Responses of cytokines to influenza vaccination in elite boy gymnasts

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Original Article

Abstract

Introduction: Many of effects of exercise are mediated through cytokines. Inflammatory cytokines are increased by exercise and inhibited by anti-inflammatory cytokines. Meanwhile, vaccination leads to an increase in the inflammatory cytokines. To test the hypothesis that exercise training might alter the cytokine response to the influenza vaccine, we measured selected cytokines in elite boy gymnasts and non-exercising subjects who were administered with the influenza vaccine and compared these against non-vaccinated gymnasts.

Methods: Forty five healthy 9- to 12- years old children (30 gymnasts and 15 untrained) were assigned to training group (TG), training-vaccine group (TVG) and vaccine group (VG). The exercise protocol consisted of a 3 hours gymnastic practice, 3 days per week, for 8 weeks. TVG and VG group were immunized with trivalent influenza vaccine in the 0(Pre), 4 and 8th weeks. Pre-vaccination, 4 and 8 week blood samples were obtained in all subjects. Data were analyzed by one-way analysis of variance (ANOVA) and repeated measures tests. Significance level was accepted at P<0.05.

Results: After the intervention, significant decreases in IL-6 and IFN-γ were observed in all the groups (P<0.05). A significant difference, in IL-6 level between TVG and TG groups at week 8, and a significant differences in INF-γ level between TVG and TG groups at week 4 were also observed (P<0.05). There was not a significant increase in the IL-2 in TVG and TG groups, but a significant increase was observed in VG (P<0.05). A significant difference in IL-2 level between TVG and VG groups, between TG and VG at 4th week and between TVG and VG at 8th week was observed.

Conclusion: The results demonstrate that gymnastic training and vaccine in healthy children lead to reduction in inflammatory cytokines. This might explain the immature immune system response in children to both vaccination and exercise training.

Key words: Influenza, Vaccination, Athletes, Cytokine, Exercise


Introduction:

Homeostasis of the immune system is, in part, regulated through production of pre-inflammatory cytokines by the T helper lymphocyte sub-class Th1 and the anti-inflammatory sub-class Th2 (1). There is a well-known effect of physiological stress,
including exercise on Th1 and Th2, changing their distribution and function (2). Concentration of cytokines like interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and tumor necrosis factor-β (TNF-β) produced by Th1 reduces severe exercise training (3). In contrast, concentrations of cytokines like IL-14, IL-5, IL-6, IL-10, and IL-13 produced by Th2 do not change or increase following long-term exercise training (3-5).

Studies have shown that intense exercise leads to inflammation and production of pre-inflammatory cytokines like IL-1 and TNF-α (1,6,7). Therefore, intense exercises can have significant effects on the regulation of Th1 and Th2 cells and may change the distribution of Th1 and Th2 cells by temporarily inhibiting Th1 and weakening or suppressing the immune system (8). Cytokines are circulating mediators of the immune system and are very significant at the onset of exercise and regulate immune responses to that exercise bout. In addition, they modulate the function of other cells and have a role on the inflammatory process (7). IL-2 and IL-6 are produced by the inflammatory CD8 cells and T helper Th respectively. Also, they stimulate T and B cells, their multiplication and differentiation to inflammatory cells, antibody production, release of INF-γ, and stimulate the killing activity of NK cells. IFN-γ is produced by inflammatory CD4, T killer and NK cells which inhibits virus replication and enhancement of CD4 gene identification by T killer and NK cells and also stimulate cell killing function (9,10).

Increases in IL-6 and IFN-γ secretions occur following exercise in response to increasing levels of of stress hormones such as epinephrine and cortisol. It is believed that there is a bilateral relationship between the immune system and these humoral factors in such a way that an increase of glucocorticosteroids leads to temporary reduction of immune system function (9).

A common issue for athletes in heavy training or immediately following competition is an increased risk of acquiring infections particularly those of the upper respiratory tract (11). These upper respiratory tract infections (URTI) include simple sore throats to influenza. Indeed, the more intense and strenuous the exercise, the greater the risk of URTI (12).

Hence, it is sensible that one way of reducing acquiring infection is to limit the exercise intensity; though this is not always feasible. Farzanegi et al. (2013) demonstrate that influenza vaccination has a significant effect on immune system and inflammatory state in prepubertal boy gymnasts (13). But Eliakim et al. (1997) shown that the exercise-induced changes in cellular and humoral immune functions among the female gymnasts were generally similar to those described in adults (14). Clearly, however, there are interactions between acute exercise and the activity of the immune system which to date are not fully understood, but are likely to involve the cytokine responses.

Based on recent studies, it is hypothesized that Th1 cell function following vaccination is higher (10,15). Also, the immediate cell responses after vaccination cause multifold increases of IL-2 and IFN-γ producing cells; that is CD8 and CD4 cells. Edwards et al. (2007) report that eccentric exercise with deltoid and arm biceps muscles causes an increase in antibody titer in women and IFN-γ in men participating in the study (16). Bruunsgaard et al. (1997) did not find any difference in antibody response to tetanus, diphtheria and pneumococcal toxoids in thri-tolon when administered following prolonged exercise (17). Different factors like intensity, duration of exercise training, sex, age, level of physical fitness, vaccine composition and measuring methods have an effect on antibody response at the time of vaccination and sport participation (18).

In the present study we have tried to investigate changes in IL-2, IL-6 and IFN-γ as pre-inflammatory cytokines in the body of gymnasts during 8 weeks exercise and being vaccinated to influenza. Gymnasts are generally younger and have a greater chance of acquiring respiratory tract infection than adults. We hypothesized that there is a type of equilibrium between pre-inflammatory and anti-inflammatory cytokines in response to exercise and vaccination for enhancement of the immune system.

**Methods:**

This study was a quasi-experimental with a control group and pre and post test design, that was conducted at the IAUSEPI (Islamic Azad
University of Sari, Exercise Physiology Institute) following IAU IRB (Institutional Review Board) approval. Thirty active pre-pubertal elite male gymnasts were recruited from competitive gymnastics clubs in Ghaemshahr. To qualify for the experimental group, gymnasts had to be competing at a minimum of a provincial level and training at least 8 h/wk. Fifteen age-matched boys were recruited from Elementary Boy School to participate as the control group. All gymnasts and controls were Tanner stage 1-2 and had serum testosterone levels at or below the detection limit of 1 pmol/l at baseline and at 12 months follow-up (19). Gymnasts and controls were excluded if they had allergy to eggs or a history of major medical illnesses before enrollment, as documented by a clinical examination at their first visit. Subjects were interviewed regarding any injuries. Exercise (swimming and cycling) was only used if a gymnast was injured.

Training involved practicing leaps, pivots, dance, strength and stretching exercises. The routine consisted of a warm up, routine training, and cool down/bearing exercise. All subjects and their parents were informed of any risks that might result from participation, and informed consent was obtained before the study started. No subjects were on chronic medication or had taken anti-inflammatory agents during the course of study. All subjects agreed to provide blood samples before and after 4 and 8 weeks protocol. Participants were divided in three groups: VG, n=15 (8.1±0.1 years), TG, n=15 (7.8±0.95 years), and TVG, n=15 (7.9±0.8 years) (Table 1).

The active pre-pubertal gymnasts were training in an elite squad in Ghaemshahr at a sub-Olympic standard [Federation International Gymnastic (FIG)]. They trained under supervision for 8–15 h/week. Most training sessions were ~3 h and consisted of a warm up, routine training, and strength and stretching exercises. The routine training involved practicing leaps, pivots, dance, acrobatic, and aerial elements singularly and in combination on each apparatus (19). Both the arms and legs were loaded. Body weight was used as the resistance in the strength component. Cross training (swimming and cycling) was only used if a gymnast was injured.

The subjects were free of major medical illnesses before enrollment, as documented by a review of their medical histories and a clinical examination at their first visit. Subjects were excluded if they had an allergy to eggs or a history of immunodeficiency. Three blood samples were collected from all subjects; the first at baseline on day 0 just prior to vaccination, the second collected approximately day 28 after the first vaccine (4 weeks), and the third blood samples collected approximately 56 days after the first vaccination (8 weeks). All blood samples were spun in a sterile heparin-coated micro centrifuge tube. Each blood collection tube used contained the appropriate anticoagulant for each variable measured. All tubes were inverted several times and stored on ice until centrifuged at 3000 × g for 15 min at 4°C. Aliquots of the resulting plasma were stored at −80°C until the day of cytokine assay.

Prior to the 2006-2007 influenza seasons, all subjects were enrolled in a multi-project influenza vaccine study during the period from September to December 2007.

Table 1 summarizes the demographic information of the study subjects in the three age groups, as well as the vaccination and blood-sampling protocols for each age group. After obtaining informed consent from the subjects or their parents and assent from children, the subjects were immunized with following current guidelines for influenza vaccination. For the current study, these subjects were immunized a second dose of the same vaccine was given at approximately 28 days after the first dose based on current vaccination guidelines. The 2006-2007 TIV contained influenza A/Wisconsin (H3N2), A/New Caledonia (H1N1), B/Malaysia like Strain. Each dose of TIV contained 45μg of HA in the recommended ratio of 15 μg from each of the three vaccine strains.

We used ELISA kits from Bomdez Med System (Austria), for all of the cytokine measurements. For IL-2 inter-assay CV% was 7.8–10.4%; intra-assay CV% was 5.6–6.1%; and assay sensitivity was 0.180 pg/mL. For IL-6 inter-assay CV% was 7.1–29.5%; intra-assay CV% was 3.8–11.1%; and assay sensitivity was 0.094 pg/mL. For IFN-γ inter-assay CV% was 5.3–9.0%; intra-assay CV% was 1.6–4.0%; and assay sensitivity was 0.059 pg/mL.

Demographic and circulating inflammatory markers data of the three groups (VG, TG, and TVG) were expressed as group means ± standard error (SE). The one-way variance and repeated measures variance of analysis (ANOVA) and a Tukey post hoc test were used to detect and then
identify changes in measured variables during exercise over time. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC). A $P \leq 0.05$ level after correction was considered as significant. All of the statistical analysis was done using SPSS 14.

**Results:**

Baseline characteristics of the study participants are displayed in Table 1. There were no differences in baseline values between groups. We concluded that the three groups were homogenous prior to study.

Significant increases for IL-2 from pre-exercise and vaccination to the 4th week post exercise and vaccination, and significant decreases to the 8th weeks post exercise and vaccination were observed in TVG group. But there was no significant difference between on pre-exercise and vaccination with 8th week post exercise and vaccination (P=0.392). No significant increases for IL-2 from pre exercise to the 4th week post exercise, and the 8th week post exercise were observed in TG group. But there was significant difference between on pre-exercise with 8 weeks post exercise (P=0.082).

Significant increases for IL-2 from pre vaccination to the 4th week post vaccination (P=0.000), and no significant decreases in 8th week post vaccination (P=0.058) were observed in the VG group. But there was significant difference between pre-vaccination with 8th week post vaccination (P=0.035). Significant differences in IL-2 level between TVG and VG groups (P=0.015), between TG and VG (P=0.001) at week 4th and between TVG and VG (P=0.014) at week 8th were observed (Figure 1).

Significant decreases for IL-6 after 4 week (P=0.000), and significant increases to 8 weeks post exercise and vaccination (P=0.031) were observed in the TVG group. Then there was a significant difference between pre-exercise and vaccination with 8 weeks post exercise and vaccination (P=0.042). Significant decreases for IL-6 after 4 week (P=0.002), and significant increases to 8 weeks post exercise (P=0.025) were observed in the TG group. Then there was significant difference between pre-exercise with 8th week post exercise (P=0.000).

Significant decreases for IL-6 after 4th week (P=0.001), and a significant increase in 8 week post vaccination (P=0.015) were observed in the VG group. A significant difference between pre-vaccination and 8th week post vaccination was observed (P=0.035). A significant difference in IL-6 level between TVG and TG groups (P=0.016) at week 8 were apparent (Figure 2).

There was a significant decrease in INFγ after the 4th week (P=0.000) and to after 8 week (P=0.082) in the TVG group. There was a significant difference between the mean pre-exercise and vaccination compared with 8 weeks post-exercise vaccination (P=0.037). There were significant decreases in INFγ from pre-exercise compared with 4 week post-exercise (P=0.003), and after 8th week (P=0.043) in the TG group. There was a significant difference between pre-exercise with those of 8th week post-exercise (P=0.000).

Significant decreases in INFγ from pre-vaccination to 4th week post vaccination (P=0.000), and after 8 week, (P=0.096) in the VG group. A significant difference was detected between pre-vaccination and 8 week post vaccination.
vaccination sample (P=0.042). A significant difference in INF-γ level between TVG and TG groups (P=0.009) at week 4 was also observed (Figure 3).

Figure 3. Effect of exercise and vaccination on INFγ

Table 2. Changes in the measured variables between three groups before and after 8 weeks

<table>
<thead>
<tr>
<th>Week</th>
<th>TVG</th>
<th>TG</th>
<th>VG</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1.5 ± 0.8</td>
<td>1.4 ± 0.18</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>Post Forth week</td>
<td>2.1 ± 0.7</td>
<td>1.5 ± 0.1</td>
<td>3.4 ± 0.5ab</td>
</tr>
<tr>
<td>Post Eight week</td>
<td>1.7 ± 0.5</td>
<td>2.1 ± 0.5</td>
<td>2.2 ± 0.35ab</td>
</tr>
<tr>
<td>Pre</td>
<td>1.3 ± 0.5</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>0.5 ± 0.4a</td>
<td>0.6 ± 0.2a</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Pre</td>
<td>1.2 ± 0.5</td>
<td>1.5 ± 0.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Post Forth week</td>
<td>0.8 ± 0.2a</td>
<td>0.3 ± 0.3ab</td>
<td>1.1 ± 0.4a</td>
</tr>
<tr>
<td>Post Eight week</td>
<td>0.2 ± 0.1a</td>
<td>0.4 ± 0.4ab</td>
<td>0.6 ± 0.1a</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation. Abbreviations as follows: TVG, training-vaccine group; TG, training group; VG, vaccine group; VO2max, Maximal rate of oxygen consumption.

Conclusion:

This study demonstrates that 8 weeks of gymnastic exercise with concurrent influenza vaccination leads to significant reductions of serum level IL-6, IFN-γ, and a brief increase in IL-2.

A number of investigations devoted to the pediatric population show that compared with adults, healthy children experience smaller overall perturbations to the immune system in response to exercise, and demonstrate a faster recovery of the immune system following exercise (6,20).

Although the role of cytokines as pathologic mediators has been studied in children, little is known about the maturation of these agents in healthy individuals (4).

Recently, one study (21) demonstrated that cytokine production was decreased in children compared with adults, and then this maturational difference might explain the increased susceptibility to infection recognized in children. Children, like adults, respond to exercise with cytokine-associated alterations in immune function characterized by increases in NK cell number and activity (22,23).

A few studies hypothesis that function of Th1 following vaccination increases and therefore the concentration of IL-2 and IFN-γ becomes elevated (24,25). Also, the T cell response immediately following vaccination leads to multifold increase in IL-2 and IFN-γ (CD4+ and CD8+) producing cells (25). Kohut et al. note a gradually increasing IL-2 concentration following influenza vaccination in the old and young subjects (26). Our findings did match the above hypothesis so that after 4 weeks IL-2 levels increased in three groups, and in the TG

Table 1. Clinical characteristics of study subjects at baseline

<table>
<thead>
<tr>
<th>Variables</th>
<th>TVG (n=15)</th>
<th>TG (n=15)</th>
<th>VG (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>8.1 ± 0.1</td>
<td>8 ± 0.7</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>21 ± 2.7</td>
<td>21.1 ± 5.2</td>
<td>22.9 ± 9.7</td>
</tr>
<tr>
<td>Height, cm</td>
<td>128.3 ± 2.1</td>
<td>130.2 ± 2.4</td>
<td>133.2 ± 9.7</td>
</tr>
<tr>
<td>Body fat, percent</td>
<td>10.3 ± 2.3</td>
<td>11.5 ± 4.4</td>
<td>12.5 ± 1.9</td>
</tr>
<tr>
<td>VO2, Max, ml/kg</td>
<td>41.1 ± 7.4</td>
<td>42.3 ± 8.9</td>
<td>39.8 ± 4.7</td>
</tr>
</tbody>
</table>
Responses of Cytokines to Influenza Vaccination

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Most pediatric studies (33,34) have investigated the impact of exercise on circulating IL-6 levels under field conditions, which do not allow for adequate experimental control to make comparisons among different groups. Other studies have shown the IL-6 response exercise at a defined work-rate are consistent, by showing that children’s responses are ~50% lower than adults under similar exercise conditions (35,36).

In agreement with this hypothesis, after 4 weeks IL-6 levels reduced in all three groups. In TVG and VG IL-6 levels increased then until the end of the eighth week, but levels were still less than the levels observed in the first week. In contrast, IL-6 levels in TG continued to decrease until the end of the eighth week such that a significant difference in IL-6 level between TVG and TG groups at week 8 were observed.

The mechanism of change in circulating IL-6 following exercise is described by a number of investigators (11,37,38). IL-6 induces expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) (38), a potent angiogenic factor, which is likely to be important in muscle adaptation to exercise. Given the constitutive expression of IL-6 in skeletal muscle and its release during contraction, comparatively smaller exercise-induced increases in IL-6 in children may be sufficient to serve an adaptive function, without compromising other anabolic mediators (IGF-1) (39). With respect to the metabolic roles of IL-6, the smaller increases in children may reflect differences in fuel selection during exercise; IL-6 is thought to be released from muscle during exercise as a potential regulatory hormone to increase liver gluconeogenesis (39). Some of studies (40) have clearly demonstrated that children preferentially oxidize fat rather than carbohydrate as a source of endogenous fuel during exercise (41), thus, children’s muscle glycogen levels are not lowered as much during exercise, and the intracellular signaling for IL-6 release may also then be attenuated.

Interestingly, after 4 weeks IFN-γ levels reduced in all three groups and in TG continued to decline until the end of the eighth week. Meanwhile, the level of IFN-γ in TVG and VG increased, but still remained less than the levels observed in the first week. Possible explanations for this unexpected result include a difference in the kinetics of the secondary responses or in the functional status of immune cells that are re exposed to vaccine (12). A similar lack of boosting effect was observed in TG in their antibody-secreting-cell responses to the exercise training (15). However, it is interesting that a similar lack of boosting effect was not observed in TVG in their antibody-
secretion of cytokines may also exist and be related to the intensity, duration, type of exercise, readiness, sex, age of the subject, time and place of sampling, the sensitivity of the instrument, and measurement method (42,44). These factors however are not easily and properly controlled in all studies, since data from different studies may not be compared due to these variables. Nevertheless, data such as that reported in the current study assist in the understanding of what is considered normal in adolescents and takes into account the effects of exercise training.

In summary, we demonstrate large decreases in inflammatory cytokines IL-6 and IFN-γ after exercise and concurrent vaccine in children. There is also a simultaneous slight increase in circulating IL-2. This relatively long period of training in gymnasts typically results in some time in a catabolic state and this separately may affect the immature immune system in children. These issues should be addressed in future studies with larger sample sizes in order to optimize the use of vaccine in this age group.

References:


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پاسخ سایتوکین‌ها به واکسن آنفلوانزا در زیمناست‌های پسر نخبه

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چکیده

پیشینه: سایتوکین‌های پیش‌تئابی اثرات ورزش را تحت‌الحمایه قرار می‌دهند. سایتوکین‌های ضد‌تئابی در پاسخ به واکسن آنفلوانزا هم اثرات خاصی دارند. این مطالعه کاهش معنی‌دار سطح سایتوکین‌های پیش‌تئابی و افزایش سایتوکین‌های ضد‌تئابی را در پاسخ به واکسن آنفلوانزا در زیمناست‌های پسر نخبه تحقیق کرده است.

روش کار:

1. 55 کودک سالم (21-3 ساله) به صورت تصادفی به گروه تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیشترین تمرین و واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واکسن بیش از واک森