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Thiol-activated Cytolysins from Gram-positive Bacteria as a Possible New Bacterial Modulin: Implication in the Immune Response

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Thiol-activated cytolysins (TACYs) are a family of 50 to 60 kDa single chain protein toxins capable of forming transmembrane pores on the target cells leading to cytolysis (1). TACYs are characterized by activation at reducing condition, reversible inactivation by oxidation and irreversible inactivation by treatment with cholesterol. A unique sequence of ECTGLAWEWWR is highly conesrved in the C-terminus of all the TACYs. Over 20 different species of Gram-positive bacteria are known to produce TACYs, among which perfringolysin O (PFO from *Clostridium perfringens*) and pneumolysin (PLY from *Streptococcus pyogenes*) have been studied extensively from the biochemical point of view. Listeriolysin O (LLO) encoded by 529 bp *hly* gene of *Listeria monocytogenes* is a 58 kDa protein toxin belonging to this family. From the observation of intracellular fate of *hly* mutant, it is established that LLO contributes to the virulence expression by virtue of the lysis of phagosomal membrane enabling this bacterium to escape from endosomal compartment into the cytosolic space in macrophages. Thus, LLO is the essential virulence factor of *L. monocytogenes*, though the biological function of other TACYs has not yet been elucidated well.

L. monocytogenes is one of the typical intracellular parasitic bacteria residing inside macrophages of the infected host. Because of the presence of pathogenicity island including *hly* in the chromosomal DNA, *L. monocytogenes* is capable of escaping from the killing mechanism of macrophage and spreading cell-to-cell. Infection of mice with *L. monocytogenes* results in the growth of bacteria in the liver and spleen in a primary infection. When mice survives the infection, a strong cell-mediated immunity develops. *Listeria*-specific protective immunity is highly effective against challenge infection. The major effector cells operating in the protective immunity are TH1 cells that produce macrophage-activating cytokines upon encountering with listerial antigen. During the course of our studies on the generation of *L. monocytogenes*-specific TH1 cell, we have found that LLO-producing ability of bacteria is the prerequisite for the induction of immunity (2). Purified LLO was found to be capable of inducing endogenous cytokines contributing to the induction of TH1 cells (3). Among various cytokines expressed in the spleen cells by stimulation with purified LLO, IFN-γ was revealed to be the most important in the induction of protective TH1 cells (4). IFN-γ production was mainly from NK cells in response to IL-12 and IL-18 produced by macrophages (5). From these results, it became clear that macrophages first respond to LLO, then macrophage-derived cytokines stimulate NK cells to produce IFN-γ. LLO, a known virulence factor of *L. monocytogenes*, appears to

play a pivotal role in the induction of protective immunity of the host through cytokine-inducing ability (6, 7).

This newly recognized activity of LLO could be applied as T-cell adjuvant to the induction of protective immunity. LLO-negative strains or killed cells of *L. monocytogenes* are not able to induce protective immunity, however, the induction of protective immunity became possible by means of administration of purified or recombinant LLO along with these non-immunogenic strains in mice (8, 9).

Various bacterial components have been known to be active in cytokine induction in an antigen-nonspecific manner. LPS, lipoprotein or peptidoglycan are bacterial modulins capable of stimulating macrophages for cytokine expression, and TLR (Toll-like receptor) is implicated in the recognition of such bacterial modulin and following signal transduction. In order to determine the structure-function relationship of LLO and related toxins of TACY family, we have constructed various mutant proteins and truncated proteins by using His-tagged recombinant technique. The recombinant full-length LLO exhibited ability for both cytolysis and cytokine induction. Cytolytic activity was completely blocked by free cholesterol, while the cytokine-inducing ability was expressed after cholesterol treatment, suggesting the dissociated mechanisms operate in these two phenomena. Recombinant proteins truncated for C-terminal portion are not cytolytic any more and replacement of any amino acid comprising the unique undecapeptide (ECTGLAWEWWR) resulted in the loss of cytolytic activity. The C-terminal portion was also important in the full expression of cytokine-inducing ability, but some truncated proteins were still active in cytokine induction. According to the reported 3D structure of PFO, all the TACYs seem to be consisting of four domains. The recombinant preparation of the 4th domain was not hemolytic but was capable of blocking the binding of full-length protein, suggesting that the domain 4 of TACYs is the site of binding to membrane (10). Some preparation of domain 1-3 with a complete lack of domain 4 still exhibited the cytokine induction. These results suggest that N-terminal portion stimulate the cytokine expression, and the binding of domain 4 initiates the membrane lysis.

The cytolysis by TACYs appears to depend on the binding of C-terminal portion to membrane cholesterol followed by oligomerization and pore formation. In contrast to the process of membrane lysis, TACYs appears to be active in the stimulation of cytokine induction probably through binding of N-terminal portion (11). The detail of molecular mechanism for binding and signaling by TACYs is under extensive study. The recognition of pore-forming cytolysins from gram positive bacteria as a possible new bacterial modulin may pave the way to a better understanding of pathophysiology and immunology of infections by Gram-positive bacteria producing TACYs.

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