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The effect of timing of influenza vaccination and sample collection on antibody titers and responses in the aged

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ABSTRACT

Antibody responses, B cell subset distribution in blood and the blood transcriptome were analyzed in younger and aged human subjects before and after vaccination with the inactivated influenza vaccine. In the aged, but not the younger, individuals we saw a clear difference in antibody titers including those at baseline depending on the time of vaccination and sample collection. Differences in baseline titers in aged individuals treated in the morning or afternoon in turn affected responsiveness to the vaccine. In both younger and aged individuals, the time of sample collection also affected relative numbers of some of the B cell subsets in blood. A global gene expression analysis with whole blood samples from the aged showed small but statistically significant differences depending on the time of sample collection. Our data do not indicate that timing of vaccination affects immune responsiveness of the aged, but rather shows that in clinical influenza vaccine trials timing of collection of samples can have a major and potentially misleading influence on study outcome. In future vaccine trials, timing of vaccination and sample collection should be recorded carefully to allow for its use as a study covariant.

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1. Introduction

Circadian regulation through clock genes affects 8–10% of the mammalian genome [1–3] including those involved in immune responses [4–8]. Circadian functions change with age [9,10]; some of the clock genes become desynchronized while a new set of genes start oscillating in a 24-h cycle [9]. Previous studies in mice and humans tested the effect of the circadian clock on immune responses with albeit contradicting results. In mice one studies showed enhanced T cell responses upon their stimulation during the day rather than at night [11] while another study reported superior innate and adaptive responses upon vaccination in the evening rather than in the morning [12]. In humans, who unlike mice are diurnal, one study reported no difference in severity of disease caused by an attenuated Venezuelan equine encephalomyelitis virus given to healthy men in the morning or evening [13]. Other studies showed that men but not women vaccinated in the morning mounted a better peak antibody response to both hepatitis A virus and the A/Panama influenza virus strain [14]. It was also reported that morning immunization of the aged improves their antibody response to the H1N1 component of the inactivated influenza vaccine [15,16].

We conducted a 5-year study in younger (30–40 years of age) and aged (≤65 years of age) humans to assess antibody responses to the two influenza virus strains of the inactivated influenza vaccine. We focused on B rather than T cell responses as neutralizing antibodies are the best-studied correlate of protection although T cells are thought to contribute [17]. Community-dwelling individuals were vaccinated in fall or early winter. Records were kept on time of vaccination and collection of pre- and post-vaccination samples. In the aged but not the younger individuals we saw differences in antibody titers including those at baseline depending on the time of sample collection. This in turn affected responsiveness to the vaccine defined as vaccine-induced increases in titers after vaccination. In both younger and aged individuals, the time of sample collection also affected relative numbers of some of the circulating B cell subsets. A global gene expression analysis conducted with whole blood samples from the aged, over the last 4 years of the study, showed small but statistically significant differences between those bled in the morning or afternoon. Differences were seen in genes encoding or affecting clock genes suggesting that

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observed differences in immune responses were controlled by circadian regulation.

2. Material and methods

2.1. Participants

Human participants from the research triangle area of North Carolina were recruited during the influenza season over a period of 5 years (2011–2015) at the Clinical Research Unit at Duke University Medical Center (Durham, NC, USA) in accordance with the local Institutional Review Boards. Participants that met our inclusion criteria were divided into two groups: the younger were 30–40 years of age and the aged were ≥65 years of age. A total of 59 young and 80 aged individuals were recruited. Several participated repeatedly in the 5-year study so that a total of 203 and 460 matched samples were obtained from the younger and aged, respectively. We collected demographic data and noted the time of vaccination and sample collection. Initially in years 1 and 2 subjects were assigned to AM or PM groups, that is individuals that were immunized before or after noon, in years 3–5 the actual time of vaccination and sample collection was recorded. Seasonal inactivated influenza vaccine (FLUARIX® Trivalent or Quadrivalent, GlaxoSmithKline) was administered intramuscularly. Blood was collected before and on days 7 and 14 or 28 following vaccination.

2.2. PBMC and plasma isolation

1.5 mL of blood was centrifuged to isolate plasma and rest of the blood was used for PBMC isolation as described [11].

2.3. Virus strains and purification

Influenza virus A/California/7/2009 (H1N1) pdm09-like virus, A/Perth/16/2009 (H3N2)-like virus, A/Victoria/361/2011 (H3N2)-like virus, A/Texas/50/2012 (H3N2)-like virus and A/Switzerland/9715293/2013 (H3N2)-like virus strains from the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia were expanded in 10 day-old specific pathogen-free embryonated eggs and virus-containing allantoic fluid was harvested, purified and titrated as described [18].

2.4. Antibody assays

Human plasma was screened for virus neutralizing antibodies (VNAs) by microneutralization assays and for antibody isotypes by ELISAs against influenza A viruses as described [18].

2.5. Flow cytometry

Multiparametric flow cytometry was performed on younger and aged PBMCs using a panel to identify mature naïve B cells ($CD19^+CD20^+IgD^+CD27^-CD38^-$), transitional B cells ($CD19^+CD20^+IgD^+CD27^{+/-}CD38^{+/-}$), non-switched memory B cells ($CD19^+CD20^+IgD^+CD27^+CD38^-$), switched memory B cells ($CD19^+CD20^{+/-}IgD^-CD27^+CD38^-$), double-negative B cells ($CD19^+CD20^+IgD^-CD27^-CD38^-$) and antibody secreting cells ($CD19^+CD20^-IgD^-$ $CD27^{++}CD38^{++}$) as described [19]).

2.6. Microarray studies

RNA was extracted using the PAXgene Blood RNA Kit IVD for isolation and purification of intracellular RNA from blood stabilized in PAXgene Blood RNA Tubes. RNA integrity was assessed using a bioanalyzer and only samples with an RNA integrity (RIN) # of >7.5 were processed for arrays. A constant amount (400 ng) of total RNA was amplified and hybridized to the Illumina H12-v4 human whole genome bead arrays. The data were submitted to ImmPort database (https://immport.niaid.nih.gov, study number N01-AI-100018C).

Illumina GenomeStudio software was used to export expression levels and detection p-values for each probe of each sample. Signal intensity data was quantile normalized and log2 transformed. Significance of expression level differences between any two groups values were done using linear regression with correction for multiple testing to estimate False Discovery Rate (FDR) done according to Storey et al. [20].

2.7. Statistical analyses

Titer data were analyzed using Wilcoxon rank-sum tests. The baseline information was taken into account using fold-change from baseline at post-vaccination. Mixed-effect models were used to examine the effect from timing of blood-draw on the level of titers adjusted for the time of visits (2nd visit vs. 1st and 3rd visit vs. 1st visit). For each studied variable in titers, log-transformed titer was used in Mixed-effect models analysis. A $p\text{-value} < 0.05$ is considered to be statistically significant.

3. Results

3.1. Study design

We conducted a 5-year study to assess antibody responses to the influenza A components of the inactivated influenza vaccine with 114 samples from younger (30–40 year-old) and 165 samples from aged (>65 year-old) individuals. At visit 1, individuals were bled to test for pre-vaccination titers and distribution of B cell subsets and then vaccinated. They were bled again 7 days later at visit 2 and 14 or 28 days later at visit 3 to assess antibody titers when according to our previous studies neutralizing antibody responses have peaked [18]. As of year 2, blood samples from the 1st and 2nd visit were analyzed for their global gene expression profiles. Records were kept at what time individuals were vaccinated or bled (AM vs. PM in years 1 and 2 of the study, actual time as of year 3). Numbers of samples from individuals that were vaccinated and or bled at the different visits in the morning or afternoon are shown in Suppl. Table 1. Other human subject characteristics such as age, gender, race, influenza vaccination history, body mass index (BMI) and use of common prescription drugs (metformin, non-steroidal anti-inflammatory drugs [NSDAIDS] and statins) that distinguished individuals vaccinated or bled AM or PM during the 3 visits were largely comparable (Suppl. Table 2). In the younger at visit 1 there was a significant difference in BMI between the AM and PM groups while in the aged there was a trend towards higher use of NSDAIDS in the visit 2 AM group. As these differences were not consistent between time points we doubt that they affected our results. There were significant and consistent differences between the two age groups regarding time of AM vaccination and sample collection; the younger individuals at all 3 visits were treated about an hour earlier than the aged. The opposite trend was seen in the afternoon.

3.2. Effect of timing of vaccination and collection of pre-vaccination blood on antibody titers and responses

We divided samples into those from individuals that were bled and vaccinated ante (8–12 am, 65 younger and 94 aged) or post (12–5 pm, 48 younger and 71 aged) meridiem. In the aged VNA titers to H1N1 were significantly higher at baseline in the PM

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