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Dependence of the lethal effect of pore-forming haemolysins of Gram-positive bacteria on cytolytic activity

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Among bacterial haemolysins, cholesterol-dependent cytolysins (CDCs) produced by various Gram-positive bacteria are known to exhibit a lethal activity in mice. In this study, recombinant CDCs of streptolysin O, pneumolysin, ivanolysin O, listeriolysin O and several listeriolysin O mutants were constructed and the relationship between cytolytic activity and the lethal activity of each recombinant protein in mice was examined. Specific activity for cytolysis was determined by a quantitative haemolytic assay. Each protein was injected intravenously into mice and the lethal activity was evaluated by measuring the time until death of the mice. The four full-length CDC proteins exhibited lethal activity and their activities were highly proportional to their cytolytic activities. Inhibition of haemolytic activity resulted in the loss of lethal activity and non-haemolytic mutants of listeriolysin O did not exhibit any lethal activity. These data clearly indicate that the lethal effect of CDC proteins is dependent on the cytolytic activity.

INTRODUCTION

Cholesterol-dependent cytolysins (CDCs) are a family of structurally related cytolytic protein toxins produced by different species of Gram-positive bacteria belonging to the genera *Streptococcus*, *Listeria*, *Clostridium* and others (Alouf, 1999; Billington *et al.*, 2000). These toxins, traditionally known as haemolysins, are characterized by a highly conserved undecapeptide sequence (ECTGLAWEWWR) near the C terminus, which is essential for their cytolytic activity (Michel *et al.*, 1990; Owen *et al.*, 1994; Sekino-Suzuki *et al.*, 1996). They are capable of binding directly to membrane cholesterol and forming transmembrane pores, resulting in cell lysis.

CDC proteins have also been known to exhibit lethal activity in experimental animals. Over 50 years ago, Howard & Wallace (1953b) reported the lethal action of streptolysin O (SLO). Since then, a similar lethal effect has also been observed in other CDC toxins – listeriolysin O (LLO), pneumolysin (PLY) and perfringolysin O (Bernheimer, 1976). The molecular mechanism of lethal activity has not been well elucidated, but the primary target of the lethal action appears to be the heart. In mice given an intravenous injection of a crude preparation of LLO, Kingdon & Sword (1970) observed a lethal arrhythmia as demonstrated by an electrocardiogram. Halbert *et al.* (1961) and Alouf & Palmer (1999) also showed that rabbits and mice given a lethal dose of SLO suffered from conduction defects and ventricular disturbances.

Although a number of previous reports suggest that cytolytic activity observed *in vitro* is related to the expression of lethal activity *in vivo*, there has been very limited information to show any direct evidence for a contribution of cytolytic activity to the lethal effect. In order to make this point clear, we constructed a variety of recombinant CDC proteins, including LLO, ivanolysin O (ILO), SLO, PLY, a non-cytolytic truncated LLO deficient for the C terminus and LLO mutants with replacement of 1 aa in the conserved undecapeptide region, which exhibited variable cytolytic activities. Each recombinant protein was examined for specific haemolytic activity *in vitro* and lethal activity in mice.

Abbreviations: CDC, cholesterol-dependent cytolysin; HU, haemolytic unit; ILO, ivanolysin O; LLO, listeriolysin O; PLY, pneumolysin; SLO, streptolysin O.

METHODS

Mice. Female C3H/HeN and C3H/HeJ mice were used for the experiments at 7 weeks of age. All experimental procedures on mice were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

Construction and purification of various recombinant proteins. We generated various CDC proteins as 6 × His-tagged recombinant proteins as listed in Table 1. Briefly, the gene encoding the mature CDC protein was amplified by PCR using the primers shown in Table 2 and ligated into the pQE31 vector (Qiagen). For the construction of LLO mutant with replacement of 1 aa in the undecapeptide region, the region containing the undecapeptide to the C terminus was amplified by PCR with a mutagenic primer and the 3′ LLO primer (Table 2). A secondary PCR was performed with the 5′ LLO primer and the PCR product in which the mutation was introduced as the 3′ primer. The resultant second PCR product was ligated into the pQE31 vector. The recombinant plasmid was electroporated into *Escherichia coli* SG13009. The recombinant *E. coli* clone was cultured in tryptic soy broth at 25 °C for 4–6 h in the presence of 1 mM IPTG. Bacterial lysate was prepared and recombinant protein was purified with a Ni–nitrilotriacetic acid agarose column (Qiagen). The buffer was replaced with PBS by passing through PD10 desalting columns (Amersham Pharmacia Biotech AB). The level of LPS was determined by a *Limulus* colour KY test (Wako Pure Chemical Industries). Purity was determined by Coomassie brilliant blue staining after SDS-PAGE. Each recombinant protein prepared in this study showed a single band of the predicted molecular mass following SDS-PAGE analysis (data not shown).

Assay for haemolytic activity of recombinant cytolysins. Recombinant protein was diluted twofold with PBS and incubated with an equal volume of 1 % sheep erythrocytes for 30 min at 37 °C. Supernatant was collected and the A_{415} of released haemoglobin was measured. One haemolytic unit (HU) was defined as the amount of recombinant protein required for 50 % haemolysis.

Determination of lethal toxicity of CDCs. Five C3H/HeN mice per group were injected intravenously with LLO, ILO, SLO, PLY or LLO mutants and survival time (t) was measured in seconds. Lethal activity of CDCs was defined as $1000/t$. In some experiments, LLO was treated with 10 μg cholesterol ml^{-1} overnight at 4 °C or by heating at 94 °C for 15 min to inhibit haemolytic activity. To exclude the possible contribution of contaminating LPS to the lethal effect of CDCs, we employed LPS-non-responsive C3H/HeJ mice for measurement of lethal activities.

Statistical analysis. Student's t-test was used to determine the statistical significance of the values obtained and a value of $P<0{\cdot}05$ was considered statistically significant.

RESULTS AND DISCUSSION

Dose-dependent expression of the lethal activity of LLO

In order to determine whether the lethal effect of LLO was exhibited in a dose-dependent manner, mice were injected intravenously with graded doses of LLO (Fig. 1). The lethal effect was not observed in mice injected with less than 12·5 pmol LLO. After injection of 100 pmol LLO (equivalent to 5·6 μg), mice quickly succumbed with a mean survival time of 55 s. The survival time decreased gradually with an increase in the dose of LLO. Mice given 800 pmol died as early as 26 s after the injection.

It is known that LPS induces a lethal shock to mice by inducing high titres of endogenous cytokines (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993). As the LLO prepared in this study contained a small amount of LPS [0·188 ng (μg LLO protein)$^{-1}$], the contaminating LPS might have been involved in the lethal activity of LLO. To rule out this possibility, we injected 100 pmol LLO into LPS-non-sensitive C3H/HeJ mice and monitored survival time. There was no significant difference between the mean survival time of C3H/HeN ($55 \pm 6{\cdot}7$ s) and C3H/HeJ ($51 \pm 6{\cdot}7$ s) mice, indicating that contaminating LPS was not involved in the lethal effect of LLO. In addition, mice did not die without showing any symptoms when given 5 ng *E. coli* LPS, roughly five times higher than the dose of LPS contaminating the standard injecting dose of LLO in this study.

Critical requirement of haemolytic activity for the lethal effect of LLO

A previous report has shown that the lethal effect of LLO is prevented by neutralization of the haemolytic activity (Geoffroy *et al.*, 1987). When 100 pmol LLO was subjected to treatment with cholesterol or was heated at 94 °C for

Table 1. Recombinant cytolysins used in this study

Cytolysin	Molecular mass (kDa)	Origin/characteristics
Full-length cytolysins		
Listeriolysin O	56	*Listeria monocytogenes* EGD
Ivanolysin O	56	*Listeria ivanovii* ATCC 19119
Pneumolysin	53	*Streptococcus pneumoniae* IID5114
Streptolysin O	60	*Streptococcus pyogenes* KY011
Mutant LLOs		
LLO 415	43	Domains 1–3, no domain 4
LLO C484S	56	Cys484Ser mutation
LLO W489A	56	Trp489Ala mutation
LLO W491A	56	Trp491Ala mutation
LLO W492A	56	Trp492Ala mutation

Table 2. Oligonucleotide primers used in this study

Cytolysin	Primer	Restriction enzyme site	Nucleotide sequence (5′→3′)*
Full-length cytolysins			
Listeriolysin O	Forward	*Bam*HI	CGATGGATCCTGATGCATCTGCATTCAATAAAG
	Reverse	*Pst*I	ACGCCTGCAGTTCGATTGGATTATCTACACTATTAC
Ivanolysin O	Forward	*Bam*HI	CGATGGATCCTGATGCCTCAGTATATAGTTAC
	Reverse	*Sal*I	ACGCGTCGACTTACTTATTGGATTATCTACAG
Pneumolysin	Forward	*Bam*HI	CGATGGATCCTATGGCAAATAAAGCAGTAAAT
	Reverse	*Kpn*I	ACGCGGTACCCTAGTCATTTTCTACCTTATC
Streptolysin O	Forward	*Bam*HI	CGATGGATCCTAACAAACAAAACACTGCTAGT
	Reverse	*Sal*I	ACGCGTCGACTCCTACTTATAAGTAATCGAA
Mutant rLLOs			
LLO 415	Forward	*Bam*HI	CGATGGATCCTGATGCATCTGCATTCAATAAAG
	Reverse	*Pst*I	ACGCCTGCAGTGTATAAGCTTTTGAAGTTGT
LLO C484S	Forward		CTAAAGAATCCACTGGTTTA
LLO W489A	Forward		GGTTTAGCTGCGGAATGGTG
LLO W491A	Forward		AGCTTGGGAAGCGTGGAGAAC
LLO W492A	Forward		TTGGGAATGGGCGAGAACGGTAA

*Restriction enzyme sites are underlined.

15 min, the haemolytic activity was reduced from 538 to 15·6 and 1·0 HU, respectively. These treatments completely abolished the lethal activity of LLO in mice (data not shown). Furthermore, we investigated the lethal effect of LLO 415, a truncated LLO mutant that was deficient for the C-terminal domain 4 and did not bind cholesterol (Kohda *et al.*, 2002). The truncated LLO 415 showed neither haemolytic activity nor lethal activity, indicating that the cytolytic activity of LLO is exclusively required for expression of the lethal effect *in vivo*. Geoffroy *et al.* (1987) reported that purified native LLO showed a lethal toxicity to mice and that this toxicity was inhibited completely by treatment with cholesterol or antiserum or by heating, which is consistent with our present findings.

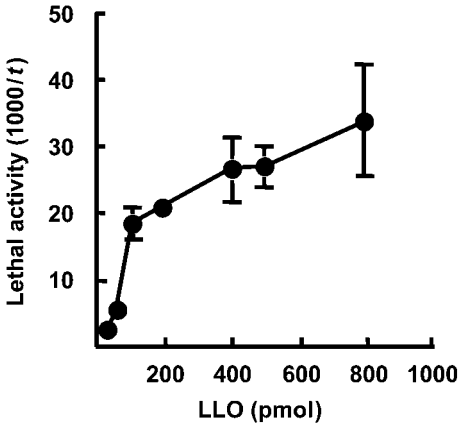

Fig. 1. Dose-dependent lethal activity of LLO. Graded doses of LLO were injected intravenously into C3H/HeN mice and survival time (*t*) was monitored in seconds ($n=5$ for each group). Lethal activity was expressed as 1000/*t*. Data represent the mean ± SD and are representative of three independent experiments.

Lethal effect and cytolytic activity in other CDC proteins

It is known that various protein toxins of the CDC family cause haemolysis by a common mechanism (Alouf, 1999; Billington *et al.*, 2000). If the cytolytic process of LLO is necessary for the lethal effect *in vivo*, it is possible that other CDC toxins also exhibit a lethal effect in proportion to their haemolytic activities. To address this point, we generated recombinant ILO, SLO and PLY. Based on the lethal activity of LLO, we injected 100 pmol each toxin and monitored the survival time (Fig. 2). As expected, all CDCs exerted a lethal effect; however, there were differences in activity among the recombinant CDC proteins examined. LLO exerted the strongest lethal effect, whilst the activity of ILO was somewhat weaker than LLO. SLO and PLY showed a significantly weaker activity than LLO. Interestingly, these lethal activities paralleled their haemolytic activities, as LLO induced a strong haemolysis, with the activity of ILO being somewhat weaker than that of LLO, and SLO and PLY showing markedly weaker haemolytic activities compared with LLO. These data indicated that there is a strong relationship between haemolytic activity and the lethal effect of CDCs.

To confirm this further, we generated several mutant LLOs exhibiting different cytolytic activities. An undecapeptide located in domain 4 in the C-terminal region is believed to be critical for binding to cholesterol on the cell membrane

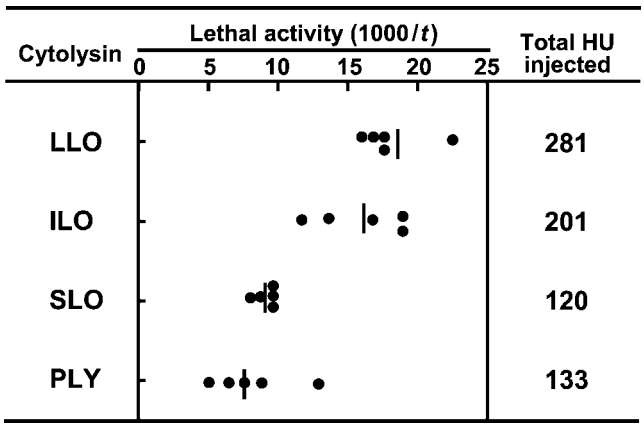

Fig. 2. Lethal effects of several CDCs. LLO, ILO, SLO or PLY (100 pmol) was injected intravenously into C3H/HeN mice and survival time (t) was monitored in seconds. Dots indicate the lethal activities of recombinant CDC proteins. Bars indicate the mean of lethal activities. The haemolytic activity of each 100 pmol haemolysin is also indicated (HU). Data are representative of three independent experiments.

and for cytolysis (Michel *et al.*, 1990; Owen *et al.*, 1994; Sekino-Suzuki *et al.*, 1996; Baba *et al.*, 2001). The requirement of domain 4 was clearly shown by the loss of cytolytic and lethal activities in a preparation of LLO containing only domains 1–3 (LLO 415) (Fig. 3). It has been reported that replacement of a unique cysteine residue located within the undecapeptide of LLO by site-directed mutagenesis resulted

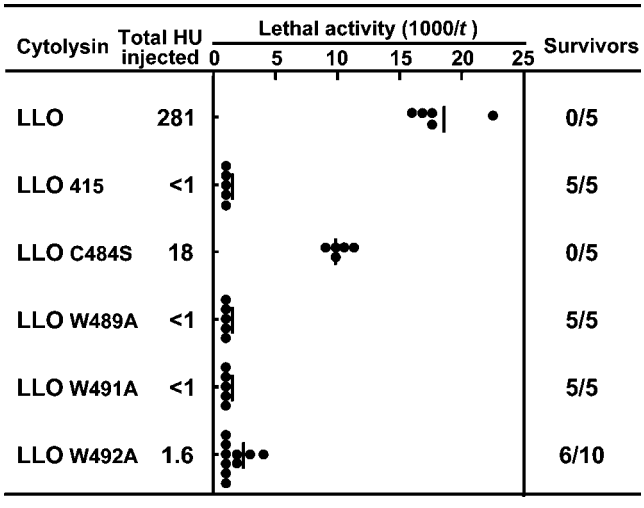

Fig. 3. Lethal effects of LLO and LLO mutants. LLO or an LLO mutant (100 pmol) was injected intravenously into C3H/HeN mice and survival time (t) was monitored in seconds. Dots indicate the lethal activities of LLO and the LLO mutants. Bars indicate the mean of lethal activities. Haemolytic activities of LLO and the LLO mutants injected into mice are indicated (HU). Data are representative of three independent experiments.

in just a minor loss of haemolytic activity. In contrast, a remarkable decrease in the cytolytic activity was demonstrated after replacement of one of the three hydrophobic tryptophan residues in the undecapeptide (Michel *et al.*, 1990). Thus, we generated four LLO mutants: LLO C484S, which showed a minor decrease in the haemolytic activity, and LLO W489A, LLO W491A and LLO W492A, in which the cytolytic activity was mostly greatly diminished. Although LLO C484S retained its lethal activity, its mean survival time was extended twofold (Fig. 3). The two non-haemolytic mutant proteins, LLO W489A and LLO W491A, did not exhibit any lethal activity. In the group of mice given LLO W492A, in which the haemolytic activity was severely affected, six out of 10 mice survived. Based on these results, it seems that the lethal effect of CDCs is highly dependent on the cytolytic process of CDCs. In order to show more clearly the correlation between the lethal effect and haemolytic activity, we plotted the lethal activity ($1000/t$) data against the cytolytic activity (HU) of CDCs and LLO mutants and found that the correlation coefficient ($r=0{\cdot}911$) was significant. Furthermore, Howard & Wallace (1953a, b) showed that rabbits were more sensitive to the lethal action of SLO than mice and that rabbit erythrocytes were also sensitive to cytolysis by SLO compared with mouse erythrocytes. Halbert *et al.* (1963) reported a similar potency of SLO. These reports support the correlation between cytolytic and lethal activities.

As mentioned above, cholesterol-treated LLO displayed no detectable lethal effect, despite retaining a haemolytic activity of 15·6 HU. In contrast, the injection of similar cytolytic activity (18 HU) of the LLO C484S mutant protein resulted in a clear, albeit reduced, lethal effect in this experiment. In this regard, it was also conflicting that LLO W492A exhibiting only 1·6 HU displayed some lethal activity. Judging from the dose-dependency of the lethal activity of LLO shown in Fig. 1, there appears to be a threshold level of $\sim$100 pmol of active molecule required for lethal activity. Therefore, it is possible that a small number of active cytolytic molecules remaining intact after cholesterol treatment were not able to cause the final cardiotoxicity, while all of the molecules of mutant proteins with a very low specific activity were engaged in affecting cardiac function. A second possible explanation for the discrepancy in the activity of cholesterol-treated LLO may be an altered *in vivo* distribution after injection. As cholesterol treatment results in the formation of protein–cholesterol complexes *in vitro*, it is conceivable that, due to hydrophobic interactions, such complexes form a microaggregation with which the remaining active LLO molecules associate and then are cleared from circulation more quickly than other soluble mutants. A third possibility is that a small proportion of active LLO remaining after cholesterol treatment is more susceptible to the blocking effect of plasma cholesterol in the circulation compared with the susceptibility of mutants with far lower specific activity. At present, we have no direct evidence to explain the abolished lethality following cholesterol treatment whilst retaining some haemolytic

activity. Due to the difficulty in completely abolishing haemolytic activity with *in vitro* cholesterol treatment, it seems that more than one of the above-mentioned mechanisms may be involved.

Many studies have suggested that CDCs affect cardiac function (Halbert, 1970; Kingdon & Sword, 1970; Bernheimer, 1976). It has been found that certain substances with antiserotonin activity protect animals against the acute lethal effects of SLO (Halbert *et al.*, 1963). The extremely rapid alteration in cardiac function elicited by SLO might result from the release of vasoactive substances rather than from the direct cytotoxic action of the protein itself (Alouf & Palmer, 1999). Kingdon & Sword (1970) reported that creatine phosphokinase levels in plasma increased after an injection of haemolysin, suggesting that haemolysin causes damage to the myocardium. In the present study, it was shown clearly that the cytolytic activity of any of the CDC proteins was indispensable for the expression of lethal activity *in vivo*. However, the molecular mechanism for the cardiotoxicity is yet to be determined at the molecular level. The various recombinant CDC proteins constructed in this study may be useful tools in this line of study.

We have shown here that CDCs are capable of exhibiting lethal activity in mice when injected intravenously in the form of purified recombinant protein. In the case of infection with bacteria producing CDC, however, it is open to argument whether CDC protein contributes to pathogenesis by exhibiting such a direct lethal effect. For instance, LLO, a major virulence factor of *Listeria monocytogenes*, enables the bacterium to escape from macrophage phagosomes. It has been reported that the cytolytic activity is exerted only in acidic conditions and not in neutral to alkaline conditions as observed in the cytoplasm (Geoffroy *et al.*, 1987). Therefore, it is unlikely that LLO, even if released into the circulation, affects the cardiac function of the host infected with *L. monocytogenes*. On the other hand, it has been shown that PLY is a virulence determinant of *Streptococcus pneumoniae* and contributes to its pathogenesis by exerting a haemolytic activity (Jounblat *et al.*, 2003). In addition, SLO is known to be required for the virulence of *Streptococcus pyogenes*; however, mutation in the gene encoding SLO resulted in little effect on lethality in infected mice and SLO may contribute to virulence by an unknown mechanism other than lethal toxicity (Limbago *et al.*, 2000). Taken together, these results indicate that the direct engagement of cardiotoxicity of the CDC protein does not seem to be essential for the lethality in the infected host, although this activity has long been known as one of the biological activities of the CDC family of toxins.

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