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The pathogenic mechanism of the diarrheal syndrome caused by *Bacillus cereus*.

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Abstract

As a contaminant of food commodities, *Bacillus cereus* may produce several enterotoxins that are responsible for the development of a diarrhaeal syndrome. Although four enterotoxins –haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), enterotoxin-T, and cytotoxin-K– have been described as possibly responsible for this syndrome, the two most important enterotoxins are the three-component HBL and NHE. However, their mode of action is unclear.

Insight into the pathogenic mechanism is of great importance. It may help to clarify questions related to dose–response relationships, such as the chance of symptoms when exposed to a certain amount of enterotoxin. Also, in combination with quantitative data on the occurrence of pathogenic *B. cereus* strains in food, it may contribute to re-evaluation of the tolerance levels set for *B. cereus* in food commodities. Suggestions are given here for further research aimed at elucidating the pathogenic mechanism of the diarrheal syndrome using human and animal cell lines.

Contents

Samenvatting

Bacillus cereus is de veroorzaker van twee verschillende soorten voedselgerelateerde aandoeningen. In het ene geval gaat het om een braaksyndroom. In het andere geval gaat het om een diarreesyndroom. Het diarreesyndroom kan veroorzaakt worden door vier enterotoxinen: enterotoxine-T, cytotoxine-K, haemolysine BL (HBL) en non-haemolytisch enterotoxine (NHE).

In dit rapport staat een beschrijving van het pathogene mechanisme van HBL en NHE centraal en wordt ingegaan op de mogelijke bijdragen van het micro-organisme en de enterotoxinen aan het pathogene mechanisme. Een beter begrip van het pathogene mechanisme is om twee redenen van belang. Ten eerste voor een betere beschrijving van dosis-respons relaties: wat is de kans om ziek te worden bij inname van een bepaade dosis. Ten tweede kan het, in combinatie met kwantitatieve gegevens omtrent voorkomen van ziekteverwekkende *B. cereus* stammen, bijdragen aan een bijstelling van de criteria die gesteld zijn ten aanzien van het mogen voorkomen van *B. cereus* in levensmiddelen.

Daarnaast wordt een voorstel gedaan voor verder onderzoek om het pathogene mechanisme achter het diarreesyndroom op te helderen.

Summary

Bacillus cereus is the etiological agent of two types of food borne disease: an emetic syndrome, and a diarrheal syndrome. Four enterotoxins have been described as cause for the diarrheal syndrome: enterotoxin-T, cytotoxin-K, haemolysin BL (HBL), and non-haemolytic enterotoxin (NHE).

In this report, factors that may contribute to the pathogenic mechanism of the diarrheal syndrome are reviewed, as well as the contribution of the microorganism and the enterotoxins to the pathogenic mechanism. A better insight into the pathogenic mechanism may not only lead to a better description of dose-response relationships (what is the chance of getting ill after intake of a certain amount of *B. cereus*), but also to re-evalution of tolerance levels set for *B. cereus* in food commodities.

Finally, suggestions for further investigation to clarify the pathogenic mechanism are presented.

1. Introduction

Bacillus (B.) cereus is an ubiquitary, spore-forming, Gram-positive microorganism. It is the etiological agent of two types of food borne disease, an intoxication (emetic form) and a toxico-infection (diarrheal form).

A peptide-like toxin causes the emetic form. The onset of symptoms of the disease starts within about 1 to 5 hours after consumption of the contaminated food, and the duration of the symptoms is 24 hours at most (Kramer and Gilbert, 1989). The pathogenic mechanism of the emetic form of disease is well understood and well described in the literature. The toxin is ingested with the contaminated food after it has been produced in the food during growth of *B. cereus*. Interaction with the nervus vagus leads to emesis (Agata et al., 1995b;Isobe et al., 1995).

The diarrheal form is caused by one or more protein enterotoxins (these will be discussed in chapter 2). The onset of symptoms of the disease starts within 8 to 12 hours after consumption of the contaminated food, and the symptoms last also 24 hours at most (Kramer and Gilbert, 1989). To date, the pathogenic mechanism of the enterotoxins has not been clarified, although some suggestions have been made (Beecher and Macmillan, 1991).

Improved knowledge of the pathogenic mechanism is necessary for two reasons. Firstly, better understanding of the pathogenic mechanism may lead to a better description of the dose-response relationship in humans. We hope to get insight into this dose-response relationship by using a so-called “parallelogram” approach. It is not an option to use human volunteer studies to elucidate the dose-response relationship. This is circumvented by using modelling of the relationship after determining such relationships in animal cells, test animals and human cells. This approach is depicted in figure 1.

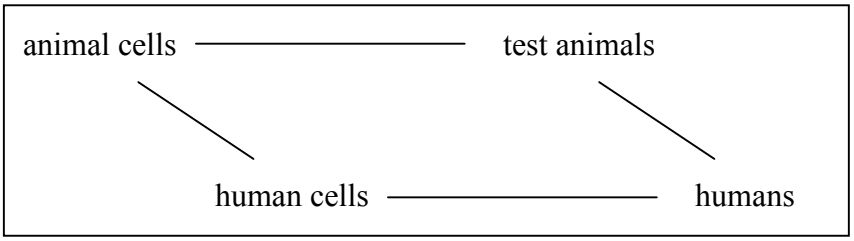

Figure 1. Parallelogram approach for modelling dose-response relationship in humans.

Secondly, a better insight into the pathogenic mechanism in combination with quantitative data concerning the occurrence of pathogenic *B. cereus* (Wijnands et al., in preparation) may lead to adjusting the tolerance level set for *B. cereus* in food commodities.

Enterotoxins are the compounds that cause the disease; the microorganism *B. cereus* is only the producer of the enterotoxins. Therefore, making statements on the amount of *B. cereus* colony forming units that cause disease is not relevant as the enterotoxin production is strain dependent. Also the type of food may influence the growth of diarrheagenic *B. cereus* strains. In this report, after a brief introduction on the enterotoxins of *B. cereus*, a review will be given of the available knowledge on the pathogenic mechanism of the enterotoxins, on factors contributing to the pathogenicity of the micro-organisms themselves, and on mechanisms involved in the development of diarrhea. Finally suggestions for further research will be presented in order to clarify the pathogenic mechanism leading to diarrhea caused by the enterotoxins haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) of *B. cereus*.

2. The enterotoxins of *Bacillus cereus*

At present four enterotoxins produced by *Bacillus cereus* have been described, haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), enterotoxin-T and cytotoxin-K (Granum, 1994;Granum and Lund, 1997;Lund et al., 2000;Agata et al., 1995a). Three of these (HBL, NHE, and cytotoxin-K) are related to outbreaks of food borne disease; enterotoxin-T has been classified as enterotoxin on the basis of genetic and structural relationship with bacterial enterotoxins (Agata, 1995a).

HBL is a haemolysin consisting of three proteinaceous subunits: B, L_1 and L_2, a binding factor and two "lytic" factors, respectively. These three subunits have been purified and characterized. The toxin shows dermonecrotic activity as well as activity towards vascular permeability, and causes fluid accumulation in ligated rabbit ileal loops (Granum and Lund, 1997). All three components are necessary for maximal enterotoxic activity (Beecher et al., 1995).
A single gene codes for each subunit; the genes from all three subunits are grouped in one operon.

NHE has recently been characterized (Lund and Granum, 1996) and, like HBL, consists of three proteinaceous subunits as well: nheA, nheB and nheC, two lytic factors and a binding factor respectively. Although binary combinations of the subunits show some biological effect, maximal activity is achieved when all three components are present (Lund and Granum, 1997). Here too, a single gene codes for each subunit, and the genes of all three subunits are grouped in one operon (Granum et al., 1999). There is substantial similarity between the proteins of the haemolytic and the non-haemolytic enterotoxin (Granum et al., 1999).
Transcription of the operon for the haemolytic enterotoxin HBL and the operon for the non-haemolytic enterotoxin NHE seems to be positively regulated by plcR, a gene that also regulates phospholipase C expression (Agaisse et al., 1999;Granum et al., 1999). This plcR gene has also been described in connection with enterotoxic properties of *B. thuringiensis*, which also belongs to the *B. cereus* goup (Agaisse et al., 1999). It has been proven that $plcR^-$-strains are not able to produce the enterotoxin HBL, while their $plcR^+$- equivalent did show HBL-production (Salamitou et al., 2000).

Enterotoxin-T has been described in 1995 (Agata et al., 1995a). This compound has been named such based on cloning and immunoblot experiments. Activity experiments have been performed with a recombinant product of the bce-T gene. To date, however, the enterotoxin

has not been related to outbreaks of food borne disease. It is a single component protein enterotoxin with activity towards vascular permeability. Also it exhibits cytotoxicity to Vero-cells, causes fluid accumulation in the ligated rabbit ileal loop test, and is lethal to mice after intravenous injection (Agata et al., 1995a). Such properties were found also by Shinagawa after the production and subsequent purification of a single non-haemolytic protein (Shinagawa et al., 1992)

Cytotoxin-K is the most recently described enterotoxin from *B. cereus*. It was detected after a food poisoning outbreak in an elderly home in France. In total 44 people were ill, 6 of these patients had bloody diarrhea, and three of these six died (Lund et al., 2000).
Cytotoxin-K is a single component protein enterotoxin showing necrotic and haemolytic activity, and is highly toxic to epithelial cells as shown with human CaCo2-cells (Hardy et al., 2001).

3. The mode of action of the enterotoxins

Investigations concerning the pathogenic mechanism of the diarrheagenic enterotoxins of *B. cereus* have been hampered by the various names attributed to the enterotoxin HBL. The nomenclature, loop fluid-inducing toxin/skin test/necrotic toxin, was primarily based on the effects observed in various tests (Turnbull, 1987). This situation ended when the HBL enterotoxin had been purified and used for tests (Beecher and Macmillan, 1991;Beecher and Wong, 1994). HBL is a vegetative growth metabolite, since it was found that loop-fluid inducing activity was first demonstrated halfway through the exponential phase of growth, with a maximum at about the time growth ceased to be exponential (Spira and Silverman, 1979).
Based on cytotoxicity tests with purified (subunits of) HBL or NHE enterotoxin it has been demonstrated that the mode of action of the two multi-component enterotoxins, HBL and NHE, is highly comparable (Lund and Granum, 1997). For these tests Vero cells were used.
Two possible working mechanisms for HBL have been suggested: either binding of the B-component and subsequent entering of the L-component(s) into the cell to alter metabolic functions, or binding of the B-component and the formation of membrane lesions through the combined binding of B and L components (Beecher and Macmillan, 1991).
In vitro tests using human CaCo-2 cells to clarify the pathogenic mechanism of *B. cereus* toxico-infection are scarce. CaCo-2 cells have been used for studying the adhesion of spores (Andersson et al., 1998). In other investigations culture filtrate of different strains has been used to investigate the pathogenic mechanism, but due to the multifactorial character of the culture filtrate no correlation could be made between any specific compound and biological activity (Minnaard et al., 2001).
Enterotoxin-T has only been produced by cloning and expression of the gene in *Escherichia coli* (Agata et al., 1995a). It has never yet been found in an outbreak of food borne disease. So, if pathogenic at all, no information on the mechanism of this enterotoxin is available.

Cytotoxin-K is the “youngest” of the enterotoxins, since it has first been described in 2000 (Lund et al., 2000). Therefore, investigations concerning the pathogenic mechanism of cytotoxin-K are limited. In vitro studies have revealed that the toxin is able to form pores in lipid bilayers which might indicate that the mode of action of the toxin is the formation of pores in the epithelial cells, causing fluid release and destruction of the epithelial cells leading to necrosis (Hardy et al., 2001). In structure as well as in mode of action cytotoxin-K resembles that of *Staphylococcus aureus* α–toxin or *Clostidium perfringens* β–toxin (Lund et al., 2000).

4. The role of the micro-organism in pathogenicity

HBL is a vegetative growth metabolite, since it was found that loop-fluid inducing activity was first demonstrated halfway through the exponential phase of growth, with a maximum at about the time growth ceased to be exponential (Spira and Silverman, 1979). As the genetic and molecular organization of HBL and NHE are very similar, NHE like HBL is a vegetative growth metabolite (Granum and Lund, 1997). Both enterotoxins consist of three proteins, and the current understanding is that neither of (the subunits of) these enterotoxins will pass unaffected the stomach barrier upon ingestion. This means that preformed and extracellular HBL or NHE does not play a role in the pathogenicity of *B. cereus*. Furthermore, the general understanding is that the majority of the vegetative cells of *B. cereus* also do not pass unaffected the stomach barrier. In most cases the pH in the stomach is too low for vegetative cells to survive. However, spores are able to reach the intestines. Therefore, germination of spores in the small intestine, growth and simultaneous production of enterotoxins is generally believed to be the route for the diarrheal syndrome caused by *B. cereus*. However, the uptake of food influences the pH in the stomach, ensuring that it is not constantly low (Russell et al., 1993;Dressman et al., 1990). Investigations have shown that dependant on the type of food and the age of individuals, the pH may, at least temporarily, reach higher values. Currently investigations are carried out in our laboratory to investigate the survival of *B. cereus* at pH-values between the normal pH of the stomach and the lowest pH for growth of *B. cereus* (data to be published). Also, investigations regarding the survival of stomach passage of the enterotoxins HBL and BHE will be carried out. These researches are undertaken to determine whether vegetative cells and/or enterotoxins are able the pass the stomach and reach the small intestine, and thus contribute to the pathogenicity of *B. cereus*.

Considering the disease-inducing route, there has to be a mechanism which enables the spores and growing vegetative cells to remain in the small intestine long enough to germinate and to produce the enterotoxins necessary for the pathogenic effect. Very few investigators have highlighted this vital part of the pathogenic mechanism. A marked difference in hydrophobicity between *B. cereus* spores and spores of other *Bacillus* species has been found (Wiencek et al., 1990). This hydrophobicity is believed to play a role in the adherence of spores not only to solid surfaces (Husmark and Ronner, 1990), but also to epithelial cells (Andersson et al., 1998). However, the hydrophobicity of vegetative cells is much less than that of spores (Wiencek et al., 1990), which raises the question whether other/more factors contribute to the adhesion of *B. cereus* to epithelial cells.

It has been suggested that appendages on the spore surface also contribute to the adhesion of spores to epithelial cells (Andersson et al., 1998). Whereas vegetative cells also have a number of appendages, fimbriae or flagella-like structures, on the surface, adhesion to epithelial cells is certainly a factor to investigate in order to clarify the pathogenic mechanism.

5. Enterotoxins and diarrhea

Infectious diarrhea may be divided into three types: the secretory, invasive, and penetrating type, respectively. Secretory diarrhea is liquid diarrhea linked to the perturbation of the movement of water and electrolytes across epithelium of the small intestine, most often related to an enterotoxin. Invasive diarrhea is characterized by the destructive invasion of the mucosa, generally at the level of the colon. In the penetrating type microorganisms (like *Salmonella typhi*) are able to penetrate the apparently intact epithelium and to proliferate there. According to this classification diarrhea caused by *B. cereus* is of the secretory type, like *Vibrio cholera* (Belaiche, 2000).

Adherence of enteropathogens to the intestinal epithelium is an essential step required for colonization (Contrepois, 1993). Attachment of the bacteria is linked to the presence of filamentous, antigenic appendages, called fimbriae, on the exterior of the microbial capsule, which recognize a specific site on the enterocyte (Belaiche, 2000).
Augmentation of intestinal secretion may be linked to the presence of enterotoxin. Bacterial enterotoxins are frequent causes of diarrhea. In a general manner these enterotoxins are controlled at a genetic level. Production of the enterotoxin components and forming of the enterotoxin-complex is believed to take place intracellularly in the bacterial cell. Then the enterotoxin-complex is either expressed on the bacterial cellmembrane or secreted into the intestinal lumen (P. E. Granum, personal communication). In the intestinal lumen, the toxin causes diarrhea through the following steps: binding to a specific membrane receptor, transmission of the signal across the membrane wall and stimulation of an intracellular messenger, and finally perturbation of the exchange of water and electrolytes (Belaiche, 2000).

For transport of electrolytes four intracellular routes are available: through calcium-ions (Ca^{2+}) dependency, through cyclic GMP (cGMP), through cyclic AMP (cAMP), and through rearrangement of the cytoskeleton (Rousset and Dubreuil, 2000). It has been suggested that *B. cereus* enterotoxin(s) use(s) the cAMP route for perturbation of the electrolyte transport. The mechanism for this route as well as for the other routes for electrolyte transport has been described in detail (Powell, 1987).

6. Suggestions for further research

The clarification of the dose response relationship and the influence of food matrix and intestinal matrix are important objectives of the project 250912, entitled: Quantitative research of *Bacillus cereus* within the scope of hazard characterisation and exposure assessment. Especially (the mechanism behind) the diarrheal syndrome caused by *B. cereus* needs further investigation.

The diarrheal syndrome is caused by enterotoxins formed intracellularly during vegetative growth of *B. cereus* in the small intestine. The enterotoxins HBL and NHE are primarily formed during the late exponential growth phase (Spira and Silverman, 1979). The general understanding is that neither the enterotoxins nor vegetative cells survive the stomach passage, due to the unfavourable circumstances in the stomach. Since the enterotoxins have to be formed in the small intestine, this implies that *B. cereus* spores passing the stomach germinate in the small intestine, grow to exponential phase and subsequently produce the enterotoxins. The median transit time in the small intestine is 8 hours. It is very likely that some kind of attachment or adhesion of spores and of the growing vegetative cells must take place to get sufficient outgrowth for enterotoxin production.

As pointed out in the previous chapters of this report very little is known about the mechanism of the diarrheal syndrome caused by the enterotoxins of *B. cereus*. However, to study the influence of food and intestinal matrix on germination, growth and adhesion of spores and subsequent expression of enterotoxins basic knowledge on the adhesion mechanism is of vital importance. Such basic knowledge is also of importance in the study of dose-response relationships concerning the amount of cells or enterotoxin necessary to induce diarrhea in humans. To investigate these phenomena adhesion/invasion and cytotoxic/lytic experiments using differentiated CaCo-2 cells (mimicking human epithelial cells of the small intestine) and IEC-18 (mimicking rat epithelial cells of the small intestine) can be applied (Pinto et al., 1983; Duizer, 1999). Similar experiments have been carried out with *Bacillus piliformi*, although this microorganism invades the epithelial cells (Franklin et al., 1993).

The two enterotoxins of interest, HBL and NHE, are three-subunit protein complexes. The current understanding is that the complexes are formed within the cell and subsequently excreted as complex into the lumen of the small intestine near the epithelium or expressed at the cell surface (PE Granum, personal communication). To confirm or reject this hypothesis fluorescent labeled monoclonal antibodies against the separate subunits of the enterotoxin HBL (Dietrich et al., 1999) can be used in combination with confocal laser scanning. As the mode of action of HBL and NHE is highly comparable (Lund and Granum, 1997), solving the hypothesis for HBL would also mean solving the hypothesis for NHE. This technique enables the determination of the position and composition of the enterotoxin complexes with respect to the *B. cereus* cell.

The current understanding that entire complexes are excreted by the cells eliminates the possibility that enterotoxin components deriving from different strains together form an active enterotoxin. This would mean that strains that possess the genes of only one or two of the components would never contribute to the development of diarrhea.
If, however, not complete three-component complexes are excreted by cells but individual components that are assembled outside the cell to three component complexes, it might be possible that strains lacking one or two enterotoxin genes contribute to pathogenesis. Also the use of confocal laser scanning with labeled monoclonal antibodies against the separate components of HBL may help to elucidate the question whether entire complexes or separate components are excreted from the *B. cereus* cells.

Both HBL and NHE consist of three components, one binding factor and two “lytic” factors (see chapter 2). As stated in chapter 3 (Beecher and Macmillan, 1991) it is unclear how binding of the enterotoxin to epithelial cells takes place and how the “lytic” factors work. Nor is it clear whether the “lytic” factors enter the host cell cytoplasm to stimulate an intracellular messenger into perturbation of the exchange of water and electrolytes (Belaiche, 2000) or whether they are inserted into the host cell membrane to form pores. The afore mentioned monoclonal antibodies against the various components of the enterotoxin HBL may help elucidate where the individual components of this enterotoxin end up in or on the hostcell. Differentiated CaCo-2 cells may be used as human host cells, IEC-18 cells may serve as animal host cells (Duizer, 1999). Using both cell types will help substantiate the “parallelogram” approach as outlined in the introduction.

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