INFECTIVITY OF CRYPTOSPORIDIUM PARVUM IN HEALTHY ADULTS WITH PRE-EXISTING ANTI-C. PARVUM SERUM IMMUNOGLOBULIN G

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Abstract. A 50% infectious dose (ID₅₀) of 132 Cryptosporidium parvum oocysts was previously determined in serologically negative individuals (ELISA). In this study, 17 healthy adults with pre-existing anti-C. parvum serum IgG were challenged with 500–50,000 oocysts. Infection and diarrhea were associated with the higher challenge doses. The ID₅₀ was 1,880 oocysts, > 20-fold higher than in seronegative volunteers. Fecal oocysts were detected in only seven (53.8%) of 13 individuals with clinical cryptosporidiosis, indicating that the host response may effectively decrease the number of oocysts produced. Subjects with the highest absorbances prior to challenge had little to no increase in IgG following challenge, whereas volunteers with lower reactivities showed significant postchallenge increases. This suggests that an upper limit of serum IgG was present in some subjects, while others were further stimulated by an additional exposure. These data indicate that prior exposure to C. parvum provides protection from infection and illness at low oocyst doses.

Cryptosporidium parvum is a recognized cause of diarrheal illness in waterborne outbreaks1–3 and in individuals with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS).4 The increased awareness of cryptosporidiosis in the general population and in specialized settings, such as day care centers, as well as the seriousness of the disease in immunosuppressed individuals have earned Cryptosporidium a place on the list of emerging diseases that are a threat to the public health.5 Furthermore, no effective chemotherapeutic agent has been demonstrated to prevent or cure C. parvum infections. Thus, there is considerable interest in the development of immunotherapies designed to limit infection. To rationally design such interventions, it is important to understand the major immunologic mechanisms that are operative in self-limited disease. Animals6–8 and humans9 with intact immune systems are typically capable of clearing the parasite within 1–3 weeks after infection. Several studies with experimental animal models have demonstrated that most species are refractory to a second oocyst challenge.6,8 Indeed, adult animals exposed to the parasite for the first time are relatively resistant to infection and often have only a transient period of oocyst shedding with few to no symptoms. In contrast, healthy adult volunteers can experience infections and diarrheal illnesses when oocyst challenges are at least one year apart.10

Many studies in animals11–15 and humans16–19 have documented the presence of antibodies to C. parvum infection. However, these investigations in humans were carried out in serologic surveys or in select persons experiencing diarrhea and only after the infections were diagnosed. Thus, the observations were necessarily limited by the lack of information on the immune status and exposure history prior to the exposure. Also, differences among the studies in antigen preparations used in the testing and definitions of positivity further complicate direct comparisons.

In volunteer studies previously published,20 an anti-C. parvum ELISA was used to identify serologically negative volunteers that were subsequently challenged with known concentrations of oocysts. Following challenge, the serum antibody response was primarily IgM and in some cases IgA. However, no significant increase in serum IgG was found in any of the volunteers.10 After one year, 19 of 29 volunteers were rechallenged with the homologous isolate, which resulted in an illness attack rate that was equal to the primary challenge and the development of specific serum IgG in only 32% of the exposed individuals. Thus, multiple exposures may be necessary for the development of anti-C. parvum IgG,10 which is typically found in one-fourth to one-third of persons in the United States. Cryptosporidium parvum seroprevalence rates vary with socioeconomic conditions, geographic location, and other factors. The identification of serum antibody levels associated with resistance to infection would be of use in assessing populations at risk for outbreaks of cryptosporidiosis.

The presence of serum antibodies and their association with protection from oocyst challenge is the subject of the present study. Cryptosporidium parvum infectivity and clinical outcome was determined in 17 healthy, adult volunteers who had pre-existing serum IgG to parasite antigens. A dose-response curve was constructed by challenging volunteers with a single inoculum of 500–50,000 C. parvum oocysts, and a 50% infectious dose (ID₅₀) was calculated as we have done for antibody-negative volunteers.20 Various clinical and parasitologic parameters were monitored, and the postchallenge serum IgG response was followed.

MATERIALS AND METHODS

Screening and enrollment of volunteers. Volunteers, who were primarily employees and students from the Texas Medical Center and surrounding area (Houston, Texas), were provided with detailed information regarding cryptosporidiosis and a precise description of the study. Only volunteers who scored 100% on a written examination, as described previously,20 were eligible for additional screening. Volunteers were excluded if they cared for infants less than two years of age, elderly individuals, persons with chronic illnesses, or those with known immunosuppression or if such persons resided within their household.

After informed consent was obtained, volunteers were re-
required to provide their medical histories and pass a physical examination. To be eligible for the study, each volunteer had to be within normal parameters for complete blood count, blood chemistry, urinalysis, chest radiography, electrocardiography, delayed-type hypersensitivity (Candida, tetanus, and mumps), T cell subsets, and immunoglobulins. In addition, negative results were required for stool ova and parasites, hepatitis B surface antigen, hepatitis C antibody, syphilis, and HIV virus, and a tuberculin skin test. Volunteer sera were further tested for antibodies to C. parvum; only those who were positive were enrolled in the study. Volunteers (n = 17) were challenged in groups of two or three with 500–50,000 oocysts between December 1994 and March 1996 and followed as previously described for six weeks postchallenge. This study was approved by the Committee for the Protection of Human Subjects, University of Texas Health Science Center.

Oocyst purification and quality control. Cryptosporidium parvum oocysts (Iowa isolate) were propagated and purified at the University of Arizona (Tucson, AZ) by one of the authors (CRS) as described. Purified oocysts were stored in 2.5% potassium dichromate at 4°C and sent to the University of Texas School of Public Health (Houston, TX). An aliquot of each oocyst preparation was delivered to the Clinical Microbiology Laboratory at Hermann Hospital (Houston, TX) for testing. The oocyst preparation was stained and cultured for bacteria, mycobacteria, and fungi. Adventitious virus detection used WI-38, MRC-5, RKC, (Houston, TX) for testing. The oocyst preparation was confirmed to be negative two weeks prior to oocyst ingestion by the volunteers.

Inoculum preparation. Oocyst preparations (100 µl) were washed twice in 10 ml of cold, sterile phosphate-buffered saline (PBS), pH 7.2, to remove potassium dichromate, resuspended in cold, sterile PBS, and serially diluted. Oocysts were counted using a hemacytometer and adjusted to the desired dose. A minimum of five additional counts was done to yield a median coefficient of variation of 4% (range = 1.3–16.4%). Oocysts in gelatin capsules were delivered within 1 hr of preparation and were ingested by volunteers with 250 ml of saline. No other foods or beverages were consumed by the volunteers for 8 hr before or 90 min after inoculum ingestion. Thereafter, no dietary restrictions were imposed.

Volunteer challenge. Prior to challenge volunteer sera were screened for anti-C. parvum IgG by an ELISA. The procedures for the anti-Cryptosporidium antibody assay were modified from those of DuPont and others. Briefly, biotinylated mouse anti-human IgG or IgM (Zymed Laboratories Inc., San Francisco, CA) was added to wells and incubated at 37°C for 1 hr. The wells were then washed with 0.15 M PBS, 0.2% Tween 20, pH 7.2. Horseradish peroxidase–labeled streptavidin (Life Technologies, Gibco-BRL, Gaithersburg, MD) was added to the wells and further incubated at 37°C for 1 hr. The reaction was visualized with peroxidase-activated 2.2’-azino-di-[3-ethyl-benzthazolinisolfonate(6)] (Boehringer Mannheim Biochemicals, Indianapolis, IN) and read spectrophotometrically at 414 nm. Positive and negative controls (in triplicate) were included on each microtiter plate. Prechallenge serum samples were considered positive if the mean absorbance value was greater than 1.5 times that of the mean negative control value from the same plate (absorbance index). The postchallenge IgG response (net absorbance) was calculated by subtracting the prechallenge absorbance from the postchallenge absorbance. All time points from each volunteer were run on the same plate.

Seventeen volunteers in groups of 2–3 were challenged with a single dose of C. parvum oocysts ranging from 500 to 50,000. Oocysts were ingested within 14–51 days of calf passage and in all cases were demonstrated to have an excretion rate of 87% or greater at the time of challenge. Actual oocyst concentrations delivered to the volunteers were typically within 5% or less of the target dose.

Collection of postchallenge samples and data. All stools passed by the volunteers were collected daily for the first 14 days following challenge. Subsequently, three 24-hr collections per week were obtained for the remaining four weeks of the study. Volunteers were instructed to keep the stool specimens on ice in insulated coolers until transport to the General Clinical Research Center (Hermann Hospital, Houston, TX). Vital signs were obtained and recorded by the nursing staff upon each visit. Volunteers were asked to fill out a daily diary for time of day and consistency of all stools passed and to document all gastrointestinal symptoms experienced. Oral electrolyte solutions along with instructions for the treatment of diarrhea were also provided to each volunteer. Household contacts were given detailed descriptions of the study and were monitored by the clinical staff for diarrheal illnesses. Cryptosporidium parvum oocysts were quantified by direct immunofluorescence assay (DFA) using procedures described previously. Stools from all episodes of diarrhea were cultured for enteric bacterial pathogens, including Shigella, Salmonella, Campylobacter, Aeromonas, and Plesiomonas. In addition, blood was collected by venipuncture from each individual at approximately days 5, 10, 30, and 42 postchallenge. Serum was separated, aliquoted, and stored at −90°C until tested.

Study definitions and statistical analysis. Symptomatic individuals were defined as those volunteers having two or more concurrent gastrointestinal complaints: abdominal pain/cramps, tenesmus, gas, nausea, vomiting, fecal urgency, and fecal incontinence. Diarrhea was defined as the production of 200 grams or more of unformed (soft to watery) stool per day, three or more unformed stools in 8 hr, or four or more unformed stools in 24 hr. Duration of diarrhea included the time (hours) from the first unformed stool to the last unformed stool before wellness. Wellness was declared after the first 24 hr in which no unformed stools were passed or symptoms occurred. Confirmed infection was defined as the presence of oocysts in the stool after 36 hr or more postchallenge. A clinical definition for infection (presumed infection) was used to include individuals with symptoms compatible with cryptosporidiosis in whom oocysts could not be identified given the limitation of the DFA (> 10,000 oocysts/ml). Presumed uninfected was defined as the absence of gastrointestinal symptoms and fecal oocysts throughout the course of the study. The term presumed is being used given the limitation of DFA to detect fewer than 10,000 oocysts/ml.

The Kruskal-Wallis nonparametric analysis of variance
test was used to evaluate the relationship between clinical outcome and IgG absorbance index or challenge dose and between challenge dose and infection (intensity, onset, or duration of oocyst excretion) or illness (onset, duration, severity, or number of diarrheal episodes) parameters. The difference in the proportion of presumed infections among seronegative volunteers and volunteers with pre-existing anti-
*C. parvum* antibodies was evaluated by Fisher’s exact test. Spearman’s rank correlation was used to evaluate the relationship between postchallenge net absorbance and all parameters of oocyst excretion and diarrheal illness. The ID$_{50}$ was calculated using the method described by Reed and Muench.21

**RESULTS**

*Cryptosporidium parvum* infectivity and oocyst excretion patterns. Volunteers with an absorbance value exceeding 1.5 times the mean absorbance of the negative control serum (absorbance index) were eligible for the study. Individual absorbance indices of volunteers with pre-existing anti-*C. parvum* IgG ranged from 1.53 to 7.22 with a median index of 1.91. These volunteers ranged in age from 20 to 44 years with a median of 30 years, and 53% were female.

Infectivity of the *C. parvum* Iowa isolate was evaluated based on the definitions of confirmed and presumed infections as described. For the present evaluation, the data previously reported on seronegative volunteers20 was reassessed based on revised definitions and a method based on cumulative percent of infected individuals.21 When volunteers with presumed infection were included, the estimated ID$_{50}$ decreased to 83 oocysts for antibody-negative volunteers (Figure 1B). In comparison, for volunteers with pre-existing anti-*C. parvum* IgG, the estimated ID$_{50}$ was 1,880 oocysts, a 23-fold increase over serologically negative volunteers. When the evaluation was limited to oocyst shedders (Figure 1A), the ID$_{50}$ was 132 for antibody-negative volunteers and 7,638 oocysts for antibody-positive volunteers, an even greater increase (57-fold) over the previous results.

Oocyst excretion patterns for all infected seropositive volunteers and their relationship to clinical category are shown in Figure 2. Seven volunteers had fecal oocysts detectable by DFA. Oocysts were initially detected as early as day 4 and as late as day 19 with a median onset of excretion at day 5, a time coincident with the incubation period of diarrheal illness. Oocysts were shed for a median of nine days (range = 1–11 days). As shown, oocysts were typically seen on consecutive days during or near the time of illness. The intensity of infection (total oocysts per six-week study) in DFA-positive individuals did not differ significantly (log mean ± SD oocysts = 7.32 ± 1.29 versus 6.32 ± 2.28, $P = 0.2$) between those with or without pre-existing antibody. However, the number of presumed infections (i.e., illness without oocysts) in volunteers with pre-existing serum antibody was three-fold greater (6 of 13, 46.2% versus 3 of 21, 14.3%; $P = 0.05$) than in volunteers without pre-existing serum antibody. Thus, while the intensity of oocysts in the DFA-positive group did not change, the number of individuals excreting oocysts did, indicating that overall (i.e., all presumed infected) fewer oocysts were excreted among individuals with pre-existing antibody.

Among the DFA-positive individuals of the present study, those with mild/no symptoms tended to excrete fewer oocysts ($8.5 \times 10^4; n = 2$) than those with diarrhea ($1.9 \times 10^8$, $n = 5$), an observation that was consistent with the earlier study.8 All volunteers cleared their fecal oocysts by day 20 postchallenge and remained oocyst-negative throughout the rest of the study period. Five volunteers experienced a diarrheal illness, but had no detectable oocysts. However, the clinical illness in these subjects was indistinguishable in onset, duration, and constellation of signs and symptoms from that seen in symptomatic volunteers who were excreting oocysts. One additional volunteer (#65) did not have diarrhea or detectable oocysts, but met the criteria for gastrointestinal symptoms, which included nausea (one day only), abdominal pain, and fecal urgency over the period from day 5 to day 15 postchallenge. Thus, all six subjects were categorized as presumed infection.

**Clinical outcome of oocyst ingestion.** A diarrheal illness was documented in 10 (58.8%) of 17 subjects (Table 1). None of these subjects were positive for bacterial enteropathogens. Parameters of illness include all periods of time in which volunteers fit the criteria for symptoms or diarrhea. The median incubation period was five days (range = 3–12 days) with a duration of diarrhea and/or gastrointestinal symptoms of approximately 155 hr (6.5 days). The total number of unformed stools produced over the duration of
illness was a median of 10 (range = 3–35). Total stool weight during diarrheal episodes was 1.27 kg (median) with the highest weight of 4.63 kg occurring over a 14-day period. Thirteen (76.5%) challenged volunteers were classified as having presumed infection. Of these, seven were positive for fecal *Cryptosporidium* oocysts. Mild symptoms without diarrhea occurred in one of the 13 volunteers with presumed infection. Two individuals were asymptomatic, but infected, since fecal oocysts were detected on day 5 and days 11–15, respectively, following challenge. Four (23.5%) volunteers challenged with 500 or 5,000 oocysts remained asymptomatic and negative for fecal oocysts throughout the six-week study.

Volunteers were assigned to a challenge dose irrespective

![Table 1](image)

<table>
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<th>Volunteer number</th>
<th>Intended dose</th>
<th>Incubation period (days)</th>
<th>Duration of diarrhea* (hours)</th>
<th>Total no. of unformed stools</th>
<th>Total stool weight (kg)</th>
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* Values given for duration of diarrhea, number of unformed stools, and total stool weight represent cumulative totals for all periods of time in which volunteers fit the criteria for diarrhea.
of their prechallenge IgG absorbance index. Analysis of volunteers by clinical outcome category (i.e., diarrhea, mild/no symptoms, presumed uninfected) showed that the mean absorbance indices did not differ \((P = 0.59)\). As in our earlier study,\(^{20}\) the likelihood of infection was significantly influenced \((P = 0.046)\) by the magnitude of the oocyst challenge dose received; a geometric mean dose in infected volunteers (confirmed or presumed) was 9,316 oocysts versus 1,585 oocysts in the presumed uninfected. Furthermore, the magnitude of the challenge dose was not associated with a variety of other infection or illness parameters, including onset, duration or intensity/severity. In contrast, the challenge dose did have an impact on the development of a diarrheal illness, which was more likely to occur \((P = 0.05)\) when subjects ingested 10,000 or more oocysts (Figure 3).

Comparison of illness in volunteers with or without pre-existing anti-\(C.\ parvum\) IgG revealed that there was no significant difference in attack rate for diarrhea (antibody+ = 51.7% versus antibody− = 58.8%). However, differences among presumed infected subjects were seen in the number of unformed stools produced (Figure 4A) and the duration of diarrhea (Figure 4B). In these subjects median values of 10 unformed stools (95% confidence interval [CI] = 5.8, 19.6) and 154.5 hr of diarrhea (95% CI = 77.6, 212.6) were significantly higher than the four unformed stools (95% CI = 1.6, 12.4) and 48 hr of diarrhea (95% CI = 35.7, 99.1) observed in serologically negative volunteers \((P = 0.014\) and 0.028, respectively). When the analysis was restricted to DFA-positive, symptomatic individuals, individuals with pre-existing anti-\(C.\ parvum\) IgG had a 2.3-fold increase in the duration of diarrhea and a 2.1-fold increase in the number of unformed stools. However, these increases were not statistically significant, due at least in part to the lower \(n\) values. Thus, these measurements show that volunteers with pre-existing antibody to \(C.\ parvum\) experienced a more severe illness. Other parameters, such as the incubation period for diarrhea and the total unformed stool weight, did not differ significantly between the two groups.

**Postchallenge serum IgG response.** The change in absorbance following challenge was calculated for each volunteer and compared with the prechallenge absorbance value (Figure 5). Volunteers having the highest prechallenge absorbance values did not show a significant increase when re-exposed to \(C.\ parvum\) oocysts, indicating that maximum response had already been attained. In contrast, individuals with lower prechallenge IgG levels (0.1–0.75 net absorbance) responded to oocyst challenge with a boost in antibody levels. Overall, postchallenge net absorbance values did not show an association with challenge dose \((P = 0.46)\), presence \((P = 0.73)\), onset \((P = 0.91)\), duration \((P = 0.71)\) or intensity \((P = 0.56)\) of detectable oocysts occurrence \((P = 0.18)\), or severity (i.e., duration, number of diarrheal ep-
The present study was undertaken to address the latter point and to further examine the effect of pre-existing antibody on the severity of illness and intensity of infection, as well as the effect of parasite challenge on the level of serum IgG. The definitions used when the volunteer studies were initiated were adopted from the traveler’s diarrhea literature. To date, Cryptosporidium studies on a total of 89 volunteers have been completed. Retrospective analysis of these subjects has prompted us to re-examine and modify our original definitions to more closely reflect our experience with Cryptosporidium infections. In this analysis, it was apparent that some individuals had experienced unformed stools and gastrointestinal symptoms, but did not meet the strict definition of diarrhea, i.e., ≥ 3 stools in 8 hr or ≥ 4 in 24 hr. Furthermore, we noted a small number of individuals who had an illness characteristic of cryptosporidiosis, but in whom we could not detect oocysts by direct immunofluorescence. Thus, after careful consideration and discussion, the definitions of diarrhea and infection that are included in this report (see Materials and Methods) were formulated.

Infection, which was previously limited to the detection of fecal oocysts, was the most difficult to define. While the presence of oocysts is an obvious indicator of replicating organisms, the detection methods that are available may not always be sensitive enough to reveal low oocyst concentrations. Indeed, when the stools of recent symptomatic volunteers were tested by the more sensitive method of flow cytometry,32 oocysts were detected in otherwise negative stools.

As stated above, despite the DFA-negative oocyst results, several volunteers exhibited a clinical picture of cryptosporidiosis and could not reasonably be ignored. These individuals have been categorized as presumed infected for the following reasons: all were negative for bacterial enteropathogens, the constellation of signs and symptoms, onset and duration of the illness were indistinguishable from DFA-positive volunteers, and the DFA method has limited sensitivity. However, to examine the findings fully, we have included the data analysis from both points of view.

In this study, the presence of pre-existing serum IgG correlated with a relative resistance to infection as demonstrated by the considerable increase in ID₅₀ in these volunteers. Indeed, only one (33%) of three individuals with pre-existing serum IgG had evidence of infection with 500 C. parvum oocysts, a proportion that may change somewhat with increased numbers of volunteers challenged at this dosage. This level of exposure (i.e., 500 oocysts) is thought to exceed usual oocyst concentrations in water. In comparison, five (83%) of six antibody-negative volunteers were infected after receiving 500 oocysts. However, this protective effect could be overcome when 10,000 or more oocysts were ingested.

Given the seroprevalence of the general population and this relative resistance to low oocyst concentrations, a significant portion of individuals may experience protection from infection in situations where low oocyst concentrations are present. The annual risk for Cryptosporidium infection in the non-AIDS population from consumption of tap water (unit concentration of 0.001 oocysts/L) was estimated to be 0.0009 (CI = 0.003, 0.0028). Applying this point estimate to a city of four million with an estimated 25% seropositivity...
would result in approximately 3,000 cases of *C. parvum* infection instead of the 4,000 cases expected in a similar seronegative population.

Furthermore, pre-existing anti-*C. parvum* serum IgG had an effect on the development of illness, which in these volunteers was correlated \((P = 0.05)\) with a high challenge dose \((\geq 10,000\) oocysts\). Pre-existing serum IgG also appeared to have an effect on the severity of illness experienced by these volunteers. Although the attack rate for diarrhea was the same in those with or without pre-existing serum antibody, the number of unformed stools passed and the duration of the diarrheal episode were significantly increased. This result was not seen in a study of volunteers who were rechallenged with the homologous isolate after one year.\(^9\) In these subjects, the number of stools and the duration of illness were decreased. This apparent discrepancy may be the result of several important differences between the two studies. First, only one of 19 volunteers had demonstrable serum antibodies to *Cryptosporidium* antigens (ELISA) at the time of rechallenge. It is possible that the lack of serum antibodies may be an indicator of a weak immune response in the intestinal mucosa. Second, the rechallenge volunteers were exposed to a homologous isolate. In contrast, the volunteers with high levels of pre-existing antibodies were likely exposed to heterologous isolates on an unknown number of occasions prior to this exposure. In other systems such as *Giardia*, the immune response to a homologous isolate results in a different level of protection than with a heterologous isolate.\(^34\) Furthermore, it now appears that *Cryptosporidium* isolates are genotypically and phenotypically diverse,\(^35-37\) which may translate to differences in virulence and vigor of the mucosal response. The increased severity of illness in the antibody-positive volunteers supports the view of diarrhea as an integral part of the protective response to enteric organisms. The enhanced response in sensitized individuals is presumably a result of increased production of cytokines and other modulators that have an effect on mucosal barrier permeability. Studies designed to test this hypothesis are in progress.

Another major finding of the present study was the significant decrease \((P = 0.05)\) in the number of symptomatic volunteers shedding detectable levels of oocysts. This result suggests that if individuals with pre-existing antibody acquire infection, the immune response often significantly limits the formation of oocysts and, thus, decreases the opportunity for secondary transmission. Serum and secretory antibody collected during the study will be useful in identifying the exact life stage(s) and antigens that are targeted in an effective immune response.

Finally, while the relative level of serum IgG in volunteers with pre-existing antibody did not appear to be an additional factor in the development of infection or illness, it did relate to the boosting effect of oocyst challenge. That is, individuals with net absorbance values of approximately 0.75 or more showed little to no increase in IgG levels after challenge, while those with net absorbances of 0.1–0.75 often had dramatic increases in IgG. This suggests that there may be an upper limit of serum IgG response that is attained with multiple exposures. It is not known how long the serum IgG persists in these individuals; however, plans to follow the volunteers on an annual basis should shed light on the decay of this protective response.

In summary, the results of this study indicate that anti-*C. parvum* serum IgG correlates with protection to exposures to low numbers of the parasite. It should be understood, however, that the study was not designed to examine a mechanism of action and cannot distinguish between a direct, neutralizing effect and a surrogate marker of another effector response. It is possible that specific serum IgG may reach the mucosal surface, as has been shown for other pathogens,\(^38\) or that serum antibody may simply be a marker of an effective secretory and/or cellular response to infection. Studies using the serum and secretory antibodies, as well as an endoscopic biopsy specimens, collected during the course of this experiment are currently being used to address these issues.

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