Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome

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Schumann, Alexandra, Sophie Nutten, Dominique Donnicola, Elena M Comelli, Robert Mansourian, Christine Cherbut, Irène Cortesy-Theulaz, and Clara García-Rodenas. Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome. Physiol Genomics 23: 235–245, 2005. First published August 30, 2005; doi:10.1152/physiolgenomics.00057.2005.—The postnatal maturation of the gut, partially modulated by bacterial colonization, ends up in the establishment of an efficient barrier to luminal antigens and bacteria. The use of broad-spectrum antibiotics in pediatric practices alters the gut bacterial colonization and, consequently, may impair the maturation of the gut barrier function. To test this hypothesis, suckling Sprague-Dawley rats received a daily intragastric gavage of antibiotic (Clamoxyl; an amoxicillin-based commercial preparation) or saline solution from postnatal day 7 (d7) until d17 or d21. Luminal microbiota composition and global gene expression profile were analyzed on samples from small intestine and colon of each group. The treatment with Clamoxyl resulted in the almost-complete eradication of Enterobacteriaceae and Enterococcus. The global gene expression analysis revealed that Clamoxyl affects the maturation process of 249 and 149 Affymetrix probe sets in the proximal and distal small intestine, respectively, and 163 probe sets in the colon. The expression of genes coding for Paneth cell products (defensins, matrilysin, and phospholipase A2) was significantly downregulated by the Clamoxyl treatment. A significant downregulation of major histocompatibility complex (MHC) class I and II genes, involved in antigen presentation, was also observed. Conversely, mast cell proteases expression was upregulated. These results suggest that early treatment with a broad-spectrum antibiotic deeply affects the gut barrier function at the sucking-weaning interface, a period during which the gut is challenged by an array of novel food-borne antigens.

THE GASTROINTESTINAL (GI) mucosa is in constant contact with a luminal environment that contains not only nutrients but also a huge array of potentially harmful microorganisms and toxins. A healthy GI mucosa is able to handle this massive collection of antigens and modulate the immune response according to the level of hazard related to each antigen. This capacity of the GI tract is defined as gut barrier function. In infants, the intestine remains immature and relatively permeable to antigens during the first weeks of life (3, 18, 50). Subsequently, the postnatal maturation of the mucosal barrier reduces drastically such transfer, so that in healthy adults, only small amounts of dietary antigens can reach the circulation (22). The maturation of the intestinal barrier is the consequence of morphological and functional changes of the mucosa, which occur under genetic and endocrine control (6). However, the onset of weaning and the impact of bacterial colonization have been shown to modulate this process (11, 28). The comparison of germ-free vs. conventional microbiota animals has brought interesting clues to further our understanding of the impact of the gut microbiota on the different mechanisms involved in the gut barrier function. In particular, the absence of intestinal microbiota results in decreased mucus layer thickness (56), lower goblet cell numbers (32), decreased motility (30), and decreased numbers of IgA-producing cells (40). In addition, the antigen presentation capacity of the intestinal mucosa appears to be affected by the gut microbiota, since germ-free animals do not express major histocompatibility complex (MHC) class II proteins, molecules that are responsible for the presentation of antigenic material to the immune T cells sitting in the lamina propria (54). The effect of intestinal microbiota perturbations, such as those produced by antibiotic treatment, on the intestinal barrier has been less extensively explored. Therapy with broad-spectrum antibiotics is frequently observed in pediatric practices (43, 49, 51), children within their first year of life being particularly affected (49). One major consequence of such early antibiotherapy is the alteration of the normal colonization process by the gut microbiota. Neonatal antibiotic treatment has been shown to reduce the biodiversity of the fecal microbiota, to delay the colonization by beneficial species such as Bifidobacteria or Lactobacilli, and to induce colonization by antibiotic-resistant or opportunistic strains (2, 7). The impact of such aberrant colonization on the maturation of the intestinal barrier, particularly in terms of host defense and presentation of the luminal antigens to the immune system, is still unknown.

We postulated that the disturbances created by antibiotic administration in early life would affect the postnatal maturation process of the intestine and impair antigen uptake, handling, and/or presentation by the gut at weaning. To assess our hypothesis, we treated suckling rat pups with a commercial preparation of amoxicillin widely employed in pediatric practices (Clamoxyl) and analyzed at the sucking-weaning interface the effect of the treatment on the global gene expression profile in the small intestine and colon.

We observed that several genes related to host defense and antigen presentation were downregulated after the antibiotic treatment, whereas genes related to mast cell activation were upregulated. These results suggest that early antibiotic treatment may disrupt the normal process of antigen presentation.
and handling in a period that is related to a massive penetration of novel food antigens in the gut.

MATERIALS AND METHODS

Animals

Adult male and female Sprague-Dawley rats were obtained from Iffa Credo (Charles River, Lyon, France) and were acclimatized to our internal facility for 2 wk before mating. The pups were born and housed under conventional conditions and were breast-fed until death. All the protocols were approved by the Ethical Committee of the Canton de Vaud (Switzerland).

Experimental Design

At postnatal day 6 (d6), rat pups were weighed and randomly assigned for fostering to standardized litters of 10 pups. To avoid a dam-related bias, each experimental group was composed of animals from different litters. Pups received by intragastric gavage a daily dose of 1 ml/100 g body wt of either Clamoxyl solution (Clamoxyl, an amoxicillin-based antibiotic preparation; SmithKline Beecham Pharmaceuticals, Toledo, Spain) (10 mg/ml; Clam group, n = 33) or saline (Cont group, n = 33) from d7 until the day of death. Eleven animals from each group were killed at d15, d17, or d21 by exhaustive bleeding after anesthesia with isoflurane (3.5%). The small intestine and the colon were removed and kept on ice. The small intestine was divided into two parts of identical length, namely proximal and distal parts. After the luminal contents from each of the three segments were flushed with 2 ml of ice-cold sterile saline solution, centered 1-cm-long sections were excised, snap-frozen and stored at −80°C until RNA extraction.

Bacterial Counts of Small Intestine and Colonic Luminal Contents

Fresh luminal contents from the three intestinal segments (n = 11/group) were processed within 30 min after collection for the enumeration of the endogenous populations of total aerobic and total anaerobic bacteria and Lactobacillus in small intestine and colon, as well as Enterobacteriaceae and Enterococcus populations in colon. Bacteria were detected on selective or semiselective media. One hundred microliters of diluted bacteria were detected on selective or semiselective media. One hundred microliters of diluted bacteria were applied on bile esculin agar (BEA) (Fluka, Buchs, Switzerland) for Enterobacteriaceae, sheep blood (TSS) medium (Biomerieux SA, Lyon, France) for total aerobes. These plates were incubated anaerobically at 37°C for 48 h. The anaerobic atmosphere was obtained using Anaerocult A (Merck, Darmstadt, Germany). Dilutions were also applied on bile esculin agar (BEA) (Fluka, Buchs, Switzerland) for Enterococcus, Drigalski medium (Bio-Rad, Marnes la Coquette, France) for Enterobacteriaceae, and TSS (Biomerieux) for total aerobes. These plates were incubated aerobically at 37°C for 24 h. Bacterial concentration is expressed as colony forming units (cfu) per gram of intestinal contents.

Microarray Analysis of Gene Expression

Global gene expression analysis was performed on the proximal and distal small intestine and on the colon of six rats per group (Clam and Cont) killed at d17 and d21, leading to three biological replicates for each condition. Total RNA was extracted from each sample independently, as previously described (12), and then DNase I treated and purified using the Nucleospin II kit (Machery-Nagel AG, Oensingen, Switzerland) according to the manufacturer’s instructions. Sample monitoring by the Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Rotkreuz, Germany) consistently demonstrated high-quality RNA (28S-to-18S ratio from 1.9 to 2.2 in proximal small intestine and from 1.5 to 1.9 in distal small intestine and colon) for all samples but one (colon, Cont group, d17), which was excluded from further analysis. First- and second-strand cDNA syntheses were performed using the SuperScript double-stranded cDNA synthesis kit (Invitrogen, Basel, Switzerland), using an oligo(dT) primer containing T7 RNA polymerase binding site. Labeled cRNA was prepared with the RNA transcript labeling kit (Enzo Biochem). Twenty micrograms of cRNA were fragmented and hybridized to the Affymetrix RA230A probe array cartridge (Affymetrix, High Wycombe, UK), which contains 15,866 probe sets, following the manufacturer’s instructions. Each sample was hybridized to one array, leading to a total of 35 chips used. The arrays were scanned at 488 nm using an Argon-ion laser (Agilent), and hybridization signals were generated with Affymetrix MicroArray Suite 5.0 (MAS 5.0). The entire data set is publicly available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) through the accession number GSE2354. Differentially expressed genes were identified using the Global Error Assessment (GEA) method (37) at a test rejection level of 0.001. This method consists in first calculating the within-group mean square error (MSE) of the four studied groups for each probe set. Because of the small sample size, the MSE of a given gene is replaced by the median MSE of 201 genes with similar level of expression. This statistic was used for all statistical analyses of the microarray data. The data sets corresponding to the three intestinal parts were treated independently. To determine the effect of Clamoxyl at each age, we compared, using a Fisher’s least significant differences (LSD) t-test, the control and antibiotic groups at d17 and d21. The effect of Clamoxyl on the genes changing with the maturation was analyzed by two consecutive LSD t-tests: the first one allowed the selection of a subset of the genes differentially expressed between d17 and d21 in the control group. The second t-test selected, within these genes, those showing a significant interaction between age and antibiotic treatment (i.e., by comparing the difference between d21 and d17 in the controls vs. antibiotic group); a comparison-wise error rate of 0.0005 was employed as a cut-off in both tests to obtain an experiment-wise error rate of 0.001. Results were visualized using the Eisen’s software (19). Selected probe sets were annotated using NetAffx (http://www.affymetrix.com/analysis/index.affx). When the same gene was represented by more than one probe set, only one was selected based on the following criteria: 1) unique chromosomal localization (http://www.ensembl.org/) and 2) specificity of hybridization, as indicated by the probe set suffix (Affymetrix GeneChip Rat Expression Set 230 technical note). To identify the biological processes modulated by the antibiotic, the selected probe sets were clustered using the Biological Process Gene Ontology (GO) database (http://www.geneontology.org) as previously described (41), but using the NetAffx instead of the Ensembl annotation. Indeed, only 13% of the probe sets contained in the RAE230A chip have an Ensembl (v29 release of this database) annotation compared with 32% in NetAffx. Further searches with Hammer (17) against Pfam (http://www.sanger.ac.uk/Software/Pfam/) and with PSI Blast (1) for murine and human homologs did not yield any additional information.

Tagman Real-Time PCR Analysis of Matrilysin and Mast Cell Protease 4

RNA was prepared as described above from the proximal and distal small intestine and from the colon of 11 rats per group (Clam and Cont) at each age (d17 and d21), including the 3 rats that had been used in the microarray analysis. Two micrograms of total RNA from each sample were reverse transcribed with the first-strand cDNA synthesis kit for RT-PCR (Catayls, Wallisellen, Switzerland) using an oligo(dT), primer. Ribonucleicprotein F was used as a housekeeping
...gene, as the microarray data showed this gene not to be affected by the age or the treatment. The primers and TaqMan probe sets for matrixlysin, mast cell protease 4, and ribonucleoprotein F were synthesized by Applied Biosystems (Foster City, CA) through their Assays-on-Demand service (assays Rn00563467_m1, Rn00820964_g1, and Rn00821053_g1, respectively). Real-time PCR amplification was then performed using an ABI 7900HT Thermocycle (Applied Biosystems) with the following conditions: 2 min at 50°C and 10 min at 95°C for the initial set-up, followed by 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and elongation). All the samples were analyzed in triplicate. Cycle-to-cycle fluorescence emission was monitored and quantified using the GeneAmp SDS 2.1 software (Applied Biosystems). Relative gene expression was calculated after normalization to ribonucleoprotein F. Statistical analysis of gene expression differences was carried out as further explained.

**Statistical Analysis of Microbiota and Real-Time PCR Analyses**

When data followed normal distribution, analysis of variance (ANOVA) at a significance level of 5% was performed. LSD was subsequently calculated to determine the differences between pairs of samples. When data did not follow a normal distribution, nonparametric Kruskal-Wallis ANOVA on ranks and multiple-comparison Z-value test were performed. Differences were considered significant when \( P < 0.05 \). Results are expressed as means ± SD.

**RESULTS**

**Clamoxyl Drastically Altered the Intestinal Microbiota Composition**

The impact of Clamoxyl treatment on the process of intestinal colonization by the resident microbiota was monitored at the suckling-weaning interface by quantifying total aerobic and anaerobic living bacteria, as well as some of the predominant species in the suckling rat intestine, namely *Lactobacillus*, *Enterobacteriaceae*, and *Enterococcus* (16, 42). Figures 1 and 2 show, respectively, the concentration of the intestinal microflora in the colon and small intestine of control (Cont) and Clamoxyl-treated (Clam) groups. In colon, total aerobic (Fig. 1A) and anaerobic (data not shown) counts were \( \sim 10^9 \) cfu/g lower in the Clam than in the Cont group at d15, d17, and d21 (\( P < 0.05 \)). Similarly, colonic *Lactobacillus* (Fig. 1B), *Enterococcus* (Fig. 1C), and *Enterobacteriaceae* (Fig. 1D) were drastically depleted after the antibiotic administration. In the small intestine, total aerobic (Fig. 2A) and anaerobic (data not shown) populations were not significantly different between the Clam and Cont groups (\( P > 0.1 \)). However, the *Lactobacillus* species were almost eradicated in the Clam group (Fig. 2B).

**Clamoxyl Affected the Maturational Changes of Gene Transcription**

A GeneArray analysis was performed on the proximal and distal small intestine and on the colon of Cont and Clam rats at d17 and d21 to determine how the Clamoxyl treatment affected the expression of genes during the maturation process. Figures 3–5 show the impact of the treatment on the age-related changes in gene expression. Probe sets whose expression was affected by age in the control group (\( P < 0.0005 \)) are shown in the first column of the heat maps, in which the fold change between d21 and d17 defines the maturation-associated profile of each gene. The changes related to maturation were quantitatively more important in the small intestine, where 9 and 12% of the probe sets showed modulation in the proximal and distal parts, respectively (Figs. 3 and 4), than in the colon (3% of the probe sets; Fig. 5). The second column of the heat maps represents the age-related fold change for the selected genes in the antibiotic-treated animals and was used to calculate the interaction of age and treatment (i.e., third column of the heat maps). Among the genes affected by age in the control group, the proportion of probe sets in which the maturation profile was modulated by the Clamoxyl treatment was higher in the colon (34%) than in the small intestine (17% in the proximal part and...
8% in the distal part). Their expression levels and annotation are given in Supplementary Table S1 (available at the Physiological Genomics web site). When clustered using the Biological Process GO database, only 25, 38, and 48% of these probe sets in the proximal small intestine, distal small intestine, and colon, respectively, were assigned to a GO node. The expression profiles of the corresponding genes are expanded in the second heat map of Figs. 3–5. Concerning the gut barrier-related functions, Clamoxyl affected significantly the maturation of endocytosis and vesicle-mediated transport in the proximal small intestine, as well as the maturation of the immune function in both distal small intestine and colon.

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1 The Supplemental Material for this article (Supplemental Tables S1 and S2) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00057.2005/DC1.
Clamoxyl Differentially Affected Gene Expression All Along the Gut

The GeneArray analysis was also performed to determine how the antibiotic treatment affected the expression of genes along the intestine at the suckling-weaning interface. The complete list of the significantly modulated probe sets is provided in Supplementary Table S2. Figure 6 summarizes the quantitative effect of the antibiotic treatment on the different intestinal parts. Only a restrained number of probe sets, nine at d17 and seven at d21, were significantly affected in the three regions (Supplementary Table S2A). The amount of probe sets showing a common behavior in two intestinal parts ranged from 17 to 48 at d17 and from 22 to 43 at d21 (Supplementary Table S2, B–D). At d17, the number of probe sets significantly affected by the treatment was higher in the proximal small intestine (894) than in the distal small intestine (420) or in the large intestine (358). Conversely, at d21, Clamoxyl induced more changes in the large (509) and distal small intestine (377) than in the proximal small intestine (137).

Clamoxyl downregulated the expression of genes related to antigen presentation. Various genes related to antigen presentation, such as MHC class II and nonclassical class Ia, were downregulated with Clamoxyl treatment in the different parts of the intestine (Table 1). In particular, the expression of MHC gene...
class II RT1Bb chain was downregulated by nine-, four-, and threefold in the proximal and distal small intestine and in colon, respectively. In addition, the expression of CD1d, a gene associated with the presentation of lipid-containing antigens, was significantly downregulated in the proximal small intestine at d21.

Clamoxyl downregulated the expression of genes coding for anti-microbial products. Some genes associated with Paneth cell secretion products were differentially regulated with the Clamoxyl treatment, especially in proximal small intestine at d17 (Table 2). Therefore, the gene expression of several anti-microbial peptides such as defensins, lysozyme, and phospholipase A2 was downregulated with the antibiotic treatment. In addition, the gene expression of matrilysin, a metalloproteinase known to hydrolyze defensin precursors into their mature and active forms, was more than sixfold lower in the proximal small intestine of Clam than in that of Cont at d17. The changes in matrilysin gene expression were confirmed by real-time PCR, which showed a significant downregulation of this gene with Clamoxyl (1.7-fold, \( P < 0.05 \)).

Clamoxyl upregulated the expression of mast cell proteases in the distal small intestine. Numerous proteases secreted by mast cells were upregulated by the treatment in the distal part of the small intestine (Table 3). Changes affected the pups at
both d17 and d21. Real-time PCR analysis of mast cell pro-

Dilusion The goal of the described study was to determine the impact

Table 1. Gene expression of antigen presentation molecules

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Gene Title</th>
<th>Clam Cont</th>
<th>P value</th>
<th>Clam Cont</th>
<th>P value</th>
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<td>CD74 antigen</td>
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<td>1370822_at</td>
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<td>7·10^-1</td>
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<td>600 523</td>
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<td>1370382_at</td>
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<td>P &lt; 1·10^-16</td>
<td>42 78</td>
<td>4·10^-3</td>
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<td>1388236_at</td>
<td>RT1-CE11 class I</td>
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<td>898 1.428</td>
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<td>1368593_at</td>
<td>CD1d1 antigen</td>
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<td>189 276</td>
<td>4·10^-4</td>
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Probe set, Affymatrix probe set no.; Gene title, NetAffx annotations; Clam and Cont, mean average difference intensity (ADI) data for the Clamoxyl (Clam) and control (Cont) groups; P value, level of significance. *Significant differences between Clam and Cont groups (P < 0.001).
compensated by the overgrowth of other nonanalyzed populations. In line with this, Mentula et al. (39) already reported a significant increase, specific to the small intestine, in the number of coliforms in dogs treated with amoxicillin, an antibiotic that, like amoxicillin, belongs to the amino-penicillin family. The different response of the microbiota in small intestine and colon may be linked to differences in the bacterial diversity (16) as well as in the ecological conditions (34) of both intestinal parts. For instance, the transit time is several times slower in the colon than in the small intestine (27), likely leading to larger periods of contact of the colonic populations with the antibiotic and, therefore, to increased efficacy of the amoxicillin in this intestinal part.

The impact of Clamoxyl on the intestinal transcriptome was analyzed with the GeneArray technology. The GeneArray analysis was performed within the interface between the suckling and weaning periods. Drastic changes in the intestinal function have been reported during this period, especially in the small intestine (13, 25, 31). Accordingly, our data show that important changes occur at the transcriptional level between days 17 and 21. Quantitatively, the small intestine appeared to be more affected than the colon, with ~10 and 3% of probe sets, respectively, undergoing significant age-related modulation (Figs. 3–5).

Interestingly, a significant proportion of the genes undergoing maturational changes (10–30% depending on the intestinal part) showed modulation by the Clamoxyl treatment so that their normal pattern of maturation was either accelerated or slowed down. The importance of the luminal environment, in particular of the intestinal microbiota, on the process of gut

Table 2. Gene expression of anti-microbial products

<table>
<thead>
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<th>Probe Set</th>
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<th>Day 17</th>
<th>Day 21</th>
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<td>α-defensin 5</td>
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<td>8</td>
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<tr>
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<td>lysozyme</td>
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<td>phospholipase A2 group IIA</td>
<td>274</td>
<td>685</td>
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<td>1368766_at</td>
<td>matrix metalloproteinase 7</td>
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<td>11</td>
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<td>defensin NP-4 precursor</td>
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<td>defensin NP-3 precursor</td>
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Table 3. Gene expression of mast cell proteases in distal small intestine

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<td>109</td>
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<tr>
<td>1371164_at</td>
<td>mast cell protease 10</td>
<td>15p13</td>
<td>18</td>
<td>7</td>
<td>4·10⁻⁴⁻</td>
<td>19</td>
<td>38</td>
<td>9·10⁻³⁻</td>
</tr>
</tbody>
</table>

Localization, chromosomal localization. *Significant differences between Clam and Cont groups (P < 0.001). For more details, see legend to Table 1.
postnatal development has been recently reviewed by Hooper (28). This group has also shown, by comparing germ-free and monocolonized mice, that commensal bacteria modulate the expression of genes involved in postnatal maturation (29). In our study, the modulatory effect of Clamoxyl was proportionally stronger in the colon, with 34% of the age-related genes affected, than in the small intestine, with only 17 and 8% of genes affected in proximal and distal small intestine, respectively. In the small intestine, probe sets modulated by the treatment clustered evenly into different functional probe sets, including endocytosis and vesicle-mediated transport in the proximal small intestine and immune response in the distal part (Figs. 3–5 and Supplementary Table S1). Moreover, in colon, most of the probe sets, in which maturation was up- or downregulated by the antibiotic treatment, were related to ion transport processes (Fig. 5 and Supplementary Table S1). The relevance of these results is limited by the fact that only 32% of the probe sets contained in the chip were associated with a biological process with the databases currently available. Nevertheless, these data give interesting hints on the modulation by the antibiotic treatment of biological functions critical in this period of life.

Interestingly, the probe sets that were altered by the treatment all along the intestinal tract belong to the MHC, a family of genes that are involved in antigen presentation. The main function of MHC molecules is to bind antigenic peptide fragments derived, for example, from pathogens and to display them on the cell surface for recognition by immune T cells (48). Both RT1 class Ib and class II genes were globally downregulated by the antibiotic treatment. These results are in agreement with the data reported by Matsumoto et al. (38), who showed that the intestinal epithelium of germ-free mice does not express MHC molecules. This paper also demonstrated that colonization of the germ-free mouse gut induced expression of MHC class II molecules in epithelial cells (38). Adequate antigen presentation by MHC class II is required for the tolerization to luminal antigens, as shown in MHC class II-deficient mice, which lack the ability to acquire oral tolerance (14). The role of RT1 class Ib molecules is less clear. A large number of MHC class Ib genes have been described (23) and are distinguished from the “classical” class Ia by their limited polymorphism (52). The specific patterns of expression of RT1 Ib suggest specialized roles in antigen presentation. For example, in mice, the class Ib locus M molecules can present peptides with NH2-formylated amino termini from infectious organisms, such as Listeria monocytogenes (52). However, other MHC class Ib loci (HLA-A) have functions unrelated to the immune system such as iron metabolism and uptake in the intestinal tract (10). Unfortunately, the specificity of the MHC class Ib locus Aw2, downregulated with Clamoxyl, is unknown.

Taken together, our results suggest that early antibiotic treatment may affect the acquisition of tolerance to luminal antigens by altering the mechanisms of antigen presentation. It is important to note that the presence of intestinal microbiota is required for the maintenance of oral tolerance, as evidenced in germ-free rodent models (55). In this regard, some population-based studies have found a positive association between the early administration of antibiotics and the risk of later development of atopy (15, 21, 58, 60) or Crohn’s disease (9), both allergy and Crohn’s disease being characterized by a hyperresponsiveness of the immune system to harmless antigenic components (i.e., protein and commensal microbiota).

Paneth cells are key players in the mucosal handling of luminal bacteria, acting specifically at the level of the small intestinal crypts (44). Indeed, they are able to synthesize and secrete compounds such as defensins, lysozyme, and type II phospholipase A2, with anti-microbial properties (24, 46, 59). The gene expression of several antimicrobial peptides, defensins 3 and 5, lysozyme, and phospholipase A2, as well as matrilysins downregulated in our study (48). Both RT1 class Ib and class II genes were globally downregulated by the antibiotic treatment, whereas the defensins predominantly found in epithelial cells (5), defensins, were not significantly affected. Contrary to our results, Putsep et al. (45) did not find any differences in the qualitative profile of prodefensin peptides present in germ-free or conventional mice. They concluded that the intestinal microbiota did not affect the production of defensins by the Paneth cells. However, this paper did not present quantitative data, and, therefore, an effect of the microbiota on the actual amount of synthesized defensins cannot be ruled out. Moreover, Ayabe et al. (4) showed a dose-dependent secretion of defensins when the crypts from germ-free mice were isolated and exposed to bacteria or bacterial antigens, indicating that, indeed, the presence of bacteria stimulates the secretion of these molecules. As indicated, the matrilysin gene was significantly downregulated in the Clamoxyl-treated group. In line with these results, Lopez-Boado et al. (33) failed to detect matrilysin protein in germ-free mice. Matrilysin is a protease expressed in Paneth cells that plays a crucial role in mucosal defense by activating the prodefensin molecules (59). Therefore, a reduction in the activity of this protease may compromise the anti-pathogenic activity of the defensins (59). We also observed reduced expression of the phospholipase A2 gene after antibiotic intervention. This enzyme has been shown to activate both gram-positive (46) and gram-negative bacteria (24). Accordingly, Qu et al. (47) showed that in vivo intraluminal perfusion with lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, increased phospholipase A2 and lysozyme activity in the luminal perfusates of the LPS-exposed rats. The changes observed in the level of expression of Paneth cell products were specific to postnatal day 17, suggesting higher susceptibility before weaning to the alterations occurring at the luminal level. Although these data should be confirmed by appropriate functional assays, they strongly suggest that an antibiotic treatment may reduce the innate ability of the host to deal with pathogens in the still-immature gut. The results appear to be related to an alteration of the secretory ability of the cell rather than to a change in the number of cells, as the immunohistochemical analysis of the intestinal tissues did not reveal any effect in the number of Paneth cells (data not shown).

Finally, our GeneArray analysis revealed increased gene expression of a number of mast cell proteases in the distal part of the small intestine of Clamoxyl-treated rats. Interestingly, these protease genes are located on the same chromosome (15q12 or p13). In contrast, mast cell protease 6, which is not located on chromosome 15, was downregulated by the antibiotic treatment. These data strongly suggest that the upregulated genes were all modulated by the same transcription factor. The
observed upregulation of mast cell protease expression is in agreement with the data reported by Enerback et al. (20), who showed that administration of the antibiotic Polymyxin to rats induced an increase of the mucosal mast cell number. Mast cells are considered effector components of the immune system, playing an important role in host defense against pathogens (35, 36). However, they also play a central role in the onset and maintenance of chronic intestinal inflammation (61) and in the clinical manifestations of gastrointestinal allergic disorders (8). Although we did not observe any signs of macro or microscopic inflammation in the intestine of the Clamoxyl-treated animals (data not shown), an increase in the mast cell number and/or activity may reflect a subinflammatory status that, as in other conditions (53), can predispose the animal to an exacerbated inflammatory reaction in the case of insult.

In summary, the Clamoxyl treatment altered the normal colonization pattern of the gut microbiota and interfered with the normal maturation profile of 10–30% of genes in the different intestinal segments. In particular, the antibiotic treatment resulted in the downregulation of genes related to innate host defense and to antigen presentation, and in the upregulation of genes related to mast cell activity. The design of our study did not allow us to determine whether the observed effects were attributable to the microbiota changes or to the Clamoxyl chemical composition (either the antibiotic itself or the vehicle). However, our results suggest that the early administration of a commercial antibiotic preparation widely employed in pediatric settings interferes with the maturation of various parameters of the mucosal barrier. These changes occur at the suckling-weaning transition, a critical period in which the gut and the subjacent immune tissue deal with an array of novel food antigens and bacteria. It would be interesting to evaluate whether these changes are associated with an increased risk of contracting infection or developing inflammatory or atopic diseases later in life.

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REFERENCES


