Original Articles

EFFECT OF ZINC SUPPLEMENTATION ON CELL-MEDIATED IMMUNITY AND LYMPHOCYTE SUBSETS IN PRESCHOOL CHILDREN

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***Objective**: In a zinc supplementation trial (with a significant impact on diarrheal morbidity), to evaluate effect of zinc supplementation on cellular immune status before and after 120 days of supplementation. **Design**: A double blind, randomized controlled trial with immune assessment at baseline and after 120 days on supplement. **Setting**: Community based study in an urban slum population. **Subjects**: Randomly selected children (zinc 38, control 48), had a Multitest CMI skin test at both times. In 66 children (zinc 22, control 34), proportions of CD3, CD4, CD8, CD16, CD20 cells and the CD/CD8 ratio were also estimated using a whole blood lysis method and flowcytometry. **Intervention**: Zinc gluconate to provide elemental zinc 10 mg daily and 20 mg during diarrhea. **Main outcome results**: Regarding CMI, the percentage of anergic or hypoergic children (using induration score) decreased from 67% to 47% in the zinc group, while in the control group it remained unchanged (73% vs 71%)(p=0.05). The percentage of children deteriorating between first and second tests was significantly lower in the zinc group (13% vs 33%, p=0.03). Regarding lymphocyte subsets, the zinc group had a significantly higher rise in the geometric means of CD3 (25%, p=0.02), CD4 (64% p=0.001), and CD4/CD8 ratio (73% p=0.004) with no difference in CD8 and CD20. The rise in CD4 was significantly higher in the zinc as compared to the control group; the ratio of geometric means was 1.45 (95%CI, 1.03-2.01). **Conclusion**: Zinc supplementation improves cellular immune status, which may have been one of the mechanisms for observed impact of zinc supplementation on diarrheal morbidity.*

Key words: *Cell mediated immunity, Diarrhea, Zinc.*

IN developing countries, malnutrition and infectious diseases account for most of the deaths among preschool children(l- These conditions have been shown to have a bidirectional causal relationship in that an infectious illness results in deterio- cycle. ration in nutritional status and undernutrition leads to greater infectious disease morbidity(5-ll). Understanding of 4). the biological mechanisms mediating the interaction is important in the development of interventions to interrupt this vicious

Compromised immunocompetence, which has been found in malnutrition (12,13), and in single micronutrient deficiencies, such as those of vitamin A, zinc, folic acid, iron or iodine (14,15), may be an important mechanism for greater morbidity (16,17). In regard to immunity and infectious disease morbidity, zinc deficiency may be particularly important. Zinc deficiency has been shown to be common in developing country children with high rates of diarrhea and malnutrition (18). Animal experiments have clearly demonstrated that selective zinc deficiency results in diminution of thymus size and function, as well as impairment in T-cell dependant immune functions(19-21). These defects can be completely corrected by zinc repletion (19,22). Decreased immunocompetence has been shown to be a risk factor for diarrheal and respiratory morbidity(23,24) and zinc supplementation has been shown to reduce this morbidity (25). This study was designed within a community-based randomized controlled trial, in which zinc supplementation resulted in a significant reduction in diarrheal morbidity(18,25,26). It studied effects of zinc supplementation on the two most commonly used markers of cellular immune status to investigate possible mechanisms for the beneficial effect of zinc.

Subjects and Methods

Methods for the Main Study

The detailed methods of the supplementation trial have been presented in detail elsewhere (18,25). Briefly, from the children with diarrhea presenting to the dispensary in Kalkaji, a low socio-economic population of New Delhi, those 6 to 35 months old with reported passage of at least 4 unformed stools in the previous 24 hours, a diarrheal duration of <7 days, and permanent residence in Kalkaji were selected for inclusion. Exclusion criteria were children enrolled previously in this study, malnutrition sufficiently severe to require hospitalization, or refused consent. Study children were followed at home until recovery of the enrollment episode. A subset, selected at the time of initial randomization continued to be followed by household morbidity surveillance visits every fifth day for another 6 months.

Liquid supplements were made by Sandoz India Ltd. (Mumbai). Each 5 ml contained vitamins A (800 units), Bl (0.6 mg), B2 (0.5 mg), B6 (0.5 mg), D3 (100 IU), E (3 mg) and niacinamide (10 mg). In addition, the zinc preparation contained zinc gluconate (10 mg elemental zinc). A fixed dose of 5 ml per child was given daily for 6 months; during diarrheal illness this was increased to 10 ml to provide for excess zinc losses. The supplements were given by field workers at daily home visits. Weight was measured to 10 g with an electronic scale (SECA Corporation, Colombia, MD) by two independent observers; length or height was measured to 0.1 cm with length boards (Shorr Productions, Olney, MD). For dehydrated children the weight was repeated after hydration. A venous blood sample for zinc estimation was collected at enrollment and after 120 days using Monovette (Sarstedt, Newton, NC) trace element-free syringes and zinc-free heparin. Plasma was separated within 15 minutes of blood collection and aliquots transferred into trace element-free Eppendorf plastic tubes for storage at —20° C.

Methods for CMI Skin Test

A sample of 86 randomly selected children (zinc 38, control 48) were tested for CMI. The cell-mediated immune competence (CMI) was assessed once at the beginning of follow up and again after 120 days of supplementation using a multiple antigen skin test (Multitest CMI, Institute

Merieux, Lyon, France). The selection of children and application of the skin test was performed masked to treatment group allocation. The skin test system consists of a plastic device with eight multi-puncture heads capable of simultaneously administering seven test antigens (tetanus, diphtheria, streptococcus, tuberculin, Candida, trichophyton, and proteus) and a control (70% glycerol) substance (27). After the skin test system came to room temperature, it was applied to the interior surface of the child's forearm or thigh. The tests were read after 48 hours by one of the two physicians using a special caliper supplied with Multitest CMI. Two measurements of induration, perpendicular to each other, were taken for each antigen and control. A test antigen was considered positive if it produced an average induration of ≥2 mm more than the induration noted with glycerol alone. To assess reliability, duplicate blind measurements were taken by the two study physicians throughout the course of the study, the number of positive responses score showed more than 98% agreement. For the analysis, positive response to none of the seven antigens was defined as anergy, to only 1 antigen was defined as hypoergy and to 2 or more antigens was called normal(27,28). In addition, a score was computed by summation of the indurations of all positive responses; a score of zero was considered anergy, a score of 2-9 mm was considered hypoergy and a score of >9 mm was considered normal(27,28). For number of antigens positive and induration score, MANOVA was used to compare the two supplementation groups for the number of children in the three categories of cellular immune response, controlling for CMI status before supplementation. MacNemar's test was used to compare the proportion of anergic or hypoergic children before and after the supplementation in the two groups.

Methods of Estimation of Lymphocyte Subsets

In a sub-sample of 66 randomly selected children (zinc 22, control 34), lymphocyte subsets (CD3, CD4, CD8, CD16, CD20, CD4/ CD8 ratio) were estimated once at enrollment and again after 120 days of supplementation. At both of these times, 2 ml venous blood samples in EDTA (ethylenediamine tetra-acetate) were obtained. The samples were transported to the laboratory the same day and were processed using a whole blood lysis method (29). A 5 tube panel of antibodies was selected. The first tube (leucogate reagent for electronic gating) had FITC (CD45)+PE-(CD14), the second tube had unstained cells as negative control, the third had (helper/inducer T cells) FITC(CD3) + PE-(CD), the fourth had (cy-totoxic/suppressor T cells) FITQCD3) +PE(CD8) and the fifth tube had (B cells) FITC (CD20). We used Becton Dickinson (San Jose, California) immuno-cytometry monoclonal antibodies. For each sample, 5 tubes were prepared to which 10 µl of each of the two monoclonals (20 µl/tube) were added. After adding 100 µl of blood to the monoclonal antibody combination, vortex-ing for 15 seconds and incubating for 20-25 minutes in the dark at room temperature, the erythrocytes were lysed by an automated lysing instrument (the Q-prep work station) with standard Q-prep reagents (Coulter Cytometry, Hiatech Florida). The samples after this step were stored at 4°C until they were analyzed later the same day.

Samples were analyzed using a Becton-Dickinson flowcytometer calibrated with CALIBRITE beads. The Becton-Dickinson lysis II programme was used for analysis. The leucogate (CD45+CD14) fluorescent information was used to set an electronic gate around the lymphoid population,

which included at least 95% lymphocytes and less than 5% non lymphocytes (granulocytes, monocytes and debris). Quadrant markers were set using negative controls (unstained cells). Finally, the instrument was compensated using two separate positive controls for each color and 5,001-10,000 gated events were analyzed per sample.

Geometric means and standard deviations for percentages of each of the subsets and a paired difference between the baseline and post supplementation samples was estimated. Differences between the two supplementation groups at baseline were evaluated by a two tailed t-test applied on log transformed data. The within group paired differences between baseline and post supplementation were evaluated by a paired t-test. The ratio of mean difference and its 95% confidence limits were estimated using the Taylor series method.

Results

The zinc and the control groups were comparable for a number of assessed baseline characteristics including age, anthropometric status, breastfeeding status, plasma zinc levels and diarrheal morbidity in 2 months before enrollment in the sample of children selected for the CMI skin test and lymphocyte subset estimation,

CMI Skin Test Results

Using the number of positive antigens, anergic or hypoergic children in the zinc group decreased from 39.5% at baseline to 26.3% after 120 days of supplementation, but remained unchanged in the control group (Table I). Using the induration score, the percentage of anergic or hypoergic children in zinc group decreased from 61% to 47%, while in the control group it remained essentially unchanged (73% vs 71%) (p=0.05). Five children (13%) in the zinc group deteriorated (from normal to hypoergy or hypoergy to anergy) during supplementation in comparison to 16 children (33%) in the control group (p=0.03).

Lymphocyte Subset Results

At baseline there were no significant

TABLE I- *Effect of Zinc Supplementation on the Cellular Immune Responses*

CMI index	Zinc group (n=38)		Control group (n=48)	
	Before	After	Before	After
I. *Effect on the Number of Positive Responses (Induration ≥2 mm) out of 7 Antigens*				
Anergy (0)$	21.1*	15.8	27.1	25.0
Hypoergy (1)	18.4	10.5	18.8	18.8
Normal (≥2)	60.5	73.7	54.2	56.3
II. *Effect on Induration Score (Total mm of Positive Induration)*				
Anergy (0)	28.9	15.8#	29.2	25.0#
Hypoergy (2-9 mm)	31.6	31.6	43.8	45.8
Normal (>9 mm)	39.5	52.6	27.1	29.2

* Values give percentages
$ Refer to number of positive antigens
#MANOVA comparing zinc and control group (p>0.05) after correction for baseline differences.

differences between the zinc and the control groups in CD4 (ratio of geometric mean (RGM) 0.91, 95% CI 0.74-1.12), CD8 (RGM 0.85, 95% CI 0.71-1.24) or for CD4/CD8 ratio (RGM 0.90, 95% CI 0.65-1.25). In the zinc group there was a significant rise in CD3 (25%; p=0.02), CD4 (64%; p=0.001) and CD4/CD8 ratio (73%; p=0.004). There was a decline in CD8, which was not statistically significant *(Table II)*. The CD4 rise was significantly higher in the zinc as compared to the control group (RGM 1.45; 95% CI 1.03-2.01).

Discussion

In this study population, oral zinc supplementation for 120 days improved two classic parameters of cell-mediated immune competence-the number of circulating T-lymphocytes, especially CD4 cells,

TABLE II— *Effect of Zinc Supplementation on the Total Lymphocytes and Lymphocyte Subsets, B-Cells and NK-Cells.*

Cell subset	Zinc group (n=22)	Control group (n=34)	Ratio of paired difference (95% CI)
CD3 (Total Lymphocytes)			
Baseline	47.0	51.9	
Post supplement	59.1.	54.1	
Paired difference	1.25(1.03-1.54)	1.03	1.21(0.94-1.55)
CD4 (Helper T Cells)			
Baseline	20.3	24.0	
Post supplement	33.4	28.2	
Paired difference	1.64(1.29-2.10)	1.14	1.45(1.03-2.01)
CD8 (Suppressor T Cells)			
Baseline	23.8	25.3	
Post supplement	22.6	21.1	
Paired difference	0.95(0.72-1.25)	0.83	1.13(0.78-2.25)
CD4/CD8 Ratio			
Baseline	0.85	0.94	
Post supplement	1.48	1.30	
Paired difference	1.73(1.21-2.49)	1.31	1.32(0.78-2.25)
CD20 (B-Cells)			
Baseline	18.9	19.5	
Post supplement	22.4	21.3	
Paired difference	1.17(0.80-1.73)	1.09	1.07(0.70-1.66)
CD16 (NK Cells)			
Baseline	*7.7*	6.2	
Post supplement	2.7	3.0	
Paired difference	0.35(0.20-0.62)	0.49	0.73(0.34-1.55)

and the cutaneous delayed hypersensitivity reaction. The deterioration of CMI immune status in children between baseline and 120 days is consistent with our finding of worsening zinc status between baseline and 120 days in the unsupplemented group (25). In the main trial to which this study was added, we documented a reduction in diarrheal morbidity with zinc supplementation (18,25). The improvement in cellular immune status supports the possibility of this being a mechanism for this effect. The improvement in immune markers should be regarded as a general indication of improved immune status, since it does not actually measure immunity against specific pathogens responsible for diarrhea.

The results of this study are consistent with earlier reports of an impairment in delayed cutaneous hypersensitivity to recall antigens or chemical agents in zinc deficient states (30-32). Golden *et al.(33)* found improvement in CMI response to Candida antigen in malnourished children by application of 1% zinc sulfate ointment. An effect of zinc supplementation on delayed cutaneous hypersensitivity in acrodermatitis enteropathica(34) and in marasmic children during rehabilitation has also been documented(35). Zinc deficiency has been shown to be associated with atrophy of lymphoid tissues, reduced lymphocyte count and proportion of CD4 T-helper cells(36-38). Decreased lymphocyte proliferation(34) and reduced cytotoxic activity(39) has also been found in zinc deficiency states. All of these respond dramatically to repletion of zinc(40).

Our data do not indicate whether the beneficial influence of zinc is secondary to the correction of a latent zinc deficiency or to immunostimulation by zinc. Although we have baseline plasma zinc levels on most of the children in the trial the sample size of children on whom immune estimations were performed is not sufficient for any meaningful subgroup analysis. Nevertheless, there are indications that this was a population with zinc deficiency. In the main trial 37% of children had low plasma levels of zinc. The possibility of an immunostimulating effect of zinc supplementation, independent of zinc depletion, is suggested by several observations: in healthy young adults oral administration of zinc increases *in vitro* lymphocyte subpopulation in patients with lymphoblastic leukemia (42) and addition of zinc to drinking water of mice is associated with a time and dose dependant increase in mitogen stimulation of their spleen lymphocytes *in vitro* (43).

The observation of an increase in the number of circulating T-lymphocytes especially CD4 cells, after zinc supplementation may be explained by direct effect of zinc ion on the lymphocyte membrane affecting maturation and differentiation of T-lymphocytes or by a stimulation of thymus endocrine function. The first possibility seems less likely because *in vitro* experiments demonstrating a direct effect of zinc on the cell membrane requires concentrations one to two orders higher than those that can be achieved *in vivo* after supplementation with the doses of zinc we used(41,44). The alternative explanation of an effect of zinc on thymic epithelial cells, leading to an improvement of their secretory activity seems attractive. There is a marked atrophy of the thymus in children with protein energy malnutrition (PEM) with defects in CMI (45,46). The T-lymphocyte immaturity can be reversed with *in vitro* incubation with thymulin (47). These changes may in fact be an effect of zinc deficiency is suggested by the (a) contradictory results for effects on the immune system between laboratory experiments on PEM (impaired humoral immunity and augmented cellular immunity) and field studies on PEM (de-

fects of both cellular and humoral immunity)(15), and (b) experimental studies showing that zinc deficiency without PEM causes profound defects in cellular immunity with thymic hypoplasia(21). Recent work has made it apparent that thymic epithelial cells are source of a number of peptides and hormones which participate in a cascade of multimolecular and multicellular events influencing T-cell maturation(48). Once T-lymphocytes leave the thymus their differentiation and maturation is thought to be regulated by zinc-thymulin and deficiency of zinc-thymulin has been associated with secondary cellular immune deficiency and with immune senescence(48). Thus terminal deoxynucletidyl transferase or zinc-thymulin have been suggested as possible mechanisms by which zinc may be affecting T-cell development and function(48).

The previous observation that cellular immune status is a risk factor for diarrhea(23,24), the observed impact of zinc supplementation on diarrhea(18,25,26) and the demonstrated effect of zinc on CMI support the hypothesis that zinc deficiency alone or in combination with other deficits increases susceptibility to infections through a depression in immune function. Improvement of zinc nutriture in children may provide an important preventive intervention for the high infectious disease morbidity and associated mortality in developing countries. As these effects have important public health implications, larger studies evaluating the effect of zinc supplementation on immune responses and relating this to changes in morbidity are required.

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