

Programmed vaccination may increase the prevalence of asthma and allergic diseases

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ABSTRACT

Background: The prevalence of asthma and allergic diseases has risen in recent decades. The etiology of asthma and allergic diseases has not been entirely elucidated.

Objective: In this study, we investigated the possibility that programmed vaccination in China may have a potential role in asthma and allergic diseases.

Methods: In this animal model, newborn BALB/c mice were randomly divided into three groups: vaccine plus ovalbumin (OVA), OVA, and control. The mice of vaccine plus OVA only group were inoculated with vaccines by following the National Vaccines Inoculation Program in China. Mice of vaccine plus OVA and OVA only groups were sensitized and challenged with OVA. Airway hyperresponsiveness was assessed by lung function and serum interleukin (IL) 4 and interferon (IFN) γ were measured.

Results: The results of lung function showed that mice of the vaccine plus OVA group exhibited an increase in enhanced pause (Penh) compared with that in the OVA group at methacholine concentrations of 6.25 and 12.5 mg/mL ($p < 0.05$). Serum IL-4 in the vaccine plus OVA group was higher than that in the OVA group ($p < 0.01$). The serum IFN- γ level in the OVA group was lower than that in the control group ($p < 0.01$), and also lower than that in the vaccine plus OVA group ($p < 0.05$). The ratio of IFN- γ to IL-4 both in the OVA and vaccine plus OVA group was lower than that in the control group ($p < 0.01$).

Conclusions: Results of our study indicated that programmed vaccination in China may have a potential role in the prevalence of asthma and allergic diseases by inducing T-helper 2 cytokine expression and may be responsible for the increasing prevalence of asthma and allergic diseases in China.

(Am J Rhinol Allergy 30, e113–e117, 2016; doi: 10.2500/ajra.2016.30.4335)

The prevalence of asthma and allergic diseases is increasing worldwide after the improvements in living conditions and health care over the past several decades.¹ Analysis of epidemiologic data shows that the incidence of asthma and allergic diseases in developed countries is higher than that in developing countries. In addition, the incidence of asthma and allergic diseases in urban areas is higher than that in rural areas.² Results of two studies have indicated that these variations may be explained by a set of complex causal factors related to changes in the environment and urbanization.^{3,4} We suggested that the T-help (Th) 1 to Th2 imbalance induced by vaccination is important in the pathogenesis of asthma and allergic diseases. Vaccination emerged and has spread in developed countries since the 1950s.^{5,6}

The prevalence of asthma and allergic diseases is increasing yearly. Although results of some studies have shown that the prevalence of asthma may have reached a plateau in some developed countries,^{7–9} the same process reappeared in developing countries. The results regarding the effects of vaccination on asthma and allergic diseases are contradictory.^{10–12} Results of two studies showed that bacillus Calmette-Guérin vaccination may prevent asthma and allergic disease by induction of the Th1 response.^{13,14} However, we were unable to find reports on the effects of programmed vaccination on asthma and allergic diseases. As a developing country, China has experienced accelerated urbanization over the past 30 years. The popularity of vaccination has increased rapidly, and the prevalence of asthma and allergic diseases has also increased significantly. Therefore, we proposed that programmed vaccination in China has a potential role in the prevalence of asthma and allergic diseases. In this study, we

investigated whether programmed vaccination is involved in the increased prevalence of asthma and allergic diseases in China.

METHODS

Animals

Newborn BALB/c mice obtained from the Department of Animal Medical Experiment, General Hospital of Shenyang Military Command, were used in this study. The mice were randomly divided into the following three groups, with eight animals in each group: vaccine plus ovalbumin (OVA) group, OVA only group, and the control group. All the animals were cared for and treated in the same animal center in our hospital, and, when not being treated, the animals had free access to food and water. All animal procedures were approved by the Animal Investigational Committee of the General Hospital of Shenyang Military Command and were performed in accordance with The Guide for Care and Use of Laboratory Animals issued by the Ministry of Health, People's Republic of China.

Inoculation of the Vaccines

In accordance with the National Vaccines Inoculation Program in China, 11 types of vaccines were included in this protocol: bacillus Calmette-Guérin vaccine; hepatitis B vaccine; poliomyelitis vaccine in dragee candy (human diploid cell) live; diphtheria, tetanus, and acellular pertussis combined vaccine; diphtheria and tetanus combined vaccine; measles and rubella combined vaccine live; measles, mumps, and rubella combined vaccine live; Japanese encephalitis vaccine live; group A meningococcal polysaccharide vaccine; group A and C meningococcal polysaccharide vaccine; and hepatitis A (live) vaccine. The vaccines inoculation program (Table 1) was carried out to imitate the human vaccines inoculation program. The doses of the vaccines for mice immunization were converted from those for humans according to the following formula: the mice dose = (human dose $\div$ human baby body weight (kg) $\div$ 1000) $\times$ mice body weight (g) $\times$ 37.5. The date of vaccine inoculation in mice corresponded to that in humans. Mice in the vaccine plus OVA group were inoculated with different vaccines by following the vaccine instructions on the date shown in Table 1. Animals in the control and OVA-only

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No external funding sources reported

The authors have no conflicts of interest to declare pertaining to this article

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Table 1 The experiment schedule of programmed vaccination and OVA sensitization and challenges

Human Month	Mice Day	Control Group	OVA-Only Group	Vaccine + OVA Group
0	0	0.9% NaCl	0.9% NaCl	BCG and hepatitis B
1	1	0.9% NaCl	0.9% NaCl	Hepatitis B
2	2	0.9% NaCl	0.9% NaCl	Poliomyelitis
3	3	0.9% NaCl	0.9% NaCl	Poliomyelitis and combined diphtheria, tetanus, and acellular pertussis
4	4	0.9% NaCl	0.9% NaCl	Poliomyelitis and combined diphtheria, tetanus, and acellular pertussis
5	5	0.9% NaCl	0.9% NaCl	Combined diphtheria, tetanus, and acellular pertussis
6	6	0.9% NaCl	0.9% NaCl	Hepatitis B
8	7	0.9% NaCl	0.9% NaCl	Combined measles and rubella, and Japanese encephalitis
6–18	8	0.9% NaCl	0.9% NaCl	Group A meningococcal polysaccharide
	14	0.9% NaCl	0.9% NaCl	Group A meningococcal polysaccharide
18	16	0.9% NaCl	0.9% NaCl	Hepatitis A
18–24	17	0.9% NaCl	0.9% NaCl	Combined diphtheria, tetanus, and acellular pertussis
	19	0.9% NaCl	0.9% NaCl	Combined measles, mumps, and rubella
24	21	0.9% NaCl	0.9% NaCl	Japanese encephalitis
36	30	0.9% NaCl	0.9% NaCl	Group A and C meningococcal polysaccharide
48	41	0.9% NaCl	0.9% NaCl	Poliomyelitis
72	61	0.9% NaCl	0.9% NaCl	Combined diphtheria and tetanus, and group A and C meningococcal polysaccharide
	68	0.9% NaCl/Imject Alum	OVA/Imject Alum	OVA/Imject Alum
	75	0.9% NaCl/Imject Alum	OVA/Imject Alum	OVA/Imject Alum
	82	0.9% NaCl/Imject Alum	OVA/Imject Alum	OVA/Imject Alum
	89	0.9% NaCl	1% OVA	1% OVA
	90	0.9% NaCl	1% OVA	1% OVA
	91	0.9% NaCl	1% OVA	1% OVA

OVA = ovalbumin; BCG = bacillus Calmette-Guérin.

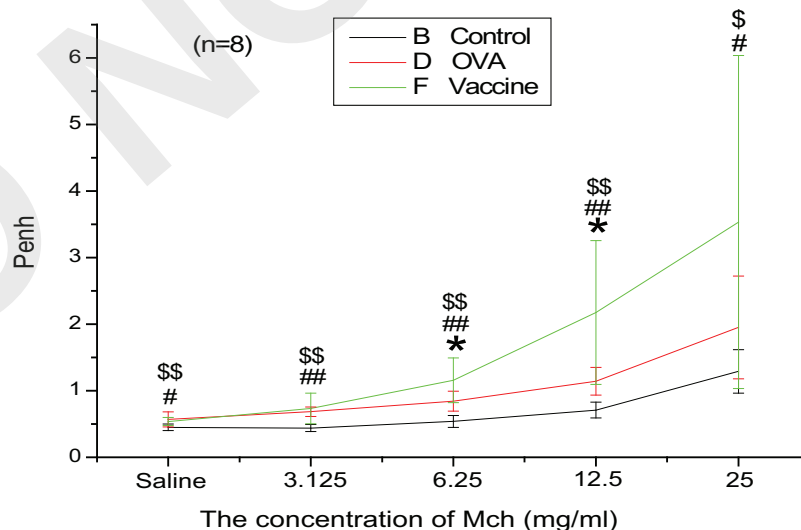


Figure 1. Airway hyperresponsiveness to methacholine (Mch) challenge is promoted by vaccination. Mice were either inoculated with vaccine and then sensitized and challenged by ovalbumin (OVA) or were treated with OVA only, or were untreated (control). Airway responsiveness in the animals was evaluated by the enhanced pause (Penh) in the pressure curves via whole-body plethysmography after Mch challenge. The results are shown as the mean (triangle for vaccine plus OVA, circle for OVA only, and square for control). The mean $\pm$ standard deviation (SD) (the error bars) of Penh ($n = 8$ for each group; Saline indicates 0.9% NaCl). * $p < 0.05$ for vaccine plus OVA versus OVA only; # $p < 0.05$ for OVA only versus control; \$\$\$ $p < 0.01$ for OVA only versus control; \$\$\$\$ $p < 0.0001$ for vaccine plus OVA versus control.

groups were treated with the same volume of 0.9% NaCl instead of the vaccines.

Figure 2. Vaccine inoculation increased serum interleukin (IL) 4 level induced by ovalbumin (OVA). Mice were either inoculated with vaccine and then sensitized and challenged by OVA, or were treated with OVA only or untreated (control). Serum IL-4 levels were measured by enzyme-linked immunosorbent assay. The serum levels of IL-4 in the control group, OVA-only group, and the vaccine plus OVA group were mean $\pm$ standard deviation (SD) 4.322 ± 3.05 , 13.048 ± 3.185 , and 31.494 ± 7.856 pg/mL, respectively. ** $p < 0.01$ for vaccine plus OVA versus OVA only; ### $p < 0.01$ for OVA only versus control.

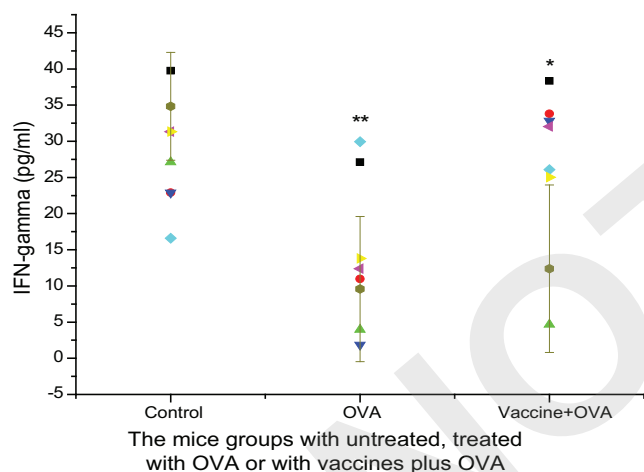
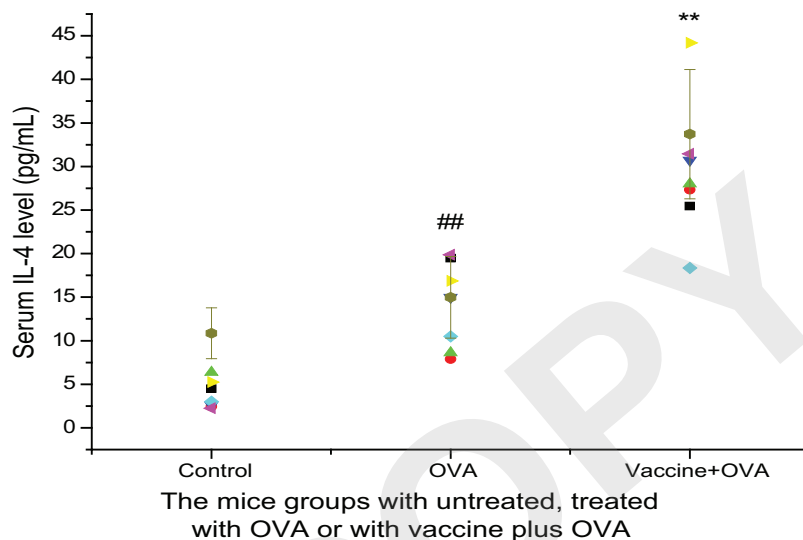


Figure 3. Serum interferon (IFN) γ level was decreased in mice treated with ovalbumin (OVA) but not in mice treated with vaccine plus OVA. Mice were either inoculated with vaccine and then sensitized and challenged by OVA or treated with OVA only or untreated (control). Serum IFN- γ levels were measured by enzyme-linked immunosorbent assay. Mean $\pm$ standard deviation (SD) serum IFN- γ levels in the control group, OVA-only group, and the vaccine plus OVA group were 28.347 ± 7.453 , 13.699 ± 10.033 , and 25.642 ± 11.568 pg/mL, respectively. * $p < 0.05$ for vaccine plus OVA versus OVA only; ** $p < 0.01$ for OVA only versus control.

Allergen Sensitization and Challenge in Mice

Mice in the vaccine plus OVA and OVA-only groups were sensitized with 50 μ g of OVA (Sigma-Aldrich, Co., St. Louis, MO) in 100 μ L of phosphate buffer saline (PBS) mixed with 100 μ L of Imject Alum (Thermo Scientific, Rockford, IL) intraperitoneally three times on 0, 7, and 14 days one week after immunization. Mice in the control group were injected with the same volume of 0.9% NaCl-Imject Alum. On 21, 22, and 23 days after sensitization with OVA, mice in the vaccine plus OVA group and OVA-only groups were challenged with an aerosol of 1% OVA in PBS in a chamber for 30 minutes per day, and the animals in the control group were treated with an aerosol of 0.9% NaCl (Table 1).

Measurement of Airway Hyperresponsiveness

Lung function in mice was tested at 18–24 hours after the last OVA challenge, during which the respiratory pressure curves were mea-

sured and recorded by using whole-body plethysmography (EMKA Technology, Paris, France), whereas methacholine (Mch) (Sigma-Aldrich) was administered by ultrasonic atomizing inhalation for 3 minutes in stepwise increasing concentrations from 0 to 25 mg/mL. Airway responsiveness was expressed as enhanced pause (Penh), a calculated unit-less value that correlates the airway resistance, impedance, and intrapleural pressure within the same animal. A total of eight mice were assessed in each group.

Serum Collection

After lung function was tested, the mice were killed, and serum samples were collected. The collected serum samples were stored at -20°C until analyzed.

Measurement of Cytokines

Serum samples were analyzed for cytokine interleukin (IL) 4 and interferon (IFN) γ levels by enzyme-linked immunosorbent assay (Quantikine ELISA, R&D Systems; Minneapolis, MN) by following the manufacturer's instructions. The minimum detectable dose of both mice IL-4 and IFN- γ was <2 pg/mL.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Significance testing was performed by using the unpaired Student's t -test. A p value of <0.05 was considered statistically significant.

RESULTS

Vaccination Is Associated with Airway Hyperresponsiveness

Lung function in the vaccine plus OVA, OVA-only, and control groups was measured to assess whether vaccine inoculation contributes to airway hyperresponsiveness. The results showed that the Mch challenge caused a dose-dependent increase in Penh, and the extent of the Penh increase in response to Mch stimulation at the same dose was different in the different groups. Mice in the OVA-only group showed a greater increase in Penh compared with mice in the control group ($p < 0.05$ for 0.95 NaCl solution [solution indicates 0.9% NaCl], 25 mg/mL of Mch, $p < 0.01$ for 3.125, 6.25, and 12.5 mg/mL of Mch). Mice in the vaccine plus OVA group also showed a greater increase in Penh compared with mice in the control group ($p < 0.05$ for 25 mg/mL of Mch, $p < 0.01$ for Saline, 3.125, 6.25, and 12.5 mg/mL of

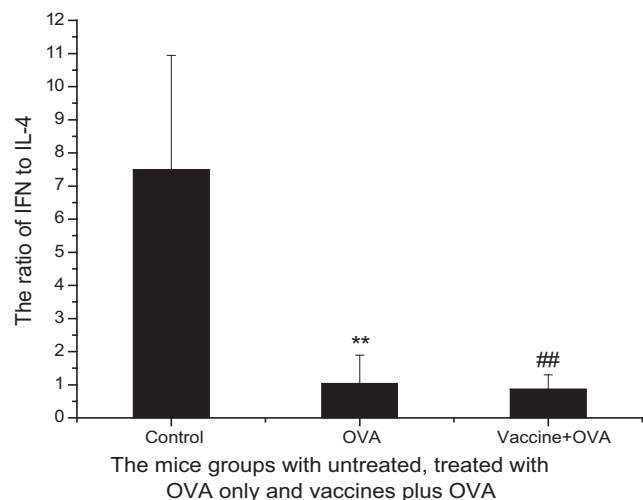


Figure 4. The ratio of interferon (IFN) γ to interleukin (IL) 4 was decreased both in the ovalbumin (OVA) only group and in the vaccine plus OVA group. The mean $\pm$ standard deviation (SD) ratios of IFN- γ to IL-4 in the control, OVA-only, and vaccine plus OVA groups were 7.493 ± 3.451 , 1.038 ± 0.853 , and 0.861 ± 0.442 , respectively. The ratios of IFN- γ to IL-4 both in the OVA-only group and in the vaccine plus OVA group were significantly lower than that in the control group. ** $p < 0.01$ for control versus OVA only; ### $p < 0.01$ for control versus vaccine plus OVA.

Mch). Mice in the vaccine plus OVA group exhibited a greater increase in Penh compared with the mice in the OVA-only group at the Mch concentrations of 6.25 and 12.5 mg/mL ($p < 0.05$) (Fig. 1).

Effects of Vaccine Inoculation on the Production of IL-4 and IFN- γ in Response to OVA

To clarify whether immunization affected the balance of Th1:Th2 and production of cytokines, the levels of IL-4 and IFN- γ in serum were measured by enzyme-linked immunosorbent assay in the three mice groups. Analysis of the results showed that the serum levels of IL-4 in the control, OVA-only, and vaccine plus OVA groups were mean $\pm$ SD 4.322 ± 3.05 , 13.048 ± 3.185 , and 31.494 ± 7.856 pg/mL, respectively. The serum level of IL-4 in the vaccine plus OVA group was significantly higher than that in the OVA-only group ($p < 0.01$), and the serum level of IL-4 in the OVA-only group was also significantly higher than that in the control group ($p < 0.01$) (Fig. 2). The results of serum IFN- γ analysis when using enzyme-linked immunosorbent assay showed that serum levels of IFN- γ in the control, OVA-only, and vaccine plus OVA groups were mean $\pm$ SD 28.347 ± 7.453 , 13.699 ± 10.033 , and 25.642 ± 11.568 pg/mL, respectively. The serum level of IFN- γ in the OVA-only group was significantly lower than that in the control group ($p < 0.01$). The serum level of IFN- γ in the vaccine plus OVA group was significantly higher than that in the OVA-only group ($p < 0.05$), and there was no statistical difference in serum IFN- γ levels between the vaccine plus OVA group and control group ($p > 0.05$) (Fig. 3).

Effects of Vaccination on the Balance between Th1 and Th2 Cytokines

To determine whether vaccination affected the balance of Th1 and Th2 cytokines, we compared the ratios of IFN- γ to IL-4 in the three mice groups. Analysis of the results showed that the ratios of IFN- γ to IL-4 in the control, OVA-only, and vaccine plus OVA groups were mean $\pm$ SD 7.493 ± 3.451 , 1.038 ± 0.853 , and 0.861 ± 0.442 , respectively. The ratios of IFN- γ to IL-4 both in the OVA-only group and in

the vaccine plus OVA group were significantly lower than that in the control group ($p < 0.01$) (Fig. 4).

DISCUSSION

The pathogenesis of asthma is unclear. One of the accepted hypotheses regarding the pathogenesis of asthma is the environment hypothesis because some studies found that the prevalence of asthma and allergic diseases in children from rural environments was lower. The epidemiology of asthma in school children was investigated in two epidemiologic studies, Parsfal study and Gabriela study.² The results of the two studies showed that children who resided in rural environments showed an inverse correlation with the prevalence of asthma and allergic diseases. This phenomenon was explained by the fact that the children who lived in rural environments were in contact with more bacteria, which led to increased expression of Th1 and benefit due to the Th1:Th2 balance.³ However, children who lived in urban environments expressed Th2, which led to an imbalance in Th1:Th2. The reason for the imbalance in Th1:Th2 may be caused not only by environmental factors but also the higher popularity of vaccination.

Infection is a serious disease that has wreaked havoc worldwide and has claimed countless lives. Analysis of data indicates that half of children < 5 years old die due to infection, even though the use of antibiotics is widespread.¹⁵ Vaccination is an important method of preventing diseases. However, many vaccines are attenuated live vaccines. The purpose of inoculation is to stimulate immune responses in humans. These immune responses may induce increased expression of Th2 lymphocytes and sensitize the organism, and then increase the prevalence of asthma and allergic diseases.¹⁶ An epidemiologic survey by Kemp *et al.*¹⁷ included 1184 subjects inoculated with vaccines and 23 subjects not inoculated with vaccines. Analysis of the results showed that the risk of asthma in subjects inoculated with vaccines was higher than that in subjects not inoculated with vaccines.

Analysis of our results showed that the Penh value in mice that were administered programmed vaccination was higher than that in the mice not given programmed vaccination. This confirmed that programmed vaccination can increase airway responsiveness. We investigated the mechanism further and found that the serum level of IL-4 in mice that were given programmed vaccination was higher than that in mice not given programmed vaccination. The ratio of IFN- γ to IL-4 in mice given programmed vaccination was less than that in the control group, although the serum level of IFN- γ in mice given programmed vaccination was not different from that in the control mice. Different to allergic airway responsiveness, the serum level of IFN- γ in the mice induced by programmed vaccination was not decreased. This may be caused by the five attenuated virus live vaccines used in the programmed vaccination, which may increase the serum level of IFN- γ in the mice induced by programmed vaccination. In this study, the mice given programmed vaccination received more alum than that of the OVA group because some vaccines contain alum.

The mechanism of action for alum in immune response is complex and is still not completely understood. The study by Mannhalter *et al.*¹⁸ showed that alum alone had no effect on T-cell activation and immune response. Recently, Rose *et al.*¹⁹ demonstrated that intraperitoneal injection of alum resulted in cell necrosis and IL-33 release. Both alum and IL-33 induced significant dose-dependent increases in IL-5, IL-6, IL-13, granulocyte-colony stimulating, keratinocyte-derived cytokine, monocyte chemotactic protein-1, monocyte inflammatory protein-1 α , and monocyte inflammatory protein-1 β . However, no significant induction of many cytokines, including IFN- γ and IL-4, was observed after injection of alum or IL-33 compared with PBS-treated mice.¹⁹ More experiments are necessary for this study to show the effect of alum on the results. The time interval between the last vaccination and the first OVA sensitization is another factor that

influences the experimental results. In the present study, we chose only 1 week after vaccination to sensitize the mice with OVA. Ideally, the time intervals of 1 week to 4 weeks should be included in this study. We will conduct this experiment in a subsequent study. In brief, the serum level of IL-4 in mice given programmed vaccination was higher than that in both the OVA group and the control group. The high Th2 response may contribute to high airway responsiveness and induce a high prevalence of asthma and allergic diseases.

CONCLUSION

Programmed vaccination in China may increase airway responsiveness in children given programmed vaccination by stimulating high Th2 response and may be responsible for the increasing prevalence of asthma and allergic diseases in China.

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