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Intestinal Flora of Animal Models of Human Diseases as an Environmental Factor

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Abstract
Genetically-engineered animals are known to be useful in clarifying the functions of many genes and as animal models for human diseases. However, it has been widely reported that pathophysiology is not expressed in these animals when they become germfree or SPF animals, i.e., the pathophysiology is not the result of genes alone and a combination of gene function and intestinal flora as an environmental factor are necessary. It is important to determine the roles of each of these two factors by pathophysiological analysis. Gnotobiotic mice were produced by establishment of specified bacterial species in germfree animals to form the intestinal flora of SPF animals and they were placed in barrier facilities. Measures have been taken against infections by bacteria such as *Pseudomonas aeruginosa* and *Enterobacter cloacae*. In addition, gnotobiotic mice with a highly normal physiology are required. Analysis of the effects of each bacterial species and combinations of bacteria on in vivo functions, i.e., the cross-talk between the host and intestinal flora, is essential in the creation of better laboratory animals. Monitoring of the intestinal flora, a key factor in the colonies produced, is a topic for future research.

Introduction

In laboratory animals, the phenotype is formed by the effect of the developmental environment on the genotype. Then the effects of the rearing environment or proximate environment are added and finally the dramatype is determined as the result of an experiment (Russel and Burch, 1959) (Figure 1).

Environmental control is performed together with genetic control not only in animal models for human disease but also as one aspect of the modernization of laboratory animals. Environmental factors include the living environment and microbiota. The former is rather well controlled in terms of both equipment and regulations. In microbiological control, it is necessary to include not only pathogenic microorganisms but also bacterial groups required for the composition of the indigenous flora (Figure 2). Table 1 shows the results of tests on fecal flora of mice with developmental abnormalities or postmortem when germfree (GF) animals were placed directly in barrier facilities. It was clear that the composition of the intestinal flora was abnormal. Enterobacteriaceae and *Pseudomonas aeruginosa* were detected at high counts. *Lactobacillus* was not detected but *Clostridium perfringens* was. The reason for this is that GF mice in barrier facilities are easily colonized by different microorganisms other than mouse indigenous intestinal bacteria (Table 1). Therefore, gnotobiotic (GB) mice with specific types of bacterial species established are used in the production of SPF animals (Itoh et al.,1986; Orcutt et al., 1987).

It has become easy to produce genetically-engineered animals such as transgenic and knockout mice, and the functions of many genes have been clarified. However, many reports have indicated that pathophysiology of these animals as human disease models can no longer be observed when the animals are cleaned by establishing them as germ-free or SPF animals (Matsumoto et al., 1979; Kullberg et al., 1998; Narushima et al., 1998; Kado

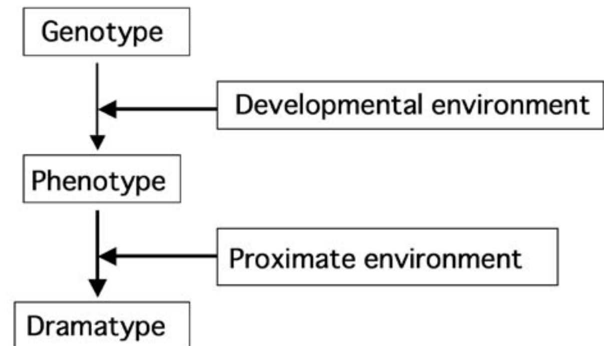

Figure 1. Control of Dramatype as the character of experimental animals. Genotype is modified by the developmental environment and forms the Phenotype. Dramatype is characterized as Phenotype-added proximate environmental factors, e.g. food, housing condition and microbiota.

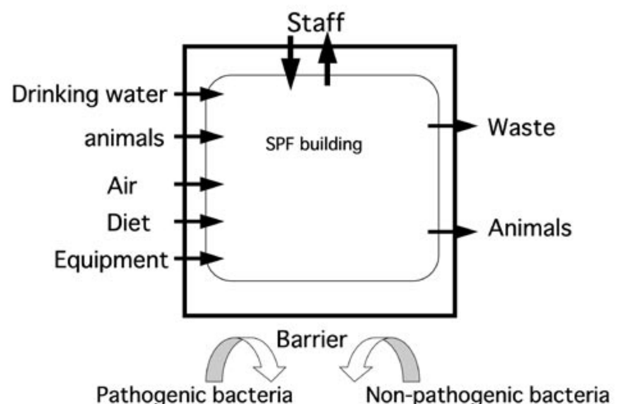

Figure 2. Barrier system.

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Table 1. Abnormal fecal flora of mice in barrier-sustained colony				
	Mice (7-week-old) [a]			
Bacteria	1	2	3	4
Enterobacteriaceae	6.5[b]	7.1	8.0	7.3
Streptococci	7.0	6.9	6.4	6.7
Staphylococci	6.2	5.4	4.3	4.0
Bacilli	3.0			
P. aeruginosa	5.3	4.7	4.8	3.5
Lactobacilli				
Bifidobacteria				
Eubacteria		9.6		
Bacteroidaceae	10.0	10.1	9.8	9.9
Clostridia	9.6	8.0	7.3	8.0
C. perfringens	8.2	7.0	7.0	8.8
Fusiform bacteria	8.7	9.5	9.9	8.9
Total count	10.4	10.3	10.1	10.1

[a] Germfree ICR mice were transferred from germfree isolators into a new established barrier-sustained colony without any association with intestinal bacteria.

[b] Log. number of bacteria/g-feces

et al., 2001; Noguchi et al., 2002). Control of the intestinal flora must be reconsidered in terms of the expression of pathophysiology. The intestinal flora is one of the accessory factors of in vivo response, suggesting that it also has a major effect on the expression of the genes in the body itself. Genetically engineered animals provide new tools for research on intestinal flora.

Intestinal flora and the dramatype

It is already well known that intestinal flora has a major effect on the in vivo physiology, but the availability of genetically engineered animals has led to a reconsideration of the important effect that the intestinal flora has on the expression of pathophysiology.

In a study in which various gnotobiotes were produced by establishing germfree C3H/He male mice and the spontaneous occurrence of liver cancer was compared, it was reported that the carcinogenesis was inhibited or promoted depending on the species of bacteria established in the intestine. The carcinogenesis rate was 30% in germfree mice and 75% in conventionalized mice. In gnotobiotic mice administered four strains including *Escherichia coli*, *Streptococcus faecalis* (currently *Enterococcus faecalis*) and two strains of *Clostridium paraputrificum* isolated from humans, the carcinogenesis rate was 100%. Carcinogenesis was promoted by intestinal flora. However, when one strain of *Bifidobacterium longum* was added to these four strains, the carcinogenesis rate was reduced to 46%, the same rate as that in mice administered only *B. longum*. It was clear that intestinal flora is involved in the expression of pathophysiology at the gnotobiote level (Mizutani and Mitsuoka, 1979).

In the relation between the intestinal flora and the expression of pathophysiology, attention is currently focused on the disease model of inflammatory bowel disease (IBD) (Matsumoto et al., 1979). Among the IBD models produced by gene targeting, severe intestinal inflammation is observed in IL-2, IL-10 and T-cell-receptor α chain knockout mice. CD4+ T-cells are involved in IBD in these mice, but the lesions cannot be observed if the animals become germfree or SPF (Sadlack et al., 1993; Sellon et al., 1998; Dianda et al., 1997), suggesting that intestinal flora plays an important role in activation of CD4+ cells. In IL-10 knockout mice, it has been reported that enteritis caused by *Helicobacter hepaticus* does not occur when they become germfree (Kullberg et al., 1998). In the SAM P1/Yit strain of progressive aging mice, a spontaneous onset model, intestinal inflammation does not occur if the mice become germfree (Kado et al., 2001).

In transgenic mice produced to analyze the pathophysiology of familial amyloidotic polyneuropathy, a form of systemic amyloidosis (mice carrying a human mutant transthyretin gene), amyloid deposits in various organs are no longer observed when the conventional mice become SPF. However, since the concentration of mutant protein in the blood does not change, it appears that environmental factors, including intestinal flora, affect the amyloid deposits (Noguchi et al., 2002). The authors found that no amyloid deposits appeared after SAM P/I mice became SPF. It has also been reported that intestinal flora plays an essential role in the formation of adenocarcinoma in the T-cell receptor β-chain and *p53* double knockout mice, a model of colorectal carcinogenesis (Kado et al., 2001).

The rasH2 mice carrying the c-Ha-ras gene, a human oncogene, show a high susceptibility to carcinogens with genotoxicity (Yamamoto et al., 1996). When rasH2 mice were inoculated subcutaneously with 1,2-dimethylhydrazine (DMH), they develop colorectal cancer at a high frequency. However, in germfree rasH2 mice, the number of mice showing carcinogenesis did not differ from that of SPF mice, but the number of tumors and tumor scores (number of tumors x tumor size) were markedly reduced. It is interesting that in control mice without the c-Ha-*ras* gene, tumorigenesis is markedly inhibited in SPF mice, but in germfree control mice, the same level of tumorigenesis as in germfree rasH2 mice was observed. When colorectal carcinogenesis in gnotobiotic mice obtained by introducing various combinations of intestinal bacteria into germfree rasH2 mice was compared with human flora associated mice administered human feces of different origins, major differences were found depending on the composition of flora present in the intestines. However, in both gnotobiotic and human flora-associated mice, the tumor scores were lower than in the SPF mice. Colorectal carcinogenesis by inoculation with DMH shows changes in the final degree based on the combination of the c-Ha-*ras* gene and intestinal flora (Narushima et al., 1998) (Table 2).

The rasH2 mice shows marked inhibition of DMH-induced colorectal cancer when apple pectin or fermentation products of *Bifidobacterium longum* were added to the ordinary diet, but these inhibitory effects are not observed in control mice (Ohno et al., 2000).

The functions of the human c-Ha-*ras* gene in the rasH2 mice have already been confirmed, but it was suggested

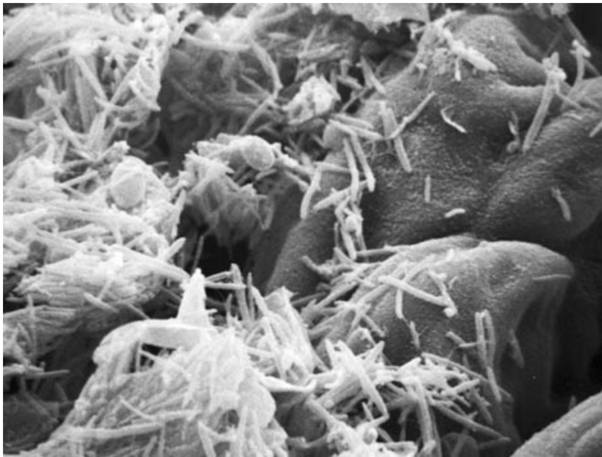

Figure 3. Fusiform-shaped bacteria on the epithelial cells of the cecum.

that environmental factors such as intestinal flora and diet ingredients, which have been overlooked in the past, have effects even on gene function.

In activation-induced cytidine deaminase (AID) deficient mice, a disease model with abnormal immune function, IgA is not produced but hypertrophic protrusion of isolated lymphoid follicles occurs through accumulation of unmutated IgM plasma cells in Peyer's patches and isolated lymph nodules in the intestine. The development of the lesions was accompanied with overgrowth of anaerobes in the small intestine. The lesions disappeared when these anaerobic bacteria were suppressed with antibiotics (Fagarson et al., 2002). This growth of anaerobic bacteria in the intestinal flora was caused mainly by segmented filamentous bacteria (SFB) (Suzuki et al., 2004). SFB have an important role in normalization of immune function in the small intestine (Umesaki et al., 1993; Umesaki et al., 1997) and the role of SFB in AID-deficient mice is an interesting topic.

Standardization of intestinal flora

Since genetically engineered animal models are strongly influenced by environmental factors, especially intestinal flora, as mentioned previously, differences in response may arise in each facility. Profiles of intestinal epithelial lymphocytes (IEL) and production of cytokines differ in different breeding facilities even in the same strain of mice, and when newborns obtained by cesarean section are fostered by parents with different intestinal flora, the IEL responses differ due to the effects of the foster parents and not the parent mice (Nagura et al., unpublished data).

Table 2. Development of colorectal tumors in transgenic CB6F1 mice carrying the c-Ha-ras gene (Tg) and control non-transgenic mice (non-Tg)

Tg	Tg	non-Tg
SPF mice [a]	+ + + [a]	–
Gnotobiotic mice	+ ~ + +	– ~ +
HFA mice	+ ~ + +	– ~ +
GF mice	+	+

[a] – ~ + + +: degree of lesions

Standardization of intestinal flora has made progress with the development of SPF laboratory animals, and the analysis has reached the gnotobiote level in mice.

Dubos et al. (Lee et al., 1968; Gordon and Dubos, 1970) found that extremely oxygen-sensitive anaerobic bacteria are predominant in the intestinal flora of mice and referred to these bacterial as tapered rods (called fusiform-shaped bacteria) (Figure 3). These bacteria appeared to be *Fusobacterium*, *Eubacterium* and *Clostridium*. The bacteria disappeared from the mucous layer of the cecum when antibiotics were administered and this induced enlargement of the cecum, indicating that the bacteria have an important effect on the physiology of mice (Savage and Dubos,1968; Savage and McAllisten, 1971).

Standardization of intestinal flora started with measures to prevent infection. This was based mainly on the colonization resistance factor (CRF) proposed by van der Waaij and it became clear that clostridia play an important role among the bacteria constituting the intestinal flora (Van der Waaij et al., 1971). Four out of five of the strains isolated from CRF mice were clostridia and three of these were fusiform-shaped bacteria (Wensinck and Ruseler-van Embden, 1971). In ex-GF mice administered only spores obtained by alcohol and heat treatment of mouse feces by Hazenberg and Custer-van Lieshout (1976), the cecum size became the same as that of conventional mice, indicating the importance of clostridia indigenous to the mouse intestine in the production of physiologically normal gnotobiote mice.

Koopman et al. studied many mouse physiological markers such as cecum size, intestinal transit rate, IgA production, production of volatile fatty acids, bile acid composition and β-aspartylglycine concentration, and composition of intestinal flora (Koopman et al., 1978, 1979, 1982; Welling et al., 1980). It was evident that cecum size is linked to many other markers and the CRF is important in normalization of germfree mice.

As shown in Figure 4, the authors tried to standardize mouse intestinal flora by investigating which bacterial species and groups were involved in normalization of

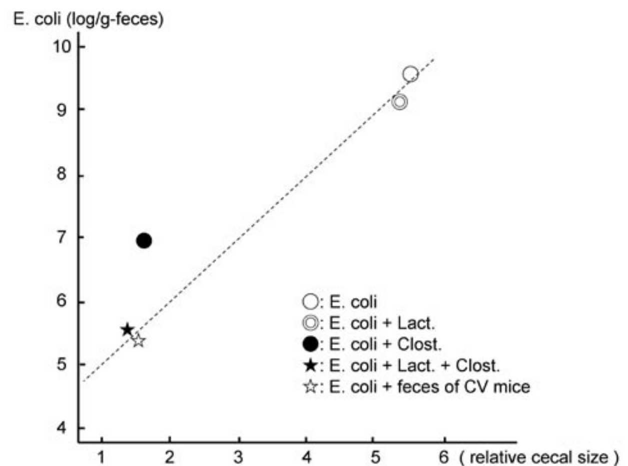

Figure 4. The number of *E. coli* in the feces and relative cecal size of ex-GF mice.

germfree mice using relative cecal weight as index of normal physiology and *E. coli* counts in feces as index of normalization of intestinal flora. The results showed that *Clostridium* spp. [ordinary mouse feces treated with chloroform (only spores survived)] alone were sufficient for normalization of cecum size, but a combination of *Clostridium* spp. and *Lactobacillus* spp. was required for normalization of *E. coli* counts in the feces (Itoh et al., 1986). When 115 strains were isolated from chloroform-treated mouse feces (CHF), almost all of the strains were found to be fusiform-shaped bacteria, which were classified into 36 groups by biological and biochemical markers. Forty-six of these strains were administered, one strain at a time, to mice monoassociated with *E. coli*. Three groups of polyassociated mice were produced by housing them together and 3 or 4 polyassociated mice of each group were housed together in an isolator. Mice with the 46 administered strains were finally prepared. In these ex-GF mice, the cecum size became small and similar to the cecum size of GF mice administered CHF of conventional mice (Itoh and Mitsuoka, 1985a). *Lactobacillus* strains including *L. acidophilus* (currently *L. johnsonii*), *L. murinus* Ia, and *L. fermutrin* IIIb and IVb (currently *L. reuteri*) are widely distributed in the intestines of mice of all strains and rearing environments (Itoh et al., 1983b). When these main bacterial species and types were combined with *Clostridium*, *E. coli* numbers in the feces were suppressed to the 10^4/g level (37). However, a mixture of *Clostridium* + *Lactobacillus* could not completely eliminate *P. aeruginosa* infected orally and it was necessary to add 17 strains of Bacteroidaceae isolated from feces of conventional mice and classified mainly as strict anaerobes to the *Clostridium* + *Lactobacillus* mixture (Itoh et al., 1986). In a comparison of fecal flora between conventional mice and SPF mice performed by the authors (Table 3), the counts and detection rates of aerobes including *P. aeruginosa* in SPF mice were higher and the counts of Bacteroidaceae, strict anaerobes, were lower than those in conventional mice (Itoh et al., 1983a). It was suggested that the high rate of contamination by *P. aeruginosa* in SPF environments is caused by poor colonization of Bacteroidaceae, especially strict anaerobes, which are maintained in the mouse intestine in a normal condition. From molecular and culture analysis of mouse intestinal flora, it is clear that many strains are difficult to culture (Itoh and Mitsuoka, 1985b; Salzman et al., 2002). Future analysis of the physiological activity of lactobacilli, clostridia and Bacteroidaceae should also include these hitherto unculturable strains, as for example, segmented filamentous bacteria (SFB) (Klaasen et al., 1991).

Umesaki et al. (1999) compared normalization of the immune function of the digestive tract using IgA production and IEL function as indices, and found that *Clostridium* plays the leading role in normalization in the large bowel, while SFB are essential in the small bowel. They reported that development of the intestinal mucosal immune system by *Clostridium* + SFB was induced in conventionalized animals. SFB become established in the lower part of the small intestines of mice (Hampton and Rosario, 1965) and rats (Savage, 1968) by adhering to the epithelial cells and host specificity is very strong (Tannock et al., 1984).

It is necessary to accumulate data on bacterial species and combinations of them involved in normalization of mice, but the basic flora, i.e., the minimum required bacteria for normalization of GF mice, is considered sufficient. However, Hashizume et al. (2003) found that SPF rats with this basic flora showed abnormal increases in lactate concentrations in the cecum when fructo-oligosaccharide (100 g/kg) was added to their diet. When these rats were administered *Megasphaera elsdenii* that can convert lactate into butyrate, the butyrate concentration increased and lactate decreased, i.e., accumulation of lactate not seen in normal rats occurs due to a deficiency in the composition of the intestinal flora. For laboratory animals used in experiments with many different objectives, it is essential to accumulate much more data and it is necessary to further improve the basic flora.

Basic flora can also be produced in rats (Yanabe et al., 2001a) by the same method as used in mice. Basic flora at the limited-flora level has also been studied for rabbits (Yanabe et al., 1999a, 1999b) and guinea-pigs (Yanabe et al., 2001b).

Necessity of monitoring intestinal flora in laboratory animals

When mice used as laboratory animals are established as SPF animals, artificially prepared flora are almost always used as breeding stock for the intestinal flora. Therefore, it is necessary to confirm if the bacteria have become established in the mice to the degree required. The intestinal flora of mice has a very complex structure in the same way as that of humans and other animal species and it consists of more than 90% strict anaerobes including fusiform-shaped bacteria. When the authors compared viable bacterial numbers in various types of media using the plate-in-bottle method (Itoh and Mitsuoka, 1985b), the highest count (5.6×10^{10}/g) was obtained with the SM10 medium and about 73% of the bacteria observed directly in feces by Gram staining could be cultured. In recent years, intestinal flora of laboratory animals has been subject to molecular genetic analysis by methods such as the FISH method (Salzman et al., 2002) using 16SrDNA sequences, ribosomal DNA restriction analysis (ARDRA)(Inoue and Ushida, 2003) and cloning (Suzuki et al., 1986; Dewhirst et al., 1999; Brook et al., 2003). However, these methods have high detection limits and bacterial counts below 10^6 - 10^7/g are difficult to detect. Monitoring of SPF intestinal flora is performed to determine if the required bacteria have become firmly established. The objective of the monitoring is not to determine if certain strains are not present as in the case of monitoring of pathogenic microorganisms but to check that they are present. However, since convenience is important in all facilities, the authors consider that a monitoring system based on the combination of FISH and culturing might be the method of choice.

Table 3. Fecal flora of SPF and conventional mice.

	Colony A	Colony B	
Bacterial groups	CV colony	CV colony	SPF colony
	(*N*=42)	(*N*=36)	(*N*=42)
Enterobacteriaceae	5.2±0.6 (29)↓	4.6±1.3 (35)	6.0±0.7 ↑ (42)
Acinetobacter	5.0±1.0 (32)↑	– (0)	5.0 (1)
Pseudomonas aeruginosa	– (0)	– (0)	4.5±0.6 (18)↑
Other gram negative aerobic rods	5.0±1.1 (13)	5.3±1.6 (11)	– (0)↓
Streptococci	6.2±1.0 (42)	5.5±0.9↓ (36)	6.0±0.6 (42
Staphylococci	4.1±0.7↓ (26)↓	4.7±0.7 (32)	5.4±0.8 ↑ (40)
Corynebacteria	4.1±1.0 (3)	– (0)	– (0)
Bacilli	3.2±0.5 (13)	3.4±0.6 (16)	– (0)
Yeasts	– (0)	– (0)	– (0)
Lactobacilli	8.9±0.8 (42)	9.3±0.4 (36)	9.0±0.5 (42)
Bifidobacteria	8.5±0.7 (21)	7.5±1.3↓ (25)	9.1±1.1 (30)
Eubacteria	8.6±0.9 (40)	8.0±1.5 (33)	8.8±0.7 (17)
Eubacteria on ES agar	7.9±0.9 ↑ (34)	6.6±1.3 (26)	– (0)↓
Bacteroidaceae	10.4±0.3 (42)	10.4±0.3 (36)	9.9±0.5↓ (35)
Bacteroidaceae on EG agar	9.7±0.4 (42)	9.8±0.3 (36)	9.6±0.6 (35)
Peptococcaceae	9.5±0.9 (20)	9.0±0.5 (16)	8.9±0.5 (16)↓
Clostridia	8.9±0.6 (29)	8.4±0.9 (26)	8.2±1.5 (38)
Coiled form clostridia	7.2±2.2 (6)	8.0±0 (3)	8.6±0.6 (22)↑
C. perfringens	– (0)	– (0)	– (0)
Anaerobic curved rods	9.0±0.1 (8)	8.8±0.7 (15)	– (0)↓
Spirochaetaceae	9.0±0 (1)	9.0±0 (1)	8.5±0.7 (2)
Fusiform bacteria	9.6±0.5 (42)	9.5±0.6 (36)	9.6±0.4 (42)
Total count	10.6±0.2	10.5±0.2	10.3±0.3↓

Mean± S.D. (Number of positives): Log/g feces, –: negative, ↑↓ Indicate significantly lower or higher counts (p<0.01) by Student's t-test (bacterial counts) and a chi-square test (frequency of occurrence) than the other groups.

Future prospects

The appearance of genetically modified animals has resulted in the clarification of the function of individual genes, but the intestinal flora has very important effects as an environmental factor in the expression of pathophysiology due to gene manipulation. When these animals are used as animal models of human disease, caution is required and stable environmental factors are important in animal experiments to confirm the functions of genes accurately. Genes and intestinal flora should be set in high precision laboratory animals and at present, it is recommended to maintain a stable intestinal flora based on basic flora. The ultimate animal models can be considered as animals with clearly-defined genes and gnotobiotes with clearly-defined intestinal flora.

With the appearance of genetically modified animals, it has become possible to analyze what role intestinal bacteria play in the expression of pathophysiology by means of new experimental techniques and the production of gnotobiotes for research on intestinal flora.

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