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Gene expression profiling toward the next generation safety control of influenza vaccines and adjuvants in Japan

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ABSTRACT

Influenza becomes epidemic worldwide every year, and many individuals receive vaccination annually. Quality control relating to safety and potency of influenza vaccines is important to maintain public confidence. The safety of influenza vaccines has been assessed by clinical trials, and animal safety tests are performed to monitor the consistent quality between vaccines used for clinical trials and marketing; the biological responses in vaccinated animals are evaluated, including changes in body weight and white blood cell count. Animal safety tests have been contributing to the quality relating to the safety of influenza vaccines for decades, but improvements are needed. Although precise mechanisms involving biological changes in animal safety tests have not been fully elucidated, the application of cDNA microarray technology make it possible to reliably identify genes related to biological responses in vaccinated animals. From analysis of the expression profile of >10,000 genes of lung in animals treated with an inactivated whole virion influenza vaccine, we identified 17 marker genes whose expression patterns correlated well to changes in body weight and leukocyte count in vaccinated animals. In influenza HA vaccine-treated animals exhibiting subtle changes in biological responses, a robust expression pattern of marker genes was found. Furthermore, these marker genes could also be used in the evaluation of adjuvanted influenza vaccines. The expression profile of marker genes is expected to be an alternative indicator for safety control of various influenza vaccines conferring high sensitivity and short turnaround time. Thus, gene expression profiling may be a powerful tool for safety control of vaccines in the future.

1. Introduction

Influenza virus triggers the highly contagious acute respiratory disease, influenza. Gradual antigenic drift in two surface antigens of influenza virus, hemagglutinin (HA) and neuraminidase (NA), occurs through influenza epidemics [1]. Global influenza virus surveillance is performed to predict trends in antigenic drift of influenza virus, which is important to inform manufacturers of vaccine strain candidates before the annual epidemic season starts. Many individuals receive vaccination annually, however, vaccination is always accompanied by adverse reactions. Although most adverse reactions are mild and local, severe systemic reactions occur in rare cases. Vaccines are manufactured in compliance with Good Manufacturing Practices to assure their quality. Furthermore, it is mandatory for manufacturers to submit vaccines of each production batch to National Regulatory Authorities, which undergo quality control testing and review to ensure the specifications relating to safety, potency, and purity are met before market release. During the manufacturing process, animal safety tests are performed as quality control tests to detect extraneous toxic contaminants or residual impurities that may cause adverse reactions.

Animal safety tests have long been performed as safety control of various vaccines. However, the procedure takes time, and a number of animals is needed. Further, sensitivity should also be considered for current vaccines with fewer impurities. It is apparent that animal safety testing should be improved in many aspects. Since the late 1990s, the field of toxicogenomics has evolved rapidly [2,3]. Toxicogenomics is the study of the toxicity and side effects of chemicals and drugs on specific organs using gene expression profiling [4,5]. The simultaneous analysis of whole genes can identify unique expression patterns associated with a drug's toxicity, which can be difficult to be evaluated with conventional

Abbreviations: ATT, abnormal toxicity test; HA, hemagglutinin; IFN, interferon; LTT, leukopenic toxicity test; PDv, pandemic influenza vaccine; PLT, platelet; RE, toxicity reference; SA, saline; WBC, white blood cell; WPv, whole virion preparation of inactivated influenza vaccine.

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procedures. At present, gene expression profiling data in chemical compound-treated animals or cells have been accumulated, and large toxicogenomic databases are available [6–10]. Further, after the completion of the draft human genome in the early 2000s [11,12], the expectation of pharmacogenomics and pharmacogenetics has increased. In the United States, genomic data related to investigational new drugs or new drug applications have been submitted to the Food and Drug Administration since 2004, either voluntary or on request [13]. Recently, approaches in genomics and transcriptomics have expanded to the field of vaccinology. Many transcriptomic studies have reported on immunological responses against vaccines in humans [14–20]. Here, we describe our studies in the last decade to apply a transcriptomic approach to the safety control of influenza vaccines in a preclinical setting.

2. Animal safety tests of influenza vaccines in Japan

2.1. Abnormal toxicity test

Abnormal toxicity test (ATT) is also known as the general safety test or innocuity test. ATT analyzes body weight changes of vaccine-treated animals to screen for general vaccine toxicity, including influenza vaccines. Influenza vaccine (5 mL) is injected into the peritoneum of guinea pigs weighing 300–400 g, and weight change is analyzed at 1, 2, 3, and 7 days after administration. None of the animals should show any abnormal signs; no statistically significant difference in weight change should be observed between the treated animals and the control group over the test period [21]. ATT plays an important role in safety control of influenza vaccines, and also ensures consistency among vaccine batches [22]. For the analysis, 8-week-old rats are intraperitoneally administered with 5 mL of samples, and the body weight change is evaluated for 4 consecutive days after administration.

2.2. Leukopenic toxicity test

Leukopenic toxicity test (LTT) analyzes peripheral white blood cell (WBC) counts in mice 12–18 h after intraperitoneal injection of influenza vaccine. LTT uses the inactivated whole virion influenza vaccine as the toxicity reference (RE), which induces a significant WBC count reduction in mice [23,24]. For LTT, physiological saline (SA), influenza HA vaccine (HA vaccine), and serial dilutions of RE are injected to mice at a dose of 0.5 mL. At least 5 and 10 mice aged four weeks are used for HA vaccine/RE-treated and SA-treated groups, respectively. The leukopenic toxicities of influenza HA vaccines are calculated relative to RE, and to pass LTT, leukopenic toxicity must be no higher than that reducing to 80% of the WBC count of the SA-treated group [21]. For the analysis, 8-week-old rats are intraperitoneally administered with 5 mL of samples, and the WBC counts is examined at 2, 16, 48, 72, and 96 h after administration, unless otherwise indicated.

3. Gene expression profiling for safety control of various influenza vaccines

3.1. cDNA microarray analysis

Animal safety tests, including ATT and LTT, have contributed to the safety control of influenza vaccines over recent decades. However, improvement and development of a new evaluation system is warranted. We have been pursuing the application of cDNA microarrays to vaccinated animals to characterize the biological effects of vaccines in terms of gene expression profiling [25–27]. Unlike the ATT guideline in Japan, we used rats for the analysis because rats are commonly used for toxicity testing, and substantial research tools for rats are available for further studies. An inactivated whole virion influenza vaccine (WPv) and an inactivated whole virion H5N1 pandemic vaccine absorbed onto an aluminum salt (PDv) were provided from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), and were injected into rat peritoneum. SA and an HA vaccine were also included in the study. We found that WPv and PDv induced significant body weight loss for 4 consecutive days, and there was a significant decrease in WBC and platelet (PLT) counts 16 h after administration.

For cDNA microarray analysis in vaccinated rats, lungs were harvested at 1, 2, 3, and 4 days after vaccine administration. Lungs were snap frozen in liquid nitrogen for storage. Thawed lung was homogenized and poly(A)$^+$ RNA purified from each sample was labeled with Cyanine 5 (Cy5). cDNA microarray analysis was performed using a set of synthetic 80-mer polynucleotides representing 11,464 rat transcripts derived from 10,490 independent genes, including most of the RefSeq clones deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan), arrayed on aminosilane-coated glass slides [28–30]. Cy5-labeled poly(A)$^+$ RNA from each sample was competitively hybridized on the slide with Cyanine 3 (Cy3)-labeled rat common reference RNA. Hybridization signals were measured, processed into primary expression ratios (Cy5 intensity of each sample/Cy3 intensity of common reference RNA), and then normalized by multiplying normalization factors calculated for each microarray feature. For data processing and hierarchical cluster analysis, the primary expression ratios were converted into $\log_2$ ratios. Those genes with $\log_2$ ratios greater than 1 or less than –1 in at least one sample were extracted from the primary data matrix, and then subjected to two-dimensional hierarchical cluster analysis for samples and genes [30]. WPv- and PDv-treated samples at day 1 showed a clear and significantly distinct pattern in gene expression compared to that found in other samples, which correlated to the results of ATT and LTT. Because gene expression profiling at day 1 could characterize biological responses in rats treated with whole virion-type influenza vaccines, the test time was considerably shortened (Fig. 1).

3.2. Identification of marker genes

Our analysis of lungs from vaccinated rats at day 1 resulted in the identification of 76 genes, whose expression levels were statistically upregulated in WPv-treated samples compared to SA-treated samples ($P < 0.005$) [30]. These 76 genes can be further categorized according to type or function, including interferon (IFN)-inducible, chemokine/cytokine, and immune response. Among these genes, 17 met the requirement for high expression and were chosen for further study [31] (Table 1). Most of the identified marker genes, and their transcripts, were related to influenza virus infection and immune responses. For example, *Irf7* and *Mx2* are considered IFN-stimulated genes. IRF7 belongs to the IFN regulatory factor family of transcription factors. IRF7 is phosphorylated upon virus infection, translocates into the nucleus, and binds to the IFN-stimulated response element within the promoter region of target genes including IFNα and IFNβ [32]. It was reported that stable knockdown of *Irf7* in Madin-Darby canine kidney (MDCK) cells enhanced production of influenza viruses [33]. Further, mouse *Mx2* was shown to be upregulated in lungs infected with the low pathogenicity H3N2 influenza strain X31, along with *Ifi47* [34]. Although rodent MX2 dose not inhibit influenza virus replication probably due to its subcellular localization in the cytoplasm [35,36], human MXA (an ortholog of rodent MX1) can bind to influenza virus nucleoprotein in the cytoplasm [37] and retain the viral genomic RNA near late endosome [38]. CXCL9 and CXCL11 are IFN-stimulated chemokines that are upregulated in human dendritic cells exposed to influenza virus [39]. *Lgals9* encodes galectin-9 that specifically binds to β-galactoside sugars. Serum

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