chemicals [8]. DNA microarray assays have also been applied to the analysis of the side effects of medicines [9]. Recently, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have, either individually or together, started to review submissions for the qualification of biomarkers for medical products for specific purposes proposed by industry [10]. The introduction of pharmacogenomics, or pharmacogenetics, to the evaluation of medicines is a global trend.

For a better understanding of the molecular toxicology regarding vaccines, DNA microarray analysis promises to be an ideal method, as has been the case for pharmaceuticals. The FDA now encourages the voluntary submission of genomic data to the FDA outside of the regular review process [11]. However, no studies similar to those described above for pharmaceuticals have yet been conducted in the field of vaccines. At the beginning of this review, we summarized the current efforts used for the control of vaccine safety using conventional animal tests. We then referred to our recent efforts using DNA microarray analysis to identify "genetic signatures" for the toxicants remaining in vaccines. Since pertussis and influenza vaccines are among the most commonly used vaccines, we tried to develop a system to evaluate the "genetic signatures" of the toxicity of these vaccines.

2. Current Vaccine Safety Test

2.1. Body Weight Change in Vaccinated Animals. To screen for general toxicity of vaccines, the body weight of vaccine-treated animals can be analyzed as the general safety test [12]. Five mL of the vaccine are injected into the peritoneum of guinea pigs weighing 300–400 g, and the weight loss experienced by the animals is analyzed at days 1, 2, 3, 4, and 7 after administration. None of the animals should show any abnormal signs; no statistically significant ($P = .01$) difference in weight loss should be observed between the treated animals and the control group on any observation day. This test has been applied to a wide variety of vaccines in a unified way, and plays an important role in ensuring the safety and consistency of vaccine batches [12]. For pertussis vaccine (inactivated whole cell formulation), the effects of vaccine treatment were also measured using test for toxicity to mouse weight gain, in addition to the general safety test. All mice were weighed on days 0, 1, 2, 3, 4, and 7 after vaccine administration. The criterion for judgment is that mean body weight 3 days after injection should be no less than that at the time of injection upon statistical analysis, and no mice showed any abnormal sign during the observation periods [12]. When the reference vaccine (RE: the inactivated whole cell pertussis vaccine) was administrated, weight loss was observed on day 1 after administration (Figure 1(a)).

2.2. Leukocytosis-Promoting Toxicity in Vaccinated Animals. To detect the toxin present in pertussis vaccines, the number of peripheral leukocytes can also be analyzed. Pertussis vaccine is injected into the peritoneum of mice at a dose of 0.5 mL. Leukocytes present in peripheral blood are then counted 3 days after injection [12]. The white blood cell (WBC) counts in peripheral blood of reference vaccine-treated mice reach approximately 2,500 cells/μL (Figure 1(b)). The standard criterion of safety for pertussis vaccine (inactivated whole cell formulation) is that the mean count of leukocytes in peripheral blood, 3 days after injection, should not exceed 10 times that before injection [12].

2.3. Leukopenic Toxicity Test in Vaccinated Animals. Quality control of influenza vaccines is performed using the general safety test and the leukopenic toxicity test (LTT), which is based on peripheral WBC counts in mice 12–18 hours after intraperitoneal injection of a vaccine. The criterion for judgment is that the leukopenic toxicity of the test sample relative to that of the toxicity reference sample should be no higher than the value corresponding to 80% of the leukocyte count of the control relative to that of the toxicity reference sample [12–14].

3. DNA Microarray-Based Safety Test

The currently used quality control and safety tests, such as the LTT and the general safety test, have been used to evaluate vaccine safety for over 50 years [3]. We are now developing a new quality control method for vaccines using DNA microarray analysis as a substitute for the conventional animal tests [15–17]. The principle of this method is to translate vaccine quality, immunogenicity, and reactogenicity, into gene expression profile data. This method is expected to be informative, rapid, and highly sensitive.

For DNA microarray analysis using vaccines, 8 week-old male rats, weighing 180–220 g, were intraperitoneally administered with 5 mL of vaccine or physiological saline (SA). Three to 6 rats were used for each group. Vaccinated rats were sacrificed to obtain whole lung, kidney, brain, and the lateral left lobe of the liver on day 1, 2, 3, and 4 postadministration (Figure 2). Tissues were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and poly(A)$^+$ RNA was purified from the lysate. Cyanine 5-labeled poly(A)$^+$ RNA was subjected to DNA microarray analysis. Blood was also collected, however, this could not be analyzed due to the low quality of purified RNA.

For DNA microarray analysis, a set of synthetic polynucleotides (80-mers) representing 11,468 rat transcripts and including most of the RefSeq genes deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan) was arrayed on aminosilane-coated glass slides [18, 19]. Cyanine 5-labeled poly(A)$^+$ RNA was competitively hybridized on the slide with cyanine 3-labeled common reference RNA. Hybridization signals were measured, processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from common reference RNA]), and then normalized by multiplying normalization factors calculated for each microarray feature.

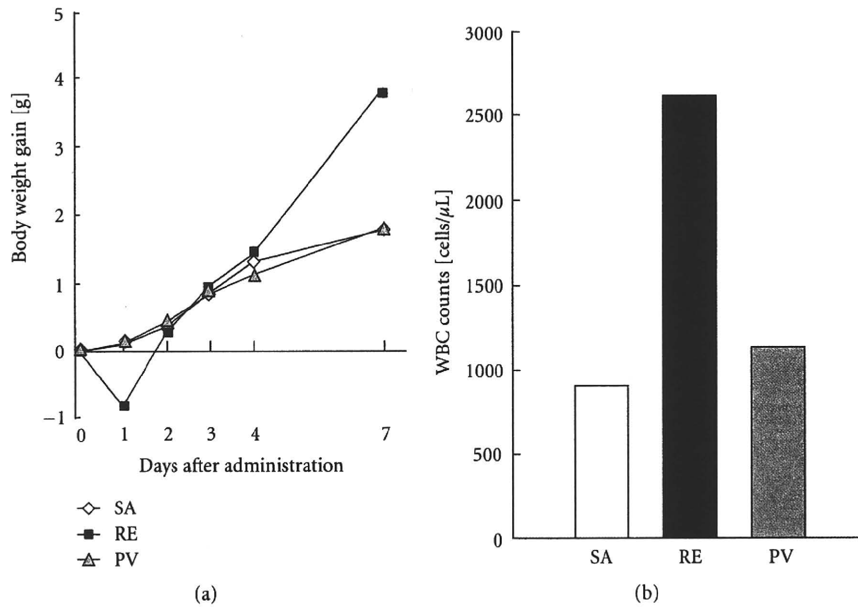

Figure 1: Safety control tests for pertussis vaccines. (a) Test for toxicity to mouse weight gain. Physiological saline (SA), an inactivated whole-cell pertussis vaccine (RE), or an acellular pertussis vaccine (PV)-administered mice were weighed on 0, 1, 2, 3, 4, and 7 days postadministration. Ten mice in each group were used, and the mean changes in body weight are indicated. (b) Leukocytosis promoting activity of various pertussis vaccines. White blood cell (WBC) counts in peripheral blood were measured 3 days after vaccine administration. Ten mice in each group were used and the mean WBC counts are indicated.

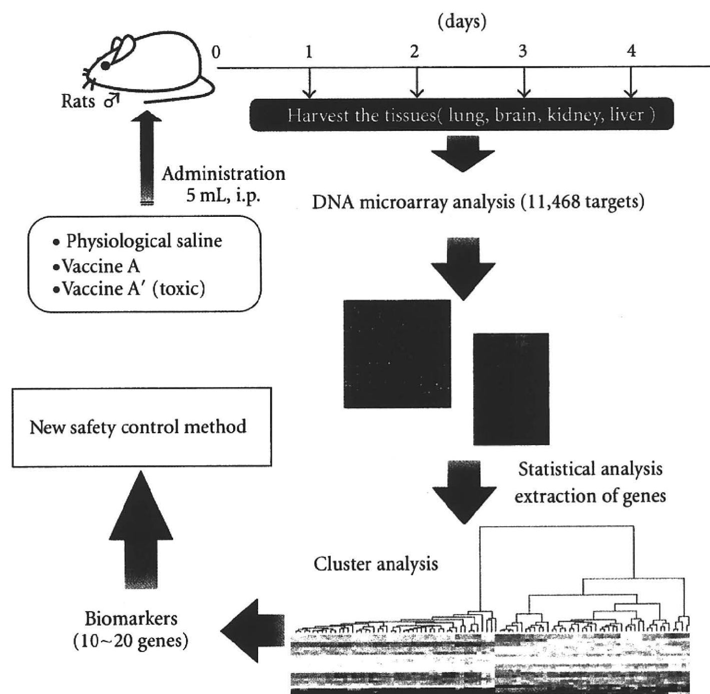

Figure 2: The gene expression analysis procedure. The detail of the procedure is described in the text.

For data processing and hierarchical cluster analysis, the primary expression ratios were converted into $\log_2$ ratios ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity). The genes with $\log_2$ ratios over 1 or under -1 in at least one sample were extracted from the primary data matrix, then subjected to two-dimensional hierarchical cluster analysis for samples and genes.

For the identification of biomarker genes for pertussis vaccines, we extracted differentially expressed genes from physiological saline and pertussis toxin-treated lung samples using the t-test ($P < .01$). Among the extracted genes, we further selected genes that exhibited mean average $\log_2$ ratio differences greater than 0.75 between the two sample groups [17]. For influenza vaccines, we extracted differentially expressed genes from physiological saline and inactivated whole-virion vaccine-treated lung samples using the t-test ($P < .005$) [16].

4. Pertussis Vaccines

Pertussis, or whooping cough, is an infectious respiratory disease caused by a Gram-negative bacillus, *Bordetella pertussis*. *Bordetella pertussis* possesses several pathogenic components, including pertussis toxin (PT) [20]. PT is known as a leukocytosis promoting factor, a major contributor to the pathogenesis of pertussis, and an antigen in immunity to pertussis [21]. At present, whole-cell pertussis vaccines and acellular pertussis vaccines containing inactivated PT are in commercial use [20].

Although pertussis vaccines are effective in the prevention of whooping cough, they have occasionally caused local reactions such as redness, swelling, and pain at the injection site. However, little is known about the overall responses to these vaccines. To address this problem, we applied DNA microarray analysis and quantification of specific genes to analyze the toxicants in pertussis vaccines [15, 17]. Three preparations, an acellular vaccine containing inactivated pertussis toxin (PV), an inactivated whole-cell vaccine (RE), and a purified pertussis toxin (PT) were prepared. RE is a reference vaccine for National Quality Control Tests of pertussis vaccines in Japan and is made from formaldehyde-inactivated *Bordetella pertussis* preparations. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, 5 mL of SA, PV, PT, and RE were each injected into 3 rats and the vaccinated tissues, lung, brain, kidney, and liver, were harvested at 1, 2, 3, and 4 days after vaccine administration. The experiments were performed twice and purified poly(A)$^+$ RNA from a total of 384 samples was subjected to DNA microarray analysis.

Of the 4 organs tested, the lung expressed genes that were extracted by DNA microarray analysis were classified sharply into clusters depending on sample treatment. From the DNA microarray analysis of vaccinated rat lungs at day 1, 13 genes for which expression levels were dynamically changed in response to PT treatment were [17] (accession numbers were updated in Table 1). Interestingly, the DNA microarray-based gene expression data correlated well with the body weight change of vaccine-treated mice (Figure 1(a)) and rats [17]. The real-time PCR quantification results of the expression levels of the 13 genes were comparable to the relative expression ratios from the DNA microarray analysis. Furthermore, cluster analysis using the 13 genes could distinguish SA- and PV-treated groups from PT- and RE-treated groups. These 13 genes are likely to be closely involved in the toxicity of pertussis vaccines. To quantify these genes in a convenient way, the QuantiGene Plex assay was applied. The QuantiGene Plex assay enabled the simultaneous analysis of the 13 genes. We evaluated the expression levels of the 13 genes in the lungs of rats vaccinated with various doses of RE. Nine genes, *S100A9*, *S100A8*, *IRF7*, *MX2*, *IFI27L*, *BEST5*, *MMP9*, *MMP8*, and *CYP2E1* (indicated in bold letters in Table 1) showed dose-dependent up-or down-regulation in response to the various doses of RE treatment. RE vaccine toxicity could be measured by the expression level in lung lysate of these 9 genes. The quantification of these 9 genes using the QuantiGene Plex assay is, we believe, a promising candidate for a new control test for pertussis vaccines.

5. Influenza Vaccines

Influenza virus triggers a highly contagious acute respiratory disease and has caused epidemics and global pandemics, partly because it possesses the capacity for gradual antigenic change in two surface antigens, hemagglutinin (HA) and neuraminidase (NA) [22]. To combat influenza, split vaccines consisting of subvirion preparations and whole-virus vaccines are manufactured using strains recommended annually by the WHO, based on the antigenic characteristics of HAs and NAs. Furthermore, the recent circulation of the highly pathogenic avian influenza A (H5N1) virus has raised concerns about the preparations for a coming influenza pandemic [23]. Many efforts are underway to develop vaccines against influenza A (H5N1).

To identify biomarkers for influenza vaccine toxicity, 3 vaccines were used: trivalent influenza HA vaccine (HAv, a split vaccine), trivalent influenza vaccine (WPv, an inactivated whole-virion vaccine), and prepandemic influenza vaccine (PDv, inactivated whole-virion (A/H5N1) absorbed onto an aluminum salt). All were produced by Kaketsuken, The Chemo-Sero-Therapeutic Research Institute, Japan. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, SA, HAv, WPv, and PDv were each injected into 5 rats, and the vaccinated tissues, lung, liver, brain, and peripheral blood, were harvested at 1, 2, 3, and 4 days after vaccine administration. Purified poly(A)$^+$ RNA from a total of 320 samples was subjected to DNA microarray analysis [16]. Based on the analysis of pertussis vaccines, described above, the gene expression profiles from lung samples were subjected to two-dimensional hierarchical cluster analysis. PDv- and WPv-treated samples at day 1 formed an independent cluster from other samples, indicating distinct profiles in gene expression of these groups. As was the case with pertussis vaccines, we tried to identify several biomarkers from the analysis of lung gene expression. The analysis of lungs from vaccinated rats at day 1 resulted in the extraction of 76 genes, whose expression levels were statistically different between SA- and

TABLE 1: Biomarkers for pertussis vaccine toxicity.

Category	Accession no.	Symbol	Brief description
Inflammation	NM_053587	***S100A9***	A calcium binding protein that may be associated with acute inflammatory processes, coupled with S100a8
	NM_053822	***S100A8***	May play a role in inflammatory responses such as cell motility, coupled with S100a9
	NM_019323	*MCPT9*	A serine protease expressed in mast cells, but the precise function has not yet been determined
	NM_031530	*CCL2*	A ligand for CCR2 that acts as a chemoattractant of monocytes
IFN inducible, immune response	NM_001033691	***IRF7***	Unknown
	NM_134350	***MX2***	Involved in inhibiting vesicular stomatitis virus
	NM_203410	***IFI27***	Induced by steroid hormone, IFN, and LPS in endometrium at implantation, dendric cells, and macrophases
	NM_001007694	*IFIT3*	May induced by IFN or virus infection
	Y07704	***BEST5***	Induced by IFN and involved in bone formation
Peptidoglycan metabolism	NM_031055	***MMP9***	Metalloproteinase involved in extracellular matrix remodeling, bone resorption, and immune responses
	NM_022221	***MMP8***	May play a role in appositional bone formation and regulation of the extracellular matrix
Xenobiotic metabolism	J02627	***CYP2E1***	Protects hepatocytes from stress-induced cell death
Others	NM_001106862	*NGP*	Unknown

TABLE 2: Biomarkers for influenza vaccine toxicity.

Category	Accession No.	Symbol	Brief description
IFN inducible gene	NM_172019	*IFI47*	Mouse homolog may be a guanine nucleotide-binding protein induced by IFN-gamma
	AF329825	*TRAFD1*	Putative TRAF-interacting zinc finger protein
	NM_019242	*IFRD1*	May be involved in proliferation of neuronal and glial precursors
IFN inducible, immune response	NM_001033691	*IRF7*	Unknown
	NM_134350	*MX2*	Involved in inhibiting vesicular stomatitis virus
Immune response	NM_172222	*C2*	Likely component of the classical pathway of the complement cascade
	NM_012708	*PSMB9*	Subunit of the proteasome complex, which may play a role in protein catabolism
	NM_032056	*TAP2*	Transports peptides into the ER lumen for binding with MHC class I molecules; plays a role in antigen processing and presentation
	NM_033098	*TAPBP*	Facilitates the binding of MHC class I molecules to the transporter associated with antigen processing (TAP) in MHC class I assembly
	NM_017264	*PSME1*	May play a role in proteasome activation
Chemokine and Cytokine function	AF065438	*LGALS3BP*	Displays differential expression in a fibroblast cell line transformed by human T-cell leukemia virus type 1 Tax protein
	NM_012977	*LGALS9*	A highly selective urate transporter/channel
	NM_053819	*TIMP1*	Acts as an inhibitor of metalloprotease activity; may play a role in vascular tissue remodeling
	NM_023981	*CSF1*	Plays a role in macrophage formation
	NM_145672	*CXCL9*	Chemokine which plays a role in the recruitment of mononuclear cells and in allograft rejection
	XM_223236	*CXCL11*	Mouse homolog is a chemokine and is involved in the immune response
Transcription activity	AJ302054	*ZBP1*	DNA binding protein; thought to bind Z-DNA, which is largely controlled by the amount of supercoiling

WPv-treated samples ($P < .005$) [16]. The cluster analysis using these 76 genes successfully distinguished WPv- and PDv-treated groups at day 1 from other groups, indicating the suitability of the 76 genes as biomarkers for influenza vaccines.

The extracted 76 genes were categorized according to function, such as interferon-inducible, chemokine and cytokine function, immune response, transcriptional activity, and so on. Among the 76 genes, 17 genes met the requirement for high expression levels and were chosen as representatives for each functional category (Table 2). Among the 17 genes, *IRF7* and *MX2* were also nominated for biomarkers of pertussis vaccine toxicity. Real-time PCR quantification results of the expression levels of the 17 genes were comparable to the relative expression ratios determined by DNA microarray analysis. We are now working to establish a rapid quantification system for these 17 biomarkers using the QuantiGene Plex assay.

6. Japanese Encephalitis Vaccines

Japanese encephalitis (JE) is a seasonal and sporadic encephalitis in East Asia caused by the JE virus. Vaccination is very important to prevent JE infection, because palliative care is the only treatment available for JE patients. Recently, a Vero cell-derived JE vaccine had been licensed in Japan as an alternative to the long-used mouse brain-derived JE vaccines. The newly developed Vero cell-derived vaccine should be at least equivalent to the mouse brain-derived vaccines, because the mouse brain-derived vaccines were considered generally safe and succeeded in the near elimination of JE in certain endemic regions. In this context, we performed DNA microarray analysis of tissues from rats administered with mouse brain-derived or Vero cell-derived JE vaccine and compared the gene expression profiles. As expected, the gene expression patterns in brain and liver were comparable between mouse brain-derived and Vero cell-derived vaccines, indicating that both vaccines possessed equivalent reactivity characteristics in rats [24].

7. Conclusions

Over recent decades, the safety control of vaccines has been assessed using several animal tests, including the body weight change test and white blood cell counts. However, conventional animal safety tests need to be improved in many aspects. For example, the number of test animals used needs to be reduced and the test period needs to be shortened. This requires the development of a new vaccine evaluation system. In this review, we showed that gene expression patterns were well correlated to the biological responsiveness of vaccinated animals. From the DNA microarray analysis of lungs from vaccinated rats, we identified 13 and 17 biomarkers to detect the toxicity of pertussis and influenza vaccines, respectively.

Furthermore, the QuantiGene Plex assay for gene expression analysis is being introduced. The QuantiGene Plex assay was revealed to be as accurate as real-time PCR and has the great benefit of being able to evaluate all biomarkers simultaneously. Using the QuantiGene Plex assay, we could rapidly and sensitively detect the gene expression changes that accompany biological reactivity in vaccinated rats.

Thus, it may be concluded that DNA microarray technology is an informative, rapid, and highly sensitive method with which to evaluate vaccine quality. Our data suggest that this new method has the potential to shorten the time for safety tests and can reduce the number of animals used. In addition, our test may contribute to the development of urgently required vaccines. Further analyses are required to confirm that gene expression changes correlate with vaccine quality.

In this review, we referred to our recent efforts of exploring new safety control methods using gene expression pattern indexes, focusing on pertussis and influenza vaccines. In the future, for the evaluation of all kinds of vaccines, microarray analysis is expected to play an important role in the new safety control test, especially for checking toxin-reactive transcripts.

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Original Article

Induction of Indistinguishable Gene Expression Patterns in Rats by Vero Cell-Derived and Mouse Brain-Derived Japanese Encephalitis Vaccines

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SUMMARY: Transcriptomics is an objective index that reflects the overall condition of cells or tissues, and transcriptome technology, such as DNA microarray analysis, is now being introduced for the quality control of medical products. In this study, we applied DNA microarray analysis to evaluate the character of Japanese encephalitis (JE) vaccines. When administered into rat peritoneum, Vero cell-derived and mouse brain-derived JE vaccines induced similar gene expression patterns in liver and brain. Body weights and blood biochemical findings were also similar after administration of the two vaccines. Our results suggest that the two JE vaccines are likely to have equivalent characteristics with regard to reactivity in rats.

INTRODUCTION

Japanese encephalitis (JE) is a seasonal and sporadic viral encephalitis in East Asia, caused by infection with the JE virus. The JE virus exists in a zoonotic cycle between mosquitoes and swine and/or water birds. Infectious mosquitoes transmit JE to humans, a dead-end host (1). The great majority of infections are not apparent; the incidence of JE is considered to be 1 case per 250 to 500 infections (2). Even if the disease becomes manifest, recovery from mild illness occurs in most cases. Severe infection can cause febrile headache syndrome, aseptic meningitis, or encephalitis after an incubation period of about 6 to 16 days (1). Once JE has developed, the fatality rate is relatively high, from 5 to 40%, depending on the outbreak. Permanent neurological or psychiatric sequelae are left in 45–70% of survivors (1–3). No specific treatments for JE are available; therefore, preventing virus infection with vaccination is the most effective form of defense.

The approved and widely used JE vaccine is manufactured from inactivated JE virus that has been propagated in mouse brain. This mouse brain-derived (MBD) vaccine is currently manufactured and used in Japan, Korea, Taiwan, Thailand, Vietnam, and India, and is licensed in the United States, Canada, Israel, Australia, and several other Asian countries. Vaccination has succeeded in the near elimination of JE in several countries.

The MBD JE vaccine is a very pure form; impurities are removed during the manufacturing process, especially brain-derived matter (3). Thus the vaccine has been considered safe. However, adverse reactions, such as local reactions and mild systemic events, may occur in 10–30% of vaccinated subjects (3). Acute disseminated encephalomyelitis (ADEM) coinciding with the administration of MBD vaccines has been reported at frequencies of 1 to 2 out of 100,000 doses (2,3). In the wake of a severe case of ADEM, the recommendation for a program of routine childhood immunization against JE was suspended in Japan in 2005 (2,4). It is of great concern that non-immunized children are not given the JE vaccine in JE-infected areas of Japan.

To replace the current MBD vaccine, Vero cell-derived (VCD) vaccines have been developed (5–10). The cessation of using mouse brain for virus propagation is expected to reduce the incidence of severe adverse reactions, including ADEM, because myelin basic protein, which is abundant in the central nervous system, is a possible substrate that provokes ADEM (11). Further, a cell culture-based technique is advantageous for large-scale production of JE vaccine. The demand for JE vaccine is growing, because the distribution of the JE virus has expanded throughout Asia and towards the northern edge of Australia over the last decade (12,13), and these newly JE virus-infected countries will require JE vaccine.

Apart from these concerns about the JE vaccine, moving towards cell culture-based vaccines is a global trend in the field of virus vaccine development (14). Primary hamster kidney cells were the first cells to be accepted for the production of JE vaccine, and continue to be used in China and some other countries (3,15). Recently, vaccine production using primary cell culture systems has been replaced by production using continuous cell lines (CCLs), including the Vero cell line (14). The Vero cell line is the most widely accepted CCL by regulatory authorities and has been used for over 30 years for the production of polio and rabies virus vaccines (16,17). In addition, VCD vaccines for rotavirus, smallpox

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virus, and influenza virus have been developed (14,18). In the case of JE vaccines, one of the developed VCD vaccines has received recent approval in the United States and Europe. Another was licensed in Japan in February 2009.

A newly licensed VCD JE vaccine must be at least equivalent to the current high-quality MBD vaccine in effectiveness. In this study, we applied conventional animal tests to demonstrate the equivalence of the MBD JE vaccine and the VCD JE vaccine. Further, based on our previous studies demonstrating that DNA microarray analysis was able to assay the features of a vaccine with high sensitivity, comprehensive gene expression analysis was performed to characterize the physiological reactivity of both JE vaccines.

MATERIALS AND METHODS

Animals: Eight-week-old male Wistar rats, weighing 160–200 g, were obtained from SLC (Tokyo, Japan). Animals were housed in rooms maintained at 23 ± 1°C, with 50 ± 10% relative humidity and 12-h light/dark cycles, for at least 1 week prior to the test challenge. All procedures used in this study complied with institutional policies of the Animal Care and Use Committee of the National Institute of Infectious Diseases.

Vaccines: The approved JE vaccine (MBD) is an inactivated, highly purified JE virus (Beijin-1 strain), propagated in mouse brain. The improved inactivated vaccine (VCD) is manufactured from the same strain in Vero cells. Both vaccines were generous gifts from Biken, The Research Foundation for Microbial Diseases of Osaka University, Japan. We administered 5 ml of MBD or VCD into rat peritoneum. Physiological saline (SA) was used as a control.

Weight check: The rat decreasing body weight test was performed according to the Minimum Requirements for Biological Products in Japan (19). After we injected 5 ml of samples into the peritoneum, animals were weighed daily. Five rats in each group were used.

Hematological test: Rats were treated with SA, MBD, or VCD, and blood samples were collected on days 1, 2, 3, and 4 after administration. Blood was immediately mixed with EDTA, and the number of erythrocytes, hematocrit level, hemoglobin value, number of leukocytes, and number of platelets (PLT) were determined using an automatic hematocytometer, the Celltac MEK-5254 (Nihon Kohden, Tokyo, Japan). Five rats in each group were used.

Serum test: Blood samples for the serum test were collected separately from the same rats used for the hematological test. After centrifugation at 3,000 rpm for 15 min, 10-fold diluted supernatants were used for subsequent tests. We measured the activity of glutamate oxaloacetate transaminase/aspartate aminotransferase (GOT/AST), glutamate pyruvate transaminase/alanine aminotransferase (GPT/ALT), alkaline phosphatase (ALP), amylase (AMYL), and creatine phosphokinase (CPK), and the quantity of blood urea nitrogen (BUN), creatinine (CRE), total cholesterol (TCHO), triglyceride (TG), glucose (GLU), and C-reactive protein (CRP) using a DRICHEM-3030 according to the manufacturer's instructions (Fujifilm, Japan). Five rats in each group were used.

RNA preparation: Rats were sacrificed to obtain the whole brain and the lateral left lobe of the liver. Tissues were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and mixed with 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, Tex., USA) according to the manufacturer's instructions.

Microarray preparation and expression profile acquisition: For microarray analysis, rats were treated with SA, MBD, or VCD (3 rats per treatment), and 2 tissue samples from each animal, brain and liver, were analyzed on days 1–4 post-treatment. A set of synthetic polynucleotides (80-mers) representing 11,468 rat transcripts and including most of the RefSeq genes 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 (20,21). Poly(A)$^+$ RNA (1.5 μg) of each sample was labeled using SuperScript II (Invitrogen, Carlsbad, Calif., USA) with Cyanine 5-dUTP. A common reference RNA (MicroDiagnostic) was labeled with Cyanine 3-dUTP (PerkinElmer, Boston, Mass., USA). Labeling, hybridization, and washes of microarrays were performed with a Labeling & Hybridization Kit (MicroDiagnostic) according to the manufacturer's instructions. The common reference RNA was purchased as a single batch and was labeled 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, Union City, Calif., USA) 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]). The GenePix Pro 3.0 software performed normalization for the median of ratios (primary expression ratios) by multiplying normalization factors calculated for each feature on a microarray.

Data analysis: Data processing and hierarchical cluster analysis were performed using Excel (Microsoft, Redmond, Wash., USA) and an 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 1 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 extracted genes by t test between SA- and MBD-, SA- and VCD-, or MBD- and VCD-treated samples ($P < 0.01$).

RESULTS

Vaccine-treated animals showed no weight loss: Vaccines for public use are all made according to Good Manufacturing Practice (GMP), and many tests must be done before releasing vaccines to assure their quality. Conventional animal tests including the decreasing body weight test are applied for the quality control of vaccines (19). To explore the effects of the JE vaccines in a conventional method, we first applied the decreasing body weight test to the MBD and VCD JE vaccines, as described in Minimum Requirements for Biological Products in Japan (19). For this test, 5 ml of the vaccine was injected into the rat peritoneum, and the weight of the treated rats was measured daily for 4 days. As shown in Fig. 1, VCD-treated rats (filled circles) did not show

any weight loss, and gained weight in a similar manner to that of the SA- and MBD-treated groups (open and gray squares, respectively). Further, no abnormalities were observed in the condition or behavior of the rats during the testing period. Severe toxicity of MBD and VCD was not detected from this test.

Hematological tests revealed no significant changes in vaccinated rats: To investigate the influence of JE vaccines on hematological parameters, we treated rats with SA, MBD, or VCD (5 rats per treatment) and collected blood samples on days 1, 2, 3, and 4 after administration. We counted erythrocytes, leukocytes, and PLT and measured hematocrit levels and hemoglobin values. At any time point, all characteristics examined were within normal ranges and showed no significant differences among SA-, MBD-, and VCD-treated groups (Fig. 2). These results indicated that neither MBD nor VCD exhibited hematotoxicity to the treated rats.

Normal levels were observed in serum tests in vaccine-treated rats: To evaluate the reactivity of JE vaccines on biological functions, we performed serum tests on vaccine-administered rats. On days 1, 2, 3, and 4 after administration of SA, MBD, or VCD, we collected blood from the same rats used for hematological tests, and isolated serum. Each serum sample was tested for liver function, renal function, muscle dysfunction, and metabolic abnormalities. No significant increase was observed in GOT/AST, GPT/ALT, ALP, or AMYL in any samples tested, indicating that no liver damage had occurred (Fig. 3 top panels). CRP values were all below detection limits (data not shown). Tests of renal (BUN and CRE) and muscle (CPK) function and of metabolism (TCHO, TG, and GLU) showed no differences among the vaccine-treated groups (Fig. 3 middle and bottom panels). These results suggested that SA, MBD, and VCD had similar biological reactivity in rats.

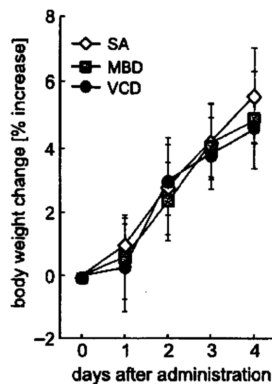

Fig. 1. Body weight analysis of the JE vaccine treated animals. The effects of mouse brain-derived (MBD) JE vaccine, Vero cell-derived (VCD) JE vaccine, and saline (SA) treatment were measured using decreasing body weight toxicity tests. All rats were weighed at days 0, 1, 2, 3, and 4. Changes in rat body weight were assessed as the percentage increase or decrease, and are indicated by the mean change ± S.D.

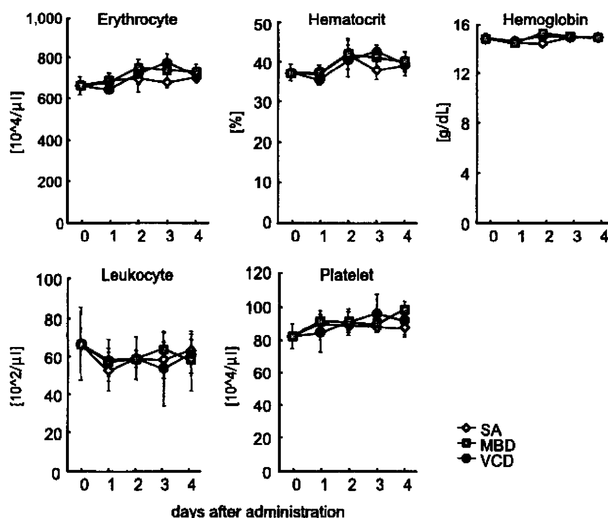

Fig. 2. Hematological tests for vaccinated rats. Blood obtained from individual rats was subjected to hematological tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean ± S.D.

Microarray analysis of tissues from vaccine-treated rats: Although the animal tests described above have long been accepted for the quality control of biological reagents (22–24), the progress of molecular biotechnology presents the possibility to improve or renew the traditional tests. Among recent technologies, the high-throughput 'omics'-based technologies have led the way to clarify immune responses to pathogens and responses of metabolic pathways, as well as to develop new vaccine candidates (25–27). Now, several efforts have been made to analyze the side effects of pharmaceuticals using one of the 'omics' technologies, transcriptomics (28,29). In this context, we performed DNA microarray analysis of the vaccinated rat tissues, liver and brain, and tried to determine the effects of MBD and VCD by analyzing gene expression patterns. The liver is thought to be one of the most appropriate organs to analyze biological alterations due to vaccination, because it is the major organ of metabolism. The brain was taken as another target tissue because a neurological effect can be one of the side effects of JE vaccination.

For the analysis, SA-, MBD-, and VCD-treated rats (3 rats per group) were sacrificed to obtain the liver and brain on days 1, 2, 3, and 4 post-administration. Thirty-six samples from each tissue type were obtained. Poly(A)$^+$ RNA purified from the samples and a rat common reference RNA were labeled with Cyanine-5 and Cyanine-3, respectively, and hybridized to microarrays representing 11,468 transcripts. Hybridization signals were processed into expression ratios as $\log_2$ values (designated log ratios) and compiled into a matrix designated as the primary data matrix (see Materials and Methods).

To predict the most obvious differences obtained from the cluster analysis, we extracted genes with log ratios over 1 or under −1 in at least 1 sample in each group. Eventually, 2,386 genes for liver and 4,075 genes for brain were extracted and subjected to two-dimensional hierarchical cluster analysis for samples and genes (Fig. 4A). With hierarchical cluster analysis, genes were grouped according to expression patterns; thus samples having a similar gene expression pattern were clustered together, and samples having a distinct gene expression pattern formed a separate cluster (Fig. 4A) (30–32). If all test samples showed similar gene expression patterns, no clear clusters were formed. Thus, whether distinct clusters were formed was the criterion for the assessment of whether treatment with the 2 vaccines induced different gene expression patterns. Each column represents a sample. Each row represents a gene, and gene expression values are typically illustrated by a colored rectangle, red for up-regulation, blue for down-regulation, and yellow for no change. As shown, no

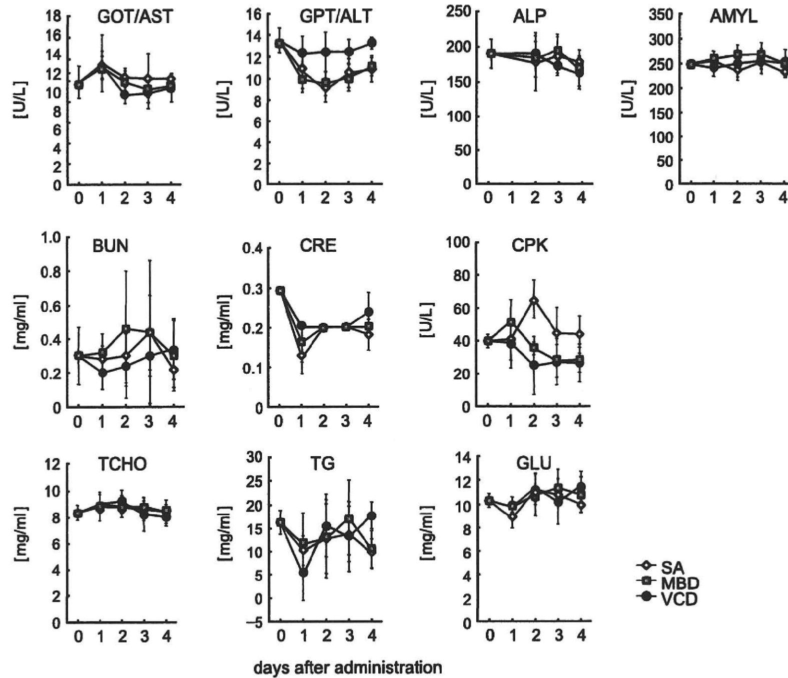

Fig. 3. Serum tests for vaccinated rats. Serum was separated from blood obtained from individual rats, and subjected to serum tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean ± S.D.

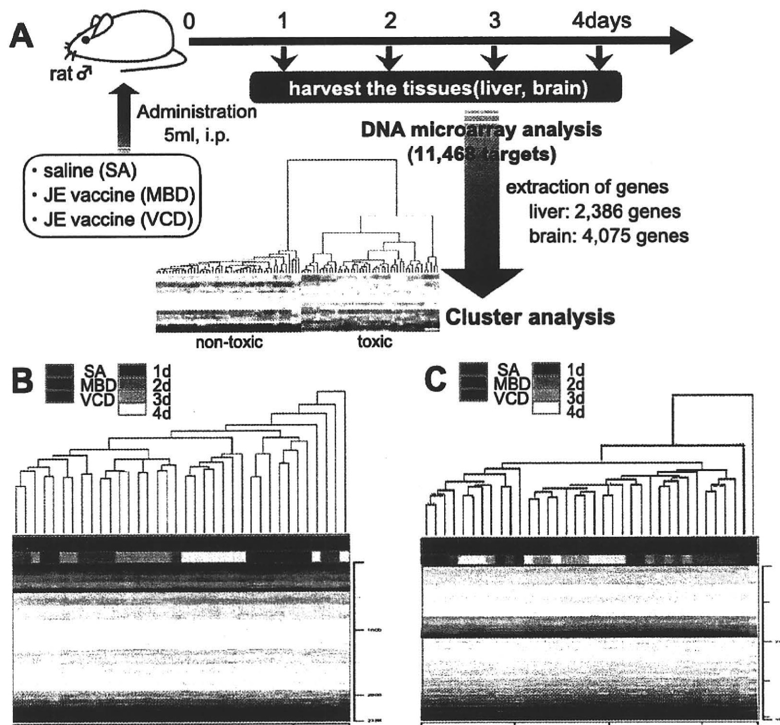

Fig. 4. Gene expression profiling and cluster analysis of vaccine-treated rat tissues. The procedure for gene expression analysis is outlined in A. For the cluster analysis, 2,386 genes for liver (B) and 4,075 genes for brain (C) were extracted from 11,468 targets and assembled in the order obtained from the results of the two-dimensional hierarchical cluster analysis. The results were drawn as a dendrogram based on the similarities of gene expression patterns of each sample. The y-axis of the dendrogram shown in (B) and (C) depicts the Euclid square distance as the dissimilarity coefficient, indicating the relationship between the samples. Red and blue indicate increases and decreases in the expression ratio, respectively.

clear clusters, corresponding to distinguishable gene expression patterns, were apparent, either in liver (Fig. 4B) or in brain (Fig. 4C). Gene expression patterns were very similar in all vaccine-treated samples.

Further, we tried to identify specific genes whose expression levels were changed following JE vaccine treatment.

However, no genes could be selected from the comparison between MBD- and VCD-treated groups. MBD and VCD treatment could not be distinguished by gene expression analyses, indicating equivalent characteristics of MBD and VCD.

DISCUSSION

Comprehensive gene expression analysis is now an established approach to analyzing the effects of any manipulation on the whole transcriptome of living organisms. The genomic data associated with drug responses are expected to aid in the analysis of inter-individual variability and the tailoring of the administration of drugs to individuals to achieve maximal efficacy and minimum risk. The US Food and Drug Administration (FDA) now encourages voluntary genomic data submissions to the agency as part of new drug applications and biologics licensing applications (33). In this context, we have been trying to introduce DNA microarray analysis to the conventional quality control tests of the pertussis and influenza vaccines. The results of DNA microarray analysis correlated well with the results of conventional animal tests, and toxicity-related biomarkers were successfully extracted from the analysis (30–32). In the present study, we further applied this DNA microarray technology to analyze the biological reactivity of the JE vaccines (MBD and VCD). In liver and brain, the overall gene expression patterns were similar between MBD- and VCD-treated rats (Fig. 4), which was in accordance with the results obtained from the decreasing body weight test (Fig. 1) or the blood and serum tests (Figs. 2 and 3).

ADEM, an adverse reaction associated with JE vaccination, is thought to be a monophasic autoimmune disorder of the central nervous system, typically following a febrile infection or a vaccination (34). The precise mechanisms of ADEM have not been fully elucidated; however, recent studies suggested the involvement of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and chemokines (35–37). Further, several genes associated with inflammation or immune responses, including Irf7, were up-regulated in JE virus-infected mouse brains (38,39). Therefore, inflammation above certain levels may be associated with adverse reactions to vaccines, that is, inflammation-related genes could be markers to detect contaminating toxicity that can cause adverse reactions. However, we found no significant changes in the expression levels of inflammatory genes between MBD- and VCD-treated rat tissues. We showed by using animal tests and comprehensive gene expression analysis that the two Japanese encephalitis vaccines, the existing MBD and the improved VCD vaccines, seemed to possess identical biological reactivity in rats.

To address concern about the reliability of the genomic data obtained from DNA microarray analysis, the FDA recently launched the MicroArray Quality Control (MAQC) project in anticipation of the regulatory submission of pharmacoinformatic and toxicoinformatic data in applications or supplements (33). The results of the MAQC project, showing interplatform reproducibility, were reported in 2006 (40–45). Subsequently, the follow-up MAQC-II project is progressing towards the development and the validation of genomic data in clinical applications. Similarly, in Japan, the Japan MicroArray Consortium (JMAC) for the standardization and the international harmonization of microarray platforms is ongoing and is coordinated with the FDA and the European Medical Agency (EMEA) (46). The efforts to achieve array platforms for the practical application of genomic data are being accelerated on a worldwide scale.

Although our experiments were limited with regard to the number of animals and vaccines examined, our DNA microarray technology was previously shown to be reproducible (30,32). The genomic data obtained in this study is, we believe, reliable. Recently, the VCD JE vaccine was licensed in Japan. It is desirable to accumulate gene expression profiles, especially data documenting the dynamics of inflammatory cytokines, in addition to generating animal testing data to enable a more reliable evaluation of the new JE vaccine.

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Applicability of bacterial endotoxins test to various blood products by the use of endotoxin-specific lysates

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ABSTRACT

Endotoxin contamination is a serious threat to the safety of parenteral drugs, and the rabbit pyrogen test has played a crucial role in controlling this contamination. Although the highly sensitive endotoxin test has replaced the pyrogen test for various pharmaceuticals, the pyrogen test is still implemented as the control test for most blood products in Japan. We examined the applicability of the endotoxin test to blood products for reliable detection and quantification of endotoxin. Nineteen types of blood products were tested for interfering factors based on spike/recovery of endotoxin by using 2 types of endotoxin-specific lysate reagents for photometric techniques. Interfering effects on the endotoxin test by the products could be eliminated by diluting from 1/2 to 1/16, with the exception of antithrombin III. However, conventional lysate reagents that also react with non-pyrogenic substances, such as (1–3)-β-D-glucan, produced results that were not relevant to endotoxin content or pyrogenicity. Our results showed that the endotoxin test would be applicable to most blood products if used with appropriate endotoxin-specific lysate reagents.

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1. Introduction

Lipopolysaccharide, which is also referred to as endotoxin, is a cell wall component of gram-negative bacteria, and is known to have various biological activities. Even small amounts of endotoxin may cause adverse physiological effects such as febrile reactions in humans [1,2]. Therefore, contamination with endotoxin is a serious threat to the safety of parenteral pharmaceuticals. For decades, the rabbit pyrogen test has played a key role in controlling the pyrogenicity of various drugs [3–5]. However, the rabbit pyrogen test has limited sensitivity and accuracy compared to the endotoxin test and requires a large number of rabbits.

The bacterial endotoxin test was first developed by Levin and Bang [6,7], and is based on highly sensitive clotting of *Limulus* amoebocyte lysate in the presence of endotoxin. Methods and lysate reagents for the test have been remarkably improved to allow its practical application to a wide range of pharmaceuticals. In particular, the specificity of the test to detect endotoxin was improved by the removal or suppression of factor G [8] in lysate reagents, which eliminated the reactivity to (1–3)-β-D-glucan and other non-pyrogenic substances [9,10]. This markedly improved its applicability to biological products by allowing pyrogenicity-based validation.

The endotoxin test in place of the pyrogen test for end products of parenteral drugs was approved in the early 1980s in the United States [3,11], and other nations have followed [4,5]. Application of the endotoxin test to antibiotics as a substitute for the pyrogen test began in 1995, and was followed by its application to a wider range

Abbreviations: APC, Human activated protein C concentrate; AT-III, Human antithrombin III concentrate; EU, Endotoxin units; FIB, Human fibrinogen; HAP, Human haptoglobin; HBs, Hepatitis B; IG, Normal human immunoglobulin; IG-D, Human anti-D(Rho) immunoglobulin; IG-H, Human anti-HBs immunoglobulin; IG-His, Normal human immunoglobulin with histamine; IG-ION, Ion-exchange-resin-treated normal human immunoglobulin; IG-PEG, Polyethylene glycol-treated normal human immunoglobulin; IG-PEG-H, Polyethylene glycol-treated human anti-HBs immunoglobulin; IG-PEG-T, Polyethylene glycol-treated human anti-tetanus immunoglobulin; IG-PEP, Pepsin-treated normal human immunoglobulin; IG-PH4, pH 4-treated normal human immunoglobulin; IG-PH4A, pH 4-treated normal human immunoglobulin (acidic); IG-SUL, Sulfonated normal human immunoglobulin; IG-T, Human anti-tetanus immunoglobulin; IM-IG, Intramuscular-immunoglobulin products; IV-IG, Intravenous-immunoglobulin products; IX, Concentrated human blood coagulation factor IX; JP, The Japanese Pharmacopoeia; JPRSE, Japanese Pharmacopoeia reference standard endotoxin; MR, Minimum Requirements for Biological Products; VIII, Concentrated human blood coagulation factor VIII.

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of antibiotics in 1998 in Japan [12]. The application of the endotoxin test to biological products also began in 1993, but has been limited to interferon injections and 2 types of blood products, namely, human plasma protein fraction (PPF) [14] and human serum albumin (HSA) [15]. The pyrogen test is still implemented as the control test for the majority of blood products in Minimum Requirements for Biological Products in Japan (Japanese-MR) [13]. In the present study, we examined the applicability of the endotoxin test to various blood products other than PPF and HSA. We present the necessity of using endotoxin-specific lysate reagents for reliable endotoxin testing of blood products and then results of the test for interfering factors based on spike/recovery of endotoxin by using 2 types of endotoxin-specific lysate reagents.

2. Materials and methods

2.1. Blood products

Nineteen types of plasma derivatives listed in Table 1 were used in this study. The blood products were kindly provided by Benesis Corp. (Osaka, Japan), CSL Behring K. K. (Tokyo, Japan), Japanese Red Cross Society (Tokyo, Japan), Kaketsuken, (Kumamoto, Japan) and Baxter Ltd. (Tokyo, Japan). IG-PEG, IG-PEG-H, IG-PEG-T, IG-PH4A, IG-PH4, IG-ION, IG-SUL and IG-PEP were categorised as intravenous-immunoglobulin products (IV-IG), and IG, IG-H, IG-T, IG-D and IG-His were categorised as intramuscular-immunoglobulin products (IM-IG).

Table 1
List of blood products.

Type	Products	Abbreviation	MVD[a]	
			ES-III	Endospecy
IV-IG	Polyethylene glycol-treated normal human immunoglobulin	IG-PEG	16	64
	Polyethylene glycol-treated human anti-HBs immunoglobulin	IG-PEG-H	53	213
	Polyethylene glycol-treated human anti-tetanus immunoglobulin	IG-PEG-T	53	213
	pH 4-treated normal human immunoglobulin (acidic)	IG-PH4A	16	64
	pH 4-treated normal human immunoglobulin	IG-PH4	16	64
	Ion-exchange-resin-treated normal human immunoglobulin	IG-ION	16	64
	Sulfonated normal human immunoglobulin	IG-SUL	16	64
	Pepsin-treated normal human immunoglobulin	IG-PEP	16	64
IM-IG	Normal human immunoglobulin	IG	80	320
	Human anti-HBs immunoglobulin	IG-H	80	320
	Human anti-tetanus immunoglobulin	IG-T	80	320
	Human anti-D(Rho) immunoglobulin	IG-D	80	320
	Normal human immunoglobulin with histamine	IG-His	80	320
Other	Human haptoglobin	HAP	32	128
	Concentrated human blood coagulation factor VIII	VIII	80	320
	Concentrated human blood coagulation factor IX	IX	80	320
	Human fibrinogen	FIB	32	128
	Human antithrombin III concentrate	AT-III	53	213
	Human activated protein C concentrate	APC	53	213

[a] Maximum valid dilution to determine the endotoxin limit.

2.2. Reference standard endotoxin

Japanese Pharmacopoeia reference standard endotoxin (JPRSE) lot 3 derived from *Escherichia coli* UKT-B strain (13,000 endotoxin units (EU)/vial) [16] was used throughout the study. A vial of JPRSE was reconstituted with 1.3 mL of pyrogen-free distilled water (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) to prepare a standard endotoxin stock solution of 10,000 EU/mL and kept at 4 °C for use within 14 days of the reconstitution. Pyrogen-free distilled water was used to dilute the standard endotoxin stock solution and test samples throughout the study.

2.3. Lysate reagents

We employed 5 commercially available lysate reagents in this study. Two of these reagents were endotoxin-specific reagents from which factor G was suppressed or removed: Limulus ES-III Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for the kinetic–turbidimetric technique and Endospecy (Seikagaku Biobusiness Corporation, Tokyo, Japan) for the kinetic–chromogenic technique. Tests for interfering factors based on spike/recovery of endotoxin were carried out using these endotoxin-specific lysate reagents. Two reagents were conventional lysate reagents containing both factor C and factor G, not specific for endotoxin: Kinetic-QCL (Lonza Japan, Tokyo, Japan) and Toxicolor (Seikagaku Biobusiness Corporation) both for the kinetic–chromogenic technique. Fungitec G test MK (Seikagaku Biobusiness Corporation), for the kinetic–chromogenic technique, is a (1–3)-β-D-glucan-specific lysate reagent and was used to assess contents of reactive substances other than endotoxin.

2.4. The bacterial endotoxin test

Bacterial endotoxins test was carried out according to Japanese-MR. In brief, JPRSE stock solution was diluted to make 5 appropriate serial dilutions at two-fold intervals as shown in Table 2. A test sample and the sample spiked with an appropriate concentration of JPRSE were diluted to make 4 serial dilutions at two-fold intervals, unless stated otherwise (Table 2). A 100 or 50 μL portion of each dilution was mixed with the same volume of a lysate reagent according to the manufacturer's instructions. The reaction mixtures were incubated at 37 °C. The time to reach a designated turbidity (ES-III) or a designated absorbance (Kinetic-QCL), or the rate of colour development (Endospecy, Toxicolor and Fungitec G test MK) of the reaction mixtures was measured by appropriate equipments

Table 2
Dilution procedures for JPRSE and test sample.

	Solution	ES-III		Endospecy	
		Dilution factor	Concentration of added endotoxin after dilution	Dilution factor	Concentration of added endotoxin after dilution
A	JPRSE	1	1	1	0.25
		2	0.5	2	0.125
		4	0.25	4	0.0625
		8	0.125	8	0.03125
		16	0.0625	16	0.015625
		32	0.03125	32	0.0078125
B	Test sample	2	–	4	–
		4	–	8	–
		8	–	16	–
		16	–	32	–
C	Test sample + endotoxin	2	0.5	4	0.25
		4	0.25	8	0.125
		8	0.125	16	0.0625
		16	0.0625	32	0.03125

listed in Table 3. Endotoxin content in a test sample was calculated with respect to JPRSE by the parallel line assay method using logarithmically transformed values of dose and reactions (time or rate of colour development), with the exception of double-logarithmically transformed time for ES-III.

2.5. Maximum valid dilution (MVD)

MVD is the maximum allowable dilution of a sample solution at which the endotoxin limit can be determined. The MVD was calculated by the following equation according to the Japanese Pharmacopoeia (JP) [5], and is listed in Table 1.

$$\mathrm{MVD} = \frac{\text{Endotoxin limit} \times \text{Concentration of sample solution}}{\lambda}$$

Endotoxin limit: The endotoxin limit for injections (5.0 EU/kg), Concentration of sample solution: Units/mL or mL/mL, λ: The lowest point (EU/mL) used in the standard regression curve of the turbidimetric (ES-III, 0.03125 EU/mL) or chromogenic (Endospecy, 0.0078125 EU/mL) technique.

2.6. The pyrogen test

The pyrogen test was carried out according to Japanese-MR. In brief, female rabbits of Japanese white strain (Kitayama Labes Co., Ltd., Nagano, Japan, or Japan Laboratory Animals, Inc., Tokyo, Japan) weighing approximately 2–3 kg were housed in separate cages in an air-conditioned animal room. Three rabbits were allocated for each treatment and were intravenously injected with a specified volume of a test sample per kg body weight, according to Japanese-MR. The rectal temperature of each rabbit was monitored for 3 h by using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa, Japan). The highest rectal temperature recorded for a rabbit during a period of 3 h after the injection was regarded as the maximum temperature. The response of the rabbit was calculated as the temperature rise between the maximum temperature and the initial temperature prior to the injection. When the temperature rise was negative, the response was interpreted as zero. The pyrogenicity of a sample was expressed as the mean response of 3 rabbits.

2.7. Statistical analysis

Analyses of the parallel line assays were performed using Finney's method [17].

3. Results

3.1. Comparison of lysate reagents

Factor G-reactive substances in various blood products were assessed using 2 types of endotoxin-specific lysate reagents (ES-III and Endospecy), 2 conventional lysate reagents (Toxicolor and Kinetic-QCL) and a glucan-specific lysate reagent (Fungitec G test MK). The content of factor G-reactive substances in test samples was calculated with respect to the Reference Standard (1–3)-β-D-glucan (Fungitec G test MK Reference Standard, Seikagaku Biobusiness Corporation) by the parallel line assay method using logarithmically transformed values of dose and rate of colour development. Results using one batch each of 17 types of blood product are shown in Table 4. No significant level of reaction was detected for any of the samples when measured by the endotoxin-specific lysate reagents. However, positive reactions were observed in several types of sample when tested by the conventional lysate reagents and the glucan-specific reagent. Although strong positive responses ranging from 38 EU/mL to 336 EU/mL were detected for 3 types of blood product by Kinetic-QCL, these samples did not cause any pyrogenic reaction in rabbits, suggesting that the positive reactions to the lysate reagents were not attributable to endotoxin. The differences in EU/mL value by Toxicolor (conventional) and by Endospecy (endotoxin-specific) were calculated and compared with (1–3)-β-D-glucan content by Fungitec G test MK to evaluate if the differences could be explained by (1–3)-β-D-glucan content. This study had the advantage for such analysis, because 3 of the lysate reagents were produced by a single manufacturer, and the rate of colour development for all the 3 reagents was measured by the same equipment. Significant correlation ($P = 0.00026$) was found between these differences and (1–3)-β-D-glucan content (Fig. 1). These results suggest that the test using conventional lysate reagents would be affected by the presence of factor G-activating substances in the products for quantification of endotoxin. Therefore, endotoxin-specific lysate reagents were employed in the endotoxin test to assay for the presence of interfering factors in blood products.

3.2. Test for interfering factors

Nineteen types of blood products listed in Table 1 were tested to examine the presence of enhancing or inhibiting (interfering) factors for the reaction based on spike/recovery of endotoxin. JPRSE and test samples were prepared according to Table 2 and measured using endotoxin-specific lysate reagents by kinetic–turbidimetric (ES-III) and kinetic–chromogenic (Endospecy) techniques. Examples of dose-response regression lines of JPRSE and a test sample spiked with endotoxin are depicted in Fig. 2. When significant deviations from linearity and/or parallelism were detected, the dilutions deviated from linearity and/or parallelism were not used for calculation as shown in Fig. 2A. The endotoxin content of test samples was calculated with respect to JPRSE by the parallel line assay method insofar as validity of the assay was ensured, whereas endotoxin content in each dilution of test samples spiked with endotoxin was calculated using the response of each dose in reference to JPRSE under the assumption that the response was on a dose-response line parallel to that of JPRSE. Recovery of endotoxin added to a test sample was calculated by subtracting the endotoxin content found in the sample (solution B) from the content in the sample spiked with endotoxin (solution C) and was shown in Tables 5 and 6. A value lower than 0.5 in solution C indicated that the recovery of spiked endotoxin was less than 50%, and dilutions showing such results were considered invalid (underlined values in the Tables). When IG, IG-H, XIII and IX products from more than one manufacturer were tested, the observed inhibition intensities in each product were homogeneous regardless of the manufacturer.

The inhibitory effect on the test was reduced by diluting test samples, and valid endotoxin recoveries within the range of 50%–200% for the test using ES-III were achieved by making dilutions of 1/2 to 1/8, with the exceptions of IG and AT-III. However, slight inhibition persisted in some samples even at these dilutions, which may hamper to attain parallel dose-response regression lines of the samples and JPRSE. Because these slight inhibitory

Table 3
Lysate reagents and equipments.

Lysate reagent	Equipment	Manufacturer
ES-III	Toxinometer ET-301	Wako Pure Chemical Industries, Ltd.
Endospecy	Wellreader SK603	Seikagaku Biobusiness Corporation
Toxicolor		
Fungitec G test MK		
Kinetic-QCL	ELx808	Lonza Japan

Table 4
Reactions of blood products to various lysate reagents.

Products	EU/mL				pg/mL	Difference[b]	Pyrogen test
	ES-III	Endospecy	Toxicolor	Kinetic-QCL	β-Glucan[a]		
IG-PEG	0.06	0.01	0.03	0.05	162.5	0.02	Negative
IG-PEG-H	0.02	0.01	0.02	0.02	79.0	0.01	Negative
IG-PEG-T	0.03	0.01	0.20	0.02	43.2	0.18	Negative
IG-PH4A	0.06	0.02	0.05	0.09	114.9	0.03	Negative
IG-PH4	0.02	0.01	0.01	0.02	4.0	0.00	Negative
IG-ION	0.04	0.02	0.13	0.17	168.8	0.11	Negative
IG-SUL	0.03	0.01	0.01	0.02	2.5	0.00	Negative
IG	0.06	0.05	0.19	38.52	153.8	0.14	Negative
IG-H	0.05	0.02	0.08	0.20	82.3	0.06	Negative
IG-T	0.05	0.07	2.66	336.85	3936.0	2.59	Negative
IG-H (Freeze-dried)	0.03	0.01	0.09	0.10	308.1	0.07	Negative
IG-T (Freeze-dried)	0.03	0.03	0.04	195.31	56.2	0.01	Negative
IG-D	0.05	0.01	0.11	0.08	334.4	0.10	Negative
HAP	0.01	0.01	0.01	0.18	6.2	0.00	Negative
VIII	0.01	0.01	0.01	0.02	12.7	0.00	Negative
IX	0.01	0.02	0.11	0.02	907.6	0.09	Negative
FIB	0.02	0.04	0.05	0.23	26.6	0.01	Negative

[a] Contents of factor G-reactive substances measured by Fungitec G test MK and expressed in terms of pg of β-glucan.
[b] Difference of EU/mL value by Endospecy from that by Toxicolor.

effects depended on the concentration of the samples, greater dilutions for these samples would be required to attain parallel regression lines to that of JPRSE. No parallelism of regression lines of endotoxin-spiked samples to that of JPRSE was observed due to the dose-dependent inhibitory effect that persisted in some IM-IGs, such as IG, IG-H and IG-His, within the dilution range from 1/2 to 1/16. Therefore, we performed further testing using ES-III with the IM-IGs and the sample spiked with endotoxin at greater dilutions (1/8 to 1/64, at two-fold intervals). Parallelism of the regression lines to JPRSE could be achieved by diluting the IM-IGs to 1/16 or 1/32 (Table 7).

In contrast, Endospecy was not shown a significant inhibiting factor for all the blood products tested at a 1/4 dilution, and parallelism of regression lines of the endotoxin-spiked samples to that of JPRSE was attained by diluting to 1/4 or 1/8 (Table 6), with the exception of AT-III.

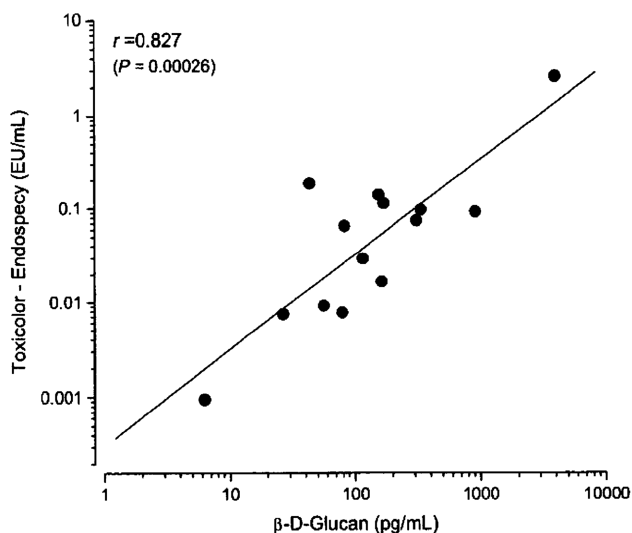

Fig. 1. Relationship of the results from 3 types of lysate reagents. The difference between EU/mL value by conventional reagent (Toxicolor) and that by endotoxin-specific reagent (Endospecy) was calculated and compared with (1–3)-β-D-glucan content by Fungitec G test MK. Each point shows this difference and the (1–3)-β-D-glucan content of each blood product.

AT-III markedly interfered with the endotoxin test using both ES-III and Endospecy, as shown in Tables 5 and 6. Therefore, dilution factors eliminating this inhibitory effect of AT-III were examined to gain a 50% recovery of spiked endotoxin for each of the lysate reagents. AT-III was serially diluted at two-fold intervals, and an equal volume of endotoxin solution was added to the AT-III dilutions at a final concentration of 0.125 EU/mL for ES-III or 0.0625 EU/mL for Endospecy. Recoveries of endotoxin added to each of the AT-III dilutions were calculated and at least diluting AT-III to 1/64 and 1/256 was required for valid tests (greater than 50% recovery of spiked endotoxin) using ES-III and Endospecy, respectively.

4. Discussion

The pyrogen test has played a crucial role in controlling endotoxin contamination in parenteral pharmaceuticals. However, the test has limited accuracy and sensitivity and is criticized for requiring a large number of animals. The endotoxin test is highly sensitive and accurate in detecting endotoxin and has been applied to antibiotic products [5,12,18], vaccines [4,13,19,20], and blood products [13–15]. Despite this, the pyrogen test is still applied to most of the blood products in Japan, and application of the endotoxin test is limited only to PPF and HSA [13]. Therefore, we examined the applicability of the endotoxin test to various other plasma derivatives for updating Japanese-MR (Table 1).

Various lysate reagents and test methods are currently available for the endotoxin test. However, conventional lysate reagents generally contain factor G, which is a protease zymogen. Factor G is autocatalytically activated by (1–3)-β-D-glucan or other non-pyrogenic substances, including components of a hollow-fibre (Cuprophan) hemodialyzer, and cellulosic materials from certain cellulose-based filters used in the production process of pharmaceuticals [21,22]. The activated factor G directly activates proclotting enzyme, which causes a positive reaction in the endotoxin test [23]. Therefore, conventional lysate reagents react with (1–3)-β-D-glucan in addition to endotoxin [8]. The positive reaction to substances other than endotoxin may lead to a conflicting result for endotoxin detection, making pyrogenicity-based validation difficult. When 17 types of blood product were tested using endotoxin-specific lysate reagents, none of them showed any detectable positive reaction. However, some of them clearly showed a positive reaction when

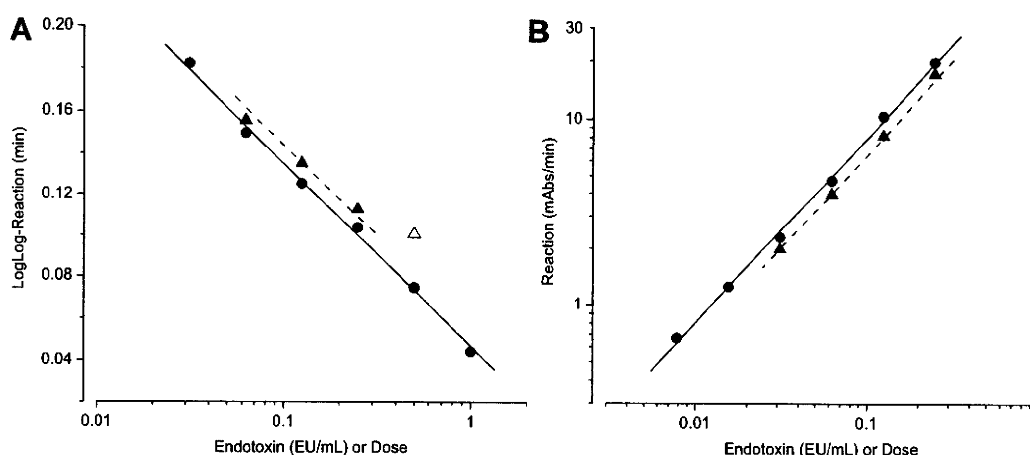

Fig. 2. Dose-response regression lines of JPRSE solution (●) and a test sample (▲) spiked with 1.0 EU/mL of endotoxin by kinetic–turbidimetric technique (ES-III) (A) and kinetic–chromogenic technique (Endospecy) (B). Double logarithmic transformation of time to reach a predetermined turbidity for ES-III and logarithmic transformation of rate of colour development for Endospecy were attained to achieve the linearity and parallelism of regression lines and allowed the application of parallel line assay. The dilution of the test sample (△) was excluded for analysis because of significant deviation from linearity and parallelism due to dose-dependent inhibitory effect.

tested using conventional lysate reagents or a glucan-specific lysate reagent. Although positive reactions ranging from 38 to 336 EU/mL were observed for 3 types of blood product using Kinetic-QCL, none of them caused a febrile response in the pyrogen test. The pyrogen test results were consistent with the endotoxin-specific reagent test results, suggesting that the positive reactions observed in these samples by conventional lysate reagents were attributable to activating factor G and were not relevant to the presence of endotoxin. Indeed, there was a significant correlation between the contents of factor G-reactive substances measured by the glucan-specific lysate reagent and the differences between EU/mL value by Toxicolor (conventional) and by Endospecy (endotoxin-specific) (Fig. 1). These results suggest that the use of conventional lysate reagents would not be appropriate because the presence of factor

Table 5
Recovery of endotoxin added to blood products using ES-III.

Products	Number of batches	Endotoxin content (EU/mL)						Recovery (%)
		Solution B	Solution C (x dilution factor)					
			All [a]	x 16	x 8	x 4	x 2	
IG-PEG	3	0.062	0.930	0.978	0.890	0.614[d]	0.238[d]	86.9
IG-PEG-H	3	0.013	0.857	0.962	0.835	0.893	0.696[d]	84.4
IG-PEG-T	3	0.035	0.929	0.977	0.918	0.795[d]	0.525[d]	89.1
IG-PH4A	3	0.066	0.901	0.998	0.824	0.463[d]	0.042[d]	83.3
IG-ION	3	0.026	0.781	0.911	0.745	0.681	0.501[d]	75.4
IG-PH4	3	0.041	0.856	0.904	0.816	0.610[d]	0.424[d]	81.5
IG-SUL	3	0.036	0.715	0.790	0.690	0.587[d]	0.509[d]	67.9
IG-PEP	1	0.016	0.644	0.764	0.595	0.610	0.425[d]	62.7
IG[b]	3	0.064	0.553	0.591	0.495[e]	0.360[d]	0.234[d]	48.5
IG[b]	2	0.038	0.646	0.679	0.619	0.426[d]	0.265[d]	60.7
IG-H[b]	3	0.063	0.613	0.629	0.508[d]	0.385[d]	0.248[d]	54.8
IG-H[b]	2	0.077	0.820	0.820	0.630[d]	0.530[d]	0.417[d]	74.3
IG-T	2	0.054	0.681	0.739	0.637	0.498[d]	0.339[d]	62.8
IG-H (Freeze-dried)	3	0.032	0.799	0.819	0.811	0.693[d]	0.576[d]	76.7
IG-T (Freeze-dried)	3	0.033	0.665	0.699	0.660	0.561[d]	0.501[d]	63.1
IG-D	3	0.047	0.650	0.654	0.646	0.488[d]	0.494[d]	59.8
IG-His	3	0.061	0.711	0.727	0.587[d]	0.481[d]	0.369[d]	64.7
HAP	4	0.012	0.752	0.773	0.757	0.765	0.720	74.1
VIII[b]	3	0.013	0.832	0.835	0.831	0.832	0.829	81.8
	1	0.367	1.165	1.079	1.248	1.216	1.216	79.8
VIII[b]	3	0.009	0.929	0.970	0.966	0.939	0.851	92.0
VIII[b]	3	0.008	0.922	0.889	0.930	0.973	0.897	91.3
IX[b]	4	0.011	1.094	1.015	1.178	1.153	1.052	108.2
IX[b]	3	0.010	1.198	1.243	1.215	1.138	1.195	118.9
FIB	4	0.023	0.873	0.912	0.865	0.826[d]	0.553[d]	84.8
AT-III[c]	3	–	–	–	–	–	–	–
APC	3	0.014	1.068	1.046	1.155	0.999	1.039[e]	105.4

Underlined values: Recoveries showing less than 50% of added endotoxin.
[a] Endotoxin contents calculated using values of dilution ranges attaining a parallel regression line to that of JPRSE.
[b] Produced by more than one manufacturer.
[c] No available data for calculation.
[d] Values of the dilution in all tested batches could not attain a parallel regression line to that of JPRSE.
[e] Values of the dilution in some tested batches could not attain a parallel regression line to that of JPRSE.

Table 6
Recovery of endotoxin added to blood products using Endospecy.

Products	Number of batches	Endotoxin content (EU/mL)						Recovery (%)
		Solution B	Solution C (x dilution factor)					
			All[a]	x 32	x 16	x 8	x 4	
IG-PEG	3	0.012	1.191	1.200	1.191	1.214	1.140	118.0
IG-PEG-H	3	0.012	1.073	1.081	1.075	1.111	1.009	106.1
IG-PEG-T	3	0.012	1.004	1.037	1.022	1.040	0.884	99.2
IG-PH4A	3	0.022	1.078	1.098	1.062	1.100	1.038	105.6
IG-ION	3	0.010	0.646	0.713	0.644	0.619	0.602	63.6
IG-PH4	3	0.021	0.772	0.778	0.781	0.803	0.720	75.0
IG-SUL	3	0.020	0.736	0.772	0.746	0.730	0.685	71.5
IG-PEP	1	0.012	0.733	0.779	0.731	0.741	0.670	72.1
IG[b]	2	0.032	0.803	0.864	0.827	0.786	0.714	77.1
IG[b]	2	0.041	0.791	0.890	0.819	0.674	0.618[c]	75.0
IG-H[b]	3	0.023	0.766	0.835	0.772	0.722	0.656[d]	74.2
IG-H[b]	2	0.031	0.848	0.941	0.882	0.760	0.724[d]	81.5
IG-T	2	0.077	1.016	1.094	1.001	1.018	0.909	93.9
IG-H (Freeze-dried)	3	0.012	0.960	0.972	0.955	0.978	0.925	94.8
IG-T (Freeze-dried)	3	0.023	0.858	0.876	0.856	0.858	0.834	83.4
IG-D	3	0.017	0.864	0.932	0.878	0.814	0.764	84.6
IG-His	3	0.016	0.570	0.628	0.557	0.548	0.532	55.3
HAP	4	0.009	0.877	0.814	0.841	0.951	0.945	86.7
	3	0.016	0.816	0.737	0.808	0.887	0.869	80.0
VIII[b]	1	0.144	0.919	0.837	0.880	1.077	0.937	77.5
VIII[b]	3	0.012	0.820	0.812	0.789	0.836	0.855	80.8
VIII[b]	3	0.011	0.840	0.810	0.820	0.862	0.887	82.9
IX[b]	4	0.019	0.984	0.905	0.951	1.108	1.128	96.5
IX[b]	3	0.012	0.908	0.885	0.857	0.922	1.006	89.6
FIB	4	0.042	1.078	0.984	1.030	1.207	1.188	103.5
AT-III	3	0.080	0.355	0.355	0.155[c]	0.033[c]	0.006[c]	27.4
APC	3	0.085	1.002	1.001	0.983	1.112	1.074	91.6

Underlined values: Recoveries showing less than 50% of added endotoxin.
[a] Endotoxin contents calculated using values of dilution ranges attaining a parallel regression line to that of JPRSE.
[b] Produced by more than one manufacturer.
[c] Values of the dilution in all tested batches could not attain a parallel regression line to that of JPRSE.
[d] Values of the dilution in some tested batches could not attain a parallel regression line to that of JPRSE.

G-activating substances in blood products may conflict with the evaluation of endotoxin content.

Nineteen types of blood product were assayed for interfering effects on the endotoxin test based on spike/recovery of endotoxin by using endotoxin-specific lysate reagents. The minimum dilution required for elimination of interfering effects was determined according to the results on serial dilutions of each product. Interfering effects of IG, IG-H, XIII and IX products were examined using batches from multiple manufacturers, and recoveries of spiked endotoxin were rather consistent for each product irrespective of the manufacturer. Inhibitory effects could be eliminated by diluting blood products from 1/2 to 1/8 for the test using ES-III, with the exceptions of IG and AT-III. However, some IM-IG preparations persistently showed dose-dependent inhibitory effects even at higher dilutions, which would hamper the application of the parallel line assay. Greater dilutions from 1/16 to 1/32 were required for such IM-IGs to attain parallelism of the regression lines to that of JPRSE for the test using ES-III. On the other hand, when tested by Endospecy, the blood products did not exhibit significant interference, and parallel regression lines to that of JPRSE could be attained at dilutions from 1/4 or 1/8, with the exception of AT-III. AT-III markedly inhibited tests using both ES-III and Endospecy, and application of the endotoxin test was not possible at a dilution not exceeding MVD.

MVDs for combinations of each blood product and lysate reagent were calculated as described in Materials and methods (Table 1), and compared with the minimum dilutions necessary for eliminating the interfering effect of each product. The MVDs were also compared with the minimum dilutions necessary for attaining parallelism of dose-response regressions to JPRSE. The endotoxin tests using the appropriate endotoxin-specific lysate reagents were applicable to all the blood products tested at dilutions not exceeding MVDs, except for AT-III. Tentative endotoxin limits were calculated from the minimum pyrogenic dose of endotoxin per kg body mass (=5.0 EU/kg), and the maximum dose of each product per kg per hour according to "Decision of Limit for Bacterial Endotoxins" in the general information of JP. It was reported that the in vivo febrile response of endotoxin was affected by the presence of biological products. Certain intravenous IgG products that spiked with endotoxin caused a significant reduction in the febrile response of rabbits when compared to rabbits that received the same dose of endotoxin in saline [24]. On the other hand, non-pyrogenic amounts of endotoxin caused a febrile response in rabbits when injected with a non-pyrogenic dose of recombinant human interferon beta [25]. However, the interfering or synergistic effects of products on the in vivo action of endotoxin cannot be evaluated by the endotoxin test. An endotoxin dose of less than 5.0 EU/kg would be an appropriate minimum pyrogenic dose for pharmaceuticals that may enhance the in vivo action of endotoxin, such as interferon beta. Thus, the endotoxin limit of each product should be carefully assessed by taking into consideration the possible synergistic effect between the product and endotoxin in order to guarantee the same level of safety as the pyrogen test.

Pyrogens other than endotoxin are not detectable by the endotoxin test, whereas the pyrogen test has the ability to detect such pyrogens in addition to endotoxin. Accordingly, a special attention should be paid for the replacement of the pyrogen test with the endotoxin test. Endotoxin is considered to be the most important pyrogen pertaining to the safety of parenteral drugs including blood products [26] because of its ubiquity in nature, stability, and potent toxicity. It was reported that the pyrogenic activity of endotoxin is approximately 1000–10,000-fold higher

Table 7
Recovery of endotoxin added to IM-IGs that interfered with the test using ES-III.

Products	Number of batches	Endotoxin content (EU/mL)						Recovery (%)
		Solution B	Solution C (x dilution factor)					
			All[a]	x 64	x 32	x 16	x 8	
IG	3	0.058	0.784	0.821	0.831	0.745[d]	0.561[c]	76.7
IG-H[b]	3	0.145	0.768	0.799	0.692[d]	0.630[c]	0.504[c]	73.0
IG-H[b]	2	0.051	0.741	0.723	0.829	0.739	0.610[d]	72.8
IG-His	3	0.057	0.588	0.613	0.579	0.571[d]	0.526[d]	57.2

[a] Endotoxin contents calculated using values of dilution ranges attaining a parallel regression line to that of JPRSE.
[b] Produced by more than one manufacturer.
[c] Values of the dilution in all tested batches could not attain a parallel regression line to that of JPRSE.
[d] Values of the dilution in some tested batches could not attain a parallel regression line to that of JPRSE.

than that of other pyrogens such as peptidoglycan, lipoteichoic acid, and β-glucan [27,28]. The extent of factor G-reactive substances detected in the blood tested products by the glucan-specific lysate reagent caused no febrile response in rabbits. However, it was reported that (1–3)-β-D-glucan strongly co-stimulated cytokine production (IL-6/IL-8) induced by ligands for Toll-like receptors (TLRs) such as endotoxin in the monocyte activation test (MAT) [29]. Glucan-specific lysate reagents could detect and quantify (1–3)-β-D-glucan in blood products, but evaluate neither its in vivo biological activity nor synergistic effect of (1–3)-β-D-glucan in the presence of endotoxin. It was also reported that peptidoglycan fragments (0.001–0.01 μg/mL), constituted of 2 stem-peptide trimers, induced pro-inflammatory cytokine release from human peripheral blood mononuclear cells showing much higher activity than insoluble and soluble peptidoglycan (≥1 μg/mL of minimal active concentration) [30]. The MAT, which is a novel test method to detect or quantify pyrogens that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, has been implemented in the European Pharmacopoeia as a replacement for the pyrogen test in 2010 [31]. It is expected that the MAT has potentials to detect non-endotoxin pyrogens and to evaluate synergistic effects between (1–3)-β-D-glucan and endotoxin or other ligands for TLRs, and may reflect clinically relevant responses. As little is known about the contamination with (1–3)-β-D-glucan or peptidoglycan in blood products, further study would be necessary on physiological and clinical impacts of the contamination with such immunomodulators. Alternative approaches such as specific quantification, the MAT or combined estimate can be important for quality control of blood products.

The endotoxin test is the most sensitive method of endotoxin detection and is much more sensitive than the pyrogen test. None of the tested samples showed a positive response in the pyrogen test, whereas the endotoxin test detected a low but significant level of endotoxin in a single batch of VIII (Tables 5 and 6). Although the contamination of endotoxin did not induce a pyrogenic response in rabbits, the results revealed inconsistent endotoxin levels among batches. These results suggest that the endotoxin test is valuable in monitoring production consistency due to its high sensitivity. Furthermore, the use of rabbits in the pyrogen test is affected by various uncontrollable factors, such as sensitivity variation among individual rabbits, which may hamper reproducibility of results among different laboratories or even within a single laboratory. Manufacturers individually performed testing for interfering effects in their blood products based on spike/recovery of endotoxin using endotoxin-specific lysate reagents, ES-III or Endospecy, according to the common operating procedure. The results were highly consistent between laboratories when the same lysate reagent was used (data not shown). Thus, the endotoxin test has several advantages such as sensitivity and reproducibility in detecting and quantifying endotoxin when compared with the pyrogen test that requires a large number of rabbits. The endotoxin test using appropriate endotoxin-specific lysate reagents was shown to be reliably applicable for various plasma derivatives, including various immunoglobulin and blood coagulation factor products even in the presence of factor G-reactive substances, with the exception of AT-III.

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