Vitamin A Supplementation Reduces the Monocyte Chemoattractant Protein-1 Intestinal Immune Response of Mexican Children

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Introduction

Diarrheal disease continues to be a considerable cause of childhood morbidity and mortality in developing countries (1). Vitamin A supplementation has been shown to offer a cost-effective means of reducing the severity of diarrheal episodes and in reducing diarrhea-associated mortality (2). This effect of vitamin A supplementation on enteric infections and pathogenesis may be mediated by its action on the inflammatory cytokines (3). Chemokines are a large family of chemotactic cytokines that aid the innate immune response in recruiting adaptive immune cells that are essential to the development of an appropriate response to infectious agents but also mediate inflammation and tissue injury (4–6). They play an important role in diarrheal disease outcomes among children in developing countries because epithelial cells transcribe and secrete chemokines that contribute to the onset of pathogenesis following exposure to such pathogens as *Campylobacter jejuni* (7–9). The clarification of the effect that vitamin A supplementation has on these chemokines may lead to a better understanding of what specific mechanisms are involved in the association between vitamin A supplementation and childhood health outcomes.

Monocyte chemoattractant protein 1 (MCP-1) belongs to the CC chemokine family, one of two broad groups of chemokines classified according to the sequence of homology and cysteine residue positions. MCP-1 is involved in the attraction of monocytes to infected tissue sites and is regulated by the immune system in response to inflammation. MCP-1 is a potent chemoattractant for monocytes and macrophages, and its expression is induced by various stimuli, including bacteria, viruses, and cytokines. MCP-1 plays a key role in the recruitment of monocytes and macrophages to inflammatory sites, where they participate in the innate immune response. MCP-1 also regulates leukocyte migration and plays a role in the development of adaptive immune responses.


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Abbreviations used: EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; IQR, interquartile range; MCP-1, Monocyte chemoattractant protein 1; OR, odds ratio.
migration of mucosal mast cells that are induced during intestinal mastocytosis following nematode infections of mice (14). MCP-1 also protects against lethal endotoxemia in mice by enhancing the anti-inflammatory response (15), but also plays an important role in innate immunity required for clearance of bacterial organisms (16). Finally, it acts as an attractant for CD4+ and CD8+ lymphocytes (17,18) and induces the maturation of dendritic cells that are involved in the activation of these T-cell populations (19,20). These multiple roles of MCP-1 in regulating the pro-inflammatory and adaptive cytokine responses make it of primary clinical relevance.

We carried out a randomized, placebo-controlled, double-blind trial to evaluate the impact of vitamin A supplementation on pathogen-induced immune response among children from peri-urban communities of Mexico City, Mexico. Here we report the impact of vitamin A supplementation on concentrations of MCP-1 in stools collected from a subsample of study children during the summer diarrheal season. We focused on this chemokine due to its role in the innate and adaptive immune responses to gastrointestinal pathogens (8,21). We also report on the regulatory effect that supplementation has on MCP-1 concentrations during infections with enteropathogenic Escherichia coli (EPEC), enterotoxigenic E. coli (ETEC), Giardia lamblia and Ascaris lumbricoïdes and diarrheal pathogenesis. The modification of the effect of vitamin A on MCP-1 by these pathogens could determine whether there is a common or unique immune response mechanism underlying these effects. Any regulatory effect of vitamin A supplementation on MCP-1 expression may have important implications for pathogen-specific health outcomes in children.

Subjects and Methods

This study was based in La Magdalena Altlicpac, a peri-urban community located along the eastern perimeter of Mexico City as described previously (22). Briefly, project health workers first carried out a census of all children between the ages of 5 and 15 mo living within these communities. Mothers of all eligible children were then invited to participate in the trial during household visits by project personnel. Children were excluded from the study if they had diseases causing immuno-suppression, or any congenital acquired alteration of the digestive tract that could change the absorption of micronutrients. Children who were taking vitamin supplements were also excluded from the study.

Two hundred children were enrolled after parents had given informed consent for their participation. Children were assigned to receive vitamin A or a placebo using a randomized sequence generated from a random number table in blocks of 20. Children <12 mo of age who were assigned to the vitamin A group were administered a solution containing 20,000 international units (IU) of retinol [3.3 IU = 1 retinol activity equivalent (μg)] at baseline and subsequently every 2 mo until the end of the study, whereas children >12 mo received a solution containing 45,000 IU of retinol. Testing was carried out at the National Institute of Medical Sciences, “Salvador Zubiran”, Mexico City, to ensure that the placebo and vitamin A water-miscible solution were similar in taste, viscosity, and color.

Children were followed for up to 15 mo. Households were visited twice each week to determine whether the child had diarrhea, and if so, we noted (as reported by the child’s mother or caretaker) the number of times and consistency of the evacuations and whether there was a presence of blood or mucus in the stools. One stool sample was collected every 2 wk from healthy children, whereas 2 stool samples were collected from children with diarrheal disease. No initial and final blood samples were taken from the study children because parents did not consent to blood being drawn.

The endpoints for this analysis were the levels of MCP-1 in the stool samples after vitamin A supplementation, as well as changes in chemokine following infections by diarrheal pathogens or following the onset of diarrheal episodes.

Laboratory methods. Fresh stools, collected from children during the summer months of June, July, and August, were placed in sealed test tubes in ice and then frozen at −20°C within 4 h after collection. We analyzed MCP-1 from a subsample of children during these months because this is the period when diarrheal E. coli are the most prevalent and when diarrheal rates reach their peak. Samples were extracted as described previously (22) and the supernatants were collected, frozen, and stored at −70°C. The samples were assayed for MCP-1 using paired ELISA specific capture and biotinylated detecting antibody (Pierce-Endogen and R&D Systems). Peroxidases conjugated to steavidin (Pierce-Endogen) were used to detect the capture antibody, and peroxidase activity was measured using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate and read at a wavelength of 405 nm. Recombinant MCP-1 was used to generate a standard curve, and levels of chemokine from the stool extracts were determined using the standard curve in 96-well plates, according to the manufacturer’s protocol. The unit for all chemokine assays was pg/mL normalized to g/L of protein per stool. The detection limit for these cytokine assays was 100 pg/L.

All stool samples were plated on selective agars for the identification of Salmonella and E. coli. E. coli isolates were characterized as either enterotoxigenic E. coli (ETEC) or enteropathogenic E. coli (EPEC) strains using a multiplex PCR assay (23). The parasites A. lumbricoïdes and G. lamblia and their ova were identified by the Kato-Katz technique (24).

Data analysis. Data were entered in Visual Fox Pro 6.0 (Microsoft) and verified and checked for range and consistency. Distributions of MCP-1 were first compared between fecal samples collected from children in the vitamin A and placebo groups using Wilcoxon’s rank-sum test. Distributions of MCP-1 concentrations were also compared among samples that were positive or negative for EPEC, ETEC, G. lamblia, A. lumbricoïdes, or collected from children with or without diarrhea. We then used multinomial logistic regression analysis, modeling the probability distributions of cytokine values categorized into 3 levels and ordered from lowest to highest: nondetectable, less than the median of positives, or greater than or equal to the median of positives (25). Each sample was assigned to a category based on the values of the chemokine assayed in that sample. This approach was used, insofar as an important proportion of samples had no detectable levels of chemokine, rendering conventional analytic techniques inapplicable. The inclusion of the vitamin A variable in the model tested the hypothesis that the probability distribution of categorized chemokine values in the supplemented group differed from the distribution in the placebo group and was expressed as an odds ratio (OR).

Using this technique, we first modeled the overall probability for these differences in categorical levels of MCP-1 among children randomized to the vitamin A and placebo groups. In this first analysis we also included variables for the presence or absence of infections by EPEC, ETEC, G. lamblia, A. lumbricoïdes, and diarrheal disease to model the probability that categorical levels of MCP-1 among infected children or children with diarrhea differed from noninfected children or children without diarrhea. A second comparison of MCP-1 levels among children in the vitamin A and placebo groups was then carried out stratified by the presence of infection. Using the presence of diarrhea for infections by ETEC, EPEC, A. lumbricoïdes, and G. lamblia. Finally, comparisons of fecal MCP-1 levels among children in the vitamin A and placebo groups were then carried out stratified by the presence or absence of diarrheal symptoms. The age of the child was included in all of the analyses as well as confounders by pathogens. A diarrheal episode was defined as the mother’s reporting of symptoms in the child and was confirmed with the passage of ≥3 liquid stools in 24 h. A period of ≥3 symptom-free days was used to define the end of an episode. A pathogen infection was defined as encompassing at least 1 pathogen-positive stool and in stools collected for 3 wk following that positive stool. Significance was set at $P < 0.05$ and $P < 0.1$ for interactions. Data were analyzed using the GENMOD procedure in the Statistical Analysis System (SAS, version 8.2) software (SAS Institute).

The sample size for the overall study was calculated assuming that the study population had a diarrheal disease rate of 3 episodes/child-y and that the vitamin A supplement would reduce the incidence rate of diarrhea by ~20%. A sample size of 100/group was required to detect a 20% difference between the control and treatment group with a power of 0.8.
of 80%, a 5% significance level, and an expected loss to follow-up of 20%. This calculation allowed for repeated measurements of the outcome and a correlation between measurements at different time points of 0.7 (26).

The study protocol was approved by the human subjects committee of the National Institute of Public Health of Mexico and Harvard School of Public Health.

Results

A total of 127 children, enrolled in the larger community trial, were included in this analysis of cytokine levels in stools collected during the summer months of June through August of 1998. The remaining 73 children were not enrolled until the fall and so were not included in the analysis. As described previously, no significant differences were found when the distributions of socio-demographic and household characteristics among children in the vitamin A and placebo group were compared using Chi-square or Wilcoxon’s rank-sum tests (22). Approximately 42–45% of households had no access to piped water, whereas 65% had no indoor toilets. Twenty five to 30% of children were stunted. The initial vitamin A status of this population was not assessed. However, the most recent national nutrition survey carried out in Mexico in 1998 reported that children from this region of Mexico City were not deficient, but that 37% had low serum retinol levels [10–20 μg/DL retinol (0.349–0.698 μmol/L)] (27). Overall, 505 stool samples were collected from children during this period for a mean of 4 stools/child. Two hundred and sixty of these samples were collected from 70 children in the placebo group and 243 samples from 57 children in the vitamin A group. For the 3-mo period of this study there was a total of 87 diarrheal episodes among 127 children: 51 in the placebo group and 36 in the vitamin A group; with an incidence rate of 3.88 episodes and 2.80 episodes/child-y, respectively.

Approximately 37% of the samples that were assayed had no detectable levels of MCP-1. The proportion of samples with no detectable MCP-1 levels did not differ between children in the vitamin A and placebo groups (Table 1). The use of multinomial regression models in the analysis of differences of fecal chemokine levels between groups was meant to address this nonnormal distribution of chemokine values.

### Table 1

Fecal MCP-1 concentrations and multinomial regression model comparing categorized chemokine values in the vitamin A and placebo groups and by the presence or absence of diarrhea

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>% Not detectable</th>
<th>Median ± IQR</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment arm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A</td>
<td>192</td>
<td>32.75</td>
<td>284.88 ± 885.35</td>
<td>0.64 (0.42–0.97)</td>
</tr>
<tr>
<td>Placebo</td>
<td>229</td>
<td>42.71</td>
<td>403.39 ± 931.16</td>
<td></td>
</tr>
<tr>
<td>Diarrheal episode</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsymptomatic</td>
<td>350</td>
<td>38.86</td>
<td>306.82 ± 889.29</td>
<td>1.71 (1.06–2.75)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>73</td>
<td>28.77</td>
<td>596.23 ± 1408.21</td>
<td></td>
</tr>
</tbody>
</table>

Overall, the distributions of MCP-1 concentrations in the vitamin A and placebo groups differed (P = 0.02) when compared using the Wilcoxon’s rank-sum test, with concentrations being lower in the vitamin A group. The distributions of MCP-1 concentrations were also found to vary among children infected with EPEC vs. non-EPEC infected children (P = 0.05) and children who had diarrhea vs. children without diarrhea (P = 0.01). In the multinomial logistic regression analysis, vitamin A supplementation was found to be associated with a 36% reduction in the odds that MCP-1 would have higher-ordered values (OR 0.64, 95% CI 0.42–0.97, Table 1). Children with diarrhea were found to have a 71% increase in the odds of having higher MCP-1 levels in their stool than children without diarrhea (OR 1.71 95% CI 1.06–2.73, Table 1). A significant increase in MCP-1 levels similar in magnitude was found among children infected with ETEC (OR 1.66 95% CI 1.06–2.60, Table 2). In contrast, the odds of having higher MCP-1 levels in the stool were reduced by 46% among children infected with EPEC (OR 0.54, 95% CI 0.33–0.97, Table 2). A similar but nonsignificant reduction was found among children infected with Ascaris. No significant differences were found between children infected with Giardia and uninfected children (P = 0.32).

A second analysis was carried out to determine whether the impact of vitamin A supplementation on MCP-1 levels was modified by different pathogen infections. In this analysis, the odds of having higher MCP-1 levels in the stool were reduced 65% among vitamin A-supplemented children who were infected with EPEC (OR 0.35, 95% CI 0.16–0.76), a reduction that differed from the impact vitamin A had on the odds among children not infected with EPEC (P for interaction = 0.08, Table 3). No such difference was found among Ascaris-infected children. The odds of having higher MCP-1 levels was reduced among vitamin A-supplemented children not infected with Ascaris compared with children in the placebo group (P for interaction = 0.08).
TABLE 3  Multinomial regression model comparing categorized MCP-1 values in the vitamin A and placebo groups by pathogens and diarrheal episode

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Vitamin A</th>
<th>Placebo</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>P-value for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No EPEC</td>
<td>183/53</td>
<td>195/70</td>
<td>1.00 (0.62–1.59)</td>
<td>0.98</td>
<td>0.08</td>
</tr>
<tr>
<td>EPEC</td>
<td>25/14</td>
<td>40/19</td>
<td>0.38 (0.18–0.80)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>No ETEC</td>
<td>183/53</td>
<td>178/70</td>
<td>0.98 (0.56–1.54)</td>
<td>0.67</td>
<td>0.82</td>
</tr>
<tr>
<td>ETEC</td>
<td>33/19</td>
<td>29/19</td>
<td>1.74 (0.63–4.94)</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>No G. lamblia</td>
<td>195/53</td>
<td>188/70</td>
<td>0.46 (0.19–1.09)</td>
<td>0.04</td>
<td>0.32</td>
</tr>
<tr>
<td>G. lamblia</td>
<td>17/12</td>
<td>19/13</td>
<td>0.25 (0.01–1.16)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>No A. lumbricoides</td>
<td>173/53</td>
<td>165/70</td>
<td>0.62 (0.41–0.94)</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>A. lumbricoides</td>
<td>7/12</td>
<td>7/13</td>
<td>1.17 (0.25–5.50)</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>No-diarrhea</td>
<td>182/53</td>
<td>168/70</td>
<td>1.22 (0.78–1.9)</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>30/22</td>
<td>40/26</td>
<td>1.47 (0.56–3.82)</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>

1 Odds ratio represents the probability that MCP-1 (categorized into 3 levels: nondetectable, < median, > median) will have a higher value among vitamin A–supplemented children. Age of child included in model.
2 P-values for interaction indicates whether the effect of vitamin A was significantly different between strata.
3 Number of stools collected from noninfected children/Number of noninfected children.
4 Number of stools collected in 3 wk period following pathogen infections/Number of infected children.
5 Number of stools collected from children without diarrhea/Number of children without diarrhea.
6 Number of stools collected within 10 d after an episode/Number of children with diarrhea.

interaction = 0.08). The effect of vitamin A on MCP-1 levels among children infected with ETEC and Giardia or with diarrhea did not differ from its effect on levels of MCP-1 among uninfected children or among children without diarrhea in the third analysis.

Discussion

We found that vitamin A supplementation, overall, significantly reduces levels of the chemokine MCP-1 in stools collected from children during the summer diarrheal disease season in a peri-urban area of Mexico City. Infections by EPEC also significantly reduced MCP-1 levels, whereas infections by ETEC and the presence of diarrheal symptoms in children were associated with significant increases of this chemokine in stool. The impact of vitamin A supplementation on MCP-1 levels was also significantly modified by EPEC and Ascaris infections. To our knowledge, this study is the first to determine levels of chemokine in stools among children in a community-based study where diarrheal disease is widely prevalent and where it is an important cause of morbidity. In addition, to our knowledge, it is the first to report that vitamin A supplementation has a clear and direct effect on a chemokine in a clinical trial. As such, these findings can provide insight into the mechanisms underlying the impact of vitamin A on the gastrointestinal immune response and health outcomes in children.

MCP-1 is produced ubiquitously by a number of cell populations, both constitutively and in response to cytokine or pathogenic stimuli (28). Our finding, that vitamin A is associated with reduced fecal concentrations of MCP-1, is not in agreement with studies reporting that vitamin A markedly induces the expression of MCP-1 in undifferentiated human monocyteic cells (29). However, another study reported that all-trans retinoic acid regulates expression of constitutive MCP-1 in mesangial cells differently from MCP-1 expression induced by IL-1β (30). IL-1β–induced MCP-1 was unaffected by retinoic acid, whereas constitutive expression was substantially suppressed. It is not clear whether such constitutive expression occurs in the gut epithelium. Constitutive expression of MCP-1 has been reported for bronchial epithelium and renal glomeruli, which may contribute to the continuous attraction of monocytes into these sites (31,32). This direct effect of vitamin A on MCP-1 suggests that the regulation of MCP-1 secretion in the gastrointestinal tract by vitamin A may be directly mediated by the activation of retinoic acid receptors (30).

Our study found that levels of MCP-1 in the stool are partly determined by the type of pathogen infecting the child. MCP-1 is upregulated and secreted by human epithelial cells in the gastrointestinal tract, after pathogenic microbial infection, as part of the mucosal inflammatory response (8,21,33,34). Invasive pathogen infections uniformly upregulate MCP-1 and other chemokine mediators, irrespective of their mechanisms of invasion and localization within the cell (35–37). Less is known of the regulatory effect of infections by noninvasive pathogens, such as EPEC, which cause pathogenesis by inducing attaching and effacing lesions (A/E), characterized by the loss of microvilli and the remodeling of the mucosa epithelial at the site of bacterial attachment (38). Khan et al. (39) reported that the infection of mice with Citrobacter rodentium, a mouse-adapted A/E bacterium, did lead to the upregulation of the MCP-1 response. The consistent effect of EPEC infections suggests that EPEC has an important immuno-regulatory effect on the intestinal cytokine response that is independent of the effect that vitamin A supplementation has on this response.

The reduction in MCP-1 levels among children infected with EPEC was unexpected. However, EPEC can have a complex effect on the host’s immune response due to its ability to subvert and manipulate this response. Klapproth et al. (40,41) reported that EPEC produces lymphostatin, a toxin that inhibits lymphocyte proliferation and suppresses IL-2, IL-4, and IFN-γ production. The reduction of MCP-1 levels among vitamin A–supplemented children infected with EPEC is similar in direction and magnitude to the reductions we found for IL-6, IFN-γ, and IL-4 in these same supplemented children (22). We suggested that these reductions among EPEC-infected children may be due to lymphostatin impairing the production of adaptive immune-response cytokines. The reduction of overall fecal concentrations of MCP-1 found among EPEC-infected children in our study may also result from the action of this toxin.

The overall reduction in fecal MCP-1 concentrations among vitamin A–supplemented children may be the mechanism underlying the association between supplementation and reductions in diarrheal disease. MCP-1 is involved in the inflammatory response for many types of pathogenic processes, and so its reduction may have led to the reduction of diarrheal disease. Although it is it is still unclear what aspects of innate immunity are elevated during gastrointestinal infections by A/E bacterium, or what role they play in intestinal host defense, other invasive pathogens induce a protective inflammatory response involving chemokines. For example, Vasquez-Torres et al. (42) showed that mice exhibiting delayed chemokine production were more susceptible to experimental Salmonella infection. As a result, the reduction in MCP-1 among vitamin A–supplemented children may reflect a general effect of vitamin A on the inflammatory response induced by a range of bacterial enteric pathogens.

The same limitations to the study that we addressed in our previous study are relevant here. The censoring that exists in the data as a result of the analysis of samples collected only during the summer months may be introducing a bias in our findings.
However, the possibility of bias may be limited because of the distinct etiologies of summer diarrheas in Mexico that makes this period a distinct and definable analytical unit. This seasonality of diarrheal pathogens may limit the external validity of our findings because it is not clear whether the effect of vitamin A on MCP-1 levels may change when different pathogens are more prevalent. The failure to consider whether the nondetection of cytokines in the stool is due to diverse sets of factors such as degradation, insensitivity of the assays, and low productivity may be an additional limitation of the study. The identical treatment of samples during collection, processing, storage, and analysis between the vitamin A and placebo groups would suggest that such systematic bias is minimized.

The reduction in MCP-1 levels seen among vitamin A supplementation, if confirmed, may have important implications on the development of new public health interventions for the control of diarrheal disease and the evaluation of children’s health status. Supplementation could be used as a prophylactic measure for effectively treating the inflammatory response induced by diarrheal pathogens and other gastrointestinal inflammatory diseases. More importantly, fecal levels of MCP-1 in children could be used as a noninvasive marker for determining the vitamin A status of children in developing countries to evaluate the effectiveness of public interventions concerned with reducing vitamin A deficiencies. There is currently no simple marker that adequately reflects children's vitamin A status.

**Literature Cited**