Intestinal Microflora and Digestive Toxicity of Irinotecan in Mice

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Abstract

Purpose: Delayed diarrhea is the most important side effect of irinotecan. The aim of this study was to investigate the role of intestinal microflora on the induction of systemic and intestinal toxicity and diarrhea, studying germ-free and holoxenic mice i.p. injected with irinotecan.

Experimental Design: To evaluate the lethal dose, starting with 100 mg/kg/4 d, we treated the holoxenic mice with 100, 80, and 60 mg/kg/4 d and germ-free mice with 60, 80, 100, and 150 mg/kg/4 d. We recorded the percentage of dead animals, diarrhea, and the epithelial damage to the jejunum, ileum, cecum, and colon at optical and scanning electron microscopy.

Results: Germ-free mice were more resistant to irinotecan than the holoxenic group. The lethal dose was between 60 and 80 mg of irinotecan for holoxenic mice and ≥150 mg for the germ-free. The intestinal damage score was higher in holoxenic than germ-free mice at 100 mg and equally diffuse in the small and large bowel. The damage in germ-free mice was less severe (8 of 40 samples) prevailing in the ileum. The differences were significant for all sites (jejunum, $P < 0.001$; ileum, $P = 0.012$; cecum, $P = 0.001$; colon, $P < 0.001$). No damage was found in germ-free mice at 60 mg. Diarrhea was present in all 100 and 80 mg holoxenic mice and in 19 of 20 cases at 60 mg whereas it was absent in 60 mg or sporadic in 80 and 100 mg germ-free mice.

Conclusions: The intestinal microflora plays a key role in the intestinal toxicity of irinotecan.

Irinotecan is an antiproliferative drug active against several tumors and mainly used in the treatment of colorectal cancer (1).

Limiting toxicities are neutropenia and diarrhea, which frequently occur together. Whereas early diarrhea occurs within 24 hours and can be avoided by prophylactic administration of atropin (because early diarrhea is a cholinergic-related effect), delayed diarrhea appears between the 2nd and 21st days, but mainly between the 6th and 10th days after drug administration (2). Diarrhea is observed in ~70% of treated patients and is higher in monotherapy and in the first cycles, where grade 3-4 diarrhea affects up to one of three of the patients (3, 4), whereas in the schedules of association it rarely exceeds 20% of cases (4, 5). The clinical importance is evident if we consider that diarrhea can induce changes to treatment and make it impossible to maintain the scheduled dose intensity. Indeed, after 1 month of therapy with a weekly regimen of Irinotecan and 5-fluorouracil/folinic acid, 55% of patients received a reduced dose mainly because of intestinal toxicity (6). Because the diarrhea is often poorly controlled by loperamide, other drugs or strategies (such as octreotide, acetorphan, antibiotics, glutamine, budesonide, IL-15, thalidomide, cyclooxygenase-2 inhibitors, ciclosporin, kampo medicines, and alkalization of intestinal lumen) have been suggested.

Delayed diarrhea has been attributed to both a secretory and exudative mechanism reflecting damage to the intestinal mucosa (2). The i.p. administration of irinotecan in mice induces diffuse mucosal damage with a correlation between the grade of diarrhea and the amount of irinotecan administered (7), as well as its concentration and that of its active metabolite SN-38 concentrations in the intestinal wall (8). The few patients with irinotecan-induced diarrhea, studied for endoscopy and histology, have shown diffuse mucosal damage to the large bowel (9). Although the action of bacterial pathogens has been ruled out, intestinal bacterial microflora have been implicated in irinotecan toxicity (10). In fact, irinotecan is a prodrug transformed into the much more active SN-38 by hepatic, serum, neoplastic, and gastrointestinal carboxylesterases. In turn, SN-38 is glucuronized in the liver and released into the intestinal lumen as a nontoxic SN-38 glucuronide.

Bacterial β-glucuronidase in the intestinal lumen may trigger the release of large amounts of active SN-38 starting from SN-38 glucuronide (10). Prophylactic use of antibiotics in rats reduced both SN-38 concentration in the intestinal lumen and diarrhea (11). Administration of antibiotics in man reduced both fecal β-glucuronidase activity and the SN-38/SN-38 glucuronide ratio (12) although available data on the efficacy

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Received 4/5/05; revised 11/4/05; accepted 12/2/05.

Grant support: "Ex-60% program" of the University of Bologna.

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doi:10.1158/1078-0432.CCR-05-0750

of this prophylactic treatment on diarrhea are inconclusive (12, 13). To settle the matter, we evaluated the direct role of intestinal microflora using holoxenic mice (bearing their own intestinal microflora) and germ-free mice comparing their respective lethal doses of irinotecan, diarrhea, and damage to the intestinal epithelium.

Materials and Methods

Animals

We used 120 adult holoxenic and germ-free C3H/HJ mice obtained from the Centre National de la Recherche Scientifique (Orleans-La-Source, France). Animals were treated at "Unité d'Ecologie et de Physiologie du Système Digestif," Institut National de la Recherche Agronomique (Jouy-en-Josas, France), in accordance with local institutional guidelines for the proper care of animals.

Germ-free mice were reared in flexible plastic Trexler type isolators (La Calhène, Velizy-Villacoublay, France), transferred to an experimental isolator, and housed in groups of five per cage. They were fed *ad libitum* with a commercial diet sterilized by γ irradiation (40 kGy) and given sterilized (20 minutes, 120°C) tap water to drink. Room temperature was 21°C and with 12-h light/dark periods.

Irinotecan was i.p. injected for 4 consecutive days from 10 a.m. to 11 a.m. to avoid any chronopharmacologic interference. The control groups for the germ-free and holoxenic mice were i.p. injected with physiologic solution at the same volume as irinotecan (0.2-0.3 mL/mouse/d). Mice had an initial mean weight of 32 g (range, 27-38 g) and were weighed daily before each administration of irinotecan. The procedure for germ-free mice was as follows: each germ-free mouse was put in a previously weighed single sterile container, then taken from the isolator, weighed, and finally returned to the isolator.

Study design

Lethal dose and time of death. To establish the lethal dose in both holoxenic and germ-free animals, we started with 100 mg/kg/d for 4 days (20 animals per group) according to the dose used by Ikuno et al. (7). Because this dose turned out to be lethal for all the holoxenic treated mice, two independent groups of holoxenic mice were respectively treated with successive doses of 80 mg/kg/d (10 mice) and 60 mg/kg/d (20 mice). The number of mice was doubled in the latter group to obtain an adequate number of animals for histology.

In contrast, because the 100-mg dose was not lethal for all germ-free mice, three independent groups of animals (10 per group) were inoculated with doses of 60, 80, and 150 mg/kg/d, respectively. Ten germ-free and 10 holoxenic control mice were also used.

The percentage deaths and the time lapsed between inoculation and death were recorded in each group of animals. The dying animals were killed immediately whereas the surviving ones were killed on the 15th day after drug administration. The mice were killed by exanguination. The abdomen was opened through a midline incision and the gastrointestinal tract was removed. Large samples from the jejunum, ileum, cecum, and colon were examined by light and scanning electron microscopy.

Diarrhea. Diarrhea was only evaluated in terms of its presence/absence in the different groups of animals. In mice, diarrhea can be indirectly assessed by the presence of perianal staining of the coat and/or sawdust around the anus; thus, it is very difficult to give a diarrhea score.

Histologic analysis. Histologic damage to the intestinal mucosa was evaluated by optical microscopy and scanning electron microscopy, comparing germ-free and holoxenic mice treated with 100 mg irinotecan.

Light microscopy. Specimens for light microscopy were fixed in 10% formalin and processed according to conventional methods. Serial 3-μm sections from each specimen were stained with H&E.

The hallmarks of intestinal damage in irinotecan treated mice are epithelial vacuolization and necrosis, presumably correlated phenomena indicating progressive damage. Damage to the epithelium was graded as follows: 0, absence of alterations; 1, focal vacuolization; 2, diffuse vacuolization; 3, focal necrosis; and 4, multifocal necrosis.

Other characteristics of the irinotecan treatment are reduced epithelial thickness, enlargement of blood and lymphatic vessels in the lamina propria, submucosal edema, hyperplasia of goblet cells, and changes in the morphology and number of Paneth cells counted in 10 crypts for each specimen.

Scanning electron microscopy. Samples of the jejunum, ileum, cecum, and colon were fixed in 5% glutaraldehyde in 0.1 phosphate buffer (pH 7.2) for 15 to 30 minutes at 4°C to 5°C. After several washings in buffer, the samples were dehydrated in serial solutions of ethanol (10%, 30%, 50%, 75%, and 95%) for 15 minutes each at 4°C to 5°C. After a passage in absolute ethanol at room temperature, samples were further dehydrated by Critical Point Drying (Balzers 010), then put on specific aluminium plates and covered with gold-conducting film with Balzers MED 010. Finally, the specimens were observed with Philips Scanning Electron Microscopy 515 at acceleration between 8 and 15 kV.

Statistical tests. The different survival between holoxenic and germ-free mice treated with irinotecan at different doses was evaluated with the Kaplan-Meier method and the log-rank test was used to compare the curves. Differences in damage to the jejunum, ileum, cecum, and colon in germ-free and holoxenic mice treated with irinotecan 100 mg were evaluated with light microscopy using Mann-Whitney *U* test. Fisher's exact test was used to assess the differences in blood vessel enlargement in both the jejunum and colon of germ-free and holoxenic mice. The number of Paneth cells in control and treated germ-free and holoxenic mice was evaluated with ANOVA test.

Results

Lethal dose and time of death

Figure 1 shows that germ-free mice were more resistant to the drug than holoxenic mice. On the 15th day, only 1 of 50 holoxenic mice was alive. All holoxenic mice treated with 100 or 80 mg/kg/d died within the 8th day from beginning of the test with percentages of survival of 45% and 80% on the 5th day and 5% and 30% on the 6th day, respectively.

All animals of the holoxenic 60 mg group were alive on the 6th day; in this group, deaths occurred later within the 7th and 10th days. The surviving animal was in apparently good health on the 15th day.

By contrast, all germ-free mice treated with 60 and 80 mg were living and in apparently good health on the 15th day. Only 30% (6 of 20) of the germ-free 100 mg animals died, all within the 8th day, whereas on the 6th day, 95% of the animals were still living versus 5% of the 100 mg holoxenic group.

Eighty percent (8 of 10) of the germ-free mice treated with 150 mg died. In this group, deaths occurred earlier and all within the 6th day (it was impossible to complete the entire cycle of irinotecan in the six animals which died before the last planned inoculation). Differences in survival between holoxenic and germ-free mice treated with irinotecan 100/80/60 mg/kg/4 d were statistically significant ($P < 0.0001$, log-rank test). In other words, the lethal dose 100 was two to three times higher in germ-free mice than in holoxenic ones, exceeding 150 mg/kg/4d in the former and ranging between 60 and 70 mg/kg/4d in the latter.

All control animals (holoxenic and germ-free) were living and healthy on the 15th day after the inoculation of i.p. physiologic solution.

Diarrhea

Diarrhea was present in all 100 and 80 mg holoxenic mice from the 4th day onward. In addition, diarrhea was present in almost all the 60 mg holoxenic mice (19 of 20). Diarrhea was

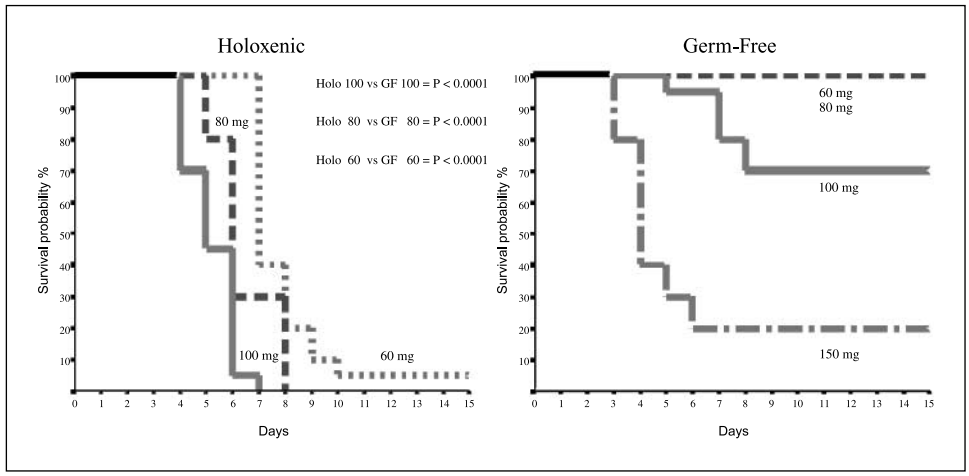

Fig. 1. Survival of holoxenic and germ-free mice treated with different doses of irinotecan.

absent in the 60 mg germ-free mice; at 80 mg, it was sporadic and moderate in only one animal. Diarrhea was already evident in 100 mg germ-free dead mice after the third inoculation of irinotecan and lasted until the animals died. By contrast, moderate diarrhea was found in only 2 of 14 surviving 100 mg germ-free animals and disappeared before they were killed on the 15th day. At 150 mg, some germ-free animals died suddenly before developing diarrhea whereas diarrhea was severe in the other dead mice.

Intestinal damage at light microscopy

Table 1 lists the epithelial damage in the jejunum, cecum, ileum, and colon of 10 holoxenic mice treated with irinotecan 100 mg and 10 surviving germ-free mice treated with irinotecan 100 mg.

Among the 40 intestinal segments of the holoxenic animals examined, only one was undamaged. The damage mainly consisted of diffuse vacuolization (16 samples) and focal necrosis (15 samples). Damage was equally diffused in the small and large bowel in mice bearing their own intestinal microflora, with a slight prevalence of necrosis in the cecum and colon (6 and 6) compared with the jejunum and ileum (4 and 4). Damage was more rare in germ-free mice (32 of 40 samples had no epithelial damage), prevailing in the small bowel (mainly ileum) and significantly less severe than that encountered in holoxenic animals (jejunum, $P < 0.001$; ileum, $P = 0.01$; cecum, $P = 0.001$; colon, $P < 0.001$; Mann-Whitney U test).

Figure 2A to H shows the most common histologic aspects found in the jejunum, ileum, colon, and cecum of the irinotecan 100 mg holoxenic and germ-free mice. In addition, lacking vacuolization or signs of necrosis, a monolayer epithelium was more frequently found in the cecum of germ-free than holoxenic animals (5 versus 2). Hyperplastic/hypertrophic goblet cells were only seen in the colon of holoxenic animals (6 of 10 cases).

Histologic analysis was possible in only four germ-free animals which died after treatment with irinotecan 100 mg between the 5th and 8th days. The jejunum of these four animals showed a slight reduction of villi with hypertrophic Paneth cells, signs of epithelial vacuolization, and focal necrosis in one mouse only. In addition to a reduction of villi and hypertrophic Paneth cells, areas of multifocal necrosis were

Table 1. Score of intestinal epithelial damage in small and large bowel of germ-free and holoxenic mice treated with irinotecan 100 mg/kg/d × 4

	No damage, 0	Focal vacuolization, 1	Diffuse vacuolization, 2	Focal necrosis, 3	Multifocal necrosis, 4
Holoxenic mice					
n	1 (2.5%)	3 (7.5%)	16 (40%)	15 (37.5%)	5 (12.5%)
Jejunum	0	2	4	3	1
Ileum	0	0	6	3	1
Cecum	0	1	3	5	1
Colon	1	0	3	4	2
Germ-free mice					
n	32 (80%)	3 (7.5%)	2 (5%)	3 (7.5%)	0 (0%)
Jejunum	9	1	0	0	0
Ileum	5	2	1	2	0
Cecum	8	0	1	1	0
Colon	10	0	0	0	0

NOTE: *n*, total number and percentage of mice with different types of damage irrespective of sites. Jejunum: holoxenic versus germ-free, $P < 0.001$; ileum: holoxenic versus germ-free, $P = 0.012$; cecum: holoxenic versus germ-free, $P = 0.001$; colon: holoxenic versus germ-free, $P < 0.001$. Mann-Whitney U test.

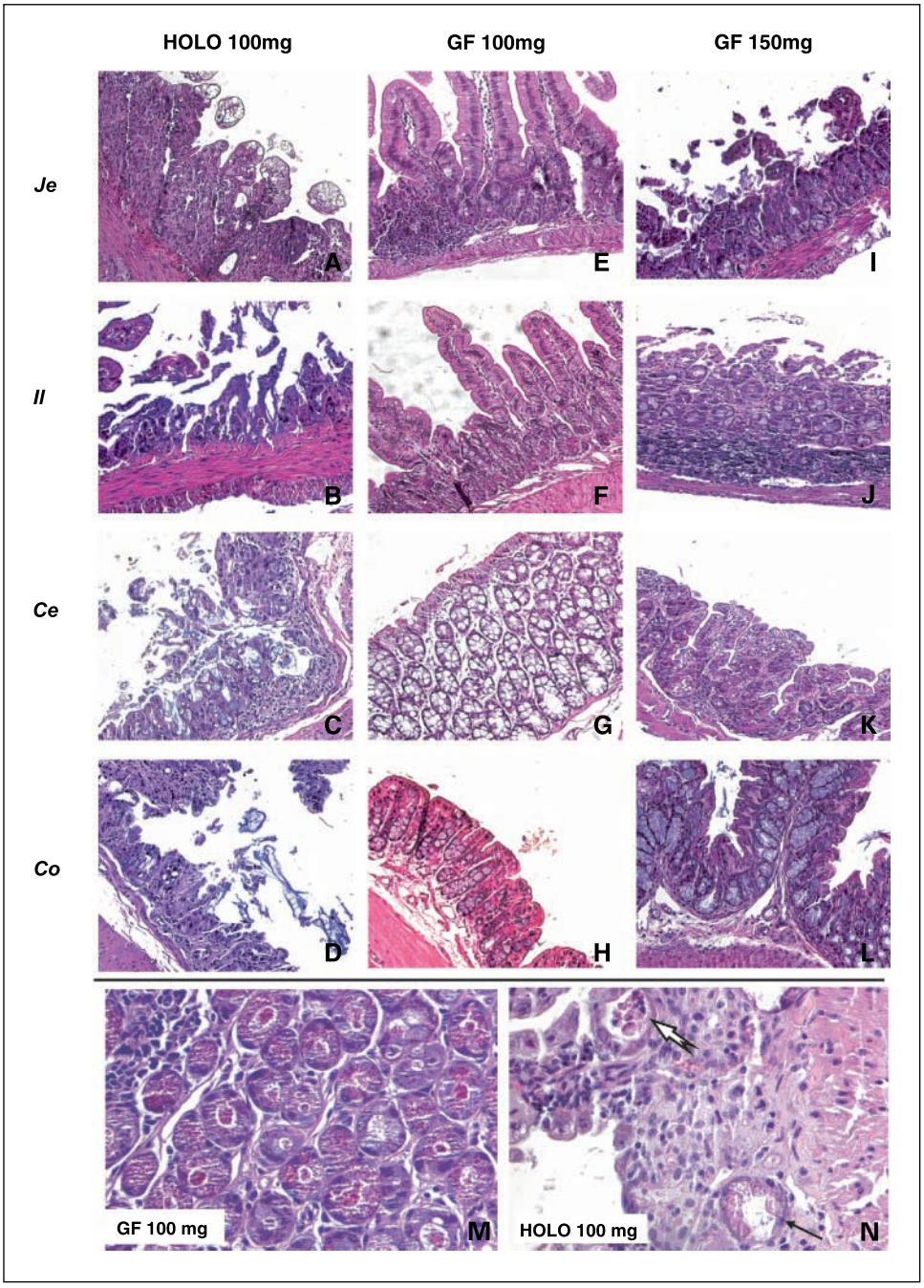

Fig. 2. *A* to *N,* histopathologic changes in the jejunum (*Je*), ileum (*Il*), cecum (*Ce*), and colon (*Co*) of holoxenic and germ-free C3H/HJ mice treated with 100 or 150 mg (germ-free) of irinotecan (H&E staining; magnification, ×250). *A,* holoxenic mice show shortened villi in the jejunum with several vacuolized epithelial cells; *B* to *D,* areas of epithelial necrosis in the ileum, cecum, and colon. *E* to *H,* germ-free mice (100 mg) have no visible damage in the small or large bowel. Germ-free 150 mg mice have visible damage only in the small bowel (necrosis of villi; *I* and *J*) whereas the large bowel is spared (*K* and *L*). *M* and *N,* features of Paneth cells in the ileum in germ-free and holoxenic 100 mg treated mice (magnification, ×400). Paneth cells are more numerous and hypertrophic in germ-free treated mice compared with 100 mg holoxenic treated mice (*black arrow,* a single degranulated Paneth cell; *empty arrow,* an enlarged vessel of the lamina propria).

present in the ileum in three of four animals. No vacuolization or necrosis was present in the cecum but only a reduction of epithelial thickness with a monolayer in two cases. The colon was spared in all mice. It was confirmed that damage was limited to the small intestine also in the dying 100 mg germ-free animals, with greater damage in the ileum than in the surviving 100 mg germ-free mice.

For the holoxenic 60 mg mice (i.e., around lethal dose; 10 evaluated by histology), the histologic damage reflected that found in the 100 mg group, whereas at 60 mg no damage occurred in the germ-free mice. Vice versa, there was severe vacuolization and necrosis in the jejunum and mainly ileum in germ-free mice irinotecan 150 mg (i.e., at around their lethal dose) without manifest damage in the large bowel (Fig. 2I-L).

No intestinal histologic damage was found in a single irinotecan 150 mg germ-free mouse, given the early death of the animal (just after the second injection of irinotecan) and presumably before the damage had time to develop. Figure 3 summarizes the topography of damage to the small and large bowel in holoxenic and germ-free treated mice at different doses of irinotecan.

Blood vessels. The enlargement of blood vessels (frequently associated with severe submucosal edema) prevailed in the holoxenic mice as opposed to the germ-free animals and this difference was statistically significant in both the jejunum and colon ($P < 0.001$ and $P = 0.007$, respectively; Fisher's exact test).

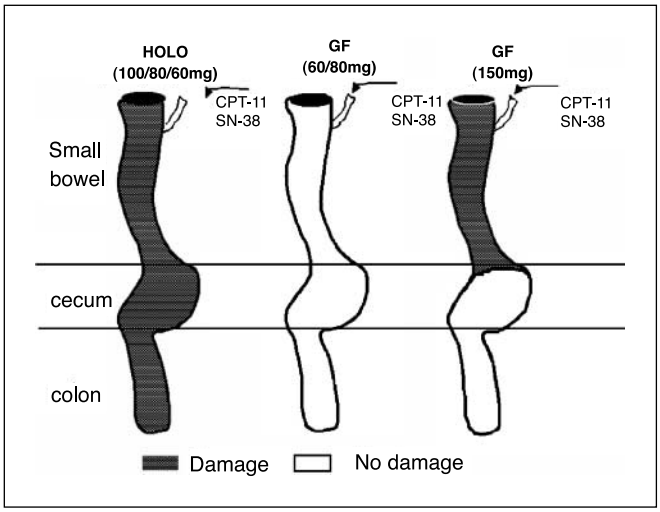

Fig. 3. Topography of the damage in the intestinal tract of holoxenic and germ-free mice treated with irinotecan at different dosages of irinotecan. The damage in holoxenic treated mice is present in small and large bowel whereas in germ-free mice no damage is found around the lethal dose for holoxenic (60-80 mg). The damage appears only in the small bowel at 150 mg irinotecan in germ-free mice whereas the large bowel is undamaged.

Paneth cells. Untreated control germ-free and holoxenic mice have a similar number of Paneth cells in the jejunum and ileum [$P > 0.05$ (NS), ANOVA] but the cells are larger in germ-free than in holoxenic mice because they contain more secretion granules. A remarkable degranulation of Paneth cells and a statistically significant decrease in their number were observed in the jejunum and ileum of 100 mg holoxenic mice compared with germ-free 100 mg ones ($P = 0.002$ and $P = 0.001$, respectively) and compared with control holoxenic mice with respect to the ileum ($P = 0.029$; ANOVA; Table 2; Fig. 2M-N). In contrast, there were no significant differences in the number of Paneth cells between controls and treated germ-free mice although the cells appeared larger in the germ-free mice, probably due to the absence of degranulation.

Scanning electron microscopy

Scanning electron microscopy allowed a detailed evaluation of the surface epithelial damage and the microbial ecology of the various intestinal segments. Figure 4A to H shows the different bacterial morphotypes along the intestinal tract of holoxenic mice: the jejunum mainly contains cocci and small bacilli. Filamentous bacteria can be found in the ileum, directly linked to the epithelium. Several bacterial populations in the cecum and colon are scattered throughout the mucosal surface. Their morphology differs from that of the small intestine and mainly consists of pointed rods and spiral bacteria referable to anaerobic strains.

Figure 4I to J shows the ultrastructural characteristics of apoptosis, the most typical expression of the damage from irinotecan. The process is characterized by a rarefaction of microvilli and the formation of blebs on the surface of the intestinal epithelial cells. The increased apoptotic rate induced by the chemotherapy drug brings about a significant loss of both single and grouped cells, which the proliferation rate is unable to replace. As a consequence, different-sized interruptions are formed in the epithelial lining.

Diffuse damage, as already assessed at light microscopy, was observed in holoxenic mice treated with irinotecan 100 mg in all sectors of the intestine with beheading of villi, holes in the epithelium due to increased cellular loss caused by severe apoptosis, and clearly visible areas of necrosis mainly in the cecum and colon. Furthermore, fibrine filaments and many bacteria infiltrated these superficial epithelial interruptions. At an equivalent dose, the damage was far less remarkable in germ-free mice and was confined to apoptotic outcomes in the ileum and sporadically in the cecum.

Histologic damage in holoxenic 60 mg irinotecan mice was similar to that in the 100 mg group whereas no damage occurred in the corresponding germ-free mice (Fig 5A-H). In contrast, scanning electron microscopy confirmed the presence of necrosis and vacuolization in 150 mg germ-free animals, already found at optical microscopy, and disclosed significant apoptotic damage mainly in areas of the jejunum and ileum apparently free at light microscopy. In addition, very slight random damage was found in the cecum not detected at light microscopy whereas the colon was also unaffected at scanning electron microscopy (Fig. 5I-L).

Discussion

Our results clearly show that bacterial factors enhance the intestinal toxicity of irinotecan. Although germ-free animals are considered more fragile than holoxenic ones, germ-free life ensures a stronger resistance to the systemic and intestinal damage induced by irinotecan. The lethal dose of irinotecan in germ-free mice is thrice higher than in holoxenic mice, being >150 mg/kg/d × 4 i.p. in the former and ~60 to 70 mg/kg in the latter. The final causes of mortality in mice have not been directly investigated (evaluating neutropenia and bacteriemia through hemograms and blood cultures) although a similarity is likely

Table 2. Number of Paneth cells in controls and in 100 mg irinotecan holoxenic and germ-free treated mice

Anatomic site	Controls		Irinotecan 100 mg	
	Holoxenic	Germ-free	Holoxenic	Germ-free
Jejunum	36.8 ± 5.8 (31-46)	40.6 ± 6.1 (31-46)	22.6 ± 20.6* (9-80)	48.7 ± 18.7 (17-80)
Ileum	44.0 ± 6.1 (29-52)	50.2 ± 7.1 (45-61)	22.8 ± 22.5†,‡ (9-83)	55.1 ± 15.4 (21-72)

NOTE: Data are expressed as mean ± SD per 10 crypts and ranges.
*$P = 0.0002$, in comparison with germ-free treated mice (ANOVA).
†$P = 0.029$, in comparison with the ileum of the control holoxenic mice (ANOVA).
‡$P = 0.001$, in comparison with germ-free treated mice (ANOVA).

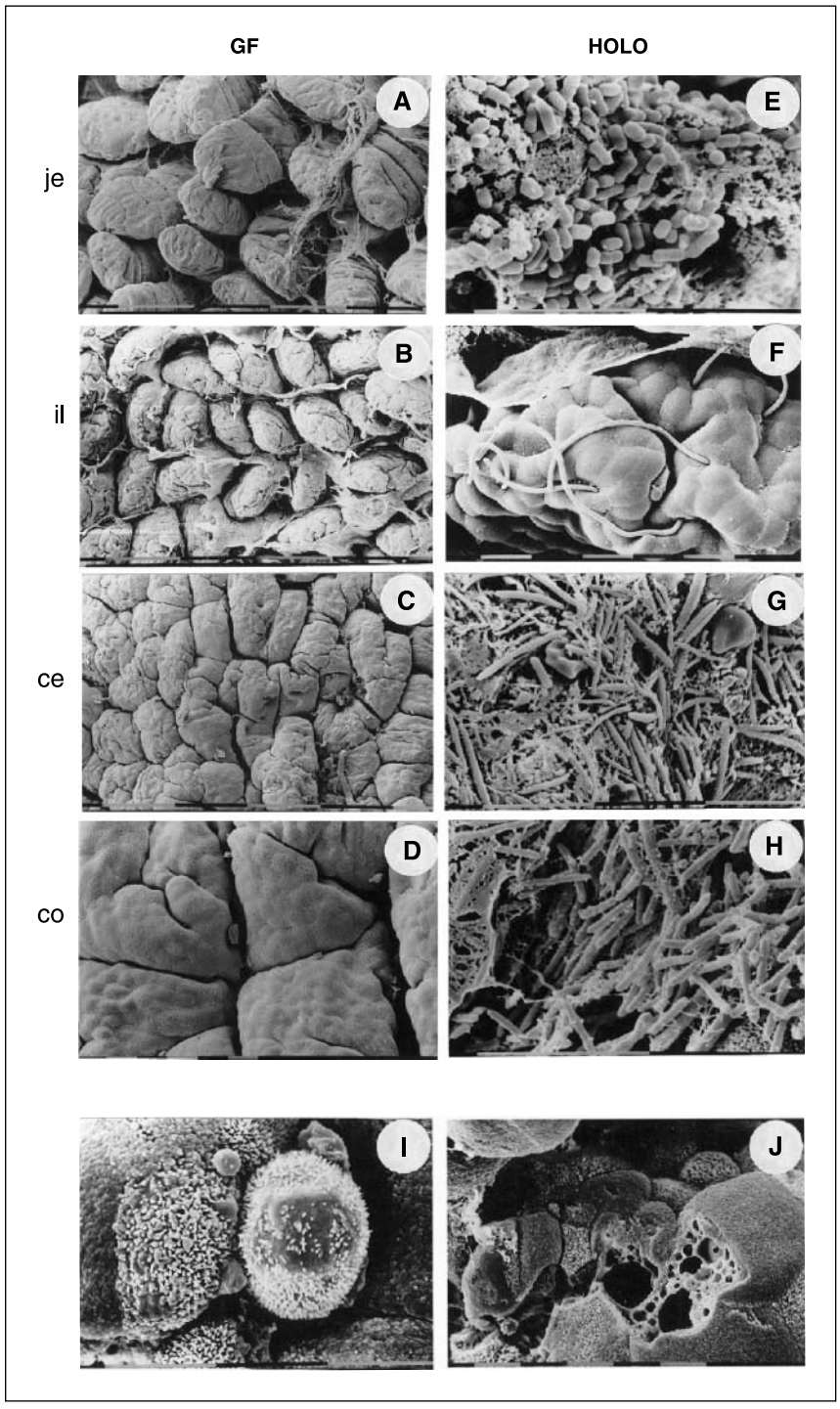

Fig. 4. Scanning electron microscopy of the jejunum, ileum, cecum, and colon of germ-free (*A-D*) and holoxenic (*E-H*) C3H/HJ control mice. Bar, 0.1 mm (*A*, *B*, and *C*); 10 μm (*E-H*). No bacteria are found on the mucosa in germ-free mice. Different types of bacteria are found in different intestinal segments in holoxenic mice. Long filamentous bacteria, confined to the mucosa, are visible in the ileum (*G*). Several morphotypes and many bacteria are visible in the large bowel (*G* and *H*). *I* and *J*, apoptotic epithelial cells. Bar, 10 μm. There is a rarefaction of microvilli and blebs on the cell surface (*I*); the residual epithelium is on the right after the loss of single cells due to apoptosis (*J*). je, jejunum; il, ileum; ce, cecum; ce, colon.

to exist between holoxenic mice and patients with irinotecan-related severe febrile diarrhea. These patients can die from septicemia due to neutropenia and mucosal damage inducing bacterial translocation. Endoscopy in patients with severe diarrhea from irinotecan always disclosed intestinal damage (hemorrhage, ulceration, and perforation; ref. 9) whereas histology showed a thinning of the colonic mucosa (14), one of the characteristics found in the large bowel of rodents.

Interestingly, holoxenic mice die mainly between the 6th and 9th days (i.e., in the same lapse of time when man shows the nadir of neutrophils and the peak of delayed diarrhea). Diarrhea was well correlated with intestinal damage in both holoxenic and germ-free mice treated with irinotecan. Obviously excluding sepsis, death may be caused in most germ-free treated mice (at high doses of irinotecan) by diarrhea/dehydration.

During therapies with most drugs inducing intestinal damage (e.g., 5-fluorouracil, ARA-C, methotrexate, etc.), the role played by the intestinal bacteria seems to be limited to causing possible sepsis. This is why the guidelines for treatments

causing severe mucosal damage, such as bone marrow transplantation, suggest an antibiotic prophylaxis (even in the absence of comparisons with placebo; ref. 15). By contrast, the microflora seems to play a dual role during treatment with irinotecan: on the one hand, it is implicated in possible septicemia and, on the other hand, it induces mucosal damage. This was also suggested by some of the histopathologic data in our study. At the dose of 60 mg irinotecan, the intestinal mucosa of holoxenic mice was severely affected whereas it was completely undamaged in the germ-free mice. In other words, at the lethal dose for holoxenic mice, germ-free animals show no mucosal damage, no diarrhea, and no death.

The damage found in the intestine of our irinotecan holoxenic mice is similar to that previously reported: vacuolization, necrosis, hyperplastic goblet cells, vessel dilation, and submucosal edema (7, 8, 10). In addition, we also found an unknown alteration to Paneth cells. Like enterocytes, Paneth cells originate from the stem cells in the neck of the crypts and their main action is thought to be the preservation of contiguous cryptal stem cells from bacterial overgrowth and infection. When adult germ-free mice are associated with microflora, their Paneth cells show fewer secretory granules (containing lysozyme, phospholipase A2, and α-defensin) than germ-free animals (16). These molecules are selectively released in the presence of bacteria or their products (lipopolysaccharide) within the crypts and, interestingly, also for cholinergic stimulation (17). Irinotecan has a well-known cholinergic action and early diarrhea is recognized through this mechanism. Our data on untreated holoxenic and germ-free mice confirm a jejunal-ileal gradient in the number of Paneth cells and the capacity of germ-free to reach the full development of these cells, notwithstanding their antibacterial role (18). In the presence of intestinal bacteria, irinotecan reduces the number of Paneth cells and induces their degranulation. On the contrary, Paneth cells were hypertrophic both in germ-free mice which survived at 100 mg and in those dying between the 5th and 8th days, suggesting that the cholinergic stimuli require the concomitant presence of the bacteria to trigger degranulation. Further, no differences were found in the number of Paneth cells between controls and treated germ-free, but the cells were severely reduced in number in treated holoxenic mice compared with controls and statistically fewer than in germ-free treated ones. Probably, this numerical reduction and the functional depletion of Paneth cells, in parallel with the severe damage to the small bowel of rodents with microflora, expose the animals to a higher risk of sepsis due to bacterial translocation. It is still unknown whether this alteration is relevant in man whose topographical distribution of microflora is different (19, 20). Bacteria are present in high concentrations in rodents, both in the small and large bowel (with a prevalence of >2 log in the large bowel), whereas high numbers of bacteria are present in man only in the colon. In other words, the human microbial ecology of

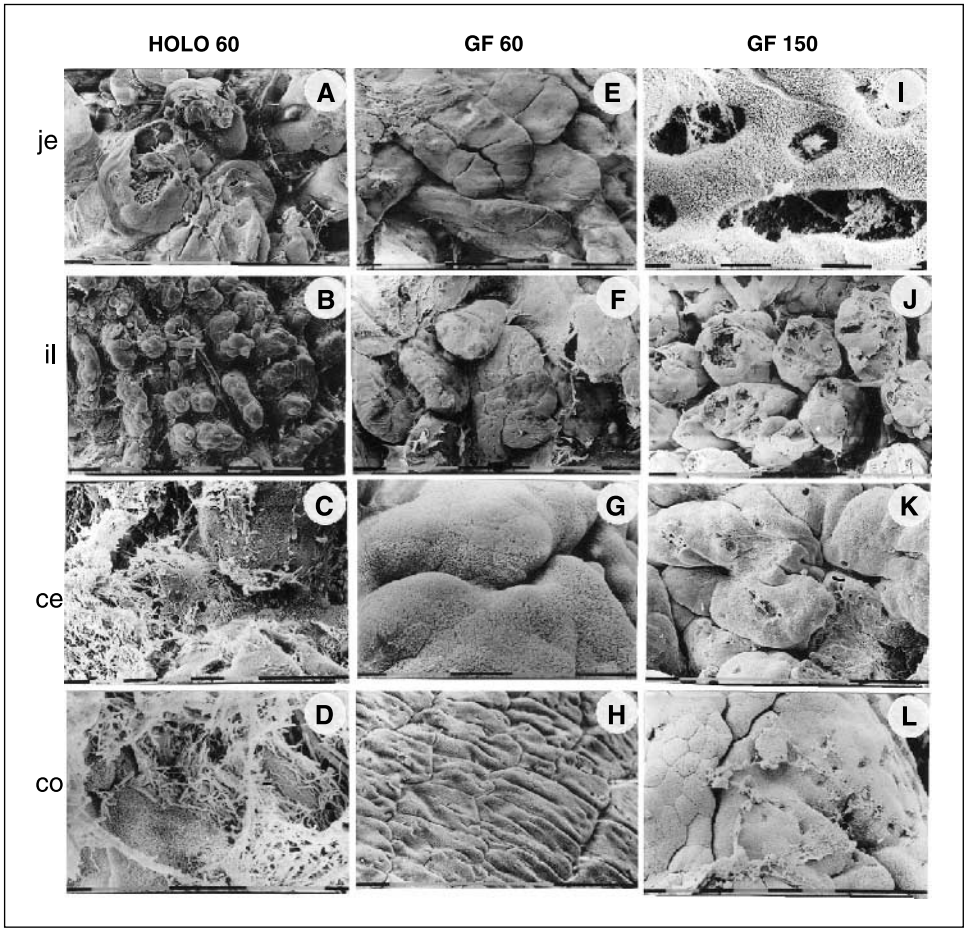

Fig. 5. Scanning electron microscopy of the jejunum, ileum, cecum, and colon of holoxenic 60 mg (*A-D*) and germ-free 60 mg (*E-H*) and 150 mg (*I-L*) irinotecan C3H/HJ treated mice. Bar, 0.1 mm (*A, B, E, F, H, J*, and *K*); 10 μm (*C, D, G, I*, and *L*). Holoxenic mice: the mucosal damage is severe and widespread in the small and large bowel with shortening and beheading of villi. Cell loss and many bacteria are seen in the cecum and colon. Germ-free 60 mg: the mucosa is preserved both in the small and large bowel whereas germ-free 150 mg mice show severe damage in the jejunum and ileum (singular cell loss and areas with beheading of villi). The large bowel mucosa is more preserved with only random single cell loss not visible at optical microscopy. je, jejunum; il, ileum; ce, cecum; ce, colon.

the large bowel is more similar to that of holoxenic animals whereas the small bowel is more similar to germ-free animals.

The damage in holoxenic mice was present in the small and large bowel and was more severe in the latter, reflecting the distribution of the intestinal microflora along the digestive tract. Light and electron microscopy showed that each intestinal sector of holoxenic mice treated with irinotecan 100 mg or even with 60 mg was severely damaged in almost all cases. The damage in germ-free mice only affected the small bowel and appeared at dosages double those in holoxenic and was full blown at dosages thrice higher (150 mg). The large bowel of irinotecan 60 mg germ-free mice (around the lethal dose for holoxenic) was undamaged and even 150 mg (around the lethal dose for germ-free) was almost safe, clearly suggesting that no mucosal damage occurs in this area in the absence of microflora.

This study does not allow any conclusions on the mechanisms involved in the reduced tolerance to irinotecan in the presence of microflora although indirect evidence suggests its action in the drug pharmacokinetics. Irinotecan is transformed by carboxylesterase into its 100 to 1,000 times more active SN-38 metabolite. Most of the circulating SN-38 is detoxified for glucuronization by the hepatic enzyme UGT1-A1 and excreted in the bile in a glucuronized form (SN-38 glucuronide) but can be reconverted into the free form by the enzyme β-glucuronidase present in both plasma and tumoral tissue (21). When the SN-38 glucuronide reaches the intestinal lumen, it comes into contact with further amounts of β-glucuronidase, mainly carried by autochthonous bacteria (10, 22). This may expose the bowel to the potential production of additional SN-38, which is the best candidate for intestinal mucosal damage due to its high antitumor and cytotoxic activity on the intestinal cell lines (23). In rats, the percentage of irinotecan remains unchanged in the biliary flow and feces whereas SN-38 increases from 2% in the bile to 12% in the feces, suggesting its direct production in the intestine (24). In patients with percutaneous biliary catheters, the concentration of free SN-38 in the bile is low, between 0.1% and 0.4% of the irinotecan administered doses, whereas in the feces the concentration of free SN-38 increases to >8%, with a parallel decrease of SN-38 glucuronide (25). A limited amount of SN-38 can also be produced by irinotecan directly in the intestinal lumen by the carboxylesterases of the brush border (24). Finally, the concentration of SN-38 in the intestinal lumen is likely to depend on several variables, such as the hepatic secretion rate of SN-38 and SN-38 glucuronide, pH of the feces, bacterial β-glucuronidase, and intestinal carboxylesterase activities.

There is a broad consensus on the correlation between diarrhea and irinotecan but mainly between diarrhea and SN-38 in the intestinal wall and lumen (8, 21). The damage topography in germ-free could reflect the fact that all free SN-38 secreted in the bile is reabsorbed completely before entering the cecum and no other SN-38 can be produced in the large bowel in the absence of bacteria (11).

The independence of the plasma compartment of SN-38 from the intestinal one suggests that the damage to the small bowel of holoxenic mice is probably not caused by an increased reabsorption of SN-38 from the intestinal lumen due to the higher production by colonic bacteria (11, 12, 26) but by the arrival of irinotecan/SN-38 through the biliary flow (as in germ-free) and further *in situ* production of free SN-38 (probably due to the microbial action). This series of events is further sustained by the fact that at a functionally equivalent dose for germ-free and holoxenic mice [i.e., at 1/3 of their respective lethal doses (60 mg for germ-free and 25 mg for holoxenic; data not shown)], the small intestine remains unchanged in germ-free mice whereas it is damaged in 40% of holoxenic animals.

The transformation of SN-38 glucuronide into SN-38 by the intestinal bacteria has also been suggested in man (27). The strong correlation between diarrhea and the intestinal damage found in mice treated with irinotecan could shed some light on human diarrhea considered as a secretory and exudative phenomenon.

The drugs used in the prevention/therapy of irinotecan-related diarrhea have different sites of action. For some the direct target is the intestinal mucosa, controlling the absorption of electrolytes and water (glutamine, acetorphan and octeotride) or modulating the immune/inflammatory reactions (budesonide, thalidomide, Celecoxib, and JBT 3002 lipopeptide; refs. 28–34). Other drugs reduce the endoluminal concentration of active SN-38 by blocking the intestinal carboxylesterase using alkalinization of intestinal lumen, baicaline, or, more significantly, antibiotics (12, 35–39).

The results obtained with antibiotics remain contradictory. Antibiotic treatments (penicillin, streptomycin, and neomycin plus bacitracin) in rodents and in two small series of patients with previous delayed diarrhea decreased the intestinal damage (in animals), improved diarrhea, and inhibited β-glucuronidase activity (11, 12, 26, 39). However, in the only randomized study, prophylactic systemic antibiotics (quinolones) failed to reduce the outbreak/score of delayed diarrhea (13). Antibiotic prophylaxis in man probably cannot entirely respond to the need to control delayed diarrhea. In this context, some peculiarities of human intestinal microflora should be highlighted: (*a*) the microflora is formed by a pool of bacteria belonging to >500 different species, some of which are dominant (>10^8 colony-forming units/g), and therefore fundamental in terms of functional capacity, and others subdominant. Under normal conditions, the balance between them is stable and actively maintained by a repressive activity of the dominant populations over the subdominant ones (40); (*b*) host specificity of microflora: some molecular methods have shown that the microflora with its own biochemical and antigenic load is specific and relatively stable in the life of the individual, representing a sort of intestinal "fingerprint" (41).

The involvement of microflora in irinotecan toxicity shown by this study and its individual characteristics may help to clarify why genetic factors can only partially explain the unpredictability of irinotecan toxicity (42). The negative action versus irinotecan might not be due to the microflora as a whole but to some of its components. For example, should the action of β-glucuronidase be recognized, it must be recalled that only 10% of bacteria isolated from the cecum and feces of rodents (*Peptostreptococcus*, *Staphylococcus*, and *Clostridium*) have β-glucuronidase activity and that *in vivo* β-glucuronidase varied widely during repeated sampling over a short time (22). The lack of information on how the microflora affects irinotecan toxicity precludes a

long-lasting and widespread prophylactic use of antibiotics (as required in irinotecan administration schedules) because of both general concerns about the use of antibiotics (i.e., the selection of resistance factors) and specific problems in this field (i.e., antibiotics might have negative effects on the single individual). In fact, the suppression of some dominant bacterial populations may allow the overgrowth of smaller populations [e.g., increasing the overall β-glucuronidase activity (carried for a subdominant bacterial species)]. Furthermore, there is recent evidence that the long-term use of neomycin in rodents increases serum bilirubin, thus potentially interfering with hepatic irinotecan metabolism (43). At present, the safest way to decrease irinotecan delayed diarrhea seems to be the oral alkalinization of the intestinal lumen because a higher pH reduces the deconjugation of SN-38 glucuronide (37).

In conclusion, the intestinal microflora plays a key role in increasing irinotecan intestinal toxicity. Further studies are necessary to understand how intestinal bacteria reduce the tolerability of this drug.

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