000). Past research has demonstrated strong correlations between GrzB ELISPOT assays and the traditional chromium release assays, making ELISPOT an ideal substitute for more traditional techniques (Bleackley et al., 1988; Doherty and Christensen, 2000; Russell and Ley, 2002; Rininsland et al., 2000). Additionally, the use of frozen, rather than fresh, peripheral blood mononuclear cells (PBMCs) allows for testing on large sample populations, which is a more practical concern for clinical trials. For these reasons, we have developed an influenza-specific GrzB ELISPOT assay using frozen PBMCs to measure CMI of older adults post influenza vaccination.

2. Materials and methods

2.1. Study subjects

As previously reported, the sample population for this study included 106 eligible subjects who expected to be available for the duration of the study, ranging in age from 50 to 74 years (Umlauf et al., 2012a). All subjects underwent thorough review of their vaccination history and were in good health throughout the duration of this study. The 2010–2011 licensed trivalent influenza vaccine, containing the A/California/7/2009 H1N1-like, A/Perth/16/2009 H3N2-like, and B/Brisbane/60/2008-like viral strains, was administered to all participants. Venipunctures were performed on these subjects once prior to vaccination (Baseline or Day 0) and three times within 75 days post-vaccination (Day 3, Day 28, and Day 75). All subjects provided written informed consent, and the study was approved by the Institutional Review Board of the Mayo Clinic.

To verify the results of our study, the assay was also performed on 11 younger subjects ranging in age from 19 to 21 years. These subjects received the seasonal trivalent influenza vaccine for the 2005/2006 influenza season. These subjects have previously been described by Poland et al. (2008).

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from each subject at each time point pre- and post-vaccination (Day 0, 3, 28 and 75) from 100 mL of whole blood using Cell Preparation Tube with Sodium Citrate (CPT™) tubes, as previously described (Umlauf et al., 2012a). Cells were resuspended at a concentration of 1×10^7/mL in RPMI 1640 medium containing L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 10% dimethyl sulfoxide (DMSO; Protide Pharmaceuticals, St. Paul, MN) and 20% fetal calf serum (FCS; Hyclone, Logan, UT), frozen overnight at −80 °C in Thermo Scientific freezing containers (Thermo Fisher Scientific, Waltham, MA) to achieve an optimal rate of cooling. Cells were then transferred to liquid nitrogen for storage, as previously reported (Umlauf et al., 2012a,b).

2.3. Growth of influenza virus

The influenza A/California/7/2009/H1N1-like strain was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA). The virus was grown on embryonated chicken eggs, and the allantoic fluid containing the virus was harvested and titered by hemagglutination (HA) and 50% Tissue Culture Infectious Doses ($TCID_{50}$) assay following infection by Madin–Darby canine kidney epithelial cells (MDCK) using standard protocols (Webster et al., 2002; WHO Global Influenza Surveillance Network, 2011; Wang et al., 2006).

2.4. Hemagglutination inhibition (HAI) assay

Influenza A/California/7/2009 (H1N1)-specific HAI titers were measured in subjects' sera at each time point pre- and post-vaccination (Days 0, 3, 28 and 75) using a standard protocol, as described elsewhere (Webster et al., 2002; WHO Global Influenza Surveillance Network, 2011; Wang et al., 2006).