Table 2. Common variance and coefficient of regression for reference and sample vaccines at 4 and 7 days

Preparation	Common variance		Regression coefficient	
	4th day	7th day	4th day	7th day
DTaP reference	6.208	6.170	7.135	7.358
DTaP vaccine	7.591	7.380	6.631	6.718
DT standard	6.470	6.335	8.491	8.531
DT vaccine	7.116	7.079	9.257	9.300
T standard	6.659	6.282	7.941	8.005
T vaccine	8.244	8.208	9.899	9.546

DTaP, adsorbed diphtheria, tetanus, and acellular pertussis combined vaccine; DT, adsorbed diphtheria and tetanus combined toxoid vaccine; T, adsorbed tetanus toxoid vaccine; reference, Japanese national reference tetanus toxoid preparation (Lot 2, 40 U/vial); standard, Japanese national standard tetanus toxoid preparation (Lot 3, 65 IU/vial).

preparation. Based on the finding that the dose-response of each sample lot and that of the reference preparation showed no significant deviation from parallelism or linearity, it can be assumed that the assay results with a 4-day observation period are reliable. A comparison of the common variances and regression coefficients of potencies on the 4th and the 7th day indicated no statistically significant difference between the results, suggesting that the results can be considered interchangeable; the statistical parameters were essentially the same on both days (Table 2). In addition, no significant difference ($P = 0.05$) was observed between the potencies on the 4th and the 7th day for any of the three types of vaccine products (i.e., DTaP, DT, and T).

In this study, the vaccine potencies on the 3rd day were not examined. The challenge toxin solution used in this study was suitable for a 7-day observation period. The toxin solution did not have sufficiently high toxicity to show 50-200 LD_{50}/mouse on the 3rd day; the toxin solution did not satisfy the Japanese minimum requirement. The potencies on the 3rd day and the 7th day were not compared.

In conclusion, a reduction of the observation period in the tetanus toxin challenge method from 7 to 4 days is acceptable from the statistical point of view. This means that the results obtained on the 4th day can be considered to be a reliable indicator of a tetanus vaccine's potency. If widely adopted, this method will reduce animal suffering and provide significant cost benefits and labor-saving. Thus, curtailing the observation period from 7 to 4 days would be fully be applicable to the toxin challenge method and adoptable in the forthcoming version of the Minimum Requirements for Biological Products of Japan.

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Recombinant cholera toxin B subunit (rCTB) as a mucosal adjuvant enhances induction of diphtheria and tetanus antitoxin antibodies in mice by intranasal administration with diphtheria–pertussis–tetanus (DPT) combination vaccine

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Abstract

Recombinant cholera toxin B subunit (rCTB) which is produced by *Bacillus brevis* carrying pNU212-CTB acts as a mucosal adjuvant capable of enhancing host immune responses specific to unrelated, mucosally co-administered vaccine antigens. When mice were administered intranasally with diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of diphtheria toxoid (DTd), tetanus toxoid (TTd), pertussis toxoid (PTd), and formalin-treated filamentous hemagglutinin (fFHA), the presence of rCTB elevated constantly high values of DTd- and TTd-specific serum ELISA IgG antibody titres, and protective levels of diphtheria and tetanus toxin-neutralizing antibodies but the absence of rCTB did not. Moreover, the addition of rCTB protected all mice against tetanic symptoms and deaths. DPT combination vaccine raised high levels of serum anti-PT IgG antibody titres regardless of rCTB and protected mice from *Bordetella pertussis* challenge. These results suggest that co-administration of rCTB as an adjuvant is necessary for induction of diphtheria and tetanus antitoxin antibodies on the occasion of intranasal administration of DPT combination vaccine.

Keywords: DPT combination vaccine; Recombinant cholera toxin B subunit (rCTB); Intranasal administration; Diphtheria and tetanus antitoxin antibodies

1. Introduction

Cholera toxin (CT), a major enterotoxin produced by *Vibrio cholerae*, is composed of two subunits—a toxigenic A subunit (CTA) which activates ADP-ribosylation activity and a pentameric B subunit (CTB) which is responsible for CT binding to the cell membrane GM1 gangliosides. The basic chemical structure of heat-labile toxin (LT) which is produced by enterotoxigenic *Escherichia coli* is almost the same as that of CT. Both CT and LT act as a mucosal immunogen and a mucosal adjuvant, and induce antigen-specific systemic IgG and secretory IgA antibody responses at various mucosal sites to unrelated vaccine antigens when administered along with them via the nasal or oral route [1–5]. Only an A subunit of LT (LTA) and various mutant LTs and CTs lacking ADP-ribosylation activity also retain the mucosal adjuvant properties of wild CT and LT [5–11]. Moreover, it has been shown that recombinant CTB (rCTB) and B subunit of LT (rLTB) also have potent adjuvant activity towards intranasally or orally administered antigens [12–14].

We demonstrated that rCTB, which is produced by *Bacillus brevis* HPD31 carrying pNU212-CTB [15] and purified with affinity chromatography using D-galactose immobilized agarose [16], effectively delivers tetanus toxoid (TTd) and diphtheria toxoid (DTd) to mucosal inductive sites and elicits antigen-specific serum IgG and mucosal IgA antibody responses when given intranasally along

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with the toxoids [17,18]. With intranasal co-administration of an acellular pertussis vaccine the adjuvant activity of rCTB was vague because of the mucosal adjuvanticity of pertussis toxoid (PTd) [19]. No distinct local histopathological reactions were observed in the muscle, the nasal cavity and the small intestinal loop given rCTB [20], and frequent nasal administrations of rCTB-containing same and different vaccine-induced antigen-specific immune responses without induction of systemic tolerance and suppression by pre-existing anti-rCTB immunity [21]. In this study we investigated the adjuvant effect of rCTB on intranasal administration of a 30 μl (15 μl per nare) of a diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of DTd, TTd, PTd, and formalin-treated filamentous hemagglutinin (fFHA) and found that rCTB has an important role in the induction of diphtheria and tetanus antitoxin antibodies.

2. Materials and methods

2.1. Animals, immunogens, immunization, and sample collection

All procedures on animals were conducted according to the Guideline for the Care and Use of Laboratory Animals of the Nagoya City University Medical School under protocols approved by the Institutional Animal Care and Use Committee at the Nagoya City University Medical School. Female BALB/c mice (SLC, Shizuoka, Japan) aged 7 weeks were used in this study. Each group consisted of 5, 10, 15 or 16 mice. TTd containing 200 Lf units ml^{-1} (66 μg protein nitrogen (PN) ml^{-1} and purity: 3025 Lf mg^{-1} PN), DTd containing 200 Lf units ml^{-1} (67 μg PN ml^{-1} and purity: 2985 Lf mg^{-1} PN), PTd containing 16 μg PN ml^{-1}, pertussis toxin (PT) containing 30 μg PN ml^{-1}, formalin-non-treated filamentous hemagglutinin (FHA) containing 32 μg PN ml^{-1} and fFHA containing 75 μg PN ml^{-1} were provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). PT and FHA were used for an enzyme-linked immunosorbent assay (ELISA) and TTd, DTd, and PTd were not adsorbed to aluminum salt. Mice were administered and boosted intranasally with 30 μl of solution consisting of 5 Lf DTd, 5 Lf TTd, 0.5 μg PTd, and 5 μg fFHA (DPT combination vaccine) with and without 10 μg rCTB under light ether anesthesia. Intranasal administrations were performed on days 0, 14, 21, and 28. Mice were sacrificed on day 35 and blood was collected. Almost the same experiments were repeated four times and designated as Experiments 1–4. Experiment 4 was mainly carried out for a challenge test of tetanus toxin (TT). In Experiment 1 collection of lung and nasal cavity lavages, small and large intestinal washes, saliva and vaginal secretions, and feces was done for measurement of mucosal IgA titres as described before [12,17]. These samples were stored at −20 °C until use.

2.2. Measurement of antigen-specific IgG, IgA, and IgE antibody levels

Antigen-specific serum IgG and IgA, and mucosal IgA antibody titres were measured using ELISA (in-house ELISA) as described before [17,22] and antigen-specific serum IgE antibody was detected by fluorometric capture ELISA as described by Sakaguchi et al. [23] except the use of 5-[5-(*N*-succinimidyloxycarbonyl) pentylamido]hexyl D-biotinamide (Dojindo Laboratories, Kumamoto, Japan) in place of *N*-hydroxysuccinimidobiotin to conjugate antigen with biotin; serum and mucosal IgA, and serum IgE antibody titres were determined only in Experiment 1. Calibration of the mean and standard deviation (S.D.) of values at 450 and 405 nm with sera and each washing sample of five non-immunized mice, setting of antibody-positive cut off values and expression of antigen-specific antibody ELISA titres and the geometric mean (G.M.) ± S.D. were carried out according to the previous description [19].

2.3. Cell culture method (CCM) for measurement of diphtheria antitoxin titres

In Experiments 1–4, the titration of diphtheria antitoxin of mouse serum was performed by CCM using Vero cells as described by Miyamura et al. [24] and diphtheria antitoxin titres were expressed as international neutralizing antibody units (IU) ml^{-1}.

2.4. Determination of anti-tetanus toxin antibody

The TT neutralization (TTN) assay was carried out in mice as described in "Minimum Requirement for Biological Products [25]". Briefly, six-fold dilution (in Experiments 2 and 3) and two-fold dilution (in Experiment 4) of pooled sera were performed and assayed for TT level of L+/1000. Tetanus antitoxin titres were calculated relatively for the standard tetanus antitoxin [26,27] and expressed in IU ml^{-1}.

2.5. TT direct challenge test

Five days after final collection of blood on day 35, mice were challenged by subcutaneous injection of approximately 100 LD_{50} of TT and was observed through out their life and death after 7 days.

2.6. Kaketsuken particle agglutination (KPA) method

The particle agglutination test with poly (γ-methyl L-glutamate), which was developed and shortened to KPA by KAKETSUKEN (The Chemo-Sero-Therapeutic Research Institute) [28], was carried out beforehand for the purpose of reference to measure tetanus antitoxin titres in

Experiments 1–4 and diphtheria antitoxin titres in Experiments 2–4, using Tetanus and Diphtheria Antibody Assay kits, respectively. The procedure was done according to the manufacturer's instructions. The highest dilution showing positive agglutination was determined and the unitage of serum sample was calculated against the reference serum and expressed as IU ml^{-1}. There is a significant correlation [28] between the level of anti-tetanus KPA titres and that of tetanus antitoxin titres by hemagglutination [29] and between the level of anti-diphtheria KPA titres, and that of diphtheria antitoxin titres by CCM [24].

2.7. Measurement of anti-PT and anti-FHA antibody titres by calibration against a reference mouse serum

Serum PT- and FHA-specific IgG antibody titres were also expressed as ELISA units (EU) ml^{-1} on the basis of a comparison of the response curve of the test serum to that of the reference mouse serum provided by the National Institute of Infectious Diseases, Tokyo, Japan (anti-PT IgG antibody: 886 EU ml^{-1} and anti-FHA IgG antibody: 21 900 EU ml^{-1}). This was done according to a modified version of the parallel line assay procedure described by Sato and Sato [30].

2.8. Intranasal challenge of Bordetella pertussis

Intranasal infection of *B. pertussis* 18 323 phase I was performed using mice immunized intranasally with DPT combination vaccine with and without rCTB according to the method described before [19] and three mice per group were used.

2.9. Statistics

Analysis of antibody titres was performed on logarithmically transformed data and the G.M. and S.D. values were calculated. Mann–Whitney's *U*-test was used to compare mean values of different groups with serum and mucosal antibody titres. Statistical significance was designated as $P < 0.01$ or $P < 0.05$.

3. Results

3.1. DTd-specific serum IgG antibody and diphtheria antitoxin responses: comparison among in-house ELISA, CCM, and KPA titres

We assessed the immune responses to DTd induced by intranasally delivered DPT combination vaccine. Mice immunized with DPT plus rCTB showed much higher DTd-specific serum IgG antibody titres by in-house ELISA than those without rCTB except Experiment 1, indicating the statistically significant adjuvant effect of rCTB in Experiments 2 and 3 (Fig. 1A).

Mice immunized with DPT plus rCTB showed the mean $\log_{10}$ diphtheria antitoxin titres ± S.D. values of 0.47 ± 0.36, 0.80 ± 0.37, and -0.21 ± 0.65 IU ml^{-1} in Experiments 1–3, respectively, which were measured by CCM (Fig. 1B). These titres were sufficiently greater than 0.01 ($\log_{10} -2$) IU ml^{-1} of serum and significantly higher than those obtained from mice treated with DPT alone, clearly showing that rCTB specifically induces diphtheria antitoxin antibody even if in-house ELISA antibody titres are almost the same irrespective of rCTB (Fig. 1A, Experiment 1). Diphtheria antitoxin titres measured by KPA in Experiments 2 and 3 also showed significant differences between mice immunized with DPT in the presence and absence of rCTB (Fig. 1C).

3.2. TTd-specific serum IgG antibody and tetanus antitoxin responses: comparison among in-house ELISA, KPA, and TTN titres

The addition of rCTB to DPT combination vaccine induced sufficiently high serum anti-TTd IgG in-house ELISA antibody titres, which were significantly high only in Experiment 3 when compared with no addition of rCTB (Fig. 2A).

The mean $\log_{10}$ tetanus antitoxin titres ± S.D. values measured by KPA were 0.65 (pool sera), -1.75 ± 0.69 and -1.47 ± 0.96 in Experiments 1–3, respectively, in the absence of rCTB and 2.53 (pool sera), 0.89 ± 0.05 and 0.75 ± 0.31 in Experiments 1–3, respectively, in the presence of

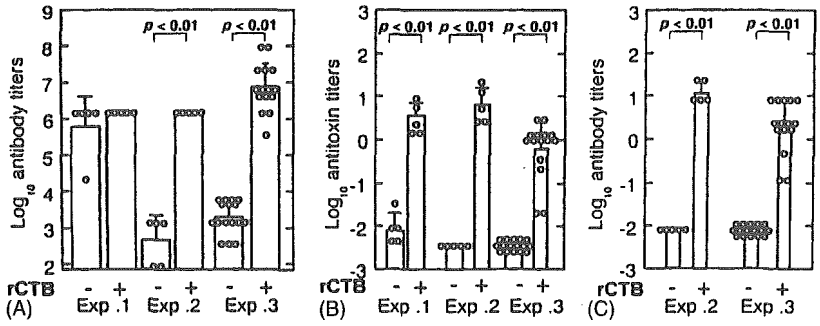

Fig. 1. Correlation among serum DTd-specific IgG in-house ELISA antibody titres (A), diphtheria antitoxin titres by CCM (B), and anti-diphtheria KPA titres (C) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times but anti-diphtheria KPA titres were not measured in Experiment 1.

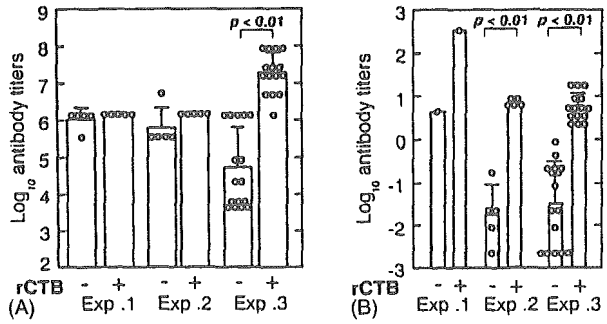

Fig. 2. Correlation between serum TTd-specific IgG in-house ELISA antibody titres (A) and anti-tetanus KPA titres (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. In Experiment 1 pooled sera from five mice were used to measure anti-tetanus KPA titres.

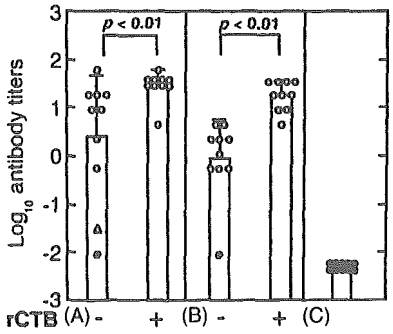

Fig. 3. Correlation between serum anti-tetanus KPA titres and life and death of mice in Experiment 4. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB (A) and with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd ± 10 μg rCTB (B) or administered intranasally with PBS (C) on days 0, 14, 21, and 28. Serum was collected from each mouse on day 35 and used for the measurement of anti-tetanus KPA titres. Pooled sera were used for TTN test. Tetanus toxin challenge test was performed on day 40 and observed through out the life and death of mice after 7 days. (○) Mice showing no symptoms, (△) paralyzed mouse, and (●) dead mice. Anti-diphtheria KPA titres were also examined in Experiment 4 but not shown because almost similar results to those of Fig. 1C were obtained.

rCTB (Fig. 2B). These tetanus antitoxin titres were significantly different between the presence and absence of rCTB unlike serum anti-TTd IgG in-house ELISA antibody titres. Subsequently, the toxin neutralization tests were carried out by six-fold dilution using the pool sera of mice obtained from Experiments 2 and 3. In Experiment 2 the TTN titres of pool sera from each five mice were less than 0.01 IU ml^{-1} without rCTB and 0.36–2.16 IU ml^{-1} with rCTB (Table 1). In Experiment 3, 15 or 16 mice were divided into two groups by KPA titres, i.e. into a higher and lower one. In mice immunized with DPT alone, TTN titres obtained from pool sera of 5 mice showing KPA titres less than 0.0025 unit ml^{-1}, and 11 mice showing KPA titres from 0.01 to 1.0 units ml^{-1} were less than 0.01 IU ml^{-1} (Table 1). On the other hand, in mice immunized with DPT in the presence of rCTB both higher group (5 mice) showing KPA titres between 8 and 16 unit ml^{-1} and lower one (10 mice) showing KPA titres between 2 and 4 unit ml^{-1} demonstrated TTN titres from 1.80 to 10.8 IU ml^{-1} (Table 1). Judging from these results, the addition of rCTB is essential to elevate tetanus antitoxin titres unlike anti-TTd antibody in-house ELISA titres.

Table 1
Correlation of serum anti-tetanus KPA titres with tetanus toxin neutralization (TTN) titres of pooled sera

Experiment number	rCTB	Number of mice	KPA titres (units ml^{-1})[a]	TTN titres (IU ml^{-1})[b]
Experiment 2	−	5	0.0025–0.16	<0.01
	+	5	6.4–8.0	0.36–2.16
Experiment 3	−	5	<0.0025	<0.01
	−	11	0.01–1.0	<0.01
	+	10	2.0–4.0	1.8–10.8
	+	5	8.0–16.0	1.8–10.8

[a] See Fig. 3B.
[b] Pooled sera were used and TTN tests were carried out by six-fold dilution.

3.3. Correlation between TTd-specific KPA and TTN titres

In Experiment 4, the correlation between TTd-specific KPA and TTN titres was investigated in detail in combination with DTd + TTd + PTd ± fFHA ± rCTB. As shown in Fig. 3, tetanus antibody titres measured by the KPA method with rCTB were significantly higher in comparison with those in the absence of rCTB irrespective of fFHA. Two of the 10 mice immunized with the mixture of DTd, TTd, PTd, and fFHA without rCTB showed very low anti-tetanus KPA titres, and one of them died on day 1 and the other one was paralyzed (Fig. 3A). Only one of the 10 mice immunized with DTd, TTd, and PTd in the absence of both fFHA and rCTB showed very low anti-tetanus KPA titre and died on day 1 (Fig. 3B). All mice inoculated with phosphate buffer saline died on day 1 (Fig. 3C). However, the addition of rCTB to DPT combination vaccine surely protected all mice against tetanic symptoms and deaths regardless of fFHA (Fig. 3A and B). TTN titres obtained from pooled sera of each ten mice immunized without rCTB were 2.77 IU ml^{-1} in the presence of fFHA and 0.36 IU ml^{-1} in the absence of fFHA. The addition of rCTB showed high TTN titres of 11.60 and 10.30 in the presence and absence of fFHA, respectively. Diphtheria antitoxin titres measured by CCM and KPA in the same samples were significantly higher in the presence of rCTB like Experiments 1–3 (data not shown).

Together with the results obtained from Experiments 1–3, these results made it clear that the addition of rCTB to DPT combination vaccine is necessary to protect against tetanus and diphtheria.

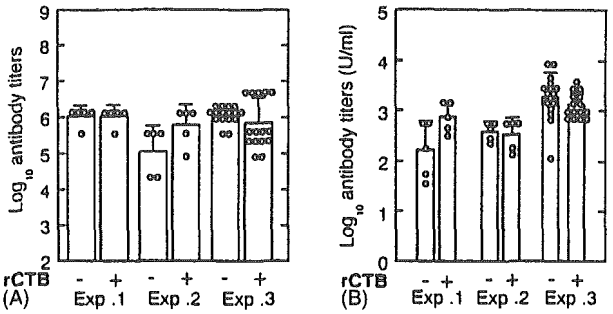

Fig. 4. Correlation between serum PT-specific antibody titres by in-house ELISA (A) and ELISA units by calibration against a reference mouse serum (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. Both titres were not measured in Experiment 4.

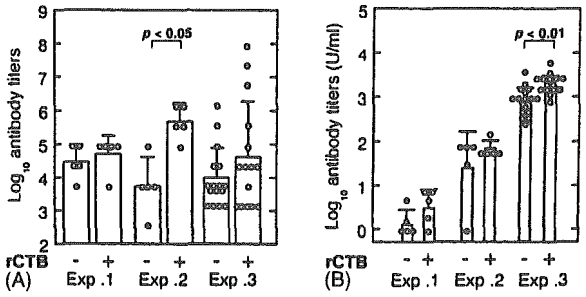

Fig. 5. Correlation between serum FHA-specific antibody titres by in-house ELISA (A) and ELISA units by calibration against a reference mouse serum (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. Both titres were not measured in Experiment 4.

3.4. Serum PT-specific IgG antibody titres by in-house ELISA and serum anti-PT ELISA units by calibration against a reference mouse serum

High levels of anti-PT serum IgG antibody titres by in-house ELISA were induced irrespective of rCTB through Experiments 1–3 and there were no significant differences in the presence and absence of rCTB (Fig. 4A). Serum anti-PT ELISA units from calibration against a reference mouse serum also increased and the presence and absence of rCTB showed no significant differences (Fig. 4B).

3.5. FHA-specific serum IgG antibody titres by in-house ELISA and serum anti-FHA ELISA units by calibration against a reference mouse serum

Serum FHA-specific IgG antibody titres by in-house ELISA increased substantially irrespective of rCTB and there was a significant difference in the presence and absence of rCTB only in Experiment 2 (Fig. 5A). Anti-FHA ELISA units obtained from calibration against a reference mouse serum were also elevated regardless of the presence or absence of rCTB and a significant increase with rCTB was observed only in Experiment 3 (Fig. 5B).

3.6. Protection from intranasal challenge of B. pertussis in mice intranasally administered with DPT combination vaccine with and without rCTB

The bacteria were not removed from the lungs of non-vaccinated control mice even on day 14 of infection. However, regardless of rCTB, intranasal administration with DPT combination vaccine showed complete clearance of the bacteria from the lungs by 6 days postinfection (data not shown).

3.7. DTd-, TTd-, PT-, and FHA-specific serum IgA and IgE antibody responses

Serum IgA antibodies specific to all components of DPT vaccine were produced independent of rCTB and anti-DTd, TTd, and FHA IgA antibody titres were significantly higher in the presence of rCTB (Fig. 6A). Serum IgE antibody responses to DTd, TTd, and PT were seen in the presence of rCTB and TTd- and PT-specific IgE antibody titres were significantly higher (Fig. 6B).

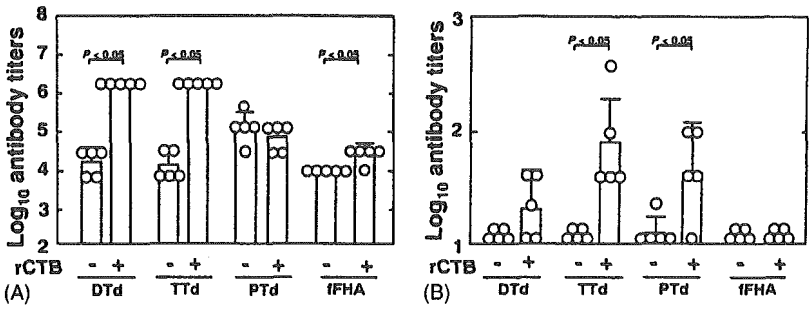

Fig. 6. Serum DTd-, TTd-, PT- and FHA-specific IgA (A) and IgE (B) antibody titres with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. Both titres were measured only in Experiment 1.

3.8. Mucosal DTd-, TTd-, PT-, and FHA-specific IgA antibody responses

Mucosal antigen-specific IgA antibody titres were measured only in Experiment 1. Significantly higher levels of mucosal DTd-specific IgA antibody titres were observed in the lungs, small intestines, feces and the saliva and vaginal secretions in the presence of rCTB (data not shown), almost coinciding with the results obtained from intranasal immunization with DTd ± rCTB [18]. Mucosal TTd-specific IgA antibody titres rose significantly in the nasal cavities, lungs, small intestines, feces, and the saliva and vaginal secretions in the presence of rCTB (data not shown), being different from the previous results from intranasal immunization with TTd ± rCTB [17]. The difference between two data may be due to the presence of PTd. High or moderate levels of PT-specific mucosal IgA antibody responses were induced in the lungs and the vaginal secretions regardless of rCTB and mucosal anti-FHA IgA antibody titres in the presence of rCTB increased significantly in the lungs and small intestines but not in the other mucosal sites (data not shown). These results were roughly in agreement with the those from intranasal immunization with PTd + fFHA ± rCTB [19].

4. Discussion

In this study the mucosal adjuvant effect of rCTB on intranasal administration of DPT combination vaccine consisting of DTd, TTd, PTd, and fFHA was investigated and it was clearly shown that co-administration of rCTB is essential for induction of diphtheria and tetanus antitoxin antibodies. Moreover, all mice intranasallly administered with a mixture of DPT combination vaccine and rCTB were surely protected from challenge of TT and diphtheria antitoxin titres above a protective level of 0.1 IU ml^{-1}, which is the smallest level necessary to protect humans from diphtheria, were induced. These results coincided with those from intranasal co-administration of rCTB and DTd or TTd except a little high serum IgE antibody titres [17,18]. Our previous studies showed that subcutaneous injection of aluminium-adjuvanted DTd elicited high levels of DTd-specific serum IgG, no or slight levels of DTd-specific serum and mucosal IgA and significantly high levels of DTd-specific serum IgE antibody responses [31], and moreover, caused severe histopathological reactions [20]. Accordingly, rCTB seems to be an excellent adjuvant. The mucosal adjuvant effect of rCTB for mixed PTd and fFHA is inconspicuous because of the mucosal adjuvanticity of PTd and induction of moderately high levels of serum PT-specific IgE antibody titres [19].

As far as we know about intranasal administration of DPT combination vaccine and a mucosal adjuvant in animal experiments, there is only one paper reported by Nagai et al. [32]. They used some onjisaponins, from the root of *Polygala tenuifolia* Willdenow, as a mucosal adjuvant and showed significant increases in serum anti-DTd, -TTd, -PTd IgG, and nasal IgA antibody titres. However, there were no data on diphtheria and tetanus antitoxin antibody and anti-DTd, -TTd, and -PTd serum IgE antibody titres.

It is generally known that mucosal IgA plays an important role in protection against infections by enteropathogens and viruses both in human and animal models [33–37]. The inhibition of bacterial adherence by mucosal IgA is considered to be one of the most important defense mechanisms against mucosal bacterial invasion and in vitro, has been shown to limit the attachment of bacteria to epitherial cells isolated from various mucosal sites [38–43]. Moreover, mucosal IgA can also neutralize toxins by blocking their binding to cell receptors [44]. On the other hand, a functional role for serum IgA is still unclear but the IgA, not the IgG, serum fraction, from patients with *Clostridium difficile* has been reported to neutralize the cytotoxic and enterotoxic properties of the major virulence factor, toxin A, of the bacterium [45]. In this study, rCTB significantly enhanced production of serum IgA antibodies and mucosal ones to DTd in the respiratory tract and the vagina. In our preliminary experiment, IgAs purified from three mouse sera with 13.0, 1.63, and 3.26 CCM IU ml^{-1}, which were intranasally administered with DTd + rCTB, showed 0.102, 0.005, and 0.026 CCM IU ml^{-1}, respectively, and mucosal IgAs purified from the pulmonary lavages of 4 mice intranasally immunized with DTd + rCTB, showed 0.006, 0.102, 0.026, and 0.026 CCM IU ml^{-1}, suggesting the possibility of neutralizing diphtheria toxin of IgA. However, it has been suggested that IgA is not essential for preventing viral infection, reduction in the severity of disease, or both because of no differences between wild-type $IgA^{+/+}$ and transgenic $IgA^{-/-}$ knockout mice [46]. The role of systemic and mucosal IgA merits further research.

There have been several studies that have tracked the tissue distribution of a marker following intranasal delivery to mice but the results obtained from these studies have been varied [47–51]. Visweswaraiiah et al. [52] reported in detail that all parameters such as level of anesthesia, position of the animal during and post dye administration, dosing schedule and total volume administered are important for a solution delivered intranasally to be retained in the nasal cavity of a mouse. In case of a total volume of 30 μl, it is important to hold a mouse under heavy anesthesia and in a supine position both during and post dye administration and to administer according to the optimal schedule of administration, i.e. 2 μl per nare at 5 min intervals [52]. However, in our experiment a 15 μl bolus of vaccine per nare (a total volume of 30 μl) was delivered intranasally to moderately anesthetized mice held upright during administration and placed on its stomach in the cage post administration. Accordingly, a part of intranasally administered vaccine may have been swallowed or aspirated into the lungs.

Phase 1 safety studies conducted with intranasally administered rCTB and LTR192G revealed only minor side effects including self-limiting increased nasal secretions, itching,

runny nose and sneezing within a certain amount of them but not visible effects on the nasal mucosa, systemic adverse events and long-term adverse events [53]. Clinical trials with an inactivated, virosome-formulated, LT-adjuvanted, intranasal subunit influenza vaccine showed solicited and unsolicited symptoms such as nasal discomfort, sneezing, nasal pain, stuffy nose, runny nose, shivering, malaise, and so on. Moreover, Bell's Palsy (facial paresis) was observed in a tiny minority [53]. However, a detailed analysis of the observed cases has not revealed a distinct pattern for an influenza vaccine-induced facial paresis. Pre-clinical studies in animal models are needed to evaluate the safety of intranasally administered CT and LT adjuvants, and derivatives prior to the initiation of clinical trials. Intranasal administration of rCTB to BALB/c mice caused no distinct local histopathological reactions in the nasal cavity [20]. If rCTB is confirmed to be completely non-toxic for humans, an intranasal DPT combination vaccine with rCTB could be easily licensed for human use. The needle-free intranasal administration is an attractive alternative procedure, especially in developing countries.

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Original Article

Tetanus Antibody Assay Combining In-House ELISA and Particle Agglutination Test and Its Serosurvey Application in a Province in Turkey

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SUMMARY: In order to determine a practically useful quantitative assay method for tetanus antibody in a large-scale seroepidemiological study, a method combining an in-house ELISA with a particle agglutination test (KPA) was evaluated in comparison with the in vivo mouse neutralization test. Serum samples with mouse neutralization antibody titers 0.01 IU/ml (the minimum protective level) or below showed considerable overestimation of antitoxin titers up to 1.0 IU/ml when studied by in-house ELISA alone. On the other hand, the KPA values were highly correlated with the mouse test, even in cases of titers equal to 0.01 IU/ml or below. The combination of these two procedures, in which in-house ELISA values of 1.0 IU/ml or below were replaced by KPA values, provided a high correlation in antibody titers with the mouse test ($r = 0.968$). We applied this combined method to a tetanus seroepidemiological survey in a province in Turkey. The survey included 347 subjects from the healthy population, and the quantitative analyses showed high antibody levels in children and young adults and significantly low levels among adults aged 40 or over. A characteristic distribution of antibody titers in each age group was also demonstrated.

INTRODUCTION

In 1997, Refik Saydam National Hygiene Center (RSHC) and the General Directorate of Primary Health Care of the Ministry of Health of Turkey, with the cooperation of the Japan International Cooperation Agency, implemented a project for infectious disease control in Turkey. Seroepidemiological surveillance on vaccine-preventable diseases including tetanus, diphtheria, pertussis, polio, measles, and hepatitis B, was conducted in 2000-2001 (1,2). Three provinces with geographically distinct locations and different socio-economic levels were selected for the survey; one each from the regions of the Black Sea (Samsun), the Mediterranean (Antalya), and the South-East Anatolia (Diyarbakir). This was a cross-sectional study involving both urban and rural health centers. Serum samples from randomly selected healthy subjects were analysed to determine the current states of immunity to vaccine-preventable diseases. The project also aimed to establish laboratory methods for routine use in seroepidemiological surveillance in RSHC.

For infectious disease surveillance in a population, seroepidemiological study on antibody prevalence is essential, and it requires a method technically and economically suitable to test a large number of samples. Also, the method should preferably be applicable to various antigens in a laboratory. ELISA is a suitable method for this purpose; it is cost effective and can evaluate antibodies against various antigens using the same procedure.

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According to a generally adopted classification, a tetanus antitoxin level of 0.01 IU/ml is considered to be the minimum protective level though it affords incomplete protection; 0.1 IU/ml is considered protective; and 1.0 IU/ml or above offers long-term protection (3-5). The reliable titration of such protective levels is necessary to predict the immunity against tetanus in a given population. ELISA has been widely used for tetanus seroepidemiology. However, human sera with low antibody titers are reportedly often overestimated when titrated by ELISA (3,6). To overcome this problem, the tetanus antibody assays with ELISA in the early studies were supplemented by a neutralizing antibody assay in mice for low-titer sera (7,8). Subsequent modifications of the ELISA with additional procedures such as a competitive assay, a toxin binding inhibition assay, and a double-antigen procedure were then described (9-12).

The purpose of the present study was to determine a practically useful method for a quantitative assay of tetanus antibodies in a large number of serum samples, covering even the minimum protective level or lower. We evaluated a combined assay method in which the antibody titers equal to or less than 1.0 IU/ml with in-house ELISA were replaced by those of a particle agglutination test (KPA). We also describe here the result of the application of this combined method to a seroepidemiological survey conducted in a province as a part of a project for infectious disease control in Turkey. Our study showed that this combined assay method made it possible to provide accurate assessment of age-specific tetanus antibody prevalence in the areas under study.

MATERIALS AND METHODS

Mouse neutralization test: Tetanus test toxin (4.4×10^6

LD_{50}/ampoule) supplied by the National Institute of Infectious Diseases, Tokyo, was used for the in vivo assay. The test was performed by the lethal end point method using Swiss mice of either sex, weighing 17-20 g, randomly bred in our own establishment. Sera were titrated at the L+/1000 dose of tetanus toxin. The range of serum dilutions to be tested depended on the tetanus antitoxin content of the sera, which had been previously determined by in-house ELISA. Three different dilutions of serum were incubated with tetanus toxin for 1 h at room temperature, and 0.4 ml of the mixtures was then injected subcutaneously into each of two mice. The mice were observed for the following 5 days, and the symptoms and time of death were recorded. A control series of mice, injected with tetanus toxin mixed with defined amounts of the International Standard for Tetanus Antitoxin (1,400 IU/ampoule, National Institute for Biological Standards and Control [NIBSC], Potters Bar, UK) were included in every experiment, and titers of the samples were calculated according to these results. The amount of tetanus antitoxin in each serum was expressed in International Units per ml (IU/ml). The cut-off value used for the test in our laboratory was 0.01 IU/ml.

KPA: KPA was performed using a kit manufactured by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. The kit was made of polypeptide artificial carrier particle (porous spheres of poly γ-methyl-L-glutamate) stained with Reactive Blue solution, sensitized with highly purified tetanus toxoid using tannic acid, and provided in lyophilized form (13,14). The test was performed according to the manufacturer's instructions. For brief, a volume of 25 μl of twofold serial dilutions of test sera was mixed with a 25 μl suspension of the coated particle in the wells of a U-bottomed microplate. The plate was held for 2 h at room temperature. Non-agglutinated particles settled to form a clear spot with a smooth circumference in the center of the well that was judged to be negative. Agglutinated particle formed a large rough ring that was judged to be positive. The reference serum of the kit was assayed in parallel in each test, and the result was used to determine the minimum detectable level. The antibody titer of the test serum was calculated by multiplying the end point dilution of positive agglutination and the minimum detectable titer of the reference serum. According to the manufacturer, the cut-off value of the test is 0.01 IU/ml.

In-house IgG ELISA (In-house ELISA): An in-house ELISA for anti-tetanus toxoid IgG antibody was conducted using 96-well flat-bottom plates (Greiner, no. 655001, Frickenhausen, Germany) with a purified tetanus toxoid (Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan). The plates were coated with tetanus toxoid (100 μl at 2 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6) overnight in a refrigerator in a humid atmosphere. Then 125 μl of blocking buffer (PBS containing 0.5% BSA) was distributed into each well and incubated for 1 h at 37°C in an Incubator/shaker (Labsystem, Helsinki, Finland). After every step, plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Eight twofold serial dilutions of test sera and the reference antiserum were prepared with PBS containing 0.5% BSA and 0.05% Tween 80, and then added to the plates. After being incubated for 1 h at 22°C, Fc-specific alkaline phosphatase-conjugated goat anti-human IgG (Seikagaku Kogyo, Tokyo, Japan) diluted in PBS-T was distributed to each well, and plates were held for 1 h at 22°C. Finally, p-nitrophenyl phosphate (Wako, Tokyo, Japam) in diethanolamine buffer (1 mg/ml, pH 9.6) was added to each well. After 1 h at 22°C, the reaction was stopped by 3M NaOH solution, and the plates were read at $A_{405/630}$ by an ELISA reader (Labsystem).

The International Antitoxin Unit was determined using the reference antitoxin (Anti-tetanus immunoglobulin, human, lyophilized, 120 IU/ml, NIBSC), using a parallel line assay (15). A standard curve was drawn for each plate, and the curve drawn by the optical densities of test serum dilutions, which was linear and parallel to the standard curve, was interpolated. Statistical significance in the test was set at a probability of $P = 0.05$. The cut-off value of the test was determined to be 0.01 IU/ml.

Study population and serum samples: This study was part of the national seroepidemiological surveillance of vaccine-preventable diseases, conducted by the Ministry of Health of Turkey in three selected provinces in 2000-2001 (1). For the surveys, study subjects were selected randomly from each age group by health centers in the given areas, and questionnaires and blood samples were collected after obtaining informed consent to participate (2).

For the comparative study between the tests, 62 serum samples from Samsun Province with different antibody levels by ELISA were used. More sera with low ELISA titers were selected and titrated by the mouse neutralization test and KPA, respectively. The sera studied to determine the applicability of the method to seroepidemiology were collected in 2001 from Antalya Province from subjects including a healthy population ranging in age from 6 months to 91 years old

Table 1. Number of subjects and vaccination history, Antalya, 2001

Age group	Total	Sex		Area		Tetanus vaccination dose				
		Male	Female	Rural HC	Urban HC	Non	1-2	3-4	5≤	unknown
0-1	28	13	15	14	14	0	2	26	0	0
2-3	31	14	17	17	14	1	0	29	0	1
4-5	26	11	15	12	14	0	0	25	0	1
6-7	27	10	17	12	15	1	0	16	10	0
8-9	32	18	14	17	15	0	2	2	27	1
10-19	33	14	19	18	15	0	6	6	18	3
20-29	41	16	25	19	22	0	12	14	6	9
30-39	40	16	24	18	22	2	10	10	12	6
40-49	42	20	22	24	18	2	14	8	3	15
50-	47	25	22	23	24	3	12	4	1	27
Total	347	157	190	174	173	9	58	140	77	63

HC: health centers.

(Table 1). The group of 347 subjects consisted of males and females, from rural and urban areas, with no statistically significant difference between groups ($P > 0.05$).

Statistical analysis: Statistical analysis was carried out using the chi-squeare test and F test. A titer of less than 0.01 IU/ml was set to 0.005 IU/ml for calculating the geometric mean titer (GMT).

RESULTS

Comparison of antibody titers measured by KPA or in-house ELISA with those by in vivo neutralization test in mouse: Sixty-two sera were used for the comparison. The in vivo neutralization test in mouse was taken as the standard test. Correlations of the values obtained by the KPA and the in-house ELISA against the mouse test are shown in Figs. 1a and 1b, respectively.

In the mouse test, 37 of the 62 sera provided antibody titers equal to or above 0.01 IU/ml, and 25 sera were below this level. The antibody values determined by KPA correlated well with those by the mouse test; 37 sera were >0.01 IU/ml and 25 were below this level (Fig. 1a). On the other hand, in the in-house ELISA, 23 of 25 serum samples that were below 0.01 IU/ml by the mouse test showed values between 0.1 IU/ml and 1.0 IU/ml (Fig. 1b). Thus, within this low-titer range, the in-house ELISA values resulted in a mixture of specific and non-specific reactions in which it was impossible to differentiate the presence of protective antibody from non-specific reactivity. The results indicated that the in-house ELISA was most useful to estimate samples above 1.0 IU/ml. Under this level, however, the method is not acceptable, and the serum titers should be determined by either the mouse neutralization test or KPA.

Combination of tetanus antibody titers of in-house ELISA above 1.0 IU/ml and KPA equal to or below 1.0 IU/ml: Figure 1c shows the correlation analysis of the combined assay method in which the tetanus antibody titers >1.0 IU/ml (17 of 62 serum samples) were based on the results of the in-house ELISA and those 1.0 IU/ml or below (45 of 62 serum samples) were based on the KPA. In order to evaluate the combination of the two methods statistically, the correlation coefficients and the regression lines against the mouse test were calculated separately for the titers from the two methods of the combined line, excluding double-negative titers in both tests (<0.01 IU/ml, $n = 24$) in order to avoid adverse affects resulting from their significant number.

In Fig. 1c, the correlation between titers of the mouse test and a range >1.0 IU/ml of the combined line (titers based on the in-house ELISA, $n = 17$) was r = 0.903, and the regression line equation was Y = 1.1509 X − 0.1569. The correlation between titers of the mouse test and a range of <1.0 IU/ml of the combined line excluding the double-negatives in both tests (titers based on the KPA, $n = 21$) was r = 0.921, and the regression line equation was Y = 0.9399 X − 0.1607. No statistically significant difference was found between the correlation coefficients of the two component tests of the combined line, which were calculated separately (r = 0.903 and r = 0.921, $P > 0.05$), nor between their intercepts (−0.1569 and −0.1607, $P > 0.05$). The correlation between the titers of the mouse test and the combined line of the two tests excluding the double-negatives in both tests was r = 0.968 ($n = 38$), and the regression line equation was Y = 1.0429 X − 0.0922.

The results indicated that the replacement of in-house ELISA values equal to 1.0 IU/ml or below with KPA values could be applicable for tetanus seroepidemiology in the whole titer range including levels as low or lower than that of minimum protection.

Application of the combined method to analysis of tetanus antibody prevalence in Antalya: The method combining the in-house ELISA and the KPA was applied for the quantitative assay of community-based seroepidemiology against tetanus in Antalya. All of the 347 serum samples were titrated by in-house ELISA and the 138 samples with titers 1.0 IU/ml or below were reassessed by KPA.

The distribution of tetanus antibody titers in each age group is illustrated in Fig. 2, which clearly shows a characteristic

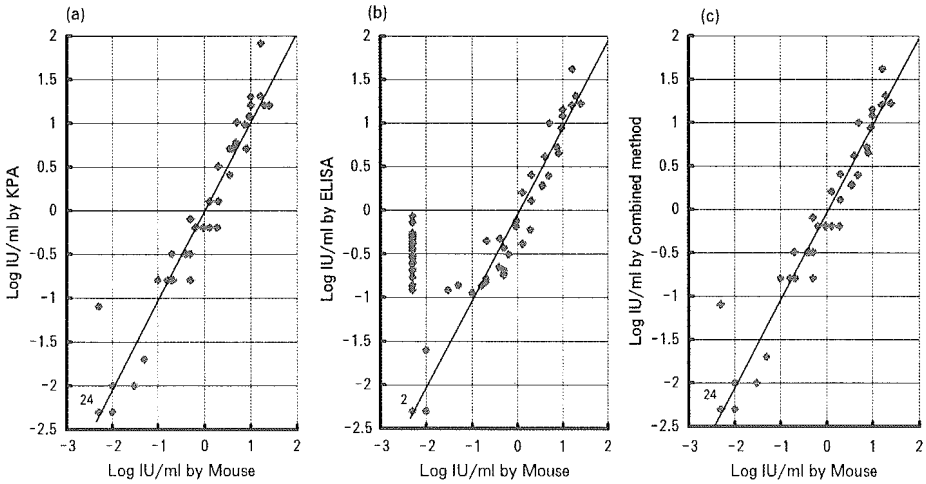

Fig. 1. Correlation of tetanus antibody titer measurements using four methods. The in vivo neutralization test in mouse, along with in vitro KPA, in-house ELISA, or the combined method of in-house ELISA with KPA, were used for measurements ($n = 62$). A titer of less than 0.01 IU/ml was set to 0.005 IU/ml in the figures. The number at the lower left in each figure indicates the number of multiple marks for the double-negatives (<0.01 IU/ml) – that is, on both tests.
(a) Correlation of antibody titers in mice and KPA. r = 0.901, excluding double-negatives (<0.01 IU/ml) in both tests. (b) Correlation of antibody titers in mouse and in-house ELISA. r =0.903 in the range of >1.0 IU/ml. (c) Correlation of antibody titers in mouse and the method combining in-house ELISA (>1.0 IU/ml) with KPA(<1.0 IU/ml). r = 0.968 excluding double-negatives (<0.01 IU/ml) in both tests.

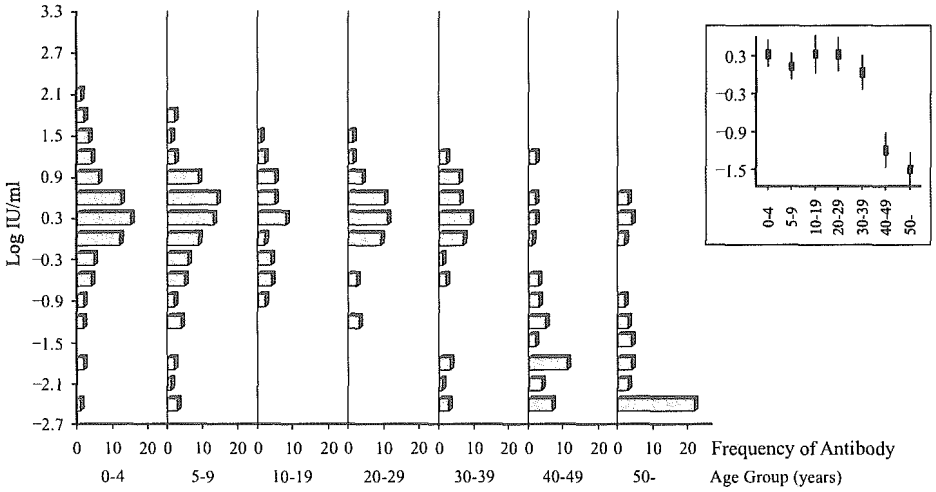

Fig. 2. Tetanus antibody prevalence in each age group in Antalya Province, Turkey, 2001. Tetanus antibody titers were determined by the combined method of in-house ELISA and KPA (n = 347). Inset shows geometric mean titers (GMTs) and confidence interval of antibody titers in each age group. A titer of less than 0.01 IU/ml was set to 0.005 IU/ml for calculating GMT. The x-axis of the inset indicates age groups in years, and the y-axis indicates Log_{10} IU/ml.

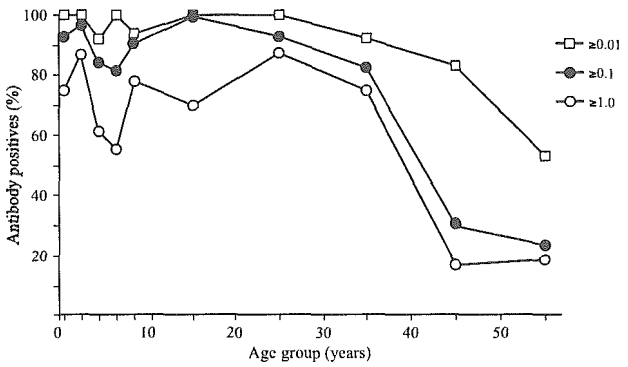

Fig. 3. Age group distribution at three different tetanus antibody levels, Antalya Province, Turkey, 2001. Tetanus antibody titers were determined by the combined assay method of in-house ELISA and KPA. n = 347.

distribution of tetanus antibody in each age group. The great majority of the children and young adults had antibody titers above the minimum protective level, and GMTs for the age groups up to those in their 30s were more than 1.10 IU/ml (log_{10} value 0.04, the inset of Fig. 2). In contrast, in groups of subjects in their 40s and those over 50, the antibody titers were scattered, and a significant number of persons lacked the minimum protective level of antibody. Seven of 42 subjects in the 40s age group (16.7%; 95% confidence interval [CI] 31.4 - 7.0%) and 22 of 47 subjects ≥50 years (46.8%; CI 61.9 - 32.1%) were revealed to have antibodies under the minimum protective level of 0.01 IU/ml. The remarkably low GMTs in these older groups compared with levels in other age groups are also demonstrated in the inset of Fig. 2 ($P < 0.01$); 0.065 IU/ml (log_{10} value - 1.19, CI 0.118 - 0.035 IU/ml) for subjects in their 40s, and 0.032 IU/ml (log_{10} value -1.494, CI 0.057 - 0.018 IU/ml) for those ≥50 years.

In order to demonstrate tetanus antibody prevalence in Antalya, three different antibody levels were used (Fig. 3). The antibody-positive rates were 89.6 % (CI 92.6 - 85.9%) for ≥0.01 IU/ml, 74.1% (CI 78.6 - 69.1%) for ≥0.1 IU/ml, and 60.2% (CI 65.4 - 54.9%) for 1.0 IU/ml in total subjects. The antibody prevalence above 0.1 IU/ml, which is considered to be a fully protective level against tetanus, was more than 82% in the groups of children and adults up to those in their 30s, but was markedly reduced to 31.0% (CI 47.1 - 17.6%) in subjects in their 40s, and 23.4% (CI 38.0 - 12.3%) in ≥50 years old ($P < 0.01$). In addition, at the higher antibody levels of 0.1 IU/ml and 1.0 IU/ml shown in Fig. 3, a certain reduction was seen in groups of those 4 - 5 and 6 - 7 years of age compared with those in the younger age group, but the rates increased again at 8 - 9 years of age. The reduction in antibody levels was statistically significant ($P < 0.05$).

A summary of the vaccination histories of the subjects collected by questionnaires are shown in Table 1. High vaccination rates of more than 90% for ≥3 doses are shown among children in groups under 10 years of age, but in groups of subjects in their 40s or over 50, only 16 of 89 subjects had ≥3 doses. Moreover, many of these older subjects had an unknown history of tetanus vaccination. In addition, a large percentage of children had, by 8 - 9 and 10 - 19 years of age, received a 5th injection of tetanus vaccine, in contrast to 3 - 4 doses received by those in the younger groups. It was observed that antibody prevalence pattern was correlated with vaccination histories throughout all subject groups.

DISCUSSION

Although ELISA is widely used for tetanus seroepidemiology, the overestimation that occurs in a significant number of low-titer sera poses problems (3,6,16). Our study confirmed that the sera with antibody titers less than 0.01 IU/ml by the mouse neutralization test gave a considerable titer variation up to 1.0 IU/ml with in-house ELISA, as described previously (9). As a result, the in-house ELISA failed to discriminate between in vivo neutralizing antibody and non-specific reactivity at low antibody levels. KPA was easy to perform, and the results were well correlated with the in vivo mouse neutralization method for all antibody examined in a serological survey. However, this method was more expensive and not cost effective for assay of a large number of samples. On the other hand, the combination of these two methods was acceptable for tetanus seroepidemiology; it can obtain reliable antibody titers quantitatively at a comparatively lower

cost. In this study, this method was applied for community-based tetanus seroepidemiology, which was done for the first time in Turkey, in contrast to the studies on limited age groups previously reported from this country (17-19).

It is generally accepted that the antibody against tetanus toxin cannot be obtained naturally and that the antitoxin detected in human sera is exclusively induced by vaccination (3). Immunization against tetanus in Turkey started in 1937 and was accelerated in 1985 with the National Vaccination Campaign. At present, based on the National Routine Immunization Program of the Ministry of Health, children receive their primary vaccination against tetanus with 3 doses of diphtheria-pertussis-tetanus vaccine (DPT) in the 2nd, 3rd, and 4th months after birth, and the 4th dose at 16-24 months. Subsequently, 2 booster doses with adult form diphtheria-tetanus vaccine (dT) are offered at primary school and another dose with tetanus toxoid (TT) is offered during high school. In addition, women receive two TT doses in their first pregnancy. Men in the army are also given additional TT. According to the data from the General Directorate of Primary Health Care, Antalya was one of the provinces with the highest immunization coverage in Turkey; the reported vaccination coverage in Antalya for the 3rd dose of DPT during the past 5 years was approximately 90% on average, against approximately 80% on average throughout the country (20).

Our study demonstrated that children and younger adults possess a high antibody level of immunity against tetanus, whereas a large proportion of the older population lacked even the minimum protective level. The evidence of low-titer antibodies in a significant percentage of these older age groups, as shown in Fig. 2 and also at the 0.01 IU/ml level in Fig. 3, may suggest the waning of the antibody during the years after immunization. Further, it is considered very likely that older groups had received insufficient vaccination during childhood, given that the national vaccination program for children was not fully in place before 1985. Also, the vaccination rates of these groups in our study were significantly low, and many subjects in these groups did not know their vaccination history.

The high rate of minimum antibody level among children with a certain reduction in antibody titers in those from 4 to 7 years of age indicated that after the four primary immunizations by 24 months old, the antibody titers decreased until the booster in the primary school period, which once more elevated the children's antibody levels. This result provides evidence for the effectiveness of booster injections during the primary school period, confirming that the national vaccination schedule implemented in Turkey is adequately providing tetanus immunity among the young population.

Our results showed that a significant portion of the general population possessed the tetanus antibody in a range not measurable by in-house ELISA, and these subjects had to be reassessed by KPA. We successfully conducted a large-scale, quantitative seroepidemiological study covering as low as the minimum protective level by applying the combined method described above. However, further improvement of in-house ELISA for tetanus seroepidemiology remains to be considered.

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Intratypic Recombination among Lineages of Type 1 Vaccine-Derived Poliovirus Emerging during Chronic Infection of an Immunodeficient Patient

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We determined the complete genomic sequences of nine type 1 immunodeficient vaccine-derived poliovirus (iVDPV) isolates obtained over a 337-day period from a poliomyelitis patient from Taiwan with common variable immunodeficiency. The iVDPV isolates differed from the Sabin type 1 oral poliovirus vaccine (OPV) strain at 1.84% to 3.15% of total open reading frame positions and had diverged into at least five distinct lineages. Phylogenetic analysis suggested that the chronic infection was initiated by the fifth and last OPV dose, given 567 days before onset of paralysis, and that divergence of major lineages began very early in the chronic infection. Key determinants of attenuation in Sabin 1 had reverted in the iVDPV isolates, and representative isolates of each lineage showed increased neurovirulence for PVR-Tg21 transgenic mice. None of the isolates had retained the temperature-sensitive phenotype of Sabin 1. All isolates were antigenic variants of Sabin 1, having multiple amino acid substitutions within or near neutralizing antigenic sites 1, 2, and 3a. Antigenic divergence of the iVDPV variants from Sabin 1 followed two major independent evolutionary pathways. The emergence of distinct coreplicating lineages suggests that iVDPVs can replicate for many months at separate sites in the gastrointestinal tract. Some isolates had mosaic genome structures indicative of recombination across and within lineages. iVDPV excretion apparently ceased after 30 to 35 months of chronic infection. The appearance of a chronic VDPV excretor in a tropical, developing country has important implications for the strategy to stop OPV immunization after eradication of wild polioviruses.

The central strategy of the World Health Organization Global Polio Eradication Initiative is widespread use of oral poliovirus vaccine (OPV) at high rates of coverage. This strategy has reduced the global incidence of polio by over 99% since the start of the Initiative in 1988 and restricted wild poliovirus circulation to countries in western and central Africa and southern Asia (87). However, use of OPV is associated with some rare adverse events, including the appearance of cases of vaccine-associated paralytic poliomyelitis among OPV recipients and contacts (76), and the occurrence of polio outbreaks associated with circulating vaccine-derived poliovirus (cVDPV) (36). While cVDPV outbreaks can be prevented by maintenance of high rates of OPV coverage, the occurrence of vaccine-associated paralytic poliomyelitis is associated with the inherent genetic instability of the live, attenuated OPV strains (56).

In immunocompetent individuals, the risk of vaccine-associated paralytic poliomyelitis is very low, estimated in the United States at 1 case per 2.4 million OPV doses distributed (75, 76). The risk of vaccine-associated paralytic poliomyelitis is over 3,000-fold higher in patients with B-cell immunodeficiencies such as common variable immunodeficiency, X-linked agammaglobulinemia, and severe combined immunodeficiency (77). Moreover, whereas the period of poliovirus excretion is usually 2 to 6 weeks in susceptible immunocompetent individuals (2), it can be prolonged for up to 10 years or more in immunodeficient patients (35, 52, 76). Chronic poliovirus excretion (>12 months) appears to be very rare (37) and appears to be largely, but perhaps not exclusively (31, 54), associated with B-cell immunodeficiencies. Fewer than 25 immunodeficient chronic excretors have so far been identified since the introduction of OPV in 1961 (27, 76), and most of the patients have been from high-income countries (27, 76) such as the United States (35, 77), the United Kingdom (52, 53), Germany (5), Italy (11), and Japan (29, 91).

In this report, we describe a case of immunodeficient vaccine-associated paralytic poliomyelitis in a child from Taiwan diagnosed with common variable immunodeficiency (18, 69). The patient was found to have excreted type 1 immunodeficient vaccine-derived polioviruses (iVDPVs) for 10 months after onset of paralysis. From the evolution rate of the iVDPV isolate genomes, we estimated that the total period of excretion was from 30 to 35 months, and that the chronic infection most likely began with administration of the fifth and last dose

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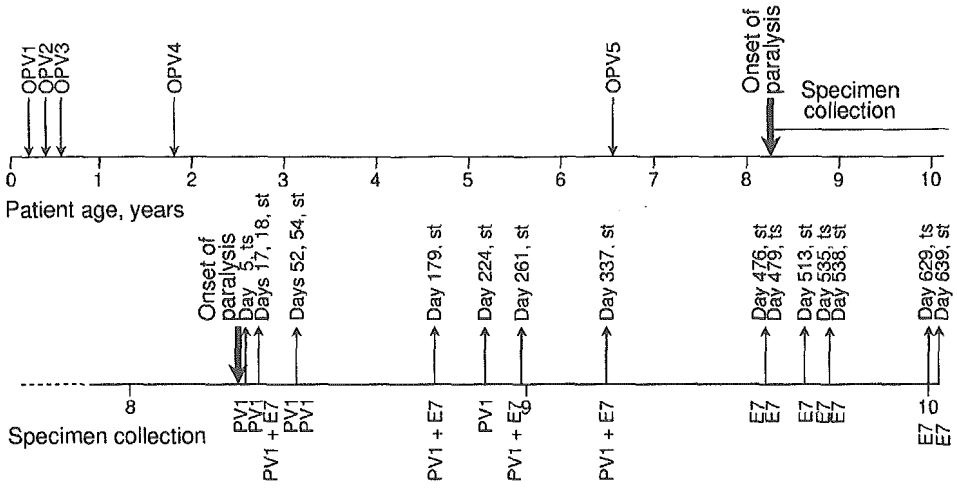

FIG. 1. Time line summarizing immunization history, age at onset of paralysis, and time of specimen collection for the immunodeficient chronic excretor (upper line). The lower line is an expansion of the time line from the patient at age 8 years to 10.1 years showing times (in days after onset of paralysis) of collection of specimens (st, stool specimen; ts, throat swab) found to contain poliovirus type 1 (PV1) iVDPV or echovirus type 7 (E7) or both.

of OPV. The iVDPV isolates obtained from the patient represented five main lineages derived from the common-source infection. All isolates were antigenic variants of the Sabin type 1 OPV strain (LSc 2ab; Sabin 1), but lineages differed in the pattern of amino acid substitution within or near neutralization antigenic sites. Divergence of the separate lineages appeared to have started very early in the chronic infection, with the earliest diverging lineage evolving largely independently. Some iVDPV isolates had mosaic genome structures indicative of recombination across and within lineages. Representative isolates from each lineage were tested for neurovirulence in PVR-Tg21 transgenic mice expressing the human receptor for poliovirus and were found to be either highly or moderately neurovirulent in this animal model. In addition, all isolates when grown in HeLa cells at 39.5°C had lost the temperature-sensitive phenotype of Sabin 1.

There was no evidence of spread of iVDPV to contacts of the case-patient. However, Taiwan currently maintains the high rates of OPV coverage needed to limit iVDPV spread. The appearance of a chronic VDPV excretor in a tropical, developing country underscores the challenges inherent to the development of a global strategy for cessation of OPV use after eradication of wild polioviruses.

MATERIALS AND METHODS

Patient. The case patient, a boy born in 1993, had received a primary series of trivalent OPV doses at ages 2, 4, 6, and 21 months and a booster dose at age 6.7 years (Fig. 1). In April 2001, at age 8 years, the patient developed acute paralysis and was diagnosed with bulbospinal poliomyelitis. Throat swabs and stool specimens were taken for virus culture, and blood specimens were taken for immunologic studies. The patient was diagnosed with common variable immunodeficiency on the basis of quantitative serum immunoglobulin readings and was placed on intravenous immunoglobulin therapy (47).

Virus isolation and typing. Clinical specimens obtained from the case patient included throat swabs taken at 5, 479, 535, and 629 days after onset of paralysis, and stool specimens taken at 17, 18, 52, 54, 179, 224, 261, 337, 476, 513, 538, 639, 688, and 752 days after onset. Stool specimens were also obtained from 62 contacts, including four siblings of the case patient. Virus was isolated by culture in RD (human rhabdomyosarcoma cell line; ATCC CCL 136), L20B (mouse L cells expressing the human poliovirus receptor) (65), and HEp-2 (human cervical carcinoma cell line; ATCC CCL 23) cells (86). Echovirus type 7 (E7) isolates were initially identified by patterns of neutralization with Lim Benyesh-Melnick pools and confirmed by neutralization with E7-specific antisera and VP1 sequencing (86). Poliovirus isolates were initially characterized by immunofluorescence assay, microneutralization, diagnostic PCR (39, 40, 88), and VP1 sequencing (35, 86). Several of the poliovirus isolates had mixed-base sequences at multiple sites, and isolates from specimens taken at days 5, 17, 54, 179, 224, 261, and 337 were plaque purified before complete genomic sequencing.

Antigenic characterization. Initial intratypic differentiation of VDPV isolates used highly specific cross-absorbed antisera in an enzyme-linked immunosorbent assay format (82). Briefly, clinical isolates were tested with two different antiserum preparations, one that reacts with Sabin 1 and another that reacts primarily with wild type 1 polioviruses. In this assay, isolates can have one of four different antigenic properties: (i) vaccine-like (reaction only with the anti-Sabin strain sera), (ii) non-vaccine-like (reaction only with anti-wild poliovirus sera), (iii) double-reactive (reaction with both anti-Sabin strain and anti-wild poliovirus sera), and (iv) nonreactive (no reaction with either anti-Sabin strain or anti-wild poliovirus sera). Only Sabin 1-related isolates have vaccine-like antigenic properties, some Sabin 1-related isolates have double reactive antigenic properties, most wild polioviruses and some Sabin 1-related antigenic variants have non-vaccine-like antigenic properties, and a small number of wild polioviruses and Sabin 1-related antigenic variants have nonreactive antigenic properties (82).

Nucleic acid sequencing. Conditions for reverse transcription-PCR amplification and cycle sequencing were as described previously (50). Sequencing was performed in both directions, and every nucleotide position was sequenced at least once from each strand. Terminal sequences were determined by using the 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE system kits (Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions.

Phylogenetic analysis. P1/capsid, P2/noncapsid, P3/noncapsid, and complete open reading frame (ORF) sequence relationships among the nine iVDPV isolates and Sabin 1 were constructed from the corresponding regions using the maximum-likelihood method implemented in the DNAml program of the PHYLIP 3.5c package (21). The topology of the trees was obtained by majority-rule consensus among 1,000 bootstrap replicates (20, 21). The corresponding branch lengths were evaluated by likelihood ratio tests among nested models of nucleotide evolution as implemented in the program Modeltest (67). The tree with the best likelihood ratio test score was rooted to the sequences of Sabin 1 and displayed using the program TreeExplorer (K.Tamura, http://evolgen.biol.metro-u.ac.jp/TE/TE_man.html). Insertion/deletion 5′ untranslated region (5′-UTR) differences were treated as single-nucleotide substitutions.

Analysis for recombination across lineages. Discontinuities across different genomic intervals among the day 5, 18, and 52 isolates and among the day 52, 179, and 224 isolates were initially visualized by using distance plot and bootscan functions of the Simplot program (51). The program DnaSP (71) was used to localize the likely sites of recombination by alignment of polymorphic nucleotide sites and analysis of the statistical significance of discontinuities in the extent of

nucleotide sequence identity. Paired estimates of corrected nucleotide substitutions and standard errors among the recombinant sequence blocks were calculated using the MEGA software package (45).

Estimation of the time of the initiating OPV dose. The time of the initiating OPV dose was estimated from the rate of fixation of nucleotide substitutions into the nine iVDPV isolates. The maximum-likelihood estimates of the number of synonymous substitutions at synonymous sites (K_s) that accumulated from the Sabin 1 sequence were computed according to the method of Goldman and Yang (25) as implemented in the CODEML program within the PAML package (90). This method corrects for the transition/transversion rate bias, the codon frequency bias, and for multiple substitutions at a site. Corrected K_s values relative to the root sequence (Sabin 1; K_s set to zero) for each iVDPV isolate were plotted as a function of the date of sample collection. The rate of accumulation of synonymous substitutions was estimated by weighted linear regression (where the weight for each data point was proportional to the reciprocal of the error variance for the corrected K_s value) using statistical applications within the SAS system, version 9 (SAS Institute, Inc., Cary, N.C.). The date of the initiating OPV dose was estimated from the intercept on the abscissa at $K_s = 0$, and the 95% confidence limits around the regression line were calculated following procedures described by Sokal and Rohlf (74).

Estimation of time of divergence of iVDPV lineages. Maximum-likelihood estimates of divergence times were obtained assuming a unique common ancestor and a single linear rate of evolution for all iVDPV lineages, as shown by the linear regression analysis. Under these assumptions, a maximum-likelihood tree was constructed using third codon positions after an exhaustive search (78), and further analyzed under the single rate dated tips model (68) implemented in the PAML package (90) with dated internodes scaled according to the time of the last OPV dose. The parental root sequence was that of the P1/capsid region of Sabin 1 and zero time was assumed to be the date of the last OPV dose (567 days before onset of paralysis).

Numbering of nucleotide and amino acid positions. The ORF sequences of all iVDPV isolates were colinear with those of Sabin 1, but their 5′-UTR sequences were not. To facilitate comparisons, numbering of nucleotide positions of all isolates followed that described for Sabin 1 (59), with insertions assigned serial letters. Amino acid positions were indicated by the name of the viral protein and numbered consecutively from residue 1 of each protein. Amino acid substitutions were indicated by the following convention: viral protein:original residue–position–substituted residue. For example, VP1:T106A indicates a threonine-to-alanine substitution at amino acid position 106 of VP1.

Neurovirulence testing in PVR-Tg21 mice. Neurovirulence tests on iVDPV isolates were carried out on PVR-Tg21 mice as previously described (43, 46, 73). The mice were purchased from the Central Laboratories for Experimental Animals (Kanagawa, Japan). The type 1 reference strains were Mahoney/USA41 (neurovirulent) and Sabin 1 (attenuated). Six or eight mice (equal numbers of males and females) were inoculated (30 μl/mouse) intracerebrally for each virus dilution (in 10-fold increments; range, 1.5 to 7.5 log 50% cell culture infectious dose ($CCID_{50}$)/mouse). Mice were examined daily for 14 days postinoculation, and the times of paralysis or death were recorded. The virus titer that induced paralysis or death in 50% of inoculated mice was calculated by the method of Kärber (32) and expressed as $CCID_{50}$/mouse.

Measurement of temperature-sensitive phenotype. The temperature sensitivity of the iVDPV isolates was measured by the efficiency of plating at 39.5°C compared with 34.5°C. The efficiency of plating values were determined by plaque assays performed on monolayers of HeLa cells as described previously (73, 89).

Nucleotide sequence accession numbers. Complete genomic sequences of the nine iVDPV isolates described in this study were submitted to the GenBank library under accession numbers AF538840, AF538841, AF538842, AF538843, AY928383, AY928384, AY928385, AY928386, and AY928387 (corresponding to the day 5, 17, 52, 337, 261, 224, 54, 179, and 18 isolates, respectively).

RESULTS

Clinical and epidemiologic investigations. The last case of poliomyelitis in Taiwan associated with circulating wild poliovirus occurred in 1982, at the end of a large outbreak (1,043 reported cases with 98 deaths) associated with an imported type 1 poliovirus (13, 41). All subsequent poliovirus isolates obtained since 1982 have been derived from the oral poliovirus vaccine. Taiwan introduced immunization with OPV in 1966 and has maintained high rates of OPV coverage since 1982 and intensive surveillance for cases of acute flaccid paralysis since 1994. In 2000, all countries within the Western Pacific Region of the World Health Organization were certified as polio-free (1, 84).

The case patient developed poliomyelitis in April 2001, 19 years after the last case associated with wild poliovirus in Taiwan. Clinical records indicated that the case patient received five doses of OPV at ages 2, 4, 6, and 21 months and 6.7 years, the last dose given 567 days (~19 months) before onset of paralysis (Fig. 1). At age 8 years, the patient developed fever and upper respiratory tract infection followed by acute left-arm paralysis. Paralysis progressed to his right arm and both legs, and further involved difficulties in swallowing, impairment of tongue and eye movement, and respiratory muscle paralysis. The clinical diagnosis was bulbospinal poliomyelitis. A month after paralysis, the patient was diagnosed with common variable immunodeficiency, a defect in antibody production (18, 69), on the basis of quantitative serum immunoglobulin readings of 270 mg/dl for immunoglobulin G (normal range: 639 to 1,349 mg/dl), 6.2 mg/dl for immunoglobulin M (normal range: 56 to 352 mg/dl), and <5.9 mg/dl for immunoglobulin A (normal range: 70 to 132 mg/dl) (15, 28). He was immediately placed on a therapeutic regimen of monthly injections of intravenous immunoglobulin. The patient continued to have atrophy and residual paralysis in both legs more than 3 years after onset of paralysis.

Clinical specimens were obtained from the patient at days 5 (throat swab), 17, 18, 52, 54, 179, 224, 261, 337, 476, 479 (throat swab), 513, 535 (throat swab), 538, 629 (throat swab), 639, 688, and 752 (all were stool specimens if not otherwise indicated) after onset (Fig. 1). Poliovirus type 1 was isolated from all specimens taken up to day 337. Echovirus type 7 and poliovirus type 1 were isolated from the day 18, 179, 261, and 337 specimens; E7 was isolated from all specimens taken from day 476 to day 639; no virus was isolated from the day 688 and day 752 specimens.

The local bureau of health investigated the polio immunization status of schoolmates of the case patient and children in his neighborhood and community (1,682 children in all). All of the children had been vaccinated with at least 3 doses of OPV, and three contact children under 5 years had received catch-up vaccinations between July and September 2000. In addition, stool specimens were taken from 62 suspected contacts of the case patient (including his four siblings) for virus isolation. None of the contacts were found to be infected with poliovirus.

Sequence properties of poliovirus isolates. Characterization of the early poliovirus isolates (from the days 5, 17, and 18 specimens) by enzyme-linked immunosorbent assay using cross-absorbed neutralizing antisera (82) showed that all were antigenically distinct (nonreactive or non-vaccine-like) from Sabin 1, a property subsequently confirmed for all nine isolates. However, characterization by diagnostic PCR and by sequencing of the VP1 region (906 nucleotides) showed that all isolates were derived from the Sabin 1 strain. The nine isolates differed from Sabin 1 at 2.43% to 3.53% of VP1 nucleotide sequences, from each other at 0.22% (excepting the day 52 and day 54 isolates, which had identical VP1 sequences) to 5.28% of VP1 nucleotide sequences, and from contemporary wild type 1 polioviruses (including representative isolates from the 1982 Taiwan outbreak) at >18% of VP1 nucleotide sequences

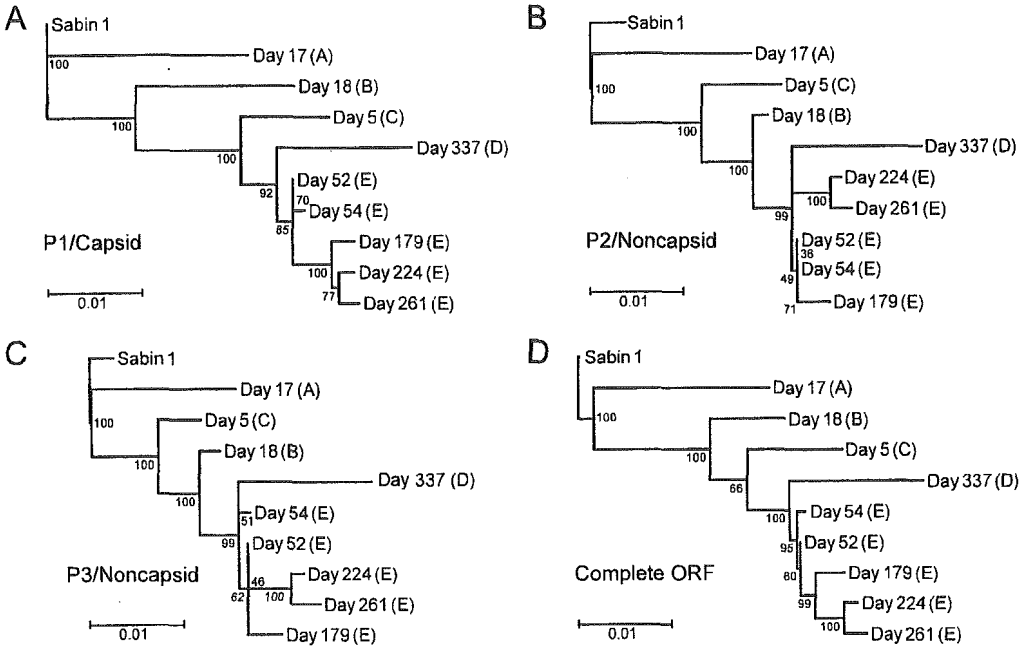

FIG. 2. Maximum-likelihood trees summarizing sequence relationships among the Sabin 1 OPV strain (root of tree) and the nine type 1 iVDPV isolates across different genomic intervals: (A) P1/capsid region (reference interval for evolution rate calculations), (B) P2/noncapsid region, (C) P3/noncapsid region, and (D) complete ORF. Major diverging lineages are labeled A to E.

(34, 73) (data not shown). The Taiwan isolates were identified as iVDPVs in accordance with the World Health Organization classification scheme, where VDPVs are poliovirus clinical isolates having 1% to 15% VP1 nucleotide sequence divergence from the parental OPV strain, and iVDPVs are VDPVs isolated from patients known to have B-cell immunodeficiencies (36).

Subsequent nucleotide sequencing of the complete genomes (7,411 to 7,441 nucleotides) of all nine isolates showed that all sequences were derived from Sabin 1. Most of the genetic differences among isolates were nucleotide substitutions, 82% of which generated synonymous codons. However, three different categories of 5′-UTR deletions were also observed. The day 17 isolate had no 5′-UTR deletions compared to Sabin 1. By contrast, all other VDPV isolates had a deletion of 8 nucleotides at positions 667 to 674, and the day 5 pharyngeal isolate had an additional deletion of 22 nucleotides at positions 695 to 716. The deletions occurred within a highly variable 5′-UTR interval that appears to have little secondary structure (63). Type 1 polioviruses with deletions within this interval have been shown to be viable in cultured cells (24, 44) and in humans (49). Four 5′-UTR nucleotide substitutions (G26A, U344C, U355C, and G480A) were shared by all isolates. All of these substitutions but U344C represent reversions back to the parental Mahoney sequence (59), and all three were consistently found among type 1 cVDPV isolates from Hispaniola (34) and the Philippines (73).

Emergence of multiple iVDPV lineages. Relationships among the complete P1/capsid region sequences of the nine iVDPV isolates were summarized in a tree constructed using the maximum-likelihood algorithm (21) and rooted to the Sabin 1 sequence (Fig. 2A). The tree had a deeply branched topology, with five major branches corresponding to lineages A to E, extending from a single main lineage. The first lineage (A) to branch off from the main lineage is represented by the day 17 isolate. This isolate differed from all others by the absence of any 5′-UTR deletions. Replication of lineage A virus may have ceased soon after onset of paralysis, as no other lineage A isolates were subsequently found. All other isolates contained the 8-nucleotide 5′-UTR deletion, which probably became fixed into the virus population very early in the chronic infection. The P1/capsid sequences of the day 17 (lineage A) and day 18 (lineage B) isolates were quite distinct, differing at 3.9% of nucleotide positions. The extensive divergence of the two isolates was surprising because the viruses originated from stool specimens taken on consecutive days. However, the VP1 sequence of the original day 17 isolate was ambiguous at several positions, indicating that the virus population was a mixture of variants, and the isolate was plaque-purified before complete genomic sequencing. By contrast, the day 18 isolate sequences were unambiguous, so no plaque purification was performed. It is possible that the day 17 specimen also contained lineage B virus that was removed by the plaque-purification step.

The day 18 isolate sequence appears at the tip of a long branch (Fig. 2A), suggesting that the day 18 isolate represents a second lineage that may have terminated soon after onset of paralysis. Two other distinct lineages, C and D, are represented by the day 5 and day 337 isolates, respectively. The 22-nucleotide 5′-UTR deletion apparently occurred after lineage C diverged from the evolutionary pathway leading to lineages D and E. Lineage D is defined by the sequence of the day 337 isolate, from the last poliovirus-positive stool specimen. Lineage E is defined by five closely related isolates from

the day 52, day 54, day 179, day 221, and day 261 specimens. Divergence among isolates of lineage E was limited and probably occurred during the period of sampling.

All sequences of the iVDPV isolates were derived from Sabin 1. Trees of the P2/noncapsid, P3/noncapsid, and complete ORF regions had deeply branched topologies similar to the P1/capsid tree (Fig. 2). However, the four trees were not congruent, as the order of branch nodes and branch lengths differed. For example, the branch nodes of the isolate sequences were ordered day 18 (B) –day 5 (C) –day 337 (D) in the P1/capsid tree, and day 5 (C) –day 18 (B) –day 337 (D) in the P2/noncapsid and P3/noncapsid trees (Fig. 2). Bootstrap support (20, 21) for the order of these nodes was high (>90%) in all trees. Moreover, the branch length of the day 18 isolate from its node was long in the P1/capsid tree and short in the P2/noncapsid and P3/noncapsid trees.

As the window for comparison moved from the P1/capsid region to the P2/noncapsid and P3/noncapsid regions, the branch node of the day 18 isolate shifted away from the root and that of the day 5 isolate shifted toward the root. By contrast, the sequence relationships among most other isolates were similar across the ORF, apart from minor differences in tree topologies that had low bootstrap support. The exception was the day 179 isolate, within lineage E, that was most closely related to the day 224 isolate in the P1/capsid tree, but most closely related to the day 52 isolate in the P2/noncapsid and P3/noncapsid trees. Although some of the differences in tree topologies may be attributable to stochastic variability in the rates of fixation of substitutions across different lineages and genetic intervals, another mechanism probably explains the more pronounced differences.

Recombination across and within iVDPV lineages. Relationships among the aligned complete ORF sequences of the day 5, day 18, and day 52 isolates were initially examined by using the distance plot and bootscan functions of the Simplot program (51), which revealed a possible recombination site between nucleotide positions 2659 and 2678, near the 5′ terminus of the VP1 region (Fig. 3B). Upstream P1/capsid region sequences (nucleotide positions 743 to 2658) of the day 52 isolate more closely matched those of the day 5 isolate (genetic distance 0.006 ± 0.002) than the day 18 isolate (genetic distance 0.035 ± 0.004), whereas the downstream ORF sequences (nucleotide positions 2679 to 7369) of the day 52 isolate more closely matched those of the day 18 isolate (genetic distance 0.007 ± 0.001) than the day 5 isolate (genetic distance 0.019 ± 0.002), a pattern suggestive of recombination.

Because recombination is difficult to detect at low levels of divergence (66), recombination was further investigated using the program DnaSP (71). The sharp discontinuity in the extent of sequence identity, clearly visualized by alignment of polymorphic nucleotide sites among the ORFs of the three isolates (Fig. 3C), was very likely produced by recombination. The likelihood that the observed sequence discontinuities arose by random substitution without recombination is very low ($P < 0.00001$; G test with Williams's correction). Although the summary alignment (Fig. 3B) shows the day 52 isolate genome as a mosaic assembled from upstream sequences similar to those of the day 5 isolate and downstream sequences similar to those of the day 18 isolate, the actual evolutionary relationships among the three isolates is likely to be more complex. For example, runs of sequence differences in the P3/noncapsid regions of the day 18 and day 52 isolates (Fig. 3C) may signal the occurrence of additional recombination events. However, short stretches of recombinant sequences are difficult to distinguish from localized clustering of substitutions (66). More importantly, because the sequences of the parental and progeny viruses directly participating in the recombination events are unknown, and only a small number of representatives of each lineage were available for analysis, the observed mosaic structures of the genomes are explainable by several alternative pathways of recombination.

Similarly, we found that the genome of the day 224 isolate also appeared to be a mosaic, with upstream ORF sequences (nucleotide positions 743 to 3366) more closely related to those of the day 179 isolate and downstream sequences (nucleotide positions 3372 to 7369) more closely related to those of the day 52 isolate (Fig. 3D). Alignment of the polymorphic nucleotide sites (Fig. 3E) revealed discontinuities in the extent of sequence identity that are most likely attributable to recombination ($P = 0.008$ for random substitution without recombination; G test with Williams's correction). The statistical support for recombination among the lineage E isolates was less robust than in the previous example because the number of polymorphic sites distinguishing the ORF sequences of the three isolates was small ($n = 60$).

Estimation of the date of OPV exposure that initiated the chronic iVDPV infection. The topologies of the trees indicated that genetic divergence from Sabin 1 increased with the time of sampling (Fig. 2). An approximately linear relationship ($R^2 = 0.84$) was obtained when the number of synonymous substitutions (K_s) in the P1/capsid region was plotted as a function of the times of collection (zero time: date of last OPV dose) for the nine specimens (Fig. 4). The linear relationship suggests that all lineages were derived from a single OPV dose. The date of the initiating OPV dose, estimated by extrapolation to the time when $K_s = 0$, was 59 days before the fifth and last OPV dose (95% confidence interval: 588 days before to 190 days after the last OPV dose). The rate of P1/capsid evolution estimated from the slope of the regression line was $(2.92 \pm 1.25) \times 10^{-2}$ synonymous substitutions per synonymous site per year, a value very similar to those previously obtained for poliovirus P1/capsid region or VP1 sequences (34, 35, 50, 53). When we performed similar analyses based upon total nucleotide substitutions (K_t) in the P1/capsid region, the intercept was 172 days before the last OPV dose and the evolution rate at all sites was estimated to be $(1.14 \pm 0.45) \times 10^{-2}$ substitutions per site per year (data not shown).

Although the two analyses yielded comparable estimates for the time of start of the chronic infection, we generally prefer to base our estimates upon the rate of fixation of synonymous substitutions, which we assume to accumulate at a nearly constant rate by random genetic drift. We also prefer to use the P1/capsid region for our calculations because it is the largest interval within the ORF that can generally be assumed to share a recent common ancestry, as recombination of noncapsid region sequences with heterologous species C enteroviruses frequently occurs in circulating wild polioviruses (10, 49) and cVDPVs (34, 70, 73, 89). However, because sequences in all genomic intervals were derived from a recent common Sabin 1 ancestor, we were also able to estimate the date of the initiat-