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Persisting Humoral Antiviral Immunity within the Japanese Population after the Discontinuation in 1976 of Routine Smallpox Vaccinations

Shuji Hatakeyama,[1]* Kyoji Moriya,[2] Masayuki Saijo,[3] Yuji Morisawa,[2] Ichiro Kurane,[3] Kazuhiko Koike,[1,2] Satoshi Kimura,[4] and Shigeru Morikawa[3]

Department of Infectious Diseases[1] and Department of Infection Control and Prevention,[2] Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, Musashimurayama,[3] and AIDS Clinical Center, International Medical Center of Japan, Shinjyuku-ku,[4] Tokyo, Japan

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Concerns have arisen recently about the possible use of smallpox for a bioterrorism attack. Routine smallpox vaccination was discontinued in Japan in 1976; however, it is uncertain exactly how long vaccination-induced immunity lasts. We sought to evaluate the seroprevalence and intensity of anti-smallpox immunity among representatives of the present Japanese population. The subjects included 876 individuals who were born between 1937 and 1982. Vaccinia virus-specific immunoglobulin G (IgG) levels were measured by enzyme-linked immunosorbent assay (ELISA), and 152 of 876 samples were also tested for the presence of neutralizing antibodies. Of the subjects who were born before 1962, between 1962 and 1968, and between 1969 and 1975, 98.6, 98.6, and 66.0%, respectively, still retained the vaccinia virus-specific IgG with ELISA values for optical density at 405 nm (OD_{405}) of ≥0.10. The corresponding figures for retained IgGs with OD_{405} values of ≥0.30 were 91.0, 90.3, and 58.2%, respectively. Neutralizing antibodies were also maintained. The sera with OD_{405} values of ≥0.30 showed 89% sensitivity and a 93% positive predictive value for detection of neutralizing antibodies (≥4). Thus, approximately 80% of persons born before 1969 and 50% of those born between 1969 and 1975 were also found to have maintained neutralizing antibodies against smallpox. A considerable proportion of the previous vaccinated individuals still retain significant levels of antiviral immunity. This long-lasting immunity may provide some protective benefits in the case of reemergence of smallpox, and the disease may not spread as widely and fatally as generally expected.

Smallpox was officially declared eradicated by the World Health Organization in 1980 after a worldwide mass vaccination campaign (22). Routine smallpox vaccination was discontinued in Japan in 1976, prior to the declaration. However, concerns have arisen recently about the possible use of variola virus, the causative agent of smallpox, as a bioweapon (14). A total of 37 million Japanese, accounting for approximately 30% of the total population, who were born after the discontinuation of the routine vaccination program are considered to be completely susceptible to smallpox (1), but the immune status of those who were vaccinated decades ago is uncertain. It had been believed that the full protective immunity conferred by smallpox vaccination lasts only 3 to 5 years and that even partial immunity fades substantially after 10 to 20 years (5, 14, 21). Recently, however, it has been suggested that the immunity may last much longer. Several epidemiological studies have shown that immunity to smallpox may still be present many years after the vaccination (8, 13, 15). The degree of residual protection in vaccinated cases was estimated (8) by analyzing data on the outbreak that occurred in Liverpool, in the United Kingdom, during 1902 to 1903 (13) and on smallpox epidemics that occurred following reintroduction to Europe between 1950 and 1971 (15). The authors concluded that protection against fatal smallpox disease was lost at the rate of 0.363% per year and, thus, that 77.6% of vaccinees were still protected even 70 years after vaccination (8). Furthermore, El-Ad et al. reported that the levels of virus-specific neutralizing antibody remain stable for at least 30 years after revaccination (9), and T-cell immunity in response to smallpox vaccination was also reported to remain constant for decades (7, 11).

It has recently been shown that B-cell and T-cell-deficient mice immunized with modified vaccinia virus Ankara, an attenuated vaccinia virus, are both protected against challenge with a pathogenic vaccinia virus, although depletion of a single component of the immune response can reduce the extent of protection (17, 24). In contrast, double-knockout mice deficient in major histocompatibility complex class I and II were not protected (24). These findings indicate that both humoral and cellular immunities make significant contributions to protection against smallpox. With respect to humoral immunities, neutralizing antibodies are believed to play a crucial role in the protection against smallpox (14, 16, 19). Several studies have shown that certain levels of neutralizing antibodies might be involved in preventing the disease or attenuating disease severity (4, 16, 19), although the actual neutralizing antibody titers considered sufficient to protect against smallpox remain to be determined. These data endorse the idea that adequate serum antibody levels might be one of the benchmarks of protective immunity.

* Corresponding author. Mailing address: Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81 3 3815 5411 (ext. 33029 or 35335). Fax: 81 3 5800 8806. E-mail: shatake-tky@umin.ac.jp.

If people who were vaccinated some decades ago still maintain some immunity against smallpox, the morbidity, mortality, and transmission rates associated with the disease might be reduced significantly compared with present expectations (3, 18, 20). This might also affect future vaccination policy. Therefore, it is important to clarify whether individuals who were vaccinated decades ago maintain any immunity to smallpox, and if so, what fraction of the population possesses the immunity and how strong the immunity is. We have used enzyme-linked immunosorbent assays (ELISA) and neutralization assays to study the actual prevalence of virus-specific antibodies among representatives of the present Japanese population.

MATERIALS AND METHODS

Study population. We used stored anonymous serum samples that had been obtained from healthcare workers at the University of Tokyo Hospital in 2002 for serological screening of measles, rubella, mumps, and varicella-zoster virus. The sera were stored at 4°C until use. The present study was conducted with the approval of the Institutional Review Board of the University of Tokyo. It was impossible to identify the actual vaccination histories of each individual, because we used anonymous specimens. The ages of the 876 participants (257 males and 619 females) ranged from 20 to 65 years as of 2002 (mean ± standard deviation, 34.4 ± 10.3 years). Routine smallpox vaccination was discontinued in Japan in 1976, and the Immunization Law at that time recommended that individuals should receive three vaccinations against smallpox; the first vaccination was conducted in infancy, and subsequent vaccinations were given at the ages of 6 and 12 years. In this study, therefore, participants were divided into four birth cohorts according to the expected number of smallpox vaccinations they had received: those born after 1975 (younger than 26 years, as of 2002; the never-vaccinated group); those born between 1969 and 1975 (aged 27 to 33 years, as of 2002; the probable once-vaccinated group); those born between 1962 and 1968 (aged 34 to 40 years, as of 2002; the probable twice-vaccinated group); and those born before 1962 (older than 41 years, as of 2002; the probable thrice-vaccinated group). Before 1970, the most widely used strain of smallpox vaccine in Japan was the Ikeda strain, whereas the Lister strain was used during the 1970s.

Detection of vaccinia virus-specific IgG antibodies by ELISA. Levels of specific antibodies against the vaccinia virus, which was used for smallpox vaccines, were measured by ELISA. HeLa cells were infected with the vaccinia virus, Lister strain, at a multiplicity of infection of 1 and cultured for 48 h. They were then lysed in 1 ml of phosphate-buffered saline (PBS) containing 1% NP-40. The lysates were clarified by centrifugation at 10,000 ×*g* for 5 min, and the supernatant fraction was used as a positive vaccinia virus antigen. The mock-infected HeLa cells were also treated in the same way as those being used to prepare the vaccinia virus antigen to produce a negative-control antigen. Half of the wells of a flat-bottomed 96-well ELISA plate (Iwaki, Asahi Techno Glass, Chiba, Japan) were coated with the vaccinia virus-positive antigen, and the other half were coated with the negative-control antigen, followed by incubation at 4°C overnight. Both of the antigens were diluted 1:1,000 with PBS before coating, a dilution level that was determined by preliminary evaluations with box titration using the positive-control serum sample. After being washed three times with PBS containing 0.05% Tween 20 (T-PBS), the wells were blocked with 200 μl of PBS containing 5% skimmed milk and 0.05% Tween 20 (M-T-PBS) for 1 h and then washed three times with T-PBS. Samples of sera (100 μl per well), which were diluted 1:400 with M-T-PBS, were added, and the plates were incubated at 37°C for 1 h. The plates were washed three times with T-PBS and then incubated with 100 μl of M-T-PBS containing horseradish-peroxidase-conjugated goat anti-human IgG antibodies (ZYMED Laboratories, San Francisco, Calif.) (1:1,000 dilution) at 37°C for 1 h. After an additional washing step, 100 μl of the substrate reagent, ABTS [2,2′-azunobis (3-ethylbenzthiazoline sulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany), was added to each well. The plates were incubated at room temperature for 30 min, and the optical density (OD) was measured at a wavelength of 405 nm (OD_{405}) with reference to that at 490 nm. The adjusted OD values (OD_{405}) were calculated by subtracting the OD values of the negative-control-antigen-coated wells from those of the corresponding wells. The negative- and positive-control sera were included for verification in each run.

Vaccinia virus neutralization assay. We conducted the neutralization assay on 152 serum samples, which were randomly selected from each of the birth cohorts (the pre-1962 cohort [$n = 49$], the 1962-to-1968 cohort [$n = 22$], the 1969-to-1975 cohort [$n = 47$], and the post-1975 cohort [$n = 34$]). Vaccinia virus, Lister strain, and the RK13 cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin, and streptomycin were used. After heat inactivation at 56°C for 30 min, 100 μl of each of the sera serially diluted fourfold (beginning at 1:4) with DMEM containing 2% FCS and an equal volume of virus suspension containing 100 PFU per 100 μl were mixed and then incubated at 37°C for 2 h. RK13 cell monolayers in 24-well tissue culture plates were inoculated with 100 μl of the mixture in duplicate, and the cells were then incubated at 37°C for 1 h with frequent shaking. Thereafter, the inoculum was removed and the cells were washed once with PBS. They were then cultured in 1 ml of the overlay medium (DMEM containing 2% FCS, penicillin, streptomycin, and 0.5% methylcellulose) at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 days. The cells were fixed with 10% formalin in PBS and stained with 0.1% crystal violet, and the number of plaques was counted. In each assay, the negative- and positive-control sera were included for verification. The neutralizing antibody titer (NT_{50}) was defined as a reciprocal of the highest dilution level of the serum demonstrating a >50% reduction in plaque count compared with the negative control results.

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by standard methods (10). The differences in OD_{405} values and the proportion comparison between the birth cohorts were evaluated by an unpaired Student's *t* test and chi-square test, respectively, using Stat Flex version 5.0 software (Artech, Osaka, Japan). The level of statistical significance was set at $P < 0.05$. Receiver operating characteristics (ROC) and two-graph ROC (TG-ROC) curves were analyzed using the Stat Flex software. Vaccinia virus-specific NT_{50} values were analyzed by log transformation to linearize the relationship between variables. The relationship between antibody titers determined by neutralization assay and OD_{405} values determined by ELISA was evaluated by linear regression analysis.

RESULTS

Detection of vaccinia virus-specific antibodies by ELISA. We determined the cutoff values in the ELISA for the detection of specific antibodies against vaccinia virus by ROC and TG-ROC curves. The OD_{405} values of sera among subjects who were born in 1977 or after were treated as negative examples, because the routine smallpox vaccination program had already been discontinued. On the other hand, the serum samples of subjects who were born in or before 1968 were treated as positive examples with the vaccination history, because the vaccination program was being strictly enforced at that time, and so the majority of these subjects were considered to have been vaccinated. Logistic regression analysis ROC and TG-ROC curves under the aforementioned conditions are shown in Fig. 1. The cutoff value was set at 0.10, at which level the ELISA system exhibited optimal sensitivity (98.3%) and specificity (99.1%) for detecting the vaccinia virus-specific IgGs elicited by past smallpox vaccination. The area under the ROC curve was 0.988, which corresponds to "excellent probability" (12), allowing us to use this test to distinguish between vaccinated and unvaccinated individuals.

The OD_{405} values that were determined by subjecting serum samples (diluted 1:400) to ELISA are plotted for each birth cohort in Fig. 2A. Of the subjects born before 1962, those born between 1962 and 1968, and those born between 1969 and 1975, 98.6, 98.6, and 66.0%, respectively, retained vaccinia virus-specific IgGs (Table 1). There were significant differences ($P < 0.0001$) in the seropositivity rate between the 1969-to-1975 cohort and the older birth cohorts, but the difference between the 1962-to-1968 cohort and the pre-1962 cohort was not statistically significant ($P = 0.984$). The geometric mean for the OD_{405} values was 1.46 in the pre-1962 cohort, 1.36 in the 1962-to-1968 cohort, and 0.88 in the 1969-to-1975 cohort. Although there was no significant difference in these means

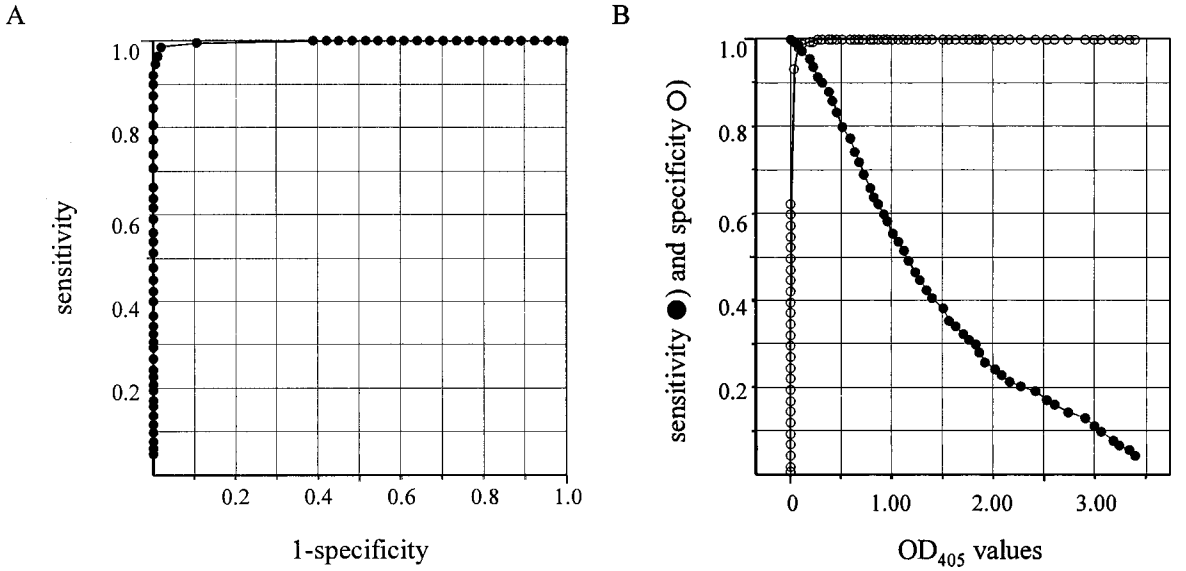

FIG. 1. Receiver operating characteristics (ROC) curve (A) and two-graph-ROC curve (B) for the detection of vaccinia virus-specific IgG. These graphs show the relationship between the sensitivity and specificity of the vaccinia virus-specific ELISA system for each cutoff OD_{405} value. The most optimal sensitivity (98.3%) and specificity (99.1%) are obtained when the cutoff OD_{405} value is set at 0.10. The area under the ROC curve is 0.988, which indicates the test has a good probability of distinguishing between vaccinated and unvaccinated individuals.

between the pre-1962 and 1962-to-1968 cohorts ($P = 0.371$), the mean OD_{405} value for the 1969-to-1975 cohort was significantly ($P < 0.0001$) lower than that for the pre-1969 cohorts (i.e., the pre-1962 and 1962-to-1968 cohorts). The year-on-year seropositivity rate for vaccinia virus-specific IgG among the 1969-to-1975 cohort was 90.0% for 1969 ($n = 29$), 93.3% for 1970 ($n = 30$), 76.2% for 1971 ($n = 42$), 88.6% for 1972 ($n = 44$), 79.3% for 1973 ($n = 58$), 45.8% for 1974 ($n = 48$), and 6.5% for 1975 ($n = 46$). The smallpox vaccination rate in Japan was reported to have already declined sharply in the final few years before the cessation of routine vaccination (information from the Ministry of Health, Labor and Welfare of Japan), which corresponds to the lower seropositivity rate in the 1974 and 1975 birth cohorts of this study. If we took into account the OD_{405} values of the seropositive subjects alone among the 1969-to-1975 cohort (i.e., excluding the seronegative subjects;

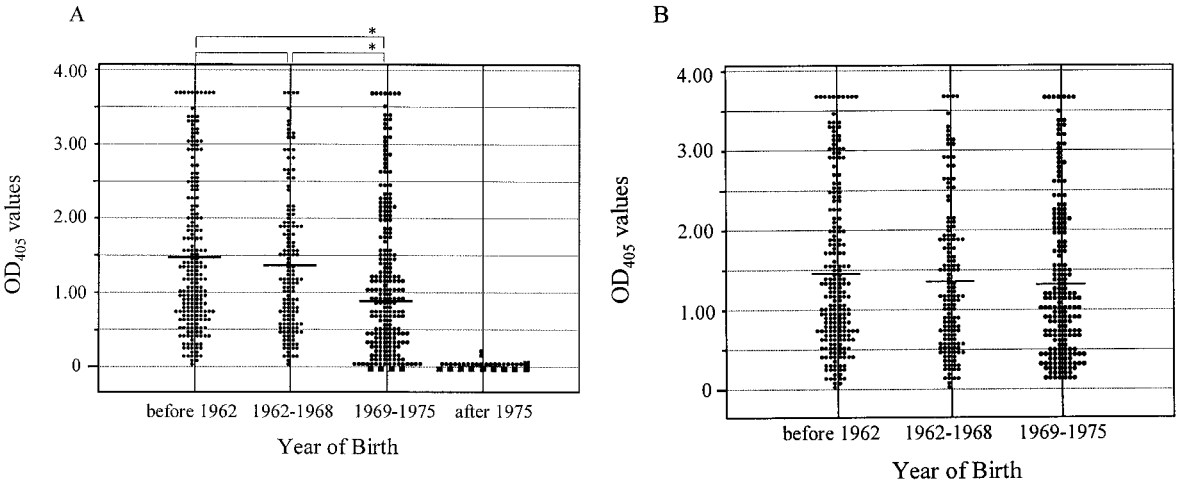

FIG. 2. Vaccinia virus-specific IgG determined by ELISA (the OD_{405} values). Panel A shows the ELISA OD_{405} values in each birth cohort. The bar indicates the geometric mean value, and the actual mean values are 1.46, 1.36, 0.88, and 0.02 for the pre-1962, 1962-to-1968, 1969-to-1975, and post-1975 birth cohorts, respectively. An asterisk indicates that the difference between the mean OD_{405} values is statistically significant. The pre-1962 and 1962-to-1968 cohorts exhibit significantly higher (unpaired Student's t test, $P < 0.0001$) mean OD_{405} values than the other two cohorts, but the difference between those of the pre-1962 and the 1962-to-1968 cohorts is not significant ($P = 0.371$). Each circle corresponds to one subject, and each square represents 20 subjects. Panel B shows the OD_{405} values in the pre-1962, 1962-to-1968, and 1969-to-1975 cohorts. Note, however, that only seropositive samples (OD_{405} values ≥ 0.10) are included for the 1969-to-1975 cohort. The mean values, between which there is no significant difference, are 1.46, 1.36, and 1.39, respectively. IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; OD_{405}, adjusted optical density.

TABLE 1. Seroprevalence of vaccinia-virus-specific IgG, as determined by ELISA, in four different birth cohorts that cover the period from before 1962 to after the cessation of routine smallpox vaccination in Japan in 1976

Birth yr	No. of subjects	No. (%) of seropositive subjects	
		$OD_{405} \geq 0.10$	$OD_{405} \geq 0.30$
Before 1962	212	209 (98.6)	193 (91.0)
1962–1968	144	142 (98.6)	130 (90.3)
1969–1975	297	196 (66.0)	173 (58.2)
After 1975	223	2 (0.9)	0 (0)
Total	876	548	495

TABLE 2. Sensitivity, specificity, PPV, and NPV of ELISA with the designated reference value for neutralization assay for vaccinia virus within a subsample of 152 sera

ELISA OD_{405}	No. of NT_{50} results		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	≥4	<4				
≥0.10	96	17	97.0	67.9	85.0	92.3
<0.10	3	36				
≥0.30	88	7	88.9	86.8	92.6	80.7
<0.30	11	46				

OD_{405} values < 0.10), who were unlikely to have been vaccinated, the mean OD_{405} value was 1.32, which was not significantly different from those of the pre-1969 birth cohorts (Fig. 2B).

Relationship between the ELISA and the neutralization assay. Neutralizing antibodies were also maintained, and a significant linear correlation was observed between the neutralizing antibody titers (NT_{50}) and the OD_{405} values determined by the ELISA ($R^2 = 0.450$; $P < 0.0001$) (Fig. 3). The sensitivity, specificity, PPV, and NPV of the ELISA testing for the presence of neutralizing antibodies ($NT_{50\geq}4$) were 88.9, 86.8, 92.6, and 80.7%, respectively, when the reference OD_{405} value in the ELISA was set at 0.30. The corresponding figures for a reference OD_{405} value of 0.10 were 97.0, 67.9, 85.0, and 92.3%, respectively (Table 2). Neutralizing antibodies were negative ($NT_{50} < 4$) in all of the 34 samples from the post-1975 birth cohort. On the basis of these analyses, the ELISA OD_{405} values of ≥0.10 provide good sensitivity and specificity (98.6 and 99.1%, respectively) for the prediction of smallpox vaccination history, and OD_{405} values of ≥0.30 provide a high PPV (92.6%) for detecting the retention of neutralizing antibodies. The OD_{405} values of ≥0.30 were demonstrated in 91.0% of subjects in the pre-1962 cohort, 90.3% of those in the 1962-to-1968 cohort, and 58.2% of those in the 1969-to-1975 cohort (Table 1). The OD_{405} values were below 0.30 in all samples from the post-1975 birth cohort. These results indicate that approximately 80% of subjects in the pre-1969 birth cohorts, and approximately 50% of those in the 1969-to-1975 cohort, also retained neutralizing antibodies against smallpox.

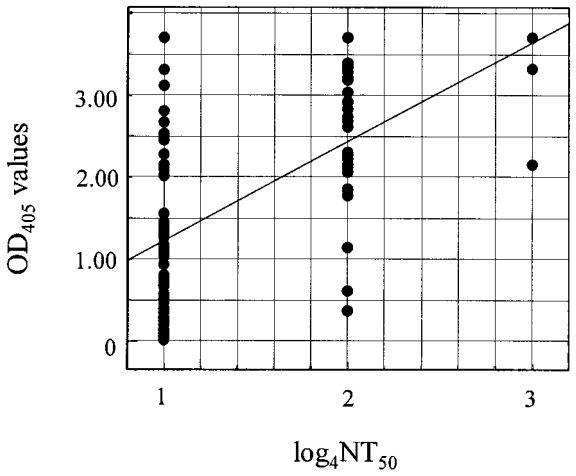

FIG. 3. Relationship between neutralizing antibody titers to vaccinia virus and ELISA OD_{405} values. The regression analysis yields a significant linear relationship ($R^2 = 0.450$; $P < 0.0001$).

DISCUSSION

Neutralizing antibodies have been thought to constitute an important correlate of protective immunity against smallpox (4, 14, 16, 19). To perform a test on a large number of samples, we developed a vaccinia virus-specific ELISA technique with high processing ability, because neutralizing assays are time and labor intensive and are not adequate for handling a large number of samples. Therefore, we evaluated the correlation between antibodies detected by the neutralization assay and those detected by ELISA, because the antibodies detected by ELISA do not always reflect the presence of neutralizing antibodies.

It has been shown that the majority of the Japanese population who were vaccinated against smallpox prior to the cessation of the routine vaccination program in 1976 still maintain certain levels of ELISA-detectable virus-specific antibodies. More than 98% of subjects in the pre-1969 birth cohorts and 66% of those in the 1969-to-1975 cohort still maintained detectable levels of IgG antibodies (OD_{405} values ≥ 0.10). One of the reasons why the subjects in the 1969-to-1975 cohort have a lower seropositivity rate than those in the older cohort is that the vaccination rate per se would have been lower, because they were given only one opportunity to receive a smallpox vaccination. In fact, the smallpox vaccination rate at each stage was reported to be around the 80% level, except in 1974 and 1975, when the rate declined markedly (information from the Ministry of Health, Labor, and Welfare of Japan). The mean OD_{405} value calculated only from the seropositive subjects among the 1969-to-1975 cohort was 1.32, and this value was not significantly different to that of the pre-1962 cohort or the 1962-to-1968 cohort. Although we do not have precise information on how many vaccinations each individual actually received, this result suggests that the additional vaccinations had little influence on the period or degree of IgG retention, as long as the first vaccination had "taken" successfully. Therefore, further investigations are required to determine whether or not multiple smallpox vaccinations are necessary for acquiring significant protection, although it is generally believed that additional vaccinations are likely to confer a stronger and

longer-lasting immune response (2, 9, 23). Hammarlund et al. reported that the mean antibody titer induced by double vaccinations was very slightly but significantly higher than that induced by a single vaccination but that additional (between 3 and 14) vaccinations did not result in any further increase in long-term antibody production (11).

In the present study, most of the individuals whose serum samples exhibited OD_{405} values of ≥0.30 also retained the neutralizing antibodies. Thus, it was demonstrated that a considerable proportion of the previously vaccinated individuals still retained neutralizing antibodies. Although lower ELISA OD_{405} values tend to associate with lower neutralizing antibody titers and higher OD_{405} values tend to associate with higher neutralizing antibody titers, the correlation between them was only moderately positive ($R^2 = 0.450$; $P < 0.0001$). It may be due to a number of epitopes on the viral proteins other than neutralizing epitopes on vaccinia virus.

This study has revealed that many individuals who were vaccinated 27 to 53 years ago retain a significant degree of antiviral humoral immunity. Although this remaining immunity may no longer provide full protection, it is highly likely to afford at least partial protection. Hammarlund et al. showed that virus-specific T-cell immunity could persist for a long time after smallpox vaccination, perhaps as long as 75 years, declining only slowly, with a half-life of 8 to 15 years (11). Furthermore, it was shown that virus-specific memory B-cells were maintained for more than 50 years after vaccination and correlated positively with circulating antibody levels (6). In addition, some epidemiological analyses have also indicated that the immunity achieved after smallpox vaccination may remain for several decades (1, 8, 13, 15). Taking these data together, it appears that the immunity conferred by smallpox vaccination persists for longer than had previously been expected.

In the present study, we found that more than 98% of the Japanese population in the pre-1969 birth cohorts and 66% of those in the 1969-to-1975 cohort still maintain the vaccinia virus-specific IgG, whereas approximately 80 and 50%, respectively, also retain detectable levels of neutralizing antibodies. These long-term persisting immunities may provide some protective benefits in the case of intentional smallpox reemergence. In addition, the present results may also contribute in making policy of vaccination priority, especially if vaccine supplies become limited in the event of a widespread outbreak.

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