Normalization of Reproductive Function in Germfree Mice Following Bacterial Contamination

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Abstract: The capacity for reproduction in germfree mice remain inferior to their conventional counterparts even after improvement of feed and other such rearing conditions. The authors provide evidence of increased reproductive capacity in germfree mice following association with bacteria. Estrous cycles were normalized in female mice accidentally contaminated with bacteria, and in mice given fecal suspensions of the accidentally contaminated mice per os. Significant rises were seen in their copulation and implantation rates, reaching levels comparable to values in conventional mice. In male mice, bacterial contamination induced significant increase in sperm motility. Bacteria were identified in the feces of the contaminated mice, and reproductive capacity was examined in mice associated with the identified bacteria. As a result, normalization of the estrous cycle, and rises in copulation and implantation rates were noted in *B. distasonis* and *C. perfringens* di-associated mice. Values from *B. subtilis* mono-associated mice were comparable to those in germfree mice. These results from our accidental contamination indicate that *B. distasonis* and *C. perfringens* are capable of normalizing estrous cycles in female germfree mice, and in increasing their reproductive capacity by raising their rates of copulation and implantation.

Key words: *Bacteroides*, *Clostridium*, Germfree mice, Reproduction

Introduction

Germfree (GF) animals, reared under sterile conditions, are devoid of any bacteria in the intestines or any other part of their bodies. Reyniers was the first to succeed in long-term maintenance of germfree albino

rats [17], and following successful production of the Swiss strain of germfree mice by Pleasants in 1958 [15], many strains of germfree mice have been produced. Today, germfree animals are being used in many fields, such as in studies on physio-morphological changes arising from the absence of intestinal flora [5],

(Received 4 November 1997 / Accepted 24 February 1998)

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the significance of intestinal flora [7], nutritional studies [21], and immunological studies taking advantage of germfree animals as organisms unsensitized by indigenous or exogenous bacteria [8].

Regarding reproduction in these GF rodents, Newton [13] and Gustaffson [6] have reported that GF Swiss albino mice and Long Evans rats breed comparably to conventional (CV) animals, respectively. On the other hand, difficulty has been reported in establishing new breeding colonies of GF CF#1 mice [22]. It was therefore a question of whether the difference between GF and CV mice in reproductive ability was dependent on the animal strain or other factors. Moreover, the rearing environment of GF mice being different from that of CV mice [6, 13, 22], it remains unclear whether the inferior reproductive capacity of GF mice is due to the deficiency of microflora, the strain of mice, or the rearing environment. Shinoda *et al.* [18] have reported that the litter size of GF ICR mice remained smaller than that of CV ICR mice, even after improvement of the rearing environment of GF animals, such as diet composition [27] and rearing conditions [16]. In breeding GF mice, we found that their reproductive capacity was greatly enhanced following bacterial contamination.

This paper reports on the effect of bacteria on reproductive function in GF ICR mice, through evaluation of GF mice contaminated with bacteria.

Materials and Methods

Mice

Conventional (CV) and germfree (GF) Jcl:ICR mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Male and female GF mice, bred and maintained in plastic isolator, were used in the experiment at 10–16 weeks. One hundred mg of feces from AC mice was suspended in 1 ml of anaerobic dilution medium, and 0.5 ml of this suspension was administered via sonde into the stomach of GF mice to systematically (intentionally) produce contaminated (SC) mice. The SC mice were examined for reproductive capacity 7 days after ingestion of the fecal suspension. *Bacillus subtilis* was incubated in Heart Infusion broth (Eiken Chemical Co., Ltd. Tokyo, Japan), and 0.2 ml of these bacteria incubated in HI broth were orally administered to GF mice. *Bacteroides distasonis* and *Clostridium perfringens* were incubated in GAM broth (DIFCO Laboratories, Detroit,

USA) for 24 hr under anaerobic condition, and 0.2 ml of each incubate was orally administered to GF mice. These mice were examined for reproductive capacity 7 days after the administration of bacteria. AC, SC, and the bacteria-associated mice were housed and maintained in plastic isolators under the same conditions as GF mice. Furthermore, AC, SC, CV and the bacteria-associated mice were provided with feed, water and bedding autoclaved at 123°C for 30 min.

Bacterial examination

Following preparation of a 10-fold dilution of feces from SC mice by suspending 100 mg of feces in 1 ml of anaerobic dilution medium, 0.1 ml was applied to Blood agar (Eiken Chemical Co., Ltd. Tokyo, Japan) and GAM agar (DIFCO Laboratories, Detroit, USA) and incubated for 24 hr at 37°C. The GAM agar was incubated under anaerobic conditions. Following incubation, the bacteria were enumerated, and identified with API kits (BIO MERIEUX S.A., Marcy-l'Etoile, France).

Examination of reproductive function in female mice Estrous cycle:

Estrous cycles were examined by using vaginal smears obtained daily in the morning over ten days. Swabs immersed in physiological saline were used for obtaining intravaginal epithelial cells from the mice. The smears prepared on glass slides were then fixed in methanol for 40–60 min. The vaginal smears were stained with Giemsa solution (E. Merck, Darmstadt, Germany) and classified as: proestrus (P), estrus (E), metestrus (M) or diestrus (D). Proestrus was determined by the presence of nucleated epithelial cells only or including a small number of cornified cells, estrus by the presence of both cornified cells and nucleated epithelial cells, and metestrus by the presence of masses of cornified cells followed by the appearance of leukocytes. Diestrus was identified by a reduction in quantity of vaginal smears, and presence of leukocytes, nucleated epithelial cells, cornified cells and mucus [2].

Copulation rate:

In the evening, female mice were mated with male mice at a ratio of two to two in one cage and were inspected for the presence of vaginal plugs the next morning. The ratio of mice with vaginal plugs over the number of mated mice was considered the copulation

ratio. Female mice were mated with male mice for five days.

Implantation rate:

Female mice were sacrificed on day 11 of gestation, and the number of implantation sites and corpora lutea were enumerated. The uterus was resected along the vertical axis, and drops of 10% ammonium sulfide were applied following removal of the fetuses. Points along the uterine wall appearing black were counted as the sites of implantation [9]. The number of corpora lutea was enumerated by resecting the ovary, and counting the number of matured corpus lutea of pregnancy under a microscope. Rates of implantation were calculated as follows: (Number of implantation sites on day 11 of gestation/Number of corpora lutea) $\times$ 100%.

Gestation:

The day when female mice were found with vaginal plugs was considered day 0. Gestation period was counted from day 0 to the day of delivery.

Examination of reproductive function in male mice:

The reproductive organs such as testis, seminal vesicle and caput epididymis were weighed. The cauda

of the epididymis which include mature sperm were removed, and all sperm within the two cauda was suspended in 4 ml of warmed phosphate buffered saline (PBS). After a further 10-fold dilution in PBS, the total number of sperm and the number of immobile sperm were counted on a counting grid under a microscope at 37°C. The number of abnormal sperm was counted after staining with Giemsa solution.

Statistical analysis:

Data were analyzed statistically by Student's *t*-test.

Results

Examination of reproductive function in AC, GF and CV mice

Figure 1 shows the estrous cycles of accidentally contaminated (AC), germfree (GF) and conventional (CV) mice recorded over ten days. The estrous cycles follow a mostly normal pattern in CV mice. In GF mice, the estrous cycles of three mice were irregular due to prolonged periods of metestrus or diestrus. The estrous cycles of AC mice became more regular than

Fig. 1. The estrous cycles of accidentally contaminated, germfree and conventional mice P, E, M and D indicate proestrus, estrus, metestrus and diestrus, respectively.

those of GF mice following contamination with bacteria, approaching a pattern similar to that of CV mice. The frequency of estrus in AC female mice was 2.2 ± 0.5 over a period of ten days, which was almost the same level as that of CV mice (2.3 ± 0.5). In comparison, estrus in GF mice was observed 1.4 ± 0.6 times over ten days, which was significantly low compared to either AC or CV mice ($p < 0.05$).

With respect to the reproductive function of male AC, GF and CV mice, Table 1 shows the weights of the reproductive organs, and the number, motility and abnormality rates of sperm. Two experiments each were performed with AC and GF mice. No significant difference in the weights of testes, seminal vesicles or caput epididymides was observed between the three groups. There was also no significant difference in the

number and the abnormality rate of sperm between the three groups, but the motility rates of sperm in AC and CV mice were 90.0% and 89.3%, respectively, but that of GF mice was significantly lower at 76.3% ($p < 0.05$).

In Table 2, rates of copulation and implantation, gestation period and litter size are given for female AC, GF and CV mice. AC and GF mice were studied in two experiments each. The copulation rate in AC female mice was 100%, which was the same remarkably high level seen in CV mice, while that of GF mice was 76.4%. Concerning the rate of implantation, AC mice had a much higher level than GF mice, 82.7% and 66.2%, respectively ($p < 0.05$). The implantation rate of CV mice being higher (92.0%), a higher level of significance was seen in the difference between CV and GF mice ($p < 0.01$). Periods of gestation in the three

Table 1. Reproduction data of accidentally contaminated, germfree and conventional male mice

Exp.			B.W. (g)	Weight (mg) / B.W. (g)			Sperm		
				Testis	Seminal vesicle	Caput epididymis	Number ($\times 10^7$)	Abnormality ratio (%)	Motility ratio (%)
AC mice	1	(n=4)	38.1 ± 0.8^a	5.7 ± 0.1	7.6 ± 2.4	1.6 ± 0.1	2.1 ± 0.5	6.0 ± 1.1	92.1 ± 4.3
	2	(n=4)	33.9 ± 2.1	5.6 ± 0.3	4.7 ± 0.8	1.5 ± 0.2	1.0 ± 0.4	8.3 ± 4.0	87.9 ± 5.0
	Average	(n=8)	$36.0 \pm 2.7^{b,c}$	5.7 ± 0.2	6.1 ± 2.3	1.5 ± 0.2	1.5 ± 0.8	7.1 ± 2.9	90.0 ± 5.4^d
GF mice	1	(n=5)	41.2 ± 1.2	5.0 ± 0.7	6.8 ± 1.1	1.3 ± 0.2	1.4 ± 1.0	9.7 ± 2.8	70.8 ± 11.6
	2	(n=6)	39.8 ± 2.6	6.0 ± 0.8	5.7 ± 2.3	1.3 ± 0.3	1.0 ± 0.7	6.5 ± 4.1	80.0 ± 11.0
	Average	(n=11)	40.4 ± 2.1	5.6 ± 0.9	6.2 ± 1.8	1.3 ± 0.3	1.2 ± 0.9	8.1 ± 3.8	76.3 ± 12.8
CV mice		(n=11)	42.1 ± 1.8	6.4 ± 0.9	6.1 ± 1.5	1.3 ± 0.1	1.6 ± 0.4	8.7 ± 2.5	89.3 ± 3.1^d

a) Mean $\pm$ S.D. b) Significant difference in body weight was seen between AC and GF mice ($p < 0.05$). c) Significant difference was seen in body weight between AC and CV mice ($p < 0.01$). d) Significant differences were observed in the motility ratio between AC and GF mice, and also between CV and GF mice ($p < 0.05$).

Table 2. Reproduction data of accidentally contaminated, germfree and conventional female mice

Experiment			Copulation ratio (%)	Implantation ratio (%)	Gestation period (days)	Litter size
AC mice	1	(n=4)	100	82.8 ± 6.1^a	19.7 ± 0.5	10.7 ± 3.3
	2	(n=4)	100	82.6 ± 6.0	20.8 ± 0.4	9.5 ± 0.7
	Average	(n=8)	100	82.7 ± 6.6^b	20.1 ± 0.7	10.3 ± 2.7^c
GF mice	1	(n=5)	72.7	57.7 ± 14.3	19.8 ± 1.0	8.0 ± 2.3
	2	(n=6)	80.0	75.0 ± 2.9	19.6 ± 0.5	6.8 ± 1.5
	Average	(n=11)	76.4	66.2 ± 14.9	19.7 ± 0.8	7.4 ± 2.0
CV mice		(n=5)	100	92.0 ± 6.3^c	19.8 ± 0.5	13.5 ± 1.3^c

a) Mean $\pm$ S.D. b) Significant difference was seen in the implantation ratio between AC and GF mice ($p < 0.05$). c) Significant differences were observed in the implantation ratio between CV and GF mice, and also in litter size between AC or CV and GF mice ($p < 0.01$).

group mice were about 20 days, and there was no significant difference between the three groups. Litter sizes of AC and CV mice were significantly larger than those of GF mice ($p < 0.01$), so that significantly higher rates of copulation and implantation, and significantly larger litter size were noted in AC mice than in their GF counterparts. Furthermore, the values obtained for AC mice were comparable to levels observed in CV mice.

Examination of reproduction in GF and SC female mice

To confirm that the increase in reproductive function in GF mice was induced by bacterial contamination, reproductive ability was examined in SC mice which were administered fecal suspensions from the AC mice. The reproductive data from SC mice are shown in Table 3. The rate of copulation in SC mice was 88.9%, whereas that in GF mice was 77.8%. The rate of implantation in SC mice was significantly superior to GF mice, being 95.4% and 78.0%, respectively ($p < 0.01$). The gestation period in SC and GF mice was about 19 days, with no significant difference between the two groups. The litter size of SC mice was 13.0, excellent in comparison to the 8.5 of GF mice ($p < 0.05$). Thus, the administration of feces from AC mice was found to raise rates of copulation and implantation and litter size in the SC mice.

Bacterial examination of feces from SC mice

To determine the contaminating bacteria, fecal bacteria from the SC mice were identified. *Bacillus subtilis* (1×10^8 cfu), *Corynebacterium* sp. (1×10^8 cfu) and *Moraxella* sp. (1×10^8 cfu) were detected in 100 mg feces under aerobic culture. As anaerobes, *Bacteroides distasonis* (4×10^9 cfu) and *Clostridium perfringens* (5×10^9 cfu) were detected in 100 mg feces (data not shown).

Examination of reproduction in GF mice contaminated by identified bacteria

Among the species isolated from the feces of SC mice, *B. subtilis* is a bacterium which is found most frequently in contamination due to incompletely sterilized diet. On the other hand, because the predominant bacteria within the mouse intestine are anaerobic, the two anaerobes isolated from SC mice, *B. distasonis* and *C. perfringens*, were selected for further study as the most likely candidates capable of normalizing reproductive function. Hence, reproductive function was examined in female mice associated with *B. subtilis*, or *Bacteroides distasonis* and *Clostridium perfringens* to further clarify which of these bacteria were contributing to increased reproduction in mice. In Table 4 are the reproduction data for *B. subtilis*-associated (mono-associated), *B. distasonis* and *C. perfringens*-associated (di-associated), and *B. subtilis*, *B. distasonis* and *C. perfringens*-associated (tri-associated) mice. In mono-associated mice, the estrous cycles were still irregular due to prolonged diestrus (data not shown) and the mean frequency of estrus was 1.3 ± 0.5 times over a 10-day period which was not significantly different from that of GF mice. Estrous cycles of di-associated and tri-associated mice, which were associated with *B. distasonis* and *C. perfringens*, became regular in comparison to mono-associated mice (data not shown). The frequency of estrus in di-associated and tri-associated mice was higher than in GF mice, and an especially significant difference was observed in frequency of estrus between Exp. 1 on di-associated mice and GF mice, 2.0 ± 0.8 and 1.4 ± 0.5 , respectively ($p < 0.05$). Copulation rates in mono-associated mice did not increase significantly in comparison to GF mice, but di-associated and tri-associated mice (92.3 and 91.7%, respectively) exhibited rates superior to those of GF mice (71.6%). The rates of implantation in di-associ-

Table 3. Reproduction data of systematically contaminated and germfree female mice

		Copulation ratio (%)	Implantation ratio (%)	Gestation period (days)	Litter size
SC mice	(n=9)	88.9	$95.4 \pm 4.6^{a, b)}$	19.5 ± 0.7	$13.0 \pm 1.4^{c)}$
GF mice	(n=9)	77.8	78.0 ± 9.0	19.0 ± 0	8.5 ± 0.7

a) Mean $\pm$ S.D. b) Significant difference was seen in the implantation ratio between SC and GF mice ($p < 0.01$). c) Significant difference was seen in litter size between SC and GF mice ($p < 0.05$).

Table 4. Reproduction data of germfree mice associated with combinations of *B. distasonis*, *C. perfringens*, and *B. subtilis*

Bacteria administered	Exp.		Frequency of estrus	Copulation ratio (%)	Implantation ratio (%)
<i>B. distasonis</i>	1	(n=5)	2.0 ± 0.8 ^{a, b)}	100	98.1 ± 3.3
and <i>C. perfringens</i>	2	(n=5)	1.9 ± 0.7	84.6	88.7 ± 13.2
	Average	(n=10)	1.9 ± 0.7	92.3	92.3 ± 11.4 ^{c)}
<i>B. distasonis</i> , <i>C. perfringens</i> and <i>B. subtilis</i>		(n=7)	1.7 ± 0.5	91.7	93.7 ± 10.8 ^{c)}
<i>B. subtilis</i>		(n=7)	1.3 ± 0.5	77.8	77.6 ± 11.8
GF		(n=5)	1.4 ± 0.5	71.6	78.9 ± 10.8

a) Mean ± S.D. b) Significant difference was seen in frequency of estrus between the *B. distasonis* and *C. perfringens* group (Exp.1), and the GF group ($p < 0.05$). c) Significant differences were observed in the implantation ratio between the *B. distasonis* and *C. perfringens* group and GF group, and also between the *B. distasonis*, *C. perfringens* and *B. subtilis* group and GF group ($p < 0.05$).

ated and tri-associated mice were much higher than those of GF mice ($p < 0.05$), but the difference was not significant in comparison to mono-associated mice. Thus, the frequency of estrus, copulation rate and implantation rate of GF mice were increased through association with *B. distasonis* and *C. perfringens*, but not by *B. subtilis*.

Discussion

In this report, the authors provide experimental evidence of increased reproductive function following accidental bacterial contamination, demonstrating involvement of microorganisms in reproduction of GF mice.

Following accidental bacterial contamination, estrous cycles were normalized in female GF mice, and male mice demonstrated an increase in the motility of sperm to values comparable to those of CV mice. The prolonged diestrus in the estrous cycles of GF mice and subsequent decrease in the frequency of estrus we observed are in agreement with the findings of Minami *et al.* [12]. Regarding the weights of the male reproductive organs, our findings are in agreement with those of Shinoda *et al.* [19] who reported no significant difference between GF and CV ICR mice in the weights of testis, epididymis or seminal vesicle, and concluded that the presence or absence of bacteria has no influence on the weight of reproductive organs. But their report does not include any analysis of sperm. We believe the

rise in the copulation rate is attributable to both the rise in frequency of estrus in female mice and increased sperm motility in male mice following bacterial contamination. The increase in litter size is believed to be a result of the rise in the implantation rate. The marked increase in the implantation rate we observed following bacterial contamination is supported by Shinoda *et al.* [18] who report significantly higher implantation rates in CV ICR mice than in their GF counterparts.

To confirm that these phenomena are due to bacterial contamination, we investigated the effects of oral administration of fecal suspensions from AC mice on reproductive function in GF mice, focusing on the probability of the effects being due to intestinal bacteria, which are the most numerous both in terms of number and species. As a result, copulation and implantation rates were increased in the mice given fecal suspensions, confirming that the rise in the reproductive functions was induced by bacteria having colonized the intestinal tract. Furthermore, to clarify which of the contaminating bacterial species were responsible for stimulating reproduction, fecal bacteria from the SC mice were identified. Five species were isolated, and from among the five, the effects of *Bacteroides* sp. and *Clostridium* sp., with consideration to the fact that they are dominant species in the intestinal tract of mice and humans [4], and *B. subtilis*, which is often found in insufficiently sterilized feed and prone to becoming a contaminant of GF rodents [personal communication], were investigated. As a result, it was found that no

increase in reproductive function arose from association with *B. subtilis*, but that significant increases in frequency of estrus, copulation and implantation rates were seen following administration of *B. distasonis* and *C. perfringens*. From this, normalization of reproductive capacity in the mice was traced to the presence of *B. distasonis* and *C. perfringens*.

One possible reason for normalization of estrous cycles in the mice following administration of *B. distasonis* and *C. perfringens* is direct infiltration of neutrophils and subsequent shortening of diestrus caused by these bacteria in the vagina. Nevertheless, although the administration of human feces to GF BALB/c mice is capable of normalizing the estrous cycles to that approaching that seen in CV mice, their intravaginal flora are comprised of facultative anaerobes such as *Staphylococcus* spp., *Enterococcus* spp. and *Escherichia coli*, with no anaerobic bacteria being isolated [12], so that the probability of intravaginal colonization of *Bacteroides* or *Clostridium* is low.

Furthermore, Minami *et al.* [12] report a much lower progesterone value for GF mice than for CV mice on day one of diestrus, indicative of involvement of hormonal changes brought about by bacteria in their estrous cycles. Additionally, Beaver [3] reports that it is possible to normalize estrous cycles by precipitating neutrophil infiltration in the absence of bacteria through the administration of progesterone to GF mice. From these observations, it is inferred that the normalization of estrous cycles following association with *B. distasonis* and *C. perfringens* is not due to direct effects on intravaginal cells due to the presence of these bacteria in the vagina, but that these bacteria colonizing the intestinal tract are somehow indirectly involved, such as through the metabolism of sex hormones.

As to implantation, it is well known that not only progesterone, but also estrogen [28] is indispensable among the steroid hormones, and that these sex steroid hormones undergo enterohepatic circulation. This has been clarified for estriol in particular, with blood estriol being conjugated in the liver, becoming estriol-3-sulfate-16-glucuronide, which is excreted in bile, becoming deconjugated into free estriol by β -glucuronidase or sulfatase produced by bacteria in the intestinal tract. The majority of the free estriol is then absorbed through the intestinal membrane, where it is reconstituted into estriol-16-glucuronide and returned to the liver [10].

The involvement of intestinal bacteria in enterohepatic circulation is also supported by reports such as the following. Plasma and urine estriol decrease in pregnant women administered ampicillin [25], and a noticeable increase is seen in conjugated estrogen in feces [1]. The same has been clarified for progesterone [11]. Furthermore, by comparing GF with CV rats, it has been found that the absence of intestinal flora results in increased excretion of estrone sulfate, indicative of suppressed intestinal absorption [20]. The following are examples of reports on the involvement of specific intestinal bacteria in enterohepatic circulation. *E. coli* and *Bacteroides* sp. produce glucuronidase [26], whereas *Clostridium* sp., *Lactobacillus* sp., *Eubacterium cylindroides*, *Peptococcus niger*, and *Bacteroides* sp. are steroid-desulfating bacteria [23]. And *Bacteroides* and *Clostridia* have been reported to be more active in deconjugation in bile acid metabolism, but *Bacilli* exhibit almost no such activity [24].

From the above, it is inferred that *Bacteroides* and *Clostridia* may be promoting the enterohepatic circulation of estrogen and progesterone, resulting in increased blood levels of the hormones which act on the uterus, the target organ, normalizing estrous cycles and promoting implantation of fertilized ova.

On the other hand, Nomura *et al.* [14] report that although production of testosterone involved in the sperm-producing function is low in the testes of GF mice, F1 mice mono-associated with *E. coli* or *Bacteroides* exhibit testosterone production comparable to CV mice. From such findings, it is possible to surmise that change in testosterone metabolism is involved in the increased motility of sperm in male mice following bacterial contamination.

This study provides evidence that normalization of estrous cycles, and increase in copulation and implantation rates occur following bacterial contamination of GF mice. It also demonstrates that the rise in reproductive function was induced by *B. distasonis* and *C. perfringens*. We have not carried out direct investigation on which of these two species has the greater effect on reproduction, and it is left to further study to determine the exact roles of the two species. But because their anaerobic character reduces the probability of their intravaginal presence (and hence their direct influence on intravaginal epithelial cells), and because *Bacteroides* and *Clostridium* are both predominant genera colonizing the intestinal

tract of CV mice involved with metabolism in enterohepatic circulation, their involvement is of interest as a possible indication of indirect influence via enterohepatic metabolites as the mechanism by which they may be stimulating reproductive function.

Acknowledgments

We express our deep gratitude to Ms. Yukane Nakazawa for advice in preparation of the manuscript, and Mr. Mitsugu Hirano for maintenance of the germ-free mice.

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