but that the interfering effect on LAL activity could be attenuated by treating with phosphate buffer (PB) [15]. We tested for endotoxin contents in DTaP and DTaP combined with inactivated polio vaccine (DTaP-IPV) purchased from European, the U.S.A and Asian markets in comparison with Japanese DTaP by the BWD toxicity test, LAL test, rabbit pyrogen test and an *in vitro* pyrogen test based on prostaglandin E_2 (PGE2) induction in rabbit peripheral blood [16].

The imported vaccines showed very strong BWD toxicity in spite of showing no significant difference in LAL test results from that of Japanese DTaP. Furthermore, one of the imported vaccine batches very strongly interfered with endotoxin activities. We made a detailed analysis on the characteristics of the strong interfering effect of the batch on endotoxin activities and its possible impact on the safety control for endotoxin in aP-based combination vaccines is discussed in the present paper.

2. Materials and methods

2.1. Reference pertussis vaccine for toxicity tests

Reference pertussis vaccine for toxicity tests Lot 2 (Reference vaccine), which is a lyophilized whole cell preparation of inactivated pertussis organisms being used for the National Control Tests of pertussis vaccines in Japan, was used [5]. Its assigned unit value of BWD toxicity was 1368 Body Weight Decreasing Units (BWDU) per vial. A vial of the vaccine was reconstituted in 12 ml of pyrogen-free physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo) and was serially diluted at four fold intervals from 1 in 1 to 1 in 64 for use.

2.2. Vaccines

Two batches each of DTaP and DTaP-IPV used in the present study were those purchased from the European, the U.S.A. and Asian markets. Japanese DTaPs were those submitted for National Control Tests during 1999 and 2005. Formulations of the vaccines are shown in Table 1.

2.3. Toxicity test in mice

The BWD toxicity test was performed according to Minimum Requirements. In brief, groups of ten mice each were intraperitoneally injected with 0.5 mL of a test vaccine or a dilution of the Reference vaccine diluted serially at four fold intervals. Body weight change of the mice during 16 h after injection was measured for testing BWD toxicity [17]. BWD toxicity of a test vaccine was calculated relative to that of the Reference vaccine according to the parallel line assay method using logarithmic dose and the body weight change to express the results as BWDU/mL [18].

2.4. Endotoxin

Japanese Pharmacopoeia Reference Standard Endotoxin Lot 3 (RSE), which is a lyophilized preparation of Westphal endotoxin extracted from *Escherichia coli* UKT-B strain (13,000 endotoxin units (EU) per vial) [19], was used as the standard preparation in the LAL test.

Lipopolysaccharide (LPS) extracted and purified from *B. pertussis* phase I Tohama strain by Westphal's phenol water method [20] (Bp-LPS) was used for spiking to test vaccines in a suppression/enhancement test to examine an interfering effect of a vaccine. The EU value of Bp-LPS was measured relative to the activity of RSE. For the suppression/enhancement test, a test vaccine was spiked with Bp-LPS at a final concentration of 100 EU/mL and kept at 4 °C for one week before measurement if not otherwise stated.

2.5. The LAL test

Test samples, RSE and Bp-LPS were serially diluted at four-fold intervals with pyrogen-free distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo). A 50 μL-volume of a dilution was mixed with the equal volume of an endotoxin specific LAL regent (Endospecy; Seikagaku Biobusiness Corp., Tokyo) of which reactivity to (1-3)-β-D-glucan was eliminated [7]. LAL activity was measured as the rate of color development using a specially equipped microplate reader (Well Reader SK603; Seikagaku Biobusiness Corp., Tokyo) [11,21]. Endotoxin content of a test sample was calculated relative to that of RSE according to the parallel line assay method using logarithmic values of dose and rate of color development to express as EU/mL.

2.6. The rabbit pyrogen test

Test vaccines were mixed with one in ten volume of 1000 EU/mL of Bp-LPS. Bp-LPS in physiological saline (100 EU/mL) was served as the control endotoxin. Female rabbits of Japanese white strain (Kitayama Labes Co., Ltd., Nagano or Japan Laboratory Animals, Inc., Tokyo) weighing approximately 3 kg were housed in cages separately in an air-conditioned animal room. Three animals were allocated to each treatment. They were intravenously injected with 1 mL/kg of a test sample. Rectal temperature of the animals was monitored for 3 h using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa, Japan). The maximum rise in rectal temperature during

Table 1
Formulation of vaccines.

	Vaccines	PT[a] μg/dose	FHA[b] μg/dose	PRN[c] μg/dose	Fimbrie μg/dose	Dtd[d]	Ttd[e]	IPV			Aluminum salt	Al^{+++} mg/dose
								Type 1[f]	Type 2[f]	Type 3[f]		
A	DTaP	25	25	8		25Lf[g]	10Lf[g]				Hydroxide	0.5
B	DTaP	10	5	3	5	15Lf[g]	5Lf[g]				Phosphate	0.33
C	DTaP-IPV	25	25	8		25Lf[g]	10Lf[g]	40	8	32	Hydroxide	0.5
D	DTaP-IPV	25	25			≧30IU[h]	≧40IU[h]	40	8	32	Hydroxide	0.3
J	DTaP	23.4	23.4			≦15Lf[g]	≦2.5Lf[g]				Phosphate	0.08

[a] PT:pertussis toxin.
[b] FHA:filamentous haemagglutinin.
[c] PRN:pertactin.
[d] Dtd:diphtheria toxoid.
[e] Ttd:Tetanus toxoid.
[f] D-antigen units of poliovirus/dose.
[g] Lf:Limit of floculation units/dose.
[h] IU:International Units/dose.

3 h was taken as the pyrogenic response of each rabbit. Pyrogenicity of a test sample was expressed as mean maximum temperature rise of 3 rabbits during 3 h after injection.

2.7. In vitro PGE2 induction assay

The assay was carried out according to the method of Ochiai et al. [16,22]. Although blood from different rabbits show different responsiveness to endotoxin in PGE2 production, results are consistent and reproducible even in blood from different rabbits so far as assessing the activity of vaccine samples in relative to the response to the standard endotoxin using blood collected from a single rabbit. Therefore, each assay was performed with blood from one rabbit and replicated using blood from the indicated number of rabbits in tables and figure. Briefly, a 100 μL-volume of an appropriate dilution of a test sample or a dilution of serially diluted Bp-LPS was gently mixed with 150 μL of heparinized fresh blood of a rabbit (Kitayama Labes Co., Ltd., Nagano or Japan Laboratory Animals, Inc., Tokyo) in a pyrogen-free centrifuge tube (Assist Co., Ltd, Tokyo) containing 750 μL of pyrogen-free physiological saline. The mixture was incubated at 37 °C for 8 h. Supernatants of the mixtures were isolated by centrifuging at 500 × g for 2 min and stored at −20 °C until use. PGE2 concentration of the supernatants was assayed by a commercial enzyme-linked immunoassay (EIA) kit (Prostaglandin E_2 High Sensitivity Correlate-EIA, Assay Designs, Inc., MI., USA) [16]. The activity of a test sample was calculated relative to that of Bp-LPS according to the parallel line assay method using logarithmic values of dose and PGE2 concentration to express as EU-equivalent/mL.

2.8. Statistic analysis

Analysis of the data of parallel line assay was carried out according to Finney's method [18]. Significance and validity tests were made at a level of $P = 0.05$.

3. Results

3.1. Toxicity to mouse body weight gain (BWD toxicity)

BWD toxicity test is based on the linear dose-dependent weight loss of mice received serial dilutions of the Reference vaccine or endotoxin [4]. The vaccines were tested for BWD toxicity as described in Materials and methods and results were summarized in Table 2. In the table, BWD toxicity unit values of the imported vaccines are represented with their 95% confidence intervals and those of Japanese DTaP and DTwP were represented as geometric mean unit values of lots tested during 1999 and 2005 and before 1981, respectively. The imported vaccine batches showed strong BWD toxicity comparing to that of Japanese DTaPs. The level of BWD toxicity of batch D was slightly higher than that of average Japanese DTaP but other three batches (A, B and C) showed the levels of BWD toxicity comparable or even excess to that of Japanese DTwPs (Table 2).

Table 2
BWD toxicities of DTaP and DTwP vaccines.

Vaccines	BWD activity	
	BWDU/mL	95% C.I.[b]
Imported vaccine		
A (DTaP)	58.86	(31.11–111.36)
B (DTaP)	100.79	(39.92–254.47)
C (DTaP-IPV)	334.05	(66.98–1666.05)
D (DTaP-IPV)	19.63	(8.98–42.89)
Japanese vaccines		
Acellular(1999 ~ 2005) ($N = 158$)	9.06	
Whole cell(~ 1981) ($N = 176$)	56.4	
Control[a]	4.88	
Japanese Minimum Requirements for DTaP	≦10	

[a] Normal mice were measured as a control group.
[b] 95% confidence interval.

3.2. Endotoxin contents in vaccines

The BWD toxicity test was initially implemented for controlling mainly residual endotoxin in DTaP [3,23] but was possibly affected by various vaccine constituents such as adjuvant gel [24]. We tested the imported vaccine samples by the LAL test to compare their endotoxin contents with that of Japanese DTaP. Amounts of endotoxin detected for imported vaccine batches A, B, C and D were 0.05, 0.13, 0.04 and 0.25 EU/mL, respectively, and were not much higher comparing to the content in Japanese DTaP of which average endotoxin content in batches tested during 1999 and 2005 was 0.024 EU/mL.

For verification of the LAL test, the imported vaccine batches and a typical Japanese DTaP batch were spiked with LPS (RSE or Bp-LPS) and kept at 4 °C to monitor the change in detectable LAL activity. The LAL tests were carried out using several dilutions of imported batches spiked with RSE or Bp-LPS and endotoxin contents of test samples were calculated according to the parallel line assay method. As a representative experiment, Bp-LPS was spiked to undiluted vaccines at 100 EU/mL and kept at 4 °C to monitor the change in detectable LAL activity for 15 days and the results were summarized in Fig. 1. In the figure, weighted means of 5 batches from 5 Japanese manufacturers are shown as the results of Japanese DTaP batches. The imported vaccine batch A markedly interfered with LAL activity of Bp-LPS, while other imported vaccines and the Japanese vaccines did not show any significant effect on LAL activity of the spiked LPS. An obvious interfering effect of batch A on Bp-LPS was seen immediately after spiking and reached the maximum level of over 90% suppression by day 3 and no further change was seen until day 15.

We previously reported that Japanese DTaPs generally do not show interfering effect on endotoxin activities except for one

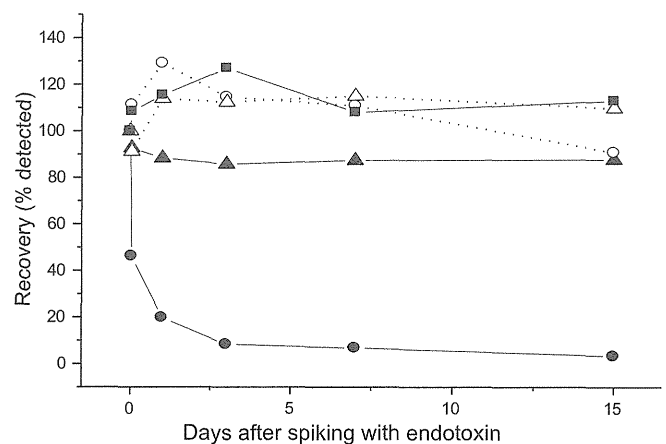

Fig. 1. Kinetics of interfering effect of acellular pertussis-based combination vaccines on LAL activity of Bp-LPS. DTaP batches A (●), B(▲) and Japanese DTaP batches (■), DTaP-IPV batches C(○) and D (△) were spiked with Bp-LPS at 100 EU/mL and stored to monitor the change in detectable LAL activity at 4 °C for 1 h, 1, 3, 7 and 15 days. The results of Japanese batches represent weighted means of 5 batches 5 five Japanese manufacturers. Batch A markedly interfered with LAL activity of Bp-LPS, while other batches showed no effect. Vertical bars represent 95% confidence intervals of recovery values.

DTaP product that interfered with LAL activity of spiked LPS without affecting pyrogenicity [14]. We, therefore, examined the effects of batch A on biological activities of endotoxin by the LAL test, pyrogen test and *in vitro* PGE2 induction assay to compare with a typical Japanese DTaP. Batch A and the Japanese DTaP were spiked with Bp-LPS at 100 EU/mL and kept at 4 °C for one week to monitor detectable LAL activity, *in vitro* PGE2 induction activity and pyrogenicity. Batch A strongly interfered with PGE2 induction activity and pyrogenicity as well as LAL activity of Bp-LPS while the Japanese DTaP did not show such interference with any of the activities (Table .3). As a result, detectable LAL and PGE2 induction activities of Bp-LPS spiked to batch A were suppressed to the level below 1.9% of the activities of the control Bp-LPS in saline, while those of Bp-LPS spiked to Japanese DTaP were not significantly suppressed but even seemed slightly enhanced as seen in Table 3.

3.3. Inhibitory factors in the vaccine

The possibility of attributing the strong interfering effect of DTaP batch A to strong adsorption of LPS to adjuvant gel was examined. DTaP batch A spiked with Bp-LPS at 100 EU/mL and kept at 4 °C for 3 or 4 days was centrifuged to separate supernatant and precipitate. When the supernatant was examined by LAL test, only 0.03 EU/mL of the activity was detected. However, without spiking with LPS, 0.04 EU/mL was detected for supernatant of batch A and, therefore, no change in LAL activity was seen for the supernatant even by spiking the vaccine with 100 EU/mL of LPS (Table 4). PGE2 induction activity was also measured for supernatant of batch A isolated after spiking with Bp-LPS at 100 EU/mL to detect only 2.20 EU-equivalent/mL (Table 4). When supernatant of batch A was isolated and then spiked with Bp-LPS at 100 EU/mL to examine the effects on the activities of LPS, LAL activity detected was only about 16 EU/mL even in the absence of aluminium gel, while pyrogenicity and PGE2 induction activity were not so much affected from the levels of their controls (Table 4).

As only trace LAL activity could be detected when directly assessed for batch A spiked with Bp-LPS at 100 EU/mL (Table 3), it was necessary to apply a special measure for attenuating the interfering effect to assess LAL activity of precipitate. Treating with 0.25 M PB could completely abolish the effect of Japanese interfering DTaP on LAL activity of spiked LPS [15]. Batch A was spiked with LPS and treated with 0.25 M PB directly but no marked effect of PB treatment was seen on the interference to LAL activity (Data not shown). For further characterization of the interfering effect, precipitate of batch A isolated after spiking with Bp-LPS at 100 EU/mL was treated with 0.25 M PB to examine the effect of PB treatment. In spite of the strong interfering effect of batch A, pyrogenicity could become detectable by treating the precipitate with 0.25 M PB, and some extents of LAL and PGE2 induction activities were also recovered by the treatment (Table 4). Other than the control Bp-LPS in saline we used also the control Bp-LPS in 0.25M PB to monitor the effect of saline and 0.25 M PB on Bp-LPS. Comparison of the two control Bp-LPS preparations indicates that although no difference in pyrogenicity and PGE2 induction activity, LAL activity of Bp-LPS in saline might have partially declined comparing to that of Bp-LPS in 0.25 M PB during one week of storage. In spite of the change in LAL activity of the Bp-LPS in saline, results of LAL test were not affected by the change so far as assessing in relative to RSE as in Materials and methods.

4. Discussion

Acellular pertussis-based combination vaccines are playing the essential role in immunization programs in many nations. When the first aP was developed and implemented in 1981, no LAL reagent that has sufficient specificity to LPS was available and the validation of the test method based on the correlation with pyrogen test was not feasible. BWD test based on endotoxin dose-dependent weight loss of mice was, therefore, implemented for controlling endotoxin in aP in Japan. The test contributed to reduce febrile response rate of DTaP vaccinees [3,25],

We tested for BWD toxicity of aP-based combination vaccines from the European, USA and Asian markets. Three out of the four imported vaccine batches showed the levels of BWD toxicity significantly excess over the limit value of 10 BWDU/mL and the levels of the toxicity were even comparable to that of Japanese DTwP (Table 2). Although BWD toxicity should basically correlate with endotoxin content [3,23], the test is not very specific for detecting endotoxin and might be affected by various vaccine constituents such as adjuvant gel and characteristics of antigens [24,26]. Therefore, higher concentrations of aluminum adjuvant in the imported vaccines could be a cause of the strong BWD toxicity but actual reason for the strong BWD toxicity could not be fully elucidated in the present study. Further investigation would be necessary for the causal mechanism of the strong toxicity to mouse weight and its possibility of relevance to reactogenicity.

Although of the contribution of BWD toxicity test in the control of residual endotoxin in Japanese DTaP batches in the 1980s [3,4], the test is not sensitive enough for evaluating recent batches of the vaccine due to marked reduction in residual endotoxin [27]. Specificity of LAL reagents to LPS was remarkably improved by eliminating or suppressing the reactivity to non-pyrogenic (1-3)-β-D-glucan in the 1980s [7] and the validation based on the correlation with pyrogen test became feasible. Accordingly, LAL test was implemented for testing DTaPs in Japan in 2004. In the course of the validation, we experienced a Japanese DTaP product that strongly interfered with LAL test without affecting pyrogenicity [14]. For such a product, LAL test is not relevant to the safety control. However, the interfering effect of the vaccine could be

Table 3
Effect of DTaP vaccines on the activities of LPS.

Samples	LAL test		Pyrogenicity		PGE_2 induction	
	EU/ml	95% C.I.[b]	Delta T (°C)[c]	95% C.I.[b]	EU-equivalent/ml[d]	95% C.I.[b]
Saline + LPS[a]	78.11	(67.41–90.50)	0.83	(0.48–1.18)	100	
Japanese DTaP	0.03	(0.02–0.03)	0.05	(−0.01–0.11)	0.95	(0.77–1.17)
Japanese DTaP + LPS[a]	141.79	(127.64–157.51)	1.32	(1.04–1.59)	153.03	(130.13–179.96)
DTaP batch A	0.05	(0.05–0.06)	0.24	(0.04–0.43)	1.87	(1.57–2.23)
DTaP batch A + LPS[a]	2.58	(2.31–2.87)	0.33	(0.14–0.52)	5.71	(5.04–6.47)

[a] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL.
[b] 95% confidence interval.
[c] Mean of maximum rise in rectal temperature of 6 rabbits during 3 h.
[d] PGE_2 induction activity calculated in reference to that of Bp-LPS to express as EU-equivalent. The results represent weighted means of 7 independent measurements.

Table 4
Effect of DTaP batch A on the activities of LPS.

Samples	LAL test		Pyrogenicity		PGE_2 induction	
	EU/ml	95% C.I.[e]	Delta T (°C)[f]	95% C.I.[e]	EU-equivalent/ml[g]	95% C.I.[e]
Saline + LPS[a]	75.81	(67.57–85.06)	1.02	(0.28–1.76)	100	
0.25M PB + LPS[a]	115.57	(104.99–127.22)	1.04	(0.62–1.46)	118.70	(56.50–249.35)
(batch A) Sup	0.04	(0.02–0.07)	N.D		N.D	
(batch A + LPS) Sup[b]	0.03	(0.02–0.04)	N.D		2.20	(1.62–2.97)
(batch A) Sup + LPS[c]	15.95	(14.20–17.91)	0.88	(0.09–1.68)	82.76	(54.40–125.88)
(batch A + LPS) ppt + 0.25M PB[d]	38.52	(35.19–42.16)	0.77	(0.40–1.15)	17.05	(11.19–25.98)

[a] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL.
[b] Kept at 4 °C for 3 or 4 days after spiking with Bp-LPS at 100 EU/mL to isolate supernatant by a centrigugation.
[c] Kept at 4 °C for 1 week before centrifuging and the supernatant was spiked with BP-LPS at 100 EU/mL to keep at 4 °C for another 1 week.
[d] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL and the centrifuged precipitate was treated with phosphate buffer at 4 °C for 1 week.
[e] 95% confidence interval.
[f] Mean of maximum rise in rectal temperature of 3 rabbits during 3 h.
[g] PGE_2 induction activity calculated in reference to that of Bp-LPS to express as EU-equivalent. The results represent weighted means of 4 independent measurements.

eliminated by treating with 0.25 M PB and LAL test became applicable [15].

We attempted to validate LAL test for the imported aP-based combination vaccines. One of the vaccine batches purchased from the European market, batch A, very strongly interfered with LAL activity of Bp-LPS while no such interference was seen for other batches (Fig. 1). The strong interference to LAL activity was also seen when used LAL reagent for kinetic-turbidimetric and gelation assay from another manufacturer. Recoveries of LAL activity of Bp-LPS spiked at 100 EU/mL to batch A were 4.8% and only 0.01% after 1 h and 1 day storage, respectively (data not shown). Batch A suppressed not only LAL activity but also *in vitro* PGE2 induction activity and pyrogenicity of Bp-LPS (Table 3). Batch A showed significant interfering effect on LAL activity of Bp-LPS immediately after spiking and the effect reached the maximum level of over 90% suppression by day 3 (Fig. 1). When batch A was spiked with Bp-LPS at 100 EU/mL and supernatant was isolated after keeping at 4 °C for 3 or 4 days, only trace amounts of LAL activity could be detected in the supernatant and the level was equivalent to the activity of supernatant of the vaccine without spiking (Table 4). Furthermore, PGE2 induction activity of spiked Bp-LPS detected in the supernatant was only 2.2 EU-equivalent/mL.

When supernatant of batch A was isolated and spiked with Bp-LPS at 100 EU/mL, LAL activity detected was only about 16 EU/mL to suggest even without adjuvant gel, supernatant alone would suppress LAL activity. However, the supernatant did not significantly affect PGE2 induction activity and pyrogenicity (Table 4). These results indicate that the strong interference by batch A was mainly attributable to strong adsorption of LPS to adjuvant gel.

As cation was reported to impede the stability of LAL activity [28], aluminium ion, if exists, may interfere with LAL activity of LPS. However, when measured aluminium content in the supernatant of DTaP batch A using aluminium analysis kit [29], no aluminium ion was detected (data not shown). Furthermore, dispersion state of LPS was reported to influence activities of endotoxin [30]. The difference in the effect of supernatant on LAL and other activities might have reflected the difference in dispersion states of LPS in reaction mixtures for LAL test and other tests. The effect of dispersion state on LAL activity may also explain the slight but significant difference in LAL activities of the two LPS controls, Bp-LPS in saline and that in 0.25 M PB, in Table 4. However, the suppression of LAL activity by supernatant was much more extensive comparing to the difference of the Bp-LPS controls to suggest involvement of another mechanism.

Batch A had the similar constituents of DTaP antigens and aluminum to those of batch C differing in combination with IPV (Table 1). Although batch A and batch C contained the equal amount of aluminium hydroxide gel, the batches behaved in significantly different ways in interference with activities of LPS. This difference and the interfering effect of batch A supernatant particularly on LAL activity may be suggesting additional ingredients that influence dispersion state of LPS in batch A such as a higher concentration of detergent. However, actual cause of the difference in interfering effect of the batches on LPS activity could not be identified in the present study. Consequently, LAL test was suppressed not only by aluminium adjuvant but also by supernatant and, therefore, LAL test could not be relevant to safety control for vaccine products with the similar property as batch A.

Batch A was spiked with Bp-LPS at 100 EU/mL and precipitate was isolated for treating with 0.25 M PB. Although the PB treatment completely abolished interfering effect of Japanese interfering DTaP, interfering effect of batch A could be attenuated only partially by PB treatment to recover 38.5 EU/mL and 17.05 EU-equivalent/mL of LAL and PGE2 induction activities, respectively. On the other hand, of approximately 1.0 °C of pyrogenicity of the control Bp-LPS in saline, 0.77 °C of pyrogenicity was recovered by treating the batch A precipitate with 0.25 M PB (Table 4).

Consequently, batch A strongly suppressed activities of spiked LPS by the strong adsorption to adjuvant gel but *in vivo* pyrogenicity might readily be recovered to the most significant extent from the precipitate by treating with PB comparing to *in vitro* activities. This may suggest that the adsorption of LPS to adjuvant gel is stable without PB treatment, but the treatment with PB may facilitate to release when further injected intraveneously to rabbits. Therefore, endotoxin contaminated in such vaccines may readily lose *in vitro* activities but may still retain *in vivo* activities such as pyrogenicity. Monocyte activation test (MAT), which is a novel test method to detect or quantify pyrogens that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, has been implemented in the European Pharmacopoeia as a replacement for the pyrogen test [9]. Therefore, we examined if the MAT using human monocytoid cell line, 28SC cells [31] can be applied to measure endotoxin activity in batch A, but the cell line assay was found not applicable due to very strong cytotoxicity of the vaccine. An appropriate *in vitro* control measure for the safety of such a vaccine could not be identified in the present study and, therefore, an appropriate *in vivo* control test would be required, at least, where control tests on final products are mandatory for approving release of vaccine batches like batch A

until a suitable *in vitro* method could be developed for testing such vaccine products.

Acknowledgments

This research was funded by Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare of Japan.

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Vaccine 30 (2012) 1191–1197

Contents lists available at SciVerse ScienceDirect

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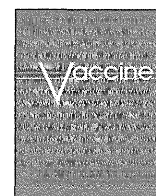

Antigen-loaded dissolving microneedle array as a novel tool for percutaneous vaccination

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ARTICLE INFO

Article history:
Received 28 August 2010
Received in revised form
14 November 2011
Accepted 29 November 2011
Available online 13 December 2011

Keywords:
Delivery system
Intradermal injection
Skin immunization

ABSTRACT

Antigen-loaded dissolving microneedle array (dMNA) patches were investigated as novel systems for vaccine delivery into the skin, where immuno-competent dendritic cells are densely distributed. We fabricated micron-scale needles arrayed on patches, using chondroitin sulfate mixed with a model antigen, ovalbumin. Insertion of dMNA effectively delivered substantial amounts of ovalbumin into the skin within 3 min and induced robust antigen-specific antibody responses in the sera of mice. The antibody dose–response relationship showed that the efficiency of dMNA patch immunization was comparable to that of conventional intradermal injections. Thus, Antigen-loaded dMNA patches are a promising antigen-delivery system for percutaneous vaccination.

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1. Introduction

Skin is not only a physical but also an immunological barrier that protects the body against exogenous insults. A large number of professional antigen-presenting cells, including Langerhans cells and dermal dendritic cells, populate epidermal and dermal layers of the skin and sentinel exogenous pathogens [1,2]. Therefore, the skin has been considered as an ideal target for vaccine delivery. Many studies have reported that intradermal vaccine delivery could result in more superior immune responses quantitatively and/or qualitatively than would subcutaneous or intramuscular vaccine routes [3–7]. However, the majority of vaccines are commonly inoculated subcutaneously or intramuscularly using needles and syringes, since the so-called Mantoux technique, intradermal administration by a conventional needle and syringe, requires special skills for correct application.

In recent years, microneedles (MNs) have emerged as a novel tool for percutaneous drug delivery [7–12]. Progress in microelectronics technology has enabled generation of micron-scale needles that can penetrate the *stratum corneum* and reliably deliver drugs into the epidermal or dermal skin layers. In particular, MN array patches carrying antigens are attractive because they can be manufactured cost-effectively and potentially allow vaccine self-administration in a simple and painless manner. The titanium MN array, Macroflux®, coated with a model antigen, ovalbumin (OVA), showed significant dose sparing effect [8]. The polyvinylpyrrolidone based dissolving MN patches encapsulating inactivated influenza virus vaccine successfully induced protective immunity against the lethal challenge with homologous influenza virus [12]. Especially, dissolving MNs are attractive because they generate no sharp biohazardous waste.

We have developed a technology to produce MNs by using water-soluble, thread-forming biopolymers, chondroitin sulfate or dextrin. MNs are assembled into a 10×10 array on a patch called a dissolving microneedle array (dMNA) patch in this study [13,14]. We have succeeded in encapsulating various bioactive macromolecules, including peptides and proteins formulated as solid dispersions into MNs. The designed MNs are strong and sharp enough to penetrate the *stratum corneum* upon insertion into the skin and dissolve within few minutes, releasing the loaded macromolecules. Thus far, we have reported that dissolving MNs can successfully deliver various bioactive macromolecules, such

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doi:10.1016/j.vaccine.2011.11.111

as insulin, erythropoietin, interferon-α, human growth hormone, or low-molecular-weight heparin percutaneously in experimental animals [15–20].

In this study, we encapsulated a model antigen, ovalbumin, into dMNA patches and investigated the potency of these patches to induce antigen-specific antibody responses in mice.

2. Materials and methods

2.1. Mice

We used female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan), aged 7–10 weeks at the time of the primary immunization.

Animals were housed in a specific pathogen-free facility and given free access to water and food. The use of animals and study protocols were approved by our institutional animal care and use committee.

2.2. Antigen and adjuvant

Ovalbumin (OVA) and cholera toxin (CT) were purchased from Sigma (St. Louis, Mo, USA).

2.3. Fabrication of antigen-loaded, two-layered dMNA patches

To make a concentrated antigen solution, "drug glue", a required quantity of OVA according to an intended dosage, and 0.5 mg Evans blue (EB; Nacalai Tesque, Kyoto, Japan) were added to 860 mg sodium chondroitin sulfate (Nacalai Tesque). Subsequently, 900 μl distilled water were added and the mixture kneaded at room temperature. The "drug glue" was then degassed under reduced pressure and dispensed into a mold containing 100 inverted, cone-shaped wells arrayed in a 1.0 cm^2 area. Each well was 500 μm deep and 300 μm across at its top. After the "drug glue" was poured into the wells, the mold was centrifuged for 5 min at 3000 rpm (Kubota 1700, Tokyo, Japan). After centrifugation, the "vehicle glue" comprising 1.0 g chondroitin sulfate and 1.0 ml distilled water was painted over the mold. It was then dried under the pressure of the stainless steel plate for 3 h. Thereafter, the plate was removed and the two-layered dMNA was detached using the supporting material, i.e. base (Fig. 1A and B).

2.4. Immunization

Mice were anaesthetized by intraperitoneal injection of a ketamine–xylazine mixture. The dorsa of mice were shaved using electric clippers followed by an electric razor. The application site on the bare dorsal skin of mice was gently swabbed with 70% ethanol and allowed to dry.

For the dMNA patch immunization, the dMNA patch was placed on the shaved dorsal skin and pressed by a thumb for 3 min to insert the MNs into the superficial dermal layer. The continuous pressing was required to keep insertion of all MNs into the skin, because the dMNA patch had size of 12 mm × 12 mm and did not fit the natural body shape of mouse, while it was easily applied human skin and fixed by adhesive tape.

Transcutaneous (TC) immunization was performed as previously described [21]. In brief, the dorsal skin of the mice was shaved carefully using a No. 40 clipper, and the mice were rested for 48 h. A 0.64-cm^2 square gauze patch with an adhesive lining (Shirojuhji, Tokyo, Japan) was soaked with 50 μl solution of antigen in physiological saline with or without adjuvant and fixed on the shaved dorsal skin using medical tape. The mouse was replaced into the cage. Eighteen hours later, the gauze patch was removed.

For intradermal (ID) administration, 50 μl antigen solution in physiological saline were injected into the shaved dorsal skin using a conventional needle and syringe according to the Mantoux technique.

2.5. ELISA for OVA-specific IgGs

OVA-specific IgG titers in the sera of mice were determined by ELISA. In brief, 96-well plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with OVA in 0.1 M carbonate–bicarbonate buffer, pH 9.0, and blocked with PBS containing 1% bovine serum albumin (Sigma). Thereafter, serially diluted serum samples were added to the OVA-coated plates which were incubated at room temperature for 1.5 h. The plates were washed 3 times with wash buffer (PBS containing 0.05% Tween 20) and peroxidase-labelled rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA) was added. After a 1.5-h incubation at room temperature, the plates were washed 3 times with the wash buffer and *o*-phenylenediamine (Sigma) in phosphate–citrate buffer containing 0.03% H_2O_2 was added. The reaction was stopped 10 min later by adding 1 N H_2SO_4 and optical densities were measured at 492 nm. Endpoint titers were expressed as reciprocal log of the limiting dilutions with an optical density greater than 0.5. The titers not less than 4 were regarded as significant.

2.6. ELISA for OVA content

The OVA content in dMNA patches was determined using a sandwich ELISA kit (Morinaga, Tokyo, Japan) according to manufacturer's instructions. The OVA content of samples was extrapolated by a standard curve prepared using the reference OVA provided in the kit.

2.7. Statistical analysis

The data are presented as the geometric means of the values obtained from individual animals. Groups were compared using unpaired, two-tailed Student's t test and $p \leq 0.05$ was considered significant.

3. Results

3.1. Skin delivery of EB by dMNA patches

To visualize the effectiveness of skin delivery of molecules loaded in the dMNA patches, the indicator dye, EB, was encapsulated into dMNA patches (Fig. 1C). EB-loaded dMNA patches were put on the shaved dorsal skin of mice and pressed manually for 3 min. After removing the patches from the skin, it was observed that most of the MNs on the patches had dissolved and lost their shape, but some MN residuals were observed on the patch (Fig. 1D). On the site of dMNA-patch application, EB spots corresponding to all MNs of the patch were obvious (Fig. 1F). The EB spots gradually diffused in the tissue surrounding the puncture sites in several hours (Fig. 1G) and disappeared 24 h later (Fig. 1H), indicating that the dMNA successfully penetrated the *stratum corneum* and delivered the EB into the epidermal and/or dermal layer. Thus, we confirmed that the dissolving MNs composed of chondroitin sulfate were sharp and strong enough to pierce the skin of mice and dissolve within few minutes.

3.2. Comparison of serum antibody responses induced by dMNA-patches with those induced by TC patch immunization or ID injection

Serum antibody responses in mice induced by antigen-loaded dMNA patches were compared with those induced by TC patch immunization or ID injection. dMNA patches containing 10 or

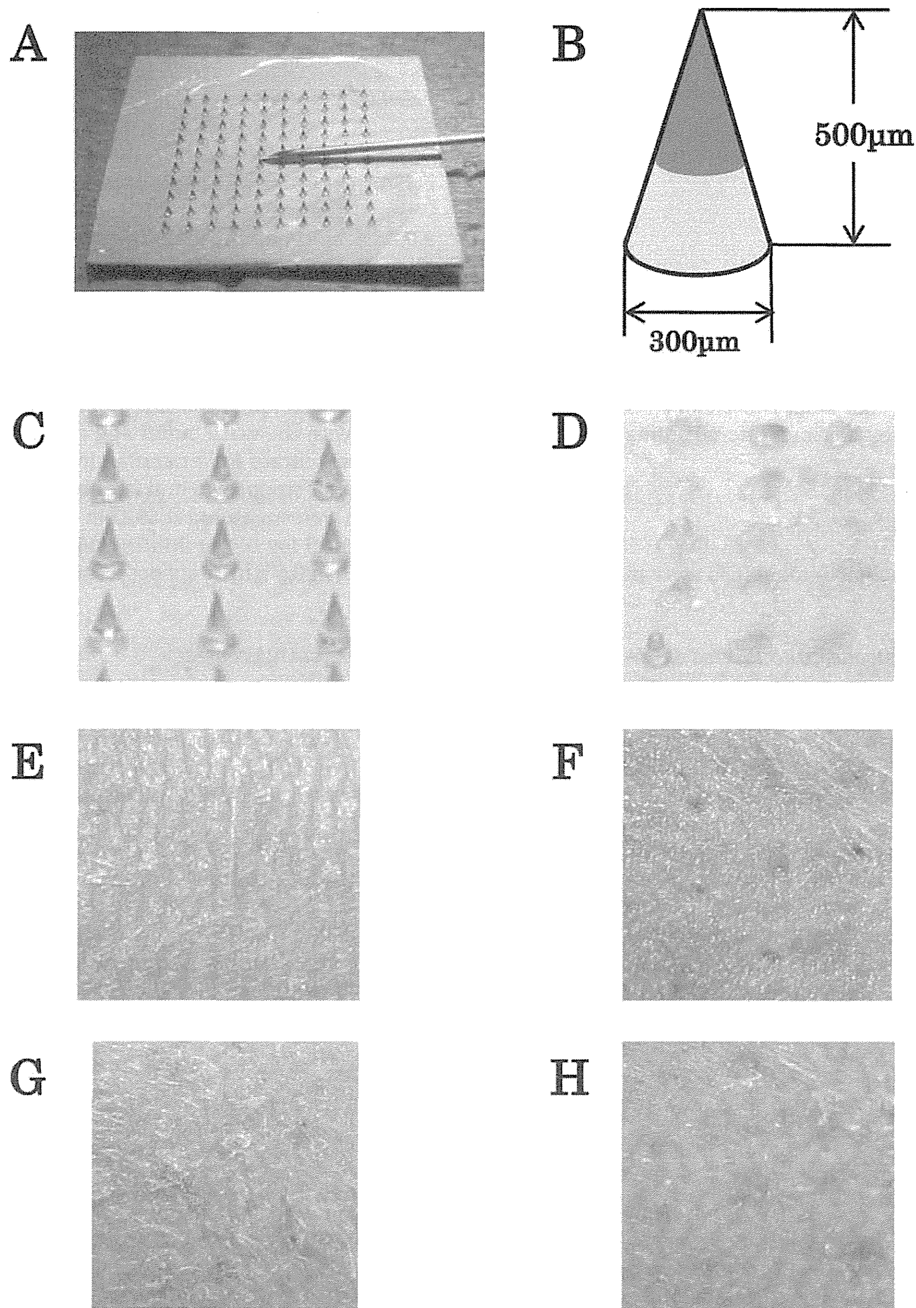

Fig. 1. An EB-loaded, two-layered dMNA patch. (A) A dMNA patch and a 26-G conventional needle. Dissolving MNs composed of chondroitin sulfate were arranged in 10 × 10 array on a patch. (B) Schematic view of the two-layered conical MN. (C–D) Close-up view of an EB-loaded, two-layered dMNA patch before (C) and after (D) skin insertion for 3 min. (E–H) Close-up view of dorsal shaved skin of a mouse before (E), immediately (F), 1 h (G), and 24 h (H) after application of an EB-loaded dMNA patch.

100 μg OVA with no adjuvant were applied in two groups of five mice. The other four groups of mice were immunized by TC route using gauze patches soaked with 50 μl solution containing 10 or 100 μg of OVA with or without 10 μg of CT as the adjuvant. The last group of mice were injected intradermally with 50 μl antigen solution containing 10 μg OVA. For boosting, all groups were re-immunized 2 weeks later and the OVA-specific IgG serum titers were determined 2 weeks after the first and the boost immunizations.

OVA-loaded dMNA patches induced substantial amounts of antigen-specific IgGs after the primary immunizations (Fig. 2A) but were boosted even more after the secondary immunizations (Fig. 2B). The dMNA containing 10 μg OVA induced comparable levels of antibody responses to those induced by ID injection of the same dose after both primary and secondary immunization. On the other hand, the TC patch immunization did not induce any significant antibody response after the primary immunization regardless of adjuvant co-application. Although the TC patches with 100 μg OVA induced significant antibody responses after the booster immunization, antibody titers were significantly less than those induced by dMNA patches. Thus, the antigen-loaded dMNA patch was far more effective than the TC patch immunization but comparably potent to ID injection in eliciting antibody responses in mice.

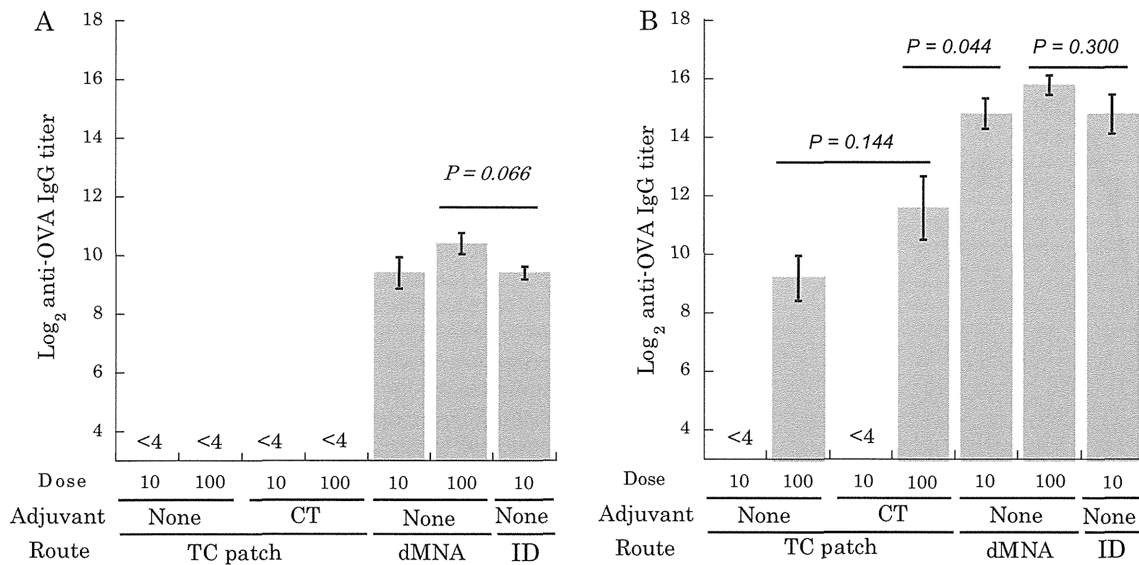

Fig. 2. Comparison of serum antibody responses induced by TC, dMNA, or ID immunization. Four groups of mice ($n = 5$) were immunized transcutaneously with gauze patches drenched with 50 μl antigen solution in physiological saline containing 10 or 100 μg OVA with or without 10 μg CT. Other two groups of mice ($n = 5$) were immunized with dMNA patches containing 10 or 100 μg OVA. Another group of mice ($n = 5$) was ID injected with 50 μl of antigen solution in physiological saline containing 10 μg OVA. Immunizations were performed at week 0 and week 2. OVA-specific serum IgG titers were determined at 2 weeks (A) and 4 weeks (B) after the primary immunization. The geometric mean and the SEM are shown for each group.

3.3. Antibody dose–response relationships induced by dMNA-patch application and ID injection

To compare the efficiency of dMNA patch immunization with that of ID injection, dose–response relationships using decreasing OVA doses were assessed. Three groups of five mice were applied with dMNA patches containing 10, 2.5, or 0.5 μg OVA. Other four groups of five mice were injected intradermally with 50 μl antigen solution containing 10, 2.5, 0.5 or 0.1 μg OVA. All groups of mice were immunized at week 0 and week 2 and their serum OVA-specific IgG titers were measured 2 weeks after each immunization.

Fig. 3 shows the result of primary (A) and secondary (B) immunization. A similar manner of dose-dependency was observed in mice undergoing dMNA patch immunization or ID injection. Thus, antibody-inducing efficiency of OVA-loaded

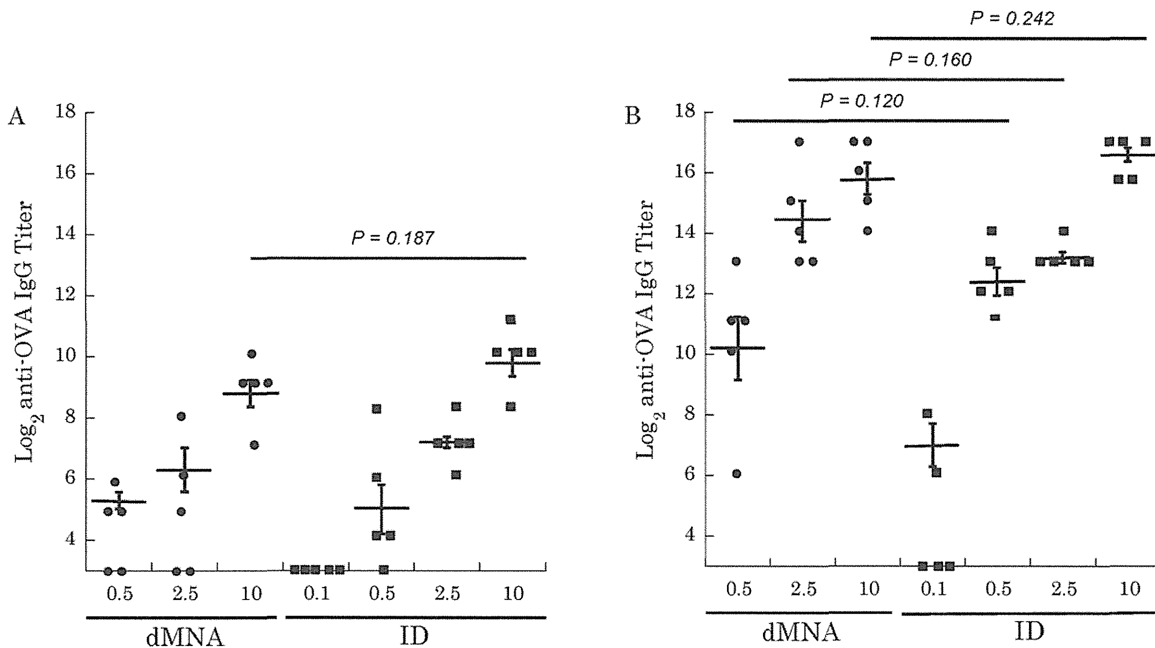

Fig. 3. Dose–response of serum antibody production by dMNA-patch application or ID injection. Groups of mice ($n = 5$) were immunized by dMNA patches containing 0.5, 2.5, or 10 μg OVA, or ID intra-dermal injection using 50 μl antigen solution in physiological saline containing 0.1, 0.5, 2.5, or 10 μg OVA. Immunizations were performed at week 0 and week 2. OVA-specific serum IgG titers were determined at 2 weeks (A) and 4 weeks (B) after the primary immunization. Each circle or square represents the serum antibody titer for each mouse. Each horizontal line and each vertical line represents the geometric mean and the SEM of the significant titers, respectively, for each group.

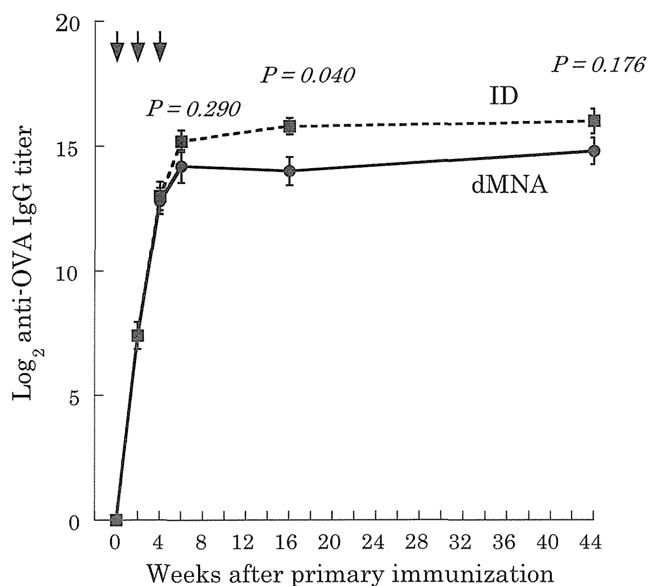

Fig. 4. Persistence of serum antibody responses induced by dMNA-patch application or ID injection. Groups of mice ($n = 5$) were immunized by dMNA patches (circles) containing 10 μg OVA or ID injection (squares) with 50 μl antigen solution in physiological saline containing 10 μg OVA at week 0, week 2, and week 4. OVA-specific serum IgG titers were determined at 2, 4, 6, 16, and 44 weeks after the primary immunization. The geometric mean and the SEM are shown for each group.

dMNA patches was apparently comparable to that of ID injection.

3.4. Persistence of serum antibody production induced by dMNA patches

To elucidate the persistence of antibody production in serum following dMNA-patch application, the time-course of OVA-specific IgG titers was recorded (Fig. 4). Two groups of mice ($n = 5$) were immunized 3 times: at week 0, week 2, and week 4 with either OVA-loaded dMNA or ID injection of OVA solutions. Both vaccination media contained 10 μg OVA. Antibody titers in mice immunized by the dMNA patches were maintained evenly during 38 weeks after the last immunization, which was comparable with those induced by ID injections.

3.5. Estimation of net OVA skin delivery by dMNA patches

Some residuals of MNs were observed on the dMNA patches after skin application (Fig. 1D), implying that not all but some parts of OVA loaded in the dMNA were delivered into the skin. In order to estimate the amount of OVA delivered into the skin, i.e. net delivery, we quantified OVA content in the dMNA patches before and after dermal application. The dMNA patches were halved, i.e. each containing 50 MNs. One half was kept intact while the other was applied to the mouse skin for 3 min. The intact MNs and the patch residuals were dissolved separately in PBS and OVA levels were measured by ELISA. The difference in OVA content between the intact MNs and the residuals indicated the net OVA delivery into the skin.

Table 1 shows the net OVA delivery by three different dMNA preparations, containing ~10-fold increasing OVA doses. The low, middle and high dose dMNA preparations (referred to as dMNA-L, dMNA-M and dMNA-H, respectively) were made from the "drug glue" composed of 860 mg of sodium chondroitin sulfate, 0.5 mg of EB and 0.4 mg, 4 mg or 40 mg of OVA, respectively according to the procedure described in Section 2. All dMNA patches with different OVA doses effectively delivered significant OVA levels comprising ~20–80% of the original dMNA OVA content. Two weeks after dMNA patch application, OVA-specific IgG titers were determined. Although the net OVA delivery into the skin of individual mice was not stable, every mouse showed significant antibody responses according to OVA levels loaded on the different dMNA patch preparation.

4. Discussion

OVA-loaded dMNA patch application to the skin efficiently induces antigen-specific antibody responses in the sera of mice. Dose–response analyses revealed that the efficiency of dMNA patch immunization was comparable to that of conventional ID injections. Although TC immunization using gauze patches drenched

Table 1
Net OVA skin delivery by OVA-loaded dMNA patches.

	Mouse no.	OVA contents (μg)		Net delivery (μg)	Net delivery (%)	Anti-OVA IgG titer
		Intact	After application			
dMNA-L1	1	0.137	0.026	0.112	81.4	7
	2	0.150	0.053	0.097	64.9	7
	3	0.173	0.130	0.044	25.1	6
	4	0.174	0.120	0.055	31.4	6
	5	0.145	0.101	0.044	30.6	7
	Mean	0.156	0.086	0.070	46.7	6.6
	SD	0.015	0.040	0.028	22.3	0.49
dMNA-M	6	1.76	1.17	0.59	33.7	7
	7	1.81	1.34	0.47	26.2	8
	8	1.30	0.97	0.34	25.9	9
	9	1.34	0.30	1.03	77.2	9
	10	1.60	1.22	0.38	24.0	8
	Mean	1.56	1.00	0.56	37.4	8.2
	SD	0.211	0.367	0.249	20.2	0.75
dMNA-H	11	18.5	13.8	4.8	25.6	10
	12	17.0	9.6	7.4	43.5	9
	13	16.5	10.8	5.7	34.7	10
	14	16.4	12.3	4.1	25.0	9
	15	14.0	8.8	5.2	36.9	8
	Mean	16.5	11.1	5.4	33.2	9.2
	SD	1.47	1.80	1.13	7.0	0.75

with OVA solutions with or without an adjuvant also successfully induce OVA-specific antibody responses, booster immunization with high antigen doses (100 μg) are required to induce significant responses, even if CT were used as the adjuvant. In general, TC immunization requires comparatively high doses of antigen (and adjuvant) to induce robust immune responses because of the barrier function of the *stratum corneum* [21–30]. The low efficiency of TC immunization has hindered its practical use. Our results illustrate that MN arrays potentially circumvent the difficulties inherent in TC immunization. MN arrays could penetrate the *stratum corneum* forming channels for antigen delivery while dramatically increasing the antigen delivery efficiency up to the level afforded by conventional needle injection. Furthermore, MN arrays could be applied by a simple and convenient technique similar to TC immunization.

Recently, various approaches for vaccination using MN systems have been proposed: (1) pretreatment of solid MNs to pierce the *stratum corneum* followed by topical vaccine application, (2) solid MN coated with dry vaccines, and (3) hollow MNs for injecting liquid vaccines [5,31,32]. We believe that dissolving MNs are another potentially promising approach affording several advantages, including (1) no sharp residuals after inoculation, (2) no risk of remaining pieces of unexpected broken MNs in the skin, and (3) a simple production procedure and single-step molding.

Practically, dissolving MNs should satisfy several properties, including (1) safety of base MN material, (2) antigen integrity during the production process, and (3) mechanical strength and sharpness enabling penetration of the *stratum corneum*. In this study, we used chondroitin sulfate as the base material for dissolving MNs. Formulations containing chondroitin sulfate as the main ingredient have been approved as drugs for osteoarthritis in Japan and Europe. These formulations have been safely used orally, intramuscularly, or intravenously [33,34]. Therefore, we could expect that chondroitin sulfate can be used safely as the base material for dissolving MNs.

Chondroitin sulfate is dissolved in water to form a highly viscous gel at ambient temperature, enabling production of MNs without heating. This characteristic of chondroitin sulfate is advantageous to make MNs containing heat-labile proteins such as antigens [35–37].

In general, water-soluble materials are mechanically weaker than metals or silicon. To acquire enough strength for penetrating skin, we reduced the MN aspect ratio, making complete skin insertion of MN shaft difficult. Therefore, we generated MNs with two layers—antigen was loaded into the "tip" layer while the "base" layer comprised only the base material, chondroitin sulfate. In our previous study, this approach improved the efficiency of insulin delivery through the skin [38].

As a result, OVA-loaded dMNA composed of chondroitin sulfate successfully penetrated the *stratum corneum* and delivered the loaded macromolecules into the skin in a short time. OVA delivered by dMNA patches induced robust antigen-specific antibody responses with dose–response efficiencies comparable to ID injections, indicating that the antigen retained its integrity during the dMNA production process. The quantitative analysis of antigen in dMNA patches before and after application revealed that significant amounts of antigen loaded in the dMNA were effectively delivered into the skin. The duration of the dMNA patch application was short (only 3 min) in this study. To prolong the period of insertion of dMNA into the skin might improve the delivery efficiency.

In conclusion, antigen-loaded dMNA patches composed of chondroitin sulfate successfully delivers the antigen percutaneously through a simple and convenient technique. This application efficiently induces robust antigen-specific antibody responses. Antigen-loaded dMNA patches are a promising antigen-delivery system for percutaneous vaccination.

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綜合臨牀　第60巻第11号
（平成23年11月1日発行　別刷）

ワクチンプログラム

Vaccine program in Japan

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解　説

特集 VPDを制御するためのわが国の課題

ワクチンプログラム

Vaccine program in Japan

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Key words　予防接種法　定期接種　子宮頸がん等ワクチン接種緊急促進事業　感染症発生動向調査　予防接種後副反応

日本で接種可能なワクチンの種類

2011年現在，わが国で接種可能なワクチンを制度別，種類別に表1に示す．表1以外のワクチン，例えば不活化ポリオワクチン，麻疹風疹おたふくかぜ混合(measles-mumps-rubella：MMR)ワクチン，麻疹風疹おたふくかぜ水痘混合(measles-mumps-rubella-varicella：MMRV)ワクチン，経鼻生インフルエンザワクチン，経口コレラワクチン，腸チフスワクチン，髄膜炎菌ワクチン，ダニ媒介性脳炎ワクチン，その他多数存在する多価混合ワクチン等の接種を希望する場合は，海外で接種を受けるか，あるいは医師が個人輸入したワクチンの接種を受ける以外，現時点では国内で受ける方法はない．

予防接種の制度をもとに分類すると，これらのワクチンは，予防接種法に基づいて接種が行われる定期接種・臨時接種・2011年に制定された新しい臨時接種と，予防接種法には基づかない定期外接種，いわゆる任意接種に分類される．また，任意接種のワクチンのなかには，子宮頸がん等ワクチン接種緊急促進事業の一環として実施されているワクチンもあり，接種の制度はさまざまである．制度によって異なる点は，接種の実施主体，接種費用の公費助成の有無，予防接種後健康被害救済

表1　日本で接種可能なワクチンの種類と予防接種の制度

制度	ワクチン
予防接種法に基づく【定期接種】(定期接種の対象年齢は政令で規定)	生ワクチン(すべて一類疾病) BCG　ポリオ　麻疹風疹混合(MR)　麻　疹(はしか)　風　疹 不活化ワクチン(インフルエンザ：二類疾病，その他：一類疾病) DPT/DT　日本脳炎　インフルエンザ
【子宮頸がん等ワクチン接種緊急促進事業】	不活化ワクチン インフルエンザ菌b型(Hib) 肺炎球菌(7価結合型) HPV(ヒトパピローマウイルス：2価，4価)
【定期外接種(任意接種)】	生ワクチン 水　痘　流行性耳下腺炎(おたふくかぜ)　ロタウイルス　黄　熱 不活化ワクチン 破傷風トキソイド　成人用ジフテリアトキソイド B型肝炎　A型肝炎　狂犬病　肺炎球菌(23価多糖体)　ワイル病秋やみ 定期接種・子宮頸がん等ワクチン接種緊急促進事業対象ワクチンを対象年齢以外で受ける場合

国立感染症研究所感染症情報センター第3室(予防接種室) 室長

0371-1900/11/¥50/頁/JCOPY

表2 予防接種法に基づく接種と対象疾病

種類	目的	対象疾病
定期接種(一類疾病)	その発生およびまん延を予防すること.努力義務あり.	ジフテリア，百日せき，急性灰白髄炎，麻しん，風しん，日本脳炎，破傷風，結核 その発生およびまん延を予防するためとくに予防接種を行う必要があると認められる疾病として政令で定める疾病
定期接種(二類疾病)	個人の発病またはその重症化を防止し，併せてこれによりそのまん延の予防に資すること.努力義務なし.	インフルエンザ
臨時接種	一類疾病および二類疾病のうち，緊急のまん延予防.努力義務あり.	天然痘や高病原性鳥インフルエンザ(A/H5N1)のように感染力が強く，病原性がきわめて高いものを想定しているが，これまでに実施されたことはない
新しい臨時接種	右記に示したような新型インフルエンザが発生した場合の緊急のまん延予防.努力義務なし.	2009年に発生したパンデミックインフルエンザ(A/H1N1)と同程度の感染力や病状を呈する新型インフルエンザ

制度，予防接種後副反応報告制度等がある.

次にワクチンの種類をもとに分類すると，生ワクチンと不活化ワクチン/トキソイドにわけられる．接種後の健康状況の観察の視点や，接種スケジュールの構築を考えるうえで，ワクチンの制度や種類は重要なポイントである.

予防接種法に基づく予防接種と，基づかない予防接種

2011年7月22日に，「予防接種法および新型インフルエンザ予防接種による健康被害の救済等に関する特別措置法の一部を改正する法律(平成23年法律第85号)」が施行された．これにより，予防接種法に基づいて実施される予防接種には，従来の定期接種(一類疾病・二類疾病)，臨時接種に加えて，新しい臨時接種が導入されることとなった(**表2**).

予防接種法に基づく定期接種(一類疾病)，または従来から制定されていた臨時接種に関しては，対象者に対して国は，当該予防接種を受けることを勧奨しており，当該対象者が16歳未満の者または成年被後見人であるときは，その保護者に対して当該予防接種を受けさせることが勧奨されている．すなわち，被接種者は予防接種を受けるよう努める責務がある.

一方，今回制定された新しい臨時接種は，2009年に発生したパンデミックインフルエンザ(A/H1N1)のように，病状の程度がそれほど重くない新型インフルエンザが発生した場合の対応に万全を期するために制定されたもので，定期接種(一類疾病)や臨時接種のように，国民に対して予防接種を受ける努力義務を課さない新しい臨時接種である．努力義務を課さない定期接種としては，これ以外に定期接種(二類疾病)があげられる.

子宮頸がん等ワクチン接種緊急促進事業

2010年11月26日から，「子宮頸がん等ワクチン接種緊急促進事業」[1]の一環として，表1にあげた3つの種類の病原体に対するワクチンが接種されている．この事業は，市町村特別区長が実施主体になって行っているもので，2011年度からは，ほとんどすべての市町村特別区がこの事業を実施している.

接種の費用に関しては，都道府県にワクチン接種緊急促進基金が作られ，その基金が活用されていることから被接種者の費用負担はないか，あるいはきわめて少ない．接種に対する法律上の努力義務はない.

それぞれのワクチンの対象年齢は，以下に示す通りであるが，この年齢で受けない場合は，本事業の一環ではなく定期外接種(いわゆる任意接種)

表3 定期接種化に向けて議論されているワクチンのファクトシート(国立感染症研究所：2010年7月7日)

ワクチンの種類	2011年7月時点 URL
水痘ワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bxqx.pdf
おたふくかぜワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bybc.pdf
B型肝炎ワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bxqf.pdf
ヘモフィルスインフルエンザ菌b型ワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bxfi.pdf
肺炎球菌コンジュゲートワクチン(小児用)	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bxqo.pdf
肺炎球菌ポリサッカライドワクチン(成人用)	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000byee.pdf
ヒトパピローマウイルス(HPV)ワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000byb3.pdf
ポリオワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000bybl.pdf
百日せきワクチン	http://www.mhlw.go.jp/stf/shingi/2r9852000000bx23-att/2r9852000000byfg.pdf

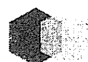
として受けることになる．

①ヒトパピローマウイルスワクチンについては，13歳になる年度(中1相当)から16歳になる年度(高1相当)の女性で，12歳になる年度(小6相当) から始める市町村特別区においては，15歳になる年度(中3相当)までの女性が対象である．

なお，医学的な理由で3回の接種が完了しなかった場合や，ワクチンの供給量不足で2010年度中に1回目の接種が行えなかった場合で2011年9月30日までに始めた場合は，17歳になる年度(高2相当)の女性も事業の対象に含まれる．

②インフルエンザ菌b型ワクチン(ヒブワクチン)については，生後2ヵ月から5歳未満の乳幼児が対象である．

③小児用肺炎球菌ワクチン(7価結合型ワクチン)についても，生後2ヵ月から5歳未満の乳幼児が対象である．



現在定期接種化が議論されている予防接種

表1に示すワクチンのうち，定期接種に含まれていないワクチンのなかにも，感染症予防ならびにその対策として重要なワクチンが多数存在する．一般の人々のなかには，定期接種に含まれていないワクチンは不要なワクチンと誤解している人もいる．海外では予防接種によってすでに患者数が激減している感染症が，国内では接種率が低いためにまだ多数の患者発生が認められていることは改善すべき点である．

2010年7月7日に国立感染症研究所は，定期接種化が必要と考えられている6疾患7ワクチン(水痘ワクチン，おたふくかぜワクチン，B型肝炎ワクチン，ヘモフィルスインフルエンザ菌b型ワクチン，肺炎球菌ワクチン(コンジュゲート，ポリサッカライド)，ヒトパピローマウイルスワクチン)について，ファクトシート[2]を発表した(**表3**)．さらに，これらに加えて，現在定期接種化はなされているが，近年の感染症発生動向をもとに，制度の変更が必要と考えられる2ワクチン(百日せきワクチン，ポリオワクチン)についても同様にファクトシート[2]を発表した(表3)．

さらに，予防接種法の対象となる疾病・ワクチンのあり方について，医学的・科学的な視点から議論を行い，予防接種法へ位置づけるかどうかについての考え方について整理し，予防接種部会に報告することを目的に，厚生科学審議会感染症分科会予防接種部会(部会長：国立成育医療研究センター総長 加藤達夫)のもとにワクチン評価に関する小委員会(委員長：国立感染症研究所感染症情報センター長 岡部信彦)が設置された．小委員会では，各疾病・ワクチンについて8つの作業チームが構成された．作業チームのメンバーはファクトシート[2]を作成した国立感染症研究所の専門家(疫学部門，製剤担当部門)，臨床の専門家，医療経済の評価に関する専門家，感染症疫学の専門家，その他各疾病・ワクチンの特性等に応じて適宜メンバーを追加して構成され，2010年末に作業チーム報告書[3]がまとめられ，小委員会から予防接種部会に報告された(2011年3月11日，5月26

表4 定期接種化に向けて議論されているワクチンのワクチン評価に関する小委員会作業チーム報告書（ワクチン評価に関する小委員会）

ワクチンの種類	2011年7月時点URL
水痘ワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rqn.pdf
おたふくかぜワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rqu.pdf
B型肝炎ワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rr1.pdf
ヘモフィルスインフルエンザ菌b型ワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rpv.pdf
肺炎球菌コンジュゲートワクチン(小児用)	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rq2.pdf
肺炎球菌ポリサッカライドワクチン(成人用)	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rq9.pdf
ヒトパピローマウイルス(HPV)ワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rqg.pdf
ポリオワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rr8.pdf
百日せきワクチン	http://www.mhlw.go.jp/stf/shingi/2r98520000014wdd-att/2r98520000016rrf.pdf

日）．その内容は，すべて上記ファクトシートともに厚生労働省のホームページに公開されているので是非一読されたい(表4)．

予防接種で予防可能な感染症の国内患者発生動向

感染症の予防及び感染症の患者に対する医療に関する法律(以下，感染症法)に基づく感染症発生動向調査では，一類感染症(7疾病)，二類感染症(5疾病)，三類感染症(5疾病)，四類感染症(42疾病)，五類感染症(44疾病)，感染症法第14条第1項に規定する厚生労働省令で定める疑似症(2疾病群)ついて，医師および指定届出医療機関の管理者に対して都道府県知事への届出を求めており，発生を監視するとともに，その対策に努めている．

このなかから，予防接種で予防可能な感染症あるいはそれを含む疾病について1999年第14週以降の患者報告数を表5に示す[4]．全数報告疾患と定点把握疾患があるため，これらの表を同時に比較することはできない．

定期接種のワクチンは定期外接種のワクチンに比べて接種率が高いことから，患者数は少ない傾向にあるが，定期外接種の対象疾病については，まだ多数の患者が国内で発生し，予防可能な感染症に罹患し，一部の人では重症化し，そのなかから死亡者が発生していることを忘れてはならない．2011年9月5日から，インフルエンザについては基幹定点医療機関からインフルエンザによる入院患者数の報告が求められることになった．

予防接種スケジュール

スケジュールを考えるに際して，最も重要な点は，発症後重症化リスクの高い感染症を第一優先に考えること，次いで，その感染症の好発年齢に達する前に接種を完了させること，周りの流行状況を考慮することである．また，スケジュールの構築にあたっては，ワクチンの種類を考える必要がある．

ワクチンには生ワクチンと不活化ワクチンがある．生ワクチンは，接種後ワクチン株が被接種者に感染し，体内で増殖する．その結果，被接種者の体内でワクチン株に対する液性免疫，細胞性免疫が産生され，経口生ワクチンでは腸管免疫も産生される．産生された免疫により，その後病原性の強い野生株が侵入してきたときに発症予防あるいは重症化予防を期待している．

以上のことから，ワクチン株が感染し増殖している間は，その病気が軽く発症したような症状が見られることがある．そのような時期に別の種類のワクチンを接種すると，それぞれのワクチン株が干渉して増殖が抑制され，免疫の獲得が不十分になったり，接種後の副反応が出現する好発時期に別のワクチンを接種することになる．これらを総合的に勘案して，生ワクチン接種後は27日以上別の種類のワクチンの接種を行わないことになっ

表5 予防接種で予防可能な感染症の国内患者発生動向（感染症発生動向調査より：2011年4月23日現在報告数）：国立感染症研究所感染症情報センターホームページ予防接種情報より抜粋

■発生累積報告数（全数把握対象疾患）

	1999年（14週〜）	2000年	2001年	2002年	2003年	2004年	2005年	2006年	2007年	2008年	2009年
痘そう*	…	…	…	…	0（11/5〜）	0	0	0	0	0	0
ポリオ**	0	0	0	0	0	0	0	0	1	2	0
結核***	…	…	…	…	…	…	…	…	21,946（4/1〜）	28,459	26,996
ジフテリア	1	0	0	0	0	0	0	0	0	0	0
コレラ	39	58	50	51	24	86	56	45	13	45	16
A型肝炎	763	381	491	502	303	139	170	320	157	169	115
黄 熱	0	0	0	0	0	0	0	0	0	0	0
狂犬病	0	0	0	0	0	0	0	2	0	0	0
日本脳炎	5	7	5	8	1	5	7	7	10	3	3
B型肝炎	510	425	330	332	245	241	209	228	199	178	178
急性脳炎****	…	…	…	…	12（11/5〜）	166	188	167	228	192	526
髄膜炎菌性髄膜炎	10	15	8	9	18	21	10	14	17	10	10
先天性風疹症候群	0	1	1	1	1	10	2	0	0	0	2
破傷風	66	91	80	106	73	101	115	117	89	123	113
風疹*****	…	…	…	…	…	…	…	…	…	293	147
麻疹*****	…	…	…	…	…	…	…	…	…	11,012	732
新型インフルエンザ等感染症******	…	…	…	…	…	…	…	…	…	0	12,654

注 *2003年11月5日から対象疾患．
**2006年4月1日からワクチン株由来の症例についても報告対象となった．それ以前は野生株ポリオのみ報告対象．
***2007年4月から対象疾患（結核統計による報告数とは異なる）．
****2003年11月4日以前は，定点把握対象疾患．急性脳炎には麻疹脳炎，風疹脳炎，インフルエンザ脳症などが含まれる．
*****2007年以前は．定点把握対象疾患．
******2008年5月12日より，新型インフルエンザ等感染症が追加され，新型インフルエンザ（A/H1N1）が全数把握対象疾患になったが，2009年8月25日より，定点把握疾患のインフルエンザとして報告されることになった．

■発生累積報告数（定点把握対象疾患）

		1999年（14週〜）	2000年	2001年	2002年	2003年	2004年	2005年	2006年	2007年	2008年	2009年
インフルエンザ	イ	65,471	769,964	305,441	747,010	1,162,290	770,063	1,563,662	900,181	1,212,042	621,447	3,068,082
感染性胃腸炎*	小	507,592	886,174	874,241	889,927	906,803	952,681	941,922	1,148,962	989,647	1,056,747	814,793
急性脳炎**	基	129	149	134	108	99（〜11/4）	…	…	…	…	…	…
細菌性髄膜炎***	基	235	256	278	300	298	379	309	350	383	410	462
水 痘	小	162,424	275,036	271,409	263,308	250,561	245,941	242,296	265,453	245,880	224,835	202,732
成人麻疹****	基	83	426	931	440	462	59	7	39	975	…	…
百日咳	小	2,653	3,804	1,760	1,458	1,544	2,189	1,358	1,504	2,932	6,753	5,208
風 疹****	小	2,972	3,123	2,561	2,971	2,795	4,239	895	509	463	…	…
ペニシリン耐性肺炎球菌感染症	基	2,129	4,321	5,254	6,132	6,447	6,692	6,233	5,294	4,840	5,257	4,773
麻疹（成人麻疹を除く）****	小	5,875	22,552	33,812	12,473	8,285	1,547	537	516	3,132	…	…
無菌性髄膜炎*****	基	1,126	1,873	1,254	2,985	1,625	1,028	773	1,140	797	744	644
流行性耳下腺炎（ムンプス）	小	69,070	132,877	254,711	180,827	84,734	127,592	187,837	200,639	67,830	65,361	104,568

イ：インフルエンザ定点 全国約5,000（内科 約2,000および小児科 約3,000）
小：小児科定点 小児科 全国約3,000
基：基幹定点 内科および小児科医療を提供する300人以上収容する病院 全国約470
2011年4月23日現在報告数．全国の定点医療機関より報告された数を示している．

*感染性胃腸炎にはロタウイルス腸炎が含まれる．
**2003年11月5日以降，全数把握対象疾患．
***細菌性髄膜炎にはインフルエンザ菌b型，肺炎球菌による髄膜炎が含まれる．
****2008年以降，全数把握対象疾患．
*****無菌性髄膜炎にはムンプス髄膜炎が含まれる．

ている．

一方，不活化ワクチンは接種後，ワクチン株が感染し体内で増殖するという生ワクチンのような過程はないため，接種後6日以上の間隔をあければ，別の種類のワクチンの接種が可能である．

ここで注意すべきことは，不活化ワクチンは生ワクチンのように1回あるいは2回の接種で長期間の免疫を維持することは困難であるため，基礎免疫に加えて，定期的に追加接種を行わなければ予防に必要な免疫を維持することが困難である．同じ不活化ワクチンを複数回接種する場合は，1週間間隔で接種するより，3〜4週間程度間隔を

空けたほうがその後の免疫獲得が良いことから，ワクチンによって接種の推奨間隔が定められている．不活化ワクチンであるという理由から同じ種類のワクチンを1週間間隔では接種しない．

予定したスケジュールで接種ができない場合もよくある．とくに年少児では，予定日に体調が優れない場合もあり，そのような場合は無理をして接種をするのではなく，体調が回復するまで待つことが重要である．また，集団接種で行われている定期接種のワクチンの場合，接種日が決められている場合が多いので，接種の予定を市町村特別区にあらかじめ問い合わせおくことも重要である．体調不良で予定通りのスケジュールで受けられなかった場合，その後の接種を忘れることが多いため，必ず受けられなかった日に次の予定を決めておくことが受け忘れ予防に繋がる．また，体調不良等で推奨された接種間隔を過ぎてしまっても最初からやり直す必要はなく，気付いた時点でなるべく早く接種を再開することが重要である．

一例として，接種スケジュール案を国立感染症研究所感染症情報センターのホームページ上に公開しているので参照して欲しい(2011年7月現在 http://idsc.nih.go.jp/vaccine/dschedule.html)[4]．また，日本小児科学会も推奨する予防接種スケジュールをホームページ上に公開しているので参照されたい(2011年7月現在 http://www.jpeds.or.jp/saisin/saisin_110427.pdf)．

複数ワクチンの同時接種

接種のスケジュールを考えるにあたって，とくに乳幼児期に接種するワクチンが近年多く承認されたこともあり，適切な時期にワクチンを接種し，発症予防に資するためには，複数ワクチンの同時接種が考慮される．現在，同時接種は医師がとくに必要と認めた場合は接種可能となっており，日本小児科学会からも同時接種に対する考え方が示されている[5]．それぞれのワクチンの接種間隔の原則を守りつつ，必要な時期になるべく早く免疫を獲得しておくためには，同時接種は必要な医療行為と考える．

ところが2011年3月初め，小児用肺炎球菌ワクチンおよびヒブワクチンを含む同時接種後の死亡報告が相次いだ．4名中3名は接種翌日の死亡，1名は接種3日後の死亡であった．これをうけて厚生労働省は，同年3月5日にこれらのワクチンの接種を一時的に見合わせると発表したが，その後2010年7月に接種を受けた者の死亡報告，接種3日後，7日後の死亡を含む3名の死亡報告があった．これらの7名のなかには，重症の基礎疾患を有する小児も含まれていた．

医薬品等安全対策部会安全対策調査会ならびに子宮頸がん等ワクチン予防接種後副反応検討会で，報告された7名についてのカルテや解剖所見など入手された情報から改めて評価が行われた結果，「現段階の情報において，いずれもワクチン接種との直接的な明確な因果関係は認められないと考えられる」との発表がなされた．

ワクチンの検定結果と品質管理についても再度調査され，「死亡報告されたロットについての試験結果は，すべて変動域内に留まり，逸脱は認められなかったこと，製造工程等の逸脱等について確認した結果も問題となる点は認められなかった」と報告された．

著者は，当時入手可能であった人口動態統計から，乳児の死亡数と乳幼児突然死症候群の報告数を月別にまとめワクチンの導入時期と比較検討した(**図1，2**)．その結果，小児用肺炎球菌ワクチンならびにヒブワクチンの導入後，導入前と比較して乳児の死亡数が多くなっているという傾向はこの調査からは認められなかった．

これ以外にも，海外での予防接種後の死亡例に関する調査が行われ[6]，いずれもワクチンとの因果関係は明確ではないが，感染症や乳幼児突然死症候群が原因の大半を占めており，小児用肺炎球菌ワクチンではおおむね対10万接種で0.1～1程度，ヒブワクチンではおおむね対10万接種で0.02～1程度の死亡報告がなされていた．今回国内で

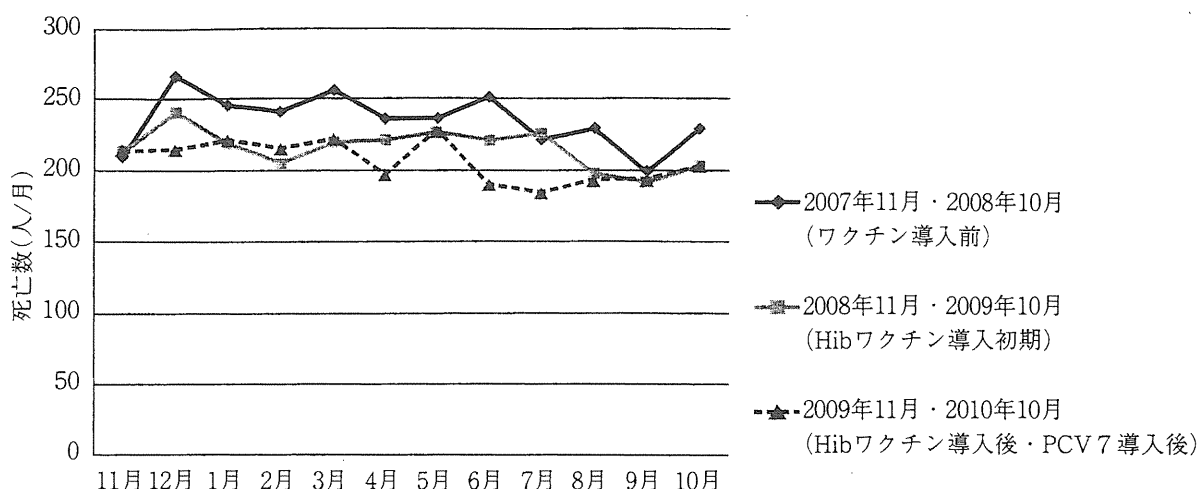


図１ 小児用肺炎球菌およびヒブワクチン導入前後の乳児死亡数(2007年11月～2010年10月)：人口動態統計より作図

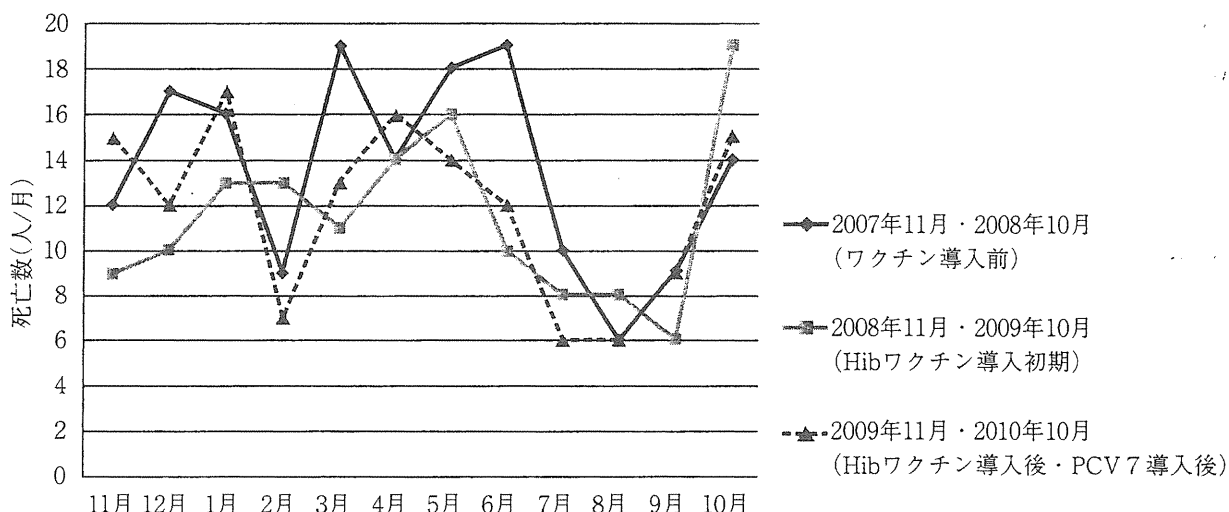


図２ 小児用肺炎球菌およびヒブワクチン導入前後の乳幼児突然死症候群による乳児死亡数
(2007年11月～2010年10月)：人口動態統計より作図

報告された死亡報告は両ワクチンとも対10万接種あたり0.1～0.2程度とされ，諸外国で報告されている状況と大きな違いは見られず，国内でのワクチン接種の安全性に特段の問題があるとは考えにくいとされた.

また,同時接種に関しては,「国内外の調査より，同時接種における副反応の発現率は，単独接種に比べて高い傾向があるとする報告もあるが，重篤な副反応の増加は認められておらず，とくに安全性上の懸念が認められない」とされた.

以上のことから，「小児用肺炎球菌ワクチンとヒブワクチンについては同時接種により短期間に効率的に予防効果を獲得できるメリットが期待され得ると同時に，それぞれ単独接種が可能であることを示した上で，同時接種を行う場合にはその必要性を医師が判断し，保護者の同時を得て実施する」とされた．また，「重篤な基礎疾患，例えば重篤な心疾患のある乳幼児については，髄膜炎等の重症感染症予防のためにワクチン接種が望まれるものであり，状態を確認して慎重に接種する，その際，単独接種も考慮しつつ，同時接種が必要な場合には医師の判断により実施する」とされた.

2011年３月当時入手可能であったさまざまな情報が収集され，審議会等で慎重に審議された結果，これらのワクチンの接種は2011年4月1日から再開された．以上の経過は，厚生労働省のホームペー