Changes in Urine Proteome Before and After Influenza Vaccination

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Abstract

Proteins in urine are enriched through multiple filtrations of plasma by the glomeruli, and the information they contain can sensitively reflect changes in the human body. Following vaccination, blood antibody titers and seroconversion rates are typically assessed at 2–4 weeks; could urine enable earlier observation of the initiation or progression of the human immune response? Are immune responses consistent across different individuals after receiving the same vaccine? We recruited 8 volunteers (4 female, 4 male) to receive the seasonal quadrivalent influenza split-virus vaccine. The vaccine was manufactured using influenza A and B virus strains recommended by the World Health Organization (WHO), which were separately inoculated in chicken embryos, followed by culture, virus harvest, inactivation, concentration, purification, and splitting. Clinical trials have validated that this vaccine provides antibody protection, i.e., it stimulates the body to produce immunity against influenza virus upon administration. We found that immune responses varied among individuals after vaccination; differential proteins associated with the immune response in urine first appeared in samples collected 24 hours post-vaccination.

Full Text

Preamble

Changes in Urine Proteome Before and After Influenza Vaccination

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Introduction

Urine proteins are enriched through multiple filtrations of plasma by the glomeruli, and the information they contain can sensitively reflect physiological changes in the human body. While antibody titers and seroconversion rates in blood are typically measured 2–4 weeks after vaccination, we asked whether urine might reveal the initiation or progression of immune responses at an earlier stage. Additionally, do individuals mount consistent immune responses after receiving the same vaccine? To address these questions, we recruited eight healthy volunteers (four female, four male) to receive the seasonal quadrivalent influenza split-virus vaccine. The vaccine was manufactured using influenza A and B strains recommended by the World Health Organization (WHO), propagated in chicken embryos, and processed through cultivation, virus harvest, inactivation, concentration, purification, and splitting. Clinical trials have confirmed its protective efficacy, demonstrating that vaccination stimulates immunity against influenza virus. We found that individual immune responses varied considerably following vaccination, with immune-related differential proteins in urine appearing as early as 24 hours post-vaccination.

Materials and Methods

Subjects and Sample Collection

Eight volunteers (four female, four male, aged 21–29) received the quadrivalent influenza split-virus vaccine from Hualan Biological Vaccine Inc. Urine samples were collected before vaccination and at 24 hours post-vaccination, followed by collection every 7 days for a total of four additional time points. This study was approved by the Ethics Committee of Beijing Normal University, and all participants provided informed consent. Urine samples at three time points were unavailable due to volunteer menstruation or scheduling conflicts.

Protein Sample Preparation and Trypsin Digestion

Urine samples (n = 45) were stored at -80°C until all collections were complete. For analysis, 20 mL of each urine sample was processed. Proteins were reduced with 20 mmol/L dithiothreitol (DTT) at 37°C for 1 hour to break disulfide bonds, followed by alkylation with 55 mmol/L iodoacetamide (IAA) in the dark for 30 minutes. Proteins were then precipitated with three volumes of ice-cold ethanol at -20°C for 2 hours, and the precipitate was collected by centrifugation at 12,000 × g for 30 minutes at 4°C. The protein pellet was resuspended in an appropriate volume of lysis buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, and 50 mmol/L Tris). Protein concentration was determined using the Bradford assay.

Using the filter-aided sample preparation (FASP) method, 100 µg of protein from each sample was digested with trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) at a 50:1 protein-to-enzyme ratio. The resulting

peptide solutions were desalted using C18 Oasis HLB columns (Waters, Milford, MA, USA) and dried by vacuum centrifugation. Dried peptides were sealed and stored at -80°C.

LC-MS/MS Analysis

Dried tryptic peptides were resuspended in 0.1% formic acid. For each sample, 1 μg of peptides was analyzed using a Thermo EASY-nLC 1200 chromatography system coupled to a Thermo Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The liquid chromatography setup included a trap column (75 m × 2 cm, nanoViper C18, 2 m, 100 Å) and an analytical column (50 m × 15 cm, nanoViper C18, 2 m, 100 Å). The injection volume was 2 L with a flow rate of 250 nL/min. Mobile phases consisted of (A) 0.1% formic acid in water and (B) 80% acetonitrile/20% water/0.1% formic acid. Peptides were separated using a 120-minute gradient: 0–3 min, 3–8% B; 3–93 min, 8–22% B; 93–113 min, 22–35% B; 113–120 min, 35–90% B.

Mass spectrometry parameters were as follows: nanoESI ion source with a spray voltage of 2.2 kV; capillary temperature of 320°C; S-lens RF level of 30; MS1 scans acquired in the Orbitrap at 120,000 resolution (@ m/z 200) with a scan range of m/z 350–1350, AGC target of 4e5, and maximum injection time of 50 ms; MS2 scans acquired in the Orbitrap at 30,000 resolution (@ m/z 200) with a starting m/z of 110, AGC target of 1e5, and maximum injection time of 50 ms. Precursor ions were selected using a 2.0 m/z isolation window with HCD fragmentation at NCE 32. Data-dependent MS/MS was performed on the top 20 most abundant precursors with a dynamic exclusion time of 15 seconds.

Protein Identification and Quantitative Analysis

Raw data files were analyzed using Proteome Discoverer (version 2.1, Thermo Scientific) and MaxQuant (version 1.6.17.0). Xcalibur Qual Browser (version 3.0.63, Thermo Fisher Scientific) was used to inspect base peak chromatograms. All RAW files were processed together in a single MaxQuant window. Database searching was performed using the Andromeda search engine against the UniProt human sequence database (March 17, 2020; 196,111 entries). Main search precursor mass tolerance was set to 4.5 ppm, and fragment mass tolerance to 20 ppm. Enzyme specificity was set to Trypsin/P with a maximum of two missed cleavages. Minimum peptide length was set to seven residues. Variable modifications included protein N-terminal acetylation and methionine oxidation, while carbamidomethylation of cysteine was set as a fixed modification. No Andromeda score threshold was applied for unmodified peptides; each identified modification required a minimum Andromeda score of 40. Peptide and protein false discovery rates (FDR) were set to 1% using a target-decoy reverse database strategy.

The “match between runs” feature was enabled with a match time window of 0.7 min and an alignment time window of 20 min to parallelize elution times across

samples. Label-free quantification (LFQ) was performed using the MaxLFQ algorithm integrated in MaxQuant.

Subsequent data processing was performed with Perseus (version 1.6.14.0). Contaminants and proteins identified with fewer than one unique peptide were removed. LFQ intensities were log2-transformed for normalization. Missing values were considered to represent low-abundance proteins below the detection limit rather than random missing data, and were imputed with random values from a normal distribution below the median. Unsupervised hierarchical clustering analysis (HCA) was performed using the Wukong platform, and biological pathway analysis was conducted using DAVID Bioinformatics Resources 6.8.

Results

Protein Identification and Differential Analysis via Bottom-Up Proteomics

For each volunteer, urine samples from different time points were analyzed together in a single MaxQuant window, yielding comparable total protein numbers per individual. Each time point was analyzed with three technical replicates, which showed Gaussian distribution and high similarity in protein numbers and intensities, indicating high reliability. A total of 32,415 tryptic peptides were identified, including those with N-terminal acetylation and methionine oxidation. These peptides mapped to 5,199 protein groups, with an average of approximately 10 peptides identified per group and a sequence coverage of 30.9%. Among these, 1,893 high-confidence proteins (Q $<$ 1% and $>$1 unique peptide) were identified. The urine proteome spanned over six orders of magnitude, demonstrating excellent depth of coverage.

Each volunteer provided one baseline sample before vaccination and subsequent samples at 24 hours, 7 days, 14 days, 21 days, and 28 days post-vaccination. We employed two comparison strategies to identify differential proteins: Method 1 compared each post-vaccination time point to the pre-vaccination baseline, while Method 2 compared adjacent time points. Differential proteins were filtered using criteria of $p < 0.05$ and fold change >2 or <-2 for both methods. Method 1 identified 25 shared differential proteins across all eight volunteers, while Method 2 identified 35 shared proteins, with 14 proteins common to both approaches. Among the 25 proteins from Method 1, five were associated with neutrophil or leukocyte immune pathways, while 13 of the 35 proteins from Method 2 were involved in leukocyte-mediated immunity. The biological pathways enriched from these protein sets are illustrated in the accompanying figures. The identification depth of immune-related proteins among the shared differential proteins is also shown. Detailed information on the number of differential proteins per volunteer at each time point and their intersections is provided in the supplementary figures.

Distinct Immune-Related Pathways Reflected in Urine Proteomes of Eight Vaccinated Volunteers

We performed self-controlled before-and-after analysis for all eight volunteers and identified differential proteins using the two methods described above, followed by biological pathway analysis for each pairwise time point comparison. Most volunteers showed enrichment of immune-related pathways in urine collected 24 hours post-vaccination, indicating vaccine-induced immune activation. However, the specific pathways varied considerably among individuals. We hypothesize that some volunteers may have had prior exposure to components of the quadrivalent influenza vaccine in their environment, resulting in a secondary immune response upon vaccination. Additionally, individual constitutional differences may contribute to varied immune responses to the same vaccine. Volunteer #3 did not provide a urine sample at 24 hours, so comparison was made between the third time point and baseline.

The table below lists immune-related pathways enriched from differential proteins between each pair of time points for all eight volunteers (pathways with FDR < 0.05 and $p < 0.05$ are highlighted; light blue indicates $p < 0.05$ but FDR < 0.5; dark blue indicates $p < 0.05$ but FDR < 1). Only immune-related pathways are shown here; complete information including p-values, FDR values, and other enriched biological pathways is provided in the supplementary tables. The pathways include diverse immune processes such as innate immune response in mucosa, defense response to Gram-negative bacteria, establishment of skin barrier, inflammatory response, leukocyte migration, acute-phase response, antibacterial humoral response, viral entry into host cell, regulation of macroautophagy, positive regulation of T cell proliferation, and many others, with distinct patterns observed for each volunteer across different time intervals.

Discussion

This study represents the first exploration of human immune responses following vaccination from the perspective of urine proteomics. We demonstrate that even after receiving the same vaccine, individuals exhibit distinct immune-related pathways enriched among differential proteins in their urine proteomes. Notably, immune-related pathways were detectable in urine within 24 hours post-vaccination for most volunteers, whereas blood antibody detection is typically performed 2–4 weeks after immunization. The vaccine manufacturer collected serum antibody titers and seroconversion rates from trial participants 28 days post-vaccination. Our findings suggest a novel approach for earlier validation of vaccine efficacy.

The observed variation in differential proteins and immune pathways among individuals may be attributed to several factors. First, the quadrivalent influenza vaccine contains four viral types, and prior exposure to these viruses may vary among individuals, potentially triggering secondary immune responses in some volunteers. Second, constitutional differences may lead to varying degrees of im-

mune activation upon encountering the same antigen. In conclusion, our study demonstrates that urine proteomics can reveal early immune responses post-vaccination, offering a new strategy for vaccine efficacy validation, while also highlighting the heterogeneity of immune mechanisms triggered by the same influenza vaccine in different individuals.

Note: Figure translations are in progress. See original paper for figures.