Abstract. A statistical analysis of clinical, nutritional, and immunological data gathered in a previous 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.
Stool and blood were collected in the usual manner, and duodenal fluid was collected by either naso-gastric intubation or string capsule (Enterotest®, 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 Mesa 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
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.**

<table>
<thead>
<tr>
<th>Serum ELISA*</th>
<th>Total antibody*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case No.</td>
<td>SelgA0</td>
</tr>
<tr>
<td>L601</td>
<td>0.14</td>
</tr>
<tr>
<td>L602</td>
<td>0.14</td>
</tr>
<tr>
<td>L611</td>
<td>0.14</td>
</tr>
<tr>
<td>L610</td>
<td>0.14</td>
</tr>
<tr>
<td>L634</td>
<td>0.13</td>
</tr>
<tr>
<td>L630</td>
<td>0.03</td>
</tr>
<tr>
<td>L603</td>
<td>0.07</td>
</tr>
<tr>
<td>L604</td>
<td>0.09</td>
</tr>
<tr>
<td>L636</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* 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

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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 3
Assay results.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Cell mediated immune factors*</th>
<th>Diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM10</td>
<td>CM16</td>
</tr>
<tr>
<td>L601</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>L602</td>
<td>0.00</td>
<td>8.00</td>
</tr>
<tr>
<td>L611</td>
<td>0.00</td>
<td>NA</td>
</tr>
<tr>
<td>L609</td>
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<td>NA</td>
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<td>L610</td>
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<td>3.00</td>
</tr>
<tr>
<td>L636</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*CMI response reported as number of reactive sites with $\geq 2$ 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:
  Age TIBC6
  
  R squared = 0.9374021
  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 Grncis, 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 response involved in cryptosporidial infection. We set disease severity, as defined by day-duration of diarrhea, 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


