Microbiota matures colonic epithelium through a coordinated induction of cell cycle-related proteins in gnotobiotic rat

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Microbiota matures colonic epithelium through a coordinated induction of cell cycle-related proteins in gnotobiotic rat. Am J Physiol Gastrointest Liver Physiol 299: G348–G357, 2010. First published May 13, 2010; doi:10.1152/ajpgi.00384.2009.—Previous studies have suggested that intestinal microbiota modulates colonic epithelium renewal. The objective of our work was to study the effects of microbiota on colonic epithelium structure and cell cycle-related proteins by using gnotobiotic rats. Colonic crypts and amount of cell cycle-related proteins were compared between germ-free (GF), conventional (CV), and conventionalized rats by histochemistry and Western blot. Ki67 and proliferating cell nuclear antigen (PCNA) were used as surrogates for proliferative cells; p21cip1 and p27kip1 were markers of cell cycle arrest; anti- and proapoptotic proteins, Bcl2 and Bax, respectively, were also studied. We observed 40% increase of the crypt proliferative area 2 days after inoculation of GF rats with a complex microbiota. This recruitment of proliferative cells may account for the 30% increase of crypt depth observed between CV and GF rats. The hyperproliferative boost induced by microbiota was compensated by a fourfold increase of p21cip1 and p27kip1 involved in cell cycle arrest and a 30% drop of antiapoptotic Bcl2 protein while Bax was unchanged. Inductions of p21cip1, p27kip1, and PCNA protein were not paralleled by an increase of the corresponding mRNA. We also showed that p21cip1 induction by microbiota was partially restored by Bacteroides thetaiotaomicron, Ruminococcus gravis, and Clostridium paraputrificum. Colonization of the colon by a complex microbiota increases the crypt depth of colon epithelium. This event takes place in conjunction with a multistep process: a hyperproliferative boost accompanied by compensatory events as induction of p21cip1 and p27kip1 and decrease of Bcl2. Gut epithelium (4, 29), which may be used as receptors for bacterial adhesins or as energy sources for the intestinal bacteria, especially in limited nutrient availability (45). Finally, our group has shown that intestinal microbiota diversifies metabolic pathways of the colonic mucosa, especially the one of butyrate, one of the end products of bacterial fermentation (5) and a major energy source for colonocytes. Hence, the cross talk between these two obligatory partners (i.e., microbiota and digestive tract) leads to the development of a stable ecosystem (24, 42).

Colonic epithelium is one of the most dynamic structures in the whole organism as it self-renews every several days. This renewal is sustained by epithelial progenitor cells that migrate upward from the bottom of the crypts undergoing proliferation, differentiation, and maturation before their extrusion into the intestinal lumen (3, 28). It is now well established that some pathogenic bacteria, probably for their own benefit, can modify the host cell cycle (35). This point has recently been revealed for Shigella, a pathogenic intestinal bacteria, which strongly reduces the number of proliferating cells in a rabbit ileal loop (16). However, this effect on cell cycle may not be produced exclusively by pathogenic strains (34).

Indeed, the commensal intestinal microbiota influences cell proliferation and cell kinetic variables of the intestinal epithelium in gnotobiotic animals. An increased duodenal epithelium proliferation (23) and enterocyte transit time from crypt to villus (43) were observed in conventional (CV) mice compared with germ-free (GF) ones. More recent results indicate that the ileal expression level of some genes involved in apoptotic and proliferative activities are modified in the presence of a complex microbiota (48). In the colon, the number of cells per crypt is reduced in GF compared with CV animals (1). Therefore, throughout the digestive tract, all indicators linked to cell production and cell turnover are slower in the absence of microbiota.

To better understand how microbiota modulates the cell cycle in the digestive tract, the objectives of our work were to study 1) the effects of microbiota colonization on colonic epithelium structure and on cell cycle-related proteins by using gnotobiotic rats and 2) whether the effect of complex microbiota on cell cycle-related proteins may be mimicked by dominant members of the intestinal microbiota: Bacteroides thetaiotaomicron, Ruminococcus gravis, and Clostridium paraputrificum. These strains belong to Bacteroidetes and Firmicutes phyla. Furthermore, effects of B. thetaiotaomicron on the host intestinal response indicate the major role of this bacteria in symbiotic host-bacteria relationships (4, 19).
This work shows that, with along colonization, the microbiota increases crypt depth in coordination with an induction of cell cycle-related proteins.

MATERIALS AND METHODS

Animals and experimental design. All procedures were carried out according to European guidelines for the care and use of laboratory animals and with permission 78–122 of the French Veterinary Services. The following groups of rats (male, Fisher 344) were used: germ-free (GF); conventional (CV); GF inoculated with a fecal microbiota obtained from CV rats (Ino-CV); GF-inoculated with pure bacterial strains: C. paraputrefaciens (strain number: 217.59) (30) isolated from the intestinal contents of CV rats (Ino-Cp); B. thetaiotaomicron (strain number: BII8) (Ino-Br) (11) and R. gnarus (strain number: FRE1) (Ino-Rg) (9), isolated from the fecal contents of humans.

To obtain Ino-CV rats, GF rats were inoculated by oral gavage with 1 ml feces freshly recovered from a CV rat and diluted 100-fold in LCY (Liquid Casei Yeast extract) medium. To obtain monoxenic rats, GF rats were inoculated by oral gavage with 1 ml of an 18- to 24-h anaerobic culture.

Animals were born and bred at the Centre de Recherches, Institut National de la Recherche Agronomique (Jouy-en-Josas, France). The GF, Ino-Cp, Ino-Br, and Ino-Rg rats were reared in Trexler-type isolators (La Calhène, Véizy, France). The CV and Ino-CV batches were reared in standard conditions. All groups of rats received the same standard diet (UAR), with the exception of a batch of Ino-Cp rats which received a specific diet containing both amylomaltose starch and lactulose to stimulate butyrate production (5, 30). GF and CV rats compared with this Ino-Cp group received the same diet. All diets were sterilized by gamma irradiation (45 kGy). All rats were euthanized at the age of 3 mo. In the group of Ino-CV, rats were euthanized 2, 14, or 30 days after the inoculation with conventional microbiota; they were named Ino-2d, Ino-14d, and Ino-30d, respectively. Monoxenic rats were euthanized 30 days after inoculation with a pure bacterial strain.

At 9 AM, rats were anesthetized with isoflurane, the colons were removed and immediately used either for cell isolation or for histological procedure.

Cell isolation procedure. Colonic epithelial cells were isolated from the whole colon according to the method described by Cherbuy et al. (6). Cells originating from the whole epithelium were obtained under this procedure (6). The cell pellet was immediately used for protein extraction.

Protein extraction. Protein extraction was made on freshly isolated cells according to Ref. 22. Briefly, the cell pellet was resuspended in a lysis buffer (22) containing 0.1% Triton X-100 and a cocktail of protease inhibitor (Roche). Lysis was performed for 1 h on a continuous rotation at 4°C. During lysis, cells were homogenized twice through a 26-gauge needle. Cells were centrifugated (10,000 g; 4°C; 20 mm), the supernatant was removed, aliquoted, and stored at −80°C until analysis. Proteins were measured according to Ref. 25.

Western blot analysis. Proteins were resuspended in Laemmli solution heated 5 min at 90°C and electrophoresed was run on a 12 or 15% SDS-PAGE. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane (Amersham Biosciences, Saclay, France). After blocking by TBS-T/5% milk, membranes were incubated overnight at 4°C with the primary antibody, followed by incubation with appropriate peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The signal was detected using the ECL + kit (Amersham Biosciences). Proteins were analyzed using anti-PCNA (GeneTex; diluted 1/1,000), anti-p21cip1 (Oncogene; 1/200 or Santa Cruz Biotechnology; 1/200), anti-p27kip1 (Santa Cruz Biotechnology; 1/200), anti-Becl2 (Santa Cruz; 1/400), anti-Bax (Santa Cruz; 1/200), anti-skp1 (BD Transduction Laboratories; 1/1,000), anti-GAPDH (assay designs, 1/5,000).

Signals detected on autoradiographic films were quantified by scanning densitometry of the autoradiograph by using a Lasplus camera (Fujiﬁlm, Paris, France) and Aida software (Raytest, Courbevoie, France).

Histology and immunohistochemistry. Colon samples were cut into 2-cm sections, fixed in 4% paraformaldehyde (5 h, room temperature), dehydrated, and embedded in paraffin according to standard histological protocols. Four-micrometer sections were mounted on SuperFrost Plus slides. Slides were stained with eosin for histological analysis. Immunological staining was done with the Envision + system-horse-radish peroxidase (Dako, France) according to the recommendations of the manufacturer. Antigen retrieval was performed by boiling slides for 40 min in 0.01 mol/l sodium citrate pH 6.0. Primary antibodies used were Ki67 (clone MIB-5, Dakocytomation, dilution 1/50) and anti-PCNA (GeneTex; dilution 1/10,000). Negative controls were performed by omitting the primary antibody from the reactions. In these conditions no signal was observed (data not shown). Only U-shaped longitudinally cut crypts with open lumina along the crypt axis were evaluated. For each section, Ki67- or PCNA-positive cells were counted on 10 crypts, and results were expressed as percent of total colonic crypt cells.

Total RNA extraction and real-time RT-PCR analysis. Total RNA was extracted from isolated colonic epithelial cells by the guanidinium thiocyanate method (7). RNA concentration and purity were determined by absorbance measurement using a nanodrop and RNA Integrity Number (RIN) was checked with the Agilent 2100 bioanalyzer and the RNA 6000 nano labChip kit (Agilent technologies). All RNAs had a RIN between 8.5 to 9.5, indicating a high RNA quality in all samples. Seven micrograms of the total RNA samples were subjected to a reverse transcription step by use of the high-capacity cDNA archive kit (Applied Biosystems, France). Real-time quantitative PCR was performed on cDNAs in a ABI PRISM 7000 Sequence Detection System by using Rn00582195-m1 and Rn00589996-m1 TaqMan Gene expression assays for p21cip1 and p27kip1, respectively (Applied Biosystems, France). Since we have already used 18S rRNA with colon samples (18), 18S rRNA was considered as housekeeping gene and measured with Hs99999901-s1 Taqman gene expression assays. Results obtained on p21cip1 and p27kip1 were normalized to 18S rRNA (reference gene) and compared with the means target gene expression of CV rats as calibrator sample. At least three rats were used for each GF, Ino-2d, Ino-14d, Ino-30d, and CV group. The following formula was used: fold change = 2−ΔΔCt, where ΔΔ threshold cycle (Ct) equals (target Ct − reference Ct) of sample minus (target Ct − reference Ct) of the calibrator.

Presentation and analysis of data. Results are presented as means ± SE for the number of animals indicated. Comparisons of group data were performed using one-way analysis of variance (ANOVA) followed by a Tukey’s Studentized range test (jmp version 7) when the ANOVA revealed differences among the groups. Differences were considered statistically significant at P < 0.05.

RESULTS

The microbiota shapes the morphology of the colonic epithelium. Colonic crypt depth was greatly affected by the bacterial status of the rats with a crypt depth 35% lower in germ-free rats (208 ± 32 μm for GF rat vs. 274 ± 19 μm for CV rats; P < 0.05) (Fig. 1A). Furthermore, in the colonic mucosa of GF rats, bifurcating crypts were observed with two flask-shaped bases joining in a single unit at the top of the crypt (Fig. 1A). These features are characteristic of crypt fission, which is a mechanism whereby intestinal crypts divide (10). Crypts with a fission form represented 25.8 ± 1.6% of total crypts in GF rat mucosa, which was significantly higher than values obtained in CV (2.7 ± 3.9% of total crypt).
The microbiota modulates cell cycle-related protein abundance. It is well known that colonic crypts are highly spatially structured with an area where the cells are proliferating and a nonproliferating portion at the top (3, 28). As expected, the proliferative cells, which were stained by two proliferative markers (Ki67 and PCNA) (Fig. 1, B and C), were restrained to the bottom of the crypt. The Ki67-positive cells represented, respectively, 41 ± 5 vs. 46 ± 4% of total colonic crypt cells in GF and CV rats. The PCNA-stained cells were 63 ± 5% in GF and 65 ± 5% of total cells in CV rats. There were thus no significant differences in the distribution of Ki67 and PCNA in CV and in GF rats. As it has been previously reported (15, 32), the PCNA-stained zone was more extended than the Ki67-positive zone.

The amount of PCNA was compared by Western blot in colonic epithelial cells from GF and CV rats (Fig. 2). Colonic PCNA protein levels were 60% higher in CV than in GF rats (Fig. 2A). It has recently been shown that Cif, a cyclomodulin produced by pathogenic strains of *Escherichia coli*, modulates the amount of two cyclin-dependent kinase inhibitors, p21\(^{cip1}\) and p27\(^{kip}\) (41). According to the key role of these two proteins in intestinal epithelial cell renewal (40), we further studied the effect of the GF state on p21\(^{cip1}\) and p27\(^{kip}\). Results...
indicated that abundance of these proteins was dramatically decreased in GF rat (see Fig. 2B for p21cip1 and Fig. 2C for p27kip1). All these quantitative data are in accordance with the fact that CV rats displayed deeper crypts with a well-preserved ratio between proliferative and nonproliferative cells as observed by immunohistochemistry (Fig. 1, B and C).

Progressive adaptation of colon epithelium to the presence of microbiota. A progressive effect of the transfer of a complex intestinal microbiota on the colonic epithelium was further studied. Ino-CV rats were euthanized 2, 14, or 30 days after inoculation. Several arguments were considered for the choice of this time course: we chose a time point preceding the total renewal of the colonic epithelium (2 days) and later points, encompassing two or three renewals of colonic epithelium (14 and 30 days) (3, 28). These periods also correspond to the progressive establishment of the biochemical functions of the transferred intestinal microbiota, which occurs within 3 wk following the transfer of the intestinal microbiota (31).

Histological analysis indicated that crypt depth progressively increased following the transfer of a complex microbiota (Fig. 3A). The crypt depth was of 208 ± 32 μm in GF rats, 234 ± 27 μm in Ino-2d, 274 ± 27 μm in Ino-14d, and 256 ± 4 μm in Ino-30d. The maximal depth of crypt was reached in Ino-14d, since there were no significant differences between values obtained in Ino-14d and CV rats. Colonic crypt structure also differed strongly between GF and Ino-CV. As previously mentioned, typical figures of crypt fission were observed in the colonic mucosa of GF rats. The number of bifurcating crypts was decreased in the colonic mucosa of Ino-2d, representing 8.33 ± 7.14% of total crypt. In Ino-14d or Ino-30d, we were not able to observe such bifurcating crypts. Furthermore, in contrast to GF, colonic crypts were tightly and individually structured in Ino-CV groups. According to these observations, one can postulate that in GF rats colonic crypts are ready to split up, which can account for the higher number of crypts in Ino-2d. Indeed, in this group of rats, the crypt number was significantly 30% higher than in GF rats. Thus the number of crypts per millimeter of colonic length was respectively of 20.0 ± 3.0 and of 26.6 ± 3.5 for GF and Ino-2d groups (P < 0.05). In Ino-14d and Ino-30d the crypt density was progressively decreased respectively to 22.8 ± 2 and to
When probing Ki67- and PCNA-positive cells (Fig. 3, B and C), the presence of complex microbiota for 2 days led to a rapid increase in the percent of stained cells. As previously mentioned, Ki67-positive cells represented 41 ± 5% of the total colonic crypt cells in GF, whereas they represented up to 73 ± 5% in Ino-2d. PCNA-positive cells ranged from 63 ± 5%
in GF to 88 ± 4% in Ino-2d rats. When microbiota was present for a longer time, i.e., 14 and 30 days, neither Ki67- nor PCNA-stained cells were significantly different from that in CV rats. These results indicated that the inoculation of a complex fecal microbiota in GF rats led to a transitory hyperproliferation boost that was detected at 2 days after inoculation. As observed by immunohistochemistry, we confirmed by Western blot that PCNA abundance was higher in Ino-2d compared with GF, thus acting as a molecular signature of the boost in cell proliferation (Fig. 4A). The first days of bacterial establishment was also accompanied by a 30% decrease of Bcl2, an antiapoptotic regulator, but had no effect on the proapoptotic protein Bax (Fig. 4B). In contrast to PCNA and Bcl2, neither p21cip1 (Fig. 4C) nor p27kip1 (Fig. 4D) was modified between GF and Ino-2d rats. However, both proteins were increased in Ino-14d, i.e., 14 days after inoculation at the first days of bacterial establishment.
same level as in CV rats (Fig. 4, C and D). The p21cip1 and p27kip1 values obtained for Ino-14d were similar to Ino-30d (see graphs of figures). We also confirmed this induction pattern by using nuclear proteins for PCNA, p21cip1, and p27kip1 (see Supplementary Fig. S1; Supplemental Material for this article is available online at the Journal website).

Thus quantitative data obtained by Western blot suggested that the inoculation of a complex fecal microbiota in GF rats led to a hyperproliferation step 2 days after inoculation and a successive induction of cell cycle arrest proteins. This was also accompanied by a decrease in the antiapoptotic protein Bcl2 whereas the proapoptotic protein Bax amount remained unchanged. Also, we did not observed cleaved forms of caspase 8 and caspase 3 nor increase in these protein amounts among group of rat (data not shown).

In contrast to protein levels, mRNA encoding p21cip1, p27kip1, and PCNA were not modified by the bacterial status. Indeed, \(2^{-\Delta\Delta C_t}\) values for p21cip1 were \(1 \pm 0.3, 0.94 \pm 0.2, 0.6 \pm 0.12\), and \(1.2 \pm 0.4\), respectively, from GF, Ino-2d, Ino-14d, and CV rats. For p27kip1, \(2^{-\Delta\Delta C_t}\) values were \(1.8 \pm 0.5, 1 \pm 0.3, 0.7 \pm 0.1, 1 \pm 0.1\), respectively, from GF, Ino-2d, Ino-14d, and CV rats. Similar mRNA amounts for p21cip1 and PCNA were also observed by Northern blot in GF and CV rats (data not shown). In parallel, we also observed that p53 is stable in colonocytes whatever the bacterial status of the rat (data not shown).

The effect of a complex microbiota is partially restored by dominant members of the adult intestinal microbiota. To define whether the effects of a complex microbiota may be mimicked by B. thetaotaomicron, R. gnarus, and C. paraputrificum, we obtained monoassociated rats (Ino-Bt, Ino-Rg, Ino-Cp). One week after the inoculation, bacteria were present in the feces at \(10^9\) to \(10^{10}\) bacteria/g and remained at a high level throughout the protocol (30 days) (see Supplementary Fig. S2 and Refs. 5, 30). Cell cycle proteins were analyzed on colon epithelial cells by Western blot (Fig. 5; see Supplementary Fig. S3 for representative autoradiographs). None of the three bacterial strains modulated PCNA protein abundance (Fig. 5, A–C). Also, p27kip1 amount was unchanged between GF, Ino-Bt and Ino-Rg (data not shown). In contrast, p21cip1 increased weakly in monoexenic rats, while remaining lower than the amount detected in CV rats (Fig. 5, D–F).

It has been previously shown that C. paraputrificum have the ability to produce butyrate when rats are fed a diet containing both amylomaize starch and lactulose (5, 30). The levels of PCNA (Fig. 5C) and p21cip1 (Fig. 5F) were unchanged between Ino-Cp rats fed either a standard (UAR diet) or an amylomaize starch- and lactulose-enriched diet (EL5 diet).

**DISCUSSION**

This work shows for the first time that microbiota modifies in vivo colonic epithelium structure in concordance with a well-orchestrated induction of proteins involved in the cell cycle. Results indicate that colonization of the colon by a complex microbiota increases the crypt depth of colonic epithelium that is related to a hyperproliferative boost and compensatory events illustrated by the drop of Bcl2 and the induction of p21cip1 and p27kip1. These events led to a controlled homeostasis of the colonic epithelium in response to the presence of a complex intestinal microbiota. We have recently also observed a controlled hyperplasia of the colonic epithelium in human patients with short bowel syndrome, displaying a drastic change of microbiota composition (17, 18). The preservation of homeostatic balance is also crucial in protecting intestine from injury such as chemical challenges or radiation (39). Since Toll-like receptors are involved in bacterial recognition and play an important role in intestinal epithelial homeostasis control (38), it would be now interesting to follow up these receptors in GF and different groups of inoculated rats.

Overall, all observations led us to conclude that the absence of microbiota is associated with an epithelium atrophy. By comparing GF, CV, and Ino-CV rats, we determined that rats harboring a complex microbiota had deeper colonic crypts (Fig. 1 and 3). Furthermore, we observed a lower number of colonic crypts in the mucosa of GF rats than in Ino-2d rats, suggesting that the implantation of microbiota contributes to crypt formation, probably through crypt fission. This result must be put in perspective with the colonic maturation through crypt fission that occurs after birth in mammals (27). The fact that microbiota is a key actor in recovering energy from food is obviously linked to the metabolic activity of microorganisms but may also be related to its trophic effect on the epithelium surface. The increase in the intestinal absorptive surface area could thus contribute to the weight gain observed in CV compared with GF rodents (2).

Our observations, in accordance with previous data (1), confirm that colonic epithelial cells of GF had a lower capacity of cycling than CV cells. The fact that GF displayed higher amount of antiapoptotic protein like Bcl2 than CV rats indicate that all life cycle steps of epithelial cells (from division to apoptosis via cell cycle arrest) can be modulated by microbiota. The effect of the commensal microbiota on cell cycle proteins could explain the acquisition of a deeper colonic epithelium structure with a well-preserved ratio between proliferating and nonproliferating compartments. In addition, it has been shown that the rate of intestinal renewal provides an important intrinsinc defense system. The intestinal cell turnover rate is one of the key factors involved in resistance toward parasitic infection, probably because it participates in its expulsion (8). The trophic effect of microbiota could thus also to be a manner for the host to stimulate the mucosal defense.

Throughout the digestive tract, the effect of microbiota on cell cycle related proteins was segment specific and particularly marked in the colon. In the ileum, PCNA, p21cip1 and p27kip1 were only 30–40% higher in CV than in GF rats (vs. 60/70% in colon), whereas in duodenum and in jejunum these proteins tended to be similar in CV and GF rats (data not shown). Thus the colon where the density of microbiota was the highest seems to be the site where the cell cycle related proteins were more sensitive to microbiota.

PCNA and Ki67 are abundant proteins in the colon with restricted detection at the bottom of the crypts. Correlated with their localization, Ki67 and PCNA play a role in proliferation (15, 20, 32); Ki67, although universally used as a proliferative marker, still has an unknown function; PCNA is a member of the DNA sliding clamp family and plays a role not only in proliferation through DNA replication but also in DNA repair (26). The fact that both Ki67 and PCNA were enhanced 2 days after microbiota transfer illustrated a rapid and strong boost in proliferation. Because the increase in the proliferative compart-
ment precedes complete renewal of the colonic epithelium, our results suggest that microbiota signals may mobilize stem cells to generate more proliferative cells. When the pattern of protein expression (Fig. 4) was paralleled with the modeling of the epithelium structure (Fig. 3), the proproliferative event was anterior to the maximal depth acquisition of the crypt (observed at 14 days after inoculation). Hence, on the basis of this kinetic, we propose that the proproliferative bacterial signal is necessary to provide deeper crypts. The boost of proliferation was also accompanied by a decreased of Bcl2 protein amount that regulates the number of cells entering the bottom/top axis in the colon (47). All these observations suggested that a tight control of epithelium homeostasis began as soon as 2 days after microbiota inoculation activating proproliferative and decreasing antiapoptotic signals. Furthermore, in addition to its role in the proliferative process, PCNA could be of physiological importance for protecting epithelial cells toward DNA damages linked to bacteria products. Indeed, it has been demonstrated that the action mechanisms of several cyclomodulins involve eukaryotic DNA damage (34, 35).

P21cip1 and p27kip1 are cyclin-dependent kinase inhibitors involved in cell cycle arrest, and they are considered tumor-suppressor genes. In the intestine, their role is probably more complex (37, 44). As an example, there are no differences between proliferation and differentiated epithelial cell lineages in p21cip1−/−, p27kip1−/−, and p21cip1−/−, p27kip1−/− mice compared with their wild-type counterparts (50). It has therefore been suggested that p21cip1 and p27kip1 play a critical role in regulating intestinal cell turnover after external stress. Indeed, disruption of these genes enhanced intestinal tumor formation after administration of a carcinogen (36) or a high-fat “Western style” diet (49). According to these data, our

Fig. 5. Analysis of PCNA and p21cip1 in colonic epithelial cells isolated from monoassociated rats. GF rats were inoculated with Ruminococcus gnavus (Ino-Rg) (A and D), Bacteroides thetaiotaomicron (Ino-Bt) (B and E), or Clostridium paraputrificum (Ino-Cp) (C and F) and PCNA (A–C) and p21cip1 (D–F) were analyzed on colonic isolated cells. All rats were euthanized 30 days after the inoculation with 1 ml of overnight bacterial culture. Ino-Rg, Ino-Bt, and a group of Ino-Cp were fed a standard diet (UAR). To promote butyrate production in Ino-Cp, another group of Ino-Cp was fed the EL5 diet. Colonic epithelial cells were isolated and proteins were extracted as described in MATERIALS AND METHODS. Proteins were size fractionated and blotted with an anti-PCNA and an anti-p21cip1 (see Supplementary Fig. S3 for representative autoradiographs). Membranes were then analyzed for densitometry analysis performed on n = 5–4 rats per group. Results are presented as means ± SE. Means with different letters are significantly different (P < 0.05).
results suggest that the colonic hyperplasia observed within 2 days after transfer of intestinal microbiota is counterbalanced by an induction of p21cip1 and p27kip1, thus restraining proliferation. Induction of p21cip1 and p27kip1, which are both involved in gating cell-cycle progression, may attenuate the microbiota-induced proliferative signals in the intestine.

We sought to use monoassociated rats to decipher mechanisms involved in the effect of complex microbiota on cell cycle-related proteins. Dominant members of the adult intestinal microbiota, i.e., *B. thetaiotaomicron* and *R. gnavus*, were challenged for their ability to mimic a complex microbiota. Furthermore, we tested the role of butyrate through *C. paraputrificum*-inoculated rats receiving two diets, producing butyrate or not. None of these conditions increased PCNA protein levels, although each pure bacterial strain had been inoculated by an induction of p21cip1 and p27kip1, thus restraining proliferation. Butyrate (a transcriptional activator of p21cip1) was not involved in the induction. In addition, mRNA content of p21cip1 was similar between GF and CV, thus reinforcing our hypothesis that P21cip1 and p27kip1 mRNAs remained stable between GF and CV rats and that many microbes interfere with different members of the adult intesti-

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

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