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Effect of Malnutrition in Ecuadorian Children on Titers of Serum Antibodies to Various Microbial Antigens

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The titers of serum antibodies to natural infection with enteric and respiratory pathogens, to a food antigen and to tetanus and diphtheria toxoid were evaluated by enzyme-linked immunosorbent assay in 1,554 Ecuadorian children younger than 5 years of age. The nutritional status of the children was assessed by anthropometry and measurement of biochemical status indicators. The children were enrolled in a representative national nutrition and health survey. Antibody titers were analyzed as a function of the nutritional status of the children. For 12 of 14 antibody concentrations tested, underweight children showed lower antibody titers than did control children. The difference was statistically significant for antibody to both T-cell-dependent antigens (tetanus toxoid, rotavirus, respiratory syncytial virus) and T-cell-independent antigens (lipopolysaccharide, polyribosyl-ribitol phosphate, capsular polysaccharide). When children with a recent episode of diarrhea were excluded, many of the differences remained significant. When these children were further classified by age, only difference in titers of antibodies to respiratory syncytial virus and tetanus toxoid remained significant. No statistically significant difference was detected between underweight and control children with respect to protective antibody levels to four bacterial antigens. Anemic children showed significantly lower antibody levels to both T-cell-dependent and T-cell-independent antigens than did control children, and a higher proportion of anemic children had diphtheria antitoxin below a conservatively defined protective antibody level. No major differences in antibody titers were seen between children with different retinol and zinc concentrations in serum.

The host response to respiratory and enteric infections, in addition to being affected by environmental and socioeconomic factors, can be altered by protein energy malnutrition and single-nutrient deficits such as vitamin A or iron deficiency. Increased susceptibility to, or severity of, respiratory and enteric disease in children in developing countries may be due to effects of malnutrition on immune function (23). In fact, it has been proposed that infants and children in the developing world constitute the largest population with an acquired immunodeficiency syndrome attributed to the consequences of malnutrition on the immune system (23). Consistent findings in protein energy malnutrition were an impairment of cell-mediated immune responses, a functional defect in the activity of the complement system, a decrease in the amount of secretory immunoglobulin A (IgA) antibody present in secretions, and elevated levels of IgE. A fifth finding of potential relevance to the host response to respiratory and enteric pathogens is a diminished serum antibody response to polysaccharide antigens in protein energy malnutrition with a relatively well preserved response to many protein antigens. Therefore, killed typhoid vaccine does not induce a strong response to the O antigen in malnourished children (38), although tetanus toxoid induces good protective levels of antibody even in children with severe protein energy malnutrition (15). Therefore, it was suggested that antibody responses, especially to polysaccharide antigens, may be improved in malnourished children by providing a protein supplement around the time of the immunization (29). Similarly, provision of a single nutrient such as vitamin A has been proposed to increase antibody response to tetanus vaccination in children suffering from vitamin A deficiency (33). However, the logistical problems in providing supplements are formidable, and the amount of gain is still uncertain. In this study, we investigated the association between the nutritional status and serum antibody response to various microbial antigens in Ecuadorian infants and children. The children were enrolled into a national cross-sectional study on nutrition and health (16). The nutritional status of the children was evaluated by anthropometry and measurement of biochemical nutritional status indicators (16). The antibody concentrations in serum to T-cell-dependent and T-cell-independent microbial antigens were measured by enzyme-linked immunosorbent assay (ELISA). These antibodies reflected both natural exposure to pathogens and a food antigen, and immune response to vaccination with tetanus and diphtheria toxoid in the past (5, 7–11, 13). Low weight for age and the presence of anemia were associated with significantly lower antibody concentrations to both T-cell-dependent and T-cell-independent antigens.

MATERIALS AND METHODS

Study design. The study protocol and sampling procedures are described in detail elsewhere (16). Briefly, a two-stage stratified, probabilistic clustered sample was drawn to study the health and nutritional status of Ecuadorian children aged 0 to 59 months. From the sample of 8,100 children representative of the 1.2 million in that age group in 1986, a subsample of 1,620 was selected for the evaluation of specific nutrient deficiencies, by using biochemical status indicators, and for the evaluation of the antibody titers in serum. Of these children, 540 lived in two metropolitan areas (Quito and Guayaquil; population, 1.1 and 1.6 million, respectively), 540 lived in 14 predominantly urban regions (cantons), and 540 lived in 14 predominantly rural cantons. Ninety-six percent of the population was represented; the remote Galapagos Islands and the thinly inhabited Amazon Basin (<4% of the total population) were excluded for logistic reasons. Data and serum samples were obtained from 1,554 children. About 50 children represented each 2-month age interval of children between 0 and 59 months of age. No significant differences with respect to gender, geographic region, population density, and socioeconomic level were detected between the different age groups (data not shown). Prematurity was not asked about in the survey.

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The survey was conducted between March and November 1986. During this period, a team of medical doctors, nutritionists, and social workers travelled through the country equipped with basic laboratory facilities to visit the preselected census areas. Serum samples were collected at the time when the anthropometric measurements and the investigations of the place of living were done. Sera were collected by finger prick in infants and by venipuncture (antecubital vein) in children older than 6 months. The samples destined to be investigated in the Swiss laboratory (ELISA, zinc and retinol concentrations in serum) were kept frozen in the mobile laboratory and sent on dry ice to the Swiss laboratory. Hemoglobin concentrations were determined in the mobile laboratory in Ecuador. During the medical examination, parents were asked whether diarrhea had occurred in the 2 weeks preceding data and sample collection. A diphtheria-tetanus vaccination history was obtained from the vaccination cards. Ecuadorian infants were vaccinated at 2, 4, and 6 months of age on average. The proportion of infants given the first two doses was high, >82%; this high coverage was confirmed in a seroepidemiological survey (8). The relationship between the time of immunization and the serum sampling is thus approximately given by the time interval: chronological age in month minus 6 months (time of last diphtheria-pertussis-tetanus vaccination).

Anthropometry. The children were weighed to the nearest 100 g in underwear and without shoes, using Salter spring balances with a capacity of 25 kg. Height was measured to the nearest millimeter in infants and children less than 2 years by using wooden infantometers. Height-for-age (HA) and weight-for-age (WA) *Z* scores were calculated in comparison with the World Health Organization reference values. *Z* scores are formed by subtracting a measured value from the mean of the reference for the appropriate age and dividing the result by the standard deviation. The resulting *Z* score is not the diagnosis of malnutrition but denotes the probability that the child is not part of the reference population. For a *Z* score of −2, this probability is 0.023.

Nutritional status indicators. The hemoglobin concentration in whole blood was determined by the cyanmethemoglobin method, using kits from Boehringer, Mannheim, Germany. Hemoglobin values were corrected for altitude (17). Anemia was defined by a sea-level-corrected hemoglobin concentration below 110 g/liter as recommended by the World Health Organization (18, 27). Zinc concentrations in serum were determined by atomic absorption after protein precipitation with trichloroacetic acid (17). Zinc levels below 65 μg/dl in serum were considered an indication of a poor zinc status (27). Serum samples for zinc measurement were taken in tubes for oligoelement determination (Sarstet, Sevelen, Switzerland). The tubes were tested with distilled water and acidified water for zinc extraction and found to be negative. Retinol concentrations in serum were determined by high-performance liquid chromatography with fluorometric detection (16). Vitamin A levels in serum of below 200 μg/liter were considered low (18, 27).

Antibody titers in serum. Titers of IgG and IgM antibodies against a range of microbial antigens were measured by ELISA. The assays were done as described previously (12), and all results have been published previously as seroprevalence studies (5, 7–11, 13). Serum samples to be tested were diluted in phosphate-buffered saline-Tween 20 (0.05%) to a standard dilution of 1:100. All samples were tested in duplicate against the test antigen fixed on microtiter plates (Greiner, Nürtingen, Germany) and on plates coated with coating buffer alone or an appropriate control antigen (see below for antigens). A blank consisting of the diluent only was included on each test plate. Sera with known relative or absolute antibody titers were incorporated as standards in all assays. The absorbance obtained on the control plate was subtracted from that obtained on the test plate. The optical density readings were then expressed with respect to the standards to account for interplate differences in the assays. One larger serum sample was added as 50 individual serum samples to the collection, and the experimenter was unaware of their code numbers. Calculation of their mean and standard deviation confirmed the reproducibility of the assays.

Bound antibodies were revealed with affinity-purified antibody to human IgG and IgM coupled to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). Sigma 104 substrate was used as specified by the manufacturer. The reagents were standardized with respect to sensitivity and specificity for the different antibody classes by the method of Yolken et al. (42) with plates to which chromatographically purified IgG, IgM, or IgA (Cappel Laboratories, West Chester, Pa.) had been added at various dilutions. Conjugate dilutions were chosen so that microtiter plates coated with 10 ng of the specific Ig class per ml gave an absorbance of 0.1 optical density unit. We confirmed that at an optical density of <1.0, the absorbance of the standards was linearly proportional to the relative antibody concentration.

For rotavirus ELISA, CsCl gradient-purified simian rotavirus SA-11 grown on MA-104 cells was used on test plates and an identically purified antigen from mock-infected MA-104 was used on control plates. For respiratory syncytial (RS) virus ELISA, the purified Long strain of RS virus grown on Vero cells was used on test plates and coating buffer was used on control plates. For a food antigen ELISA, purified crystallized β-lactoglobulin (Sigma) was used on test plates and coating buffer was used on control plates. In these assays, sera with known relative antibody titers, obtained from adults in Germany, were used as standards (12).

In tetanus and diphtheria antitoxin ELISA, purified tetanus and diphtheria toxoids from a World Health Organization reference laboratory (Statens Seruminstitut, Copenhagen, Denmark) were used on test plates and coating buffer was used on control plates. A human serum sample with a known tetanus antitoxin titer was obtained from Institute Pasteur, Paris, France, and a human serum sample with a known diphtheria antitoxin titer was obtained from the Swiss Serum and Vaccine Institute, Bern, Switzerland; these were used as reference sera.

In *Escherichia coli* ELISA, pooled lipopolysaccharides from the 13 most common O serogroups identified in enterotoxigenic *E. coli* diarrhea, prepared as described previously (6), were used on test plates and core glycolipid of *E. coli* J5 (Sigma) was used on control plates. Purified heat-labile enterotoxin from *E. coli* was purchased from the Swiss Serum and Vaccine Institute and used on test plates. Heat-labile enterotoxin-specific ELISA was done as described by Levine et al. (26). Sera from American volunteers who were orally challenged with strain O78:H11 (39) served as reference sera.

In polyribosyl-ribitol phosphate (PRP)-specific ELISA, purified PRP antigen obtained from the Swiss Serum and Vaccine Institute was used on test plates. In capsular polysaccharide-specific ELISA, purified pneumococcal capsular polysaccharides from 12 serotypes of *Streptococcus pneumoniae* were obtained from the American Type Culture Collection, Rockville, Md., and used on the test plates. The purified pneumococcal group antigen C polysaccharide was purchased from Statens Seruminstitut and fixed on test plates. In these ELISAs, plates fixed with coating buffer only were used as controls. Sera with known relative antibody titers, obtained from adults in Germany, were used as standards (12).

RESULTS

Low HA and antibody titers. Serum samples were obtained from 1,570 Ecuadorian children less than 5 years old, and complete anthropometric data were available for 1,553 children. Of these, 486 (31%) showed HA *Z* scores of <−2. If not indicated otherwise, infants and children younger than 1 year old were excluded from the analysis because their serum antibody partly reflects passively acquired maternal antibody and many children have not yet experienced an exposure to the relevant antigens. Of the 1,263 children older than 1 year, 461 (36.5%) showed HA *Z* scores of <−2. Antibody concentrations in serum were compared in children showing HA *Z* scores of <−2 (stunted children) and >−1 (control children). Children with HA *Z* scores in the intermediate range of >−2 and <−1 were excluded to obtain two clearly separated groups. Statistical comparisons of the means and their standard deviations by *t* test showed significant differences between the two groups of children for antibodies to eight antigens (Table 1). In four cases, stunted children showed significantly lower mean antibody concentrations than did control children (IgG antibody to the T-cell-dependent antigens tetanus toxoid and RS virus and IgM antibody to T-cell-independent antigens lipopolysaccharide and PRP. However, in four cases (implicating antibody to T-cell-dependent and T-cell-independent antigens), stunted children showed statistically higher antibody levels than did control children (Table 1). For antibodies to four antigens analyzed in this study (tetanus and diphtheria toxoid, PRP of *Haemophilus influenzae*, and capsular polysaccharide of *S. pneumoniae*), protective levels of antibody in serum have been defined in the literature (19, 20, 25, 30, 31, 36, 41). We chose conservatively to take 0.1 IU of tetanus antitoxin per ml (30, 31) and 0.15 IU of diphtheria antitoxin per ml (36, 41) as the protective levels. The case is less clear for antibodies to the bacterial polysaccharide antigens capsular polysaccharide and PRP. We had not international reference sera, but we have calculated absolute antibody concentrations. In addition, a mixture of capsular polysaccharides from 12 serotypes of *S. pneumoniae* was used; therefore, the concentrations of antibody to individual serotypes could not be calculated. We chose 1.5 μg of IgG antibody to PRP (20) and capsular polysaccharide (19, 25) per ml as the protective level.

No difference was detected between stunted and control children with respect to the proportion of children with antibody concentrations below the protective level in serum (Table 2). One might object that the age range of 1 to 5 years

TABLE 1. Antibody titers in serum of Ecuadorian children older than 1 year as a function of their nutritional status defined by anthropometry

Nutritional parameter	Cutoff	*n*	Mean antibody titer and standard error against[a]:															
			T-cell-dependent antigen								T-cell-independent antigen							
			IgG					IgM			IgM			IgG				
			TT	DT	LT	BLG	RV	RS	RV	RS	LPS	PRP	CPS	C	PRP	CPS
HA *Z*	<-2	461	0.49 ± 0.02	0.37 ± 0.02	0.54 ± 0.01	0.12 ± 0.003	0.56 ± 0.02	0.32 ± 0.007	0.20 ± 0.004	0.31 ± 0.006	0.31 ± 0.008	0.19 ± 0.004	0.16 ± 0.005	0.14 ± 0.004	0.17 ± 0.004	0.17 ± 0.006
	>-1	418	0.55 ± 0.02	0.34 ± 0.02	0.51 ± 0.01	0.12 ± 0.04	0.53 ± 0.02	0.36 ± 0.007	0.19 ± 0.004	0.32 ± 0.006	0.34 ± 0.008	0.21 ± 0.005	0.15 ± 0.005	0.13 ± 0.004	0.15 ± 0.003	0.15 ± 0.005
P[b]			0.04*	0.21	0.05	0.59	0.21	0.000*	0.10	0.15	0.002*	0.05*	0.23	0.009	0.001	0.05
WA *Z*	<-2	215	0.48 ± 0.02	0.36 ± 0.03	0.52 ± 0.02	0.13 ± 0.005	0.49 ± 0.02	0.31 ± 0.009	0.20 ± 0.007	0.30 ± 0.009	0.30 ± 0.01	0.18 ± 0.007	0.14 ± 0.007	0.12 ± 0.006	0.15 ± 0.005	0.14 ± 0.007
	>-1	507	0.55 ± 0.02	0.38 ± 0.02	0.52 ± 0.009	0.12 ± 0.003	0.56 ± 0.01	0.36 ± 0.006	0.19 ± 0.004	0.32 ± 0.005	0.34 ± 0.007	0.21 ± 0.004	0.16 ± 0.005	0.14 ± 0.004	0.16 ± 0.003	0.16 ± 0.005
P			0.02*	0.56	0.82	0.31	0.01*	0.000*	0.79	0.03*	0.003*	0.002*	0.02*	0.05*	0.24	0.008*
WA *Z* corr[c]	<-2	169	0.49 ± 0.03	0.37 ± 0.03	0.51 ± 0.02	0.12 ± 0.005	0.50 ± 0.03	0.31 ± 0.01	0.20 ± 0.007	0.31 ± 0.01	0.31 ± 0.01	0.18 ± 0.008	0.15 ± 0.008	0.13 ± 0.007	0.15 ± 0.005	0.15 ± 0.008
	>-1	489	0.56 ± 0.02	0.38 ± 0.02	0.52 ± 0.009	0.12 ± 0.03	0.56 ± 0.02	0.36 ± 0.006	0.19 ± 0.004	0.32 ± 0.005	0.34 ± 0.008	0.21 ± 0.005	0.16 ± 0.005	0.14 ± 0.004	0.16 ± 0.003	0.17 ± 0.005
P			0.06	0.89	0.87	0.85	0.03*	0.000*	0.80	0.38	0.02*	0.004*	0.19	0.20	0.29	0.02*

[a] Abbreviations of the antigens: TT, tetanus toxoid; DT, diphtheria toxoid; LT heat-labile enterotoxin of *E. coli*; BLG, β-lactoglobulin; RV, rotavirus; LPS, lipopolysaccharide of *E. coli*; CPS, capsular polysaccharide of *S. pneumoniae*; C, C polysaccharide of *S. pneumoniae*.
[b] Probability by Student's *t* test. Comparisons that are different at the 5% significance level are underlined. *, comparisons showing significantly higher means in the children with better nutritional status ($P = 0.000$ means $P < 0.0001$).
[c] WA *Z* corr, WA *Z* score of children who did not experience a recent episode of diarrheal disease.

TABLE 2. Proportion and percentage of Ecuadorian children, with nutritional status defined by anthropometry, who showed antibody concentrations below the protective level

Parameter	Cutoff	No. of children below protective level/total no. (%) with antibody to following antigen[a]:			
		TT	DT	CPS	PRP
HA *Z*	<-2	17/426 (4.0)	65/426 (15.3)	77/417 (18.5)	51/376 (13.6)
	>-1	8/383 (2.1)	49/382 (12.8)	79/376 (21.0)	49/341 (14.4)
P[b]		0.15	0.36	0.37	0.83
WA *Z* corr[c]	<-2	7/157 (4.5)	25/157 (15.9)	33/151 (21.9)	19/134 (14.2)
	>-1	10/466 (2.1)	49/463 (10.6)	88/457 (19.3)	57/357 (16.0)
P		0.15	0.09	0.48	0.68

[a] Abbreviations: TT, tetanus toxoid; DT, diphtheria toxoid; CPS, capsular polysaccharide of *S. pneumoniae*.
[b] Probability in two-tailed Fisher's test.
[c] See Table 1, footnote *c*.

old in one group is to great to allow real evaluations of effects. Therefore, antibody levels in stunted and control children were compared for each 1-year age group interval. Only for IgG antibody to PRP antigen, enterotoxin, and RS virus did two or more 1-year subgroups show significant differences in titer. For IgG antibody to RS virus, three 1-year age group intervals showed significantly higher mean levels in control children than in stunted children (Table 3), while for IgG antibody to *E. coli* enterotoxin, three 1-year age group intervals showed significantly higher mean levels in stunted children than in control children (data not shown).

Low WA and antibody titers. Over all age groups, 231 of 1,554 children (15%) showed WA *Z* scores <-2 (underweight children). Of the 1,263 children older than 1 year, 215 (17%) were underweight. Antibody concentrations in serum were compared between underweight and control children showing WA *Z* scores >-1. Children with WA *Z* scores in the intermediate range of >-2 and <-1 were excluded to obtain two clearly separated groups. For 12 of the 14 antibody levels measured in this study, underweight children older than 1 year showed consistently lower antibody titers (Table 1). The difference was statistically significant for nine different antibody concentrations including antibody to both T-cell-dependent (tetanus toxoid, rotavirus, RS virus) and T-cell-independent (lipopolysaccharide, PRP, and capsular polysaccharide) antigens.

Multivariate analysis by stepwise logistic regression revealed rural and high-altitude place of living, lack of a sanitation system, and a recent episode of diarrhea as independent

TABLE 3. Comparison of IgG antibody levels to RS virus in 1-year age groups of Ecuadorian children classified according to nutritional status assessed by anthropometry

Age (yr)	Mean OD ± SD for HA *Z* of:		*P*[a]	Mean OD ± SD for WA *Z* corr[b] of:		*P*
	<-2	>-1		<-2	>-1	
<1	0.20 ± 0.13	0.25 ± 0.13	0.09	0.14 ± 0.10	0.24 ± 0.13	0.007
<2	0.25 ± 0.13	0.30 ± 0.13	0.006	0.24 ± 0.12	0.29 ± 0.14	0.03
<3	0.30 ± 0.13	0.34 ± 0.13	0.03	0.31 ± 0.14	0.35 ± 0.13	0.06
<4	0.33 ± 0.12	0.39 ± 0.11	0.0002	0.31 ± 0.11	0.39 ± 0.12	0.0003
<5	0.39 ± 0.12	0.41 ± 0.12	0.11	0.39 ± 0.11	0.40 ± 0.11	0.53

[a] *P*, probability in *t* test; significant differences are underlined.
[b] See Table 1, footnote *c*.

factors associated with low weight. None of the factors that related to the place of living (zone, altitude, sanitation) confounded the association between low weight and decreased antibody titers (data not shown). To eliminate the acute effects of diarrhea on weight as a result of water loss and anorexia, the association between low weight and decreased antibody titers was reanalyzed for children who had not experienced a recent episode of diarrhea. In control children, significantly higher concentrations were found for IgG antibody to RS virus, rotavirus, and capsular polysaccharide and for IgM antibody to lipopolysaccharide and PRP than in underweight children (Table 1). The difference was at the borderline of significance for tetanus antibody levels. When the results were broken down into 1-year age group intervals, only antibody to RS virus (Table 3) and tetanus toxoid showed significantly higher antibody levels in at least two age groups when control children were compared with underweight children. No statistically significant difference was detected between underweight and control children with respect to the proportion of children with antibody concentrations in serum below the protective level against four bacterial antigens (Table 2).

Anemia and antibody titers. Of 1,535 children in all age groups analyzed for hemoglobin concentration, 435 (28%) showed altitude-corrected hemoglobin concentrations below 110 g/liter, fulfilling the World Health Organization criterion for anemia. Of the 1,162 children older than 1 year for whom hemoglobin measurements were available, 249 (21%) showed low hemoglobin concentrations. Anemic children older than 1 year had significantly lower antibody titers to both T-cell-dependent antigens (tetanus toxoid, diphtheria toxoid, rotavirus, and RS virus) and T-cell-independent antigens (lipopolysaccharide, PRP, capsular polysaccharide, and C polysaccharide) than did control children (Table 4). A significantly higher proportion of anemic children than control children had antibody levels that were below a conservatively defined protective antibody level for diphtheria antitoxin; this was not observed for tetanus antitoxin or antibodies to capsular polysaccharide and PRP (Table 5). When the results were broken down into 1-year age group intervals, no antibody showed significantly higher levels in at least two age groups when control children were compared with anemic children (data not shown).

Vitamin A deficiency and antibody titer. Of 1,267 children 0 to 5 years old tested for vitamin A deficiency, 173 (14%) showed retinol levels in serum below 200 μg/liter. When the analysis was restricted to children older than 1 year, 136 of 1,064 children tested (13%) had low retinol concentrations in serum. These children, however, did not differ from control children with retinol concentrations in serum above 200 μg/liter in terms of antibody titers in serum, except for IgG antibody to RS virus (Table 4). The difference between vitamin A-deficient and control children remained significant for RS virus-specific IgG antibody in two 1-year age group intervals (Table 6). This was not observed for any other antibody specificity (e.g., tetanus antitoxin [Table 6 and data not shown]). No difference was detected between the two groups for the proportion of protective antibodies (Table 5).

Zinc concentration and antibody titer. Over all age groups, 198 of 818 children tested (24%) showed zinc concentrations in serum below 65 μg/dl, indicative of a poor zinc status. When the analysis was restricted to children older than 1 year, 190 of 773 children tested (25%) had zinc concentrations in serum below 65 μg/dl. These children did not differ from control children with respect to antibody titers in serum (Table 4). No difference was detected between the two groups in the proportion of protective antibodies (Table 5), and none of the

TABLE 4. Antibody titers in serum of Ecuadorian children older than 1 year as a function of selected nutrient deficiencies

Nutritional parameter	Cut-off	*n*	Mean antibody titer and standard error against[a]:											
			T-cell-dependent antigen						T-cell-independent antigen					
			IgG				IgM		IgM				IgG	
			TT	DT	LT	BLG	RV	RS	RV	RS	LPS	PRP	CPS	C	PRP	CPS
Zinc	<65 μg/dl	190	0.50 ± 0.03	0.35 ± 0.03	0.52 ± 0.01	0.12 ± 0.004	0.57 ± 0.02	0.33 ± 0.01	0.19 ± 0.006	0.32 ± 0.009	0.33 ± 0.01	0.20 ± 0.006	0.16 ± 0.007	0.14 ± 0.006	0.16 ± 0.005	0.18 ± 0.008
	>65 μg/dl	583	0.55 ± 0.02	0.38 ± 0.02	0.53 ± 0.009	0.11 ± 0.003	0.59 ± 0.01	0.35 ± 0.005	0.20 ± 0.004	0.33 ± 0.005	0.35 ± 0.007	0.21 ± 0.004	0.17 ± 0.004	0.14 ± 0.004	0.16 ± 0.003	0.18 ± 0.004
P[b]			0.17	0.26	0.33	0.17	0.64	0.64	0.21	0.08	0.08	0.09	0.54	0.47	0.80	0.94
Vitamin A	<200 μg/liter	136	0.52 ± 0.03	0.38 ± 0.03	0.52 ± 0.02	0.12 ± 0.005	0.56 ± 0.03	0.32 ± 0.01	0.19 ± 0.009	0.31 ± 0.01	0.32 ± 0.01	0.19 ± 0.007	0.15 ± 0.009	0.14 ± 0.008	0.16 ± 0.006	0.18 ± 0.01
	>200 μg/liter	928	0.52 ± 0.01	0.37 ± 0.01	0.52 ± 0.007	0.12 ± 0.002	0.56 ± 0.01	0.34 ± 0.004	0.20 ± 0.003	0.32 ± 0.004	0.33 ± 0.005	0.20 ± 0.003	0.16 ± 0.003	0.13 ± 0.003	0.16 ± 0.002	0.17 ± 0.003
P			0.73	0.80	0.79	0.67	0.81	0.02*	0.45	0.62	0.52	0.08	0.46	0.43	0.53	0.45
Hemoglobin	<110 g/liter[c]	249	0.47 ± 0.02	0.32 ± 0.02	0.55 ± 0.01	0.13 ± 0.004	0.47 ± 0.02	0.31 ± 0.009	0.18 ± 0.006	0.30 ± 0.007	0.30 ± 0.01	0.18 ± 0.006	0.14 ± 0.007	0.12 ± 0.005	0.15 ± 0.005	0.14 ± 0.006
	>110 g/liter	913	0.54 ± 0.01	0.38 ± 0.01	0.52 ± 0.007	0.12 ± 0.002	0.57 ± 0.01	0.34 ± 0.004	0.20 ± 0.003	0.32 ± 0.004	0.33 ± 0.005	0.21 ± 0.003	0.16 ± 0.003	0.14 ± 0.003	0.16 ± 0.002	0.16 ± 0.003
P			0.01*	0.007*	0.05	0.02	0.000*	0.004*	0.000*	0.004*	0.02*	0.000*	0.01*	0.009*	0.44	0.006*

[a] Abbreviations of the antigens: TT, tetanus toxoid; DT, diphtheria toxoid; LT, heat-labile enterotoxin of *E. coli*; BLG, β-lactoglobulin; RV, rotavirus; LPS, lipopolysaccharide of *E. coli*; CPS, capsular polysaccharide of *S. pneumoniae*; C C polysaccharide of *S. pneumoniae*.

[b] Probability by Student's *t* test. Comparisons that are different at the 5% significance level are underlined; *, comparisons showing significantly higher means in the children with better nutritional status.

[c] Hemoglobin concentration after altitude correction.

TABLE 5. Proportion and percentage of Ecuadorian children, with nutritional status defined by biochemical status parameters, who showed antibody concentrations below the protective level

Parameter	Cutoff	No. of children below protective level/total no. (%) with antibody to following antigens[a]:			
		TT	DT	CPS	PRP
Zinc	<65 μg/dl	6/189 (3.2)	31/189 (16.4)	33/188 (17.6)	23/159 (14.5)
	>65 μg/dl	18/582 (3.1)	67/582 (11.5)	101/574 (17.6)	79/578 (13.7)
P		1.0	0.10	1.0	0.80
Vitamin A	<200 μg/liter	2/130 (1.5)	19/130 (14.6)	25/129 (19.4)	16/116 (14.3)
	>200 μg/liter	30/925 (3.2)	126/925 (13.6)	176/905 (19.4)	123/861 (14.3)
P		0.42	0.79	1.0	1.0
Hemoglobin	<110 g/liter	7/248 (2.8)	45/248 (18.1)	54/243 (22.2)	32/215 (14.9)
	>110 g/liter	27/909 (3.0)	113/905 (12.5)	167/886 (18.8)	116/816 (14.2)
P		1.0	0.03	0.24	0.83

[a] TT, tetanus toxoid; DT, diphtheria toxoid; CPS, capsular polysaccharide of *S. pneumoniae*.
[b] Probability in two-tailed Fisher's test.

antibody specificities showed significant differences in more than one 1-year age group interval (data not shown).

DISCUSSION

Height and weight measure two different effects of malnutrition (22). Low HA is the result of reduced skeletal growth (stunting), while low WA signifies a low total body mass (underweight) without indicating whether this is due to small size, to thinness, or to a combination of the two. Multivariate analysis by stepwise logistic regression showed that rural and high-altitude place of living, lack of sanitation, anemia, and low zinc and retinol concentrations in serum were independently associated with stunted growth in the Ecuadorian children. In multivariate analysis, low weight in Ecuadorian children was associated independently with rural and high-altitude place of living and lack of sanitation, but not with any of the biochemical nutritional status indicators; in contrast to stunting, it was also associated with a recent episode of diarrhea.

In addition to nutritional parameters, we tested the antibody response to major childhood pathogens in children from developing countries. *S. pneumoniae* and *H. influenzae* are the two major respiratory pathogens in children from developing countries (34). The role of RS virus is less well documented, but it is certainly an important pathogen (35). In many epidemiological investigations from Latin America, rotavirus and enterotoxigenic *E. coli* are two major enteric pathogens in children (see reference 4 and references therein). Diphtheria and tetanus rank as the fourth and fifth most important causes, respectively, of vaccine-preventable diseases in Ecuador (28). Adding more clinical relevance to the antibody measurements, at least four serum antibodies have been linked with clinical protection against disease (tetanus and diphtheria antitoxin and antibodies to capsular polysaccharide of *S. pneumoniae* and PRP of *H. influenzae*).

Nearly one-third of the Ecuadorian children investigated showed stunted growth. No consistent association between low HA *Z* scores and antibody concentrations in serum was seen. With respect to four antibody specificities covering both T-cell-dependent and T-cell-independent antigens, control children showed significantly higher antibody concentrations than stunted children, while with respect to four other antibody specificities, stunted children showed significantly higher antibody concentrations than control children, once again covering both T-cell-dependent and T-cell-independent antigens. Curiously, IgG antibody to PRP antigen was present at higher concentration in stunted than in control children, whereas the converse was true for IgM antibody to the same antigen. These observations raise doubts about the biological meaning of these statistical significances. The standard deviations for the optical density readings were relatively small for the ELISA antibody measurements in the Ecuadorian children (see Fig. 1 in references 9 and 11 and Fig. 4 in reference 5). Because of the relatively small standard deviations and the large number of children tested, the *P* values became significant even for minor differences in mean optical density. When smaller numbers of subjects were analyzed by stratifying for 1-year age group intervals, only three antibody specificities showed significant differences between stunted and control children in more than one age subgroup. Note that here, once again, the differences were not consistent: control children showed higher IgG antibody titers to PRP and RS virus than did stunted children, stunted children showed higher IgG antibody titers to enterotoxin than did control children.

TABLE 6. Comparison of IgG antibody levels to tetanus toxoid and RS virus in 1-year age groups of Ecuadorian children classified according to vitamin A status

Age (yr)	Mean OD ± SD for anti-tetanus IgG level		*P*[a]	Mean OD ± SD for anti-RS virus IgG level		*P*
	Vit A < 200 μg/liter	Vit A > 200 μg/liter		Vit A < 200 μg/liter	Vit A > 200 μg/liter	
<1	0.23 ± 0.26	0.33 ± 0.43	0.08	0.23 ± 0.14	0.21 ± 0.12	0.42
<2	0.53 ± 0.45	0.45 ± 0.40	0.43	0.26 ± 0.15	0.27 ± 0.14	0.65
<3	0.63 ± 0.38	0.59 ± 0.42	0.65	0.34 ± 0.11	0.32 ± 0.14	0.50
<4	0.51 ± 0.41	0.54 ± 0.44	0.71	0.31 ± 0.10	0.37 ± 0.12	<u>0.001</u>
<5	0.45 ± 0.31	0.53 ± 0.41	0.22	0.36 ± 0.09	0.40 ± 0.12	<u>0.03</u>

[a] *P*, probability in *t* test; significant differences are underlined.

In contrast to the HA parameter, low weight (WA *Z* scores of <-2) was consistently associated with lower antibody titers when compared with control children. Note, however, that weight is a less reliable parameter than height of low nutrition, as it can change quickly. Multivariate analysis revealed an association between low weight and occurrence of recent diarrhea (see above). Because gastrointestinal infection is at least transiently associated with a loss of weight as a result of loss of appetite and loss of water from an increased number of liquid stools and vomiting, a low WA value in a cross-sectional study design is not a hard indicator of nutritional status. Therefore, in a second analysis, children with a recent episode of gastrointestinal infection were excluded. It is important that when this was factored out, many of the antibody differences remained statistically significant. However, when the children were also stratified for age, control children showed significantly higher concentrations only for tetanus antitoxin and IgG to RS virus than did underweight children.

The differences in mean antibody concentrations between underweight and control children are relatively small, even in cases where they are statistically significant. This could indicate lack of importance of these differences and thus a lack of association between the nutritional status defined by WA values and immune response defined by antibody concentrations in serum. However, the fact that the differences in antibody titer were small is likely to reflect the heterogeneity of the subjects in this type of population study. Furthermore, there are often differences between ex vivo and in vitro measures, such that small but real in vitro differences can be more significant in vivo.

In the Ecuadorian children, the antibody response to protein antigens was affected by malnutrition in the same way as was the antibody response to polysaccharide antigens. Several investigators found a decreased response to typhoid immunization with a T-cell-independent antigen in malnourished individuals (14, 38, 40), whereas a normal antibody response to tetanus toxoid was observed in the same class of individuals (14, 15). However, other studies reported a normal antibody response to typhoid vaccination in children suffering from kwashiorkor when compared with control children (32). Therefore, conflicting results have been obtained by different workers, and no clear-cut selective effects of malnutrition on antibody response to T-cell-independent antigens can be expected.

During the last decade, certain nutrient deficiencies and their effect on immune response have been the focus of considerable interest. Several clinical reports suggested an association between marginal zinc deficiency and impaired immune response (21). No significant difference was detected in antibody titers between Ecuadorian children showing normal and low zinc levels in serum. This is not surprising, because to our knowledge no differences in antibody titers in serum to infectious agents have been described for children differing in zinc status. Our data should, however, be interpreted with caution, because the zinc concentration in serum is only a proxy measure of the zinc status in the body as a whole.

The clearly established association between hypovitaminosis A and microbial infections (37) suggests that nutritional vitamin A deficiency impairs immune responsiveness. With one exception, we did not detect a difference in antibody titers between children with low and normal retinol levels. The exception was IgG antibody to RS virus. Paradoxically, in the literature there is little evidence that vitamin A deficiency affects immune status in humans. No difference in the IgG response to tetanus toxoid was seen in vitamin A-deficient children from Bangladesh (3) and India (2) compared with control children. Only one study from Indonesia (33) showed a depressed immune response to tetanus in children with vitamin A deficiency.

If a negative iron balance persists over some time, a measurable reduction in the hemoglobin concentration will occur. We are not aware of detailed studies in the literature which analyzed the effect of iron deficiency on human antibody response (1). Ecuadorian children with normal hemoglobin concentrations showed higher antibody titers to some but not all antigens than did anemic children. In addition, the proportion of anemic children with diphtheria antitoxin levels below 0.15 IU/ml was significantly lower than the proportion of control children. When the children were subdivided into 1-year age group intervals, none of these differences remained significant. Note, however, that the number of subjects in these subgroups was often relatively small (≤ 30); consequently, the statistical power to detect minor differences was small.

The statistical analysis supports a relationship between nutritional status and concentrations of antibody to major childhood pathogens in the serum of Ecuadorian children. The astonishing fact about this study is that differences were detected given the probable heterogeneity in the exposure history and age range of the children. Note that malnourished children are more exposed to parasitic infections which are known to trigger a polyclonal B-cell activation and also a marked increase in Ig levels (24). This effect could mask a stronger relationship between malnutrition and decreased antibody levels. Whether the observed differences have clinical relevance cannot easily be decided from these data. With respect to tetanus and diphtheria vaccination, where protective antibody levels are well defined, very few children ($<5\%$) showed antitoxin levels below 0.1 IU/ml, and the prevalence was independent of their nutritional status. Somewhat more children showed diphtheria antitoxin levels below 0.15 IU/ml (about 15%), and here a trend toward association with poorer nutritional status was observed. However, clinical protection is usually still conferred in children with antitoxin levels above 0.01 IU/ml.

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