Effect of zinc or zinc plus arginine supplementation on antibody titre and lymphocyte subsets after influenza vaccination in elderly subjects: a randomized controlled trial

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Abstract

Objective: to evaluate whether oral supplementation with zinc or zinc/arginine increases the antibody response to influenza vaccine or modulates the lymphocyte phenotype in elderly subjects.
Design: a randomized controlled trial with two supplemented groups and one control group.
Setting: a community nursing home.
Participants: 384 subjects aged 64-100 (mean age 82 years) examined in three separate studies.
Intervention: oral supplementation with zinc (400 mg/day) or zinc plus arginine (4 g/day) for 60 days starting 15 days before influenza vaccination. The control groups received vaccine only.
Measurements: haematological and nutritional indices, antibody titre against influenza viral antigens, lymphocyte phenotype.
Results: supplementation with zinc or zinc plus arginine increased zinc plasma concentrations restoring the age-related impairment in zinc concentrations to values found in younger people. The antibody titre against influenza viral antigens was not increased in zinc or zinc/arginine supplemented groups in comparison with subjects receiving vaccine alone. The number of CD3, CD4 or CD8 lymphocytes was not affected by zinc or zinc/arginine supplementation.
Conclusion: prolonged supplementation with zinc or zinc/arginine restores zinc plasma concentrations but is ineffective in inducing or ameliorating the antibody response after influenza vaccination in elderly subjects.

Keywords: *immune response, influenza vaccine, randomized controlled trial, zinc*

Introduction

Influenza is a major cause of morbidity and mortality world-wide, with most of the serious complications occurring in the elderly population and particularly in individuals living in high-density environments, such as nursing homes [1, 2]. Annual vaccination against the influenza viruses is recommended for older people but the protection offered by standard influenza vaccination in preventing illness is low in elderly nursing home subjects [3]. Influenza vaccination is not very effective in conferring adequate antibody protection in elderly subjects [2, 4, 5]. One of the main causes of this low antibody response is age-related impairment in immune functioning with thymus involution and decline in various peripheral immune functions. Advancing age is

also associated with reduced immune responsiveness to foreign antigens, particularly to those that are T cell-dependent, such as influenza viral antigens [6].

One way to improve the immunological responses to vaccination is by enhancing antibody responses with immuno-potentiating substances [7], particularly for those whose endogenous levels are reduced with advancing age. Either thymic hormones [8], interleukin-2 [9] or other immunomodulants [10, 11] can increase the serum antibody response to influenza viral antigens in elderly subjects, particularly in very old subjects.

We were interested in improving antibody response to influenza vaccine using zinc and arginine, two immunopotentiating agents.

Zinc plays a crucial role in the development and maintenance of immune competence [12]. Zinc metabolism is altered during ageing and a reduction of serum zinc concentrations is commonly found in healthy old age, and particularly in institutionalized elderly people [13, 14]. Furthermore, zinc turnover is altered in trauma, exercise, inflammation and with tumours and burn injury [12].

Zinc deficiency produces rapid and severe depression of immune function. Hypoplasia of the thymus, decrease of T lymphocyte number, reduction of T-helper lymphocyte function and of the cytotoxic activity of natural killer (NK) cells occur in humans and animals [12, 15, 16]. The zinc-induced immune impairment has been associated with Down's syndrome, cystic fibrosis, acrodermatitis enteropathica, sickle cell anaemia, AIDS and leukaemia [12]. In most cases, zinc treatment reversed the immune deterioration in these diseases [12, 17]. Stimulation of both T cells and B-lymphocytes, as well as NK cell activity, antibody formation and intracellular killing of parasites is triggered or enhanced by zinc supplementation [18-20]. Similarly, various age-related immune deteriorations have been corrected by increasing the reduced zinc concentrations [17]. Increased antibody response to tetanus toxoid vaccination occurs in healthy elderly people after zinc supplementation, but no beneficial effects on the antibody formation to influenza virus vaccine was found in a previous study [21, 22].

Arginine is a basic amino acid with immuno-modulating effects [23, 24]. It restores several immune indices that are decreased during ageing: oral arginine supplementation in old age restores the reduced thymic endocrine activity [25], improves peripheral immune efficiency (such as mitogen responses and NK activity) and increases the percentage of peripheral blood T-helper lymphocytes [26].

Some immunomodulating effects of either zinc or arginine are exerted directly on lymphocytes, although an indirect action through the modulation of endogenous cytokine production has also been demonstrated [27, 28].

In some studies, the combination of zinc and arginine supplementation has been demonstrated to be more effective than the administration of the single nutrient alone in increasing immune functioning [26].

We report the result of a survey performed in three consecutive winters on the effectiveness of zinc or zinc plus arginine supplementation on the specific production of antibodies against influenza viral antigens.

Methods

Subjects and immunization procedure

The studies performed during the winters of 1991-92, 1992-93 and 1994-95 included 63, 223 and 98 institutionalized elderly people respectively. The subjects enrolled in the winters of 1991-92 and 1992-93 were split into two groups: one received vaccine alone (36 and 123 subjects respectively) and the other received zinc sulphate oral supplements (27 and 100 subjects respectively). The 98 elderly subjects in the 1994-95 study were split into three groups receiving vaccine alone (31 subjects), zinc sulphate (33 subjects) or zinc sulphate plus arginine (34 subjects). The mean age ($\pm$ SD) of the subjects in each vaccine group was the same (82 $\pm$ 7 years). In all groups, ages ranged from 64 to 100 years. Ninety, 70 and 75% of the subjects from the 1991-92, 1992-93 and 1994-95 studies respectively were female and the gender ratio in the vaccine groups was equivalent.

The elderly subjects were in a stable clinical condition. Their main clinical problems were neurological (52%), psychiatric (21%) and cardiovascular (22%). The main associated problems were cardiovascular (12%), psychiatric (17%), neurological (11%), locomotor (26%) and respiratory (1%). Elderly subjects in poor health with neoplastic or degenerative diseases were excluded. The groups did not differ in major clinical problems, mental impairment or drug therapy.

Subjects were vaccinated with standard influenza vaccine (Sclavo, Italy) containing B/Yamagata, A/Taiwan and A/Beijing in the winter season 1991-92, A/Taiwan, A/Beijing and B/Panama in the winter season 1992-93, while the influenza vaccine used in the 1994-95 study contained A/Shangdong, A/Singapore and B/Panama. The minimal vaccine concentration was of 15 μg for each strain.

The subjects in the zinc supplemented group received 200 mg of zinc sulphate (IDI Pharmaceutics, Italy) twice daily for 60 days, starting 15 days before vaccination. Arginine (Damor Pharmaceutics, Italy) was given at a dose of 2 g twice daily for 60 days, starting 15 days before vaccination.

Evaluation of influenza antibody titre and nutritional parameters

Blood samples were obtained on days −15, 0 and 45

from vaccination. After clotting and centrifugation, the sera were stored at −20°C until analysis of antibody titre and nutritional parameters.

Sera from each of the blood samples were assayed for influenza virus antibody titres using a standard haemagglutination inhibition assay [29].

All serological titres were expressed as the reciprocal of the serum dilution starting from a 1:10 dilution. The anti-influenza virus antibody titres were converted to log to base 2.

Serum zinc concentrations were determined by atomic absorbance spectrophotometry according to the method of Fernandez and Kahn [30] and the reference standard procedure of Evenson and Warren [31] was used.

Folic acid and vitamin B12 concentrations were determined by conventional radio-immuno assay systems (Johnson and Johnson, Italy). Total protein and albumin concentrations were determined using colorimetric assay (Poli, Italy).

Lymphocyte phenotype

The phyco-erythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies anti-Leu4 (CD3), anti-Leu3 (CD4) and anti-Leu2 (CD8) were purchased from Becton Dickinson (San Jose, CA, USA).

1×10^5 PBMCs were washed twice in PBS containing 0.1% NaN_3 + 10% FCS and then labelled with 20 μl of MoAb in a final volume of 100 μl of PBS for 30 min in ice. At the end of the incubation, cells were washed twice in PBS + 0.1% NaN_3, re-suspended in Isoton II (Coulter, Eurodiagnostics, Hialeah, FL, USA) and immediately analysed with a Coulter Epics V flow cytometer.

Statistical analysis

Data were analysed for statistical significance by using parametric or non-parametric tests according to the distribution of the data.

Comparisons of variables among groups were made by analysis of variance (ANOVA) or Kruskal-Wallis ANOVA.

The two-tailed paired Student's t-test or the Wilcoxon test were used to test differences before and after treatment in the same group. The one-way repeated measures analysis of variance or the Friedman repeated measures analysis of variance on ranks were used to evaluate differences on days −15, 0 and 45. When significant differences were found, statistical analysis was made by Student-Newman-Keuls method. Pearson correlation coefficients were computed for selected variables. Unless otherwise indicated, significance was set at the 5% level ($P < 0.05$). The statistical analysis was performed with SigmaStat software version 1.03 (Jandel Scientific, Germany).

Results

Haematological and nutritional parameters of elderly subjects

Tables 1 and 2 show the haematological and the nutritional parameters of the subjects enrolled in the study conducted during the winter season 1994-95. As shown in Table 1, the haematological values evaluated before vaccination and/or zinc or zinc/arginine supplementation were not significantly different in the three groups of subjects. No blood result was modified after 2 months of zinc or zinc/arginine supplementation.

The values of total proteins, albumin, folate and vitamin B12 were in the normal range and were not significantly different in the groups of subjects who received vaccine, vaccine plus zinc supplementation or vaccine plus zinc/arginine supplementation (Table 2).

The serum zinc concentrations of the subjects enrolled in the winter season 1992-93 were significantly increased after 15 days of zinc supplementation ($P < 0.0001$) without further significant changes at the end of zinc administration (day 60; Table 3). The serum zinc concentrations were not modified in the group of

Table 1. Haemanalysis of elderly subjects before and after zinc or zinc/arginine supplementation

	Mean value (± SD), by group					
	Vaccine		Vaccine + zinc		Vaccine + zinc + arginine	
	Before	After	Before	After	Before	After
Erythrocytes (×10⁶/mm³)	4.1 ± 0.6	4.0 ± 0.6	4.3 ± 0.5	4.0 ± 0.5	4.3 ± 0.4	4.2 ± 0.5
Haemoglobin (g/100 ml)	12.1 ± 1.7	11.7 ± 1.7	12.7 ± 1.6	11.9 ± 1.9	12.7 ± 1.6	12.3 ± 1.5
Haematocrit (%)	37.5 ± 5.2	35.9 ± 4.9	38.8 ± 4.2	36.0 ± 5.1	38.9 ± 4.4	36.5 ± 7.6
Leucocytes (×10²/mm³)	63 ± 24	72 ± 23	76 ± 26	86 ± 35	67 ± 22	73 ± 29
Lymphocytes (×10²/mm³)	18 ± 6	18 ± 6	17 ± 5	18 ± 7	27 ± 37	19 ± 9
Neutrophils (×10²/mm³)	39 ± 20	54 ± 39	54 ± 30	61 ± 32	59 ± 11	49 ± 29

Statistical analysis to test differences before and after treatment was performed using the Wilcoxon test.

Table 2. Nutritional parameters before vaccination in elderly subjects

| | Mean value ± SD, by group | | | |
Parameter	Vaccine	Vaccine + zinc	Vaccine + zinc + arginine	Range
Total proteins (g/dl)	7.7 ± 0.3	8.2 ± 0.8	8.0 ± 0.8	6-8
Albumin (g/dl)	4.7 ± 0.4	4.9 ± 0.3	4.6 ± 0.5	3-5
Folate (ng/ml)	1.2 ± 1.1	1.2 ± 1.2	1.5 ± 1.4	3-17
Vitamin B12 (pg/ml)	343 ± 348	333 ± 182	391 ± 327	180-710

Statistical analysis was performed using Kruskal-Wallis one-way Anova on ranks.

subjects who received only vaccine. In the course of the winter season 1994-95 a similar increase in serum zinc levels was present in zinc supplemented subjects ($P < 0.0001$); the increase of serum zinc concentrations was higher in subjects who received zinc/arginine supplements than in those who received zinc alone, but this difference was not significant (Table 3).

Antibody titres against influenza viral antigens

The antibody titre against the influenza virus was slightly increased in both vaccine and vaccine plus zinc groups of subjects 45 days after vaccination in the study conducted during the winter season 1991-92 ($P < 0.01$ and $P < 0.003$ respectively; Table 4).

The antibody titre against influenza viral antigens was not modified 45 days after vaccination in the groups of subjects receiving either vaccine alone or vaccine plus zinc supplementation in the winter season 1992-93 (Table 5).

In the study conducted during the season 1994-95, the antibody titre against viral antigens increased significantly in subjects who received vaccine, vaccine plus zinc or vaccine plus zinc/arginine ($P < 0.001$; Table 6).

The analysis of the antibody titre as a mean of log of increment for each subject revealed no significant differences in the different groups studied in the winter seasons 1991-92 and 1994-95. The antibody titres were not correlated with the serum zinc concentrations either before or after supplementation.

The percentage of responders and non-responders to each viral antigen after vaccination did not significantly differ with different treatments. The mean percentage of responders in the groups treated with vaccine and vaccine plus zinc were 66 and 76% in the winter of 1991-92, 6 and 5% in the winter of 1992-93 and 57 and 52% in the winter of 1994-95. In the group treated with vaccine plus zinc and arginine in 1994-95, 41% responded.

The increment in antibody titre after zinc or zinc plus arginine supplementation did not differ from vaccine alone treatment in responders and non-responders when analysed separately.

Table 3. Zinc serum levels in elderly subjects receiving zinc or zinc/arginine supplementation 15 days before treatment (T_{-15}) at supplementation (T_0) and 45 days after treatment (T_{45})

| | | Mean zinc serum level ± SD | | |
Season	Treatment	T_{-15}	T_0	T_{45}
1992/93	Vaccine	56.1 ± 13.0 (25-90/56[a])	–	52.7 ± 12.0 (22-85/55)
	Vaccine + zinc	55.7 ± 15.5[b] (20-92/58)	68.2 ± 19.9[b,c] (30-120/68)	74.7 ± 26.2[b,c] (24-150/68)
1994/95	Vaccine	69.7 ± 18.8 (32-111/69)	–	5.8 ± 14.8 (24-83/58)
	Vaccine + zinc	66.1 ± 13.6[b] (39-96/64)	89.2 ± 18.7[b,c] (48-129/85)	89.0 ± 26.5[b,c] (50-150/83)
	Vaccine + zinc + arginine	72.0 ± 17.3[b] (39-103/70)	95.3 ± 17.6[b,c] (48-127/100)	98.8 ± 24.0[b,c] (52-150/98)

[a] Minimum-maximum/median value.
Statistical analysis was performed using Friedman RM Anova on ranks.
[b] $P < 0.0001$.
[c] $P < 0.05$ versus T_{-15} by Student-Newman-Keuls method.

Table 4. Antibody titre to influenza virus before and after vaccination during winter 1991/92

Group	Day[a]	Mean antibody titre (Log-2)
Vaccine	0	5.66 ± 1.91
	45	6.69 ± 1.78[b]
Vaccine + zinc	0	5.26 ± 1.48
	45	6.57 ± 1.59[c]

[a]From vaccination.
[b]$P < 0.01$ and [c]$P < 0.003$ *versus* value on day 0 by Wilcoxon test.

Lymphocyte phenotype

The absolute number of peripheral lymphocytes was no different before and after vaccination either in subjects receiving vaccine alone (2638 ± 651 *versus* 2586 ± 804 per mm^3) or in subjects receiving vaccine and zinc supplementation (2609 ± 951 *versus* 2688 ± 1201 per mm^3). Figure 1 shows the phenotype of

blood lymphocytes of 15 vaccinated and 48 vaccinated and zinc supplemented subjects. The percentages of CD3, CD4 or CD8 lymphocytes were not modified 45 days after vaccination in both groups of subjects.

Discussion

Institutionalized elderly people are at high risk of acquiring influenza and developing related complications [1, 2]. Age-related immune impairment, underlying disease and crowded living conditions contribute to the transmission of influenza [3].

Although annual influenza vaccination is recommended in elderly communities, the efficacy of current inactivated influenza vaccine declines with age, this being related in part to an age-associated immune defect [2, 4, 5].

Our study confirms the low effectiveness of standard influenza vaccination in conferring adequate antibody response against influenza viral antigens in elderly people and demonstrates the inability of either zinc or

Table 5. Antibody titre to influenza virus before and after vaccination during winter season 1992–93

Group	Day[a]	Mean antibody titre (Log-2) ± SD, by viral antigen		
		Taiwan	Beijing	Panama
Vaccine	0	8.64 ± 1.00	7.35 ± 0.72	6.60 ± 0.95
	45	8.38 ± 0.94	7.50 ± 0.75	6.55 ± 0.90
Vaccine + zinc	−15	8.09 ± 1.12	6.70 ± 0.88	6.31 ± 0.95
	0	8.07 ± 1.10	6.67 ± 0.96	6.25 ± 1.04
	45	7.77 ± 1.12	6.68 ± 0.89	6.03 ± 0.95

Statistical analysis was performed using Friedman RM Anova on ranks.
[a]From vaccination.

Table 6. Antibody titre to influenza virus before and after vaccination during winter 1994/95

Group	Day[a]	Mean antibody titre (Log-2) ± SD, by viral antigen		
		Panama	Singapore	Shangdong
Vaccine	−15	6.7 ± 1.2[c]	7.5 ± 1.3[c]	6.8 ± 0.9[c]
	0	–	–	–
	45	7.7 ± 1.5[c]	8.4 ± 1.5[c]	7.5 ± 1.3[c]
Vaccine + zinc	−15	7.2 ± 1.4[b]	7.7 ± 1.4[b]	7.2 ± 1.4[b]
	0	7.3 ± 1.5[b]	7.8 ± 1.4[b]	7.2 ± 1.4[b]
	45	8.0 ± 1.4[b]	8.6 ± 1.2[b]	8.3 ± 1.4[b]
Vaccine + zinc + arginine	−15	7.3 ± 1.3	8.2 ± 1.4[b]	7.5 ± 1.8[b]
	0	7.4 ± 1.4	8.3 ± 1.4[b]	7.6 ± 1.7[b]
	45	7.8 ± 1.3	9.1 ± 1.2[b]	7.9 ± 1.5[b]

[a]From vaccination.
Statistical analysis was performed using Friedman RM Anova on Ranks; [b]$P < 0.05$ by the Student-Newman-Keuls method; [c]$P < 0.0001$ by the Wilcoxon test.
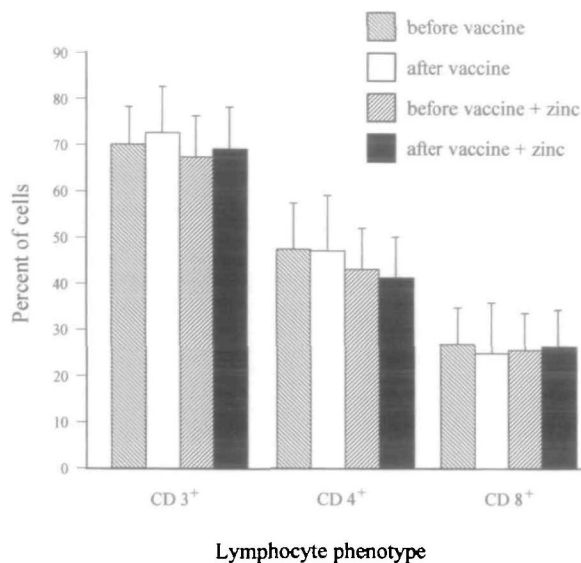
Figure 1. Lymphocyte phenotype before and 45 days after influenza vaccination with or without zinc supplementation. Peripheral blood lymphocytes were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies anti-Leu 4 (CD3), anti-Leu 3 (CD4) and anti-Leu 2 (CD8).

zinc/arginine supplementation to ameliorate the antibody response to influenza vaccine.

We and others have previously reported the poor effectiveness of standard vaccination procedures in ageing, suggesting that there may be differences in the ability of elderly people to respond to influenza vaccines compared with younger people [2, 4, 5]. The data presented in this paper confirm and expand the previous observations by demonstrating the lack of an adequate antibody response in elderly people and, particularly, in institutionalized subjects. Although an increase in antibody titre was obtained in the studies conducted in the winter seasons 1991–92 and 1994–95, the increase was very small and not comparable with that obtained in younger ages. Compared with young adults in whom significant rise in antibody titre (greater than fourfold) has been reported [32], we found a smaller rise in antibody titre (lower than twofold) in elderly subjects. The statistically significant increase in antibody titre found in zinc-treated subjects was concomitant with a significant increase also in control subjects treated with vaccine alone (Tables 4 and 6).

This suggests that the low positive effect on antibody titre was not related to zinc, but, rather, to the vaccine alone or to immunization consequent to influenza disease.

Furthermore, we observed no increase in the 1992–93 winter season (the study including most of the subjects examined), confirming previous data on the reduced effectiveness of influenza vaccine in the elderly [2, 4, 5, 9].

The main cause for this low response is probably linked to the age-diminished antibody response, particularly to the T-cell-dependent antigens, such as the influenza viral antigens.

To correct the age-related immune impairment in antibody production after influenza vaccination, several immuno-potentiating approaches have been successfully used: thymic hormones, IL-2, RU41740 or the immunomodulator Imuthiol increase the magnitude of serum antibody response to influenza viral antigens in elderly subjects [8–11].

Both clinical and experimental studies have demonstrated that zinc can act in an immuno-modulating manner either by restoring the zinc concentrations in conditions of deficiency or by increasing zinc concentrations in diseases, such as AIDS, cancer or the common cold, that are not apparently related to zinc deficiency [12, 33, 34]. The immune impairment found in human diseases such as Down's syndrome, cystic fibrosis, acrodermatitis enteropathica, sickle cell anaemia characterized by zinc deficiency has been improved by zinc treatment [12, 17]. Similarly, various age-related immune deteriorations have been corrected by optimizing the reduced serum zinc concentrations in old age [17]. Experiments on rodents, involving dietary zinc supplementation over the animals' whole life span, have demonstrated that many of the age-related immune modifications, including decreased NK cytotoxicity, can be prevented [35]. Trials in elderly humans have shown that zinc supplementation is able to increase the cutaneous sensitivity to various antigens and the antibody response to tetanus toxoid [19] and to recover thymic endocrine activity [36]. These data have indicated that zinc might provide a simple but powerful means of improving immunocompetence in large populations.

Our results clearly show that neither zinc nor zinc/arginine supplementation increase antibody response after influenza vaccination in elderly subjects. Despite the increase in serum zinc concentrations, neither zinc or zinc/arginine were effective. The fact that serum zinc concentrations were restored on day 15 (i.e. at the time of vaccination) excludes the possibilities that zinc ineffectiveness was related to a late rise in serum concentrations or that the administration of vaccine was not accompanied by increased zinc concentrations.

Our data agree with those of Remarque et al., who showed the ineffectiveness of zinc supplementation in ambulant elderly people in increasing antibody response to influenza virus vaccine [22]. Since several authors have reported a reduced zinc status in elderly nursing home residents and hospital patients compared with ambulant elderly subjects [13, 14], it is possible that the higher proportion of subjects with a zinc deficiency present in our study resulted in enhanced antibody response after correction of zinc levels. Although we found lower mean concentrations in our subjects compared with the ambulant elderly

subjects studied by Remarque and co-workers (9-10 μmol/l *versus* 11.9 μmol/l), we found no increase in antibody titre after influenza vaccination in our institutionalized elderly subjects.

A role for arginine in immunological ageing has been suggested because of its stimulatory action on growth hormone that, in turn, modulates various immune functions during development and ageing. Arginine supplementation in old age restores thymic endocrine activity, peripheral immune efficiency and increases the percentage of peripheral blood T-helper lymphocytes. The combination of zinc and arginine is more effective than single nutrients in restoring thymic endocrine activity and the cytotoxic activity of NK cells. We used 4 g arginine per day as similar doses have induced a significant increment of thymulin blood concentrations and CD4$^+$ lymphocytes in elderly subjects [37]. The lack of effect of the zinc/arginine supplementation on antibody response to influenza vaccine suggests that arginine *per se* does not act at the level of humoral immunity, nor does it enhance potential immunologial effects of zinc.

In conclusion, neither zinc nor zinc/arginine supplementation is effective in inducing or increasing antibody responses after influenza vaccination.

Key points

- Influenza vaccine is not always effective in older people.
- Zinc is important in immune competence and some age-related immune deterioration can be reversed by zinc supplementation. Arginine can also modulate the immune response.
- In a randomized controlled trial of zinc (with and without arginine), there was no improvement in the rsponse of elderly people to influenza vaccination.

Acknowledgements

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