Effect of multiple micronutrient supplementation during pregnancy on inflammatory markers in Nepalese women

Laura J Hindle, Rachel Gitau, Suzanne M Filteau, Katie J Newens, David Osrini, Anthony M Costello, Andrew M Tomkins, Anjan Vaidya, Raj Kumar Mahato, Birendra Yadav, and Dharma S Manandhar

ABSTRACT
Background: Multiple micronutrient supplementation of Nepalese women during pregnancy is associated with a significant increase in birth weight.

Objective: We tested the hypothesis that improved birth weight in infants of mothers supplemented with micronutrients is associated with a decrease in inflammatory responses and an increase in the production of T helper 1 cells and T helper 2 cells.

Design: The study was embedded in a randomized controlled trial of 15 micronutrients, compared with iron-folate supplementation (control), given during pregnancy with the aim of increasing birth weight. Blood samples were collected at 32 wk of gestation, 12–20 wk after supplementation began, for the measurement of inflammatory markers. Breast-milk samples were collected 1 mo after delivery for the measurement of the ratio of milk sodium to potassium (milk Na:K). In an opportunistically selected subgroup of 70 women, mitogen-stimulated cytokine production was measured ex vivo in whole blood.

Results: Blood eosinophils; plasma concentrations of the acute phase reactants C-reactive protein, α1-acid glycoprotein (AGP), neopterin, and ferritin; milk Na:K; and the production of interleukin (IL) 10, IL-4, interferon γ, and tumor necrosis factor α in whole blood did not differ significantly between the supplemented and control groups. Plasma C-reactive protein and AGP were higher in women who had a preterm delivery, and AGP was higher in women who delivered a low-birth-weight term infant than in women who delivered a normal-birth-weight term infant.

Conclusions: The results indicate an association between systemic inflammation in late pregnancy and compromised delivery outcome in Nepalese women but do not support the hypothesis that multiple micronutrient supplementation changes cytokine production or inflammatory markers.

KEY WORDS Micronutrients, pregnancy, cytokine, inflammation, interleukin 4, acute phase proteins, subclinical mastitis

INTRODUCTION

Low birth weight is a major global cause of infant morbidity and mortality, and interventions to decrease its prevalence are a current focus of research and program development. Improvement of maternal micronutrient status is one potential intervention (1), and several trials in different countries are currently evaluating the effect of a supplement providing approximately the recommended dietary allowance of 15 micronutrients on low birth weight (2). The present work was part of one of these trials, in which the supplementation of pregnant Nepalese women resulted in a significant 77-g (95% CI: 24, 130 g) increase in birth weight and a 25% decrease in the incidence of birth weight <2.5 kg as well as a nonsignificant increase in duration of gestation (3). The trial also found a slight but not significant increase in perinatal mortality among infants of supplemented women. When these mortality figures were combined with those from a different trial in Nepal with a similar micronutrient supplement (4, 5), a worrisome significant increase in neonatal mortality occurred (6). Thus, the trials have found both positive and negative effects of great public health importance after the micronutrient supplementation of pregnant women. There is a clear need to investigate the mechanisms of these effects to understand these disparate results and to aid in policy making and practice.

Numerous potential mechanisms whereby maternal micronutrient supplementation might affect infant birth weight and survival exist, although these have been relatively little researched (7). This article focuses on a likely candidate mechanism: immune function and inflammation. Maternal infection is a major cause of preterm delivery (7, 8) and evidence indicates that this may be mediated in part by cytokines (8). Inflammatory responses, even in the absence of infection, may result in preterm delivery (9), and the balance of type 1 (eg, interferon-γ, IFN-γ) and type 2 (eg, interleukin 4; IL-4) cytokines is believed to be important for healthy pregnancy (10, 11). Some of the best evidence that these cytokine mechanisms are important for human health comes from studies of malaria in pregnancy. Among Malawian women, malaria was associated with increased tumor necrosis factor α (TNF-α) in placental blood, and high TNF-α was associated with infant low birth weight (12). Among Kenyan (13) and Cameroonian (14) women, malaria was associated with increased TNF-α and type 1 cytokines, which were associated...
with infant low birth weight, although the reports showed different effects of malaria on the immunoregulatory cytokine IL-10. Considerable experimental evidence indicates that micronutrient deficiencies or supplementation can modulate immune and inflammatory responses (15–17), including type 1 and type 2 cytokine balance (18–21). Furthermore, because inflammation is a 2-edged sword, with both beneficial and harmful effects well described (22), it could potentially be associated with either beneficial effects on birth weight or detrimental effects on infant survival.

This study took advantage of the infrastructure of a large maternal micronutrient supplementation trial in Nepal (3). We analyzed the effect of randomized double-blind trial of supplementation with multiple micronutrients compared with iron and folate on immune and inflammatory markers and also investigated how these markers were associated with pregnancy outcomes.

SUBJECTS AND METHODS

The study was conducted in Janakpur, Nepal, with subject recruitment from August 2002 to October 2003. The trial was approved by the Nepal Health Research Council and the ethics committee of the Institute of Child Health and Great Ormond Street Hospital for Children and was conducted in collaboration with His Majesty’s Government Ministry of Health, Nepal. In Nepal, maternal nutritional status is poor and the incidence of low birth weight is approximately 40% in rural and 25% in urban pregnancies (3). Pregnant women (n = 1,200) were enrolled when they came to the study clinics for antenatal care at a mean (±SD) of 16.3 ± 3.0 wk gestation in the control group and of 16.1 ± 2.9 wk in the supplementation group. Inclusion criteria were gestation <20 wk, no preexisting maternal illness of a nature likely to affect the pregnancy, single live pregnancy detected by obstetric ultrasound, and residence potentially accessible for home follow-up. Women were randomly assigned to receive either routine iron (60 mg/d) and folic acid (400 µg/d) tablets or multiple micronutrient supplements in the form of tablets (3) for the remainder of their pregnancies. Compliance within both treatment groups was approximately 95%. Detailed methods and primary outcomes of the study—birth weight and gestational age—were published previously (3).

Secondary outcomes for the present study were investigated in subsamples of different participants. We concentrated on low-technology assays of markers of inflammation (23) that were suitable for the limited laboratory facilities available at the trial site in Janakpur, Nepal. First, blood samples were collected at 32 wk of pregnancy and were used to measure eosinophils, which reflect type 2 cytokine production (22) and the acute phase reactants C-reactive protein (CRP), α1-acid glycoprotein (AGP), ferritin (a marker of iron status and an acute phase protein), and neopterin. Acute phase proteins reflect production of inflammatory cytokines such as TNF-α, IL-1, and IL-6 (24), and neopterin is a marker of IFN-γ production (25, 26). Second, 1 mo after delivery we collected breast-milk samples for measurement of milk Na:K, which reflects mammary inflammation (27) and increases as a nonspecific marker of poor postpartum health (28). For the abovementioned markers, 2 related subsamples were used. The first subsample was a randomly selected group of 250 participants investigated at enrollment and at 32 wk gestation (~16 wk after supplementation) for mainly micronutrient status: plasma vitamins A and E, triacylglycerols, and ferritin. The results for vitamins were published previously (3); only the results for ferritin are presented here. Immunologic outcomes—CRP, AGP, and neopterin at 32 wk gestation and milk Na:K at 1 mo after delivery—were measured in a larger random subsample of 700 participants, which included the first subsample.

The third type of immune marker was the ex vivo production of the cytokines IL-4, IL-10, IFN-γ, and TNF-α in whole blood, which were measured in a subset of women at 32 wk of gestation. We wished to investigate potential cytokine mediators of any changes we found in the other inflammatory markers. Whole blood cytokine production was measured, rather than production in isolated enumerated leukocytes, because it represents a person’s overall production and is technically easier in a field site with limited laboratory facilities (29). For logistical reasons, whole blood cytokine production was measured in 70 women at their 32-wk gestation study visit during a month (July or August 2003) when one of the authors (RG) was in Janakpur to run the stimulation part of the assay.

Laboratory analyses

Stimulation of whole blood for cytokine production was conducted in Nepal with fresh samples according to established methods (30). Phytohemagglutinin (from Phaseolus vulgaris, no. L9132; Sigma, Poole, United Kingdom) in doses of either 1 or 5 µg/mL or lipopolysaccharide (from Escherichia coli, no. L2654; Sigma) in doses of 1 ng/mL or 1 µg/mL, were diluted under sterile conditions in London in 100 µL RPMI medium, portioned into 5-µL tubes, and frozen for transport to Nepal. Vials containing only 100 µL RPMI were also prepared for control, unstimulated samples. In Janakpur, where the laboratory facilities were very limited, tubes were opened briefly under clean but not sterile conditions for the addition of 100 µL fresh whole blood and then incubated at 37 °C for either 20 or 72 h. The times and stimulant doses were chosen as a result of initial studies conducted on blood from the laboratory staff in London and for convenient sampling times in Nepal. After incubation, the tubes were centrifuged at 3000 rpm for 5 min at room temperature, and the supernatant fluid was removed and frozen at −20 °C for <1 mo until transport to London in a liquid nitrogen cooler for cytokine analyses. In London, storage was at −80 °C.

Supernatant fluid was diluted 1:1 in dilution buffer, and cytokine concentrations were measured by enzyme-linked immunosorbent assay (ELISA) with the use of compact kits (Mast Diagnostics Pelikine, Bootle, United Kingdom). Standard ranges for the kits were as follows: IL-4, 0.6–450 pg/mL; IL-10, 1.2–300 pg/mL; TNF-α, 1.4–100 pg/mL; and IFN-γ, 2–500 pg/mL. Any samples above these ranges were repeated at higher dilutions. The quality-control sample used was a pooled supernatant fluid sample from cultures obtained from the laboratory staff in the United Kingdom. The interassay CVs were as follows: IL-4, 13.6%; IL-10, 14.1%; TNF-α, 15.5%; and IFN-γ, 15.1%.

Initially, ELISAs were run with the use of all samples from 5 arbitrarily selected Nepalese women to see which stimuli provided the best responses (data not shown). The higher stimulant doses (5 µg phytohemagglutinin and 1 µg lipopolysaccharide) were selected because they resulted in higher cytokine concentrations. The supernatant fluid concentrations of IL-4, IL-10, and TNF-α at 72 h were lower than those at 20 h, so only the 20-h samples for these cytokines were analyzed for the remaining
Subjects. IFN-γ concentrations were higher at 72 h than 20 h; thus, the remaining 72-h samples were selected for analysis. Production of IL-4 and IFN-γ after lipopolysaccharide stimulation was undetectable in the samples of the 5 Nepalese women; therefore, IL-4 and IFN-γ were investigated only after phytohemagglutinin stimulation. In each case, supernatant fluid samples from unstimulated cultures were run on the same ELISA plate as those from stimulated cultures, and the results were calculated as the difference between stimulated and unstimulated cytokine concentrations.

Plasma CRP concentrations were measured by sandwich ELISA with the use of antibodies and control serum from Dako (Ely, United Kingdom; 31). N-rheumatology standard serum from Dade Behring (Schwalbach, Germany) was used for calibration. The assay was standardized against an external control: NIBSC Control Human C-reactive Protein 1st international standard (code 85/506) version 02 (NIBSC, Hertfordshire, United Kingdom). An internal control of pooled plasma from a UK blood bank was also used with each assay. Interassay CVs were 10.1% and 10.7% for the external and internal quality controls, respectively.

Plasma AGP was measured by turbidimetry (31) with a COBAS FARA autoanalyzer (Roche, Welwyn Garden City, United Kingdom), antibodies from Dako (Ely), and a standard from Dade Behring (Schwalbach, Germany). An internal control of pooled plasma from a UK blood bank was used with each assay and the interassay CV was 5.7%.

Plasma neopterin concentrations were measured by ELISA (BRAHMS, Henningsdorf, Germany). The assay was standardized against an external control (NIBSC). The interassay CV for pooled plasma was 4.3%.

Plasma ferritin was measured by ELISA with antibodies and standards from Dako (32). The interassay CV for pooled plasma was 26%; ferritin assays were done last, and an insufficient amount of sample remained to repeat the analyses to lower the CV. Eosinophil counts were conducted in Nepal by microscopy, after application of Wright’s stain to blood smears, and were expressed as percentages.

Breast milk was obtained separately from the left and right breasts by manual expression. Milk Na:K was measured by flame photometry (33). The interassay CV for a pooled sample of milk from healthy UK women was 5.2% for sodium and 4.7 for potassium.

Sample size

The sample size for the main study was selected to detect, at 5% significance and 90% power, a 100-g difference in birth weight between treatment groups. For the nested study of inflammation, the size of the subset was chosen to detect 20% differences in plasma acute phase proteins or milk Na:K, at 5% significance and 80% power, between treatment groups or between women with or without adverse pregnancy outcomes such as preterm delivery or low-birth-weight infants. Sample sizes are lower for some blood analytes than for others, mainly neopterin and ferritin, because of an insufficient sample volume and for milk Na:K because some women were unable to provide milk samples from one or both breasts. Sample size calculations were not done for whole blood cytokine analysis because, for logistical reasons, this work could be done only on an opportunistic non-random, sample. However, we estimated that 70 women would be sufficient because similar numbers of persons, or fewer, have been sufficient to detect differences in the ex vivo production of similar whole blood cytokines in micronutrient-supplemented Indonesian infants (21) and pregnant women with or without pregnancy complications (10).

Data analysis

Plasma inflammatory markers were log-normally distributed, so the analyses were conducted with the use of log₁₀-transformed data, and geometric means are presented. We did not analyze acute phase proteins categorized as normal or elevated according to standard cutoffs because evidence indicates that standard cutoffs are inappropriate during pregnancy (MA Dijkhuizen, FT Wieringa, CE West, unpublished observations, 2004; 34). For eosinophil counts, many of which had the value zero, a value of one was added to all before log transformation and subtracted again after conversion to the original scale for presentation. Standard statistical analyses (correlations, t tests) were performed by using SPSS 12.0.1 (SPSS Inc, Chicago, IL). Because of the small sample size for whole blood cytokines, these results were analyzed by using nonparametric statistics. Medians and 25th and 75th percentiles are presented with comparisons by Mann-Whitney U test.

Similar nonparametric statistics were run for milk Na:K, which, unusually (27, 28, 33), did not achieve a normal distribution after log transformation; however, results were virtually identical if transformed values were analyzed with the use of parametric statistics. Not all women were able to donate milk from both breasts. In the 331 women for whom milk samples from both breasts were available, milk Na:K correlated (r = 0.40, P < 0.001) but was significantly higher (P = 0.03) in the right than in the left breasts, as we observed previously in Zambian women (28). Results of statistical analyses were very similar for the right and left breast samples, so only the analyses for the left breast samples are provided.

To determine whether plasma inflammatory markers at 32 wk gestation were associated with adverse pregnancy outcomes, we compared values from women with normal or adverse outcomes using a t test or ANOVA for normally distributed data and the Mann-Whitney U test for milk Na:K. Because of the extensive overlap between preterm birth and low birth weight, which resulted in very few normal-weight preterm infants, these outcomes were analyzed together with infants categorized as normal-weight term, preterm, or low-birth-weight term. Whole blood cytokines were not compared with pregnancy outcome results because of nonrandom selection and low numbers of adverse outcomes.

RESULTS

Characteristics of mothers at enrollment and infants at delivery for the present subsets of the main cohort are shown in Table 1. Randomization initially to treatment and later for subsetting resulted in balanced treatment groups for the larger group for plasma marker analysis. The opportunistically selected small group for whole blood cytokine production had an excess of primiparous women in the iron + folate group. The increase in birth weight and decrease in proportion of low birth weight with maternal micronutrient supplementation in the main trial (3) was reflected within the subsets; however, group differences were not always statistically significant, probably because of a smaller sample size.
Maternal markers of inflammation at 32 wk gestation showed the expected correlations (data not shown). Plasma AGP, CRP, neopterin, and ferritin were all positively intercorrelated, whereas the percentage of blood eosinophils correlated significantly and negatively only with plasma ferritin. Milk Na:K results were from samples collected several months after the blood inflammatory marker results and were not significantly correlated with any of them.

Micronutrient supplementation had no significant effects on any of the maternal inflammatory markers measured (Table 2). Note that milk was not available from women whose infants had died before 1 mo of age; this was unlikely to have seriously biased the results because the number of deaths was similar in both treatment groups (Table 1). Although not statistically significant, the lower plasma ferritin concentration in the micronutrient-supplemented group may reflect the fact that ferritin is a marker of both iron status and inflammation and the micronutrient group received only half the dose of iron that the iron + folate group received.

Whole blood production of IL-4, IL-10, IFN-γ, and TNF-α did not differ significantly between the micronutrient and iron + folate groups (Table 3). Spearman correlations of cytokine production and blood inflammatory markers were run for this subset. AGP did not correlate significantly with any of the cytokines, ferritin correlated significantly only with phytohemagglutinin-stimulated IFN-γ (r = −0.51, P = 0.046; n = 16), neopterin correlated significantly only with phytohemagglutinin-stimulated IL-4 (r = −0.34, P = 0.005; n = 64), and milk Na:K correlated significantly at 1 mo after delivery only with lipopolysaccharide-stimulated TNF-α (r = 0.45, P = 0.014; n = 29). Plasma CRP correlated significantly with phytohemagglutinin-stimulated IFN-γ (r = −0.31, P = 0.010; n = 68) and with phytohemagglutinin-stimulated IL-4 (r = −0.35, P = 0.004; n = 65). The percentage of blood eosinophils correlated significantly with the type 2 cytokine IL-4, produced in response to phytohemagglutinin (r = 0.29, P = 0.04; n = 49).

The results of comparisons of the inflammatory markers with the more common adverse events—preterm delivery (<37 wk) or birth weight <2.5 kg—and normal-birth-weight term delivery are shown in Table 4. AGP was higher at 32 wk gestation in both women with a preterm delivery or with a low-birth-weight term infant, and CRP was higher in women with a preterm delivery. No significant differences in blood inflammatory markers (milk was not available from women whose infants died) were seen between women whose pregnancies did or did not result in a live birth or whose infants did or did not die during the first month of

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Subset for plasma markers</th>
<th>Subset for whole-blood cytokine production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 368)</td>
<td>(n = 32)</td>
</tr>
<tr>
<td>Iron + folate (µg/L)</td>
<td>5.2 ± 2.0</td>
<td>5.0 ± 1.9</td>
</tr>
<tr>
<td>Micronutrients (µg/L)</td>
<td>5.7 ± 0.13</td>
<td>5.8 ± 0.71</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.71</td>
</tr>
<tr>
<td>Hemoglobin at enrollment (g/L)</td>
<td>19.9 ± 2.4</td>
<td>19.9 ± 2.4</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.94</td>
<td>0.29</td>
</tr>
<tr>
<td>Primiparous (n [%])</td>
<td>115 ± 15</td>
<td>116 ± 16</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.87</td>
<td>0.10</td>
</tr>
<tr>
<td>Live births (n [%])</td>
<td>304 ± 13</td>
<td>359 ± 11</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.88</td>
<td>0.04</td>
</tr>
<tr>
<td>Neonatal deaths (n [% of live births])</td>
<td>8 ± 2.3</td>
<td>6 ± 1.8</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>Preterm, &lt;37 wk (n [%])</td>
<td>30 ± 8.5</td>
<td>27 ± 7.3</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.71</td>
<td>0.52</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.74 ± 0.42</td>
<td>2.81 ± 0.43</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.46</td>
</tr>
<tr>
<td>Low birth weight, &lt;2.5 kg (n [%])</td>
<td>76 ± 24</td>
<td>61 ± 19</td>
</tr>
<tr>
<td>P&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.07</td>
</tr>
</tbody>
</table>

<sup>1</sup> Tests were used for continuous variables and chi-square tests were used for categorical variables.

### Table 2

Effect of micronutrients on blood inflammatory markers at 32 wk gestation and ratios of milk sodium to potassium (milk Na:K) at 1 mo after delivery<sup>1</sup>

<table>
<thead>
<tr>
<th></th>
<th>Iron + folate</th>
<th>Micronutrients</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.33 (1.17, 1.50)</td>
<td>1.35 (1.18, 1.53)</td>
</tr>
<tr>
<td>AGP (g/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.46 (0.44, 0.48)</td>
<td>0.45 (0.43, 0.47)</td>
</tr>
<tr>
<td>Neopterin (mmol/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8.90 (8.56, 9.25)</td>
<td>8.97 (8.61, 9.35)</td>
</tr>
<tr>
<td>Ferritin (µg/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>29.0 (25.8, 32.7)</td>
<td>25.0 (22.4, 27.9)</td>
</tr>
<tr>
<td>Eosinophils (%)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.6 (2.1, 3.1)</td>
<td>2.5 (2.0, 3.0)</td>
</tr>
<tr>
<td>Milk Na:K&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.38 (0.31, 0.49)</td>
<td>0.38 (0.30, 0.49)</td>
</tr>
</tbody>
</table>

<sup>1</sup> CRP, C-reactive protein; AGP, α<sub>1</sub>-acid glycoprotein.
<sup>2</sup> Tests.
<sup>3</sup> All values are geometric x; 95% CI in parentheses.
<sup>4</sup> All values are medians; 25th and 75th percentiles in parentheses. Samples taken from the left breast.
life, possibly in part because of the low numbers of these adverse events (Table 1).

DISCUSSION

The results do not support an important role for alterations in systemic inflammatory responses or production of IL-4, IL-10, TNF-α, or IFN-γ in the mechanism of the increased birth weight of infants of mothers supplemented during pregnancy with multiple micronutrients at levels of about one recommended dietary allowance. Sample sizes for the plasma acute phase proteins were large enough to have detected any biologically important differences and, indeed, differences in plasma CRP and AGP were observed between women who delivered preterm low-birth-weight term infants. The sample size for whole blood cytokine production was, of necessity, low; thus, conclusions should be made with caution. Nevertheless, similar sample sizes were sufficient to detect differences in cytokine production in whole blood cultures between pregnant Italian women with normal and poor pregnancy outcomes (10), between vitamin A or zinc deficient to detect differences in cytokine production in whole and, indeed, differences in plasma CRP and AGP were large enough to have detected any biologically important differences, 21), and between vitamin A–supplemented and placebo-treated Ghanaian women (35).

The interpretation that inflammatory responses or type 1 versus type 2 cytokine production are not important mediators of the effect of micronutrient supplementation on birth weight depends on the validity of the indicators used. Plasma CRP and AGP are well-established markers of systemic inflammation of both clinical and public health importance (24). It is notable that these acute phase markers were the only ones significantly associated with birth outcome. These acute phase proteins merit further exploration as low-cost markers of high-risk pregnancies, but this will require definition of normal plasma concentrations during pregnancy because CRP concentrations are elevated even in healthy pregnancy (MA Dijkhuizen et al, unpublished observations, 2004; 34). Plasma neopterin is less frequently used as an acute phase marker, but it has been recommended as an adjunct to CRP as an indicator of infection (36). Although ferritin is well known to increase during acute phase responses (37), its association with iron status confounds its use as an acute phase protein and, in fact, it was originally measured in our study as an indicator of iron status. We included it with the other inflammatory markers in this study because of concerns that high plasma ferritin is associated with preterm delivery (38) and low birth weight (39); however, no such association was seen in the present study. The measurement of cytokines that elicit inflammatory responses is generally more difficult and expensive than is the measurement of these downstream acute phase markers of cytokine

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stimulus</th>
<th>Value</th>
<th>No. of samples</th>
<th>Value</th>
<th>No. of samples</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (pg/mL)</td>
<td>Phytohemagglutinin</td>
<td>10.2 (5.0, 27.4)</td>
<td>37</td>
<td>15.9 (3.9, 25.1)</td>
<td>29</td>
<td>0.97</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>Lipopolysaccharide</td>
<td>172 (82, 387)</td>
<td>38</td>
<td>153 (66, 285)</td>
<td>31</td>
<td>0.35</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>Phytohemagglutinin</td>
<td>77 (27, 122)</td>
<td>38</td>
<td>50 (17, 113)</td>
<td>31</td>
<td>0.41</td>
</tr>
<tr>
<td>IFN-γ (pg/mL)</td>
<td>Phytohemagglutinin</td>
<td>306 (163, 556)</td>
<td>37</td>
<td>349 (111, 897)</td>
<td>32</td>
<td>0.49</td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
<td>Lipopolysaccharide</td>
<td>2.08 (1.48, 3.52)</td>
<td>37</td>
<td>2.18 (1.10, 3.85)</td>
<td>32</td>
<td>0.79</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>Phytohemagglutinin</td>
<td>259 (108, 367)</td>
<td>38</td>
<td>203 (88, 376)</td>
<td>32</td>
<td>0.44</td>
</tr>
</tbody>
</table>

1 Median amounts (25th and 75th percentiles) of cytokine produced by lipopolysaccharide- or phytohemagglutinin-stimulated cells in the 2 treatment groups. See Subjects and Methods for assay details.
2 Mann-Whitney U tests.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Value</th>
<th>n</th>
<th>Value</th>
<th>n</th>
<th>Value</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/L)</td>
<td>1.23 (1.11, 1.37)</td>
<td>438</td>
<td>1.45 (1.16, 1.83)</td>
<td>91</td>
<td>2.37 (1.69, 3.33)</td>
<td>44</td>
<td>0.001</td>
</tr>
<tr>
<td>AGP (g/L)</td>
<td>0.44 (0.43, 0.46)</td>
<td>433</td>
<td>0.48 (0.45, 0.52)</td>
<td>93</td>
<td>0.50 (0.44, 0.57)</td>
<td>44</td>
<td>0.014</td>
</tr>
<tr>
<td>Neopterin (mmol/L)</td>
<td>8.88 (8.59, 9.18)</td>
<td>408</td>
<td>8.96 (8.28, 9.69)</td>
<td>87</td>
<td>8.85 (8.01, 9.78)</td>
<td>40</td>
<td>0.97</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>27.6 (25.2, 30.2)</td>
<td>150</td>
<td>28.0 (21.3, 36.8)</td>
<td>29</td>
<td>23.5 (16.5, 33.5)</td>
<td>19</td>
<td>0.54</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>2.49 (2.10, 2.94)</td>
<td>258</td>
<td>2.66 (1.77, 3.84)</td>
<td>51</td>
<td>2.45 (1.29, 4.21)</td>
<td>27</td>
<td>0.95</td>
</tr>
<tr>
<td>Milk Na:K</td>
<td>0.38 (0.31, 0.48)</td>
<td>350</td>
<td>0.37 (0.30, 0.44)</td>
<td>92</td>
<td>0.41 (0.32, 0.57)</td>
<td>30</td>
<td>0.22</td>
</tr>
</tbody>
</table>

1 CRP, C-reactive protein; AGP, α1-acid glycoprotein.
2 Defined as <2.5 kg.
3 Defined as <37 wk.
4 ANOVA for all variables except milk Na:K, for which nonparametric statistics and Mann-Whitney U tests were used.
5 All values are geometric x; 95% CI in parentheses.
6,7 Significantly different from normal-birth-weight term delivery (t test): 6 P < 0.001, 7 P < 0.05.
8 Ratio of milk sodium to potassium. All values are medians; 25th and 75th percentiles in parentheses.
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production; furthermore, interpretation of the results is less clear. For example, many cytokines have short plasma half-lives or their plasma concentrations are not significantly associated with clinical outcomes (40). Ex vivo cytokine production in whole blood can indicate the potential of blood cells to respond in the presence of autologous plasma, but this may not be closely related to the extent that they actually have responded in vivo. Furthermore, most inflammatory or immune responses occur in tissues, not blood, and blood and relevant tissue production may not be correlated (40). For these reasons, in addition to the low sample size, we feel that the simpler measures of CRP, AGP, neopterin, and eosinophils are actually preferable for the purpose of assessing immune function in public health intervention trials such as this one.

CRP and AGP were higher in women who delivered preterm or low-birth-weight term infants, although geometric mean values were low, even in women with these adverse outcomes. We did not examine the associations of low-birth-weight or preterm delivery with CRP and AGP in categorical analyses with acute phase proteins above or below certain cutoffs, because it is not clear which cutoffs should be used in pregnancy. The association we observed is perhaps not surprising given the known link between reproductive tract infections and chorioamnionitis with outcomes such as preterm birth and low birth weight. Unfortunately, it was not possible to perform a placental histology in our trial. Further research is in progress to investigate the sensitivity and specificity of different acute phase proteins as predictors of adverse outcomes. The assays for these proteins are relatively simple and cheap and, if useful for identifying high risk pregnancies, are potentially feasible even in settings with poor resources.

We also examined a nonspecific marker of poor maternal health after delivery, milk Na:K, to determine whether improving maternal micronutrient status could reduce breast inflammation, even though the inflammation could not have been a causal factor for adverse birth outcome. Although previous studies have shown that multiple micronutrient supplementation of HIV-infected, but not uninfected Zimbabwean women, (41) or antioxidant-rich food supplementation of Tanzanian women reduced milk Na:K (42), micronutrient supplementation did not decrease milk Na:K in the Nepalese women in the present study. The reason for this difference is unclear and may depend on underlying micronutrient status or other health indicators. However, it is also notable that the sample size of the present study was larger than the sample sizes of the previous studies; therefore, previously observed differences may have resulted from chance.

Trials using the same UNICEF-derived multimicronutrient supplements have produced variable but not completely conflicting results (2). In general, birth weights were slightly higher, but not always significantly so, probably because of both study design factors and the underlying nutritional and health status of the population. Little mechanistic data, and apparently no data on inflammation, have been published from these trials; therefore, the reasons for the effects or lack of effects are unknown. Potential mechanisms, other than inflammation, whereby multiple micronutrient supplementation might increase birth weight are as follows: direct contributions to fetal tissue synthesis, improvements in placental function (43, 44), and improvements in the efficiency of the metabolic handling of macronutrients, eg, by improving maternal plasma volume expansion (45), by modifying the hypothalamic-pituitary-adrenal axis (7, 9), or by decreasing oxidative stress (43). Further research is required to determine whether these or other mechanisms mediate the increased birth weights observed in some trials of micronutrient supplementation.

LJH performed the cytokine and biochemical assays, conducted the preliminary data analysis, and wrote the manuscript. RG developed the whole blood cytokine assays and conducted the blood stimulation assays in Nepal. SMF designed the inflammation component of the studies, conducted the final statistical analyses, and drafted the manuscript. KJN conducted the biochemical analyses. DO supervised the main supplementation trial in Nepal. AMC was the principal investigator for the main study. AMT contributed to the design and interpretation of the inflammation component of the study. AV was the clinical coordinator of the study and supervised the clinical aspects of the laboratory work. RKM was the supervising pathologist for laboratory work at Janakpur Zonal Hospital. BY was responsible for collecting and processing the specimens and for the laboratory work in Nepal. DSM was the principal investigator in Nepal. All authors contributed to the final version of the manuscript. None of the authors had any conflicts of interest in relation to this work.

We are grateful to the trial participants and to the members of the study team who supported the laboratory component: Ram B Baniya, Puspa Baniya, Shiv Shanker Chaube, Bechan Chaudhary, Pravin Jha, Shyam Sundar Jha, Binaya Karki, Sushila Karki, Pusker Manandhar, and Gunaman Sah. We thank the Medical Superintendents of Janakpur Zonal Hospital: Bijay Kumar Singh, Lakhan Lal Sah, and Hukum Dev Sah.

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