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Short Communication

Vitamin A status is associated with T-cell responses in Bangladeshi men

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Recommendations for vitamin A intake are based on maintaining liver stores of ≥0·070 μmol/g, which is sufficient to maintain normal vision. We propose that higher levels may be required to maintain normal immune function. To test this hypothesis, we conducted an 8-week residential study among thirty-six healthy Bangladeshi men with low vitamin A stores. Subjects were randomised to receive vitamin A (240 mg in four doses) or placebo during study weeks 2 and 3. Vitamin A stores were estimated by isotopic dilution at week 8. Total T-cells, the naive T-cells:memory T-cells ratio and mitogen-induced lymphocyte proliferation were positively and significantly correlated with vitamin A stores ($P<0·05$). Mitogen-stimulated IL-2, IL-4 and TNFα increased significantly ($P<0·05$) in the vitamin A but not placebo group after supplementation, while IL-10 production was significantly and negatively correlated with vitamin A stores ($P<0·05$). Segmented linear regression analysis revealed that naive T-cell counts and T-cell blastogenesis were positively associated with vitamin A stores above but not below 0·070 μmol/g liver. These data show that increasing vitamin A stores above the level that maintains normal vision enhances some measures of T-cell-mediated immunity, suggesting a difference in requirements for maintaining vision and immune function.

Vitamin A: Vitamin A stores: [^{2}H]retinol dilution: T-cell phenotypes: T helper type 1 and 2 cytokines: RDA

Several aspects of both innate and adaptive immunity are compromised by clinical and subclinical vitamin A deficiency[1]. Animal studies and *in vitro* experiments show that vitamin A affects many aspects of T-lymphocyte development and function. Vitamin A metabolites increase the expression of anti-apoptotic proteins in naive T-cells[2], inhibit apoptosis of activated T-cells[3] and augment the early expression of key genes involved in T helper type 2 (Th2) development from naive lymphocytes *in vitro* [4,5]. Deficiency *in vivo* has been shown, using different experimental approaches, to diminish Th2 development[6] and enhance IL-10-producing Th2 cells while decreasing IL-2- and interferon γ (IFNγ)-producing T helper type 1 (Th1) memory cell development[7]. Vitamin A metabolites inhibit IFNγ response in the absence of Th1 cytokines while enhancing IFNγ in the presence of Th1 cytokines without modifying IL-4 response in either case[8]. In the presence of Th2 cytokines, vitamin A increases IL-4 secretion and Th2 cell frequency even when only antigen-presenting cells are treated[8] and can also act as a cofactor in CD3-induced activation of human T-cells[9] and IL-2-mediated proliferation[10].

Human studies on the impact of vitamin A status on immune functions are limited. Vitamin A supplementation increases total lymphocyte and CD4 T-cell count among HIV-positive infants[11], total lymphocyte count among infants with measles[12] and the proportion of naive CD4 T-cells among severely deficient children[13]. Non-significant changes in total lymphocytes or lymphocyte subpopulations have also been reported among young infants[14] and non-pregnant HIV-positive women[15]. In contrast, long-term daily supplementation among HIV-positive women during pregnancy resulted in a negative effect on total lymphocytes and a positive effect on CD8 T-cells[16]. Few studies have examined the *in vitro* response of isolated lymphocytes after vitamin A supplementation. Supplementation among common variable immunodeficient patients with low vitamin A enhances mitogen-stimulated peripheral blood mononuclear cell (PBMC) proliferation[17] and whole-blood stimulation with mitogens shows depressed production of IFNγ among vitamin A-deficient children[18]. A potential shortcoming of these supplementation trials is that the study subjects have had infectious diseases, genetic disorders or severe vitamin A deficiency and thus were likely

Abbreviations: ICDDR,B, International Centre for Diarrhoeal Disease Research, Bangladesh; IFNγ, interferon γ; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; SI, stimulation index; Th1, T helper type 1; Th2, T helper type 2.

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to have other nutritional deficiencies, which might have confounded the study results.

We hypothesised that vitamin A supplementation to increase liver vitamin A stores above the minimum recommended level of 0·070 μmol/g[19] will affect circulating T- and B-lymphocyte numbers and the proliferative and cytokine response of T-lymphocytes to mitogenic stimulation. To test this hypothesis, we conducted a controlled dietary study that provided recommended levels of all major micronutrients and energy (except vitamin A) and used high-dose vitamin A supplementation in a double-blind, randomised, placebo-controlled trial in which we estimated the total body vitamin A pool size by the [^{2}H]retinol dilution technique. We have previously reported the effect of this intervention on vaccine responses and markers of innate immunity[20,21].

Experimental methods

Study site, subjects and diet

The present study was carried out at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh. Recruitment and the diet for this residential study have been described[20,21]. Briefly, thirty-six healthy men (aged 20–30 years) were recruited based on having low serum retinol (< 1·22 μmol/l) and normal haematological and C-reactive protein (< 5 mg/l). Subjects stayed at the designated research area for 12 h/d, 7 d/week, where they received a diet low in vitamin A (about 40 mg retinol equivalents per d) but otherwise adequate[20,21]. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects or patients were approved by the Institutional Review Board of the University of California, Davis and the Ethics Review Committee of the ICDDR,B. Written informed consent was obtained from all subjects.

Study design

After 1-week stabilisation (days 1–7) subjects were randomised to receive vitamin A (4 × 60 mg retinol equivalents) or placebo (maize oil) (days 7, 12, 17 and 22). On day 36, a single 10 mg dose of stable isotope-labelled vitamin A was given orally. Venous blood was obtained after an overnight fast before (day 7) and at 1 week after supplementation (day 29). Serum retinol was measured by HPLC[22]. To estimate vitamin A pool size[23], blood was collected at 3 weeks (day 57) after isotope dosing. The vitamin A and placebo groups will be referred to as the high-vitamin A and low-vitamin A groups, respectively.

Immunophenotyping of cells in whole blood

Multi-test reagent kits with Trucount tubes (BD Bioscience, San Jose, CA, USA) were used to determine the absolute counts (per litre whole blood) of B-cells, naive (CD45RA$^+$), memory (CD45RO$^+$) and total T-cells (CD3$^+$ cells in lymphocyte gate based on forward and side scatter) as well as subsets (CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$) in a four-colour flow cytometer using CELLQUEST software (FACSCalibur; BD Bioscience). The reagents included: CD45RA$^-$FITC/CD45RO$^-$PE/CD3$^-$PerCP/CD4$^-$APC (catalogue no. 34076), CD45RA$^-$FITC/CD45RO$^-$PE/CD3$^-$PerCP/CD8$^-$APC (catalogue no. 340770) and CD3$^-$FITC/CD16$^+$CD56$^-$PE/CD45$^-$PerCP/CD19$^-$APC (catalogue no. 340492).

Peripheral blood mononuclear cell proliferation and cytokine assay

PBMC were separated from fresh heparinised blood by a standard density gradient method (Ficoll-Paque-PLUS; Amersham Biosciences, Uppsala, Sweden), cultured in standard Russ-10 media[4] supplemented with 10 % heat-inactivated (56°C for 30 min) autologous plasma. Triplicate cultures (5×10^4 PBMC in a ninety-six-well U-bottomed plate) were stimulated with three levels of phytohaemagglutinin (PHA)-P (Sigma, St Louis, MO, USA) at 5·0, 2·5 and 1·25 mg/l for 3 d at 37°C and 5 % CO_2. Cell proliferation was measured by incorporation of [^{3}H]thymidine. Results were expressed as stimulation index (SI), the ratio of mitogen-stimulated to unstimulated wells. An additional well of the highest mitogen dose was collected at 3 d for analysis of supernatant fraction levels of IL-2, IL-4, IL-10, IFNγ, TNFα and IL-5 by Luminex assay (Bio-Plex system; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical analyses were done in SigmaStat 3.1 (Systat Software, Inc., San Jose, CA, USA) and SPSS 13.0 for windows (SPSS Inc., Chicago, IL, USA). As previously discussed[20,21], vitamin A pool size estimates were unreliable for three subjects. Data from these three subjects were not used in the analysis where the estimated total body vitamin A pool size was used (i.e. correlation and regression analyses). Two-phase segmental linear regression was used to estimate the relationship between liver vitamin A concentration and functionally similar clusters of immune response variables below and above the current recommendation for minimum adequate liver vitamin A stores of 0·070 μmol/g. Functionally similar immune responses were converted to *z*-scores (mean of 0, SD of 1) and grouped together into the same regression. Clusters used in this analysis were: (a) naive T-cells (CD4$^+$ and CD8$^+$); (b) memory T-cells (CD4$^+$ and CD8$^+$); (c) three levels of PHA-induced PBMC SI; (d) PHA-induced Th1 responses (IL-2, IFNγ and TNFα) in PBMC; (e) PHA-induced Th2 responses (IL-4, IL-5 and IL-10) in PBMC. GraphPad Prism 5 for Windows (GraphPad Software Inc., San Diego, CA, USA) was used for this analysis.

Results

Vitamin A status improved with supplementation

The mean age of the study subjects in the low- and high-vitamin A groups was 24·3 (SD 2·70) and 23·5 (SD 3·05) years, respectively. BMI, serum retinol and haematological measurements at baseline did not differ between the groups[21]. Vitamin A status improved following supplementation as indicated by increases in serum retinol and higher whole-body vitamin A stores in the high-vitamin A group, as previously reported[21]. The mean total vitamin A pool sizes at 1 week after vitamin A supplementation in the

low- and high-vitamin A groups were 0·060 (SD 0·042) and 0·224 (SD 0·060) mmol, respectively ($P<0·001$). The estimated liver vitamin A concentrations in the low- and high-vitamin A groups were 0·039 (SD 0·029) and 0·158 (SD 0·042) μmol/g, respectively ($P<0·001$).

Vitamin A stores positively correlated with peripheral blood naive T-cell counts

Lymphocyte counts did not differ between treatment groups at baseline, including total, memory and naive CD3, CD4 and CD8 T-cells, and total B-cells (data not shown). In both treatment groups total CD3 T-cells, and total and naive CD8 T-cells, decreased significantly ($P<0·05$) during the supplementation period. Total CD3 T-cells decreased by 14 %, from 2479 (SD 712) to 2141 (SD 561) $\times 10^3$/l, total CD8 T-cells decreased by 21 %, from 982 (SD 469) to 775 (SD 304) $\times 10^3$/l and naive CD8 T-cells decreased by 16 %, from 465 (SD 205) to 390 (SD 167) $\times 10^3$/l (n 36). Decreases tended to be greater in the high-vitamin A than the low-vitamin A group, but were not statistically significant. For example, the high-vitamin A group had a marginally greater decrease ($P=0·088$) in memory CD4 T-cells (11·1 %; from 641 (SD 210) to 570 (SD 127) $\times 10^3$/l) than did the low-vitamin A group (2·7 %; from 767 (SD 220) to 746 (SD 269) $\times 10^3$/l) after adjusting pre-supplementation values by analysis of covariance. Although changes over time did not differ by treatment group, significant correlations between vitamin A stores and post-supplementation T-cell counts were seen for total and naive CD3 and CD8 T-cells and for naive:memory ratios of CD3, CD4 and CD8 T-cells (Fig. 1(a)).

Vitamin A stores positively correlated with T-cell proliferation

The change in PBMC SI in response to supplementation ranged from −8 to 9 % for the low-vitamin A group and from 8 to 45 % for the high-vitamin A group (Table 1). The only statistically significant increase (45 %) was seen in the high-vitamin A group at the highest mitogen dose ($P<0·05$). When the groups were compared directly (7 *v.* 45 %), the difference in SI was marginally significant ($P=0·092$). In addition a significant positive correlation ($P<0·05$) was seen between vitamin A pool size and SI at the highest mitogen dose and a marginally significant ($P=0·06$) positive correlation at the lowest dose (Fig. 1(b)).

Vitamin A supplements increased mitogen-stimulated IL-2, IL-4 and TNFα production while vitamin A stores were negatively correlated with IL-10

The high-vitamin A group had significantly increased ($P<0·05$) production of IL-2 (45 %), IL-4 (88 %) and TNFα (57 %) by mitogen-stimulated PBMC cultures following supplementation, while the corresponding changes in the placebo group (8, 33 and 47 %, respectively) were not statistically significant (Table 1). A significant negative correlation ($P=0·01$) was seen between vitamin A stores and the IL-10 response following supplementation (Fig. 1(b)).

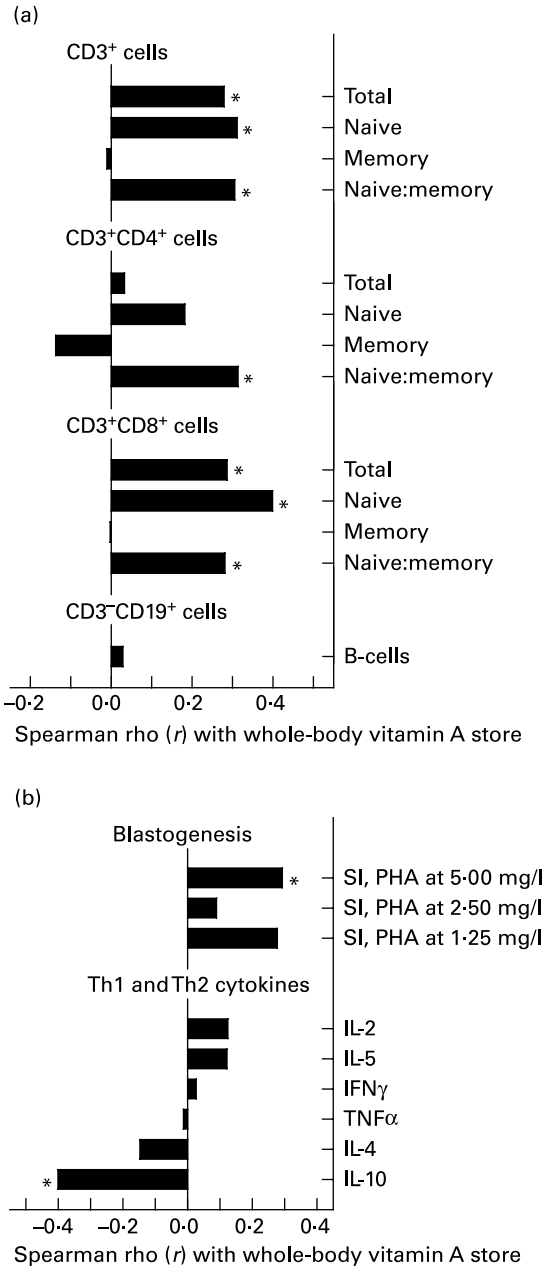

Fig. 1. Spearman correlations between whole-body vitamin A pool size and measures of cell-mediated immunity (n 33): (a) absolute counts (per litre whole blood) of peripheral blood lymphocytes primarily involved in adaptive immunity; (b) peripheral blood mononuclear cell blastogenesis as stimulation index (SI) obtained from 5×10^4 cells treated with phytohaemagglutinin (PHA) at 5·0, 2·5 and 1·25 mg/l; and PHA-stimulated T helper type 1 (Th1) and T helper type 2 (Th2) cytokine responses in 3 d culture. Very similar correlation coefficients were seen when these immune response variables were correlated with estimated liver vitamin A stores (data not shown). *$P\leq 0·05$. IFN, interferon.

Association of T-cell counts and proliferation with vitamin A stores seen above but not below critical level for vision

Segmental linear regression analysis comparing vitamin A stores with immune response variables identified two clusters of response variables (naive CD4 and CD8 T-cells, and T-cell proliferation) with slopes near 0 (flat line) below 0·070 μmol/g

Table 1. Different levels of phytohaemagglutinin (PHA)-induced peripheral blood mononuclear cells (PBMC) stimulation index (SI) and PHA-induced T helper type 1 and 2 cytokine responses in men before and at 1 week after supplementation with placebo (low vitamin A stores) or vitamin A (high vitamin A stores)

(Mean values with their standard errors)

	Low vitamin A stores (*n* 18)				High vitamin A stores (*n* 18)				
	Pre-supplementation		Post-supplementation		Pre-supplementation		Post-supplementation		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P*†
PBMC SI									
PHA at 5·00 mg/l	121	18·2	130	19·3	124	15·7	180*	20·0	0·092
PHA at 2·50 mg/l	40·2	6·06	43·7	7·45	34·0	4·07	44·9	5·66	0·552
PHA at 1·25 mg/l	13·5	2·40	12·4	2·95	12·8	1·56	13·8	2·59	0·609
PBMC cytokines (ng/ml)									
IL-2	137	57·5	235	80·0	92·8	15·6	491*	157	0·171
IL-4	2602	641	3452	772	1681	195	3166*	791	0·404
IL-10	6624	1184	6893	1027	5750	1209	5876	1175	0·663
IFNγ	7695	1817	9703	1722	7487	1940	8353	1583	0·582
TNFα	1379	271	2024	298	1043	144	1639*	185	0·422
IL-5	633	132	723	107	687	172	975	242	0·379

IFN, interferon.

* Mean value was significantly different from that pre-supplementation ($P<0·05$; paired *t* test, within group).

† Between-group significance levels at post-supplementation after adjustment for pre-supplementation values by analysis of covariance.

liver and significant positive slopes above this level. For the naive CD4 and CD8 cluster the slope (β coefficient) above 0·070 μmol/g was 2·15 (95 % CI 0·66, 3·6). For T-lymphocyte proliferation an inflection point was found at 0·080 μmol/g, above which the slope was 1·68 (95 % CI 0·35, 3·00).

Discussion

RDA are defined as the level of intake that will prevent signs of deficiency in about 97·5 % of the population. For this purpose, vitamin A 'deficiency' has been defined as diminished retinal sensitivity to light[19]. However, immune function is also affected by vitamin A status[1] and might be used to define a different RDA for vitamin A, one to prevent immunodeficiency. A difficulty with this approach is that there are many ways to measure immune function, as recently discussed[24]. Thus one might find different associations of vitamin A with immune function depending on the immunological indicator selected for analysis.

We have examined the effect of vitamin A status on measures of adaptive immunity using two approaches. First, we identified changes within treatment groups (i.e. vitamin A *v.* placebo) before and after supplementation. This approach is useful for identifying the effect of the intervention, but has the disadvantage of considering vitamin A status as a bimodal variable. For this reason we also assessed vitamin A status using stable-isotope dilution in order to have a continuous variable reflecting vitamin A stores for each individual. This approach is also useful because it provides a means to compare our data with the RDA, which uses a value of 0·070 μmol/g for liver vitamin A stores to define the RDA[19]. Estimated liver stores for subjects in the present study ranged from 10 % (0·007 μmol/g) to 400 % (0·286 μmol/g) of this target level.

The present study found a significant positive correlation between vitamin A stores and total or naive T-cells in peripheral blood. In addition, two-step segmented linear regression analysis found a positive association of vitamin A stores with naive T-cells above 0·070 μmol retinol/g liver. Since our analysis was limited to observations on cell frequencies in peripheral blood, which represent a small percentage of the total lymphocyte population, it is possible that these differences represent redistribution of lymphocytes rather than true changes in frequencies. However, previous observations from human studies and from animal studies, where researchers are not limited to the pool of lymphocytes found in the blood, support our observations and argue that the observed differences represent underlying changes in immune function. With regard to previous studies, lower percentages of naive T-cells have previously been reported for vitamin A-deficient children, with subsequent increases following vitamin A supplementation[13]. Similar studies have also shown increases in total lymphocyte counts with supplementation[11,12]. These observations for naive T-cells suggest an association of vitamin A status with enhanced thymopoietic capacity. Circulating naive T-cell levels correlate with both antigen-specific function[25,26] and thymic size as measured by volumetric computerised tomographic measurements[27,28]. In support of the present observation, *in vitro* studies with the vitamin A metabolites all-*trans*- and 9-*cis*-retinoic acid show that vitamin A inhibits activation-induced apoptosis of immature thymocytes[29,30]. In addition, retinoic acid can directly inhibit pro-apoptotic[30] and induce anti-apoptotic[2] signalling pathways in naive T-lymphocytes. These mechanisms may account for the positive association of vitamin A stores with naive T-cell numbers in the present study.

A greater proliferative response to the T-cell mitogen PHA was also seen in the present study, as well as a positive association of vitamin A stores with the PHA response. Our data agree with previous *in vitro* observations that all-*trans*-retinoic acid enhances T-cell proliferation[9,10]. Retinoic acid enhances retinoic acid receptor-α-mediated IL-2 receptor expression in thymocytes[31] and also amplifies IL-2-induced signalling downstream of the receptor[10]. This mechanism is also

supported by the fact that retinoic acid can stimulate IL-2-induced DNA synthesis in resting human thymocytes[32]. In the present study we detected a significant increase in PHA-stimulated IL-2 concentration in the group receiving vitamin A but not placebo. Since autologous plasma was used in the PBMC cultures, this enhanced response could be due to the higher retinol concentration found in plasma from the subjects treated with vitamin A compared with placebo[21] or it could be due to an intrinsic difference in the T-cell populations. In either case, we speculate that *in vivo* T-cell proliferation would also be greater in subjects with higher vitamin A stores.

We did not find consistent difference in PHA-stimulated Th1 or Th2 cytokine responses between the treatment groups. In addition to IL-2, higher IL-4 and TNFα responses were seen in response to vitamin A supplementation but not placebo. Although the increase in IL-4 suggests a Th2 bias, as might be expected based on previous data[4–6,33], TNFα is typically produced by Th1 cells. Thus the increase in both of these cytokines could be secondary to enhanced proliferation of both Th1 and Th2 cells in the vitamin A group.

A significant negative correlation was seen between vitamin A stores and PHA-stimulated IL-10 response in PBMC. This finding is reminiscent of results from the our research showing a similar negative association of vitamin A stores with both tetanus toxoid-specific IL-10 production by T-cells[21] and lipopolysaccharide-stimulated IL-10 from whole-blood cultures[20], as well as results from a mouse study in which vitamin A deficiency increased the number of IL-10-producing T-cells[22]. IL-10 is a regulatory cytokine that diminishes the development of pro-inflammatory immune responses, including responses mediated by both innate and adaptive immune cells[34]. Overproduction of IL-10 could thus represent a mechanism by which vitamin A deficiency down-regulates or 'impairs' some immune responses.

Previous observations from the our research suggest that the current 0·070 μmol/g index level for setting the RDA is adequate to maintain protective response to immunisation, but that increases above this level may 'enhance' antigen-specific T-cell proliferation and production of some cytokines, such as IL-5[21], and may also enhance some innate immune responses[20]. The present study shows that vitamin A stores above the 0·07 μmol/g level were associated with higher naive T-cell numbers and greater polyclonal T-cell proliferation. These observations raise the question of whether even higher vitamin A stores, such as those seen in adults in the USA and other industrialised countries[23,35–39], would further enhance these responses. Such responses might, under some circumstances, contribute to the development of T-cell-mediated inflammatory conditions, such as asthma[40]. This possibility suggests that future evaluation of the tolerable upper intake level (UL) for vitamin A intake might consider risk of adverse immune-reactivity, at least in populations at risk of T-cell-mediated chronic diseases, in addition to the indicators used in setting the current UL[19].

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S. M. A. helped formulate the study design and implemented both clinical and laboratory aspects of the study. M. J. H. collaborated on the study design and was responsible for assessing vitamin A status using the stable-isotope dilution method. R. R. supervised laboratory work in Bangladesh. C. B. S. oversaw the development of the study design and its implementation.

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