Vitamin A supplementation modifies the association between mucosal innate and adaptive immune responses and resolution of enteric pathogen infections[1–3]

Kurt Z Long, José Ignacio Santos, Jorge L Rosado, Teresa Estrada-Garcia, Meredith Haas, Abdullah Al Mamun, Herbert L DuPont, and Nanda N Nanthakumar

ABSTRACT

Background: The efficacy of vitamin A supplementation on diarrheal disease morbidity may reflect the divergent effects that supplementation has on pathogen-specific immune responses and pathogen-specific outcomes.

Objective: We examined how vitamin A supplementation modified associations between gut-cytokine immune responses and the resolution of different diarrheal pathogen infections.

Design: Stools collected from 127 Mexican children who were 5–15 mo old and enrolled in a randomized, placebo-controlled vitamin A supplementation trial were screened for enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and *Giardia lamblia*. Fecal concentrations of interleukin (IL)-6, IL-8, IL-4, IL-5, IL-10, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured by using an enzyme-linked immunosorbent assay. Hazard models that incorporated categorized cytokine variables (ie, nondetectable, less than the median of detectable concentrations, and at least the median of detectable concentrations) were fit to the length of pathogen infections stratified by treatment group.

Results: Vitamin A–supplemented children with fecal MCP-1 or IL-8 concentrations less than the median of detectable concentrations and IL-10 concentrations of at least median concentrations had longer durations of EPEC infection than did children in the placebo group. In supplemented children, detectable fecal TNF-α or IL-6 concentrations were associated with shorter ETEC infection durations, whereas MCP-1 concentrations of at least the median were associated with longer infection durations. Children in this group who had IL-4, IL-5, or IFN-γ concentrations of at least median detectable concentrations had shorter durations of *G. lamblia* infection.

Conclusion: The effect of supplementation on associations between fecal cytokine concentrations and pathogen infection resolution depends on the role of inflammatory immune responses in resolving specific pathogen infections. *Am J Clin Nutr* 2011;93:578–85.

INTRODUCTION

Vitamin A–supplementation clinical trials have reported that supplementation is associated with significant reductions in infant mortality (1–3). However, supplementation does not consistently reduce the overall incidence and prevalence of diarrheal disease (3–7). These inconsistent effects may result from the treatment of diarrheal disease as a single disease outcome although it is caused by an extensive and diverse group of pathogens (8, 9). Each of these pathogens induces a unique immune response that can lead to the resolution of the infection or lead to pathogenesis if the response is inadequate or inappropriate. Vitamin A may partly determine the appropriateness of generated responses because it differentially regulates the innate and adaptive immune responses (10, 11). Therefore, the efficacy of supplementation in specific communities may depend on how appropriate the vitamin A–regulated responses are for the array of pathogens prevalent in those communities. The simultaneous misclassification of diarrheal disease as an outcome and the failure to consider the pathogen-specific effect of vitamin A may bias findings of associations between vitamin A supplementation and diarrheal disease and thus be responsible for previously reported inconsistent effects.

We have addressed these issues by carrying out a randomized, placebo-controlled, double-blind trial concerned with the efficacy of vitamin A supplementation on specific pathogen outcomes in children living in peri-urban areas of Mexico City (12, 13). Supplementation in this trial was shown to be associated with distinct pathogen-specific innate and adaptive cytokine responses in stools and with divergent pathogen-specific clinical outcomes. We subsequently addressed the hypothesis that the distinct

[1] From the Nutrition, Environmental Health, Disease and Injury Control Unit, School of Population Health, University of Queensland, Queensland, Australia (KZL and AAM); the Infectious Diseases, Microbiology and Clinical Immunology Unit, Department of Experimental Medicine, School of Medicine, National Autonomous University of Mexico, Mexico City, Mexico (JIS); the Department of Nutrition, Universidad Autónoma de Querétaro, Querétaro, Mexico (JLR); the Department of Molecular Biomedicine, CINVESTAV-IPN, Mexico City, Mexico (TE-G); DePauw University, Greencastle, IN (MH); the University of Texas School of Public Health and Medical School, Houston, TX (HLD); St Luke's Episcopal Hospital, Houston, TX (HLD); the Baylor College of Medicine, Houston, TX (HLD); and Harvard Medical School, Boston, MA (NNN).

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[3] Address correspondence to KZ Long, School of Population Health. University of Queensland, Herston Road, Herston, Queensland 4006, Australia. E-mail: k.long@uq.edu.au.

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pathogen-specific outcomes associated with supplementation resulted from the differential regulation of the gut-cytokine immune responses by vitamin A. To do so, we first examined the role of specific cytokine responses in resolving infections by enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and *Giardia lamblia*, which are 3 pathogens that produce pathology through quite different mechanisms and induce very different cytokine profiles. Differences in proinflammatory and regulatory cytokine concentrations were shown to be associated with contrasting differences in the resolution of inflammatory and noninflammatory pathogen infections in children followed during the summer months (14). These results confirmed that the unique immune-response pathways induced by each of the pathogens in young children could lead to the resolution of infection if adequate or prolong infection if inadequate.

In the current study, we further addressed our hypothesis by comparing associations between cytokine concentrations and the resolution of infection in children in vitamin A and placebo groups for these 3 pathogens during the same 3-mo summer period. This comparison allowed us to confirm that the distinct pathogen-specific results in supplemented children resulted from the differential regulation of these pathogen-specific pathways by vitamin A.

SUBJECTS AND METHODS

Study population

Community health workers conducted a census of all children <2 y of age living in 9 neighborhoods (colonias) within the community of La Magdalena Atlicpac, which is located along the eastern perimeter of Mexico City. Mothers of all children from 5 to 15 mo of age were invited to participate in the study as previously described (12, 13). We targeted this population so that we could determine the effect of vitamin A supplementation on the children's developing immune responses during and immediately after weaning. Children were excluded if they had diseases causing immunosuppression or any congenital or acquired alteration of the digestive tract, such as chronic diarrhea, that could alter the absorption of micronutrients. Children who were already taking vitamin supplements were also excluded. Overall, 200 children living in this community were identified and enrolled over a 10-mo period after their parents consented to their participation in the study

Study design

Once enrolled, each child was randomly assigned to receive vitamin A or a placebo by using a randomized sequence generated from a random-number table by an epidemiologist at the National Institute of Public Health. Children <12 mo of age who were assigned to the vitamin A group were administered a solution that contained 20,000 international units (IU) of retinol [3.3 IU retinol = 1 retinol activity equivalent (in μg)] at baseline and subsequently every 2 mo until the end of the study, whereas children ≥ 12 mo of age received a solution that contained 45,000 IU retinol. This design allowed the evaluation of the effect of frequent low doses of vitamin A on the pathogen-specific immune response and diarrheal disease. The testing and preparation of the placebo and vitamin A solutions were carried out by personnel at the National Institute of Nutrition to ensure that the solutions were similar in taste, viscosity, and color and to ensure that the field personnel and principal investigator were unaware of the regimens. The field team was in charge of administering the supplements and placebos to children from identical opaque, plastic droplet bottles.

During the baseline visit, information was collected regarding the number of household members, their ages, education levels, and incomes, household construction materials, the source and quality of household water, the type of household sanitation facilities, and household possessions. Information was also collected during the first visit to determine the child's feeding and weaning patterns as well as morbidity experience in the previous 2 wk. A previously validated questionnaire was used in the collection of these data by project personnel who had received training in its application.

Recruited children were followed prospectively for ≤ 15 mo during which time households were visited twice a week. At each visit, mothers or child caretakers were interviewed to determine the presence of the following symptoms: diarrhea, the number and consistency of evacuations, the presence of blood and mucus in stools, fever, cough, and respiratory difficulty. A stool sample was collected twice a month in healthy children and ≤ 3 stools were collected in the week after a diarrheal episode. Project supervisors accompanied $\approx 5\%$ of all household visits to ensure the quality of data collection. Children were referred to the study physician for diagnosis and treatment when the fieldworker or caregiver was concerned about the child's health status.

Laboratory methods

All stool samples were plated onto Salmonella Shigella, MacConkey, and MacConkey-tellurite agars for the identification of *Salmonellae* spp. *Shigella* and *E. coli* (15). The Kato-Katz technique and trichrome staining of wet mounts of concentrated stools were carried out to identify *Ascaris lumbricoides*, *Entamoeba histolytica*, and *G. lamblia* ova in stools (16). Five lactose-fermenting colonies with morphologies that resembled that of *E. coli* (when present) were selected from MacConkey agar plates and speciated biochemically. Diarrheagenic *E. coli* were characterized by a single multiplex technique as previously described (17) that detected the following pathogenic genes: heat-stable and heat-labile enterotoxins (*st*, *lt*) for ETEC, intimin (*eaeA*), and bundle-forming pilus (*bfp*) for EPEC, Shiga toxin 1 and 2 (*stx*1, *stx*2) and intimin (*eae*A) for Shiga-toxin producing *E. coli* (STEC), and invasive-associated loci (*ia*1) for enteroinvasive *E. coli* (EIEC).

An aliquot from each fresh stool collected from children was frozen ≤ 4 h after collection at -20°C. For the analysis of fecal cytokines, we selected stools collected from a subsample of children during the summer months of June, July, August, and early September when diarrheal rates reach their peak and when diarrheal *E. coli* were the most prevalent. Samples were extracted as previously described (13) by homogenization and centrifugation (15 min at 10,000 $\times$ *g*) at 48°C with phosphate-buffered saline that contained protease inhibitors (benzenesulfonyl, pepstatinA, leupetin, and aprotinin; 1:10 wt:vol; Sigma, St Louis, MO). The resulting supernatant fluids were

collected, frozen, and stored at −70°C. These supernatant fluids were assayed for chemokines monocyte chemoattractant protein 1 (MCP-1) and interleukin (IL)-8, proinflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-6, the T helper (Th) 1 cytokine interferon-γ (IFN-γ), Th2 cytokines IL-4 and IL-5, and the T regulatory cytokine (Treg) IL-10 with an enzyme-linked immunosorbent assay by using paired enzyme-linked immunosorbent assay specific capture and biotinylated detecting antibody (Pierce-Endogen, Rockford, IL and R&D Systems, Minneapolis, MN). Peroxidases conjugated to stepavidin (Pierce-Endogen, Rockford, IL) were used to detect the capture antibody, and peroxidase activity was measured by using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) substrate and read at a wavelength of 405 nm. Recombinant cytokines and chemokines were used to generate a standard curve, and concentrations of these cytokines and chemokines from the stool extracts were measured by using the standard curve in 96-well plates according to the manufacturer's protocol. The unit for all cytokine assays was picograms per milliliter normalized to milligrams per milliliter of protein per stool. The detection limit for these cytokine assays was 10 pg/mL.

Outcome definitions

The primary endpoints for this analysis were the durations of infections by diarrheagenic *E. coli* and *G. lamblia* in children. An infection by *G. lamblia* was defined as a stool that was positive for the parasite. Infection by any of the specific diarrheagenic *E. coli* was defined as any lactose-positive bacteria isolated from stool that had pathogenic genes for the respective pathogens. The beginning of each pathogen infection was defined as the midpoint in time between a stool that was negative for that pathogen and a subsequent positive stool. The end of the episode was defined as the midpoint between the last sequentially positive stool and the subsequent negative stool. Durations of pathogen infections were defined as encompassing the time between these 2 midpoints.

Statistical analyses

Results from the screening of stools collected from children followed during the summer months were analyzed in 2 stages. In a previous article, we first fit Weibull's parametric regression survival-time models that incorporated categorized chemokine and cytokine variables (ie, nondetectable, less than the median of detectable concentrations for each chemokine or cytokine, and at least the median of detectable chemokine or cytokine concentrations) to durations of pathogen infections to test how differences in these concentrations were associated with infection duration (14). For the current article, we compared associations between pathogen durations and chemokine and cytokine concentrations in children in vitamin A and placebo groups to test how vitamin A modified these associations. As such, separate models were also run within vitamin A and placebo groups to determine how the effects of these cytokines were modified within these different strata by vitamin A. The mean pathogen infection duration within these categories were also separately estimated from the data. A variable for the presence or absence of diarrheal symptoms associated with infections was included in each pathogen-duration analysis to control for the modifying effect of the onset of symptoms. Statistical significance was set at $P < 0.05$ and < 0.1 for interactions. Analyses were carried out by using the STREG procedure from STATA software (version 9.0; StataCorp, College Station, TX).

For the overall study, it was calculated that a sample size of 100 samples per group was required to detect a 20% reduction between control and treatment groups with a power of 80% and a 95% significance level and an expected loss to follow-up of 20 children (18). The current analysis used a subsample of children from a 3-mo summer period of the study when diarrheal rates were highest. The sample size needed for this subanalysis was calculated to be 60 samples per group on the basis of the formula developed by Hsieh and Lavori (19) for hazards analysis and by using our previous findings of the effect of vitamin A on the length of pathogen infections (12). The study was approved by the ethical review committees from the National Centre for the Health of Infants and Adolescences of Mexico and the Harvard School of Public Health.

RESULTS

A sample of 127 children followed during the months of June, July, and August were included in the study (**Figure 1**). A total of

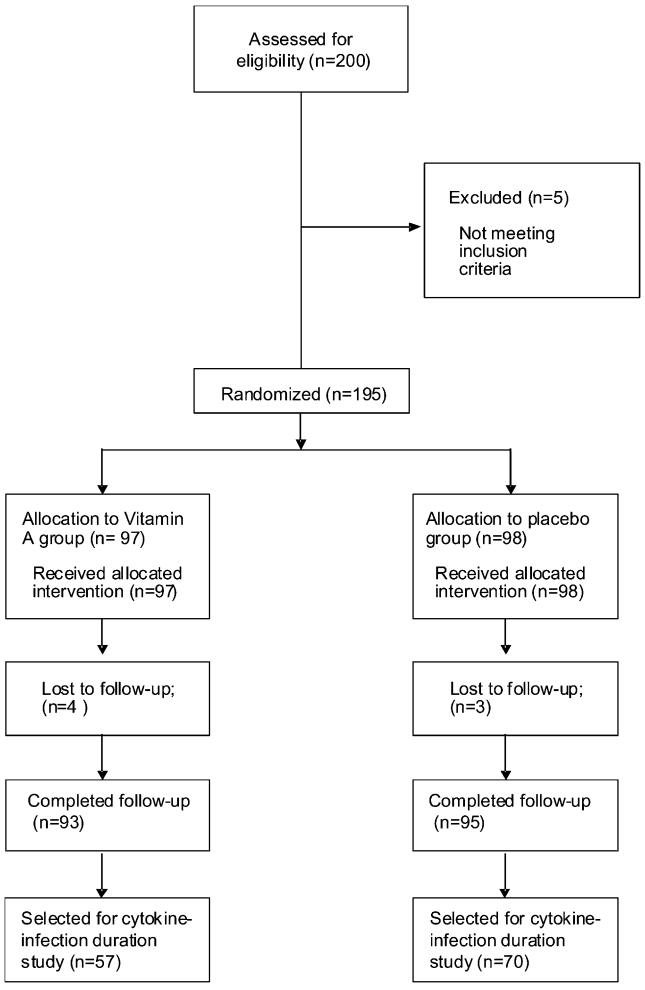

FIGURE 1. Trial profile of children selected for the substudy who were enrolled during the months of June, July, and August when diarrheal rates were highest.

554 stool samples were collected from these 127 children (291 samples from 70 children who were administered the placebo and 263 samples from 57 children who were administered the vitamin A supplement). The distributions of sociodemographic characteristics of study children and households from this study period, which previously were described (13), did not differ between children who were administered vitamin A and children in the placebo group.

A total of 149 pathogens were identified in stools collected during the 3-mo period of this analysis that represented 131 separate episodes of infection. ETEC infections were the most frequent (50 episodes) with 28 and 22 episodes identified in children in the placebo group and vitamin A groups, respectively, followed by EPEC infections (42 episodes) with 25 and 17 episodes identified in the same respective groups. Thirty *Giardia* episodes were identified, and 18 of these episodes occurred in the placebo group and 12 episodes occurred in the vitamin A group. These episodes included in the subgroup analyses were all single episodes from different subjects. The remaining infections were due to STEC, EIEC, *A. lumbricoides*, and *E. histolytica.* Approximately 11 of 50 ETEC infections (22%) were associated with a diarrheal episode, whereas 23 of 47 EPEC infections (49%) were associated with an episode.

There were significant pathogen-specific differences in associations between infection durations and fecal cytokine and chemokine concentrations when stratified by treatment arm. Vitamin A–supplemented children with MCP-1 or IL-8 fecal concentrations less than median detectable concentrations for each of these chemokines or IL-10 concentrations of at least median detectable concentrations of this cytokine had either marginally or fully significant lower risks of an EPEC infection ending (P for interaction = 0.06, 0.02, and 0.05, respectively; **Table 1**). As a result, these children had longer infection durations than did children in the placebo group. Vitamin A–supplemented children with IFN-γ concentrations less than median detectable IFN-γ concentrations had a higher risk of an EPEC infection episode ending and, thus, had shorter infection lengths than did children in the placebo group (P for interaction = 0.04; Table 1).

Important associations were shown between durations of ETEC infection and innate cytokines and chemokine responses that were different in direction from those shown for EPEC. Children in the placebo group with TNF-α or IL-6 concentrations of at least the median of detectable concentrations had lower risks of an ETEC infection ending and so had longer lengths of infection than did children in this group with no detectable concentrations. This association was reversed in vitamin A–supplemented children in whom fecal TNF-α concentrations less than median detectable concentrations were associated with higher risks of an ETEC infection ending. As a result, these children had a marginally significant higher risk of an ETEC infection ending than did children in the placebo group (P for interaction = 0.08). This risk of an ETEC infection in supplemented children with IL-6 concentrations of at least median detectable concentrations was significantly higher than the risk of an ETEC infection for children in the placebo group (P for interaction $\leq$ 0.01; **Table 2**). As a result, these supplemented children had shorter infection lengths than did children in the placebo group. In contrast, supplemented children with fecal MCP-1 concentrations less than median detectable fecal MCP-1

TABLE 1
Associations between fecal concentrations of innate immune-response cytokines and chemokines and risk of enteropathogenic *Escherichia coli* infection ending by treatment arm[1]

	Placebo group				Vitamin A group				
Cytokine	No. of infections	Infection duration	Hazard ratio (95% CI)	P	No. of infections	Infection duration	Hazard ratio (95% CI)	P	P for interaction
		d				*d*			
MCP-1									
ND	5	17.00 ± 2.48[2]	1	—	8	10.92 ± 4.68	1	—	—
Less than the median	6	12.49 ± 1.60	2.75 (0.65, 11.56)	0.16	6	17.45 ± 4.63	0.77 (0.26,2.21)	0.63	0.06
At least the median	4	22.77 ± 2.24	0.86 (0.23, 3.25)	0.83	1	10.92 ± 4.06	—	—	—
IL-8									
ND	8	18.90 ± 4.0	1	—	9	21.9 ± 5.9	1	—	—
Less than the median	5	12.10 ± 2.67	2.57 (0.66, 10.00)	0.17	4	37.20 ± 10.38	0.37 (0.10, 1.31)	0.12	0.02
At least the median	9	16.43 ± 3.62	1.51 (0.47, 4.87)	0.48	3	24.72 ± 6.89	0.80 (0.18, 3.45)	0.75	0.93
IFN-γ									
ND	10	12.91 ± 3.4	1	—	13	22.05 ± 4.75	1	—	—
Less than the median	5	31.15 ± 5.02	0.40 (0.09, 1.67)	0.21	3	10.25 ± 3.7	1.53 (0.35, 6.63)	0.56	0.04
At least the median	5	17.96 ± 4.95	0.51 (0.16, 1.60)	0.25	2	38.94 ± 5.66	0.07 (0.00, 0.67)	0.02	0.09
IL-10									
ND	11	18.86 ± 4.0	1	—	8	14.41 ± 5.99	1	—	—
Less than the median	2	15.19 ± 4.0	0.90 (0.15, 5.35)	0.9	4	25.97 ± 6.11	0.60 (0.18, 1.96)	0.4	0.22
At least the median	5	23.92 ± 4.96	0.97 (0.30, 3.09)	0.95	4	45.5 ± 13.82	0.18 (0.04, 080)	0.02	0.05

[1] MCP-1, monocyte chemoattractant protein 1; ND, nondetectable; IL, interleukin; IFN-γ, interferon-γ. Risk of infection ending, infection duration, and P values were calculated after fitting Weibull's hazard model to the length of infection duration, which was defined as the time from the midpoint between a negative stool sample and a subsequent positive stool sample and between the last sequentially positive stool sample and subsequent negative stool sample. Reference groups were stool samples with no detectable concentrations of cytokine or chemokine; the presence or absence of diarrheal symptoms was included in models.

[2] Mean ± SD (all such values).

TABLE 2
Associations between fecal concentrations of innate immune-response cytokines and chemokines and risk of enterotoxigenic *Escherichia coli* infection ending by treatment arm[1]

	Placebo group				Vitamin A group				
Cytokine	No. of infections	Infection duration	Hazard ratio (95% CI)	*P* value	No. of infections	Infection duration	Hazard ratio (95% CI)	*P*	*P* for interaction
		d				*d*			
MCP-1									
ND	5	22.82 ± 0.5[2]	1	—	4	18.01 ± 2.02	1	—	—
Less than the median	8	15.94 ± 0.36	2.20 (0.70, 6.90)	0.18	4	24.43 ± 2.85	0.47 (0.12, 1.88)	0.29	0.07
At least the median	5	21.21 ± 0.41	1.17 (0.33, 4.09)	0.8	7	17.71 ± 1.77	1.04 (0.28, 3.85)	0.95	0.8
TNF-α									
ND	13	14.46 ± 0.32	1	—	10	16.58 ± 1.44	1	—	—
Less than the median	2	24.91 ± 0.49	0.29 (0.09, 0.90)	0.03	3	30.7 ± 2.51	0.20 (0.03, 0.99)	0.05	0.7
At least the median	7	26.61 ± 0.67	0.24 (0.08, 0.74)	0.1	3	17.84 ± 1.87	0.82 (0.21, 3.13)	0.77	0.08
IL-6									
ND	10	14.30 ± 0.19	1	—	8	20.94 ± 0.28	1	—	—
Less than the median	5	21.36 ± 0.53	0.36 (0.08, 1.64)	0.18	2	15.70 ± 0.41	2.0 (0.42, 9.44)	0.38	0.08
At least the median	4	26.70 ± 0.54	0.23 (0.08, 0.64)	<0.01	6	13.5 ± 0.3	2.89 (0.66, 12.65)	0.15	<0.01

[1] MCP-1, monocyte chemoattractant protein 1; ND, nondetectable; TNF-α, tumor necrosis factor-α; IL, interleukin. Risk of infection ending, infection duration, and *P* values were calculated after fitting Weibull's hazard model to the length of infection duration, which was defined as time from the midpoint between a negative stool sample and a subsequent positive stool sample and between the last sequentially positive stool sample and subsequent negative stool sample. Reference groups were stool samples with no detectable concentrations of cytokine or chemokine; the presence or absence of diarrheal symptoms was included in models.

[2] Mean ± SD (all such values).

concentrations had marginally significantly lower risks of an ETEC infection episode ending and, thus, had longer ETEC infection lengths than did children in the placebo group (*P* for interaction = 0.07; Table 2).

Very different patterns of associations were shown between cytokine concentrations and durations of *G. lamblia* infections. Children in the placebo group with any detectable IL-5 concentrations in stools or concentrations of IL-4 of at least median detectable concentrations had significantly lower risks of episodes ending and, thus, had longer lengths of *G. lamblia* infections than did children in this group with no detectable concentrations (**Table 3**). In contrast, vitamin A–supplemented children with fecal IL-4 concentrations of at least median detectable concentrations or any detectable fecal IL-5 concentrations had significantly shorter lengths of *G. lamblia* infection than did children in the placebo group with these same concentrations. In addition, vitamin A–supplemented children with fecal IFN-γ concentrations of at least median detectable

TABLE 3
Associations between fecal concentrations of adaptive immune-response cytokines and risk of *Giardia lamblia* infection ending by treatment arm[1]

	Placebo group				Vitamin A group				
Cytokine	No. of infections	Infection duration	Hazard ratio (95% CI)	*P*	No. of infections	Infection duration	Hazard ratio (95% CI)	*P*	*P* for interaction
		d				*d*			
IL-4									
ND	6	13.16 ± 1.16[2]	1	—	6	18.80 ± 4.24	1	—	—
Less than the median	5	23.51 ± 4.24	0.30 (0.07, 1.25)	0.1	3	15.95 ± 4.24	1.43 (0.34, 5.98)	0.62	<0.01
At least the median	6	36.80 ± 3.24	0.12 (0.24. 0.58)	<0.01	3	12.3 ± 3.82	2.64 (0.61, 11.32)	0.19	<0.01
IL-5									
ND	10	17.36 ± 21.79	1	—	8	15.56 ± 5.79	1	—	—
Less than the median	3	56.73 ± 12.30	0.02 (0.003, 1.18)	<0.01	2	19.44 ± 13.30	0.94 (0.18, 4.91)	0.95	<0.01
At least the median	3	44.90 ± 14.95	0.05 (0.01, 0.31)	<0.01	6	12.12 ± 7.46	1.94 (0.33, 11.41)	0.46	<0.01
IFN-γ									
ND	10	16.23 ± 5.0	1	—	8	19.8 ± 4.24	1	—	—
Less than the median	5	12.37 ± 3.0	0.60 (0.14, 2.40)	0.47	2	13.42 ± 0.86	0.19 (0.03, 1.21)	0.08	0.21
At least the median	4	27.16 ± 1.07	0.02 (0.004, 0.10)	0.65	6	16.8 ± 3.41	1.77 (0.19, 15.84)	0.6	<0.01

[1] IL, interleukin; ND, nondetectable; IFN-γ, interferon-γ. Risk of infection ending, infection duration, and *P* values were calculated after fitting Weibull's hazard model to the length of infection duration, which was defined as the time from the midpoint between a negative stool sample and a subsequent positive stool sample and between the last sequentially positive stool sample and subsequent negative stool sample. Reference groups were stool samples with no detectable concentrations of cytokine or chemokine; the presence or absence of diarrheal symptoms was included in models.

[2] Mean ± SD (all such values).

concentrations had significantly shorter infection lengths than did children in the placebo group.

DISCUSSION

We have shown that vitamin A supplementation differentially modified associations between gut chemokine and cytokine responses and durations of infections by EPEC, ETEC, and *G. lamblia*. The presence of fecal proinflammatory and regulatory cytokines in supplemented children was shown to be associated with a prolongation of EPEC infections but a more rapid resolution of ETEC infections. For *G. lamblia* infections, adaptive cytokines were strongly associated with a more rapid resolution of infection in vitamin A–supplemented children. These findings suggested that the effect of vitamin A supplementation on these associations depended on the role of proinflammatory or noninflammatory immune responses in the resolution of specific pathogen infections and whether vitamin A up-regulated or down-regulated these responses.

Proinflammatory chemokines and cytokines associated with EPEC infection durations in vitamin A–supplemented children in our study have previously been shown to be elevated after infections by this pathogen and may play a role in its resolution (14). For example, IL-8 is one of the primary chemokines induced as part of the inflammatory response during EPEC infections (20, 21). MCP-1 and IFN-γ responses are also up-regulated by epithelial cells in the gastrointestinal tract after infections in the gut by *Citrobacter rodentium*, which is a mouse-adapted bacterium related to EPEC and may be important in its control (22–24). In contrast, greater IL-10 concentrations have been associated with increased durations of asymptomatic EPEC infections but decreased durations of symptomatic infections (14).

The associations between reduced risks of an EPEC infection ending and concentrations of inflammatory and anti-inflammatory cytokine concentrations in vitamin A–supplemented children may reflect the down-regulation of inflammatory responses and up-regulation of antiinflammatory responses. For example, Dong et al (25) reported that that *all-trans* retinoic acid promoted the increased gene expression of the antiinflammatory regulatory cytokine IL-10 in cultured Peyers patches. Vitamin A supplementation was also associated with reduced concentrations of the proinflammatory cytokine MCP-1 overall and after EPEC infection in animal models and clinical trials (13, 26). This down-regulation may underlie the marginal association we observed between lower MCP-1 concentrations and reduced EPEC infection resolution in vitamin A–supplemented children. In contrast, all-*trans* retinoic acid has been reported to increase the production of the inflammatory chemokine IL-8 in cultured normal human keratinocytes and skin (27, 28).

The positive association shown between greater TNF-α or greater IL-6 fecal concentrations and ETEC infection durations in children in the placebo group suggested that these components of inflammatory responses were inadequate for the resolution of ETEC infections. In vitro and in vivo studies have reported that IL-6 concentrations were significantly increased after ETEC infections (29–31). IL-6, in conjunction with TGF-β, plays an important role in the induction of Th17 cells that clear extracellular infections but also promote tissue inflammation and damage (32–34). The heat-labile enterotoxin from ETEC is associated with enhanced concentrations of IL-6 and a suppressed Th1 response, which creates an environment where Th-17 responses are dominant (35). The results of our study indirectly suggested that the induction of this pathway may lead to a reduced clearance of ETEC.

In supplemented children, increased IL-6 concentrations were associated with reduced ETEC infection durations, which was a result that may partly explain our previous findings that vitamin A supplemented children had significantly reduced durations of ETEC infections than did children in the placebo group (12). IL-6, along with TGF-β, is essential for the generation of a strong Th2 regulated secretory immunoglobulin A response in the gut, which plays an important role in ETEC resolution (36). Vitamin A may enhance this effect because of its effect on the Th2 response. In vitro and in vivo studies have shown that retinoic acid plays a key role in inhibiting the IL-6– and TGF-β–derived induction of proinflammatory Th17 cells in the gastrointestinal tract that results in the promotion of antiinflammatory Treg cells (37, 38). Also, Ahmed et al (39) recently reported that liver vitamin A stores in Bangladeshi men were negatively associated with serum IL-6 and -17 concentrations. We hypothesized that the associations shown in our study between IL-6 and TNF-α concentrations and the increased resolution of ETEC infections in supplemented children may have resulted from the down-regulation of the proinflammatory response and up-regulation of the Treg regulatory response by vitamin A.

The marginally significant reduction in ETEC durations in supplemented children with increased concentrations of MCP-1 may be related to findings that ETEC infections enhanced the expression of MCP-1 (31). We have shown that the MCP-1 response was down-regulated in vitamin A–supplemented children. Similarly, a number of studies have reported a reduced MCP-1 production in *all-trans*-retinoic acid–treated mice with collagen-induced arthritis (40, 41). More research is needed to clarify how MCP-1 expression relates to ETEC resolution and how vitamin A may modify this association.

Increased *G. lamblia* infection durations observed in children with either increased fecal IL-4 or IL-5 concentrations are not consistent with reports that these cytokines were elevated during the acute and elimination phases of infection (42). Overall, intestinal immunoglobulin A antibodies that are up-regulated by Th2 cytokines are important in eradicating *G. muris* or *G. lamblia* infection (43). However, a strong Th1 response is also important in reducing *G. lambia* intestinal trophozoites and fecal cyst counts (44, 45). The associations between greater fecal concentrations of these cytokines and shorter infection lengths in supplemented children suggested that Th1 and Th2 cytokines are important for resolving *G. lamblia* infections (10, 46).

These associations may not represent the direct effect of vitamin A on the gut-cytokine immune responses and infection resolution because vitamin A may have produced these effects through other mechanisms. However, we are confident that the reported results represented a more direct causal relation because the use of parametric hazard parametric regression models in this analysis was able to directly link the effects of vitamin A on these cytokine responses with differences in the length of pathogen-specific infections. Also, the vitamin A status of children was not determined at baseline, and thus, it was not possible to address whether deficient children may have benefited more from supplementation than did nondeficient children. However, it was shown that overt vitamin A deficiency and subclinical deficiency in children living in Mexico City was minimal (47).

In conclusion, there is need to further clarify how vitamin A modifies the different immune-response components that play a role in outcomes associated with infections by EPEC, ETEC, and *G. lamblia*. If these findings can be confirmed and extended to include other pathogens, they may explain why vitamin A has not been shown to have a consistent effect on diarrheal disease in previous studies. The contrasting pathogen-specific effects of vitamin A supplementation may produce diverse effects in different community settings where there is a variation in the prevalence of different types of diarrheal pathogens. This complexity may explain the inconsistent effects of vitamin A on different health outcomes reported in previous studies that have contributed to a lack of consensus regarding the effectiveness of vitamin A supplementation (5, 7, 48, 49). These results have important implications for the development of more cost-effective supplementation programs because they suggested that communities that differ in indicators of pathogen prevalence and diarrheal disease burden should be targeted for distinct supplementation interventions.

The authors' responsibilities were as follows—KZL, JIS, and JLR: were involved in the design of the project, procurement of funds, and project supervision; KZL and AAM: were involved in data analyses; TE-G: was responsible for the characterization of bacterial isolates; NNN: developed the assays for measurement of fecal cytokine concentrations and participated in the interpretation results; MH: contributed to the measurement of fecal cytokine concentrations; KZL: was responsible for the preparation of the manuscript, had full access to all study data, and had final responsibility for the decision to submit the manuscript for publication; and JIS, JLR, and HLD: provided input for the preparation of the article. None of the authors had a conflict of interest.

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