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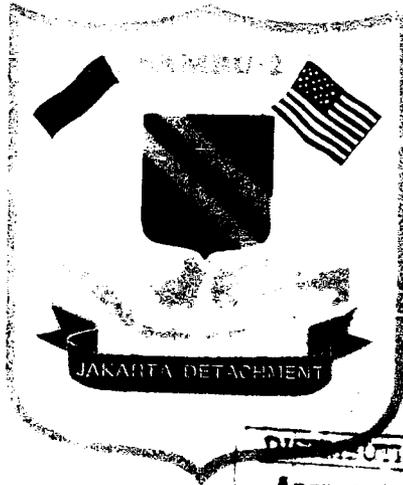
IMMUNE RESPONSE TO CRYPTOSPORIDIOSIS IN PHILIPPINE CHILDREN

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## IMMUNE RESPONSE TO CRYPTOSPORIDIOSIS IN PHILIPPINE CHILDREN

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**Abstract.** An ELISA was used to measure the *Cryptosporidium*-specific IgA, IgG, and IgM antibody levels in serum, stool, and duodenal fluid of 15 Filipino children. Antibody levels were measured on admission to the hospital, 1 week later, and at a 6 week follow-up examination. Delayed type hypersensitivity skin tests were used to assay cell mediated immunity (CMI), iron status was measured by serum iron tests and total iron binding capacity, and the degree of malnutrition was determined by clinical examination. Antibody response to *Cryptosporidium* was qualitatively and quantitatively strong and maintained over time. All subjects showed impaired CMI early with some reconstitution after 6 weeks. All subjects showed some degree of malnutrition and/or depleted iron status.

*Cryptosporidium* ssp. is generally accepted as a pathogen of the digestive tract in humans and in animals.<sup>1</sup> Although there is a considerable body of literature covering the biology and epidemiology of *Cryptosporidium*,<sup>1-8</sup> there is little known about the immunity to cryptosporidial infection in humans. In other coccidian infections, it is believed that either cell-mediated immune response (CMI) or an antibody-dependent cell mediated cytotoxic effect (ADCC) is the primary defense.<sup>9,10</sup> Recent investigations on immunity to *Cryptosporidium* advance similar findings.<sup>11,12</sup> We investigated the immune response to *Cryptosporidium* in infants and young children with diarrhea admitted to San Lazaro Hospital (SLH), a large infectious disease hospital, in Manila. We were interested in the antibody response over time, the cell mediated immune response, and the correlation between the level of immunity and the state of nutrition, particularly iron status. The study period was July 1986–October 1987, and we were able to update the existing literature on the epidemiology of cryptosporidiosis in the Philippines.<sup>2</sup>

### MATERIALS AND METHODS

#### Subjects

Children 1–24 months of age with diarrhea who were admitted to SLH were screened by stool examination for *Cryptosporidium*. Those found positive by modified Kinyoun stain were

considered for enrollment in the study. Although there were several cases of infection with multiple enteric pathogens, the subjects selected for enrollment were infected only with *Cryptosporidium* ssp. Upon obtaining informed consent from the parents or legal guardians, the children were formally enrolled. Fifty-six age- and sex-matched controls were selected from patients admitted to the hospital for any condition other than gastrointestinal disease.

#### Specimens

Immediately following enrollment, a stool sample, 3–5 ml venous blood, and a duodenal fluid sample were obtained. Stool and blood were collected in the usual manner, and duodenal fluid was collected by either naso-gastric intubation or string capsule (Entero-Test®, HDC Corp., Mountain View, CA). Stool specimens were analyzed by Rotazyme ELISA kits (Abbott Labs, North Chicago, IL) for rotavirus and plated on appropriate enteric media; colonies were identified by the API System (API Systems, SA, La Balme les Grottes, France). Specimens were labeled and frozen at –60°C for future use. This procedure was repeated 1 week and 6 weeks post-enrollment for each subject that could be located.

#### ELISA

To determine the levels of *Cryptosporidium*-specific IgA, IgG, and IgM antibodies in the stool,

TABLE I

Enteric pathogens identified from pediatric diarrhea cases admitted to San Lazaro Hospital, Manila, July 1986–October 1987

Organism isolated	Total number positive	Percent positive
1. Rotavirus	487	59.2
2. <i>Ascaris lumbricoides</i>	110	31.4
3. <i>Trichuris trichiura</i>	76	9.2
4. <i>Salmonella</i> group B	71	8.6
5. <i>Cryptosporidium</i> ssp.	70	8.5
6. <i>Vibrio cholera</i>	55	6.7
7. <i>Shigella flexneri</i> (B)	39	4.7
8. <i>Salmonella</i> group E	26	3.2
9. <i>Aeromonas hydrophila</i>	22	2.7
10. <i>Blastocystis hominis</i>	9	1.1
11. <i>Campylobacter jejuni</i>	7	0.85
12. <i>Entamoeba coli</i>	6	0.72
13. Hookworm	6	0.72
14. <i>Salmonella</i> group C <sub>2</sub>	3	0.4
15. <i>Vibrio fluvialis</i>	3	0.4
16. <i>Giardia lamblia</i>	3	0.4
17. <i>Salmonella</i> group C <sub>1</sub>	2	0.24
18. <i>Shigella sonnei</i> (D)	1	0.12
19. <i>Enterobius vermicularis</i>	1	0.12

Total number of patients = 823.

serum, and duodenal fluid samples, an enzyme-linked immunosorbent assay (ELISA) was performed. The method was modified from that of Ungar and others.<sup>13</sup> *Cryptosporidium parvum* oocysts used as solid phase antigen were supplied by Bruce Anderson, University of Idaho, Caldwell, ID and Ron Fayer, USDA, Beltsville, MD. Oocysts in bovine fecal material were cleaned and concentrated by discontinuous density gradient centrifugation in Percoll as previously described.<sup>14</sup> After concentration, 250–300 k oocysts suspended in coating buffer were placed in each well of 96-well Immulon-2<sup>®</sup> flat-bottomed plates (Dynatech Laboratories, Chantilly, VA). Plates were allowed to coat overnight at 4°C. Plates were then blocked by the addition of 5% horse serum in phosphate buffered saline (PBS) and stored at –60°C until ready for use. Prior to assay, plates were washed 2 times in washing buffer. Samples to be assayed (serum, stool, and duodenal fluid) were first processed to separate the IgG fractions from the IgA and IgM fractions. This was done using the Quik-Sep System II<sup>®</sup> column chromatography kit (Isolab Inc., Akron, OH). After separation, samples were diluted as follows: all serum antibodies, 1:100; stool and duodenal fluid IgG, 1:26; and stool and duodenal fluid IgA and IgM, 1:10. These samples, in 50  $\mu$ l

portions, were then added to appropriate wells, incubated for 1 hr at 37°C, then washed 3 times in buffer. Conjugates of anti-human IgA, IgG, and IgM with horseradish peroxidase (Sigma, St. Louis, MO) in 50  $\mu$ l portions of 1:1,000 dilution were then added to the wells, incubated for 1 hr at 37°C, and washed as before. Substrate OPD (Abbot Laboratories, North Chicago, IL) was then added to the wells, allowed to react for 30 min, and then stopped with H<sub>2</sub>SO<sub>4</sub>; the absorbance was read at 402 nm on a Titertek<sup>®</sup> Multiscan plate reader (Titertek, Flow Labs, Inglewood, CA). Known positive and negative control samples plus PBS were run. To establish cutoff values, serum samples were obtained from 12 healthy infants at a U.S. Air Force Hospital in the Philippines. Normal control sera from SLH were not used because of high reactivity to *C. parvum* oocysts, suggesting prior exposure to the organism. Multiple portions of each of the samples from the 12 healthy infants were assayed for IgA, IgG, and IgM by our ELISA procedure.

#### Indirect immunofluorescent antibody assay

Samples of stool, serum, and duodenal fluid were screened by indirect immunofluorescent antibody assay (IFA) using methods previously described.<sup>15</sup> Briefly, *C. parvum* oocysts were air-dried and methanol fixed on glass slides. Samples, after Quik Sep treatment, were added to the smear and incubated. Serum samples were diluted 1:100, stool and duodenal fluid IgG samples were diluted 1:26, and stool and duodenal fluid IgA and IgM samples were diluted 1:10. Slides were washed thoroughly, and a 1:5 dilution of goat anti-human IgA, IgG, and IgM conjugated to FITC (Sigma) were added and incubated for 30–60 min at 37°C. Slides were washed again, mounted, and observed on a fluorescence microscope.

#### Total antibody

Total antibody levels for IgA, IgG, and IgM, expressed in mg/dL, were obtained for each sample by the radial immunodiffusion (RID) method. RID assays for the 3 subclasses of human antibodies were performed using the Accra Assay<sup>®</sup> series (ICN Biomedicals, Costa Mesa, CA) and were computed using the end point method as described in the product insert.

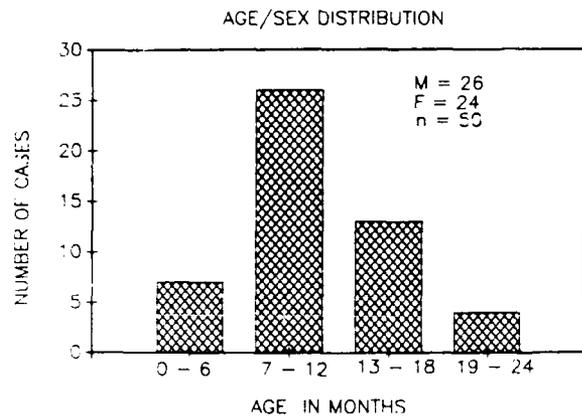


FIGURE 1. The number of cases of cryptosporidiosis was greatest in children 7-18 months of age. There was no detectable difference in sex distribution.

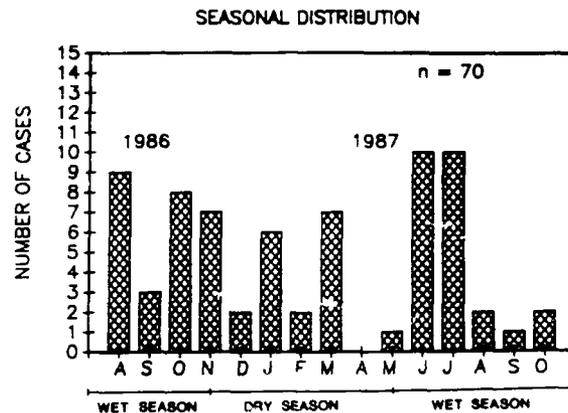


FIGURE 2. In support of previous studies, we detected a slight seasonality for cryptosporidiosis, with more cases occurring during the warmer, rainy months.

#### Cell mediated immunity

The subjects' ability to mount a cell mediated response (CMI) was evaluated using a delayed type hypersensitivity (DTH) test administered to the forearm or thigh. The resulting indurations were measured after 48 hr and a score derived. In order to standardize the procedure, the Multitest CMI kit (Merieux Institute, Miami, FL) was used. The 7 pre-measured antigens provided in the kit cover a broad range of substances that should give a reasonably accurate assessment of CMI status.

#### Serum iron and total iron binding capacity

Acute, 1 week, and 6 week serum samples from each subject were analyzed to obtain serum iron and total iron binding capacity (TIBC) using the Gemini serum iron/TIBC reagent kit and the Gemini Miniature Centrifugal Analyzer (Electro Nucleonics, Fairfield, NJ).

## RESULTS

#### Epidemiological data

Our study in July 1986-October 1987 showed a prevalence of 8.5% for *Cryptosporidium* among 823 subjects sampled making it the fifth most common isolate (Table 1). Because SLH is a large charity hospital, the patients represent the lowest income class in the metropolitan area. These patients often live with poor hygiene and sanitation, resulting in maximum exposure to enteropathogens early in life. The largest number of

cases were in the 7-18 month age group with almost equal sex distribution (Fig. 1). To evaluate the possibility that some of the isolates could reflect asymptomatic carriage of *Cryptosporidium*, we sampled 74 non-diarrheic individuals including some immediate family members of the enrolled subjects. No oocysts were found. We found a slight indication of seasonality in the occurrence of cases (Fig. 2), with the higher numbers appearing during the rainy months.

#### Humoral immunity

**ELISA.** Among the 70 diagnosed cases of cryptosporidiosis, 21 subjects completed the entire course of specimen collection. Samples of serum, stool, and duodenal fluid from acute, 1 week, and 6 week collection times of all completed subjects were analyzed by ELISA for levels of *Cryptosporidium* oocyst-specific IgA, IgG, and IgM antibodies (Table 2). The serum samples showed mean optical density (OD) values significantly higher than controls for all 3 subclasses at the 3 different collection times, although there was no significant difference between the acute, 1 week, and 6 week levels. The duodenal fluid samples only showed higher mean OD values for IgA at the 6 week collection. Stool samples showed significantly higher mean OD values for IgM at all 3 collections and for IgG at the acute collection; however, the values decreased over time. We were able to locate 6 subjects at 6 months post-study and collected serum and stool samples. We found an elevated antibody response to *Cryptosporidium* among these samples (Table 3).

TABLE 2  
Cryptosporidium specific IgA, IgG, and IgM antibody levels in serum, duodenal fluid, and stool as determined by ELISA\*

		Examination			Normal control†
		Acute	1 week	6 weeks	
Serum	IgA	0.18 (0.177)‡	0.18 (0.183)‡	0.172 (0.125)‡	0.012 (0.005)
	IgG	0.396 (0.260)‡	0.46 (0.248)‡	0.515 (0.229)‡	0.283 (0.096)
	IgM	0.445 (0.159)‡	0.461 (0.033)‡	0.484 (0.137)‡	0.136 (0.015)
Duodenal fluid	IgA	0.101 (0.132)	0.16 (0.182)	0.231 (0.193)§	0.05 (0.021)
	IgG	0.077 (0.066)	0.048 (0.046)	0.089 (0.116)	0.037 (0.025)
	IgM	0.23 (0.101)	0.258 (0.132)	0.288 (0.134)	0.285 (0.036)
Stool	IgA	0.293 (0.368)	0.254 (0.268)	0.222 (0.2)	0.154 (0.019)
	IgG	0.07 (0.068)§	0.04 (0.031)	0.037 (0.028)	0.027 (0.012)
	IgM	0.32 (0.183)¶	0.27 (0.119)‡	0.254 (0.098)‡	0.153 (0.019)

\* Mean (SD) of optical density values read at 402 nm absorbance.

† Controls for serum were a pool of 12 healthy infants from Clark Air Force Base, Philippines. Controls for stool and duodenal fluid were from healthy infants from SLH.

‡ Statistically significant difference between sample mean and normal control mean,  $P < 0.001$ , Student's *t*-test (2-tailed).

§ Statistically significant difference between sample mean and normal control mean,  $P = 0.01$ , Student's *t*-test (2-tailed).

¶ Statistically significant difference between sample mean and normal control mean,  $P = 0.001$ , Student's *t*-test (2-tailed).

IFA. To visualize the antigen-antibody reactions and to help support the ELISA results, an IFA was run. The results of the IFA appear in Figure 3. Clear evidence of antibody binding can be seen on both the oocyst walls and, in some cases, on the sporozoites within.

Total antibody. Total antibody levels in serum, stool, and duodenal fluid were assayed by RID. Only the IgA subclass showed a significant decrease from normal controls, and this appeared at all 3 collection times in serum and duodenal fluid (Table 4).

CMI. Delayed-type hypersensitivity skin reaction was used as an indicator of subjects' CMI response. The test was performed on 7 of the 21 subjects at enrollment and at the 6 week follow-up (Table 5). Age matched controls were tested once. On initial testing, all subjects showed total skin energy compared to controls who reacted to a variety of antigens. At the 6 week follow-up, the mean score for the experimental group was still significantly lower than the controls, although there was an increase in DTH response from the initial test.

Iron status. Serum samples drawn at the acute, 1 week, and 6 week collection times were analyzed for serum iron, TIBC, and percent saturation of transferrin. Serum iron levels at all 3 collection times were significantly lower than control values, although there was no significant fluctuation of values between collection times (Fig. 4). Over the 6 week time span, TIBC increased to the point where the 6 week values were significantly higher than control values. Corre-

spondingly, the percent saturation of transferrin decreased from 18.3% to 13.1% over the 6 week period. The normal control value for percent saturation was 39.4% (Fig. 5). Subjective and objective evaluations of these patients by our clinical team indicated the existence of some degree of malnutrition in every case.

#### DISCUSSION

The immune responses to coccidial infections have been characterized as primary CMI with strong T cell dependency,<sup>9,16</sup> as antibody mediated (citing the enhancement of primary in-

TABLE 3  
Six month follow-up assay for Cryptosporidium antibodies from 6 return subjects\*

		IgA	IgG	IgM
Serum	Subjects	0.35† (0.227)	0.335† (0.126)	0.433† (0.136)
	Controls‡	0.261 (0.024)	0.239 (0.027)	0.281 (0.124)
Stool	Subjects	0.095† (0.065)	0.044† (0.01)	0.101† (0.062)
	Controls§	0.491 (1.152)	0.05 (0.011)	0.055 (0.023)

\* ELISA results reported as mean optical density read at 402 nm. Mean (standard deviation) of 3 readings of a single test of each pooled sample from the 6 subjects.

† Statistically significant difference between sample mean and normal control mean,  $P < 0.001$ , Student's *t*-test.

‡ Normal controls were 12 healthy infants from Clark Air Force Base, Philippines.

§ Normal controls were 6 healthy children from San Lazaro Hospital/NAMRU-2 staff.

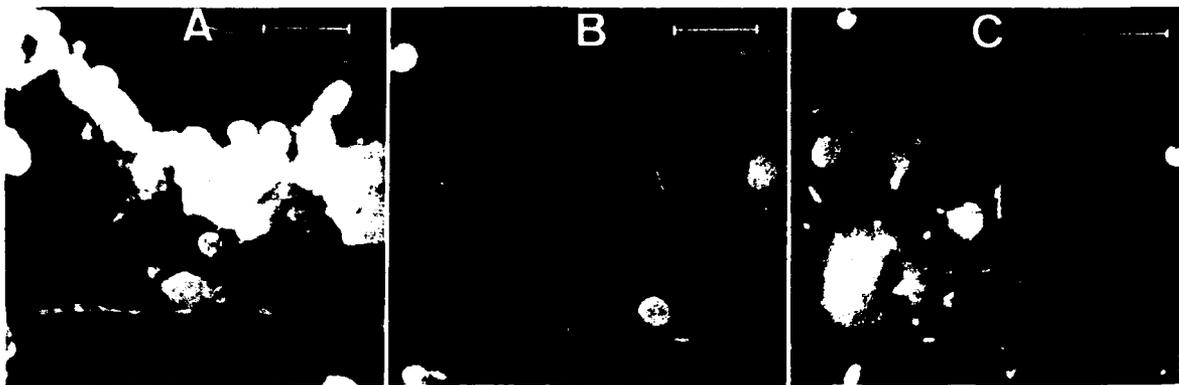


FIGURE 3. Results of IFA using *Cryptosporidium parvum* oocysts and subject sera and stool antibodies. A. Serum IgA shows strong reaction to oocysts. B. Stool IgG shows strong reaction to oocysts. C. Stool IgA shows distinct reaction to oocysts and sporozoites. Bars = 10  $\mu$ m.

fection in antibody deficient mice), or as an interplay of humoral and CMI with ADCC as a strong possibility.<sup>10</sup> The immune response to *Cryptosporidium* has similarly been described as either CMI, as evidenced by the course of the disease in AIDS patients, or humoral,<sup>12,17</sup> with reference to the increased susceptibility of hypogammaglobulinemic patients.<sup>11</sup>

Our longitudinal study of 21 children with cryptosporidiosis shows a marked cryptosporidial antibody response for IgA, IgG, and IgM in serum. This response was maintained over time, as seen in 6 subjects in a 6 month follow-up screening (Table 3). These findings were consistent with previously reported work,<sup>1,17</sup> and probably indicate continued exposure to the organism with resulting boosting effect. The total serum antibody response for IgG and IgM was com-

parable to controls, although there was a lower value of total serum IgA in the subjects.

In stool samples, IgM was found in significant amounts over a period of 6 weeks, although the values decreased over time. IgA and IgG were found in detectable levels, though there was no difference from controls. The decreased antibody levels in stools may have resulted from the flushing action of prolonged, severe diarrhea, rather than from decreased production or secretion.<sup>18</sup>

In duodenal fluid samples, only IgA was found at significant levels and only at the 6 week sampling time. We do not believe that the results for duodenal IgG and IgM antibody levels reflect a true picture of the situation. Due to delays in processing, a considerable amount of IgG and IgM may have been degraded by enzymatic activity present in the fluid prior to freezing, stor-

TABLE 4

Total antibody concentration (mg/dL) over time, in serum, stool and duodenal fluid, as determined by radial immunodiffusion assay (RID)

		Acute	1 week	6 weeks	Normal controls	Normal range†
Serum	IgA	97	87	102	280*	19-119
	IgG	1,383	1,343	1,375	2,042	258-1,393
	IgM	282	275	253	150	14-114
Duodenal fluid	IgA	1	1	2	582†	§
	IgG	5	5	8	1	§
	IgM	1	1	0	1	§
Stool	IgA	11	14	11	2	§
	IgG	5	11	3	1	§
	IgM	2	7	4	1	§

\* Statistically significant difference between 3 sample means and control mean,  $P = 0.02$ , 1-way ANOVA.

† Statistically significant difference between 3 sample means and control mean,  $P < 0.001$ , 1-way ANOVA.

‡ Normal ranges for children up to 24 months old.

§ Normal values not reported.

TABLE 5

Cell mediated immune response as determined by delayed type hypersensitivity reaction using the Multitest\* CMI kit

	Mean scores*	
	Subjects	Controls
Initial test	0/0†	4/15
Follow up test (6 weeks)	3/6‡	ND

\* Composite score is the total number of antigens showing positive reaction sum of diameters in mm of measured induration. Antigens used were tetanus, diphtheria, Streptococcus, tuberculosis, glycerin control, Candida, Trichophyton, Proteus.

† Statistically significant difference between sample mean and control mean  $P < 0.001$ , Student's  $t$ -test (2-tailed).

‡ Statistically significant difference between sample mean and control mean  $P = 0.03$ , Student's  $t$ -test (2-tailed).

age, and assay. Previous work has shown considerable IgG and IgM secretion into the duodenum.<sup>19,20</sup> We feel that a similar situation existed in our study, although it may have been masked by problems in technique.

The antibody response to *Cryptosporidium* in our subjects was remarkable qualitatively as well as quantitatively. The 3 subclasses showed distinct affinity for the oocyst wall and the pellicle, or outer membrane, of the sporozoites. As with other coccidia, surface structures on *Cryptosporidium* are probably involved in infectivity and may be targets of a protective immune response.<sup>21</sup> These structures on the oocyst have been characterized as carbohydrate moieties, either alone or in conjunction with lipids or proteins.<sup>22</sup> Serum IgM showed the strongest binding response to surface structures on both the oocysts and sporozoites as determined by immunoelectron microscopy (data not shown).

We wanted to evaluate the CMI status of our subjects. We initially intended to perform T4/T8 ratios to measure CMI competence. However, the amount of blood required was considered excessive for the age and nutritional state of the children, and so an alternative method was employed. DTH as expressed by skin reaction was the method of choice. The CMI Multitest uses a battery of 7 common antigens and is regarded as an adequate test of CMI in infants and young children.<sup>23</sup> On initial testing, all 7 subjects showed total skin energy. At 6 weeks follow-up, there was a slight increase in the test scores, but they were still significantly below the normal control values. We were also able to test 6 subjects at a 6 month follow-up; at this time there were some increases in skin reactivity, indicating a partial reconstitution of CMI competence.

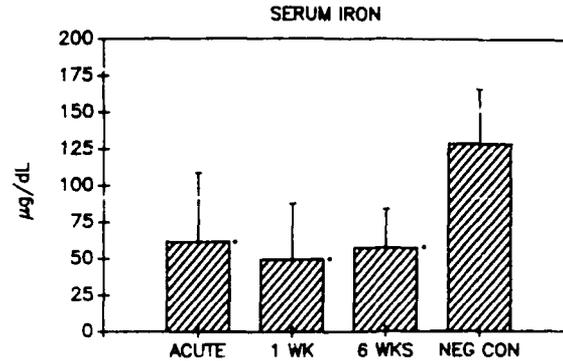


FIGURE 4. Subjects showed decreased serum iron levels at 3 different sampling times as compared to controls.

\* $P < 0.001$ , Student's  $t$ -test. Statistically significant difference between sample mean and negative control mean.

The finding of impaired CMI in our subjects was not unexpected.<sup>24-26</sup> These children displayed varying degrees of malnutrition (as determined by Gomez's classification)<sup>27</sup> which, based on their economic status and lifestyle, was probably a pre-existing condition to their cryptosporidiosis. The relationship between nutritional state and immunity is well documented. Malnutrition appears to have little or no effect on the quality or quantity of antibody production, but it does impair CMI.<sup>28</sup>

We were interested in the role of iron and its effect on CMI. Previous studies have shown that iron malnutrition was correlated with CMI impairment, particularly lymphocyte function,

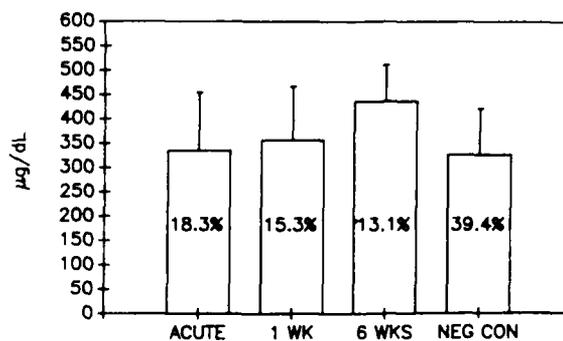


FIGURE 5. Subjects showed increasing total iron binding capacity and decreasing percent saturation of transferrin over time, indicative of iron deficiency anemia, as compared to controls.

\* $P = 0.002$ , Student's  $t$ -test. Statistically significant difference between sample mean and negative control mean.

phagocytosis, and neutrophil bactericidal activity, while antibody responses and complement were normal.<sup>29-31</sup> Our results (Figs. 4, 5) show decreased serum iron, increased TIBC and decreasing percent saturation of transferrin. This is the classical picture of iron deficiency anemia. These results support the existing work on iron status and immunity.

A question that we could not resolve was the role of *Cryptosporidium* in iron malnutrition. In a population suffering from protein calorie malnutrition (PCM), there is probably concomitant iron deficiency and impaired CMI. We speculate that *Cryptosporidium*, by virtue of its preference for parasitizing the brush border of enterocytes, may incapacitate enough absorptive surface and molecular transport mechanisms to further disrupt iron absorption, thus precipitating a vicious circle of nutrient deficit, impaired CMI, increased and prolonged infection, and further nutrient deficit.

In our study of cryptosporidiosis in Philippine children, we found a prevalence rate of 8.5% in a sample population consisting of subjects 1-24 months of age, with no apparent or documented immunodeficiency disorders. There was no significant difference in sex distribution. The greatest number of cases occurred in the 7-18 month age group. The highest number of cases occurred in the warm wet months. Our subjects were almost all from low income families where sanitation and personal hygiene were minimal. Our epidemiological findings are in agreement with previously published studies on children from developing countries,<sup>1-4,6-8,32</sup> except we show an increase in prevalence (2.9% vs. 8.5%) for the Philippines since the initial 1985 study.<sup>2</sup> This may be due to a difference in methodology. In 74 non-diarrheic individuals, we found no asymptomatic carriage of *Cryptosporidium parvum*. This may be a reflection of the small sample size, since other studies have shown evidence of asymptomatic carriage.<sup>33</sup>

In conclusion, we examined a sample population of pediatric subjects diagnosed as having cryptosporidial diarrhea but with no other documented immunodeficiencies. We found a marked and prolonged antibody response to *Cryptosporidium*, but impaired CMI. The infection cleared spontaneously over time and CMI was reconstituted. The prolonged antibody response, and specifically the IgM response, may reflect a situation of continuous exposure to the

organism in a contaminated environment with a subsequent boosting effect. The CMI deficit may have been related to general PCM or, more specifically, to iron deficiency which was found in all subjects. These findings, and the published results of other workers,<sup>34,35</sup> lead us to conclude that the immune response to cryptosporidiosis in our subjects is probably an antibody dependent cell-mediated cytotoxic effect of unknown mechanism. For clinicians in developing countries who are faced with pediatric cases of cryptosporidiosis we recommend, in addition to routine supportive care, the monitoring of CMI status by skin testing and an aggressive program of nutritional therapy aimed at reconstituting the cell mediated immune response. Although iron may be an important factor in rebuilding ADCC immunity, intensive iron supplementation should be approached with caution.<sup>36</sup>

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<p>An ELISA was used to measure the <i>Cryptosporidium</i>-specific IgA, IgG, and IgM antibody levels in serum, stool, and duodenal fluid of 15 Filipino children. Antibody levels were measured on admission to the hospital, 1 week later, and at a 6-week follow-up examination. Delayed type hypersensitivity skin tests were used to assay cell mediated immunity (CMI), iron status was measured by serum iron tests and total iron binding capacity, and the degree of malnutrition was determined by clinical examination. Antibody response to <i>Cryptosporidium</i> was qualitatively and quantitatively strong and maintained over time. All subjects showed impaired CMI early with some reconstitution after 6 weeks. All subjects showed some degree of malnutrition and/or depleted iron status.</p>					
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