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Chapter 6

Genomic Approaches Enable Evaluation of the Safety and Quality of Influenza Vaccines and Adjuvants

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76361

Abstract

Vaccination is an effective means for prevention of the progression and spread of influenza virus infection. Nonetheless, there is a risk of adverse reactions, such as pain and fever, during the vaccination. In addition, because people from a wide age range, that is, from children to the elderly, are inoculated with vaccines, safety confirmation of these vaccines is important. Safety assessments of a vaccine, in the form of quality controls, have been carried out on animals. For example, the abnormal toxicity test is based on body weight changes as a toxicity index, and the leukopenic toxicity test can evaluate hematological toxicity. Meanwhile, since the 2000s, safety evaluation of drugs and chemicals by the genomic approach has been conducted frequently. The benefits with respect to safety evaluation are high sensitivity and abundant information about toxicity profiles. In this chapter, we describe the genes that are helpful as safety assessment markers and their usefulness for safety testing and vaccine development. In addition, this information may provide toxicity profiles, help understand the reactogenicity of nasal vaccines or adjuvants, and explain the prospects of genomic analyses in the development of novel vaccines and adjuvants.

Keywords: influenza vaccine, safety test, biomarker, preclinical test, quality control

1. Introduction

The current toxicological assays for chemicals and biological therapeutics (biologicals) involve high costs, are time-consuming, and require a large number of animals. Thus, such a project becomes a substantial investment in the development of a drug or biological therapeutic [1, 2]. There is a need to improve these preexisting safety-testing strategies.

The microarray technology was recognized in the toxicology research community after its introduction in the 1990s [3–5]. Subsequently, toxicological studies using the microarray technology have given rise to a new field termed toxicogenomics [6]. By integrating the genomic technology and bioinformatics, toxicogenomics has garnered a great deal of attention as an alternative means of addressing drug safety by studying the fundamental molecular mechanism of toxicity, which was difficult to detect with conventional toxicological methods [6]. In fact, microarray-based toxicogenomics remains a major breakthrough. Using microarrays, we can monitor the expression levels of tens of thousands of genes at the same time and evaluate gene expression profiles altered by various compounds or changes in gene expression profiles associated with different physiological conditions. Moreover, testing a large number of genes together gives the opportunity to identify genetic patterns and signatures that provide unique insights into drug toxicity, which are difficult to obtain by conventional animal-based techniques [7]. Thus, toxicogenomics is expected to revolutionize the traditional approaches to toxicity assessment and has been considered a paradigm shift in toxicology. To date, many studies have revealed the value of toxicogenomics [8–15]. For example, it has been suggested that toxicogenomic biomarkers can identify drug candidates that are more likely to cause toxicity in susceptible patient populations despite the lack of conventional toxicity indicators, such as hematological parameters, body weight changes, blood biochemical data, and histopathological data, which are examined in preclinical studies [16, 17]. Similarly, more sensitive biomarkers for the detection of early toxicity can be analyzed at "subtoxic doses" of a candidate therapeutic agent, where the injury is at the genetic level, but does not occur at the phenotypic level or cannot be detected by clinical-chemistry measurements [18].

Just as chemical and synthetic drugs, biological therapeutics are evaluated for their toxicity by safety tests involving animal experiments, as part of preclinical studies. In addition, to guarantee the quality and homogeneity of the preparation, a portion of the biological preparation is subjected to toxicity tests for each lot [19, 20]. These toxicity tests are based on the aforementioned conventional assays, and phenotypic alterations such as body weight changes, hematological changes, pathological changes, and similar parameters are the evaluation criteria [19, 21]. Tests of the safety and quality control of vaccines include the abnormal toxicity test (ATT, also known as a general toxicity test) [21], and the leukopenic toxicity test (LTT) [19]. In all preclinical trials, in addition to these tests, a pathological examination is carried out. Although these tests have historically been practiced for a long time, it is expected that genomics techniques will be incorporated into these tests to improve their sensitivity and to obtain information on toxicity. For biologicals, however, toxicity studies using the genomics technology have not yet been actively carried out, when compared to the testing of chemical and synthetic drugs.

Therefore, we have been using the genomics technology to search for vaccine safety assessment markers since the late 2000s. In particular, we have been conducting research on the use of genomics technology for studying pertussis vaccine [22, 23], Japanese encephalitis vaccine [24], and influenza vaccine [25]. This chapter provides an introduction to the genomics technology in the safety assessment and quality control evaluation of influenza vaccines and describes a new evaluation method involving the biomarkers obtained by the genomics technology.

http://dx.doi.org/10.5772/intechopen.76361

2. Current vaccine safety tests

This section describes the lot release safety and quality control testing methods implemented for the influenza vaccine. The ATT has been conventionally conducted as an animal-based test to evaluate the contamination by phenol, which is used in the process of inactivating endotoxins, viruses, and bacteria [26, 27]. The method of ATT is simple: 5 mL of a sample is injected into the abdominal cavity of a guinea pig, and its survival and 7-day body weight changes are measured [19]. It has been suggested that these 7-day body weight changes reflect the biological activity of the vaccine. Indeed, if the animals were inoculated with a different type of influenza vaccine, their body weight changes would show different profiles [21]. The whole-particle inactivated influenza vaccine (WPV) that has high reactogenicity [28]. Nevertheless, it causes highly frequent adverse reactions, such as pain, swelling, and fever [29]; on the other hand, the hemagglutinin-split influenza vaccine (HAV), whose effectiveness is inferior to that of the WPV [28], has been reported to cause almost no adverse reactions [29]. The current seasonal influenza vaccines are based on the HAV, and the WPV is manufactured only as a pandemic vaccine. This approach also includes avoiding adverse reactions caused by WPVs. Therefore, the WPV also serves as a toxicity index in the quality control testing of the HAV by the LTT, which is described later.

The LTT is a safety test that assesses the leukopenic toxicity induced by the WPV as a toxicity index [19]. In this method, mice are inoculated with 0.5 mL of a WPV as a toxicity reference vaccine, and the leukopenic activity rate induced by the WPV at that time point is set to 100% leukopenic activity. At that time point, the test sample confirms whether the leukopenic activity rate is within 20% or not. The test criterion is as follows: the leukopenic activity rate should be less than 20% of the toxicity control. On the other hand, the ATT is an assay that evaluates the body weight loss of guinea pigs, and the transition during their recovery. When the same experiment was carried out in rats, WPV-injected rats showed a severe body weight loss, unlike HAV-injected rats [21]. A vaccine showing a statistically significant body weight loss in a population, when compared with a homogeneous preparation, would be rejected in terms of its quality.

Thus, safety and quality of the HAV are mainly ensured by two tests. Safety assessments of the HAV have been conducted using WPVs, which is one of the safety indices.

3. The genomic approach to identifying novel biomarkers of influenza vaccine safety

The search for new biomarkers that can reflect the bioactivity assessed in the ATT and LTT was conducted by performing comprehensive gene expression analyses on major organs via the microarray technology [25]. Inactivated influenza vaccines have been widely used for preventing infections and the spread of infections; they can roughly be subdivided into two types: the HAV and WPV [30]. The HAV mainly contains hemagglutinin (HA). This type of vaccine has no strong bioactivity; it does not contain substances other than HA proteins that act as antigens, thereby leading to no adverse reactions. Nevertheless, their ability to induce

antibody production is considered insufficient to prevent the progression of influenza virus infection [30]. Historically, however, vaccines have been more effective than the existing split vaccines. The WPV is considered effective against influenza virus infections. This type of vaccine contains the whole influenza virus particle, including lipid and single-stranded RNA, and therefore, drives various immune responses. On the other hand, various WPV-induced immune responses also cause adverse reactions in humans [29]. Therefore, although WPV is a highly effective vaccine, it has lately not been employed as a seasonal influenza vaccine and is only partially manufactured as a pandemic influenza vaccine. We have carried out the searches for safety assessment marker genes of the HAV using two types of vaccines: the WPV and split influenza vaccine (SV). The WPV has high reactogenicity (effectiveness and toxicity) and therefore serves as a toxicity reference. The SV has low reactogenicity and frequency of adverse reactions and is therefore employed as a safety control. As a result, the clearest clustering of gene expression patterns in the lungs of animals by different types of vaccines was obtained [25]. In particular, the gene expression patterns in the lungs differed between the SV-treated and WPV-treated animals. Furthermore, the gene expression levels, which showed large differences between the SV- and WPV-treated animals, were estimated. As a result, 18 genes expressed in the lungs were identified as biomarker genes (**Table 1**) [25].

Symbol	Official full name	Accession
Cxcl11	Chemokine (C-X-C motif) ligand 11	NM_019494
Cxcl9	Chemokine (C-X-C motif) ligand 9	NM_008599
Zbp1	Z-DNA binding protein 1	NM_021394
Mx2	MX dynamin-like GTPase 2	NM_013606
Irf7	Interferon regulatory factor 7	NM_016850
Lgals9	Lectin, galactoside-binding, soluble, 9	NM_010708
Ifi47	Interferon gamma inducible protein 47	NM_008330
Tapbp	TAP binding protein (tapasin)	NM_001025313
Csf1	Colony stimulating factor (macrophage)	NM_007778
Timp1	Tissue inhibitor of metalloproteinase 1	NM_001044384
Trafd1	TRAF type zinc finger domain containing 1	NM_001163470
Lgals3bp	Lectin, galactoside-binding, soluble, 3 binding protein	NM_011150
Psmb9	Proteasome (prosome and macropain) subunit, beta type, 9	NM_013585
C2	Complement component 2	NM_013484
Tap2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	XM_006525355
Ifrd1	Interferon-related developmental regulator 1	NM_013562
Psme1	Proteasome (prosome and macropain) activator subunit 1	NM_011189
Ngfr	Nerve growth factor receptor	NM_033217

Table 1. Marker genes for safety evaluation of influenza vaccines.

http://dx.doi.org/10.5772/intechopen.76361

Functionally, these biomarker genes tend to correlate with the white blood cell (WBC) count in the peripheral blood of animals (leukopenic toxicity) that were treated with the inactivated influenza vaccine [31, 32]. It has been known that intraperitoneal injection of the WPV causes leukopenic toxicity in mice, and this bioactivity has been used as an index for the safety evaluation of inactivated influenza vaccines in Japan, which is termed the LTT [19]. In addition, WPV-induced body weight loss may be reflected in biomarker gene expression levels [25]. Thus, WPV-like bioactivity assessed by the ATT could be predicted by the expression levels of biomarker genes. Furthermore, the biomarker genes can partially measure biological activities that cannot be quantitated by means of body weight changes. For example, the identified biomarker gene expression increases with leukopenia and weight loss; however, some genes show increased expression even in a state without body weight loss and without a decreased WBC count (without leukopenic toxicity) [33]. The SV hardly induces the elevated expression of biomarker genes. By contrast, if SVs produced at different manufacturing plants were evaluated using the biomarker gene expression levels, variations in their quality can be reflected in the expression levels [33]. This variation is not indicated by body weight changes or by the WBC count [33]. This case is an example where the gene expression level can detect biological reactions that cannot be detected by phenotypic changes. This is the advantage of the toxicogenomics technology. Thus, it is likely that the genomics technology is also useful for the safety assessment of vaccines. Furthermore, there is a possibility that the assay involving the newly identified biomarker genes can be widely adopted as an alternative method to the currently popular methods: the ATT and LTT.

4. Safety evaluation of influenza vaccines on the basis of biomarker gene expression

The utility of the identified biomarker genes has been verified. For seasonal influenza vaccines, the ATT has been regarded as a test for safety and quality control. Therefore, a safety assessment of SVs manufactured at four manufacturing plants was conducted by means of biomarker gene expression and body weight as parameters (ATT) and by the LTT, with a WPV as a control [33]. With respect to phenotypic changes, body weight loss rates of all the SVs were found to be equivalent, and leukocyte number reduction was hardly observed for the HAVs from all the manufacturers. Nevertheless, in case of one manufacturer's HAV, analyses of the expression of 18 biomarker genes in lungs showed a significant difference in gene expression levels from other manufacturers' HAV [33]. This result suggests that the biomarker genes identified by the microarray analysis can capture biological changes that cannot be detected by body weight changes and leukocyte number reductions. This finding indicates that the analysis of expression of biomarker genes is a more sensitive assay than the conventional safety and quality control tests (ATT and LTT). This evaluation method can be applied not only to predict the toxicity but also to evaluate the homogeneity among vaccines produced in different batches.

Subsequently, the safety assessment of trivalent virosome-type influenza vaccine (Inflexal Berna V) currently licensed in several European countries such as Switzerland and Italy was

performed by means of the biomarker genes. The virosome-type influenza vaccine is similar to the WPV but does not contain viral RNA. Leukopenic reactions were not noticeable when the animals were vaccinated with the virosome-type influenza vaccine; however, a body weight loss was observed, accompanied by an increase in the expression of some biomarker genes [31]. It is thought that some biological activities of this vaccine may be close to those of the WPV, because just like the WPV, Inflexal Berna V consists of a virosomal formulation. Genes whose increased expression levels were induced by the virosomal type influenza vaccine include *Tap2* and *Psmb9*, which are involved in antigen presentation and antigen digestion, suggesting that the antigen-presenting ability is higher for the virosomal-type influenza vaccine than for the HAV [31]. Consequently, it is likely that biomarker genes obtained by genomic analysis can elucidate the mechanistic details of bioactivity and toxicity.

5. Safety evaluation of the nasal influenza vaccine using biomarker gene expression

Nasal vaccines have been attracting attention as promising strategies against influenza virus infection. This is because nasal vaccines can predominantly induce mucosal immunity, when compared with conventional subcutaneous vaccines or intramuscularly injectable vaccines [34]. In nasal vaccines, IgA antibody production and secretion in the bronchial and intranasal cavities are observed, and this approach seems to be effective for the prevention of influenza infection [35–37]. For this reason, several newly developed vaccines have been designed on the premise of nasal inoculation. It is important to develop a safety assessment method for nasal vaccines by assays that are different from the conventional intramuscular and subcutaneous injections. This is because there are case control studies on the use of the inactivated intranasal influenza vaccine, which is composed of influenza antigens in a virosomal formulation with an *E. coli*-derived LT adjuvant, and the risk of Bell's palsy in Switzerland [38]. Therefore, to determine the relation between the expression of the 18 biomarker genes and the safety evaluation of the nasal inoculation influenza vaccine, an assay was devised. Mice were nasally inoculated with an influenza vaccine, and biomarker gene expression levels in the lungs were analyzed [39]. As described earlier, this biomarker gene has been identified based on the gene expression profile obtained when the vaccine was inoculated intraperitoneally. After the administration of the nasal influenza vaccines, there was an increase in the WPV-dependent expression of the biomarker gene; the evaluation of the HAV based on WPV was shown to be possible by nasal inoculation and by analysis of marker genes [39]. Furthermore, the biomarker expression level positively correlated with lymphoproliferation in nasal-associated lymphoid tissue [39], and it was inferred that this formulation induces the activity of mucosal immunity. Furthermore, in recent years, the development of an adjuvant-containing vaccine has been advanced for the purpose of enhancing the effectiveness of SVs [37]. The same trend in nasal vaccines has also been seen [37] because of the ability to induce IgA production by SVs alone is not enough to prevent infection with an influenza virus. Therefore, there has been active development of adjuvanted influenza vaccines. Although adjuvants increase the effectiveness of vaccines, strong adjuvant bioactivity is thought to lead to toxicity.

The strong bioactivity of the adjuvant will ensure increased effectiveness of vaccines. In some cases, however, highly reactogenic adjuvants can cause toxicity in humans. For example, poly I:C is known to function as an excellent vaccine adjuvant. On the other hand, it is known to cause exothermic reactions and cytokine storms [40–42]. Additionally, in the past, poly I:C has been discontinued due to adverse reactions such as a fever and arthritis in clinical trials [40]. Even other adjuvants such as R848, a Toll-like receptor (TLR)7/8 agonist, are known to cause cold-like symptoms, including a fever [43–45]. Such compounds are **excellent in terms of enhancing the** effectiveness of the vaccine; however, the risk of developing toxicity remains high. Therefore, we hypothesized that the expression of 18 biomarker genes could be applied to the safety assessment of adjuvanted vaccines. The objective of this safety test is to identify an adjuvant that has high reactogenicity and toxicity such as poly I:C and R848. The risks of adverse reactions caused by adjuvanted vaccines as test products were compared with those of the WPV. In the case of a nasal vaccine, expression of some biomarker genes was higher when animals were inoculated with the TLR9 agonist CpG K3-adjuvanted HAV, than when the animals were inoculated with the HAV alone [39]. Nonetheless, the marker gene expression levels were markedly lower than those of the WPV. Thus, the CpG K3 adjuvant did not have high reactogenicity accompanied by toxicity. The CpG K3 adjuvant is under development for use with malaria vaccines [46]; no adverse reactions have been reported so far. The authors of these reports presumed that the risk of toxicity would not be high in humans. Currently, the authors are working on building a database for constructing an adjuvant evaluation system based on an influenza vaccine that includes various adjuvants including poly I:C and R848, an oil/water emulsion adjuvant, and various other TLR-related adjuvants.

6. Development of an alternative assay for the leukopenic toxicity test based on biomarker gene expression

The biomarker genes for the safety assessment of an influenza vaccine are characterized by the biological activity that can be detected by the ATT and LTT. Specifically, biomarker gene expression levels and the WBC count with body weight changes show a negative correlation [31]. Momose *et al*. (2015) reported that a virosomal influenza vaccine caused only a body weight loss and did not cause leukopenia; however, some of the marker genes showed increased expression levels at that time point [31]. In other words, it seems that all the marker genes cannot respond uniformly via the same mechanism of action. Therefore, we considered whether the leukocytopenic activity could be evaluated with the expression of the marker genes responsible for the leukopenic activity, and we searched for biomarker genes associated with leukocytopenic activity. Furthermore, we devised a method for WBC count-predicting systems involving only the biomarker gene expression levels. If this method is established, it will be possible to set up the WBC number prediction using the biomarker gene expression and body weight loss evaluation by the ATT in one test system. This strategy will reduce the number of animals required and shorten the testing duration. We tried to identify the genes useful for the prediction of the WBC count from the biomarker gene set by multiple linear regression analysis and a stepwise method [32]. In the multiple regression analysis method,

a linear equation expressed by the following formula was derived, and a predicted value was calculated. In particular, the leukocyte count of the animals and data on the expression levels of all the biomarker genes were acquired. The animals were inoculated with the WPV or HAV, and the expression levels of marker genes and numbers of WBCs were then determined. Multiple regression analysis was performed on the acquired data. A linear equation was then derived. The regression equation is shown below.

$$(\text{Predicted WBC}) = (\text{intercept}) + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n \quad (1)$$

In this equation, "x" is substituted by the marker gene expression that corresponds to its coefficient (β); "n" indicates the number of factors corresponding to the number of selected genes. The intercept was used for calculation of the WBC number in the multiple regression analysis.

Precision of the prediction differs depending on the combination of marker genes. Therefore, by the stepwise method, a linear equation was derived that contains the combinations of marker genes having the highest prediction accuracy. As a result, some gene combinations (models) were selected (**Table 2**). Predicted leukocyte numbers produced by these models can predict leukopenia caused by WPVs with high accuracy (**Figure 1**). Even if the predicted

Predictor variable	Model 1	Model 2	Model 3	Model 4
	β	*β*	*β*	*β*
Intercept	2141.2	5390.6	4222.5	5293.7
C2	—	—	—	502.8
Trafd1	−3196.2	−2886.6	—	−1131.1
Irf7	—	—	—	−94.7
Csf1	—	—	—	1118.1
Ngfr	−1344.8	—	—	−360.1
Ifi47	—	—	—	472.3
Ifrd1	—	—	—	−1628.5
Psme1	4099.9	—	—	—
Tap2	3084.6	1839.1	—	—
Cxcl11	−0.3847	−0.1217	—	—
Lgals9	−8.0607	—	—	—
Zbp1	−197.49	—	—	—
Cxcl9	—	—	1.8226	—
Lgals3bp	—	—	−552.93	—
Tapbp	—	—	349.20	—

Table 2. Multiple regression by the stepwise forward selection method for the leukopenic reaction prediction model (cells/μl blood).

http://dx.doi.org/10.5772/intechopen.76361

values of individuals are analyzed, the deviation between the predicted value and the measured value is small [32]. In addition, variations in the WBC count owing to individual differences are reproduced with high accuracy [32]. Therefore, it was shown that the leukopenic activity can be predicted by means of the identified marker gene set. With this method, it is expected that it will be possible to carry out WBC count reduction assays and abnormal toxicity negative tests by expression analysis of one biomarker gene. As mentioned in the previous section, the development of adjuvanted vaccines has advanced in recent years. Some adjuvants, like WPVs, exert a leukocytopenic activity. Leukopenic activity is also present in compounds with an excellent adjuvant activity such as Poly I:C and R848 [43]. Therefore, we analyzed the CpG K3 adjuvant, which manifested a slight leukopenic activity according to the newly developed multiple regression Equation [32]. As a result, a slight leukopenic activity was observed in animals that received the CpG K3 adjuvant in combination with the SV. Furthermore, when the expression levels of the marker genes were analyzed, and the predicted WBC count was calculated from their expression levels, the leukocyte count reduction by the CpG K3 adjuvant could be predicted with high accuracy [32]. At the same time, however, it became clear that leukocytosis is unpredictable in this system [32]. When the CpG K3 adjuvant was inoculated at low concentrations, the leukocyte count tended to

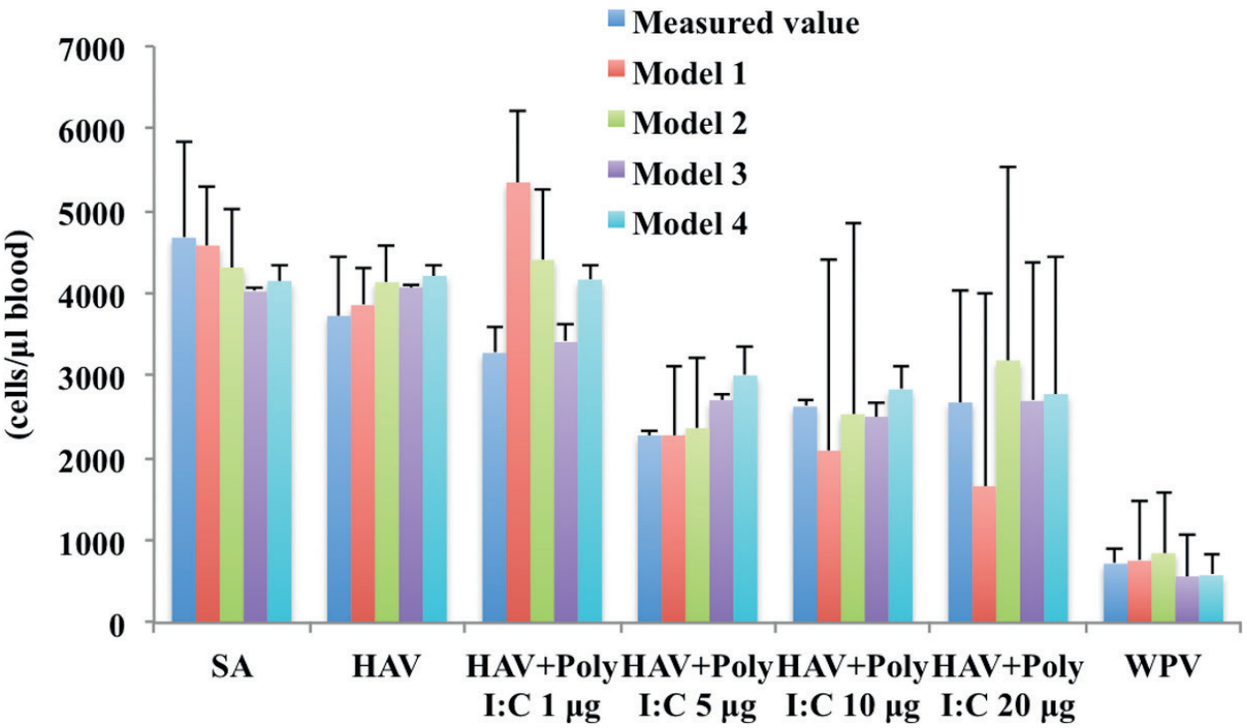

Figure 1. Prediction of a leukocyte count reduction by means of marker gene expression levels in mice treated with influenza vaccine. In the leukopenic toxicity test (LTT), mice were intraperitoneally injected with 0.5 mL of one of influenza vaccines. The dosing of whole-particle inactivated influenza vaccine (WPV) and hemagglutinin-split vaccine (HAV) was 15 μg hemagglutinin (HA)/0.5 mL. Poly I:C was reconstituted in an appropriate volume of the HAV solution to obtain concentrations of 1, 5, 10, and 20 μg poly I:C per dose. Saline (SA) served as the cssssontrol. Sixteen hours after vaccination, blood was collected to assess the numbers of leukocytes, and the lungs were immediately excised. The collected lung tissue was subjected to gene expression analyses. The predicted values were calculated via the multiple regression equation. The coefficient values and values serving as the intercept are indicated in **Table 2**. The error bar indicates standard deviation.

increase. This increase in the WBC count could not be predicted from the biomarker gene expression levels. The possible reason is that multiple linear regression analysis was performed on the animals inoculated with vaccines with leukopenic activity. The physiological association of the extracted biomarker gene with leukopenia could be another reason for this phenomenon. We have identified biomarker genes highly correlating with leukocyte counts in mathematical terms, ignoring the functions of the genes. There is a report that apoptosis of leukocytes caused by type 1 interferons (IFNs) could be a mechanism underlying the WBC count reduction by WPVs [47]. The marker gene set contains many genes related to type 1 IFN. By contrast, in model 4 (**Table 2**), more than a half of the genes related to type 1 IFN were omitted because they lacked predictability. As a result, it is conceivable that a correlation cannot be obtained from only a simple expression level because of the time lag between gene expression and actual leukocyte depletion; furthermore, a gene itself forms a complicated network.

7. Possibility of application of genomic approaches to *in vitro* safety evaluation methods

Currently, safety evaluation and parts of quality control of vaccines are carried out in animal experiments. The ATT and LTT are representative tests in this regard; they have been practiced for more than 50 years [48]. Due to the nature of the test, the ATT excludes the unintended toxicity of the vaccine; the test of lots having reactogenicity different from that of other reference lots is a final confirmatory test designed to prevent serious toxicity in humans [21]. Actually, the ATT has been incorporated into the lot release of vaccines, especially for inactivated influenza vaccines in Japan [19]. On the other hand, tests on animals are being replaced with *in vitro* evaluation systems involving cultured cells, from the viewpoint of animal welfare and cost [48]. In the safety evaluation of chemically synthesized medicines, there has been a notable development: human cultured hepatocytes and cardiomyocytes prepared from induced pluripotent stem cells [48–52]. The benefit of the *in vitro* evaluation system is not only the reduction in the number of animals used, but also the possibility of using human tissue or fluid samples, so that extrapolation to humans can be expected. For biologicals, a part of the rabbit pyrogen test was replaced with an endotoxin quantitative test. Nevertheless, this replacement has not yet been achieved for all biologicals. The endotoxin test could not be performed on some preparations because of the presence of interfering substances [53, 54]. Therefore, a rabbit pyrogen test has been carried out for these biologicals. In this test, the causative agent of the fever has been recognized as the endotoxin in some biologicals. Thus, switching to a method of directly quantitating endotoxins was introduced as an alternative for the rabbit pyrogen test. Nonetheless, because the ATT evaluates the weight loss of animals, it is difficult to determine from body weight changes what types of molecular signaling pathways or physiological reactions have been affected. Therefore, it has not been easy to develop an *in vitro* assay as an alternative for the ATT.

We have tried to create a safety evaluation system for an influenza vaccine using the genomics technology in animal models [22–25]. The marker genes identified in animal experiments are believed to be involved in the body weight loss of animals after WPV injection [25]. When considering an

alternative assay, phenotypic changes in animals, such as body weight loss, cannot be assessed in cultured cells. On the contrary, marker genes linked to these bioactivities can be identified at the cultured-cell level. Biomarker genes can make it possible to link cellular data with biological reactions observed in animal phenotypes. We are currently working on demonstrating the usefulness of marker genes and their expression mechanisms. Most of the marker genes are involved in an immune response and are related to type 1 IFN signaling and innate immune responses [39]. According to these findings, it is possible that the usefulness of biomarker genes evaluated in animals can be extrapolated to cultured cells, if such cell lines as peripheral blood mononuclear cells, immune cells, and alveolar epithelial cell lines are employed in the assays. If an alternative (*in vitro* method) for the ATT and LTT is developed, it will be possible to secure the safety and quality of the current ATT and LTT by animal-free testing. This approach is expected to reduce the number of animals tested and to shorten the testing period.

8. Establishment of a new evaluation method for vaccine or adjuvant bioactivity based on biomarker gene function

Analysis by the genomics technology can be applied not only to the search for biomarkers but also to mechanistic analyses. Besides, it is possible to classify each biological reaction by hierarchical clustering analysis, according to microarray analysis results. Microarray analysis is the most information-rich assay; however, it is inefficient in terms of cost and labor. In the case of a clear-purpose test such as safety evaluation and quality control, it is expected that robust results will be obtained by using only selected highly important genes for evaluation. Therefore, if we consider the function of the genes identified as safety or quality evaluation markers for influenza vaccines, then the biological activity profile of the vaccine may be predicted. For example, genes such as *Irf7* are induced by type 1 IFN [55], genes such as *Psmb9* and *Tap2* are involved in antigen presentation [56], and *Csf1* participates in the activation of monocytes and macrophages [57]. Thus, expression levels of these genes could serve as indicators of the mode of action and help in the development of a biological activation assessment tool. These genes are thought to be involved in innate immunity, in which responses are observed at a relatively early time point after vaccination. Indeed, expression of these genes was assayed 16 h after vaccination. Therefore, long-term toxicity due to the vaccine (e.g., autoimmune and chronic inflammatory reactions) cannot be assessed. Safety evaluation by means of these biomarker genes is helpful for the development of adjuvant-containing vaccines. This is because most adjuvants are designed to activate the innate immune system. Adjuvants enhance innate immunity via cytokine production and activation of antigen-presenting cells; however, strong activation of innate immunity causes uncontrollable inflammatory reactions. This problem could lead to a fever, pain, and swelling, which appear as adverse reactions. Thus, adjuvants are required to have strong innate-immunity–activating effects, but at the same time, good safety. On the other hand, it is difficult to distinguish between the effectiveness and safety of vaccines. For example, interleukin (IL)-6 and type 1 IFN are important for the induction of adaptive immunity and are favorable for vaccine efficacy [58, 59]. Nevertheless, excess production of IL-6 or type 1 IFN causes a cytokine storm. Thus, safety can be evaluated with the same biological vector as that of effectiveness. In other words, if the factor of effectiveness becomes excessive, toxic effects

may appear. We are currently working on establishing a safety assessment system based on the WPV as a toxicity indicator [60]. The WPV is an effective and excellent vaccine, but the frequency of adverse reactions is high, and currently, it is only rarely used, especially as a pre-pandemic vaccine. Therefore, we believe that the WPV can be a safety indicator. In other words, we think that there is a high probability that adjuvants and vaccines with innate-immunity–inducing activities that exceed the activity (toxicity) of WPVs will cause adverse reactions [60].

9. Future perspectives of safety evaluation of vaccines and adjuvants

According to the abovementioned concept, various evaluations of adjuvanted vaccines have been carried out. Furthermore, we have focused on the functions of biomarker genes. We have attempted to compile gene clusters based on the function of each gene. Such an assay is currently at the development stage, and further examination of the evaluation method and validation should be conducted in the future. Such an assay is considered applicable to the development of novel adjuvants.

For the development of low-molecular-weight synthetic drugs, a seed compound having a desired bioactivity is searched for by a screening system in compound libraries [61]. For promoting adjuvant development, to create as many prominent novel adjuvants as possible, finding seed compounds that are likely to become adjuvants is a crucial step in adjuvant development. Conventionally, to demonstrate whether a compound has adjuvant activities, animals are inoculated with one of the compounds, and the antibody titer and infection prevention rate are then assessed. This evaluation is not as efficient as the seed search and compound screening because the assessment process takes more than 1 month. In contrast, if we introduce an evaluation method involving a biomarker gene or genes, then prediction of safety and of the biological activity profile for compounds may be achieved in animals or cultured cells. Such an assessment may make it possible to search for effective adjuvant seeds that are safer than WPVs and more effective than the HAV.

10. Extrapolation of the safety evaluation results to humans according to biomarkers' gene function

Given that the evaluation of the quality and safety of vaccines assumes a reaction with humans, the evaluation result must reflect the human biological response. Generally, there are species differences in immunological responses between humans and rodents. Therefore, it is necessary to interpret the results carefully.

In case of safety evaluations based on genomic analyses, estimating the difference between experimental animals and humans with reference to the function of genes may be partially possible. For example, in the case of the WPV, leukopenic toxicity and body weight loss are observed in rodents, but these effects cannot be verified in humans. Nevertheless, at the gene level, if a gene is conserved among species, it is possible to estimate whether similar biological reactions can be observed between humans and animals. All our identified marker genes are homologous

http://dx.doi.org/10.5772/intechopen.76361

to their human counterparts, except for *Ifi47*. This observation suggests that some phenomena common to the tested animals and humans may be identified via the animal experiments by means of marker genes. To test this hypothesis and to develop an *in vitro* assay system, the use of human peripheral blood mononuclear cell-based or alveolar-epithelial-cell-based methods is necessary. Thus, extrapolation of the results of these evaluations to humans can be partially achieved by bridging the species differences with the marker genes.

11. Conclusions

It has been established that information that could not be obtained by conventional phenotypic analyses can be obtained by genomic analyses. Research conducted on the safety of vaccines and adjuvants using toxicogenomics has been less likely to be reported, and such data about chemically synthesized drugs have mostly been limited. Since the late 2000s, we have been trying to apply the genomics technology to the safety assessment of vaccines, and to demonstrate its sensitivity, ability to yield abundant toxicological and mechanistic information, the possibility of extrapolating its results to humans, and its potential for application to *in vitro* evaluation systems. In addition, we have shown that the newly developed evaluation system may be employed in analyses involving a biomarker gene(s) as an indicator, instead of the conventional quality control or safety test. It can also be assumed that these technologies can be utilized for adjuvant development, and it is expected that a wide range of genomics technologies will be applied in the future to the development of quality control and safety testing.

Acknowledgements

The research summarized in this review was supported by AMED, grant numbers JP17ak0101071 and JP17fk0108124.

Conflict of interest

The authors declare that they have no conflicts of interest.

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