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Received April 26, 1962. P.S.E.B.M., 1962, v110.

Effect of a *Clostridium* Species Upon Cecal Size of Gnotobiotic* Mice.† (27548)

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(Introduced by Morris Pollard)
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One of the frequent morphological anomalies of the axenic(1) rat and mouse is enlargement of the cecum(2) which may occupy 30-90% of the abdominal cavity. The wall of the cecum is thin(2) and the contents are fluid. Such enlargement simulates the appearance of pregnancy, and may lead to a fatal volvulus(3). Similar distention of the cecum has been reported for the conventional rat maintained either on a diet of raw unboiled potato starch(4), or lactose(5), or one deficient in potassium(6). Evidence that the cecal anomaly may be unrelated to diet has been reported(7). Addition of antibiotics to the diet of conventional rats also caused occasional enlargement of the cecum (8,9). This report describes the effect of a *Clostridium* species on the restoration of the distended cecum of axenic mice to normal size.

Materials and methods. Axenic and conventional ("normal") albino ND-2 mice§ (male and female, 21-365 days old) were housed in flexible film isolators which were provided with sterile supplies of San-i-cel bedding,‖ Purina chow 5010C,¶ and water. Animals and materials were introduced into the closed system through a "lock" sterilized with 2% peracetic acid spray(10). Preliminary experiments were designed to determine if any organisms in the aerobic microflora of conventional mice would restore the enlarged cecum of axenic mice to normal size. Dilutions of cecal contents of conventional mice were incubated aerobically on Desoxycholate agar, Endo agar, S. S. agar, *Staphylococcus* Medium No. 110, MacConkey agar, L.B.S. medium, and trypticase soy agar at 26, 37, and 55°C. Cultures of aerobic bacteria were then isolated by routine bacteriological technics. Selected isolates were propagated for 24 hours in thioglycollate broth to which a small chip of calcium carbonate had been added. Axenic mice which had been starved for 6 hours were fed a diet soaked in the broth culture. Both axenic and conventional control animals were maintained on similar but untreated diets. Animals were sacrificed at various time intervals by cervical fracture. Ceca were excised and weights were expressed as percentage of total body weight. Bacteriological tests were made to

* The term "gnotobiote" refers to an animal of known microbial content.

† This investigation supported in part by a grant from the Office of the Surgeon General, Dept. of the Army.

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§ Originally derived from the Rabstein colony, Fort Detrick, Md.

‖ Paxton Processing Co., Paxton, Ill.

¶ Ralston Purina Co., St. Louis, Mo.

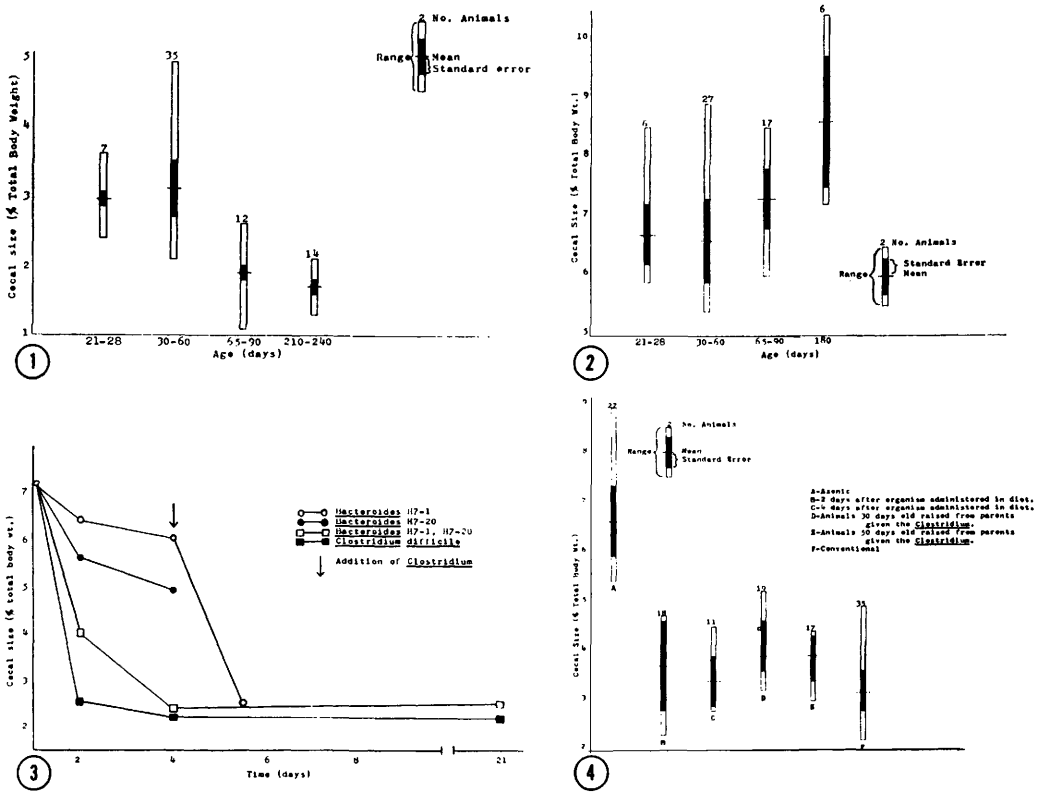

FIG. 1. Relation of age to cecal size of conventional ND-2 mice. No. of animals noted at top of each column.

FIG. 2. Relation of age to cecal size of axenic ND-2 mice. No. of animals examined noted at top of each column.

FIG. 3. Effect of anaerobic isolates upon cecal size of gnotobiotic ND-2 mice.

FIG. 4. Comparison of cecal size of axenic, conventional, and *Clostridium* gnotobiotic DN-2 mice (30-60 days of age).

ascertain the presence of the test inoculum which was added to the diets and for presence of extraneous contaminants. In other experiments, the microbial flora of conventional animals was introduced into axenic mice by first soaking the entire cecal contents of one conventional mouse in the diet of an axenic mouse colony. Thereafter, one mouse from this exposed colony was transferred to another colony of axenic mice. Subsequently, 5 more such transfers were made. Cecal size of animals from each passage was determined and contents were plated on brain heart infusion agar supplemented with 5% bovine blood and 1% cystine. Cultures were incubated for 4 days at 37°C in a sealed desiccator, rendered anaerobic with ignited yellow phosphorus.

Results. There was a significant difference in cecal size of conventional mice of the age groups 21-60 days as compared with the age groups 65-240 days (Fig. 1); furthermore, the cecum of conventional mice decreased in size with age. There was no significant difference in cecum size among any of the age groups (21-180 days) of axenic mice (Fig. 2). At one year of age, axenic mice had a mean cecal size of 18% of total body weight. In contrast, cecal size of the axenic mice of each age group was significantly larger than that of the conventional counterpart.

In preliminary experiments, the enlarged cecum of axenic mice of all age groups was not reduced after introduction of the aerobic bacterial isolates recovered from conventional mice. In later experiments, all animals passaged from the colony associated with the cecal contents of a conventional mouse had ceca

TABLE I. Microbes Isolated from First and Fourth Passages.

	Incubation temp. (°C)	Media	1st passage Colonies/g cecal contents	1st passage Cultures	4th passage Colonies/g cecal contents	4th passage Cultures
Aerobes	26	Endo agar	9.0×10^7	*Proteus* *E. coli* *Aerobacter*	1.6×10^7	*E. coli* *Aerobacter*
		T.S.A.*	1.0×10^8	*Proteus* *Micrococcus* Coliforms	1.7×10^6	Coliforms
	37	Endo agar	1.0×10^7	*Proteus* *E. coli* *Aerobacter*	1.2×10^6	*E. coli* *Aerobacter*
		T.S.A.*	1.4×10^8	*Proteus* *Micrococcus* Coliforms	1.3×10^7	Coliforms
	55	Endo agar	.0		.0	
		T.S.A.*	8.7×10^6	*Proteus* *Micrococcus*	.0	
Anaerobes	26	B.H.I.A.†	8.5×10^7	*Bacteroides* *Clostridia* *Micrococcus*	2.0×10^6	*Bacteroides* *Clostridia*
	37	"	4.7×10^7	*Bacteroides* *Clostridia* *Micrococcus*	4.2×10^7	*Bacteroides* *Clostridia*
	55	"	2.0×10^5	*Micrococcus*	.0	

* Trypticase soy agar.
† Brain heart infusion agar, 5% bovine blood and 1% cystine in anaerobic phosphorus chamber.

which were within "normal" limits of size. In addition, a change in the microbial flora was noted in each group (Table I). Ceca of first passage animals contained *E. coli, Aerobacter, Proteus, Bacteroides, Micrococci* and *Clostridia*. The flora of the fourth passage lacked *Proteus* and *Micrococci*. A *Clostridium* species and 2 strains of *Bacteroides* were isolated from the brain heart infusion plates under anaerobic conditions. The addition of both strains of *Bacteroides* to the diet of axenic mice produced a reduction in cecal size, whereas neither strain alone was effective. After 48 hours of exposure to the *Clostridium* isolate, the enlarged cecum of axenic mice attained normal dimensions (Fig. 3). This microbe was identified as *Clostridium difficile* (11). A comparison of cecal size of the axenic, conventional, and *Clostridium* gnotobiote is shown in Fig. 4. There was no significant difference between any animals in the latter 5 categories of Fig. 4. A culture of *Clostridium difficile* (A.T.C.C. 90556) produced results similar to those obtained with our isolate. Addition of *C. difficile* to the flora of mice containing a single inactive *Bacteroides* culture, reduced the cecum to normal size within 36 hours (Fig. 3).

Discussion. Enlargement of the cecum is one of the most obvious anatomical changes associated with the axenic rodent. The etiology of this anomaly has been related either to deletion of an essential labile nutrient from the diet following heat sterilization or to absence of intestinal flora. In this study, the sterilized diet caused no cecal anomaly to develop in conventional mice. Exposure of axenic mice to the cecal contents from conventional animals was consistently associated with a restoration of "normal" cecal dimensions. Using anaerobic culture technics, *Clostridium difficile* was isolated. When established in the intestinal tract of axenic mice, this *Clostridium* produced a reduction of the cecum to "normal" size. A similar reduction of cecal size has been observed following ad-

ministration of a combination of 2 strains of *Bacteroides*. The specific effect of *Clostridium difficile* on the cecum of the axenic animal has not yet been determined, other than that it will restore the cecum to normal size.

Summary. An anaerobic, gram-positive, spore-forming bacillus has been isolated from the cecal contents of conventional mice. This organism causes reduction in size of the enlarged cecum usually associated with axenic mice. It has been identified as *Clostridium difficile*. A combination of 2 strains of *Bacteroides* isolated from cecal contents of conventional mice was also effective in this respect.

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Received May 7, 1962. P.S.E.B.M.. 1962, v110.

Curtain Electrophoretic Separation of Phenyl Acetate Hydrolysing Esterases of Human Serum. Demonstration of an EDTA-Dependent Enzyme.* (27549)

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Using cellulose column electrophoresis for separation of plasma from various species, Augustinsson(1-4) found A- and C-esterases in human plasma. The A-esterase hydrolyzes phenyl acetate at a high rate and is resistant to inhibitors such as organophosphorus compounds and physostigmine; however, it does not cleave aliphatic esters. This esterase was found to migrate as a separate fraction with an electrophoretic mobility similar to that of albumin in a veronal buffer. The C-esterase of human plasma was shown to be a typical butyrylcholinesterase with low substrate specificity and sensitive to various inhibitors, physostigmine being the most potent one.

In a large number of electrophoretic preparations of human serum we found high phenyl acetate hydrolyzing activity corresponding to the A-esterase as described by Augustinsson (1-4). Using continuous flow curtain electrophoresis this A-esterase was found to migrate slightly faster than the albumin fraction; furthermore since the prealbumin region is relatively low in protein content, high specific A-esterase activity was thus obtained. Studies of the resistance to heat and EDTA of this fraction revealed that more than one distinct A-esterase was present. Based upon these observations(5) Lundblad(6) separated serum on Sephadex and by using differential heat resistance as a criterion, he was able to confirm the existence of at least 2 A-esterases in human serum.

In the present study repeated continuous flow curtain electrophoresis of the A-esterase fraction yielded 2 distinct A-esterases of high specific activities.

Material and methods. Human serum was obtained from healthy blood donors. The blood was drawn directly into sterile bottles without additions. Each bottle was kept at 35°C for 1 hour to secure maximum clot formation. The clot was then loosened aseptically from the glass wall and the specimen left overnight in a refrigerator. The next day

* This study was supported by Research Grant from Nat. Cancer Inst., Nat. Inst. Health, U.S.P.H.S.