

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 hematology analyzer, 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

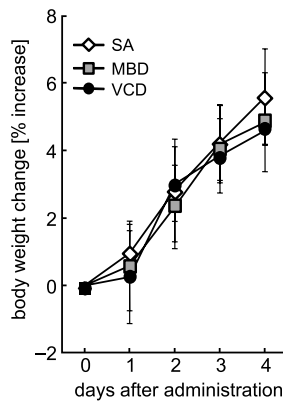


Fig. 1. Body weight analysis of the JE vaccine treated animals. The effects of mouse brain-derived (MBD) JE vaccine, Vero cell-derived (VCD) JE vaccine, and saline (SA) treatment were measured using decreasing body weight toxicity tests. All rats were weighed at days 0, 1, 2, 3, and 4. Changes in rat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

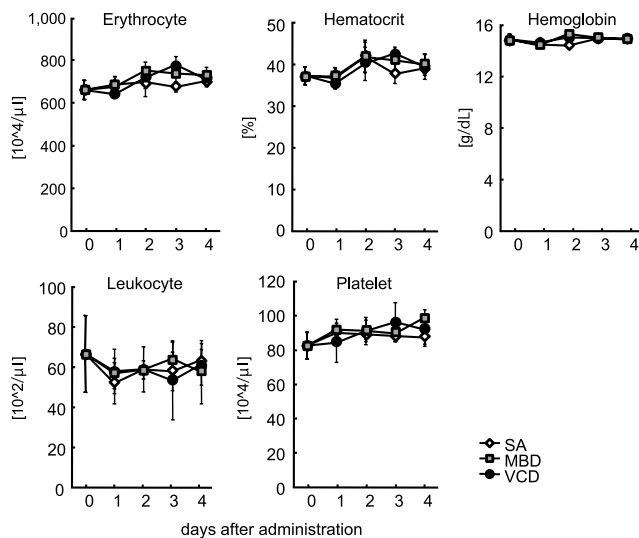


Fig. 2. Hematological tests for vaccinated rats. Blood obtained from individual rats was subjected to hematological tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean \pm S.D.

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 \log_2 values (designated log ratios) and compiled into a matrix designated as the primary data matrix (see Materials and Methods).

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

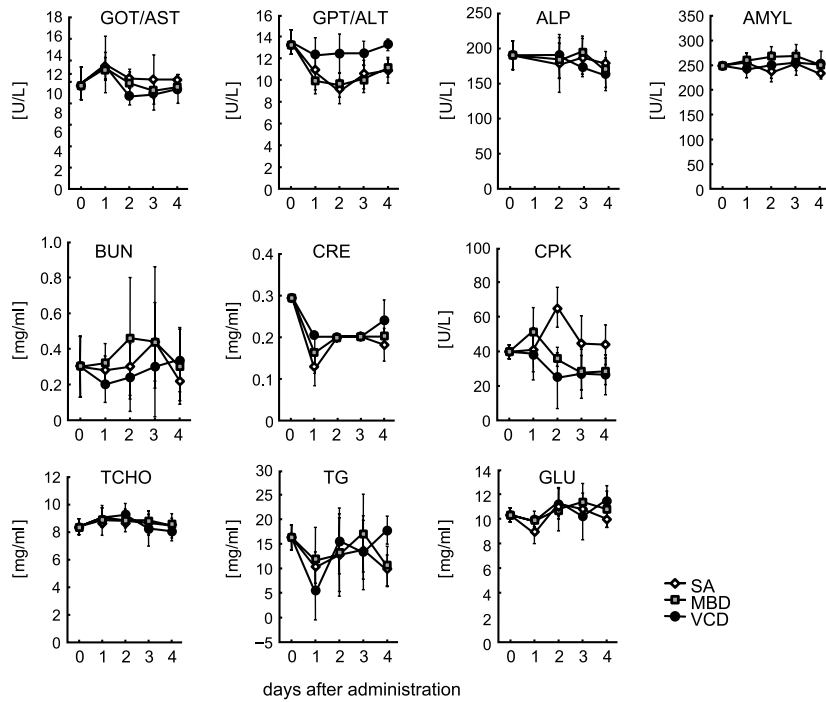


Fig. 3. Serum tests for vaccinated rats. Serum was separated from blood obtained from individual rats, and subjected to serum tests. The tests were performed for 4 consecutive days after SA (open square), MBD (gray square), and VCD (filled circle) administration. Values are expressed as mean \pm S.D.

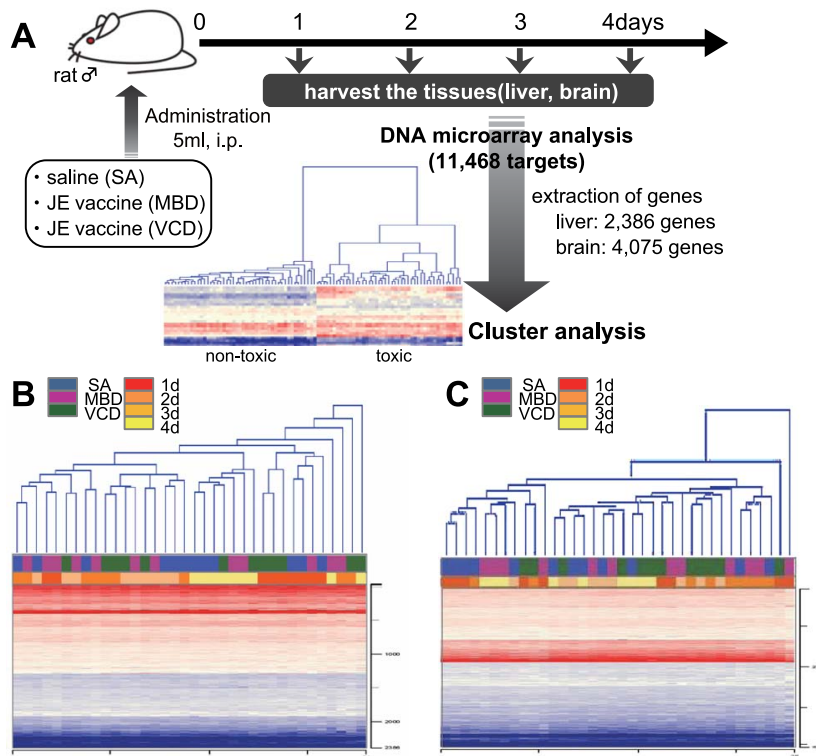


Fig. 4. Gene expression profiling and cluster analysis of vaccine-treated rat tissues. The procedure for gene expression analysis is outlined in A. For the cluster analysis, 2,386 genes for liver (B) and 4,075 genes for brain (C) were extracted from 11,468 targets and assembled in the order obtained 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 Euclid square distance as the dissimilarity coefficient, indicating the relationship between the samples. Red and blue indicate increases and decreases in the expression ratio, respectively.

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

in all vaccine-treated samples.

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

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

DISCUSSION

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

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

To address concern about the reliability of the genomic data obtained from DNA microarray analysis, the FDA recently launched the MicroArray Quality Control (MAQC) project in anticipation of the regulatory submission of pharmacoinformatic and toxicoinformatic data in applications or supplements (33). The results of the MAQC project, showing interplatform reproducibility, were reported in 2006 (40–45). Subsequently, the follow-up MAQC-II project is progressing towards the development and the validation of genomic data in clinical applications. Similarly, in Japan, the Japan MicroArray Consortium (JMAC) for the standardization and the international harmonization of microarray platforms is ongoing and is coordinated with the FDA and the

European Medical Agency (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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