Published in final edited form as:
Nutr Res. 2007 July ; 27(7): 400–407.

Association of vitamin A deficiency with decrease in TNF-α expressing CD3-CD56+ NK cells in Ghanaians

Yi Jiang[1], **Francis Obuseh**[1], **William Ellis**[2], **Chandrika Piyathilake**[3], and **Pauline Jolly**[1,4]

1 *Department of Epidemiology, University of Alabama at Birmingham, Birmingham, AL*

3 *Nutrition Sciences – Nutritional Biochemistry and Genomics, University of Alabama at Birmingham, Birmingham, AL*

2 *Department of Biochemistry, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana*

Abstract

Although low plasma vitamin A concentrations are associated with increased incidence or severity of infections such as respiratory tract infection and measles in children, there is a paucity of data on the effect of vitamin A deficiency on the distribution of, and cytokine production by, the different cellular immune subsets in humans. We conducted a cross-sectional study in a district in Ghana to characterize cellular subsets and functional capacity of peripheral blood mononuclear cells from vitamin A deficient and vitamin A sufficient (normal) individuals, and evaluated the relationships between vitamin A concentration in plasma and cellular immune status. We measured the percentages of selected cellular phenotypes and intracellular cytokine expression and describe the differential cellular subset distributions and alterations in cytokine expression in participants with normal and deficient vitamin A concentrations. The major change observed in the constitution of cellular subsets was a decrease in TNF-α expressing CD3-CD56+ NK cells in those with vitamin A deficiency compared with normal individuals. CD4+ T cell proliferation and production of IFN-γ and IL-4 were not statistically different between the two groups. These results support previous studies that demonstrated decreased NK cell activity in vitamin A deficient animals. The decrease in TNF-α expressing NK cells observed in vitamin A deficient individuals in this study could help to explain the decreased resistance to infections observed in those with vitamin A deficiency.

Keywords

vitamin A; cellular immunity; T-cells; NK cells; humans

1. Introduction

Vitamin A deficiency is one of the world's major malnutrition problems. An estimated 124 million children are vitamin A deficient and are at higher risk of death from infectious diseases [1]. Studies in humans and evidence from animal experiments show that there is an association between vitamin A status and immune function [2]. The immune efficacy of vitamin A supplementation on infection rates has been examined in several randomized, double-blind,

[4]Corresponding Author: Pauline E. Jolly, PhD, MPH, Professor, Department of Epidemiology, School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama 35226, Tel: 205 934-1823, Fax: 205 975 3329, Email: jollyp@uab.edu.

placebo-controlled trials of malnourished children in various developing countries [3–6]. Providing vitamin A supplements to malnourished children has been found to reduce mortality associated with measles and diarrhea [3–4] but not pneumonia [4–6]. These studies do not, however, provide insight into how vitamin A supplementation results in decreased morbidity and mortality associated with measles or diarrheal infections.

Experiments using animals suggest that deficiency of vitamin A and related retinoids modulate many different immune response elements. These include inhibition of mitogen-stimulated T-cell proliferation [7–9], antigen-specific antibody production [10], and the ability to produce immunoglobulins IgA and IgG [11–12]; reduction in the ability of CD4+ T cells to provide B-cell stimulus for antigen–specific IgG1 responses [12]; limitation of Th-2-type cytokine-gene expression [13]; decrease in ability of neutrophils to phagocytose infectious organisms and generate active oxidant molecules [14]; and reduction in the resistance to several infectious organisms [9–10]. Although much data are available from animal studies on the association between vitamin A status and impaired immune function, there is a paucity of data on the effect of vitamin A deficiency on the distribution and function of different cellular subsets in the human immune system. This information is urgently needed, especially as immune suppression is a significant global health problem, and immune suppressive/immune modulating diseases, such as human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), malaria, measles, and parasitic infections are rampant in developing tropical countries or the world where millions of people experience chronic nutritional deficiency. We examined the relationship between plasma vitamin A concentration and the percentages of different immune cellular subsets and intracellular cytokine expression in humans in order to better define the role of vitamin A in immune function.

Thus, we measured plasma vitamin A concentrations, characterized cellular subsets and intracellular cytokine expression in peripheral blood mononuclear cells (PBMCs) of study participants and evaluated the relationship between vitamin A concentration and the distribution and potential cytolysine activity of cellular subsets. We found an association between vitamin A deficiency and a decrease in TNFα-expressing CD3-CD56+ NK cells. This is the first report of this association in the humans and is a significant finding that will contribute to advancement of the study of vitamin A deficiency and impairment of immune function.

2. Materials and Methods

2.1. Study population

The study population consisted of a 56 adults from the Ejura Sekyedumase district in the Ashanti Region of Ghana. The Institutional Review Board of the University of Alabama at Birmingham (UAB) and the Medical School Ethics Committee of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana, gave approval for the study. Since serum retinol decreases transiently during the acute phase response and can thus interfere with the use of serum retinol as an indicator of vitamin A status [15–16], those with acute illness were excluded from participation. After written informed consent was obtained, a 20 ml blood sample was taken by venipuncture from each participant in EDTA vacutainer tubes and separated into plasma and PBMCs (17). The plasma was stored at −80°C. Peripheral blood mononuclear cells were separated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density centrifugation and stored in Cell Freezing Medium II (Atlanta Biologicals, Norcross, GA) in liquid nitrogen [17]. These samples were shipped to UAB for analysis. Plasma samples were used for determination of vitamin A, vitamin E, hepatitis B virus (HBV) surface antigen (HBsAg), hepatitis C virus (HCV) antibody and malaria antibody and antigen. PBMCs were used for determination of percentages of different leukocyte subsets. These methods are detailed below.

2.2. Simultaneous determination of vitamins A and E in plasma

A modified version of the high performance liquid chromatography (HPLC) procedure developed by Stacewicz-Sapuntzakis et al. [18] was used to measure both vitamins A and E in plasma. The HPLC system included a 150 × 3.9 mm Nova-pak C18 (4 microns) column with a guard pak pre-column (both from Waters, Milford, MA), Waters Millipore TCM column heater, Waters 490 multi-wavelength detector, Hitachi 655–61 processor, Hitachi 655A-11 liquid chromatography, and BioRad autosampler AS-100. The mobile phase consisted of methanol/acetonitrile/methylene chloride (50:45:5, v/v/v; Mallinckrodt Specialty Chemical Co., Paris, KY) run at 1 ml/min. Vitamin A (all trans retinoic acid) was obtained from Sigma Chemical Co. (St. Louis, MO), and vitamin E (dl-alpha tocopherol) and tocol were obtained from Hoffmann-La Roche Inc. (Nutley, NJ). Tocol is a tocopherol derivative that is used as an internal standard to correct for any loss in retinol and tocopherol during the extraction procedure because it is well separated from retinol under normal phase conditions. In preparation of the standards, vitamins A and E were dissolved in ethanol and concentrations were measured at 325 nm and 292 nm, respectively using a programmable multi-wavelength detector (Waters 490). Tocol was dissolved in ethanol (0.3 μg/ml). All procedures were performed in subdued yellow light. Fresh standards were prepared for each assay and standard curves were constructed by plotting peak heights against the concentrations of vitamin standards.

Plasma samples from study participants were thawed and 200 μl of each placed in a test tube, 100 μl of the internal standard (tocol) and 100 μl ethanol for protein precipitation were added and the tubes were vortexed for 2 minutes. For extraction, 1 ml of hexane (EM Science, Cherry Hill, NJ) was added and the mixture was vortexed for 5 minute and centrifuged at 8000 RPM for 10 minutes. The top hexane layer was carefully removed with a Pasteur pipette into another microcentrifuge tube and dried using a rotary speed-vac concentrator/evaporator (Savant Instrument Inc, Farmingdale, NY) heated to 37°C for 25 minutes. The residue was dissolved in 200μl mobile phase and votexed for 30 seconds. Twenty microliters of this extract was injected for chromatographic analysis.

Tocol internal standard was used to determine the percentage recovery in samples. For quality control, pooled normal human plasma samples were divided into two portions of high and low concentration for vitamin A and E and prepared for analysis in the same manner as the patient samples. These were run in each assay. Evaluation of the laboratory performance was assessed by comparing the results of the quality control samples with the mean and standard deviations (SD) calculated from the results of several runs of the assay. The run was rejected if any value fell outside the range of ± 2 SD from the mean.

2.3. Test for antibodies to HBV surface antigen

Antibody to HBV surface antigen (HBsAg) in plasma samples was determined using the Bio-Rad Enzyme Immunoassay according to the manufacturer's directions (Bio-Rad, Redmont, WA, USA). A sample was considered initially reactive for anti-HBs if the absorbance value was greater than or equal to the mean absorbance value of the negative controls plus 0.07 according to the manufacturer's instructions. Positive samples were determined by repeated reactivity in duplicate tests.

2.4. Test for HCV antibody in plasma

Qualitative detection of antibody to HCV in plasma was conducted using the Abbott HCV Enzyme Immunoassay according to the manufacturer's directions (Abbott Laboratories, Abbott Park, IL, USA). Test samples with an optic density (OD) greater than or equal to the mean absorbance of the negative controls plus 0.25 times the mean absorbance of the positive controls were considered initially reactive by the criteria of ABBOTT HCV EIA 2.0. Positive samples were determined by repeated reactivity in duplicate tests.

2.5 Determination of malaria antigen and antibody in plasma

To determine malaria infection, we conducted a Malaria Antigen Celisa assay (Cellabs Pty Ltd., Brookvale, Australia). This is a monoclonal antibody-based assay specific for detection of a *P. falciparium* merozoite antigen that circulates for up to 14 days post-infection in the plasma. The assay detects *P. falciparum* infection at parasitemias as low as 0.001% and has a sensitivity of 98% and a specificity of 96%. A test for malaria antibody in plasma using a Malaria Antibody ELISA (DIA.PRO Diagnostic Bioprobes Srl, Milano, Italy) was also conducted. These microplates were coated with purified recombinant proteins of *P. falciparum* and *P. vivax* that account for approximately 80% and 15% of all cases of malaria, respectively, worldwide. This test has a sensitivity of 98% and a specificity of 98% on plasma and sera.

2.6. Determination of percentages of leukocyte immunophenotypes using flow cytometry

The percentages of T cells (CD3+), subsets of T cells (CD4+ and CD8+), B cells (CD19+), NK cells (CD3-CD56+) and macrophages (CD14+) were measured by flow cytometry. CD8 + T cell subset classification has been proven useful in monitoring the immune system in several clinical situations [19,20]. Therefore, we classified CD8+ T cell subsets into naïve (CD8 +CD45RA+CD27+), memory (CD8+CD45RA-CD27+) and CTL effector (CD8+CD45RA +CD27-) cells by flow cytometry. Subtypes of NK cells CD3-CD56+CD16+ and CD3-CD56 +CD16- were also determined.

PBMCs were incubated with combinations of fluorescein FITC-, PE-, PerCP-labeled monoclonal antibodies against CD3 (clone SK7), CD4 (clone RPA-T4), CD8 (clone SK1), CD14 (clone MδP9), CD16 (clone 3G8), CD19 (clone 4G7), CD27 (clone MT271), CD45RA (clone HI100), and CD56 (clone NCAM16.2) (BD PharMingen, San Diego, CA) for 30 min at 4°C [21]. Isotype-matched irrelevant FITC-, PE-, and PerCP-labeled MAbs (BD PharMingen, San Diego, CA) were used as controls in the experiments. After washing the cells three times in PBS, cell fluorescence for each phenotype was analyzed using Becton Dickinson FACS and CELLQuest software.

2.7. Determination of cytokine production by CD4+, CD8+ and CD3-CD56+ cells

CD4+ T helper cell (TH) and CD8+ T cell cytokine profiles (IL-4, IFN-γ) were assessed by flow cytometric detection of mitogen-induced intracellular cytokines. CD8+ T cell cytokine production (perforin and granzyme A) was measured by intracellular cytokine staining and multi-parameter flow cytometry [22–24]. Also, the presence of intracellular cytokines perforin and TNF-α expression in phenotypically defined NK cells (CD3-CD56+) was examined.

For intracellular cytokine staining, PBMCs (1×10^6) were placed in 12×75 mm tissue culture tubes containing 2 ml of medium containing 0.5 μg each of CD28 and CD49d monoclonal antibodies and phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, MO). These cultures were incubated at 5-degree slants at 37°C in a humidified 5% CO_2 atmosphere for 6 hours. In the last 5 hour, 10 μg/ml of the Golgi inhibitor, Brefeldin A (Sigma, St. Louis, MO) was added. After incubation, the cells were collected in phosphate-buffered saline (PBS) and washed once with cold PBS containing 1% bovine serum albumin (BSA). Cells were then re-suspended in 100 μl of staining buffer (PBS supplemented with 0.1% sodium azide and 1% FBS pH 7.4) and the phenotypic monoclonal antibodies (CD3, CD4, CD8, and CD56) and incubated at 4° C for 30 mins. After staining, the cells were washed with staining buffer and fixed in 1 ml of fix/perm buffer (BD PharMingen, San Diego, CA) for 30 mins at 4°C. The cells were then washed with perm staining buffer (BD PharMingen) and incubated with cytokine antibodies (anti-IFN-γ, clone 4S.B3; anti-IL-4, clone 8D4-8; anti-perforin, clone 8G9; anti-granzyme A, clone CB9 and anti-TNF-α, clone MAb 11) (BD PharMingen) in the presence of perm staining buffer for 30 mins at 4°C. After washing with perm buffer, immunophenotypic patterns were

investigated using a Becton Dickson FACS with CELLQuest software. At least 10,000 events were acquired and positive cells were expressed as percentages among the different subsets (CD4+, CD8+, CD3-CD56+).

2.8. Determination of CD4 lymphoproliferative response

CD4+ T cells proliferation was measured using bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) incorporation assay by flow cytometry [25]. PBMCs (1×10^6) were placed in 12×75 mm tissue culture tubes containing 2 ml of medium with or without pytohemagglutinin (PHA) (Sigma, St. Louis, MO). These cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 days. BrdU was added to the cultured cells at a final concentration of 40 μM, 16 h before the end of the 3-day incubation period. After incubation, cells were collected and stained with an anti-BrdU MAb (clone B44) conjugated to FITC (BD PharMingen) together with a PE-coupled anti-CD4 MAb in the presence of DNase (Sigma). Thereafter, the frequency of CD4+ T cells with BrdU incorporation was measured by flow cytometry with CELLQuest software.

2.9. Determination of monocyte phagocytic function

We examined phagocytosis of peripheral blood monocytes by flow cytometry using the method of Steinkamp *et al.*, [26] with some modifications. The concentration of cell suspension was adjusted to 4×10^6 cells/ml in medium without serum. Fluorescent latex particles (2.5% latex solids, 2.0 μm in diameter; Polyscience, Warrington, PA) were diluted 10 times with RPMI 1640 media and 4 μl of the suspension of these particles added to 1 ml of cell suspension. The mixture was allowed to react at 37°C for 90 min with gentle shaking in a water bath. The cells were then washed three times with cold PBS (pH 7.4) to terminate the reaction and stained with PE-labeled anti-CD14 MAb. The final pellet was re-suspended with PBS and the percentage of CD14+ cells with phagocytosed fluorescent particles was measured by flow cytometry. The phagocytic rate, which represents the percentage of cells with ingested particles relative to the total number of CD14+ cells, was determined.

2.10. Statistical analysis

Data were entered and analyzed using Windows SPSS version 10.0 and expressed as the means ± SD. An unpaired t test was used to compare two groups when the variances were equal or the Mann-Whitney U test when the variances were unequal. Both the univariate and multivariate statistical methods were employed to assess correlation. Associations between continuous variables were examined using Pearson's correlation coefficient, a P value of <0.05 was considered statistically significant.

3. Results

3.1. Vitamin A concentrations and demographic characteristics of study participants

Study participants ranged in age from 19 to 86 years, with a mean of 43.7 ± 18.3 years (Table 1). The median vitamin A concentration for the total group was 20.39 μg/dL, the mean was 22.22 ± 1.07 μg/dL, and the range 8.3–48.7 μg/dL. Participants were categorized as having normal vitamin A concentration (≥20 μg/dL), moderate vitamin A deficiency (10 μg/dl to <20 μg/dL) or severe vitamin A deficiency (<10 μg/dL) [27]. Two of the study participants (3.6%) had severe vitamin A deficiency with vitamin A concentrations of 8.3 μg/dL and 9.4 μg/dL respectively, 25 (44.6%) had moderate vitamin A deficiency (mean 16.81 ± 2.65 μg/dL, range 11.7–19.96 μg/dL), and 29 (51.8 %) had normal vitamin A concentrations (mean 27.8± 7.00 μg/dL, range 20.1–48.7 μg/dL). Thus, approximately 48% of participants had severe or moderate vitamin A deficiency. Fifty of the 56 participants had deficient vitamin E levels (<0.5 mg/dL; Table 1). Sixteen percent of participants were positive for HBV infection, 11 percent

for HCV infection and 18% were positive for malaria antigen indicating current or very recent infection. All participants were positive for malaria antibody. Study participants were divided into normal (≥20 μg/dL) and deficient vitamin A groups (<20 μg/dL) for analysis of immune cell subsets. The two groups did not differ significantly with regard to age and gender.

3.2. Association of percentages and function of leukocytes with vitamin A concentration

When immune cellular subsets of the study group were examined according to normal or deficient vitamin A concentrations, we found that the vitamin A deficient group had non-significant decreases in the percentages of CD3+, CD4+, CD8+, CD14+, CD19+, CD8+CD27-CD45RA+and CD3-CD56+ cells compared with the normal vitamin A group (Figure 1).

The percentage of IFN-γ expressing CD4+ T cell was lower in the vitamin A deficient group (mean ± SD =6.73 ± 4.54) compared with the normal vitamin A group (mean ± SD =10.27 ± 10.39; Table 2) but this difference was not statistically significant ($P = 0.121$). Both groups of participants demonstrated a Th1 profile and the IFN-γ: IL-4 ratio in the vitamin A deficient group was 2.52:1 compared with 3.78:1 in those with normal vitamin A concentrations. No statistically significant difference in lymphoproliferative response of CD4+ T cells was observed between the two groups.

When the mean percentages of CD8+ T cells expressing IFN-γ were compared for the two groups, the percentage of IFN-γ expressing CD8+ T cells was significantly lower in the vitamin A deficient group (13.07±7.76) compared to the normal group (19.51 ± 10.00.)($P = 0.041$) (Table 2). By contrast, the percentage of IL-4 expressing CD8+ T cells in the vitamin A deficient participants was slightly higher compared to participants with normal vitamin A concentrations but the difference was not statistically significant.

When CD8+ cells were analyzed for the presence of intracellular perforin and granzyme A without stimulation, we found that the percentages of CD8+ T cells containing perforin, and granzyme A were slightly lower (but not statistically significant) in participants with vitamin A deficiency compared with those with normal vitamin A concentrations (Table 2).

In our study group, the percentage of CD3-CD56+ NK cells was 4.27 ± 3.13 % (median = 3.77, range, 1–17 %). The vitamin A deficient group of participants had a slightly lower percentage of NK cells compared to the sufficient vitamin A group. The percentages of CD3-CD56+CD16- (primarily $CD56^{bright}$subset) and CD3-CD56+CD16+ (primarily $CD56^{dim}$subset) were not different between the two groups (Figure 1).

When perforin and TNF-α expressing CD3-CD56+ NK cells were measured in the two groups, we found a significant decrease in the percentage of TNF-α expressing CD3-CD56+ NK cells in the vitamin A deficient group (12.24 ± 6.66%) compared to the normal group (19.03 ± 10.56%)($P = 0.009$) (Table 2; Figure 2). The percentages of perforin expressing NK cells were not different between the two groups.

There was no difference in the phagocytic rates between the two groups, and no relationship between the phagocytic rates and vitamin A concentrations (Table 2).

3.3. Univariate and multivariate analysis for correlation of vitamin A with demographic and immune variables

Several factors (age, gender, vitamin E levels, percentages of T and B cells and other immune parameters) were examined for correlation with vitamin A concentration using univariate analysis. We found that low vitamin E level (Pearson correlation=0.356, $P = 0.010$) and low percentage of TNF-α expressing NK cells (Pearson correlation =0.384, $P = 0.006$) were significantly associated with vitamin A deficiency. Multiple linear regression analyses that

included each of the variables showed that these two parameters remained significantly related to vitamin A deficiency. After adjusting for vitamin E level there was still a strong association between the percentage of TNF-α expressing NK cells and vitamin A concentration (coefficient = 0.328, $P = 0.025$). We also conducted multiple linear regression analysis with TNF-α expressing NK cells as the dependent variable after adjusting for other immunity parameters and found a significant association between the percentage of TNF-α expressing NK cells and vitamin A concentration (coefficient =0.259, $P = 0.026$).

4. Discussion

Vitamin A deficiency increases susceptibility to diseases. Animal studies have shown that vitamin A deficiency impairs both humoral and cellular mediated immunity [7–14]. There have been few reports on the potential role of vitamin A deficiency in modifying the peripheral distribution of lymphocyte subsets in humans. The main observations in the studies of Semba *et al.* [28] were that children with xerophthalmia had lower ratios of helper to suppressor lymphocytes, and lower proportions of the naïve subset of helper T cells than children with normal vitamin A concentrations. In this cross-sectional study we showed non-significant decreases in percentages of the CD3+, CD4+, CD8+, CD14+ and CD19+ cells in vitamin A deficient participants. Other investigators have shown a reduced frequency of T helper cells in vitamin A-deficient mice [11], however, T cell numbers were not markedly altered. Although a small reduction in the relative number of T cells has been reported in vitamin A deficient children [28], significant reductions have not been observed in mice [29] or rat [30–31], even when functional responses were greatly impaired.

In animals, changes in vitamin A status or the administration of natural or synthetic retinols have been shown to significantly affect T-cell functions [32]. However, the effects of vitamin A status on T cell function in humans are not clearly defined. Moreover, epidemiological evidence is scarce regarding a relationship between vitamin A status and T cell function in humans. A signal report suggested a positive association between plasma retinol concentration and delayed-type hypersensitivity in the elderly [33], while other studies did not find any relationship between plasma retinol concentrations and T cell function [34–35]. In this study, there were no statistical differences in the means of the lymphocyte, monocyte, or NK cell subsets between the vitamin A deficient and normal groups except in the proportions of IFN-γ expressing CD8+ T cells and TNF-α expressing NK cells. Vitamin A-deficient participants were more likely than those with normal vitamin A concentrations to have lower percentages of IFN-γ expressing CD8+ T cells and TNF-α expressing NK cells. However, by univariate analysis, only the percentage of TNF-α expressing NK cells showed a significant association with vitamin A concentration. Using multiple linear regression analysis we found that the low percentage of TNF-α expressing NK cells was strongly associated with a lower plasma vitamin A concentration after adjusting for other variables.

NK (CD3-CD56+) cells comprise about 5–20% (~15%) of all circulating lymphocytes in humans [36–39]. Although the range of NK cells in our study participants was 1–17%, the mean percentage of these cells was lower than the reported mean. This may be due to heterogeneity [40–41] and overall health in different populations as well as variability in the methodologies used. NK cells can kill target cells recognized through their NK receptors, as well as cells coated with specific antibody which binds to the NK cell Fc receptor [42]. Upon contact with their targets, NK cells degranulate and release a pore-forming protein, perforin, into the intercellular spaces resulting in colloid-osmotic injury to the target. Besides perforin, granzymes, TNF and Fas also are involved in NK-mediated target cell killing. NK cells also produce cytokines such as interferon-γ, TNF, and GMCSF and therefore also help in the defense against bacterial, protozoan and fungal infections through activation of phagocytic cells. Moreover, NK cells are thought to play an important role in tumor-immune surveillance. In

this study, we did not find any association between perforin expressing NK cells and vitamin A deficiency. However, vitamin A deficiency was associated with a relatively lower proportion of TNF-α expressing NK cells. Several studies in experimental animal models have demonstrated that vitamin A deficiency decreases NK cell lytic activity [43]. Our study in humans supports this finding and provides initial evidence to justify further investigations on the effect of vitamin A on NK cell function.

In conclusion, we describe the differential subset distributions between vitamin A deficient and sufficient individuals as well as cytokine expression in specific lymphocyte subsets. The major change in the constitution of lymphocyte subsets is a decrease in TNF-α expressing CD3-CD56+ NK cells in individuals with vitamin A deficiency compared to those with normal vitamin A concentrations. The results obtained indicate that there are alterations in cellular immunity in vitamin A deficient participants which could decrease host resistance to infections. These findings should be considered exploratory, given the cross-sectional design of this study and because each variable was subjected to multiple comparisons. Also, it is important to recognize that because of the limited sample size, the study may not have been powered to identify other immune parameters potentially associated with vitamin A deficiency. Further clarification of these other possible immune correlates of vitamin A deficiency might be afforded by additional investigation.

Acknowledgements

This research was supported by an USAID grant LAG-G-00-96-90013-00 for the Peanut Collaborative Support Research Program and Minority Research Training Grant TW37 T00077 from the Fogarty International Center and the National Center on Minority Health and Health Disparities, National Institutes of Health. We are grateful to the District Health Director, Physician, Pharmacist, Environmental Field Officers, and other health personnel at the Ejura District Hospital for their assistance. We especially thank the participants who made this study possible. We thank Thomas Kruppa, Ohene Adjei and other laboratory personnel at the Kumasi Center for Collaborative Research (KCCR) in Tropical Diseases, KNUST for assistance with cell separation, storage and shipping.

References

1. Humphrey JH, West KP Jr, Sommer A. Vitamin A deficiency and attributable mortality among under-5-year-olds. Bull WHO 1992;70:225–32. [PubMed: 1600583]
2. Semba RD. Vitamin A, Immunity, and Infection. Clin Infect Dis 1994;19:489–99. [PubMed: 7811869]
3. Fawzi WW, Chalmers TC, Herrera MG, Mosteller F. Vitamin A supplementation and child mortality. JAMA 1993;269:898–903. [PubMed: 8426449]
4. Barreto ML, Santos LM, Assis AM, Araujo MP, Farenzena GG, Santos PA, Falccone RL. Effect of vitamin A supplementation on diarrhea and acute lower-respiratory-tract infections in young children in Brazil. Lancet 1994;344:228–31. [PubMed: 7913157]
5. The Vitamin A, Pneumonia Working Group. Potential interventions for the prevention of childhood pneumonia in developing countries: a meta-analysis of data from field trials to assess the impact of vitamin A supplementation on pneumonia morbidity and mortality. Bull WHO 1995;73:609–19. [PubMed: 8846487]
6. Dibley MJ, Sadjimin T, Kjolhede CL, Moulton LH. Vitamin A supplementation fails to reduce incidence of acute respiratory illness and diarrhea in preschool-age Indonesian children. J Nutr 1996;126:434–42. [PubMed: 8632216]
7. Friedman A, Sklan D. Impaired T lymphocyte immune response in vitamin A depleted rats and chicks. Br J Nutr 1989;62:439–49. [PubMed: 2479411]
8. Friedman A, Sklan D. Antigen-specific immune response impairment in the chick as influenced by dietary vitamin A. J Nutr 1989;119:790–5. [PubMed: 2786059]
9. Sklan D, Melamed D, Friedman A. The effect of varying dietary concentrations of vitamin A on immune response in the turkey. Br Poult Sci 1995;36:385–92. [PubMed: 7583372]

10. Friedman A, Mjeidovsky A, Leitner G, Sklan D. Decreased resistance and immune response to Escherichia coli infection in chicks with low or high intakes of vitamin A. J Nutr 1991;121:395–400. [PubMed: 2002410]
11. Carman JA, Smith SM, Hayes CE. Characterization of a helper T lymphocyte defect in vitamin A-deficient mice. J Immunol 1989;142:388–93. [PubMed: 2463304]
12. Gangopadhyay NN, Moldoveanu Z, Stephensen CB. Vitamin A deficiency has different effects on immunoglobulin A production and transport during influenza A infection in BALB/c mice. J Nutr 1996;126:2960–7. [PubMed: 9001362]
13. Cantorna MT, Nashold FE, Hayes CE. Vitamin A deficiency results in a priming environment conducive for Th1 cell development. Eur J Immunol 1995;25:1673–79. [PubMed: 7614995]
14. Twining SS, Schulte DP, Wilson PM, Fish BL, Moulder JE. Vitamin A deficiency alters rat neutrophil function. J Nutr 1997;127:558–65. [PubMed: 9109605]
15. Jolly PE, Moon TD, Mitra AK, Rosario GR, Blount S, Clemons TE. Vitamin A depletion in hospital and clinic patients with acquired immunodeficiency syndrome- a preliminary report. Nutr Res 1997;17:1427–41.
16. Stephensen CB, Gildengorin G. Serun retinol, the acute phase response, and the apparent misclassification of vitamin A status in the third National Health and Nutrition Examination Survey. Am J Clin Nutr 2000;72:1170–8. [PubMed: 11063445]
17. Jolly PE. Replicative characteristics of primary isolates of the human immunodeficiency virus type 1 in peripheral blood mononuclear cells, primary macrophages and CD4+ transformed T-cell lines. Cell Mol Biol 1997;43:1057–65. [PubMed: 9449539]
18. Stacewicz-Sapuntzakis M, Bowen PE, Kikendall JW, Burgess M. Simultaneous Determination of Serum Retinol and Various Carotenoids: their Distribution in Middle-aged Men and Women. J Micronutr Anal 1987;3:27–45.
19. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997;186:1407–18. [PubMed: 9348298]
20. Takata H, Takiguchi M. Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules. J Immunol 2006;177:4330–43. [PubMed: 16982867]
21. National Committee for Clinical Laboratory Standards. Clinical applications of flow cytometry: quality assurance and immunophenotyping of peripheral blood lymphocytes; Tentative guideline. Villanova, PA: National Committee for Clinical Laboratory Standards; 1992. NCCLS Document H42-T
22. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood 1995;86:1408–19. [PubMed: 7632949]
23. Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J Exp Med 1995;182:1357–67. [PubMed: 7595206]
24. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differrential production of interferon-r and interleukin-4 in response to Th1- and Th2-stimulation pathogens by ã/ä T cells in vivo. Nature 1995;373:255–7. [PubMed: 7816142]
25. Mehta BA, Maino VC. Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. J Immunol Meth 1997;208:49–59.
26. Steinkamp JA, Wilson JS, Saunders GC, Stewart CC. Phagocytosis: Flow cytometic quantitation with fluorescent microspheres. Science 1982;215:64–6. [PubMed: 7053559]
27. Jason P, Archibald LK, Nwanyanwu OC, Sowell AL, Buchanan I, Larned J, Bell M, Kazembe PN, Dobbie H, Jarvis WR. Vitamin A levels and immunity in humans. Clin Diagn Lab Immunol 2002;9:616–21. [PubMed: 11986269]
28. Semba RD, Muhilal Ward BJ, Griffin DE, Scott AL, Natadisastra G, West KP Jr, Sommer A. Abnormal T-cell subset proportions in vitamin A deficient children. Lancet 1993;341:5–8. [PubMed: 8093291]

29. Smith SM, Levy NL, Hayes CE. Impaired immunity in vitamin A-deficient mice. J Nutr 1987;117:857–65. [PubMed: 3495650]

30. Bowman TA, Goonewardene IM, Pasatiempo AMG, Ross AC, Taylar CE. Vitamin A deficiency decreases natural killer cell activity and interferon production in rats. J Nutr 1990;120:1264–73. [PubMed: 1698953]

31. Zhao Z, Ross AC. Retinoic acid repletion restores the number of leukocytes and their subsets and stimulates natural cytotoxicity in vitamin A-deficient rats. J Nutr 1995;125:2064–73. [PubMed: 7643240]

32. Ross, AC.; Hammerling, UG. Retinoid and the immune system. In: Sporn, MB.; Roberts, AB.; Goodman, DS., editors. The Retiniods: Biology, Chemistry and Medicine. New York: Raven Press; 1994. p. 521-43.

33. Chavance M, Herbeth B, Mikstacki T, Fournier C, Vernhes G, Janot C. Nutritional support improves antibody response to influenza virus vaccine in the elderly. Br Med J 1985;291:1065–6.

34. Goodwin JS, Garry PJ. Lack of correlation between indices of nutritional status and immune function in elderly humans. J Gerontol 1988;43:46–9.

35. Gardner EM, Bernstein ED, Dorfman M, Abrutyn E, Murasko DM. The age-associated decline in immune function of healthy individuals is not related to changes in plasma concentrations of b-carotene, retinol, a-tocopherol or zinc. Mech Ageing Dev 1997;94:55–69. [PubMed: 9147360]

36. Meier U, Owen RE, Taylor E, Worth A, Naoumov N, Willberg C, Tang K, Newton P, Pellegrino P, Williams I, Klenerman P, Borrow P. Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. J Virol 2005;79:12365–75. [PubMed: 16160163]

37. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, Hooijkaas H, van Dongen JJ. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997;130:388–93. [PubMed: 9063413]

38. McNerlan SE, Alexander HD, Rea IM. Age-related reference intervals for lymphocyte subsets in whole blood of healthy individuals. Scand J Clin Lab Invest 1999;59:89–92. [PubMed: 10353321]

39. Tsegaye A, Messele T, Tilahun T, Hailu E, Sahlu T, Doorly R, Fontanet AL, Rinke de Wit TF. Immunohematological reference ranges for adult Ethiopians. Clin Diagn Lab Immunol 1999;6:410–4. [PubMed: 10225845]

40. Kassu A, Tsegaye A, Petros B, Wolday D, Hailu E, Tilahun T, Hailu B, Roos MT, Fontanet AL, Hamann D, Rinke de Wit TF. Distribution of lymphocyte subsets in healthy human immunodeficiency virus-negative adult Ethiopians from two geographic locales. Clin Diagn Lab Immunol 2001;8:1171–6. [PubMed: 11687459]

41. Kassa E, Rinke de Wit TF, Hailu E, Girma M, Messele T, Mariam HG, Yohannes S, Jurriaans S, Yeneneh H, Coutinho RA, Fontanet AL. Evaluation of the World Health Organization staging system for HIV infection and disease in Ethiopia: association between clinical stages and laboratory markers. AIDS 1999;13:381–9. [PubMed: 10199229]

42. Whiteside TL, Herberman RB. Role of natural killer cells in health and disease. Clin Diagn Lab Immunol 1994;1:125–33. [PubMed: 7496932]

43. Ross A, Stephensen C. Vitamin A and retinoids in antiviral responses. FASEB J 1996;10:979–85. [PubMed: 8801180]

List of Abbreviations

BrdU

5-bromo-2-deoxyuridine

BSA

Bovine serum albumin

DNase

Deoxyribonuclease

FACS

Fluorescent-activated cell sorting

FBS
Fetal bovine serum

FITC
Fluorescein isothiocyanate

GMCSF
Granulocyte-macrophage colony-stimulating factor

HBsAg
Hepatitis B surface antigen

HBV
Hepatitis B virus

HCV
Hepatitis C virus

HIV/AIDS
Human immunodeficiency virus/Acquired immune deficiency syndrome

IFN-γ
Interferon gamma

IgA
Immunoglobulin A

IgG
Immunoglobulin G

IL-4
Interleukin 4

MAbs
Monoclonal antibodies

NK cells
Natural killer cells

PBMC
Peripheral blood mononuclear cells

PBS
Phosphate buffered saline

PE
Phycoerythrin

PerCP
Peridinin Chlorophyll Protein

Th-2
T helper cell type 2

TNF-α
Tumor necrosis factor alpha

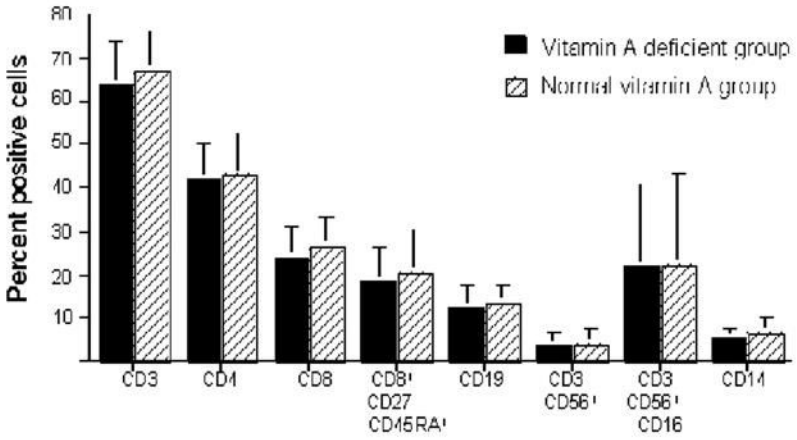

Figure 1.

Percentages of leukocyte immunophenotypes in relation to vitamin A concentration. The mean percentages of various leukocyte subsets (CD3, CD4, CD8, CD8+CD27-CD45RA+, CD19, CD3-CD56+, CD3-CD56+CD16-, and CD14 cells) were determined and examined according to vitamin A concentration. There were no significant differences in leukocyte subsets between the vitamin A deficient and normal groups.

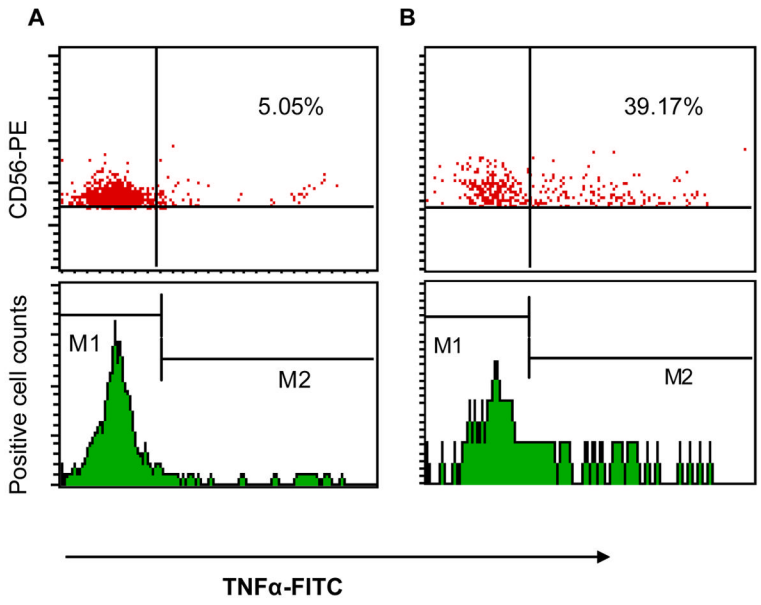

Figure 2.

Flow cytometric analysis of intracellular TNF-α expression in CD3-CD56+ NK cells from participants with different vitamin A concentrations. The percentages of TNF-α expressing CD3-CD56+ NK cells were significantly lower in participants with vitamin A deficiency compared with those with normal vitamin A. Representative flow cytometry profiles demonstrated lower TNF-α expression on CD3-CD56+ NK cells in a vitamin A deficient individual (A) than in an individual with normal vitamin A concentration (B; 5.05% vs 39.17% respectively). Samples were first gated on the CD3-CD56+ lymphocyte populations then the percentage of TNF-α positive cells were determined.

Table 1

Age, gender, HBV, HCV, malaria antigen status and vitamin A and E levels of study participants

	Vitamin A deficient group (n=27)	Normal vitamin A group (n=29)	Total (n=56)
Age (years)			
Mean	46.70± 18.42	40.97±18.05	43.73±18.29
Median	45.0	36.0	41.0
Range	19–86	21–80	19–86
Gender			
Male	13 (48%)	17 (59%)	30 (54%)
Female	14 (52%)	12 (41%)	26 (46%)
HBV positive	6(67%)	3(33%)	9 (16%)
HCV positive	4(67%)	2(33%)	6 (11%)
Malaria antigen positive	7(70%)	3(30%)	10 (18%)
Vitamin A (μg/dL)			
Mean ± SD	16.22±3.32*	27.81±7.01	22.22±1.072
Median	17.4	25.7	20.4
Moderate deficiency(<20μg/dL)	25(93%)		
Severe deficiency(<10μg/dL)	2(7%)		
Vitamin E (mg/dL)			
Mean ± SD	0.3492±0.118	0.3961±0.087	0.3735±0.105
Median	0.35	0.37	0.36
Normal vitamin E (≥0.5mg/dL)	2(7.4%)	4(13.8%)	
Vitamin E deficient (<0.5mg/dL)	25(92.6%)	25(86.2%)	

n=sample size

* Significant at $p<0.05$

Range of vitamin A levels for all participants = 8.3–48.7μg/dL

Range of vitamin E levels for all participants = 0.055–0.5995mg/dL

SD = standard deviation

HBV = Hepatitis B Virus

HCV = Hepatitis C Virus

P<0.05 compared to normal vitamin A group using unpaired t test.

Table 2

Percentages of leukocyte subsets of participants according to normal and deficient vitamin A concentrations

Cell subset	Vitamin A deficient group (<20μg/dl) (n=27)	Normal vitamin A group (≥20 μg/dl) (n=29)	*p* value
BrdU-CD4+			
mean ± SD	13.33±6.56	12.22±4.37	0.505
median	12.28	11.06	
IL-4 CD4+			
mean ± SD	2.67±2.13	2.72±2.10	0.934
median	1.96	2.17	
IFN-γ CD4+			
mean ± SD	6.73±4.54	10.27±10.39	0.121
median	6.09	7.16	
IL-4 CD8+			
mean ± SD	3.80±4.41	2.75± 2.14	0.334
median	2.41	1.97	
IFN-γ CD8+			
mean ± SD	13.07±7.76	19.51±10.0	**0.041** *
median	12.90	18.81	
Perforin CD8+			
mean ± SD	32.39±14.88	34.60±19.23	0.644
median	32.40	32.01	
Granzyme A CD8+			
mean ± SD	62.03±15.83	64.67±14.37	0.517
median	64.30	68.25	
Perforin CD3-CD56+			
mean ± SD	69.26±16.37	68.34±14.18	0.834
median	73.27	66.67	
TNF-α CD3-CD56+			
mean ± SD	12.24±6.66	19.03±10.56	**0.009** *
median	9.81	18.82	
Phagcytosis rate			
mean ± SD	10.74±9.09	9.19± 6.75	0.471
median	7.30	6.89	

n=sample size

BrdU = bromodeoxyuridine; IL = interleukin; IFN-γ = interferon- γ; TNF-α = tumor necrosis factor- α; CD = cluster of determination/differentiation; SD = standard deviation

* P<0.05 compared to normal vitamin A group using unpaired t test