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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 $23 \pm 1^\circ\text{C}$, with $50 \pm 10\%$ 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 hemacytometer, 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 instruc-

tions. 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

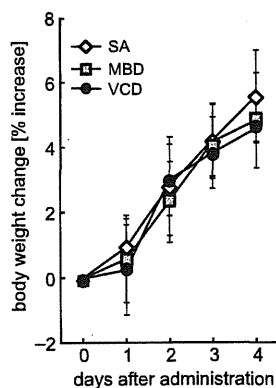


Fig. 1. Body weight analysis of the JE vaccine treated animals. The effects of mouse brain-derived (MBD) JE vaccine, Vero cell-derived (VCD) JE vaccine, and saline (SA) treatment were measured using decreasing body weight toxicity tests. All rats were weighed at days 0, 1, 2, 3, and 4. Changes in rat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

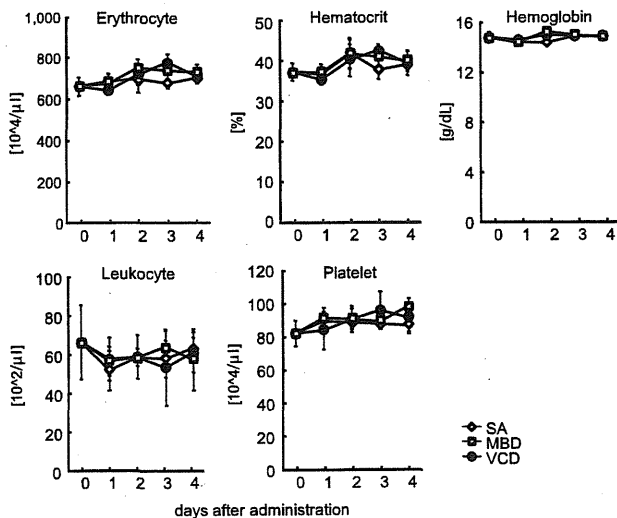


Fig. 2. Hematological tests for vaccinated rats. Blood obtained from individual rats was subjected to hematological tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean \pm S.D.

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.

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 log₂ 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

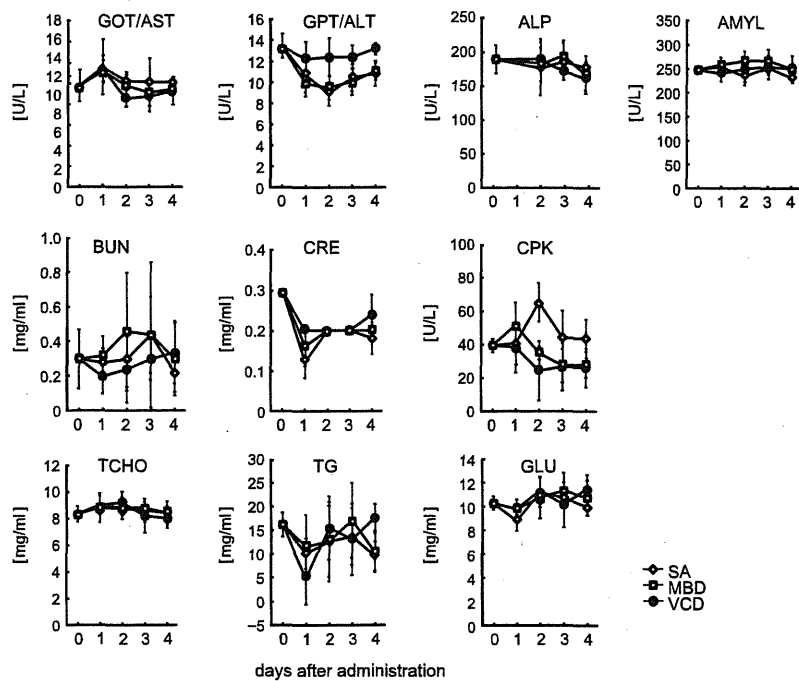


Fig. 3. Serum tests for vaccinated rats. Serum was separated from blood obtained from individual rats, and subjected to serum tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean \pm S.D.

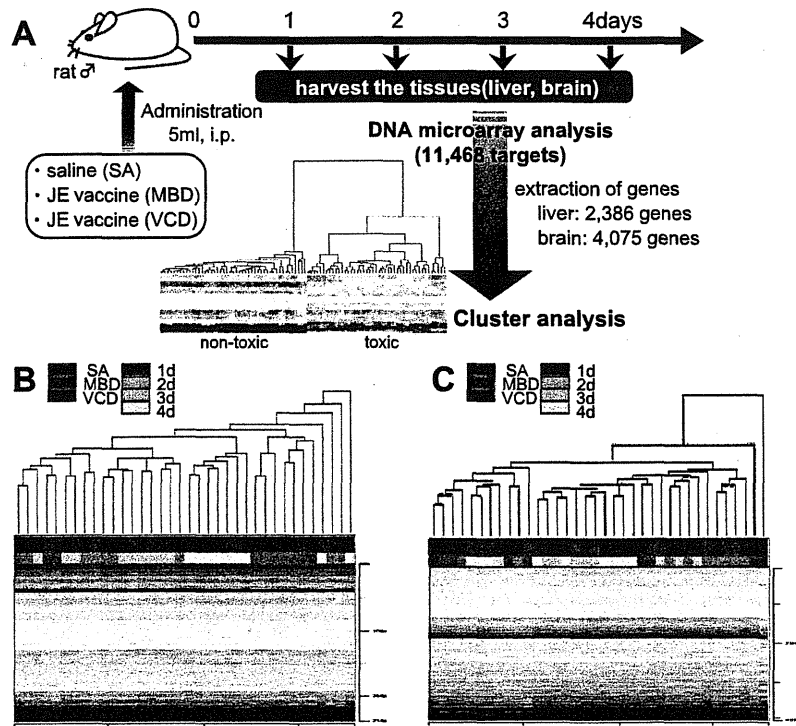


Fig. 4. Gene expression profiling and cluster analysis of vaccine-treated rat tissues. The procedure for gene expression analysis is outlined in A. For the cluster analysis, 2,386 genes for liver (B) and 4,075 genes for brain (C) were extracted from 11,468 targets and assembled in the order obtained 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 Euclidean 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 (EMA) (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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An improved abnormal toxicity test by using reference vaccine-specific body weight curves and histopathological data for monitoring vaccine quality and safety in Japan

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Abstract

Vaccines differ from other pharmaceutical products. The quality and safety of batches are regulated to high standards by national regulatory authorities. Various quality control and safety tests have been developed, including the abnormal toxicity test (ATT), which is described in the World Health Organization (WHO) guidelines and in each country's pharmacopoeia. However, the criteria for abnormal results are not well defined in these guidelines. In addition, the animal grade to be used in ATT, classified on the basis of microbial colonization, was not designated in either guideline.

In this study, we report a new and improved method of performing ATT, including statistical, histopathological analysis and hematological findings. It is based on the observation that there are body weight changes characteristic to each vaccine, and such standardized changes can be used as references for evaluating test vaccines. In addition, histopathological data are useful for determining vaccine quality and safety. Combined with histopathological examination, the improved ATT will be of great use for evaluating the consistency, quality and safety of different batches of vaccine. The results of these analyses were similar using either 'clean' or specific pathogen-free guinea pigs.

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Keywords: Abnormal toxicity test; General safety test; Vaccine; Quality control

1. Introduction and history

Vaccination is one of the most effective and beneficial strategies for preventing and controlling human infectious diseases around the world. Various vaccines have been developed and can successfully prevent infectious diseases such as diphtheria, Haemophilus influenza b (Hib), pertussis, tetanus, polio, and influenza [1]. Vaccines are considered to

differ from other pharmaceutical products because they are primarily intended for use in healthy people, including children. Vaccine production processes are also highly variable, depending on the target infectious disease. Current vaccines consist of inactivated, killed or live attenuated, antigenic components purified directly from organisms or produced by recombinant DNA technology. In addition, adjuvant is added to some vaccines to induce increased immunogenicity. On account of the complex production processes, vaccines are regulated by the national regulatory authorities of individual countries, on a lot-to-lot or batch-to-batch basis, and require high levels of quality and safety control.

To control and maintain vaccine quality, various quality control and safety tests have been developed, and are used to

Abbreviations: ATT, abnormal toxicity test; MRBP, minimum requirements for biological products; CFR, Code of Federal Regulations; SPF, specific pathogen-free.

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test the starting materials, materials under processing and the final products [2]. Among them, the abnormal toxicity test (ATT) (also known as the general safety test, innocuity test, or test for freedom from abnormal toxicity) has been developed to detect non-specific toxicity and/or contamination exogenous substances, and its inclusion in the national vaccine control regime is recommended by the WHO [3]. Many countries, including Japan [4] and the United States (21 CFR 610.11) [5], (but not EU countries) require ATT as a batch release testing to be performed on mice or guinea pigs for vaccines, sera and immunoglobulins [6]. In the EU, ATT is still included in some pharmacopeia European monographs and is required for the licensing and product validation process.

According to the WHO guidelines [3], the method of ATT is simple: 5 ml of vaccine is injected intra-peritoneally into guinea pigs and the animals are observed for 7–12 days. If there are no abnormal health signs, the product passes the test. The most frequently used signs to determine the safety of the vaccine are death rate and abnormal body weight changes.

In Japan, ATT was introduced in 1948 after immunization with a certain lot of diphtheria toxoid caused severe adverse events claiming 84 deaths in Kyoto and Shimane prefectures [7]. Since then, ATT has been conducted to test the safety of vaccine and blood products, both by the manufacturers and by the National Regulatory Authority, National Institute of Infectious Diseases (NIID).

ATT was described in the “minimum requirements for biological products (MRBP)” in article 42(1) of the Pharmaceutical Affairs Law in Japan, which defines the manufacturing methods, quality control and test methods applicable to pharmaceuticals [4]. According to the MRBP, the guinea pigs are required to survive without any abnormal signs during the observation and the body weight should return to its starting level within a specified time [8]. However, the criteria for abnormal signs are not detailed in either guideline. In addition, the required grade (defined by microbial colonization) of guinea pigs to be used in ATT is not designated in these guidelines. It has been suggested that animal grade is important in order to minimize the variation between test results and the effects of infectious agents on experimental results. When ATT was introduced in Japan in 1948, hemolytic streptococcal infection in guinea pigs was one of the most serious problems affecting body weight changes and test results. In 1971, a ‘clean’ grade of animals derived from specific pathogen-free (SPF) guinea pigs were introduced in ATT, which cleared problems caused by the infections. Nevertheless, it was thought that the clean grade guinea pigs were generated from SPF animals and were maintained under the SPF condition; they were fed with non-sterilized food and not monitored regularly for pathogens. Ideally, SPF and inbred animals should be used for ATT [9] and better methods could reduce the number of animals needed for research [10]. In Japan, inbred guinea pigs sufficiently well-characterized with respect to vaccine quality control testing are not available. In addition, it has been suggested that conventional guinea pigs are not suitable for evaluating liver toxicity, due to the natural occurrence of focal liver necrosis in clinically normal guinea

pigs [11,12]. Thus, a change from “clean” to SPF animals for ATT is urgently required in Japan.

In this study, we report an improved method of ATT, including statistical and histopathological analyses. The trend in body weight changes following the inoculation of each vaccine, together with statistical and pathological analyses, were used to evaluate consistency among different vaccine batches. We also report that body weight changes and histopathological trends were not affected by changing the animal grade.

2. Materials and methods

2.1. Animals

According to the minimum requirements for biological products (MRBP) in Japan [4], we purchased female Hartley guinea pigs (280–300 g body weight, ‘clean’ grade and SPF grade) from SLC (Shizuoka, Japan). Both clean and SPF guinea pigs were derived from same Nakaizu colony. They were housed in solid-bottomed metal cages and fed on standard guinea pig chow. The temperature was maintained between 24–26 °C. Five guinea pigs were caged together during the tests. All of our experiments were performed as national control tests for vaccines and in accordance with the guidelines for animal experiments of the National Institute of Infectious Diseases, Japan.

2.2. Abnormal toxicity test (ATT)

Prior to ATT, the body weights of the guinea pigs (clean grade and SPF grade) were monitored for 7 days. Only animals with no abnormal signs were used for the test. Each injection group consisted of two animals selected randomly from the healthy animals. Vaccines (Table 1) at a dose of 5 ml were injected intra-peritoneally into each guinea pig. Vaccines used in this study were test specimens for vaccine testing and some vaccines were provided from manufactures for detailed examination. Body weight was monitored for 7 days after the injection. At day 7, the guinea pigs were anesthetized with pentobarbital (50 mg/kg) and processed for histopathological analysis. For hematological analysis, 2 ml peripheral blood was collected from the heart and immediately submitted to automatic measurements of peripheral blood leukocytes (PBL), mean corpuscular hemoglobin (HGB), red blood cells (RBC), hematocrit (HCT), mean corpuscular volume (MCV) and platelets (PLT) by automatic hemacytometer, the Celltac MEK-5254 (Nihon Kohden, Tokyo, Japan).

Animals were considered to have passed the test if there was no abnormal histopathological changes and no significant difference ($P < 0.01$) in the body weight curves between the test vaccine and reference population.

2.3. Statistical analysis

Significant differences were calculated using the z-test for the body weight changes and Student’s *t*-test for other

Table 1
Body weight change after inoculation of various vaccines

Biological name	N	Body weight change after inoculation							
		Day 1		Day 2		Day 3		Day 4	
		AVE	VAR	AVE	VAR	AVE	VAR	AVE	VAR
Control (non-treatment, NT)	138	2.065	40.777	11.261	43.056	19.355	61.807	52.297	175.145
Control (saline, SA)	68	-2.382	23.046	8.544	35.714	16.176	36.386	48.456	147.326
Influenza HA vaccine (Flu-HA)	460	-5.098	59.160	8.148	63.372	16.467	65.914	52.507	173.819
Adsorbed diphtheria-purified pertussis-tetanus combined vaccine (DPT)	274	-4.704	40.495	6.215	44.199	14.712	61.898	47.588	179.100
Japanese encephalitis vaccine (JEV)	248	-9.734	45.370	6.520	46.639	15.210	47.470	47.895	148.119
Adsorbed hepatitis B vaccine (CHO) (HBV)	81	-6.074	27.119	6.568	35.248	14.074	62.519	50.235	168.307
Adsorbed tetanus toxoid (ATT)	79	-3.633	51.671	7.544	68.636	15.759	88.236	49.595	200.039
Adsorbed diphtheria-tetanus Combined Toxoid (ADCT)	55	-6.164	54.325	4.709	53.877	14.218	69.433	47.545	192.808
Pneumococcal vaccine polyvalent (PVP)	48	-38.813	65.092	-44.375	90.282	-27.021	97.340	21.667	101.163
Cholera vaccine (CV)	44	-14.568	79.879	-3.045	106.789	6.227	54.087	38.000	179.349
Freeze-dried inactivated tissue culture rabies vaccine (RV)	29	-11.241	40.047	4.276	68.778	12.448	68.399	43.345	105.448
Freeze-dried inactivated tissue culture hepatitis A vaccine (HAV)	28	-0.964	58.554	9.107	39.284	14.357	38.608	47.321	142.078
Yellow fever vaccine (YFV)	12	-1.000	24.545	10.917	24.992	18.500	47.364	55.417	142.629
Well's disease and Akiyami combined vaccine (WDACV)	12	-15.083	85.174	-7.750	171.477	2.917	101.902	37.583	341.174
Adsorbed habu-venom toxoid (HT)	10	-7.000	33.333	3.800	22.400	10.700	23.789	40.500	134.722
Adsorbed diphtheria toxoid for adult use (ADT)	8	-12.750	36.500	-0.375	42.554	7.250	42.214	47.000	143.143
Freeze-dried mamushi antivenom, equine (MA)	6	-1.500	16.300	6.667	47.867	14.500	30.300	51.833	224.967
Freeze-dried habu antivenom, equine (HA)	6	2.500	14.300	14.167	116.167	25.833	198.167	55.667	435.067
Freeze-dried botulism antitoxin, equine (BA)	4	1.500	13.667	12.000	22.000	18.750	18.917	56.250	47.583
Freeze-dried gas gangrene Antitoxin, equine (GGA)	2	-8.000	128.000	14.500	60.500	23.000	0.000	53.500	112.500
Gas gangrene antitoxin, equine (GGaE)	2	8.000	2.000	16.000	18.000	22.500	84.500	38.500	40.500

The average (AVE) and variance (VAR) of body weight change 1, 2, 3, and 7 days after various vaccines treatment were shown.

statistical analyses, according to the MRBP in Japan [12]. To evaluate difference between clean grade and SPF grade guinea pigs, Spearman's rank correlation coefficient (r) was calculated. All statistical analyses were performed using GraphPad Prism (version 4, GraphPad Software, San Diego, CA), Excel 2008 (Microsoft Japan, Tokyo, Japan) and JMP Statistical Software (version 5, SAS Institute Inc., Cary, NC).

2.4. Histology

At day 7 after vaccination, animals were anesthetized with pentobarbital (50 mg/kg). Afterwards, pancreas, spleen and liver specimens were excised and weighed. Tissues were fixed in Bouin's solution (Sigma, St. Louis, MO) and 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 24 h. After fixation, tissues were dehydrated through a series of graded ethanols and xylene and embedded in paraffin. Paraffin-embedded specimens were cut into 4 µm sections and stained with hematoxylin and eosin. The surface area of focal necrosis and aluminum deposition were measured using a slide gauge.

3. Results

3.1. Selection of healthy animals in the preliminary test

Prior to performing ATT, guinea pigs (280–300 g body weight) were monitored for 7 days under normal conditions (Fig. 1A). Fig. 1B shows the data of body weight change monitored for 7 days before the start of ATT, which was

obtained from previous tests done in NIID using 8000 guinea pigs. As shown in Fig. 1C, the increase in body weight followed a linear regression ($Y = 14.899X - 13.054$, $R^2 = 0.9655$) and the daily increase in body weight was normally-distributed at days 2 and 3 after the animals arrived at the laboratory. Body weights were increased by 10.87 ± 6.58 g at day 2 and by a further 7.963 ± 5.51 g at day 3. Body weight changes at day 1 were highly variable due to transportation stress and dietary conditions prior to delivery. Thus, we have not taken body weight changes at day 1 into consideration (data not shown). Based on these preliminary test data above, we judged and selected animals with coefficients of regression of 5 and coefficients of correlation to the parent population of 0.866 as being suitable for further experimentation. After selection, the guinea pigs were classified according to their final body weights and randomized to different vaccine-treated groups. Vaccines was injected intra-peritoneally and body weights were monitored for the next 7 days.

3.2. Body weight changes after vaccine injection

Table 1 shows types of vaccines used in this study. We used two control groups, non-treated (NT) and saline-treated (SA) one. Fig. 2A-1 shows body weight changes after the vaccine injection over the 7 day period. In the NT group, body weight increased by 2.065 g at day 1, then by 11.261 g (day 2), 19.355 g (day 3), and 52.297 g (day 7) (Fig. 2A). In the SA group, body weight initially decreased by 2.382 g at day 1, but then increased by 8.544 g (day 2), 16.176 g (day 3), and 48.456 g (day 7) (Fig. 2B). As in the SA group, body weights

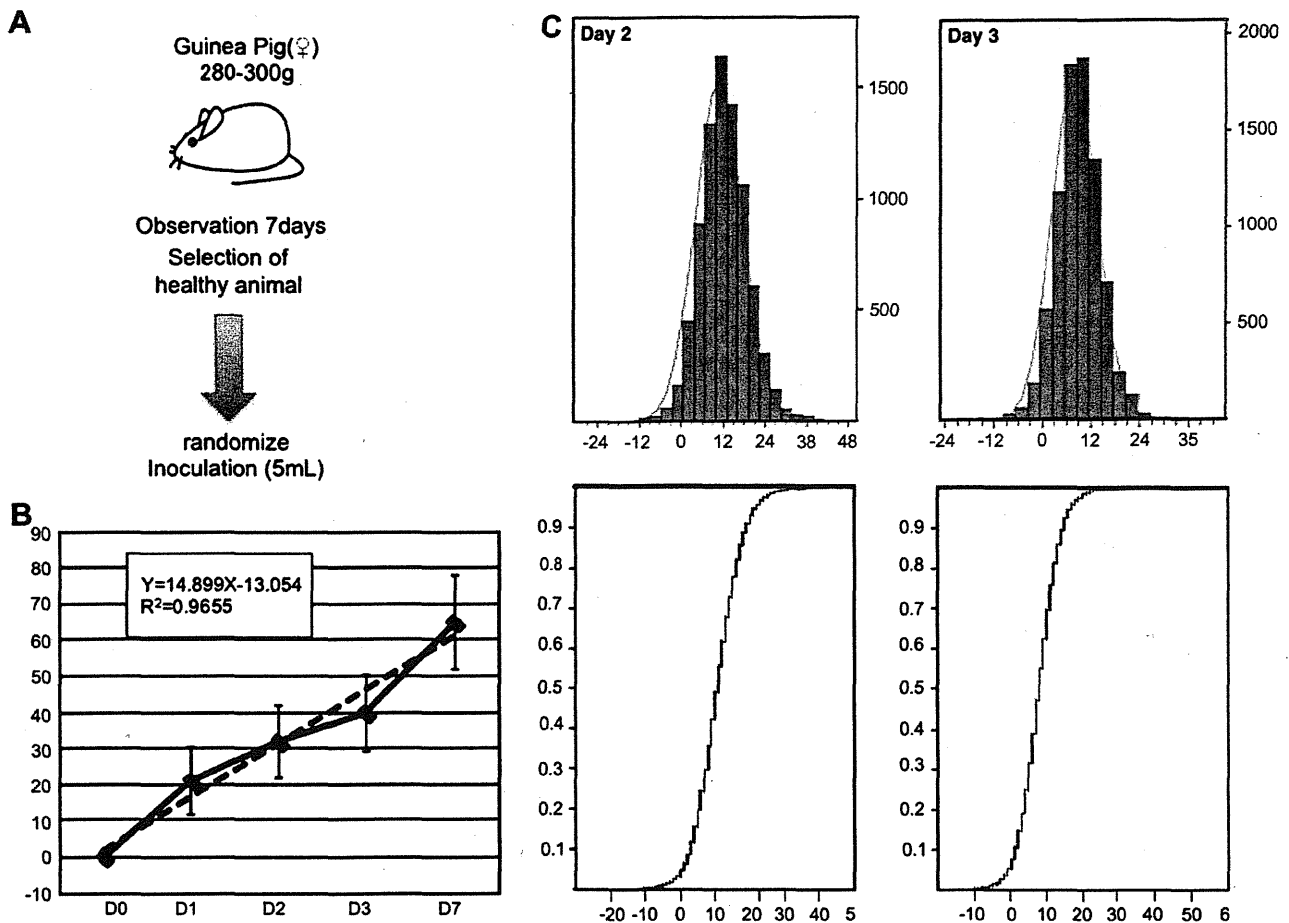


Fig. 1. Preliminary phase of abnormal toxicity test. (A) Summary of preliminary test. (B) Body weight changes after the arrival of guinea pigs at our animal facility. Collinear approximation is $Y = 14.899X - 13.054$ and coefficient of determination $R^2 = 0.9655$. (C) Upper panel: normal distribution of daily body weight change at day 2 and 3. Lower panel: cumulative distribution of daily body weight changes at day 2 and 3.

initially declined in several vaccine-treated guinea pigs: -5.098 g (influenza vaccine; Flu-HA), -4.704 g (adsorbed diphtheria-purified pertussis-tetanus combined vaccine, DPT), -9.734 g (Japanese encephalitis vaccine, JEV), -6.074 g (recombinant adsorbed hepatitis B vaccine, HBV), -3.633 g (adsorbed tetanus toxoid, TT), -6.164 g (adsorbed diphtheria-tetanus combined vaccine, ADCT), and -38.813 g (pneumococcal vaccine polyvalent, PCV) (Fig. 2C–J). At day 2, body weights increased to reach and exceed the initial body weights in the Flu-HA-, DTP-, JEV-, HBV-, TT- and ADCT-treated guinea pigs. Body weights continued to decline at day 2 only in the PVP-treated guinea pigs (-44.375 g), and although body weights had increased by day 3, they had still not reached the initial body weight. At day 7, body weights had increased and recovered to the initial body weights in the PVP-treated guinea pigs. These trends in body weight changes followed a specific pattern with an initial reduction in body weight followed by an increase to the original body weight, with the increase being almost linear. Body weight changes at day 1 for various vaccines are summarized in Fig. 2J. Using the body weight curve data for each vaccine of at least 50 batches of vaccine, we constructed standard body weight change values for each vaccine. The population of animals

with these values are known as the “reference population” (RP) (Table 1). Deviations from RP values were calculated by z-test, and if the values are within the reference range ($P < 0.01$) and no pathological signs were evident, we judged that the test specimen passed ATT. If the body weights fell below the reference values, ATT was repeated twice more before final judgment.

3.3. Pathological changes in vaccine-treated guinea pigs

Pathological changes, such as hemorrhagic ascites (Fig. 3A) and inflammation of the pancreas (Fig. 3B, C) were seen with guinea pigs receiving DPT, aluminum adsorbed vaccine and toxoid. Such changes were associated with a significant decrease of the body weight in comparison with RP ($P < 0.01$). However, the pathological effects varied among different batches of vaccines. The statistical analysis of ATT results improved the identification of pathological changes in the liver, spleen and pancreas. We measured the number of leukocytes (Fig. 3F), pancreas (Fig. 3G) and spleen weights (Fig. 3H), the area of focal necrosis in the liver (Fig. 3I) and the area of aluminum deposition in the pancreas (Fig. 3J), using more than 10 batches of each vaccine. These

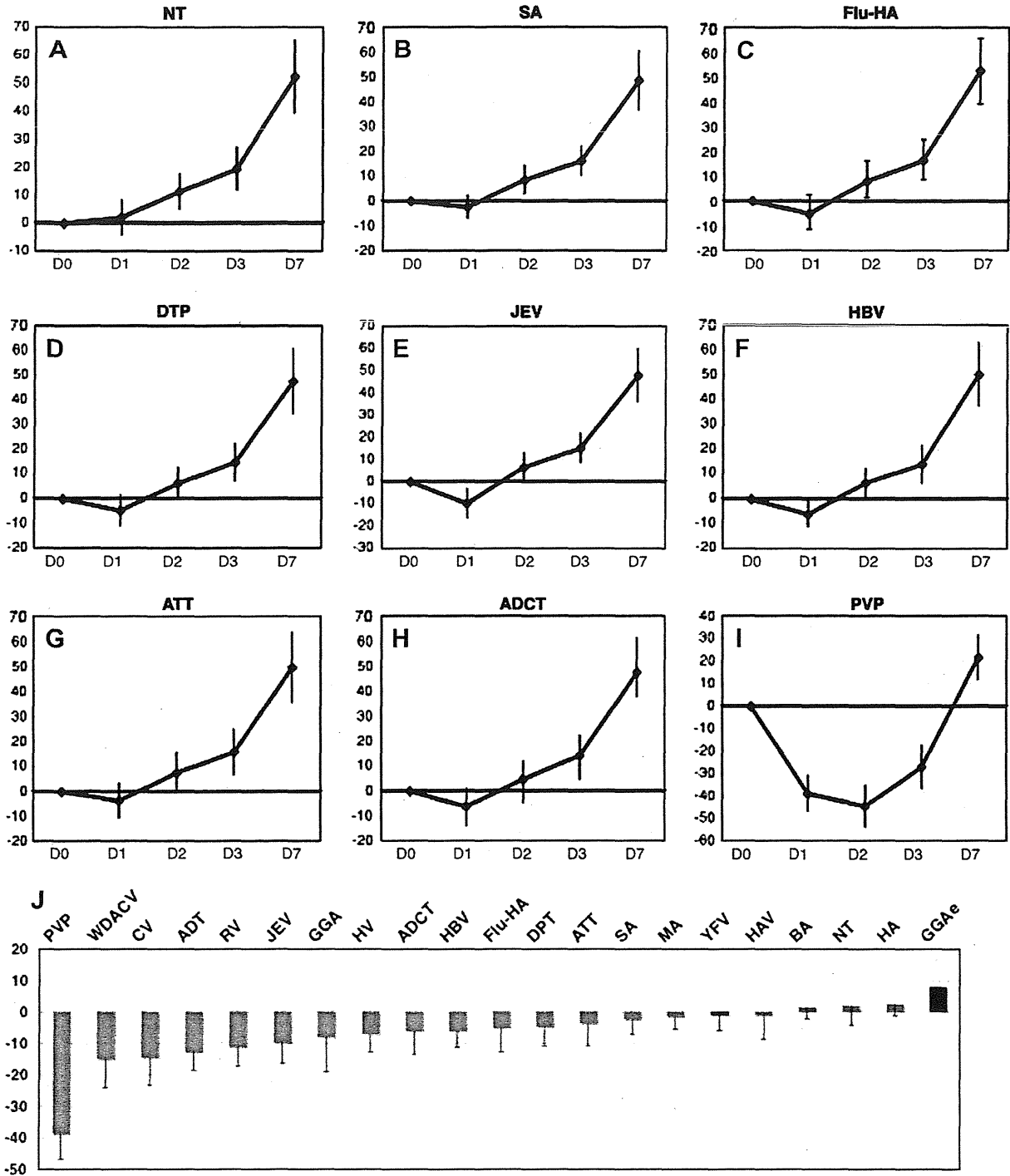


Fig. 2. Effects of several vaccine treatments on guinea pig body weight. Body weight changes after vaccination. (A) non-treated (NT) and (B) saline (SA)-treated animals as a control. (C) influenza HA vaccine (Flu-HA), (D) adsorbed diphtheria-purified pertussis-tetanus combined vaccine (DTP), (E) Japanese encephalitis vaccine (JEV), (F) hepatitis B vaccine (HBV), (G) adsorbed tetanus toxoid (TT), (H) adsorbed diphtheria-tetanus combined toxoid (ADCT), (I) pneumococcal vaccine polyvalent (PVP). (J) Body weight changes at day 1. WDACV, Weil's disease and Akiyami combined vaccine; CV, cholera vaccine; RV, rabies vaccine; GGA, freeze-dried gas gangrene antitoxin, equine; HV, adsorbed habu-venom toxoid; MA, freeze-dried mamushi antivenom, equine; HAV, freeze-dried inactivated tissue culture hepatitis A vaccine; BA, freeze-dried botulism antitoxin, equine; HA, freeze-dried habu antivenom, equine; GGAE, gas gangrene antitoxin, equine.

pathological changes were characteristic to each vaccine, and occurred not only in animals receiving the re-tested and rejected vaccines, but also in the passed vaccines, especially in the DPT, aluminum adsorbed vaccine and toxoid. The degree

of pathological change varied, though no significant differences were found between controls and the test vaccines ($P < 0.05$). These data indicate that the pathological changes were due to the reactogenicity of the vaccines, rather than their

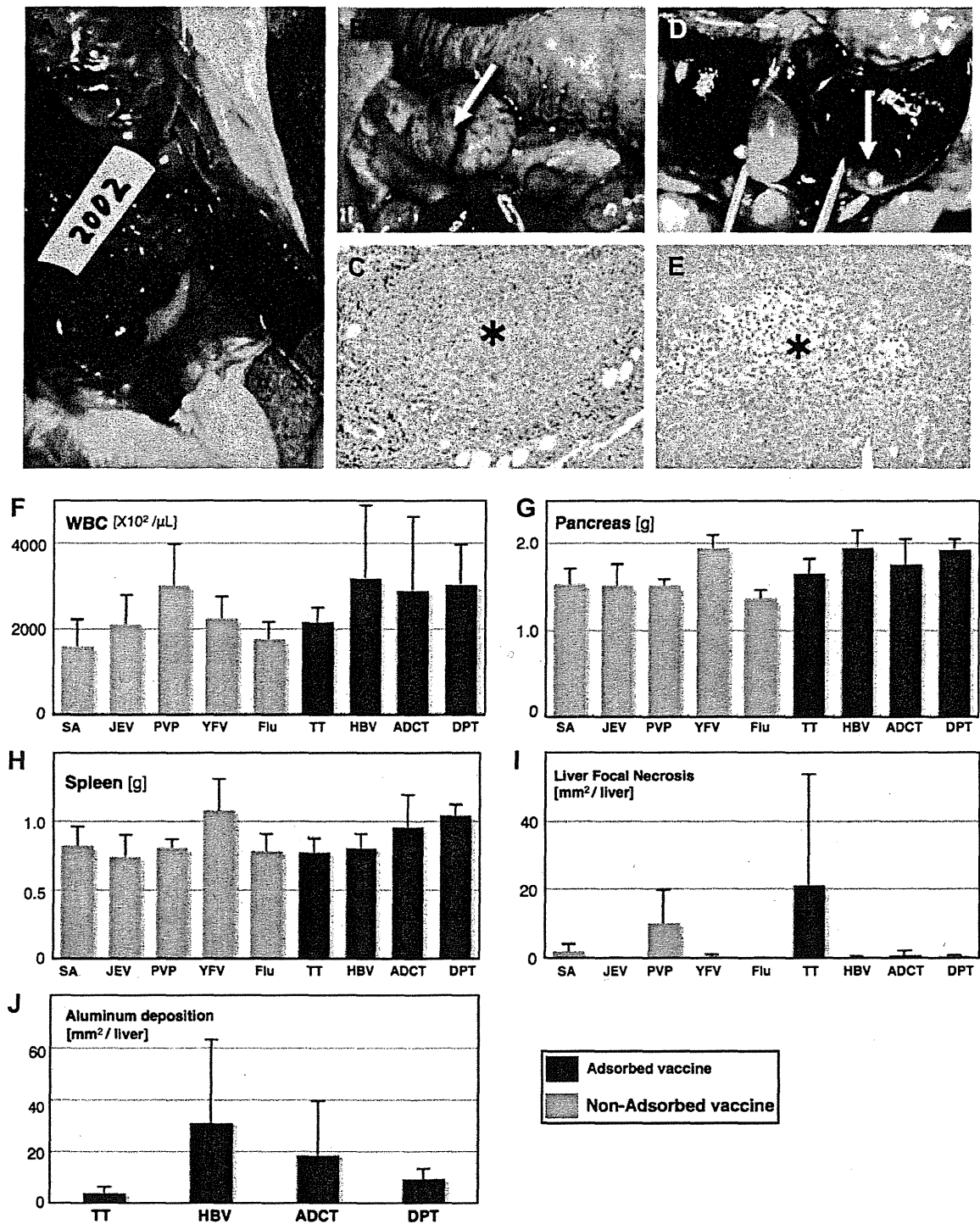


Fig. 3. Histopathological analysis in the guinea pig after vaccination. (A) Specific toxic and pathological changes, such as hemorrhagic ascites in the abdomen of the DPT-treated guinea pigs at day 7. (B) Inflammation in the DPT-treated pancreas at day 7. (C) Histological analysis of inflammation in the DPT-treated pancreas by hematoxylin and eosin staining (H&E). (D) Focal necrosis in the DPT-treated liver at day 7. (E) Histological analysis of focal necrosis in the DPT-treated liver. H&E staining. (F) The number of leukocytes in the peripheral blood after vaccination at day 7. (G) Pancreas weight after vaccination at day 7. (H) Spleen weight after vaccination at day 7. (I) The area of focal necrosis in the vaccine-treated liver (mm^2). (J) Total area of aluminum deposition in the pancreas (mm^2). NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent.

toxicity. These histopathological reference data will help us to define the criteria for abnormal toxicity levels in histopathological analyses.

3.4. Effects of animal grade change on ATT

To improve the quality of histopathological analyses, 'clean' animals need to be substituted by SPF animals. Our body weight RP was based on 'clean' animals, and we needed to determine the effect of changing the animal grade on the results of ATT. To validate the reliability and reproducibility of our reference population based on 'clean' animals, we focused on the vaccines (Flu-HA, DTP, YFV, JEV, HBV, TT, ADCT, PVP) for which a RP of body weights and histopathological features had already been established and confirmed in our current ATT.

Compared with the RP based on 'clean' guinea pigs, no significant differences in body weight changes were observed in NT, SA, Flu-HA, DTP, JEV, HBV, TT, ADCT and PVP-treated

SPF guinea pigs ($P < 0.01$). Strong correlations between body weight changes in the 'clean' and SPF-based RPs were demonstrated (Fig. 4). Using the SPF-based RP, the conclusions from ATT based on the 'clean' RP were not changed.

3.5. Effects of animal grade change on pathological changes

To validate our modified ATT, we compared the data obtained with clean animals with those obtained with SPF animals. No significant differences in body weight changes were observed in NT, SA, Flu-HA, DTP, JEV, HBV, TT, ADCT and PVP-treated SPF guinea pig ($P < 0.01$) (Fig. 4). Pathological changes were similar for the both group injected with JEV, PVP, YFV, Flu-HA, TT, HBV, ADCT and DTP. The WBC (Fig. 5A), the pancreas (Fig. 5B) and spleen weights (Fig. 5C), and the area of focal necrosis in the liver (Fig. 5D) and area of aluminum deposition (Fig. 5E) were similar in both sets of animals ($P < 0.05$).

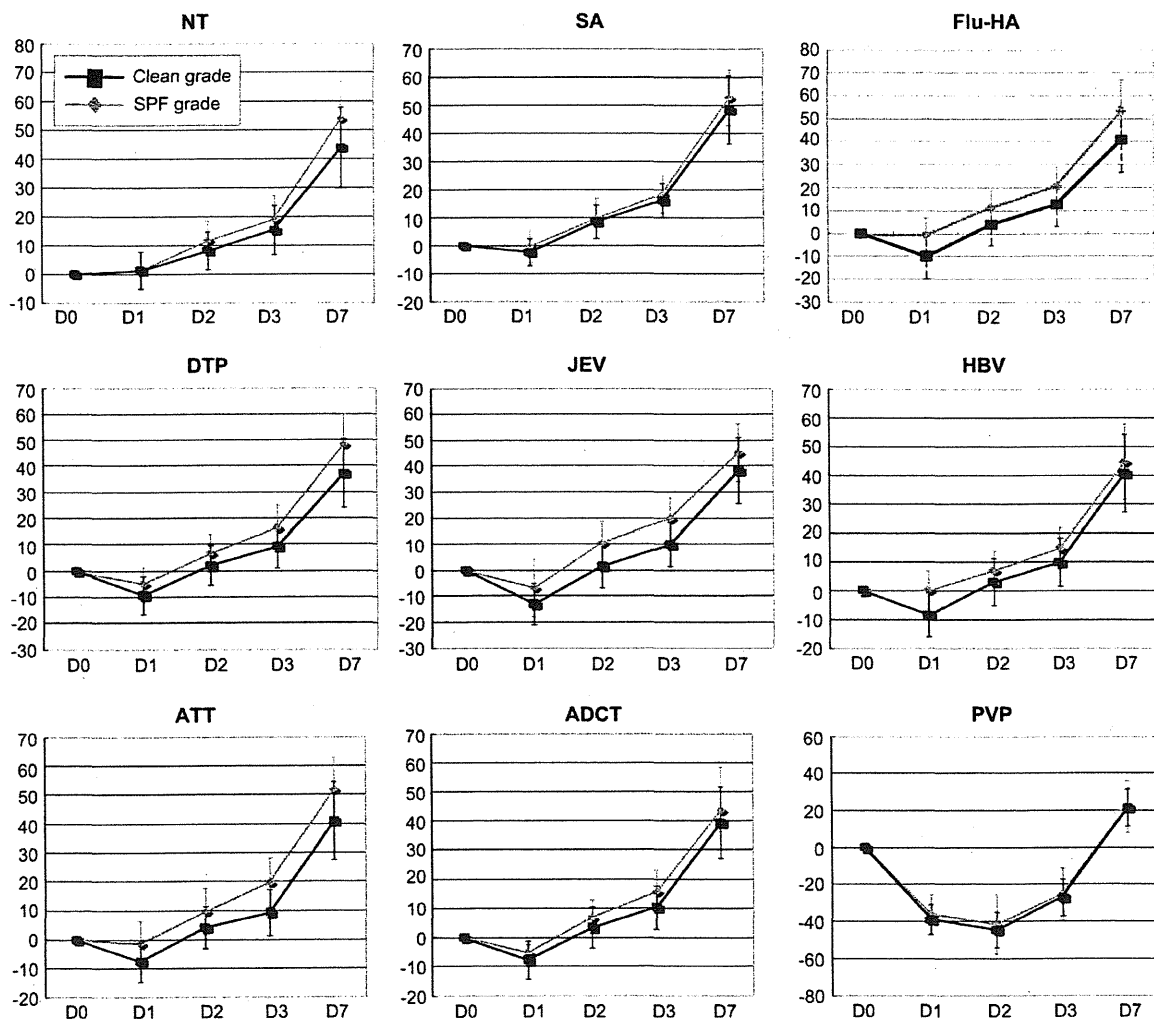


Fig. 4. Effects of animal grade changes from 'clean' to specific pathogen-free (SPF). The body weight changes after vaccination in the SPF reference group were similar to those in the 'clean' reference group. A strong correlation in body weight change was observed between the 'clean' and SPF-based reference populations. NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent.

3.6. Proposal of improved ATT (Fig. 6)

The diagram of the improved protocol for ATT is shown in Fig. 6. It has two steps: first, arriving animals weighing 280–300 g were monitored for 7 days and only healthy animals are used for ATT. The animals are then randomly allocated to

control and vaccine-treated groups, each consisting of two animals. After vaccine injection, the body weight is monitored for 7 days. If there is no significant deviation ($P < 0.01$) from RP values in Table.1, vaccines are considered to have passed the test. If a significant difference is found, histopathological analyses should be performed and the test should be repeated

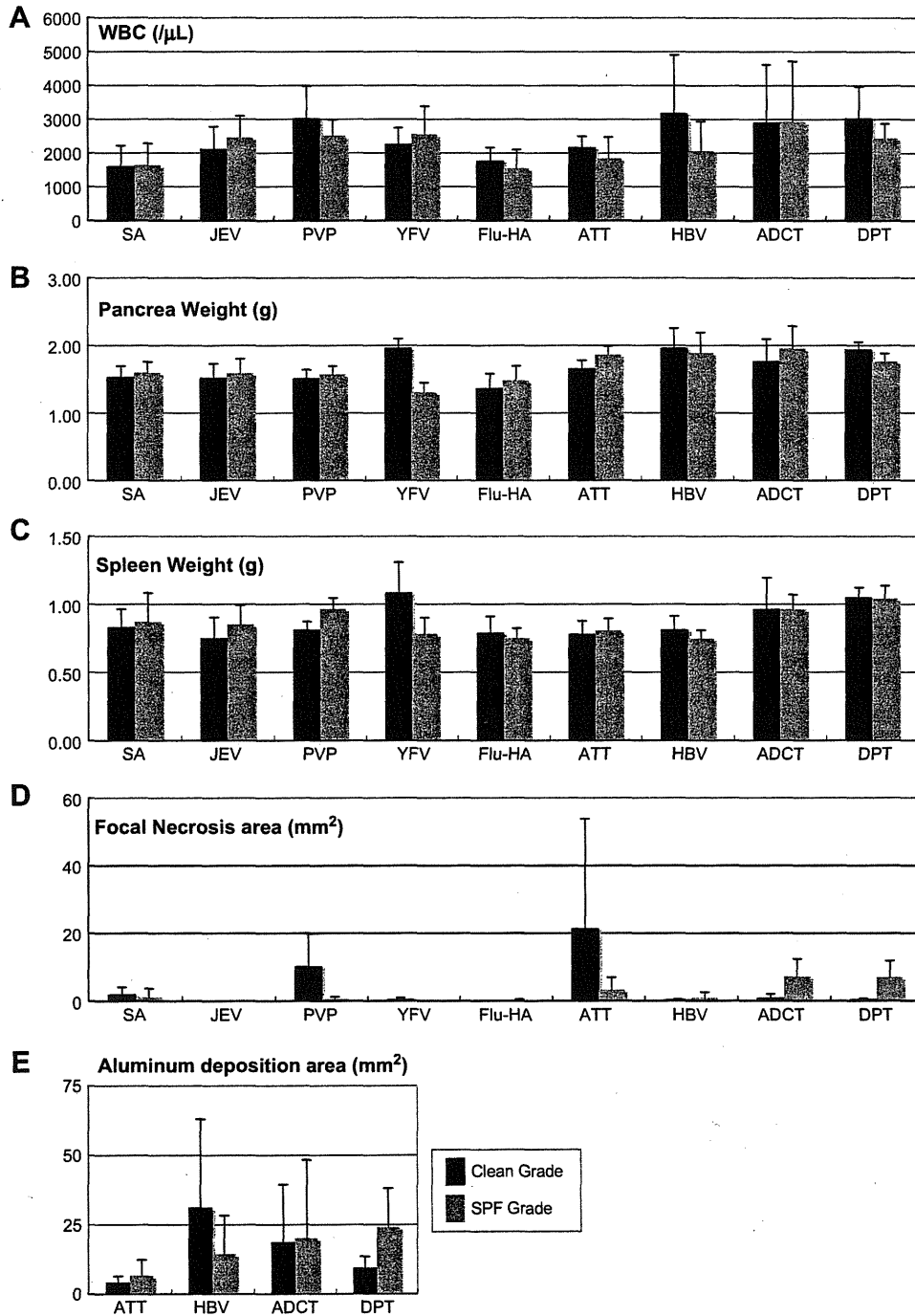


Fig. 5. Comparison of histopathological data between 'clean' and specific pathogen-free (SPF) reference groups. (A) The number of leukocytes, (B) pancreas weight, (C) spleen weight, (D) the area of focal necrosis in the liver, (E) total area of aluminum deposition in pancreas were similar in the 'clean' and SPF-based reference populations. NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent; YFV, yellow fever vaccine.

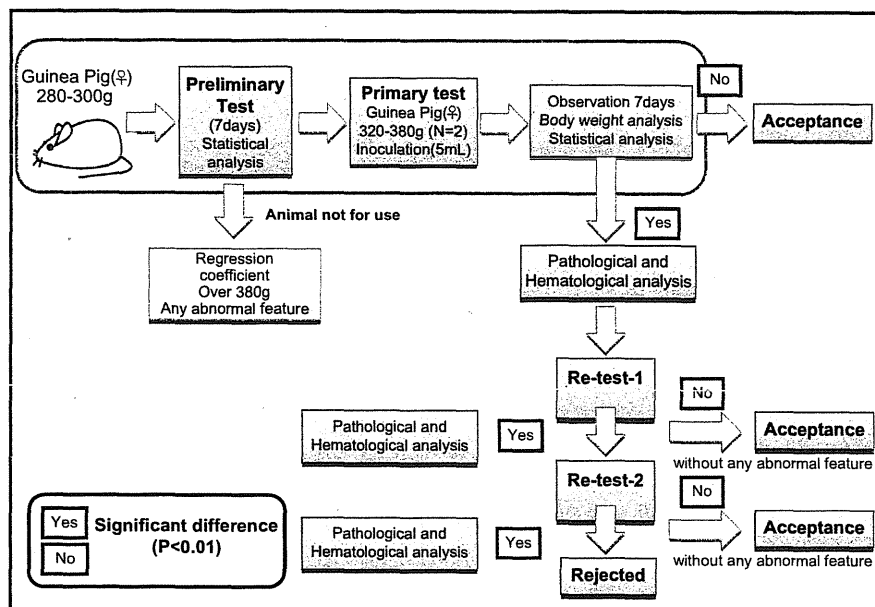


Fig. 6. Criteria of Japanese abnormal toxicity test.

at least twice. If, after two re-tests, the results are still significantly different, the vaccine is considered to have failed.

4. Discussion

ATT is performed as a safety test, in accordance with the WHO guidelines [3]. These tests ensure that the minimum requirements for vaccine safety and consistency between vaccine batches are met in Japan [4] and in other countries [5]. In Japan, tests performed by the National Regulatory Authority are important for ensuring the minimum safety requirements for vaccines. In this study, we improved the current ATT by using combined statistical and histopathological analyses and by comparing the results with RPs based on previously-passed vaccines. Since introducing these new methods to ATT, the number of re-tests has decreased and the number of animals used in ATT for DPT has been reduced from 3 to 2. Using statistical and histopathological analyses, the accuracy of the test has been improved. These test methods will enhance vaccine safety and quality control. Our method can be easily applied and is useful for vaccine manufacture by constructing a vaccine specific reference population based on the currently passed 50 lots of vaccines. Together with our data, these methods will help to understand internal quality control and future validation study for manufactures.

In the EU, in order to conform to the '3R' principle [13], animal safety tests for vaccines, sera and immunoglobulins were abolished after the introduction of Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP) principles. In the US, however, the general safety test is still required by the FDA for vaccines for human use. Whether or not ATT is relevant for determining the safety of vaccines is an ongoing discussion [14]. In the US, whole blood, red blood cells, cryoprecipitated antihemophilic factor (AHF), platelets, plasma, and cellular therapy are not required to pass ATT and

are described in US the Code of Federal Regulations (CFR) (21 CFR 610.118) [15]. Recently, the FDA amended the 21 CFR 610.118(g), such that manufacturers can now submit a request for exemption from ATT requirements to the director of the Center for Biologics Evaluation and Research (CBER), if they believe that their product is not appropriate for ATT, or if they can demonstrate alternative methods to ensure the safety of their products [5].

In Japan, ATT are aimed at ensuring the safety and consistency of vaccine batches. It has been suggested that adsorbed toxoid [16] and DPT [17] cause specific histopathological effects in treated animals. We proposed the aforementioned modified protocol of ATT, whose result was not affected by using the clean animal in place of SPF animals. ATT with additional statistical and histopathological analyses should therefore be the first choice for determining vaccine safety and quality.

The use of ATT for blood products was abolished in Japan in 2005, more than 10 years after the introduction of GMP regulations covering these products. This abolition resulted in an 80% reduction in animal use for ATT in Japan (data not shown). This refinement [18] in the use ATT is consistent with the spirit and concept of the '3R' principle [13].

It has been suggested that ATT cannot detect target substances causing adverse events and body weight changes. Although the mechanism causing body weight change is still unclear, Hamaguchi et al. reported that the reactogenicity and toxicity of pertussis vaccine in the rat was strongly correlated with global gene expression patterns in the liver, and they identified biomarkers relating to the pertussis vaccine-related toxicity [19]. Mizukami et al. also reported that global gene expression patterns were strongly correlated with ATT and the other current regulatory test, the leukopenic toxicity test, performed in Japan [20]. It has been suggested that reductions in body weight were due to nutritional changes caused by

changes in water consumption by animals after vaccination [21]. The two gene profiles established following pertussis vaccine and influenza vaccine treatment were different, suggesting that body weight reduction occurred as a result of changes in the expression of different genes in animals treated with different vaccines. Furthering improvements in ATT is one of the most important roles for the NRA in Japan.

In this study, we have reported on an improved method for ATT, including statistical and histopathological analyses. It based on the observation that there is body weight change characteristic to each vaccine, and such standardized changes can be used as references for evaluating test vaccines. Our improved method can both evaluate the degree of vaccine toxicity itself and lot-to-lot difference in vaccine. These improvements will help to ensure the safety and quality of vaccines.

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Identification of a site-specific tyrosine recombinase that mediates promoter inversions of phase-variable *mpl* lipoprotein genes in *Mycoplasma penetrans*

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Mycoplasma penetrans has the ability to change its surface lipoprotein profiles frequently. The P35 family lipoproteins encoded by the *mpl* genes are key players in this profile variation. The *M. penetrans* HF-2 genome has 38 *mpl* genes that form three gene clusters. Most of these *mpl* genes have an invertible promoter sequence that is responsible for the ON/OFF switching of individual *mpl* gene expression. Here, we identified the recombinase that catalyses inversions of the *mpl* gene promoters. We focused on two open reading frames of the *M. penetrans* HF-2 genome, namely MYPE2900 and MYPE8180, which show significant homology to the tyrosine site-specific recombinase (Tsr) family proteins. Since genetic tools for *M. penetrans* are still not developed, we cloned the MYPE2900 and MYPE8180 genes and expressed them in *Mycoplasma pneumoniae* and *Escherichia coli*. The promoter regions of the *mpl* genes [*p35* (MYPE6810) or *p42* (MYPE6630) genes] were also introduced into *M. pneumoniae* and *E. coli* cells expressing MYPE2900 or MYPE8180. Inversion of these promoters occurred in the presence of the MYPE2900 gene but not in the presence of the MYPE8180 gene, indicating that the MYPE2900 gene product is the recombinase that catalyses *mpl* gene promoter inversions. We used a PCR-based method to detect *mpl* promoter inversion. This method also enabled us to detect inversions of 10 *mpl* gene promoters in *M. penetrans* HF-2 cells. All these promoter inversions occurred at the 12 bp inverted repeat (IR) sequences flanking the promoter sequence. The consensus sequence of these IRs was proposed as TAAYNNNDATTA (Y=C or T; D=A, G or T).

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INTRODUCTION

Mycoplasmas belong to a group of bacteria with no cell wall and have the minimum range of genome sizes that are necessary for self-replication. Their small genomes (580–1350 kb) sequenced to date lack numerous genes required for biosynthetic pathways, thus reflecting their parasitic lifestyle with a dependence on host organisms for nutrient acquisition. Mycoplasmas usually inhabit the mucosal tissues of specific host organisms. Almost 200 mycoplasma species have been isolated from a wide range of host

organisms, including humans. Several of these species are well recognized as pathogens (Sasaki, 2006; Waites *et al.*, 2005). As parasitic bacteria, mycoplasmas can continue to colonize their host even in the presence of specific immune responses. The molecular mechanisms responsible for this immune evasion are not fully understood; however, a number of recent studies have suggested probable mechanisms for continuous infection of mycoplasmas; these include molecular mimicry of host cell components (Jacobs *et al.*, 1995), modulation of host immunity by mycoplasmal cell components (Rottem, 2003), invasion of host cells (Baseman *et al.*, 1996) and generation of surface antigen variants of mycoplasmas (Denison *et al.*, 2005; Rosengarten *et al.*, 2000). Among these strategies, surface antigenic variation is a commonly observed phenomenon in many mycoplasma species (Citti *et al.*, 2005; Yogev *et al.*, 2002). Surface variations may play important roles in interaction between mycoplasmas and host cells during

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Abbreviations: Tsr, tyrosine site-specific recombinase; Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; IR, inverted repeat.

Two supplementary tables, listing the oligonucleotides used in this study, are available with the online version of this paper.

infection. In most of the known cases, the variable surface molecules of mycoplasmas are lipoproteins. Depending upon the species, these lipoproteins are encoded by single or multiple genes and undergo frequent phase and size variation during mycoplasma growth (Citti *et al.*, 2005). Various genetic mechanisms are used to modulate the expression of these lipoprotein genes. These mechanisms include DNA rearrangements (Glew *et al.*, 2002; Shen *et al.*, 2000), nucleotide insertions and deletions in the gene regions (Glew *et al.*, 1998; Yogev *et al.*, 1991), gene conversions (Noormohammadi *et al.*, 2000), and site-specific DNA recombinations (Chopra-Dewasthaly *et al.*, 2008; Lysnyansky *et al.*, 2001). Characterization of these genetic mechanisms may provide us with not only a detailed understanding of the surface antigenic variation mechanism of mycoplasmas but also an insight into the survival strategy of minimalist bacteria through utilization of their small genomes.

Mycoplasma penetrans was first isolated from a urine sample of a human immunodeficiency virus (HIV)-infected patient (Lo *et al.*, 1992). It was also isolated as a potential aetiological agent from a primary antiphospholipid syndrome patient without HIV infection (Yáñez *et al.*, 1999). Although the pathogenicity of *M. penetrans* to humans remains questionable, the characteristics of this bacterium (i.e. invasion of eukaryotic cells, toxicity to chick embryo, and haemolytic and haemoxidative activity) (Girón *et al.*, 1996; Kannan & Baseman, 2000; Lo *et al.*, 1993) suggest its potential pathogenicity to humans. *M. penetrans* also has the ability to change its surface antigenicity frequently by changing the expression pattern of the P35 family lipoproteins (Neyrolles *et al.*, 1999; Röske *et al.*, 2001). The P35 family lipoproteins are encoded by the *mpl* genes and, thus far, 38 *mpl* genes have been found in the *M. penetrans* HF-2 genome. Of these 38 genes, 30 form a large gene cluster in a 50 kb region of the genome (Horino *et al.*, 2003; Sasaki *et al.*, 2002). *M. penetrans* uses a unique mechanism to modulate the expression of these *mpl* genes. Most of the *mpl* genes have an independent promoter for their expression. These promoter sequences are present in approximately 135 bp DNA regions and are flanked by 12 bp inverted repeats (IRs). Inversion of these promoters causes ON/OFF switching of individual *mpl* genes. The invertible promoter elements also contain unique sequences that form a terminator-like structure depending on the direction of promoters (see Fig. 4a). This terminator-like structure may serve to prevent readthrough transcription from preceding genes or antisense transcription from OFF configuration promoters (Horino *et al.*, 2003). Thus, the *mpl* genes have unique genetic switches, which possess promoter and terminator functions in the short DNA region. To further characterize these unique genetic switches and understand the nature of antigenic variation of *M. penetrans*, in this study we attempted to identify the factors involved in *mpl* gene promoter inversions. For this purpose, we reconstructed the *mpl* promoter inversion system in *Mycoplasma pneumoniae* and *Escherichia coli* cells.

METHODS

Bacterial strains and culture conditions. *M. penetrans* strain HF-2 (Yáñez *et al.*, 1999) and *M. pneumoniae* strain M129 (Lipman *et al.*, 1969) were cultured in PPLO medium at 37 °C as described previously (Horino *et al.*, 2003; Kenri *et al.*, 2004). Transformation of *M. pneumoniae* M129 with the staphylococcal transposon Tn4001mod vectors was performed by the electroporation method (Hedreyda *et al.*, 1993). *M. pneumoniae* transformants were selected in PPLO liquid medium containing 18 µg gentamicin (Gm) ml⁻¹ or 15 µg chloramphenicol (Cm) ml⁻¹. Genomic DNAs of the mycoplasma strains were extracted by the QIAamp mini kit (Qiagen) and were used as templates for PCR to construct the plasmids. *E. coli* strains were grown in Luria-Bertani (LB) medium with or without 50 µg ml⁻¹ of ampicillin (Ap), kanamycin (Km) or spectinomycin (Sp), or 15 µg Cm ml⁻¹ to select the plasmid markers.

Cloning of the p42 and p35 promoter regions. PCR primers used for cloning are listed in Supplementary Table S1 (available with the online version of this paper). An approximately 3.2 kb region containing the *p42 mpl* gene (MYPE6630) and its invertible promoter region (see Figs 1a and 5a) was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers p42-F and p42-R. To minimize mutations caused by PCR amplification, a high-fidelity DNA polymerase (PyroBest; TaKaRa) was used. The corresponding region of the *p35 mpl* gene (MYPE6810) was also amplified from *M. penetrans* HF-2 by PCR with primers p35-F and p35-R. The amplified *p42* and *p35* regions were inserted into the pENTR/D-TOPO plasmid by using the TOPO cloning system according to the manufacturer's instructions (Invitrogen), resulting in plasmids pAH501 and pAH502 (Table 1). The cloned *p42* and *p35* promoter sequences on these plasmids were in the ON direction. To introduce the *p42* promoter sequence into *M. pneumoniae* cells, the cloned *p42* region on plasmid pAH501 was transferred to the *Sma*I site of the Tn4001mod vector plasmid pISM2062.2 (Knudtson & Minion, 1993) by using the Gateway cloning technique (Invitrogen). Briefly, pISM2062.2 was converted into the Gateway destination vector by inserting the Gateway vector conversion system (reading frame cassette A) at the *Sma*I site. The *p42* sequence of the pAH501 plasmid was transferred to the pISM2062.2 destination vector by the LR reaction of the Gateway system. The resulting plasmid was designated pAH511 (Table 1) and introduced into *M. pneumoniae* M129 by electroporation. The *p42* and *p35* promoter sequences on the pAH501 and pAH502 plasmids, respectively, were also transferred to the *Sma*I site of the pCL1920 plasmid, which has a pSC101 replicon (Lerner & Inouye, 1990), by using the Gateway cloning system for introduction into *E. coli* cells. These plasmids were designated pAH521 and pAH522, respectively (Table 1).

Construction of the MYPE2900 and MYPE8180 expression clones. PCR primers used for plasmid construction are listed in Table S1. MYPE2900 was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers 2900-F-TOPO and 2900-R-Asc. MYPE8180 was also amplified using primers 8180-F-TOPO and 8180-R-Asc. The amplified MYPE2900 and MYPE8180 fragments were inserted into the plasmid pENTR/D-TOPO by using the TOPO cloning system, resulting in plasmids pAH301 and pAH302 (Table 1). The cloned MYPE2900 and MYPE8180 on the pAH301 and pAH302 plasmids were sequenced to confirm that no mutations were incorporated during the PCR cloning. To express MYPE2900 and MYPE8180 in *M. pneumoniae* cells, the *tuf* promoter (*P_{tuf}*) sequence of *M. pneumoniae* was linked to MYPE2900 and MYPE8180. The *P_{tuf}* sequence was amplified from *M. pneumoniae* M129 genomic DNA by PCR using primers *tuf*-F-Bam-TOPO and *tuf*-R-Afl. The amplified *tuf* promoter fragment was digested with *Bam*HI and *Afl*III and ligated into the *Bam*HI–*Nco*I sites of pAH301 and pAH302, resulting in plasmids pAH303 and pAH304 (Table 1). The MYPE2900 and

Table 1. Plasmids constructed in this study

Plasmid	Vector	Marker	Gene
pAH301	pENTR/D-TOPO	Km ^R	MYPE2900
pAH302	pENTR/D-TOPO	Km ^R	MYPE8180
pAH303	pENTR/D-TOPO	Km ^R	P _{tuf} -MYPE2900
pAH304	pENTR/D-TOPO	Km ^R	P _{tuf} -MYPE8180
pAH311	pKV104 (Tn400I mod)	Ap ^R , Cm ^R	P _{tuf} -MYPE2900
pAH312	pKV104 (Tn400I mod)	Ap ^R , Cm ^R	P _{tuf} -MYPE8180
pAH331mut	pColdI	Ap ^R	P _{cspA} -MYPE2900mut
pAH332	pColdI	Ap ^R	P _{cspA} -MYPE8180
pAH332mut	pColdI	Ap ^R	P _{cspA} -MYPE8180mut
pAH501	pENTR/D-TOPO	Km ^R	<i>p42</i> and promoter region
pAH502	pENTR/D-TOPO	Km ^R	<i>p35</i> and promoter region
pAH511	pISM2062.2 (Tn400I mod)	Ap ^R , Gm ^R	<i>p42</i> and promoter region
pAH521	pCL1920 (pSC101 replicon)	Sp ^R	<i>p42</i> and promoter region
pAH522	pCL1920 (pSC101 replicon)	Sp ^R	<i>p35</i> and promoter region

MYPE8180 genes on the pAH303 and pAH304 (P_{tuf}-MYPE2900 and P_{tuf}-MYPE8180, respectively) were then transferred to the *Sma*I site of the Tn400I mod vector plasmid pKV104 (Hahn *et al.*, 1999) by using the Gateway cloning technique. The resulting Tn400I mod plasmids pAH311 and pAH312 (Table 1) were used to transform *M. pneumoniae*. To express the MYPE2900 and MYPE8180 genes in *E. coli*, these genes were amplified from *M. penetrans* HF-2 genomic DNA by PCR with the primer pairs 2900-F-SacTGG and 2900-R-Xho or 8180F-Nde and 8180-R-Eco, respectively. The amplified MYPE2900 fragments were digested with *Sac*I and *Xho*I and inserted into the *Sac*I-*Xho*I site of plasmid pColdI (TaKaRa), resulting in plasmid pAH331mut (Table 1). The MYPE2900mut gene on plasmid pAH331mut was sequenced, and the conversion of the codon UGA to UGG was confirmed (nt position 28–30 of the MYPE2900 gene). The amplified MYPE8180 fragments were digested with *Nde*I and *Eco*RI and inserted into the *Nde*I-*Eco*RI site of plasmid pColdI, resulting in pAH332 (Table 1). Two UGA codons present in MYPE8180 sequences (at nt positions 145–147 and 517–519) were converted to UGG by using the GeneTailor site-directed mutagenesis system according to the manufacturer's instructions (Invitrogen). After mutagenesis, the nucleotide sequence of the MYPE8180mut gene was confirmed by sequencing. The plasmid carrying the MYPE8180mut gene was designated pAH332mut (Table 1). Plasmids pAH331mut and pAH332mut were introduced into *E. coli* BL21(DE3) to express MYPE2900 and MYPE8180.

Protein analysis. *E. coli* BL21(DE3) strains harbouring plasmids pAH331mut, pAH332mut or pColdI were grown at 37 °C until the mid-exponential phase. The cultures were maintained at 15 °C for 30 min, and 0.2 mM final concentration of IPTG was added. After the addition of IPTG, the cultures were maintained at 15 °C with shaking for 24 h. *E. coli* cells were collected from a 1 ml volume of the cultures by centrifugation at 20 000 *g* for 2 min and lysed by adding 150 µl of a sample loading buffer for SDS-PAGE. The samples were then subjected to SDS-PAGE at a load of 15 µl per lane. The proteins were visualized by Coomassie brilliant blue staining. For Western blot analysis, the proteins, separated by SDS-PAGE, were transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal antibody specific for His₆-tag (Cell Signaling Technology) was used at a 1:1000 dilution to detect His₆-tagged MYPE2900 and MYPE8180 proteins. The reacting antibodies were detected with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulin G; Promega) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) Colour Development Substrate (Promega) according to the manufacturer's instructions.

PCR detection of *mpl* gene promoter inversion. The PCR primer sets designed for the detection of inversions of the *mpl* gene promoters are listed in Supplementary Table S2. Each primer set consists of three primers [promoter (P), forward (F) and reverse (R) primers]. Two PCRs were performed using these primer sets (with FP primers or RP primers) for each of the corresponding *mpl* gene promoters to detect the inversion event (see Figs 1 and 5). Genomic DNAs from *M. penetrans* HF-2 or *M. pneumoniae* M129-*p42* were analysed with this PCR. The *E. coli* plasmid DNAs carrying the *p42* or *p35* promoter regions were also analysed with this method. Genomic DNAs of *M. pneumoniae* M129-*p42* transformants were extracted from 25 ml of culture by the QIAamp mini kit. The genomic DNAs were diluted and 1 ng of each (about 1 × 10⁶ copies of *M. pneumoniae* M129 genome) was examined by PCR. For purification of *M. penetrans* HF-2 genomic DNA, a single colony of *M. penetrans* HF-2 on a PPLO agar plate [derived from the same culture stock used for genome sequencing (Sasaki *et al.*, 2002)] was picked up and cultured in 2 ml PPLO liquid medium at 37 °C. After growth, 100 µl of the culture was added to 25 ml fresh PPLO liquid medium and cultured until the medium colour changed to orange. Genomic DNA was extracted from 1 ml of the culture by the QIAamp mini kit. One nanogram of the purified genomic DNA (about 6 × 10⁵ copies of *M. penetrans* HF-2 genome) was subjected to the PCR analysis. *E. coli* plasmid DNAs were extracted from 4 ml of the *E. coli* cultures by the QIAprep Spin Miniprep kit (Qiagen). One microlitre of plasmid solution was subjected to PCR examination. The PCR mixture *Premix Ex Taq* Hot Start Version (TaKaRa) was used for the PCR examination, and the PCR was performed under the following conditions: 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2.5 min. The PCR products were analysed by 0.8% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. For sequencing analysis, the PCR products were excised from agarose gels and extracted by using the MinElute gel extraction kit (Qiagen). The extracted DNAs were sequenced by the dye-termination method by using corresponding P, F, or R primers and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

RESULTS

MYPE2900 belongs to the tyrosine site-specific recombinase (Tsr) family and catalyses *p42* promoter inversion in *M. pneumoniae* cells

The gene annotation process for the complete genome sequence of *M. penetrans* HF-2 indicated that two open