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CLOSTRIDIUM DIFFICILE: AN OLD FRIEND IN OUR GI-TRACT

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SUMMARY

Clostridium difficile is an anaerobic, sporeforming Gram-positive rod commonly occurring in the environment as well as in the gastro-intestinal tract of most mammals.It may produce at least 3 different biologically active substances, i.e. toxin A and B and a motility enhancing substance,

In humans it is established in nearly all infants and in this age group the presence of toxin-producing strain(s) is seldom associated with clinical symptoms. Detection-problems may be a major reason for an assumption that *Cl. difficile* is more rare in intestinal microbiota from adults. Data from individuals receiving one dose of antibiotics before undergoing minor elective surgery indicate that around half of adults may harbour *Cl. difficile* in their intestinal microbiota.

In the following some evidence for an assumption that *Cl. difficile* is involved in establishment and maintenance of intestinal motility is summarized. Experiments, carried out in mono-associated rats, showed that the presence of toxins caused a dramatic, but temporary reduction in mitotic activity without causing clinical signs or symptoms.

It is hypothesized that the presence of secondary "troublemakers" may be needed for the establishment of clinical symptoms. In the future, more efforts should be allocated to questions around identifying the possible "troublemakers".

THE MICROBE

Clostridium difficile is a strictly anaerobic Gram-positive rod with elongated spores of about the same width as the rod itself. When grown on blood agar, the colonies are irregular, rough and non-haemolytic. It ferments glucose, mannose and mannitol, but not galactose, lactose, saccharose, raffinose and inulin. It is indole-negative, but some strains may produce hydrogen sulphide. Some strains produce a filterable, thermo-labile toxin which induces local oedema and convulsion in guinea pigs. "The toxin is lethal on injection into dogs, rats, guinea pigs, rabbits and pigeons, but has no effect by mouth in the rat, guinea-pig and dog". This information is taken from the leading book in medical microbiology 35 years ago (*Topley and Wilson's principles of bacteriology, virology and Immunity*, 1975) in which it was placed in a subchapter, entitled "Notes on less important strains". Basically, the infor-

Table 1: Incidence in faeces of *Cl. difficile* and its cytotoxin after one dose of a prophylactic antibiotic to patients undergoing minor elective surgery

Antibiotic	Dose	Incidence of *Cl. difficile* (%)	Toxigenic strains (%)
Mezlocillin	2 g	3	100
Cefoperazone	2 g	44	57

"No patient experienced diarrhoea" (*Privitera* et al., 1991)

mation is still true, but much more has been added since then.

Clostridium difficile was firstly isolated from the stools of healthy newborns by Hall and O'Toole as early as in 1935 (*Hall* and *O'Toole*, 1935). They referred to the organism as *Bacillus difficile* because of the difficulties that they encountered during the isolation of the organism. Shortly afterwards, the isolates were correctly renamed to *Clostridium difficile*. Interestingly, they proposed that the strain has some biological activity because filtrates produce muscular activity in some animal experiments.

Cl. difficile - either as spores or vegetative forms - is widely distributed. It has been isolated from soil, sand and intestinal contents of most mammalian species. In hospitals, spores are always found when thoroughly investigated for. Thus, attempts to totally protect an individual from being exposed to *Cl. difficile* will usually fail.

Over the years, many approaches have been used for the isolation of *Cl. difficile.* The most commonly used medium for isolation of *Cl. difficile* from stools is the cycloserine-cefoxitin-fructose-egg-yolk medium developed in 1979 (*George* et al., 1979). This medium serves as a selective and differential medium for *Cl. difficile* and is reported to detect as few as 2000 organism in a total number of more than a billion other organisms per gram wet weight of faeces. It is uncertain whether molecular microbiological methods can increase the sensitivity of detection.

In general, the carrier-rate of *Cl. difficile* in healthy adults is reported to be up to 5%.However, even a negative cultivation result does not exclude the presence of Cl. *difficile* in the large intestine. As is indicted in a study from Italy (*Privitera* et al., 1991) the carrier rate may be higher (Table 1).

In infants, the carrier rate is much higher, in some places up to nearly 100%. In fact, it has been estimated that "50% or higher of infants are colonized with toxigenic *Cl. difficile* and are asymptomatic" (*Lyerly* et al., 1988). This strongly indicates that *Cl. difficile* may have a physiological role to play.

It has been known for a long time that some strains of *Cl. difficile* might produce biologically active substances, as toxin A, toxin B, and substance(s) with motility enhancing properties. Some biological effects of these substances will be highlighted in the following paragraphs.

CLOSTRIDIUM DIFFICILE AND INTESTINAL MOTILITY

Over the years, the results of studies in different animal species have described a considerable enlargement of the caecum in germfree animals in comparison with conventionally reared animals (Figure 1). In the 1960-ties, it was also

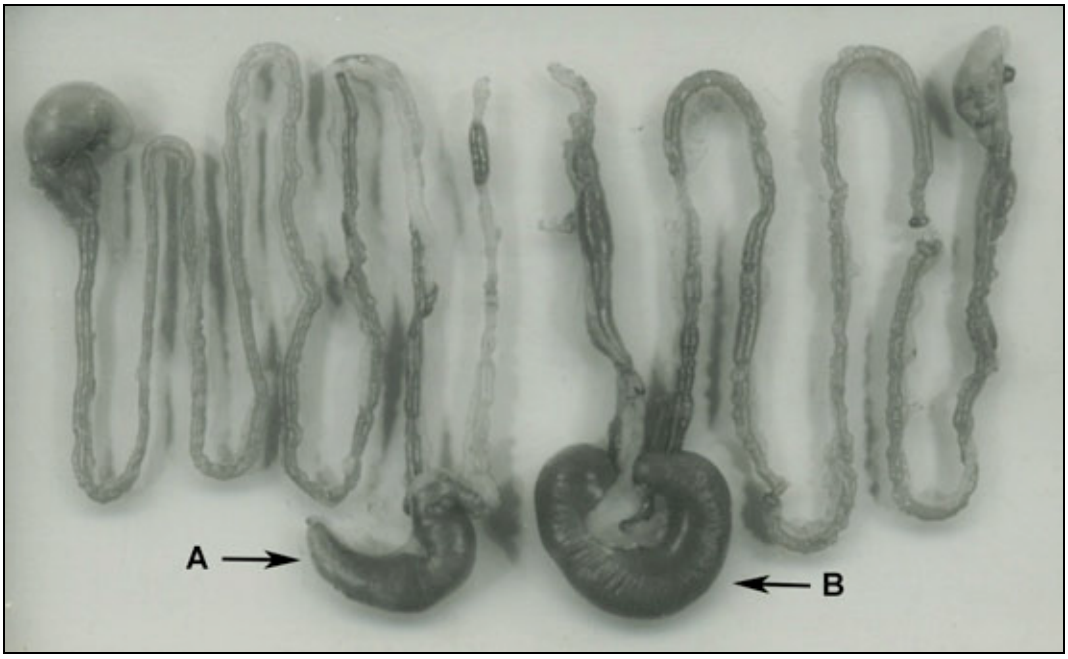

Figure 1: Intestinal tract of a conventional and a germfree rat of the same age. Note the difference in caecum-size between conventional (A) and germfree (B).

well established that when germfree animals were given intestinal content from their conventional counterparts, their caecum-size was gradually reduced, At that time, however, neither the mechanisms behind, nor the microbial species involved were known. It was also an everyday experience for those working with germfree and conventional animals, that spontaneous contractions of the large intestine were commonly observed in conventional animals, but that they were absent in their germfree counterparts. Similarly, the intestinal wall was more reactive to mechanical stimuli in conventional than in germfree animals. These observations created the background for the two following series of experiments:

- The amount of, and reactivity to some biogenic amines in the caecum of germfree versus conventional animals was determined (*Strandberg* et al., 1966).
- Caecal walls from four age-matched germfree and conventional rats, reared on the same autoclaved semi-synthetic diet (D7-diet), were investigated for the presence of some biogenic amines. Mean values, expressed a mcg/wet tissue in germfree and conventional rats respectively, were as follows: noradrenaline 0.56/0.98, l-adrenaline <10/<10, dopamine <10/<10, serotonin 5.8/6.5, acetylcholine 8.7/10.7, histamine 17.9/17.6. As is evident from these data, the concentrations of biogenic amines were virtually the same in both groups.

When investigating smooth muscle sensitivity towards these biogenic amines, strips from the same two groups of animals were prepared from the medium part of the caecum along the great curvature and movements were recorded isotonically on a smoked drum with a linear frontal writing level. Threshold dose was defined as the

smallest dose causing measurable concentration of the strip. Threshold doses, given as median value, mcg/ml bath fluid, for the four biogenic amines tested in groups of germfree and conventional animals were as follows: l-adrenaline 1.0/0.25, serotonin 0.75/0075, acetylcholine 1.5/0.005 and histamine >100/1.24. Thus, strips of caecal smooth muscles were 1-3 log less sensitive for biogenic amines than strips from their conventional counterparts.Also the types of contractions varied between the two groups of animals, most pronounced regarding serotonin. Strips from germfree animals subjected to serotonin showed an initially rapid, then slowly proceeding contraction. The strips from the conventional rats on the other hand reacted by rapid, rather twisting contractions.

From these experiments it was quite obvious that germfree rats had normal amounts of main biogenic amines in their colonic tissue. However the muscular sensitivity towards these amines was markedly reduced. As the focus at that time was on the microbes and mechanisms behind caecum enlargement, a second series of experiments was performed: Effect of conventionalization and mono-association with *Cl. difficile* of germfree rats on the caecum enlargement and reactivity to some biogenic amines (*Gustafsson* et al., 1970). In these experiments, age and gender matched young (at weaning) when included on the experiments and old(around 1 year old) germfree and conventional rats were used.They were all given the same autoclaved D7-diet. For Conventionalization, the faeces from 3-5 conventional rats were collected, suspended 1:19 in sterile saline, and each animal received aliquots of this suspension (1-2 ml) given both orally and rectally. After conventionalization, the animals were housed in the conventional animal room.

For mono-assosiation the strain *Cl. difficile* ATCC 0689 was grown anaerobically in Brain Liver Heart broth (Difco) for 3 days at 37°C, and aliquots of the broth were given to germfree rats as described above. These mono-associated rats were kept in the isolators during the experimental period. Female rats were only used for the comparison of smooth muscle activity in different organs while males were used in all other experiments. The most striking results were found in young animals. Conventionalization of germfree animals at weaning reduced caecum-size to conventional values within the observation period. Interestingly, the sensitivity towards the two biogenic amines acetylcholine and serotonin were similar in germfree and conventional rats at weaning, but were strikingly different 8 weeks later. The values found in conventionalized ex-germfree rats were comparable to those found in the conventional rats, whereas the values found in rats mono-associated with *Cl. difficile* were in-between the two groups. Interestingly, one of the rats in the *Cl. difficile* groups exhibited spontaneous contractions as those seen in conventional rats. In the old animals, the caecum-size was significantly reduced within the first week in both ex-germfree groups. The sensitivity towards the two biogenic amines was only modestly altered. Reactivity to biogenic amines in the other organs tested were similar, irrespectively of age and microbial status.

Before taking a glance in the historical mirror it has to be underlined that the focus for the research was on possible mechanisms behind the enlargement of caecum. *Cl. difficile* was chosen as a test organism because it was assumed that it belonged to an important part of the normal microbiota in young, healthy mammals. *Skelly* et al. (1962) found a full reduction of cae-

cum-size after mono-contamination with *Cl. difficile*, whereas *Wiseman* and (1965) found a transient reduction after mono-contamination of germfree rats with either *Cl. difficile* or *Salmonella typhimurium*. None of these investigators, however, studied alterations in sensitivity towards biogenic amines

When *Cl. difficile* in the 1970ties came up a causative microbe for antibiotic associated diarrhoea, the focus was changed. Out of the close to 6500 articles referred in Med-Line on *Cl. difficile*, only a very few are commenting on its motility properties, and here will only be mentioned a few. In 1998, Justus found that "*Cl. difficile* produces a heat labile substance or substances that altered the motility of the small intestine independent of the proteins responsible for *in vivo* tissue damage and cytotoxin assay positivity" (*Justus* et al., 1998). The substance(s) had a high molecular weight, were obtained from culture filtrates and "induced significantly more burst of action potentials (41.1/h) than all agents studied". In 2001, Huseby published studies on migrating myoelectric complexes (MMC), in the small intestine of germfree, conventional and ex-germfree rats mono-associated with *Clostridium tabificum*, *Lactobacillus acidophilus, Bifidobacterium bifidum, Echerichia coli* or *Micrococcus luteus* (*Huseby* et al., 2001). *Cl. tabificum* had the most pronounced effect, significantly reducing MMC-time in the mono-associated rats. *Cl. tabificum*, closely related to *Cl. difficile*, was chosen because it was known that it could influence upon some biogenic amines *in vitro*. Unfortunately the authors did not test *Cl. difficile* as a mono-contaminant. In 2000 it was shown in some *in vitro* studies that cytotoxin A was involved in "both direct excitation of enteric neurons and suppression of norepinephrine release from postganglionic sympathetic nerve fibres in the enteric nervous system" (*Xia* et al., 2001).

The results mentioned above clearly show that the intestinal microbiota do influence upon sensitivity towards biogenic amines and that *Cl. difficile*, by various mechanisms, most probably is involved in establishment and probably also maintenance of intestinal myelo-electric activity. The peculiar observation that both germfree as well as conventional rats at weaning had the same low sensitivity towards biogenic amines (*Gustafsson* et al., 1971) fits very well in a hypothesis that some basic functions are established at birth. If an intestinal microbiota is lacking, they might either be switched off or "re-started" when specific microbes are introduced (*Bry* et al., 1996).

CLOSTRIDIUM DIFFICILE AND INTESTINAL CELL KINETICS

In all mammals, the intestine is characterized with a very rapid turnover rate of enterocytes, supposed to be a major defence mechanism. In some comparative studies on germfree, conventional and mono-associated rats, we found that age, gender and microbial status influenced upon intestinal cell kinetics and morphology in an compartmentalized manner, and that most influences were related to the microbial status of the gut (*Banasaz* et al., 2000, 2002). In another series of experiments, groups of young, male germfree rats were mono-associated with either a toxin producing (Strain 70-685, gift from Prof. P. Bourlioux, Dept. of Microbiology, University Paris-Sud, Paris,

Table 2: Mitotic index (% of cells blocked in mitosis during 4 hours) in samples from jejunum, ileum, caecum and colon taken from rats mono-associated with a toxin producing and a non-toxin producing strain of *Clostridium difficile*

Day of mono-association	Toxigenic strain	Jejunum	Ileum	Caecum	Colon
0	yes	31	32	16	12
3	yes	39	37	25	20
7	yes	7	6	13	8
21	yes	33	32	18*	9
7	no	35	31	19	12

France) or a non-toxin producing (Strain CCUG 37785, Culture Collection, University of Gothenburg, Gothenburg, Sweden) strain of *Cl. difficile* for 3, 7 and 21 days, respectively. The mitotic activity was blocked, eight parts of the intestine were taken for microscopic examination, and aliquots from intestinal content were analysed for the presence of *Cl. difficile* and its toxins. All animals looked healthy and no diarrhoea was observed in any animal throughout the experimental period. *Cl. difficile* was found to be established in equal numbers in both groups. Toxins were found only in the group mono-associated with the toxin-producing strain, and highest values were recorded after 21 days (Table 2).

After 3 days of mono-associated, the mitotic index was found to be slightly elevated in all compartments in both groups. This is a common phenomenon observed when germfree animals are mono-associated with microbes including probiotic strains (*Banasaz* et al., 2002), and is assumed to reflect a general microbial triggering mechanism by substances present in both Gram-negative as well as Gram-positive microorganisms(*Oleya* et al., 2001). After 7 days, a dramatic reduction in mitotic activity was seen in the rats mono-associated with the toxin-producing strain. After 21 days, however, a normal mitotic activity was established again, irrespectively of presence of high amounts of toxins.

The clinical consequences of these findings can be outlined as follows: An increase in the number of toxin-producing *Cl. difficile* might cause a rapid and dramatic reduction in production of new enterocytes, i.e. a major defence mechanism in the intestine. However, for establishment of a disruption of the epithelial cell lining and development of diarrhoea, presence of other microorganisms might be needed. In some preliminary - and not yet published - experiments, we tried unsuccessfully to find such secondary troublemakers.

FUTURE ASPECTS

In future experiments, due considerations should be paid to combine the temporary increase in mitotic activity often found when some probiotic microbial strains are given together with the temporary decrease when toxin-producing strains are established. The results found in such models might be of prophylactic clinical importance. Additionally, characterization of possible secondary troublemakers should be intensified.

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