Development of *Cryptosporidium parvum*-Induced Gastrointestinal Neoplasia in Severe Combined Immunodeficiency (SCID) Mice: Severity of Lesions Is Correlated with Infection Intensity

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Abstract. We reported previously that *Cryptosporidium parvum* was able to induce intestinal tumors in severe combined immunodeficiency (SCID) mice treated with corticoids. To further characterize this *Cryptosporidium*-induced cell transformation, SCID mice treated with dexamethasone were challenged with *C. parvum* oocysts, and euthanatized sequentially after infection for histologic examination. Ki-67 was used as a marker of cellular proliferation. Our previous results were confirmed, and it was also found that mice receiving higher inocula (10^6 – 10^7) experienced more severe neoplastic development. Additionally, neoplastic changes were observed not only in the caecum but also in the stomach and duodenum of some animals. Interestingly, SCID mice (6/6) inoculated with 10^5 – 10^7 oocysts showed high grade intraepithelial neoplasia or adenomas with high grade dysplasia in the caecum after Day 46 post-infection (PI). Immunohistochemistry for Ki-67 staining indicated the neoplastic process associated to cryptosporidiosis, and evidenced the first immunohistochemical alterations at early stages of the process, even at 3 weeks PI.

INTRODUCTION

Cryptosporidium apicomplexan protists known to be causative agents of diarrhea in animals, have emerged as major causes of diarrhea in humans.¹ *Cryptosporidium* species are able to cause self-limiting diarrhea in immunocompetent adults or life-threatening diarrhea in immune suppressed persons, particularly in acquired immunodeficiency syndrome (AIDS) patients.² Within the genus *Cryptosporidium*, many morphologically similar species have been reported, but with varying degrees of disease severity according to host susceptibility to infection and to diversity in isolate pathogenicity.³ Interestingly, *Cryptosporidium* species have been related to carcinogenesis. The association of cryptosporidiosis and colonic adenocarcinoma was speculated in the case of a Spanish patient carrying both, who died rapidly after the onset of symptoms.⁴ More recently, an epidemiologic study in Poland reported a high frequency of cryptosporidiosis in patients with colorectal cancer.⁵ However, in these reports it was unclear if *Cryptosporidium* sp. behaved as a carcinogenesis factor or simply as an opportunistic agent whose development was enhanced by host immunosuppression. In this work, we described the potential role of *Cryptosporidium parvum* in the development of colon neoplasia in experimentally infected severe combined immunodeficiency (SCID) mice treated with dexamethasone.⁶ Herein, the ability of *C. parvum* to induce neoplastic changes could be established experimentally. In addition, we showed that deep immunosuppression alone did not entail neoplasia in uninfected hosts. Taking into account that *C. parvum* is able to modulate apoptosis of intestinal cells during its replication,⁷ a mechanism known to be involved in the carcinogenesis processes,⁸ and that SCID mice developed a chronic *C. parvum* infection with dose-dependent pathologic

effects,⁹ we further studied *C. parvum*-induced gastrointestinal neoplasia using variable doses of *C. parvum* inocula and the cell proliferation marker Ki-67. The Ki-67 nuclear antigen has been shown to be expressed in proliferating cells during G₁, S, G₂, and mitosis stages of the cell cycle. In the normal gastrointestinal epithelium, Ki-67 is expressed in the nuclei of replicating cells around the base of the crypts; in dysplastic epithelia and advanced neoplasms, Ki-67 expresses a markedly abnormal pattern of proliferation in the middle and upper zones of the crypts.¹⁰

MATERIALS AND METHODS

Overall study design and procedures. The study targeted the ability of different inoculum sizes of *C. parvum* oocysts to induce gastrointestinal neoplastic changes in dexamethasone-treated or untreated SCID mice. Follow-up included evaluation of infection intensity (according to oocyst shedding counts and semi-quantification of parasites in the tissues, see below), histology, immunohistochemistry, and semi-quantitative assessing of the severity of lesions. The SCID mice are susceptible to *Cryptosporidium* infection and develop chronic disease caused by their defect in T and B lymphocytes,⁹ but dexamethasone treatment can also inhibit the priming of the innate immune response and interferon- γ (IFN- γ)-regulated gene expression.¹¹

Animals and experimental design. *Cryptosporidium parvum* IOWA oocysts were purchased from Waterborne TM, Inc. (New Orleans, LA). The suspension of oocysts was stored in a solution containing phosphate buffered saline (PBS), penicillin, streptomycin, gentamicin, amphotericin B, 0.001% Tween 20. Infective doses were prepared and were inoculated by oral-gastric gavages using 18–20 gauge feeding tubes to 7-week-old female CB17-SCID mice obtained from a colony bred at Pasteur Institute of Lille (France). Oocyst viability before inoculation was determined by a trypsin-taurocholate excystation test¹² and absence of bacteria or fungi was assured by testing the oocyst suspensions on plate count agar (37°C,

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1 week) and on Sabouraud plates (37°C, 1 week). When needed, animals were administered with Dexamethasone sodium phosphate (4 mg/L drinking water) (Dex) (Merck, Lyon, France). Dex administration started 2 weeks before inoculation and was maintained during the whole experimentation as previously described.⁶ Thirty-two SCID mice were randomly divided into eight groups of four in capped cages according to the dose of *C. parvum* oocysts inoculated and to the administration or not of Dex. Four groups of mice without Dex were inoculated by oral route with 10⁵, 10⁶, 10⁷, or 10⁸ oocysts, respectively, in 200 µL of PBS. The other four groups were inoculated similarly but they received oral Dex as explained previously (Table 1). Eight SCID mice constituted two control groups: four mice received oral Dex and were inoculated with PBS (CDex group), and four mice received Dex and an inoculum from which oocysts (equivalent to 10⁶) were removed by filtration with a Nanosep MF tube (Pall Corporation, Port Washington, NY) with a 0.45 µm pore-size membrane (CDV group). On Days 20, 35, 46, and 57 post-inoculation (PI), one mouse from each group (including CDex and CDV) was euthanatized by a

sodium pentobarbital (Ceva, Libourne, France) intra-cardiac injection. The rationale for selecting dates for euthanasia was based on our previous results.⁶ The SCID mouse colony source of the mice used in this work is regularly evaluated for the absence of microbial or parasitologic infections, including *Helicobacter*. Experiments were performed according to the European guidelines (Council directives on the protection of animals for experimental and other scientific purposes. J. Off. Communautés Européennes, 86/609/EEC, 18 December 1986, L358).

Collecting mouse fecal specimens. Specimens were collected from the first day post-infection (PI) until the end of the experiment. Every 1–3 days each group of animals (initially four per group) was transferred to a clean cage during 30 to 60 minutes. Freshly collected fecal pellets (between 5 and 12 pellets) at each time point from each cage were placed in 1.5 mL conical tubes, weighed, and suspended in 500 to 1,000 µL deionized (Milli-Q, Millipore Corp.) water.

Processing of mouse fecal specimens and quantification of the oocyst shedding. The feces were homogenized by

TABLE 1

Experimental cryptosporidiosis in severe combined immunodeficiency (SCID) mice: testing the influence of inoculum size and Dex administration on the severity of digestive lesions

Mouse no.	Dexamethasone*	Inoculum	Day of euthanasia (PI)	Log ₁₀ of oocysts/mg feces (number of mice)†	Histopathologic findings‡ (Presence of <i>C. parvum</i> organisms)										Score of severity§
					Stomach		Liver		Duodenum		Ileocaecal region		Colon		
1	No	10 ⁵	20	2.87 (4)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0
2			35	3.21 (3)	0	(0)	1	(0)	0	(0)	0	(0)	0	(0)	1
3			46	3.91 (2)	0	(0)	1	(+2)	0	(0)	0	(0)	0	(0)	1
4			57	4.49 (1)	2	(+3)	1	(0)	1	(0)	0	(0)	0	(0)	4
5	Yes	10 ⁵	20	3.7 (4)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0
6			35	4.01 (3)	0	(0)	0	(0)	0	(0)	0	(+1)	0	(0)	0
7			46	4.94 (2)	0	(0)	0	(0)	0	(0)	3	(+3)	2	(+3)	5
8			57	4.87 (1)	0	(0)	1	(0)	2	(+2)	3	(+3)	0	(0)	6
9	No	10 ⁶	20	4.21 (4)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0
10			35	3.75 (3)	0	(0)	1	(0)	1	(0)	0	(0)	0	(0)	2
11			46	4.32 (2)	0	(0)	1	(0)	1	(0)	0	(0)	0	(0)	2
12			57	4.32 (1)	2	(+2)	1	(0)	0	(0)	0	(0)	0	(0)	3
13	Yes	10 ⁶	20	4.52 (4)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0
14			35	4.23 (3)	0	(0)	0	(0)	0	(0)	0	(+1)	0	(0)	0
15			46	5.08 (2)	0	(0)	0	(0)	0	(0)	3	(+3)	2	(+1)	5
16			57	5.17 (1)	3	(+3)	1	(0)	1	(0)	3	(+3)	3	(+2)	11
17	No	10 ⁷	20	2.65 (4)	0	(0)	1	(+1)	0	(0)	0	(0)	0	(0)	1
18			35	4.42 (3)	2	(+2)	1	(0)	0	(0)	0	(0)	0	(0)	3
19			46	4.41 (2)	2	(+2)	1	(0)	1	(0)	0	(0)	0	(0)	4
20			57	4.81 (1)	2	(+3)	1	(0)	1	(0)	0	(0)	0	(0)	4
21	Yes	10 ⁷	20	4.33 (4)	0	(0)	0	(0)	0	(0)	0	0	0	(0)	0
22			35	4.73 (3)	0	(0)	0	(0)	0	(0)	0	(+1)	0	(0)	0
23			46	5.27 (2)	0	(0)	0	(0)	1	(0)	3	(+3)	0	(+1)	4
24			57	5.01 (1)	2	(+2)	0	(0)	1	(0)	3	(+3)	0	(+1)	6
25	No	10 ⁸	20	3.77 (4)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0
26			35	4.39 (3)	2	(+2)	1	(0)	1	(0)	0	(0)	0	(0)	4
27			46	4.78 (2)	2	(+3)	1	(0)	1	(0)	0	(0)	0	(0)	4
28			57	4.87 (1)	2	(+3)	1	(0)	1	(0)	0	0	0	0	4
29	Yes	10 ⁸	20	3.7 (4)	1	(0)	0	(0)	0	(0)	0	(0)	0	(0)	1
30			35	4.1 (3)	2	(+3)	0	(0)	0	(0)	0	(0)	0	(0)	2
31			46	4.24 (2)	0	(0)	1	(0)	1	(0)	0	(0)	0	(0)	2
32			57	4.01 (1)	2	(+2)	0	(0)	3	(+1)	0	(0)	0	(0)	5

* Dexamethasone (4 mg/L of drinking water) administration was started 2 weeks before inoculation and maintained during the whole experimentation. Control mice (uninfected Dex-treated SCID mice, and Dex-treated SCID mice inoculated with an inoculum from which *Cryptosporidium* oocysts were removed by filtration, see Materials and Methods) were euthanatized at the same dates as experimental mice. All mice of control groups did not develop *Cryptosporidium* infection or histologic gastrointestinal lesions.

† The amount of oocysts was calculated according to the number of mice in each cage at each time point (showed in brackets). One mouse was euthanatized at each of the indicated times. ‡ According to the lesions more frequently found, each site was scored as follows: 0 = no lesion, 1 = inflammation, 2 = intraepithelial neoplasia low grade, and 3 = high-grade intraepithelial neoplasia or adenoma with high-grade dysplasia. In parentheses were added the results of the histologic semi-quantitative assessment of *C. parvum* organisms in the host tissues (see Materials and Methods). Parasite rates were scored according to previous studies as follows: 0 = no detectable parasite; 1+ = small number of parasites focally distributed; 2+ = moderate number of parasites widely distributed; 3+ = high number of parasites widely distributed throughout the tissue.

§ The degree of severity of histologic damage was calculated by the sum of individual scores over the five organs.

extensive vortexing. To concentrate the oocysts, immunomagnetic separation (IMS) was done using Dynabeads anti-*Cryptosporidium* kit (Invitrogen, Cergy-Pontoise, France) according to the supplier recommendations. Briefly, 300 μ L collected from each pool of fecal specimens were incubated with 100 μ L of the Dynabeads anti-*Cryptosporidium*. The samples were then placed in a magnetic particle concentrator (MPC-1) to separate the bead-oocyst complex from the contaminating debris. The beads were resuspended and transferred into a 1.5 mL tube, and separated by using a magnetic particle concentrator (MPC-2). To dissociate the bead-oocyst complex 50 μ L of 0.1 N HCl was used. Afterward, 30 μ L of the purified oocyst suspension were placed in each well of a 10-well slide (Thermo Scientific, Portsmouth, NH) and allowed to dry. Slides were incubated at 37°C for one hour, fixed with methanol, and processed for immunofluorescence (IF) using an FITC conjugate anti-*Cryptosporidium* monoclonal antibody (Cellabs Pty. Ltd., Croissy-Beaubourg, France). The whole surface of each well was examined, the average number of fluorescing oocysts identified in 10 randomly selected microscopic fields at a magnification of $\times 400$ was recorded, and the number of oocysts per milligram of pooled feces was calculated. The oocyst number per milligram of feces from all the mice in each cage was obtained. The mouse number in each cage decreased from 4 to 3 to 2, and finally to 1; therefore, the number of oocysts shown in Table 1 did not necessarily represent the specific shedding of each animal. It was assumed that the IMS technique using the Dynabeads anti-*Cryptosporidium* kit captured 100% of the oocysts in the suspension of feces.

Histologic examination. Stomach, liver, pancreas, duodenum, samples of proximal, medium and distal parts of jejunum, ileocaecal region, colon, and lungs were removed from each mouse, fixed in 10% neutral formalin and embedded in paraffin. Sections 5 μ m thick were either stained with hematoxylin and eosin (H&E) stains or used in immunohistochemistry (see below). Several stained sections of each solid organ and many longitudinal and transversal sections of gastrointestinal organs were examined microscopically using a Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID software, Cambridge, UK). The presence of parasites in the tissues was scored as follows¹³: 0, no parasites; 1+, small number of parasites focally distributed; 2+, moderate number of parasites widely distributed; 3+, abundant presence of parasites widely distributed throughout the tissue. Pathologic changes were classified according to the Nomenclature for Histologic Assessment of Intestinal Tumors in the Rodent.¹⁴ The importance of lesions was scored at the following sites: stomach, liver, duodenum, ileocaecal region, and colon. Lesions were scored as follows: 0, no lesion; 1, inflammation; 2, low grade intraepithelial neoplasia; 3, high grade intraepithelial neoplasia or adenoma with high-grade dysplasia (AHGD). The degree of severity of histologic damage for each mouse was calculated by the sum of individual scores over the five organs. This rating score, which was developed specifically to this work as no neoplastic lesions were reported before in *Cryptosporidium* infection, was based in the rationale of scoring for intestinal inflammation used previously.¹⁵

Immunohistochemistry. The expression of Ki-67 antigen in mouse intestinal sections was assessed using a monoclonal rat anti-mouse Ki-67 antibody (dilution 1:25) (M7249, Dako, Denmark), following the procedure recommended

by the supplier. Briefly, tissue sections were deparaffinized, rehydrated, and treated with a pH 6 citrate buffer for 40 minutes at 99°C. After incubation with the primary antibody, and after the secondary biotinylated rabbit anti-rat antibody (E0468, Dako)/streptavidin steps, the visualization was carried out by using DAB+ (Dako) as chromogen. The Ki-67 index was scored by counting ($400\times$ magnification) the total number of epithelial cell nuclei and that of Ki-67 immunostained nuclei in 10 randomly selected representative areas.¹⁶ Above all, rate and topography of Ki-67 immunostaining were carefully compared with the intestinal sections of control mice (Dex-treated or untreated uninfected SCID mice). For the calculation of the Ki-67 index, the areas were chosen in the sections where dysplastic lesions were identified. The Ki-67 index was scored according to the number of cells with positive nuclear staining per 1,000 cells in each area: +1, less than 10% of cells with positive nuclei; +2, 10–50% with positive nuclei; and +3, more than 50% of cells with positive nuclei and/or presence of proliferation in the upper third of the crypts and in the surface epithelium.^{10,17}

Statistical analysis. An analysis of variance (ANOVA) was conducted to account for the effects of relevant factors (inocula, Dex administration, day PI) and their interactions on average daily oocyst excretion. Data analysis was performed with the statistical software S-PLUS 2000 (MathSoft, Seattle, WA). Logarithmic transformations of oocyst excretion values were used. An average amount of oocysts excreted per day and per group of mice was estimated from all the unitary values. As stated previously, the amount of oocysts/mg of feces was estimated per day and per group of mice, with the number of mice per group decreasing over time. Significance was defined as $P \leq 0.05$.

RESULTS

Influence of *C. parvum* inoculum size and of dexamethasone administration on the intensity of oocyst shedding. The shedding of oocysts in the feces determined by IMS-IF was positive in all groups after the first day of collection PI until the end of the study. However, the median oocyst excretion during the experiments increased relative to inoculum size (in the range between 10^5 and 10^7 oocysts), and was substantially higher in Dex-treated mice than in the untreated mice (Figure 1). The highest oocyst excretion was observed in Dex-treated SCID mice inoculated with 10^7 oocysts. Unexpectedly, SCID mice inoculated with 10^8 oocysts (especially in Dex-treated animals), shed lower oocyst numbers compared with mice inoculated with lower oocyst doses (Figure 1). Additionally, ANOVA of the whole dataset showed that the day PI, the administration of Dex, and the interaction between Dex and the inoculum size, significantly influenced the oocyst excretion ($P < 0.001$, $P = 0.005$, $P = 0.05$, respectively).

Histologic alterations. In Dex-untreated SCID mice, regardless of the *C. parvum* inoculum size, mild histologic lesions were mostly observed in the stomach, liver, and duodenum (Table 1). In 8/16 Dex-untreated SCID mice (50%), parasites were detected in the antro-pyloric region in association with a slightly modified mucosa including the presence of glandular tubular structures with cellular atypias. These mucosal changes were found to be typical of low-grade intraepithelial neoplasia, and appeared as early as Day 35 PI with the highest inocula (10^7 and 10^8) (Table 1). In the liver of 13/16 (81.3%)

FIGURE 1. Daily *Cryptosporidium* oocyst excretion (natural logarithm of oocyst/mg feces) in different groups of severe combined immunodeficiency (SCID) mice over the 57 days of the study. Each box represents the median 50% of data, white line is the median. Whiskers represent the extreme values within 1.5 times the box height. Extra lines above and below the whiskers are the outliers.

Dex-untreated SCID mice, mild periportal inflammation, associated in some cases with parasite invasion of intrahepatic bile ducts, were detected. Interestingly, neither parasites nor inflammatory nor other lesions were detected in the large intestine (including caecum) of Dex-untreated SCID mice. When severity of histologic lesions was scored, Dex-untreated mice exhibited scores ≤ 4 (Table 1).

With regard to Dex-treated SCID mice, on the whole, they showed more extended and severe lesions than the untreated ones (Table 1). In 6/16 animals (37.5%) (mice nos. 7, 8, 15, 16,

24, and 32), lesions were detected in more than one organ with a maximum score of histologic severity of 11 in mouse no. 16 (Table 1). Specifically, 3 mice (nos. 24, 30, and 32) had stomach lesions suggestive of low-grade intraepithelial neoplasia, and one (no. 16) presented high-grade intraepithelial neoplasia characterized by an increasing architectural distortion associating glandular crowding, prominent cellular atypias, and pseudostratified nuclei, without stromal invasion (Figure 2 A and B). Mice nos. 8 and 32 (Table 1) had low- and high-grade intraepithelial neoplasia in the duodenum, respectively. In the ileocaecal regions, 6/16 Dex-treated SCID mice (37.5%) (nos. 7, 8, 15, 16, 23, and 24) (Table 1) presented adenomas with high-grade dysplasia after Day 46 PI (Figure 2 C and D, Figure 3), coinciding with the period of highest oocyst shedding. Three mice (18.8%) (nos. 7, 15, and 16) (Table 1) had also this kind of lesion but located in the colon. Lesions in the large intestine of Dex-treated SCID mice were characterized by adenomatous masses (Figure 3) that appeared closely packed, branching sometimes dilated tubular structures, separated by normal or inflammatory lamina propria. Focal cystic dilation was observed (Figure 2C). Some tubules were covered by a low- or high-grade dysplastic epithelium, which showed mucin depletion and nuclear stratification. In some areas, architectural distortion was associated with cellular atypias. Epithelial cells showed loss of normal polarity. As well, abnormal nuclear changes consisting of prominent nucleoli and irregularly scattered chromatin were recorded. Foci of merged glands typical of high-grade dysplasia invading into lamina propria were found (Figure 2).

On the whole, ileocaecal neoplastic lesions were found after Day 46 PI and only in Dex-treated mice (Table 1). However, gastric dysplastic lesions were observed as early as Day 35 PI (Table 1) in both Dex-untreated (nos. 18 and 26) and Dex-

FIGURE 2. Dex-treated severe combined immunodeficiency (SCID) mice infected with 10^6 oocysts of *C. parvum* (euthanasia Day 57 post-infection [PI]). **A**, Antro-pyloric region showing high-grade intraepithelial neoplasia characterized by an architectural distortion of glands (Bar = 80 μ m). **B**, Antro-pyloric region showing cellular atypias, loss of normal polarity of epithelial cells, and presence of numerous parasites inside the glands (Bar = 15 μ m). **C**, Ileocaecal region showing a projection of a polypoid structure with focal cystic dilation developing inside the intestinal lumen (Bar = 200 μ m). **D**, Areas of high-grade dysplasia (arrow) inside a neoplastic polypoid structure with numerous parasites (arrowhead) (Bar = 15 μ m), Hematoxylin & Eosin.

FIGURE 3. Ileocaecal region of a severe combined immunodeficiency (SCID) mouse infected with 10^5 oocysts of *C. parvum* and euthanized after Day 45 PI. Arrows show the presence of adenomatous masses in the intestinal lumen (Bar = 1,000 μ m).

treated mice (no. 30). Hepatobiliary inflammation was more frequent in Dex-untreated mice (13/16 versus 3/16) and it was observed with parasite colonization in two Dex-untreated mice (nos. 3 and 17).

A significant correlation (Figure 4) was observed between the histologic lesion severity scores and oocyst excretion rate ($r: 0.622, P < 0.001$). Consistently, most severe lesions were systematically associated with high parasite scores (all life cycle stages) in the tissues (Table 1). Thus, for lesions with severity scores of 2 or 3, the presence of parasites in the tissues was always either moderate or abundant, respectively, and this correlation was highly significant ($r = 0.83, P < 0.001$).

FIGURE 4. Correlation between the severity of histologic lesions and the intensity of daily oocyst excretion. Each point represents one mouse. Circles: Dex-untreated mice, Triangles: Dex-treated mice. Note that most animals euthanized between Days 20 and 35 post-infection [PI] have a score of severity < 2 (see Materials and Methods). All animals euthanized after Day 35 PI had a severity score ≥ 2 (= neoplastic lesions). There was a significant correlation between severity score and oocyst excretion ($r = 0.622, P < 0.001$).

At the histologic examination of the jejunum, a small number of parasites attached to the epithelial cells and villus atrophy and crypt hyperplasia were found without significant differences between groups of Dex-untreated or treated SCID mice. These infections were not associated with dysplastic changes. No parasites or morphologic changes were observed in other organs, such as lungs or pancreas. Mice of control groups did not develop *Cryptosporidium* infection or histologic gastrointestinal lesions.

Immunohistochemical assessment of Ki-67 antigen expression. An apparent increase of the mitosis number was observed in H&E stained sections of gastric (Dex-treated or untreated mice) or ileocaecal (Dex-treated mice) epithelia between Days 20 and 35 PI, before histologic evidence of neoplasia. To confirm that the dysplastic compartment at the top of the crypts represented abnormally proliferating cells, we stained the sections with an anti-Ki-67-specific antibody. In control or low infected animals euthanized at Day 20 PI, Ki-67 staining was observed at the lower third of gastric (data not shown) or ileocaecal epithelium crypts (Figure 5A–D). Among infected animals, the extension of Ki-67 staining increased progressively after 20 days PI, especially in mice with higher parasite loads, leading to proliferation in the upper third of the crypts and in the surface epithelium after 35 days PI (Figure 5E–H). Thus, immunohistochemical results using Ki-67 indicate that the alteration in cell proliferation occurs before the histologic diagnosis, which was currently established at Day 35 PI in the stomach, and at Day 46 PI in the ileocaecal region (Table 2).

Occurrence of gastrointestinal neoplasia. On the whole, gastrointestinal neoplastic lesions were observed in mice euthanized at Day 46 or after in both Dex-untreated and Dex-treated SCID mice (Table 1). Gastric intraepithelial neoplasia of low- or high-grade was more frequent in Dex-untreated SCID mice (6/8, 75%) than in Dex-treated animals (3/8, 37.5%). In contrast, only Dex-treated SCID mice developed duodenal (2/8, 25%) or colonic (3/8, 37.5%) intraepithelial neoplasia (of low or high grade).

With regard to the ileocaecal region (Table 1), only Dex-treated SCID mice developed neoplastic lesions described as high-grade intraepithelial neoplasia (or AHGD) in all the cases. Thus, 6/6 of Dex-treated SCID mice (100%) infected with 10^5 – 10^7 oocysts and euthanized after Day 46 PI showed ileocaecal lesions with a severity score of 3 (Figure 2; Table 1). Neoplastic lesions in more than one organ were observed in 5/6 of these mice (nos. 7, 8, 15, 16, and 24) (Table 1).

Furthermore, efforts to identify biotic or abiotic factors in the oocyst inoculum responsible for the observed neoplastic lesions were unsuccessful. No tissue lesions and no evidence of cryptosporidial infection were observed in either CDex or CDV control mice inoculated with filtrates of the oocyst inocula.

DISCUSSION

This work belongs to a series of experiments exploring the potential of *C. parvum* to induce neoplastic changes in the digestive epithelium of SCID mice treated or not with Dex. We chose this animal model based on the lack of functional B and T cells in SCID mice. Consequently, they are unable to have antibody or cell mediated immune responses, remaining infected by *Cryptosporidium* for long periods.⁹ However, it

FIGURE 5. Expression of Ki-67 in the epithelium of Dex-treated severe combined immunodeficiency (SCID) mice infected with *C. parvum*. **A**, Dex-treated uninfected mouse (control) euthanatized at Day 57 post-infection (PI): Ki-67 immunoreactive nuclei are observed at the lower third of the crypts (2–6 immunoreactive cells per glandular section) (Bar = 100 μ m). **B**, Detail of **A** (delimited area) (Bar = 50 μ m). **C**, Infected mouse euthanatized at Day 20 PI: Ki-67 immunoreactive cells confined to the lower third of the crypts (7–10 immunoreactive cells per glandular section) (Bar = 100 μ m). **D**, Detail of **C** (delimited area) (Bar = 50 μ m). **E**, Infected mouse euthanatized at Day 35 PI: increased extension of the staining (20–30 immunoreactive cells per glandular section) (Bar = 100 μ m). **F**, Detail of **E** (delimited area). Proliferating cells are observed in the upper third of the crypts and in the surface epithelium (Bar = 50 μ m). **G**, Infected mouse euthanatized at Day 57 PI: Ki-67 immunoreactive cells are present all over the crypt including the surface epithelium. The architecture of the mucosa is highly altered by the neoplastic process (Bar = 100 μ m). **H**, Detail of **G** (delimited area) (Bar = 50 μ m).

has been shown that some SCID mice can express detectable levels of immunoglobulin (leaky mice), especially when they get older.¹⁸ Treatment of animals with Dex was then based on previous reports showing that Dex administration reinforces immunosuppression, and strongly expands parasite rates in SCID mice infected with *Pneumocystis*, another opportunistic agent.¹⁹ Furthermore, a Dex-treated adult SCID mouse

model supported good propagation of at least two species of *Cryptosporidium*.⁶ Indeed, SCID mice treated with Dex showed less signs of inflammation and had higher risk of developing severe *C. parvum* infections.⁶ Consistently, in this study, regardless of the inoculum size, a slightly increased inflammatory response was observed in Dex-untreated mice. This observation is in agreement with the capacity of corticosteroids

TABLE 2
Immunohistochemical results using Ki-67

Mouse no.	Dexamethasone*	Inoculum	Number of days post-infection (PI)	Ki-67† (Presence of <i>C. parvum</i> organisms)			
				Stomach		Ileocaecal region	
1	No	10 ⁵	20	+1	(0)	+1	(0)
2			35	+1	(0)	+1	(0)
3			46	+1	(0)	+1	(0)
4			57	+3	(+3)	+1	(0)
5	Yes	10 ⁵	20	+1	(0)	+1	(0)
6			35	+1	(0)	+3	(+1)
7			46	+1	(0)	+3	(+3)
8			57	+2	(0)	+3	(+3)
9	No	10 ⁶	20	+1	(0)	+2	(0)
10			35	+2	(0)	+2	(0)
11			46	+2	(0)	+3	(0)
12			57	+3	(+2)	+3	(0)
13	Yes	10 ⁶	20	+1	(0)	+2	(0)
14			35	+2	(0)	+3	(+1)
15			46	+3	(0)	+3	(+3)
16			57	+3	(+3)	+3	(+3)
17	No	10 ⁷	20	+1	(0)	+1	(0)
18			35	+2	(+2)	+1	(0)
19			46	+3	(+2)	+1	(0)
20			57	+3	(+3)	+1	(0)
21	Yes	10 ⁷	20	+1	(0)	+2	(0)
22			35	+1	(0)	+2	(+1)
23			46	+2	(0)	+3	(+3)
24			57	+2	(+2)	+3	(+3)
25	No	10 ⁸	20	+2	(0)	+1	(0)
26			35	+2	(+2)	+1	(0)
27			46	+3	(+3)	+1	(0)
28			57	+3	(+3)	+1	(0)
29	Yes	10 ⁸	20	+3	(0)	+2	(0)
30			35	+3	(+3)	+3	(0)
31			46	+3	(0)	+3	(0)
32			57	+3	(+2)	+3	(0)

*Dexamethasone (4 mg/L of drinking water) administration was started 2 weeks before inoculation and maintained during the whole experimentation.

†Ki-67 index was scored according to the number of positive cells per 1,000 cells in each zone: +1 = less than 10% of cells with positive nuclei; +2 = 10–50% with positive nuclei; and +3 = more than 50% of cells with positive nuclei and/or presence of proliferation in the upper third of the crypts and in the surface epithelium (see Materials and Methods). In parentheses were added the results of the histologic semi-quantitative assessment of *C. parvum* organisms in the host tissues (see Materials and Methods). Parasite rates were scored according to previous studies as follows: 0 = no detectable parasite; 1+ = small number of parasites focally distributed; 2+ = moderate number of parasites widely distributed; 3+ = high number of parasites widely distributed throughout the tissue.

to inhibit innate immunity decreasing IFN- γ gene expression,¹¹ and with the ability of *Cryptosporidium* infection to induce increased interleukin-15 (IL-15) and IFN- γ expression to contribute to the elimination of parasites.²⁰

The first part of the study examined the influence of *C. parvum* inoculum size on the intensity of parasite shedding with or without Dex treatment. It was found that oocyst excretion increased according to inoculum size ranging between 10⁵ and 10⁷ and was substantially higher in Dex-treated mice than in the untreated ones. Thus, this animal model (especially with inoculum < 10⁷) could improve current models to perpetuate and amplify parasite isolates of *C. parvum* or *Cryptosporidium muris*. Moreover, another advantage of this model is that animals become chronically infected, and have a significant oocyst shedding during at least 120 days (Certad G and others, unpublished data).

Of interest, in our study the mean number of excreted oocysts tended to decrease in Dex-treated mice receiving

the highest challenge inoculum (10⁸). A likely similar phenomenon was evoked in *C. parvum* experimental infection of healthy humans.²¹ Furthermore, in studies of *Eimeria* infection of chickens and rats, it was especially noticeable that with the greatest infecting dose, the number of oocysts produced per oocyst fed was smaller.²² Sequestration of the parasites out of the intestine and into other sites could also explain a diminished detection of *Cryptosporidium* oocysts in the faeces.²³ However, in our study, mice infected with 10⁸ oocysts showed no more evidence of extra-intestinal parasites or more clinical signs than mice in other groups (Table 1).

Traditionally, in SCID mice *C. parvum* develops usually in enterocytes of small intestine and colon; gastric and duodenal locations are reported less often.⁹ In our study, the examination of tissue sections revealed marked differences between groups in relation with parasite distribution in the gastrointestinal tract. In Dex-untreated *C. parvum*-infected mice, parasites were localized predominantly in the stomach, as early as 35 days PI, and in less degree in the liver. Mead and others²⁴ noted gastric colonization in *C. parvum*-infected SCID mice (without corticosteroid administration) since the eighth week, with an intensity of the infection that increased henceforth.²⁴ Others authors have reported that liver involvement occurs later, in some cases 13–26 weeks PI.²⁵ However, we found that *C. parvum* parasites under Dex develop predominantly in the ileocaecal region and in the colon of SCID mice (Reference 6 and present work) (Table 1).

As described in our previous work, we confirmed that *C. parvum* induced neoplastic changes. Particularly notable, the association between *C. parvum* infection and the generation of ileocaecal tumors was shown in most animals, as we reported before.⁶ Additionally, for the first time *C. parvum*-induced neoplastic changes were also found in the stomach (12/16 SCID mice) and in the duodenum (2/16). Neoplastic lesions in the stomach were more frequent in Dex-untreated (8/16) than in Dex-treated mice (4/16) (Table 1). Indeed, when we assessed the intensity of the infection according to both oocyst shedding and extension of parasite invasion in the digestive tract (Table 1), a highly significant correlation (Figure 4) was found between intensity of cryptosporidiosis and severity of neoplastic lesions in Dex-treated or untreated mice. This observation suggests a direct role of *C. parvum* in the development of neoplastic lesions. Although Mead and colleagues⁹ did not report neoplastic changes in SCID mice experimentally infected with *C. parvum* (without corticosteroid administration), their results also suggested a correlation between inoculum size and severity of disease/clinical signs. After a challenge with different inoculum sizes, they noted that the extension of the infection, including colonization of gallbladder and hepatobiliary duct epithelium, was pronounced in SCID mice receiving the highest oocyst dose (10⁷).⁹

Our study showed that animals not treated with Dex had alterations in the liver and duodenum rather associated with inflammation than with neoplasia (13/16 and 8/16 Dex-untreated mice had inflammation in liver and duodenum, respectively). However, hepatic histopathologic changes were associated with parasite detection in only two Dex-untreated mice and no parasite was detected in duodenum (Table 1). Inflammatory changes in the same organs were absent in Dex-treated SCID mice (Table 1). This divergence could be explained by the anti-inflammatory effect of Dex. Differences in the severity score of lesions between the groups of Dex-untreated

and Dex-treated animals would therefore be more marked if purely inflammatory changes were not taken into account.

The present experiments show also that *C. parvum* infection induced early the emergence of an abnormal pattern of enterocyte proliferation, even before the identification of histologic changes revealing neoplasia (Figure 5). Indeed, the actual presence of neoplasia either in the stomach or in the ileocaecal region was consistent with the extension of Ki-67 staining above the basal third of the crypt. It has been suggested that a Ki-67 abnormal staining reflects an altered proliferative epithelium rather connected with neoplasia pathogenesis than with hyperplastic lesions.¹⁷ According to the standards for histologic assessment of intestinal tumors in mice,¹⁴ mitosis in hyperplastic epithelium are typically located in the lower two-thirds of the mucosa, nuclei lack significant atypia, are basally located, ovoid to round, and are usually uniformly dark with occasionally visible nucleoli.¹⁴ In our study, the dysplastic epithelium at the top of the crypts exhibited a markedly abnormal pattern of proliferation, similar to that observed in advanced neoplasms.²⁶ Additionally, early increases of Ki-67 scores preceded local detectable parasite proliferation in about half of mice.

According to the current classification of intestinal tumors in rodents, the *C. parvum*-induced changes found in Dex-treated SCID mice correspond to gastrointestinal neoplasia (= microadenoma, microcarcinoma, carcinoma *in situ*, focal areas of dysplasia or adenoma, i.e., circumscribed broad-based, sessile, or pedunculated tumors lined by dysplastic epithelium, with either low-grade or high-grade dysplasia).¹⁴ As stated by the Vienna classification of human gastrointestinal neoplasia most severe lesions we found correspond to subcategory 4.2: "non-invasive carcinoma (carcinoma *in situ*)."^{27,28} On this last point, the histologic changes described here can be considered as putative precursors to digestive neoplasia. Actually, human colorectal tumorigenesis is believed to involve a series of genetic changes leading to the progression from normal epithelium to carcinoma, via the intermediate steps of dysplasia and adenoma.²⁹ Consistently, we have recently observed invasive adenocarcinoma in a larger series of Dex-treated SCID mice infected with *C. parvum* and euthanatized at Day 120 PI (Certad G and others, unpublished data).

Previous studies have associated cryptosporidiosis with the development of tumor lesions in vertebrates. One report described the association between *Cryptosporidium* sp. and aural-pharyngeal polyps in iguanas.³⁰ Cystic hyperplasia of the colonic mucosa was also described in nude mice.³¹ None of these studies has described the presence of pre-malignant lesions associated to cryptosporidiosis. However, the presence of portal fibrosis, biliary sclerosis, and necrosis with dilation of ductlike structures lined by highly atypical biliary epithelial cells was found in IFN- γ knockout mice infected with *Cryptosporidium*.²⁵ These changes, classified as low-grade dysplasia,²⁵ are consistent with our results. Interestingly, similar histologic findings (especially Figure 3 in Mead and others²³) were associated with chronic *C. parvum* infection in NIH-III nu/nu mice.²³

Despite the scarcity of information about links between human cryptosporidiosis and digestive neoplasia, some previous data suggested a possible causal association. An epidemiologic study of 55 patients with colorectal cancer and before chemotherapy, reported 18% of cryptosporidiosis prevalence.⁵

No data about the mechanism of *C. parvum*-induced neoplasia are available. Nevertheless, it is well known that *Theileria parva*, another apicomplexan parasite, is responsible for a lymphoproliferative disorder of cattle.³² This organism infects and transforms bovine lymphocytes resulting in tumors with metastatic/invasive potential by a mechanism associated to an inhibition of apoptosis.³² Inhibition of apoptosis has also been reported in other apicomplexan protozoa including *Cryptosporidium*³³ that is able to activate NF- κ B pathway, preventing the induction of cell death early after infection. Apoptosis prevention probably benefits the parasite by stabilizing the host cell long enough to permit the completion of the life cycle.³³ Interestingly, resistance to apoptosis could be an essential step in the progression to malignancy.⁸ Therefore, persistent infection with *C. parvum* could be a risk for gastrointestinal neoplasia as an adverse effect caused by the inhibition of intestinal cell apoptosis.

In summary, despite the relatively small number of mice we used, statistical correlations were found between the intensity of *Cryptosporidium* infection and the score of severity of dysplastic lesions, especially in Dex-treated SCID mice. Moreover, adenomas with low- or high-grade intraepithelial neoplasia associated with numerous *C. parvum* life stages were observed in different areas of the digestive tract, including stomach, duodenum, and ileocaecal region. The use of Ki-67 supported the neoplastic nature of the described *Cryptosporidium*-induced epithelial transformation, and showed that potential neoplastic alterations begin before histopathologic lesions can be detected with standard stains. Further studies should be done to characterize at molecular level the *Cryptosporidium*-induced gastrointestinal neoplasia, and to explore its potential occurrence in humans.

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