Both whole cell and acellular pertussis vaccines are now available against pertussis infection. The whole cell pertussis vaccine is composed of a suspension of formalin-inactivated *B. pertussis* cells. In contrast, the acellular pertussis vaccine contains purified, inactivated components of *B. pertussis* cells. Acellular pertussis vaccines now in use contain primarily pertussis toxin (PT) and filamentous hemagglutinin [3]. Pertussis vaccines cause local reactions, such as pain, redness, or swelling, in 20–40% of treated children, and systemic events, such as fever of 105 °F or higher, febrile seizures, and hypotonic–hyporesponsive episodes, have been reported [4]. The Japanese Ministry of Health Labour and Welfare reported that the adverse reactions, such as fever, pain, edema, and purpura, were observed in 237 people in 5.4 million vaccine-treated people in 2003.

While the mechanisms underlying adverse reactions remain unknown, vaccines should undergo extensive safety and efficacy tests to control vaccine quality. Fundamental is the assurance that any vaccine destined for public use is manufactured under Good Manufacturing Practices (GMP) and has passed appropriate pre-release lot testing for purity and potency, for which manufacturers must submit samples of each vaccine lot and their own test results. In Japan as in other countries, all the vaccines must conform to the "Minimal Requirement of Biological Products" and are obliged to pass national control tests [5].

Pertussis vaccine toxicity causing adverse reactions, is evaluated by the conventional animal toxicity tests, which are based on the peripheral white blood cell (WBC) counts in mice after subcutaneous or intra-peritoneal injection [6,7]. In mice, PT blocks extravasation reactions that mediate lymphocyte homing from peripheral blood to solid lymphoid tissues [8,9]. For a vaccine to be considered safe, an increase in the WBC count in mice should not exceed 0.5 LPU (leukocytosis promoting unit)/ml at day 3 after injection [5]. Pertussis vaccine toxicity is also typically assessed by the histamine sensitization test. In this test, mouse rectal temperatures are monitored after histamine challenge on the fourth day of vaccine injection [10]. To develop more sensitive and more reproducible methods, we undertook comprehensive gene expression analysis of rats after administration of the pertussis vaccine by using DNA microarrays [11]. Hybridization of labeled nucleic acid from a sample to the microarrays identifies genes expressed in the specific sample. This approach allows simultaneous analysis of expression of the multiple genomes in a single cycle experiment. This approach has already been used for evaluation of side effects of drugs [12].

2. Materials and methods

2.1. Animals

Male Wistar rats (8 weeks) were obtained from SLC (Tokyo, Japan). All animals were housed in rooms maintained at 23 ± 1 °C, with 50 ± 10% relative humidity, and 12-h light:12-h dark cycles at least 1 week prior to the test challenge. Rats typically weighed 160–200 g on arrival.

2.2. Vaccines and toxin

Reference pertussis vaccine (reference vaccine; RE) was a lyophilized whole cell preparation of pertussis organisms incompletely inactivated by formaldehyde, used for National Quality Control Tests on pertussis vaccine in Japan since 1981. It was reconstituted in 12 ml of physiological saline, and 5 ml were injected intra-peritoneally (IP). PT (pertussis toxin) are prepared and purified by ammonium-sulfate fractionation and sucrose density gradient centrifugation, and treated with formaldehyde to destroy the toxic activity, and added with aluminum salt. PT (Wako Chemicals, Osaka, Japan) and PV (a generous gift of The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were adjusted to 5 μg/ml (PT concentration). RE, PV, and different concentrations of PT were injected into rats IP (5 ml/rat). Five milliliters of saline (SA) were injected as a control.

2.3. WBC counts

A leukocytosis-promoting test was performed according to the Minimum Requirements of Biological Products [5]. Ten microliters of each blood samples were used for WBC counting. The count was performed with a Z1 coulter particle counter (Beckman Coulter, Fullerton, CA). Three rats per group were treated by RE, PT, PV, and SA, and were analyzed on day 1–4 post-treatment. The experiments were performed three times.

2.4. Histology

Vaccine-treated livers were harvested from rats and fixed in Bouin's Solution (Sigma, St. Louis, MO) and 4% (w/v) paraformaldehyde at 4 °C for 48 h. After fixation, tissues were dehydrated through a series of graded alcohols and xylene and embedded in paraffin. Chilled paraffin blocks were cut into 4–6 μm sections, which were floated onto glass slides and dried overnight and stained with Hematoxylin and eosin (HE) and periodic acid Schiff (PAS). Cellular polysaccharide deposits were detected using the PAS reaction. Three rats per group were treated by RE, PT, PV, and SA, and were analyzed on day 1–4 post-treatment. The experiments were performed twice. Immunohistochemical staining was carried out as described [13]. Briefly, after blocking with 3% BSA in PBS, 4 μm sections were incubated overnight with anti-α1-acid-glycoprotein (Agp) (AgriSera, Vännäs, Sweden) at 4 °C overnight. Signals were detected with a VECTASTAIN ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Nuclei were stained with hematoxylin.

In situ hybridization was performed essentially as described [14]. *In situ* hybridization using liver sections was carried using digoxygenin (DIG)-labeled RNA probes specific for *Agp* (Genebank accession number

NM053288, 0.77 kb) and *Hpx* (NM053318, 1.48 kb). All cRNA probes were generated from the corresponding coding sequences. *Agp* forward primer: 5-tgcacatggttcttgtcgtt-3, reverse primer; 5-gaatcgaggtgcacaggagt-3, *Hpx* forward primer; 5-cgctactactgcttccaggg-3, reverse primer; 5-atgctgttcactttctgggg-3. *In situ* hybridization with the antisense or sense probes was incubated at 42 °C for 24 h in a humidified chamber. Hybridized DIG-labeled cRNA was detected using AP labeled-anti-DIG mouse fab fragments (Roche Diagnostics, Lewes, UK). The sections were treated with BCIP/NBT and mounted in Gel mount (Biomeda, Foster City, CA). Three rats per group were treated by RE, PT (5 μg/ml), PV, and SA, and were analyzed on day 1–4 post-treatment. The experiments were performed twice.

2.5. RNA preparation

Rats were sacrificed to obtain the lateral left lobe of the liver. Organs were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and mixed with an ISOGEN reagent (NIPPON GENE, Tokyo, Japan). Total RNA was prepared from the lysate in accordance with the manufacturer's instructions. Poly(A) + RNA was prepared from total RNA with a Poly(A) Purist Kit (Ambion, Austin, TX), according to the manufacturer's instructions.

2.6. Microarray preparation and expression profile acquisition

For the microarray analysis, three rats per group were treated by RE, PT (5 μg/ml), PV, and SA, and livers from each group were analyzed on day 1–4 post-treatment. Totally 48 liver samples were analyzed for this experiment.

A set of synthetic polynucleotides (80-mers) representing 11,464 rat transcripts derived from 10,490 independent genes and including most of the RefSeq clones deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan) was arrayed on aminosilane-coated glass slides (Type I; Matsunami, Kishiwada, Japan) with a custom-made arrayer [15,16]. Poly(A) + RNA (2 μg) was labeled with SuperScript II (Invitrogen, Carlsbad, CA) and Cyanine 5-dUTP for each sample or Cyanine 3-dUTP (Perkin-Elmer, Boston, MA) for a rat common reference RNA (MicroDiagnostic). Labeling, hybridization, and washes of microarrays were performed with a Labeling & Hybridization Kit (MicroDiagnostic) according to the manufacturer. The rat common reference RNA was purchased as a single batch and labeled as an aliquot with Cyanine-3 for a single microarray side by side with each sample labeled with Cyanine-5. Hybridization signals were measured using a GenePix 4000A scanner (Axon Instruments, Whipple Road Union City, CA) and then processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from common reference RNA], which are indicated as 'median of ratios' in GenePix Pro 3.0 software (Axon Instruments)). Normalization was performed for the median of ratios (primary expression ratios) by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software.

2.7. Data analysis

Data processing and hierarchical cluster analysis were performed using Excel (Microsoft, Redmond, WA) and a MDI gene expression analysis software package (MicroDiagnostic). The primary expression ratios were converted into $\log_2$ values ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity) (designated log ratios) and compiled into a matrix (designated primary data matrix). To predict the most obvious differences obtained from cluster analysis of the primary data matrix, we extracted genes with $\log_2$ ratios over 1 or under −1 in at least one sample from the primary data matrix and subjected them to two-dimensional hierarchical cluster analysis for samples and genes. To identify genes demonstrating significant changes in expression, we undertook the following: (i) mean averages of $\log_2$ ratios were calculated for each gene from data sets of day 1 SA- and RE-treated samples; (ii) standard deviations were calculated for each gene; (iii) the difference in mean averages between day 1 SA- and RE-treated samples was calculated for each gene and divided by the sum of the corresponding standard deviation values. A value of the difference of the mean averages/the sum of the standard deviations was defined as an expression signal/noise index for each gene. We chose the top 150 genes exhibiting the highest expression signal/noise indexes and extracted expression data corresponding to the 150 genes from the primary data matrix for all the samples, which was subsequently subjected to two-dimensional hierarchical cluster analysis for samples and genes.

2.8. Quantitative RT-PCR analysis

Total RNA was used to synthesize first strand cDNA using a First-strand cDNA Synthesis Kit (Life Science, Inc., St. Petersburg, FL), according to the manufacturer's instructions. Expression levels of Agp and Hpx were analyzed by quantitative (Q) reverse transcriptase–polymerase chain reaction using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with 7500 Fast System SDS Software Version 1.3. cDNA was amplified for Q-PCR using SYBR Green I (Molecular Probes, Inc.) to detect PCR product. One microliters of six-fold diluted cDNA was used in a 20-μl final volume reaction containing 10 μl SYBR Green® PCR Master Mix (Applied Biosystems), 0.2 μM Agp fwd primer (5′-GCTGGAGCTGGAGAAGGAGACT-3′), and 0.2 μM Agp rev primer (5′-ACAGTCCCCGGAGTTCAGAGA-3′). The 7500 Fast System was programmed to run an initial polymerase activation step at 95 °C for 10 min followed by 40 cycles of denaturation (95 °C for 15 s) and extension (60 °C for 1 min), and product synthesis was monitored at the end of the extension step of each cycle. The same conditions were used with primers 0.05 μM Hpx fwd (5′-CTGCCTCAGCCCCAGAAAGT-3′) and 0.05 μM Hpx rev (5′-GGGTGGGCTGGGCTAATTC-3′). Agp and Hpx values were normalized against rat β-actin (0.1 μM fwd 5′-ACCGTGAAAAGATGACCCAGATC-3′; rev 5′-GACCAGAGGCATACAGGGACAAC-3′).

2.9. *Western blot analysis*

One day after treatment, livers were rapidly removed from diethylether anesthetized rats, washed in PBS, and weighed. After dicing, tissue was homogenized in PBS containing protease inhibitors and lysed in PBS containing protease inhibitors, 1% (w/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, and 10 mM Na-EDTA. Supernatants were collected after centrifugation at 10,000 × *g* for 20 min and used as a whole liver lysate. Fifty micrograms of the lysate was subjected to SDS-PAGE (10% acrylamide) and the separated proteins were transferred to an Immobilon-P membrane (Millipore, Watford, UK). After incubation in TBS (20 mM Tris–HCl (pH 8.0) and 100 mM NaCl) containing 5% (w/v) BSA, the membrane was incubated with anti-AGP (AgriSera) or anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 1 h and further incubated with HRP-conjugated anti-goat or anti-rabbit IgG. Peroxidase activity was visualized with a LAS 3000 bioimaging analyzer (Fuji Film, Tokyo, Japan).

2.10. *Statistical analysis*

To evaluate the statistical significance of the difference in expression level of Agp and Hpx, the Student's *t*-test was used to calculate the *P*-value.

3. Results

3.1. *Vaccine-treated rats demonstrated leukocytosis in peripheral blood*

Animals were treated with 5 ml of reference pertussis vaccine (RE), purified pertussis vaccine (PV), pertussis toxin (PT), or saline (SA), and the peripheral WBCs were counted at days 1, 2, 3, and 4. RE is an incompletely inactivated whole cell vaccine for the reference of PT toxicity. PT is a purified pertussis toxin. To evaluate dose-response to PT, mixture of a constant amount of PV and varying amount of PT, 0.2, 1.0, and 5.0 μg/ml were used for injection (coded as PV + PT0.2, PV + PT1, and PV + PT5, respectively). Three rats per group were analyzed in each day after sample injection. RE-treated rats started to show leukocytosis at day 2; the leukocyte count continued to increase reaching values three times higher than the baseline at day 4 (Fig. 1). PV + PT5-treated rats also demonstrated leukocytosis, as did RE-treated rats. By contrast, PV- and SA-treated animals showed normal WBC counts. WBC counts of both PV + PT0.2- and PV + PT1-treated rats were within the normal range.

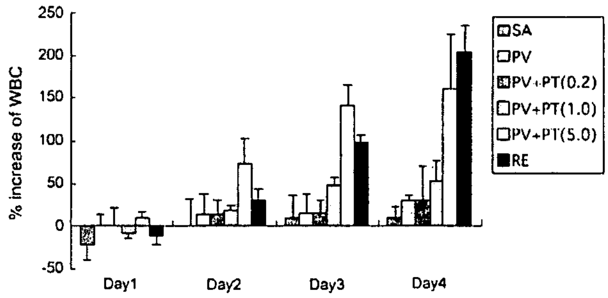

Fig. 1. Leukocytosis-promoting activity in RE- and PT-treated animals. The effects of RE-, PT-, PV-, SA-treatment were analyzed by leukocytosis-promoting tests. Various PT concentrations (0.2–5.0 μg/ml) were added to PV and a WBC count was performed at days 0, 1, 2, 3, and 4. Changes in WBC count are indicated by the mean increase ± S.D. of three independent experiments. Each value was compared to the corresponding control SA.

3.2. *Histological analysis in vaccine-treated rats*

To analyze the effect of pertussis vaccine on rat liver, we performed histological study on day 1–4 post-treatment twice. As shown in Fig. 2, SA-, PV + PT-, and RE-treated livers showed no significant change in HE stained sections. Since periodic acid Schiff (PAS)-positive glycogen granules in hepatocytes are sensitive to strong stresses by drugs, toxic agents, starvation, and hypoxic conditions, PAS staining was also evaluated. At day 1, we have remarkable change in RE samples by PAS staining, however we could not detect the same change in other samples (Fig. 2). The same results that only RE affect the PAS staining in the hepatocyte were confirmed in the other time point (day 2–4, data not shown). These findings indicated that histological analysis could be useful to monitor toxicity effects induced by RE vaccine, but did not reflect toxicity induced by PT when added to PV.

3.3. *Microarray assay of vaccine-treated liver*

To evaluate the effect of pertussis vaccine on the gene expression in the liver, we prepared three rats per group. RE-, PT- (5 μg/ml), PV-, and SA-treated groups were sacrificed to take liver samples each at days 1, 2, 3, and 4. Total 48 independent liver tissue samples were analyzed. We labeled poly(A) + RNA purified from the samples and from a rat common reference RNA with Cyanine-5 and Cyanine-3, respectively. Next, we hybridized them to microarrays representing 11,464 transcripts derived from 10,490 independent genes including most of the RefSeq clones deposited in the NCBI database. Hybridization signals were processed into expression ratios as $\log_2$ values (designated $\log_2$ ratios) and compiled into a matrix designated as the primary data matrix (see Section 2). To extract genes whose expression levels altered specifically to RE-administration at day 1 from the primary data matrix, we conducted the statistical operations described in Section 2. When the cluster analysis for liver samples was performed, two large clusters were obtained, and

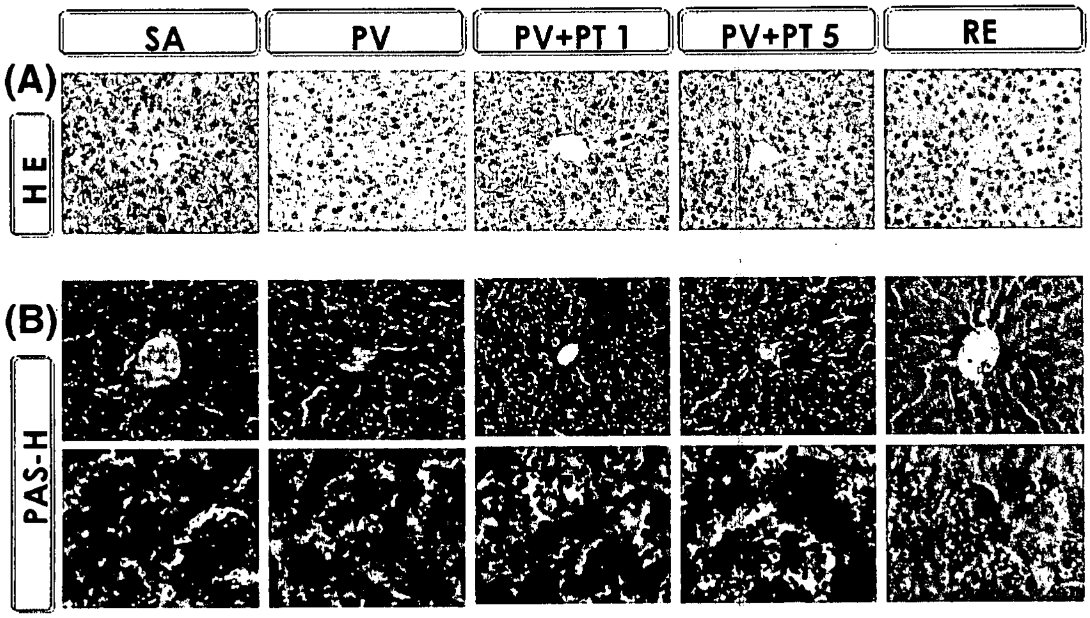

Fig. 2. Histochemical study of RE- and PT-treated liver. The left lobes of sample treated liver were sectioned and stained with HE and PAS. (A) RE-, PV-, PV + PT1- (PT 1.0 μg/ml), PV + PT5- (PT 5.0 μg/ml), and SA-treated liver were stained with HE. (B) Sectioned samples were stained with PAS plus HE and analyzed at low (upper panel) and high (lower panel) magnification.

RE-treated samples (1 day after RE administration, RE/1d) showed cluster different from others (Fig. 3A). We identified top 150 genes exhibiting the highest scores whose expression was either increased or decreased by RE-administration at day 1 (RE-1d in Fig. 3B). Of those genes, 61 are related to metabolism, 21 encode signal transduction molecules, and the rest are classified as toxic response-, inflammation-, and human-disease-related genes (Fig. 3C). Functions of about one-third of these150 genes are unknown.

3.4. Identification of toxicity-specific genes

To identify toxicity-related genes from the microarray data, we compared results from RE-treated samples with SA-treated ones. Agp, S100a8, Phyh, Got1, Lbp, and Hpx genes showed the greatest increase in expression level in RE-treated samples (Table 1). When PT-treated animal samples were compared with PV-treated ones, expressions of Hpx, Acmsd, Tat, and Cyp8b1 were significantly increased (Table 1). Phyh, Got1, Acmsd, Tat, and Cyp8b1 participated in metabolism. On the other hand, Agp, S100a8, Lbp, and Hpx participated in inflammation. To quantify expression of candidate genes, the liver cDNAs were analyzed by quantitative RT-PCR (Fig. 4, lower panel), and compared with the microarray data (Fig. 4, upper panel). Liver cDNA were prepared from two or three rats, and two independent quantification experiments were performed. Agp and Hpx genes demonstrated high expression in RE- and PT-treated liver, and their expression was low in PV- and SA-treated liver (Fig. 4). Both were then chosen for further quantitative analysis.

3.5. Agp and Hpx expression in the liver

For confirmation, we performed *in situ* hybridization and immunohistochemistry. Specific probes against Agp clearly detected Agp mRNA in RE-treated liver at day 1 (Fig. 5G and H). Western blot analysis of the cell lysates showed markedly increase of AGP expression in RE-treated liver in comparison with SA-treated liver (Fig. 5Q). Strong AGP protein expression was also detected in RE-treated livers in comparison to SA-treated livers (Fig. 5R–U). From these data, we conclude that AGP is induced in liver by RE-treatment. Since there is no appropriate antibody against rat Hpx, we analyzed Hpx expression by *in situ* hybridization. Hpx expression was rapidly induced in hepatocytes following RE-treatment, and its expression pattern was similar to Agp (Fig. 5I–P). Thus, of the nine genes identified by microarray analysis, histological analysis with specific RNA probes and antibodies demonstrated the highest correlation with Agp and Hpx. Thus, we identify Agp and Hpx as toxicity-related genes and suggest that these genes could be used as biomarkers.

3.6. Detection of PT activity using Q-PCR analysis

To detect alterations in Agp and Hpx gene expressions after vaccine-treatment, we injected various concentrations of PT (0.008–5.0 μg/ml) and RE into rats. Three rats per group were analyzed. As shown in Fig. 6, expression of Agp and Hpx was tremendously high in RE-treated livers, and both genes showed a strict dependence on the concentration of PT. These genes were clearly up-regulated by PT at

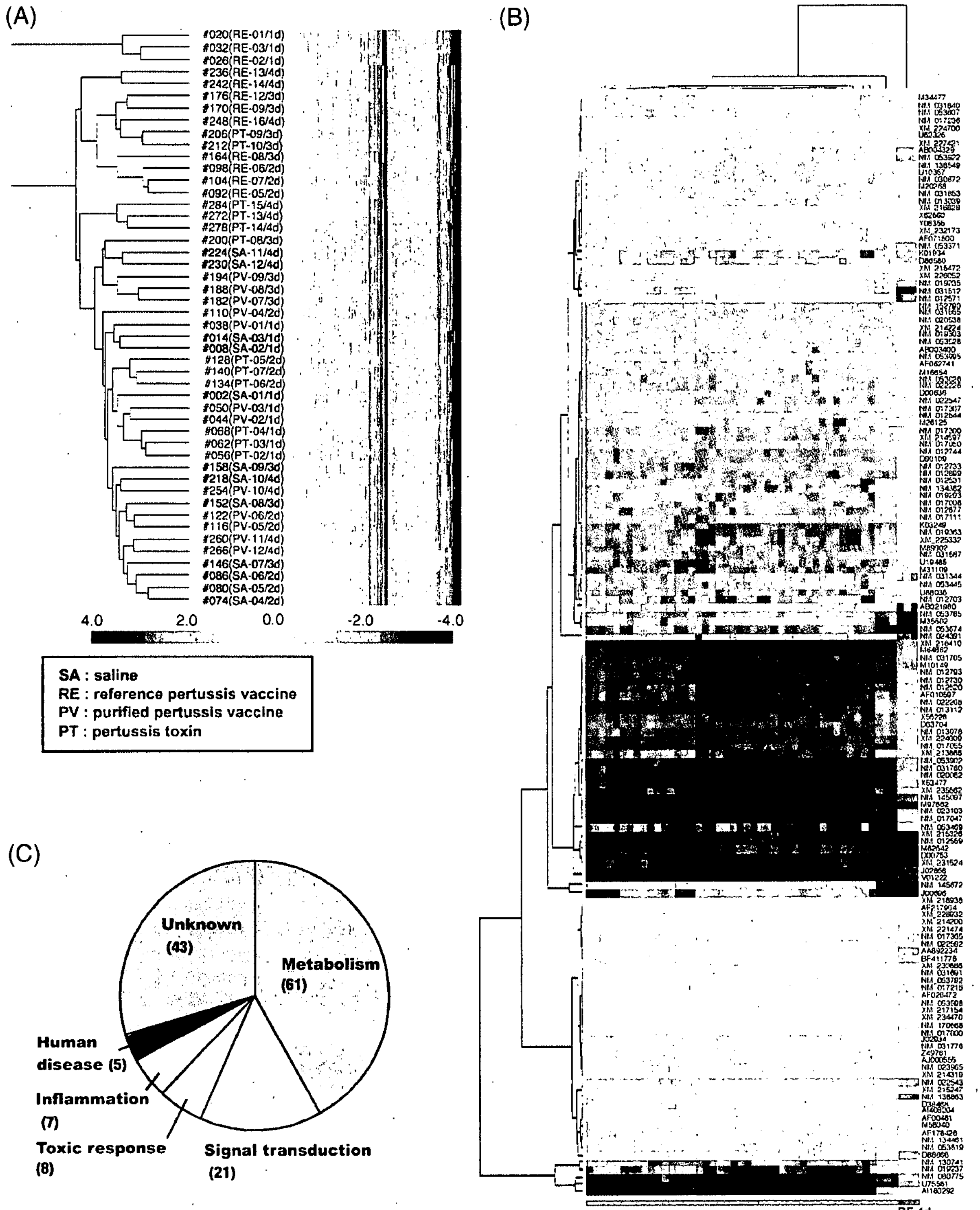

Fig. 3. Gene expression profiles obtained from RE-, PT-, PV-, and SA-treated rats. (A) Genes expressed in sample-treated liver are assembled in the order obtained from the results of cluster analysis. The color bar at left shows the expression ratio *vs.* the common reference RNA in $\log_2$; red and blue indicate increases and decreases in the expression ratio, respectively. (B) One hundred and fifty genes represented showed significant alterations in RE-treated liver at day 1 (RE-1d). The expression pattern of the 150 genes in other samples (pink bar) showed a clear difference from RE-1d (green bar). The *y*-axis of the dendrogram depicts the Euclid square distance as the dissimilarity coefficient. Red and blue indicate increases and decreases in the expression ratio, respectively. (C) Genes are classified in the circle graph.

Table 1
Transcripts upregulated in RE- and PT-treated samples

Accession no.	Symbol	Definition	Molecular function	Expression ratios			
				SA	PV	PT	RE
AB018596	Cyp8b1	Rattus norvegicus mRNA for sterol 12-alpha hydroxylase, complete code	Metabolism	1.76 ± 0.15	1.86 ± 0.48	2.70 ± 0.40	3.61 ± 0.96
J00696	Agp/Orm1	Rat alpha1-acid glycoprotein (AGP) mRNA, complete cds	Inflammation	1.34 ± 0.67	1.58 ± 0.36	4.11 ± 0.38	6.30 ± 0.17
M62642	Hpx	Rat (clone pRHx1) hemopexin mRNA, complete cds	Inflammation	2.74 ± 0.37	2.82 ± 0.15	4.08 ± 0.23	5.30 ± 0.07
NM_012571	Got1	Rattus norvegicus glutamate oxaloacetate transaminase 1 (Got1), mRNA	Metabolism	0.11 ± 0.34	0.18 ± 0.24	1.12 ± 0.35	2.86 ± 0.53
NM_012668	Tat	Rattus norvegicus tyrosine aminotransferase (Tat), mRNA	Metabolism	2.55 ± 0.16	2.34 ± 0.58	3.06 ± 0.41	4.18 ± 0.55
NM_017208	Lbp	Rattus norvegicus lipopolysaccharide binding protein (Lbp), mRNA	Inflammation	1.49 ± 0.24	1.47 ± 0.18	3.59 ± 0.41	4.84 ± 0.17
NM_053674	Phyh	Rattus norvegicus phytanoyl-CoA hydroxylase (Phyh), mRNA	Metabolism	2.03 ± 0.19	2.08 ± 0.12	3.22 ± 0.37	3.78 ± 0.16
NM_053822	S100a8	Rattus norvegicus S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA	Inflammation	−5.94 ± 0.03	−5.89 ± 0.23	−5.25 ± 0.30	−3.07 ± 0.29
NM_134372	Acmsd	Rattus norvegicus 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (acmsd), mRNA	Metabolism	2.50 ± 0.42	2.10 ± 0.21	3.32 ± 0.84	4.04 ± 0.40

Cyanine 5-labeled liver RNA and Cyanine 3-labeled rat common reference RNA were competitively hybridized to microarrays. Hybridization signals were processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from common reference RNA], and normalized (primary expression ratios). The primary expression ratios were converted into $\log_2$ values ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity) as described in Section 2. Log_2 values for each sample were taken an average and calculated S.D.

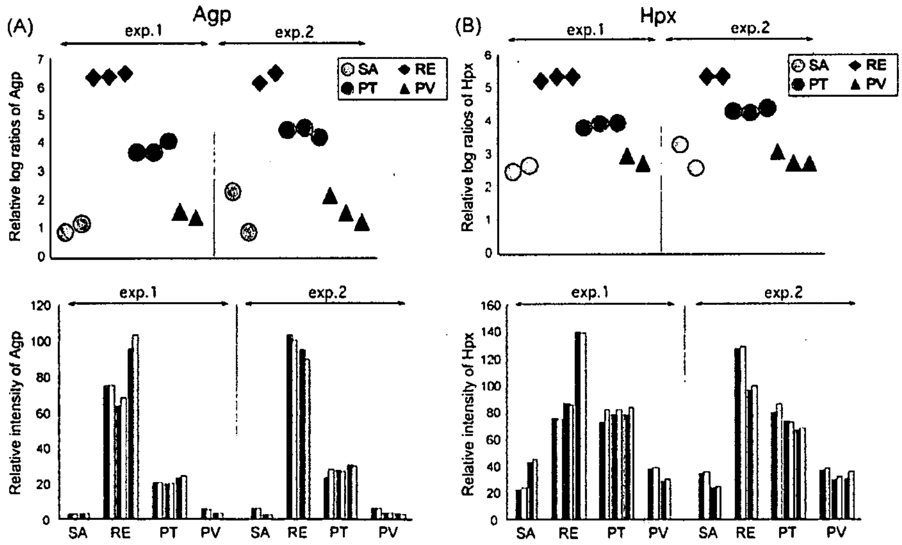

Fig. 4. Comparison of microarray data and quantitative PCR analysis. Expression of Agp (A) and Hpx (B) from DNA microarray analysis (upper panel) is compared with real-time quantitative PCR data (lower panel). (*Upper panels*) Relative $\log_2$ ratios were extracted from the secondary data matrix for Agp and Hpx. Each symbol represents data from individual animals. Two independent experiments in microarray analysis are shown. (*Lower panels*) Quantitative PCR analysis of treated livers from individual animals is shown. Duplicate data from each animal is shown as black and white bars. Agp and Hpx expression was assessed relative to rat β-actin.

concentrations of 5 μg/ml ($P < 0.05$) in comparison with SA, PV, and PT (0.008–0.2 μg/ml). At concentration of 1 μg/ml, some animals showed slight high expression of these genes in comparison with the animals treated with SA, PV or PT (0.008–0.2 μg/ml). These findings suggest that Agp and Hpx may be good candidates to monitor the PT-induced toxicity.

4. Discussion

In this study, we identified genes whose expression is affected by PT-related toxicity using DNA microarray analysis. Although the principle of nucleic acid hybridization is not new, microarrays have opened the way for parallel

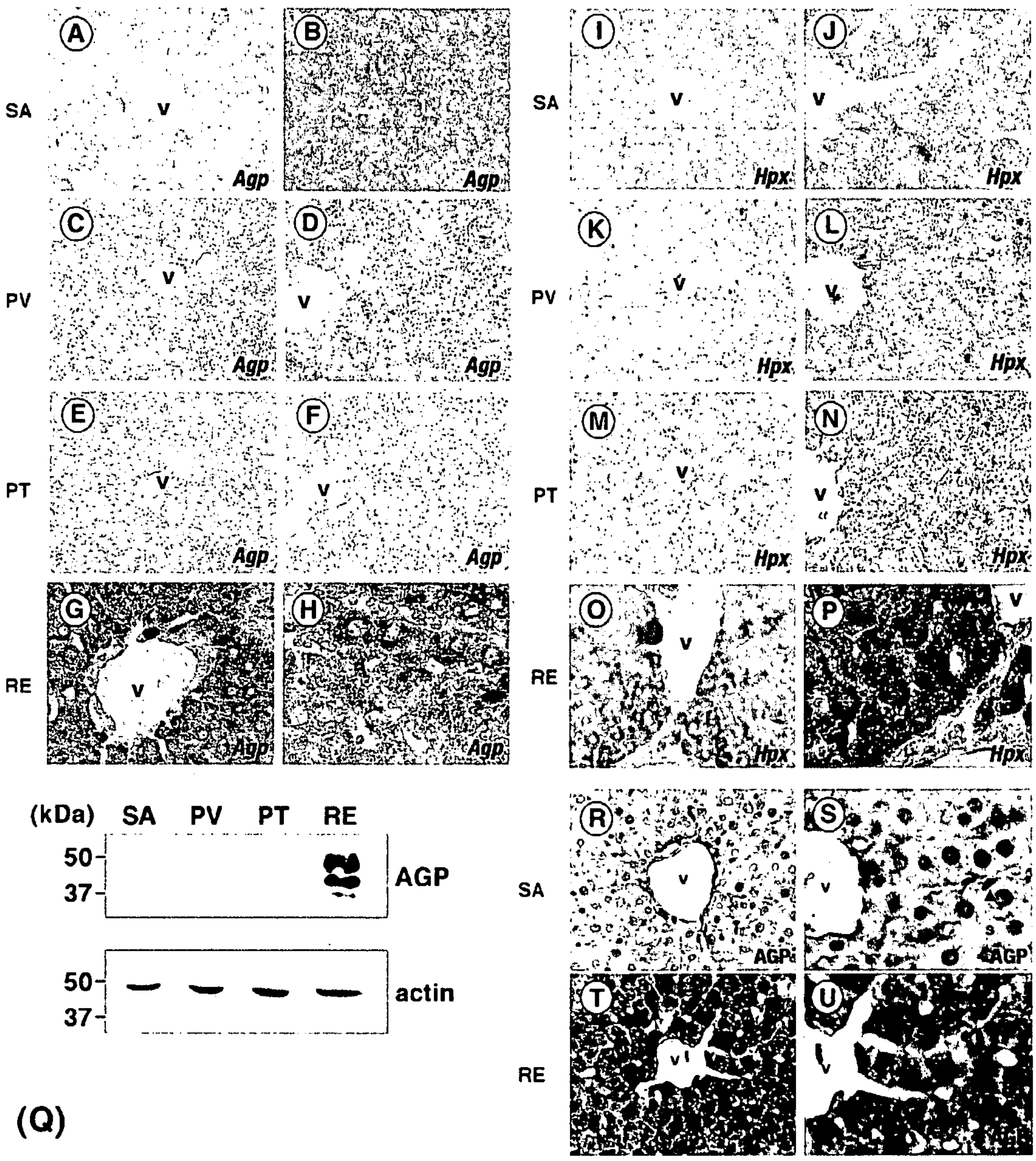

Fig. 5. Expression of Agp and Hpx in RE-, PT-, PV-, and SA-treated liver. Expression of Agp mRNA analyzed by *in situ* hybridization. SA-, PV-, PT-, and RE-treated liver was analyzed at low (A, C, E, and G) and high (B, D, F, and H) magnification. (Q) AGP protein expression in the liver was analyzed by Western blotting. (R–U) Expression of AGP protein in the liver was analyzed by immunohistochemistry. The nuclei were stained with hematoxilin (blue). SA-treated liver was analyzed at low (R) and high (S) magnification. RE-treated liver was analyzed at low (T) and high (U) magnification. Brown indicated AGP protein expression. (I–P) Expression of Hpx mRNA in liver analyzed by *in situ* hybridization. Sections of SA-, PV-, PT-, and RE-treated liver were hybridized with Hpx-specific probes and analyzed at low (I, K, M, and O) and high (J, L, N, and P) magnification. Brown signals represent Hpx expression.

detection and analysis of expression patterns of thousands of genes in a single experiment, and its sensitivity allows detection of subtle differences otherwise difficult to detect. In addition, DNA microarray-based approaches allow us to interrogate toxin-related genomes without bias as to which genes might be altered in expression.

Our histological study showed that RE-treatment severely decreased PAS-stained glycogen granules in hepatocytes. Since hepatocytes are normally full of such granules, their loss suggests a severe load of toxic substances in liver cells [17]. As liver is a major detoxifying organ and analysis of pharmaceutical toxicity using the DNA array has been undertaken in liver [18], we considered liver an appropriate organ to analyze biological alterations with pertussis vaccine.

Based on analysis of 10,490 of rat genes, we identified 150 genes demonstrating significant changes either upward

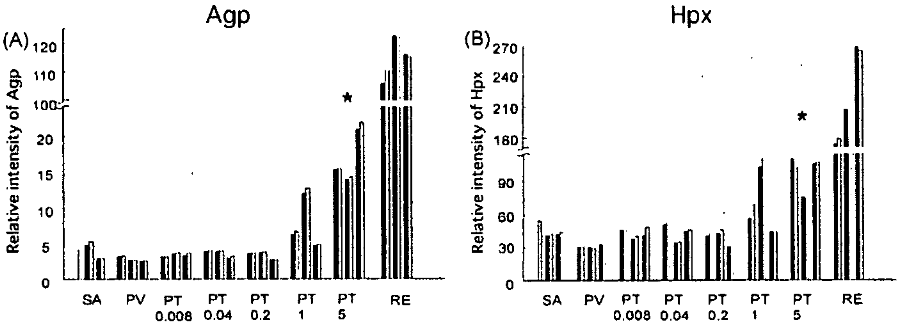

Fig. 6. Quantitative PCR analysis of Agp and Hpx expression in RE-, PT-, PV-, and SA-treated liver. Gene expression of Agp (A) and Hpx (B) in liver treated with various concentrations of PT and RE were analyzed by Q-PCR. Agp and Hpx expression was assessed relative to rat β-actin. Duplicate data from each animal is shown as black and white bars. Three animals per group were analyzed. PT0.008, PT 0.008 μg/ml; PT0.04, PT 0.04 μg/ml; PT0.2, PT 0.2 μg/ml; PT1, PT 1.0 μg/ml; PT5, PT 5.0 μg/ml. Asterisk denotes significant differences ($P < 0.05$) by the Student's *t*-test.

or downward in gene expression after RE-treatment. Among them, several metabolism-relate genes, such as FABP (fatty acid-binding protein; U7558) and NaCT (Na+-coupled citrate transporter; NM_170668) showed the great increase in expression level. FABP participates in the uptake, intracellular metabolism and/or transport of long chain fatty acids. NaCT plays a role in cellular utilization of citrate in blood for the synthesis of fatty acids and cholesterol and for the generation of energy. The great increase of these genes may be correlated to the toxin-related state of starvation that requires urgent energy derived from fatty tissues.

Reduction in PAS-positive glycogen granules in the liver (Fig. 2) after RE-treatment is consistent with this finding. Gordon et al. have reported that toxic substrates induce abnormal PAS-staining in liver cells due to reduced glycogen granules [19]. Inflammation-related genes, such as MIF (U62326), AGP (J00695), and IL-1β (NM_031512) are also up-regulated in RE-treated liver, suggesting that acute inflammation is induced by RE-treatment. In addition, many forms of P450 (NM_19303, M16654, NM_012730, X53477, J02868) are induced in the liver by RE treatment. Comparing gene expression among SA-, PT-, and RE-treated animals, we identified nine genes that were up-regulated in toxic conditions. As shown in Table 1, five genes participated in metabolism, and four genes participated in inflammation. Among them, Agp and Hpx showed unequivocal correlation with RE treatment both in the DNA microarray and histological analysis. AGP is a 41–43 kDa glycoprotein with several activities, such as immunomodulating effects and the ability to bind steroid hormones and other molecules [20,21]. Reportedly, AGP serum concentrations, which remain stable in physiological conditions (about 1 g/l in human and 0.2 g/l in rats), increase several-fold during acute-phase inflammatory reactions and AGP is considered as a major member of the positive acute phase protein family. HPX is a serum glycoprotein with a high affinity for heme, and it is produced by and secreted from the liver. It is also known as a scavenger/transporter of heme [22]. Previous studies indicate that purified HPX is an acute-phase reactant with serum levels increasing several-fold following experimentally induced inflammation [23,24]. These two genes likely react to RE and PT through acute-phase inflammatory reactions. These findings suggest that these biomarkers may be useful to detect PT-induced toxicity in pertussis vaccines.

So far, conventional leukocytosis promoting tests can detect active PT contamination at concentrations greater than 5 μg/ml. While PAS staining of glycogen granules in RE-treated hepatocytes was weak, hepatocytes treated with a high concentration of PT (5 μg/ml) were normally stained with PAS. With our method, we detected Agp and Hpx at a 5 μg/ml PT and the expression level of both genes was dose dependent on PT concentration (Fig. 6). These findings suggest that the gene expression analysis should be useful to detect the PT activity specifically as well as leukocyte-promoting tests. We could detect the different time course of the toxic effects observed as measured by gene expression analysis and leukocytosis. The specific gene expression of Agp and Hpx were observed at day 1 post-treatment, however the increase in WBC were only observed after 2 days. These findings suggest that new test takes the shorter testing period.

Many manufacturers are improving vaccine quality by new methods, such as by using cultured cell lines. However improvements in quality control are continuously required. Since microarray analysis can cover thousands of genes rapidly, it may revolutionize vaccine safety tests and in doing so increase our understanding of molecular mechanisms underlying vaccine toxicity. Given the small variability among test animals, such an approach could potentially reduce the number of animals assayed, alleviating in part ethical problems related to animal tests, which are important issues for regulators worldwide. This method can be used for safety evaluation of different vaccines, such as the emerging vaccine for high pathogenic influenza. So far, safety assessment of vaccines before licensing requires a long time period. Our present assay, with its high reproducibility and reliability, could considerably shorten that period.

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References

[1] Bordet J, Gengou D. Le micrrobe de la coqueluche. Ann Inst Pasteur 1909;23:415–9.

[2] Cone TJ. Whooping cough is first described as a disease suigeneris by Baillou in 1640. Pediatrics 1970;46:522.

[3] Sato H, Sato Y. *Bordetella pertussis* infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. Infect Immun 1984;46(2):415–21.

[4] Pertussis. In: Atkinson W, Hamborsky J, McIntyre L, Wolfe S, editors. Epidemiology and Prevention of Vaccine-Preventable Diseases. 9th ed. Washington DC: Centers for Disease Control and Prevention; 2006. p. 79–96.

[5] Ministry of Health and Welfare, J.G. The minimum requirements of biological products of Japan 1986. Tokyo: Ministry of Health and Welfare; 1986.

[6] Horiuchi Y, Takahashi M, Konda T, Ochiai M, Yamamoto A, Kataoka M, et al. Quality control of diphtheria tetanus acellular pertussis combined (DTaP) vaccines in Japan. Jpn J Infect Dis 2001;54(5):167–80.

[7] Iwasa S, Ishida S, Asakawa S, Kurokawa M. Lymphocytosis-promoting factor produced by *Bordetella pertussis*. Jpn J Med Sci Biol 1968; 21:363–8.

[8] Morsel S. Studies on the lymphocytosis induced in mice by *Bordetella pertussis*. J Exp Med 1965;121:49–68.

[9] Cyster JG, Goodnow CC. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. J Exp Med 1995;182(2): 581–6.

[10] Ishida S, Kurokawa M, Asakawa S, Iwasa S. A sensitive assay method for the histamine-sensitizing factor using change in rectal temperature of mice after histamine challenge as a response. J Biol Stand 1979;7(1):21–9.

[11] Sakamoto A, Imai J, Nishikawa A, Honma R, Ito E, Yanagisawa Y, et al. Influence of inhalation anesthesia assessed by comprehensive gene expression profiling. Gene 2005;356:39–48.

[12] Ejiri N, Katayama K, Kiyosawa N, Baba Y, Doi K. Microarray analysis on Phase II drug metabolizing enzymes expression in pregnant rats after treatment with pregnenolone-16alpha-carbonitrile or phenobarbital. Exp Mol Pathol 2005;79(3):272–7.

[13] Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech Dev 2000;93(1–2): 139–49.

[14] Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, et al. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. Proc Natl Acad Sci USA 1994;91(25):12258–62.

[15] Kobayashi S, Ito E, Honma R, Nojima Y, Shibuya M, Watanabe S, et al. Dynamic regulation of gene expression by the Flt-1 kinase and Matrigel in endothelial tubulogenesis. Genomics 2004;84(1):185–92.

[16] Ito E, Honma R, Imai J, Azuma S, Kanno T, Mori S, et al. A tetraspanin-family protein, T-cell acute lymphoblastic leukemia-associated antigen 1, is induced by the Ewing's sarcoma-Wilms' tumor 1 fusion protein of desmoplastic small round-cell tumor. Am J Pathol 2003;163(6): 2165–72.

[17] Purushotham KR, Lockard VG, Mehendale HM. Amplification of chloroform hepatotoxicity and lethality by dietary chlordecone (kepone) in mice. Toxicol Pathol 1988;16(1):27–34.

[18] Shipkova M, Spielbauer B, Voland A, Grone HJ, Armstrong VW, Oellerich M, et al. cDNA microarray analysis reveals new candidate genes possibly linked to side effects under mycophenolate mofetil therapy. Transplantation 2004;78(8):1145–52.

[19] Gordon GJ, Coleman WB, Hixson DC, Grisham JW. Liver regeneration in rats with retrorsine-induced hepatocellular injury proceeds through a novel cellular response. Am J Pathol 2000;156(2):607–19.

[20] Fournier T, Medjoubi NN, Porquet D. Alpha-1-acid glycoprotein. Biochim Biophys Acta 2000;1482(1–2):157–71.

[21] Nishi K, Sakai N, Komine Y, Maruyama T, Halsall HB, Otagiri M. Structural and drug-binding properties of alpha(1)-acid glycoprotein in reverse micelles. Biochim Biophys Acta 2002;1601(2):185–91.

[22] Tolosano E, Altruda F. Hemopexin: structure, function, and regulation. DNA Cell Biol 2002;21(4):297–306.

[23] Delanghe JR, Langlois MR. Hemopexin: a review of biological aspects and the role in laboratory medicine. Clin Chim Acta 2001;312(1–2): 13–23.

[24] Muller-Eberhard U. Hemopexin. Methods Enzymol 1988;163:536–65.

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Transcutaneous immunization by merely prolonging the duration of antigen presence on the skin of mice induces a potent antigen-specific antibody response even in the absence of an adjuvant

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Abstract

Transcutaneous immunization (TCI) is a promising needle-free technique for vaccination. In this method, strong adjuvants, such as the cholera toxin, are generally crucial to elicit a robust immune response. Here, we showed that prolonged antigen presence on the skin of mice during TCI could effectively enhance the immune response. Substantial antigen-specific antibodies were produced in the sera of mice even after non-adjuvanted TCI when the antigen presence was for longer than 16 h. This non-adjuvanted TCI method was applied using the tetanus toxoid, and potent tetanus toxoid-specific antibodies were successfully induced in the sera of mice; they survived a lethal tetanus toxin challenge with no clinical signs. Thus, non-adjuvanted approach might be a possible option for TCI, and this method might improve the safety and practicality of transcutaneous vaccination.

Keywords: Transcutaneous immunization; Adjuvant; Tetanus toxoid

1. Introduction

The skin is one of the first lines of defense of the body. During the course of evolution, the skin has developed a dense immune system comprising draining lymph nodes and various immunocompetent cells such as Langerhans cells, keratinocytes, dermal dendritic cells, and mast cells [1,2]. Together with its high accessibility, the skin's immunocompetence makes it an ideal target for vaccination.

Recently, there have been reports of needle-free vaccinations that target the intact skin surface and use peptides, proteins, or virus particles as antigens [3–7]. These novel methods, referred to as transcutaneous immunization (TCI), are performed by the topical application of antigens along with adjuvants. It is generally considered that some amount of a potent adjuvant is crucial in order to elicit a robust immune response against antigens co-administered via skin delivery [8,9]. The most common adjuvants used in the TCI method are the cholera toxin (CT) or the heat-labile enterotoxin (LT) from *Escherichia coli* [10].

Needle-free vaccination methods are desirable because they are convenient, painless, and relatively safe. In particular, vaccinations in developing countries and mass vaccinations against expected pandemics would benefit greatly from needle-free approaches [11]. Although TCI is a promising needle-free approach, the indispensable use of potent bacterial toxins as adjuvants might raise some concerns regarding the safety of this method; however, CT and LT may be less toxic when applied on the skin surface [12].

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In this study, we merely prolonged the duration of antigen presence on the skin of mice during the TCI procedure and observed that the serum antibody titre increased in a duration-dependent manner. We induced substantial serum antibody responses by a TCI of 16-h duration even in the absence of an adjuvant. In this report, we applied a modified TCI method termed "prolonged TCI" that involved no adjuvant. In this method, the tetanus toxoid (Ttd) was used as a model vaccine antigen, and this method successfully induced potent tetanus toxoid-specific antibody responses in the sera of mice; these mice survived a lethal tetanus toxin challenge without any clinical signs. These results indicate that the non-adjuvanted approach might be a possible option for TCI. This might improve the safety and practicality of transcutaneous vaccination.

2. Materials and Methods

2.1. Mice

We used female C57BL/6, BALB/c, and C3H/He mice (Japan SLC Inc., Hamamatsu, Japan) aged 7–8 weeks at the primary immunizations.

The animals were housed in a specific pathogen-free facility and provided with free access to water and food. The use of the animals and the study protocols were approved by the institutional animal care and use committee.

2.2. Antigens and adjuvant

Ovalbumin (OVA) and CT were purchased from Sigma (St. Louis, MO, USA). The Ttd was kindly provided by Kaketsuken (Kumamoto, Japan).

2.3. Conventional TCI

Conventional TCI was performed as previously described [10]. In brief, the abdomen of the mice was shaved using a No. 40 clipper, and the mice were rested for 48 h. They were anaesthetized intraperitoneally with a ketamine–xylazine mixture to prevent self-grooming. The bare abdominal skin was gently swabbed with 70% ethanol and allowed to dry. Next, 50 μl of antigen solution in PBS was placed on the bare abdominal skin over an approximate area of 1 cm^2 for 2 h. The mice were then washed extensively with lukewarm tap water and patted dry with paper towels.

2.4. Prolonged TCI

Mice were shaved and anaesthetized in the same manner as that used for the conventional TCI. The bare abdominal skin was gently swabbed with 70% ethanol and allowed to dry. Next, a 0.64-cm^2 square gauze patch with an adhesive lining (Shirojuhji, Tokyo, Japan) was soaked with 50 μl of antigen solution, and was fixed to the bare abdominal skin using medical tape (Fig. 1A and C). The mice were placed back in the cage and left for 16 h or more. Then, the medical tape and gauze patch were removed, and the abdominal skin was extensively washed with lukewarm tap water and patted dry with paper towels.

Prolonged TCI of the dorsal side of the ear was performed as described above without shaving (Fig. 1B and D).

2.5. Fecal extract

Fecal samples were collected and weighed. A hundred milligram of feces were suspended in 400 μl of PBS containing 100 μg/ml of soybean trypsin inhibitor (Wako, Ohsaka, Japan), 50 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride (Sigma), 1% of bovine serum albumin (Sigma), 5% of fetal bovine serum (Sigma), and 0.05% of sodium azide, were vigorously vortexed to homogeneity, centrifuged at 6000 × *g* for 5 min and the supernatants were collected and stored at −20 °C until assayed.

2.6. ELISA for antigen-specific IgG and IgA antibodies

Antigen-specific IgG and IgA antibody titres of the sera and fecal extracts were determined by ELISA. In brief, 96-well plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with antigen in 0.1 M carbonate/bicarbonate buffers, pH 9.0, and blocked with PBS containing 1% bovine serum albumin (Sigma). After blocking, serial dilutions of the serum samples or fecal extracts were added to the plates, which were incubated at room temperature for 1.5 h. The plates were washed 3 times with wash buffer (PBS containing 0.05% Tween 20), and peroxidase-labeled rabbit anti-mouse IgG antibodies (Zymed, San Francisco, CA, USA) or peroxidase-labeled goat anti-mouse IgA antibodies (Zymed) were added. After 1.5-h incubation at room temperature, the plates were washed 3 times with the wash buffer, and *o*-phenylenediamine (Sigma) in phosphate/citrate buffer containing 0.03% H_2O_2 was added. The reactions were arrested 10 min later by adding 1 N H_2SO_4, and optical densities were measured at 492 nm. Endpoint titres were expressed as reciprocal log_2 of the limit dilutions that recorded an optical density greater than 1.0.

2.7. Tetanus toxin challenge

The left hind thigh of the mice was injected subcutaneously with 10 median lethal dose (LD_{50}) of tetanus toxin in 0.5 ml PBS, and the mice were observed daily for up to 7 days. The mice that developed severe paralysis were euthanized.

2.8. Statistical analysis

The data are represented as the geometric means of the values obtained from individual animals. The groups were compared using unpaired two-tailed Student's *t*-tests, and *p*-values ≤ 0.05 were regarded as significant.

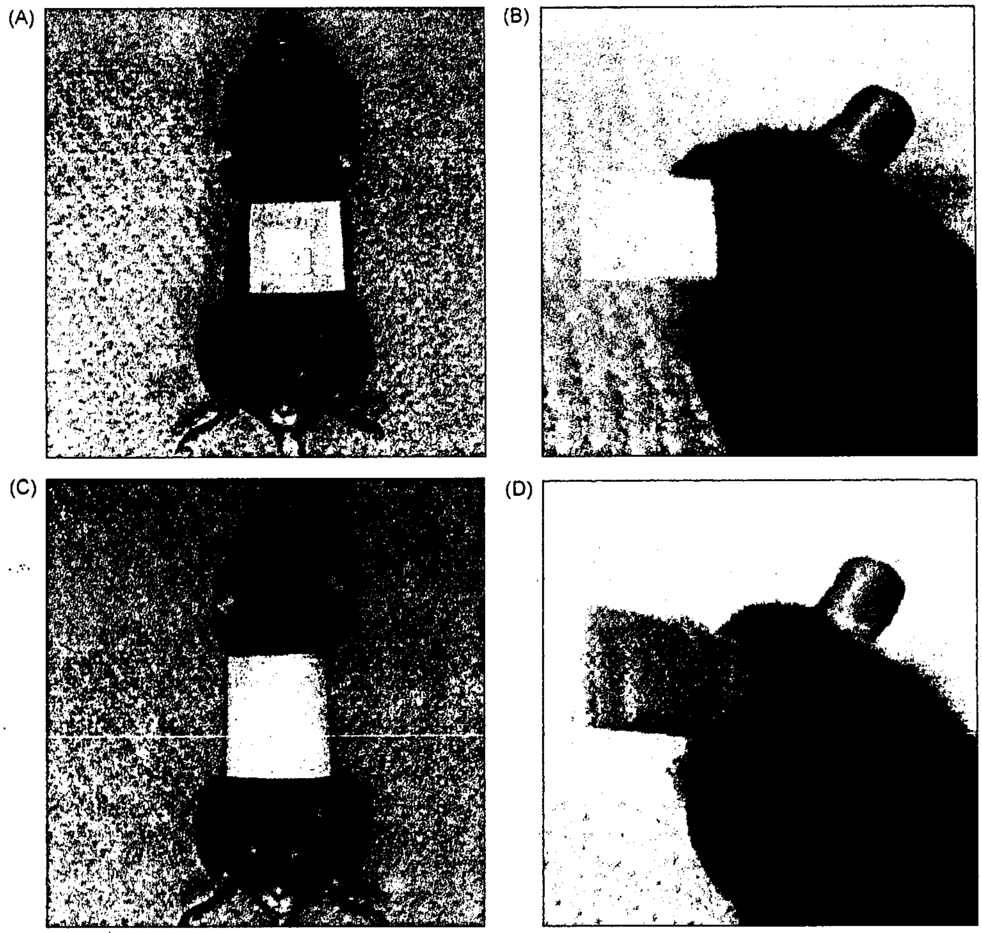

Fig. 1. Prolonged TCI using gauze patch. The gauze patch soaked with 50 μl of antigen solution was applied to the bare abdominal skin (A) or the dorsal side of the ear skin (B), and fixed with medical tape (C, D).

3. Results

3.1. Conventional TCI induced a substantial antigen-specific serum antibody response; however, the use of an adjuvant was crucial

We confirmed the crucial roll of CT as an adjuvant in the conventional TCI method.

The C57BL/6 mice were immunized by the conventional TCI procedure with 100 μg of OVA with or without 10 μg of CT as an adjuvant. Another group of mice was injected intradermally with an identical dose of OVA solution without CT. They were immunized in an identical manner 3 times with 2-week intervals. At 2 weeks after the last immunization, we collected serum samples and determined the OVA-specific and CT-specific IgG antibody titres by ELISA. When an adjuvant was used, as in the conventional TCI, we observed that the level of OVA-specific IgG antibodies induced was comparable to that induced by the intradermally injected OVA solution (Fig. 2A). CT-specific IgG antibody responses were also induced by the adjuvanted TCI (Fig. 2B). In contrast, the non-adjuvanted conventional TCI induced no significant serum antibody response.

3.2. Prolonged TCI induced a substantial antigen-specific serum antibody response even in the absence of an adjuvant

We examined the effect of prolongation of the duration of antigen presence on the skin to the immune response induced by TCI.

The bare abdominal skin or the dorsal side of the ears of the C57BL/6 mice were immunized with 100 μg of OVA with or without 10 μg of CT as an adjuvant according to the prolonged TCI procedure; the duration of the patch immunizations varied from 2 to 32 h. Booster immunizations were performed in an identical manner 2 times with 2-week intervals. At 2 weeks after the last immunization, we collected serum samples from the mice and determined their OVA-

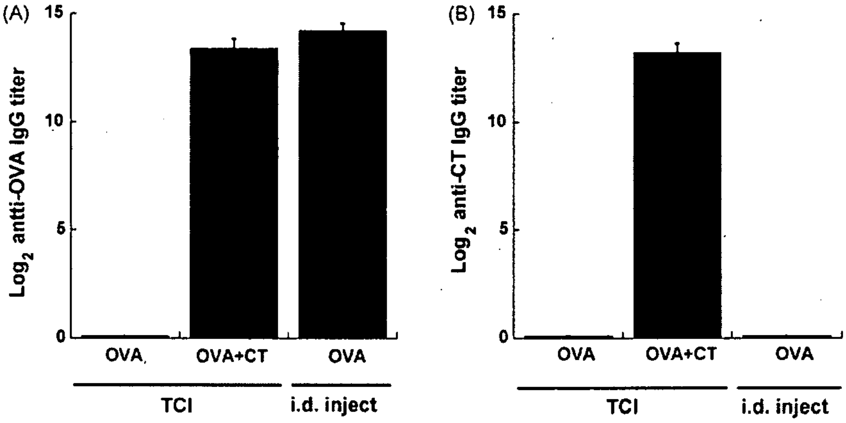

Fig. 2. Antigen- or adjuvant-specific serum IgG antibody response after conventional TCI. The bare abdominal skin of C57BL/6 mice ($n = 5$) was applied directly with 50 μl of OVA solution (2 mg/ml) with or without CT (200 μg/ml) as an adjuvant for 2 h. Another group of mice ($n = 5$) was injected intradermally with an identical dose of OVA solution without CT. Immunizations were performed in an identical manner for each group of mice at 0, 2, and 4 weeks. OVA-specific (A) or CT-specific (B) IgG titres in the serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

specific IgG antibody titres. The 2-h patch immunization with an adjuvant to the abdomen induced substantial OVA-specific serum IgG antibody production, while the 2-h immunization without an adjuvant did not induce a significant antibody response. However, the 16-h patch immunization without an adjuvant also induced a significant antibody response (Fig. 3A). On the other hand, the 2-h patch immunization of the ear induced a significant antibody response even without an adjuvant, and the titres of serum OVA-specific IgG antibodies increased with the duration of the patch immunization. The antibody titres produced by the non-adjuvanted 16-h and 32-h patch immunizations of the ear were comparable to that produced by the adjuvanted 2-h patch immunization (Fig. 3B). Thus, the prolonged TCI with patch immunization for greater than 16 h induced a substantial antigen-specific serum antibody response even when it was non-adjuvanted.

Next, we compared the immunization to the bare abdominal skin with that to the dorsal side of the ear skin for determining the most suitable target site for the prolonged TCI.

C57BL/6 mice were immunized using the prolonged TCI procedure with 10 or 100 μg of OVA without an adjuvant. We boosted the mice 2 times at 2-week intervals using the same manner of immunization as that used for the primary TCI and determined the OVA-specific serum IgG antibody titres

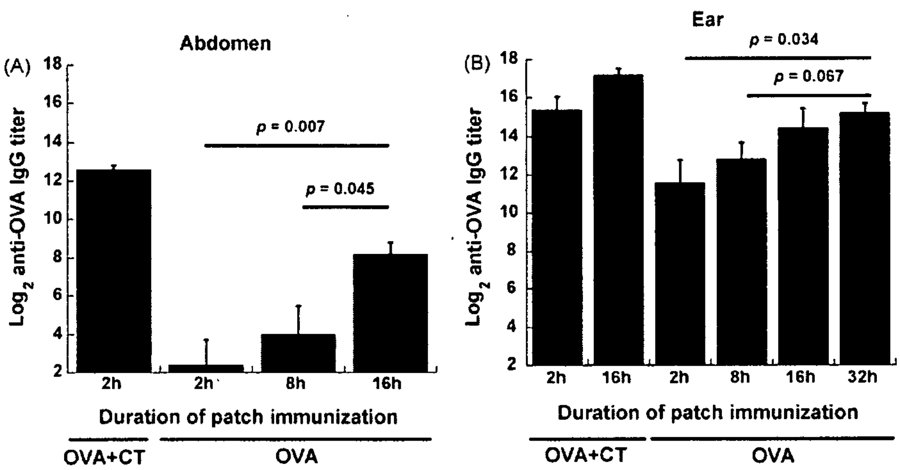

Fig. 3. Prolonged antigen presence on the skin. Gauze patches soaked with 50 μl of OVA solutions (2 mg/ml) with or without CT (200 μg/ml) as an adjuvant were taped to the bare abdominal skins (A) or the dorsal side of the left ears (B) of C57BL/6 mice ($n = 5$) for 2, 8, 16, or 32 h. Immunizations for each group of mice were performed in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

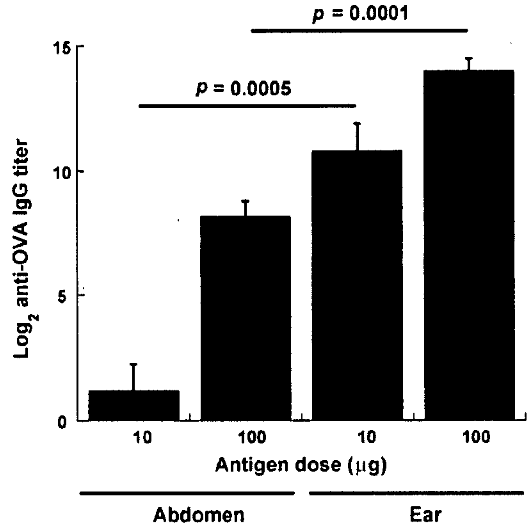

Fig. 4. Comparison between the abdominal skin and the ear skin for determining the most suitable target site for prolonged TCI. The bare abdominal skins or the dorsal side of the left ears of C57BL/6 mice ($n = 5$) were immunized by non-adjuvanted prolonged TCI procedure with 10 or 100 μg of OVA for 16 h. Immunization of each group of mice was performed in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

2 weeks after the last immunization. We found that, with both doses of OVA, the immunization of the dorsal sides of ears induced higher titres of antibodies than that of the bare abdominal skin (Fig. 4). Thus, the prolonged TCI procedure immunized the ear skin more efficiently than the abdominal skin.

3.3. Non-adjuvanted prolonged TCI of the ear skin induced a serum antibody response comparable induced by the adjuvanted TCI

We compared the serum antibody responses induced by the non-adjuvanted prolonged TCI and adjuvanted TCI by using dose–response and time-course studies.

Patches containing 1.0, 12.5, 25, 50, or 100 μg of OVA and with or without 10 μg of CT were taped to the dorsal side of the ears of the C57BL/6 mice for 16 h according to the prolonged TCI procedure. We boosted the mice twice with 2-week intervals in a manner identical to that used for the primary immunization and determined the serum OVA-specific IgG antibody titres 2 weeks after the last immunization. The titres of the serum IgG antibodies increased in a dose-dependent manner, and there was no significant difference between the magnitudes of the serum antibody response induced by the non-adjuvanted prolonged TCI and that induced by the adjuvanted TCI (Fig. 5A).

In the time-course experiment, we immunized the ears of the C57BL/6 mice with100 μg of OVA with or without 10 μg of CT according to the prolonged TCI procedure and boosted the mice twice at 2-week intervals in a manner identical to that used for the primary immunization. We collected serum samples prior to the immunization and every week for 1–6 weeks after the primary immunization. Further, we determined the time-course of the OVA-specific IgG antibody productions. Significant serum anti-OVA antibody production was observed after the first booster immunization, and this production was enhanced by the second booster immunization. There were no significant differences between the serum antibody responses induced by the non-adjuvanted prolonged TCI and the adjuvanted one, except with regard to

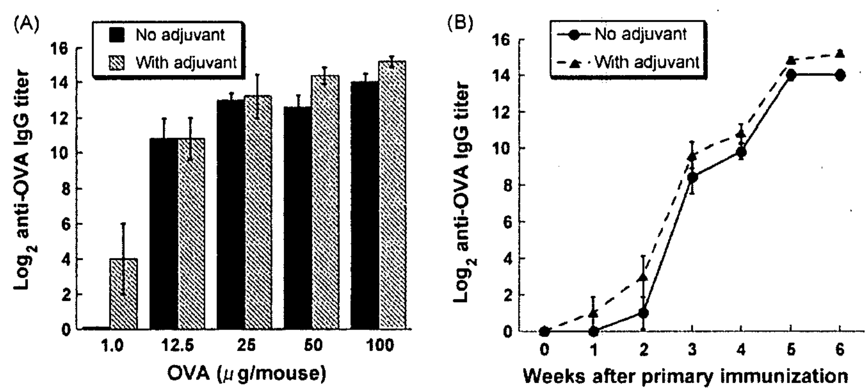

Fig. 5. Dose–response (A) and time-course (B) of serum antibody production by the non-adjuvanted or adjuvanted prolonged TCI procedure. (A) The ears of C57BL/6 mice ($n = 5$) were immunized by prolonged TCI procedure for 16 h with the indicated doses of OVA from 1.0 to 100 μg with (hatched bars) or without (solid bars) 10 μg of CT as an adjuvant. Each group of mice was immunized in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization using ELISA. The geometric mean and the standard error of the mean are shown for each group. (B) The ears of C57BL/6 mice ($n = 5$) were immunized by the prolonged TCI procedure for 16 h with 100 μg of OVA with (closed triangles and a broken line) or without (closed circles and an unbroken line) 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined every week up to 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group and time.

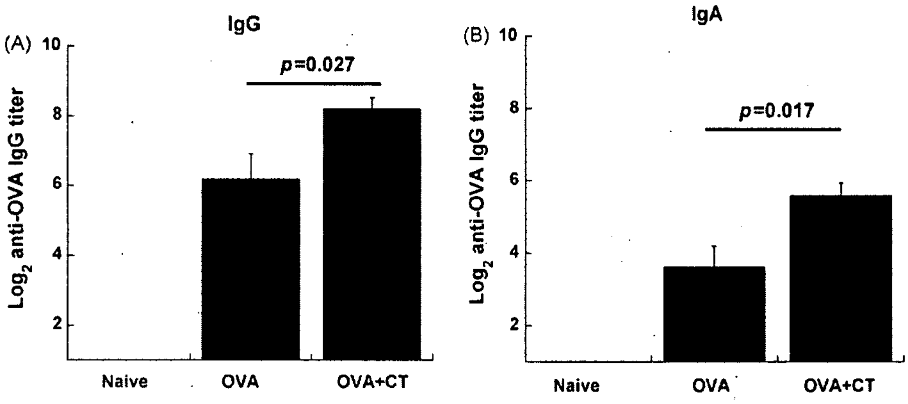

Fig. 6. Fecal antibody responses induced by the non-adjuvanted or adjuvanted prolonged TCI procedure. The ears of C57BL/6 mice ($n = 5$) were immunized by prolonged TCI procedure for 16 h with 100 μg of OVA with or without 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. Another group of mice ($n = 5$) was inoculated nothing as a negative control. The fecal samples were collected at 6 weeks after the primary immunization and OVA-specific IgG (A) and IgA (B) titers in the fecal extracts were determined. The geometric means and the standard error of the means are shown for each group.

the antibody titres at 6 weeks after the primary immunization (Fig. 5B).

Thus, the non-adjuvanted prolonged TCI comprising the 16-h antigen presence induced serum antigen-specific antibody responses of a level comparable to that observed with the adjuvanted TCI.

3.4. Non-adjuvanted prolonged TCI induced significant fecal antibody responses

Previous studies demonstrated that CT-adjuvanted TCI elicited not only systemic but also mucosal antibody responses. We examined the fecal IgG and IgA antibody responses after adjuvanted and non-adjuvanted TCI.

Patches containing 100 μg of OVA and with or without 10 μg of CT were applied to the dorsal side of the ears of the C57BL/6 mice for 16 h. We boosted the mice twice with 2-week intervals in a manner identical to that used for the primary immunization. Other group of mice were inoculated nothing as a negative control. We collected the fecal samples at 2 weeks after the last immunization.

Both groups of the immunized mice produced significant fecal IgG and IgA antibodies (Fig. 6). The CT-adjuvanted TCI group indicated approximately 4-fold higher titers of antibody production than the non-adjuvanted group.

3.5. Non-adjuvanted prolonged TCI of the ear skin induced a significant serum antibody response, regardless of mice strains

We examined strain differences with regard to the serum antibody responses induced by the prolonged TCI procedure. The ears of the C57BL/6, BALB/c, or C3H/He mice were immunized with 100 μg of OVA with or without 10 μg of CT according to the prolonged TCI procedure. The mice were boosted twice at 2-week intervals, and the OVA-specific IgG antibody production in their sera was determined 2 weeks after the last immunization. We observed significant differences in the magnitude of the serum OVA-specific IgG antibody responses among the different strains of mice. Among the 3 strains, the C57BL/6 mice produced the highest amounts of serum antigen-specific IgG antibodies, while the C3H/He mice produced the least amounts. Besides, both the non-adjuvanted prolonged TCI and the adjuvanted one induced substantial serum antibody responses in all the strains of mice that were examined (Fig. 7).

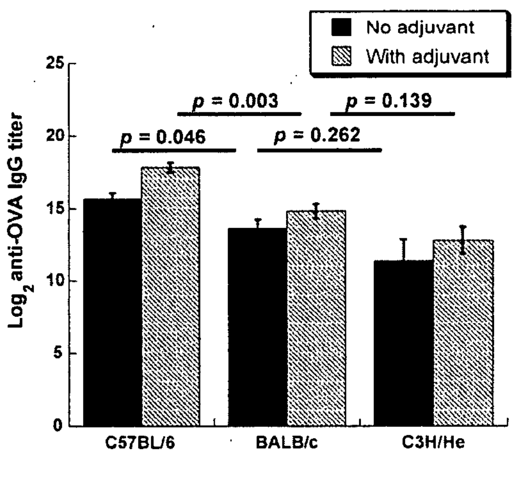

Fig. 7. Differences between the mice strains with regard to the serum antigen-specific IgG responses induced by the non-adjuvanted or adjuvanted prolonged TCI procedure. The ears of C57BL/6 ($n = 5$), BALB/c ($n = 5$), or C3H/He ($n = 5$) mice were immunized with 100 μg of OVA with (hatched bars) or without (solid bars) 10 μg of CT as an adjuvant according to the prolonged TCI procedure for 16 h at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

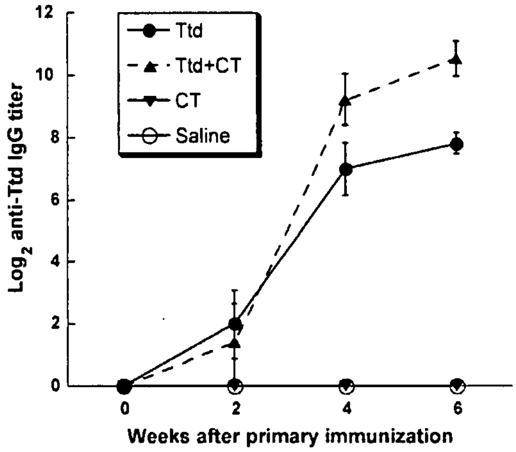

Fig. 8. Immunization using the tetanus toxoid (Ttd) for the prolonged TCI procedure with or without adjuvant. The ears of C57BL/6 mice ($n = 5$, but $n = 4$ for the "with adjuvant" group) were immunized by the prolonged TCI procedure for 16 h with 10 Lf of Ttd with (closed triangles and a broken line) or without (closed circles and an unbroken line) 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. The negative controls were immunized with 10 μg of CT (reverse closed triangles and an unbroken line) or vehicle (PBS) alone (open circles and an unbroken line) in an identical manner. OVA-specific IgG titres in serum samples were determined every 2 weeks for up to 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group and time.

3.6. Prolonged TCI using the Ttd induced a robust antibody response and provided protection against tetanus toxin challenge

We immunized the ears of the C57BL/6 mice with 10 flocculation units (Lf) of Ttd with or without 10 μg of CT according to the prolonged TCI procedure. As controls, we also immunized another group of mice with 10 μg of CT or vehicle (PBS) alone. We boosted the mice twice at 2-week intervals in a manner identical to that used for the primary immunization. We collected serum samples every alternate week for up to 6 weeks after the primary immunization and determined the titres of Ttd-specific IgG antibodies in the sera. Regardless of the use of the adjuvant, significant levels of Ttd-specific IgG antibodies were induced in the sera of the mice immunized with the Ttd, and these levels increased after the booster immunizations (Fig. 8). In contrast, in the mice immunized with either the CT or vehicle alone, no Ttd-specific IgG antibodies were induced at all the times examined.

Next, we challenged these mice with a lethal dose of tetanus toxin at 7 weeks after the primary immunization. All the control mice (only CT or vehicle immunized) died within 2 days of the challenge with signs of tetanus (Table 1). In contrast, all the mice immunized with the Ttd survived and demonstrated no clinical signs regardless of adjuvant use.

Table 1
Tetanus toxin challenge[a]

	Survival/total	% Survival
Saline	0/5	0
CT	0/5	0
Ttd	5/5	100
Ttd + CT	4/4	100

[a] C57BL/6 mice were immunized by prolonged TCI procedure with 10 Lf of Ttd with or without 10 μg of CT at 0, 2, 4 weeks. Ten micrograms of CT or vehicle (saline) alone were immunized to the other groups of mice by the same way. Ten LD50 of tetanus toxins were challenged at 7 weeks after primary immunization.

4. Discussion

In the recent decade, many studies have demonstrated the feasibility of TCI using various antigens [3–7], adjuvants [10,13–16], skin treatments [7,17–19], and animals [20–24]. Under these various settings, most experiments followed a protocol in which antigens were topically applied for 1–2 h and reported the critical role of adjuvants for the induction of robust immune responses. Skin pretreatments such as skin abrasion [25], application of penetration enhancers [7,26], use of electrophoresis [17] or sonophoresis [18] techniques, and the use of lipid carriers [20,27] were applied in some experiments;, these pretreatments might promote antigen penetration through the skin. Overall, some potent adjuvants and/or some skin penetration-enhancing methods are believed to be necessary to induce robust immune responses by TCI. Nevertheless, a few papers [28–30] reported that substantial immune responses were successfully induced in the absence of any adjuvants or penetration-enhancing methods. Further, in all of these experiments, the antigens were applied topically for a comparatively long- period of above over night. These results suggest that the duration of antigen presence on the skin during the TCI procedure might be an important parameter affecting the magnitude of the immune responses. However, thus far, the relationship between the duration of antigen presence on the skin and the magnitude of the immune response remains to be clearly elucidated. In this report, we applied OVA as an antigen for varying durations of 2–32 h on the intact skin of mice and observed that if the antigen was present on the skin for a prolonged duration, the serum antibody response was enhanced in a duration-dependent manner. Surprisingly, patch immunization on intact skin for above 16 h (referred to as prolonged TCI in this paper) induced substantial serum antibody responses even in the absence of any adjuvants. Dose–response and time-course experiments revealed that non-adjuvanted prolonged TCI to the mice ear induced serum antibody responses comparable in magnitude to those induced by adjuvanted prolonged TCI. Thus, our observations clearly indicated that the duration of antigen presence on the skin is an important factor influencing the effectiveness of TCI.

Several reports assume that some danger signals from bacterial adjuvants are necessary to activate Langerhans cells and trigger immune responses [8]. However, our data indicate that additional adjuvants are not prerequisites for the induction of immune responses. This result might imply that the antigen itself stimulates the Langerhans cells or that some substances from skin bacterial flora play the role of danger signals. It is possible that a wet gauze patch stuck on the stratum corneum for a prolonged period stimulates the Langerhans cells. In fact, there are reports that some physical stimuli to the stratum corneum, such as tape-stripping or low frequency ultrasound, activate the Langerhans cells [18,37].

Our experiments clearly indicated that the ear skin was immunized more efficiently than the abdominal skin by the prolonged TCI procedure. It is known that the differences in skin structure, such as thickness of the stratum corneum and the density of Langerhans cells, among anatomic skin sites influence the penetration efficiency of low molecular percutaneous drugs [31,32]. The difference between the structures of the ear skin and abdominal skin may explain our observation. Thus, our observation indicates that skin anatomy is an important factor influencing the efficiency of TCI.

CT or LT has strong mucosal adjuvanticity. CT-adjuvanted or LT-adjuvanted TCI induces the mucosal antibody response, in addition to the systemic immune response [6,7,15,28,33,34]. Interestingly, we observed that non-adjuvanted prolonged TCI with OVA induced substantial antigen-specific IgG and IgA antibodies in the feces of mice although the magnitude of antibody production was significantly less as compared to that induced by CT-adjuvanted TCI. Our observation indicated that the mucosal immune response to TCI could be also induced even in the absence of CT or LT.

We observed substantial antibody responses in all the 3 strains of mice immunized by the prolonged non-adjuvanted TCI. This observation suggests that this modified TCI would be feasible for a wide range of genetic backgrounds. However, there were some significant differences among the 3 strains with regard to the magnitude of serum antibody productions. The C57BL/6 mice were the most sensitive to the prolonged TCI, while the C3H/He mice were the least sensitive. The response of the BALB/c mice was intermediate. Another group has previously reported a similar hierarchy in the sensitivity to TCI [38]. They used CT as an adjuvant and hypothesized that the hierarchy reflected the difference in the sensitivity to CT based on former experiments which indicated that the plasma IgG response to CT following oral or parenteral administration was under the genetic control of the I-A region of the H-2 major histocompatibility complex [39–41]. Here, we observed the same hierarchy regardless of the use of CT, suggesting that other mechanism(s) govern the sensitivity to TCI.

We applied the prolonged TCI method by using the Ttd as a model vaccine antigen and successfully induced significant serum antibody responses. Indeed, CT had an adjuvant effect in the prolonged TCI using Ttd, but non-adjuvanted prolonged TCI using the Ttd also induced substantial Ttd-specific antibodies in the sera of mice after the second booster immunization. The mice immunized with the Ttd according to the prolonged TCI method, adjuvanted and non-adjuvanted, survived with no clinical symptoms after challenge with a lethal dose of the tetanus toxin. These results suggest that non-adjuvanted prolonged TCI method is a feasible vaccination method for infectious diseases.

Thus far, the TCI method has been shown to be functional in various disease models, including bacterial and viral infections, malignancies [26,35], and Alzheimer's disease [36], using various animals, e.g., mice, rats [20], sheep [22], cattle [24], chickens [21], and humans [12,19]. To develop an effective TCI strategy for different aims, the TCI protocol with regard to antigens, adjuvants, skin treatments, etc. requires optimization. In this study, we pointed out the importance of the duration of antigen presence on the skin during the TCI procedure and showed that substantial antibodies were induced by non-adjuvanted TCI of 16-h duration. Our observations improve the understanding regarding the TCI mechanisms and offer a practical option for developing a safe and effective method for transcutaneous vaccination.

References

[1] Bos JD. The skin as an organ of immunity. Clin Exp Immunol 1997;107(Suppl. 1):3–5.

[2] Chuong CM, Nickoloff BJ, Elias PM, Goldsmith LA, Macher E, Maderson PA, et al. What is the 'true' function of skin? Exp Dermatol 2002;11:159–87.

[3] Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin. Nature 1998;391:851.

[4] Beignon AS, Briand JP, Muller S, Partidos CD. Immunization onto bare skin with heat-labile enterotoxin of *Escherichia coli* enhances immune responses to coadministered protein and peptide antigens and protects mice against lethal toxin challenge. Immunol 2001;102:344–51.

[5] Godefroy S, Goestch L, Plotnicky-Gilquin H, Nguyen TN, Schmitt D, Staquet MJ, et al. Immunization onto shaved skin with a bacterial enterotoxin adjuvant protects mice against Respiratory Syncytial Virus (RSV). Vaccine 2003;21:1665–71.

[6] Berry LJ, Hickey DK, Skelding KA, Bao S, Rendina AM, Hansbro PM, et al. Transcutaneous immunization with combined cholera toxin and CpG adjuvant protects against Chlamydia muridarum genital tract infection. Infect Immun 2004;72(2):1019–28.

[7] Skountzou I, Quan FS, Jacob J, Compans RW, Kang SM. Transcutaneous immunization with inactivated influenza virus induces protective immune responses. Vaccine 2006;24:6110–9.

[8] Hammond SA, Guebre-Xabier M, Yu J, Glenn GM. Transcutaneous immunization: an emerging route of immunization and potent immunostimulation strategy. Crit Rev Ther Drug Carrier Syst 2001;18(5):503–26.

[9] Partidos CD, Beignon AS, Briand JP, Muller S. Modulation of immune responses with transcutaneously deliverable adjuvants. Vaccine 2004;22:2385–90.

[10] Glenn GM, Scharton-Kersten T, Vassell R, Matyas GR, Alving CR. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect Immun 1999;67(3):1100–6.

[11] Warger T, Schild H, Rechtsteiner G. Initiation of adaptive immune responses by transcutaneous immunization. Immunol Lett 2007;109:13–20.

[12] Glenn GM, Taylor DN, Li X, Frankel S. Montemarano A, Alving CR. Transcutaneous immunization: a human vaccine delivery strategy using a patch. Nature Med 2000;6(12):1403–6.

[13] Rechtsteiner G, Warger T, Osterloh P, Schild H, Radsak MP. Cutting edge: priming of CTL by transcutaneous peptide immunization with imiquimod. J Immunol 2005;174(5):2476–80.

[14] Tierney R, Beignon AS, Rappuoli R, Muller S, Sesardic D, Partidos CD. Transcutaneous immunization with tetanus toxoid and mutants of *Escherichia coli* heat-labile enterotoxin as adjuvants elicits strong protective antibody. J Infect Dis 2003;188(5):753–8.
[15] John M, Bridges EA, Miller AO, Calderwood SB, Ryan ET. Comparison of mucosal and systemic humoral immune responses after transcutaneous and oral immunization strategies. Vaccine 2002;20(21–22):2720–6.
[16] Scharton-Kersten T, Yu J, Vassell R, O'Hagan D, Alving CR, Glenn GM. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. Infect Immun 2000;68(9):5306–13.
[17] Zhao YL, Murthy SN, Manjili MH, Guan LJ, Sen A, Hui SW. Induction of cytotoxic T-lymphocytes by electroporation-enhanced needle-free skin immunization. Vaccine 2006;24(9):1282–90.
[18] Tezel A, Paliwal S, Shen Z, Mitragori S. Low-frequency ultrasound as a transcutaneous immunization adjuvant. Vaccine 2005;23:3800–7.
[19] Mckenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, et al. Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. Vaccine 2007;25(18):3684–91.
[20] Mishra V, Mahor S, Rawat A, Dubey P, Gupta PN, Singh P, et al. Development of novel fusogenic vesosomes for transcutaneous immunization. Vaccine 2006;24(27–28):5559–70.
[21] Heckert RA, Elankumaran S, Oshop GL, Vakharia VN. A novel transcutaneous plasmid-dimethylsulfoxide delivery technique for avian nucleic acid immunization. Vet Immunol Immunopathol 2002;89(1–2):67–81.
[22] Chen D, Colditz IG, Glenn GM, Tsonis CG. Effect of transcutaneous immunization with co-administered antigen and cholera toxin on systemic and mucosal antibody responses in sheep. Vet Immunol Immunopathol 2002;86(3–4):177–82.
[23] Yu J, Cassels F, Scharton-Kersten T, Hammond SA, Hartman A, Angov E, et al. Transcutaneous immunization using colonization factor and heat-labile enterotoxin induces correlates of protective immunity for enterotoxigenic *Escherichia coli*. Infect Immun 2002;70(3):1056–68.
[24] Morrow CK, Colditz IG, Cope RB. Stimulated solar UVB exposure inhibits transcutaneous immunization to cholera toxin via an irradiated skin site in cattle. Vet immunol immunopathol 2001;83(1–2):107–14.
[25] Godefroy S, Peyre M, Garcia N, Muller S, Seardic D, Partidos CD. Effect of skin barrier disruption on immune responses to topically applied cross-reacting material, CRM(197), of diphtheria toxin. Infect Immun 2005;73(8):4803–9.
[26] Frankenburg S, Grinberg I, Bazak Z, Fingerut L, Pitcovski J, Gorodetsky R, et al. Immunological activation following transcutaneous delivery of HR-gp100 protein. Vaccine 2007;25(23):4564–70.
[27] Paul A, Ceve G, Bachhawat BK. Transdermal immunization with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. Vaccine 1998;16(2–3):188–95.
[28] Ghose C, Kalsy A, Sheikh A, Rollenhagen J, John M, Young J, et al. Transcutaneous immunization with Clostridium difficile toxoid A induces systemic and mucosal immune responses and toxin A-neutralizing antibodies in mice. Infect Immun 2007;75(6):2826–32.
[29] Matyas GR, Friedlander AM, Glenn GM, Little S, Yu J, Alving CR. Needle-free skin patch vaccination method for anthrax. Infect Immun 2004;72(2):1181–3.
[30] Baca-Estrada ME, Ewen C, Mahony D, Babiuk LA, Wilkie D, Foldvari M. The haemopoietic growth factor, Flt3L, alters the immune response induced by transcutaneous immunization. Immunology 2002;107(1):69–76.
[31] Ya-Xian Z, Suetake T, Tagami H. Number of cell layers of the stratum corneum in normal skin—relationship to the anatomical location on the body, age, sex and physical parameters. Arch Dermatol Res 1999;291:555–9.
[32] Bronaugh RL, Maibach HI. Topical absorption of dermatological products. New York: Marcel Dekker, Inc.; 2002.
[33] Hickey DK, Bao S, Ikeda LT, Carey AJ, Beagley KW. Induction of anti-chlamydial mucosal immunity by transcutaneous immunization is enhanced by topical application of GM-CSF. Curr Mol Med 2005;5(6):599–605.
[34] Partidos CD, Moreau E, Chaloin O, Tunis M, Briand JP, Desgranges C, et al. A synthetic HIV-1 Tat protein breaches the skin barriers and elicits Tat-neutralizing antibodies and cellular immunity. Eur J Immunol 2004;34(12):3723–31.
[35] Seo N, Tokura Y, Nishijima T, Hashizume H, Furukawa F, Takigawa M. Percutaneous peptide immunization via corneum barrier-disrupted murine skin for experimental tumor immunoprophylaxis. Proc Natl Acad Sci USA 2000;97(1):371–6.
[36] Nikolic WV, Bai Y, Obregon D, Hou H, Mori T, Zeng J, et al. Transcutaneous beta-amyloid immunization reduces cerebral beta-amyloid deposits without T cell infiltration and microhemorrhage. Proc Natl Acad Sci USA 2007;104(7):2507–12.
[37] Nishijima T, Tokura Y, Imokawa G, Seo N, Furukawa F, Takigawa M. Altered permeability and disordered cutaneous immunoregulatory function in mice with acute barrier disruption. J Invest Dermatol 1997;109(2):175–82.
[38] Scharton-Kersten T, Glenn GM, Vassell R, Yu J, Walwender D, Alving CR. Principles of transcutaneous immunization using cholera toxin as an adjuvant. Vaccine 1999;17:S37–43.
[39] Elson CO, Ealding W. Genetic control of the murine immune response to cholera toxin. J Immunol 1985;135:930–2.
[40] Elson CO, Ealding W. Ir gene control of the murine secretory IgA response to cholera toxin. Eur J Immunol 1987;17:425–8.
[41] Elson CO. Cholera toxin as a mucosal adjuvant: effects of H-2 major histocompatibility complex and lps genes. Infect Immun 1992;60:2874–9.

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Application of complementary DNA microarray technology to influenza A/Vietnam/1194/2004 (H5N1) vaccine safety evaluation

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Summary We propose that cDNA microarray analysis can be used in the quality control of pandemic and endemic influenza vaccine. Based on the expression profiles of 76 genes in the rat lung one day after inoculation of influenza vaccine, we can distinguish whole-virion influenza vaccine (PDv: pandemic influenza vaccine and WPv: whole particle vaccine) and sub-virion vaccine (HA vaccine) from saline. Among these 76 genes, we found genes up-regulated by influenza infection, as well as genes involved in the immune response, and interferon. Hierarchical clustering of each influenza vaccine by the expression profiles of these 76 genes matched data from current quality control tests in Japan, such as the abnormal toxicity test (ATT) and the leukopenic toxicity test (LTT). Thus, it can be concluded that cDNA microarray technology is an informative, rapid and highly sensitive method with which to evaluate the quality of influenza vaccines. Using DNA microarray system, consistent with the results of the ATT and LTT, it was clarified that there was no difference in vaccine quality between PDv and WPv.

Introduction

Influenza virus triggers a highly contagious acute respiratory illness, which can lead to high fever, muscle aches, sore throat, non-productive cough, and sometimes lead to death.

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Influenza virus is a member of the *Orthomyxoviridae* family and is divided into Type A, B and C viruses. Type A influenza viruses are candidates for annual epidemic and occasional pandemics, and are sub-divided by hemagglutinin (HA) and neuraminidase (NA) [1]. Among the various types of HA, H1 and H3 subtypes are found in human and mutations in these enable the virus to evade the immune system. Recently, a highly pathogenic avian influenza A virus (H5N1) has been identified in poultry, migratory birds and human beings, which has resulted in severe disease or death around the world. As of August 16, 2007, there have been 321 confirmed human cases of avian influenza A (H5N1) reported to the WHO and 194 of these have died [2]. Although transmission from human to human is inefficient and limited, the virus has the potential to cause the next influenza pandemic, and it is essential to prepare for such a possible pandemic [3]. Among various strategies for dealing with this possibility, vaccination is the principal defense strategy for reducing morbidity and mortality during a pandemic. However, conventional split influenza vaccines might be unsuitable against a pandemic caused by influenza type H5N1. Thus, development of a pandemic influenza vaccine is urgently required. The Japanese Ministry of Health Labour and Welfare (MLHW) have released guidelines for fast-track licensing of pandemic influenza vaccine. During the pre-pandemic period, submission of a mock-up pandemic vaccine were formally assessed and approved by national regulatory authorities.

Several influenza vaccines are now being developed and pre-clinically and clinically assessed by several vaccine manufactures around the world [4]. In clinical trials, an immune response is induced by two shots of high doses of an inactivated sub-virion vaccine based on H5N1 virus isolated in 2004 [5,6] and a recombinant hemagglutinin based on H5N1 virus isolated in 1997 [7]. To induce a high level of immunity after one dose, several countries have tried to develop a whole-virion vaccine. Whole-virion vaccines are more immunogenic than split-virion vaccines [8]; however the reactogenicity of whole-virion vaccines is higher than that of split vaccines [9]. A clinical trial in China clearly showed that antibody responses were well induced after the first dose, and that no serious adverse event was reported [10]. Despite the evidence that there are differences in immunogenicity and reactogenicity between whole and sub-virion vaccines, there have been few pre-clinical trials and animal safety tests. It is important to address whether pre-clinical and animal safety tests can predict and correlate to clinical trials [11], and a rapid and more sensitive safety test must be developed.

In Japan, the MLHW decided to develop and save whole-virion H5N1 vaccine adjuvanted with aluminum hydroxide. In Japan, as in other countries, all vaccines for human use must conform to the "Minimum Requirements for Biological Products" and are obliged to pass national control tests [12]. Quality control of influenza vaccines is performed by the abnormal toxicity test (ATT) and the leukopenic toxicity test (LTT), which are based on body weights and peripheral white blood cell (WBC) counts in mice after subcutaneous or intra-peritoneal injection [13,14]. However, it was not known whether these tests (ATT and LTT) could evaluate the safety of pandemic influenza vaccine, and these tests require a lot of animals and days. In addition, several researchers have discussed the safety of aluminum adjuvant-containing influenza vaccines [15]. Although aluminum hydroxide is thought to be safe and has long been used as a vaccine adjuvant, aluminum adjuvants have resulted in macrophagic myofasciitis and delayed-type hypersensitivity [16]. Thus, we must investigate the safety and toxicity of whole-virion H5N1 vaccine adjuvanted with aluminum hydroxide.

In this study, we compare pandemic vaccine (PDv; whole-virion H5N1 vaccine adjuvanted with aluminum hydroxide), whole virion-particle vaccine (WPv) without any adjuvant, and HA vaccine (HAv). In addition, to develop rapid and more sensitive and reproducible methods, we performed a comprehensive gene expression analysis of rats after administration of the various type of influenza vaccine, using DNA microarrays. Our previous study clearly shows that cDNA microarrays are a useful technology with which to evaluate the safety and quality of pertussis vaccine, and we can successfully identify the genes related to vaccine toxicity [17]. In the present study, we developed the cDNA microarray technology and applied it to the safety evaluation of Influenza A/Vietnam/1194/2004 (H5N1) vaccines.

Materials and methods

Animals

Male Fisher-344 (F344) rats (8 weeks) were obtained from SLC (Tokyo, Japan). All animals were housed in rooms maintained at 23 ± 1 °C, with 50 ± 10% relative humidity, and 12-h light: 12-h dark cycles, at least 1 week prior to the test challenge. Rats typically weighed 160–200 g on arrival.

Vaccines

The following vaccines were used in this study. (1) PDv: inactivated monovalent A/H5N1 whole-virion influenza vaccine (derived from NIBRG-14: A/Vietnam/1194/2004) adjuvanted with aluminum hydroxide, containing 30 μg HA/ml. NIBRG-14 is constructed by reverse genetics, using A/Vietnam/1194/2004 and PR-8 (H1N1). (2) WPv: inactivated whole trivalent influenza vaccines (A/Newcaledonia/20/99 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004), containing 30 μg HA/ml each strain. (3) HAv: trivalent HA influenza vaccine (A/Newcaledonia/20/99 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004), containing 30 μg HA/ml each strain. All vaccines were produced and manufactured by The Chemo-Sero -Therapeutic Research Institute, Kaketsuken (Kumamoto, Japan). All vaccines complied with the minimum requirement for biological products in Japan. Each 5 ml vaccine was intra-peritoneally (*i.p.*) injected into rats. Five milliliters of saline (SA) (Otsuka normal saline; Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima, Japan) was intra-peritoneally injected as a control.

Abnormal toxicity test and leukopenic toxicity test

According to the minimum requirement for biological products in Japan [12], we performed an abnormal toxicity test and a leukopenic toxicity test for influenza vaccine (PDv,

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