Fliedner et al. (eds.): Clin. and Experim. Gnotobiotics, Zbl. Bakt. Suppl. 7
Gustav Fischer Verlag - Stuttgart - New York - 1979

Author's Version

Antagonistic Effect of Intestinal Bacteria from the Microflora of Holoxenic (Conventional) Piglets, Against *Clostridium Perfringens* in the Digestive Tract of Gnotoxenic Mice and Gnotoxenic Piglets

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Abstract

Antagonistic effect of piglet microflora against *Clostridium perfringens* was studied in germfree mice, to isolate bacterial strains responsible for this colonization resistance. The 1:100 dilution of the feces of a 2 day-old conventional piglet, given per os to germfree mice already harboring *C. perfringens,* led to the elimination of *C. perfringens*. From this piglet flora, 8 bacterial strains were selected, belonging to the genera *Bacteroides, Clostridium, Eubacterium, Bifidobacterium, Lactobacillus* and a strain belonging to the class of Mollicutes. When the 8 strains were given to germfree mice 3 days after *C. perfringens* inoculation, they led to rapid elimination of *C. perfringens* from feces. Sixteen other mixtures of 2 to 7 strains were similarly tested, but none was able to fully antagonize *C. perfringens*. When the 8 strains were given per os to germfree piglets after *C. perfringens* inoculation, they led to the rapid elimination of *C. perfringens* from pig feces, and to a quick recovery from diarrhea. This study led to the identification of a simplified fraction of gut microflora, able to exert a barrier effect against *C. perfringens* comparable to the entire flora of the piglet. This study suggests that gnotoxenic mice can be a suitable model for simplifying the flora responsible for a given effect in another host, animal or human.

Introduction

The barrier effect exerted by gastrointestinal microflora, is one of the basic mechanisms protecting the host against enteric infections. Bacterial strains responsible for a barrier effect against *Shigella flexneri* (4) and *Escherichia coli* (9) in the mouse, have already been isolated. But little work has been done on the antagonistic effect exerted by the microflora of animals other than rodents.

The purpose of this study was to determine whether there was an antagonistic effect of the microflora of the holoxenic piglet against *Clostridium perfringens*, and to isolate bacterial strains responsible for this effect, using gnotoxenic mice. *C. perfringens* bas been recognized as the cause of enteritis in young pigs (1), and a study on the number of *C. perfringens* in suckling piglets, has shown a great variability in this number (2). So, the barrier effect against *C. perfringens* in piglet must be quite variable.

Materials and Methods

Maintenance and Inoculation of Mice

Adult balb mice were fed a commercial diet (UAR) or a solid milk replacer for piglet, sterilized by irradiation (4 Mrad). For short term studies, the mice were maintained in glass jars as previously described (4). For long term studies, the mice were maintained in plastic Trexler type isolators. The mice were inoculated by injecting 2 ml of the appropriate bacterial culture, containing 10^8 viable cells, or 2 ml of a 1:100 dilution of feces directly into the individual drinking water tubes, after each animal had been deprived of water for one day.

Obtaining, Maintenance and Inoculation of Piglets

Axenic (germfree) newborn piglets were obtained by decontamination immediately after birth as previously described (5), and maintained in plastic Trexler type isolators. Absence of bacteria in the piglet's digestive tract was controlled for 4 days after birth (5). The baby pigs were fed concentrated cow milk ("Gloria"

milk), diluted with autoclaved water, and admixed with a vitamin mixture. The piglets were inoculated per os, by giving them 10 ml of bacterial culture, or 10 ml of a 1:100 dilution of feces.

Fecal Sampling

In the short term experiments on mice, freshly passed feces were collected from individual animal at the end of the experiments, 1 week after introduction of the last inoculum. In the case of mice housed in the same cage in an isolator for long term experiments, pooled, freshly passed feces were used for the enumeration of bacteria. Each pooled sample contained one fecal pellet from every animal in the group. In the case of piglets, freshly passed feces were collected from individual animals. All quantitative bacterial counts were made immediately after sample collection.

Bacterial Strains, Culture Media, and Enumerations

The strain of *C. perfringens* used to initiate these experiments was an asporogenous mutant of a strain previously described (3) and was cultured in the medium B' containing 0,2% agar (8). A second strain of *C. perfringens* had originally been isolated from the digestive tract of a holoxenic piglet, and a third one (strain C.p. A. 78) had been obtained from the Institut Pasteur (Paris). *C. perfringens* was enumerated in GCN medium containing per liter : enzymatic casein hydrolysate (ATBC) : 35 g, agar (Touzart and Matignon) : 10 g. The pH of the medium was adjusted to 6.5, and 0.013% Neomycin (NBC) were extemporarily added.

The strains of *Bacteroides, Clostridium, Eubacterium, Bifidobacterium, Lactobacillus*, and a strain belonging to the class of Mollicutes, were originally isolated by us from the feces of a holoxenic (conventional) 48 hour-old piglet.

Enumeration of the two *Bacteroides* strains and the two *Clostridium* strains in the feces, was made in medium B', previously described (8). For enumeration of the Mollicutes strain, the same medium was used after addition of 0.0075% Bacitracin (NBC). *Eubacterium* was counted after addition of 1 % of glucose and 0.09% of Streptomycin (Specia) to medium B'. For enumeration of *Bifidobacterium* and *Lactobacillus*, medium B' was used after addition of 1 % glucose, and after five minutes contact between the dilution and 0,2 ml of a 1,5% sodium azide solution, as previously described (7). For all the strains, serial 10-fold dilutions were poured into these selective media, placed in 8 by 400 mm tubes (7), and incubated at 37 ° C for 18 hours in the case of *C. perfringens*, and for 7 days in the case of the other strains.

Results

Barrier Effect against *C. perfringens* Exerted in Mice by the Conventional Piglet Flora

C. perfringens inoculated into axenic mice, became rapidly established in the digestive tract of the animals, and stayed then at a high level (Fig. 1). The 1:100 dilution of the feces of a 48 hours-old holoxenic (conventional) piglet, inoculated into gnotoxenic mice, monoassociated with *C. perfringens* led to a drastic decrease in the number of *C. perfringens*. This number reached within 5 days the level below 100 viable cells by gram of wet feces.

Simplification of the Microflora Exerting the Barrier Effect

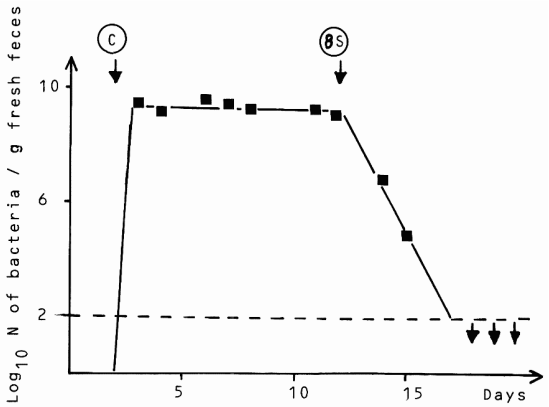
86 bacterial strains were isolated from the dominant microflora of the feces of these mice, on the different selective media.

Among these strains, 8 were selected, easily differentiated by their morphology, and their ability to grow on various selective media. These strains were belonging to the genera *Bacteroides* (strains B1 and B2), *Clostridium* (strains En and P1), *Eubacterium* (Eu), *Bifidobacterium* (Bf), *Lactobacillus* (La) and a strain belonging to the class of Mollicutes (Mo). This last strain, strictly anaerobic but not Extremely Oxygen Sensitive, showed a mycoplasm morphology and no wall in electron microscopy, typical incrusted colony on Petri dishes, but was cholesterol independent and did not pass through a Millipore membrane of 0,45 µm.

These 8 strains, together associated with axenic (germfree) mice were found to decrease drastically the number of *C. perfringens* in the feces of animals when *C. perfringens* was given per os to the gnotoxenic mice.

The barrier effect was also achieved when the 8 strains were inoculated into monoxenic mice, already monoassociated with *C. perfringens* (Fig. 1).

Fig. l: Kinetics of the establishment of *C. perfringens* in axenic mice, and of the elimination of *C. perfringens* when the 8 strains are inoculated into the monoxenic mice.



Symbols:
(Black square) *C. perfringens*,
(-----) level below which the bacterial strains are considered as absent;
(Arrow) day of inoculation of (C) *C. perfringens* and (8S) the 8 strains.

Two other strains of *C. perfringens* were eliminated in the same way by this simplified flora. When the mice were fed the milk replacer, the barrier effect exerted by the 8 strains still remained when *C. perfringens* was inoculated after the 8 strains.

Partial Barrier Effect exerted by less than 8 Strains

When only one of the 8 strains was associated with *C. perfringens* in axenic (germfree) mice no one was able alone to eliminate *C. perfringens* from the digestive tract of mice. But when the strains B2 and En were inoculated 6 days and 13 days, respectively, after *C. perfringens*, they led to a partial decrease in the number of *C. perfringens* (Fig. 2). The same result was obtained when the order of inoculation of strains B 2 and En was inversed. The mixtures of more than 2 and less than 8 strains tested in mice housed in jars, did not lead to a better elimination of *C. perfringens* (Table 1).

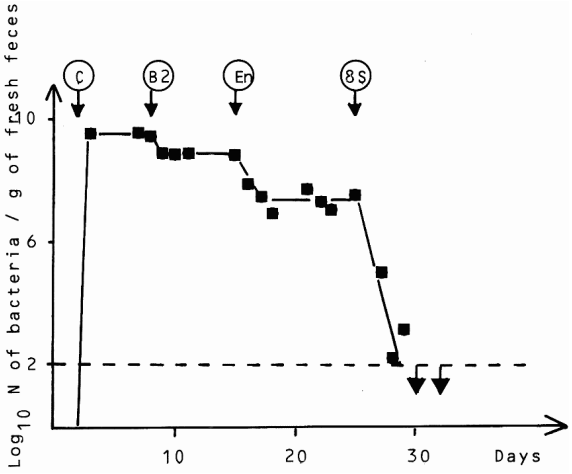
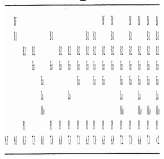


Fig. 2: Kinetics of the decrease in number of *C. perfringens* in gnotoxenic mice when *Bacteroides* (B2) and *Clostridium* (En) are successively inoculated, and elimination of *C. perfringens* when the 8 strains are added. Symbols: See Fig. 1.

Table 1: Number of *C. perfringens* in the feces of gnotoxenic mice housed in jars, inoculated with various mixtures of strains, chosen among the 8 strains. In the upper part of the table, the abbreviations designate the strains present in each mixture; figures on the last horizontal line are the $\log_{10}$ of the number of *C. perfringens.*



Barrier Effect of the Simplified Flora in Gnotoxenic Piglets

C. perfringens inoculated into an axenic piglet became rapidly established in the digestive tract of the animal, at the level of 10^8 bacteria/g fresh feces and induced diarrhea in the pig. When an axenic piglet was inoculated with a 1:100 dilution of the feces of the octoxenic mice (harboring the 8 strains), and 24 hours later with *C. perfringens*, there was a rapid decrease in the number of *C. perfringens* (Fig. 3). *Lactobacillus*, inoculated into a

piglet monoassociated since 3 days with *C. perfringens*, was unable to eliminate *C. perfringens*, or to decrease the pH of the feces. But when the 8 strains were added 3 days later, they led to a rapid decease in the number of *C. perfringens*, and to a quick recovery from diarrhea (data not shown).

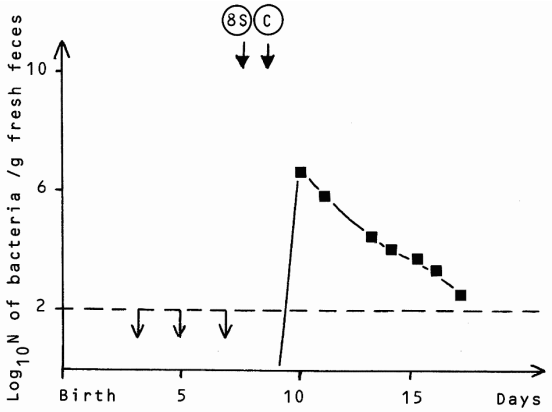


Fig. 3: Kinetics of the elimination of *C. perfringens* in a gnotoxenic piglet previously inoculated with the 8 strains. Symbols: See Fig. 1.

Discussion

The experiment described above led to the identification of a simplified, defined fraction of holoxenic (conventional) microflora, able to exert a barrier effect with regard to *C. perfringens* comparable to the entire flora of the piglet. The ecological barrier effect is likely to be exerted by the association of a few different strains, and not by a single one, as pointed out by different authors (4, 9). It is unlikely that all the 8 bacterial strains used in this experiment are necessary for this effect, and it would be interesting to find out the minimum number of strains exerting the same effect. This would allow further studies on the mechanisms involved in such a barrier effect. From another point of view, it would be easier to detect the presence of the bacteria responsible for the barrier effect in all the holoxenic piglets, and on the basis of this to try to understand why the barrier effect is not present in each animal.

Generally speaking, it would be very interesting to maintain all the potential pathogenic bacteria at a low level in the digestive tract of man and animals. But such a control requires intensive further studies to determine which are the bacteria involved in the different barrier effects, and in which conditions they eliminate the pathogens. The data presented here pro-vide the necessary tools for such studies: they show that the bacteria involved in the control process can be isolated in pure culture, and can be reassociated with axenic once to test the barrier effect. When this effect is obtained with the minimal flora, the strains can be inoculated into axenic piglets and exert the same barrier effect. Thus, gnotoxenic mice seem to be a suitable model for simplifying the flora responsible for a given effect in another host (man or animal).

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