tems in the above-noted two companies, have been successfully operating for an extended period. Hence, it was expected that an investigation and evaluation of operational data and control protocols for membrane-based water processing adopted by the Japanese pharmaceutical companies including the above noted two companies, would provide much useful information.

The protocol adopted for the surveillance study is as follows:

Outline of protocol for surveillance visit study

1. Candidate companies for the study

1–1. Protocol for company selection

a. Companies that were receptive and replied to a questionnaire submitted by an earlier study granted by the Ministry of Health, Labor and Welfare

b. Companies with extended experiences using membrane-based water processing systems

c. Companies using membrane-processed water in drug product preparation or for final rinsing

1–2. Requested respondents in the companies
Members of quality control section, operational control section, and manufacturing section

1–3. Visitors to the companies
To be discussed and decided for each company

2. Plant visit preparation

a. Visit request form.

b. Company request form.

i. General information

1) List of products manufactured and intended use of pharmaceutical water

2) Drug product markets (domestic and/or for export)

ii. System information

1) System supplier

2) System block flow diagram (PFD—from water feed source to membrane unit)

3) Water processing system P&ID (inclusive of membrane modules)

4) Membrane module: Supplier, brand, type, model and specification

5) Length/period of operation

iii. Operational information

1) Operational data (product water flow rate, temperature, pressure)

2) Total on-line time and rate of processing

3) Method of control when off-line

4) Method and frequency of disinfection, sterilization and/or cleaning

5) Membrane module integrity test protocol and testing frequency

6) Frequency and protocol of membrane module replacement

7) Analysis data of membranes

iv. Water quality information

1) Items selected for quality control-test methods and acceptance criteria, and testing frequencies adopted for monitoring the quality of feed in-take water, discharge water of membrane modules, and the water sampled at points of use

2) Quality data for feed in-take water and discharge water of membrane module (for an extended period-preferably several years)

v. References

1) Regulatory inspection history (FDA, EU, other)

2) Quality and performance confirmation procedures for membrane modules during acquisition and installation (UF and/or RO)

3) On-site inspections

According to this protocol, the study group asked more than 10 Japanese pharmaceutical companies to accept a surveillance visit by the group. In July–September 2005, the study group members visited 10 Japanese pharmaceutical plants to investigate their water processing systems and long-term operational data of the systems.

3.3 Surveillance visit results summary

3.3.1 Description of the water processing systems in 10 visited pharmaceutical plants

Descriptions of the water processing system in 10 visited plants are shown in Table 2. Table 3 shows their operation histories and disinfection methods. It was found that plants D, E, G and H have used membrane-processed water for WFI in some parts of their products.

Table 2 Pharmaceutical Water Processing Systems in 10 Visited Plants

Visited Plant	Pretreatment Unit		Membrane Module		Water Processing Capacity (m^3/h)
	Clarification	Deionization	Endotoxin/Bacteria Removal (Operation Mode)	Disinfection and/or Sterilization	
Plant A		DG[3] + RO + CED	(Hot Water Operation)	Steam Sterilization	6
Plant B	CSSF[1] + MF	IER[2]	UF (Room Temp. Operation)	Hot Water Disinfection	15×2 lines
Plant C		RO + CEDI	UF (Room Temp. Operation)	Hot Water Disinfection	7.2×2 lines
Plant D	Sand Filtration	IER[2]	RO + RO (Room Temp. Operation)	NaClO Disinfection	5.6
Plant E	Sand Filtration	IER[2]	RO + RO (Room Temp. Operation)	NaClO Disinfection	3.3
Plant F		IER[2]	UF + UF (Room Temp. Operation)	Hot Water Disinfection	5
Plant G			RO + IER[2]/CEDI	Disinfection with Hot Water (UF) and Formalin (RO)	4.2×3 lines
Plant H	MF	IER[2]	UF (Hot Water Operation)	Disinfection	10
Plant I	Sand Filtration	IER[2]	UF + UF (Room Temp. Operation)	Hot Water Disinfection	12
Plant J		Softener + RO + MD[4] + CEDI	UF + UF (Room Temp. Operation)	Hot Water Disinfection	1.7

*1 CSSF: Coagulation, sedimentation and sand filtration, *2 IER: Ion exchange resin unit including towers and mix bed, *3: Decarbonation, *4: Membrane Degassing

Table 3–1 Operational Accomplishments of Pharmaceutical WaterProcessing Systems at Each Surveyed Plant

Plant	Operational Period	Membrane Unit		Membrane Replacement Interval
		Disinfection Method	Disinfection Frequency	
Plant A	Sept. 1990–Present	UF (Steam)	3 Times per Year	2 Years
Plant B	Apr. 1985–Present	UF (Hot Water)	Daily	6 Years
Plant C	Sept. 1999–Present	UF (Hot Water)	Once 2 Weeks	5 Years
Plant D	1991–2005 (Plant Relocated)	RO + RO (NaClO)	Weekly	3–4 Years
Plant E	Apr. 2001–Present	RO + RO (NaClO)	Once 9 Days	2–3 Years

For example, plant A started production of pharmaceutical water with UF membrane–based water processing system since September 1990. UF membrane is sterilized with steam on every 4 months. Replacement of UF membrane module is about every 2 years.
Plant D and E have used membrane–processed water as WFI.

Table 3–2 Operational Accomplishments of Pharmaceutical Water Processing Systems at Each Surveyed Plant (continued)

Plant	Operational Period	Membrane Unit		Membrane Replacement Interval
		Disinfection Method	Disinfection Frequency	
Plant F	Aug. 1999–Present	UF + UF (Hot Water)	1st UF: Weekly 2nd UF: Daily	4.5 Years
Plant G	Aug. 1998–Present	UF + RO (Hot Water/ Formalin)	Monthly	3 Years (1/3 Annually)
Plant H	Jan. 1991–Present	UF (NaOH)	Twice a Year	1 Year
Plant I	May 1990–Present	UF (Hot Water)	Every 20 Days	3–6 Years
Plant J	Feb. 1999–Present	UF (Hot Water)	Daily	2.5 Years

Plant G and H have used membrane–processed water as WFI.

Necessity for applying water clarifying apparatus was dependent on the water source quality. Such apparatus was not employed in the case of clear (non-turbid) water sources such as tap water and well water. Sand filtration was commonly used when water clarification was required; however, in some situations, MF was employed. Although

not noted in Table 2, in some systems, activated carbon was used to remove hypochlorite ions.

Commonly employed for deionization are ion exchange resin units or systems where an ion exchange resin unit is the primary component. In some systems, a RO unit is added to reduce the load on the ion exchange unit, and in other systems, a CEDI unit is added in lieu of ion exchange. Decarbonators and degasifiers were employed to remove carbon dioxide, and water softeners were employed to reduce hardness.

Single-staged UF modules were most commonly used for membrane-based water processing systems. Also employed were double-staged UF modules, UF+RO modules, and double-staged RO modules. Most of RO and UF modules were supplied by Japanese membrane manufacturers, but there were also some imported modules. Capacities of the systems ranged from 1.7 m^3/hr (small scale) to 30 m^3/hr (large scale). It was impressive that large scale systems (>10 m^3/hr) were employed in 5 out of 10 visited plants.

3.3.2 Membrane module maintenance

Membrane module maintenance was primarily performed by periodic quality analysis of processed water. Quality parameters selected for the analysis were viable cell count, endotoxin content, purity test, TOC, conductivity, and particulate count. Water test samples were collected periodically at the membrane module inlet and outlet. Test results were used to confirm the absence of quality abnormalities in processed water.

In-line monitoring for conductivity and TOC were carried out at the membrane module outlet. Conductivity was monitored at 9 out of 10 visited plants, and TOC was monitored at 8 out of 10 plants.

Additionally, pressure gauges were installed at the membrane module inlet to monitor water pressure. Flow meters were installed at the outlet of the unit to monitor flow fluctuation through the unit. In most of the plants, gauges and flow meters were monitored daily by the operators. The data was recorded and used to determine requirements for membrane module maintenance.

As transparent housings were used for UF modules, it was possible to conduct daily visual inspections to detect membrane failure.

An air leak integrity test on the modules was performed every 3 to 12 months at most of the visited plants.

In several of the plants, in addition to the inspection and testing noted above, the extent of membrane deterioration was determined and used to indicate requirements for membrane module replacement. Replaced UF modules (old modules) were returned to the supplier to examine membrane tensile strength, presence of air leaks, and filtration capacity. The old module data was then compared with new module data to assess extent and degree of deterioration. Scheduling of membrane module replacement was determined based on these data and past operational records, in consideration of the supplier' s recommendation.

For RO modules, desalination capacity tests were performed every 3 to 6 months during operation to determine membrane module replacement requirements.

The timing for membrane module replacement used in high temperature filtration and disinfection was also determined based on past operational records.

3.3.3 Some examples of membrane-based water processing system operation experiences

《Example 1: System operation data at plant H》

Figs. 2–6 show system operation data at plant H where endotoxin-removal capability of UF module was clearly shown. Fig. 2 shows plant H's system block flow diagram. Figs. 3 through 6 show conductivity and endotoxin levels at the outlet of the ion exchange unit, endotoxin level at the

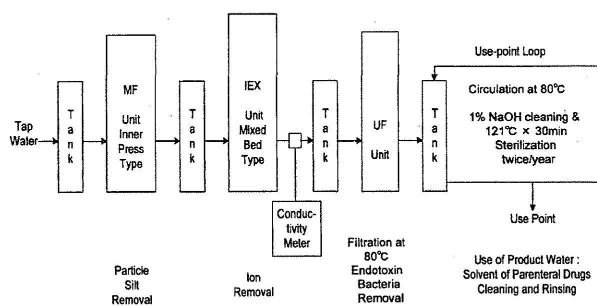

Fig. 2 Constitution of Water Processing System of Plant H MF unit is used for clarification of tap water, IEX is for demineralization and UF membrane unit is for purification. After passing UF unit, water is stored in a tank and water in the tank is circulated at 80℃. Production capacity of purified water is 10 ton/hr. Service term of this system is longer than 15 years.

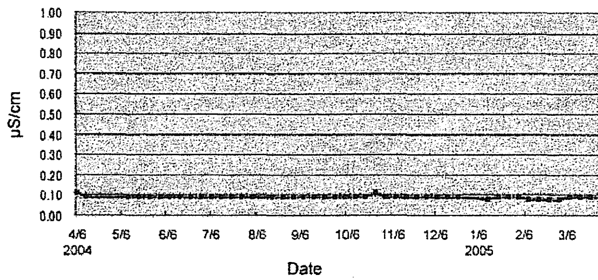

Fig. 3 Conductivity Test Results of Plant H from April 2004 to March 2005
IEX has been well operated and conductivity data were satisfactory.

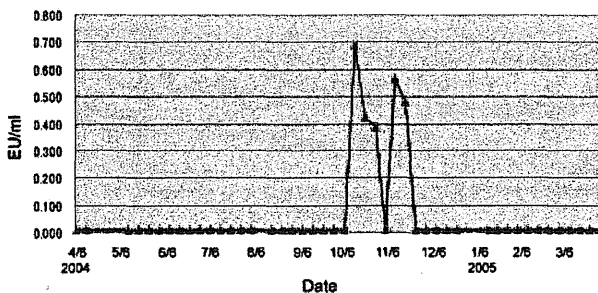

Fig. 4 Endotoxin Test Results at IEX–outlet of Plant H from April 2004 to March 2005
Endotoxin was found in water at IEX–outlet in the period from October 2004 to December 2004.

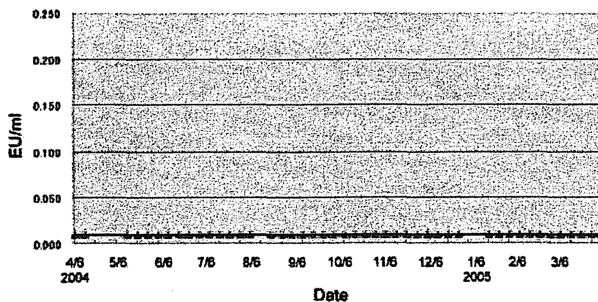

Fig. 5 Endotoxin Test results at UF–outlet of Plant H from April 2004 to March 2005
Endotoxin was not found in water at UF–outlet even in the period from October 2004 to December 2004.

outlet of UF module, and viable cell count at the point of use in the processing system, respectively.

Fig. 3 demonstrates that the ion exchange unit worked well for desalination. However, Fig. 4 indicated that there was a period when endotoxin contamination was present in the deionized water, and that this condition was present for about 2 months. Fig. 5 shows that, even during this period,

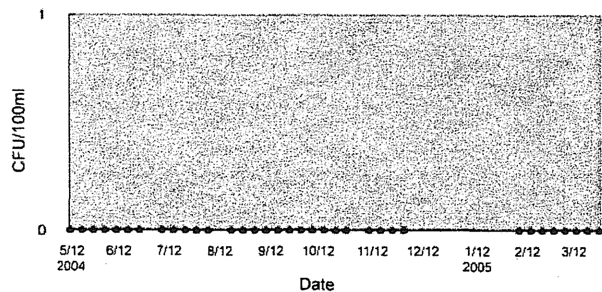

Fig. 6 Viable Bacteria Test Results at Use-point of Plant H from May 2004 to March 2005
Viable bacteria were not found at use point even in the period from October 2004 to December 2004. As far as we surveyed, viable bacteria counts were almost zero at use points of any plants.

endotoxin was not present in the processed water from the UF module located downstream of the ion exchange unit. Fig. 6 shows that no viable cells were counted at the point of use in the system during this period. This example illustrates that membrane-based water processing systems can maintain the quality of processed water even when the water source is contaminated with endotoxin.

《Example 2: An experience at plant A》

Many situations were reported where membrane failures were successfully detected via quality analysis of processed water. However, there was a situation where the pretreatment unit had performed so well in providing water with good quality that quality analysis did not reveal abnormalities in processed water even though a membrane failure existed at the time.

In plant A, UF module has not been replaced since initial operation of its water processing system, because endotoxin has not been detected in the samples taken at the UF module outlet. After 44 months, the UF module was replaced and the repalaced module was analyzed by the supplier, who found that there was a membrane failure.

The explanation for the absence of endotoxin is supposed that the deionization RO pretreatment unit at upstream of the UF module reduced the endotoxin level to below detection limits. Hence, any UF membrane failure could not be detected by checking the endotoxin level at the UF module outlet (as described above) .

Upon discovering this, plant A tried to establish a protocol for replacing UF modules. The plant replaced a UF

module after 16 months and an another module after 24 months of operation. Then the plant asked the UF module supplier to analyze these modules. Based on the test results of these modules, plant A decided to replace UF modules every two years. Since then, the plant has been replacing modules periodically.

《Example 3: An experience at plant I》

On the other hand, plant I has an experience for effectively detecting UF membrane failure by checking the endotoxin levels in the processed water from the UF module. Here purified water was prepared by the water processing system and then delivered via a 200 m long pipeline to a bulk drug facility. This caused the increase of endotoxin level of the water supplied to the UF module upto 2–20 EU/mL. By removing endotoxin with the UF module, the endotoxin level of processed water was reduced to below detection limits (0.01 EU/mL). However, there was an instance where processed water from the UF module showed detectable endotoxin levels, but still remained below the 'standard' 0.25 EU/mL. In this situation, air leak testing successfully detected a membrane failure.

From this study, it was concluded that pharmaceutical water processing systems using RO and/or UF membranes are consistently successful in preparing water suitable as WFI. However, it is desirable to develop a continuous microorganism monitoring technique in order to overcome concerns for microorganism contamination from a potential membrane failure.

3.3.4 Filtration temperatures and UF module replacement frequency

This surveillance study found that 8 out of 10 plants used heat-resistant UF membranes. At plant A and plant H, filtration was conducted at elevated temperature (80℃). Other plants operated at ambient temperature, but used the membrane's heat-resistance capacity during periodic high temperature disinfection.

As the UF membranes used are resistant up to 90℃, operating at 80℃ would not cause problems as long as the system pressures do not exceed allowable limits provided by the membrane supplier.

However it is clear, from the result of this study that the membrane replacement interval with elevated temperature operation is shorter (1 to 2 years) than with ambient temperature operation (2.5 to 6 years). This shortening of membrane life may be due to the difference of temperatures applied to the membrane modules.

From the observations noted above, filtration temperatures are considered to be an important parameter for deciding frequency of membrane replacement. If longer membrane life is desired, a combination of ambient temperature filtration and disinfection at elevated temperatures may be a good choice.

3.3.5 Comments from companies visited

1) An advantage of a membrane-based water processing system is that apparatus necessary for the system is smaller and lighter than that for a distillation system. This feature simplifies building site selection for the system, even when a large capacity system is required. When compared to a distillation system, the disadvantages of a membrane-based system are relatively more complex maintenance and relatively higher operational costs.

2) As many components of a distillation system are made of metals, membrane-based water processing is preferred, when the efficacy, safety, and quality of drug products is affected by the presence of trace amount of metals.

3) Since there are no rapid methods for detecting microorganism, two-stage UF system were employed to lower the probability of microorganism contamination, especially when there might be a membrane failure.

4) For WFI production, it is important to develop a total quality system encompassing from source water pretreatment to prepared water supply. This is required for both distillation and membrane-based processing.

3.3.6 Quality of pharmaceutical water prepared by membrane-based processing systems

Quality of membrane-processed water was found to be good and suitable for manufacturing drug products in all of the investigated plants.

Membrane-based pharmaceutical water processing systems have some pretreatment units for the source water. Some of the pretreatment units can remove microorganism and endotoxin to provide bacteria- and endotoxin-free water to membrane module. With such pretreatment, it is natural that microorganism or endotoxin is not detected in the water prepared by these systems. When two or more

water processing systems shared a common pretreatment unit, it was found that the water contaminated with bacteria and/or endotoxin was occasionally supplied to the membrane module. Even in such situations, it was proved that membrane modules did remove bacteria and endotoxin to provide water suitable for manufacturing drug products.

As the results of the evaluation of the data obtained from 10 visited plants, it was concluded that:

1) In all the plants investigated, the quality of water prepared by the pharmaceutical water processing systems based on RO and/or UF membranes, was found to be suitable for the manufacture of drug products.

2) Maintenance of membrane modules was fundamentally carried out by 1) periodic quality analysis of the membrane-processed water and 2) scheduling of membrane module replacement based on the analytical data with replaced modules and past operational records, in consideration of the supplier's recommendation.

■4. Conclusions

Based on the protocol prepared in 2004 for the surveillance visit study, in 2005 the study group visited 10 Japanese pharmaceutical plants that have been preparing water for pharmaceutical use employing membrane-based water processing systems. The operational data from the past several years were investigated and evaluated to determine if these systems consistently and reliably produced water suitable as WFI.

Based on the result of the evaluation of obtained data, it was concluded that all of membrane-based water processing systems investigated were designed, operated, and controlled such that they consistently prepared water suitable as WFI.

The main purpose for using membrane modules in a water processing system, is the removal of endotoxin and/or microorganism. As membrane modules are significantly smaller than distillation units, it becomes practical to use membrane modules for large scale preparation of pharmaceutical water. Additionally, employing multi-stage membrane treatment modules provides a multi-barrier to ensure the quality and safety of prepared water.

Membrane-based water processing is energy efficient compared with distillation. This becomes increasingly important for large scale pharmaceutical water preparation.

In conclusion, pharmaceutical water processing systems based on RO and/or UF membranes, are working well and are suitable for preparing water used for manufacturing of drug products. However, it is desirable to develop a technique for continuous microorganism monitoring in order to overcome concerns for microorganism contamination from a potential membrane failure.

■5. Acknowledgement

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For details of the results obtained in this study, see 2005 Health, Labor & Welfare Research Report No. 200501114A (in Japanese) 〔平成17年度厚生労働科学研究“日本薬局方等医薬品基準の國際ハーモナイゼーションに関する研究”(主任研究者：川西 徹 国立医薬品食品衛生研究所生物薬品部長/分担研究“膜法により製した水の信頼性に関する検討”(分担研究者：小嶋茂雄 (独)医薬品医療機器総合機構顧問)報告書〕

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Review Article

A New Method for the Evaluation of Vaccine Safety Based on Comprehensive Gene Expression Analysis

Haruka Momose,[1] Takuo Mizukami,[1] Masaki Ochiai,[2] Isao Hamaguchi,[1] and Kazunari Yamaguchi[1]

[1] *Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan*
[2] *Division of Quality Assurance, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan*

Correspondence should be addressed to Kazunari Yamaguchi, kyama@nih.go.jp

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For the past 50 years, quality control and safety tests have been used to evaluate vaccine safety. However, conventional animal safety tests need to be improved in several aspects. For example, the number of test animals used needs to be reduced and the test period shortened. It is, therefore, necessary to develop a new vaccine evaluation system. In this review, we show that gene expression patterns are well correlated to biological responses in vaccinated rats. Our findings and methods using experimental biology and genome science provide an important means of assessment for vaccine toxicity.

1. Introduction

Vaccination effectively enables the control of many infectious diseases. However, we cannot always avoid the problem of adverse reactions accompanied by vaccination. While most adverse reactions are mild and local, some vaccines have been associated with very rare but severe systemic reactions. Therefore, all vaccines for public use are made in compliance with Good Manufacturing Practices (GMP) to prevent safety problems. Furthermore, manufacturers must submit samples and results of their in-house tests for each vaccine batch to the national control authorities before vaccines are released into the market. Among many quality control tests, conventional animal safety tests are performed to detect vaccine toxicity because residual vaccine toxicity has the potential to cause adverse reactions. For example, the animal body weight change test is the most commonly used test to evaluate the toxicity of vaccines [1]. Although a good correlation of the body weight loss with a vaccine's toxicity has been shown [2, 3], a greater understanding of the molecular mechanisms involved in the reaction to a vaccines' toxicity is needed. We, therefore, attempted to measure animals' responses to vaccines by determining changes in gene expression profiles.

Gene expression profiling is a unique way to characterize how cells or tissues are affected by abnormal conditions. The measurement of gene expression levels upon exposure to toxicants can be used to identify toxic products, and to provide information about the mechanism of toxicity [4]. DNA microarray technology has opened the way for the parallel detection and analysis of expression patterns of thousands of genes in a single experiment. Furthermore, the development of high-quality gene arrays has allowed DNA microarray technology to become a standard tool in molecular toxicology. Recently, the field of toxicogenomics has validated the concept of gene expression profiles as "signatures" of toxicant classes [5–7]. These signatures have effectively directed the analytical search for predictive toxicant biomarkers and they have contributed to the understanding of the dynamic responses of molecular mechanisms associated with toxic responses. In fact, many studies of gene-expression profiles have now been reported in the toxicology field. For example, Hamadeh et al. reported patterns of gene expression in liver tissue taken from rats exposed to different

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.

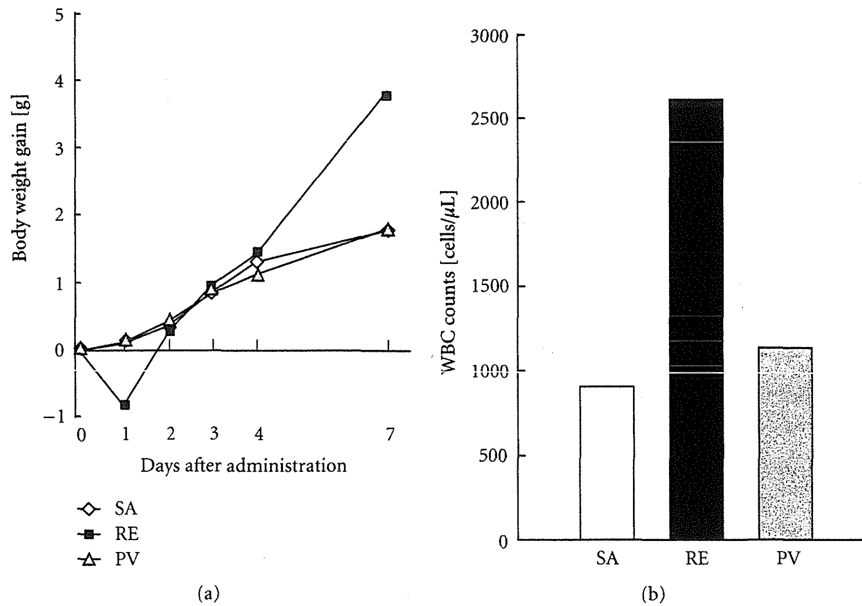

FIGURE 1: Safety control tests for pertussis vaccines. (a) Test for toxicity to mouse weight gain. Physiological saline (SA), an inactivated whole-cell pertussis vaccine (RE), or an acellular pertussis vaccine (PV)-administered mice were weighed on 0, 1, 2, 3, 4, and 7 days postadministration. Ten mice 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.

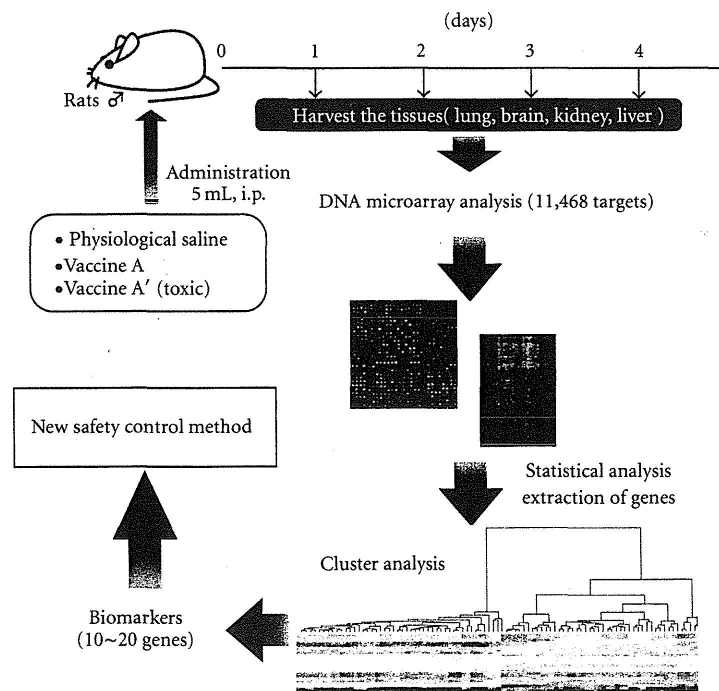

FIGURE 2: The gene expression analysis procedure. The detail of the procedure is described in the text.

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, *S10QA9*, *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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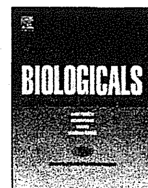

Applicability of bacterial endotoxins test to various blood products by the use of endotoxin-specific lysates

Masaki Ochiai [a,*], Akihiko Yamamoto [b], Seishiro Naito [a], Jun-ichi Maeyama [c], Atsuko Masumi [c], Isao Hamaguchi [c], Yoshinobu Horiuchi [b], Kazunari Yamaguchi [a,c]

[a] *Division of Quality Assurance, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan*
[b] *Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan*
[c] *Department of Safety Research on Blood and Biologics, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan*

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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.

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 565 3315.
E-mail address: masakio@nih.go.jp (M. Ochiai).

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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.

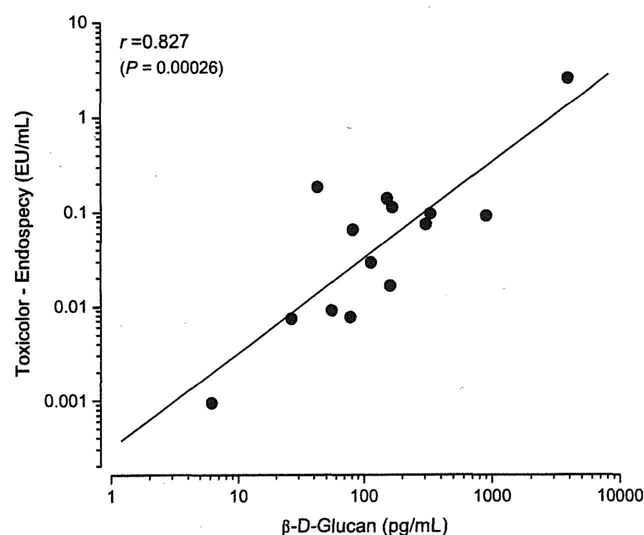

Fig. 1. Relationship of the results from 3 types of lysate reagents. The difference between EU/mL value by conventional reagent (Toxicolor) and that by endotoxin-specific reagent (Endospecy) was calculated and compared with (1–3)-β-D-glucan content by Fungitec G test MK. Each point shows this difference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

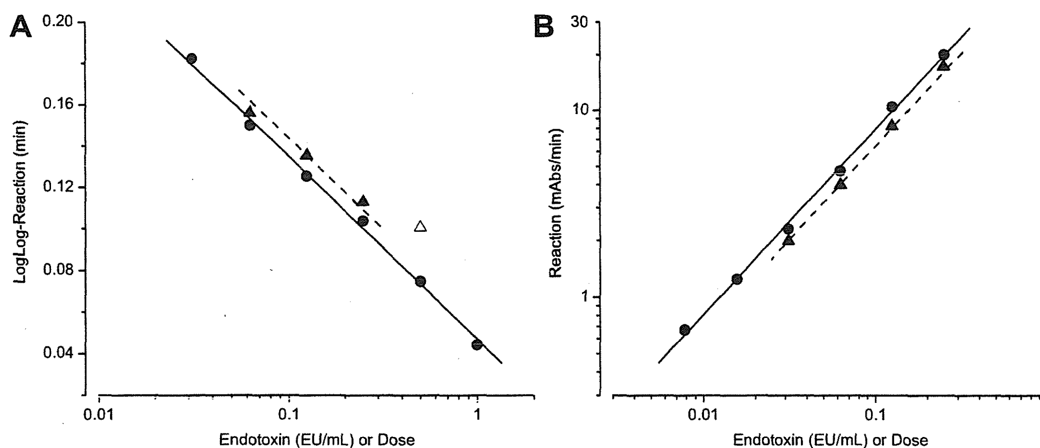

Fig. 2. Dose-response regression lines of JPRSE solution (●) and a test sample (▲) spiked with 1.0 EU/mL of endotoxin by kinetic–turbidimetric technique (ES-III) (A) and kinetic–chromogenic technique (Endospecy) (B). Double logarithmic transformation of time to reach a predetermined turbidity for ES-III 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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