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Influence of the Normal Flora on Mucosal Morphology and Cellular Renewal in the Ileum

A Comparison of Germ-Free and Conventional Mice*

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The concept of rapid and continuous renewal of the epithelium of the normal small intestine has been established by morphologic study[19] and by kinetic analysis employing radioactive deoxyribonucleic acid precursors and autoradiographic techniques.[9-16] Experimentally, cellular proliferation and migration in the intestinal epithelium are known to be influenced by such factors as endocrine deficiency,[8] starvation,[3] exposure to ionizing radiation,[17] partial resection of the small intestine,[12] and age of the animal.[11] However, the mechanisms by which epithelial renewal is regulated in the normal state, and the factors which determine the normal pace of the renewal process, are largely unknown.

The character of the intraluminal environment of the intestine might reasonably be expected to influence the life cycle of mucosal cells. The bacterial flora, a significant component of this environment, has been shown by comparison of germ-free and conventional animals to be an important determinant of mucosal structure. Morphologic study of the guinea pig[1, 19] and metric studies, particularly of the rat,[2-4] have established that many "normal" characteristics of the mucosa fail to develop fully in the absence of a bacterial flora. In fact, the normal state of affairs in the mucosa has been described as one of "physiological inflammation."[18]

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In the course of a study extending these observations to various parts of the gastrointestinal tract of the mouse, certain features of the epithelium and the lamina propria suggested that their rates of cellular renewal might be influenced by the presence or absence of a living flora. Therefore, a detailed analysis, employing tritiated thymidine as a tracer in germ-free and conventional mice, was undertaken to delineate the role of the normal flora in establishing these rates. The present report concerns the original morphologic observations and the subsequent autoradiographic findings in the ileum of these animals.

MATERIALS AND METHODS

Animals

Mice of the Swiss Webster (ND-2) strain were used. Preliminary morphologic observations were made on animals between 3 and 6 months of age, whereas the mice used in the autoradiographic study were all 3 months old. All animals, including those of the conventional group, were maintained in flexible plastic isolators,[20] with the use of standard gnotobiotic techniques,[5] and were fed the L-462 diet.[21] The germ-free and the conventional animals, housed under identical conditions and fed the same sterilized diet, differed only by virtue of the presence of a living bacterial flora in the conventional animals.* The germ-free state was defined in terms of the absence of protozoan, fungal,

* The fecal flora of conventional mice raised as in this study is known usually to include anaerobes of the Clostridium and Bacteroides groups, diphtheroids, [illegible] lactobacilli, micrococci, [illegible] Gram negative bacilli, including members of [illegible] Escherichia, Aerobacter, and Pseudomonas groups.

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bacterial, or metazoan agents demonstrable by standard techniques.[6] Cultures from the germ-free isolators, which always included fecal material in the inoculum, were consistently negative before and after the experiments.

Autoradiographic Techniques

Sterile tritiated thymidine (0.36 c. per mmole) was taken into the isolators in glass ampules, through a lock sterilized with peracetic acid. Each mouse was given 40 μc. intraperitoneally in 0.4 ml. of isotonic saline solution. The animals were sacrificed immediately upon exposure to the conventional environment at intervals of 1, 11, 24, 36, and 48 hours after the administration of tritiated thymidine. Each time group included four to seven germ-free mice and approximately as many conventional mice, both sexes being represented.

At necropsy, the terminal 8 cm. of ileum were divided into three segments which were opened and flattened on a card and fixed in formalin. The paraffin-embedded tissue was sectioned at 4 μ, and one slide from each block was stained with hematoxylin and eosin while two additional slides were coated with nuclear track emulsion (Kodak NTB-2) by the dipping method.[7] These autoradiographs were developed after a 1-month period of exposure, and stained with hematoxylin.

Quantitative Evaluation

In all of the quantitative histologic evaluations the segments of ileum from a given animal were separately scored at different times to minimize the bias inherent in performing counts on consecutive samples from the same animal. The data presented for a given mouse represent the counts derived from the three blocks of ileum, pooled after completion of the counting.

In comparing mucosal dimensions, crypts and villi were measured in terms of numbers of epithelial cells rather than in linear dimensions in order to eliminate variables related to differential shrinkage and distortion. By this method, similar to that of Quastler,[15] the depth of a crypt, for instance, is measured in terms of the number of epithelial cells in a single-file column extending vertically from the bottom of the crypt to the level of the crypt-villus junction. Only the crypt-villus units that were sectioned so as to include, in continuity, the bottom of the crypt, the lumen of the crypt, a continuous crypt-villus junction, and the tip of the villus were included in such counts.

In the autoradiographs, cells were scored as labeled if three or more silver grains were present over their nuclei. In the mice sacrificed 1 hour after injection of tritiated thymidine, the mean number of labeled cells per crypt section was determined by counting cells in 40 ideally sectioned crypts per animal. In the remaining time groups, the position of the label in each animal was scored in terms of the average number of epithelial cells in single-file columns extending vertically from the bottom of the crypts up to the leading labeled epithelial cells in ideally sectioned crypt-villus units. An average of 30 such units was evaluated for each mouse, and the mean count was recorded as the value for that animal. Plotting these values as functions of time allowed a statistical comparison of the rate of epithelial turnover in germ-free and conventional animals.

RESULTS

Morphologic Observations

The morphogenetic impact of the normal flora on the mucosa is apparent from the comparison of the ileum of the germ-free mouse with that of its conventional counterpart.

The lamina propria in the germ-free mouse (Fig. 1) consists of only a sparse stroma surrounding the blood vessels and lymphatics, and it contains few lymphocytes and mononuclear cells. In the conventional mouse (Fig. 2) the lamina propria is expanded to its more familiar state, and lymphocytes, mononuclear cells, and plasma cells are more numerous. Similarly, the Peyer's patches in the germ-free mouse are smaller than in the conventional animal, and they show fewer reaction centers, fewer plasma cells, and relatively little mitotic activity.

The epithelium of the germ-free animal tends to be more regular than that of the conventional animal, and, especially in sections stained with the periodic acid Schiff reagent, the columnar absorbing cells appear to have a wider brush border. The number of lymphocytes associated with the epithelium is much greater in the conventional than in the germ free animal.

Comparison of over all mucosal architecture in the two groups of mice indicates

1

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Fig. 1. Ileum of germ free mouse. The lamina propria is poorly developed, with relatively few cells in its interstices. The crypts are shallow. Compare with Figure 2. Hematoxylin and eosin. ×111

Fig. 2. Ileum of conventional mouse. The lamina propria is well developed and contains numerous lymphocytes, reticuloendothelial cells, and plasma cells. The crypts are deeper than those of the germ free mouse, and division figures are more numerous. Hematoxylin and eosin. ×111

TABLE 1. DIMENSIONS OF ILEAL MUCOSA IN GERM-FREE AND CONVENTIONAL MICE

	Total mucosal thickness[a]	Length of villi[a]	Length of crypts[a]	Ratio, length of villus to length of crypt
Germ free (24 mice)	49.2 ± 0.9	36.0 ± 0.8	13.2 ± 0.1	2.73 ± 0.06
Conventional (20 mice)	57.7 ± 0.8	40.4 ± 0.6	17.3 ± 0.3	2.34 ± 0.04
P value of difference	<0.001	<0.001	<0.001	<0.001

[a] Expressed in numbers of epithelial cells.

that the total mucosal thickness is somewhat less, and that the crypts, in particular, are shallower in the germ-free than in the conventional animal. This impression is borne out by actual cell counts, summarized in Table 1, where the presence of the conventional flora is seen to be associated with an increase in all mucosal dimensions, but with a relatively greater increase in the length of crypts. This apparent shift in favor of the generative rather than the functional compartment of the epithelium is also reflected in mitotic counts done on five germ-free and five conventional mice. The mean total number of division figures *per 100 ideally sectioned* crypts in the two groups are 66.6 ± 6.2 and 93.0 ± 6.0, respectively, the difference being statistically significant ($P < 0.02$).

These data, suggesting as they do, rather basic alterations in the mucosa brought about by the flora, led to a more detailed kinetic analysis of cellular renewal in the two groups of animals.

ANALYSIS OF CELLULAR RENEWAL

Epithelium. In animals sacrificed 1 hour after the administration of tritiated thymidine, the label is found within cells that are in or immediately beyond the premitotic phase of deoxyribonucleic acid (DNA) synthesis. Thus, in the ileal epithelium of both germ-free and conventional animals sacrificed at this interval, the label is seen exclusively within the crypts (Figs. 3 and 4). The mean number of labeled cells *per crypt section*, based on a count of 40 ideally sectioned crypts per animal, is 7.6 ± 0.6 in the germ-free group as compared to 11.0 ± 0.4 in the conventional group, the difference being significant at the 0.01 level. These data, paralleling the mitotic counts given above, indicate that the presence of a bacterial flora is associated with increased cellular proliferation in the ileal epithelium.*

With passage of time following administration of tritiated thymidine, the label is known to be distributed to the progeny of the cells originally labeled, and to remain within these cells once they no longer divide. Thus, labeled cells in the intestinal epithelium, once having reached the villi where normally no further division occurs, remain labeled during their ascent of the villi. The advance of the labeled epithelium with time is shown in Figures 5 to 10.

Eleven hours after administration of tritiated thymidine, labeled epithelial cells have increased in number and have reached the area of the crypt-villus junction in both germ-free and conventional animals (Figs. 5 and 6). At 24 hours the leading edge of the population of labeled cells has traveled approximately half the length of the villi in conventional animals, whereas in germ-free animals a slight lag has become apparent. At successive intervals of time after the injection of tritiated thymidine this difference in position of the advancing edge of the labeled epithelium in the two groups of animals becomes even greater (Figs. 7 and 8). Finally, at 48 hours labeled cells are found at or very near the tips of most villi in conventional mice (Fig. 9), whereas half or slightly less of the length of villi remains yet to be traversed in their germ-free counterparts (Fig. 10).

These observations, expressed quantitatively, are shown in Figure 11, which also

* A given labeling index is greater than the corresponding mitotic index because of the relatively greater duration of DNA synthesis as compared to the duration of visible mitosis. The ratio of the two indices has been estimated to be approximately 10.[34]

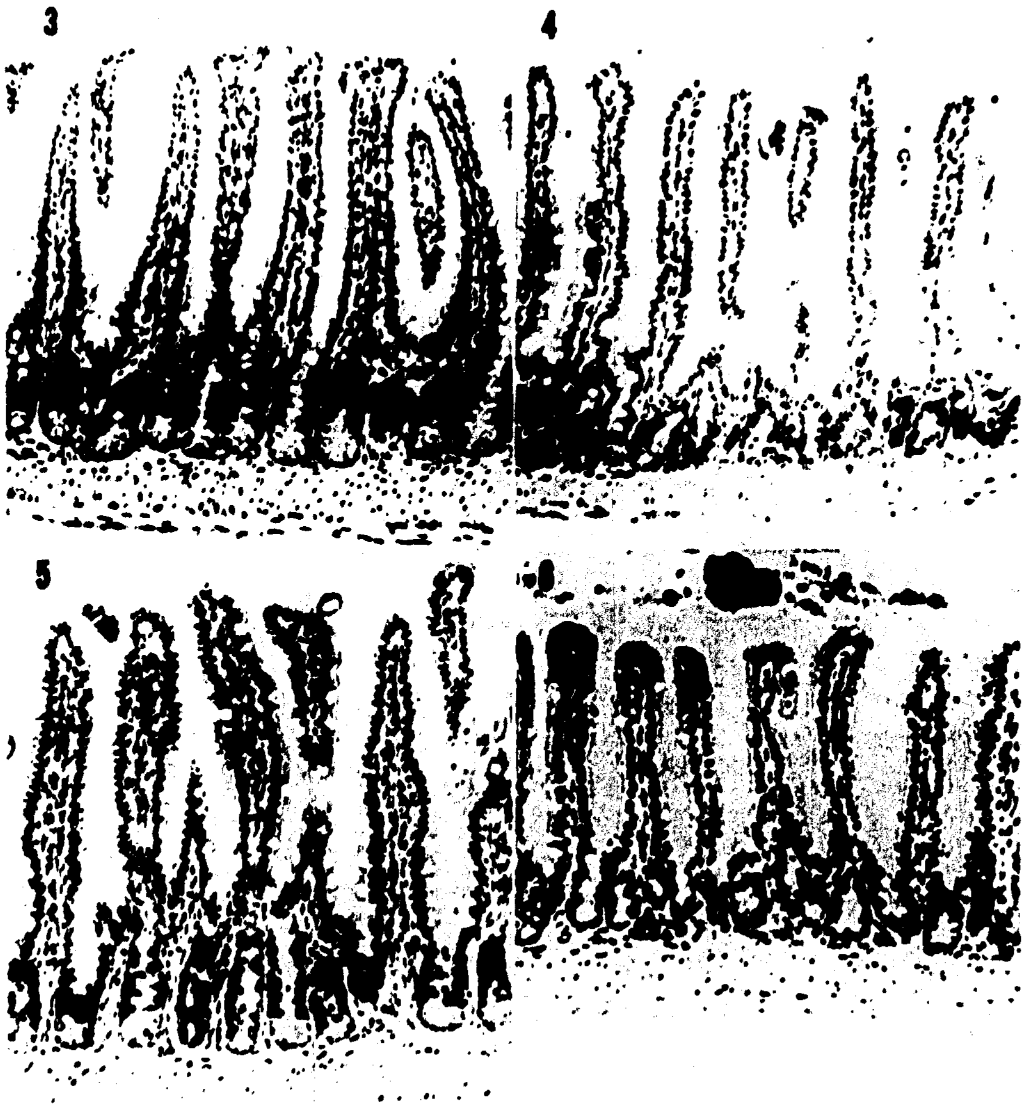

FIG. 3. Ileum of conventional mouse, 1 hour after administration of tritiated thymidine. Labeled epithelial cells are exclusively within crypts. Occasional lymphocytes and reticuloendothelial cells within the lamina propria are labeled. Autoradiograph; hematoxylin; ×105.

FIG. 4. Ileum of germ-free mouse, 1 hour after administration of tritiated thymidine. In both the epithelium and the lamina propria fewer cells are labeled than in the conventional mouse. Autoradiograph; hematoxylin; ×105.

FIG. 5. Ileum of conventional mouse, 11 hours after administration of tritiated thymidine. Labeled epithelial cells have reached the area of the crypt-villus junction. Autoradiograph; hematoxylin; ×105.

FIG. 6. Ileum of germ free mouse, 11 hours after administration of tritiated thymidine. Labeled epithelial cells have reached the area of the crypt-villus junction. The crypts, however, are shallower than in the conventional animal. Autoradiograph; hematoxylin; ×105.

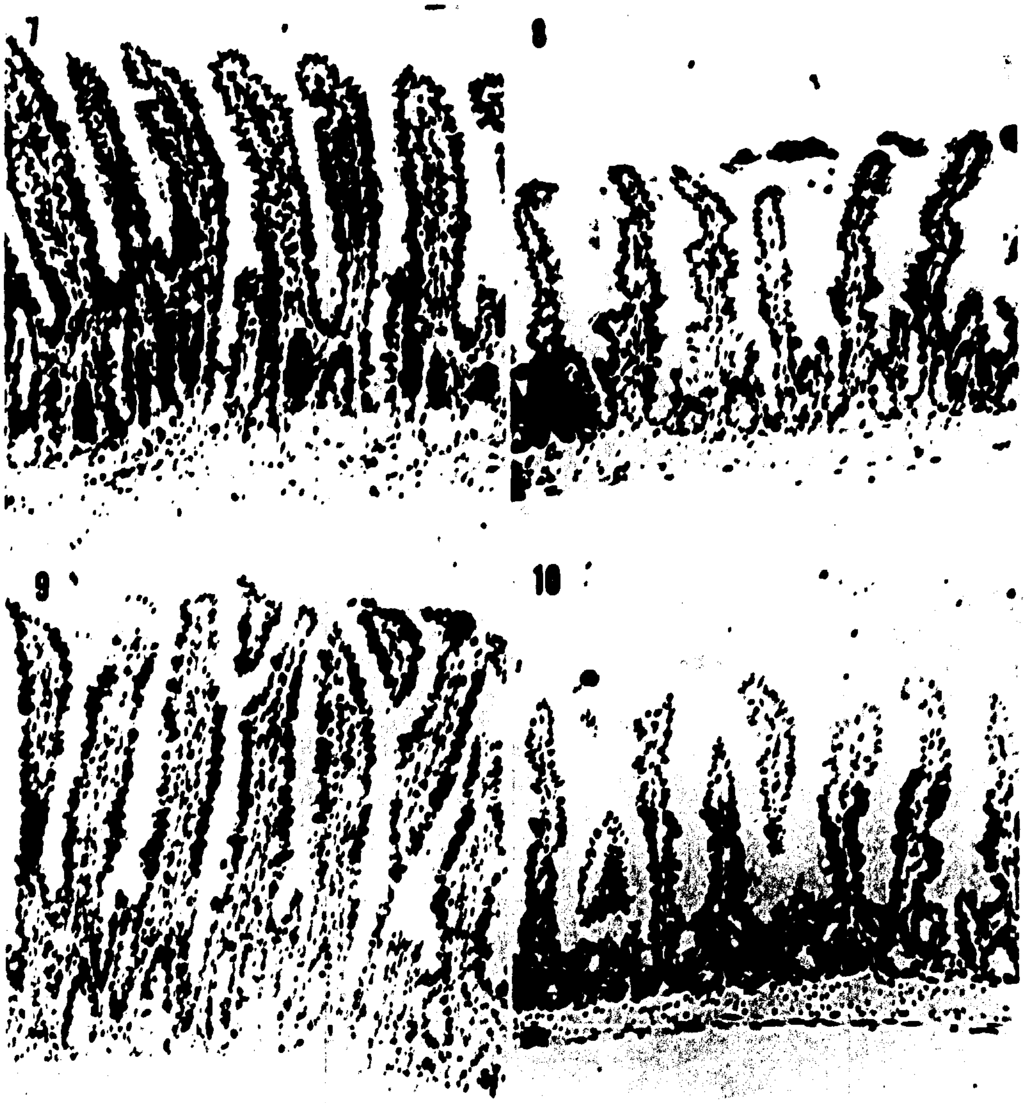

FIG. 7. Ileum of conventional mouse, 30 hours after administration of tritiated thymidine. The labeled epithelium has traversed almost three fourths of the length of villi. Autoradiograph; hematoxylin; ×105.

FIG. 8. Ileum of germ-free mouse, 30 hours after administration of tritiated thymidine. The advancing edge of the labeled epithelial population has not yet reached the level of midvillus. Autoradiograph; hematoxylin; ×105.

FIG. 9. Ileum of conventional mouse, 18 hours after administration of tritiated thymidine. The labeled epithelium is nearing the tips of villi generally, and has actually reached the tips of a few. Autoradiograph; hematoxylin; ×105.

FIG. 10. Ileum of germ-free mouse, 18 hours after administration of tritiated thymidine. Even though villi are somewhat shorter than in the conventional animal, the labeled epithelium extends only to midvillus level. Autoradiograph; hematoxylin; ×105.

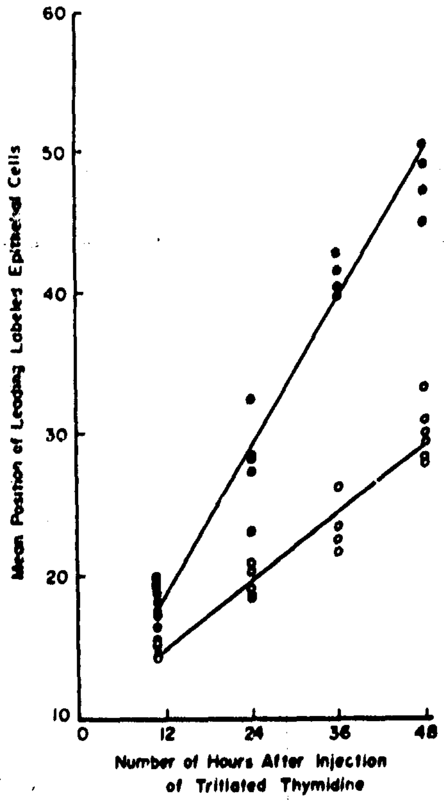

FIG. 11. Migration of the labeled epithelial population after administration of tritiated thymidine to germ-free (○ — ○) and conventional (● — ●) mice. Each point represents the value for one mouse, expressed as the mean number of epithelial cells in vertical columns extending from the bottoms of crypts to the highest labeled epithelial cells on corresponding villi. The 11-hour interval marks approximately the time the epithelium begins its ascent of the villi, after emerging from the crypts.

demonstrates graphically the extent of variation in position of the label in the several members of each time group. The rate of migration of epithelium in each of the two groups of mice appears to be constant, *i.e.*, the relationship between time and position of labeled epithelium in each case is linear. The regression lines, fitted to the data, diverge sharply (Fig. 11), having coefficients of 0.393 and 0.872. Analysis of covariance (Table 2) shows that the difference between the slopes of the lines (representing the difference between the epithelial turnover rates in the two groups) is highly significant ($F = 98.62$; *df*, 1 and 40; P, < 0.01).

Using the average mucosal dimensions recorded in Table 1 and extrapolating from the above data, it is seen that epithelial renewal is complete, *i.e.*, the labeled cells reach extrusion zones, in the ileum of conventional mice in somewhat over 2 days, whereas roughly 4 days are apparently required in germ-free mice, despite their somewhat shorter villi.

Lamina Propria. Within the lamina propria and its lymphoid tissue there is neither an anatomically defined generative compartment nor a single path of outflow of cells. Therefore, with the present experimental design, analysis of cellular renewal and migration cannot be as precise in these areas as in the epithelium. Thus, in conventional mice sacrificed 1 hour after administration of tritiated thymidine, varying numbers of labeled cells, predominantly lymphocytes and reticuloendothelial cells, are scattered irregularly in the lamina propria, and occasionally appear to be mi-

TABLE 2. ANALYSIS OF COVARIANCE*

Line	Status	N	Σx^2	Σxy	Σy^2	Regression coefficient	df	Deviations from regression	Mean square
1	Germ free	23	1827.00	1806.20	801.80	0.39283	22	30.61	
2	Conventional	20	4012.55	3240.00	2781.28	0.87198	18	108.06	
3	Within variance						40	161.67	4.12
4	Regression coefficient						1	408.03	408.03
5	Common variance	42	8740.55	5130.20	3565.78	0.58703	41	570.70	13.92
6	Adjusted means						1	1280.11	1280.11
7	Total variance	43	8705.73	4918.91	4067.00		42	1850.82	

* Height of column of labeled epithelial cells, Y, at various times, X, in germ-free and conventional mice.

grating through the epithelium at various levels of the villi (Fig. 3). At subsequent intervals of time no consistent change in the population of labeled cells can be discerned. This is likewise found to be the case with labeled lymphocytes and reticuloendothelial cells in Peyer's patches. However, comparison of the entire series of germ free and conventional animals reveals that, in all time groups, the number of labeled cells in the lamina propria, the number associated with or migrating through the epithelium, and the number in the lymphoid tissue are regularly lower in the germ free than in the conventional animals.

DISCUSSION

This study establishes that the rapid rate of cellular renewal ordinarily seen in the ileal epithelium is due, at least in part, to the presence of a living bacterial flora. In the absence of such a flora, *i.e.,* in the germ-free animal, the renewal process proceeds at a significantly slower pace. Since the basic mechanisms involved in the continuous turnover of intestinal epithelium are poorly understood, it is not possible to state precisely how the presence of bacteria results in acceleration of the process.

The continuous migration of the intestinal epithelium does not seem to depend upon a "push" from dividing cells in the crypts, as it continues even when mitotic activity is markedly depressed.[17] Rather, it appears to represent, as in other epithelia, a response to the loss of cells, with an adjustment of the mitotic rate corresponding to the need thus established. Accordingly, the present data suggest that living bacteria may act to accelerate the rate of loss of cells from the so-called extrusion zones at the tips of villi. Thus, in the ileum of the conventional mouse, maintenance of an intact epithelium requires a higher rate of cellular proliferation in the crypts than is required in the germ-free mouse. Correspondingly, in the presence of greater rates of cellular loss and replacement, the rate of epithelial migration in the conventional mouse exceeds that in its germ-free counterpart.

Comparison of the mucosal dimensions of the two groups of animals in this study, even taking into account the limitations inherent in such a two dimensional estimate, suggests that the presence of a bacterial flora results not only in acceleration of cellular turnover, but also in an increase in size of the epithelial population. These figures are essentially in accord with measurements obtained in the rat,[2] which showed the surface area of the intestinal mucosa to be greater in the conventional than in the germ-free animal. How this effect is mediated is unknown. Conceivably, the size of the epithelial population may somehow be linked to the volume of the lamina propria, which has been shown in this and other studies[3] to be strikingly responsive to the presence of a living flora.

Whatever the precise determinants of the size and renewal rate of the epithelial population, the presence of a living microbial flora clearly results in a marked reduction in the life span of ileal epithelial cells. Thus, in the conventional mouse, the epithelial cells reaching the extrusion zones are somewhat over 2 days of age, whereas in the germ-free mouse, even with its shorter villi, the corresponding cells probably reach approximately 4 days of age before they are lost into the lumen.

The microbial flora exerts a profound influence upon population dynamics, not only in the epithelium but in the lamina propria as well. This influence is evident from the differences between germ-free and conventional animals with respect to the total number of cells, the apparent rate of transepithelial loss of cells, and the number of labeled cells within the lamina propria and its lymphoid tissue.

The physiologic consequences of these differences in mucosal cell dynamics have not as yet been explored. It has been emphasized that, during its migration, the epithelial cell is undergoing constant change with respect to enzyme systems, chemical constituents, ultrastructural organization, and probably functional capacity.[18] Thus, with regard to absorptive function, for instance, the flora may exert an influence beyond simply affecting the total surface area of the mucosa, changing as it does the actual life cycle of the epithelial cell. Similarly, through its profound influence on the lamina propria, a highly reactive lymphoreticular tissue, the microbial flora may shape the defensive functions of the mucosa. This latter possibility is suggested by the discovery that the germ-free guinea pig, which, like the germ-free mouse, has a poorly developed lamina propria, succumbs rapidly to an oral challenge with *Shigella flexneri* 2a, an organism that produces no significant disease in the unaltered, conventional guinea pig.[19]

The ileum was selected for the present study by virtue of the morphologic evidence of its great responsiveness to the bacterial flora and because of the consideration that the maximal bacterial concentration in the normal small intestine occurs in this area. If the effects of the flora upon the mucosa are mediated locally, it is possible that at other levels, in the presence of different local bacterial concentrations, the influence of the flora could be of a different magnitude. Alternatively, the effects of the bacterial flora could be mediated systemically and could be related, for instance, to the much higher bacterial concentration present in the cecum. These problems are currently being investigated in our laboratory.

The present study serves to emphasize the dynamic character of the intestinal mucosa. The histologic appearance of both the epithelium and the lamina propria must be viewed as but a static representation of complex, moving events; as a reflection of the existing state of equilibrium among the rate of generation, the life span, and the rate of loss of cells from the renewal system. The normal bacterial flora is seen to be one factor that plays a definitive role in the establishment of the "normal" equilibrium of this complex system.

SUMMARY

By comparison of germ-free and conventional mice, many of the morphologic characteristics of the ileal mucosa ordinarily recognized as "normal" were demonstrated actually to develop in response to the presence of the bacterial flora of the natural environment.

Autoradiographic study of the ileum at various times after administration of tritiated thymidine to the two groups of mice revealed that cellular renewal rates in the mucosa are likewise sharply influenced by the normal microbial flora. The rate of turnover of the ileal epithelium in the germ-free state was found to be significantly lower than in the presence of the conventional flora. Although not measured as precisely, turnover of cells in the lamina propria and Peyer's patches appeared to be affected similarly by the living flora.

The possible implications of these effects of the flora were discussed and the concept of the intestinal mucosa as a dynamic system was emphasized.

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