A HIGH-YIELD OUTBRED SUCKLING MOUSE MODEL OF CRYPTOSPORIDIOSIS

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Summary :

Outbred suckling mice (NMRI strain) were used as hosts. They were initially inoculated with oocysts of human origin, and subsequently with parasites recovered from the mouse ileal mucosa. Cryptosporidia were counted in an aliquot of whole-ileum homogenate. Parasite load was expressed as cryptosporidia per centimeter of ileum. Serial passage of *C. parvum* in NMRI mouse litters led to a gradual amplification of parasite burden relative to animals initially inoculated with the human isolate.

KEY WORDS : *Cryptosporidium*, suckling mice, high-yield model.

MOTS CLES : *Cryptosporidium*, souriceau non sevré, modèle à haut rendement.

Résumé : DÉVELOPPEMENT D'UN MODELE D'INFECTION MASSIVE À *CRYPTOSPORIDIUM* CHEZ LE SOURICEAU NON CONSANGUIN, NON SEVRÉ

Des souriceaux non sevrés, non consanguins (souche NMRI) sont utilisés comme hôtes. L'infestation initiale est réalisée à partir d'oocystes d'origine humaine alors que les réinfestations sont effectuées avec des parasites récupérés dans la muqueuse iléale des souriceaux. La quantification des cryptosporidies est pratiquée sur un aliquot d'homogénéisat de la totalité de l'iléon. La charge parasitaire est exprimée par détermination du nombre de cryptosporidies par cm d'iléon. Les passages successifs de *C. parvum* chez des portées de souriceaux NMRI aboutissent à une amplification progressive de la charge parasitaire par rapport à celle obtenue après infestation initiale des souriceaux avec des oocystes d'origine humaine.

Several experimental models of infestation by *Cryptosporidium parvum* have been developed in newborn animals (Current and Reese, 1986; Novak and Sterling, 1991; Reese *et al.*, 1982; Scaglia *et al.*, 1991; Sherwood *et al.*, 1982).

Adult immunocompetent mice are almost completely resistant to *C. parvum* parasitization (Sherwood *et al.*, 1982), with the exception of the estrus mouse (Liebler *et al.*, 1986), the naturally infected guinea-pig (Chrisp *et al.*, 1990) and the aged hamster, the latter infection probably being due to age-related immunodeficiency (Rasmussen and Healey, 1992). Experimentally infected immunosuppressed rodents (Brasseur *et al.*, 1988; Rossi *et al.*, 1990; Rehg, 1991; Ungar *et al.*, 1991) and congenitally immunodeficient strains of mice, i.e. nu/nu (T cell-deficient) and SCID (which lack both T and B cells) develop chronic infections which are initially mild but eventually lead to morbidity and death (Ungar *et al.*, 1990; Mead *et al.*, 1991).

Cryptosporidiosis has also been obtained in both consanguineous (Liebler *et al.*, 1986; Novak and Sterling, 1991; Scaglia *et al.*, 1991; Sherwood *et al.*, 1982; Ungar *et al.*, 1990) and outbred mouse strains

(Current and Reese, 1986; Sherwood *et al.*, 1982; Ungar *et al.*, 1990).

With the exception of guinea pigs spontaneously infected by *Cryptosporidium parvum*, the model used by Chrisp *et al.* (1990), experimentally infected rodents do not develop physical signs of infection. However, these animal models are useful for studying the replicative cycle, parasite ultrastructure (Current and Reese, 1986; Scaglia *et al.*, 1991), and various aspects of infection (Novak and Sterling, 1991; Sherwood *et al.*, 1982). The efficacy of disinfectants (Korich *et al.*, 1990), drugs (Rehg, 1991) and immunological treatments (Arrowood *et al.*, 1989), as well as the prophylactic effects of certain drugs (Fayer and Ellis, 1993) have been studied in animal models of *Cryptosporidium* infection.

Infection is proven by the presence of oocysts in the feces or by the detection of various stages of the parasite in sections of the ileum; parasites can also be counted in intestinal mucosal scrapings (Current and Reese, 1986; Scaglia *et al.*, 1991). Quantification of the parasite load is essential for testing anticryptosporidial activity and for studying the natural history of the infection. However, oocyst shedding is related to fecal output and does not take into account intracellular development of the parasite, while histological studies depend on the distribution of the parasite along the intestinal tract.

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Fig. 1. – Ileal parasite load (steps of 5000/cm of ileum) in a selected batch of suckling mice inoculated with a homogenate of infected suckling mouse ileum or human feces. The stippled bars represent one experiment.

litter a1 (n=6): oocysts from human feces

litter b1 (n=11): cryptosporidia from murine ileal mucosa after 1 passage

litter c1 (n=11): cryptosporidia originating from murine ileal mucosa after 2 passages

litter d1 (n=10): cryptosporidia originating from murine ileal mucosa after 3 passages.

The hatched bars represent another experiment.

litter a2 (n=7): oocysts from human feces

litter b2 (n=11): cryptosporidia from murine ileal mucosa after 1 passage

litter c2 (n=13): cryptosporidia from murine ileal mucosa after 2 passages

litter d2 (n=9): cryptosporidia from murine ileal mucosa after 3 passages

MATERIAL AND METHODS

The aim of this work was to develop an early, high-yield model of cryptosporidiosis in outbred mice by serial reinoculation of suckling animals, with a view to drug screening and studies of the immunological response to *C. parvum*.

It is based on 4-day suckling NMRI mice. The mice were initially inoculated with oocysts of *C. parvum* isolated from fresh human feces. Debris were eliminated by serial passage through stainless-steel screens

with a final mesh size of 32 μ m. The oocysts were then concentrated and purified on a sucrose density gradient (1.04 to 1.10), and collected from the interface (Buraud *et al.*, 1991). After washing in PBS, they were stored in 2.5% potassium dichromate at 4°C for between 8 to 20 days until use. Prior to inoculation, oocysts were washed three times in 0.025 M PBS (pH 7.2) to remove potassium dichromate. They were then resuspended in 1.0 ml of PBS and counted using an haemocytometer. Suckling mice were inoculated by gavage (d0) with 100 μ l of concentrated material containing 10^6 oocysts. Infection was checked on

day 6 post-inoculation. Parasite load was determined by counting cryptosporidia in an ileal homogenate. As the terminal ileum is the intestinal section most heavily infected with cryptosporidia in consanguineous BALB/c mice (Novak and Sterling, 1991), we focused on this region to evaluate the severity of infection. Mice were killed by chloroform asphyxiation. The small intestine was removed from the abdomen, and the ileum, easily recognizable by its color and smaller diameter than the jejunum, was isolated and measured without traction. The ileum was then split lengthwise and homogenized for 25 seconds in 1 ml of PBS using a glass tissue grinder with a Teflon pestle mounted on a drill press operating at 300 rpm (Potter apparatus). An aliquot (20 µl) of the homogenate was smeared onto a glass slide (2 cm²) and stained by using a modified Ziehl-Neelsen technique. Cryptosporidia are easily detected at magnification of ×200 (Henriksen and Pohlenz, 1981). Parasite load was expressed as cryptosporidia per centimeter of ileum, as follows :

$$N = n \times 50 / L$$

where n is the total number of parasites counted in the smear, L the total length of ileum (cm) homogenized in 1 ml of PBS, and 50 the coefficient used to transform the number of cryptosporidia in 20 µl into the number present in the entire ileum (1 ml).

Four consecutive litters of 4-day-old NMRI mice were then infected by ingestion of a day-6 ileal homogenate. The homogenates were kept for 7 to 15 days at 4°C after the addition of penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml), until the next inoculation. Subsequent inoculations consisted of 10⁴ cryptosporidia (all stages) in 100 µl of PBS. All experiments were performed in duplicate [litter a: mice inoculated with human oocysts (a1, n = 6; a2, n = 7); litter b: mice inoculated with cryptosporidia from ileal homogenate after 1 passage in mice (b1, n = 11; b2, n = 11); litter c: mice inoculated with cryptosporidia from ileal homogenate after 2 passages (c1, n = 11; c2, n = 13) and litter d: mice inoculated with cryptosporidia from ileal homogenate after 3 passages (d1, n = 10; d2, n = 9). Parasite load was checked as previously described. A histogram was constructed for each successive litter, showing the number of animals in the different parasite load categories (steps of 5000 cryptosporidia per centimeter of ileum).

RESULTS

All 10-day mice were positive for *C. parvum* in all experiments. Parasite load varied considerably from within a given litter, from 100 to 4000 after inoculation with oocysts isolated from

human feces (litters a1 and a2, fig. 1) and from 300 to 82000 after inoculation with cryptosporidia originating from murine intestinal mucosa (litters b to d, fig. 1). Parasite load increased regularly from litter b (2nd passage in mice) to litter d (4th passage) and was significantly increased after passage 4 (ANOVA; $p < 0.05$).

Expressed as the cumulative mean per litter, the results were as follows : litter a1 (n = 6), 5000; litter a2 (n=7), 5000; litter b1 (n = 11), 7272; litter b2 (n=11), 8636; litter c1 (n = 11), 14090; litter c2 (n=13), 8076; litter d1 (n = 10), 23500; litter d2 (n=9), 17777 cryptosporidia per cm of ileum.

Few parasites were found when the homogenates were used 48 hours after collection, whereas all the animals were successfully inoculated with homogenate stored in PBS at + 4°C for 5 to 7 days, with a maximum of 60 days.

DISCUSSION

Parasite load can be determined by counting the number of parasites present on longitudinal villi in two randomly selected high-power fields (X 400) (Novak and Sterling, 1991). However, when few parasites are present, they are usually concentrated in one area. Fayer *et al.* (1993) examined five areas within histologic sections of ileum, caecum and colon and assigned a score for the percentage of epithelium parasitized. We considered that counting an aliquot of the entire homogenized ileum, which contains both free and intracellular forms of the parasite, is more representative of the degree of infection than the use of stained 5-µm slices of ileum. We obtained a mean value for parasite load in the whole ileum, rather than point values dependent on the section examined, particularly when few parasites are present. Furthermore, this counting method is not influenced by intestinal transit speed or irregular fecal excretion.

We used outbred mice to obtain larger litters; outbred and inbred mice have similar susceptibility to *C. parvum* infection (Sherwood *et al.*, 1982).

The use of suckling mouse ileum homogenates for inoculation rather than oocysts isolated from human feces means that the inoculum can be 100 times smaller and yet provide 20 times as many cryptosporidia. Indeed, the ileal homogenate from a heavily infected mouse contains several hundred thousand cryptosporidia. The regular increase in parasite load observed between passage 2 (from a to b) and passage 4 (from c to d) points to increased infectivity. The gradual increase in parasite load is not only due to amplifica-

tion of the normal life cycle by repeated penetration into enterocytes, but also to an increase in virulence through serial passage in the host (a common phenomenon in the experimental setting).

A further advantage of this model is that oocyst purification from mouse ileal homogenate is far easier than from human or calf feces. This high-yield model should prove useful for studying the natural history of *C. parvum* infection, drug screening and studies of immunological approaches aimed at eliminating the infection.

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