Cooperative, synergistic and antagonistic haemolytic interactions between haemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from *Bacillus cereus*

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Haemolysis of erythrocytes from different species (sheep, bovine, swine and human), caused by various combinations of phosphatidylcholine (PC)-preferring phospholipase C (PC-PLC), sphingomyelinase (SMase) and the three-component, pore-forming toxin haemolysin BL (HBL) from *Bacillus cereus* was analysed. The lytic potency of HBL did not correlate with phospholipid (PL) content, but lysis by the individual or combined enzymes did. SMase alone lysed ruminant erythrocytes, which contain 46–53 % sphingomyelin (SM). The cooperative action of PC-PLC and SMase was needed to lyse swine and human erythrocytes (22–31 % PC and 28–25 % SM). SMase synergistically enhanced haemolysis caused by HBL for all erythrocytes tested, which all contained >25 % SM. PC-PLC enhanced HBL haemolysis only in cells containing significant amounts of PC (swine, 22 % PC; human, 31 % PC). Unexpectedly, PC-PLC inhibited HBL lysis of sheep erythrocytes (<2 % PC) and enhanced the discontinuous haemolysis pattern that is characteristic of HBL in sheep blood agar. Inhibition and pattern enhancement was abolished by washing PC-PLC-treated erythrocytes or by adding EDTA, suggesting that enzymic alteration of the membrane is not involved, but that zinc in the active site is required, perhaps to facilitate binding. These observations highlight the potential for cooperative and synergistic interactions among virulence factors in *B. cereus* infections and dependence of these effects on tissue composition.

Keywords: haemolysin BL, phospholipase, sphingomyelinase, synergy, cooperativity

INTRODUCTION

Bacillus cereus causes diarrhoeal food poisoning, emetic food poisoning and a variety of necrotic infections. Haemolysin BL (HBL) is comprised of three components designated B, L₁ and L₂. It is a potent dermonecrotic/enterotoxin that appears to play a significant role in diarrhoeal food poisoning and endophthalmitis caused by this organism (Beecher *et al.*, 1995a, b; Beecher & Wong, 1997). However, *B. cereus* secretes a wide variety of other potentially membrane-damaging

toxins and enzymes that constitute a significant proportion of the proteins secreted by *B. cereus*. Among these are at least three haemolysins, called cereolysin O, haemolysin II and haemolysin III (Baida & Kuzmin, 1995; Bernheimer & Grushoff, 1967; Budarina *et al.*, 1994; Cowell *et al.*, 1976; Sinev *et al.*, 1993), several proteases and three phospholipases C (PLC). The phospholipases preferentially hydrolyse sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylinositol. The majority of these potential virulence factors, including HBL, sphingomyelinase (SMase) and PC-PLC, are under the control of a pleiotropic regulator called *plcR* and are expressed simultaneously (Agaisse *et al.*, 1999). There has been little research to elucidate the contributions of these multiple toxins or enzymes to the multifactorial virulence of *B. cereus*.

Tissue necrosis is characteristic of *B. cereus* infections (Drobniowski, 1993); therefore, membrane-damaging

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Abbreviations: HBL, haemolysin BL; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-preferring phospholipase; PL, phospholipid; PLC, phospholipase C; RBC, red blood cell; SM, sphingomyelin; SMase, sphingomyelinase.

Fig. 1. Spectrophotometric assays demonstrating haemolytic cooperativity and synergy. (a) Cooperative haemolysis of swine RBCs by SMase and PC-PLC, with a requirement for Ca²⁺/Mg²⁺. Either SMase or PC-PLC was added to swine RBCs at $t = 0$. The complementary enzyme was added at 11 min as indicated by the arrows. In one experiment, Ca²⁺/Mg²⁺ was added at $t = 0$ min with SMase as indicated. In the other experiment, it was added at $t = 16$ min, as indicated by the arrow. The RBCs were standardized as described in Methods and haemolysis was at 37 °C. [SMase] = 28 nM (0.18 U ml⁻¹); [PC-PLC] = 35 nM (2 U ml⁻¹); [Ca²⁺/Mg²⁺] = 0.5 mM each. (b) Haemolytic synergy between HBL and SMase on swine blood. Samples consisting of HBL, SMase or HBL+SMase were added simultaneously to standardized swine RBCs containing 0.5 mM Ca²⁺/Mg²⁺ at 37 °C. [HBL] = 3.6 nM; [SMase] = 29 nM.

factors most likely contribute directly to pathogenesis. Here, we examined the modulation of lytic effects caused by various combinations of HBL, SMase and PC-PLC, using erythrocytes from different species as models for membranes with varied phospholipid (PL) contents, as might occur in different tissues. The observed reactions can be characterized as cooperativity, synergy or antagonism, depending on proteins combined and the erythrocyte species. We use a descriptive definition of cooperativity as the lysis of red blood cells (RBCs) by a combination of proteins that individually are unable to cause lysis (Fehrenbach & Jürgens, 1991). In synergy, one or more factors in a system has intrinsic lytic activity, and the combined factors cause lysis at a greater rate than the sum of the individual rates.

The observations reported here highlight the importance of keeping in mind the potential involvement of multiple toxic factors in *B. cereus* virulence and suggest that the relevant interactions between factors during infections may vary in different tissues in a manner dependent on membrane composition.

METHODS

Erythrocytes, HBL components and enzymes. All protein and RBC preparations were in Tris-buffered saline (TBS), pH 7.4, containing 50 mM Tris/HCl and 150 mM NaCl. HBL components were purified (> 95%) and quantified as described previously (Beecher *et al.*, 1995a; Beecher & Wong, 1994b). Defibrinated blood from sheep, cows and swine was purchased from Crane Laboratories. Human blood was drawn from a volunteer. RBCs were washed in TBS by centrifugation until the supernatant was colourless, then held on ice until used the same day.

For the haemolysis experiments shown here, SMase was purchased from Sigma and PC-PLC was purchased from Boehringer Mannheim. When testing SMase, 0.5 mM CaCl₂ and 0.5 mM MgCl₂ (0.5 mM Ca²⁺/Mg²⁺) were added to erythrocyte suspensions unless otherwise indicated.

All HBL and enzyme concentrations were chosen empirically, based on preliminary dose-response experiments, so that lysis would occur within a desired time, or to be certain that enzymes would not lyse RBCs within the assay period unless in appropriate combinations. The PLC concentrations of 0.8–35 nM, and the HBL concentrations from 1 to 100 nM used here are within the secretion capabilities of *B. cereus* in culture. Typically, toxigenic strains such as *B. cereus* F837/76 secrete about 100–200 nM concentrations of each protein in complete media such as brain heart infusion broth (unpublished observations).

Purity of commercial enzymes. The purity of the commercial enzymes was analysed by densitometry of SDS-PAGE gels and by Western blot analysis with antibodies specific for each enzyme (the antibodies will be described elsewhere). PC-PLC was comprised of approximately 80% intact PC-PLC (28 kDa) and about 20% of a 25 kDa PC-PLC degradation product, which reacted with specific antibodies to the enzyme. No other bands were evident and the preparation did not react with antibodies to SMase. This profile was consistent from batch to batch over several years. The commercial SMase consisted of about 90% intact SMase and 10% of a 30 kDa SMase degradation product, which reacted with SMase antibodies. A minor band that did not react with the antibodies ran with the dye front (< 14 kDa). The enzyme preparation contained no PC-PLC activity. The purity of this product varied from batch to batch and was therefore analysed before use. Both enzymes used here exhibited some degradation but were sufficiently pure to rule out interference from non-specific proteins.

Gel-diffusion assays. The gel-diffusion assays were performed as described earlier and agar gels containing blood or phosphatidylcholine were prepared as described previously (Beecher & Macmillan, 1990).

Turbidometric determination of haemolysis. Lysis of RBC suspensions was measured either in a spectrophotometer (model U-2000 UV/Vis Spectrophotometer; Hitachi Instruments) at 630 nm through a 1 cm path length, or in a SpectraFluor microplate reader (Tecan) at 620 nm through 200 µl samples in 96-well plates. The RBC suspensions were diluted into TBS until the optical densities in the respective instruments were 0.7. The suspensions for the spectrophotometer contained approximately 1.3 × 10⁷ sheep RBC ml⁻¹, 8.2 × 10⁶ bovine RBC ml⁻¹, 6.3 × 10⁶ swine RBC ml⁻¹ and 7.5 × 10⁶ human RBC ml⁻¹. The microplate samples had 4.7 times as many cells per millilitre. The instruments and cell suspensions were warmed to the desired lysis temperature prior to initiation of lysis. In the spectrophotometer assay, 7 µl samples stock HBL or enzyme solutions were added to cuvettes containing 0.7 ml standardized RBC

suspension. In the microplates, 5 µl samples were added to 200 µl RBC suspensions. Haemolysis was monitored continually over time and haemolysis rates are reported as the decrease in OD₆₃₀ or OD₆₂₀ per minute multiplied by 1000 (e.g. ΔmOD₆₃₀ min⁻¹).

RESULTS

PC-PLC and SMase interactions are dependent on membrane PL content

Fig. 1(a) shows cooperative haemolysis of swine erythrocytes by the combined action of PC-PLC and SMase. Neither enzyme lysed RBCs on its own, but when combined, lysis was rapid and complete in the presence of 0.5 mM Ca²⁺/Mg²⁺. These ions cause optimal SMase binding and haemolysis (Tomita *et al.*, 1991).

In the presence of 0.5 mM Ca²⁺/Mg²⁺, bovine erythrocytes were slowly lysed by SMase, but not by PC-PLC (Table 1). Combining the enzymes did not

appreciably enhance lysis over that caused by SMase alone. Similar results were obtained with sheep RBCs (data not shown). This is expected from the known PL content of ruminant erythrocytes, which have a high proportion of SM in their membranes and little PC (Crowell & Lutz, 1989; Fehrenbach & Jürgens, 1991).

Haemolytic synergy between HBL and SMase

HBL and SMase were synergistic in the lysis of sheep, bovine, swine and human erythrocytes (Figs 1b, 2 and 3; Table 1). Fig. 1(b) shows an eightfold enhancement of HBL-induced lysis of swine RBCs by non-lytic SMase in the presence of 0.5 mM Ca²⁺/Mg²⁺. The synergy between HBL and SMase was even more pronounced on bovine erythrocytes (Table 1).

Human and swine erythrocytes have very similar PL compositions (Crowell & Lutz, 1989; Fehrenbach & Jürgens, 1991), but swine RBCs are about three times

Table 1. Effect of HBL, PLC and HBL + PLC combinations on lysis of erythrocytes from different species, and PL content of the erythrocyte membranes

Lysin or combination*	Erythrocyte lysis					
	Bovine		Swine		Human†	
	Rate‡	EF§	Rate‡	EF§	Rate‡	EF§
HBL	40		120		0	
PC-PLC	0		0		0	
SMase	15		0		0	
HBL + PC-PLC	32	0.8	480	4.0	0	0
HBL + SMase	1280	23.0	960	8.0	10	c
PC-PLC + SMase	17	1.1	340	c	74	c
HBL + PC-PLC + SMase	NT		NT		11¶	c
PL	PL content (%)#					
	Bovine		Swine		Human	
PC	3.8		21.7		30.9	
SM	44.6		28.1		25.0	

NT, Not tested.

* [HBL] was 5, 3.6 and 20 nM in bovine, swine and human RBCs, respectively; [PC-PLC] and [SMase] were, respectively, 35 and 28 nM in bovine and swine RBCs, and 18 and 14 nM in human RBCs.

† See Fig. 2 for dose response with respect to HBL.

‡ Lysis rate, ΔmOD min⁻¹.

§ Enhancement factor, rate for combined lysins divided by additive value of individual lysin components. c, Cooperative haemolysis at the concentrations tested. See text for definition of cooperative lysis.

|| Rate attained after treatment of cells with PC-PLC for 10 min prior to addition of HBL. PC-PLC and HBL added simultaneously resulted in a rate of 140 ΔmOD min⁻¹ (1.2 times HBL control).

¶ Cooperative haemolysis caused by 20 nM HBL in the presence of sublytic concentrations of PC-PLC (1 nM) and SMase (0.8 nM).

Data from Crowell & Lutz (1989).

Fig. 2. Effects of PC-PLC and SMase on the HBL dose response for human RBC haemolysis. HBL was added to human RBCs containing $0.5 \text{ mM Ca}^{2+}/\text{Mg}^{2+}$ at 40°C at the concentrations indicated on the x axis. Filled circles, HBL controls containing no added enzymes; open circles, all samples contained 18 nM PC-PLC (1 U ml^{-1}); open squares, all samples contained 14 nM SMase (0.09 U ml^{-1}); open triangles, all samples contained PC-PLC and SMase at 1.0 and 0.8 nM (0.055 and 0.005 U ml^{-1}), respectively. Samples were in duplicate. Where error bars are not visible, the size of the symbol is larger than the error.

Fig. 3. Gel-diffusion assay demonstrating haemolytic synergy between HBL and SMase on sheep blood. Each well contained SMase (S) and/or HBL components B, L_1 and L_2 as indicated. Protein loads were: B, 2.5 pmol (95 ng); L_1 , 0.15 pmol (5.7 ng); L_2 , 0.15 pmol (5.1 ng); SMase, 1.14 pmol (39 ng). The L components were added at limiting concentrations to minimize HBL activity between wells. The altered appearance of RBCs around the well containing SMase and L_1 also occurred in the absence of L_1 . The gel was held at 24°C for 14 h prior to photography.

more sensitive to HBL than bovine erythrocytes (Table 1), and six to eight times more sensitive than sheep RBCs (unpublished observations), whilst human erythrocytes are much less sensitive (see Figs 2 and 5). Fig. 2 shows significant enhancement of both the haemolysis rate and the dose-response range of HBL on human RBCs by a non-lytic dose of SMase.

Fig. 4. Gel-diffusion assay demonstrating enhancement by PC-PLC of the discontinuous haemolysis pattern caused by HBL in sheep blood. The well on the left contained 1 pmol of each of the three HBL components, the well on the right contained 1 pmol HBL + 5 pmol PC-PLC. The gel was held at 30°C and photographed after 7 h .

Fig. 3 illustrates a manifestation of synergy between HBL and SMase in an agar-diffusion assay in the absence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ on sheep erythrocytes, which have a SM content of 53.1% and a PC content of $< 2\%$ in their membranes (Crowell & Lutz, 1989), similar to bovine erythrocytes. A quantity of HBL exhibiting little activity in the absence of SMase caused complete lysis only within the zone affected by SMase activity. In turbidometric and gel-diffusion assays, all three HBL components were required to cause enhanced lysis with SMase (Fig. 3 and data not shown), suggesting that the mechanism of HBL is not altered despite enzymic alteration of the cell surface.

PC-PLC and HBL interactions

Unlike SMase, PC-PLC did not shift the dose-response range of HBL on human RBCs, and it only slightly enhanced lysis rate (Fig. 2). A similar rate of enhancement occurred upon simultaneous addition of HBL and PC-PLC to swine erythrocytes. However, pretreatment of the swine cells with 2 U ml^{-1} PC-PLC enhanced HBL lysis fourfold (Table 1).

We did not expect PC-PLC to have an effect on HBL lysis of sheep RBCs because PC is nearly absent from these cells (Crowell & Lutz, 1989; Fehrenbach & Jürgens, 1991). However, the presence of PC-PLC in blood agar markedly enhanced and stabilized the discontinuous haemolysis pattern of HBL (Fig. 4). This effect appears to be due to inhibition of HBL by PLC. In a suspension assay, PC-PLC partially inhibited haemolysis at all HBL concentrations, but more or less so at different concentrations in a manner that enhanced the paradoxical dose-response behaviour of HBL (Fig. 5).

The discontinuous haemolysis pattern in blood agar gels was stabilized if HBL was added to wells with PC-PLC at 0.01 – 0.05 U well^{-1} (approx. 5 – 25 ng well^{-1}). Higher concentrations of PC-PLC inhibited development of any haemolysis for the duration of the assays. The pattern could be similarly enhanced and stabilized by incorporating PC-PLC directly into the blood agar between approximately 0.15 and 2.5 U ml^{-1} .

Fig. 5. Enhancement by PC-PLC of the discontinuous haemolysis pattern caused by HBL in sheep blood suspensions. All samples in wells of a microtitre plate contained HBL at the concentrations indicated on the x axis either in the presence (open circles) or absence (filled circles) of 35 nM PC-PLC at 37 °C. Samples were in triplicate. Where error bars are not visible, the size of the symbol is larger than the error.

The active site of PC-PLC contains zinc, and the presence 10 mM of EDTA inhibited enzyme activity only fourfold in PC agar gels, but completely eliminated the effects on HBL haemolysis in gels and in suspension (data not shown). Adding ZnSO₄ to blood agar did not mimic the action of the enzyme. In addition, an experiment was performed in which a suspension of sheep RBCs was treated for 30 min at 37 °C with 2 U PC-PLC ml⁻¹ and another suspension was not treated with PC-PLC. Both suspensions were then washed three times to wash free PLC from the cells. Blood agar gels were prepared with each cell suspension. Upon addition of HBL to wells, the PC-PLC-treated cells did not produce enhanced zones compared with the untreated cells, addition of PC-PLC to wells with HBL enhanced the pattern in both gels, and EDTA prevented the enhancing effect. This suggests that the inhibitory effect is caused by the physical association of PC-PLC with the membrane and not by enzymic alteration of the cells.

Synergy between HBL and a sublytic combination of PLCs

In the binary HBL and PLC combinations in Fig. 2, SMase was at 18 nM and PC-PLC was at 14 nM (0.5 µg ml⁻¹ each). At these concentrations, the combined PLC enzymes caused lysis of human RBCs at 74 ΔOD₆₂₀ min⁻¹ (Table 1). A combination of 1 nM PC-PLC and 0.8 nM SMase was not lytic for the 2 h duration of these experiments. However, when this non-lytic, low concentration of the two enzymes was added to HBL, it had almost the same effect as 18 nM SMase, which shifted the HBL dose-response range and enhanced lysis rates (Fig. 2).

DISCUSSION

Cooperative haemolysis is a well-known phenomenon, particularly between different PLCs, and between PLCs and non-lytic proteins (Bernheimer, 1996; Fehrenbach & Jürgens, 1991). Haemolytic synergy is also well known between numerous pore-forming toxins, and between PLCs and pore-forming toxins (Bashford *et al.*, 1986; Cajal & Jain, 1997; Crowell & Lutz, 1989; Tapsall & Phillips, 1984). The first such interaction described was the CAMP (Christie–Atkins–Munch–Petersen) reaction, i.e. haemolysis that occurred between adjacent colonies on blood agar of group B streptococci and SMase (β-toxin)-producing *Staphylococcus aureus* (Christie *et al.*, 1944). The great majority of cooperative and synergistic lytic systems that have been studied are between proteins produced by different species. Such systems might have relevance in disease processes only in special circumstances of mixed infections. Here, we examined interactions on membranes between three factors from a single organism that may contribute directly to the severity of necrotic infections. Erythrocytes from different species provide model membranes with different PL compositions, as may be encountered in different mammalian tissues.

Cooperativity between *B. cereus* PC-PLC and SMase in the lysis of human RBCs was described qualitatively by Gilmore *et al.* (1989), who noted a CAMP-like reaction between clones expressing the separate genes. The combination is sometimes referred to as cereolysin AB because the PLC genes are arranged in tandem on the *B. cereus* chromosome (we avoid this usage because each enzyme has its own well-characterized activity). Not surprisingly, we found that these enzymes also cooperatively lyse swine RBCs (Fig. 1), which have PL compositions similar to human RBCs. Neither of these erythrocyte types has greater than about 30 % SM or PC, and neither the SMase nor the PC-PLC could lyse the cells on its own. However, the substrate PL for the combined enzymes was about 50 and 56 % for swine and human RBCs, both of which were rapidly lysed by the enzyme combination. SMase alone was able to lyse ruminant erythrocytes, which contain about 45–53 % SM. Lysis presumably will occur only if the target substrates are located primarily on the outer surface of the membrane, as are PC and SM (Fehrenbach & Jürgens, 1991).

HBL alone is a cooperative toxin, composed of three separately secreted proteins whose genes are arranged in tandem on the chromosome (Beecher & Wong, 1994b, 1997; Ryan *et al.*, 1997). Erythrocytes from different species vary widely in sensitivity to HBL with the following profile: guinea pig > swine > bovine > sheep > rabbit > goat > human (unpublished observations). There is no correlation between known membrane composition and sensitivity. The presence of SMase greatly enhanced the lysis of RBCs from all species tested (Table 1 and Fig. 3) and lysis was enhanced to a lesser extent by PC-PLC in cells containing appreciable PC (swine and human). Of particular

interest is that human RBCs – the least sensitive cells – became sensitive to the low HBL concentrations to which sheep RBCs are susceptible (see Figs 2 and 5) provided appropriate cations were present (Fig. 1a).

Even though the PC contents of swine and human RBCs are comparable to the SM contents (Table 1), the effect of PC-PLC on HBL lysis of these cells was less dramatic than the effects of SMase. Swine cells became notably more sensitive to HBL only after prior treatment with PC-PLC, and lysis of human RBCs was only marginally enhanced. PC-PLC may have slower kinetics than SMase or may inefficiently hydrolyse PC in biological membranes. In membranes, SM may sterically obscure PC and cleavage of ceramide head groups from SM by SMase may expose PC to the action of PC-PLC by reducing the lipid surface pressure of the membrane (Fehrenbach & Jürgens, 1991; Zwaal *et al.*, 1973). Consequently, a very low concentration of PC-PLC (1 nM) could effectively enhance lysis of human RBCs by HBL when a similarly low concentration of SMase (0.8 nM) was present (Fig. 2).

PC-PLC had an unexpected, but useful effect on haemolysis of sheep RBCs by HBL. The dose-dependent discontinuous haemolysis pattern typical of HBL in sheep and bovine blood was enhanced in gels and in cell suspensions (Figs 4 and 5). This effect appears to be caused by a general inhibition of HBL lysis, which was less pronounced in a narrow HBL concentration range (approx. 2.5–10 nM) (Fig. 5). The effect was not expected because PC constitutes < 2% of sheep RBC membranes (Crowell & Lutz, 1989). *B. cereus* PC-PLC also hydrolyses phosphatidylethanolamine and phosphatidylserine (Möllerby, 1978; Slein & Logan, 1965). However, these either make up small fractions of the total sheep RBC PL, or are primarily located on the inner-membrane leaflet (Fehrenbach & Jürgens, 1991). Our data suggest that the mechanism is non-enzymic because washing the PC-PLC-treated cells eliminated the effect. In addition, PC-PLC did not enhance the lysis of sheep RBCs by SMase.

EDTA eliminated the effect of PC-PLC, suggesting that one or more of the three zinc atoms in the active site of the enzyme is involved in its association with the membrane. We saw about a 75% reduction in PC-PLC activity against PC vesicles under the conditions used for the gel-diffusion haemolysis assay. This was enough to reduce the effective enzyme concentration to a level too low to inhibit HBL. The active site zinc is required to maintain the native conformation of the enzyme (Little, 1978; Little & Johansen, 1979) and it is possible that, in the haemolysis assays, a sufficient amount of zinc was removed to disrupt the conformation of much of the enzyme, thereby preventing it from binding to the membrane. However, it is quite difficult to remove enough metal to disrupt the enzyme structure and the zinc may have still been present but shielded to prevent the association of the enzyme with the membrane, particularly since it essentially contained no PC. It is also possible that the EDTA altered the membrane surface potential in a manner that obviated enzyme binding.

Unfortunately, there is little published information regarding the binding of PC-PLC to membranes, particularly those lacking its substrate. The data presented here suggest that this enzyme is capable of binding to a membrane interface with little or no specific substrate present.

Identifying the specific mechanism by which PC-PLC and HBL interact on a membrane will require significant advances towards understanding the mechanisms of both. However, our most recent model of the cause of discontinuous haemolysis provides a reasonable mechanism. The discontinuous haemolysis pattern of HBL occurs because excess concentrations of the B and L₁ components inhibit haemolysis (Beecher & Wong, 1997). The apparent mechanism is that the B and L₁ components self-associate at high concentrations, forming inactive homo-oligomers on the membrane surface and thus preventing the formation of competent transmembrane pores (unpublished observations). If significant amounts of PC-PLC bind to the membrane surface without altering its character, the effect will be to reduce the membrane volume available to bound HBL components. The effective increase in component concentration on the membrane would drive the formation of inactive complexes, particularly near the diffusion source.

The physical basis for synergistic enhancement of HBL lysis is also not clear from the present data. PL hydrolysis may promote binding of HBL components as suggested for the synergy between *B. cereus* SMase and *Pseudomonas aeruginosa* cytotoxin (Crowell & Lutz, 1989). Otherwise, enhanced lysis may simply be due to altered fluidity or decreased mechanical stability of the membrane.

The effects on HBL haemolysis of SMase and PC-PLC have some important practical consequences. We previously described a method to identify HBL-producing *B. cereus* strains by discontinuous haemolysis surrounding wells containing crude culture supernatants or directly surrounding colonies in HBL agar, a specially formulated sheep blood agar (Beecher & Wong, 1994a). We have found that this diagnostic characteristic is enhanced, particularly for crude culture supernatants, by adding 0.3 U PC-PLC ml⁻¹ to the gel. In addition, the effects observed here may be instrumental in deciphering structure–function relationships of HBL and the PLCs.

One problem with interpreting discontinuous haemolysis patterns around crude samples or growing colonies is that continuous haemolysis extending outward from the well or colony often overtakes or obscures the discontinuous pattern specific for HBL. The present observations suggest that much of this interfering lysis may be due to synergy between HBL and SMase. We have found that adding antisera specific for SMase to crude *B. cereus* culture supernatants eliminates much of the interfering continuous lysis (not shown). Further modification of these agar-diffusion methods that include specific inhibitors of SMase may improve their performance in identifying HBL-producing strains.

The molecular basis of *B. cereus* virulence is still largely unknown. We have demonstrated the potential modulating effects on the impairment of membrane integrity between three *B. cereus* toxins. These represent only a portion of the known membrane-active proteins produced by this organism (see Introduction). Deciphering the basis of *B. cereus* virulence will require an awareness of the contributions from a variety of potential virulence factors as well as an appreciation of the composition of the infected tissues.

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