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Duration of neutralizing antibody persisting in Thai individuals after childhood vaccination against smallpox

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Abstract

Background: Although smallpox was completely eliminated by 1980, it remains possible that variola virus could be intentionally released in an act of bioterrorism. Thus, several studies have been performed to detect antibody levels after smallpox vaccination of the current population in various countries to indicate the duration of maintenance of immunological memory. Our study endeavored to investigate the level of neutralizing (Nt) antibody responses of Thai individuals who had been immunized with smallpox vaccine during childhood.

Methods: The plaque reduction neutralization test (PRNT) was used to study vaccinia Nt antibody responses in sera of individuals ranging in age from 35–44, 45–54, 55–64, 65–74, 75–84 and >84 years old, referred to as groups 1–6, respectively. Each group included 200 sera: 100 male sera and 100 female sera.

Results: An incubation time of 15 hours for sera and vaccinia virus was confirmed to be the optimal incubation period for PRNT. Positive Nt antibody titers (≥32) were detected in 135 (11.25%) of 1,200 sera: 81 (6.75%) male sera and 54 (4.5%) female sera. There were 4 (2%), 11 (5.5%), 19 (9.5%), 16 (8%), 33 (16.5%), and 52 (26%) positive sera in groups 1–6, respectively. Interestingly, the oldest individual with positive Nt antibody was a 98-year-old female. Two males aged 96 and 91 years old had the highest Nt antibody titers.

Conclusions: Our data suggests that the vaccinia-specific Nt antibody response in the current Thai population could be maintained for more than 90 years after vaccination. However, the majority of the Thai population aged ≥35–74 years old is still highly susceptible to infection.

Keywords: smallpox vaccine; childhood immunization; duration; neutralizing antibody; PRNT

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Introduction

Smallpox is a contagious disease caused by variola virus. It is considered to be one of the most serious bioterrorist threats. Variola virus can spread from person to person by respiratory droplets or direct contact with body fluids. The infectious dose is small with a high fatality rate. Various vaccinia virus strains such as EM-63, Lister, Temple of Heaven, Western Reserve and New York City Board of Health (NYCBH) strains have been used as successful vaccines.[1-3] However, international health care authorities are concerned about the intentional release of variola virus. This has prompted researchers around the world to study the level of immunity in current populations.[4] Hammarlund *et al*. evaluated the duration of antiviral immunity after smallpox vaccination.[5] More than 90% of volunteers vaccinated 25–75 years ago still maintain immunity against vaccinia virus. Vaccinia-specific neutralizing (Nt) antibody titer was used to estimate the immunity provided by smallpox

vaccination.[6] Taub *et al.* also examined the magnitude and duration of antiviral antibody immunity conferred by smallpox vaccination in participants of the National Institute on Aging Baltimore Longitudinal Study of Aging.[7] These individuals showed the persistence of relatively stable vaccinia IgG titers for periods up to 88 years after the initial vaccination. Moreover, the vaccinia-specific Nt antibody titer ranged from 1:256 to 1:512 and remained stable. Putz *et al.* reported long-lasting antibody with similar titers against vaccinia virus in citizens aged between 30 and 100 years old.[8] Liu *et al.* demonstrated that vaccinia-specific Nt antibody was maintained for more than 40 years post vaccination in a Chinese population.[9]

The plaque reduction neutralization test (PRNT) is the standard method for studying anti-viral Nt antibody.[10] Several studies have used this assay to investigate antibody level after smallpox vaccination in volunteers.[7-9] In our study, the final Nt antibody titers were assessed in sera of Thai individuals of different ages who had been immunized with smallpox vaccine during childhood. The results indicate the length of time that Thai individuals can maintain Nt antibody against smallpox virus. This information should be useful for public health strategies against potential bioterrorism threats.

Methods

Serum

A batch of 1,200 leftover sera obtained randomly from either healthy individuals or patients during 2013 by the Department of Microbiology was analyzed with approval from the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University. These anonymous sera were negative for HIV antibody, HBsAg of HBV and HCV antibody according to previous testing. They were grouped into six groups according to individual age, ranging from ≥35–44, ≥45–54, ≥55–64, ≥65–74, ≥75–84 and ≥85 years old. Each group was composed of 200 sera: 100 male sera and 100 female sera. In addition, we collected sera from 50 females and 50 males aged less than 30 years old, representing naïve-vaccinated individuals. The negative-control serum sample was the naïve-vaccinated human pool sera. The positive-control serum sample was a post-vaccination serum. Pre- and post-vaccination sera from a volunteer who received ACAM2000™ smallpox vaccine as a primary immunization with written informed consent was collected to determine the optimal condition for PRNT. All sera were heat-inactivated at 56°C for 30 minutes, aliquoted, and stored at -20°C until use within a month.

Cell line

The thymidine kinase (TK) cell line is susceptible to vaccinia virus. Cells were cultured at 37°C/5%CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate.

Reference virus

Vaccinia virus vP1170, strain WR (Virogenetics Corp., Troy, NY, USA) was propagated in TK cells, and frozen and thawed for three freeze-thaw cycles. Vaccinia virus titer was then determined in terms of plaque forming units (PFU)/ml. The virus was then stored at -80°C until use.

Plaque reduction neutralization test (PRNT)

Serial two-fold dilutions of sample sera and control sera were prepared in serum-free DMEM with antibiotics. Sera at various dilutions (1:4, 1:8, 1:16, 1:32 and 1:64) were mixed with an equal volume of vaccinia virus, containing approximately 100 PFU. Serum–virus mixtures were incubated at 37°C/5%CO_2 for 1 hour[10] or 15 hours.[11] A back titration of viral control for each assay was prepared by mixing vaccinia virus diluted to 50, 100, and 200 PFU with equal volumes of DMEM. An overnight-prepared monolayer of TK cells in 24-well tissue culture plates was washed once with 2% DMEM. Serum–virus mixtures were then inoculated onto the cell monolayer in duplicate and allowed to adsorb at 37°C/5%CO_2 for 1 hour. Then, 2% DMEM was added and the plates were returned to the incubator for 48 hours. Tissue culture plates were stained with 1% crystal violet for 5 minutes and air-dried. Plaques in each well were manually counted under a microscope. The total plaque count for each serum dilution was defined as the mean plaque count of duplicate wells. The endpoint for serum Nt antibody titer was defined as the reciprocal of the highest dilution of the serum with a mean plaque count less than or equal to the 60% plaque reduction cutoff value. Sera that had an Nt antibody titer >64 were further diluted to ratios of 1:128, 1:256, 1:512, 1:1,024, etc. Then, the diluted sera were again subjected to PRNT to determine the final titers.

Statistical analysis

PASW Statistics (SPSS) version 18.0 was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). Comparison of the presence of NtAb in sera of males and females was performed using Chi-square test. A p-value ≤0.05 was considered statistically significant.

Results

The optimal incubation time for PRNT

The optimum incubation period for the serum and virus mixture (1 h versus 15 h) in the neutralization step of PRNT was evaluated. The pre-vaccination and post-vaccination sera of a naïve individual who received smallpox vaccine as a primary immunization were serially diluted two-fold starting at dilutions of 1:4 and 1:10. The PRNT Ab titer in the pre-vaccination serum from the 1 h and 15 h incubation periods was < 4 and < 10, respectively, as shown in **Table 1**. Interestingly, the PRNT Ab titer in post-vaccination serum at day 60 was 4 and 10, respectively, for the 1 h incubation time, whereas the PRNT Ab titer was 64 and 80, respectively, for the 15 h incubation time. This result indicated that the 15 h incubation period increased the PRNT titer of post-vaccination serum when compared to the 1 h incubation period by 16x and 8x, respectively. Thus, the 15 h incubation period was the optimal duration of the serum–virus mixing step, and was used to perform PRNT of all studied sera.

Table 1. Comparison of PRNT antibody titers using two incubation times: 1 h vs 15 h with 2 fold serial dilution starting at dilution 1:4 vs 1:10 in serum-virus mixture step of pre-vaccinated- and post-vaccinated-sera from a naïve volunteer who received primary smallpox vaccination.

Subject	Serum dilution starting at	Incubation time (h)	PRNT titers	
			Pre-vaccination	Post-vaccination
			(Day 0)	(Day 60)
001	1:4	1	<4	4
		15	<4	64
	1:10	1	<10	10
		15	<10	80

Evaluation of Nt antibody titers of individual sera from different age groups

One thousand, two hundred sera of Thai individuals (600 males and 600 females) post childhood vaccination against smallpox were evaluated for the maintenance of virus-specific humoral immunity, i.e., final titers of NtAb according to PRNT. The population in this study was stratified by age group according to the last smallpox vaccinations in Thailand, which took place in 1977/1978. The sera of individuals aged ≥35–44, ≥45–54, ≥55–64, ≥65–74, ≥75–84 and ≥85 years old were assigned as groups 1–6, respectively. Each group included 100 male and 100 female sera. A PRNT titer ≥ 32 was considered to indicate positive NtAb serum. Two to 26% of the various groups of tested sera demonstrated the maintenance of NtAb against vaccinia virus as shown in **Table 2**. Positive NtAb were detected in 135 (11.25%) of 1200 sera: 81 (6.75%) male sera and 54 (4.5%) female sera. In terms of gender, male sera (81/135) were significantly more likely to contain NtAb than female sera (54/135), with a *p*-value of 0.001. Group 1 contained 4 (2%) NtAb-positive sera (2 male and 2 female); group 2 contained 11 (5.5%) NtAb-positive sera (4 male and 7 female); group 3 contained 19 (9.5%) NtAb-positive sera (12 male and 7 female); group 4 contained 16 (8%) NtAb-positive sera (11 male and 5 female); group 5 contained 33 (16.5%) NtAb-positive sera (18 male and 15 female); and group 6 contained 52 (26%) NtAb-positive sera (34 male and 18 female). Two sera of group 6 with NtAb titers of 2,048 and 4,096 belonged to males aged

Table 3. Serum neutralizing antibody titers of non-vaccinated Thai individuals at variety of ages.

Age (years)	Gender	PRNT antibody titers*					
		< 4	4	8	16	32	64
≤11	M	10	9	1	-	-	-
	F	10	10	-	-	-	-
12-21	M	20	19	1	-	-	-
	F	20	18	2	-	-	-
22-31	M	20	20	-	-	-	-
	F	20	19	1	-	-	-
	Total	**100**	**95**	**5**	**0**	**0**	**0**

* The PRNT titer was considered to be positive for NtAb at ≥32
M= male, F= female

Table 2. Serum neutralizing antibody titers of Thai individuals at variety of ages after childhood vaccination against smallpox.

Group	Age (years)	Gender	N	PRNT titers*											#Positive NtAb		
				<4	4	8	16	32	64	128	256	512	1024	>1024	#	Total	%Total
1	≥ 35-44	M	100	81	7	9	1	2	-	-	-	-	-	-	2	4	2
		F	100	83	8	5	2	1	-	1	-	-	-	-	2		
2	≥ 45-54	M	100	75	11	7	3	3	-	1	-	-	-	-	4	11	5.5
		F	100	69	11	9	4	2	4	-	1	-	-	-	7		
3	≥ 55-64	M	100	42	21	15	10	6	2	2	-	2	-	-	12	19	9.5
		F	100	62	14	9	8	3	2	1	-	1	-	-	7		
4	≥ 65-74	M	100	49	24	8	8	3	4	2	2	-	-	-	11	16	8
		F	100	64	22	5	4	2	1	-	2	-	-	-	5		
5	≥ 75-84	M	100	40	13	20	9	14	3	-	1	-	-	-	18	33	16.5
		F	100	47	18	13	7	7	3	1	3	-	1	-	15		
6	≥ 85	M	100	29	14	18	5	16	4	9	1	2	-	2[a]	34	52	26
		F	100	46	9	17	10	7	2	6	3	-	-	-	18		
	Total		**1200**	**687**	**172**	**135**	**71**	**66**	**25**	**23**	**13**	**5**	**1**	**2**	**135[b]**	**135**	**11.25**

* The PRNT titer was considered to be positive for NtAb at ≥32; M= male, F= female
a The final PRNT titers were 2048 and 4096.
b Total male = 81; total female = 54; There was a statistically significance between genders (male vs female) on the positive of NtAb at *p*-value 0.001.

96- and 91-years-old, respectively. The oldest individual whose serum had a positive NtAb titer at 128 was a 98-year-old female.

One hundred sera of non-vaccinated individuals aged from ≤11, 12–21 and 22–31 years old were examined for NtAb titers. No positive NtAb titers were identified as shown in **Table 3**.

Discussion

WHO officially declared the eradication of smallpox in 1980, following a global campaign.[12] Vaccination had been used to prevent variola virus infection. The maintenance of long-term antibody responses is critical for protective immunity against many pathogens. Although both humoral and cellular immunities are long-lived after vaccinia virus immunization,[5,13] the last smallpox fatality in 1978 in Birmingham, England, occurred despite the patient having been previously vaccinated twice; first as a child and again aged 28.[14] Due to concerns about the risk of bioterrorist activity involving the intentional release of variola virus, several countries have evaluated the immune response to smallpox/vaccinia virus remaining in their population.[5,7,9] The last smallpox vaccination with the Lister strain in Thailand occurred in 1977/1978.[15] Thus, we investigated the remaining humoral immunity, i.e., neutralizing antibody, to smallpox/vaccinia virus in a Thai population born before 1979. In this study, the levels and duration of NtAb in Thai individuals at a variety of ages were examined by PRNT. Initially, the optimal incubation time of the neutralization step of PRNT was investigated. We compared the serum–virus mixtures for the standard 1 h vs 15 h incubation at 37°C/ 5%CO_2. Pre- and post-vaccination sera of a volunteer who received the ACAM2000™ smallpox vaccine as a primary immunization were examined. Interestingly, the 15 h incubation demonstrated a much higher serum PRNT titer than the 1 h incubation. In addition, the vaccinee demonstrated successful vaccination via the presence of a major cutaneous reaction at the inoculation site. However, the 1 h incubation did not produce a positive Nt antibody response. A 1 h incubation may not have been long enough for the serum to bind to the virus completely in this study. This showed that the longer the incubation time, the higher the sensitivity of the assay.

Our result confirms a study by Newman *et al.*, who reported the superiority of the 15 h incubation of the serum–virus mixture compared to the standard 1 h incubation.[11] However, Katz reported that PRNT titers determined following a 2 h, 37°C virus–serum incubation period did not differ significantly from those determined following an 18 h incubation period (2 h, 37°C incubation followed by a 16 h, 4°C incubation period).[16] Thus, we chose this 15 h incubation period for further study of PRNT titers in all sera.

Based upon reports from the smallpox pre-eradication period, an anti-vaccinia Nt antibody titer of >20 or >32 was protective.[17-18] We used a titer of ≥32 as a cut off for positive NtAb. Thus, positive NtAb were detected in 135 (11.25%) of 1200 sera: 81 male sera (6.75%) and 54 female sera (4.5%). All six groups of individuals of different ages showed the maintenance of NtAb against vaccinia virus. However, there were some differences in the final NtAb titers among these groups. Interestingly, the sera from older groups (group 5: aged ≥75–84 and group 6: aged ≥85) showed a higher number of positive NtAb than those of the other four groups, i.e., 16.5–26.0% vs 2.0–9.5%, respectively. The low number of positive NtAb presenting in total and the increase in number of positive NtAb sera with increasing age were concordant with a previous study in China by Liu *et al.*, who investigated the NtAb in the general population.[9] They reported that the overall percentage of positive NtAb in their study was 7.6% (21/278) and increased with age, i.e., 5.56% (9/162) vs 10.35% (12/116) of 31–40 and 41–56 years, respectively. In addition, a study by Hatakeyama *et al.* in Japan demonstrated that 80% of persons born before 1969 and 50% of those born between 1969 and 1975 had maintained Nt antibodies against smallpox.[19] Nt antibodies have been revealed to decline substantially during a 5–10 year period after natural smallpox infection or vaccination.[20-21] Thus, individuals who received the recommended single-dose vaccination during childhood may not have lifelong immunity. The percentage of individuals retaining long-term vaccinia-specific antibodies was lower among individuals who were vaccinated once, compared with individuals who received multiple vaccinations.[22-23] It is possible that in our study, most people in the younger age groups (groups 1–4) had received only one single-dose vaccine while most people in the older age groups (groups 5–6) had received more than one dose. Moreover, the duration and times of exposure to natural smallpox of the older age groups during the era of circulating smallpox may have been longer/higher than those of the younger age groups. Repeated exposure to smallpox could result in subclinical infection that may have continued to boost immunity over time.[5]

In our study the oldest individual with NtAb-positive serum (1:128) was a 98-year-old female. This indicates that NtAb could be maintained for more than 90 years after the initial vaccination. This is consistent with a study conducted in Baltimore, USA, in which vaccinia-specific IgG were found to remain for a period of up to 88 years post vaccination.[7] NtAb responses to smallpox/vaccinia virus vaccine may be related to many factors including the host's genetic make-up, the strain of vaccinia virus in the vaccine, the dose of vaccine, the times of vaccination, age, gender, etc. In our study, males (81/135; 60%) were significantly more likely to have NtAb (*p-value* = 0.001) than females (54/135; 40%). On the other hand, Liu *et al.* reported no statistically significant association between gender and the presence of NtAb, i.e., 12/21 males (57.14%) vs 9/21 females (42.86%) in Chinese individuals.[9]

In conclusion, the durability of immunity to vaccinia/ smallpox vaccine in the Thai population is lifelong for older individuals (>74 years old) who were successfully vaccinated on more than one occasion or were exposed to circulating smallox in the smallpox era. However the majority of the younger Thai population (≥35–74 years old) is highly susceptible to infection.

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Conflict of interest

The authors hereby declare no personal or professional conflicts of interest regarding any aspect of this study.

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