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第 10 回日本ワクチン学会学術集会・平成１８年１０月

2. 水上拓郎、今井順一、浜口功、河村未佳、百瀬暖佳、内藤誠之郎、前山順一、益見厚子、倉光球、滝沢和也、望月雅代、落合雅樹、山本明彦、堀内善信、野村信夫、渡辺慎哉、山口一成
網羅的遺伝子発現解析によるインフルエンザワクチンの新しい安全性評価法開発の試み
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H. 知的財産権の出願・登録状況

１．特許

特願 2006-020432:「百日咳毒素の検出方法」(2006.1.30)（加藤博史、浜口功、山口一成）

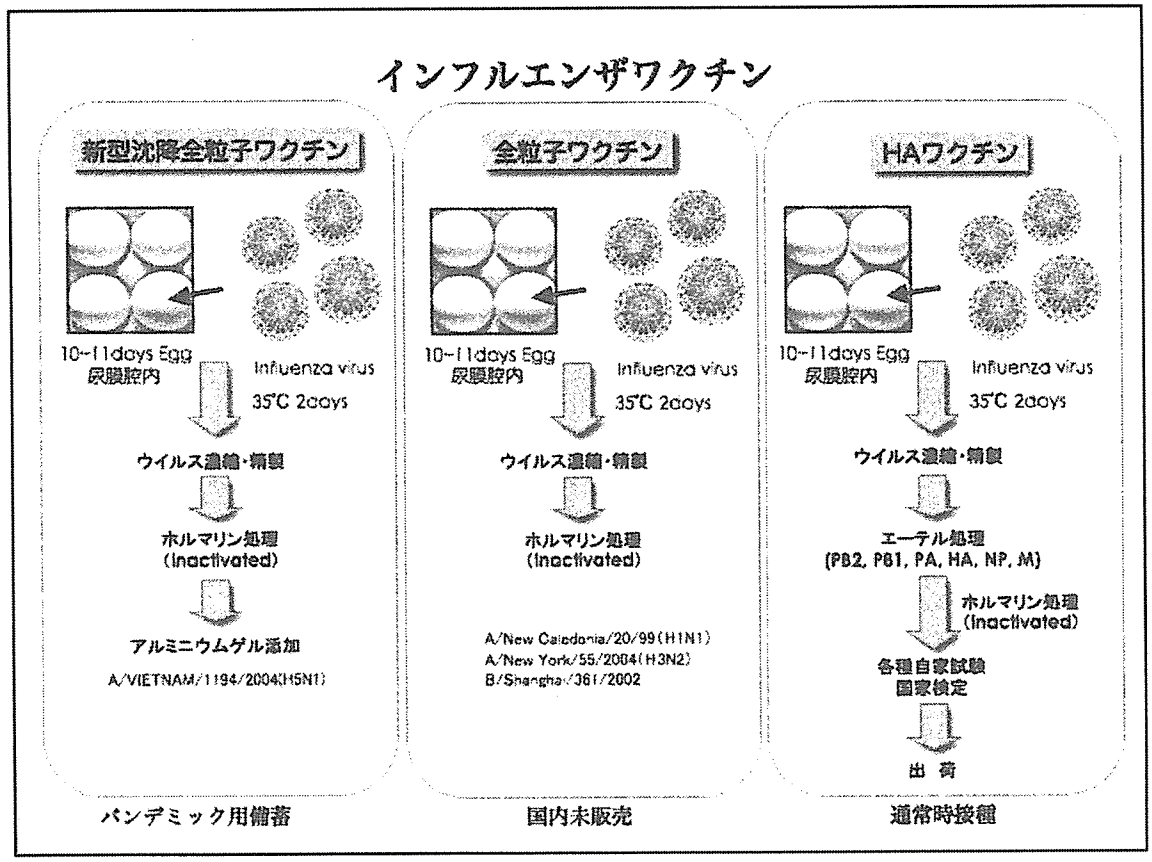

図5：インフルエンザワクチンの製造

現在広く用いられている不活化ワクチン（HA ワクチン）は、ウイルス粒子を濃縮精製し、エーテル処理によって膜脂質成分を取り除き、さらにホルマリンにより不活化したものである。一方全粒子ワクチンはエーテル処理を加えず、ウイルスの膜成分を取り除いていない、さらに新型沈降全粒子ワクチンはホルマリン処理後にアルミニウムゲルの添加が加えられている。

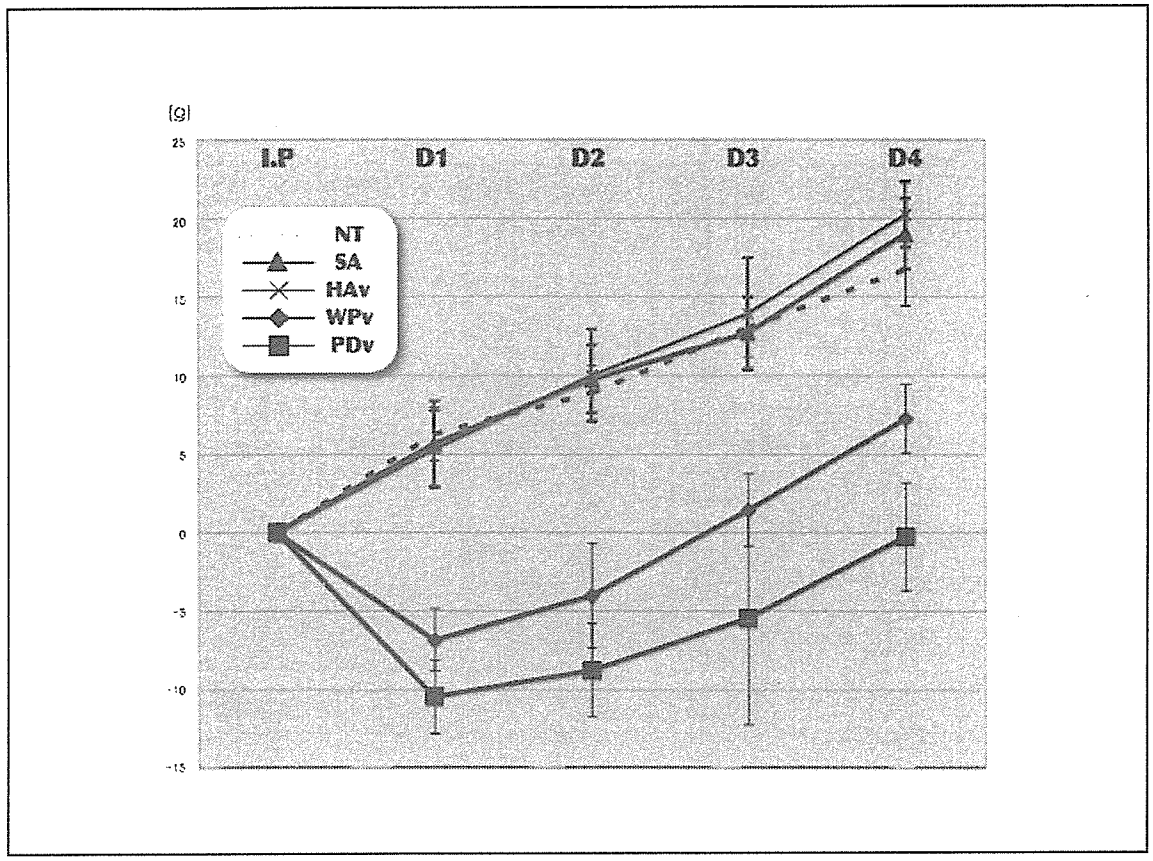

図６：インフルエンザワクチン接種後の体重変化

ラット腹腔に SA（生理食塩水）、HAV（HA ワクチン）、WPV（全粒子ワクシン）および PDV（沈降新型インフルエンザワクチン）を投与し、経時的に体重の変動を測定した。

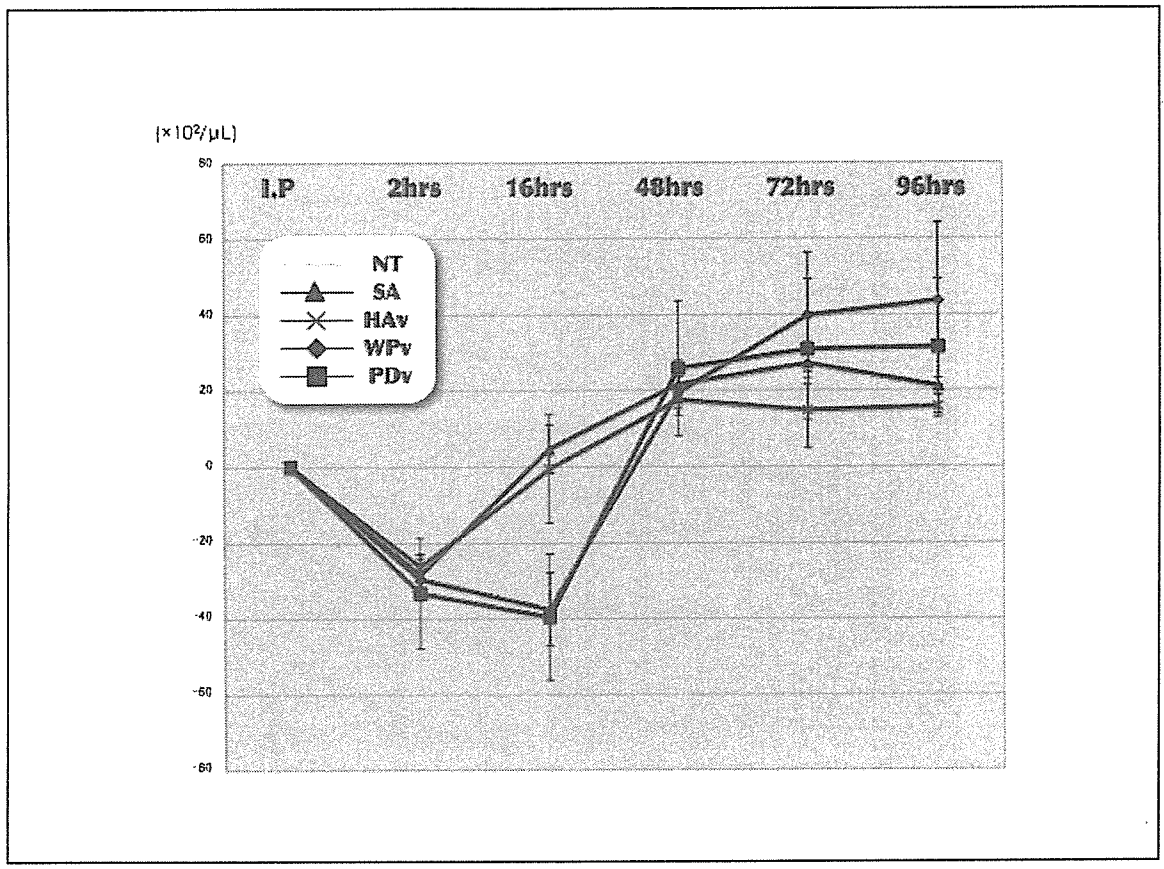

図７：インフルエンザワクチン接種後の白血球数の変化

ラット腹腔に SA（生理食塩水)、HAV（HA ワクチン)、WPV（全粒子ワクシン）および PDV（沈降新型インフルエンザワクチン）を投与し、経時的に末梢白血球数の測定を行なった。。

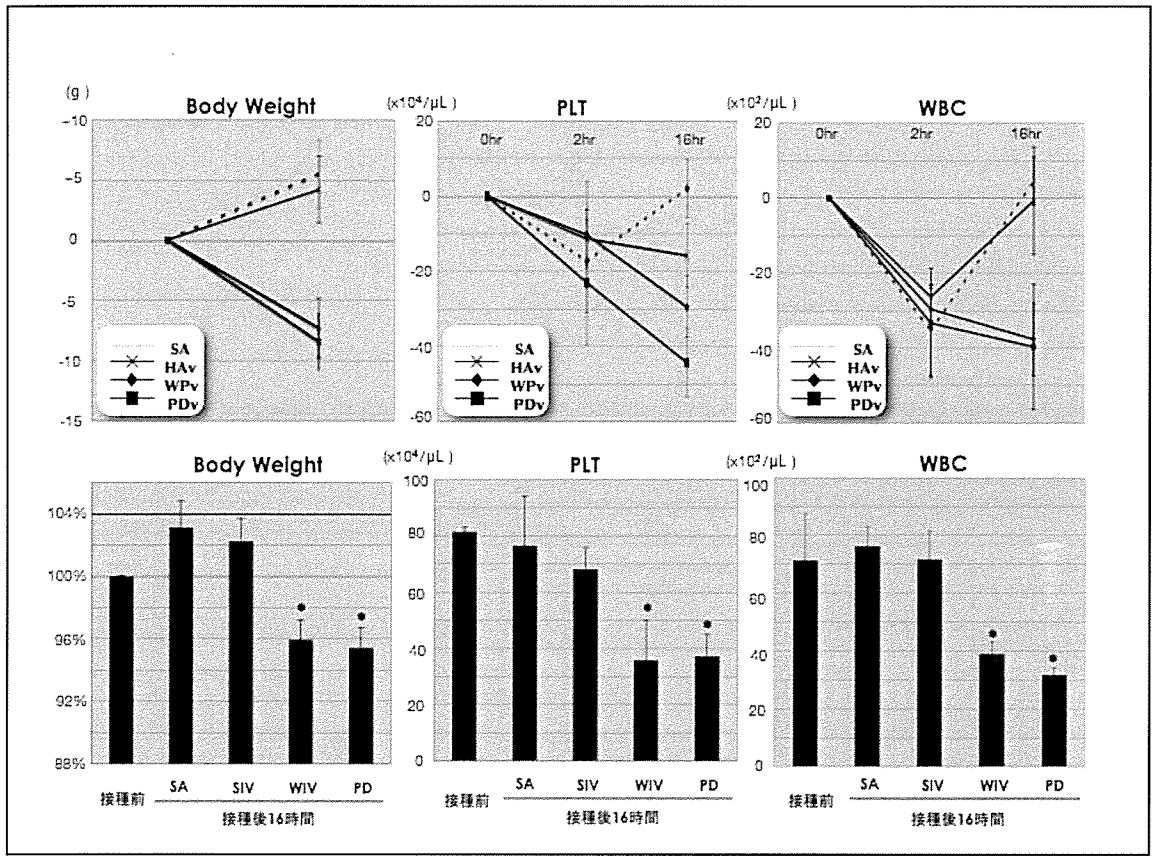

図８：インフルエンザワクチン接種後１６時間の変化

ラット腹腔に SA（生理食塩水)、HAV（HA ワクチン)、WPV（全粒子ワクシン）および PDV（沈降新型インフルエンザワクチン）を投与した。投与後 16 時間までについて、体重、血小板、白血球数の変化を解析した。

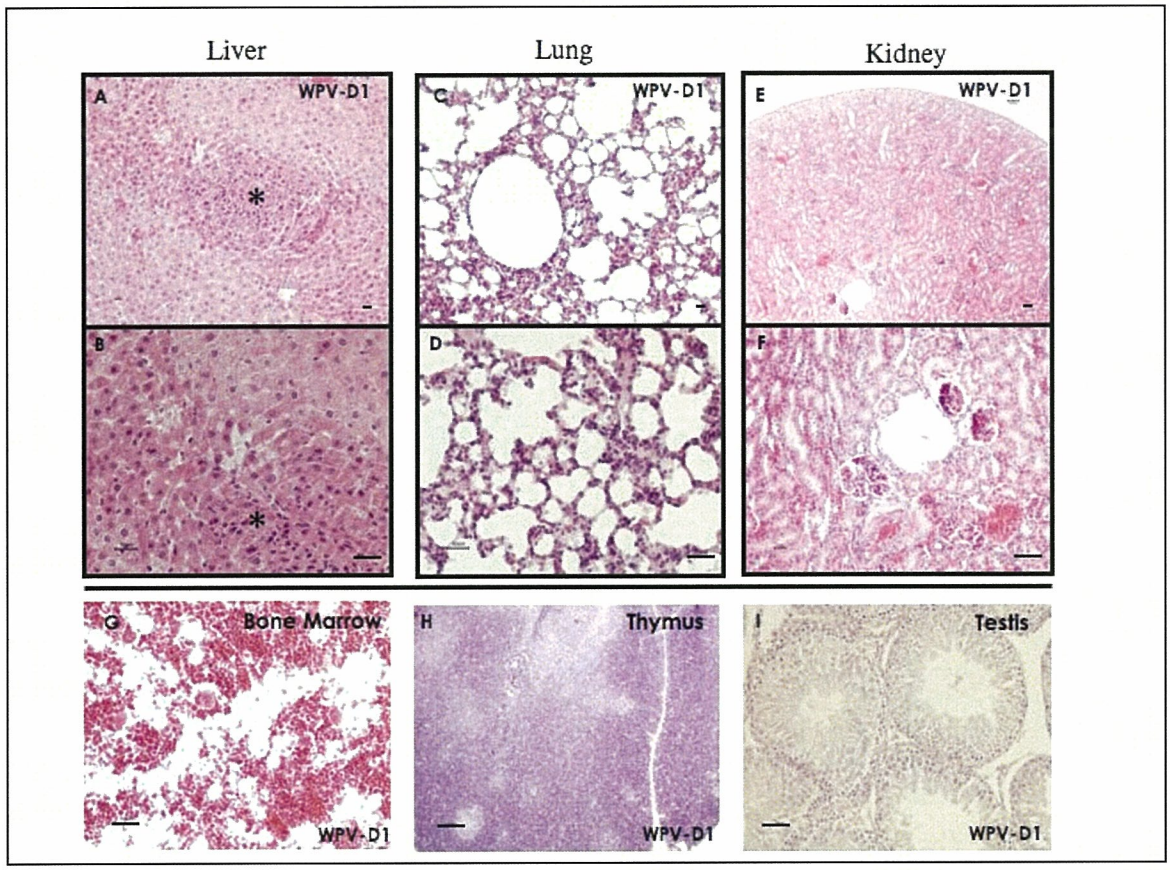

図９：インフルエンザワクチン接種後の病理組織学的変化

ラット腹腔に SA（生理食塩水）および PDV（沈降新型インフルエンザワクチン）を投与した。投与後１日目の肺、肝臓、腎臓、骨髄、胸腺、精巣を HE 染色を用いて組織学的検索を行なった。

厚生労働科学研究費補助金（トキシコゲノミクス研究事業）
（総括・分担）研究報告書

遺伝子発現の網羅的解析によるワクチンの新しい安全性評価に関する研究

研究課題：DNA マイクロアレイクラスター解析によるインフルエンザワクチンの毒性関連遺伝子の同定

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研究要旨

近年、新型インフルエンザの発生に備えて、パンデミックに対するワクチン製造がなされている。この沈降新型インフルエンザワクチンは緊急性が高く、効果を増強するために免疫増強剤（アルミニウムゲル）を通常用いるワクチンとは異なり加えている。このように、主反応に加え副反応が予測されるインフルエンザワクチンに対し、迅速でより科学的な安全性評価法の開発が急務となっている。そこで我々は現在行われている安全性試験に加えて、インフルエンザワクチンの迅速で詳細な安全性評価を可能とする試験法の開発するために、ラットの DNA マイクロアレイを用いた網羅的遺伝子解析を行った。

A. 研究目的

インフルエンザウイルスはオルソミクソウイルス科に属する直径 70-120nm のウイルスで、表面上の赤血球凝集素（HA:Haemagglutinin）とノイラミニダーゼ（NA: Neuraminidase）という２つの糖タンパク質の抗原性の違いによって分類される。インフルエンザはこの抗原変異（連続抗原変異）により短い周期の小規模流行を引き起こし、38 度以上の発熱、頭痛、関節痛、筋肉痛など全身症状を引き起こす。また、HA の大変異によって抗原型が大きく変化すると、パンデミックとなる。20 世紀において H1N1 型（スペイン風邪）、H2N2 型（アジア風邪）、H3N2（ホンコン風邪）、そして再度 H1N1 型（ロシア風邪）の大流行が発生し、1918 年のスペイン風邪では、2000-5000 万人が死亡した。

現在新型インフルエンザの発生に備えて、

パンデミックに対するワクチン製造がなされている。このパンデミック用のワクチンは有効性の観点から、過去にわが国で使用されていた全粒子型のワクチンをベースとしている。これに効果を増強するために免疫増強剤であるアルミニウムゲルを加えて製造している（**図６**）。全粒子ワクチンに関しては、現行の HA ワクチンに比べて副反応の発生頻度が高く、安全性に面で、十分な検討が必要である。本研究では DNA マイクロアレイによる解析を用いて、ワクチン投与に伴う遺伝子発現の変化を網羅的に解析し、安全性評価を可能とする新しい試験法の開発を行なう。

B. 研究方法

1）動　物

８週齢の F334/N 系統のラット（オス）を用い、生物学的製剤基準の一般試験法の異常毒性否定試験法に準じて行った。一群は各５匹用いた。

2）ワクチン

本研究課題に用いたワクチンは、（財団法人）化学及血清療法研究所より供与して頂いた、HA ワクチン、全粒子不活化ワクチン(WPV)、沈降新型インフルエンザワクチン(PDV)を用いた。HA ワクチン(HAV)、全粒子不活化ワクチンは A/New Caledonia/20/99 （ H1N1 ） 株 、 A/New York/55/2004 （ H3N2 ） 株 及 び B/Shanghai/361/2002 株の３型を混合し、沈降新型インフルエンザワクチンは A/VIETNAM/1194/2004(H5N1)株を用いた。ヒトに接種する最終濃度に合わせて調整し、5mL をモルモットに腹腔内投与した。

3）RNA 抽出

ワクチンおよび毒素が投与されたラットから肝臓左外葉、左肺、左腎臓、脳を接種後１～４日に採取した。臓器は即座に液体窒素中で凍結させ、ISOGEN 試薬（Nippon Gene）中で溶解させた。Total RNA を抽出し、Ambion 社の Poly(A) RNA Purist kit を用いて Poly(A)+RNA を精製した。

4）DNA マイクロアレイ解析

Poly(A)+RNA から逆転写酵素を用いた逆転写反応を行なう際に、共通リファレンスは Cyanine3 を、サンプル接種した RNA は Cyanine5 を取り込ませてサンプルをラベルした。ラベルされたサンプルをスライドグラス上に固定された 5,346 個の遺伝子特異的配列オリゴ DNA（80 mer）と結合（ハイブリダイゼーション）させた。各スポットの蛍光強度の比率（Cyanine3 と Cyanine5 の比率）をスキャナーおよび解析ソフトで数値化することにより、共通リファレンスに対する各遺伝子の発現量比を検出した。

C. 研究結果

1）DNA マイクロアレイ解析

インフルエンザワクチンの毒性に関して網羅的遺伝子発現解析を行なうために、接種後１、２、３、４日目に肝臓、肺、

脳、血液を採取し、合計 320 サンプルから poly(A)+RNA を抽出した。その中から肺のサンプルについて逆転写酵素を用いた逆転写反応を行なう際に、共通リファレンスを Cyanine3 で、サンプル接種した RNA を Cyanine5 でラベルした。その後それらを混合し、スライドグラス上に固定された 11,464 個の遺伝子特異的配列オリゴ DNA（80 mer）と結合（ハイブリダイゼーション）させた。各スポットの蛍光強度の比率（Cyanine3 と Cyanine5 の比率）をスキャナーおよび解析ソフトで数値化することにより、共通リファレンスに対する各遺伝子の発現量比を検出した。

肺のサンプルから取得した遺伝子発現プロファイルを基にして二次元階層クラスター解析を行なった。その結果、図10に示す様に、1日目のWPvとPDvが他のサンプルと別クラスターを形成しており、他のサンプルとは異なる挙動を示していた。

2）ワクチン毒性に関連した遺伝子群

そこで、1日目のWPv群とSA群（各3サンプル）の間で発現に有意差のある遺伝子群を統計的に抽出した後に、その抽出した43個の遺伝子だけでクラスター解析をしなおした結果が図11である。解析の結果をみると、1日目のWPvとPDvに対して、残りのサンプルが大きく2つのクラスタにわかれ、さらに、残りのサンプルのクラスタのうち2日目のWPvとPDvが別クラスターを、さらにその隣（下層）に1日目のHAvのクラスターを形成していた。3日以降のすべてのサンプルは１、２日目のWPv、PDvのクラスターからはずれていた。HAワクチンは図11で示す様に、１日目のサンプルにおいて、WPvとPDv投与後、３、４日目のサンプルに近い遺伝子発現様式を示す。また生理食塩水を投与されたサンプルが毒性が含まれるサンプルと混在していないことが明らかとなった。

D. 考察

肺の全サンプルの遺伝子発現変動を指標にしてクラスター解析を行なったところ、1日目のWPvとPDvが他のサンプルと別クラスターを形成しており、他のサンプルとは異なる挙動を示していた。このことは分担研究者浜口らがラットの体重変化および白血球数の変化で示している様に、WPvとPDvインフルエンザワクチンが投与初期に著しい生体変化をきたすことと相関していると考えられる。一方で、３日以降のWPvとPDv投与サンプルではほとんど特徴的な遺伝子発現様式を示していない。これは白血球値が投与後２日目にほぼ正常値をとるのと相関している。これらのことから図11で抽出された43遺伝子はWPvおよびPDvインフルエンザワクチンの持つ毒性反応を鋭敏に検知できる可能性を示している。またこの遺伝子セットを使用することにより、1日目（および2日目）のWPv、PDv群と、

HA v、SA群を明確に区別できることが判明した。

今後ワクチン投与量の増減による遺伝子発現量の定量的測定を行ないこれらの遺伝子を用いた安全性試験の確立を行なう。

E. 結論

インフルエンザワクチンの迅速で詳細な安全性評価を可能とする試験法の開発するために、ラットの DNA マイクロアレイを用いた網羅的遺伝子解析を行った。WPv および PD v インフルエンザワクチンの持つ毒性反応を鋭敏に検知できる 43 遺伝子の同定に成功した。

F. 健康危険情報

該当なし

G. 研究発表

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6. 内藤誠之郎、今井順一、加藤博史、河村未佳、浜口功、水上拓郎、前山順一、落合雅樹、山本明彦、堀内善信、野村信夫、渡辺慎哉、山口一成：DNA マイクロアレイを応用したワクチンの新しい安全性評価法の開発：第３２回日本トキシコロジー学会（東京）平成１７年６月

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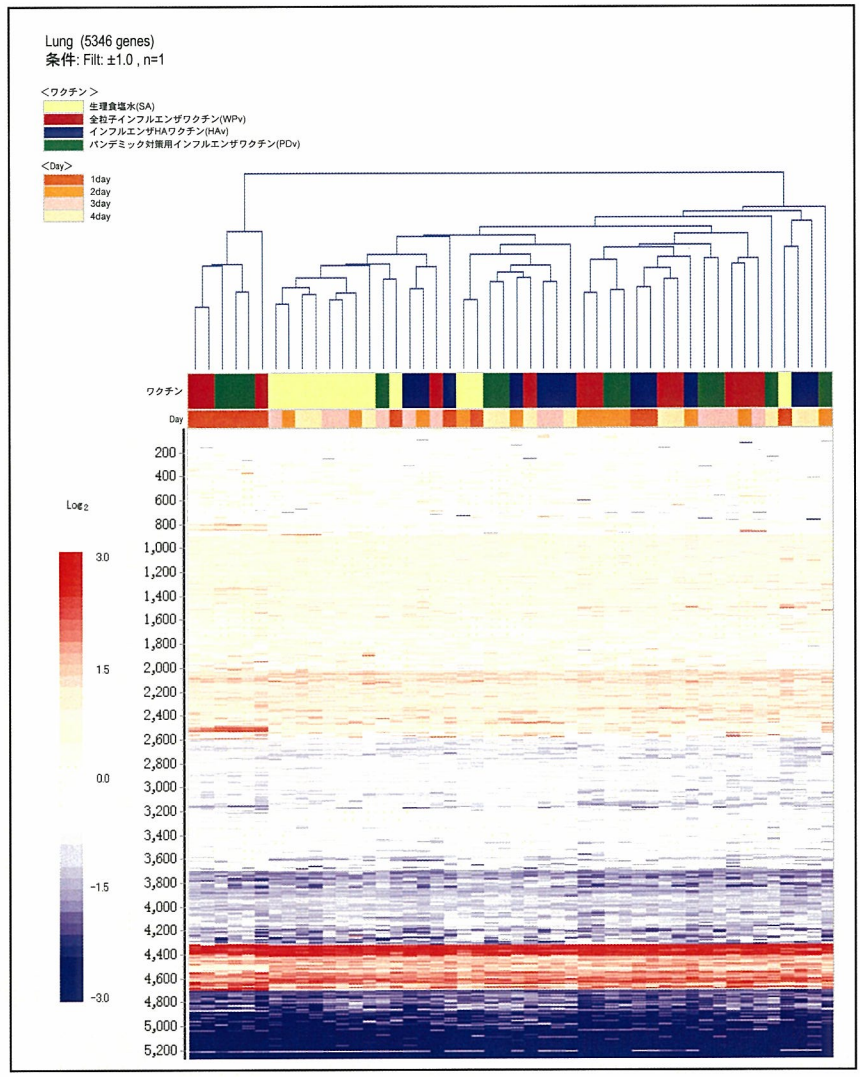

図１０．ワクチン接種ラットの肺における遺伝子発現様式のクラスター解析

ラット腹腔に SA（生理食塩水)、HAV（HA ワクチン)、WPV（全粒子ワクシン）および PDV（沈降新型インフルエンザワクチン）を投与した。投与ラットより肺を１～４日に摘出し、5346 個の遺伝子発現様式についてクラスター解析を行なった。

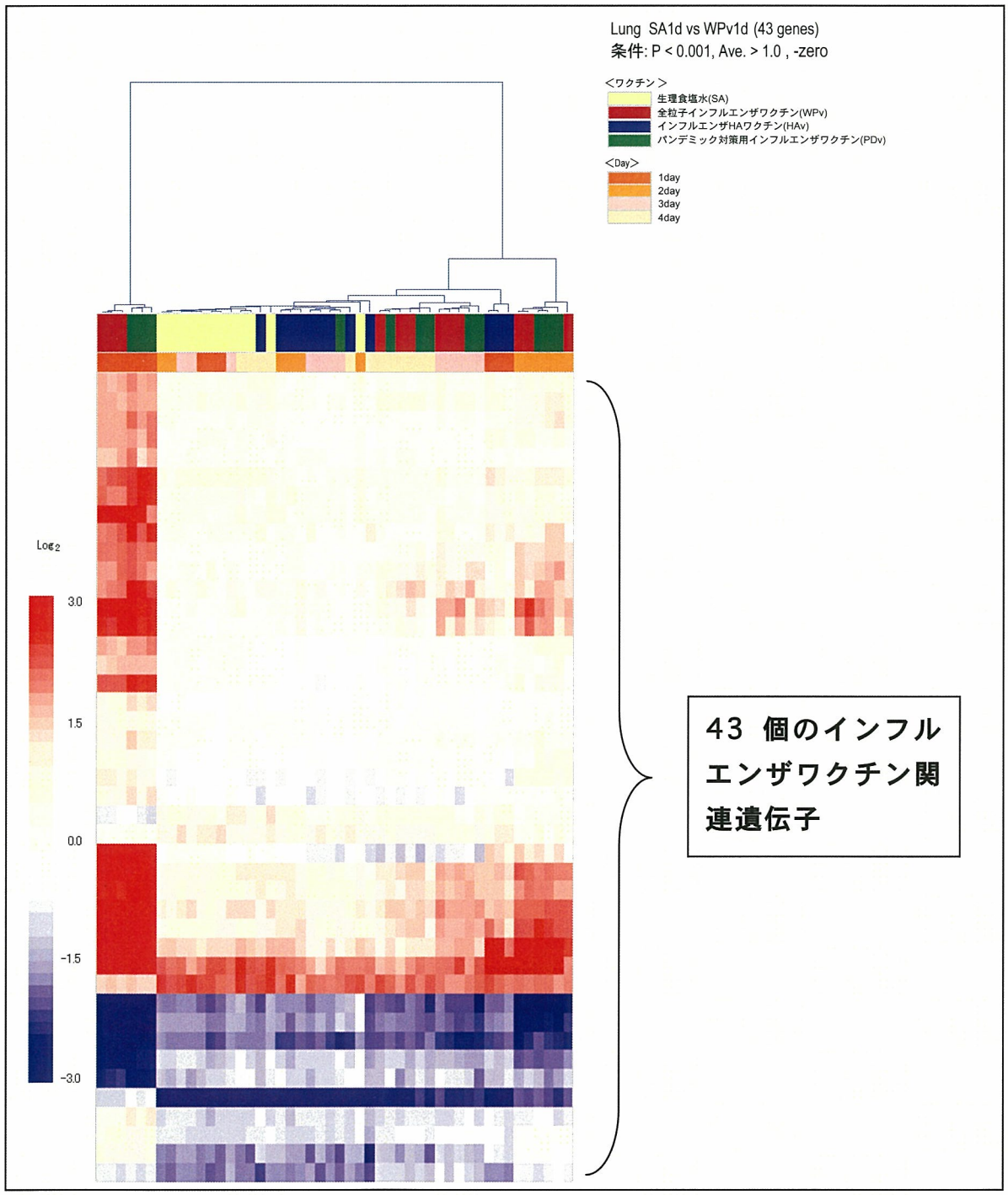

図１１．毒性関連遺伝子による肺の遺伝子発現様式

１日目の WPV（全粒子ワクチン）群と SA（生理食塩水）群の間で有意差のある遺伝子群を統計学的に 43 個抽出した後に、これらの遺伝子の発現変動をもとにクラスター解析を行なった。

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
Hamaguchi I, Imai J-I, Momose H, Kawamura M, Mizukami T, Kato H, Naito S, Maeyama J-I, Masumi A, Kuramitsu M, Takizawa K, Mochizuki M, Ochiai M, Yamamoto A, Horiuchi Y, Nomura N, Watanabe S, Yamaguch K	Two vaccine toxicity-related genes Agp and Hpx could prove useful for pertussis vaccine safety control	Vaccine			印刷中
Miura A, Honma R, Togashi T, Yanagisawa Y, Ito E, Imai J, Isogai T, Goshima N, Watanabe S, Nomura N.	Differential responses of normal human coronary artery endothelial cells against multiple cytokines comparatively assessed by gene expression profiles	FEBS Lett	580	6871-6879	2006
Yanagisawa Y, Sato Y, Asahi-Ozaki Y, Ito E, Honma R, Imai J, Kanno T, Kano M, Akiyama H,	Effusion and solid lymphomas have distinctive gene and protein expression profiles in an animal model of primary effusion	J Pathol.	209	464-473	2006

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Two vaccine toxicity-related genes Agp and Hpx could prove useful for pertussis vaccine safety control

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Abstract

Conventional animal tests such as leukocytosis promoting tests have been used for decades to evaluate toxicity of pertussis vaccine. Here, we examined gene expression in relation to the vaccine toxicity using a DNA microarray. Comparison of conventional animal test data with the DNA microarray-based gene expression data revealed a gene expression pattern highly correlated with leukocytosis in animals. Of 10,490 rat genes analyzed, two genes, α1-acid-glycoprotein (Agp) and hemopexin (Hpx), were found up-regulated by the toxin administration in a dose-dependent manner (assayed by a quantitative PCR based on the microarray). Variation of the gene expression was very small amongst the test animals, and the results were highly reproducible. These findings suggest that gene expression analysis of vaccine-treated animals can be used as an accurate and simple method of pertussis vaccine safety assessment.

Keywords: Pertussis vaccine; Microarray; Quantitative RT-PCR; Agp; Hpx; Safety test; Pertussis toxin

1. Introduction

Although regarded as one of the great public health successes, vaccines are not absolutely safe. While most adverse events associated with vaccines are minor and self-limiting, some vaccines have been associated with rare but serious health consequences.

Pertussis, or whooping cough, is an acute infectious disease caused by the bacterium *Bordetella pertussis*, which was first isolated in 1906 [1]. Outbreaks of pertussis were first described in the 16th century [2], and in the 20th century, pertussis was one of the most common childhood diseases and a major cause of childhood mortality. Prior to the availability of whole-cell pertussis vaccine in the 1940s, annual morbidity of pertussis exceeded 200,000 cases. Pertussis vaccine has long been used in many countries as the effective protective measure against the disease, leading to a dramatically decreased incidence of the disease.

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

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

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

2. Materials and methods

2.1. Animals

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

2.2. Vaccines and toxin

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

2.3. WBC counts

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

2.4. Histology

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

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

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

2.5. RNA preparation

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

2.6. Microarray preparation and expression profile acquisition

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

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

2.7. Data analysis

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

2.8. Quantitative RT-PCR analysis

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

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fwd (5′-CTGCCTCAGCCCCAGAAAGT-3′) and 0.05 μM Hpx rev (5′-GGGTGGGCTGGGCTAATTC-3′). Agp and Hpx values were normalized against rat β-actin (0.1 μM fwd 5′-ACCGTGAAAAGATGACCCAGATC-3′; rev 5′-GACCAGAGGCATACAGGGACAAC-3′).

2.9. Western blot analysis

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

2.10. Statistical analysis

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

3. Results

3.1. Vaccine-treated rats demonstrated leukocytosis in peripheral blood

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

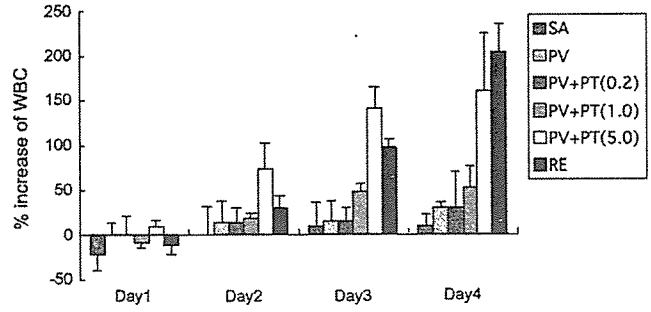

Fig. 1. Leukocytosis-promoting activity in RE- and PT-treated animals. The effects of RE-, PT-, PV-, SA-treatment were analyzed by leukocytosis-promoting tests. Various PT concentrations (0.2–5.0 μg/ml) were added to PV and a WBC count was performed at days 0, 1, 2, 3, and 4. Changes in WBC count are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

3.2. Histological analysis in vaccine-treated rats

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

3.3. Microarray assay of vaccine-treated liver

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

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