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# ORIGINAL COMMUNICATION

# Reduced production of immunoregulatory cytokines in vitamin A- and zinc-deficient Indonesian infants

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**Objective:** To determine effects of vitamin A, zinc and iron deficiency in Indonesian infants on the ability to produce immunoregulatory cytokines.

**Design, setting and subjects:** Immunological asssessment was done in 59 infants participating in a cross-sectional nutritional survey in rural West Java, Indonesia. Production of T-helper cell type-1 (Th1, cell-mediated) cytokines interferon-$\gamma$ (IFN-$\gamma$), interleukin-12 (IL-12), interleukin-18 (IL-18) and T-helper cell type-2 (Th2, humoral) cytokine interleukin-6 (IL-6) were measured after stimulation with lipopolysaccharide and phytohemagglutinin in an *ex vivo* whole blood culture system. Circulating neopterin concentrations were determined as an indicator of *in vivo* macrophage activity.

**Results:** Of the infants, 48% were vitamin A deficient, 44% were anemic (with 17% having iron deficiency anemia), and 17% were zinc deficient. Vitamin-A deficient infants had significantly reduced *ex vivo* production of IFN-$\gamma$, but also significantly higher circulating neopterin concentrations. Production of IFN-$\gamma$ and IL-12 were strongly correlated, IFN-$\gamma$ and IL-18 production were not. Zinc deficiency was accompanied by significantly reduced white blood cell counts and reduced *ex vivo* production of IL-6. Iron status was not related to cytokine production.

**Conclusions:** This study shows that in vitamin A deficiency there is Th1 dominance in a steady state, combined however with impairment of the Th1 response after stimulation, whereas in zinc deficiency, there is a decreased Th2 response. Overall, vitamin A deficiency and zinc deficiency have marked albeit different effects on the immunocompetence of infants, affecting both cell-mediated and humoral components of the immune system.

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## Introduction

Micronutrient deficiencies are prevalent worldwide and especially affect vulnerable groups such as infants and pregnant women (FAO/WHO, 1992). It is becoming increasingly clear that effects of micronutrient deficiencies are more subtle and widespread than previously thought. Profound effects on growth, development, and immunocompetence can be present in even marginal micronutrient deficiency (Bates, 1995; Shankar & Prasad, 1998). For example, vitamin A deficiency leads to markedly increased rates of morbidity and mortality from infectious diseases, and vitamin A supplementation can reduce infant mortality by as much as 25%, even in populations with a low prevalence of xerophthalmia (Bates, 1995).

It is tempting to attribute the immune defects in vitamin A deficiency to a disturbed response of immunoregulatory cytokines. Studies in animals have given conflicting results however. Depression of T-cell activation is reported, supported by findings of decreased interferon-$\gamma$ (IFN-$\gamma$) production and decreased natural killer (NK) cell activity (Bowman *et al*, 1990; Ross & Stephensen, 1996). Other studies report however overproduction of IFN-$\gamma$ and reduced antibody production, fitting with a Th1 predominance (Wiedermann *et al*, 1993; Cantorna *et al*, 1994). Although there are few reports on the effects of vitamin A deficiency on immune function in humans, decreased cell-mediated immune function including reduced type-1 cytokine production, as well as reduced natural killer cell activity, impaired phagocytosis and reduced antibody titers, have all been reported (Semba, 1994; Jason *et al*, 2002). Hence, the underlying immune defects in vitamin A deficiency remain elusive.

Another micronutrient with profound effects on the immune system is zinc. Zinc supplementation has been shown to markedly reduce morbidity of diarrheal diseases, respiratory infections, as well as malaria (Shankar & Prasad, 1998; Bhutta *et al*, 2000). Zinc deficiency in both humans and animals causes a striking depletion of both B and T cells, and also of neutrophilic granulocytes, NK cells and macrophages. Furthermore, depressed cytokine production and antibody response and reduced T-cell function have been reported (Beaton *et al*, 1992; Driessen *et al*, 1995; Beck *et al*, 1997; Shankar & Prasad, 1998).

Iron deficiency can also profoundly affect immunocompetence (Oppenheimer, 2001). However, components of the immune system are affected in different ways, depending on the degree of deficiency and concommitant infections. Hence, there is still confusion about the effect of iron on immune function (Oppenheimer, 2001).

Immunocompetence depends upon close coordination between innate and acquired immunity. IFN-$\gamma$ plays a central role, and is produced by T cells and NK cells after stimulation, activating macrophages. Interleukin-12 (IL-12) and interleukin-18 (IL-18), produced by activated macrophages, synergistically stimulate IFN-$\gamma$ production by T cells and NK cells (Okamura *et al*, 1998). Besides IL-12 and IL-18, activated macrophages produce other cytokines including interleukin-6 (IL-6).

This study investigates the effect of vitamin A, zinc, and iron deficiency in infants on the production of immunoregulatory cytokines. Cytokines were measured after stimulation of whole blood with LPS and PHA in an *ex vivo* culture system. Given the putative role of IFN-$\gamma$, IL-12, and IL-18 in cell-mediated immunity, and IL-6 in humoral immunity, these cytokines were measured in a cohort of infants with carefully defined nutritional status. In addition, circulating neopterin concentrations were determined as an indicator of *in vivo* macrophage activity induced by IFN-$\gamma$.

## Materials and methods

### Subjects and procedures

In all, 60 infants, 3–10 months old, were recruited from two adjacent villages in rural West Java, Indonesia for a nutritional survey, and further immunological testing was done when field circumstances allowed (availability of electricity). All children studied were healthy by clinical assessment. All infants still received breastfeeding, although most infants ($n=47$; 78%) also received complementary foods or formula. Mothers were informed of the procedures and purpose of the study. After written informed consent was given by the mother, a short history was obtained, infants were anthropometrically assessed, and a blood sample was taken from the infants.

### Sample collection

A nonfasting 5 ml venous blood sample was taken using a closed-tube vacuum system (Becton and Dickinson, Leiden, The Netherlands). The EDTA blood sample aliquot (2 ml) was used for hematological analyses as well as for the *ex vivo* whole blood cultures. The heparinized blood sample aliquot (3 mL) was used for the measurement of plasma concentrations of retinol, zinc, ferritin, C-reactive protein (CRP), and neopterin.

### *Ex vivo* whole blood cytokine production

Cytokine production was measured using an *ex vivo* whole blood cytokine production after stimulation assay as described by Van Crevel *et al* (1999). In short, within 15 min of collection of the blood sample, a mixture of phytohemagglutinin (PHA-P, Sigma, Zwijndrecht, The Netherlands) in a final concentration of 30 mg/l, and lipopolysaccharide (LPS, *Escherichia coli* serotype 055:b5; Sigma) in a final concentration of 10 μg/l, was added directly to the closed vacuum EDTA blood collection tube and then incubated at 37°C for 10 h. The concentrations used, as well as the incubation time, were chosen to give an optimal response in this system. After incubation, blood was centrifugated and the supernatant was collected for cytokine concentration determination. Blood was incubated with a combination of PHA and LPS in order to achieve a maximally effective stimulation.

### Hematological and biochemical analyses

Hemoglobin concentration was measured by standard cyanoblue method (Humalyzer, Germany). Total white blood cell (WBC) and differential counts were performed by an experienced technician using standard microscopy methods. Retinol was analyzed using high-performance liquid chromatography (Thermo Separation Products, San Jose, CA, USA) and zinc was analyzed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic., Australia) using trace-element free procedures, as described in an earlier paper (Dijkhuizen *et al*, 2001). The CV for both retinol and zinc analyses was $<5\%$. Ferritin, C-reactive protein (CRP), and neopterin were measured using

commercial ELISA-kits (MP Products, Amersfoort, The Netherlands) according to the guidelines of the manufacturer. The cytokines IFN-$\gamma$, IL-6, IL12-p70, and IL-18 were also measured with ELISA (Pelikine, CLB, Amsterdam, The Netherlands and (IL-18 only) R&D Systems, Minneapolis, MN, USA) according to the manufacturer's guidelines. IL-12 p70 was determined because this is the heterodimer responsible for the biological activity (Jansen *et al*, 1996). The CV for all ELISA assays was <10%. Deficiency of vitamin A was defined as a plasma retinol concentration <0.70 μmol/l. Zinc deficiency was defined as a plasma zinc concentration <9.95 $\mu$mol/l (nonfasting blood samples). Anemia was defined as a hemoglobin concentration <110 g/l, and iron deficiency anemia was defined as anemia combined with a plasma ferritin concentration <15 μg/l (Gibson, 1990).

### Ethical approval

The protocol was approved by the ethical committee of the National Health Research and Development Institute of Indonesia, and by the ethical committee of the Royal Netherlands Academy of Arts and Sciences.

### Statistical analysis

Data were checked for normal distribution using Kolmogorov–Smirnov test of normality. The concentration of the cytokines IFN-$\gamma$, IL-12, and IL-18 were transformed to natural logaritm to achieve normality. For data not normally distributed (IL-6 production and WBC counts) nonparametric tests were used. Relationships between normally distributed data was analyzed with Pearson's correlation, and between not normally distributed data with Spearman's rank test. Infants were assigned dummy variables for being vitamin A deficient or not, zinc deficient or not, and iron deficient or not. Cytokine production was first analyzed with analysis of variance or covariance, controlling for age (ANOVA or ANCOVA). However, there were significant interactions between vitamin A deficiency and iron deficiency, and between zinc deficiency and iron deficiency. Hence, differences between deficient and not-deficient groups were analyzed with the Student's *t*-test for parametric data and Mann–Whitney *U*–test for nonparametric data, excluding for other micronutrient deficiencies if possible. When necessary to control for confounding variables, analysis of covariance was used, with age as a covariate. Statistical analysis was carried out with SPSS 10.0 software package (SPSS Inc, Chicago, IL, USA).

## Results

In total, blood for the measurement of *ex vivo* cytokine production was collected from 60 infants, and complete data were available from 59 infants. The infants (32 boys and 27 girls) ranged in age from 3 to 10 months, and the age distribution was the same for both sexes.

Plasma concentrations of retinol and zinc fall, and those of ferritin rise during an acute-phase reaction, and thus these indicators may not reliably reflect micronutrient status in the presence of inflammation (Wieringa *et al*, 2002). As clinical examination can often not exclude the presence of minor or low-grade chronic infection, seven infants with an acute-phase response, as indicated by a plasma CRP concentration >10 mg/l, were excluded from the statistical analyses of micronutrient indicators. Plasma retinol concentrations were significantly lower in the excluded subjects, as was expected (mean 0.44 *vs* 0.69 μmol/l, $P<0.01$, Student's *t*-test), but plasma zinc and ferritin concentrations were not significantly different (Student's *t*-test and Mann–Whitney *U*-test, respectively).

Of the 52 infants, 25 (48%) were vitamin A deficient, nine (17%) were zinc deficient (of which seven were also vitamin A deficient, Table 1). There were 23 (44%) infants anemic of which nine (17%) had iron deficiency anemia (of which five were also vitamin A deficient and two were also zinc deficient) (Table 1). There were two infants (4%) deficient for all three micronutrients, whereas 23 infants (44%) were not deficient for any of these three micronutrients.

The production of the proinflammatory cytokines IFN-$\gamma$, IL-12, IL-18, and IL-6 after *ex vivo* stimulation of whole blood with LPS and PHA was measured. There was a strong positive correlation between *ex vivo* production of IFN-$\gamma$ and IL-12 ($r=0.54$, $P<0.001$, Pearson's correlation), and IFN-$\gamma$ and IL-6

**Table 1** Indicators of micronutrient status in the infants, and characteristics of the vitamin A- and zinc-deficient infants

| | | *Vitamin A*[c] | | *Zinc*[c] | |
|---|---|---|---|---|---|
| | *Infants* | *Deficient* | *Nondeficient* | *Deficient* | *Nondeficient* |
| *n* | 59 | 25 | 27 | 9 | 43 |
| Plasma concentrations | | | | | |
| Retinol (μmol/l)[a] | 0.66 (±0.24) | 0.49 (±0.12)[d]** | 0.88 (±0.14)[d]** | 0.56 (±0.16) | 0.72 (±0.24) |
| Zinc (μmol/l)[a] | 12.6 (±2.8) | 11.6 (±2.8)[d]* | 13.3 (±2.5)[d]* | 8.7 (±0.7)[d]** | 13.3 (±2.3)[d]** |
| Ferritin (mg/l)[b] | 20.7 (11.5–50.0) | 19.2 (10.0–61.0) | 17.9 (9.9–50.0) | 14.7 (8.0–76.3) | 19.8 (10.3–46.0) |
| Hemoglobin concentration (g/l)[a] | 109 (±12) | 106 (±13) | 112 (±11) | 107 (±11) | 109 (±12) |

[a]Mean (±s.d.) or [b]median (IQR).
[c]Deficiencies were defined as plasma retinol concentration <0.70 mmol/l for (marginal) vitamin A deficiency and plasma zinc concentration <9.95 mmol/l for zinc deficiency. Infants ($n=7$) with plasma C-reactive protein concentrations >10 mg/l were excluded.
[d]Significant difference between groups, Student's *t*-test. ** $P<0.01$, * $P<0.05$.

($\rho = 0.48$, $P < 0.001$, Spearman's rank). In contrast to IL-12, there was no correlation between *ex vivo* production of IFN-$\gamma$ and IL-18 ($r = -0.19$, $P > 0.1$, Pearson's correlation). IL-18 production was negatively correlated to IL-12 ($r = -0.29$, $P < 0.05$, Pearson's correlation) and IL-6 production ($\rho = -0.30$, $P < 0.05$, Spearman's rank). The production of IL-12 was significantly related to increasing age ($r = 0.30$, $P < 0.05$, Pearson's correlation), whereas the production of the other cytokines was not related to age.

In vitamin A-deficient infants the production of IFN-$\gamma$ was significantly lower than in infants with adequate vitamin A status (Figure 1, $P < 0.05$, Student's *t*-test ), also after exclusion of the nine infants who were zinc deficient. Further exclusion of the seven infants who were also iron deficient, so that infants with only vitamin A deficiency were compared with infants not deficient for any of these micronutrients, made the results even stronger with vitamin A-deficient infants having an *ex vivo* IFN-$\gamma$ production of only one-fourth of the production of nondeficient infants (antilog of means ($\pm$s.d.) 22.0 ($\pm$4.1) ng/l *vs* 90.9 ($\pm$3.6) ng/l, $n = 13$ *vs* 23, $P < 0.01$, Student's *t*-test). Also, there was a trend towards lower IL-6 production in vitamin A-deficient infants ($P = 0.07$, Mann–Whitney *U*-test). Although IL-12 production of vitamin A-deficient infants was less than half of the production of nondeficient infants (antilog of means ($\pm$s.d.) 30.5 ($\pm$3.8) *vs* 66.5 ($\pm$5.7) ng/l, $n = 13$ *vs* 23), the difference failed to reach statistical significance ($P = 0.12$, ANCOVA controlling for age). There were no significant differences between vitamin A-deficient and nondeficient infants in the production of IL-18.

Although only nine infants were zinc deficient, IL-6 production was significantly decreased in the zinc-deficient infants as compared to nondeficient infants (Figure 2, $P < 0.05$, Mann–Whitney *U*-test). Exclusion of the nine infants with iron deficiency anemia made these results even stronger, with zinc-deficient infants ($n = 7$, medians (IQR): 15.0 (7.5–24.9 ng/l) having an *ex vivo* IL-6 production of half of the production of infants without zinc deficiency ($n = 36$, medians (IQR): 31.4 (23.3–45.5) ng/l, $P < 0.05$, Mann–Whitney *U*-test). As five of these infants were also vitamin A deficient, it was not possible to exclude vitamin A-deficient infants, as the numbers became too low for meaningful statistical analysis. There was no significant effect of zinc deficiency on IFN-$\gamma$, IL-12, or IL-18 production.

Neither anemia nor iron deficiency anemia were associated with significant changes in cytokine production. The exclusion of the seven infants with a raised CRP did not change the observed relationships between cytokine production and vitamin A or zinc status.

WBC counts and differentiation were similar in vitamin A-deficient and nondeficient infants (Table 2) as well as in infants with or without iron deficiency anemia (results not shown). Zinc-deficient infants however, had significantly decreased WBC counts (Table 2, $P < 0.05$, Mann–Whitney *U*-test), with especially lower counts of neutrophilic granulocytes ($P < 0.05$, Mann–Whitney *U*-test). Eosinophilic granulocytes also tended to be lower, but not significantly so ($P = 0.08$, Mann–Whitney *U*-test). In all infants, total lymphocyte, monocyte and neutrophilic granulocyte counts correlated significantly with IL-6 production ($\rho = 0.37$, $P < 0.01$; $\rho = 0.51$, $P < 0.01$; and $\rho = 0.31$, $P < 0.05$, respectively, Spearman's rank), but not with the *ex vivo* production of the other cytokines. To determine whether the lower IL-6 production in zinc-deficient infants was caused by the lower WBC counts, or by a lower production of IL-6 per cell, a multiple regression model with the WBC differential counts included was tested, using a backward deletion method if $P > 0.2$. In this model, zinc deficiency remained an important determinant of IL-6 production ($r = -0.23$, $P < 0.10$, multiple regression analysis for zinc deficiency) indicating that production per cell is also affected.

Plasma neopterin concentrations were significantly related to the age of the infants ($\rho = 0.30$, $P < 0.05$, Spearman's rank). Vitamin A-deficient infants had significantly higher plasma neopterin concentrations than nondeficient infants (Table 2, $P < 0.05$, ANCOVA, controlling for age), also after exclusion

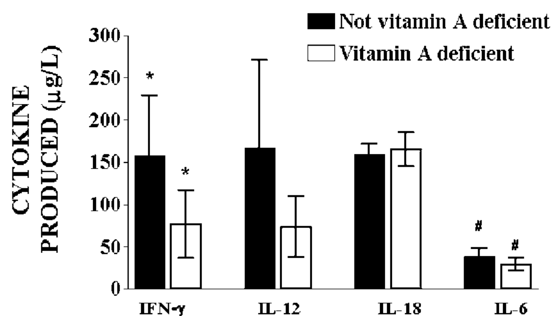


**Figure 1** *Ex vivo* whole blood cytokine production after stimulation with LPS and PHA. Mean production ($\pm$95% CI), in vitamin A deficient infants ($n = 25$) *vs* nonvitamin A-deficient infants ($n = 27$). Differences between groups: *: $P < 0.05$ (Student's *t*-test), #: $P < 0.1$ (Mann–Whitney *U*-test). IFN-$\gamma$: interferon-$\gamma$; IL:interleukin.

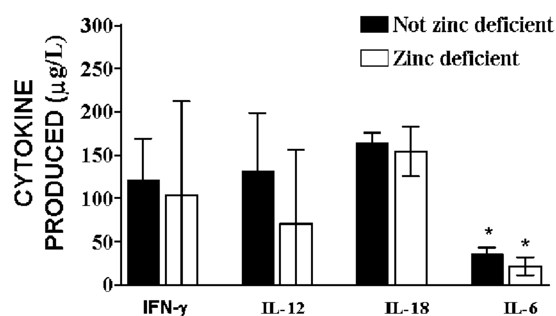


**Figure 2** *Ex vivo* whole blood cytokine production after stimulation with LPS and PHA. Mean production ($\pm$95% CI), in zinc deficient infants ($n = 9$) *vs* non-zinc-deficient infants ($n = 43$). Differences between groups: *: $P < 0.05$ (Mann–Whitney *U*-test). IFN-$\gamma$: interferon-$\gamma$; IL: interleukin.

**Table 2** Plasma neopterin concentration, WBC count and differentiation for all infants, and for the vitamin A- and zinc-deficient groups

| | | *Vitamin A*[c] | | *Zinc*[c] | |
|---|---|---|---|---|---|
| | *Infants* | *Deficient* | *Nondeficient* | *Deficient* | *Nondeficient* |
| *n* | 59 | 25 | 27 | 9 | 43 |
| Neopterin concentration (nmol/l)[a] | 20.9 (16.1–26.9) | 21.4 (17.5–27.7)[d]* | 17.1 (11.6–24.7)[d]* | 19.3 (16.1–24.4) | 19.4 (15.0–26.8) |
| Total leukocyte count ($\times 10^9$/l)[a] | 7.9 (6.2–10.5) | 7.3 (5.4–10.0) | 7.9 (6.2–10.0) | 6.6 (4.8–7.3)[e]* | 8.0 (6.2–10.3)[e]* |
| Differentation ($\times 10^9$/l)[a,b] | | | | | |
| Eosinophilic granulocytes | 0.1 (0.0–0.3) | 0.1 (0.0–0.2) | 0.1 (0.0–0.4) | 0.1 (0.0–0.2) | 0.2 (0.1–0.4) |
| Neutrophilic granulocytes | 2.9 (1.8–3.7) | 2.6 (1.6–3.4) | 3.0 (2.0–3.7) | 2.0 (1.6–2.4)[e]* | 3.1 (1.8–3.8)[e]* |
| Lymphocytes | 4.8 (3.4–6.5) | 4.7 (3.1–6.5) | 4.6 (3.5–6.2) | 4.4 (3.0–4.7) | 5.1 (3.4–6.5) |
| Monocytes | 0.1 (0.0–0.2) | 0.1 (0.0–0.2) | 0.1 (0.0–0.2) | 0.1 (0.0–0.1) | 0.1 (0.0–0.2) |

[a]Medians (IQR).
[b]Basophilic granulocytes were zero in all groups.
[c]Deficiencies were defined as plasma retinol concentration $<0.70\,\mu$mol/l for (marginal) vitamin A deficiency and plasma zinc concentration $<9.95\,\mu$mol/l for zinc deficiency. Infants ($n=7$) with plasma C-reactive protein concentrations $>10$ mg/l were excluded.
[d]Significant difference between groups, ANOVA with age as covariate. *$P<0.05$.
[e]Significant difference between groups, Mann–Whitney *U*-test. *$P<0.05$.

of the nine infants with zinc deficiency and also after further exclusion of infants with iron deficiency anemia ($n=7$). There was no relation between zinc deficiency, anemia or iron deficiency anemia and plasma neopterin concentrations.

## Discussion

This study shows that infants who are vitamin A deficient have a reduced production of proinflammatory cytokines upon stimulation, especially the type-1 cytokine IFN-$\gamma$. Circulating plasma neopterin concentrations were higher in vitamin A-deficient infants, however. Also, whereas vitamin A deficiency did not affect cell counts, zinc-deficient infants had lower WBC, as well as lower *ex vivo* production of the type-2 cytokine IL-6. Iron status was not associated with differences in *ex vivo* cytokine production.

The production of IFN-$\gamma$ is under control of more proximal cytokines such as IL-12 and IL-18, but in this study, only IL-12 was strongly correlated to IFN-$\gamma$ production. Also, the production of IL-18 in vitamin A-deficient infants was not different from infants with a normal vitamin A status. These data suggest that the lower production of IFN-$\gamma$ in the vitamin A-deficient infants is due to a decreased production of IL-12, and not to changes in IL-18 production. The lower production of IFN-$\gamma$ would imply less activation of the cell-mediated arm of the immune system. In this respect, our finding that circulating neopterin concentrations were higher in the vitamin A-deficient infants is intriguing. It is generally believed that elevated neopterin concentrations reflect increased macrophage activity, and this is regulated by IFN-$\gamma$ (Huber *et al*, 1984). How can this paradox of decreased *ex vivo* IFN-$\gamma$ production and increased *in vivo* neopterin concentrations be explained? One likely explanation for the apparent discrepancy of *ex vivo* IFN-$\gamma$ production and circulating neopterin concentrations is an upregulation of IFN-$\gamma$ receptors and/or of the IFN-$\gamma$ signalling pathway in vitamin A deficiency, which leads to a blunted response upon stimulation. Another explanation could be that the 10 h culture system used, reflects only the first nonproliferative phase of the cellular response. Whether more prolonged cultures with proliferating T cells would give a different IFN-$\gamma$ response remains to be investigated.

Irrespective of the mechanism, our findings suggest a type-1 cytokine dominance in a steady state in vitamin A deficiency, as indicated by elevation of circulating neopterin concentrations, accompanied by an impairment of IFN-$\gamma$ response after stimulation, as may occur during infection. In this context, it is important to note that these infants were selected to have no acute-phase reaction, in order to exclude coincident active infection or inflammation, as this could modify not only indicators of nutritional status but also the immune response itself. The findings of the present study are in line with the results of Jason *et al* (2001), who reported that vitamin A-deficient children had a relative type-1 cytokine dominance with significantly more T cells producing IFN-$\gamma$, and less macrophages producing interleukin-10. Unfortunately, only clinical markers of acute infection were including in the study by Jason *et al*, which might not be specific enough to exclude an acute-phase response (Wieringa *et al*, 2002). Furthermore, 25% of the infants had HIV infection, making interpretation of the effects of vitamin A deficiency on immune function more complicated.

Even though only nine infants were zinc deficient, a significant reduction of IL-6 production in whole blood cultures after stimulation was found. Moreover, IL-6 production was reduced not only because of a reduced WBC count, but also because of an impaired IL-6 production by each leucocyte. There was no significant effect of zinc deficiency on either IFN-$\gamma$, IL-12, or IL-18 production, but the small group size may have obscured an effect. These results suggest that especially the type-2 cytokine response is decreased in zinc deficiency. However, contrasting findings have been

reported with respect to zinc deficiency and IL-6 production. Some earlier reports on zinc deficiency, both in humans and animals, showed impaired production of antibodies and reduced antibody response, which seem to point more towards a reduction in type-2 responses rather than type-1 responses. Antibody production however is not exclusively driven by type-2 cytokines but also affected by type-1 cytokines (Shankar & Prasad, 1998). In contrast, some other studies found no effect on humoral immunity, but a significant reduction of type-1 cytokine production in zinc deficiency (Beck *et al*, 1997), and one study reports a reduction of IL-6 production after addition of zinc to certain cell lines (Hatakeyama *et al*, 2002). An important reason for the inconsistency among studies are differences in study design (*in vitro vs in vivo*, human *vs* animal studies, experimental *vs* field studies), and the manner in which zinc deficiency is defined by the different studies. In the present study, immune function is related to the zinc status of the infants as measured with plasma zinc concentrations, and not after experimentally induced zinc deficiency, and neither were intracellular zinc concentrations used as measure for zinc deficiency. Furthermore, the whole blood cultures were performed in the presence of EDTA, which binds most soluble zinc ($>99\%$) in the culture. Hence, the lower IL-6 production in the zinc-deficient infants in the present study is due to pre-existent effects of zinc deficiency in these infants.

A limitation of the present study in this respect is the use of plasma zinc concentrations for the determination of zinc status. Zinc status is difficult to measure, as there are no known functional stores of zinc in the body. Plasma zinc concentrations are at present the most widely accepted measure of zinc status, especially when indicators of the acute-phase response are taken into account (Brown, 1998). However, plasma zinc concentrations may not accurately reflect individual zinc status, as the variation in plasma concentrations of zinc due to changes in zinc status are small, and can be overshadowed by other factors such as diurnal and interindividual variations. Many other indicators of zinc status have been studied, however, none have been able to supplant plasma zinc as the most practical indicator (Dijkhuizen *et al*, 2003). Isotope dilution techniques have been used to measure body pools of zinc, which give a more precise estimate of zinc status, but the cost prevents widespread use of this technique (Lowe *et al*, 2001). Also thymulin and metallothionein have been used as indirect indicators of zinc ion availability, but proved to have similar or more severe limitations as plasma zinc. As this was a community-based field study with healthy infants, plasma zinc concentrations were used for the assessment of zinc status, and additional measures of zinc status were not possible for practical or ethical reasons. Therefore, the interpretation of the findings of the present study are limited to the effects of zinc deficiency as reflected by low plasma zinc concentrations in otherwise healthy infants.

Although iron has been shown to play an important role in immunocompetence, in the present study there was no clear relationship between iron status and immune function. The large physiological changes in iron status indicators during the first year of life, with relatively high iron stores in the first months of life, decreasing rapidly towards the end of the first year, may have obscured true iron status, and thereby hampered the interpretation of the effects of iron on immune function.

This study shows that vitamin A deficiency has marked effects on the cell-mediated immune response, with decreased *ex vivo* type-1 cytokine production after stimulation, and with increased *in vivo* macrophage activity. Zinc deficiency also has distinct effects on immune function, resulting in reduced circulating leukocytes, and reduced *ex vivo* IL-6 cytokine production after stimulation. This reduction in IL-6 production is caused by both the reduction in WBC count, as well as by a reduced production per leukocyte. Concurrent deficiency of micronutrients may attenuate or aggravate effects on the different components of the immune system. Therefore, when infants are deficient for one or more micronutrients, it is likely that their immune function is also impaired. The extent to which either the cellular or humoral immunity is impaired appears to be determined by the specific micronutrient deficiency, and probably bears direct relevance for susceptability of the infant to infections. Considering the high prevalence of micronutrient deficiency in infants in developing countries, which is also reflected in the high prevalence of vitamin A deficiency in the present study, programs to combat micronutrient deficiency are most needed. Whether supplementation with vitamin A and zinc can restore these immune defects, or can have additional effects on the immune response still needs to be investigated.

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