Experimental *Cryptosporidium parvum* Infections in Immunosuppressed Adult Mice

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Five strains of adult mice were immunosuppressed with the synthetic glucocorticosteroid dexamethasone (DEX), administered either orally or intraperitoneally. The strains of mice used were C57BL/6N, DBA/2N, CBA, C3H/HeN, and BALB/cAnN. All mice were evaluated for susceptibility to *Cryptosporidium parvum* after intragastric inoculation with 10^6 oocysts per mouse. The DBA/2N, CBA, C3H/HeN, and BALB/cAnN mice given 0.25 μg of DEX per g per day orally (the dose and route previously used to infect rats with *C. parvum*) failed to develop chronic infections. However, the C57BL/6N mice sustained light infections during the entire 28-day experiment. The five strains of mice were also administered DEX intraperitoneally at concentrations ranging from 62.5 to 500 μg/day. Only the C57BL/6N mice given DEX at 125 μg/day developed chronic infections which persisted over 10 weeks, suggesting that the genetic background of the mouse plays a role in determining susceptibility to cryptosporidiosis following immunosuppression with DEX. We believe that the C57BL/6N mouse model will prove to be superior to other animal models for evaluating potential anticryptosporidial agents, as well as for elucidating the immunological defects that allow *C. parvum* to establish chronic infections, because of cost effectiveness and ease in maintenance, breeding, and handling. We also evaluated the C3H/HeJ/beige mouse (lacks natural killer cell activity) and the C57BL/6N mouse maintained on a low-protein diet to induce immunosuppression. Neither of these mice exhibited heavy cryptosporidial infections.

Cryptosporidium parvum is a coccidian parasite that infects the microvillous region of epithelial cells lining the digestive and respiratory tracts of vertebrates (11, 22). In immunocompetent hosts, *C. parvum* generally causes a short-term diarrheal illness that resolves spontaneously (7, 30). However, in immunocompromised hosts, *C. parvum* may cause a life-threatening, prolonged, choleralike illness. No effective therapy for cryptosporidiosis is available, and the prognosis for immunocompromised patients, especially those with AIDS, is usually inauspicious (10).

A suitable small animal model for chronic cryptosporidiosis is necessary to identify effective anticryptosporidial agents as well as to determine the immune abnormalities that allow persistent infections to develop. Immunocompetent laboratory animal models have been limited to neonatal mice (9, 24, 28), neonatal rats (24), and neonatal hamsters (16). The disease has also been studied in infant primates (21, 22, 24), fetal lambs (17), piglets (1), and calves (8, 11, 13, 24, 29). Cryptosporidiosis has been reported in adult wild mice (18) and adult guinea pigs (6). However, because of variations in the clinical presentation and patency of infection, determining the species of *Cryptosporidium* in guinea pigs remains controversial. The brevity of infection in all of these animal models does not allow sufficient time to suitably evaluate potential anticryptosporidial agents. As a result, a chronic-infection model for cryptosporidiosis would be extremely useful.

Chronic cryptosporidiosis has been reported for immunosuppressed rats (5, 26) and hamsters (27), athymic mice (14, 31), germfree adult mice (12), T-cell subset-depleted severe combined immunodeficient (SCID) mice, and the National Institutes of Health (NIH) III (*bg/nu/xid*) mice (20, 31).

Earlier attempts to establish chronic infections in adult mice by the use of immunosuppressive therapy have failed (24, 28). The aim of this study was to determine the susceptibility of five strains of mice immunosuppressed with the synthetic glucocorticosteroid dexamethasone (DEX), using various regimens of drug administration. The results revealed that C57BL/6N mice immunosuppressed by intraperitoneal injections of DEX at a dosage of 125 μg/day developed chronic cryptosporidiosis. The length and severity of the infections were determined by monitoring oocyst shedding intensities in fecal pellets and parasite colonization in mouse tissues.

MATERIALS AND METHODS

Animals. Female C57BL/6N, C3H/HeN, BALB/cAnN, DBA/2N, and CBA mice (Simonsen Laboratories, Gilroy, Calif.), age 6 to 8 weeks and weighing 15 to 20 g, were used. The mice were maintained in the Utah State University Laboratory Animal Research Center and isolated from other animals. A pilot study was performed to determine whether any of the five different strains of adult mice could be infected with *C. parvum* following immunosuppression with DEX (Sigma Chemical Co., St. Louis, Mo.). One group of mice (10 per strain) received DEX in the drinking water at a concentration equivalent to 0.25 μg/g/day. This dose had previously been shown to be effective in immunosuppressing rats and rendering them susceptible to infection by *C. parvum* (26). The drug was administered for 14 days prior to intragastric inoculation with 10^6 *C. parvum* oocysts. The weights of the mice and the volumes of drinking water they consumed were measured every other day to ensure a proper dosage regimen. A second group of mice (20 per strain) was equally divided into four subgroups, with mice in each

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subgroup receiving DEX intraperitoneally at a dose of 62.5, 125, 250, or 500 μg/day for 14 days prior to oocyst inoculation. All mice continued to receive DEX either orally or intraperitoneally following oocyst inoculation throughout the duration of the experiment.

An additional study was designed to determine the chronicity of *C. parvum* infections in C57BL/6N mice compared with nonimmunosuppressed mice. The first group of mice was given DEX for 14 days prior to oocyst inoculation. The second group of mice was retained as nonimmunosuppressed controls. Mice in both groups were inoculated intragastrically with 10^6 oocysts. Each group consisted of 20 mice, and the experiment was repeated twice.

Another study was designed to determine whether C3H/HeJ/beige mice, which lack natural killer cell activity, are susceptible to a *C. parvum* infection. Female C3H/HeJ/beige mice (Jackson Laboratory, Bar Harbor, Maine), age 6 to 8 weeks and weighing 15 to 20 g, were used. The group consisted of 10 mice that were inoculated intragastrically with 10^6 oocysts.

Another group of 10 C57BL/6N mice were maintained on an 8% protein diet (ICN Biochemicals, Inc., Costa Mesa, Calif.) (suitable for immunosuppression induction [15]) for 2 months prior to intragastric inoculation of 10^6 *C. parvum* oocysts.

Parasites. The *C. parvum* oocysts (Iowa isolate) used in this study were originally obtained from Harley Moon (U.S. Department of Agriculture, Ames, Iowa). The oocysts were produced in experimentally infected Holstein calves and purified from feces by using discontinuous sucrose gradients (3). Purified oocysts were stored in 2.5% potassium dichromate at 4°C. Oocysts (stored for less than 4 months) used for inoculation were prepared by washing with RPMI 1640 base medium (Sigma Chemical Co.) to remove the potassium dichromate. Each mouse was inoculated intragastrically with 10^6 purified oocysts in a volume of 100 μl of RPMI 1640 base.

Determination of oocyst shedding in infected mice. Fecal pellets were collected from mice to monitor oocyst shedding throughout the experiment. Pellets were resuspended in a volume of 2.5% potassium dichromate approximately equal to twice that of the feces and stored at 4°C. Fecal suspensions were smeared onto microscope slides and observed for the presence of oocysts by an oocyst-specific monoclonal antibody-based direct immunofluorescence assay (4). Smears were examined microscopically in a blind fashion and scored 0 to 4+ based on oocyst numbers. Scoring was as follows: 0, no oocysts detected; 1+, <5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; and 4+, >100 oocysts per smear.

Histologic examination of tissues. Mice were necropsied at weekly intervals postinoculation (p.i.). Tissue samples harvested from the lungs, liver, gall bladder, pancreas, spleen, stomach, duodenum, jejunum, ileum, cecum, and large intestine were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Stained sections were examined for *C. parvum* localization by using bright-field microscopy in a blind fashion and scored qualitatively according to parasite load. Because of the patchiness of cryptosporidial colonization, 10 microscope fields were examined. Scoring was as follows: 0, no parasites observed; 1+, small numbers of parasites focally distributed in the tissue (<10% of the tissue colonized); 2+, moderate numbers of parasites widely distributed throughout the tissue (10 to 50% of the tissue colonized); and 3+, large
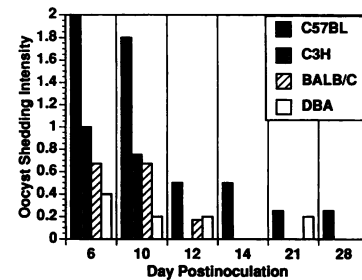

FIG. 1. Patterns of *C. parvum* oocyst shedding intensity in four strains of mice. The mice were immunosuppressed by administering DEX orally at 0.25 μg/g/day for 14 days and then inoculated intragastrically with 10^6 oocysts. Oocyst shedding intensity was determined as follows: 0, no oocysts detected; 1+, <5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; 4+, >100 oocysts per smear.

numbers of parasites widely distributed throughout the tissue (>50% of the tissue colonized).

Statistical analysis. Treatment differences were statistically compared for significance at $P < 0.01$ utilizing analysis of variance (19).

RESULTS

The results of the study designed to determine whether any of the five different strains of adult mice could be infected with *C. parvum* following immunosuppression with DEX are presented in Fig. 1 and 2. Oocyst shedding intensities of mice given DEX orally at a dose of 0.25 μg/g/day are shown in Fig. 1. All CBA mice died prior to inoculation with *C. parvum*. In the other four strains of mice, oocyst shedding was initially detected on day 6 p.i. Oocyst shedding was no longer detectable in the C3H/HeN and BALB/cAnN mice after 10 and 12 days p.i., respectively. Oocyst shedding also ceased in the DBA/2N mice after 21 days p.i. However, the C57BL/6N mice continued to shed oocysts throughout the duration of the 28-day experiment, even though shedding was particularly light in these mice after 14 days p.i.

Oocyst shedding intensities of mice given DEX intraperitoneally at a dose of 125 μg/day are shown in Fig. 2. As before, all CBA mice given DEX at the four doses died prior to inoculation with *C. parvum*. Furthermore, all mice of the
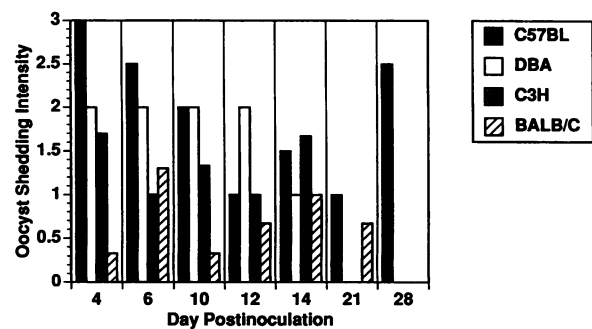

FIG. 2. Patterns of *C. parvum* oocyst shedding intensity in four strains of mice. The mice were immunosuppressed by administration of DEX intraperitoneally at 125 μg/day for 14 days and then inoculated intragastrically with 10^6 oocysts. Oocyst shedding intensity was determined as follows: 0, no oocysts detected; 1+, <5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; 4+, >100 oocysts per smear.
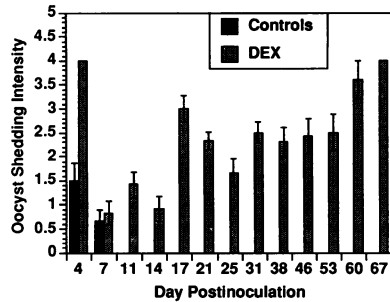
FIG. 3. Patterns of *C. parvum* oocyst shedding intensity in immunosuppressed (DEX) and nonimmunosuppressed (controls) C57BL/6N mice. The mice were immunosuppressed by administration of DEX intraperitoneally at 125 μg/day for 14 days prior to intragastric inoculation with 10^6 oocysts. Oocyst shedding intensity was determined as follows: 0, no oocysts detected; 1+, <5 oocysts per smear; 2+, 5 to 50 oocysts per smear; 3+, 50 to 100 oocysts per smear; 4+, >100 oocysts per smear.
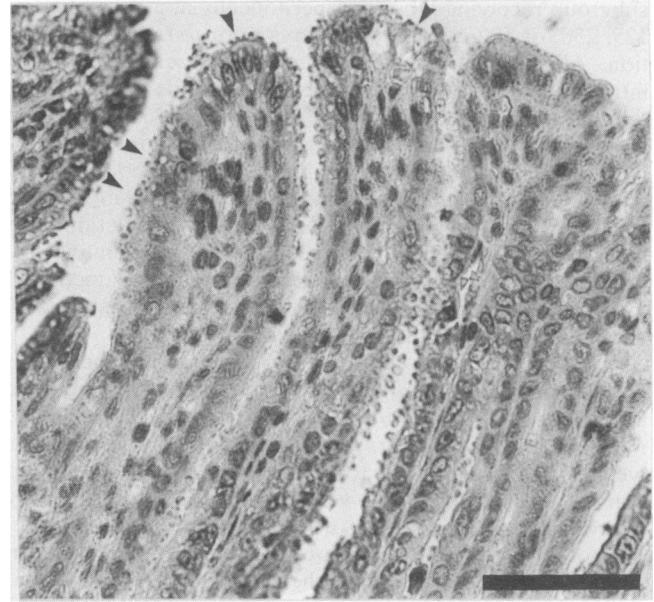

FIG. 4. Histologic section of the ileum from a DEX-immunosuppressed mouse. Note the numerous parasites (arrowheads) associated with the villous brush border. Bar = 70 μm.

remaining four strains receiving DEX at doses of 250 and 500 μg/day died prior to inoculation with *C. parvum*. Mice given DEX at a dose of 62.5 μg/day were inconsistent in their shedding of oocysts, and only a few developed infections. Oocyst shedding intensities were greater in mice receiving DEX intraperitoneally at a dose of 125 μg/day than in mice receiving DEX orally. Oocyst shedding was initially detected on day 4 p.i. in all four strains of mice. Shedding was no longer detectable in C3H/HeN mice and BALB/cAnN mice after 14 and 21 days p.i., respectively. All DBA/2N mice died by day 14 p.i. The entire group of C57BL/6N mice continued to shed oocysts until the end of the 28-day experiment. These results indicate that C57BL/6N mice receiving DEX at a dose of 125 μg/day are the most susceptible to infection by *C. parvum*.

After finding the susceptibility of the C57BL/6N mice given DEX intraperitoneally to *C. parvum* infections, we designed a study to determine the chronicity of *C. parvum* infections in C57BL/6N mice compared with nonimmunosuppressed mice. The results are presented in Fig. 3. The intensities of oocyst shedding were significantly greater in the immunosuppressed than in the nonimmunosuppressed mice. Both groups of mice commenced oocyst shedding on day 4 p.i. The nonimmunosuppressed mice stopped shedding after day 7 p.i., whereas the group of immunosuppressed mice continued to shed oocysts throughout the 10-week experiment. There appeared to be a second, but lower, peak in mean oocyst shedding intensities on day 17 p.i. in the immunosuppressed mice. This may have been the result of autoinfection (8, 30). Chronically infected mice exhibited soft (nondiarrhetic) stools, lethargy, and dehydration.

Microscopic examination of tissue sections revealed that *C. parvum* was localized predominantly in the small and large intestines of the DEX-immunosuppressed mice (Fig. 4). Specifically, cryptosporidia were noted in the gastric glands of the stomach, duodenum, jejenum, ileum, and colon collected on day 60 p.i. The greatest levels of parasites were located in the jejenum, ileum, terminal ileum, and colon. The colonization levels increased toward the end of the experiment. No parasites were observed in the nonimmunosuppressed mice.

Three of the 10 C3H/HeJ/beige mice exhibited only mild oocyst shedding (1+) for 5 days and subsequently ceased to shed oocysts by day 6 p.i. No cryptosporidia were localized

in the intestines of any of these animals on day 7 p.i. (data not shown). Two of 10 C57BL/6N mice given the 8% protein diet for 2 months exhibited only mild oocyst shedding (1+) for 6 days and subsequently ceased to shed oocysts by day 7 p.i. No cryptosporidia were localized in the intestines of any of these animals on day 7 p.i. (data not shown).

DISCUSSION

This study demonstrates the first successful attempt to produce chronic cryptosporidiosis in adult mice following drug-induced immunosuppression. The impetus for these experiments was the lack of both an in vitro culture system and a suitable adult small animal model to study the disease and evaluate potential anticryptosporidial agents. The most widely used laboratory animal model for research on *C. parvum* has been the neonatal mouse (2, 9, 23, 28). Unfortunately, the small size and the short duration of the parasitic infection in the neonatal mouse (only 2 to 3 weeks) constitute significant disadvantages of this animal model. Attempts to develop germfree adult mice as a model for *C. parvum* have failed because of the low level of parasite colonization in the gut (12).

An immunocompromised animal model for chronic cryptosporidiosis is necessary because the most severe infections have been reported for immunocompromised or immunosuppressed individuals. This is particularly true for AIDS patients. Such a model will be required to study the effectiveness of anticryptosporidial agents as well as the immunological defects which allow for susceptibility to disease. Immunosuppressed models have been characterized for the hamster and the rat by using cyclophosphamide (25), hydrocortisone acetate (5, 27), or DEX (26). Ungar et al. (31) demonstrated chronic infections in neonatally infected BALB/c mice treated with anti-CD4 monoclonal antibodies. However, they were unable to produce chronic infections in similarly treated adult mice. The genetically immunocompromised nude BALB/c mouse has been shown

to establish chronic infections (14, 31). Moreover, Mead et al. (20) demonstrated chronic *C. parvum* infections in immunocompromised SCID and NIH III (*bg/nu/xid*) adult mice. The NIH III mouse has depressed natural killer cell activity and no T-cell-mediated, T-independent B-cell-mediated, or lymphokine-activated killer cell responses. The lack of susceptibility we found in the C3H/HeJ/beige mice suggests that the resistance to *C. parvum* infections is not dependent on the presence of natural killer cell activities. The major disadvantages in using the SCID and NIH III mice are their individual costs and their stringent housing requirements.

We demonstrated that DEX-immunosuppressed adult mice of various strains respond differently to inoculation with *C. parvum*. The dose of DEX administered is critical when attempting to produce an infection. For example, the CBA mice were particularly susceptible to DEX and died shortly after the drug was given. The BALB/cAnN, C3H/HeN, and DBA/2N mice immunosuppressed by intraperitoneal injections of DEX developed only light parasitic infections which cleared within 3 weeks. However, the C57BL/6N mice immunosuppressed by intraperitoneal injections of DEX at a dosage of 125 µg/day proved very susceptible to *C. parvum* and developed chronic infections. These results suggest that the genetic background of the mouse plays a significant role in susceptibility to cryptosporidiosis following immunosuppression with DEX. The effects of DEX on the immune system may differ in certain strains. The lack of susceptibility to *C. parvum* noted in the low-protein diet-fed mice may be the result of an incomplete immunosuppression in these mice. Feeding the mice the low-protein diet and assessing their immune functions for a longer period should be considered.

A major limitation of the DEX-immunosuppressed mouse model for cryptosporidiosis may simply be that the beneficial effects of this model are limited to it and are not applicable to all immunocompromised hosts. Cost effectiveness is the primary advantage of using the DEX-immunosuppressed C57BL/6N mouse as a model for cryptosporidiosis compared with other methods of immunosuppression and other immunodeficient mouse models. A second advantage is that this mouse can be infected as an adult and will establish chronic infections with *C. parvum*. Consequently, the C57BL/6N mouse holds great promise of being a superior animal model in which to test anticryptosporidial agents as well as to discern the immunological defects which allow for the development of chronic infections.

ACKNOWLEDGMENTS

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