Suppressive effect of zinc on antibody response to cholera toxin in children given the killed, B subunit-whole cell, oral cholera vaccine

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Abstract

In a previous study, children aged 2–5 years old in Bangladesh were supplemented orally with a single dose of Vitamin A (200,000 IU) and a placebo for zinc (zinc equivalent to 20 mg of elemental zinc) everyday for 42 days (group A), zinc and a placebo for Vitamin A (group Z), and zinc and Vitamin A (group AZ) or both placebos (group P). All children were orally immunised with two doses of the killed cholera vaccine containing whole cells and a recombinant B subunit of cholera toxin (CT). The number of children who responded with ≥4-fold vibriocidal antibody (a proxy indicator of protection against cholera) was significantly greater among the zinc-supplemented groups than among the non-zinc-supplemented groups, while Vitamin A supplementation did not appear to have any effect. The sera from these children were assayed for antibody to CT. Antibody to CT is known to exert a synergistic protective effect against cholera in animal studies, and offer significantly higher short-term protection against cholera and significant short-term protection against enterotoxigenic Escherichia coli diarrhoea in humans on oral immunisation with the cholera vaccine. Children who received zinc had significantly reduced levels of serum antibodies to CT than children who received placebos only. Factorial analysis showed a trend for zinc showing a reduction in the number of children responding with CT-antibody, while Vitamin A did not appear to have any effect. Thus, zinc enhanced vibriocidal antibody response, but suppressed CT-antibody response, suggesting that zinc supplementation has different modulating effects on vibriocidal antibody response and CT-antibody response.

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1. Introduction

Micronutrients, Vitamin A and zinc are important for immunocompetence [1,2]. Supplementation of these micronutrients in deficient individuals restores immune function including increased immune response to vaccines [3,4]. In a recent study, the effect of Vitamin A and zinc supplementation on vibriocidal antibody response to a killed, oral Vibrio cholerae O1 vaccine was investigated in children in Bangladesh. Zinc, but not Vitamin A supplementation significantly increased seroconversion rate to vibriocidal antibody [5], which is an indirect marker of protection against cholera [6,7]. In natural cholera and in immune response studies to vaccination, it is customary to measure antibody response to cholera toxin (CT), the most important virulence factor of V. cholerae that induces watery diarrhoea, the hallmark of cholera [7–9]. In the present report, we present data on the effect of Vitamin A and zinc supplementation on immune response to CT in the same children on whom the vibriocidal antibody data to oral cholera vaccination was reported previously [5].

2. Materials and methods

The details of the study have been reported previously [5]. The subjects were 2–5-year-old children with Vitamin A deficiency (serum retinol, <20 μg/dl). In this double-blind trial, a total of 256 children were randomised to receive Vitamin A and a placebo (group A), zinc and a placebo (group Z), both Vitamin A and zinc (group AZ) or both placebos (group P). Vitamin A was administered as a single dose (200,000 IU) and zinc in the form of zinc acetate was
administered daily (equivalent to 20 mg elemental zinc, twice the recommended daily allowance) for 42 days. Two doses of a killed, oral vaccine, Cholerix (SBL Vaccin AB, Stockholm, Sweden) containing killed cells of O1 serogroup of \( V. cholerae \) of both biotypes and serotypes, and a recombinant B subunit of CT (rCTB) was given to all children with a 2-week interval between doses [5]. Venous blood was collected at baseline before supplementation (sample 1), 1 week after first vaccine dose (sample 2) and 1 week after the second vaccine dose (sample 3). The scheme of the study is summarised in Fig. 1. Two serum samples (samples 1 and 3) from each subject were assayed for zinc [10] and retinol [11]. All three serum samples from each subject were analyzed for CT antibodies. For measuring CT-specific antibodies of IgA (CT-IgA) and IgG (CT-IgG) isotypes, the GM1-CT assay [9] was used, with rCTB as the coating antigen in microtitre plates (Nunc, Roskilde, Denmark). Tenfold diluted serum samples were added in duplicate wells, and serial threefold dilutions were carried out. The end-point titre was determined by ELISA using a computer-based programme Multi (DataTree Inc., Walthams, MA, USA). Seroconversion to CT was defined as a twofold or higher rise in reciprocal titre from the baseline to post-vaccination levels. Multiple samples from the same individuals were tested in the same batch. The antibody levels were expressed as median and range (25–75th percentile).

Statistical analyses were carried out using the statistical package for Social Sciences (SPSS/PC+, Chicago, IL, USA). Categorical analysis was done using the \( \chi^2 \)-test or Fisher’s exact test as appropriate. Comparison of continuous variables was done with ANOVA for normally distributed data and the Kruskal–Wallis one-way ANOVA on ranks for skewed data. A paired \( t \)-test was done to compare the post-supplementation serum levels of retinol and zinc with the baseline values. The relationship of post-immunisation CT-antibody response to baseline CT antibody and Vitamin A and zinc, and age, gender and nutritional status was explored by ANOVA. Statistical significance was set at a probability level of <0.05.

3. Results

The numbers of children who completed the study were 61 in group A, 63 in group Z, 62 in group AZ and 63 in group P. These children had taken the complete schedule of Vitamin A and zinc supplements and two doses of the vaccine, and had three samples of blood collected. All these children who completed the study only were analysed. As reported previously, the anthropometric levels of the groups were similar among the four groups [5]. The pre (first bleed, Fig. 1) versus post-supplementation (third bleed, Fig. 1) levels of Vitamin A (μg/dl) and zinc (mg/dl) were: 14.2 versus 23.4 and 0.63 versus 0.66 (group A), 12.7 versus 16.0 and 0.63 versus 1.03 (group Z), 14.4 versus 24.2 and 0.61 versus 0.94 (group AZ), and 14.2 versus 15.3 and 0.61 versus 0.60 (group P). The differences in the levels of Vitamin A were significant for all groups including the group P (\( P = 0.024 \) to <0.001). The differences in the levels of zinc were significant for Z (\( P < 0.001 \)) and AZ group (\( P < 0.001 \) only).

All children were Vitamin A-deficient at recruitment. After micronutrient supplementation, the numbers of Vitamin A-deficient children were: 16 (26.2%, group A), 41 (65.1%, group Z), 15 (24.2%, group AZ), and 39 (62.0%, group P). At recruitment, 124 (49.8%) of children were zinc-deficient (<0.6 mg/dl of serum). After supplementation, the numbers of deficient children were: 21 (34.4%, group A), 7 (11.1%, group Z), 3 (4.8%, group AZ), and 32 (50.8%, group P).
The CT-IgA and CT-IgG antibodies increased significantly in all groups of children after the first and second dose of the vaccine compared to the baseline levels (Table 1). However, supplementation with zinc alone or that with both zinc and Vitamin A resulted in lower increases in antibody titres compared to the increases in the P and A groups. After the first vaccine dose, the median CT-IgA titre in the AZ group was significantly lower than those in the A and P groups, and the median CT-IgG titre in the AZ group was significantly lower than that in the A group. After the second vaccine dose, the median CT-IgA titres in the Z and AZ groups were significantly lower than that in the P group.

The numbers of children with ≥2-fold increase in serum antibody to CT are shown in Table 2. The responders were generally lower in Z and AZ groups compared to A and P groups. This difference was significant for group A versus group Z for CT-IgA after the second dose.

The immune response to CT in relation to initial zinc status in groups Z and AZ children was analysed. There were 29 deficient children in group Z and 30 deficient children in group AZ. The CT-IgA and CT-IgG responses were not significantly different between children who were zinc-deficient and zinc-sufficient in the two groups (data not shown). After supplementation, 22 children in group Z and 26 children in group AZ became zinc-sufficient. The CT-IgA and CT-IgG responses were analysed in children who remained deficient against those who became sufficient after supplementation. Among group Z children, the median titres increased 6.0–6.3-fold for CT-IgA in children who remained deficient against 3.1–4.2-fold increase in children who became sufficient (P > 0.05). The corresponding increases in CT-IgG for the deficient and sufficient children were 1.7–3.5- and 1.5–1.8-fold, respectively (P > 0.05).

Similar analysis of data for group AZ children did not show significant differences in CT-IgA and IgG levels between children who remained deficient and who became sufficient (P > 0.05).

Factorial analysis of the effect of micronutrient supplementation on CT-antibody response was carried out (Table 3). After the first vaccine dose, there were no significant differences between Vitamin A versus non-Vitamin A groups, and zinc versus non-zinc groups in the proportion of CT-IgA antibody responders. After the second vaccine dose,
the response rate for zinc groups was much lower than that
for non-zinc groups with the difference approaching statis-
tical significance ($P = 0.052$). The seroconversion rates for
CT-IgG were similar between Vitamin A and non-Vitamin
A groups. However, the rates were lower for zinc groups
versus non-zinc groups even though the differences were
not significant. After adjustments for age, gender, nutri-
tional status, and baseline antibody, Vitamin A and zinc
levels, the effect of zinc remained the same ($P > 0.05$).

4. Discussion

Vitamin A deficiency is associated with increased mor-
bidity and mortality in children [12,13]. Vitamin A sup-
plementation substantially reduces childhood mortality
[14]. The exact mechanism for Vitamin A in reducing in-
fec tion and mortality is not known. However, improved
immune response has been suggested as a possible mecha-
nism [15]. Vitamin A and its metabolites have been shown
to increase antibody response to T-cell-dependent and
T-independent antigens, increase lymphocyte prolifera-
tion and cytokine production, inhibit apoptosis, and restore
mucosal function and maintain mucosal integrity [2,4].

Zinc deficiency is associated with disruption of epithe-
lial barrier function, immunocompetence and linear growth
[16]. This decrease in immunological competence may
lead to a higher risk of infectious diseases or a greater
severity of illness [16,17]. In zinc deficiency, there is re-
duced cell-mediated immune response, reduction in T
cells, abnormal T-helper and/or suppressor functions, im-
paired macrophage function, and reduced killer cells and
antibody-dependent cytotoxicity. B lymphocyte antibody
responses are inhibited by zinc deficiency. T-dependent an-
tibody responses are more affected by zinc deficiency than
the T-independent ones [1].

The pre-supplementation serum levels of Vitamin A and
zinc among the four groups were similar. As expected,
the post-supplementation levels of Vitamin A increased in
groups A and AZ, and the greatest increase was seen in
the latter group. This is due to the synergistic interaction
between Vitamin A and zinc [18,19]. The increase in Vi-
tamin A level in group P may be due to regression to the
mean (because the subjects were selected based on extreme
values, repetition of these values per se would be expected
to result in less extreme values). In addition, the surveil-
lance would have picked up morbidity episodes promptly
and facilitated care and/or changed care-seeking patterns
thereby reducing micronutrient loss. As expected, groups Z
and AZ children had increases in serum zinc concentration
after supplementation.

Immunisation with cholera vaccine significantly increased
CT-IgA and CT-IgG antibody levels in all four groups of
children. However, these increases were in most cases sig-
nificantly depressed in groups Z and AZ compared to
groups A and P (Table 1). Analysis of responder frequency to CT
also showed generally a lower response in Z and AZ groups
compared to in groups A and P (Table 2). Factorial analysis
of responder frequency to CT also suggested a depressive
effect of zinc on both CT-IgA and CT-IgG responses after
both first and second doses of the vaccine, although the dif-
ference approached significance in the case of CT-IgA re-
sponse only after the second dose of the vaccine (Table 3).
The effects remained unaltered after controlling for relevant
variables. On the other hand, Vitamin A supplementation
did not appear to have any effect either on the level of, or
responder frequency, to CT antibody.

There did not appear to be a dose effect in terms of
initial zinc level or change in zinc level and antibody
response to CT. Analysis of antibody levels in relation to
initial zinc status did not show significant differences
among zinc-supplemented children. Even though there was
a higher magnitude of CT-IgA response among children
in group Z who remained deficient after supplementation
compared to those who became sufficient after supplemen-
tation, the difference was not statistically significant. The
small number of deficient children (seven children) might
have contributed to lack of significance. Future studies
involving a larger sample size would clarify this.

These CT-antibody responses are in contrast to vibrio-
cidal antibody responses in the same children reported earlier
[5]. The vibriocidal antibody levels were similar in all four
groups of children in response to vaccination. The number
of children responding with ≥4-fold rise in vibriocidal an-
tibody was significantly greater in group AZ compared to
group P. This responder frequency to vibriocidal antibody
was significantly greater in the zinc-supplemented groups than in the non-zinc-supplemented groups. Thus, zinc supplementation appeared to have a beneficial effect on improving the vibriocidal antibody response to cholera vaccination, which is the proxy indicator of protection against cholera [6,7]. However, as with CT-antibody response, Vitamin A supplementation did not appear to influence vibriocidal antibody response [5]. This lack of effect of Vitamin A supplementation on antibody levels to CT in children is similar to the finding in rats deficient in Vitamin A, which on supplementation with Vitamin A did not have improved antibody levels to CT upon oral immunisation with cholera vaccine and CT [20].

The differing effects of zinc on CT-antibody and vibriocidal antibody responses are intriguing. Vibriocidal antibody assay measures antibodies directed predominantly against the lipopolysaccharide (LPS) antigen of *V. cholerae* [7]. LPS is normally considered to be a T-cell-independent antigen [21]. However, in cholera, LPS antibody switch occurs from IgM isotype to IgG isotype [22], which suggests that LPS is not strictly a T-cell-independent antigen. On the other hand, CTB, being a protein, is strictly a T-cell-dependent antigen [21] and zinc deficiency is expected to have a greater effect on antibody response to it [1]. It appears that zinc has different modulating effects on antibody response to the two antigens of *V. cholerae* investigated in this study. While seroconversion rate to vibriocidal antibody is increased [5], the antibody level increase to CT is suppressed. This is reflected in reduced seroconversion rate to CT-IgA. It is possible that the reduction in CT-IgA in the zinc-supplemented groups may have resulted from increased epithelial transport of IgA. This would result in increased CT-IgA response at the mucosal surface where protection is needed. This possibility needs to be addressed in a future study. In animal studies, antibodies to LPS (measured as vibriocidal) and CT antibodies appear to have synergistic effect on protection against cholera [23]. Comparison of protective efficacies of killed, oral cholera vaccines showed that the formulation containing CTB and killed whole cells had superior protection than the one with the killed whole cells alone over the short term [24], even though this difference disappeared over the long term [25]. Moreover, the formulation with CTB also protected against enterotoxigenic *Escherichia coli* (ETEC) diarrhoea over the short term, because of the cross-reaction between CT and the heat-labile enterotoxin (LT) of ETEC [26]. In spite of these observations, in human studies, only serum vibriocidal antibody, not CT antibody has been shown to be a proxy marker of protection against cholera [6]. Mucosal antibodies provide protection against mucosal infections such as cholera and ETEC diarrhoea. Serum antibodies seem to reflect mucosal antibodies. It appears that CT antibodies may provide protection against cholera and ETEC diarrhoea. Even though zinc supplementation affected CT-antibody response in comparison to the placebo group, the increase in the level of CT antibodies in response to vaccination was nevertheless significant.

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