Comparative Toxinology of *Loxosceles reclusa* and *Corynebacterium pseudotuberculosis*

Abstract. *In contrast to other kinds of phospholipases, phospholipases D that are toxic for humans and animals are not commonly encountered as constituents of venoms or as products of pathogenic microorganisms. Toxic phospholipases D are present, however, in the venom of the brown recluse spider* (Loxosceles reclusa) *and in supernatants or filtrates of cultures of* Corynebacterium pseudotuberculosis. *Although the two enzyme toxins are derived from phylogenetically disparate entities, they are similar in molecular weight, charge, substrate specificity, and in several biological activities. They are immunologically distinguishable.*

The brown recluse spider (*Loxosceles reclusa*) inflicts painful bites on many humans in the United States each year. The toxicity of its venom is partially or wholly due to a phospholipase D (PLD) (*1, 2*). Phospholipases, especially phospholipases A_2, are frequently found as constituents of animal venoms, including those of snakes, bees, and other arthropods. In contrast, PLD (*3*) occurs rarely in venoms.

Among bacteria, extracellular toxic PLD's are produced by certain pathogenic corynebacteria (*4*), namely by the causative agent of pseudotuberculosis in sheep (*Corynebacterium pseudotuberculosis*, formerly called *Corynebacterium ovis*) and by *Corynebacterium ulcerans*, which is associated with bovine mastitis and which also inhabits the normal human pharynx where it occasionally gives rise to a diphtheria-like disease.

The PLD of *L. reclusa* (Lox-PLD) is similar to that of *C. pseudotuberculosis* (Cor-PLD) in several ways. The two enzymes are physically similar: both are basic proteins having *p*I values of 8.7 (major peak) for Lox-PLD and 9.6 (*5*), 9.8 (*6*), or 9.1 (*7*) for Cor-PLD. The molecular weight of Lox-PLD has been estimated independently to be 32,000 (*1*) and 34,000 (*2*) and we estimate that of Cor-PLD to be 31,000 (*6*).

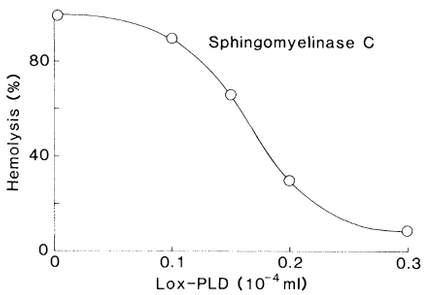
In contrast to most phospholipases, the two enzymes have a similar but unusual substrate specificity. Of the four major phospholipids in mammalian cell membranes, only sphingomyelin is hydrolyzed by Cor-PLD (*8, 9*) and the same is true for Lox-PLD (*10*). In addition, both enzymes are capable of splitting lysophosphatidylcholine.

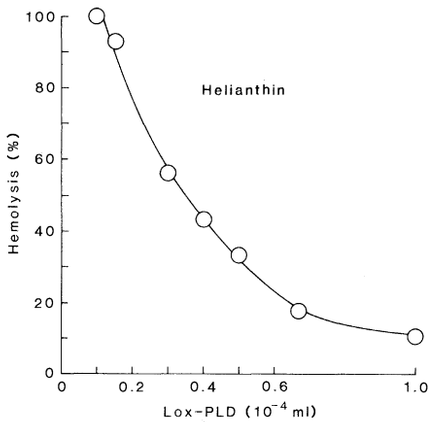
Treatment of sheep erythrocytes with minute amounts of Cor-PLD renders the cells totally resistant to lysis by staphylococcal sphingomyelinase C (*6, 11*). Inhibition of hemolysis is the basis of a simple and rapid method for assaying Cor-PLD (*6*). Lox-PLD is also highly effective in inhibiting lysis of sheep erythrocytes induced by sphingomyelinase C (Fig. 1). When either of these PLD's is used, the membrane sphingomyelin of the erythrocytes is converted to *N*-acylsphingosyl phosphate (ceramide phosphate), which is not a substrate for staphylococcal sphingomyelinase C. Although this enzymic alteration of membrane sphingolipid can account for loss of sensitivity to lysis by the staphylococcal enzyme, a different mechanism may operate in that both Cor-PLD and Lox-PLD readily bind to membranes, steric hindrance in binding may occur. It is not clear to what extent each of these two mechanisms accounts for loss of sensitivity to lysis by the staphylococcal enzyme.

Cor-PLD also protects erythrocytes from hemolysis by the nonenzymatic cytolysin (helianthin) from the sea anemone *Stoichactis helianthus* (*6, 12*). Helianthin is thought to bind specifically to sphingomyelin (*6, 12*). We found that Lox-PLD, like Cor-PLD, is a potent inhibitor of helianthin-induced lysis of sheep erythrocytes (Fig. 2).

When either Cor-PLD or Lox-PLD was mixed with sheep erythrocytes at 0°C and then centrifuged, most of the PLD (as measured by loss of sensitivity of the cells to staphylococcal sphingomyelinase C) disappeared from the supernatant (Fig. 3). Although other explanations are possible, the likely one is that the enzyme binds to the cell surface. Independent evidence for a strong affinity of isolated cell membranes for both PLD activity and dermonecrotic activity of *L. reclusa* venom has recently appeared (*2*).

Is immunological cross-reactivity between the two PLD's demonstrable? Decreasing amounts of the immunoglobulin G (IgG) fraction of sheep antiserum to *C. pseudotuberculosis* toxoid (*13*) were mixed with a constant amount, in terms of activity of either Cor-PLD or Lox-PLD and allowed to stand for 10 minutes at 25°C, after which washed sheep erythrocytes were added, and the mixtures





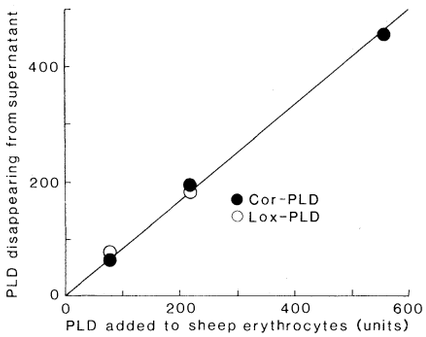


Fig. 1 (top left). Inhibition of hemolytic action of staphylococcal sphingomyelinase C (beta-toxin) by Lox-PLD. Conditions of the experiment were those described for the antihemolytic assay of Cor-PLD (*4*). Lox-PLD was isoelectrically purified sphingomyelinase D (fraction A) (*1*), containing 0.9 μg/ml as estimated from absorbance measurements. The concentration of sphingomyelinase C was approximately 5 ng of pure protein per milliliter, and the final concentration of erythrocytes was 0.35 percent by volume. The plotted data indicate that this preparation contained 23,500 beta-toxin inhibition units per milliliter. Fig. 2 (right). Inhibition of helianthin-induced lysis of sheep erythrocytes by Lox-PLD. Conditions of the experiment resembled those of Fig. 1 except that helianthin (0.14 μg/ml) (*12*) was substituted for staphylococcal sphingomyelinase C. Fig. 3 (bottom left). Removal of sphingomyelinase D by washed sheep erythrocytes at 0°C as measured by inhibition of hemolysis induced by sphingomyelinase C. Suspensions (4 ml) of sheep erythrocytes (0.7 percent by volume) in tris-buffered saline (*p*H 7.2) were mixed with PLD in tris-buffered saline containing 0.2 percent gelatin and 0.01*M* $MgCl_2$ and then centrifuged.

were held at 25°C for an additional 30 minutes. Sphingomyelinase C was added, and the mixtures were placed in a 37°C bath for 30 minutes, and then in an ice bath for 10 minutes. After brief centrifugation, hemolysis was estimated from the quantity of hemoglobin in the supernatants. The amount of IgG that prevented Cor-PLD from inhibiting sphingomyelin-induced hemolysis was 28 μg, whereas 200 times as much IgG did not neutralize the inhibitory activity of Lox-PLD. The two enzymes are therefore immunologically distinguishable. Bacteriological examination of venom from live *L. reclusa* did not reveal the presence of corynebacteria.

Cor-PLD splits the sphingomyelin of the vascular endothelial membrane and this may account for increased vascular permeability concomitant with the development of lesions (*14*). Vascular damage is also considered to play an important role in the genesis of lesions by Lox-PLD because, presumably through splitting of sphingomyelin in platelet membranes, platelet aggregation accompanies thrombus formation. This effect is probably accompanied by release of inflammatory substances (*1, 2*). Further work is needed before a conclusion can be drawn as to the precise degree of similarity that may exist between the pathological changes produced by the two PLD's.

ALAN W. BERNHEIMER
Department of Microbiology, New York University School of Medicine, New York 10016
BENEDICT J. CAMPBELL
LAWRENCE J. FORRESTER
Departments of Biochemistry and Microbiology, University of Missouri, Columbia 65212

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13. Antitoxic serum from a sheep immunized with *C. pseudotuberculosis* toxoid was supplied by H. R. Carne in 1973. The IgG was prepared from it by DEAE-cellulose chromatography by R. Linder. The solution of IgG used contained 140 mg of protein per milliliter as measured optically by the method of J. R. Whitaker and P. E. Granum [*Anal. Biochem.* **109**, 156 (1980)].
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15 November 1984; accepted 9 January 1985

Ethanol Neurotoxicity: Effects on Neurite Formation and Neurotrophic Factor Production in Vitro

Abstract. *The effects of ethanol on chick embryo sensory and spinal cord neurons growing on one of several biological substrates (poly-D-lysine, laminin, or neuron-produced neurite-promoting materials) were examined. Ethanol inhibited process formation by the neurons in a dose-dependent manner and inhibited the production of neurotrophic factors. Neuronal attachment to the substrates, survival of attached neurons, and receptor interactions of sensory neurons with nerve growth factor were not influenced by ethanol. It appears that ethanol alters certain metabolic characteristics of developing neurons.*

Prenatal exposure to ethanol is associated with characteristic effects in offspring (*1*), ranging from craniofacial dysmorphology and central nervous system abnormalities [the fetal alcohol syndrome (*2*)] to subtle neurological and behavioral disturbances. Neuropathological observations suggest that the effects of ethanol on the developing nervous system result from errors in neuronal migration (*3*). Migratory events are a very early component of neurogenesis and involve interactions of neurons with a matrix upon which they move and extend processes. These events depend on the ability of the neuron to interact with the extracellular matrix of growth and with diffusible trophic factors in its milieu. We measured matrix interaction and process formation in vitro in order to examine directly the effects of ethanol on embryonic neurons.

Dissociated neuronal cultures were prepared from 8-day chick embryo dorsal root ganglia (DRG) and spinal cord (*4*). Sensory neurons from DRG were plated in wells with nerve growth factor (NGF; 90 pg/ml) and ethanol (1, 10, 25,

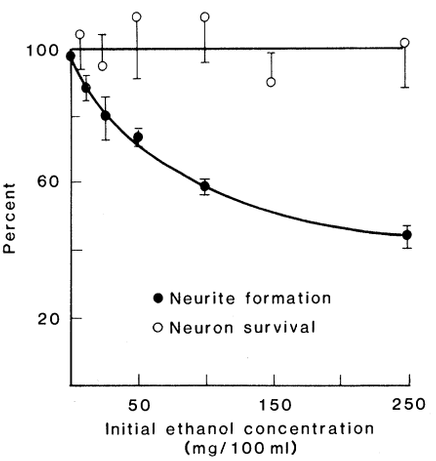

Fig. 1. Effect of ethanol on neuronal process formation and survival on poly-D-lysine. DRG neurons were obtained from 8-day chick embryos (*4*) and incubated in Ca^{2+}- and Mg^{2+}-free buffer with 0.01 percent trypsin and deoxyribonuclease (0.05 mg/ml; Worthington) for 10 minutes at 37°C. The reaction was stopped by removing the incubation solution and adding Ham's F12 medium containing 5 percent fetal calf serum (FCS). After trituration with a 5-ml pipette, the cell suspension was centrifuged (750*g* for 8 minutes at 4°C) through a concentrated FCS gradient, suspended in Ham's F12 with 5 percent FCS, plated on a Falcon culture dish, and incubated for 2 hours at 37°C in 5 percent CO_2. The nonadherent, neuron-enriched population was then seeded on 96-well Falcon microculture plates coated with poly-D-lysine (0.1 mg/ml for 24 hours) at a plating density of 10^4 cells per well in the presence of NGF (90 pg/ml) and ethanol. After 24 hours of incubation at 37°C in 5 percent CO_2, the wells were examined at ×200 and the number of cells with processes greater than 1.5 cell diameters were counted on a representative diameter of the culture well. Results for neurite formation are expressed as percentages (means ± standard deviations; $n = 3$) of the maximum response to NGF. When process formation was scored 10 μl of MTT (5 mg/ml) was added to 100 μl of medium in each well, and the wells were incubated for 4 hours at 37°C. Then 100 μl of 0.04*N* HCl in isopropanolol was added to all wells with thorough mixing. After a few minutes at room temperature the wells were read on a Titertek Multiskan MC Microelisa reader at a wavelength of 570 nm. Absorbance was directly proportional to cell number over the range 1×10^3 to 5×10^4 cells per well. Results for neuron survival are expressed as percentages (means ± standard deviations; $n = 4$) of control absorbance at 570 nm.