Supplementation with Zinc, but Not Vitamin A, Improves Seroconversion to Vibriocidal Antibody in Children Given an Oral Cholera Vaccine

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To investigate whether micronutrient supplementation could improve the vibriocidal antibody response of children to a killed oral cholera vaccine, 2–5-year-old children were randomly assigned to receive vitamin A and zinc (AZ group), vitamin A and a placebo (A group), zinc and a placebo (Z group), or both placebos (P group). All children received 2 doses of the vaccine. The number of children who had a ⩾4-fold increase in vibriocidal antibody was significantly greater in the AZ group than in the P group ($P = .025–.028$). Factorial analysis suggested that the proportion of children with a ⩾4-fold increase in vibriocidal antibody titer was significantly greater in the zinc-supplemented groups than in the groups that did not receive zinc ($P = .013–.048$) and that vitamin A supplementation did not have a significant effect. Thus, supplementation with zinc improves seroconversion to vibriocidal antibody and, hence, has the potential to improve the efficacy of oral cholera vaccine in children.

The protective efficacy of a killed oral cholera vaccine [1] and serum vibriocidal antibody levels [2], a marker of protection against cholera, have been found to be low in 2–5-year-old children (the age group most susceptible to cholera) in Bangladesh. Some vaccines stimulate a better immune response in populations in developed countries than in those in developing countries [3, 4]. A possible cause is the poor nutritional status of the individuals in developing countries. Micronutrients such as vitamin A and zinc play an important role in immunocompetence [5, 6], and micronutrient deficiencies are widespread among children in developing countries, including Bangladesh [7, 8]. The objective of the present study was to determine whether supplementation with vitamin A, zinc, or both would increase the vibriocidal antibody response to oral cholera vaccine in children.

SUBJECTS AND METHODS

Subjects. The study was conducted in an area of low socioeconomic status in Dhaka, the capital city of Bangladesh, during June 1998 through May 2000. Children 2–5 years old of either sex who were recruited from consecutive households were eligible for the study if they had vitamin A deficiency (serum retinol level, <20 μg/dL; determined by testing of a blood sample obtained for preenrollment screening) and had nutritional

status corresponding to a weight-for-age score that was ≥61% of the median National Center for Health Standards standard. Children who had received vitamin A supplementation during the preceding 6 months or who had a history of night blindness or sickness due to underlying illnesses such as diarrhea, respiratory tract infections, or other infections were excluded.

Study plan. The children were randomly assigned to 1 of 4 groups: the A group received vitamin A and a placebo; the Z group received zinc and a placebo; the AZ group received both vitamin A and zinc; and the P group received both placebos. Our assumption was that supplementation would increase vibriocidal antibody response ≥4-fold. We calculated that a sample size of 54 subjects/group would be required. A total of 256 subjects were enrolled to allow for the possibility of an attrition rate of up to 15%.

Supplementation and vaccination. The vaccine was delivered to a field clinic through a "cold chain" system. The vaccine and micronutrient supplements were administered orally. Subjects in all 4 groups received 2 doses of a killed oral cholera vaccine, Cholerix (SBL Vaccin), with a 2-week interval between doses. The vaccine consisted of 10^{11} heat- or formalin-killed whole cells of *Vibrio cholerae* O1, classical and El Tor biotypes, Inaba and Ogawa serotypes, and 1 mg of purified recombinant cholera toxin B subunit. This vaccine differed from the vaccine used in the previous trial in Bangladesh [9]; in the vaccine used in the present study, the bacterial cells expressed mannose-sensitive hemagglutinin and toxin-coregulated pilus and contained the recombinant cholera toxin B subunit, instead of the chemically purified cholera toxin B subunit. The vaccine was mixed with bicarbonate–tartaric acid buffer (each sachet contained 2.6 g of buffer [Samarin; Cederroth]). One buffer sachet was dissolved in 100 mL of water, and 1 dose of the vaccine was dissolved in 50 mL of buffer. Food and drink were not allowed for 1 h before and 1 h after vaccination. Patients were monitored for side effects such as diarrhea, vomiting, abdominal pain, fever, and rash for the next 72 h.

The common ingredients in vitamin A, zinc, and placebo syrups were coloring agent, polysorbate 80, saccharin, glycerin, orange flavor, and purified water. The vitamin A syrup contained vitamin A palmitate, and the zinc supplement contained zinc acetate. The placebos did not contain either vitamin A or zinc. The zinc syrup and its placebo syrup looked very similar, as did the vitamin A syrup and its placebo syrup (Acme Laboratories). The randomization code was broken after completion of the study. Bottles of syrup were serially numbered according to the randomization list, and this numbering corresponded to the study serial numbers. Enrolled children were assigned numbered bottles in the order in which they were recruited.

Children in the A group received 5 mL (200,000 IU) of vitamin A syrup 1 week before administration of the first dose of the vaccine and received 5 mL of a placebo syrup (in place of zinc) every day for 42 days starting 3 weeks before administration of the first dose of vaccine and ending 1 week after the second dose of vaccine. Children in the Z group received 5 mL of zinc acetate syrup (containing 20 mg of elemental zinc) daily and a single dose of a placebo syrup (in place of vitamin A), according to the same schedule used for the children in the A group. Children in the AZ group received supplementation with both vitamin A and zinc, and children in the P group received placebos in place of both vitamin A and zinc, according to the same schedule used for the children in groups A and Z.

The children received vitamin A or its placebo at a study-site clinic. Mothers were instructed to feed zinc or its placebo daily at home. Each household received one 50-mL bottle of syrup for each participating child at a time. The reliability of feeding was verified 7 days later by a home visit and measurement of the amount of syrup that had not been consumed. A health assistant collected morbidity data from every household by visiting every other day.

Testing of blood and stool samples. Approximately 3 mL of venous blood was collected from each child at 3 time points during the study period: at baseline, before supplementation (sample 1; this sample was used for preenrollment screening); 1 week after the first dose of vaccine had been administered (sample 2); and 1 week after the second dose of vaccine had been administered (sample 3).

Serum samples were tested for vibriocidal antibody response using *V. cholerae* O1, El Tor, Ogawa (strain 25049) as the target organism [10]. Seroconversion was defined as a ≥4-fold increase in reciprocal titer from the baseline level. Multiple samples from the same individual were tested in the same batch. Serum retinol levels were assayed by high-performance liquid chromatography (Waters Millipore) [11], and serum zinc levels were assayed by atomic absorption spectrophotometer (Shimadzu) [12]. Diarrheal stools were cultured for *V. cholerae* O1 and O139, according to standard methods [13].

Statistical analysis. Statistical Package for Social Science software (SPSS/PC+) was used for analysis of data. Categorical analysis was performed by means of the χ^2 test or Fisher's exact test. Comparison of continuous variables was performed using Student's *t* test, for normally distributed data, or either the Mann-Whitney *U* test or the Wilcoxon signed-rank test, for skewed data. The antibody levels were measured as median and range (25th–75th percentiles). A paired *t* test was used to compare postsupplementation serum levels of vibriocidal antibodies, retinol, and zinc with the baseline values. Before pairwise comparisons were performed, the overall significant differences were determined by either analysis of variance (ANOVA) or ANOVA on ranks, as appropriate. Correlation of data was analyzed by Spearman's rank order test. The relationship of post-

immunization vibriocidal antibody responses to baseline serum vibriocidal antibody, vitamin A, and zinc levels and to age, sex, and nutritional status was explored by ANOVA. $P < .05$ was considered to be statistically significant.

RESULTS

Anthropometric data. A total of 673 children were screened for serum retinol deficiency (< 20 μg of retinol/dL of serum), and 268 were found to have a retinol deficiency; 256 retinol-deficient children were randomly assigned to a study group. Seven children did not complete the study. The final group sizes were 61 children in the A group, 63 children in the Z group, 62 children in the AZ group, and 63 children in the P group. The anthropometric data for children in the 4 groups were similar. The ratio of boys to girls was 33:28 in the A group, 28:26 in the Z group, 35:27 in the AZ group, and 39:24 in the P group. The ratios of mean age in months to weight in kilograms were 41:12, 39:11, 41:12, and 39:11, respectively; the ratios of mean age in months to height in centimeters were 41:89, 39:88, 41:89, and 39 :88; and mean weight/height z scores were -1.10, -1.42, -1.24, and -1.28.

No significant side effects were attributed to vaccine or placebo. At follow-up examinations for morbidity, 6 children were found to have developed diarrhea, but none had *V. cholerae* O1 or O139 infection.

Micronutrient levels. Pesupplementation (blood sample 1) and postsupplementation (blood sample 3) levels of vitamin A and zinc were 14.2 versus 23.4 μg/dL and 0.63 versus 0.66 mg/dL (A group), 12.7 versus 16.0 μg/dL and 0.63 versus 1.03 mg/dL (Z group), 14.4 versus 24.2 μg/dL and 0.61 versus 0.94 mg/dL (AZ group), and 14.2 versus 15.3 μg/dL and 0.61 versus 0.60 mg/dL (P group), respectively. The differences in the levels of vitamin A were significant for all groups, including the P group ($P = .024$ to $P < .001$). The differences in the levels of zinc were significant for groups Z ($P < .001$) and AZ ($P <$ .001) only (P values were determined by the Mann-Whitney rank sum test or the Wilcoxon signed-rank test).

All study children had vitamin A deficiency at recruitment. After micronutrient supplementation, the number of vitamin A–deficient children was 16 (26.2%) in the A group, 41 (65.1%) in the Z group, 15 (24.2%) in the AZ group, and 39 (62.0%) in the P group. At recruitment, 124 (49.8%) of all children were zinc-deficient (≤ 0.6 mg/dL of serum). After supplementation, the numbers of zinc-deficient children were 21 (34.4%; A group), 7 (11.1%; Z group), 3 (4.8%; AZ group), and 32 (50.8%; P group).

Vibriocidal antibody response. The median vibriocidal antibody titers (to Ogawa serotype) of the first, second, and third serum samples were 20, 320, and 640 in the A group; 20, 320, and 320 in the Z group; 40, 640, and 320 in the AZ group; and 20, 80, and 160 in the P group. The increases in antibody levels between the first and second samples and between the first and third samples were significant for all groups ($P =$ .015–.001, by the Mann-Whitney rank sum test or the Wilcoxon signed-rank test). No significant difference was found between the antibody titers in the second and third samples. There were no significant differences among the groups at the 3 sampling times.

Factorial analysis showed that after intake of the first or second dose of the vaccine, the proportion of children with vibriocidal antibody responses was significantly greater in the zinc-supplemented groups than in the groups that did not receive zinc (table 1). No significant difference was seen between vitamin A–supplemented groups and groups that did not receive vitamin A. Zinc supplementation resulted in a 43% increase in the number of children with vibriocidal antibody responses after the first dose of the vaccine, whereas vitamin A supplementation resulted in a 12% increase after the first dose, compared with children who received placebo only. The combined administration of vitamin A and zinc resulted in a 59% increase in the number of children with antibody re-

Table 1. Proportion of children receiving supplementation with vitamin A, zinc, or both who had a vibriocidal antibody response ($\geq$4-fold increase in antibody) to the oral cholera vaccine.

Comparison	No. of children	After first vaccine dose		After second vaccine dose	
		Children with response, %	P^a	Children with response, %	P^a
Vitamin A vs. no vitamin A			.416		.322
Vitamin A with or without zinc	123	50		61	
No vitamin A (zinc or placebo only)	126	40		54	
Zinc vs. no zinc			.013		.048
Zinc with or without vitamin A	125	55		64	
No zinc (vitamin A or placebo only)	124	39		51	

[a] Calculated using the χ^2 or Fisher's exact test.

Table 2. Number of children receiving supplementation with vitamin A, zinc, or both who had a vibriocidal antibody response (≥4-fold increase in antibody) to the oral cholera vaccine.

Supplement	Total no. of children	Children with response, no. (%) After first vaccine dose	Children with response, no. (%) After second vaccine dose
Vitamin A and placebo	61	25 (41.0)[a]	34 (55.7)[a]
Zinc and placebo	63	33 (52.4)[a]	39 (61.9)[a]
Vitamin A and zinc	62	36 (58.1)[b]	41 (66.1)[c]
Placebo only	63	23 (36.5)[d]	29 (46.0)[e]

[a] $P > .05$, vs. all other groups (χ^2 or Fisher's exact test).
[b] $P = .025$, vs. placebo-only group; $P > .05$, vs. all other groups (χ^2 or Fisher's exact test).
[c] $P = .025$, vs. placebo-only group; $P > .05$, vs. all other groups (χ^2 or Fisher's exact test).
[d] $P = .025$, vs. vitamin A and zinc group; $P > .05$, vs. all other groups (χ^2 or Fisher's exact test).
[e] $P = .025$, vs. vitamin A and zinc group; $P > .05$, vs. all other groups (χ^2 or Fisher's exact test).

sponses (that approximates the 58.1% of children who received vitamin A and zinc supplementation and had responses after the first vaccine dose; table 2), which indicates that the effect was additive.

The individual and interactive effect of micronutrients on vibriocidal response frequency is shown in table 2. A significantly greater number of children in the AZ group than in the P group had responses (≥4-fold increase in antibody titer). This was true after both the first dose and the second dose of the vaccine. Other comparisons were not significant. After supplementation, vitamin A and/or zinc status (deficient or sufficient) did not correlate with vibriocidal antibody titers ($P > .05$, by Spearman's rank order correlation test) or the proportion of children with vibriocidal antibody responses ($P > .05$, by the χ^2 test). After adjustment for age; sex; nutritional status; and baseline vibriocidal antibody, retinol, and zinc levels, the proportion of children with vibriocidal antibody responses after the first and second doses of the vaccine in the AZ group remained significantly greater than that in the P group ($P < .05$, by ANOVA).

DISCUSSION

Presupplementation serum levels of vitamin A and zinc were similar among the 4 groups. As expected, postsupplementation levels of vitamin A increased in the A group and in the AZ group, and the greatest increase was seen in the latter group. This is due to the synergistic interaction between vitamin A and zinc [14]. The increase in vitamin A level in the P group may be due to regression to the mean. In addition, the surveillance would have picked up morbidity episodes promptly and facilitated care and/or changed care-seeking patterns,

thereby reducing micronutrient loss. As expected, the Z group and the AZ group had increases in serum zinc concentration after supplementation.

Even though all groups had significant vibriocidal antibody responses after vaccination, with the highest proportion in the AZ group, there were no significant differences among the groups. Factorial analysis of the proportion of children with vibriocidal antibody responses showed that zinc, not vitamin A, had a significant positive effect. However, the effect of combined supplementation was additive. The beneficial effect of the combination of vitamin A and zinc remained after the analysis was controlled for relevant variables.

Other studies have found immune responses to the killed oral vaccine in developing countries to be weaker [15] but immune responses to live oral vaccine to be stronger, although these responses did not translate into long-term protection [16]. It is noteworthy that vibriocidal antibody levels in the P group in the present study were greater than the antibody levels seen in the previous study in Bangladesh [1], and the response after the first vaccine dose was robust in all groups. These differences could be due to differences in the formulation of the vaccine and the effect of micronutrients in the present study. It is possible that a single-dose vaccine can replace a multidose vaccine.

To our knowledge, this is the first study in which the effect of combined supplementation of vitamin A and zinc on immune response to an oral vaccine was explored in a nutritionally deprived population of children. The results of our study have suggested that interventions to improve micronutrient deficiency by supplementation or fortification will have a beneficial effect on cholera vaccination programs.

Acknowledgments

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