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稀釋倍數痘苗之人體臨床反應評估

研 究 報 告

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中文摘要

關鍵詞：生物恐怖攻擊，天花，痘苗

為了能使國內的天花疫苗足以應付可能的生物恐怖攻擊，我們進行稀釋天花疫苗(痘苗)的人體試驗。在此人體試驗中，共有二百一十九位健康的志願受試者參加，以隨機分組的方式，對九十七位以前從未接種者，測試 WHO 標準建議劑量，五倍稀釋劑量，及十倍稀釋劑量的效果；對一百二十二位孩童時期曾接種過天花疫苗的受試者，測試標準建議劑量，十倍稀釋劑量及三十倍稀釋劑量的效果。並以新式雙叉針頭進行皮下接種(用量僅需傳統方法的四分之一)。初步臨床觀察結果顯示，對以前從未接種者而言，其接種標準建議劑量，五倍稀釋劑量及十倍稀釋劑量的效果皆為 100%；對孩童時期曾接種過天花疫苗的受試者而言，其接種標準建議劑量，十倍稀釋劑量及三十倍稀釋劑量的效果則為 100%，100%及 96.2%。各組效果及副作用之間並沒有統計學上有意義的差異，所有志願受試者都沒有出現嚴重的併發症。這結果呈現以下數點意義：第一，這些經冰封超過二十年的天花疫苗仍保有良好的活性及安全性，仍符合 WHO 的標準；第二，過去國內未曾使用雙叉針頭來接種天花疫苗，但在此次試驗中顯示出雙叉針頭的可行性及方便性；第三，對以前從未接種者而言，十倍稀釋劑量的效果並不比標準建議劑量的效果差(只要稀釋後病毒量還維持在 10^8 pfu/mL 以上)；對孩童時期曾接種過天花疫苗的受試者而言，標準建議劑量即使稀釋到三十倍，其效果與標準建議劑量的效果仍沒有明顯的差別(只要稀釋後病毒量還維持在 $10^{7.5}$ pfu/mL 以上)。這些結果並不受年齡的影響。依此結果，我們便可將國內現存的七十萬劑疫苗以此簡單的方式擴充至足敷全國所需的安全存量。

英文摘要

關鍵詞: Bioterrorism, Smallpox, Vaccinia virus, Lister strain

Background: Because the vaccine was not manufactured over 20 years ago in most countries, the potential for a bioterrorism using smallpox has led to an alarm whether current stocks of smallpox vaccine are enough.

Objective: To evaluate the potential to increase the supply of smallpox vaccines by diluting the vaccinia virus of Lister strain in vaccination of vaccinia-naïve and previously vaccinated subjects.

Design and subjects: A total of 219 enrolled subjects (97 vaccinia-naïve and 122 previously vaccinated) were enrolled from February 10 to October 31, 2003 in this prospective, single-blinded study. Vaccinia-naïve subjects (aged 20 to 23 years) were randomized to receive either undiluted or diluted (1:5, 1:10) smallpox vaccines, and previously vaccinated subjects (aged 24 to 65 years) were randomized to receive either undiluted or diluted (1:10, 1:30) smallpox vaccines.

Setting: a national university hospital

Measurements: The main measure was the rate of clinical success of vaccination, defined as the presence of a vesicular or pustular lesion at the inoculum site following vaccination. The second measure was immunological success rate, including significant T cell or antibody responses to vaccinia virus after vaccination.

Results: Except two subjects who received 1:30 diluted vaccine, the vaccination of all of the vaccinia-naïve and previously vaccinated subjects, who received undiluted or diluted vaccines, was successful clinically. All enrolled subjects had

significant vaccinia-specific T cell and antibody responses. No difference in response to vaccine in different age group. The diluted vaccines were not associated with decreased local reactions or adverse events when compared with undiluted vaccines. However, local reactions were more marked and most adverse events were more frequently observed in vaccinia-naïve subjects than observed in previously vaccinated subjects. No serious or life-threatening events were noted.

Conclusion: With Lister strain of vaccinia virus, the smallpox vaccine can be safely used as 1:10 dilution in vaccinia-naïve subjects and 1:30 dilution in previously vaccinated subjects if viral titer $\geqq$ 10^8 and $10^{7.5}$ pfu/mL after dilution, respectively.

本文

(1)前言

Eradication of smallpox was declared in 1980. Routine smallpox vaccination with vaccinia virus was discontinued and the vaccine was not manufactured over 20 years ago in most countries. Because the vaccinia-specific T cell immunity declines gradually after vaccination [1-3], the potential for a bioterrorism using smallpox has led to an alarm whether current national stocks of smallpox vaccine are enough or not. Fortunately, the published clinical trials conducted in the United States have shown that ≦ 10 times dilution of Dryvax (New York City Board of Health strain) remains effective [4, 5]. However, in addition to U.S., any other countries in the world may be indirectly involved or even be the target of bioterror attack with smallpox. Thus U.S. is not the only country which has the need to prepare enough smallpox vaccine for the possible intentional outbreak.

In Taiwan, nationwide routine and compulsory smallpox vaccination with Lister strain had been conducted for more than 5 decades and stopped in 1979. Because the smallpox vaccines were not manufactured any more since 1981, the supply of smallpox vaccines is severely limited in Taiwan if an outbreak occurs and if mass vaccination is needed. Our current vaccine stocks, even if diluted 10 times, would not fulfill the demands to meet the possible requirement for nationwide vaccination. Thus we conducted this study to know whether the diluted vaccines with vaccinia virus of Lister strain, not approved in U.S. but licensed in England and used in many other countries [5, 6], are effective in vaccinia-naïve persons as Dryvax does, and to evaluate the efficacy the highly diluted (1:30) vaccine in persons who have been vaccinated in the childhood, and to assess the vaccinia-specific memory responses after vaccination in these

subjects.

(2)材料與方法

Subjects

The study was approved by the institutional review board of the study site. Subjects were enrolled from February 10 to October 31, 2003 after providing written informed consent. Subjects were enrolled through public announcement. Healthy adult volunteers born after stopping nationwide smallpox vaccination (between 20 to 23 years of age) were eligible as vaccinia-naïve subjects if no vaccination scar, and no history of smallpox vaccination. Healthy adult volunteers born before stopping nationwide smallpox vaccination (between 24 to 65 years of age) were eligible as previously vaccinated subjects if they had received smallpox vaccination in childhood and had a typical vaccinia scar.

Exclusion criteria included pregnancy, severe eczema, human immunodeficiency virus (HIV) infection, a history of vaccination with any type of live attenuated virus within 60 days before the study, the receipt of blood products or immune globulin within 6 months before the study, and house-hold contact, sexual contact, or occupational exposure to pregnant women.

Vaccine

The smallpox vaccine with Lister strain (Lister vaccine; the Elstree strain of vaccinia virus) used in this study was produced in 1981 or earlier and was provided by the Centers for Disease Control in Taipei, Taiwan. The diluent to reconstitute or to dilute the lyophilized vaccine consisting of 0.01M citric acid, 0.01M sodium phosphate and 40% glycerol in sterile water. Vaccine was used immediately after reconstitution or dilution. An aliquot from each dilution of vaccine was collected at the time of preparation and frozen for subsequent

determination of the viral titer by plaque assay on the chorio-allantoic membrane of chick embryos [7].

Study design

The study was a randomized, single-blind trial conducted at the National Taiwan University Hospital. Eligible vaccinia-naïve subjects were randomly assigned to receive undiluted vaccine, a 1:5 dilution of vaccine, or a 1:10 dilution of vaccine. Eligible previously vaccinated subjects were randomly assigned to receive undiluted vaccine, a 1:10 dilution of vaccine, or a 1:30 dilution of vaccine. The subjects were inoculated with a bifurcated needle that held a drop of vaccine and that was pressed 15 times into the skin of the deltoid region of the upper arm. The vaccination site was covered with folded gauze and a semipermeable adhesive membrane. Dressings were changed and the vaccination sites were assessed and adverse events were evaluated every three to five days until 15 days after vaccination by evaluators who were blinded to the subjects assignation. The maximal local reaction and adverse events were asessed and recorded within 15 days of postvaccination. Subjects were followed for 12 weeks after vaccination. Vaccinia-specific antibody titers were determined before and 8 weeks after vaccination. Vaccinia-specific CD4+ and CD8+ T cell responses were determined before and 12 weeks after vaccination.

The primary measure was the clinical success rate of vaccination. Clinical success was defined by the presence of a vesicular or pustular lesion at the inoculum site following vaccination [3, 4]. The second measure was immunological responses (vaccinia-specific T cell and antibody responses) to vaccination defined as following.

Determination of vaccinia-specific T cell responses

The T cell responses were evaluated by determining the frequencies of vaccinia-specific CD69 expression on T-cell subsets as previously described [1]. Briefly, peripheral blood mononuclear cells (PBMC) (10^6 /mL) were suspended in complete RPMI (Gibco, Grand Island, NY) supplemented with penicillin / streptomycin and 10% fetal calf serum (Gibco) onto 24-well plates (1 mL /well). PBMC were incubated with 5 μL vaccinia virus (1.0 pfu/cell, prepared as 10^8 pfu in reconstituting fluid 500 μL) or 5 μL reconstituting fluid alone, in a final volume of 1 mL complete RPMI. After 24-hour incubation at 37℃, PBMC were stained with fluorescein-conjugated monoclonal antibodies to CD4 (CyChrome), CD8 (PE), and CD69 (FITC) (PharMingen, San Diego, CA, USA), and then were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). PBMC were gated into lymphocyte population (by forward and side scatter) and sequentially gated into CD4+ or CD8+ cells, and then were analyzed for the frequencies of CD69 expression. A total of 50000 events were analyzed for each sample. The frequencies of vaccinia-specific CD69 expression were defined as [the frequencies of CD69 expression when incubated with vaccinia virus] – [the frequencies of CD69 expression when incubated with reconstituting fluid alone]. We calculate the times of increase in vaccinia-specific responses of CD4+ and CD8+ T cell subsets after vaccination as the T cell responses to vaccination.

Determination of vaccinia-specific neutralizing antibody titers

The concentration of virus suspension in the neutralization test was

approximately 40 pfu per 0.1 mL in reconstituted fluid. Four serial twofold dilutions of serum were performed with reconstituted fluid from 1:20 to 1:160 in pre-vaccination serum samples and from 1:200 to 1:1600 in post-vaccination serum samples. Each serum dilution was incubated with an equal volume of virus suspension in a 16-h period at 37°C. After incubation, 0.1 ml of serum dilution was inoculated in membranes of chick embryos. Each dilution group inoculated in 5-6 chick embryos. The infected chick embryos were further incubated for 2 days at 37°C. The pocks formed on membranes of chick embryos were counted. In each assay, a known negative control serum was incubated. The neutralizing titer was defined as a reciprocal dilution of serum that caused a 60% reduction in pock count compared with a negative control. We arbitrarily defined the significant antibody response to vaccination as a positive seroconversion or a $\geqq$ 4-fold elevation of titer of vaccinia-specific antibody.

Statistical analysis

The 95% confidence interval was calculated for the vaccination success rates. Mann-Whitney U-test was used to assess the difference of times of increase in T cell responses and the difference of local reactions (the sizes of vesicle/pustule, erythema and induration) between each group, and one-sided Fisher exact test was used to assess the difference of the proportions of the subjects who had satellite lesions or adverse events between each group.

(3)結果

Subjects and vaccines

A total of 219 enrolled subjects were eligible: 97 were vaccinia-naïve subjects (age, 20 to 23 years; median, 22 years; sex: M/F = 51/46) and 122 were previously vaccinated subjects (age, 24 to 65 years; median, 39 years; sex: M/F = 67/54). They were randomized to receive undiluted or diluted vaccines (**Table 1**).

The mean titers of vaccinia virus were $10^{9.0}$ pfu/mL in the case of undiluted vaccine (range, $10^{8.9}$ to $10^{9.1}$), $10^{8.4}$ pfu/mL in the case of the 1:5 dilution (range, $10^{8.1}$ to $10^{8.5}$), $10^{8.0}$ pfu/mL in the case of the 1:10 dilution (range, $10^{7.8}$ to $10^{8.2}$), and $10^{7.5}$ pfu/mL in the case of the 1:30 dilution (range, $10^{7.4}$ to $10^{7.7}$) (**Table 1**).

Clinical and immunological success rate

Among the vaccinia-naïve subjects, the clinical success rate was 100% in those who received undiluted or diluted vaccines (1:5 or 1:10 dilution) (**Table 1**). Among the previously vaccinated subjects, except two subjects who received 1:30 diluted vaccine, the vaccination of all the other subjects was successful clinically (**Table 1**). No significant difference in response to vaccine in different age groups (P value not shown).

Before vaccination, all of the 97 vaccination-naïve subjects are seronegative for vaccinia-specific neutralizing antibody; of the 122 previously vaccinated subjects, 114 (93.4%) had pre-existing antibody. Eight weeks after

vaccination, all subjects, including the two subjects without vesicle/pustule following vaccination, had a significant antibody response (105 subjects had a positive seroconversion, and 114 subjects had a $\geqq$ 4-fold elevation of titer of vaccinia-specific antibody) (**Table 2**).

Twelve weeks after vaccination, all subjects had an increase in vaccinia-specific T cells responses when compared with those before vaccination (range of times of increase: CD4+ T cell response, 2.7 – 21.3 in vaccinia-naïve subjects and 1.6 – 12.8 in previously vaccinated subjects; CD8+ T cell response, 2.8 – 25.4 in vaccinia-naïve subjects and 1.7 – 11.9 in previously vaccinated subjects) (**Table 2**). The elicited vaccinia-specific CD4+ and CD8+ T cell responses in subjects who received diluted vaccines were similar to those in subjects who received undiluted vaccines (P value not shown, **Table 2**), both in vaccinia-naïve and previously vaccinated subjects. However, the times of increase in CD4+ and CD8+ T cell responses in the previously vaccinated subjects receiving undiluted vaccine were significantly smaller than those in the vaccinia-naïve subjects receiving undiluted vaccine (P = 0.01 and 0.02, respectively).

Local reactions

The local reaction of vaccination site in the subjects who received diluted and undiluted vaccines within 15 days after vaccination were not significant different both in vaccinia-naïve and previously vaccinated subjects (**Table 3**, each P value not shown). That is, the diluted vaccines were not associated with significantly smaller diameter of the maximal sizes of the vesicle/pustule,

erythema, and induration. The proportions of subjects with satellite lesions were not significantly different between groups with diluted and undiluted vaccines (among the vaccinia-naïve subjects, 2/20 (10%) in undiluted group, 6/37 (13.5%) in 1:5 dilution group, and 4/40 (10%) in 1:10 dilution group; among the previously vaccinated subjects, 3/35 (8.6%) in undiluted group, 4/35 (11.4%) in 1: 10 dilution group, and 3/52 (5.8%) in 1:30 dilution group). However, the mean diameter of the maximal sizes of the vesicle/pustule, erythema, and induration among the previously vaccinated subjects receiving undiluted vaccine were significantly smaller than those observed among the vaccinia-naïve subjects receiving undiluted vaccine (P = 0.04, 0.01 and 0.02, respectively).

Adverse events

Among the vaccinia-naïve and previously vaccinated subjects, the frequency and severity of adverse events were similar in those given diluted and in those given undiluted vaccines (**Table 4**, each P value not shown). In previously vaccinated subjects, the frequency and severity of adverse events were similar in those with age 24 ~ 40 years and in those with age 41~65 years. However, most adverse events, including fever, headache, muscle ache, fatigue and regional lymphadenopathy, were more frequently observed in vaccinia-naïve subjects receiving undiluted vaccine than observed in previously vaccinated subjects receiving undiluted vaccine (P = 0.053, 0.014, 0.02, 0.043 and 0.001, respectively). No serious or life-threatening events were observed in all subjects within 3 months of follow-up.

(4)討論

In this study, we evaluated the safety and efficacy of diluted and undiluted smallpox vaccines with Lister strain. Our results showed the vaccination can achieve a high clinical and immunological success rate when the smallpox vaccine was used as 1:10 dilution in vaccinia-naïve subjects and 1:30 dilution in previously vaccinated subjects if viral titers $\geqq 10^8$ and $10^{7.5}$ pfu/mL after dilution, respectively. The results suggest that it is possible to provide enough supply of smallpox vaccines in Taiwan and much more supply in other countries if an outbreak occurs or if mass vaccination is needed.

However, a previous study showed the smallpox vaccine with 1:32 dilution had an unreliable efficacy, with a success rate of only 52.6% (95% confidence interval 29-76%) in previously vaccinated adults [5]. Because no significant difference in biological activity was indentified between Dryvax (New York City Board of Health strain) and Liter strain [6], the difference in the success of highly diluted vaccine between the two studies may be explained by the different viral titers in vaccinia virus stocks between the U.S. and Taiwan. In the study conducted in U.S. by Frey et al. [5], the viral titer of undiluted stock was $10^{7.5}$ pfu/mL thus the 1:32 diluted vaccine had a low viral titer of $10^{6.3}$ pfu/mL. However, our mean viral titer of undiluted stock was $10^{9.0}$ pfu/mL thus our 1:30 diluted vaccine had a mean viral titer of $10^{7.5}$ pfu/mL. Therefore, our 1:30 diluted vaccine remained highly effective. Because the titers of vaccinia virus in the stocks of smallpox vaccine may be variable in different countries, the critical point to determine the vaccination success rate of diluted vaccines may be the

viral titer after dilution, rather than the dilution ratio. That is, the higher viral titers in the vaccinia virus stocks in a certain country, the higher dilution ratio is possible to maintein the efficacy of smallpox vaccine.

In contrast to the studies by Frey et al. [4, 5], in which the use of diluted vaccines was associated with less marked local reactions and lower incidences of some adverse events, our data showed that the diluted vaccines did not decrease local reactions and the incidences of adverse events when compared with undiluted vaccines. The difference between these studies may also be explained by higher viral titers in our stocks and diluted vaccines. We propose that when the viral titers in the diluted and undiluted vaccines are higher than a certain threshold, the reactogenicity of vaccinia virus may not be a dose-related response. The clinical observations are consistent with our results of T cell responses to vaccination: the responses of T cell subsets in subjects receiving undiluted vaccine were not more vigorous than those in subjects receiving diluted vaccines. Consistent with previous studies [4, 5], our data also showed that more marked local reactions and higher incidences of most adverse events in vaccinia-naïve subjects than observed in previously vaccinated subjects. No serious or life-threatening events were observed in this study.

There has been a debate about the durability of protective immunity against smallpox from vaccination. The immunity acquired from smallpox vaccination is assumed to decline rapidly by some experts but some studies suggest that protection may persist for many decades [8-12]. In recent three published papers [1-3], which addressed the issue about the decay kinetics of immunity against vaccinia virus after vaccination, showed consistently that the immunological memory to smallpox vaccines may decline with age though this immunity is

detectable even decades post-vaccination. However, we do not know how much immunological memory to vaccinia virus is enough to protect against smallpox or to reduce the disease severity. Thus, in our opinion, we should assume that most people in the world may be more susceptible to smallpox than previously expected [8, 10, 12] in lack of herd immunity because most countries had stopped smallpox vaccination over 20 years ago. The detectable but declined immunity should alert us to prepare enough amount of smallpox vaccine. The viral titer of vaccinia virus stocks in any countries under bioterrorism threats should be determined as soon as possible to estimate the national maximal supply of smallpox vaccine through the dilution policy.

(5)結論與建議

In conclusion, with Lister strain of vaccinia virus, the smallpox vaccine can be used as 1:10 dilution in vaccinia-naïve subjects and 1:30 dilution in previously vaccinated subjects if viral titer $\geqq$ 10^8 and $10^{7.5}$ pfu/mL after dilution, respectively. The data may help to counter the threat of intentional smallpox outbreaks by increasing the ability to provide many more smallpox vaccines, especially in countries which can not produce or purchase new tissue-culture vaccine.

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(7)表格

Table 1. Rates of clinical success of vaccination with diluted or undiluted smallpox vaccines in vaccinia-naïve and non-naïve subjects

Subjects	Vaccine (mean viral titer)	Number of eligible subjects	Clinical success rate
Vaccinia-naïve	Undiluted ($10^{9.0}$ pfu/mL)	20	100% (20/20)
	1:5 diluted ($10^{8.4}$ pfu/mL)	37	100% (37/37)
	1:10 diluted ($10^{8.0}$ pfu/mL)	40	100% (40/40)
Previously vaccinated	Undiluted ($10^{9.0}$ pfu/mL)	35	100% (35/35)
	1:10 diluted ($10^{8.0}$ pfu/mL)	35	100% (35/35)
	1:30 diluted ($10^{7.5}$ pfu/mL)	52	96.2% (50/52)*

*95% confidence interval: 91.0% – 100.0%

Table 2. Vaccinia-specific immune responses after vaccination with diluted or undiluted smallpox vaccines in vaccinia-naïve and non-naïve subjects

Subjects	Vaccine	Times of increase in T cell responses: median (range)		Rate of significant antibody responses*
		CD4+	CD8+	
Vaccinia-naïve	Undiluted	5.2	4.8	100%
	(n = 20)	(3.1 – 17.5)	(3.0 – 16.8)	
	1:5 diluted	4.7	5.1	100%
	(n = 37)	(2.7 – 21.3)	(3.2 – 15.3)	
	1:10 diluted	5.0	4.7	100%
	(n = 40)	(2.8 – 18.4)	(2.8 – 25.4)	
Previously vaccinated	Undiluted	2.8	3.1	100%
	(n = 35)	(1.8 – 10.3)	(2.1 – 10.5)	
	1:10 diluted	3.0	2.9	100%
	(n = 35)	(2.0 – 11.5)	(1.7 – 10.6)	
	1:30 diluted	2.7	3.4	100%
	(n = 52)	(1.6 – 12.8)	(1.8 – 11.9)	

*We defined the significant antibody response to vaccination as a positive seroconversion or a $\geqq$ 4-fold elevation of titer of vaccinia-specific antibody.

Table 3. Local reactions within 15 days after vaccination with diluted or undiluted smallpox vaccines in vaccinia-naïve and non-naïve subjects

Subjects	Vaccine	Maximal diameter within 15 days: Mean (range), mm		
		Vesicle/ Pustule	Erythema	Induration
Vaccinia-naïve (n = 97)	Undiluted (n = 20)	9.8 (5–15)	34 (12–65)	31 (14–62)
	1:5 diluted (n = 37)	9.1 (4–20)	36 (12–70)	32 (12–54)
	1:10 diluted (n = 40)	9.8 (3–20)	32 (10–60)	29 (11–65)
Previously vaccinated (n = 122)	Undiluted (n = 35)	7.3 (2–11)	19 (0–35)	16 (0–40)
	1:10 diluted (n = 35)	7.2 (2–10)	17 (0–30)	18 (0–45)
	1:30 diluted (n = 52)	7.4 (0 – 13)	20 (0–42)	17 (0–38)

Table 4. Adverse events within 15 days after vaccination with diluted or undiluted smallpox vaccines in vaccinia-naïve and non-naïve subjects

Subjects	Vaccine	Age (years)	Fever	Headache	Muscle aches	Fatigue	LAP*	Rash†
Vaccinia-naïve (n = 97)	Undiluted (n = 20)		4 (20%)	10 (50%)	8 (40%)	11 (55%)	13 (65%)	0
	1:5 diluted (n = 37)		4 (11%)	10 (27%)	10 (27%)	18 (49%)	25 (68%)	0
	1:10 diluted (n = 40)		8 (20%)	12 (30%)	17 (43%)	15 (38%)	21 (53%)	1 (3%)
Previously vaccinated (n = 122)	Undiluted (n = 35)	24 – 40 (n = 20)	1 (5%)	4 (20%)	3 (15%)	5 (25%)	4 (20%)	0
		41 – 65 (n = 15)	0	2 (13%)	1 (7%)	4 (27%)	2 (13%)	0
	1:10 diluted (n = 35)	24 – 40 (n = 19)	1 (5%)	3 (16%)	5 (26%)	7 (37%)	3 (16%)	0
		41 – 65 (n = 16)	1 (6%)	1 (6%)	1 (6%)	3 (19%)	4 (25%)	0
	1:30 diluted (n = 52)	24 – 40 (n = 30)	2 (7%)	7 (23%)	12 (40%)	15 (50%)	10 (33%)	1 (3%)
		41 – 65 (n = 22)	1 (5%)	1 (5%)	4 (18%)	4 (18%)	5 (23%)	2 (9%)

*Regional lymphadenopathy; †Rash at sites other than vaccination sites