STATISTICAL ANALYSIS OF CLINICAL, IMMUNOLOGICAL AND NUTRITIONAL FACTORS IN PEDIATRIC CRYPTOSPORIDIOSIS IN THE PHILIPPINES*

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Abstract. A statistical analysis of clinical, nutritional, and immunological data gathered in a previ-us study suggest that nutritional factors, and in particular, iron status, appeared to be of significance in mounting an effective immune response to *Cryptosporidium* infection in young children. The primary protective mechanism seemed to be cell-mediated; humoral immunity was intact in all the study subjects, however, CMI was initially impaired but improved over six weeks.

INTRODUCTION

From the early 1980s to the present, interest in *Cryptosporidium* as a significant human pathogen has steadily increased. Recognized as the putative agent of the severe, wasting diarrhea associated with acquired immunodeficiency syndrome (AIDS), this coccidian protozoan is now accepted as an enteropathogen affecting immunocompetent as well as immunocompromised individuals. The organism is found in both developing and developed countries, and displays a greater predilection for the pediatric age groups (Fayer and Ungar, 1986). In the Philippines, the prevalence of pediatric cryptosporidiosis has been reported as 2.9% (Cross *et al,* 1985). In our recent study (Laxer *et al,* 1990), we have found the prevalence rate to be 8.5% in a similar population.

The human immune response to *Cryptosporidium* is not well understood. Both humoral and cell-mediated mechanisms have been proposed, and there is evidence to support each (Casemore *et al,* 1985; Janoff and Reller, 1987). There are conflicting reports in the literature on the role of nutrition in cryptosporidiosis, although the relationship between nutrition and the intensity of immune response in general is well documented (Puri and Chandra, 1985).

The purpose of this study was to conduct a statistical analysis of clinical and laboratory data collected in a concurrent investigation of pediatric cryptosporidiosis (Laxer *et al,* 1990) and present evidence that would support a reasonable model of the immune response to this infection. In addition, we wanted to determine if there was a significant relationship between iron status and the relative strength and efficacy of the immune response.

MATERIALS AND METHODS

Subject

The clinical records of ten children, 7 males and 3 females ranging in age from 5 to 17 months, admitted to San Lazaro Hospital, Manila, with the diagnosis of cryptosporidiosis, were selected from a concurrent study because of complete documentation of medical histories, laboratory results, and follow-up visits.

Specimens

Immediately following enrolment, a stool sample, 3 to 5 ml of venous blood, and a duodenal fluid sample were obtained from each subject.

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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 USA). Specimens were labeled and frozen at -70°C for future use. This procedure was repeated at one week and six weeks post admission.

Assays

To determine the levels of *Cryptosporidium*-specific IgA, IgG, and IgM antibodies in the stool, serum, and duodenal fluid samples, an enzyme-linked immunosorbent assay (ELISA) was performed. The method was modified from that of Ungar *et al* (1986). Total antibody levels were measured by radial immunodiffusion (RID), using the Accra Assay® system (ICN Biomedicals, Costa Mesam CA USA), and expressed as mg/dl. The cell-mediated immune status was evaluated by delayed type hypersensitivity skin reaction using the Multitest CMI® kit (Merieux Institute, Miami, FL USA). Serum iron and total iron binding capacity (TIBC) were measured using the Gemini® Miniature Centrifugal Analyzer (Eletro Nucleonics, Fairfield, NJ USA). All assay methods are described in detail in the report of our other study (Laxer *et al,* 1990).

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Clinical evaluation

The nutritional status of the children was reported as percentage of the ideal body weight for age (Gomez' classification of malnutrition) (Gomez *et al,* 1956). Pertinent signs and symptoms relevant to diarrhea were recorded, specifically nausea, vomiting, abdominal cramps, irritability, anorexia, consciousness and skin turgor. Emphasis was placed on the daily follow-up of changes in stool character and frequency. These characteristics were used to measure the severity of the disease and were reported as days duration of diarrhea.

Clinical evidence of malnutrition and impaired immune status such as pallor, skin and mucous membrane lesions, organomegaly, edema and ascites, was noted and recorded. CMI testing and measurement of results were performed by a single physician.

Statistical analysis

ELISA data were reported as the mean optical density (OD) of 3 trial wells for each sample from each subject. These were then pooled into sample groups (all serum IgA; all serum IgG, etc). Total antibody data were reported as mg/dl for each sample from each subject and pooled. Iron data was reported as the mean of 3 trial runs for each serum sample from each subject, then pooled. CMI data were reported as the total number of positive reactions to a battery of 7 antigens for each subject. These data were analyzed by stepwise multiple regression using the Pro DOS statistical package (Conceptual Software Inc, Houston, TX USA).

RESULTS

Assays

The results of the ELISA, RID, serum iron, TIBC, and CMI assay are presented in Tables 1 - 3.

Clinical evaluation

Dehydration was the prominent sign on admission. Three children required intravenous fluid replacement. Symptoms included watery stool, anorexia, drowsiness, irritability and abdominal cramps. All the children were pale, though none exhibited organomegaly, edema or ascites. Detailed examination of the skin and mucous membranes yielded one child with furunculosis (patient no. 601), one child with scabies (patient no. 599) and one child with oral candidiasis (patient no. 634). These children were treated accordingly in addition to the symptomatic management of their diarrhea and dehydration and nutritional support consisting of milk formula, solid foods and vitamins that were given routinely to all subjects. CMI tests were non-reactive for all subjects on admission.

Six weeks after admission, all subjects except one were seen to the clinically improved with no

Table 1

Assay results.

Case No.	Nutritional Factors										
	Age[a]	Sex[b]	Weight[c]	Dofmaln	SeFe0	SeFe1	SeFe6	TIBC0	TIBC1	TIBC6	DDiarrhea
L601	6.00	1.00	13.00	76.00	61.00	5.00	NA	NA	NA	NA	20.00
L602	17.00	1.00	20.00	83.00	46.00	47.00	28.00	399.00	420.00	439.00	7.00
L611	11.00	0.00	17.00	77.00	30.00	117.00	63.00	274.00	320.00	438.00	21.00
L609	10.00	1.00	18.00	86.00	69.00	54.00	44.00	460.00	476.00	361.00	13.00
L610	8.00	1.00	12.00	63.00	37.00	37.00	62.00	506.00	473.00	535.00	32.00
L634	5.00	0.00	11.00	69.00	56.00	95.00	72.00	219.00	NA	292.00	14.00
L599	13.00	1.00	13.00	57.00	54.00	15.00	58.00	153.00	276.00	394.00	14.00
L603	8.00	1.00	9.00	50.00	21.00	20.00	53.00	405.00	357.00	436.00	24.00
L604	13.00	1.00	11.00	50.00	48.00	37.00	69.00	313.00	261.00	560.00	24.00
L636	12.00	0.00	10.00	44.0	35.00	42.00	75.00	237.00	409.00	477.00	19.00

[a] in months;
[b] 0 = F, 1 = M;
[C] in pounds
Dofmaln = degree of malnutrition as %
SeFeo = serum iron at acute collection
SeFe1 = serum iron at 1 week collection
SeFe6 = serum iron at 6-week collection
TIBC0 = total iron binding capacity acute sample
TIBC1 = total iron binding capacity 1 week sample
TIBC6 = total iron binding capacity 6-week sample
Diarrhea = days duration of diarrhea

recurrence of diarrhea, no pallor, no skin or mucous membrane lesions, and were playful and alert with variable degrees of reconstitution of CMI response. Only one subject (patient no. 601), a six-month old male, showed no improvement, remaining pale, irritable, with recurrent diarrhea and persistent scalp furunculosis despite treatment. His CMI remained unreactive.

Statistical analysis

In order to examine the effects of humoral and cell-mediated immunity, and nutrition and iron status on the immune response to cryptosporidiosis, the tabulated data for all variables were entered into the Prodos® computer program and analyzed by a stepwise multiple regression routine.

Table 2
Assay results.

	Serum ELISA*									Total antibody+								
Case No.	SeIgA0	SeIgA1	SeIgA6	SeIgG0	SeIgG1	SeIgG6	SeIgM0	SeIgM1	SeIgM6	TSeIgA0	TSeIgA1	TSeIgA6	TSeIgG0	TSeIgG1	TSeIgG6	TSeIgM0	TSeIgM1	TSeIgM6	DDiar#
L601	0.14	0.17	0.14	0.08	0.74	0.71	0.25	0.27	0.26	10.00	10.00	20.00	3900.00	2800.00	2525.00	143.00	218.00	293.00	20.00
L602	0.14	0.11	0.19	0.10	0.09	0.06	0.43	0.46	0.39	125.00	260.00	75.00	500.00	0.00	9.00	1062.00	0.00	9.00	7.00
L611	0.16	0.11	0.14	0.37	0.35	0.44	0.48	0.44	0.56	500.00	188.00	263.00	1188.00	1438.00	1595.00	270.00	120.00	283.00	21.00
L609	0.14	0.09	0.09	0.48	0.43	0.61	0.30	0.30	0.39	50.00	50.00	50.00	3350.00	4700.00	1125.00	300.00	505.00	125.00	13.00
L610	0.13	0.15	0.15	0.58	0.31	0.38	0.58	0.66	0.68	87.00	125.00	250.00	800.00	1150.00	1150.00	287.00	525.00	287.00	32.00
L634	0.83	0.85	0.50	0.91	0.75	0.74	0.64	0.65	0.58	88.00	9.00	88.00	850.00	9.00	900.00	188.00	9.00	163.00	14.00
L599	0.07	0.06	0.07	0.07	0.06	0.09	0.30	0.37	0.39	50.00	87.00	800.00	900.00	1400.00	187.00	187.00	187.00	187.00	14.00
L603	0.24	0.11	0.08	0.09	0.05	0.07	0.66	0.56	0.31	20.00	0.00	0.00	325.00	0.00	0.00	285.00	375.00	338.00	24.00
L604	0.09	0.07	0.08	0.17	0.25	0.41	0.32	0.29	0.30	110.00	85.00	85.00	1360.00	2000.00	2520.00	240.00	280.00	240.00	24.00
L636	0.10	0.11	0.16	0.26	0.58	0.61	0.76	0.69	0.66	50.00	0.00	0.00	2150.00	1125.00	1125.00	300.00	300.00	300.00	19.00

+ Mean optical density of three sample wells per specimen per patient read at 402 μm
+ Mg/dl total antibody per specimen as determined by RID
# Days duration of diarrhea
0 = acute collection
1 = 1 week collection
6 = 6-week collection

Table 3

Assay results.

Case No.	Cell mediated immune factors*		
	CMI0	CMI6	Diarrhea
L601	0.00	0.00	20.00
L602	0.00	8.00	7.00
L611	0.00	NA	21.00
L609	0.00	8.00	13.00
L610	0.00	NA	32.00
L634	0.00	3.00	14.00
L599	0.00	8.00	14.00
L603	0.00	0.00	24.00
L604	0.00	3.00	24.00
L636	0.00	0.00	19.00

*CMI response reported as number of reactive sites with $\geqslant$ 2 mm diameter from 8 pointed applicator.

Table 4

Results of stepwise multiple regression of variables.

Dependent variable = Days duration of diarrhea

Step I: independent variable selected:
total iron binding capacity 6 (TIBC6)
R squared = 0.6568179
Standard error = 0.017632
F value = 11.483428
P value = 0.0147
B-estimate = 0.059752

Step II: independent variable (s) selected:
R squared = 0.9374021

	Age	TIBC6
Standard error =	0.295685	0.009436
F value =	22.411625	74.488303
P value =	0.0052	0.0003
B-estimate =	-1.399798	0.081442

Examination of Table 4 shows that of the entire list of independent variables from Tables 1 - 3 that were analyzed, only age and total iron binding capacity at six weeks (TIBC6) were selected by the stepwise regression programs built-in criteria for significance. TIBC6 was selected in the first pass, with an F value of 11.483 and a value of 0.0147. On the second pass, age was selected with an F value of 22.411 and a p value of 0.0005. Also on the second pass, TIBC6 was co-selected with age, having an F value of 74.488 and a p value of 0.0003.

DISCUSSION

The immune response to coccidian infections has been characterized as primarily humoral, cell-mediated, or as antibody dependent cell-mediated immunity (ADCC) (Casemore *et al,* 1985; Janoff and Reller, 1987; Wakelin and Grencis, 1987; Lillehoj, 1987). The human immune response to *Cryptosporidium* infection has been similarly described. There is evidence to support a role for the humoral component, as seen in the increased severity and duration of infection in hypogammaglobulinemic individuals (Janoff and Reller, 1987) and complementary evidence for the cell-mediated role as seen in AIDS patients (Berkowitz, 1985). The relationship between nutritional status and immune competence is well established, and there is considerable support for the role of iron in maintaining a viable cell-mediated immune system (Chandra, 1983; Joynson *et al,* 1972).

The results of our study of pediatric cryptosporidiosis among underprivileged children living in Manila showed that, despite low serum iron levels and chronic malnutrition, the humoral immune response was vigorous. Specific anti-cryptosporidial antibody levels, and total antibody levels in general, were high. The cell-mediated response was depressed in every case on admission to the study. The clinical findings were consistent with chronic malnutrition, dehydration and impaired CMI.

We wanted to analyze our data statistically in order to detect trends that might show the type of immune reponse involved in cryptosporidial infection. We set disease severity, as defined by day-duration of dirarrhea, as the dependent variable. Various combinations of independent variables reflecting either humoral, cell-mediated, or ADCC weighted mixes were analyzed in stepwise fashion by the statistical program. Nutritional factors were also added as independent variables. The stepwise regression analysis (Table 4) selected

TIBC6 and age as the two independent variables having the most significant effect on the dependent variable. Age bore an inverse relationship to disease severity (B-estimate = -1.399), and it agrees with previous studies and our own observations. Total iron binding capacity bore a direct relationship to disease severity (B-estimate = 0.081); as iron stores are depleted and iron binding capacity increases, the severity of the infection worsens. The F and p values for both selected independent variables showed a high degree of significance.

Although our study was not a randomized examination of a large sample group and could, therefore, only show general trends, we believe that it lends support to the hypothesis that iron, with general nutrition, plays a critical role in the maintenance of cell mediated immunity, and that CMI, probably in concert with humoral immunity in an ADCC relationship, is the key element in the immune response to cryptosporidiosis.

In the population we studied, pre-existing malnutrition likely contributed to a decreased efficacy of CMI functions. This may have predisposed the individuals to a greater risk of getting the infection, which is endemic and easily spread given the local conditions of suboptimal sanitation and hygiene. Once infected, the prolonged, profuse diarrhea exacerbated the malnutrition, thereby preventing reconstitution of effective CMI and subsequent clearance of the parasite.

Since at present there are no effective chemotherapeutic agents for treating cryptosporidiosis, we think that our findings on iron status and nutritional intervention, particularly in severe pediatric cases, may have some relevance for clinicians practicing in developing countries.

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REFERENCES

Berkowitz CD. AIDS and parasitic infections, including *Pneumocystis carinii* and cryptosporidiosis. *Pediatr Clin North Am* 1985; 32 : 933 - 52.

Casemore DP, Sands RL, Curry A. *Cryptosporidium* species: a "new" human pathogen. *J Clin Pathol* 1985; 38 : 1321.

Chandra RK. Nutrition, immunity, and infection: present knowledge and future directions. *Lancet* 1983; 26 : 688 - 91.

Cross JH, Alcantara AK, Alquiza L, Zaraspe G, Ranoa CP. Cryptosporidiosis in Philippine children. *Southeast Asian J Trop Med Public Health* 1985; 16 : 257 - 60.

Fayer R, Ungar BLP. *Cryptosporidium* sp and cryptosporidiosis. *Microbiol Rev* 1986; 50 : 458 - 83.

Gomez F, Galvan RR, Frenr S, Munoz JC, Chavez R, Vasguez J. Mortality in second and third degree malnutrition. *J Trop Pediatr* 1956; 2 : 77.

Janoff EN, Reller LB. *Cryptosporidium* species, a protean protozoan. *J Clin Microbiol* 1987; 25 : 967 - 75.

Joynson DHM, Jacobs A, Walker DM, Dolby AE. Defect of cell-mediated immunity in patients with iron deficiency anaemia. *Lancet* 1972; 18 : 1058 - 9.

Laxer MA, Alcantara AK, Laxer, MJ, Menorca D, Fernando MT, Ranoa CP. Immune response to cryptosporidiosis in Philippine children. *Am J Trop Med Hyq* 1990; 42 : 131 - 9.

Lillehoj HS. Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates host protective immunity. *Infect Immun* 1987; 55 : 1616 - 21.

Puri S, Chandra RK, Nutritional regulation of host resistance and predictive value of immunologic tests in assessment of outcome. *Pediatr Clin North Am* 1985; 32 : 499 - 516.

Ungar BL, Soave R, Fayer R, Nash TE. Enzyme immunoassay detection of immunoglobulin M and G antibodies to *Cryptosporidium* in immunocompetent and immunocompromised persons. *J Infect Dis* 1986; 153 : 570 - 8.

Wakelin D, Grencis, RK. Immunological responses to intestinal parasite infection. In: Miller K, Nicklin S, eds. Immunology of the Gastrointestinal Tract. Boca Raton, Florida: CRC Press, 1987; 2 : 1 - 24.