Effects of zinc supplementation as adjunct therapy on the systemic immune responses in shigellosis1–3

Muhammad J Rahman, Protim Sarker, Swapan K Roy, Shaikh M Ahmad, Jobayer Chisti, Tasnim Azim, Minnie Mathan, David Sack, Jan Andersson, and Rubhana Raqib

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 ≥4-fold increase in serum shigellocidal antibody titers from baseline used as the cutoff, the proportion of children with shigellocidal antibody response was greater in the zinc group than in the control group (P < 0.03). There was a significant (P = 0.02) treatment × 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 shigellocidal antibody response and increased the proportions of circulating B lymphocytes and plasma cells.

KEY WORDS Zinc, adjunct therapy, Shigella, serum killing, B cells, rectum

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 due to tissue 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.

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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 (±SD) difference of 3.5 ± 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 ± 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 ± 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 multivitamins 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 pimecillinum at 60 mg · kg body wt. · 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 · kg body wt. · d. · 1. The zinc content of the consumed diet was calculated to be ≈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 ≥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-Uipjoh, 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 formaldehyde 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 Glasebearbeitungswerk GmbH & Co, Braunschweig, Germany). Two pieces were collected in Histocon (Histolab, Goteborg, 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 °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 shigellicidal assay**

The serum shigellicidal 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 × 10⁸ 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 × 10⁹ colony-forming units/mL); 20 μL of this suspension (final dilution, 1:150; 1 × 10⁶ 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 ≥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 × 10⁶ 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 at 37 °C and 5% CO₂. After being washed in phosphate-buffered saline (pH 7.4), cells were suspended (1 × 10⁷ 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), and anti-CD56 [neural cell adhesion molecule, pan natural killer (NK) cells, and PE], anti-CD16 (NK cells, Fc γ receptor III, and FITC), anti-CD157 (human NK-1, large granular L lymphocytes and NK cells, and FITC), 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/](https://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 antidiogoxigenin 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 antiangiucil nitric oxide synthase (1:250; Santa Cruz Biotechnology Inc, CA), ant Superoxide 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.

**Semi-quantitative 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^-3-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 covariance (ANCOVA) 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

<table>
<thead>
<tr>
<th>Features</th>
<th>Zinc group (n = 28)</th>
<th>Control group (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>20 ± 1.9</td>
<td>25 ± 2.4</td>
</tr>
<tr>
<td>Duration of diarrhea (d)</td>
<td>5 ± 0.6</td>
<td>4 ± 0.4</td>
</tr>
<tr>
<td>Duration of fever (d)</td>
<td>4.8 ± 0.7</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Stool (no/d)</td>
<td>5.2 ± 1.2</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Serum zinc (μmol/L)</td>
<td>9 ± 0.01</td>
<td>8.68 ± 0.01</td>
</tr>
<tr>
<td>Weight-for-height z score</td>
<td>-1.91 ± 0.75</td>
<td>-1.85 ± 0.99</td>
</tr>
<tr>
<td>Weight-for-age z score</td>
<td>-2.49 ± 0.91</td>
<td>-2.62 ± 0.78</td>
</tr>
<tr>
<td>Height-for-age z score</td>
<td>-1.68 ± 1.3</td>
<td>-1.94 ± 0.83</td>
</tr>
</tbody>
</table>

1 All values are geometric x ± SEM, except z scores, which are x ± 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
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 0.397, respectively). No significant main effect of time or treatment was observed on the percentage of markers associated with pan T cells, 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 2
Serum shigellacidal antibody titers in *Shigella*-infected patients receiving or not receiving zinc therapy at various intervals after the onset of diarrhea

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>Time after onset of diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Zinc group</td>
<td>14 (10–20)</td>
</tr>
<tr>
<td>Control group</td>
<td>21.6 (14.6–32)</td>
</tr>
</tbody>
</table>

All values are geometric 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.

### 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

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>Time after onset of diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>CD20⁺</td>
<td></td>
</tr>
<tr>
<td>CD20⁺CD38⁺</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>14.2 ± 2.9⁺ [15]</td>
</tr>
</tbody>
</table>

All values are geometric x ± 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 = 0.15) 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).

### 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)

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>Children with response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>Zinc group</td>
<td>52 (14 of 27)</td>
</tr>
<tr>
<td>Control group</td>
<td>36 (9 of 25)</td>
</tr>
</tbody>
</table>

¹ Number of subjects in parentheses.
² 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 5
Morphometric analyses of the rectal mucosa of *Shigella*-infected patients who did or did not receive zinc therapy

<table>
<thead>
<tr>
<th>Histopathologic features</th>
<th>Zinc group (n = 28)</th>
<th>Control group (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe-to-moderate colitis [n (%)]</td>
<td>16 (57)</td>
<td>26 (10)</td>
</tr>
<tr>
<td>Mild colitis [n (%)]</td>
<td>9 (32)</td>
<td>10 (38)</td>
</tr>
<tr>
<td>No colitis [n (%)]</td>
<td>3 (11)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>Mucus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depletion [n (%)]</td>
<td>21 (75)</td>
<td>19 (73)</td>
</tr>
<tr>
<td>Increased [n (%)]</td>
<td>4 (14)</td>
<td>6 (23)</td>
</tr>
<tr>
<td>Mitosis, increased [n (%)]</td>
<td>26 (93)</td>
<td>26 (93)</td>
</tr>
<tr>
<td>Cryptitis, present [n (%)]</td>
<td>19 (68)</td>
<td>16 (62)</td>
</tr>
<tr>
<td>Crypt abscess, present [n (%)]</td>
<td>13 (47)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>Surface epithelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short [n (%)]</td>
<td>17 (61)</td>
<td>16 (62)</td>
</tr>
<tr>
<td>Longer [n (%)]</td>
<td>0</td>
<td>3 (12)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Zinc group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
<td>Day 1 (n=28)</td>
<td>Day 7 (n=25)</td>
</tr>
<tr>
<td>MPO</td>
<td>2.3 ± 0.39</td>
<td>0.49 ± 0.44</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.0 ± 0.2</td>
<td>0.6 ± 0.24</td>
</tr>
<tr>
<td>SOD</td>
<td>0.27 ± 0.06</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>CD8</td>
<td>1.34 ± 0.23</td>
<td>2.43 ± 0.7</td>
</tr>
<tr>
<td>Ki67</td>
<td>1.34 ± 0.45</td>
<td>1.55 ± 0.5</td>
</tr>
<tr>
<td>TUNEL</td>
<td>0.07 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>1.87 ± 0.34</td>
<td>1.54 ± 0.14</td>
</tr>
</tbody>
</table>

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 $12.6 \times 10^6 \mu m^2$ (±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 two 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 serum conversion to shigellosis 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 abscesses 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 *Shigella* 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) 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, antimicrobial 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 saliva 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, caspase-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.

We gratefully acknowledge the participation of the patients in the study. MJR processed specimens, performed laboratory experiments, and collected data. PS performed flow cytometry–associated experiments and fluorescence-associated cell sorting (FACS) data analyses. SMA performed the statistical analysis. SKR designed the study, provided substantial advice on analyses of data, and supervised patient management and clinical evaluation. TA provided significant advice on the design of the study and helped to secure funding. JC conducted patient selection and recruitment and clinical management of study patients. MM performed histopathological analyses. JA provided significant advice on immunological assays and flow cytometry. DS provided significant advice on the design and implementation of the study. RR was involved in designing the study, securing funding, supervising laboratory experiments, compiling and analyzing data, and writing the manuscript. None of the authors had personal or financial conflicts of interest.

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