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Establishment of a Biochemically Active Intestinal Ecosystem in Ex-Germfree Rats

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A time course study for the establishment of some biochemical microbial intestinal functions was undertaken in ex-germfree rats conventionalized, i.e., colonized with conventional flora, in three different ways: untreated (group 1); contact with visitor rats (group 2); inoculated with intestinal contents from conventional rats (group 3). The first two groups of rats were inoculated with the intestinal contents from conventional rats after being out of the germfree isolators for 4 weeks. The biochemical parameters studied were degradation of mucin, inactivation of tryptic activity, conversion of cholesterol to coprostanol and of bilirubin to urobilinogen, degradation of beta-aspartylglycine, and formation of short-chain fatty acids. The results showed that the way in which the microbes were introduced and the microbial biochemical functions themselves were of importance. In several cases, social contacts, i.e., contact with visitor rats, were just as effective for the functionally adequate establishment of microbial intestinal functions as was inoculation with intestinal contents from conventional rats. Some of the biochemical parameters studied were established after a few days, whereas the establishment of others was markedly delayed. When inoculated after 4 weeks, all rats in the first two groups were colonized with conventional flora within 1 week. The results indicate that the model system described is suitable when studying buildup mechanisms in intestinal ecosystem(s).

The intestinal tracts of humans and other mammals harbor a wide variety of microbial species, usually described as normal intestinal microflora (for a review, see reference 6). Together with the host, this flora constitutes ecological system(s) that are of benefit to the host as well as the microbes. The systems are established during postnatal life, when various microbial species find their way into the intestinal tract. When germfree animals are conventionalized, i.e., colonized with conventional flora, similar ecosystems are established. Results of studies on ex-germfree animals have demonstrated that some microbial species may act synergistically and others may act antagonistically with each other (4). The exact mechanisms involved in these interactions, however, have not been elucidated.

One approach for studying the establishment of an intestinal microflora is related to estimation of its functional interactions with host-influenced parameters. Such interactions have been described as microflora-associated characteristics (MACs), which are defined as the recording of any anatomic structure or physiological or biochemical function in a macroorganism which has been acted upon by the microflora (13, 14). When the microbes that actually influence the parameter under study are absent, as in germfree animals, newborn individuals, and sometimes, patients receiving antibiotics, this is defined as a germfree animal characteristic (GAC).

In the present investigation we wanted to study the time course in the establishment of some MACs in ex-germfree rats that were colonized with conventional flora by various routes. The parameters studied were reduction of cecum size to follow the microbial interaction on an anatomic structure, degradation of mucin to study an intestinal function of major importance in preserving the integrity of the intestinal mucosa, inactivation of tryptic activity to study pancreatic and intestinal cofunction, conversion of cholesterol to coprostanol and of bilirubin to urobilinogen to follow hepatic and intestinal interactions, and the absence of beta-aspartylglycine and the presence of short-chain fatty acids (SCFAs) to elucidate microbial interactions with compounds mainly derived from the diet of the rats.

MATERIALS AND METHODS

Animals. Germfree and conventional rats of the AGUS strain (8) (weight, 300 to 400 g) were reared on a commercially obtained, steam-sterilized diet (R-3; Ewos Company, Södertälje, Sweden). The germfree technique has been described elsewhere (7).

Study design. Sixteen germfree rats of both sexes were randomly divided into three groups before they were taken out of their germfree isolators and placed separately in clean metabolic cages which were kept in a room with conventional animals with a temperature of 24 ± 2°C and a relative humidity of 55 ± 10%. The three groups were handled as follows. For group 1 (rats randomly colonized from the environment; six rats), the rats were left in their cages until day 29, when they received 1 ml of a homogenate of cecum content (HCC). HCC was obtained from six conventional rats, and was diluted 1:10 in Todd-Hewitt broth (Oxoid Ltd; Basingstoke, England) and given both intragastrically and as an enema. For group 2 (contact with visitor rats; five rats), each rat was allowed to have a separate conventional rat visitor nightly during the first week. On day 29, they received 1 ml of HCC, as described above. For group 3 (infusion group; five rats), immediately after the rats were taken out of the isolators, each animal received 1 ml of HCC, as described above.

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Each group of animal cages was kept on a separate shelf, and throughout the experiment, care was taken to avoid intra- and intergroup transportation of contaminated material. Before the start of the study, all the germfree rats (16 rats), the conventional rats used as visitors (five rats), and donors of cecum content (six rats each time HCC was prepared) were investigated for their GACs and MACs.

During the first week that rats were colonized with conventional flora feces were collected each day at hourly intervals for 12 h; thereafter they were collected once a week at hourly intervals for 12 h. After 6 weeks, all rats were exsanguinated under slight ether anesthesia, and their body and cecum weights were measured. After all samples were taken, they were immediately frozen in closed vials and kept at −20°C until analysis, which was done within 3 weeks. The samples were then gently thawed and mixed, and fractions were taken for determination of urobilinogen and SCFAs. Another fraction of about 1 g was mixed thoroughly with saline (1:2), placed at 4°C for 2 h, and centrifuged (35,000 × *g*, 4°C, 30 min). The mucin, tryptic activity, and beta-aspartylglycine parameters were determined with the supernatant; and the remaining supernatant plus sediment was used for determination of the degree of cholesterol conversion.

Degradation of mucin. The supernatants were analyzed by agar gel electrophoresis as described elsewhere (8). The mucin pattern was visualized by staining the agar plates with toluidine blue and periodic acid Schiff, and the protein pattern was visualized by staining with Coomassie brilliant blue R. Fecal supernatants from germfree and conventional rats were run on each gel as controls. An electrophoretic pattern with specific bands similar to that found in germfree rats was defined as the GAC, and a pattern similar to that found in conventional rats, i.e., without any bands, was defined as the MAC.

Determination of fecal tryptic activity. A supernatant fraction of 0.1 ml was added to 2.9 ml of Tris buffer (pH 8.2) containing 4.4 g of calcium chloride per liter. The reaction took place at 20°C and was initiated by adding 0.6 ml of 0.003 M *N*-benzoyl-DL-arginine-*para*-nitroanilide hydrochloride (Sigma Chemical Co., St. Louis, Mo.) and was stopped after 10 min by adding 0.6 ml of 5 M acetic acid. Bovine pancreas trypsin type III (Sigma) diluted in 2 mM hydrochloric acid was used for the construction of a standard curve. All samples and standards were analyzed in parallel with blanks at 405 nm on a spectrophotometer (150-20; Hitachi). Fecal tryptic activity (FTA) was expressed as milligrams of tryptic activity per kilogram of feces. By this method, values found in adult germfree and conventional AGUS rats on R3 diet were always higher than 700 and lower than 100 mg/kg of feces, respectively. Consequently, these values were chosen as GAC and MAC breakpoints.

Conversion of cholesterol to coprostanol. The combined supernatant and sediment was mixed with 2 ml of a solution of 95% ethanol and 10 M NaOH (2:1) and placed in a water bath at 60°C for 45 min. The hydrolysate was extracted twice with 10 ml of hexane. The hexane phase was extracted with 70% ethanol until the pH was neutral, evaporated, and analyzed by gas-liquid chromatography (104; Pye Unicam, England) at 290°C on 3% OV-17 (Supelco, Inc., Bellefonte, Pa.). The results were expressed as the percentage of coprostanol of the total amount of cholesterol and coprostanol that was present. By this method, peak areas less than 5% were neglected as impurities. The values in germfree rats never exceeded that value, whereas in conventional rats the values were always above 25%. Consequently, 5 and 25% were chosen as GAC and MAC breakpoints, respectively.

TABLE 1. MAC electrophoretic mucin and protein patterns in feces

Group[a]	No. of days (range) before appearance of MAC pattern[b]: Mucin	Protein
1	30 (21–30)	28 (21–31)
2	4 (2–7)	7 (4–14)
3	1	1

[a] On day 29, the animals in groups 1 and 2 received 1 ml of a homogenate of cecum content from conventional rats (HCC).

[b] Values are medians.

Conversion of bilirubin to urobilinogen. Urobilinogen was determined spectrophotometrically by applying the Ehrlich aldehyde reaction with *p*-dimethylaminobenzaldehyde directly to an aqueous extract of feces, followed by reduction of urobilin to urobilinogen with ferrous hydroxide (21). By using this method, it was regarded that values less than or equal to 0.05 mmol/kg of feces were caused by impurities. The values in germfree rats never exceeded that value, whereas the values in conventional adult rats always exceed 0.11 mmol/kg (unpublished observation). Consequently, 0.05 and 0.11 mmol/kg were used as GAC and MAC breakpoints, respectively.

Determination of beta-aspartylglycine. Supernatant fractions of 30 μl were subjected to high-voltage paper electrophoresis as described previously (24). Commercially obtained beta-aspartylglycine (Sigma) and germfree and conventional rat fecal supernatants were applied onto each sheet of paper as controls. Beta-aspartylglycine was always present in adult germfree rat feces (GAC), but was always absent from adult conventional rat feces (MAC).

Determination of SCFAs. Fractions of feces (0.5 to 0.6 g) were mixed with distilled water containing 3 mmol of 2-ethylbutyric acid (internal standard) per liter, acidified with H_2SO_4 (0.5 mmol/liter) to pH 2, and homogenized. The homogenates were vacuum distilled and analyzed by gas-liquid chromatography as described previously (10).

Statistics. The results were analyzed by the Wilcoxon two-sample rank test, and significance was assigned to a two-tailed *P* value of less than 0.05.

RESULTS

Animals. All germfree animals demonstrated GACs when reared in isolators, and the conventional rats used as visitors and suppliers of cecum contents demonstrated MACs. One animal in group 1 died of an intercurrent disease (malformations in the urinary tract) a few days after it was taken out of the isolator, and the results for that rat are excluded. Otherwise, all animals remained healthy until the experiment was terminated. Mean values of cecum weights, expressed as a percentage of body weight, were 1.41, 1.37, and 1.43 for rats in groups 1 to 3, respectively, i.e., there were no significant differences.

Degradation of mucin. The ability of mucin-degrading bacteria to be functionally established was followed, and the results are shown in Table 1. In group 1 rats, two of the five rats developed a MAC mucin pattern on day 21. In the other three rats the mucin pattern was not completely conventionalized until 24 h after they received 1 ml of HCC on day 29.

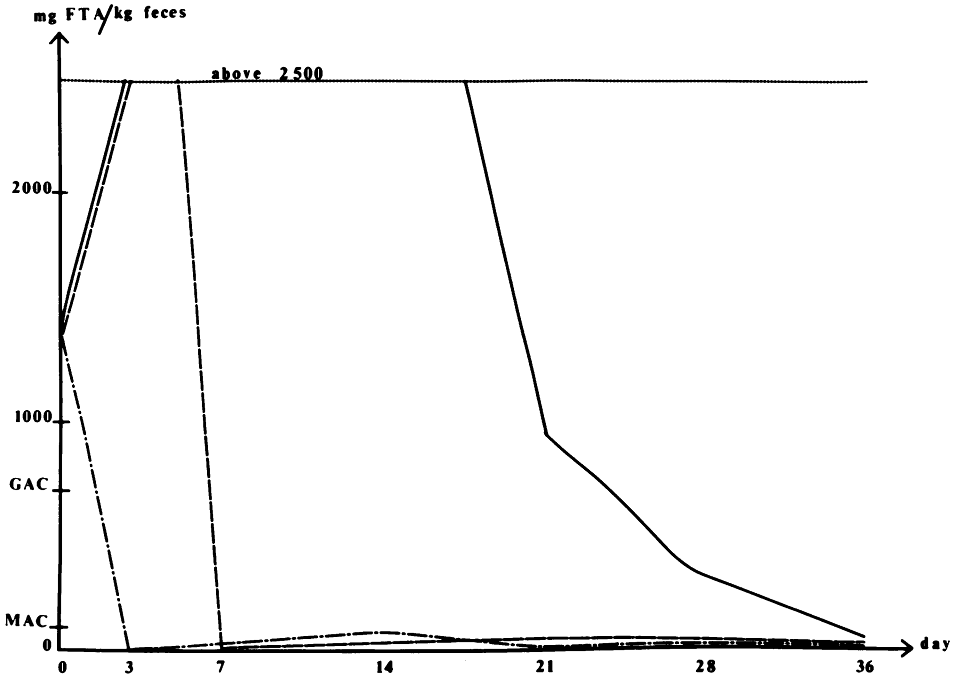

FIG. 1. Alterations of median values of FTA levels in the three groups of rats. Symbols: ——, randomly colonized rats (group 1); ——, visitor group (group 2); –·–·–, infusion group (group 3). The GAC value was higher than 700 mg/kg of feces (the range on day 0 in material from 15 rats was 1,250 to 1,550). The MAC value was lower than 100 mg/kg of feces (the range on day 36 in material from 15 rats was 0 to 90).

The protein pattern followed the mucin pattern closely in all rats except one, in which a shift to a MAC pattern was not seen until 2 days after it received HCC. In group 2 rats, i.e., those that had visitors, two rats developed a MAC mucin pattern 2 days after the transfer, one rat 4 days after the transfer, and the other two rats 7 days after the transfer. A MAC protein pattern was developed in all rats within 14 days. In group 3 rats, all five rats showed a GAC to MAC shift of both mucin and protein patterns within 24 h after they received 1 ml of HCC. The observed differences among the three groups for the establishment of MACs were statistically significant.

Determination of tryptic activity. In group 1 rats, the median FTA level was markedly increased on day 3, and remained increased for 3 weeks (Fig. 1). The MAC value was not obtained for any of these rats until they received HCC on day 29. In group 2 rats, a similar high median value was found on day 3. However, on day 7 the median value, i.e., the MAC value, was 0 and remained so throughout the observation period. In group 3 rats, all rats showed a GAC to MAC shift on day 3. (for groups 1 versus 3, $P < 0.05$ from days 3 to 28).

Conversion of cholesterol to coprostanol. The conversion of cholesterol to coprostanol was rapidly established in group 3 rats but more slowly in the other two groups of rats (Table 2). Occasionally, however, a transient MAC to GAC switch was observed in a few of the animals.

Conversion of bilirubin to urobilinogen. All fecal samples taken on day 3 contained urobilinogen (Table 3). Group 1 rats had only a very slight increase in urobilinogen production from days 3 to 28, whereas the values for group 2 and 3 rats increased to a level closer to that found in the conventional rats used as visitors. Significant differences in the values, however, were not observed among the three treatment groups when samples taken on the same day were compared. The fecal urobilinogen values were at the MAC level (i.e., higher than 0.11 mmol/kg of feces) in all rats on day 36.

Degradation of beta-aspartylglycine. The MAC pattern was established after 3 days in group 3 rats, whereas it took up to

TABLE 2. Conversion of cholesterol to coprostanol

Group	% Coprostanol on day[a]:					
	3	7	14	21	28	36
1	0	0	18	49	58	39
	0	18	26	31	57	31
	0	0	10	0	27	60
	0	0	0	0	0	31
	0	0	0	0	0	31
2	58	52	49	62	0	55
	40	27	33	42	68	39
	0	18	23	47	63	56
	26	23	31	0	0	38
	0	39	0	0	57	42
3	0	24	53	48	60	0
	14	24	27	14	70	25
	7	24	34	33	56	44
	10	13	27	61	43	45
	27	0	22	65	28	39

[a] Values expressed as the percentage of coprostanol of the total amount of cholesterol and coprostanol present. On day 0 there was no coprostanol present. On day 29 the animals in groups 1 and 2 received 1 ml of a homogenate of cecum content from conventional rats (HCC). In conventional rats the median value was 47% coprostanol (range, 39 to 49%).

TABLE 3. Urobilinogen in feces

Group	Urobilinogen (mmol/kg [range]) on day[a]:						
	0	3	7	14	21	28	36
1	0.04 (0.03–0.04)	0.11 (0.07–0.15)	0.09 (0.07–0.15)	0.09 (0.08–0.12)	0.10 (0.08–0.17)	0.14 (0.09–0.18)	0.14 (0.12–0.15)
2	0.03 (0.03–0.04)	0.13 (0.08–0.21)	0.14 (0.12–0.18)	0.14 (0.10–0.15)	0.15 (0.12–0.16)	0.19 (0.11–0.27)	0.16 (0.15–0.25)
3	0.04 (0.03–0.05)	0.11 (0.10–0.16)	0.12 (0.10–0.17)	0.12 (0.11–0.23)	0.17 (0.15–0.24)	0.19 (0.15–0.23)	0.17 (0.16–0.20)

[a] Values are medians. On day 29, the animals in groups 1 and 2 received 1 ml of a homogenate of cecum content from conventional rats (HCC). In conventional rats the median value was 0.21 mmol/kg (range, 0.17 to 0.25 mmol/kg).

14 and 21 days for rats in groups 2 and 1, respectively (data not shown). The differences between groups 1 and 3 were statistically significant on day 3.

A summary of the biochemical parameters mentioned above is given in Fig. 2. Values for GACs, those between GAC and MAC, and MACs were given scores of 0, 1, and 2, respectively, giving a total group score of 60 if a GAC to MAC switch took place in all rats. In general, the rates in the rats in groups 2 and 3 were rather close, being higher than the rate in rats in group 1. Rats in groups 1 and 2 had to receive HCC before a total GAC to MAC conversion was obtained.

Production of SCFAs. Intestinal production of SCFAs was rapidly established in all three groups of rats (Table 4). High concentrations of acetic acid were found in feces on day 3, whereas the other acids appeared somewhat later, especially in group 1 rats. However, all animals had a normal pattern of SCFAs before they received HCC.

DISCUSSION

A principal objective for ecologists is a better understanding of the patterns in which living organisms are distributed in space and time. It is a growing view that "an ecosystem can neither be understood by taking it apart and studying the part piecemeal, nor can it be dealt with by conceptually enclosing it" (25). The present study was an approach that was used to study the intestinal ecosystem. The efforts were concentrated on the establishment of some of the biochemical functions that are performed by the microbial flora. The results indicate that the way in which the microbes are introduced, as well as the functions themselves, are of importance (Fig. 2). Social contacts, i.e., visitors, in several cases were just as effective for the adequate establishment of flora as was the loading of a huge dose of intestinal contents from another rat. In group 1 rats, which were in no direct contact with conventional animals but which were exposed to bacteria from the environment (floor dust, drinking water, etc.), the delayed establishment of most microbial functions was demonstrated.

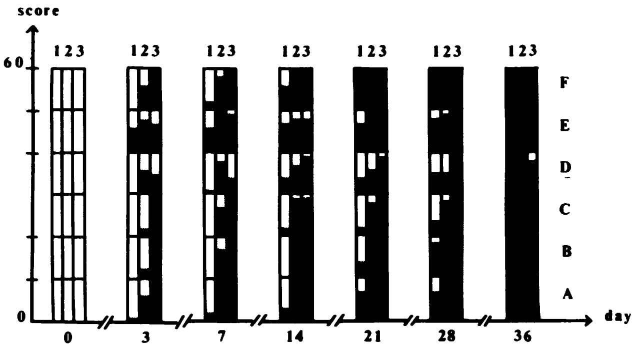

FIG. 2. Time course in the establishment of a biochemically active intestinal ecosystem. The three different groups of rats are indicated by arabic numerals above each bar. A filled box represents a complete MAC score and an open box represents a GAC value for each of the following parameters in five rats: mucin, carbohydrate staining (A); mucin, protein staining (B); inactivation of tryptic activity (C); conversion of cholesterol to coprostanol (D); conversion of bilirubin to urobilinogen (E); determination of beta-aspartylglycine (F). For further details, see text.

The time course variations by which the individual microbial functions were established may, to some extent, reflect characteristics of the microbial species involved in the particular function. This can be exemplified as follows. The ability to degrade mucin has been found among various groups of microorganisms, as in the genera *Bifidobacterium, Ruminicoccus, Peptostreptococcus,* and *Bacteroides* (for a review, see reference 3). *Peptostreptococcus* sp. and *Bacteroides* spp. can be classified as rapid-growing organisms that are present in high numbers in intestinal contents of humans and most other mammals (6). When a mucin-degrading *Peptostreptococcus* sp. was introduced into germfree rats, more than 10^8 microbes were present per g of feces after 24 h, and a GAC to MAC switch was established within 48 h (3). A similar rapid establishment of a mucin-degrading flora was found in group 3 rats, i.e., animals that received an infusion with intestinal contents from conventional rats.

The parameters FTA, beta-aspartylglycine, and SCFAs fit into the same time schedule for establishment. Although the specific microbial strains involved in the first two parameters are virtually unknown, it might be reasonable to assume that the capability of performing these reactions is not rare among intestinal organisms. A transient increase in FTA, as was found in rats in groups 1 and 2, is often observed when germfree animals are colonized with conventional flora (unpublished data) and may also be seen in conventional animals that receive antibiotics, for example, clindamycin (B. Carlstedt-Duke, L. Alm, T. Höverstad, A.-C. Midtvedt, K. E. Norin, M. Steinbakk, and T. Midtvedt, FEMS Microbiol. Ecol., in press). In fact, FTA is a rather complex parameter, reflecting the net sum of interactions between inactive pancreatic trypsinogen and host-, diet-, and microbial-derived activators and inactivators (15, 17–19). Additionally, alterations in passage time, which are brought about by the flora itself, may play a role. The presence of intestinal SCFAs indicates that anaerobic bacterial metabolism has taken place. The small amounts of some acids in feces from germfree rats probably originates mainly from the diet (11). The production of acetic acid is a very common property among intestinal microbes (12), whereas the capability of producing some of the other acids might be more rare.

The capability of transforming bilirubin to urobilinogen seems to be a rare property among intestinal microorganisms. So far, this reaction has been related to one species

TABLE 4. SCFAs in feces

Group	Day	SCFAs (mmol/kg [range]) for[a]:						
		Acetic	Propionic	Isobutyric	*n*-Butyric	Isovaleric	*n*-Valeric	Total
1	0	0.1	0.0	0.0	0.0	0.0	0.0	0.1
	3	21.3	1.1	0.5	2.4	0.5	0.0	29.1
	7	41.2	11.8	0.5	4.4	0.4	0.1	60.8
	14	50.8	14.0	1.1	6.2	1.0	0.2	73.8
	28	36.8	9.7	1.4	9.3	1.3	0.3	59.6
	36	64.6	15.7	0.9	11.5	0.8	0.8	91.7
2	0	0.1	0.0	0.0	0.0	0.0	0.0	0.1
	3	32.0	12.2	0.4	2.1	0.6	0.0	48.4
	7	37.1	12.2	0.3	2.4	0.2	0.1	57.7
	14	73.0	23.1	0.4	10.6	0.3	0.3	90.3
	28	43.2	11.8	0.8	7.3	0.8	0.2	64.4
	36	39.8	11.6	0.7	8.5	0.5	0.5	56.4
3	0	0.1	0.0	0.0	0.0	0.0	0.0	0.1
	3	26.9	13.5	0.3	1.8	0.4	0.1	41.6
	7	48.5	20.9	0.4	5.1	0.2	0.1	77.5
	14	37.2	14.9	0.8	12.3	0.6	0.8	64.3
	28	55.6	14.9	1.0	11.7	1.0	0.7	83.0
	36	44.1	10.4	0.8	6.1	0.7	0.8	65.8
Conventional rats		36.5 (27.4–49.8)	11.2 (8.9–13.1)	1.0 (0.7–1.1)	15.5 (8.4–28.0)	1.1 (0.7–1.3)	1.4 (0.8–1.8)	67.8 (47.3–90.0)

[a] Values are medians. On day 29, the animals in groups 1 and 2 received 1 ml of a homogenate of cecum content from conventional rats (HCC).

only, i.e., *Clostridium ramosum* (16). As a spore-forming organism, it might be distributed all around in the room housing the animals and, thereby, also rather easily picked up by the rats in group 1. In all three groups of rats, however, there was a tendency to produce a somewhat lower level of urobilinogen than that which was found in the conventional rats used as visitors. This is in accordance with previous findings (2, 22) and indicates that an optimal formation of urobilinogen is a result of interactions between various specific organisms.

Microbial intestinal transformation of cholesterol to coprostanol so far has been demonstrated to occur only in strains belonging to the genus *Eubacterium*, i.e., strict anaerobic, non-spore-forming, gram-positive rods (5, 20). At present, it has not been established whether these strains are motile or not. Working with motile and nonmotile variants of *Roseburia cecicola*, Stanton and Savage (23) have demonstrated that motility is "apparently advantageous to this bacterium when other micro-organisms are present with it in the mouse caecum." If the coprostanol-producing bacteria are nonmotile, at least in the intestinal environment they might be dependent on "refill" mechanisms for their continuous presence. Refill variations may then be an explanation for the periods of GACs found in a few of the animals. The cholesterol-coprostanol parameter is of considerable interest, since the microbes transform absorbable cholesterol into nonabsorbable coprostanol in all mammals, including humans, thereby having an influence on the regulation of endogenous cholesterol production. Additionally, the microbes seem to be markedly suppressed by several oral antibiotics (14, 15).

Principally, the same colonization pattern occurs when a germfree animal is introduced to a conventional counterpart or is given infusions with a suspension of intestinal content, as occurs in connection with normal birth (17). In both instances the germfree individual faces a flora in balance with the species, and establishment of the flora is a physiological process, usually not creating any disorder. Initially, bacterial species with a high intrinsic rate of increase are favored, and this is described as an r-selection (1). If these microbes are capable of performing the reaction studied (degradation of mucin, etc.), the parameter is rapidly established. With the passing of time, the premium is on competitiveness, and the flora is more multifaceted and consists of organisms that utilize even small metabolic niches (K-selection), such as transforming cholesterol and bilirubin. Sooner or later, all, or nearly all, niches are filled; and then the host harbors a conventional flora.

In fact, the present investigation was an attempt to apply a hierarchical strategy for studying the establishment of intestinal ecosystems. The germfree animals represent "empty boxes," to be filled with microbes that are capable of performing all reactions known to be operating in a normal flora. Results of the present study indicate that there are marked variations in the time course of establishment of several functions. This might have been caused by a temporary delay in the exposure of the animals to the microbes that were actually involved in the parameter(s), as indicated by the results with the untreated rats. On the other hand, it might depend on a more sophisticated microbial interspecies succession or relationship, as has been shown previously (9) to be the case in the 7-alpha-dehydroxylation of bile acids. Similarly, it is not known at present whether the bacteria that transform bilirubin to urobilinogen depend on free bilirubin or can act on conjugated bilirubin as well. If urobilinogen formation takes place on free bilirubin only, then bilirubin-deconjugating organisms also must be established. Additionally, it seems reasonable to assume that the establishment of mucin-degrading microbes should ease the establishment of microbes that are able to survive only in a close relationship with cell surfaces (as might be the case for the cholesterol-converting organisms). A microbial breakdown of mucin might also be a source of substrate for several of the microbes that are involved in the production of SCFAs, and so on.

In sum, the functional approach described in this report should allow further evaluation of the mechanisms involved in the complicated buildup of an intestinal ecosystem. Additional work is needed, however, to determine the extent to which succession does occur. Also, the physical and chemical nature of the mechanisms that actually govern each parameter warrant further investigation.

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