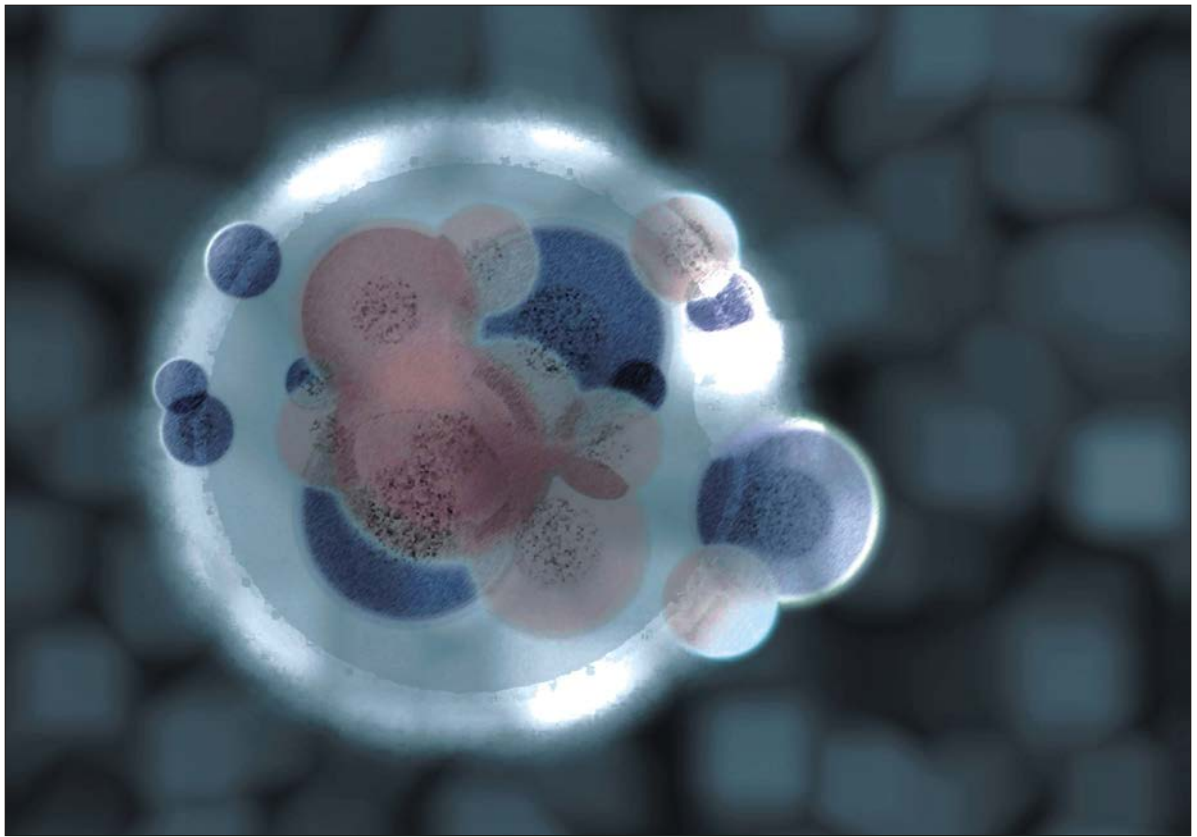




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Vitamin A and Th1 Response in Children with Acute Respiratory Infections

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Abstract: Animal studies have shown that in addition to generalized effects on immune function, zinc and vitamin A can influence Th1 and Th2 cytokine responses and thus have a profound impact on the outcome of infectious illnesses. However, vitamin A supplementation studies on children with respiratory infection are controversial. It is hypothesized that vitamin A may modulate the Th1 and Th2 bias and alter the course of respiratory infection. Therefore in this study, the micronutrient status of children during acute respiratory infection and their association with local cytokine response was determined. In addition, to study the impact of large dose vitamin A on Th1 and Th2 modulation, cytokine response was studied after oral administration of 2 lacs IU of vitamin A in normal children. Seventy two children aged 10 months to 3 years with ARI were recruited from Niloufer Hospital, Hyderabad. Cytokine response, hemoglobin status, serum zinc and vitamin A levels were determined in these children with vitamin A deficiency and compared with thirty apparently normal children of similar age group and socioeconomic status. In ten normal children, cytokine response was studied after oral administration of 2 lakhs IU of vitamin A. Correlation coefficient on log-transformed data showed a significant ($p < 0.05$) inverse association of serum vitamin A and nasopharyngeal aspirate IL2 in children with acute respiratory infection. Other nutritional parameters (weight for age, hemoglobin and zinc) showed no correlation with nasopharyngeal aspirate IL2. Children supplemented with vitamin A showed a significant ($p < 0.05$; paired t test) decrease in IL2 response from peripheral blood mononuclear cell culture supernatant compared to the baseline concentration. The study shows that vitamin A modulates TH1 response, which may be responsible for the increased morbidity seen when children with ARI are supplemented with Vitamin A.

Key words: Cytokines, Th1, Th2, vitamin A, nutritional status, respiratory infection

INTRODUCTION

Cytokines play a key role as messenger molecules in communication signals during immune response that may be beneficial or injurious to the host depending upon the type of cytokine produced at the site of infection (Román *et al.*, 1997; Anderson and Heilman, 1995). During the pathogen specific immune response, the T helper (CD4+) cells evolve into Th1 and Th2 cells each with a characteristic cytokine profile. The Th1 cytokine response is characterized by IL2 and IFN- γ , that supports cytotoxic T lymphocyte activity, which is essential to control viral and intracellular bacterial infection. In contrast, Th2 response is characterized by IL4 and IL5 that supports antibody immunity and is particularly beneficial in extracellular bacterial and parasitic infections (Anderson and Heilman, 1995; Bottomly, 1988). Apart from many other factors such as genetic, dose and

virulence of microorganism and cytokines at the vicinity of antigen and immune cell contact, nutritional factors might play a substantial role in promoting Th cells to Th1 or Th2 subtypes (Munoz *et al.*, 1995).

Micronutrients can influence and modulate immune response and alter the course and outcome of most infectious illnesses. Poor nutritional status, or selective nutrient deficiencies like zinc and vitamin A, has been shown to suppress several facets of immune response (Munoz *et al.*, 1995). Among the various nutrients, vitamin A, iron and zinc have a significant role in immune response. Animal studies have shown that in addition to generalized effects on immune function, zinc and vitamin A can influence Th1 and Th2 cytokine responses and thus have a profound impact on the outcome of infectious illnesses (Pauline *et al.*, 1985; Semba, 1998). Studies have documented low serum retinol and zinc during acute infection (Velasquez-Melendez *et al.*, 1995; Bahl *et al.*,

1998). Based on these findings clinical trials were conducted on the assumption that infectious disease outcome might improve with zinc or vitamin A supplements. However, vitamin A supplementation studies on children with respiratory infection have been disappointing (Stephensen *et al.*, 1998; Bresee *et al.*, 1996; Fawzi *et al.*, 1998). Some researchers suggested that vitamin A (VA) should not be used therapeutically in patients with pneumonia unless there is clinical evidence of vitamin A deficiency or concurrent measles infection (Fawzi *et al.*, 1998). Considering the above views, it is hypothesized that vitamin A may modulate the Th1 and Th2 bias and alter the course of respiratory infection. Thus in this study the micronutrient status of children during Acute Respiratory Infection (ARI) and their association with local cytokine (Th1, Th2) response was determined. In addition, to study the impact of large dose vitamin A on Th1 and Th2 modulation, cytokine response was studied after oral administration of 2 lacs IU of vitamin A in normal children.

MATERIALS AND METHODS

Study design: Children aged 10 months to 3 years, suffering from ARI (pneumonia, bronchiolitis and upper respiratory tract infection), with a history of illness for not more than 5 days, were recruited from Niloufer Hospital, Hyderabad. Clinical diagnosis of Pneumonia was confirmed by X-ray chest and bronchiolitis was diagnosed in infants based on the classical clinical signs of wheezy cough, dyspnoea and irritability, with or without x-ray evidence of hyperinflation of lungs (Haddad and Palazzo, 2000). Children with cough, fever and rhinitis were grouped as Upper Respiratory Tract Infection (URTI) Children with congenital heart disease, chronic lung disease or family history of asthma were excluded from the study. Sample size estimate was based on mean and SD of IL2 cytokine in vitamin A deficient ARI children with 90% power and 5% significance. This yielded a sample size of 20 ARI children with low vitamin A ($\leq 20 \mu\text{g dL}^{-1}$). Thus, 72 children with ARI were recruited, of whom there were sufficient number of children with vitamin A deficiency for comparison of cytokine response. Thirty apparently normal children of similar age group and socioeconomic status were taken as control group. The control group was taken to compare micronutrient status in the ARI children.

Nutritional status: Anthropometrics measurements were taken to assess their weight for age using Gomez classification (Gomez, 1982). After obtaining an informed consent from parents, 2 mL of blood sample was collected from children and their hemoglobin (Hb) status, serum

zinc and vitamin A levels were determined. Serum retinol was measured by HPLC. Briefly the procedure consists of extracting retinol with 0.5 mL hexane, evaporating the hexane and reabsorbing in 0.1 mL ethanol (Selvaraj and Susheela, 1970). HPLC analysis was performed using a reverse phase C18 column (Waters). Two nanogram retinol in 20 μL of ethanol was used as standard. Retinyl acetate was used for internal standard for calculation of extraction losses. Retinol peaks were detected by measuring absorbance at 325 nm and calculated against the retinol standard. Serum zinc was measured using Atomic Absorption Spectrophotometry (AAS) after diluting (1 in 5) serum in deionised water (Butrimoritz and Purdy, 1977). Hemoglobin was determined by cyanmethemoglobin method.

Systemic and local, Th1/Th2 response in children with ARI: Nasopharyngeal aspirates (NPA) were collected aseptically by passing size 5 feeding tube into the nasopharynx and applying gentle suction with a syringe. NPA secretions were rinsed into collecting vials containing 1 mL phosphate buffer. After centrifugation of nasopharyngeal secretions to precipitate cells, the supernatant was frozen at -70°C till analyzed for cytokines by ELISA (Rofalo *et al.*, 2001). An aliquote of serum was also preserved at -70°C to analyze cytokines at a later date.

ELISA (Diaclone research) was used to determine Cytokines (IL2, IL4 and IFN- γ) from NPA and serum. Recombinant cytokines of known concentrations were used to produce the standard curves. Flat-bottomed microtiter plates were coated with 100 μL (2 $\text{mg}\cdot\text{L}^{-1}$ coating buffer) of monoclonal antibody to human IL2, IL4 or IFN- γ and stored at 4°C overnight; 200 μL PBS with 10% calf serum was added to each well and the plates were allowed to incubate for 2 h at room temperature to block nonspecific binding. Then, 100 μL of serum or culture supernatant or recombinant IL2, IL4 or IFN- γ standard that had been diluted in twofold steps in PBS with 10% serum was added to each well and incubated overnight at 4°C . This was followed by the addition of biotinylated anti-human IL2 or IL4 or IFN- γ , which was allowed to stand at room temperature for 1 h. Hundred microliter of streptavidin-horseradish peroxidase was added and the mixture was allowed to stand for another hour. Plates were washed with PBS Tween between each incubation. Finally 100 μL of substrate was added to each well and the reaction was stopped by adding H_2SO_4 . The plates were read at 450 nm. Cytokine concentrations were calculated from the standard curve run on each assay plate and were expressed for milligram of total protein in the NPA. The lower limit of detection for IL2, IL4 and IFN- γ were 5.6, 1.1 and 12.5 pg mL^{-1} , respectively, with intra

and inter assay variability of less than 10 and 5%, respectively. The total protein from NPA was determined by modified Lowry's method (Lowry *et al.*, 1951).

Th1/Th2 response after 2 lacs IU of oral vitamin A in normal children: In ten apparently normal children of same age group and weight for age, blood sample was collected initially and 15 days after 2 lacs IU of vitamin A orally. The blood samples were processed for isolation of Peripheral Blood Mononuclear Cells (PBMC) (Boyum 1968). PBMC was isolated on Ficoll hypaque and stimulated with PHA for 18 hours at 37°C and 5% CO₂. After 18 h culture, the supernatant was harvested and IL2, IL4 and IFN- γ were analyzed by ELISA. Cytokine production by cultures without PHA was below the limit of detection of ELISA.

Statistical analysis: Statistical analysis was done with SPSS PC soft ware. A p-value of 0.05 was used to determine significance. Students t-test was used to compare nutritional variables (WFA, Hb, zinc and vitamin A) between ARI and control groups. Relationship of cytokine response and vitamin A concentration in ARI children was done using the nonparametric Mann-Whitney test, as the cytokine values were not distributed normally. Correlation coefficient was done after log transforming the cytokine data. Paired t test was used to compare cytokine response from PBMC of normal children before and after 2 L IU of vitamin A.

RESULTS

The mean age in months and Weight for Age (WFA) was comparable between ARI and the control group. Mean serum zinc, vitamin A and Hb were significantly lower in the ARI children compared to the control group (Table 1). Vitamin A was <20 $\mu\text{g dL}^{-1}$ in 38 of 72 ARI children and 30 children had hemoglobin less than 9 g dL^{-1} . Low zinc (<70 $\mu\text{g dL}^{-1}$) level was seen in 32 ARI children (Table 1). Thus, more than 50% of the ARI children had low vitamin A.

Local cytokine response: NPA IL2 was detectable in 53 (73.6%) of 72 ARI children and ranged from nondetectable to 472.4 pg-mg protein. The total mean and CI was 103 (64.1, 142.9) pg-mg total protein.

NPA IL4 was detectable in 84.7% of ARI children and ranged from nondetectable to 103.4 pg-mg protein. The total mean and CI was 9.3 (-5.2, 23.9) pg-mg protein. On the other hand, IFN- γ was detectable in only 7 of 72 ARI children.

Serum cytokine response: Of the 72 ARI children, only 8 and 21 showed serum IL2 and IL4 in the detectable range, with mean (CI) of 8.3 (-6.7, 23.5) and 2.3 (0.2,4.4) respectively. Serum IL2 was not correlated with NPA IL2 levels, while IL4 showed a negative correlation between NPA and serum. Serum IFN- γ was below detectable level in all the ARI children.

Nutritional status and cytokine response in children with ARI: When the mean values of NPA IL2 was compared between children with Hb of ≥ 9 and <9 g dL^{-1} , there was no significant difference. Similarly a cut off value of zinc at 70 $\mu\text{g dL}^{-1}$ showed no difference in IL2 response, while WFA and vitamin A were inversely related with NPA IL2 concentration (Table 2). However, the inverse association of IL2 with WFA disappeared when the data was controlled for vitamin A, while the association with vitamin A ($p < 0.05$) remained strong when controlled for other nutritional parameters. Correlation coefficient on log-transformed data showed a significant ($p < 0.05$) inverse association of serum vitamin A and NPA IL2 in children with ARI (Table 3). A similar association was seen in children with pneumonia; that is after excluding

Table 1: Nutritional status of children with Acute Respiratory Infection (ARI)

Group (n)	Age (months)	Weight for Hemoglobin age (%)	Hemoglobin (g dL^{-1})	Vitamin A ($\mu\text{g dL}^{-1}$)	Zinc ($\mu\text{g dL}^{-1}$)
Control (30)	24.1 $\pm$ 2.7	78.5 $\pm$ 1.22	10.3 $\pm$ 0.30	25.4 $\pm$ 1.01	114.6 $\pm$ 6.29
ARI (72)	23.7 $\pm$ 3.29	76.1 $\pm$ 1.03	8.9 $\pm$ 0.17*	19.3 $\pm$ 1.00*	76.3 $\pm$ 2.53*

Values mean $\pm$ SE; * $p < 0.001$ (Students t-test) compared to control children

Table 2: Nutritional status and NPA cytokine response in acute respiratory infections

Nutritional status (n)	IL2	IL4
WFA		
<75 (35)	134.1* (57.8,210.3)	5.1 (-2.77,12.9)
≥ 75 (37)	47.3 (31.9,62.6)	0.8 (0.5,1.0)
Hb (g dL^{-1})<9 (30)	72.4 (22.1,122.7)	4.65 (-3.48,12.79)
≥ 9 (41)	89.5 (44.1,134.9)	1.26 (0.25,2.28)
Serum VA ($\mu\text{g dL}^{-1}$)		
<20 (38)	104.8* (55.4,154.1)	4.1 (-2.6,10.87)
≥ 20 (29)	35.8 (19.2,52.4)	1.3 (-0.3,2.6)
Serum zinc ($\mu\text{g dL}^{-1}$)		
<60	121.0 (-21.8,263.98)	0.69 (0.4,1.0)
≥ 60	75.67 (43.2,108.1)	3.04 (-1.55, 7.6)

Values are mean and 95% CI; Expressed in pg/mg total protein in nasopharyngeal aspirate (NPA), * $p < 0.05$ compared to the respective better-nourished group (nonparametric Mann-Whitney test), WFA: Weight for Age; Hb: Hemoglobin; VA: Vitamin A

Table 3: Correlation coefficient for nutritional status and NPA IL2 response in children with acute respiratory infections and in children with pneumonia alone

	r-value (ARI, No. 50)	r-value (Pneumonia, No. 14)
WFA	0.069	0.337
Hb	0.175	0.134
VA	-0.324*	-0.563*
Zinc	0.142	0.108

* $p < 0.05$, WFA: Weight for Age; Hb: Hemoglobin; VA: Vitamin A

Table 4: Effect of Vitamin A (VA) supplementation on cytokine response in normal children

	Serum VA ($\mu\text{g dL}^{-1}$)	IL2 ($\text{pg}/1 \times 10^6$ cells)	IL4 ($\text{pg}/1 \times 10^6$ cells)	IFN- γ ($\text{pg}/1 \times 10^6$ cells)
Before VA supplementation	26.2 $\pm$ 3.46	21.9 $\pm$ 9.4	11.2 $\pm$ 1.84	1914.2 $\pm$ 608.98
After VA supplementation	35.7 $\pm$ 3.36	8.2 $\pm$ 3.92*	8.6 $\pm$ 2.17	1915.9 $\pm$ 494.68

Effect of vitamin A (2 lakhs IU orally), on IL2 response from PHA stimulated PBMC of 10 normal children. * $p < 0.05$ (Paired t test); compared to baseline value

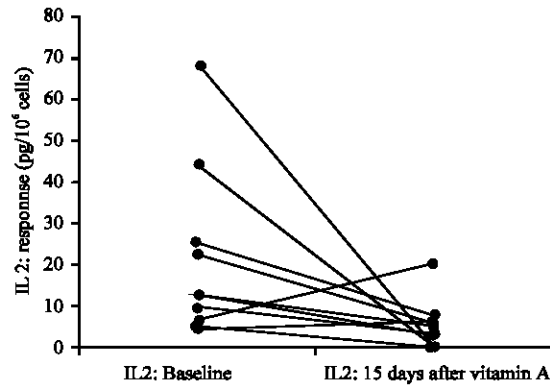


Fig. 1: Effect of vitamin A (2 lakhs IU orally), on IL2 response from PHA stimulated Peripheral Blood Mononuclear Cells (PBMC) of 10 normal children. IL2 response after 15 days of vitamin A was significantly decreased ($p < 0.05$; Paired t-test) compared to baseline response

cases of URTI and bronchiolitis. Other nutritional parameters (WFA, Hb, zinc) showed no correlation with NPA IL2. NPA IL4 was not related either with WFA, Hb and vitamin A or zinc levels. Correlation of IFN- γ with nutritional status was not attempted as very few children had detectable levels. Serum cytokines were not associated with nutritional parameters.

Oral vitamin A and IL2, IL4 and IFN- γ response with PHA: To examine the direct effect of vitamin A supplementation and since vitamin A cannot be supplemented to children with ARI the effect of large dose vitamin A was studied in normal children without ARI. The baseline vitamin A status of these children was adequate, however, serum vitamin A increased 15 days after vitamin A (2 lakhs IU) supplementation, while the IL2 concentration in the PBMC culture supernatant reduced significantly ($p < 0.05$; paired t-test) from the baseline concentration. The concentration of IL4 and IFN- γ were comparable to the baseline response (Table 4 and Fig. 1).

DISCUSSION

Vitamin A and zinc are important immunomodulators and are critical in determining the outcome of infectious disease (Munoz *et al.*, 1995; Pauline *et al.*, 1985; Semba, 1998). However, while the role of zinc has been well

defined as protective, the precise role of vitamin A has remained poorly understood (Black and Sazawal, 2001; Bhandari *et al.*, 2002). Our results show low serum retinol and zinc in children with ARI. Other investigators who have shown low retinol during acute condition have documented increased excretion of retinol in urine of such patients (Mitra *et al.*, 1998). In addition, retinol has been shown to be negatively associated with the concentration of acute phase proteins (Butler *et al.*, 1993). However, retinol concentration has been shown to increase after the acute phase even without vitamin A supplements, thus suggesting an adaptive response similar to the decrease in serum iron during acute infection (Neuzil *et al.*, 1994). Increased morbidity and mortality noted in vitamin A deficient population and reduction in all cause mortality after the administration of vitamin A, have lead to the belief that vitamin A plays a protective role in infectious diseases (Sommer, 1993; Sommer *et al.*, 1983). However, this notion has been confronted by vitamin A supplementation studies on respiratory infections (Stephensen *et al.*, 1998; Bresee *et al.*, 1996; Fawzi *et al.*, 1998). Children given high dose vitamin A showed increased symptoms of respiratory illness and increased mortality. These observations lead to the speculation that the effect of vitamin A could be disease specific, showing beneficial effects for measles, but not for pneumonia (Fitch and Neville, 2002).

Our current study suggests the possible interaction between vitamin A and immune response in acute respiratory infection (URTI, pneumonia and bronchiolitis). Though WFA, Hb and zinc did not show any association with Th1 or Th2 cytokines, serum vitamin A was inversely associated with NPA IL2 concentration in acute respiratory infection. The association remained strong when the data was controlled for other nutritional parameters. Furthermore, when the effect of large dose vitamin A was studied on the production of cytokines from PBMC, there was a significant reduction in IL2 concentration 15 days after vitamin A supplementation in apparently normal children, while IL4 and IFN γ remained within normal range. Vitamin A has been hypothesized to influence the balance between Th1 and Th2 responses. The present study suggests down regulation of Th1 response by vitamin A.

The findings of this study are consistent with previous reports that have shown that vitamin A down regulates Th1 response (Carman *et al.*, 1992; Cui *et al.*, 2000). Vitamin A deficient mice were shown to produce

high concentration of IFN- γ and IL12, that are Th1 cytokines that promote the secretion of IL2 cytokine (Carman *et al.*, 1992). In conformity, high dietary vitamin A in mice showed down regulation of Th1 response (Cui *et al.*, 2000). In humans, there was increased production of TGF β from vitamin A treated PBMC's and from skin biopsies of individuals using topical retinoic acid (Szabo *et al.*, 1994; Fischer *et al.*, 1992). TGF- β is a regulatory cytokine that is known to suppress Th1 cells (Anderson and Heilman, 1995; Bottomly, 1988). However, more recent reports have shown that physiological levels of all-trans retinoic acid increases binding of IL2 to IL2 receptors (IL2-R) that will increase T cell stimulation, but reduce the levels of free IL2 (Ertesvag *et al.*, 2002).

Taken together these data show that the Th1 and Th2 cytokines respond to dietary vitamin A and to vitamin A supplementation. However, it is unclear if vitamin A decreases IL2 concentration by down regulation of Th1 cells or whether vitamin A simply decreases IL2 concentration by increasing its binding to IL2-R. Down regulation of Th1 response might lead to poor clearance of intracellular pathogens; in contrast, excess binding of IL2 to IL2-R may lead to immunopathogenic disease. Either way, vitamin A might aggravate respiratory symptoms by altering IL2 response. This is consistent with the finding that Well-nourished preschool children who received high dose vitamin A supplementation had a higher incidence of respiratory infection, while there was no increase in respiratory infections among stunted children who had received vitamin A (Stephensen *et al.*, 1998; Bresee *et al.*, 1996; Fawzi *et al.*, 1998).

IL2, a Th1 cytokine appears to be more sensitive to nutritional status than other cytokines. In conformation with this, earlier studies from our laboratory showed a diminished IL2 response in children with protein energy malnutrition and iron deficiency anemia, while IFN γ and Th2 cytokines were not altered (Bhaskaram *et al.*, 2003). Th1 response is critical for the control of viral infections and intracellular bacterial pathogens. Decreased Th1 response will impair recovery from viral infections, as IL2 is a vital cytokine for T cell proliferation, differentiation and cytotoxic function. On the contrary heightened stimulation of T cells due to increased binding of IL2 to IL2-R will lead to immunopathogenic diseases. Whatever the underlying cause, vitamin A alters IL2 response, which may be responsible for increased morbidity associated with vitamin A supplements in children with ARI.

Though the present study does not show the effects of vitamin A on respiratory morbidity, it does signify that vitamin A modulates Th1 response and thus might alter the course and outcome of infectious disease. More

studies are needed to delineate the role of vitamin A on Th1 and Th2 response and its effect on IL2-R in acute respiratory infection.

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