ment 1.

(E SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date May 05,1993

18.5.93(Revised

Principal Investigator Dr.S.K.Roy

Trainee Investigator (if any) _____

Application No. 93-012(Revised)

Supporting Agency (if Non-ICDDR,B) _____

Title of Study A study on immunological effect of Vitamin A and zinc in a placebo controlled 4 cell trial.

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects (Yes) No
   (c) Minors or persons under guardianship (Yes) No
2. Does the study involve:
   (a) Physical risks to the subjects Yes (No)
   (b) Social Risks Yes (No)
   (c) Psychological risks to subjects Yes (No)
   (d) Discomfort to subjects Yes (No)
   (e) Invasion of privacy Yes (No)
   (f) Disclosure of information damaging to subject or others Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of organs or body fluids (Yes) No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used (Yes) No
   (c) Physical risks (Yes) No
   (d) Sensitive questions (Yes) No
   (e) Benefits to be derived (Yes) No
   (f) Right to refuse to participate or to withdraw from study (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure (Yes) No
5. Will signed consent form be required:
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

S.K.Roy

Principal Investigator

Trainee

1. Title: A study on immunological effect of Vitamin A and zinc in a placebo controlled 4 cell trial

2. Principal Investigator: Dr.S.K.Roy,
Clinical Sciences Division
Co-PI: Dr.Tasnim Azim, LSD,

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Consultant: Prof. Demissie Habte, Director, ICDDR,B

4. Starting date: July 1, 1993

5. Completion date: June 30, 1994.

6. Total direct cost: US $ 97,500

7. Source of fund: WHO

8. Scientific programme: This protocol has been approved by the clinical sciences division.

Dilip Mahalanabis

Associate Director
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ICDDR,B

May 18, 1993

Abstract Summary

Vitamin A deficiency in children is associated with increased mortality and morbidity due to respiratory tract and diarrhoeal infections. Vitamin A supplementation has been shown in some studies to reduce morbidity due to respiratory diseases. However, other studies could not document such benefit from vitamin A supplementation. The role of vitamin A on immunity in humans is not yet clear due to inconclusive results. To evaluate immune changes and compare those with of a known immunopotent agent like zinc, a randomized double blind study will be carried out in 1-3 year aged children without acute illness and wt/age between 61% and 70% of NCHS standard. Base line anthropometry and vitamin A status will be determined using MRDR test and immune status will be estimated. Each group consisting of 50 children will either receive vitamin A 200,000 IU over 7 days or 40 mg elemental zinc daily for 7 days or both or placebo. After 8 weeks immunity test will be repeated. Immunity tests will include serum IgA, IgM, IgG and lymphocyte subset proportions, lymphocyte stimulation and 8 antigen multiple skin test. Unimmunised children will be given measles vaccine and serum titre will be measured before and after supplementation. Vitamin A status will be estimated by MRDR test. Vitamin A2 will be given and 1 ml blood sample will be collected after 5 hours to see the ratio of vitamin A1 and A2 (<0.06 as cut off) as the modified relative dose response (MRDR test). Doses of vitamin A or zinc will be repeated at the completion of 2 month. The results will be compared between groups and within groups at baseline and after 6 weeks. The study will generate information which will help to examine the immune response of vitamin A therapy in children as an underlying factor for reduction in mortality or morbidity. The study will be completed within a year.

9. Reviews

a. Ethical Review Committee:----------

b: Research Review committee:----------

c. Director:----------

Introduction

1. Objectives:

A. To see the immunological changes following administration of zinc and vitamin A in children with PEM (<70% wt/age up to 61% of NCHS standard) without any acute illness.

2. Background:

Summary of the research question

Vitamin A

In recent years, a number of studies have shown association between clinical vitamin A deficiency and mortality in children. A few studies have also documented excess morbidity in deficient children. Some of the vitamin A supplementation studies have shown reduction in mortality and morbidity. The underlying mechanism has been less known in humans, animal studies have shown repair of epithelium and changes in immunity with vitamin A supplements. But there is no strong evidence of improvement in immunity in humans in regard to vitamin A status or vitamin A supplementation. It is therefore necessary to study the role of vitamin A on immunity in children and establish the scientific basis of protection of diseases specifically related to vitamin A.

ZINC

The other important micronutrient, zinc, has been reported to be esential for protein synthesis specially immunoglobulins and is responsible for immunocompetence in cell mediated immunity. Recent studies showed reduction in death and reduced morbidity from diarrhoea and ARI in malnourished children with zinc supplementation (Roy et al 1990). Delayed parasitic clearence in zinc deficient animals (Fenwick et al 1990) and reduced pyogenic infections in zinc supplemented children with improved response to PPD and candida antigens and Blastogenic response to PHA (Castillo-Duran et al 1987). It is therefore important to identify the specific role of immunoprotection from Vitamin A and zinc and quantitafy their synergistic action in prevention of infection and reduce child morbidity and thereby reduce growth faltering. Newer strategies could be formulated to maximize the benefits by a combined supplementation project at suitable interval and dosing schedules.

Background

Vitamin A deficient (night blindness and/or Bitot's spots) children have been shown to have three times higher attack rate of diarrhoea and twice the rate of respiratory tract infections compared to non xeropthalmic children (Sommer et al 1984).

Forty-nine percent (49%) greater mortality was found in children without vitamin A supplementation compared to the treatment group receiving 200, 000 IU of vitamin A at 6 month intervals in a study in Sumatra, Indonesia (Sommer et al, 1986) (1989). However, the methodological issues of these results were questioned with critical views (Gopalan 1986). Reports by the same authors on the effect of vitamin A supplementation on mortality showed that there was 4 to 12 times higher death rate among the children with mild xeropthalmia which persisted even after controlling the effects of wasting, respiratory disease, gastroenteritis, pedal oedema (Sommer et al 1983). In Thai children, Kramer et al (1991) showed that blastogenic response to PPD of mononuclear cells was higher in children with suboptimal zinc or vitamin A nutriture supplemented with less than 2 times RDA of these nutrients. Rahmatullah et al reported that a weekly supplementation of low dose vitamin A reduced 46% death in the supplemented group. A simultaneous 200,000 IU of vitamin A supplementation in Hydrabad, India has shown no difference in mortality between the supplemented and unsupplemented groups (Bijayraghavan et al 1990). A subsequent report by Rahmatullah et al from south India also showed that there was no difference in morbidity between the groups (Rahmatullah et al 1991). Milton et al (1987) showed that preschool aged children who developed mild xerophthalmia during follow up had double the risk of respiratory tract infections but there was no change in diarrhoeal attack rates.

Evidence of vitamin A deficiency in malnourished children

In a recent study of vitamin A levels in non diarrhoeal children below 1 year, vitamin A deficiency was found in high proportion. 132 infants out of 171 (77%) had serum vitamin A less than 20 ug/dl; < 10 ug% n = 64, 11-20 ug% n=68, 21-30ug% n=27, >30 ug% n=12) (Whahed et al 1993). In the other on going study of children aged 1-3 yrs of wt/age between 61% and 70% showed that 70% of these children had vitamin A level less than 20 ug/dl one week after recovery from diarrhoea (Wahed et al 1993). Severely malnourished children with signs of xerophthalmia had higher mortality and more positive urine cultures than non xeropthalmic children (Brown et al 1974). In children with mild vitamin A deficiency but without obvious malnutrition, there was no evidence of increased infection.

Vitamin A deficiency is a significant public health problem in Bangladesh (Cohen et al 1986). The role of vitamin A in the reduction of morbidity in children especially from infectious diseases is being discussed over the last few years. Young infants get most of their vitamin A supply from breastmilk, whereas concentration of vitamin A in mothers milk decreases by 3 months after birth specially in malnourished mothers with a risk of reduced vitamin A intake in their infants (Roy et al 1989).

Effect of vitamin A on infection may be mediated through two major ways; one, through improving the epithelial repair and second through immunological protection. However, the morbidity and mortality due to infections are mainly mediated through the immunological pathway. Reduction in mortality after vitamin A supplementation might be related to improvement of immunological indicators dependent on vitamin A. Since infection or death is more common in the severely malnourished children who are often deficient in many micronutrients such as zinc, folate, copper or vitamin A, malnutrition appears to be a proxy indicator of both macro and micronutrient deficiency. Malnourished children are invariably found to be immunodeficient. The effect of vitamin A supplementation in terms of decrease in morbidity or mortality is questioned.

Evidence of Vitamin A not in favour of immunocompetence

However, the role of vitamin A as a protective factor such as on immunocompetence needs to be clearly understood. It is not known whether humoral or cell mediated immunity or both are depressed in vitamin A deficiency and whether there are definite improvements after the supplementation. The confounding variables for immunity such as rate of infection, protein energy malnutrition, and trace element deficiency also need to be addressed. Reduction in morbidity and mortality is expected to involve a pathway of immunological improvement, which has not been conclusively shown in human studies with vitamin A supplementation. Chandra and Au have observed only a slight difference in weight of thymus and spleen between vitamin A deficient and control animals (Chandra and Au 1981). Vitamin A modified humoral response to T dependent antigen and the number of plaque forming cells in vitamin A deficient rats were significantly lower. Although some evidence of relationship between vitamin A deficiency and immunity has been found in animal models, there are not much evidences in human to support this. Bhaskaram and Reddy found that children with xerophthalmia had lower serum vitamin A levels, and T lymphocytes, but there was no change in thymidine incorporation when compared with normal children. Five of 9 children with xerophthalmia, had reduced response to PHA stimulation (Bhaskaram and Reddy 1975). In 123 children aged 1-5 years, most

functions of the immune response were not found to differ significantly between those with serum retinol < 20 µg/dl and those with serum retinol above 20 µg/dl. However, the number of T cells was depressed in the latter. The ratio of T- helper/T suppressor-cytotoxic cells was not altered. It was concluded that this does not offer a strong immunologic basis for increased susceptibility to infection in mild vitamin A deficiency. (Bhaskaram, 1989 IVACCG). 110 mg vitamin A was given to marginally nourished Thai children, after two or four months there was no rise in secretory IgA in the tears but there was significantly higher vitamin A level in the supplemented group compared to the controls (Agtmaal EJV et al 1989, IVACCG). Kutty et al. have shown that in xeropthalmic children who were above 80% of wt/age, B-lymphocyte population, titre of serum IgA,IgM,IgG and antibody titre to tetanus toxoid were normal compared to children with normal eye sight (Kutty et al 1981). The effect on immunity of vitamin A supplementation was seen by Brown et al. in 20 children given 200,000 IU Vitamin A and 45 control children. There was no difference in cutaneous immunity or titre of tetanus toxoid (Brown et al 1980).

Evidence of Vitamin A in favour of immunocompetence

Recently Semba et al (1991) reported that immune response to tetanus toxoid was impaired in children with vitamin A deficiency. In Indonesia, a study in 236 preschool children of 3-6 yrs age showed that cinically normal and xeropthalmic children receiving vitamin A had significant rise in Tetanus toxoid induced IgG responses after 3 weeks compared to groups receiving placebo and unsupplemented xeropthalmic children. These children were not malnourished (> 80% wt/age) and titre among the groups of unsupplemented children (with or without xeropthalmia) were equal. The effect of vitamin A on repair of epithelium is well known. The re-epithelialization can be enhanced in deficient animals by supplementation. The effect of vitamin A on reduction in mortality may not be fully explained only by the epithelial repair but that the effect of vitamin A on immunity needs to be studied. So far, the evidences on immunological effect are somewhat positive in animal models, but those in human are either negative or marginal.

Role of zinc in vitamin A metabolism

Zinc has been shown to cure night blindness in vitamin A resistant cases, for example in patients with alcoholic cirrhosis (Morrison et al 1978). Infantile PEM is also more responsive to zinc therapy compared to Vitamin A in regard to metabolic improvement (McClain et al 1979). In patients with cirrhosis of the liver, nightblindness was corrected only with zinc supplementation.

The role of zinc in immunocompetence has been studied in greater detail (Beisel 1982, Chandra 1983). Patients of acrodermatitis enteropathica had significant improvement in immunity when zinc supplementation was given. Significant reduction in infection in zinc deficient animals occurred with zinc supplementation. Zinc deficiency in mice caused depressed natural killer cell activities and the killing activity of T lymphocyte against inoculated allogenic tumour cells (Fernandes et al 1997). In zinc deficient subjects without coexisting PEM, lymphocytes showed increased in-vitro response to mitogen but decreased or no response to Candida antigen (Vangool et al 1976). Zinc therapy restored cell mediated immunity to normal levels in patients with acrodermatitis enteropathica (Oleske et al 1979). In a recent study, Fenwick et al (1990) showed that zinc deficient animals were unable to expel intestinal load of Trichinella spiralis compared to pair fed and ad libitum zinc fed animals. The size of thymus and spleen was reduced in the deficient animals suggesting a T-cell mediated immune defect. The impairment of thymus dependent immune responses in zinc deficient mice was readily reversed by zinc supplementation.

Severe depression of serum IgG has been reported in patients with zinc deficiency and coexisting PEM (Cunningham-Rundles et al 1979). The response of zinc supplementation in young children has resulted in increased secretory IgA (Lothi 1982). During nutritional rehabilitation of severely malnourished children, zinc supplementation was associated with increase in serum IgA in comparison with the unsupplemented group (Castillo-Duran et al 1987). In those children, there was significant reduction of pyogenic infection in the zinc supplemented group.

During weekly follow up of persistent diarrhoea patients, it was noticed that further diarrhoeal attack rate and duration of diarrhoea were significantly lower in the children who received zinc supplementation during a previous diarrhoeal episode (Roy et al 1990). Reduction in both diarrhoeal and respiratory tract infection was seen among the stunted children during subsequent follow up of children who received zinc supplements during an earlier episode of acute diarrhoea (Roy et al 1990). This protective effect observed in children after zinc supplemention is further supportive of improved immunity with zinc supplementation.

In view of the above knowledge, an intervention trial is proposed to evaluate the immune effect of vitamin A, zinc, or both, in a randomized trial in young children with second degree malnutrition. Malnourished children will be considered as they are likely to have reduced reserve of micronutrients which may influence their immunity status.

Hypothesis

1. Administration of zinc in zinc malnourished children improves immunological status.

2. Vitamin A administration enhances immunity in young children of a population known to have vitamin A deficiency.

3. Zinc and vitamin A may have an additive effect in immunity of malnourished children.

Rationale:

Vitamin A supplementation has been reported to reduce mortality and, in some cases morbidity from diarrhoea, ARI and other infections. The benefit may be mediated through immunity. The lack of substantial data on immunological effect of vitamin A in young children indicates a paucity of scientific basis on the mechanism of resistance to infection after vitamin A supplementation. Selection of malnourished children will also help to explore the potential of vitamin A on both immunity and morbidity. This study will also examine the comparative effect on immunity between zinc and vitamin A or a synergistic effect of these two micronutrients which may direct better or new strategies of intervention. Such a study would be able to identify the extent of immunological effects of zinc and vitamin A and compare the effects with placebo and these two micronutrients. The study will further establish the relationship between vitamin A status (MRDR) and immunity in children and strengthen the scientific basis for intervention with vitamin A or zinc or both.

5. **Specific aims**

To measure the changes in immune functions after supplementation of zinc, vitamin A or both to young children aged between 1 and 2 years which will include the following:

a. To measure delayed type hypersensitivity responses using a multi-test CMI kit.

b. To phenotype lymphocytes for assaying proportions of T & ß lymphocyte and lymphocyte subsets.

c. To assess lymphocyte function by Lymphocyte transformation assays using mitogens such as PWM, PHA,

supplementation with zinc, Vitamin A or placebo.

d. To measure the tear secretory IgA before and after supplementation with zinc, Vitamin A or placebo.

Secondary Aim:

To evaluate effects of supplementation on their 2 months' morbidity following the supplementation.

Methods of procedure:

Immunological indicators will be monitored at the beginning and after 8 weeks of giving vitamin A or zinc in a double blind randomized trial. A clinic based study will be done to see the effect of zinc or vitamin A supplementation in malnourished subjects. Vitamin A status will be estimated using the newer indicator, modified relative dose response (MRDR).

Procedure:

Study population:

Children aged 1 to 3 years will be included. Forty children in each group will be assigned to either group A or group B and group C and group D.Children will be selected from families within Dhaka city who come to the diarrhoea treatment centre of ICDDR,B.

Inclusion criteria

1. Children aged between 1 and 3 years having weight for age between 70% and 61% of NCHS standard

2. Who come to the out patient department of ICDDR,B for treatment of acute watry diarrhoea with

3. No signs of vitamin A deficiency (non invasive diarrhoea and without systemic infection) and has not received vitamin A during last 4 months

4. Who has not received measles vaccine and did not have measles will be primarily identified for the study.

5. Children who reside in and arround Dhaka city

After one week of recovery from diarrhoea,

1. baseline immunological profile will be studied

2. intervention for one week at the scheduled doses and immunologic profile will be repeated after 8 weeks.

Study groups are A. Zinc B. Vitamin A
C. Zinc and vitamin A, D. Placebo

Exclusion criteria:

1. children who needs immediate Vitamin A supplementation (clear sign of vitamin A deficiency)

2. children who received Vitamin A within the last 4 months.

3. Children with other systemic infection

Subjects who develop any kind of sign and symptoms of vitamin A deficiency will be given vitamin A and will be analysed separately.

Randomization procedure:

Parents who give their consent for the study will be selected. Children will be randomized into four groups to receive either of the treatments. A

master randomization chart will be prepared using random table in permutted block design by dividing serial numbers into four equal groups. There will be no need for stratification but according to the selection criteria, better nourished (70% wt/age) or severely malnourished (< 60%) children will be excluded. Severely malnourished patients with diarrhoea may immediately need vitamin A or well nourished will not show effect of supplements. Steps will be taken to keep equal number in each group. A longer list of numbers will be kept to compensate dropouts.

The intervention and non-intervention groups will receive indentical bottles and syrups with some flavour and only single unique serial number will be written outside the bottles. The number will indicate an exact subject of study. The randomisation list will be prepared by an expert outside of the study and will be kept out of the reach of the investigators. The children will receive the 2nd dose at 8 weeks with vitamin A or zinc who has not received that in the first dose without breaking the randomization code. The code will be broken only after completion of study and analysis according to groups.

Base line survey will be done to take socioeconomic information, dietary habits, previous illness, immunization, washing of hands and water use, frequency of cooking of food containing Vit A , cooking methods, and frequency of use of green leafy vegetables.

In the beginning of the study, baseline vitamin A status will be determined using MRDR test. Anthropometry will be done to select children at the base line, at the end of 8 weeks, and every subsequent months for 6 months.

Duration, frequency, dose:

One teaspoonful (5 ml) syrup will be given in a twice daily schedule for 7 days.

Composition of syrup:

For all groups, the main vehicle and bulk of the base syrup will consist of the same chemicals; in addition to this, each group will have either of the treatment substance (i.e zinc or vitamin A or both or none).

Syrup A:	Quantity per 5 ml
Zinc acetate	20 mg elemental
Syrup B:	
Vitamin A palmitate	15,000 IU

Syrup C:

Zinc: 20 mg elemental + Vitamin A: 15,000 IU
Syrup D: y base substance

Base substance:

Ascorbic acid	30 mg
Glycerine USP	1.2 ml
Propylene glycol USP	0.75 ml
Sorbitol 70% BP	2.0 ml
Methyl Paraben USFN	3.5 mg
Polysorate-80 (Tween-80) BPC	50 mg
Lemon oil pH grade	0.0125 ml
Caramel Brown colour powder Ph Grade	1 mg
Purified water	0.75 ml

Organisation of study

The study will be conducted among the children who will attend the out patient department of the clinical research centre of ICDDR,B. Children will be selected at the acute diarrhoea phase who will attend only with non invasive simple or mild diarrhoea whithin the nutritional status defined earlier; Wt/Age 61-70% 50th centile of NCHS standard. The children will be taken for study 1 week after the recovery of diarrhoea and the first immunological test at base line will be done at this time. Mothers will be instructed on the doses. A twice daily dose of syrup for one week will be scheduled for better compliance of the children. After completion of doses at 8 weeks, the immunity test will be repeated at 8 weeks. Tears will be collected in a small piece of sponge and will be used for secretory IgA estimation. Skin test for Cell Mediated Immunity will be performed in each child regardless of treatment group with multitest kit developed by Institute Merieux. These children will be followed up every alternate day for the 1st week and then weekly for development of any illness for 2 months. Necessary treatment measures will be undertaken.

Clinical care:

Patient care will be provided on clinical problems identified during treatment of diarrhoea or follow up. A team of medical officers and paramedics will examine and record the vitamin A deficiency symptoms and other medical problems on a predesigned questionnaire and provide the necessary care. Any

child who develops an acute symptom of night blindness or Bitot's spot will be given a Vitamin A capsule (200,000 iu) which will be recorded.

Base line test on immunity will be performed by the following tests:

Components of immunity and other variables

1. Eight antigen multiple skin test measured by 48 hours

2. Tears IgA, IgM

3. MRDR, plasma IgA, IgM, IgG.
(This will require 2 ml blood)

4. Lymphocyte proliferation assays.
For T lymphocytes - PHA, Con A antigens will be used
For T and B lymphocytes - PWM (Poke weed Mitogen)
(This will require 3 ml blood in heparinized tube).

5. Lymphocyte Phenotype using monoclonal antibodies to CD3,CD4, CD8, CD20.

Schedules: for immunity test, skin test before dose and after 8 weeks. MRDR will be done at base line and at 8 weeks.

6. Base line titre of measles antibody IgG will be mesured and will be followed by a dose of measles vaccine and titre will be measured at 8 weeks of supplementation.

7. 'Serum Zinc, RBP, albumin & alkaline phosphatase at baseline after 8 weeks.

Anthropometry will be done with weight, height, MUAC at base line, at 8 weeks .

Body weight - will be measured (nude) with an electronic scale with a 1 g sensitivity.

Length:- will be measured in knee-heel vernier with 1 mm sensitivity.

Data collection on morbidity

A predesigned questionnaire will be used to record incidence and duration of illness during the follow up period.

Outcome variables to be collected as follows:

1. Immunization test results

IgA (tears), T-lymphocyte number, proportion, phenotype, CD4 %, CD8%, CD20% PHA, CON-A, PWM at baseline and after 6 weeks.

2. Skin test CMI

3. Morbidity: New attacks of respiratory infection + duration of respiratory infection

4. Attacks of diarrhoea and duration

5. Fever without respiratory infection

6. MUAC

7. Height

8. Weight

9. Age

Sample size calculation

Change in immunity will be taken as major outcome variable. The following formula will be followed to calculate sample size according to Kirkwood B (1988)

$$n = \frac{2 \times SD^2}{d^2} \times f\ (\alpha, \beta)$$

Where 'd' is the difference between the vitamin A and placebo groups and SD is standard deviation of immunity indicators (Chandra RK 1989) and cotrol value has been taken from recently accomplished studies in ICDDR,B (Azim T, 1992). (α= significance, 1-β= power). We have considered the variance of different immunological marker and that a sample size of 50 is sufficient to

limit the significance level 5% and with 80% power. A total of 160 children in 4 groups will suffice for the study including 20% as dropout.

With Vitamin A supplementation,

Calculation on T lymphocytes, CD4
Control mean=42 , sd 12
expected difference: 10
n=30 + 5 =35

Sample size for detecting change in CD8 : Control mean=21, sd=8
expected reduction is 5, n=30.

Size of sample for PWM: n=35
Size of sample for ConA n=30
Size of sample for PHA n=35
For Zinc therapy, calculated n for T-lymphocytes CD4 = 35
n for Con A, PHA =30

Calculated sample size for 20% difference in secetory IgA in saliva, is= 32
Total number of samples in 4 groups are 35 x 4 = 140 plus 10% drop out = 14, final requirement 140+14 = 154.

Sample size adjustment for vitamin A deficiency in malnourished children.

CORRECTION FACTOR:

Since the children who will be selected by anthropometric index wt/age between 61% and 70% of NCHS. About 70 per cent of the children of this nutrition have been found to be deficient in serum vitamin a level i.e <20 ug/dl. 70% children selected with this anthropometric criteria will then have vitamin A deficiency, hence the to get the required sample size, the calculated number will be multiplied by this factor.

$$\frac{35 \times 100}{70} = 50+5 \text{ (10\% drop out)} = 55 \text{ in each group,}$$

in 4 groups total sample size will be 55 X4=220

Detailed procedure

Immunology

1. Lymphocyte Phenotype : Peripheral blood mononuclear cells will be phenotyped by indirect immunoflurescence for CD3 (T cells),CD4 (helper-T cells), CD8 (suppressor /cytotoxic T cells), CD20 (B cells).

2. Lymphocyte stimulation tests :

a. Phytohaemagglutinin (PHA) (a T lymphocyte mitogen) stimulation will be measured by culturing cells with PHA in the presence of foetal calf serum for 72 hours and assessing proliferation by HTDR incorporation.

b. Pokeweed mitogen (PWM) (A T-dependent B lymphocyte mitogen) stimulation will be measured by culturing cells with PWM in the presence of foetal .032 calf serum for 7 days and assessing proliferation by 3 HTDR incorpora tion. A similar experiment will be conducted with conconavalin A (con A) (a T lymphocyte mitogen). Where cell numbers are inadequate, PHA stimulation will be excluded from the study.

Specific Antigen: Measles vaccine will be given i.m. before the dose and titre will be measured before and after 8 weeks.

DELAYED TYPE OF HYPERSENSITIVITY:

DTH will be tested using Multitest CMI kit whereby 7 antigen and a control will be introduced intradermally into the forearm using a multiple puncture device. An induration of 2 mm or more diameter after 48 hours will be counted as a positive reaction. The antigens that will be tested include:

1. Tetanus antigen 550,000 Merieux units/ml
2. Diphtheria antigen 1100,000 Merieux units/ml
3. Streptococcus (antigen Group c) 2,000 Merieux units/ml
4. Tuberculin antigen 300,000 IU/ml
5. Glycerin control: solution of glycerine 70% w/v
6. Candida antigen (albicans) 2,000 Merieux units/ml
7. Trychophyton antigen (mentagrophytes) 150 Merieux units/ml
8. Proteous antigen (mirabilis) 150 Merieux units/mlp.

Biochemistry:

1. **Zinc** will be estimated after collection of plasma in a zinc free container using an atomic absorption spectrophotometer.

2. **Vitamin A** will be estimated using high performance liquid chromatography (HPLC) from plasma stored in a dark container.

3. **RBP** will be estimated using radial immunodiffusion plates containing monospecific antisera against human RBP or Cobas Bio auto analyzer by turbidimetric principle.

4. **Serum albumin** will be estimated along with anthropometry to assess nutritional status.

5. **Serum immunoglobulins** such as IgA, IgG and IgM will be estimated using Cobas Bio auto analyzer using respective monospecific antisera. Fractions of immunoglobulin will be measured as these are important componet of immunity and may be altered in malnutrition.

MRDR:

Children will be given 100 ug/kg BW of synthetic Vitamin A2 (di-dehydro retinol synthesized from retinoic acid) in the morning. A vitamin A free high fat containing diet will be fed, and a 1 ml blood sample will be collected after 5 hours. The concentration of Vitamin A1 Retinol palmitate and that of vitamin A2 i.e 3,4 di-dehydro retinol will be estimated. The ratio between of Vitamin A2 to A1 will be calculated and if that is less than 0.06, a significant vitamin A deficiency will be considered (cut-off point). Reference (Tanumehardjo S et al. Am J Clin Nutr 1990;52:1064-7).

Anthropometry:

Children will be weighed with minimum clothing during each visit using a 1g sensitive electronic weighing scale.

Thorough training will be given to the field staff on collection of data and anthropometry. Investigators will perform the skin tests and undertake immunity test.

Layout of study plan

Base line survey, anthropometry and selection of population

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Randomization

¦

Baseline immunity test and baseline MRDR test and biochemical test

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Allocation of treatment to each group

¦

at 8 weeks repeat immunity test

¦

at 8 weeks, a dose of Vit A and zinc in required group

¦

Laboratory test at baseline, at 8 weeks.

|

Data entry 3 months (concurrent)

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Data analysis 2 months

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Report writing 2 months.

Analysis plan

Factorial analysis will be carried out to assess the effects of Vitamin A and Zinc and their interaction on immunity.

Comparison between baseline and at 8 weeks within groups will be done.

Comparison between groups of treatment with ANOVA analysis

Appropriate tests will be used to calculate the statistical significance of difference between variable.

Relationships will be estimated using linear regression and multiple regression analysis.

Group A will be compared with group C and D
Group B will be compared with group C and D
Group A will be compared with group B

ICDDR,B diarrhoea patients at discharge from the outdoor

Facilities required

Staff: Only a few new staff will be hired and trained.
Material: Kit, logistic materials etc will be purchased.
Hood for clean laboratory environment for immunology.
Transport: ICDDR,B facilities + hiring.
Laboratory service :ICDDR,B.

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Budget

A. Professional Scientific Staff

Sl.	Details	No	% time	# months	U.S.$	
1.	Dr. S.K. Roy		25%	12	3,000	
2.	Dr. T. Azim		20%	12	2,000	
3.	Dr. D. Mahalanabis		5%	12	1,000	
4.	Dr. S.M. Akramuzzaman		10%	12	1,000	
5.	Mr. M.A. Wahed		10%	12	1,500	

B. Technical Staff

1.	Research Physician	2	100%	18	6,000	
2.	Research Officer	1	100%	18	3,000	
3.	Senior Health Assistant	2	100%	18	6,000	
4.	Female Volunteer	3	100%	12	1,000	
5.	Secretarial service	1	50%	12	800	
					25,300	

Travel: International		2,500
Local		2,500
Supplies and Materials		
Medical supply	(year 1=1,000)	1,000
Office supply	3 x $ 350	1,000
Equipment, lab supply	1 x $ 5,000	5,000
Weighing Scale	2 x $ 5,00	1,000
Electronic knee-heel vernier	1 x $ 4,000	4,000
Biochemical tests:		
Zinc assay	400 x $ 8	3,200
Vitamin A2 assay	400 x $ 10	4,000
RBP	400 x $ 8	3,200
Serum albumin	400 x $ 5	2,000
Vitamin A	400 x $ 7	2,800
		15,200
Immunology Tests		
Biohazard hood	1 x $ 6,000	6,000
Multiple skin test kit	400 x 9	3,600
Serum IgA, IgM, IgG	400 x 5	2,000
Reagents, pippettes etc.	400 x 10	4,000
Phenotyping of lymphocytes	400 x 20	8,000
Transformation antigens	400 x 20	8,000
Measles serology test	400 x 5	2,000
		33,600
Micro-Computer	1 x $ 2,000	2,000
Printing and publications	$ 800	800
Communications	$ 600	600
Telex, Fax	$ 500	500
Papers and stationery	$ 500	500
Miscellaneous	$2000	2,000
		6,400
	Total	$97,500

Abstract Summary for Ethical Review Committee

1. This study aims to evaluate the beneficial effects of supplementation of zinc and vitamin A on the immunity in children with moderate degree of malnutrion and associated beneifits on morbidity from supplementation. Many studies have documented reduction in mortality and morbidity with vitamin A and zinc supplementation but there is little evidence in human studies on changes in immunological indicators. Since children and infants are more vulnerable to malnutrition and infection and since their micronutrient deficiency leads to increased susceptibility to infection and related nutrional consequences, 1 to 3 year old children will be selected as they are more deficient compared to the breastfed younger ones. Children will receive syrup of vitamins and zinc for one week and monitoring will continue for 6 months.

2. There is no potential risk to infants and young children in this study.

3. The children will not be recruited with acute symptoms or illness, more over constant and regular care will be provided through efficient clinical team with the follow up system.

4. Informed consent in a consent form will be obtained from the legal guardian or parents before inclusion in the study. The mother will be fully informed and explained about the objectives and benefits of the study.

5. Confidentiality of childrens' record will be maintained and a unique study number will be used.

6. Interview will be taken by Health Assitants and work will be supervised by the Principal Investigator. At each time interview will be for 10 ninutes at the maximum. Data will be collected on socioeconomic status and child's feeding practice, morbidity and anthropometry. 5 ml of blood will be collected with maximum precausion by the PI in hospital or clinic set up for immunological tests on phentyping, Lymphocyte transformation and humoral immunity. This blood test will be repeated at 8 week of vitamin A, zinc or placebo supplementation.

7. Subjects of the study will receive regular home visits and treatment for illnesses. They will be advised on better child care, feeding practice and immunizations. The study will generate important scientific data to reduce knowledge gap on the specific immunity effects of micronuriets which have tremendous potential on child health, development and survival. Newer strategy for child health with added benefit from Vitamin A and Zinc supplementation at periodic intervals will reduce health worker's and mother's time but improve child health.

Vitamin A -Zinc Immunity study

CONSENT FORM

(Will be read and explained clearly before consent is obtained)

The international centre for Diarrhoeal Disease Bangladesh is going to undertake a study to know the specific protective role of vitamin A and zinc in moderately malnourished children who are known to be deficient in these micronutrients. Your young child who suffer from diarrhoea or acute respiratory infection from time to time is likely to be more deficient and more likely to benefit by participating into this study. Your child will receive a vitamin and mineral mixute which are called essential trace elements from us for one wek period and we believe this will greatly help your child but the underlying mechanism is not kown. Mainly your child will be closely examined at daily or two weekly intervals by the pysicians and trained health workers. Your child will be assessed for health status and necessary medical and nutrirtional advice will be given. The vitamins and mineral that will be given to your child has proven greater benefits for other children. There is no risk in participation in this study. Whenever your child suffer from any problem like "rat kana" we will immediately provide necessary treatment.

We would appreciate you to allow your child to participate in this study. If you agree, the following procedure will be followed for your child.

1. Your child will be examined thoroughly for any illness, presence of malnutrion, presence of vitamin and mineral deficiencies.

2. He/she will be given a bottle of either of the vitamins or mineral syrup to be given twice daily for one week.Information on Socioeconomic status will be collected.

4. A multiple skin antigen will be tested and wiil be repeated after 8 weeks.

5. 5 ml of venous blood will be collected for assaesment of nutrient deficiency, need for drug and test the disease-preventive capacity of the child. This will be checked again after the therapy at the end of 8 weeks.

6. We may visit your child at home on alternate days for 7 days and weekly for development of any illness for 2 months.

7. There is no risk to the child in participating into this study. we shall maintain confidentiality of the information of your child. At any stage you will have the full right to withdraw your child from the study; but his/her treatment by us will not be hampered. If you wish to further know about the benefits or risk of the study, we will be happy to explain them.

8. If you wish to participate in this study, please put your signature below.

Signature of the
Principal Investigator

Signature /Left thumb impression
of the legal guardian

Date ----------