IMMUNE RESPONSIVENESS ASSOCIATED WITH EXPERIMENTAL ENCEPHALITOZOOON INTESTINALIS INFECTION IN IMMUNOCOMPETENT RATS

*ICJ Omalu, DD Duhlinska, GI Anyanwu, VA Pam, PU Inyama

Abstract

Purpose: Microsporidial infections have been recognized as an increasingly important infection in immunocompromised patients, particularly those infected with HIV/AIDS. This study was designed to study immune responses associated with experimental Encephalitozoon intestinalis infection in immunocompetent rats. Materials and Methods: Thirty-four Rats in 3 groups, A (Control), B (Intraperitoneal) and C (Oral) were given injections of 0.5 ml of 2 x 10^6 of purified spores of Encephalitozoon intestinalis spores and were observed for serum specific IgG for 21 days using both direct and indirect ELISA. Results: In indirect ELISA, specific IgG were detected on days 7, 14 and 21 for the group B rats and on day 21 for group C and in direct ELISA method, specific IgG were detected in-group B rats on days 7 and 21, for group C rats on day 21 only, while in the control rats, specific IgG were not detected. There was no significant difference between the direct and indirect methods (df=1, X^2, P>0.05). E. intestinalis was observed in stool samples of rats in 1/12 (08.33%) on days 14 and 21 in group B, and in 4/10 (33.33%), 3/10 (25.00%) and 2/10 (16.67%) on days 7, 14 and 21 respectively in group C. In group A, which is the control rats, no microsporidia were observed on days 0, 7, 14 and 21. Conclusions: There were no changes in the T-lymphocyte counts of rats prior to and after inoculation with spores. Extensive lesions were observed along the intestinal walls especially on the middle and lower sections of group C rats only.

Key words: Microsporidia, Encephalitozoon intestinalis, ELISA, T-Lymphocytes

Microsporidia have long been recognized in many invertebrate and vertebrate species. They have been regarded as organisms of low pathogenicity in most mammalian species, causing spontaneous natural disease only under unusual circumstances. In mammals, three basic types of host/parasite responses have been described: a) A hypersensitive immune response reported in very young carnivores, b) A latent or clinically undetectable infection seen in euthymic mice and rabbits and, c) Clinical disease due to parasite proliferation in immuno suppressed animals and athymic mice.

Immune responses are designed to recognize and eliminate foreign agents including microorganisms. The immune systems of mammals have evolved the ability to generate responses that are acquired, specific and anamnestic so that the microorganisms are eliminated more efficiently after second and subsequent exposures. Immune responses expressed in mammals principally involve the interactions of leukocytes, which are regulated by B and T lymphocytes. The time required for specific serum antibodies to be expressed after experimental inoculations of Encephalitozoon cuniculi into euthymic mice or rabbits is affected by the route of infection. Microsporidia - specific antibodies were first detected in the sera of rabbits and mice approximately 7-10 days after parenteral inoculations (intravenous, intraperitoneal, subcutaneous or intracerebral) and were not detected until approximately 21 days after oral inoculation. Immunoglobulin M (IgM) responses occurred first and then declined as the IgG responses increased. The IgG levels in serum tended to peak about four to six weeks after parenteral inoculation and usually remained high (> 800 as measured by ELISA) for the life of the host.\(^2\,^3\) The expression of antibody responses in immunologically immature animals infected with microsporidia generally was delayed but these animals eventually achieved the same levels of specific serum IgG expression as observed in animals infected as adults.\(^4\) Mice infected with E. cuniculi remained chronically infected and continued to express specific serum antibodies, whereas mice inoculated with killed E. cuniculi only transiently expressed specific antibodies.\(^5\) Mice inoculated with viable E. intestinalis organisms cleared their infections and also transiently expressed antibodies, whereas IFN-γ receptor knocked out mice, which did become chronically infected and survived experimental E. intestinalis infections, did continue to express antibodies.\(^6\) This study shows the detection of serum specific IgG and other immune responses to E. intestinalis.

Materials and Methods

Antigens

Encephalitozoon intestinalis were obtained from stool,
washed by repeated centrifugation and purified by density gradient centrifugation with percoll® and stored in phosphate buffered saline (PBS) at 4°C for use in coating the plates for enzyme-linked immunosorbent assay (ELISA).

**Experimental animals and organism**

Six to ten week old male and female rats were used in the study. Three groups of 12 rats per cage were provided food and water and housed in a micro-insulator environment. Rats were purchased from the University of Jos, animal farm.

**Inoculation and monitoring of infections**

Twelve rats (group B) were given intraperitoneal (IP) injections of 0.5 mL volumes of $2 \times 10^6$ E. intestinalis spores, 12 rats (group C) were given 0.5 mL volumes of $2 \times 10^6$ of E. intestinalis spores orally and the other 10 rats (group A) served as control.

Sera were obtained on days 0, 7, 14 and 21 and assayed by both direct and indirect ELISA for specific IgG antibodies to E. intestinalis.

**Stool analysis and CD4+ cell count for rats**

Stool specimens were analysed on days 0, 7, 14 and 21 for the presence of Microsporidia by modified Giemsa staining technique.8

CD4+ cells were counted using the Cyflow machine on days 0 and 21 only.

**Direct and indirect ELISA**

To measure the relative levels of E. intestinalis serum specific antibodies a modified and indirect ELISA method9,10 was employed. For direct ELISA, E. intestinalis spores was adjusted to $2 \times 10^6$/mL in PBS as antigens and 100 µL was added to each of 96-micro ELISA plate and incubated with 100 µL/well of IgG (whole molecule) peroxidase conjugate (Sigma product. No. A4416) containing bovine serum albumin (BSA: 3%w/v) with O-phenylenediamine dihydrochloride (OPD) 0.4 mg/mL in 0.05 M phosphate citrate buffer, pH 5.0 containing 0.03% sodium probate. ELISA plates were read at 405 nm. ELISA readings greater than the positive control from calculation were declared positive.

**Examination of intestinal mucosae for lesion**

Three sections of the intestinal mucosa such as upper, middle and lower, were processed and examined for lesions. All rats infected intraperitoneally and orally as well as controls were examined.11

**Results**

**Stool samples of rats**

In group A (control rats), no microsporidia were observed on days 0, 7, 14 and 21 when their stool samples were examined. For group B, which were given intraperitoneal injections, E. intestinalis was observed in 1(08.33%) of the 12 rats on days 14 and 21, while in the 10 rats in group C, E. intestinalis occurred in 4(33.33%), 3(25.00%) and 2(16.67%) on days 7, 14 and 21 respectively (Table 1). There was no significant difference ($\chi^2, P > 0.05$) in the number of spores shed by the rats inoculated with spores and those by the control group.

**CD4+ cells/mL of rats**

There were no changes in the T-lymphocytes counts of rats prior to and after inoculation with spores. The mean CD4+ cells/mL of rats remained 90 cells/mL except for group C that had 89 cells/mL on day 21. There were no significant differences ($\chi^2, P > 0.05$) in the cell counts before and after inoculation with spores of E. intestinalis in the three groups (Table 2).

**Serum-specific IgG of rats**

Prior to inoculation, rats were screened for antibodies against intestinal microsporidia spores by both the direct and the indirect ELISA, with E. intestinalis spores as antigens. Pre-inoculation sera and healthy-rats-control sera did not react with any of the parasite spores. Rats were also screened for serum parasite specific antibody response 7-21 days post inoculation.

For the indirect ELISA, specific IgG was detected on days 7, 14 and 21 for the group B with rats given intraperitoneal injections. In group C with rats given oral inoculation, specific IgG were detected on day 21, while none was detected in the control rats (Fig. 1)

<table>
<thead>
<tr>
<th>Table 1: Prevalence of Encephalitozoon intestinalis in stool specimen of rats on days 0, 7, 14, and 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats/batch Days No.(%)+ ve No.(%)+ ve No.(%)+ ve</td>
</tr>
<tr>
<td>A (Control, n=10) 0 - 0 - 0 -</td>
</tr>
<tr>
<td>B (Intraperitoneal, n=12) 0 - 1 (08.33) 1 (08.33)</td>
</tr>
<tr>
<td>C (Oral, n=12) 0 - 4 (33.33) 3 (25.00) 2 (16.67)</td>
</tr>
<tr>
<td>Total (34) 0 - 4 (11.76) 4 (11.76) 3 (08.82)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2: Mean CD4+ cells/mL of rats on days 0 and 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats/batch Days</td>
</tr>
<tr>
<td>(n=34) 0 cells/mL 21 cells/mL</td>
</tr>
<tr>
<td>A (Control 10) 90 90</td>
</tr>
<tr>
<td>B (Intraperitoneal 12) 90 90</td>
</tr>
<tr>
<td>C (Oral 12) 90 89</td>
</tr>
</tbody>
</table>
In the direct ELISA method, specific IgG to *E. intestinalis* were detected in group B rats on days 7 and 21. For group C rats IgG was detected on day 21 only, while in the control rats, specific IgG were not detected (Fig. 2). There were no significant differences between the direct and indirect method (df=1, $\chi^2$, $P>0.05$) in the detection of serum specific IgG.

**Tissue examination of small intestinal mucosae**

*Encephalitozoon intestinalis* were not detected in the tissues of rats from groups A, B and C, however, rats in group C given oral inoculation had focal to extensive lesions along the intestinal walls especially on the middle and lower sections. Lesions were predominantly characterized by scattered miliary necroses and granulomas. Diffuse or focal lesions in the intestinal wall consist of inflammatory oedema with numerous macrophages. Rats in group B given intraperitoneal injections and group A (controls) showed no lesions along the intestinal mucosa.

**Discussion**

Microsporidia are common parasites of laboratory animals and recently are being recognized more frequently in humans, particularly in AIDS patients. As such, information gleaned from natural and experimental infections of laboratory animals provides a basis for understanding the host-parasite relationship of microsporidiosis in humans.

The usefulness of an animal model depends upon similarities with the human disease. Such comparisons are difficult at present because relatively little is known about the pathogenesis and clinical spectrum of disease in humans infected with microsporidia. To date, *Encephalitozoon* and *Nosema* are commonly reported in laboratory animals and insects and are only recently being reported with greater frequency in humans. Information from experimental microsporidial infections of mice and rats should increase our understanding of the pathogenesis and clinical course of microsporidiosis in humans. Microsporidiosis in mice and monkeys appears to parallel what we do know about human microsporidiosis.9

Since there is no *in vitro* culture system presently available, it has been impossible to produce enough antigens to screen for specific antibodies. This problem was circumvented by developing a procedure for the isolation, purification and sterilization of parasite spores from human stools. Apparently, the best preservation of the spore antigens is obtained when using gentle filtration, centrifugation in isotonic conditions and gradual addition of low concentrations of antibodies to the final faecal suspensions.

Healthy rats given *E. intestinalis* by intraperitoneal and oral inoculations displayed no clinical signs of disease and only sporadically shed spores in stools, which conforms to an earlier report on *E. cuniculi* and *E. hellem* on healthy monkeys.9 Monkeys given oral inoculation with microsporidia, which were immunocompetent at the time of microsporidia inoculation, still maintained a normal peripheral blood CD4+/CD29+ T lymphocyte levels (15-30%) and the clinical signs of immunodeficiency were absent. In the present study, the CD4+ T lymphocytes remained same prior to and after inoculation.9 This intact immune system was able to keep the parasites in check and prevented clinical disease though there were sub-clinical signs, which were detected at necropsy, which included inflammatory lesions occurring along the walls of the intestine. They also observed lesions in the pancreas, lung and spleen of athymic mice and ascites was common as well. These tissue sites of infection parallel several reports of microsporidiosis due to *Encephalitozoon* - associated hepatitis, peritonitis, sinusitis and nephritis which are usually observed in immunodeficient patients.13-15

*Encephalitozoon* is a microsporidia parasite that has a wide range of hosts including rodents, lagomorphs, carnivores and primates.16 Experimental *E. cuniculi* infections in
immunocompetent hosts produced only chronic asymptomatic brain and kidney lesions, in contrast to the inoculation of immunodeficiency animals such as athymic mice, resulting in lethal disease.9,15

The animals in this study expressed relatively high levels of specific antibodies, especially in the rats infected intraperitoneally, than those inoculated orally, which was earlier observed.9,16 Numerous inoculation routes have been used for establishing systemic microsporidial infections in laboratory animals, including intravenous, intranasal oral, intraperitoneal, intrarectal and intracerebral inoculations, although the lengths of time required for inducing systemic infections and the pathogenicity for these routes varied.16 Because autopsies are rarely permitted in immunodeficient patients, it is difficult to fully appreciate the clinical spectrum of Encephalitozoon - associated microsporidiosis and to ascertain which route of infection for microsporidiosis primarily occurs in immuno compromised patients.

The rat model used in this study has some advantages. The study has established Encephalitozoon infections in rats by both intraperitoneal and oral inoculations. The animals have expressed high antibody levels to microsporidia and parasites have been demonstrated in stools. Additional studies are required in evaluating diagnostic and chemotherapeutic methods in context of progressing immunodeficiency. Presently, antibodies are considered diagnostic for microsporidiosis in immunocompetent animals and parasite detection is required for diagnosing microsporidiosis in immunodeficient individuals. Additional studies are needed for comparing intravenous, intraperitoneal and oral inoculations in immunocompetent rats and mice with different species of microsporidia. Although it is believed that most natural infections in human occur by ingestion of organisms, inhalation has been reported as a likely route of infection18 and trauma may lead to infection.

Several studies using immunodeficient animals have described features similar to the infections observed in immunodeficient human patients. The disseminated lesions and tissue sites of infection that developed in SCID mice were similar to lesions that were reported in AIDS patients with Encephalitozoon infections. Athymic mice as a model to study host parasite interactions during E. cuniculi infections have been used. The liver and spleen were two preferential sites of infection and mean survival time of athymic mice inoculated intraperitoneally was three weeks.3,17,19 Microsporidiosis in immunodeficient animals will provide a useful model for studies of the microsporidial pathogenesis, mechanisms of resistance, immunotherapy and in evaluating potential antimicrosporidal agents.

This present study using healthy rats was particularly important for demonstrating that resistance to lethal disease is dependent on a functioning immune system which is consistent with the increased reports of disease associated with microsporidiosis in AIDS patients and other immunodeficient patients.

**Acknowledgement**

We wish to express our profound gratitude to Mr. Raphael of University of Jos Animal Farm for his assistance.

**References**


Source of Support: Nil, Conflict of Interest: None declared.