Effects of zinc supplementation as adjunct therapy on the systemic immune responses in shigellosis[1–3]

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ABSTRACT

Background: Zinc is lost during diarrheal diseases, and zinc deficiency induces intestinal morphology–altering inflammatory responses that zinc supplementation can correct.

Objective: We assessed the in vivo effect of zinc supplementation on systemic and mucosal responses in mildly to moderately malnourished (defined as <-1 but >-2 and <-2 but >-3 weight-for-height z scores, respectively, based on the National Center for Health Statistics growth reference) children with shigellosis.

Design: A double-blind placebo-controlled trial was conducted in *Shigella flexneri*–infected children aged 12–59 mo. Daily for 14 d, elemental zinc (20 mg) and multivitamins (vitamins A and D, thiamine, riboflavin, and nicotinamide) plus calcium were given at twice the US recommended dietary allowance to the zinc group ($n = 28$), and multivitamins plus calcium were given to the control group ($n = 28$). All subjects received standard antibiotic therapy.

Results: There was no significant interaction between zinc supplementation and time, but zinc supplementation showed a significant effect on serum zinc concentrations. With a $\geq$4-fold increase in serum shigellacidal antibody titers from baseline used as the cutoff, the proportion of children with shigellacidal antibody response was greater in the zinc group than in the control group ($P < 0.03$). There was a significant ($P = 0.02$) treatment $\times$ time interaction for the proportions of circulating $CD20^+$ and $CD20^+CD38^+$ cells, which were higher on day 7 in the zinc group than in the control group ($P < 0.007$). No effect was seen on histopathologic features or the expression of innate and inflammatory mediators in the rectum.

Conclusion: Adjunct therapy with zinc during acute shigellosis significantly improved seroconversion to shigellacidal antibody response and increased the proportions of circulating B lymphocytes and plasma cells. *Am J Clin Nutr* 2005;81:495–502.

INTRODUCTION

Shigellosis, a dysenteric illness, is responsible for significant morbidity and growth faltering in children aged <5 y in developing countries. A major public health problem in developing countries such as Bangladesh is malnutrition, which is closely associated with shigellosis and a high mortality (1, 2). Children with severe malnutrition were found to have lower immune responses during acute *Shigella* infection than did children with moderate malnutrition (3).

A distinct feature of shigellosis is the occurrence of an acute inflammatory reaction of the intestinal mucosa that results from the invasion of the epithelial layer and lamina propria by *Shigellae* (4). Some of the key events in the inflammatory process in shigellosis are activation of neutrophils, apoptosis of macrophages, release of proinflammatory mediators, and oxidative damage of the tissue (5–7). Zinc is known to affect multiple aspects of the immune system, from the skin barrier to normal cell development and growth. Zinc is a major intracellular regulator of apoptosis that acts as an antioxidant, protecting cells from oxidative damage during immune activation (8, 9). Cellular zinc concentration is important in maintaining membrane integrity (8). The role of zinc as an antiinflammatory agent has long been suggested. Zinc improves intestinal wound healing and epithelial repair in zinc-deficient patients with inflammatory bowel diseases as well as in experimental animals (10, 11). Zinc supplementation trials showed reduction in the incidence, severity, and duration of acute and persistent diarrhea and dysentery (12). Zinc supplementation for >1 mo was shown to increase cell-mediated immunity in malnourished children (13) and increase seroconversion to vibriocidal antibody response after vaccination with oral cholera vaccine (14, 15). Recently, we showed that a 14-d course of zinc supplementation during acute shigellosis in moderately malnourished children significantly enhanced the lymphocyte proliferation response and increased antigen-specific antibody titers (16). The aim of this study was to investigate the in vivo modulating effects of zinc on the serum killing of *Shigella*, the phenotypic characteristics of peripheral blood mononuclear cells (PBMCs) and innate mediators contributing to tissue damage, and the resolution of infection during shigellosis in the same group of patients.

SUBJECTS AND METHODS

Subjects

Pediatric patients aged 12–59 mo who were attending the Clinical Research Service Center at the International Centre for Health and Population Research, Bangladesh (ICDDR,B) and who had bloody mucoid stool and severe abdominal cramps were screened for the study. Stool samples were examined microscopically and cultured for enteric bacterial pathogens (7). Malnutrition in children was assessed by using the US National Center for Health Statistics growth reference chart (17). Children with mild to moderate malnutrition (defined as < -1 but > -2 and < -2 but > -3 weight-for-height z scores, respectively) who had experienced diarrhea for 0–5 d and whose stool tested positive for *Shigella flexneri* were included in the study. Exclusion criteria included the following: measles infection in the previous 6 mo; presence of obvious systemic illnesses such as pneumonia, meningitis, septicemia, leukemoid reaction, and hemolytic uremic syndrome; previous zinc supplementation; severe malnutrition (defined as < -3 weight-for-height z score); living beyond > 2 h travel distance from the Clinical Research Service Center; and refusal of consent.

The number of subjects required per group was calculated by using the normal approximation of 2 independent means of equal sample size. On the basis of a mean ($\pm$SD) difference of 3.5 $\pm$ 4.7 cm in linear growth, to achieve a target difference of 30% with 5% significance and 80% power, the number of patients needed for study in each group was estimated to be 30. To achieve a target mean difference of 3.5 $\pm$ 4.7 cm in linear growth, with 5% significant and 80% power, the number of patients needed for study in each group was estimated to be 30. To achieve a 30% higher stimulation index of lymphocyte proliferation response to mitogen—considering the mean difference of 22 $\pm$ 36 cpm (13) on the basis of the same statistical power—our estimated sample size was 27 subjects per group.

The study was approved by the Ethical Review Committee of the ICDDR,B. According to the guidelines of the committee, written informed consent was obtained from the guardian of each child before enrollment. Samples from these patients have been used in another study (16).

Study design

The study was designed as a double-blind, placebo-controlled trial in which patients were randomly assigned to 1 of 2 treatment groups: the zinc group received elemental zinc with multivitamins plus calcium syrup, and the control group received multivitamin plus calcium syrup but not the elemental zinc. A permuted block randomization procedure was performed to ensure balance over time by removing the chances of seasonal effects (18). Briefly, starting with a block size of 6, there were 15 possible arrangements with 2 treatments for equal number of subjects. For each block, 1 of the 15 possible arrangements was randomly chosen. The pharmaceutical company that supplied the supplements (Acme Laboratories, Dhaka) was responsible for the blinding. Double blinding was done by labeling identical bottles of syrup with numbers and allocating the bottles to 2 groups of children. The zinc syrup containing zinc and multivitamins plus calcium was masked for taste and flavor and was indistinguishable in consistency, appearance, and taste from the placebo syrup containing multivitamins plus calcium.

Each 5 mL of the syrup contained 3000 IU vitamin A, 600 IU vitamin D, 1.2 mg thiamine, 2.0 mg riboflavin, 6.0 mg nicotinamide, and 6.0 mg calcium. The zinc preparation contained zinc acetate (20 mg elemental zinc/5 mL) mixed in multivitamins plus calcium syrup. The experimental group received the zinc syrup containing multivitamins plus calcium (given at twice the US RDA; 19) in 2 divided doses given daily between meals for 14 d starting from the day of admission. The control group received multivitamins plus calcium. All pediatric patients received pivmecillinum at 60 mg $\cdot$ kg body wt^{-1} $\cdot$ d^{-1} in 4 divided doses for 5 d. During hospitalization, all children received a standardized diet of 100–125 kcal and 3–4 g protein in mixed food $\cdot$ kg body wt^{-1} $\cdot$ d^{-1}. The zinc content of the consumed diet was calculated to be $\approx$7–8 mg/d on the basis of zinc values for the nonenriched single food items derived from Bangladesh food composition (20) and the USDA Nutrient Database for Standard References (*see* http://www.nal.usda.gov/fnic/foodcomp/). Health assistants fed the children with the supplements during their stay at the study ward. The zinc group thus received 280 mg zinc in the supplement and $\geq$49 mg during the 7-d stay in the hospital. The control group received only 49 mg zinc during the 7 d in the hospital. At the time of discharge on day 7, the mothers were given bottles of syrup and instructions to feed the supplement to the child daily between meals at home for the next 7 d. On day 14, a health assistant visited the household, measured the amount of syrup taken, and asked whether the mother had encountered any problems when feeding the syrup.

Collection of specimens

Venous blood samples were obtained from each child at admission (day 1, which was 3–5 d after the onset of diarrhea), before any supplementation was given, and at 7 and 30 d after admission, when the child was brought to the hospital for follow-up sampling. Blood was collected in heparin-coated sterile vials (Vacutainer System; Becton Dickinson, Rutherford, NJ) in the study ward of the Clinical Research Service Center and was brought to the laboratory immediately after collection at room temperature. PBMCs were isolated from heparinized blood with the use of Ficoll-Paque density gradient centrifugation at 1700 rpm for 25 min at 24 °C (Pharmacia-Upjohn, Uppsala, Sweden). Blood was also collected in trace element–free vials (Venoject II; Terumo Europe NV, Leuven, Belgium), serum was separated, and an aliquot was frozen at −70 °C. The time between blood collection and serum separation was usually <2.5 h. Rectal biopsy specimens, taken 10–12 cm from the anus, were obtained during sigmoidoscopy (model CLE-10, Olympus Optical Co, Ltd, Tokyo) with the use of Megabite endoscope biopsy forceps (Microvasive Division, Boston Scientific Corporation, Boston) in patients on days 1 and 7. At each time point, a total of 4 pieces of rectal pinch biopsy tissue were obtained. Two pieces were fixed in buffered formaline and embedded in paraffin, sectioning was done in a microtome (RM 2055; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) at 3-μm thickness, and the section was mounted on glass slides (Superfrost/plus; Menzel-Glaser Glasbearbeitungswerk GmbH & Co, Braunschweig, Germany). Two pieces were collected in Histocon (Histolab, Goteberg, Sweden), snap-frozen in liquid nitrogen, and kept at −70 °C until they were used for cryostat sectioning (CM 3000; Leica Microsystems Wetzlar GmbH). Frozen rectal tissues were sectioned at 6-μm thickness, mounted on glass slides (Superfrost/plus), and fixed with freshly prepared 2% (wt/wt) formaldehyde (Sigma

Chemicals, St Louis) in phosphate-buffered saline (PBS; pH 7.4). After being washed, the slides were air-dried and kept at $-20\ ^{\circ}C$ until their use.

Serum zinc concentrations

Serum zinc concentrations were measured by using flame atomic absorption spectrometry on a Flame Atomic Absorption Spectrophotometer (Model 6501S; Shimadzu Corporation, Tokyo) and using zinc standard solution (BDH Spectrosol, Poole, United Kingdom). Data were expressed as μmol/L. Routine CVs of the analytic method for serum zinc concentration were based on high and low values ranging from 12.23 to 30.59 μmol/L. The CV for serum zinc concentration was 0.84 with precision at 2 SD.

Serum shigellacidal assay

The serum shigellacidal assay similar to that used for *Vibrio cholera* O1 (21) was modified to optimize it for *Shigella flexneri*. Guinea pig complement (Sigma) was used at a dilution of 1:5, and *Shigella* bacteria were used at a concentration of 1×10^3 colony-forming units/mL. *Shigella flexneri* Y strain, a patient isolate from the ICDDR,B hospital that showed the typical biochemical and serologic traits of *S. flexneri* and tested positive for Congo red binding and on the Séreny test, was chosen for the bactericidal assay. Bacteria grown in trypticase soy broth (Difco, Detroit) at 37 °C for 3 h was suspended in physiologic saline to an optical density of 0.4 at 600 nm (1×10^8 colony-forming units/mL); 20 μL of this suspension (final dilution, 1:150; 1×10^3 colony-forming units/mL) was added to 600 μL of guinea pig complement (final concentration, 1:5) and 2.38 mL of Muller Hinton broth to make a final volume of 3 mL. The mixture was applied to microtiter plates (Nunc, Roskilde, Denmark) containing serum samples (starting dilution, 1:10) that were serially diluted by 50% (50 μL/well) serum samples (starting dilution, 1:10). Serum samples were previously heat treated at 56 °C for 30 min to inactivate complements. Muller Hinton broth (100 μL) was added to each well, and the mixture was incubated in a shaker incubator (200 rpm) at 37 °C for 16 h. The optical density of the plates was measured at 595 nm. The titer of the serum was defined as the reciprocal of the last dilution in which no growth was evident by visual inspection (20). A $\geq$4-fold increase in antibody titers from baseline was defined as seroconversion (15, 22).

Cell preparation, staining, and flow cytometric analysis

Lymphocyte subpopulations were identified by immunophenotyping through the analysis of specific cell surface markers using flow cytometry. PBMCs (1×10^6 cells/mL) were either stained directly or stimulated with phorbol myristate acetate (25 ng/mL) in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine sera, 2 mmol glutamine/L, 1 mmol sodium pyruvate/L, 100 U penicillin/mL, and 100 μg streptomycin/L (GIBCO BRL) for 4 h in 37 °C and 5% CO_2. After being washed in phosphate-buffered saline (pH 7.4), cells were suspended (1×10^5 cells/mL) in PBS containing 2% heat-inactivated pooled human AB serum and stained with triple combinations of monoclonal antibodies for 30 min at 4 °C. After washing, the cells were resuspended in PBS containing 1% paraformaldehyde and analyzed within 4 h. Negative controls were included in each experiment. Three-color fluorescence analysis was performed by using a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA). Before data acquisition, instrument settings were checked and optimized by using CaliBRITE beads (Becton Dickinson). Data acquisition and analysis were conducted with CELLQUEST software (version 3.3; Becton Dickinson). All samples were analyzed by setting appropriate forward and side scatter gates around the lymphocyte population with back gating on CD45$^+$CD14$^-$ cells.

Monoclonal antibodies

The following antibodies were used in the study for the phenotype analysis by 3-color flow cytometry: anti-CD45 (pan leukocyte), anti-CD14 (monocytes), immunoglobulin G (IgG) 1, IgG2 (negative isotype control), anti-CD3 [pan T cells, peridinin chlorophyll protein (PerCP), and fluorescein isothiocyanate (FITC)], anti-CD4 [helper or inducer T cells, phycoerythrin (PE), and PerCP], anti-CD8 (suppressor or cytotoxic T cells, FITC, PE, and PerCP), anti-CD20 (pan B cells and FITC), anti-CD38 (activated or immature T and B cells and PE), HLA-DR (antigen-presenting cells, B cells, activated T cells, and PerCP), anti-CD25 (interleukin 2R, activated T and B cells, and PE), anti-CD69 (early activation marker and PerCP), anti-CD45RA (naïve T cells and FITC), anti-CD45RO (memory T cells and PE), anti-CD56 [neural cell adhesion molecule, pan natural killer (NK) cells, and PE], anti-CD16 (NK cells, Fc γ receptor III, and FITC), and anti-CD57 (human NK-1, large granular T lymphocytes and NK cells, and FITC). All antibodies were purchased from Becton Dickinson. The following combinations were used to define the different cell populations: CD3-FITC/CD4-PE/CD8-PerCP (CD4 and CD8 cells among pan T cells), CD45RA-FITC/CD45RO-PE/CD4-PerCP (naïve and memory cells within helper T cells), CD45RA-FITC/CD45RO-PE/CD8-PerCP (naïve and memory cells within cytotoxic T cells), CD20-FITC/CD38-PE/HLA-DR-PerCP (activated B cells), CD3-FITC/CD8-PE/CD69-PerCP (early activation in T cells), CD8-FITC/CD25-PE/CD4-PerCP (activated cytotoxic and helper T cells), CD57-FITC/CD56-PE/CD8-PerCP (NK cells and cytotoxic cells), CD16-FITC/CD56-PE/CD3-PerCP (NK cells), CD8-FITC/CD56-PE/CD69-PerCP (activated NK cells), and CD8-FITC/CD57-PE/CD69-PerCP (23; also, *see* www.ncbi.nlm.nih.gov/prow/).

Histopathologic tests

Formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin. The histopathologist (MM), unaware of the supplementation type, examined coded sections from each specimen. For evaluation of the biopsy specimen, histopathological features as described earlier were selected (24). On the basis of these features, histopathological changes were evaluated as normal, chronic, mild, moderate, or severe.

Detection of apoptotic cells in tissues

Apoptotic cells were visualized by using the terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end-labeling (TUNEL) method with the ApoTACS in situ DAB apoptosis detection kit (R&D Systems, Minneapolis) (25). Briefly, after nonspecific peroxidase activity in tissue was blocked with the use of hydrogen peroxide, terminal deoxynucleotidyl transferase and dUTP digoxigenin were added to the slides and incubated for 1 h at 37 °C. After the reaction was stopped, slides were incubated in

peroxidase-labeled antidigoxigenin and then developed in 3,3′-diaminobenzidine (Sigma Chemical Co). Slides were counterstained with hematoxylin and mounted.

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and stained with the following antibodies as described previously (7): monoclonal mouse anti-human myeloperoxidase (1:80; Dako A/S, Glostrup, Denmark), rabbit antiinducible nitric oxide synthase (1:250; Santa Cruz Biotechnology Inc, CA), antisuperoxide dismutase, anti-Ki67, anti-caspase-3, and anti-CD8 (all: Dako A/S). As a control, specific antibodies were replaced by irrelevant isotype-matched control antibodies.

Semiquantitative imaging

Immunohistochemical staining of specific enzymes in rectal tissues was examined with the use of a DMLB microscope (Leica Microsystems Wetzlar GmbH) equipped with a 3CCD color camera (Sony Corporation, Tokyo). Each image was examined in a Quantimet 570 image analyzer (Leica Microsystems Wetzlar GmbH) that was directed by a PC computer system. The standards were set for positive as well as negative cells. The positive staining of enzymes in rectal tissue sections were defined by computer-assisted analysis of video microscopic images as described earlier (7). The acquired image was divided into 512-by-512 pixels, and each pixel was expressed in square micrometers (area) after calibration with the current magnification. The data acquired were imported to Microsoft EXCEL. For each tissue section, at 40× magnification of ≥20, 0.4-by-10^5–μm^2 fields were investigated, and the average was used for staining each enzyme in each tissue section. The automated video microscopic analysis allowed for quantification of positive immunoreactivity relative to the total cell area of the tissue section, and the results were expressed as the percentage of the ratio of positive to total pixels.

Statistical analysis

Statistical analyses were done by using the statistical software packages SIGMASTAT (version 2.03; Jandel Scientific, San Rafael, CA) and SPSS for WINDOWS (release 10; SPSS Institute, Chicago). Data were expressed as geometric means ± SEMs or ranges. The chi-square test was used for histologic data and serum shigellacidal responses. Repeated-measures analysis of variance (ANOVA) was used to determine the proportion of cells in the blood and the antibody titers in serum in the zinc and control groups. In this procedure, responses on days 1, 7, and 30 were used as within-subject variables, and treatment was used as the between-subject factor. Greenhouse-Geisser epsilon values were used in all analyses. The main effect of treatment was compared by using Bonferroni's procedure for CI adjustment. In the variable estimate, zinc group was used as the reference category. In all cases, the error variance of the dependent variable was obtained equally across groups and was not significant by Levene's test of homogeneity. For evaluating group differences in the different immunoreactive tissue markers, analysis of covariance was used after adjustment for subject variability on entry into the study. For within-group (between-days) comparisons, one-factor ANOVA was performed.

TABLE 1
Baseline characteristics of pediatric patients with shigellosis receiving zinc therapy or placebo[1]

Features	Zinc group ($n = 28$)	Control group ($n = 28$)
Age (mo)	20 ± 1.9	25 ± 2.4
Duration of diarrhea (d)	5 ± 0.6	4 ± 0.4
Duration of fever (d)[2]	4.8 ± 0.7	4.4 ± 0.4
Stool (no./d)	5.2 ± 1.2	6.9 ± 1.4
Serum zinc ($\mu mol/L$)	9 ± 0.01	8.68 ± 0.01
Weight-for-height z score[3]	-1.91 ± 0.75	-1.85 ± 0.99
Weight-for-age z score	-2.49 ± 0.91	-2.62 ± 0.78
Height-for-age z score	-1.68 ± 1.3	-1.94 ± 0.83

[1] All values are geometric $\bar{x} \pm$ SEM, except z scores, which are $\bar{x} \pm$ SD. There were no significant differences in the baseline characteristics between the two groups.

[2] Fever was defined as a temperature >37.8 °C.

[3] Malnutrition in children was assessed by using the National Center for Health Statistics growth reference (17). Mild to moderate malnutrition in children was defined as <−1 but >−2 and < −2 but > −3 weight-for-height z scores, respectively.

RESULTS

Subjects

A total of 60 patients who were infected with different *S. flexneri* species (ie, *S. flexneri* 1b, 1c, 2a, 2b, 3a, Y, and IV-1) were initially enrolled in the study. Four patients did not complete the follow-up visits. At the end of the study, there were 28 patients in the zinc group and 28 in the control group, and their mean age was 23 mo (25th–75th percentiles: 15–30 mo). Baseline characteristics of patients are given in **Table 1**. There were no significant differences in any of the observed baseline features between the zinc and the control groups. As reported earlier in the same group of patients, there was no significant zinc supplementation × time interaction for serum zinc concentrations, but there was a significant main effect of time and a significant main effect of treatment (both: $P < 0.01$) (16). C-reactive protein was also measured in the serum of these patients; zinc supplementation did not have a significant effect on serum C-reactive protein concentrations, and only time showed a significant effect.

Serum-mediated killing of *Shigella*

Repeated-measures ANOVA was used to determine the antibody titers in the zinc and control groups. No significant interaction or treatment effects were obtained (**Table 2**). With the use of a ≥4-fold increase in shigellacidal antibody titers from the baseline as cutoff for seroconversion to shigellacidal antibody response, the proportion of children with serum bactericidal activity against *Shigella* was found to be significantly higher in the zinc group than in the control group on day 30 (**Table 3**). Zinc supplementation resulted in seroconversion in 52% of children at day 7 and in 73% of children at day 30. In the control group, seroconversion was seen in 36% of the children at day 7, and that proportion was unchanged at day 30 (Table 3).

Lipopolysaccharide-specific (LPS-specific) IgG titers were determined previously (16). Lipopolysaccharide from *S. flexneri* Y (that represents the backbone structure of all but the *S. flexneri* serotype 6 O repeating unit) was used for measuring LPS-specific antibody titers (26). When a ≥2-fold increase in LPS-specific serum IgG titers among those who seroconverted was

TABLE 2
Serum shigellacidal antibody titers in *Shigella*-infected patients receiving or not receiving zinc therapy at various intervals after the onset of diarrhea[1]

Comparison groups	Time after onset of diarrhea		
	Day 1	Day 7	Day 30
Zinc group	14 (10–20)	45.7 (30–69)	34.4 (21.5–55)
Control group	21.6 (14.6–32)	49.8 (34–73)	28.3 (19.8–40.5)

[1] All values are geometric $\bar{x}$; minimum–maximum in parentheses. $n =$ 27, 27, and 26 for the zinc group and 26, 26, and 25 for the control group on days 1, 7, and 30, respectively. Data were log transformed, and two-factor repeated-measures ANOVA was used to measure serum shigellacidal antibody titers in the zinc and the control groups. No significant interaction or treatment effects were found.

examined, 73.6% (14 of 19 subjects) responded in the zinc group compared with 56% (5 of 9 subjects) in the control group after 30 d. However, the differences were not significant.

Effect of zinc supplementation on the proportion of circulating B cells

Repeated-measures ANOVA was used to determine the proportions of phenotypic markers of PBMCs in the zinc and the control groups. In this procedure, treatment was used as the between-subject factor, and responses on days 1, 7, and 30 were used as the within-subject variable.

For CD20$^+$ responses, within-subject effects showed a significant ($P = 0.02$) time × treatment interaction, and between-subjects effects showed a significant ($P = 0.029$) treatment effect, as shown in **Table 4**. The proportion of CD20$^+$ lymphocytes in the zinc group on day 7 was significantly ($P = 0.001$) higher than that in the control group. However, the proportions of CD20$^+$ cells on day 1 and day 30 did not differ significantly ($P = 0.054$ and 0.86, respectively) between these 2 groups.

For CD20$^+$CD38$^+$ responses, the within-subject effect showed a significant ($P = 0.021$) time × treatment interaction, but between-subjects effects showed a nonsignificant ($P = 0.15$) treatment effect (Table 4). The proportion of CD20$^+$CD38$^+$ cells in the zinc group on day 7 was significantly ($P = 0.007$) higher than that in the control group, but the differences were not significant on day 1 and day 30 ($P = 0.172$ and 0.379, respectively). No significant main effect of time or treatment was observed on the percentage of markers associated with pan T cells,

TABLE 3
Proportion of *Shigella*-infected pediatric patients receiving zinc therapy or placebo who had shigellacidal antibody response (≥4-fold increase in antibody titers from baseline)[1]

Comparison groups	Children with response			
	Day 7	P[2]	Day 30	P[2]
	%		%	
Zinc group	52 (14 of 27)	0.3	73 (19 of 26)	0.01
Control group	36 (9 of 25)		36 (9 of 25)	

[1] Number of subjects in parentheses.
[2] Chi-square test or Fisher's exact test was used for determination of P value. Difference was significant at $P < 0.05$.

TABLE 4
Proportions of B lymphocytes and plasma cells in *Shigella*-infected patients who did or did not receive zinc therapy at various intervals after the onset of diarrhea[1]

	Time after onset of diarrhea		
	Day 1	Day 7	Day 30
	%		
CD20$^+$			
Zinc group	29.6 ± 4.6^a [15]	29.7 ± 2^a [15]	16.3 ± 3.3^a [14]
Control group	20.6 ± 4^a [15]	14.6 ± 2.5^b [14]	19.3 ± 3.8^a [14]
CD20$^+$CD38$^+$			
Zinc group	22.6 ± 4.9^a [15]	25.6 ± 2^a [15]	14.6 ± 3^a [14]
Control group	14.2 ± 2.9^a [15]	14 ± 2.4^b [14]	18.3 ± 3.7^a [14]

[1] All values are geometric $\bar{x} \pm$ SEM; n in brackets. Responses on days 1, 7, and 30 were used as the within-subject variable, and treatment was used as the between-subjects factor. For CD20$^+$ responses, the within-subject effects showed a significant ($P = 0.02$) treatment × time interaction, and the between-subjects effects showed a significant ($P = 0.029$) treatment effect. For CD20$^+$CD38$^+$ responses, the within-subject effects showed a significant ($P = 0.021$) treatment × time interaction, but the between-subjects effects showed a nonsignificant ($P = 0.159$) treatment effect. For a given day, means with different superscript letters are significantly different, $P < 0.05$ (two-factor repeated-measures ANOVA with Bonferroni's procedure for CI adjustment).

helper T cells, cytotoxic T cells, NK cells, activated T and NK cells, or the ratio of naïve to memory T cells.

Morphometric analyses

The number and percentage of patients with different grades of inflammation and histopathological features during the acute and convalescent stages of the disease are shown in **Table 5**. No significant differences in any of the histopathological features studied were observed between the 2 groups.

TABLE 5
Morphometric analyses of the rectal mucosa of *Shigella*-infected patients who did or did not receive zinc therapy[1]

Histopathologic features	Zinc group		Control group	
	Day 1 ($n = 28$)	Day 7 ($n = 25$)	Day 1 ($n = 28$)	Day 7 ($n = 26$)
Severe-to-moderate colitis [n (%)]	16 (57)	6 (24)	17 (61)	7 (27)
Mild colitis [n (%)]	9 (32)	10 (40)	9 (32)	9 (35)
No colitis [n (%)]	3 (11)	9 (36)	2 (7)	10 (38)
Mucus				
Depletion [n (%)]	21 (75)	3 (11)	19 (67)	7 (25)
Increased [n (%)]	4 (14)	23 (82)	6 (21)	18 (64)
Mitosis, increased [n (%)]	26 (93)	21 (75)	26 (93)	26 (93)
Cryptisis, present [n (%)]	19 (68)	12 (43)	16 (57)	7 (25)
Crypt abscess, present [n (%)]	13 (47)	6 (21)	5 (18)	0
Surface epithelial cells				
Short [n (%)]	17 (61)	2 (7)	16 (57)	2 (7)
Longer [n (%)]	0	13 (46)	3 (11)	12 (43)

[1] Chi-square test or Fisher's exact test was applied in comparing groups who did or did not receive zinc therapy. No significant differences in any of the histopathologic features studied were observed between the groups.

TABLE 6
Comparative analyses of cell surface expression of enzymes and protein markers in the rectum of *Shigella*-infected patients who did or did not receive zinc therapy[1]

	Zinc group		Control group	
Markers	Day 1 (n = 28)	Day 7 (n = 25)	Day 1 (n = 28)	Day 7 (n = 26)
MPO	2.3 ± 0.39	0.49 ± 0.44	1.2 ± 0.4	0.2 ± 0.11
iNOS	1.0 ± 0.2	0.6 ± 0.24	2.9 ± 1.3	1.2 ± 0.6
SOD	0.27 ± 0.06	0.13 ± 0.06	0.15 ± 0.08	0.11 ± 0.04
CD8	1.34 ± 0.23	2.43 ± 0.7	1.2 ± 0.6	1.8 ± 0.7
Ki67	1.34 ± 0.45	1.55 ± 0.5	0.9 ± 0.34	0.93 ± 0.15
TUNEL	0.07 ± 0.02	0.03 ± 0.01	0.06 ± 0.03	0.04 ± 0.04
Caspase-3	1.87 ± 0.34	1.54 ± 0.14	1 ± 0.18	1.95 ± 0.25

[1] All values are geometric $\bar{x}$ ± SEM. MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; CD8, cytotoxic T cells; Ki67, proliferation marker; TUNEL, apoptotic cells. Quantification of immunoreaction-positive area relative to the total tissue section was determined by a computerized image–analyzing technique, and the results were expressed as the percentage of the ratio of positive area to the total area. The average studied area for each section was 12.6×10^5 μm^2 (±10%). After adjustment for day 1 values (as covariate), analysis of covariance was applied to evaluate group differences in the various protein and enzyme markers. No significant differences in the quantitative tissue expression of these markers were found between the two groups.

Effect of zinc supplementation on the tissue expression of innate mediators in rectum

Tissue expression of markers for myeloperoxidase, superoxide dismutase, inducible nitric oxide synthase, CD8, proliferation marker Ki67, apoptotic (TUNEL-positive) cells, and caspase-3 was examined in the rectal biopsy specimens obtained from patients on days 1 and 7. After adjustment for day 1 values (as covariate), analysis of covariance was applied to evaluate group differences in the various tissue markers. No significant differences in the quantitative tissue expression of the above markers were found between the 2 groups (**Table 6**).

DISCUSSION

The study showed that a 14-d course of elemental zinc given as an adjunct therapy to antimicrobial treatment during the acute phase of bacillary dysentery enhances seroconversion to shigellacidal antibody titers and increases the proportions of B lymphocytes and plasma cells. On its own, zinc has an in vitro antimicrobial effect on various bacteria (27, 28), and zinc in combination with certain antimicrobials can also potentiate the actions of those antimicrobials (29, 30). Local application of zinc at the site of infection or abscess was also shown to inhibit bacteria growth (31, 32). However, literature on the in vivo effects of zinc supplementation on the antibacterial activity in humans is limited. Greater serum bactericidal activity against *Vibrio cholera* spp was observed in healthy children and adults immunized with a killed oral cholera vaccine who were given zinc supplementation before vaccination than in those who were not given zinc (14, 15). The findings of the present study suggest that zinc supplementation given as adjunct therapy may improve recovery by boosting the pathogen-specific functional antibody response.

Serum factors responsible for killing of *Shigellae* have been shown to consist predominantly of antibodies and the complement factors (33). In the current study, serum samples were heat-treated for inactivation of complements. Lipopolysaccharides and their components were shown to confer sensitivity of *Shigella* to killing action of serum (34, 35). However, we did not observe any correlation between LPS-specific IgG titers (16) (data not shown) and bactericidal antibody titers, probably because lipopolysaccharide from serotype-specific *S. flexneri* strains was not used. Instead, lipopolysaccharide from *S. flexneri* Y (representing the backbone structure of all but the *S. flexneri* serotype 6 O repeating unit) was used for measuring antibody titers (26). Because the serum bactericidal antibody response increased with time in both groups, it appears that specific immunity may play a substantial role. However, factors such as phagocytosis, antibacterial peptides, and other intrinsic molecules also play a significant role in the innate immunity to shigellosis (36–38). Zinc deficiency is known to affect certain mediators of innate immunity, such as the function of neutrophils, NK cells, and complements (9, 39).

In the current study, zinc therapy during shigellosis had a significant effect on the phenotypic distribution of B lymphocytes and plasma cells. This was in line with previous findings of greater antigen-specific antibody responses in zinc-supplemented patients with shigellosis than in the control group (16). Antibody production by B cells is dependent on zinc. During zinc deficiency, antibody production is disturbed, the absolute number of B cells is reduced, mature B lymphocytes undergo apoptosis, and the proportion and functions of antigen-presenting cells are altered (39–41). Evidence of the effect of zinc supplementation on the increase in salivary and serum total IgA has been found in malnourished infants (13, 42). The increase in the proportion of B lymphocytes and plasma cells in circulation after zinc therapy in shigellosis may indicate a reversal of the above processes that is due to zinc repletion or to restoration of impaired T cell function (43). The number and activity of NK cells are dependent on serum zinc concentrations (44). Zinc supplementation results in significantly greater numbers of cytotoxic and helper T and NK cells than are seen in the control group (45, 46). Immunologic memory is also influenced by zinc (9, 39). However, zinc therapy in the current study showed no effect on either the proportions of NK and cytotoxic cells or the ratios of helper to suppressor T cells and of naïve to memory cells in blood.

Zinc given as an adjunct therapy in the moderately malnourished children with shigellosis did not show a significant effect on histopathological features in the rectum. Our earlier studies in patients with shigellosis found that, even after clinical recovery, >1 mo is required for the inflammation in the rectum to subside to normal (47). The short period of 7 d in this study may be insufficient for the effect of zinc supplementation on reducing inflammation in the rectum to be seen. Zinc deficiency induces apoptosis (48) and enhances caspase-induced apoptosis that can be corrected by zinc supplementation (49). The application of a nitric oxide synthase inhibitor markedly reduced both the activity of inducible nitric oxide synthase and the inflammatory changes in the intestines in a zinc-deficient rat model (11). However, in the current study, no effect of zinc supplementation was seen on the reduction of expression of TUNEL-positive cells, capsase-3, or inducible nitric oxide synthase.

In conclusion, the findings suggest that a short period of zinc supplementation given as adjunct therapy during acute shigellosis had beneficial effects both clinically and in modulating the systemic humoral and cellular immune responses for increased host defense. Evidence of a possible role of zinc in attenuating intestinal tissue damage was not found.

REFERENCES

1. Bennish ML, Wojtyniak BJ. Mortality due to shigellosis: community and hospital data. Rev Infect Dis 1991;13(suppl 4):S245–51.
2. Teka T, Faruque AS, Fuchs GJ. Risk factors for deaths in under-age-five children attending a diarrhoea treatment centre. Acta Paediatr 1996;85:1070–5.
3. Azim T, Sarker MS, Hamadani J, et al. Effect of nutritional status on the lymphocyte responses in children with Shigella flexneri infection. Immun Infect Dis 1996;6:151–8.
4. Sansonetti PJ. Pathogenesis of shigellosis: from molecular and cellular biology of epithelial cell invasion to tissue inflammation and vaccine development. Jpn J Med Sci Biol 1998;51(suppl):S69–80.
5. Sansonetti PJ. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. III. Shigellosis: from symptoms to molecular pathogenesis. Am J Physiol Gastrointest Liver Physiol 2001;280:G319–23.
6. Raqib R, Wretlind B, Andersson J, Lindberg AA. Cytokine secretion in acute shigellosis is correlated to disease activity and directed more to stool than to plasma. J Infect Dis 1995;171:376–84.
7. Raqib R, Mia SM, Qadri F, et al. Innate immune responses in children and adults with Shigellosis. Infect Immun 2000;68:3620–9.
8. Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. Free Radic Biol Med 1990;8:281–91.
9. Shankar AH, Prasad AS. Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 1998;68(suppl):447S–63S.
10. Cario E, Jung S, Harder D'Heureuse J, et al. Effects of exogenous zinc supplementation on intestinal epithelial repair in vitro. Eur J Clin Invest 2000;30:419–28.
11. Cui L, Takagi Y, Wasa M, Sando K, Khan J, Okada A. Nitric oxide synthase inhibitor attenuates intestinal damage induced by zinc deficiency in rats. J Nutr 1999;129:792–8.
12. Bhutta ZA, Bird SM, Black RE, et al. Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. Am J Clin Nutr 2000;72:1516–22.
13. Castillo-Duran C, Heresi G, Fisberg M, Uauy R. Controlled trial of zinc supplementation during recovery from malnutrition: effects on growth and immune function. Am J Clin Nutr 1987;45:602–8.
14. Karlsen TH, Sommerfelt H, Klomstad S, et al. Intestinal and systemic immune responses to an oral cholera toxoid B subunit whole-cell vaccine administered during zinc supplementation. Infect Immun 2003;71:3909–13.
15. Albert MJ, Qadri F, Wahed MA, et al. Supplementation with zinc, but not vitamin A, improves seroconversion to vibriocidal antibody in children given an oral cholera vaccine. J Infect Dis 2003;187:909–13.
16. Raqib R, Roy SK, Rahman MJ, et al. Effect of zinc supplementation on immune and inflammatory responses in pediatric patients with shigellosis. Am J Clin Nutr 2004;79:444–50.
17. Jackson AA. Severe malnutrition. In: Warrel DA, Cox TM, Firth JD, Benz EJ Jr, eds. Oxford textbook of medicine. 4th ed. Oxford, United Kingdom: Oxford University Press, 2003:1054–61.
18. Pocock SJ. Methods of randomization. In: Pocock SJ, ed. Clinical trials: a practical approach. London: Wiley Medical Publication, 1987:66–89.
19. Food and Nutrition Board, Institute of Medicine. Dietary reference intake for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC: National Academy Press, 2001.
20. Hill D, Hassan N, Karim R, Duthie MR, eds. Tables of nutrient composition of Bangladeshi food, English version. Dhaka, Bangladesh: Helen Keller International, 1988.
21. Benenson AS, Saad A, Mosley WH. Serological studies in cholera. 2. The vibriocidal responses of cholera patients determined by microtechnique. Bull World Health Organ 1968;38:277–85.
22. Kotloff KL, Noriega F, Losonsky GA, et al. Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live oral Shigella flexneri 2a vaccine candidate attenuated by deletions in aroA and virG. Infect Immun 1996;64:4542–8.
23. Barclay AN, Brown MH, Law SKA, et al. The leukocyte antigen facts book. San Diego: Academic Press Inc, 1997.
24. Mathan MM, Mathan VI. Ultrastructural pathology of the rectal mucosa in Shigella dysentery. Am J Pathol 1986;123:25–38.
25. Raqib R, Ekberg C, Sharkar P, et al. Apoptosis in acute shigellosis is associated with increased production of Fas/Fas ligand, perforin, caspase-1, and caspase-3 but reduced production of Bcl-2 and interleukin-2. Infect Immun 2002;70:3199–207.
26. Ewing WH, Lindberg AA. Serology of Shigella. London: Academic Press, 1984.
27. Podbielski A, Spahr A, Haller B. Additive antimicrobial activity of calcium hydroxide and chlorhexidine on common endodontic bacterial pathogens. J Endod 2003;29:340–5.
28. Sawai J. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. J Microbiol Methods 2003;54:177–82.
29. Brading MG, Cromwell VJ, Jones NM, Baldeck JD, Marquis RE. Antimicrobial efficacy and mode of action studies on a new zinc/Triclosan formulation. Int Dent J 2003;53:363–70.
30. McCarthy TJ, Zeelie JJ, Krause DJ. The antimicrobial action of zinc ion/antioxidant combinations. J Clin Pharm Ther 1992;17:51–4.
31. Sohnle PG, Hahn BL. Effect of zinc-reversible growth-inhibitory activity in human empyema fluid on antibiotic microbicidal activity. Antimicrob Agents Chemother 2000;44:139–42.
32. Cho YH, Lee SJ, Lee JY, et al. Antibacterial effect of intraprostatic zinc injection in a rat model of chronic bacterial prostatitis. Int J Antimicrob Agents 2002;19:576–82.
33. Reed WP. Serum factors capable of opsonizing Shigella for phagocytosis by polymorphonuclear neutrophils. Immunology 1975;28:1051–9.
34. Doroszkiewicz W. Mechanism of antigenic variation in Shigella flexneri bacilli. IV. Role of lipopolysaccharides and their components in the sensitivity of Shigella flexneri 1b and its Lac+ recombinant to killing action of serum. Arch Immunol Ther Exp (Warsz) 1997;45:235–42.
35. Hong M, Payne SM. Effect of mutations in Shigella flexneri chromosomal and plasmid-encoded lipopolysaccharide genes on invasion and serum resistance. Mol Microbiol 1997;24:779–91.
36. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532–5.
37. Islam D, Bandholtz L, Nilsson J, et al. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat Med 2001;7:180–5.
38. Morgan DR, DuPont HL, Gonik B, Kohl S. Cytotoxicity of human peripheral blood and colostral leukocytes against Shigella species. Infect Immun 1984;46:25–33.
39. Ibs KH, Rink L. Zinc-altered immune function. J Nutr 2003;133:1452S–6S.
40. DePasquale-Jardieu P, Fraker PJ. Interference in the development of a secondary immune response in mice by zinc deprivation: persistence of effects. J Nutr 1984;114:1762–9.
41. Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J Nutr 1998;128:20–7.
42. Schlesinger L, Arevalo M, Arredondo S, Diaz M, Lonnerdal B, Stekel A. Effect of a zinc-fortified formula on immunocompetence and growth of malnourished infants. Am J Clin Nutr 1992;56:491–8.
43. Kreft B, Fischer A, Kruger S, Sack K, Kirchner H, Rink L. The impaired

immune response to diphtheria vaccination in elderly chronic hemodialysis patients is related to zinc deficiency. Biogerontology 2000;1: 61–6.

44. Ravaglia G, Forti P, Maioli F, et al. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged >90 y. Am J Clin Nutr 2000;71:590–8.

45. Sazawal S, Jalla S, Mazumder S, Sinha A, Black RE, Bhan MK. Effect of zinc supplementation on cell-mediated immunity and lymphocyte subsets in preschool children. Indian Pediatr 1997;34:589–97.

46. Chandra RK. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. Lancet 1992;340: 1124–7.

47. Raqib R, Lindberg AA, Wretlind B, Bardhan PK, Andersson U, Andersson J. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect Immun 1995;63:289–96.

48. Sunderman FW Jr. The influence of zinc on apoptosis. Ann Clin Lab Sci 1995;25:134–42.

49. Truong-Tran AQ, Ruffin RE, Zalewski PD. Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am J Physiol Lung Cell Mol Physiol 2000;279:L1172–83.