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Rapid Communication

Fulminant cryptosporidiosis associated with digestive adenocarcinoma in SCID mice infected with *Cryptosporidium parvum* TUM1 strain

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ABSTRACT

We recently demonstrated that *Cryptosporidium parvum* IOWA strain induces in situ ileo-caecal adenocarcinoma in an animal model. Herein, the ability of another *C. parvum* strain to induce digestive neoplasia in dexamethasone-treated SCID mice was explored. SCID mice infected with *C. parvum* TUM1 strain developed a fulminant cryptosporidiosis associated with intramucosal adenocarcinoma, which is considered an early histological sign of invasive cancer. Both evidence of a role of *C. parvum* in adenocarcinoma induction and the extended prevalence of cryptosporidiosis worldwide, suggest that the risk of *C. parvum*-induced gastro-intestinal cancer in humans should be assessed.

The genus *Cryptosporidium* comprises several species and genotypes which infect a wide range of hosts, including humans (Xiao, 2010). These parasites are ubiquitous and the usual lack of morphological differentiation between the species has made it difficult to track down sources of human and animal infections. Molecular typing has shown that the main agents of human cryptosporidiosis are *Cryptosporidium hominis* and *Cryptosporidium parvum*. However, *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium suis*, *Cryptosporidium muris* and several infra-specific *Cryptosporidium* genotypes have also been reported in humans (Xiao, 2010). Humans and animals with cryptosporidiosis can exhibit diverse expression of disease severity with regard to either host susceptibility to infection or virulence of the parasite isolate (Okhuysen and Chappell, 2002). To investigate these biological divergences and to contribute to the understanding of the dynamics of the infection, we developed a reproducible animal model of chronic cryptosporidiosis using dexamethasone (Dex)-treated adult severe combined immunodeficiency (SCID) mice (Certad et al., 2007, 2010). Using such a model, oocyst shedding, clinical impact, location of the infection, associated histopathological changes in the gastro-intestinal tract and virulence could be assessed. Unexpectedly, we found that *C. parvum* IOWA strain was able to induce the development of intestinal adenomas with areas of intraepithelial neoplasia in chronically infected SCID mice (Certad et al., 2007, 2010). This important observation stimulated our interest and led us to perform a series of in vivo experiments to determine whether non-IOWA strains of *C. parvum* or other *Cryptosporidium* spp. are able to both propagate and induce gut neoplasia in the Dex-treated SCID mouse model.

Cryptospordium oocysts of different species and strains were obtained from sources indicated in Table 1. In particular, oocysts of *Cryptosporidium molnari* (the sole non-mammalian species tested) were obtained from mucosal scrapings of gilthead sea bream stomachs heavily infected by the parasite, as previously described (Alvarez-Pellitero and Sitja-Bobadilla, 2002). Oocyst viability

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Table 1
Testing SCID mouse susceptibility to *Cryptosporidium* spp.: experimental design.

Experiment no.	*Cryptosporidium* spp.	Strain, origin	No. of mice							
			Inoculum size (number of oocysts)[a]							
			10^5		10^6		4×10^6		10^7	
			Dexamethasone							
			–	+	–	+	–	+	–	+
1	*Cryptosporidium parvum*	TUM1[b], Tufts Cummings School of Veterinary Medicine, USA (Dr. S. Tzipori)	4	4	4	4	ND	ND	4	4
2	*Cryptosporidium molnari*	Instituto de Acuicultura Torre la Sal (CSIC), Ribera de Cabanes, Castellón, Spain (Dr. P. Alvarez)	6	6	ND	ND	ND	ND	ND	ND
3	*Cryptosporidium hominis*	TU502, Tufts Cummings School of Veterinary Medicine, USA (Dr. S. Tzipori)	4	4	4	4	4	4	ND	ND

ND, not done.
[a] Oocysts were inoculated in 200 µl of PBS.
[b] Isolated from calf approximately 18 months prior to the present study.

before inoculation was assessed (Certad et al., 2007, 2010) and the absence of bacteria or fungi was assured by testing the oocyst suspensions on Plate Count Agar and on Sabouraud plates (37 °C, 1 week).

CB17-SCID mice between 6 and 8 weeks of age were obtained from a colony bred and regularly controlled for assessing microbial or parasitological infections, including *Helicobacter* infection, at the Pasteur Institute of Lille (France). Animals were maintained under aseptic conditions in an isolator with standard laboratory food and water ad libitum. All animal experiments were performed in accordance to the ethical European guidelines (number 86/609/EEC) and recommendation of the French Agricultural Office for the care of animal subjects. Experiments were carried out in the accredited research animal facility of Institut Pasteur de Lille (accreditation number, A59107). All animal protocols were approved by the locally appointed investigational review board. SCID mice were housed in groups, in covered cages. Infective doses were prepared and were inoculated as indicated in Table 1 by oral-gastric gavage (18–20 gauge feeding tubes). When needed, SCID mice received 4 mg/L of Dex (Merck, Lyon, France), via the drinking water (Certad et al., 2007, 2010) (Table 1). Control groups comprised four animals inoculated only with PBS without or with Dex administration. Faecal specimens were collected from the first day p.i. until the end of the experiment and were processed as previously described (Certad et al., 2010). The amount of oocysts/mg of faeces was estimated per day and per group of mice, with the number of mice per group decreasing over time. Logarithmic transformation of oocyst excretion values was used. An HEM-Sign-3 test (V6063, Servibio, Meudon, France) was used to detect faecal occult blood in Experiment 1 (Table 1). On days 20, 35, 45 and 60 p.i., or when signs of imminent death appeared, mice were euthanised by cervical dislocation.

Stomach, liver, duodenum, samples of proximal, medium and distal parts of jejunum, ileo-caecal region and colon were removed from each mouse, fixed in 10% buffered formalin, processed using standard histological techniques and embedded in paraffin. Sections 5 µm thick were stained with H & E. Immunohistochemical assays were performed on deparaffinised and rehydrated 5 µm sections using the BenchMaerk XTstaining module. Following instructions from suppliers, a rabbit polyclonal antibody to laminin (dilution 1:15) (PU078-UP, BioGenex, Netherlands) was employed as a marker of the basement membrane integrity; a mouse monoclonal antibody to cytokeratin (without dilution) (AM071–5 M, Biogenex, Netherlands) was used to visualise epithelial cells; to stain muscle fibres monoclonal antibody anti alpha smooth muscle actin (dilution 1:100) (M0851, Dako, Denmark) was used. Parasite load in digestive sections was scored as follows: 0, no parasites; +1, small number of parasites focally distributed; +2, moderate number of parasites widely distributed; +3, abundant parasites present and widely distributed throughout the section (Certad et al., 2010). Lesions at different sites were scored according to the "Nomenclature for Histologic Assessment of Intestinal Tumours in the Rodent", and compared to the "Vienna classification of the epithelial neoplasia of the gastro-intestinal tract for humans", as previously described (Certad et al., 2010) with slight modifications. Briefly: 0, no lesion; 1, inflammation and/or regenerative changes; 2, low grade intraepithelial neoplasia (LGIEN) or low grade dysplasia; 3, high grade intraepithelial neoplasia (HGIEN) or high grade dysplasia. In this category, adenoma with HGIEN, carcinoma in situ (limited to the epithelium) or intramucosal adenocarcinoma (invasion of the basal membrane of glands) was also included; 4, suspicion of invasive adenocarcinoma or invasive adenocarcinoma (penetration of dysplastic glands through the muscularis mucosae with desmoplastic stromal response). The degree of severity of histological damage for each mouse was calculated by the sum of individual scores over the five organs as previously described (Certad et al., 2010). In order to include spontaneous death as a factor of disease severity, each mouse that died before planned euthanasia was assigned a number of points equivalent to: 20 +((60 – day of death)/2), where 20 corresponded to the maximum score of severity in animals euthanised as planned, and 60 corresponded to the end time of the experiment (Table 2). Sections were examined using a Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID software, Cambridge, UK). Data analysis was performed with the statistical software S-PLUS 2000 (MathSoft, Seattle, WA, USA). Significance was defined as $P \leqslant 0.05$.

An experiment was developed using *C. parvum* TUM1 strain isolated from a calf approximately 18 months prior to this study (Donna Akiyoshi, personal communication). Regardless of inoculum size, all groups of SCID mice infected with *C. parvum* TUM1 strain showed oocysts in the faeces from day 1 p.i. until the end of the experiment (Table 2). On the whole, the shedding intensity increased over time ($P < 0.001$). ANOVA of the whole dataset showed that administration of Dex, day p.i., and the interaction between both, significantly increased the severity of the infection ($P < 0.001$, $P < 0.001$, $P = 0.006$, respectively). The group of Dex-treated animals exhibited diarrhoea, in contrast to infected non-Dex-treated mice. Diarrhoea appeared at 4 weeks p.i. in animals infected with 10^7 oocysts, at 5 weeks p.i. in mice infected with 10^6 oocysts and at 6 weeks p.i. in mice receiving 10^5 oocysts. Faecal blood in diarrhoeic stools was established using the HEM-Sign-3 test. After day 34 p.i., seven Dex-treated mice out of 24 died unexpectedly (29%), and additional animals (mice numbers six and 14) with evident signs of clinical aggravation (bloody