α-HL belongs to the category of membrane pore forming toxins which are water soluble proteins capable of forming channels in the membrane. The assembly of proteins into the membranes is an intricate phenomenon in which these proteins undergo a large degree of conformational changes in exerting the biological functions. Unlike ion channels, membrane associated proteins and cell surface receptors, these toxins do not have well defined stretches of hydrophobic amino acids in their sequences. Hence the localized environment experienced by the stretches of amino acids that insert into the membranes appear to control the assembly and function of some of these toxins. A detailed study of such toxins would provide valuable information regarding the spatial and structural organization of the polypeptide segments that span the membrane layer. Through previous studies, it has been shown that the pore formation takes place through a series of intermediates and the activity of α-HL is critically dependent on intact amino and carboxy domains. In the recently reported crystal structure of α-HL heptamer, the amino terminus has been shown to form a latch that participates in interprotomer interactions. The amino terminal sequence was earlier shown to be accessible to site specific modification in water soluble monomer, membrane bound monomer and prepore heptamer. In order to gain insight into how a well exposed segment controls the assembly and the role it plays during the pore formation, a detailed study of amino terminal deletion mutants of α-HL is warranted. Unfortunately, all N-terminal deletion mutants known so far have not provided any significant information regarding the extent of involvement of amino terminal in assembly and pore formation. Present study describes an amino terminal deletion mutant α-HL(5-293), which for the first time is capable of undergoing all the conformational changes that are necessary to form the functional pore. The biochemical studies showed that it assembles as fast as native full length α-HL but shows retardation in the kinetics of assembly i.e. α-HL(5-293) takes longer time to achieve same degree of lysis caused by α-HL. This implicates the involvement of N-terminus in the final opening up of the pore. Electron microscopic studies also showed that pores formed by α-HL(5-293) are similar to those of α-HL. α-HL(5-293) like molecules which does not cause instant damage to the membrane may be useful for studying various characteristics of target cells e.g. permeabilization characteristics, intracellular events which takes place when such membrane pore formers bind to the cell surface. Signal transduction events can also be studied since no damage would

occur to a cell in first 45-60 mins inspite of the cell being under stress condition. We have exploited this molecule to study the signal transduction pathway in epidermal cells.

Previous studies on PC12 cells have shown that treatment of the cells with α-HL leads to reduction of binding of ^{125}I-EGF to EGF receptors and inhibition of autophosphorylation of EGF receptors. These observations are interesting but they could not be explained. A common mechanism for α-HL action can not be proposed because it evokes different kind of responses in different kind of cells. To study the effect of α-HL on epidermal cells, A431 epidermoid carcinoma cell line was chosen which overexpresses EGF receptors on cell surface. Since α-HL is a membrane damaging toxin, it can act by (i) forming pores on the cell membrane (ii) intercepting signal transduction pathway (iii) both the mechanisms simultaneously. To narrow down the possibilities, we created three different molecules (a) full length α-HL(1-293) (b) N-terminal deletion mutant α-HL(5-293) (c) C-terminal deletion mutant α-HL(1-289) by amplifying the genes and cloning them in *E.coli* JM109 followed by their expression in JM109(DE3) and subsequent purification of proteins as described.

Detailed studies on these mutants showed that these three molecules arrest three different stages in the assembly of α-HL. α-HL(1-289) remains as water soluble monomer for a long time. α-HL(5-293) assembles to a prepore heptamer as fast as full length α-HL but conversion to final lytic pore is very slow hence it stays in prepore form for longer duration. Native α-HL undergoes all the steps of assembly rapidly to form a functional pore. When A431 cells were treated with 60.0 nM α-HL and α-HL(5-293), both of them inhibited phosphorylation of EGF receptors in 2 min. α-HL(1-289) which remains predominantly in membrane bound monomer form as shown by ^{35}S labeled protein and does not show any oligomerization or lysis within this time frame did not show any inhibition of EGF receptor phosphorylation with purified α-HL(1-289) because the purified protein was found to be unstable and prone to aggregation. When it was added to A431 cells suspended in DPBS, most of it was found in precipitated form. Using α-HL and its deletion mutants, our studies prove that pore formation is not at all required to inhibit phosphorylation of EGF receptors on A431cells which is a major early event in signal transduction pathway mediated by

EGF receptors. Two possibilities have been suggested by which EGF receptor phosphorylation inhibition takes place : (a) the process of binding and assembly of the toxin on membranes is sufficient to cause membrane perturbations which indirectly affect EGF receptor intercepting its function. (b) the crystal structure of α-HL heptamer has shown that each monomer is involved in several hydrophobic interactions, salt bridges and hydrogen bond formation. It seems, once a monomer binds the membrane, it undergoes a conformational change and aquires a state which can participate in variety of interactions. On a membrane surface where it has equal probability of encountering another such monomer or EGF receptor, direct interaction of membrane bound α-HL monomer with the receptor cannot be ruled out.

Besides being hemolytic, cytotoxic and lethal, α-HL is also dermonecrotic. But the phenomenon of dermonecrosis is not very well understood. Since skin epidermis also possess EGF receptors on cell surface, the study of interaction of α-HL with EGF receptor may shed some light on the process of dermonecrosis caused by α-HL. Present study for the first time laid a foundation for understanding the process of dermonecrosis caused by α-HL. α-HL and its deletion mutants have clearly demonstrated that α-HL not only damages the cell membrane but also interferes with signal transduction pathway involving EGF receptor by inhibiting their phosphorylation. At the same time, it also gives rise to many interesting questions : (i) Does α-HL actually binds to the receptor? If yes, what kind of interactions are involved (ii) Does α-HL prevents dimerization of the receptors? (iii) Does inhibition of phosphorylation in signal transduction pathway triggers apoptosis? (iv) Does α-HL affect other receptors on other cell lines in similar fashion? Studies in this direction will help in understanding the mechanism further.

This data is of immense significance as inhibitors of receptor tyrosine kinases are in great demand at present. Receptor tyrosine kinases have been found to be overexpressed on various cancer cells. Thus, they act as target for therapy. α-HL can be directed specifically to the target cells by attaching them to a guiding moiety in the form of immunotoxin. For immunotoxins, it is always desirable to have a molecule which has minimum effect on normal cells and causes maximum damage to target cells.

α-HL mutants are ideal candidates for this purpose since they can be easily engineered further to obtain minimum portion which is required for inhibiting signal transduction pathway.