6-1985

Cryptosporidiosis in the Immunosuppressed Mouse and in Cell Culture

Mercy Prabhu Das

Abstract

CRYPTOSPORIDIOSIS IN THE IMMUNOSUPPRESSED MOUSE AND IN CELL CULTURE

by Mercy Prabhu Das

Cryptosporidiosis is a parasitic infection primarily of animals that has been recently recognized as an important disease in immunocompromised humans. Most cases to date have occurred in patients with acquired immunodeficiency syndrome (AIDS). Persons with abnormal humoral or cellular immune function, and even normal subjects, have also been affected.

To determine whether or not mice, when experimentally immunosuppressed, would develop cryptosporidiosis, a test was designed using the AKR/J strain. This strain was selected because, it was previously reported that it was the strain shown to respond most readily to the production of disease caused by the opportunistic protozoan, Pneumocystis carinii. Tests were done with these mice housed in a room with other mice. In another test the were housed in a vinyl isolator in a separate room. These mice were fed a low (8%) protein diet and dexamethasone in their drinking water. Results show that the mice kept in the room with

other mice, but in separate cages, developed intestinal cryptosporidiosis as well as Pneumocystis carinii in their lungs. However, only seventeen out of thirty mice that were placed in the isolator developed the infection. These results suggest that the organisms were latent in the mouse host.

In addition to the development of cryptosporidiosis in the immunosuppressed mice, an attempt was made to demonstrate the life cycle of the organism in cell culture. The results showed that Cryptosporidium completed its life cycle in a human foreskin cell line (LFS). Cryptosporidium had previously been successfully cultured by others in primary chicken kidney, porcine kidney and human fetal lung cells. This experiment demonstrates that an additional cell line can be used to grow these organisms in cell culture.

Loma Linda University

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by

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A Thesis in Partial Fulfillment

of the Requirements for the Degree

Master of Science in Microbiology

June 1985

Each person whose signature appears below certifies that this thesis, in his opinion, is adequate in scope and quality, as a thesis for the degree Master of Science.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people who have given of their time and effort to help make this project possible:

Dr. Edward D. Wagner, my major professor, for his encouragement, guidance, and interest in making this project a success.

Dr. Robert L. Nutter, for his suggestions, support and contribution of materials for the cell culture.

Drs. Bruce Wilcox and John Lewis, for their guidance in the research and in helping prepare this manuscript.

Ellen Stellhorn, technician of the Histology Laboratory, Autopsy Service of Campus Pathology, for preparing histological sections of the mice and chicken embryos.

Raydolfo Aprecio, for instruction and technical assistance in cell culture methods.

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INTRODUCTION

Cryptosporidium species is a coccidial protozoan of the intestinal tract. It has been identified in a wide range of vertebrates, having been reported in snakes (Szabo and Moore, 1984); avians: turkeys and chickens (Hoerr, et al., 1978), and mammals: rabbits, mice, guinea pigs, dogs, calves, sheep and monkeys, and man (Heine et al., 1984b) Fayer et al., 1985; Tzipori, 1983). The infection causes enterocolitis in turkeys, guinea pigs and calves and gastritis in snakes. Diarrhea and malabsorption are the outstanding clinical manifestations of the disorder produced in humans (Anderson and Hall, 1982).

Coccidia are common intestinal tract parasites composing the suborder Eimeriina and are generally transmitted by ingestion of contaminated food or water. The sporozoan parasite, *Cryptosporidium* was first discovered and described in the gastric epithelia of the laboratory mouse by Tyzzer (1907), and was named *Cryptosporidium muris*. It inhabits the brush border of the small intestinal mucosa in the variety of hosts described above.

Protozoa are classified in four main classes: Rhizopoda (amebae), Ciliata(ciliates), Mastigophora(flagellates) and Sporozoa. Sporozoa are non-motile, spore-forming, obligatory parasites. Cryptosporidium is classified under the following scheme (Levine, 1984):

Kingdom	Animalia
Subkingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoa
Subclass	Coccidia
Order	Eucoccidida
Suborder	Eimeriina
Family	Cryptosporidiidae
Genus	Cryptosporidium

Initially, Cryptosporidium was thought to be markedly host-specific, and new species were named as the organism was found in new hosts. Further research revealed that the mammalian species, at least, could be transmitted to a variety of different hosts, and some investigators even suggested that Cryptosporidium may be a single-species genus (Tzipori, et al., 1980). This could be due to its lack of host-specificity (Tzipori and Campbell, 1981).

So far, no attempts have been made to transmit Cryptosporidium of birds, reptiles, or fish to any host other than the one in which they were found. All cross-transmission attempts have been with Cryptosporidium of mammalian origin. According to Levine (1984), four species of each host class should be considered valid, at least at this time. They are C. crotali in the rattlesnake, C. meleagridis in birds; C. muris in mammals, and C. nasorum in the fish. At this time the species designation appears uncertain. Therefore, the procedure used by the majority of authors will be used, that is, to refer to the generic designation.

LIFE-CYCLE

The organisms within the suborder Eimeriina possess a reproductive life-cycle which entails a phase of sexual differentiation, or gametogony, which supplements the process of asexual multiple fission, or merogony (Figure 1). In this way, the female form, the macrogamete, can be fertilized by the male form, the microgamete, resulting in a zygote. The zygote secretes a protective lipid and protein shell, while transforming into an oocyst. The oocyst is the infectious form passed in host feces which may remain viable in the soil for up to one year (Lasser, et al., 1979; Trier, et al., 1974).

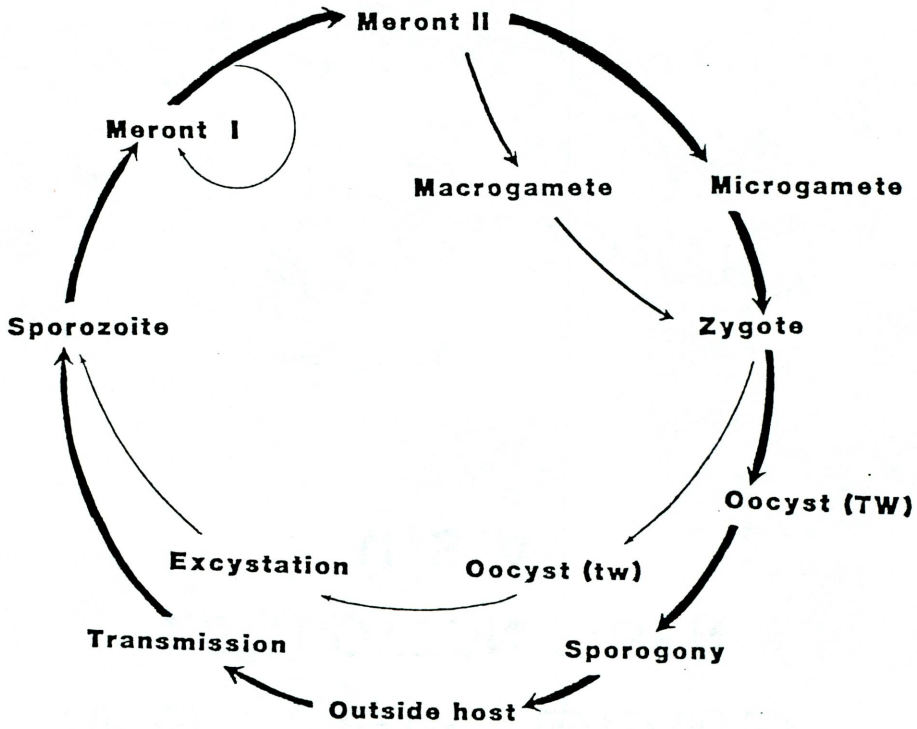

Figure 1. Life Cycle of Cryptosporidium

Two types of oocysts have recently been described. They are thick-walled and thin-walled oocysts. The thick-walled oocyst is passed outside the host and is responsible for transmission of the disease to a new host. The thin-walled oocyst is autoinfective, and is made up of a partly degenerate unit membrane. This oocyst will never leave the host, and in the absence of fecal-oral contamination and immune response, the infection may remain indefinitely (Current and Haynes, 1984).

During sporogony, two to four sporozoites are produced within an oocyst. When specific animal hosts ingest the oocysts, the sporozoites are released. These in turn invade the host intestinal epithelium and initiate the life-cycle (Bird and Smith, 1980). Ingested sporozoites undergo merogony at the host cell membrane forming a meront with eight first- generation merozoites. The merozoites invade adjacent cells and undergo merogony again to form four second-generation merozoites. The second-generation merozoites develop into macrogametes or microgametes after invasion of adjacent epithelial cells. The microgamete fertilizes the macrogamete to form an oocyst which is the infective form transmitted in fecal material to another host animal (Weisburger, et al., 1979). A characteristic that differentiates cryptosporidia from other coccidia is that these organisms infect only the microvillar area of intestinal epithelial cells, while others are found deep

within the cytoplasm of intestinal epithelial cells (Pitlik, et al., 1983; Booth, et al., 1980).

HISTOLOGY

In a duodenal biopsy from a patient with cryptosporidiosis, the villi are slightly reduced in height, and the crypts are lengthened. The lamina propria may contain an increased number of plasma cells and polymorphonuclear leukocytes. Some epithelial cells lining the villi may be slightly decreased in height and vacuolated. The organisms are two to six micrometers in size. They are usually found in the jejunum and ileum, but are also found in the stomach, duodenum and rectum. The organisms are usually seen attached to the brush border of the columnar absorptive cells (Lasser, et al., 1979; Bird and Smith, 1980). The morphologic changes that occur are believed to cause impaired digestion, malabsorption and diarrhea (Guarda, et al., 1983; Heine, et al., 1984).

PATHOLOGY

Disease: Most of the cases that have been reported were in immunosuppressed patients, for example, a renal-transplant patient with IgA deficiency (Weisburger, et al., 1979); a child with hypogammaglobulinemia (Lasser, et al., 1979); a woman with disseminated toxoplasmosis (Stemmerman, et al., 1980);and a cryptosporidiosis case

complicated by disseminated cytomegalovirus infection (Weinstein, et al., 1981). This shows that in cryptosporidial infections there is a frequency of immunosuppression as a predisposing factor, and the tendency to secondary infection (Meisel, et al., 1979). In addition, there have been cases in which cryptosporidiosis has been reported in healthy persons without any immunodeficiency. These are self-limited (Current, et al., 1983; Jokipii, et al., 1983).

Symptoms: Watery diarrhea is usually accompanied by no abdominal pain. There was a case of one patient, a child, however, who had chronic diarrhea for six years, and the organism was detected from a jejunal biopsy only two days before death (Sloper, et al., 1982). In addition to the major symptom, diarrhea, anorexia, weight loss, nausea, hepatomegaly and splenomegaly have also been found in individual cases, but these vary from one individual to another.

Treatment: A wide variety of drugs has been used in the treatment of cryptosporidiosis. These include paromomycin (Andreani, et al., 1983), sulfadiazine, sulfathalidine, sulfamethoxazole, pentamidine, pyrimethamine, trisulfapyrimidine, quinacrine, amphotericin B, metronidazole and trimethoprim. None of these drugs has

proven effective in the treatment of the disease(Stemmerman, et al., 1980; Weinstein, et al., 1981; Centers for Disease Control, 1982a). Spiramycin therapy has been recently tried in patients with cryptosporidial infections. Six out of ten patients had complete resolution of the diarrhea. Among the six patients, the amount of time varied from five weeks to sixteen weeks for resolution of the diarrhea. Treatment with spiramycin may result in significant clinical improvement even if the parasite is not eradicated from the stool (Portnoy, et al., 1984). The extent to which a drug may or may not be of help to the patient depends on the immunologic status of the patient. If there is immunodeficiency, the prognosis is grave. Death may be caused by water loss and electrolyte imbalance, malabsorption, or other complications such as toxoplasmosis or cytomegalovirus infection (Stemmerman, et al., 1980; Weinstein, et al., 1981).

DIAGNOSIS

Originally, diagnosis in humans was made only by intestinal biopsy (Nime, et al., 1976). Biopsy is no longer necessary since oocysts can be detected by direct stool examination. Since oocysts are small (half the size of a red blood cell) and of inconspicuous morphology, underdiagnosis was likely. Phase-contrast microscopy

permits observation and recognition of unstained oocysts. Sheather's sugar solution is used to concentrate the oocysts after which they can be observed under the phase-contrast microscope (Reese, et al., 1982; see Appendix B). Oocysts appear refractile and can be detected under 400X magnification. In wet mounts the oocyst contains one to six dark, large granules and many small granules. A typical oocyst is identified with one prominent dark granule and is surrounded by many small granules. In a mature oocyst, two to four sporozoites can be seen with or without the single prominent granule (Current, et al., 1983).

Certain authors found the formalin-ether sedimentation technique more satisfactory in terms of oocyst yield. The cyst wall also becomes permeable after formalin fixation so that the detailed structure of the oocyst is better recognized (Zakowski, et al., 1982). However, if viable oocysts are required, then the Sheather's sugar flotation method is the technique of choice.

The auramine O stain is as effective as the acid fast stain when a fluorescence microscope is available (Payne, et al., 1983). It is good for rapid screening. However, it is not specific for Cryptosporidium oocysts. Extraneous material and debris can also be stained and fluoresce. Nevertheless, oocysts can be differentiated on the basis of the size and structure of the oocyst wall. The acid fast stain demonstrates both the cyst wall and the internal

structures more definitely than the auramine O stain.

Iodine, which does not stain the oocyst, can be used to differentiate the oocyst of Cryptosporidium from yeast. Budding cells that are present also help to rule out Cryptosporidium (Ma and Soave, 1983).

The modified acid-fast stain is recommended for rapid screening. The modified Ziehl-Neelsen stain is often used. The oocyst stains red while the rest of the material on the slide stains blue.

ANTIBODIES TO CRYPTOSPORIDIUM

Serum antibodies were found in normal and immunodeficient persons with confirmed cryptosporidial infections. The indirect immunofluorescence (IIF) test was used to detect serum antibodies in these persons (Campbell and Current, 1983). This particular study demonstrated the presence of circulating antibodies to Cryptosporidium in the sera of immunologically normal individuals who had recovered from cryptosporidiosis, and in the sera of persons with the acquired immunodeficiency syndrome (AIDS) and persistent cryptosporidiosis.

The specificity of the antibodies was demonstrated by the marked reduction in the IIF titer. Antibodies were adsorbed onto the surface of Cryptosporidium oocysts. No reduction in titer was noted using other coccidian species. Also noted was the absence of overlap in IIF titers between

subjects who had recovered from cryptosporidiosis and subjects with no known exposure (Campbell and Current, 1983). The prevalence of antibody in a number of host species has been reported by Tzipori and Campbell, (1981). Evidence indicates that cryptosporidia infecting different species of animals have a common antigen detectable by immunofluorescence.

ZOONOSIS

Humans acquire cryptosporidial infection by the ingestion of oocysts. Transmission from animals is possibile by way of oral ingestion of the oocyst stage of the organism. After initiating a survey of Cryptosporidium in calves, Tzipori and Campbell (1981), and Babb, et al., (1982) reported a healthy male who became infected. Evidence that cryptosporidiosis is a zoonosis resulted from the finding that it occurred in caretakers of young calves with cryptosporidial diarrhea (Anderson and Hall, 1982; Blagburn and Current, 1983; Reese, et al., 1982; Centers for Disease Control, 1982b).

Successful completion of the life-cycle, from sporozoite to infective oocyst, of isolates of Cryptosporidium from humans and calves was demonstrated in endoderm cells of the chorioallantoic membrane of chicken embryos maintained at 37C (Current and Long, 1983). It was found that the inoculation of sporozoites on to the

chorioallantoic membrane was the only one resulting in complete development of Cryptosporidium. The only site of parasite development was within the microvillous region of endoderm cells of the chorioallantoic membrane. Since isolates from a human and a calf were used, it was found that they are morphologically and developmentally indistinguishable.

OBJECTIVES

Since cryptosporidial infections were reported in immunosuppressed persons, a test was designed to determine whether or not mice, when immunosuppressed, would develop cryptosporidiosis. The AKR/J strain of mice was selected because it was the strain shown to respond most readily to the production of disease caused by the opportunistic protozoan Pneumocystis carinii (Walzer, et al., 1979). It is the most common life-threatening opportunistic infection in AIDS patients. Transmission of Pneumocystis carinii is air-borne, although congenital infection has been reported. The dormant infection can be activated and elicited in mice and rats by immunosuppressing them.

During the first part of the experiment, the mice were kept in separate cages with other mice in a mouse room. For the second part of the experiment, the mice were housed in air-filtered isolators in order to determine whether

Cryptosporidium is air-borne or whether it was transmitted via some other route.

Since successful cultivation of Cryptosporidium was accomplished by others in the chicken embryo (Current and Long, 1983), development of the parasite in cell culture was attempted. Several flasks of mouse kidney fibroblasts were grown and maintained in a 5% CO_2 atmosphere at 37C. When the cells were monolayered on the bottom of the flask, sporozoites excysted from oocysts were introduced into these cultures.

During this time an article describing the development of Cryptosporidium in cell culture was published (Current and Haynes, 1984). In that paper it was shown that Cryptosporidium was grown in cultured human fetal lung cells, primary chicken kidney and porcine kidney cells. The various stages of development were recognized under a Nomarski differential interference contrast microscope.

In my experiment, primary mouse kidney, human lung and human foreskin cultures were used. Due to the uneven growth of the human lung and mouse kidney fibroblasts, they were discontinued from the experiment. Only the human foreskin cultures were used to culture Cryptosporidium.

MATERIALS AND METHODS

A. CRYPTOSPORIDIOSIS IN THE IMMUNOSUPPRESSED MOUSE

Part 1. Mice maintained in a general animal room

The AKR/J mice that were used in this study were obtained from The Jackson Laboratory, Bar Harbor, Maine. There were 22 females weighing between 18 and 20 grams. All 22 of them were placed on a normal 23% protein pelleted mouse diet and tap water for two weeks. Thereafter, 17 were separated, with 5 or 6 mice per cage, and fed a low (8%) protein diet. They were given drinking water ad libitum containing dexamethasone (1 mg/liter) plus tetracycline (1 mg/ml) to inhibit the growth of bacteria. The remaining 5 mice were placed in a cage to serve as controls and were continued on the normal diet and water.

Fecal material of each mouse was examined on the day of arrival as well as on day 14 following the two-week acclimatization period. Thereafter, they were checked on a weekly basis. The fecal smears were stained with a modified acid-fast stain (Henricksen and Pohlenz, 1981) and with a fluorochrome stain (Lennette, et al., 1980; see Appendix A). From day 42 onward, when some mice became moribund, they were killed, and the lungs and portions of small intestine were fixed in 10% buffered formalin. The intestines were sectioned and stained by the Gomori's trichrome, Periodic

Acid-Schiff, hematoxylin and eosin, and the May-Grunwald Giemsa stains. The lungs were removed from 15 mice that were killed, and lung impression smears and tissue sections were stained either with Gomori's methenamine silver or Giemsa stains.

Part 2. Mice in air-filtered isolators

A second experiment was performed in order to determine whether or not AKR/J mice would develop cryptosporidiosis when placed in air-filtered isolators. Thirty-five mice were obtained from The Jackson Laboratory. They were divided into seven cages with five mice per cage. Thirty mice were used as principals, and five mice were used as controls. The principals were fed a low (8%) protein diet and drinking water with dexamethasone and tetracycline. These seven cages of mice were placed in a vinyl isolator in which the in-coming air was filtered. Another surgical isolator was used when fecal pellets were collected and the mice weighed. Prior to use, both these isolators were disinfected and cleaned thoroughly with Wexcide. Several blood agar plates and tubes of thioglycollate broth and Sabouraud agar slants were placed in the two isolators to determine if there were any bacteria or fungi present. It was found that there were no bacteria or fungi present. The

isolators were placed in a new animal room that had not previously been used, nor had any animals been housed there.

Fecal pellets were collected twice weekly, and fecal smears were made of each specimen. Fecal smears were stained with the modified Ziehl-Neelsen stain as well as the auramine O stain (Casemore, et al., 1984). Moribund mice were killed so that their intestines and lungs could be retrieved and histologic sections made from them. The intestinal sections were stained with hematoxylin and eosin or the May-Grunwald Giemsa stains. The sections of lung were stained with Gomori's methenamine silver stain or with the hematoxylin and eosin stain.

Lymphocyte transformation tests were performed on a number of mice in order to determine their immune status. Spleens were aseptically removed and teased from the mice, and a single-cell suspension was prepared by mashing the spleen with a sterile applicator stick. The spleen cells were then washed with Roswell Park Memorial Institute (RPMI) 1640 medium by centrifugation at 1000 rpm for five minutes. One ml of ammonium chloride potassium (ACK) lysing buffer was added to the cells in the test-tube in order to remove the red cells. The cells were refrigerated for four minutes, at a temperature of 4C, after which 3 ml of RPMI 1640 medium was added and the cells centrifuged at 1000 rpm for 5 minutes. The cells were washed a second time. The cell concentration was adjusted to 2×10^6 viable cells/ml,

using 0.2% trypan blue as an indicator of cell viability. One hundred microliters of cells were added to the wells in a microtiter plate.

Spleen-cell medium control wells were included in this experiment. In addition, the general immunocompetence of the spleen-cell population was monitored with the mitogen phytohemagglutinin (PHA, 0.1 micrograms/well). The mitogen-treated cultures were terminated after 48 hours of incubation. During the final 8 hours of incubation, cultures were pulse-labeled with 1 microcurie/well of ^{3}H-thymidine. Cells from each microculture were harvested with a cell harvester onto strips of fiber-glass scintillation paper, and washed with saline. Circular discs containing cells were removed and transferred to glass vials containing scintillation fluid. The cell-containing discs were counted in a liquid scintillation counter (LS 250, Beckman Instruments, Fullerton, CA) for 10 minutes. Data were expressed as mean counts per minute for each mouse as well as a stimulation index. The results for the experimental animals were compared with that of the controls.

B. CRYPTOSPORIDIOSIS IN CELL CULTURE

The human foreskin cell cultures (LFS) were grown on 12mm sterile glass coverslips which were placed in individual wells in a 24-well tissue culture plate. When

the cells were monolayered, sporozoites were inoculated onto each coverslip. Cells were grwon and maintained in Dulbecco's Modified Eagle's Medium (Grand Island Biological Company, Grand Island, New York). The medium was supplemented with 10% fetal calf serum (Irvine Scientific Company, Santa Ana, California), 100 units of Penicillin/ml (Pfizer, Inc., New York, New York), 100 micrograms/ml streptomycin (Eli Lilly and Company, Indianapolis, Indiana), and 100 micrograms/ml fungizone (E.R. Squibb and Sons, Inc., Princeton, New Jersey). The medium was prepared in double distilled water and filter sterilized. The sporozoites were also maintained in the same medium. Sporozoites were excysted from oocysts that were incubated in trypsin and sodium taurocholate. Oocysts used for this experiment were obtained from a human, passed three times in calves, and three times in goats. These oocysts were provided in 2.5% potassium dichromate by Dr. William Current of Eli Lilly Research Laboratories, Indiana. Below is the excystation procedure used to obtain sporozoites from sporulated oocysts (Current and Haynes, 1984):

1. Oocysts stored in 2.5% potassium dichromate.
   The oocysts are viable and infective for up to 6 months.
2. 5 ml of oocyst suspension in 35ml Sheather's sugar solution (10 ml if oocyst count is less than 20 per field).

3. Mixture is stirred and centrifuged at 1000 rpm for 10 minutes.
4. Oocysts will be concentrated at the surface of the sugar solution, and they are removed from the top layer with a pasteur pipette and placed in a sterile centrifuge tube. Caution: Infectious material.
5. Oocysts are then washed three times by centrifugation with sterile phosphate-buffered saline (PBS, see Appendix B).
6. Washed oocysts are placed in antibiotic solution for three hours at 37C. 7. Oocysts are washed three times in PBS.
8. They are then placed in excystation medium (0.25% trypsin & 0.75% sodium taurocholate) for 30-60 minutes.
9. Sporozoites are released and washed twice in PBS, and resuspended in culture medium.
10. Twenty-four coverslip cultures, each in a well with a total volume of 2 ml, were inoculated with 1 ml of sporozoite suspension. The well was then filled with maintenance medium to bring the total volume up to 2 ml.

After inoculation of sporozoites, the cell cultures were incubated for four hours, at 37C in a 5% CO_2 atmosphere. This four-hour incubation period was to allow attachment and penetration of sporozoites into the cells. The culture medium was then removed and fresh medium was

added. Cell cultures were kept in a 5% CO_2 atmosphere at 37C throughout the study.

Each coverslip culture that was inoculated was examined after 12, 16, 24, 48, 72, 96 hours for the various developing stages of the parasite.

In order to prepare each cell monolayer for examination under the Nomarski differential interference contrast microscope, the coverslip was removed from the well, placed on a slide with another coverslip over it, and examined under oil immersion.

RESULTS

A. Cryptosporidiosis in immunosuppressed mice

The results of the first part of the experiment are given in Table I. Of the 17 mice used for the test on immunosuppressed mice that were kept in separate cages in an animal room along with other mice, 16 survived the test period of up to 64 days. One mouse died on day 42 and was lost to the study. It presumably died due to the pneumocystis-induced infection. All 15 remaining test mice developed the Cryptosporidium infection during the course of the test period. The diagnoses were based on positive fecal-pellet smears stained by the modified Ziehl-Neelsen staining procedure (Figure 2a). Fifteen of the 16 test mice that became moribund due to the pneumocystis infection, were killed so that the intestines and lungs could be fixed, sectioned and stained to determine the tissue infection status of Cryptosporidium and Pneumocystis carinii, respectively. All of the mice revealed parasite stages of Cryptosporidium as stained by the Giemsa method (Figure 2b). The level of infections in both the fecal material and sectioned intestinal tissues was high. The positive findings for the intestinal parasite was intermittent, with only a few mice showing the parasites as being present in the fecal material on consecutive examination days.

TABLE I. Occurrence of cryptosporidiosis in female AKR/J mice on an immunosuppressive diet/drug regimen

Mouse No.	At arrival	2 weeks on control diet	Number of days on regimen and infection status: positive (+) and negative (-)									
			13	20	27	34	42	49	55	62	64	75
1	-	-	+	-	+	-	+Killed					
2	-	-	-	-	-	+	+K					
3	-	-	-	-	-	+	+K					
4	-	-	+	-	-	+	+	+K				
5	-	-	-	-	-	+	+	+	+	Died		
6	-	-	+	-	-	+	+	+K				
7	-	-	-	-	-	-	+K					
8	-	-	-	-	-	+	-	+	+	-	-K	
9	-	-	-	-	-	-	+K					
10	-	-	-	-	-	-	+	+	+	-	-K	
11	-	-	-	-	-	-	D					
12	-	-	-	-	-	-	+	+	+	+K(d60)		
13	-	-	-	-	-	-	+	+	+	-	+K	
14	-	-	-	-	-	-	+	+	+	-	+K	
15	-	-	-	+	-	-	-	+	+	+K(d56)		
16	-	-	-	+	-	-	-	+K				
17	-	-	-	+	-	-	-	+K				
Controls												
18	-	-	-	-	-	-	-	-	-	-	-K	
19	-	-	-	-	-	-	-	-	-	-	-K	
20	-	-	-	-	-	-	-	-	-	-	-K	
21	-	-	-	-	-	-	-	-	-	-K		
22	-	-	-	-	-	-	-	-K				

Figure 2a. Mouse fecal smear showing oocysts of Cryptosporidium stained red by the modified Ziehl-Neelsen method (1000X).

Figure 2b. Intestinal histologic section of a mouse showing Cryptosporidium developmental stages on the villi. Section is stained by the May-Grunwald Giemsa stain (1000X).

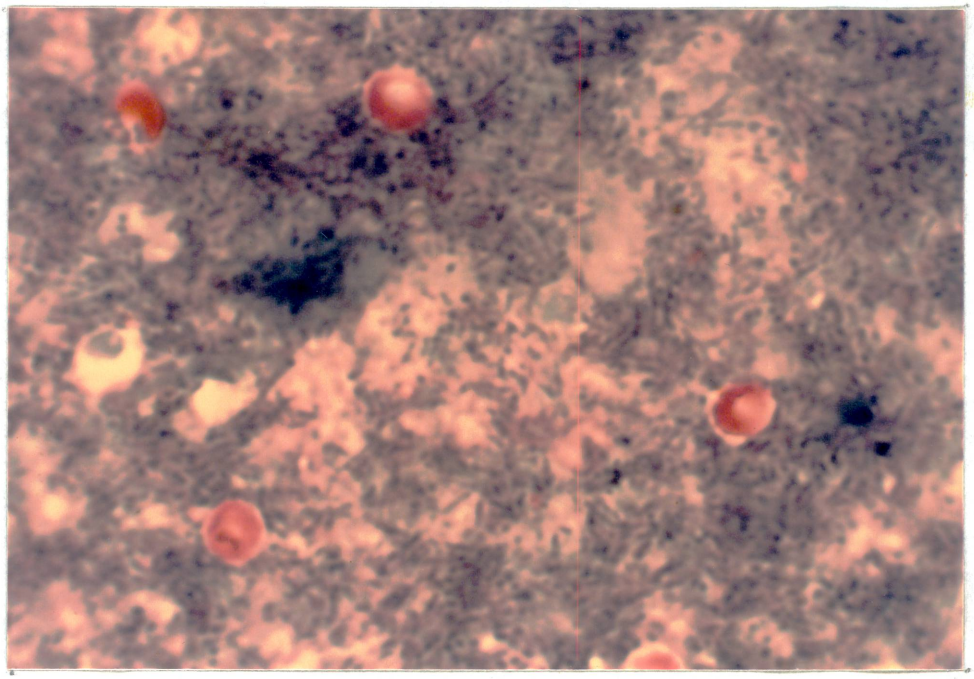

Figure 2a. Fecal smear stained by the modified Ziehl-Neelsen stain.

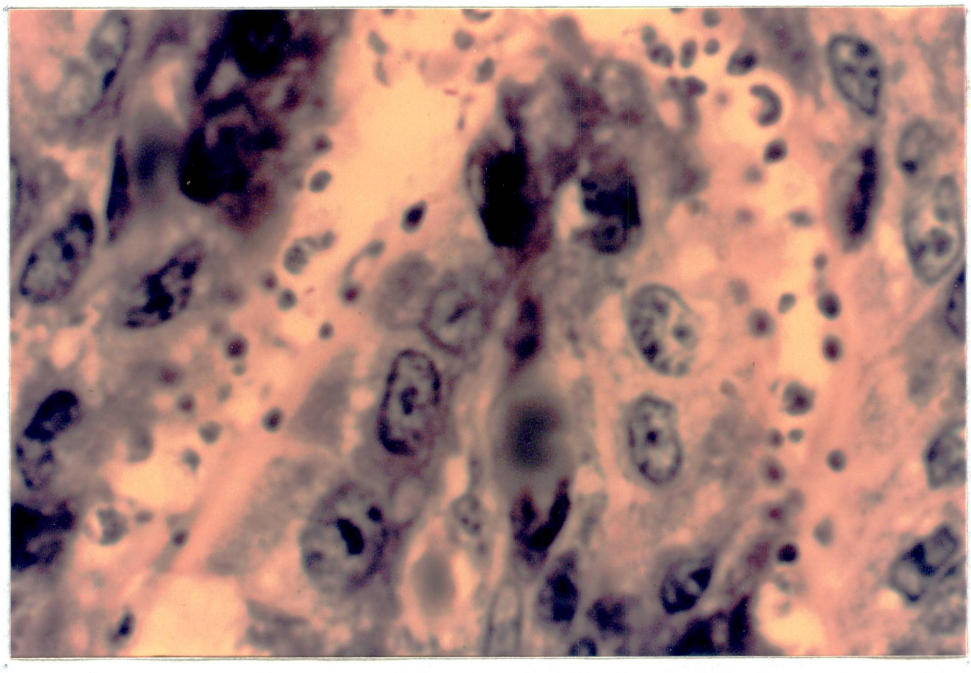

Figure 2b. Intestinal section stained by the May-Grunwald Giemsa method.

Mouse 5, which was consistently positive since day 34, was placed on the regular diet, with no dexamethasone on day 54. It died on day 57, and the intestinal tissue could not be used for histologic sectioning. Nevertheless, this mouse excreted oocysts in its fecal material until the day it died. Mice 8 and 10 were also placed on the regular diet on day 54, and they were found to be negative on days 62 and 64, respectively, when they were killed. Replacing the mice on the regular diet appears to have caused the cessation of oocyst shedding in the fecal material. However, since only two mice out of three showed no oocyst shedding, there is insufficient data to positively conclude that replacement on the regular diet did indeed cause fecal oocyst shedding to stop. There were Cryptosporidium organisms that were detected in the intestinal sections of these mice, namely, numbers 8 and 10.

All the immunosuppressed mice developed Pneumocystis carinii in their lungs. However, since mouse 11 and mouse 5 died and the tissues could not be used, it can only be assumed that they died due to this infection. This positive infection status was confirmed by the stained lung tissues. None of the five control animals was positive for Cryptosporidium. There were no symptoms seen in the infected mice, which agrees with published accounts of the infection in mice.

The results of the second part of the experiment in which the mice were placed in isolators revealed that over a period of 60 days, seventeen out of thirty mice were positive at some time or other during the course of the experiment. None of the control mice was found to be positive for Cryptosporidium. The results are tabulated in table II. There was only one mouse (VI-2) which was shedding oocysts from day 14 to day 39 during every test day until the day it was killed (day 42). Cage VII was the cage in which every mouse, when placed on the immunosuppressive diet, became positive. As indicated, the day of positive diagnosis varied considerably. Except for cage VII, oocysts were not detected in every mouse in a particular cage. The number of oocysts seen in the fecal smears was very low, ranging from three to four per slide.

Mouse VI-2 had the infection for a longer period of time than any of the other mice. All the rest of the mice were either positive during one check and negative during the next check, or did not have any trace of the infection. Intestinal histologic sections were negative for Cryptosporidium. No Pneumocystis carinii was detected in the lung tissue sections of these same mice.

Normal response to mitogenic stimulation occurred in the experimental as well as the control mice. The average counts per minute (CPM) for the PHA-stimulated wells of the control mice was 42,425, and the average CPM for the

TABLE II. Immunosuppressed mice in isolators.

Five mice per cage. Only positive mice are shown on test days.

Cage	Mouse No.	Number of days on test									
		7	14	18	21	25	28	32	35	39	42
I*											
II	1		+								
III	4	+									
	5	+	+				+				K**
IV	1									+	K
	5								+	+	K
V	1				+						K(d50)
	4								+		
	5						+				K(d48)
VI	1				+						
	2		+	+	+	+	+	+	+	+	K
	4						+				K
	5							+		D***	
VII	1			+							K(d46)
	2				+			+	+		K
	3							+			K(d46)
	4							+			D(d46)
	5		+		+					+	

* Cage 1, control mice, all negative
** K=killed, K(d no.)=killed on test day
*** D=died, D(d no.)=died on test day

TABLE III. Spleen weights (in grams) of selected control and principal mice.

Mouse No.	Weight of spleen
*I-3	0.56
*I-4	0.58
III-5	0.01
IV-2	0.02
VI-2	0.10
VII-2	0.01

* Control mice

TABLE IV. Results of lymphocyte transformation test using phytohemagglutinin (PHA). Results expressed as a mean count and a stimulation index (SI). SI calculated as follows:
$$\frac{\text{mean CPM in PHA culture - mean CPM in non-PHA culture}}{\text{mean CPM in non-PHA culture}}$$

CONTROL MICE

Mouse No.	Counts per minute (CPM)	Mean Count	Stimulation Index (SI)
I-1	PHA		
	81153.3	76546.3	4.12
	65239.8		
	83246.0		
	Non-PHA		
	18518.0	14949.9	
	11601.2		
	14730.6		
I-2	PHA		
	80566.6	70234.8	2.21
	47460.6		
	82677.2		
	Non-PHA		
	29341.8	21873.2	
	20964.6		
	15313.2		
I-3	PHA		
	28606.0	34622.3	9.14
	36916.8		
	38344.0		
	Non-PHA		
	2891.4	3413.7	
	3782.8		
	3566.8		
I-4	PHA		
	3322.3	7628.8	9.43
	6360.0		
	13204.2		
	Non-PHA		
	552.8	731.7	
	512.2		
	1130.0		
I-5	PHA		
	33405.0	23091.7	10.57
	34974.2		
	895.8		
	Non-PHA		
	2395.0	1995.3	
	2138.2		
	1452.8		

Average CPM (PHA)	42,425
Average CPM (Non-PHA)	8,593

Table IV- Continued

EXPERIMENTAL MICE

Mouse No.	Counts per minute (CPM)	Mean Count	Stimulation Index (SI)
III-5	PHA		
	70061.0	72868.2	79.6
	75675.4		
	74564.8		
	Non-PHA		
	885.6	904.0	
	980.2		
	846.3		
IV-2	PHA		
	7401.5	8659.1	52.4
	9898.6		
	8677.2		
	Non-PHA		
	126.4	162.3	
	199.5		
	160.9		
V-4	PHA		
	25708.2	40097.0	43.8
	48956.0		
	45628.9		
	Non-PHA		
	699.0	894.6	
	715.2		
	1269.9		
VI-1	PHA		
	25191.8	24555.2	56.9
	24524.8		
	23949.0		
	Non-PHA		
	383.6	423.4	
	487.7		
	399.0		
VI-2	PHA		
	45762.4	30663.9	83.6
	17766.0		
	28463.2		
	Non-PHA		
	220.6	362.3	
	421.0		
	445.3		
VII-2	PHA		
	98879.6	74384.3	82.8
	55275.0		
	68998.3		
	Non-PHA		
	927.6	887.5	
	835.6		
	899.2		

Average CPM (PHA)	41,866
Average CPM (Non-PHA)	606

experimental mice was 41,866. The average CPM of the non-PHA-stimulated wells of the experimental animals was 605.7, and that for the control animals was 8,593. This indicates that the corticosteroid and the low-protein diet did not affect the response to PHA. There was no difference in the PHA responses because there were equivalent counts in both the experimental as well as the control mice. From Table IV, it can be seen that the stimulation index ranged from 2.21 to 10.57 for the control mice, and that for the experimental animals was in the range of 43.8 to 83.6. The stimulation index was higher in the experimental mice than in the controls instead of vice versa.

Therefore, in order to compare the immunocompetence of the controls (I-1 to I-5) and the experimentals, the mean counts in the non-PHA-treated wells of each mouse can be compared. The mean counts per minute of the control mice were higher than those of the experimentals.

It was also found that the weights of the spleens of the immunosuppressed mice ranged from one-fifth to one-fiftieth the size of the controls (Table III).

B. Cryptosporidiosis in cell culture

Twenty-four cell cultures were inoculated with sporozoites of Cryptosporidium. Cell cultures were examined at different times in order to determine the development of

various stages of the life-cycle of Cryptosporidium. Each coverslip culture was placed on a slide with a coverslip over it, and examined under oil immersion, 1250X, using a Nikon Nomarski differential interference contrast microscope. Photographs were taken of the respective stages (Figure 3). The time of appearance and presence of the various stages are presented in table V.

At about eight hours after inoculation, there were a few free sporozoites seen in the culture medium. Most of the sporozoites had penetrated the human foreskin cells. Type I meronts were the first stage observed at 12 hours post-inoculation. Merogonous development took place, and by 14 hours, immature type I meronts with six or eight merozoites were seen. Immature type I meronts were characterized by a central refractile body with nuclei around the periphery of the organism. Mature type I meronts had merozoites within them as well as a refractile body. Mature type II meronts with four merozoites were observed at 24 hours post-inoculation. Mature type II meronts were seen with merozoites and a refractile body at 48 hours.

By 48 hours, sexual stages were present (macrogametes and microgametes); oocysts undergoing sporogony could also be observed at 72 hours after inoculation of sporozoites. There were more type 1 meronts than type 2 meronts that were seen in the cell culture.

Figure 3. Representative stages of development of Cryptosporidium in human foreskin cell cultures after inoculation of sporozoites. Stages were observed under a Nikon Nomarski differential interference contrast microscope (1250X). (A) Oocyst showing 3 sporozoites, (B) sporozoite above a yeast cell; (C) immature type I meront with central refractile body and nuclei around the periphery; (D) type II merozoite in the process of leaving the host cell; (E) mature type I meront with merozoites; (F) mature type II meront with merozoites; (G) macrogamete with prominent refractile body; (H) oocyst at 7 days post-inoculation.

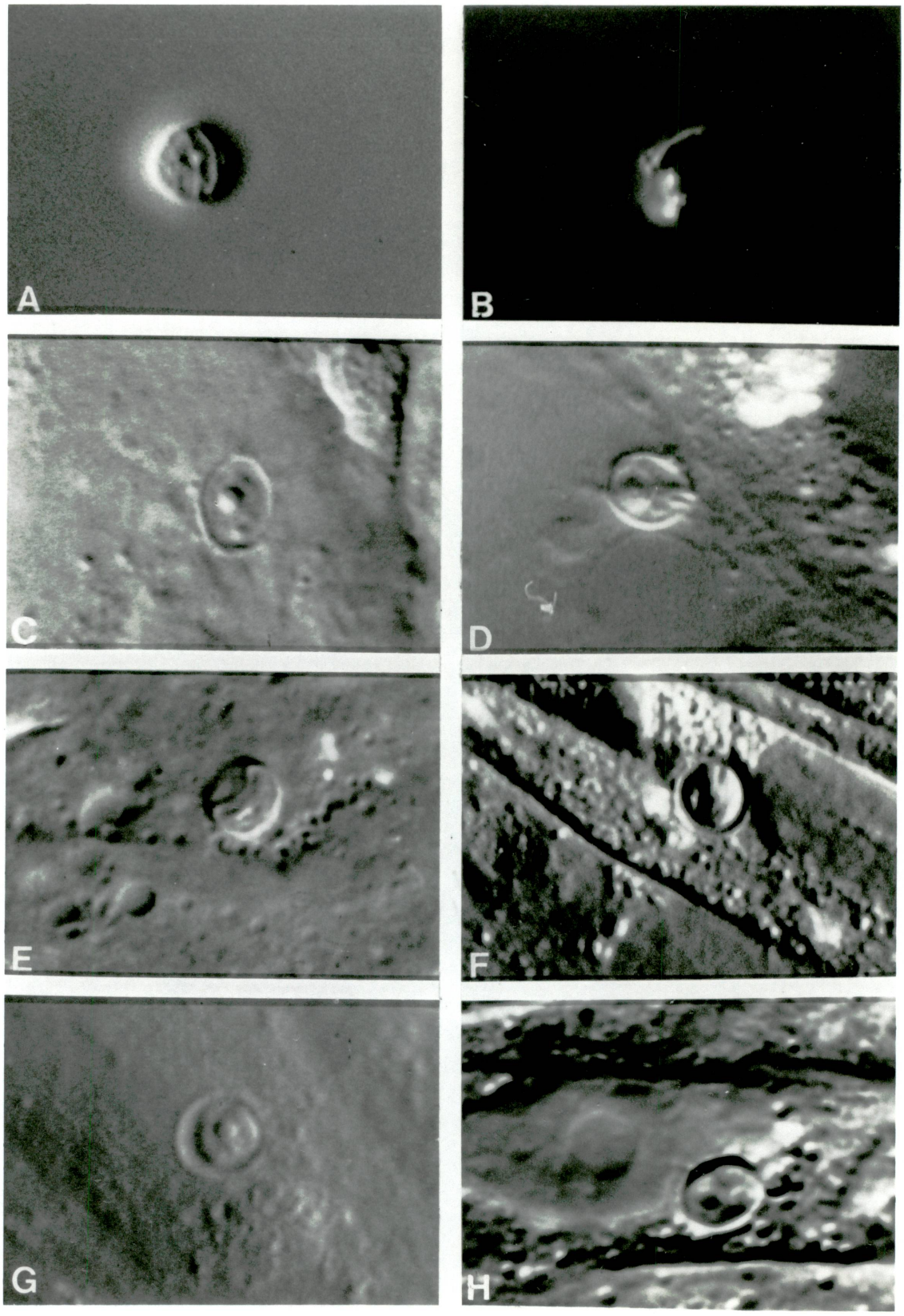
A
B
C
D
E
F
G
H

TABLE V. Developmental stages of Cryptosporidium in human foreskin cell cultures at selected times after inoculation of sporozoites. Stage seen in culture (+); stage not seen (0).

Time after inoculation (hours)	Meronts		Macrogametes/ microgametes	Oocyst
	Type 1	Type 2		
12	+	0	0	0
14	+	0	0	0
18	+	0	0	0
24	+	+	0	0
48	+	+	+	0
72	+	+	+	+
96	+	+	+	+
120	0	+	+	+
144	0	+	+	+
168	0	0	0	+
192	0	0	0	+

DISCUSSION

The results from the experiment with the immunosuppressed mice placed in the room with other mice show that these mice developed cryptosporidiosis without being inoculated with the parasite. In order for the mice to develop the infection, the parasite had to be air-borne or latent within the mouse host. The immunosuppression diet and drug regimen that were administered caused a depression in the immune status, thereby making it possible for the infection to increase. Dexamethasone, a corticosteroid, affects both the T and B cells, and hence the cell-mediated and humoral immune responses. With high dosages, there is a reduction in the size of various lymphoid tissues, for example, the spleen and lymph node (Claman, 1975). This was seen in the mice which were under the immunosuppressive diet and drug regimen and were used for the lymphocyte transformation test. When compared to the spleens of the control mice, the spleens of the immunosuppressed mice were approximately between one-fifth and one-fiftieth the size of the controls (Table III).

From the results of the lymphocyte transformation test, it can be seen that there was relatively more proliferation in the non-PHA-treated wells of the control mice than the experimentals. The mitogen, PHA, was used to measure the immune competence of the mice. Since there were equivalent

counts found in both the experimental and the control mice (Table IV), these reults could not be used to determine the immune status of the mice. PHA did not show any effects on the T-lymphocytes. Therefore, the average mean counts per minute of the non-PHA-treated wells were used as a measure of the immune competence ofthe mice. Control wells were not stimulated by PHA, nevertheless, there was slow division of the lymphocytes in these wells. Tritiated thymidine that was added would be incorporated into the DNA of these newly-dividing cells, and the lower radioactive counts seen in the experimental mice indicate that proliferation of T-lymphocytes was decreased. This is an indication of the suppressed immune status of the mice. The results from my experiment show equivalent counts in the PHA-treated wells of the control and the experimental mice.

According to Claman (1975), there would not be any difference in the proliferation of the T-lymphocytes between the immunocompetent and the immunosuppressed hosts when the immunosuppressed host has been treated with corticosteroids for an extended period of time. This can be seen in the results in Table IV.

The administration of the corticosteroid and the high carbohydrate diet resulted in the oocysts of Cryptosporidium to be detected in the feces by the modified acid-fast stain and the fluorochrome stain. To my knowledge, the results of this experiment are the first record of Cryptosporidium

infection developing in immunosuppressed mice which were not inoculated with the organism. The exact transmission of the organism in this experiment is not known, however it is known that it is transmitted by fecal-oral contamination. There have been no reports on air-borne transmission of Cryptosporidium.

In the experiment in which the mice were placed in air-filtered isolators, it was found that 56.7% developed the infection as detected in the fecal smears. This environment was controlled, and the air was filtered from the outside. Therefore this would rule out air-borne transmission from outside the isolator, and would suggest the presence of a latent infection.

If Cryptosporidium is transmitted through fecal-oral contamination, it would be expected that the oocysts that were excreted would be transmitted to the others in the same cage. However,if the number of excreted oocysts is low, then the possibility of transmission is small. In addition, if the concentration of oocysts is high, air-borne transmission within the isolator would be possible.
From the results, it can be seen that the number of oocysts that were found in the fecal smears was only about three or four per slide, and therefore the probability of air-borne transmission would be very low.

It can be seen from the table of results (Table II) that not all the mice in the same cage developed the

infection. A reason for this could have been due to the fact that if the mice ingested oocysts, the internal development of the parasite would have resulted in autoinfective oocysts that did not leave the host. Therefore, those mice that did excrete oocysts as detected in the feces, would have had the organism present in their intestine.

The histologic sections of these mice did not show any parasites in the microvillous region of the intestinal epithelial cells. Even in mouse VI-2 that did excrete oocysts that were detected during every consecutive fecal check, its intestinal section did not have any evidence of the parasite. Therefore, the infection must have been at a very low level, resulting in the organism failing to attach to the epithelial cells of the small intestine. Since Cryptosporidium is known for the extensive damage that it causes in intestinal epithelial cells which results in malabsorption, the mechanism by which it destroys or accelerates the loss of epithelial cells is unknown. Perhaps it damages the epithelial cells directly through some toxic effect or metabolic or physical effect. It has been suggested that the initial contact between the organism and the host-cell involves the shortening or absence of microvilli directly under the parasite (Figure 4). There is then the fusion of the parasitic envelope with the plasma membrane of the epithelial cell. A "feeder organelle"

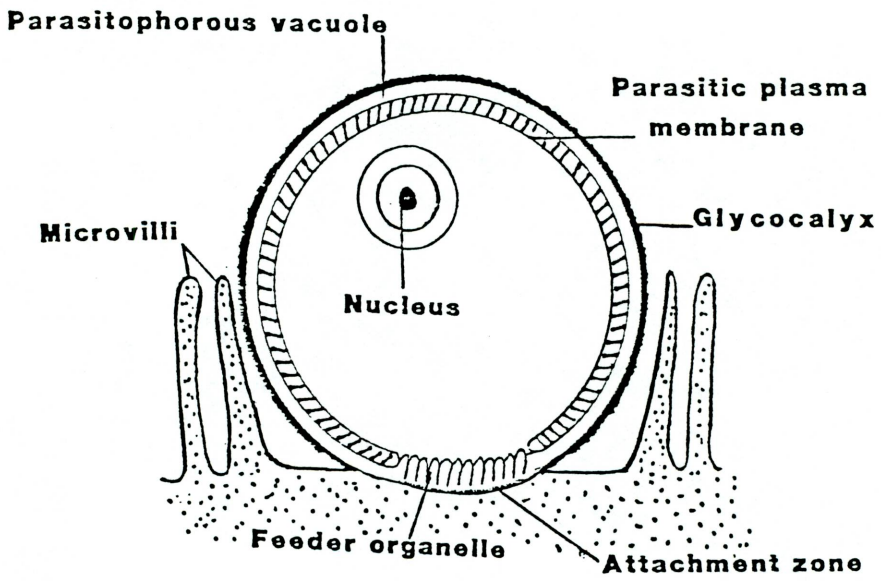

Figure 4. Basic ultrastructure of Cryptosporidium
(Adapted from Garza, D. 1983. Lab. Med. v. 14)

develops in the attachment zone and microvilli completely degenerate (Garza, 1983).

From the experiment in which Cryptosporidium was shown to develop in cell culture, it can be seen that isolates in goats have little or no host specificity since the human foreskin cells supported complete development from sporozoite to oocysts. It was also observed that there were more type 1 meronts than type 2 meronts. This is due to the fact that type 1 meronts have greater reproductive potential because of cyclic development and also because sporozoites that are released from thin-walled oocysts reinitiate the endogenous cycle. This is also one of the reasons that mice which may have the infection but do not excrete oocysts in the feces are found to have the organisms in their intestinal histologic sections.

This experiment describes an additional cell line in which Cryptosporidium can be cultured. The human foreskin cells grow uniformly and form monolayers in a relatively short period of time (three to four days).

SUMMARY AND CONCLUSIONS

Cryptosporidium is among the opportunistic agents implicated in intestinal disease, primarily in immunosuppressed persons. Data also support the contention that cryptosporidiosis occurs not only in immunocompromised patients, but also in immunocompetent individuals. Previously, the majority of cases were diagnosed after examination of small or large bowel biopsy material. However, recent cases have been diagnosed by recovering oocysts in the fecal material by flotation techniques (Garcia et al., 1983). In my experiment the Sheather's sugar flotation technique was used to concentrate the oocysts, and fecal smears were stained with the modified Ziehl-Neelsen technique and the auramine O stain.

Sherwood et al (1982) immunosuppressed inbred Porton mice and then inoculated them by gastric gavage with the oocysts of Cryptosporidium. However, the mice failed to become infected. On the other hand, my strain of mice that were immunosuppressed did develop the infection even though they were not given the infective stage oocysts. The AKR/J strain I selected was on the basis of the published results of Walzer, et al (1979) who showed this strain to become much more readily infected with Pneumocystis carinii

than did the several other strains tested. They did not check for Cryptosporidium.

If one correctly assumes that in my finding the parasite was latent in the mice tested, thus an opportunistic parasite, perhaps it would not follow that all mice tested for Cryptosporidium will have this condition. This would be suggested in the mice that were immunosuppressed and maintained in the air-filtered isolators where only one or two mice in a cage of five developed the infection. The very low level of infection in the "isolator" mice is a variation noted.

The growth of Cryptosporidium in cell culture in a cell line other than the ones already noted provides a means of studying its behaviour, development, and metabolism. In addition, it also provides a way in which potentially useful therapeutic agents can be evaluated.

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A P P E N D I X

APPENDIX A

Stains

1. Modified Ziehl-Neelsen stain

Carbol fuchsin solution

Solution A

Basic fuchsin	0.3 g
Ethyl alcohol (95%)	10.0 ml

Solution B

Phenol	5.0 g
Distilled water	95.0 ml

Solutions A and B are mixed.

Loeffler's methylene blue solution

Solution A

Methylene blue	0.3 g
Ethyl alcohol (95%)	30.0 ml

Solution B

Potassium hydroxide	0.01g
Distilled water	100 ml

Mix solutions A and B.

Decolorizer

Sulfuric acid	5% (volume/volume)

Staining procedure for the modified Ziehl-Neelsen stain (Henricksen and Pohlenz, 1981)

a. Heat fix smear on heating block (60C) for 5-7 minutes or flame fix.
b. Flood slide with carbol fuchsin; gently heat slide with bunsen burner until stain begins to steam. Do not let slide dry out.
c. Rinse with water.
d. Decolorize with 5% sulfuric acid.
e. Rinse with water.
f. Counterstain with methylene blue.
g. Rinse with water.
h. Air dry.

2. Auramine O stain

Solution 1

Auramine O	0.1 g
Ethyl alcohol (95%)	10.0 ml

Solution 2

Liquefied phenol	3.0 ml
Distilled water	87.0 ml

Combine and mix auramine-O solution with the phenol-water solution. Store in amber bottle; filtering stain is not necessary.

Staining procedure for the auramine O stain (Payne, et al., 1983)

a. Flood slide with auramine O stain for 15 minutes.
b. Rinse with water.
c. Decolorize with 0.5% HCl in 95% ethanol.
d. Counterstain with 2.5% potassium permanganate for 3 minutes.
e. Rinse with water.
f. Air dry.

APPENDIX B

Sheather's sugar solution

Sucrose	500.0 g
Water	320.0 ml
Phenol	6.5 g

1. Boil sugar solution until clear
2. Carefully add phenol and stir (under a fume hood)
3. Cool to room temperature before using

Phosphate-buffered saline (PBS, 0.01 M; pH 7.2)

Solution A

Sodium phosphate (monobasic)	1.38 g
Sodium chloride	8.50 ml
Water (double distilled)	1.00 liter

Solution B

Sodium phosphate (dibasic)	1.42 g
Sodium chloride	8.50 g
Water (double distilled)	1.00 liter

Mix 280 ml of solution A with 720 ml of solution B. Check pH and adjust if necessary.

APPENDIX C

Composition of antibiotic solution in which oocysts were placed.

Penicillin	5000 IU/ml
Streptomycin	5 mg/ml
Amphotericin B	20 micrograms/ml
Glucose	0.1 %

The antibiotics were mixed in phosphate-buffered saline.

Low-protein diet (obtained from ICN Nutritional Biochemicals, Cleveland, Ohio)

Casein high nitrogen	8%
Sucrose	78%
Vegetable oil	10%
Salt mixture	4%