Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03082.x

Vitamin A supplementation increases ratios of proinflammatory to anti-inflammatory cytokine responses in pregnancy and lactation

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Accepted for publication 24 February 2006
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Summary

Vitamin A supplementation reduces child mortality in populations at risk of vitamin A deficiency and may also reduce maternal mortality. One possible explanation for this is that vitamin A deficiency is associated with altered immune function and cytokine dysregulation. Vitamin A deficiency in pregnancy may thus compound the pregnancy-associated bias of cellular immune responses towards Th-2-like responses and exacerbate susceptibility to intracellular pathogens. We assessed mitogen and antigen-induced cytokine responses during pregnancy and lactation in Ghanaian primigravidae receiving either vitamin A supplementation or placebo. This was a double-blind, randomized, placebo-controlled trial of weekly vitamin A supplementation in pregnant and lactating women. Pregnancy compared to postpartum was associated with a suppression of cytokine responses, in particular of the proinflammatory cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α. Mitogen-induced TNF-α responses were associated with a decreased risk of peripheral parasitaemia during pregnancy. Furthermore, vitamin A supplementation was significantly associated with an increased ratio of mitogen-induced proinflammatory cytokine (IFN-γ) to anti-inflammatory cytokine (IL-10) during pregnancy and in the postpartum period. The results of this study indicate that suppression of proinflammatory type 1 immune responses and hence immunity to intracellular infections, resulting from the combined effects of pregnancy and vitamin A deficiency, might be ameliorated by vitamin A supplementation.

Keywords: clinical trial, cytokines, Ghana, humans, IFN-γ, IL-10, immunity, malaria, nutrition, pregnancy, pregnancy-associated malaria, TNF-α, vitamin A

Introduction

In animals, vitamin A deficiency is associated with alterations in the balance of type 1 (proinflammatory) to type 2 or type 3 (non-inflammatory or anti-inflammatory) cytokine responses and can lead to inappropriate responses to infection [1,2]. If similar immune dysregulation occurs in humans, the ability to control infections such as toxoplasmosis, tuberculosis, leishmaniasis and malaria, where the cytokine balance is crucial to control of the infection, may be compromised [3–8]. To date, relatively few studies have attempted to assess how vitamin A status affects immune function in humans. However, there is substantial evidence from community trials in young children that vitamin A supplementation is associated with large reductions in child mortality – mainly from infectious causes – [9] and a trial of maternal vitamin A supplementation in Nepal reported a 44% reduction in all cause maternal mortality [10]. Data from studies designed to assess markers of cell-mediated immunity in individuals with vitamin A deficiency have tended to support evidence from animal studies that vitamin A deficiency is associated with a bias towards type 1 immune responses [11,12], but the studies are somewhat unrepresentative as they were conducted in individuals with underlying immune deficiencies. In otherwise healthy women, vitamin A deficiency has been associated with increased markers of inflammation in breast milk, but these were not reversed by vitamin A supplementation [13,14]. A study in vitamin

A-deficient but otherwise healthy children in Indonesia gave ambiguous results, as vitamin A deficiency was associated with a type 1 bias in the steady state but also with reduced production of interferon (IFN)-γ after antigen stimulation [15].

During pregnancy a delicate balance between type 1 and type 2 or type 3 immune responses is required to maintain tolerance of the antigenically foreign fetus while continuing to provide protection from infection [16]. Proinflammatory immune responses can damage the placenta leading to placental insufficiency and premature delivery or low birth weight babies; in successful pregnancies anti-inflammatory cytokines are secreted at the materno–fetal interface [16–18]. On the other hand, suppression of type 1 responses to commonly encountered antigens [19–22] may explain the increased risk of infection with intracellular pathogens during pregnancy [23]. The accumulated evidence suggests that vitamin A deficiency, and consequently vitamin A supplementation, during pregnancy is likely to disturb this balance, but the outcome is impossible to predict from available data. Thus, if vitamin A supplementation promotes anti-inflammatory immune responses this might increase the risk of infection; alternatively, if vitamin A supplementation promotes proinflammatory responses it might increase the risk of poor birth outcomes. As there are currently two large intervention trials investigating the effects of vitamin A supplementation on maternal morbidity and mortality in Bangladesh and Ghana, we felt that it was important to evaluate the impact of vitamin A supplementation on cellular immune responses in pregnant and lactating women.

Subjects, materials and methods

Study design

Study subjects were pregnant primigravid women enrolled in a randomized, double-blind, placebo-controlled trial of vitamin A supplementation and immunity to pregnancy-associated malaria, the details of which are reported elsewhere [24]. At enrollment, 51% (45/89) of women were marginally vitamin A-deficient (plasma retinol <30 μg/dl), while 15% were deficient (< 20 μg/dl). Mild to moderate anaemia (plasma Hb <11 g/dl) was common [86% (83/97)] but severe anaemia (< 7 g/dl) was observed in only 7%. Women were recruited from antenatal clinics at Nkoranza District Hospital and three rural health clinics, in Brong Ahafo region, Central Ghana, during the period March–June 2001. Women were assigned randomly, using balanced block randomization (eight women/block), to receive weekly vitamin A (10 000 IU as retinyl palmitate in groundnut oil, plus tocopherol as a preservative) or placebo (groundnut oil plus tocopherol) from enrollment until 6 weeks postpartum. Fieldworkers delivered supplements to women, in person each week, and directly observed their consumption. Primigravid women were eligible for inclusion in the study if they were pregnant (as assessed by urine test or palpation), resident within the study area, in good health and less than approximately 24 weeks pregnant, as determined by fundal height measurements. In practice, fundal height estimates were inaccurate and gestational ages at recruitment were thus recalculated at the end of the study from known dates of full term deliveries. According to available health records, none of the women were suffering from clinical HIV infection or tuberculosis but diagnostic tests were not performed. The estimated prevalence of HIV infection in pregnant Ghanaian women in the Brong Ahafo region for 2000 was 1.6% [25]. Individual informed consent was obtained from all study participants.

Enrollment of 35 women into each arm of the study was calculated to give 90% power at the 5% level to detect a two-fold difference in mean cytokine responses between the vitamin A and placebo groups.

Ethical approval for the study was obtained from the ethics committees of the London School of Hygiene and Tropical Medicine and the Ghanaian Ministry of Health.

Laboratory assays

Venous blood samples (4 ml) were collected into heparinized vacutainers (Greiner, Labortechnik, Austria) on three occasions from each woman: at enrolment ($n = 50$ in the placebo group, $n = 48$ in the vitamin A group), in late pregnancy ($n = 43$ in the placebo group, $n = 45$ in the vitamin A group) and at 6 weeks postpartum ($n = 49$ in the placebo group, $n = 46$ in the vitamin A group). At the same time, finger-prick blood samples were obtained for assessment of plasma haemoglobin (Hb) concentrations (HemoCue, Ångelholm, Sweden) and determination of malarial parasitaemia by microscopic examination of Giemsa-stained thick blood films. Plasma retinol was measured in baseline samples by high performance liquid chromatography using standard methods [26] from plasma stored in UV-protected vials for a maximum of 5 months at −30°C.

Whole blood culture

Whole blood assays were carried out as described previously. Although this method does not allow for cytokine responses to be standardized by the number of leucocytes per well, this assay has been shown previously not to be sensitive to normal variation in leucocyte numbers [27,28]. In brief, blood was diluted 1 : 10 with RPMI-1640 tissue culture medium (Invitrogen, Paisley, UK), containing 100 U/ml of penicillin and streptomycin and 0·002 M L-glutamine (Invitrogen) and aliquoted (180 μl) into 96-well flat-bottomed tissue culture plates (SLS Ltd, Nottingham, UK). Twenty μl of purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statens Serum Institute, Copenhagen, Denmark), phytohaemaglutinin (PHAp; Sigma, Dorset, UK) or tissue culture medium (negative control) were added to replicate wells to

give a final concentration of 1 μg/ml and cultures were incubated at 37°C in 5% CO_2 for 1, 3 or 6 days. Aliquots of culture supernatant (150 μl) were removed from wells on day 1 for assessment of tumour necrosis factor (TNF)-α, day 3 for interleukin (IL)-10 and day 6 for interferon (IFN)-γ, based on previous data of optimum yields using the same whole blood culture method [28,29]. Samples were stored at −40°C until required.

Cytokine immunoassays

Supernatants were shipped to the United Kingdom and analysed for IFN-γ, TNF-α and IL-10 using a sandwich enzyme-linked immunosorbent asay (ELISA) technique with commercially available monoclonal antibodies (Pharmingen, Oxford, UK) as described [30]. The lower limits of detection (LLD) were 62 pg/ml for IL-10 and 125 pg/ml for IFN-γ and TNF-α. The upper limit of detection (ULD) was 3000 pg/ml; samples containing more than 3000 pg/ml cytokine were retested at a 1 : 25 dilution, resulting in an ULD of 75 000 pg/ml. A positive-control supernatant was tested on each ELISA plate; coefficients of variation between plates were 12% for IL-10, 7·7% for IFN-γ in response to PHA, 18·5% for IFN-γ in response to PPD and 36% for TNF-α. Samples were grouped and tested by time of collection (enrolment, late pregnancy and postpartum) to minimize variation between the treatment groups.

Statistical analysis

Statistical analysis was performed in Stata 8 (Stata Corporation, Texas, USA). Cytokine responses were categorized as either detectable (≥ LLD) or non-detectable (< LLD) and analysed by logistic regression or, where a sufficient proportion of the values were within the detection limits, analysed by linear regression of logarithmically transformed data, censored for values that lay outside the detection limits (i.e. the model accepts them as being somewhere between 0 and the LLD, or more than the ULD). For samples where both values lay within the detection limits of the assay, $\log^e$ ratios of cytokine concentrations were calculated and analysed by linear regression. Analyses assessing changes in cytokine concentrations due to pregnancy status were performed in the placebo group only, by linear regression of values within the detection limits, and robust standard errors to account for repeated measurements within individuals. We hypothesized that vitamin A supplementation could affect immune responses differently in pregnancy and postpartum and therefore effects of vitamin A supplementation were assessed in two separate models: at late pregnancy, with responses controlled for those at enrolment in early pregnancy; and at postpartum, with responses controlled for those at late pregnancy. As the number of doses of vitamin A or placebo varied depending on gestational age at enrolment, analyses were initially stratified by the number of doses received at late pregnancy (7–11, 12–16 or 17–26) and at postpartum (15–25, 26–38). However, as no evidence of a dose–response effect was observed, results are presented for all groups combined.

Results

The study was designed to assess the effect of pregnancy on cytokine responses to a mitogen (PHA) and an antigen (PPD) and to determine the effect of vitamin A supplementation on these during pregnancy and postpartum. Table 1 shows baseline characteristics of the two groups. Although mean levels of most variables were similar there was a marked difference between the groups in educational level. Nutritional status at baseline (as indicated by the variables reported in Table 1) was similar in the two treatment groups. Gestational ages, back-calculated from recorded gestational age at delivery, ranged from 4 to 29 weeks (median = 16 weeks) at enrolment and 25–36 weeks (median = 31 weeks) at the late pregnancy blood collection and did not

Table 1. Baseline characteristics of women allocated randomly to receive vitamin A supplementation or placebo.

Variable	Placebo ($n = 50$)	Vitamin A ($n = 48$)
Mean plasma retinol μg/dl (s.d.)	30·6 (9·1) $n = 47$	28·5 (11·1) $n = 42$
Mean haemoglobin g/dl (s.d.)	9·76 (1·6) $n = 50$	9·26 (1·3) $n = 47$
Mean BMI (kg/m^2) (s.d.)	21·3 (1·8) $n = 49$	21·2 (2·0) $n = 48$
Mean MUAC (cm) (s.d.)	26·4 (2·6) $n = 50$	25·8 (2·0) $n = 47$
Mean age (years) (s.d.)	21·0 (2·9) $n = 50$	21·0 (2·9) $n = 48$
Mean gestational age (weeks) (s.d.)[1]	15·0 (5·6) $n = 50$	17·0 (4·3) $n = 48$
Peripheral parasitaemia	18/46 (39%)	20/44 (45%)
Temperature > 37·4°C	22/50 (44%)	17/48 (35%)
Recruited in peak malaria transmission[2]	33/50 (64%)	31/48 (65%)
Resident in Nkoranza town	33/50 (66%)	31/48 (65%)
Level of education		
None	9/48	2/47
Primary	7/48	7/47
Junior	28/48	26/47
Secondary	4/48	12/47
Enrolment[3]		
Early	20/46	10/42
Mid	15/46	20/42
Late	11/46	12/42

[1]Gestation was estimated by palpation and measurement of fundal height. [2]Although malarial transmission was perennial, transmission peaked in the months at the end and after the two rainy seasons. [3]Enrolment. Women who enrolled at different times during their pregnancies had different numbers of weeks between the data collections at enrolment and late pregnancy: early, more than 16 weeks; mid, 12–16 weeks; late, 7–11 weeks. BMI: body mass index; MUAC: mid-upper-arm circumference.

differ significantly between the treatment groups. Compliance was uniformly high, with 99·1% of all scheduled doses of vitamin A or placebo being received. Women who enrolled earlier in their pregnancies had a greater number of total weekly doses of vitamin A or placebo and these were similar between the treatment groups, ranging from 16 to 37 (median = 27) for the vitamin A supplementation group and 15–38 (median = 24) for the placebo group.

Cytokine responses to PHA and PPD are suppressed during pregnancy

Proportions of women with detectable cytokine responses (i.e. > LLD) were assessed at the three time-points – enrolment in early pregnancy, late pregnancy and 6 weeks postpartum (Table 2). Cells from the majority of women made detectable IFN-γ and IL-10 responses to the mitogen PHA but fewer made detectable levels of TNF-α. The proportions of detectable responses to PPD were lower than for PHA, especially for TNF-α and IL-10.

Comparisons of the proportions of detectable responses at the three time-points within the vitamin A-supplemented and placebo groups indicated strong evidence of an effect of pregnancy status. The proportions of detectable responses to PHA for all tested cytokines were significantly lower in late pregnancy than in either early pregnancy or at postpartum ($P < 0{\cdot}01$). The proportions of detectable responses to PPD were reduced in both early and late pregnancy compared to postpartum ($P < 0{\cdot}01$) and were reduced further in late compared to early pregnancy, reaching statistical significance for IFN-γ ($P < 0{\cdot}001$).

Analysis of the effect of pregnancy status on the levels of positive cytokine responses was performed in the placebo group only (Table 3). Concentrations of IFN-γ and IL-10 decreased in early and late pregnancy compared to postpartum; for IFN-γ and TNF-α, levels were lowest in late pregnancy while for IL-10 levels were lowest in early pregnancy. Furthermore, pregnancy-associated suppression of cytokine concentrations was more marked for IFN-γ than for either TNF-α or IL-10.

The differences in the extent of suppression of the different cytokine responses prompted us to assess the effect of pregnancy on the ratios of cytokine responses to PHA (Table 3). As expected, the ratio of IFN-γ to IL-10 was significantly lower in both early and late pregnancy compared to postpartum. In contrast, the ratio of TNF-α : IL-10 was highest in early pregnancy and lowest in late pregnancy. The ratio between the two proinflammatory cytokines IFN-γ and TNF-α was reduced significantly in both early and late pregnancy compared to postpartum, indicating that IFN-γ responses are more severely suppressed during pregnancy than TNF-α responses.

Levels of mitogen-induced TNF-α but not IFN-γ or IL-10 were associated with peripheral parasitaemia

There was no association between peripheral parasitaemia and IFN-γ or IL-10 responses at any of the time-points tested (data not shown). However, lower TNF-α responses were observed in women with peripheral parasitaemia during pregnancy but not postpartum. At enrolment in early pregnancy, peripheral parasitaemia (36/84) was associated non-significantly with a decreased likelihood of a detectable response to PHA (OR = 0·34, $P = 0{\cdot}06$) and in those with a detectable response, lower levels of TNF-α (29% lower, $P = 0{\cdot}06$). In a multivariable analysis controlling for an effect of plasma retinol and other factors (see below), the presence of peripheral parasitaemia was associated with 40% lower TNF-α concentrations ($P = 0{\cdot}01$). In late pregnancy the presence of peripheral parasitaemia (26/85), controlling for vitamin A supplementation, was associated with 36% lower TNF-α concentrations ($P = 0{\cdot}05$), while the ratio of TNF-α : IL-10 was non-significantly lower (37%, $P = 0{\cdot}11$) in those with peripheral parasitaemia. There was no evidence of

Table 2. Prevalence of detectable cytokine responses in whole blood assays after stimulation with phytohaemagglutinin (PHA) or purified protein derivative (PPD).

	Responses to *PHA* N > LLD/total samples (%)			Responses to *PPD* N > LLD/total samples (%)		
	IFN-γ	TNF-α	IL-10	IFN-γ	TNF-α	IL-10
Placebo group						
Early pregnancy	46/46 (100%)	39/46 (85%)	48/48 (100%)	23/43 (54%)‡	3/46 (7%)‡	7/48 (15%)‡
Late pregnancy	37/39 (95%)†	24/40 (60%)†	38/42 (90%)†	17/41 (42%)‡	0/40 (0%)‡	2/42 (5%)‡
Postpartum	44/44 (100%)	32/44 (73%)	45/45 (100%)	35/44 (80%)	18/44 (41%)	18/44 (41%)
Vitamin A supplemented group						
Early pregnancy	46/46 (100%)	37/48 (77%)	48/48 (100%)	32/47 (68)‡	2/47 (4%)‡	4/48 (8%)‡
Late pregnancy	43/45 (96%)†	24/44 (55%)†	40/44 (91%)†	17/45 (38)‡§	1/44 (2%)‡	2/43 (5%)‡
Postpartum	44/45 (98%)	36/42 (86%)	46/46 (100%)	38/47 (81%)	20/44 (46%)	20/46 (44%)

†Proportions of positive responses to PHA were significantly reduced in late compared to both early pregnancy and postpartum ($P < 0{\cdot}01$). ‡Proportions of positive responses to PPD were reduced in early and late pregnancy compared to postpartum ($P < 0{\cdot}01$). §Proportion of IFN- γ responses to PPD was further reduced in late compared to early pregnancy ($P < 0{\cdot}001$). Statistical analysis was by *Z*-test of proportions.

Table 3. Pregnancy-associated changes in PHA-induced absolute cytokine concentrations and ratios of geometric mean responses (placebo group only).

	IFN-γ[1]		TNF-α[2]		IL-10[3]		IFN-γ : IL-10		TNF-α : IL-10		IFN-γ : TNF-α	
	% change (95% CI)	*P*-value	% change (95% CI)	*P*-value	% change (95% CI)	*P*-value	% change (95% CI)	*P*-value	% change (95% CI)	*P*-value	% change (95% CI)	*P*-value
Late *versus* early pregnancy	−10% (−40/+33)	0·58	−17% (−35/+6)	0·13	−9% (−13/+37)	0·44	−21% (−56/+17)	0·24	−38% (−56/+9)	0·014	+4% (−38/+73)	0·88
Early pregnancy *versus* postpartum	−64% (−75/−48)	< 0·001	−38% (−48/+0·1)	0·051	−33% (−45/−18)	< 0·001	−47% (−62/−25)	0·001	+26% (−15/+88)	0·24	−57% (−70/−36)	< 0·001
Late pregnancy *versus* postpartum	−68% (−76/−56)	< 0·001	−69% (−57/−17)	0·003	−26% (−45/−2)	0·034	−58% (−71/−39)	< 0·001	−21% (−50/+27)	0·33	−55% (−72/−26)	0·002

Analysis is by linear regression of log-transformed values for samples within the detection limits of the assays using robust standard errors to account for within subject correlation of the three repeated measurements in individuals. [1]Percentage of samples within levels of detection = 100% at early pregnancy, 95% at late pregnancy and 99% at postpartum. [2]Percentage of samples within levels of detection = 81% at early pregnancy, 57% at late pregnancy and 76% at postpartum. [3]Percentage of samples within levels of detection = 100% at early pregnancy, 91% at late pregnancy and 100% at postpartum.

an association between TNF-α responses and peripheral parasitaemia (34/91) at postpartum ($P = 0{\cdot}80$).

Mitogen-induced cytokine production is positively correlated with plasma retinol concentration at enrolment in early pregnancy

To determine whether vitamin A status at enrolment, as measured by plasma retinol, affected cellular immune responses, PHA-induced cytokine responses at enrolment (early pregnancy) were compared with baseline plasma retinol concentrations. Although we did not measure plasma concentrations of markers of the acute phase response, which is known to be associated with decreased plasma retinol concentrations [31], there was no evidence of a relationship between plasma retinol and either reported morbidity in the previous 2 days, increased temperature (> 37·4°C) or peripheral parasitaemia (data not shown) to suggest that infection was likely to confound the relationship between plasma retinol and cytokine responses. Other possible confounding factors including baseline nutritional status (as assessed by mean upper arm circumference), level of education, urban or rural area of residence or season (high or low malaria transmission) were investigated, but no evidence was found of associations with cytokine responses. After controlling for age (which was significantly positively associated with IFN-γ and IL-10 responses) and peripheral parasitaemia in all models, plasma retinol concentration was positively and significantly associated with IFN-γ and IL-10 mitogen-induced cytokine responses. For every 0·01 μg/dl increase in plasma retinol, IFN-γ production increased by 3·5% ($P = 0{\cdot}002$), IL-10 production increased by 1·3% ($P = 0{\cdot}04$) while for TNF-α there was no significant increase ($P = 0{\cdot}18$). In addition, plasma retinol was also positively associated with the ratio of IFN-γ : IL-10 ($P = 0{\cdot}05$).

At enrolment, very few PPD-induced responses were above the LLD and the only cytokine for which there were sufficient data points for analysis was IFN-γ (55/90 > LLD), which when analysed by logistic regression did not show any significant association with plasma retinol concentration [odds ratio (OR) = 0·68, $P = 0{\cdot}42$] or peripheral parasitaemia (OR = 0·82, $P = 0{\cdot}72$). Furthermore, IFN-γ responses to PPD were not associated with presence or absence of a bacille–Calmette–Guérin (BCG) scar (OR = 1·42, $P = 0{\cdot}42$).

Vitamin A supplementation increases the ratios of proinflammatory to anti-inflammatory cytokine responses

Comparisons of cytokine responses between the vitamin A and placebo group were analysed as ratios of proinflammatory to anti-inflammatory cytokines. Cytokine ratios had been shown previously to be more informative than absolute levels [30], and comparison of ratios avoided differences in cytokine responses being due to possible effects of vitamin A

Table 4. Geometric mean cytokine ratios in response to stimulation of whole blood with phytohaemagglutinin (PHA) in the placebo and vitamin A supplemented groups.[1]

	Geometric mean cytokine ratios (95% CI)					
	Placebo group			Vitamin A supplemented group		
	IFN-γ : IL-10	TNF-α : IL-10	IFN-γ : TNF-α	IFN-γ : IL-10	TNF-α : IL-10	IFN-γ : TNF-α
Early pregnancy	25·8 (19·9- 5·33)	0·68 (0·51–0·91)	42·3 (28·3–63·2)	28·9 (22·8–36·8)	0·70 (0·50–0·97)	65·3 (42·9–99·5)
Late pregnancy	20·5 (15·4–27·2)	0·42 (0·31–0·57)	62·3 (44·3–87·5)	28·9 (21·4–39·2)	0·50 (0·34–0·73)	67·7 (43·1–106·2)
Postpartum	48·6 (37·3–63·5)	0·50 (0·36–0·70)	113·7 (71·9–179·9)	53·5 (41·7–68·8)	0·94 (0·60–1·50)	95·0 (68·5–131·8)

[1]Ratios were calculated only for samples within the detection limits. LLD: lower limit of detection. ULD: upper limit of detection. Numbers of total samples tested varied between the cytokines and time-points due to some samples having insufficient volumes for complete testing.

supplementation on the numbers of leucocytes in whole blood cultures, rather than effects on cytokine production *per se*.

At enrolment, as expected, geometric mean PHA-induced cytokine concentrations and PHA-induced cytokine ratios were similar in the two treatment groups (Table 4). In response to PPD, the prevalence of IFN-γ responses to PPD did not differ between the vitamin A and placebo groups, while 99% of TNF-α responses and 95% of IL-10 responses were below the LLD and could not be analysed (Table 4).

We tested for an effect of vitamin A supplementation on cytokine responses in pregnancy and at postpartum compared to late pregnancy. Level of education (unevenly distributed between the vitamin A and placebo groups) and presence of peripheral parasitaemia were included a priori in multivariable models. Other variables that were determined to have a significant effect in some models included baseline nutritional status [as determined by body mass index (BMI)] and place of residence (urban or rural), and these were thus included in all models.

We examined the effect of vitamin A supplementation on the ratios of proinflammatory IFN-γ and TNF-α to the anti-inflammatory cytokine IL-10 (Table 5). The ratio of IFN-γ to IL-10 production was significantly higher in the vitamin A-supplemented group compared to the placebo group in late pregnancy and at postpartum (61% and 57%). The ratio of TNF-α : IL-10 was also higher in the vitamin A-supplemented group at both time-points, but did not reach statistical significance (90% and 57% increases, respectively).

Discussion

The purpose of this study was to determine whether vitamin A deficiency, or conversely vitamin A supplementation, was associated with altered immune function in a cohort of primigravid African women at high risk of both vitamin A deficiency and infection by a number of intracellular pathogens, including malaria and tuberculosis. Cytokine responses measured in whole blood assays confirmed and extended previous reports that antigen-specific cell-mediated immune responses are down-regulated during

Table 5. Effect of vitamin A supplementation on ratios of geometric mean cytokine production in response to stimulation with phytohaemagglutinin at late pregnancy (compared to early pregnancy) and postpartum (compared to late pregnancy).[1]

Time	Cytokine ratio	% change (95% CI)	*P*-value
Late pregnancy adjusted for early pregnancy and other possible confounders and co-factors[2]	IFN-γ : IL-10 ($n = 59$)	+61% (+1%/+257%)	$P = 0{\cdot}045$
	TNF-α : IL-10 ($n = 32$)	+56% (−10%/+257%)	$P = 0{\cdot}11$
Postpartum adjusted for late pregnancy and other possible confounders and co-factors[2]	IFN-γ : IL-10 ($n = 58$)	+57% (−1%/+244%)	$P = 0{\cdot}043$
	TNF-α : IL-10 ($n = 28$)	+90% (−13%/+414%)	$P = 0{\cdot}10$

[1]Analysis by linear regression of ratios of log-transformed cytokine responses, calculated for supernatants with concentrations within the detection limits of the assays. [2]Level of education (unevenly distributed between the vitamin A and placebo groups), and presence of peripheral parasitaemia were included a priori in multivariate models. Other variables that were determined to have a significant effect in some models included: baseline nutritional status as determined by body mass index and place of residence, urban or rural, and were consequently included in all models.

pregnancy but that non-specific (mitogen-induced) responses affected are less severely [21,22,32] These data also support the hypothesis that pregnancy induces a bias towards anti-inflammatory cytokine responses, as IFN-γ and TNF-α responses were more severely suppressed than IL-10 responses and ratios of IFN-γ : IL-10 and TNF-α : IL-10 were significantly reduced during pregnancy. Furthermore, our results suggest that vitamin A supplementation of subclinically vitamin A-deficient women during pregnancy and the postpartum period may increase the production of the proinflammatory cytokine IFN-γ (and possibly TNF-α) and decrease production of the anti-inflammatory cytokine IL-10, resulting in significant skewing of the immune response towards a type 1 cytokine balance during both pregnancy and the postpartum period.

The observation that IFN-γ production was suppressed more severely during pregnancy than was TNF-α production may indicate that macrophages, an important source of TNF-α, are affected less by pregnancy than are T cells and natural killer (NK) cells, which are the major sources of IFN-γ. In addition, as the decrease in the ratio between TNF-α and IL-10 was only significantly reduced in late pregnancy it appears that TNF-α is not suppressed until later in pregnancy; this is consistent with reports that TNF-α plays an important role in placentation early in pregnancy [33]. Importantly, the observation that lower TNF-α responses in late pregnancy were associated with increased risk of malarial parasitaemia confirms previous reports of the relationship between cytokine production and risk of malaria infection [30], and supports the notion that suppression of proinflammatory cytokine responses during pregnancy might increase the mother's susceptibility to infection.

The hypothesis that vitamin A status influences cellular immune function is strongly supported by the significant positive associations between plasma retinol concentration and production of IFN-γ and IL-10 at baseline. Furthermore, vitamin A supplementation led to skewing of the cytokine response, significantly increasing the ratios of IFN-γ to IL-10 (and tending to increase ratios of TNF-α : IL-10) during pregnancy and postpartum. Thus, contrary to some animal studies [1,34,35], and studies in immunocompromised humans [11,12], our data suggest that vitamin A supplementation tips the balance of cytokine responses towards proinflammatory rather than anti-inflammatory immune responses. The differences between our data and those published previously might be explained by physiological differences between study populations (pregnant or not), extent of vitamin A deficiency prior to supplementation and presence or absence of concurrent infections. Our results are however, consistent with those of Wieringa *et al.* [15] who reported vitamin A deficiency to be associated with decreased IFN-γ responses after mitogen stimulation in Indonesian children, and Allende *et al.* [36], who found enhanced IFN-γ and IL-2 transcription and enhanced expression of cell surface activation markers in human PBMCs cultured with retinol. Furthermore, our results fit with data from studies in mice, in which vitamin A deficiency was associated with decreased proportions of IFN-γ and IL-2 positive T cells and increased proportions of IL-10 positive T cells [37]. Also, other studies have demonstrated decreased delayed-type hypersensitivity (DTH) responses in vitamin A-deficient rats [38,39], increased PPD-induced IL-2 production and enhanced DTH responses in vitamin A-supplemented *M. bovis*-infected mice [40], enhanced cell-mediated responses to tetanus toxoid, diptheria and PPD in Bangladeshi infants receiving vitamin A supplementation at the time of vaccination [41] and enhanced Th-1-dependent, IgG1 antibody responses to tetanus toxoid in children receiving vitamin A supplementation [42].

Potentiation of type 1 (proinflammatory) responses during pregnancy is, potentially, a double-edged sword. While stronger Th-1 responses might reduce the risk of infection during pregnancy, providing protection against opportunistic viral, bacterial and protozoal infections that target the placenta and/or the developing fetus, inappropriate overproduction of type 1 cytokines may cause placental damage and threaten the viability of the fetus and the mother [43–45]. Alternatively, in mouse models, insufficient IFN-γ production at the materno–fetal interface has been associated with poor placental maturation and modification of the uterine spiral arteries, leading to fetal loss [46,47]. The tendency for vitamin A supplementation during pregnancy to tip the cytokine balance towards type 1 responses suggests that care must be taken to evaluate the potential deleterious side effects of this intervention. However, in this study the dose of vitamin A was low (equivalent to <4 days recommended safe intake [48]) and no adverse effects on fetal and infant survival or on preterm birth were observed in a similar low-dose maternal vitamin A supplementation trial [49].

One potential confounder of the effect of vitamin A supplementation on cytokine responses is maternal infection, which might potentiate Th-1-like responses. In this community, placental malaria infection is common and 87% of the women in this study showed evidence of past or current placental malaria infection by histology [24]. We were unable to measure malaria-specific cytokine responses, as the whole blood assay used here is not suitable for detecting T cell responses to malaria parasites [50] but we did not find any difference in PHA-induced or PPD-induced cytokine production between women who showed evidence of placental malaria and those without. However, the presence of peripheral malarial parasitaemia in late pregnancy was associated with low ratios of Th-1 to Th-2/3 cytokines and we did find weak evidence to suggest that vitamin A supplementation reduces the risk of chronic placental malaria infection [24], suggesting that potentiation of type 1 responses may enhance anti-malarial immunity in pregnancy.

In conclusion, this study has shown that vitamin A supplementation in a subclinically vitamin A-deficient population of primigravid African women increases

proinflammatory cytokine responses during pregnancy and during the postpartum period. These findings, especially when taken together with the data on peripheral and placental malaria infection, suggest that in countries with a high prevalence of subclinical vitamin A deficiency, low-dose vitamin A supplementation has the potential to improve pregnancy outcomes by enhancing resistance to pregnancy-associated infections. Given the low cost and ease of distribution, vitamin A supplementation may represent a cost-effective intervention to improve maternal health in low-income countries.

Acknowledgements

S. E. C. was a recipient of a Medical Research Council, UK Studentship (G78/6363) and the work was supported by grants from the University of London Central Research Fund, the Nestle Foundation, the Parkes Foundation and awards including the Denis Burkit Study Award and a Jeremy Chadwick Traveling Fellowship. We thank the computer centre staff at KHRC, especially Boniface Batosona and Seeba Amengo-Etego, the laboratory technician Kofi Tchwum and the fieldworkers Kwasi Osei Owusu, Betty Baazing, Doris Asare and Prosper Owusu. We also thank Dr Harry Tagbor, then the Nkoranza District Medical Officer and Chief Medical Officer of St Theresa's Hospital, for his support and practical guidance; Eddy Addo (Nutrition Unit, Noguchi Memorial Institute of Medical Research, University of Ghana) for the high-performance liquid chromatography assessment of plasma retinol and Maxwell Appawu (Parasitology Unit, NMIMR, University of Ghana) for assessment of the malarial parasitaemias. We would also like to express our immense appreciation for the co-operation of all of our study participants.

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