MOLECULAR AND BIOCHEMICAL STUDIES ON ANTHRAX LETHAL TOXIN

THESIS

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOSCIENCES AND BIOTECHNOLOGY

BY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MOLECULAR AND BIOCHEMICAL STUDIES ON ANTHRAX LETHAL TOXIN** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biosciences and Biotchnology, University of Roorkee is an authentic record of my own work carried out during a period from July 1993 to April 1996 under the supervision of **Dr. Yogendra Singh,** Centre for Biochemical Technology, Delhi and **Dr. Rajesh P. Singh,** Department of Biosciences and Biotechnology, University of Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other university.

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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

Anthrax lethal toxin consists of two proteins, protective antigen (PA), and lethal factor (LF). These proteins are individually non-toxic. However, combination of PA and LF is toxic to animals and some macrophage cell lines and is known as lethal toxin (LT). In the process of cytotoxicity, PA (binding moiety) binds to the cell surface receptors and facilitates the internalization of LF (catalytic moiety) into the cytosol for its activity. In the present study, LF gene was subcloned, expressed and purified from E. coli. The biological activity of E. coli expressed LF was comparable to LF purified from *B. anthracis*. LF is the cytotoxic molecule of the toxin, because when PA and LF alone or in combination were internalized into the cytosol through virosomes, LF or PA+LF were toxic to the macrophage cells. Like other bacterial toxins, which require intracellular processing and activation for their biological activity, PA of LT is proteolytically cleaved for internalization and translocation of LF into the cytosol. No proteolytic activation or intracellular processing of LF was required for its biological activity. Intracellular distribution studies using ^{125}I-LF indicated that large fraction of LF remains associated with lysosome and cytosol. Studies using brefeldin A suggested that golgi has no role to play in the activity of LT. Inhibition of protein synthesis was not the primary event in the toxicity of LT as reported for several bacterial protein toxins. However, inhibition of host cell protein synthesis by cycloheximide provided protection to the macrophages against LT challenge. These results suggest that host cell factors are essential for the cytotoxicity of LT. In the process of toxicity Ca^{++} influx precedes the cytolysis of the macrophages. LT enhances the inositol phosphate production in the cell, which could be responsible for the increased influx of Ca^{++} and cell lysis.

Studies on the structure of the toxin proteins suggest that PA is predominantly a β-class protein, whereas LF has both α-helix and β-sheets in its secondary structure. Spectrofluorometric studies reveals that LF exposes hydrophobic residues at acidic pH, which may facilitate the translocation of the

protein into the cytosol. Stoichiometric studies suggest that PA and LF combine in an equimolar ratio.

These studies will be helpful in elucidating the mechanism of action of anthrax lethal toxin and could be useful in designing and developing new therapies against the disease.

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ABBREVIATION

A	Absorbance
ANS	Anilino naphthalene sulfonate
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
B. anthracis	*Bacillus anthracis*
BCA	Bicinchoninic acid (4,4'-dicarboxy-2,2'-biquinoline)
bp	Base pair(s)
^{o}C	Degree celsius
C	Concentration in mole
CD	Circular Dichroism
Ci	Curie
cAMP	Cyclic adenosine monophosphate
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediamine-tetraacetic acid
EF	Edema factor
FCS	Foetal calf serum
g	Relative Gravitational Force
GST	Glutathione-S-transferase
HBSS	Hanks balanced salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRP	Horse radish peroxidase
IL	Interleukine
IPTG	Isopropyl-B-D-thio-galactopyranoside
IP	Inositol phosphate
kb	Kilobase
kDa	Kilo dalton

L	Path length in nanometer
LB	Luria Bertani medium
LF	Lethal factor
LT	Lethal toxin
mg	milligram
min.	Minute(s)
ml	Millilitre
MTT	3-(4-5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide
nm	nanometer
ng	nanogram
OD	Optical density
PA	protective antigen
PAn	Trypsin nicked PA
PAGE	Polyacrylamide gel electrophoresis
PI	Phosphatidyl Inositol
PMSF	Phenyl methyl sulfonate fluoride
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethyl ethylene diamine
TMB	Tetramethyl benzedine
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) amonium methane
μg	Microgram
μm	Micrometer
μl	Microliter
μCi	Micro Curie
ψ	Observed ellipticity
θ	Molar ellipticity
α	Alpha
β	Beta
γ	Gamma

INTRODUCTION

INTRODUCTION

Anthrax is a bacterial disease which continues to be a threat for both human and animal lives. Sporadic incidences of the disease are being reported from different parts of the world even today. Anthrax is a major cause of mortality and threat to several endangered species in enzootic areas (Pienaar 1967, Ebedes, 1976). It is primarily a disease of herbivores and before the Sterne's vaccine in late 1930s, it was one of the foremost causes for heavy loss of cattle, sheep and goats leading to major economic set-back to livestock farmers. The etiological agent of anthrax is a Gram positive, aerobic, spore forming, non- motile bacilli, *Bacillus anthracis*. It is the only virulent species found in the entire genera bacillus, infecting a wide range of animals including humans (Merchant and Packer, 1968). Man is infected directly by inhalation of spores, indirectly by handling infected animals or by consuming contaminated animal products.

In 1870's several outbreaks of anthrax caused severe economic loss to farmers and leather industries, which led to the studies on anthrax and development of vaccine against the disease. *B. anthracis* produces spores which can survive indefinitely in a dry environment. The spores are highly resistant to temperature and most of the disinfectants. These features favoured the use of anthrax spores as a potential agent for biological warfare. Pasteur with Chamberland and Roux in 1881, proved that immunity did exist after using two vaccines which contained attenuated strains of *B. anthracis*. Pasteur's vaccine, which consisted of capsulated non-toxigenic strain of *B. anthracis*, is of historical importance as the first widely acknowledged bacterial vaccine. The Pasteur's vaccine was widely used in cattle and sheep over the next fifty years. In the 1920s and 1930s several modifications to the Pasteur's vaccine were introduced to increase longivity and immunogenicity. In 1937 Sterne formulated a live spore vaccine which is still in use throughout the world (Leppla, 1991). The protection studies, quality control tests and field reports indicated a residual virulence in

Sterne vaccine. The live spore vaccine is considered unsuitable for administration to humans in several parts of the world.

The search of the disease causing factors of *B. anthracis*, led Smith and Keppie to discover anthrax toxin in 1954. Subsequently it was shown that there are at least two virulence factors in *B. anthracis*, a three component protein toxin (Protective antigen, PA; Edema factor, EF; Lethal factor, LF) and poly D-glutamic acid capsule.

The importance of PA in vaccine was established in 1950s but only recently with the purification and genetic manipulation of the toxic components, it became apparent that antibodies to PA are essential for protection against anthrax. Though PA plays the major role in providing the humoral response, there are reports indicating a significant contribution of LF and EF towards the immunity of the disease (Ivins and Welkos, 1988; Pezard et al., 1995).

Thus, studies were carried out to characterize the protective epitopes and functional domains on the toxin proteins. The present study was undertaken with following objectives:

1. Expression and purification of lethal factor (LF) from Escherichia coli (E. coli).
2. Introduction of LF into cytosol in order to examine its ability to lyse the eukaryotic cells.
3. Intracellular trafficking of LF in the cytosol.
4. Processing and activation of LF in the cytosol, and
5. Biophysical studies of the toxin proteins.

These studies will increase our knowledge in understanding the mechanism of action of lethal toxin which could lead to the development of an effective therapy against anthrax.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Anthrax disease has been known since antiquity, as its reference can be seen in the ancient texts of Hippocratic era (Greek physician of 5th Century BC), and is still prevalent in different parts of the world. For the first time the organism *B. anthracis* was seen as a non-motile, filiform body in the blood of animals which died due to anthrax (Ray and David, 1850). Delafond in 1860 first demonstrated these small bodies in the blood of animals infected artificially or naturally with anthrax. Brauell, in 1857 reported that the disease was intertransmissible between human and animals and could be reproduced in healthy animals by injecting them with infected blood. Davaine, in a series of studies between 1863-1868 showed the presence of filiform bodies as a consistent feature in the blood of infected animals or malignant postules of patients suffering from anthrax. He designated these bodies as **bacterides** and reproduced the disease by injecting these bacterides in rabbits and guinea pigs. It was Robert Koch in 1876, who put forward his famous **postulations** and established Bacillus anthracis as the causative organism of anthrax. Louis Pasteur used *Bacillus anthracis* in his earlier studies on the **germ theory** and gave the first live, heat attenuated anthrax vaccine for field trial in 1881. Bail, in 1904 found that sterile filtrates of edematous fluid or macerated lesion carrying tissue extract of anthrax produced identical lesion when injected into healthy animals. In the process, inoculated animals were immunized actively against anthrax. Gladstone in 1946 demonstrated that the soluble protective antigen of anthrax was the component responsible for immune response against the disease and could be extracted from the culture supernatant of *B. anthracis*. It was later identified as a protein, as it was susceptible to heat ($56^{o}C/30$ min) as well as trypsin (Gladstone,1946 and Watson et al., 1947). Smith and Keppie in 1954 developed anthrax in healthy guinea pigs by injecting sterile plasma from the infected blood (Smith and Keppie, 1954). Watson and his group in 1947 showed that the crude soluble filtrate of *B. anthracis* is toxic to the recipient animals and contains an additional factor responsible for toxicity which is different from the

protective immunizing agent. Such toxic factor could be removed by adsorption on calcium phosphate (Watson et al., 1947). Later, the factor responsible for causing edema in the skin was separated from PA by adsorption to glass filters (Stanley and Smith, 1961; Beall et al., 1962). The third component in anthrax toxin mixture responsible for the killing of rats and guinea pigs was identified and designated as LF (Stanley and Smith, 1961; Smith and Stanley, 1962; Beall et al., 1962). These observations led to the conclusion that the anthrax toxin consists of multiple protein factors and there is an interdependency between the factors to elicit the lethal effect in animals (Smith et al., 1956).

B. anthracis is a Gram positive, cylindrical (1-1.2 μm x 3-8μ m in size), flagellated, capsulated and non-motile organism which grows in the form of chains. It is a facultative aerobic organism and produces spores in adverse growth conditions. Under favourable conditions, the spores germinate to produce vegetative bodies. These vegetative bodies of anthrax are susceptible to heat, disinfecting agents, and to the defence system of the body, whereas the spores are highly resistant to heat and many disinfecting agents. Spores remain viable in the soil for several years. In semisolid agar *B. anthracis* produces dull, opaque, grayish-white, irregular border colonies with strands of cells growing parallel to give a **Medusa head** type growth.

PATHOGENESIS

Anthrax in cattle, sheep, horse and mules can lead to septicemia which can be further categorized either as apoplectic, acute or subacute depending upon the severity of the disease. However, massive hemorrhagic swellings are observed in subacute type of anthrax. Anthrax is manifested as pharyngitis in swine and as acute gastroenteritis in dogs and cats. In human beings depending upon the route of entry and dose of the organism, anthrax can be characterized into three forms pulmonary, cutaneous, and intestinal. Inhalation of spores causes pulmonary anthrax. Infection through wound or cracks in the skin produces cutaneous form leading to malignant pustules. Intestinal form of anthrax occurs

on consumption of contaminated or undercooked meat form infected animals. The most studied form of anthrax in human being is the cutaneous form which starts as a small pimple and gradually spreads to form a large swelling with a dark centre surrounded by small vesicles. Regional lymph nodes swell and become highly painful. In untreated cases the bacteria invades the blood stream resulting in septicemia and finally death.

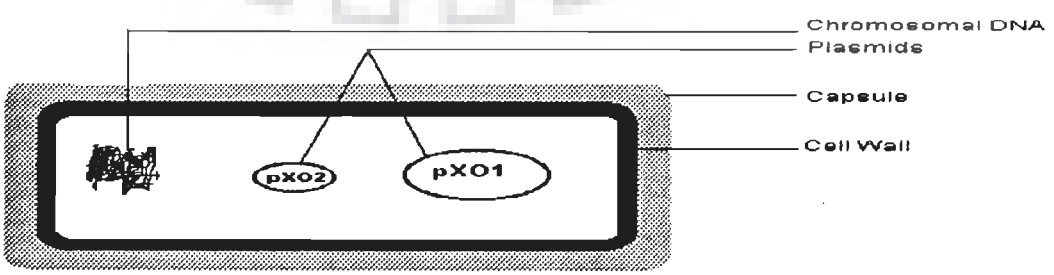
Mouse and guinea pigs are susceptible to anthrax among the laboratory animals. These animals are commonly used as a model for experiments and diagnosis of anthrax. Rabbits are less susceptible, but do succumb to anthrax, whereas rats (except Fischer 344) and birds are resistant to anthrax.

VIRULENCE FACTORS

Virulence of B. anthracis is chiefly attributed to two major factors (i) A poly D-glutamic acid capsule and (ii) protein exotoxins. Exotoxins are secreted in active bacteraemic stage of the disease. Other proteinaceous material like phospholipases, proteases and thiol activated hemolysin secreted by the organism aid in enhancing the virulence effect in the host (Leppla, 1991). But nothing is known regarding their independent contribution in the pathogenesis of anthrax.

The virulence factors are encoded on two separate plasmids pXO1 and pXO2 (Mikesell et al., 1983; Thorne, 1985; Green et al., 1985; Uchida et al., 1985). Strains devoid of either plasmid lose their virulence significantly. Plasmid pXO1 (184 kb) contains the gene for toxin proteins whereas plasmid pXO2 (92 kb) carries the genes encoding the capsule (Fig. I).

Figure: I



SCHEMATIC DIAGRAM OF VIRULENT *BACILLUS ANTHRACIS*
(cited from Leppla, 1981)

These genes are cap A, cap B and cap C encode the necessary enzymes to synthesize the capsule of *B. anthracis* (Makino et al., 1989; Makino et al., 1988; Robertson et al., 1990, Ivins et al., 1986, Uchida et al., 1986, Uchida et al., 1985 and Green et al., 1985). The capsule plays an important role in preventing opsonization and phagocytosis of the bacteria (vegetative forms) by the host leucocytes at the initial stage of infection (Keppie et al., 1963, Thorne et al., 1960, Welkos, 1991). It has been proposed that the presence of pXO1 plasmid helps in the survival of bacteria inside the host body (Pezard et al., 1995). The genes encoding three single chain proteins which form the exotoxin are present on the pXO1 plasmid (Mikesell et al., 1983, Thorne et al., 1985). These proteins have been designated as protective antigen (PA), edema factor (EF) and lethal factor (LF).

In the classical sense, anthrax is a toxigenic disease like tetanus and diphtheria (Hambleton et al., 1984), since injection of the toxin alone produces symptoms identical to those of natural infection with the organism (Leppla, 1995, Turnbull et al., 1990, Stephen, 1986). The tripartite nature of anthrax toxin was first reported by Stanley and Smith (1961). In an attempt to find effective immunogen for anthrax, Gladstone in 1946 discovered PA which was initially designated as factor II of anthrax toxin. The associated component initially designated as factor I, present in the soluble protective antigen mixture was named as edema factor as it produced edema at the site of injection along with PA in animals (Stanley and Smith, 1961, Beall et al., 1962). Other then producing edema, the antigen complex killed rats and guinea pigs when injected parenterally. It led to the identification of a third component of the toxin, responsible for the lethality of animals. The component was initially designated as factor III and is now known as lethal factor (LF) (Stanley and Smith, 1961; Smith and Stanley, 1962; Beall et al,. 1962). Individual protein components are not pathogenic. PA along with LF or EF produces specific pathological symptoms in animal and susceptible cells. PA and EF (Edema toxin) produce edema, whereas PA and LF in combination (Lethal toxin) kill susceptible animal and macrophage

cells (Friedlander, 1986). Both protein exotoxins of anthrax, edema toxin and lethal toxin, are similar to many other protein toxins in that they have a binding or B component responsible for receptor binding and A component which determines the enzymatic activity.

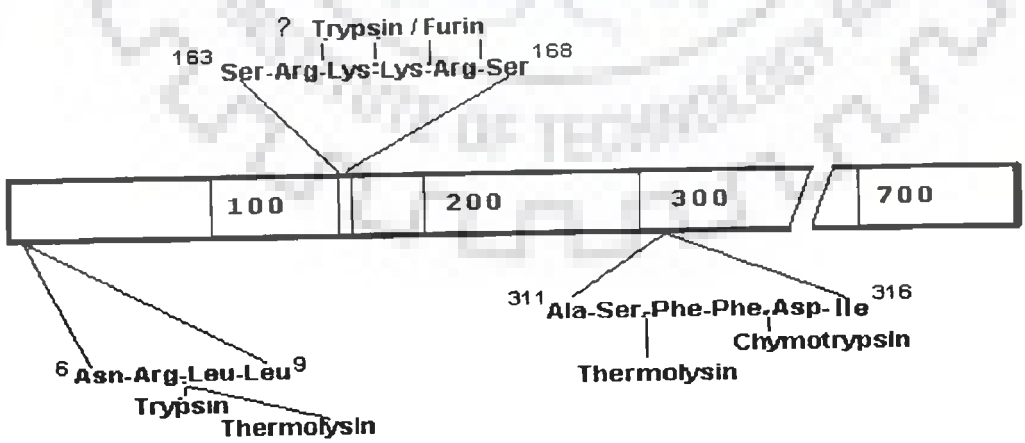
PROTECTIVE ANTIGEN

Protective antigen (PA) is the receptor binding component of the toxin (B component) which translocates LF or EF into the cytosol of the mammalian cells. It was the first molecule to be identified in the anthrax toxin mixture (Gladstone, 1946). The gene coding for PA protein has been cloned (Vodkin and Leppla, 1983) and sequenced (Welkos et al., 1988). The total G+C content of PA was found to be 31% similar to the genomic DNA of B. anthracis (Welkos et al., 1988). The DNA sequence contains 764 codons of which 29 correspond to signal peptide. PA protein does not contain any cysteine residue which is probably an advantage for stabilization of the protein against oxidation in the extracellular environment as reported for many other extracellular proteins (Pollack and Richmon, 1962). The comparison of PA sequences to protein data bases revealed similarity to *Clostridium perfringens* iota toxin. The iota toxin is one of a group of several clostridial toxins that share with the anthrax toxin the unusual property of containing separate proteins that must interact to intoxicate cells. The cell binding subunit **Ib** of iota toxin has got extensive sequence similarity to the stretch of protein starting from the amino acid sequence 170- 590 of the PA of anthrax (Perelle et al., 1993). Ib subunit of iota toxin is proteolytically activated and is involved in the translocation of the catalytic protein into the cells (Perelle et al., 1993). Furthermore in iota toxin presence of an Arg residue and closely spaced Phe-Phe residues at the possible catalytic site suggested that the protein may need some clostridial protease rather than the eukaryotic proteases for its normal activation. However, in PA, ^{312}Phe-Phe^{313} is recognized by chymotrypsin/thermolysin (Novak et al., 1992) and has been reported to be involved in the channel formation and translocation of LF (catalytic molecule) into the cytosol (Singh et al., 1994).

There are two protease sensitive sites uniquely placed and dividing the total PA protein into 3 distinct functional regions (Fig. II). A site in PA that is highly sensitive to trypsin is the sequence of 4 basic amino

acids, ^{164}Arg-Lys-Lys-Arg^{167}. A eukaryotic cell surface furin protease cleaves receptor bound PA at this sequence to generate a 20 kDa and 63 kDa fragment (Leppla et al., 1988). Analysis of several PA mutants suggests that LF dependent toxicity requires the sequence Arg-X-X-Arg, which is recognized by furin protease present at the cell surface (Klimpel et al., 1992). A similar fragment of PA can be obtained in solution by controlled proteolysis of the protein with trypsin (Leppla , 1991; Novak et al., 1992). Proteolytic cleavage of PA at this site causes a change in the conformation (Koehler and Collier, 1991) of PA_{63} which exposes sites for the binding of LF or EF (Singh et al., 1989a). Specific nicking of PA in plasma even in the absence of any cellular contents could be due to the presence of soluble forms of furin which work like a calcium dependent protease in vitro (Ezzell and Abshire, 1992). There are some ambiguities regarding the possible cell surface protease(s) involved in the proteolytic cleavage of PA. When this site was altered to SSRR it retained some toxicity when added onto the cells along with LF but the effect was inhibited in the presence of leupeptin, a protease inhibitor. It is proposed from these experiments that the protease responsible for the cleavage of PA has substrate specificity to two basic amino acids in the recognition sequence which are different from the proposed substrate sequence of furin (Leppla, 1995). PA (having RAAR sequence) has been reported to be cytotoxic to Chineas hamster ovary (CHO) cells which do not produce furin suggesting that additional protease(s) involved in the proteolytic activation of PA may be different from furin (Leppla, 1995).

Figure: II



PROTEASE SENSITIVE SITES OF PROTECTIVE ANTIGEN (Leppla, 1991)

The second proteolytic site consists of sequence ASFFD^{315}, and being rich in hydrophobic amino acids, is susceptible to chymotrypsin and thermolysin. Chymotrypsin cleaves at the carboxyl side of Phe^{314} whereas thermolysin cleaves between residues Ser^{312} and Phe^{313}. Both the proteases cleave PA_{83} into two polypeptide fragments of 37 kDa and 47 kDa (Leppla, 1991a). The 47 kDa COOH-terminus fragment of PA retains the binding property to the cell surface receptors equipotently and specifically as PA_{83} (Novak et al., 1992; Leppla, 1991a). Deletion of 3, 5, or 7 amino acid residues from the COOH-terminus reduces the binding of this altered PA to cell surface receptors and subsequent toxicity of the lethal toxin. The receptor binding activity is completely lost with 12 to 14 residues deletion at the COOH-terminus of PA suggesting its role in receptor binding activity (Singh et al., 1991). These observations were further confirmed by the crystallographic structure of the PA protein. Crystal studies suggest that the 14 COOH-terminal amino acids of PA form the complete outer β-strand of the 4 strand β- sheets present on the binding domain. Deletion of 14 amino acids abolishes the outer β-strand completely, thereby severely affecting the conformation and rigidity of the preceding hairpin loop which is an important part of interdomain interface (Leppla personal communication).

Recently a PA protein altered at both trypsin and chymotrypsin site has been engineered which is more stable in *B. anthracis* culture supernatant (Singh et al., 1994). The altered PA is completely non-toxic and is expected to retain nearly all epitopes of PA. The nontoxic nature of mutant PA should make it possible to include LF/EF in the vaccine to obtain antibodies against all the components of the toxin.

LETHAL FACTOR

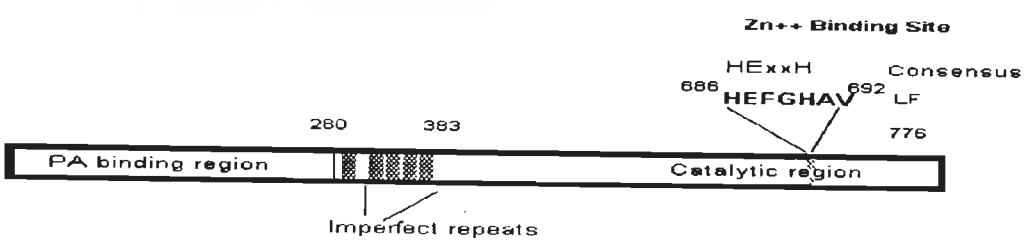
Lethal factor (LF) is the second component of the tripartite exotoxin of B. anthracis, which plays the major role in pathogenesis (Stanley and Smith, 1961; Smith and Stanley, 1962). Several macrophage derived cell lines and Fisher 344 rats are sensitive to lethal toxin (Friedlander, 1986). The gene encoding LF has been cloned and sequenced (Robertson et al., 1988, Bragg and Robertson,

1989). The DNA sequence contains an open reading frame (ORF) of 809 codons. The precursor protein of LF contains a 33 amino acid signal peptide which is similar to other leader peptides present in secreted proteins. Mature LF is secreted as a 776 amino acid protein with a molecular mass of 90.2 kDa (Bragg and Robertson, 1989). The first 254 amino acids of the protein are necessary for binding to PA and its translocation into the cytosol (Arora et al., 1993). This region of LF has a substantial homology with the amino acid sequnces and hydrophilic profiles of the corresponding region of EF. LF and EF compete for PA binding because of this sequence similarity (Robertson et al., 1988, Bragg and Robertson, 1989). Although there is a remarkable homology in the nucleotide sequence between the first 300 amino acids of LF and EF, there is very little serological cross reactivity between the two proteins. Three cross reactive monoclonal antibodies have been described that inhibit the association of LF and EF to PA_{63} (Little et al., 1990). Mutations in the COOH-terminus region affect the catalytic activity of LF significantly without affecting PA binding property (Quinn et al., 1991). One of the most unique features of the LF gene is the presence of five imperfect repeats, each 19 amino acids long located between nucleotides 879-1596 (Bragg and Robertson, 1989). Mutations in repeat regions disrupted the ordered structure of LF leading to the destabilization (proteolytic degradation) of the protein (Quinn et al., 1991). This area is thought to serve as a structural element separating the two, the binding and the enzymatic domains or interacts with protein or lipid surface of the cells. This region has homology with a calcium binding protein (Lowe et al., 1990).

Comparison of carboxy terminus amino acid sequences of LF with metalloprotease recognition sites of other proteins led to the identification of a short region on LF ^{686}HEFGHAV^{692} (Klimpel et al., 1994) containing the Zn^{++} binding consensus sequence, HExxH (Jongeneel et al., 1989) (Fig. III). These sequences are present in three different proteins, *Bacillus stearothermophilus* neutral proteinase (thermolysin), rat soluble metallo-endopeptidase and *Bacillus cereus* protease (Kubo and Imanaka, 1988; Watt and Yip, 1989; Wetmore et al., 1992). Sequence homologies were studied between LF and several known

metalloendopeptidases (Thermolysin, *Listeria monocytogens* metalloprotease, Elastase, *Serratia marcescens* neutral protease, Tetanus toxin, *Clostridium* botulinum neurotoxin type D and rat aminopeptidase N). Alignment of sequences using PILEUP and GAP programmes (Genetics computer group, 1991) revealed a high similarity between the residues 686- 718 of LF with 142-172 of thermolysin. The sequence identity and similarity were calculated to be 65.4% and 88.5% respectively by both the programmes (Klimpel et al., 1994). The presence of sequences for the zinc binding is one of the critical requirements of zinc metallopeptidases. The sequence HExxH has been designated as the conserved zinc binding domain in most of the metalloendopeptidases (Jongeneel et al., 1989; Fairweather and Lyness, 1986). The two histidine residues present in the sequence chelate zinc together and with the third COOH-terminal residue which should preferably be histidine, cysteine or glutamic acid (Vallee and Auld, 1990 a, b). The glutamic acid between two histidines acts as a nucleophilic molecule during the catalytic process (Klimpel et al., 1994). PROSITE database with the program MOTIFS was used (Genetics Computer Group, 1991) for aligning and detecting the matches in LF sequences. A ten amino acid residue stretch in LF was detected matching to the zinc metalloprotease signature, when a single mismatch was permitted (Klimpel et al., 1994). The G in the first position was the only mismatch from the 10-residue motiff and is chemically similar to the accepted A. It is incidentally common for other toxin proteins of known metalloprotease activity (Schiavo et al., 1992a, b, c, 1993; Bairoch, 1993)like tetanus toxin with a mismatch at position 1 and botulinum neurotoxin type A with the mismatch at position 10 of the signature sequence. Several protease inhibitors including bestatin and captopril were reported to inhibit intoxication of

Figure: III



SCHEMATIC DIAGRAM OF LF SHOWING DIFFERENT FUNCTIONAL DOMAINS
(cited from Quinn et al.,1991; Klimpel et al., 1994)

macrophages by lethal toxin which indirectly proves a simillarity to zinc dependent metalloproteases (Klimpel et al., 1994).

In a subsequent study, substitution of Glu^{687} to Cys in LF reduced its zinc binding property and catalytic activity significantly (Klimpel et al., 1994) but substitution of Ala for Glu^{720} and Glu^{741} has no effect on the catalytic or Zn^{++} binding activity of LF (Klimpel et al., 1994). These results strongly supported the hypothesis that LF is a zinc dependent enzyme. However, protease activity or its cellular substrate has not been identified.

Macrophages are the only cell type reported to mediate the lethal activity of anthrax toxin (Hanna et al., 1993). Out of several murine species tested only C3H and HeJ species of mice and Fischer 344 rats were susceptible to LT (Welkos et al., 1986; Ezzell et al., 1984; Friedlander et al., 1993). J774 and RAW 264.7 are the murine macrophage cell lines widely used for cytotoxicity assay to study the biological activity of LT (Friedlander, 1986). In contrast A/J mouse and IC-21 cells were completely resistant to cytotoxic action of LT (Welkos et al., 1986). Comparison of sensitive and resistant cells indicated that most of the cell types have receptors for PA. Infact several resistant cell lines have more receptors for PA in comparison to sensitive macrophage cells suggesting that receptor number is not the reason for making the cell resistant to anthrax lethal toxin. Internalization of LF directly into cytosol by osmotic lysis of pinocytic vesicles led to the lysis of the only sensitive cells. In these experiments very high concentration of LF was required to obtain toxicity. These observations suggest that LF may require some activation or processing in the cytosol for expression of its toxic activity (Singh et al., 1989a, b).

Action of LF on macrophages leads to enhanced permeability of cell membrane as observed by the increased movement of K^+ and Na^+ ions within 45 min of addition of the toxin followed by ATP depletion leading to gross changes in the balance of electrolyte concentration of cells within 60 min (Hanna et al., 1992). In 75 min morphological changes were seen with influx of calcium into the cells which peaks at about 150 minutes followed by lysis of cells

(Bhatnagar et al., 1989). There are several proteases which cause damage to K^+ channel proteins, thus the channel pore remains open permanently. As suggested by the reports, the damage to certain peptide "chain" in the "ball-and-chain" arrangement of the channel could cause the channel to remain open (Miller, 1991). These activities disturb the electrolyte balance of the cell. PA_{63} forms channel in the endolysosomal membrane without any remarkable adverse effect on the cellular morphology (Milne and Collier, 1993). Thus it can be inferred that the cytotoxic action of LT is due to lethal factor.

EDEMA FACTOR

Edema factor (EF) was the first enzymatic molecule isolated in anthrax toxin. It is an adenylyl cyclase which requires calmodulin for its activity in eukaryotic cells (Leppla, 1982). The gene is located in pXO1 and ORF consists of 800 codons, of which 33 correspond to signal peptide (Leppla, 1991). The mature protein consists of 767 amino acids with a total molecular mass of 88.8 kDa (Leppla, 1991). The protein has got sequence similarity with LF of anthrax and adenylyl cyclase of *Bordetella pertussis* (Robertson, 1988). Similarities between nucleotide sequence level and hydrophilic behaviour of 1-250 amino acids of LF and EF justifie their binding to PA in a competitive manner (Leppla, 1982). The carboxy terminal fragment of EF (300-767 amino acids) essentially retains the catalytic domain of the protein. Studies on the sequence homologies with other proteins have shown that 309-324 amino acids of EF match significantly with 54-66 amino acids of B. pertussis adenylyl cyclase. The stretch of amino acids includes the consensus sequence, GxxxxGKS, present in most of the nucleoside triphosphate binding proteins (Higgins et al., 1986) (Fig. IV).

Figure: IV

Consensus	Gx xxxGKS
EF	GVATKGLNVHGKSSDW
BPCYA	GVATKGLGVHAKSSDW

Sequence similarity of anthrax toxin edema factor (EF) and *Bordetella pertussis* adenylyl cyclase (BPCYA). (cited from Leppla, 1995)

It can be infered that the sequence partially includes the site responsible for ATP binding. To find the area responsible for the catalytic activity of EF several mutation studies have been conducted, mutation of Lys^{320} to His, in the ATP binding signature sequence completely abolishes the activity of the protein (Xia and Storm, 1990). The Lys residue present preceding to the consensus has also been implicated in ATP binding activity of the protein. Substitution of $K^{313}Q$ also makes the protein catalytically inactive (Labruyere et al., 1991). A synthetic peptide corresponding to 499-532 residues of EF, binds tightly to calmodulin (Munier et al., 1993). It could be the possible calmodulin binding site in the EF. This site has got a homology with 239-273 residues of *B. pertussis* adenylate cyclase which hasidentical calmodulin binding activity (Munier et al., 1993).

EXPRESSION AND PURIFICATION OF TOXINS

B. anthracis (V770-NP1-R (ATCC # 14185) is generally used for the production of toxin proteins. However, a rifampicin resistant mutant of the strain, SRI-1, was reported to secrete 50-75% more toxins (Leppla, 1988) when the culture was grown in "R" media (Ristroph and Ivins, 1983). Ion-exchange and hydrophobic interaction chromatography have been employed for the purification of toxin proteins (Wilkie and Ward, 1967; Quinn et al., 1988). Monoclonal and immunoadsorbent columns (Larson et al., 1988) have also been used to purify individual toxin components. Avirulent/attenuated strains of *B. anthracis* secreting individual toxin component have been developed by Pezard et al., 1993. PA is either expressed in *B. subtilis* using plasmid pUB110 containing PA gene (Ivins and Welkos, 1986), or a plasmid pYS5 (shuttle vector) derived from pUB110 and pVC8f(+)T expressing an enhanced amount of PA (50 ug/ml) in the culture supernatant of both *B. anthracis* and *B. subtilis* (Singh et al., 1989a). Application of modern genetic tools has allowed to design several protease deficient strains of *B. subtilis* like DB- 104 (Kawamura and Doi, 1984) and WB 600 (Wu et al., 1991) which increased the yield of PA in the culture supernatant (Singh et al., 1989a, Ivins et al., 1990). Recently, PA has been expressed and purified from

periplasm of *E. coli* (Sharma et al., 1995), an easy system to work in the laboratory.

Recovery of LF is approximately four fold less as compared to PA when expressed and purified from *B. anthracis* (Quinn et al., 1988). Recently LF was expressed as fusion protein consisting of 1-164 amino acids of PA at NH_2-terminus of LF protein in *B. subtilis*. The chimeric protein was expressed having a recognition sequence for factor Xa separating PA and LF proteins (Klimpel et al., 1994; Leppla, 1995). In other approaches the proteins, PA and LF genes have been cloned and expressed in *E. coli* (Singh et al., 1991; Arora et al., 1992). Several mutants of LF protein have been expressed as glutathione-s-transferase fusion proteins, which can be purified in single step using glutathione affinity column (Arora and Leppla, 1994).

MECHANISM OF ACTION OF ANTHRAX TOXIN

Both exotoxins of anthrax, edema toxin and lethal toxin fit into the "AB" model of toxin, similar to the other bacterial toxins (Gill, 1978)for their activity in suseptible hosts. With few exceptions to the proposed "AB" model, anthrax exotoxin is unique in having A and B moieties as separate proteins. PA, the B-moiety binds to cell surface receptors and acts as a common vehicle for the translocation of two separate A-moieties (LF and EF) to the cytosol of the eukaryotic cells (Fig. V and VI). LF and EF express the biochemical/cytotoxic activity of anthrax toxin. A high degree of affinity exists (K_d 1nM) for the PA to the receptors on the cell surface (Leppla et al., 1988; Friedlander et al., 1993). The number of receptors vary from cell to cell (5,000-50,000) irrespective of the origin of the organ or host (Escuyer and Collier, 1991; Friedlander et al., 1993).

In the process of cytotoxicity, PA binds to cell surface receptors and gets proteolytically cleaved by furin, a membrane associated protease present in most cell types (Klimpel et al., 1992). Cleavage leads to the release of a 20 kDa (amino terminus) fragment into the media and large Carboxy-terminal 63 kDa fragment remains bound to the cell. Cleavage exposes a site on PA for binding to LF or EF (K_d 10pM) (Leppla et al., 1988). Both the proteins EF and LF compete for the binding site on PA. The toxin complex is endocytosed and through the acidic vesicles is delivered in the cytosol. Recently, it has been

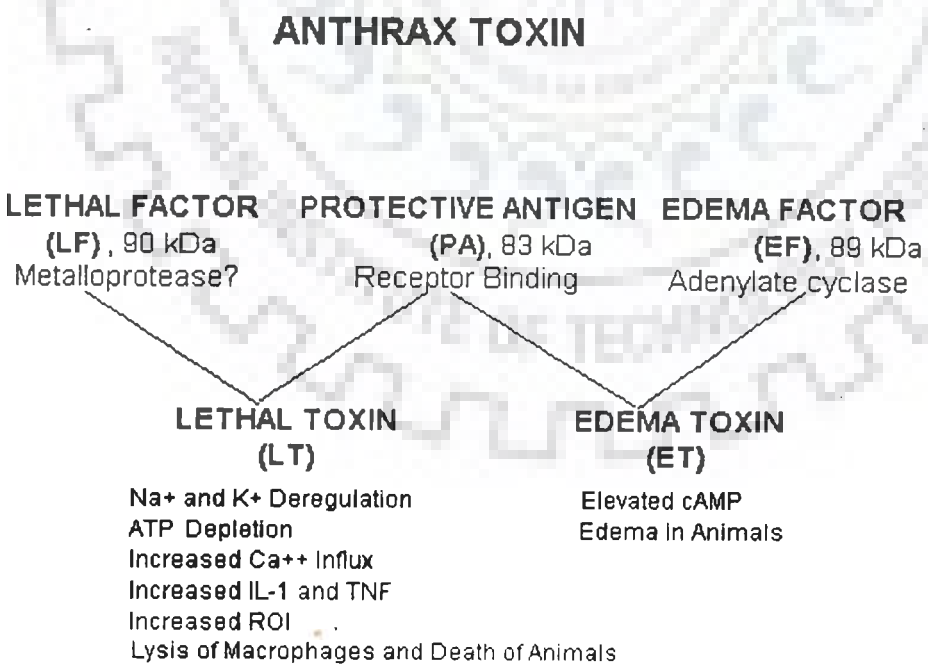
demonstrated that acidic pH induces formation of heptameric oligomer of PA_{63}, which is responsible for the formation of membrane spanning channels required for the translocation of LF/EF to the cytosol (Milne and Collier, 1993; Milne et al., 1994). EF is a calmodulin and Ca^{++} dependent adenylyl cyclase which elevates cAMP in the cytosol of the cells (Leppla, 1982). The exact function of LF is not known but it has been proposed to act as a metalloprotease.

There are several hypotheses regarding LT's possible mode of action in killing animals. According to Hanna et al., 1993 macrophages are the only cell type which mediate the lethal toxin intoxication in the susceptible host. A low concentration (LF 10^{-10} ug/ml) of lethal toxin triggers formation of a large amount of IL-1 and TNF (10^5 molecules/macrophage cell). The high level of cytokines produced in the process could be the major cause of shock leading to death of animals infected with anthrax.

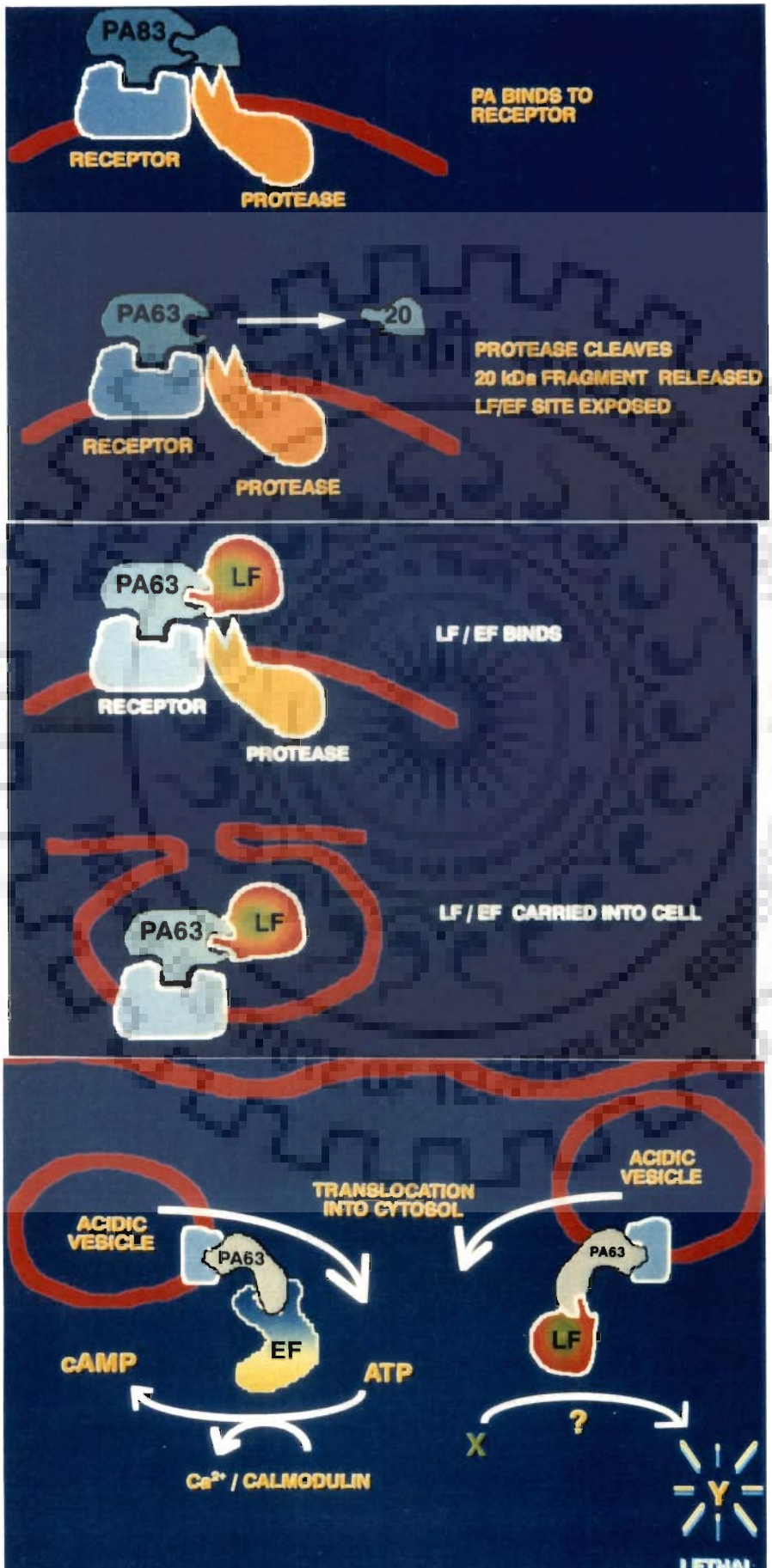
Hanna et al., 1994 have also shown that macrophage like cell lines RAW264.7 and J774.16 when incubated with lethal toxin increase the levels of reactive oxygen intermediates (ROI). The high level of ROI production may be one of the causes for lethal toxicity of anthrax toxin.

Figure: V

COMPONENTS OF ANTHRAX TOXIN



SCHEMATIC DIAGRAM SHOWING ACTION OF ANTHRAX TOXIN



MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture medium and fetal calf serum were purchased from GIBCO-BRL (Gaithersburg, MD). SM2 Bio beads were obtained from BioRad (Richmond, California), Triton X-100 was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Radioactive ^{14}C-leucine (60.12 mCi/m mole) and Na^{125}I (2 Ci/m mole) were obtained from BARC (Trombay, Bombay). ^{3}H-myo-Inositol (10 Ci/m mole) was purchaged from DuPont NEN. Dowex 1 ion- exchange matrix was obtained from Sigma Chemical Co. Sendai virus (Z-strain) was provided by Dr. D. P. Sarkar (South Campus, University of Delhi).

Cell Culture

A macrophage like cell lines J774A.1 and RAW 264.7 (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% foetal calf serum, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were incubated at 37^{o}C and 5% CO_2.

Cytotoxicity Assay

Macrophage cells (RAW264.7 or J774A.1) were incubated with toxin (PA and LF, 0.001-1 μg/ml) for specified time periods at 37^{o}C. At the end of the experiment, cell viability was determined using MTT (3-4, 5-dimethylthiazole-2-yl 2,5,-diphenyltetrazolium bromide) assay (Quinn et al., 1991). MTT dissolved in DMEM was added to the cell culture to a final concentration of 0.5 mg/ml. Incubation was continued for another 60 min at 37^{o}C to allow uptake and oxidation of the dye to produce the purple precipitate (formazan derivative) by viable cells. The media was replaced with 100 μl of 0.5% SDS, 25 mM HCl in

90% isopropyl alcohol to solubilize the precipitate. The absorbance at 590 nm was read using a microtitre plate reader (Nunc, GMBH)

Protein Synthesis Inhibition Assay

The protein synthesis inhibition was studied by monitoring the incorporation of ^{14}C-leucine in the acid precipitable proteins of the cell. Cells were grown in a 24-well tissue culture plates (Corning, NY) and incubated in the media containing toxin (PA and LF, 0.5 μg/ml each) at 37^{o}C for various time periods. At the end of incubation, ^{14}C-leucine (5 μCi/ml) was added and further incubated for 2.5 hours. The cells were solubilized in 0.1 N NaOH solution after which filter paper discs (Schleicher and Schuell, Germany) were dropped into each well. Discs from each well were recovered, air dried and soaked in chilled 12% TCA solution for 10 min for complete precipitation of the proteins on the filter paper. The discs were washed once with cold 6% TCA solution and three times with cold ethanol:acetone (1:1, v/v) to remove unincorporated leucine from the samples. The paper discs were dried, suspended in scintillation fluid and radioactivity determined in a beta counter (LKB, Rack beta).

Preparation of Loded Virosomes

Virosomes were prepared by the method of Bagai et al., 1993; Bagai and Sarkar, 1994). A suspension of 10 mg of sendai virus was centrifuged at 1,00,000 x g for 1 hour at 4^{o}C. The pellet was resuspended in 200 μl of phosphate buffer containing 20 mg of triton X-100, incubated for 1 hour at 20^{o}C followed by centrifugation at 1,00,000 x g for 1 hour at 4^{o}C. The clear supernatant containing the virus envelopes was mixed either with LF (200 μg), PA (200 μg) or PA + LF (200μg each) and triton X- 100 was removed by stepwise addition of methanol washed SM2 Bio- beads in a ratio of 1:8 (detergent : beads, w/w) for a period of 6 hours. The turbid suspension was separated from the bio-beads by passing through a syringe fitted with a 26 gauge needle and then centrifuged. The amount of entrapped toxin protein was

calculated on the basis of entrapment of iodinated lysozyme as described by Bagai and Sarkar, 1994. Blank virosomes were prepared by recovering triton X-100 generated envelopes in the absence of added protein.

Radio-Iodination of Protein

PA or LF (50 μg/ml) was incubated with 1 μg of chloramine T and 1 mCi of $Na^{125}I$ in 100 ul of 0.1 M sodium phosphate buffer, pH 7.0 at 4°C for 5 minutes. The reaction was terminated by adding 2 μg sodium metabisulfite in 0.1 M sodium phosphate buffer, pH 7.0. Iodinated protein was passed through a Sephadex G-25 column (30 cm x 1 cm) to separate the free iodine from the protein. An aliquot from each fraction was precipitated with TCA. Fractions showing radioactivity in the pellet were pooled and stored at 4°C for further use.

Receptor Binding Assay

The binding of toxin protein to cell surface receptors was measured in 24 well plates using the radioiodinated proteins. RAW264.7 cells were cooled by placing the plates at 4°C for 15 minutes. The medium was replaced with cold essential media containing Earle's salt (EMEM) without bicarbonate and supplemented with 1% bovine serum albumin (BSA) and 25 mM Hepes, pH 7.4, with varying concentrations of ^{125}I-PA (0.1 μg/ml, specific activity 1 x 10^6 cpm). For binding assays at 37°C, cells were incubated with toxin proteins (0.5 μg/ml) for 2 hrs. Binding of LF to PA protein was measured by incubating iodinated LF (0.5 μg/ml of specific activity 1 x 10^6 cpm) and PA (0.5 μg/ml) with the macrophage cells. The cells were incubated with the radiolabeled proteins at 4°C or 37°C for 12 hrs or 2 hrs, respectively. The cells were washed thrice with cold Hank's Balanced salt solution (HBSS) and then dissolved in 0.1 N NaOH solution. The radioactivity was measured in gamma counter (EC India Ltd.).

Competent Cell Preparation

Competent cells were prepared by the method of Cohen et al., 1972. A

single colony was inoculated into 5 ml of LB medium and grown overnight at 37°C with shaking at 200 rpm. The overnight culture was diluted 100 fold in 25 ml of LB medium and grown at 37°C and A_{600} was measured every 30 minutes. When absorbance reached 0.3-0.4, culture was chilled on ice and centrifuged at 5000 rpm (Sigma rotor 12150) for 10 minutes at 4°C. The supernatant was decanted and the pellet was suspended gently in 12.5 ml of 50 mM $CaCl_2$. The cells were incubated in $CaCl_2$ for 1 hr on ice and centrifuged at 3500 rpm (Sigma rotor 12150) for 10 minutes at 4°C. The supernatant was decanted and pellet resuspended in 2 ml of 50 mM $CaCl_2$ containing 20% glycerol and stored at -70°C in aliquots for subsequent use.

Transformation

Transformation was carried out by the method described by Mandel and Higa, 1970. To 100 μl of competent cells 1-2 μl of DNA (50-100 ng) was added and incubated on ice for 45 minutes. The cells were subjected to heat shock at 42°C for 90 seconds on a heating block and immediately chilled on ice for 5 minutes. After addition of 400 μl of LB medium, the cells were incubated on a shaker at 37°C for 1 hr. The cells (50-100 μl) were plated on LB agar plates containing 100 μg/ml ampicillin and incubated overnight at 37°C.

Plasmid Preparation (Mini Prep)

A single transformed colony was inoculated in 5 ml of LB medium containing 100 μl/ml of ampicillin and shaken overnight at 37°C. 3 ml of the culture was pelleted at 6000 rpm (Sigma rotor 12154) for 4 minutes and supernatant aspirated out. The pellet was suspended in 150 μl of solution I (25 mM Tris buffer, pH 8.0, 10 mM EDTA, 50 mM Glucose and 100 μg/ml lysozyme) and kept at room temperature for 10 minutes. To the tube 300 μl of solution II (1% SDS and 0.2 N NaOH) freshly prepared, was added and mixed gently. The tube was incubated on ice for 10 minutes. Cold ammonium acetate (150 μl of 7.5 M) was added to the tube and incubated further for 10 minutes

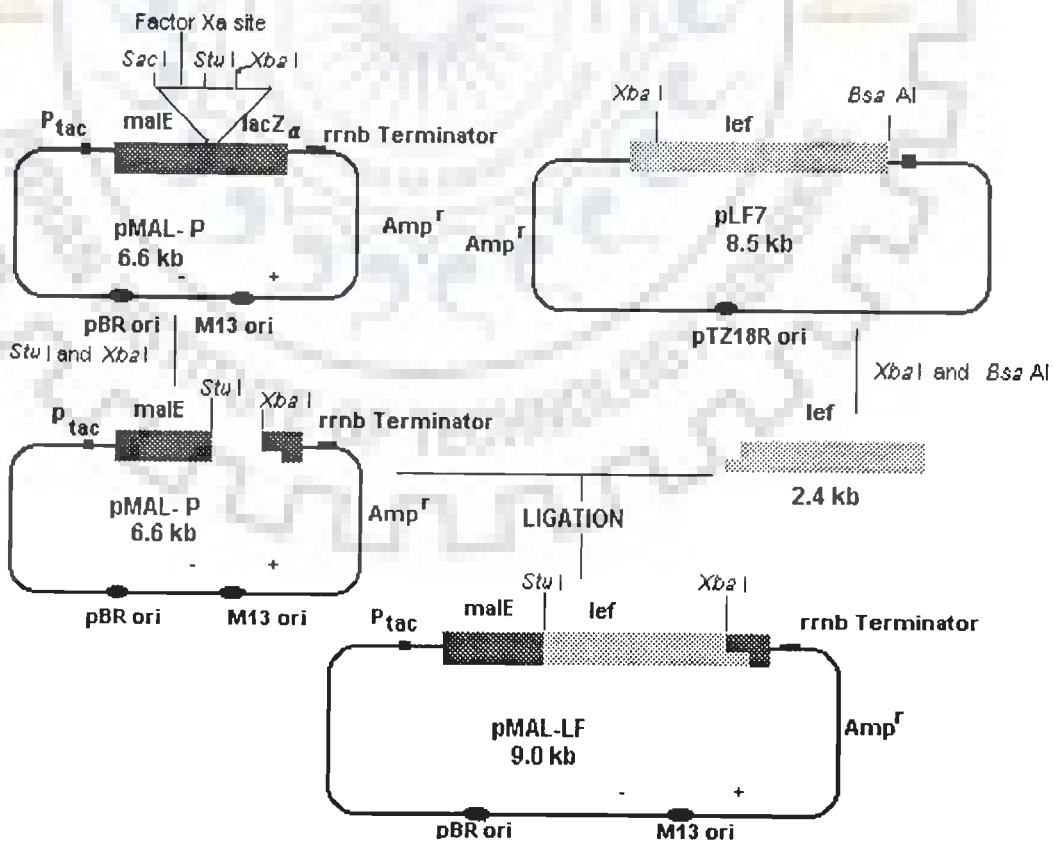
Figure 1: Construction of plasmid used for expression of LF in *E. coli.* Plasmid pMAL-LF contain the entire lef gene for LF protein and malE for encoding the maltose binding protein in the chimeric protein MBP-LF. P_{tac} Taq polymerase promotor; Ori, origin of replication; Amp^r, ampicillin resistance; malE, gene encoding MBP.

on ice. The mixture was centrifuged at 12,000 rpm (Sigma rotor 12154) for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and equal volume of isopropanol was added, mixed and kept on ice for 30 minutes. The DNA was pelleted by centrifugation at 18 000 rpm (Sigma rotor 12154) for 15 minutes. The pellet was washed in 70% ethanol and dried in Speed Vac (Heto). The dried pellet was suspended in 40 μl of TE buffer.

Plasmid Construction

In this study LF was expressed as MBP-LF fusion protein. LF gene was isolated from plasmid pLF7 (Robertson and Leppla, 1986). To clone the LF gene in vector pMAL-P, the pLF7 plasmid was digested with *Stu* I and *Bsa* A1. The fragment containing LF gene was isolated from agarose gel and ligated to the pMAL-P vector digested with *Stu* I and *Xba* I (Fig. 1). *E. coli* DH_5 α cells were transformed with the resulting plasmid pMAL-LF and plated on LB plates containing 100 μg/ml ampicillin.

CONSTRUCTION OF PLASMID FOR EXPRESSION OF LF IN *E. COLI*



Expression and Purification of LF

LF was expressed as a fusion protein with maltose binding protein (MBP) using pMAL vector (Yanisch-Perron et al., 1985). E. coli (BL21,λDE3) was transformed with plasmid pMAL-LF carrying the LF gene. The culture was grown at 37°C in LB medium containing 100 μg/ml ampicillin. When A_{600} reached 1.0, IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to a final concentration of 1 mM and the culture was incubated for an additional 2 hours. The cells were harvested by centrifugation at 4000 x g for 15 minutes. The pellet from 1 litre culture was suspended in 50 ml buffer A (20 mM Tris pH 8.0 containing 200 mM NaCl and 5 mM EDTA) and sonicated (5 cycles of 30 seconds each at 4°C). The sonicate was centrifuged at 15,000 x g for 30 minutes and supernatant was used for purification of LF. The supernatant was diluted five times with buffer A and chromatographed on 10 ml amylose column previously equilibrated with buffer A. The column was washed with 100 ml of buffer A and bound protein was eluted with 10 mM maltose in buffer A. The fractions containing the fusion protein were pooled and dialysed against buffer B (10 mM Tris, pH 8.0 containg 100 mM NaCl and 2 mM $CaCl_2$). The dialysed protein sample was incubated with factor Xa (1 unit/50 μg protein) for 4 hours at 25°C and again passed through the amylose column to remove MBP. The final purification was performed on a 90 ml Sephadex G-100 column using 10 mM Tris buffer, pH 7.5 containing 50 mM NaCl.

Gel Electrophoresis

Purified protein preparations and cell associated radioactivity were analyzed by SDS-PAGE (Laemmli, 1970). The Western blots were developed by transferring the proteins to nitrocellulose and then probing with polyclonal rabbit anti-LF antiserum. The gels were stained in Coomassie Brilliant blue R-250 and destained with solution containing 10% acetic acid and 30% methanol (v/v). To analyse the radiolabeled proteins, gels were dried, exposed to X-ray film (Indu, India) and developed.

Protein Estimation

The concentration of the protein was estimated using BCA reagent (Pierce Chemical co.). Bovine serum albumin (BSA) was used as a standard protein to obtain the calibration/reference curve for quantitation of the protein in the unknown samples. Varying concentrations of BSA were taken in a clean eppendorf tube and volume was adjusted to 0.5 ml with water. BCA stock reagent solutions A, B and C were mixed to get the final working solution and 0.5 ml of the solution was added to each tube containing protein samples. Samples were mixed by vortexing and incubated for 1 hour at 60°C. The absorption at 562 nm was measured spectrophotometrically (Shimadzu).

Subcellular Fractionation

Macrophage cells were scrapped into 1.5 ml of 0.25 M sucrose solution containing 10 mM Tris, pH 7.5 and 1 mM EDTA. The cell suspensions were transferred into a 5 ml glass homogenizer and homogenized gently (medium speed). Total 10-15 strokes were required to get 90% rupture of the macrophage cells. Homogenized cell samples were stained by 0.25% trypan blue to determine cell viability. Homogenized samples were subjected to differential centifugation to separate the cell organelles (Bijsterbosch and Van Berkel, 1992). Cell homogenates were centrifuged at 1200 x g for 5 minutes to separate the nucleus and intact cells. The supernatant was collected and centrifuged at 9,000 x g for 5 minutes to obtain mitochondria. Supernatant collected at this step was again centrifuged at 22,000 x g for 15 min to get lysosomal fraction in the pellet. Supernatant was collected and subjected to ultracentifugation at 1,00,000 x g for 30 min to obtain microsomes in pellet. All the fractionation procedures were performed at 4°C. The radioactivity associated with different fractions was counted in a gamma counter (EC India Ltd.).

Proteolytic Cleavage of PA in Solution

PA was incubated with trypsin (2.5 ng/μg of protein) for 25 minutes at room temperature in 25 mM HEPES buffer, pH 7.5 containing 1 mM $CaCl_2$ and 0.5 mM EDTA. Trypsin was inactivated by adding 1 mM p-amidinophenylmethylsulfonyl fluoride (PMSF). The trypsin cleaved PA was designated as PA-nicked (PAn).

Binding of LF to Trypsin Nicked PA (PAn) in Solution

. Trypsin nicked PA was incubated with equimolar concentration of LF in 25 mM Tris buffer, pH 7.5 containing 1 mM $CaCl_2$ and 0.5 mM EDTA for 20 minutes at room temperature. To study the effect of pH on the binding of LF to PAn, the pH was adjusted by adding dilute HCl.

The binding of LF to PAn was determined by analysing the samples on gradient gels with plastic backing (8-25%, Phast gel, Pharmacia). Buffer strips were made from 2% agarose (SeaKem GTG, FMC) dissolved in 0.88 M L-alanine, 0.25 M Tris, pH 8.8, containing 2 mg/ml CHAPS.

ANS Binding Studies

ANS (8-anilino-1-naphthalene sulfonate) binding studies were monitored by spectrofluorescence measurements conducted on a Hitachi 650-60 spectrofluorimeter equipped with a Hitachi 056 recorder. A quartz cell of 1 cm path length, 5 nm slit width for excitation and emission wave lengths was used for all the measurements. The spectrum of buffer containing 8-anilino-1-naphthalenesulfonic acid (ANS, Sigma) (5 μg/ml) dissolved in 10 mM Tris acetate, pH 6.8, was recorded. Protein was added in the cuvette after the spectrum was corrected for buffer. Studies at several pH values were performed after adjusting the pH of the sample in the cuvette. The excitation wavelength was 380 nm and the emission spectrum was recorded at 400-540 nm after being corrected for sample dilution.

Circular Dichroism Studies

Circular dichroism (CD) spectra were recorded at room temperature (23°C) with a Jasco J500A spectro-polarimeter equipped with a DP 500N data processor. All the CD measurements were carried out in a 1 mm path length cell at protein concentration 200-500 µg/ml in 10 mM Tris buffer, pH 7.2, containing 1 mM $CaCl_2$. Each spectrum was recorded atleast 4 times and averaged after subtracting the values obtained with the buffer. The spectra was recorded at several pH values. The data was expressed as molar ellipticity

$$[\theta] = \frac{\psi \times s \times 10^{-3} \times 100}{C \times L}$$

Where $[\theta]$ is the molar ellipticity in deg cm^2 $dmol^{-1}$.

ψ is the observed ellipticity.

C is the concentration in mole/litre of peptide residue.

L is the path length in cm.

s is the sensitivity in milli-degrees.

Assay of the Inositol Phosphate

Inositol phosphates were measured according to the procedure described by Beaven et al., 1984 and Berridge et al., 1983. Macrophage cells (J774A.1) were labeled with ^{3}H-inositol (4 μCi/ml) for 12 hours at 37°C. The medium was removed and washed two times with Earle's balanced salt solution containing 10 mM lithium chloride. Cells were incubated with several concentrations of toxin (PA, 0.1 μg/ml and LF 0.001-0.1 μg/ml) for various time periods. At the end of incubation period 750 μl of chloroform/ methanol/ 4 N HCl (100:200:2) was added in the wells to get a monophasic extract of the culture. After transferring the entire extract to a tube, 250 μl of chloroform and 250 μl of 0.1 N HCl were added. Samples were vortex-mixed and centrifuged at 100 x g for 2-3 minutes to separate the two phases (upper aqueous and lower

chloroform).

An aliquot (500 μl) of the upper aqueous phase was loaded onto a Dowex resin column (100-200 mesh, converted to formate form by extensive washing with 1 M sodium formate). The column was washed 2 times with 3 ml of 5 mM inositol in water to remove ^{3}H-inositol. The inositol phosphates were eluted with 1.5 ml (2 x 750 μl) of 1 M sodium formate. The radioactivity in the eluate was determined by liquid scintillation counting.

The chloroform phase containing phosphatidylinositol (PI) was mixed with 3 ml of chloroform:methanol (2:1). The solution was washed three times with 2 ml of methanol containing 1 M KCl and 10 mM inositol in the ratio of 1:1. Chloroform was removed by evaporation at 65^{o}c and the associated radioactivity was determined by liquid scintillation counting.

RESULTS

RESULTS

EXPRESSION AND PURIFICATION OF LF

To investigate the role of individual components of anthrax toxin, purified protein is required. *B. anthracis* secretes PA as the major protein component of the exotoxin. *B. subtilis* and *B. anthracis* transformed with plasmids carrying PA gene have been reported to express and secrete substantial amount of PA protein in cultures. PA has also been expressed and purified from *E. coli.* In contrast, recovery of LF is less from the culture supernatant of *B. anthracis.* Subcloned LF gene, expressed in *E. coli* gives low yield of the protein irrespective of promoters used (Robertson and Leppla, 1986; Quinn et al., 1991). Susceptibility of LF to the *E. coli* proteases further decreases the yield of LF. Recently, several mutant LF proteins have been expressed as chimeric proteins containing glutathione-S-transferase. Addition of glutathione-S-transferase aids in purification and improves the protein recovery. In the present study LF was expressed in E. coli as a chimeric protein fused with maltose binding protein (MBP). Plasmid pLF7 was digested with *Xba* I and *Bsa* AI to get the entire LF gene and then ligated with *Xba* I and *Stu* I digested pMAL-P vector fragment, to get the plasmid pMAL-LF (Fig. 1). *E. coli* (BL-21 λDE3) was transformed with plasmid pMAL-LF and induced with IPTG to express LF. To obtain maximum amount of LF, bacteria were induced at the late log phase of its growth (Fig. 2). The duration of induction period was optimized by inducing cultures with IPTG for different time periods and then analysing the expressed fusion protein by Western blotting (Fig. 3). It was observed that one litre media when inoculated with 1% of 12 hour grown culture reached A_{600} = 0.8 (late log phase) after 4.5 hours of incubation at 37^{o}C. Induction with 1 mM IPTG for 2.5 hours was optimum for maximum recovery of the intact MBP-LF protein. The expressed MBP-LF protein was localized in the cytosol. To purify LF from *E. coli,* cells were collected by centrifugation, sonicated in 20 mM Tris buffer, pH 8.0 containing 200 mM NaCl and 5 mM EDTA and loaded on the amylose column.

The bound MBP-LF was eluted with 20 mM Tris buffer, pH 8.0 containing 200 mM NaCl and 10 mM maltose. After purification on the amylose column, the fusion protein (MBP-LF) migrated as a band of 130 kDa (Fig 4A and B, lane 2). The intact LF was obtained by cleaving the MBP-LF protein with factor Xa (Fig 4A and B, lane 3) and passing again through amylose column to remove free MBP. The final purification of LF was performed on Sephadex G-100 column which removed all low molecular weight proteins. Purified LF migrated as a single band of 89 kDa on gel (Fig 4A and B, lane 4). The cells obtained from 1 litre of culture yielded 200 μg of purified LF protein.

Several murine macrophage cells are used to assay the biological activity of lethal toxin which cytolyse the macrophage cells. The cell viability was measured by the ability of the cells to oxidise MTT to a blue colored precipitate. Macrophage cells (J744A.1) were incubated with LF (0.01-1 μg/ml) purified from B. anthracis and *E. coli* in combination with PA (0.5 μg/ml) at 37^{o}C for three hours. MTT (0.5 μg/ml) was added and incubated for an additional 60 minutes at 37^{o}C. The *E. coli* purified LF lysed 50% of the cells at 30 ng/ml as compared to 25 ng/ml of LF from *B. anthracis* (Fig. 5).

INTRODUCTION OF LF IN THE CYTOSOL THROUGH VIROSOMES

Earlier studies have shown that nanogram quantity of lethal toxin is required to lyse the J774A.1 cells. Introduction of LF by osmotic lysis of pinocytic vesicles lysed the J774A.1 cells at a very high concentration of LF (Singh et al., 1989b). The low potency of LF when internalized through osmotic lysis of pinocytic vesicles suggested that PA may be facilitating the killing process after LF is internalized into the cells. In this study LF was introduced directly into cytosol through Sendai virus envelopes. Figure 6 shows the sensitivity of J774A.1 cells to LF when delivered directly into the cytosol through virosomes. Cell viability was measured by oxidation of MTT after incubating the cells with varying concentrations of virosome entrapped LF. A concentration dependent lysis of J774A.1 cells was observed after introduction of LF through

Figure 2 : Growth curve of *E. coli* transformed with pMAL-LF. LB media was inoculated with *E. coli* (BL-21λDE3) transformed with pMAL-LF and incubated at 37°C. Cultures were removed at various time points and $A_{600\ nm}$ was recorded ⊙ BL-21 (λDE3), o, YS2, ●, MAL-LF.

GROWTH CURVE OF *ESCHERICHIA COLI* (BL-21 λ DE$_3$) TRANSFORMED WITH pMAL-LF

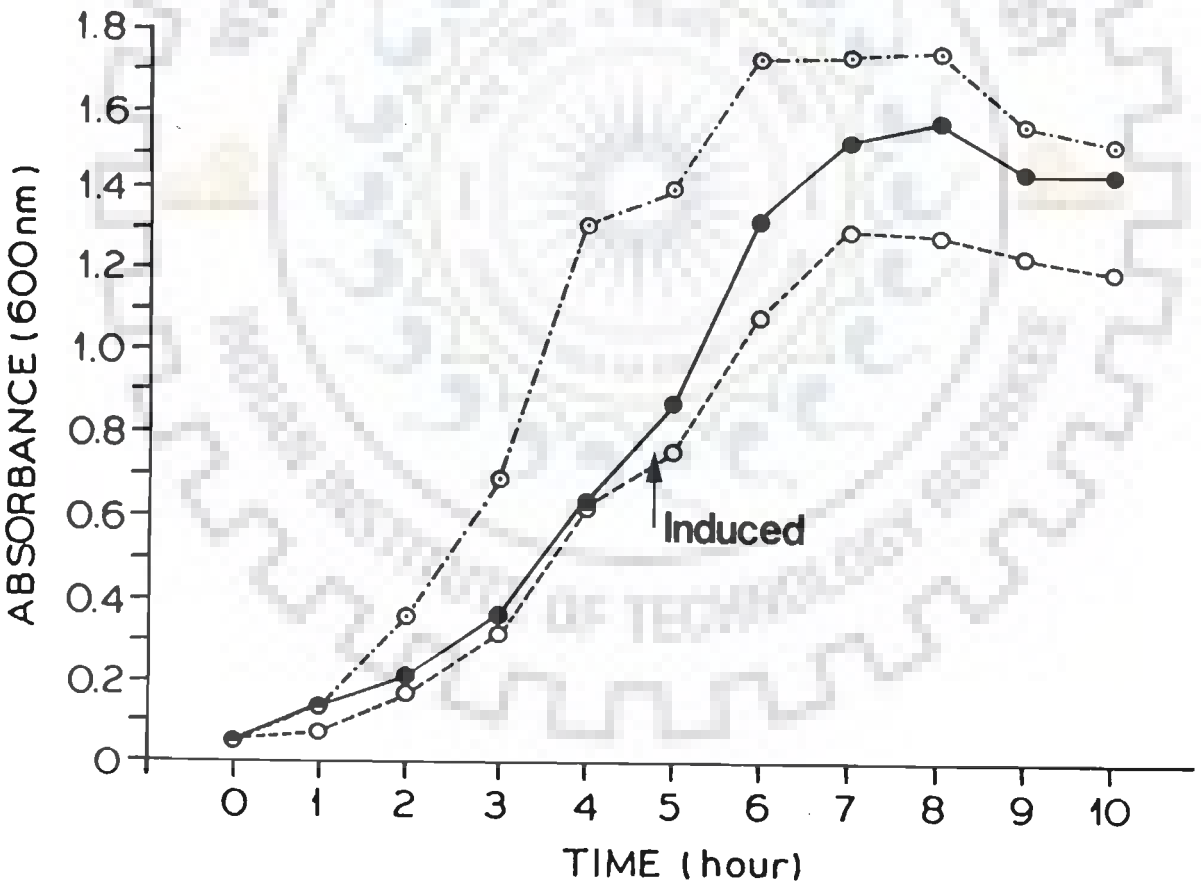


Figure 3 : Immunoblot of LF expressed in *Escherichia coli*. *E. coli* cells transformed with pMAL-LF and grown in LB medium. After induction with 1 mM IPTG bacterial pellets at various time points were solubilized in SDS-sample buffer. Proteins were separated by 10% SDS-PAGE and electro-transferred on nitrocellulose. Blots were developed using polyclonal LF antibodies. Lane 1, LF from *B. anthracis*; lane 2-5, Total *E. coli* cells after induction with IPTG at 1; 2; 3; and 4 hours, respectively.

IMMUNOBLOT OF LF EXPRESSED IN *ESCHERICHIA COLI*

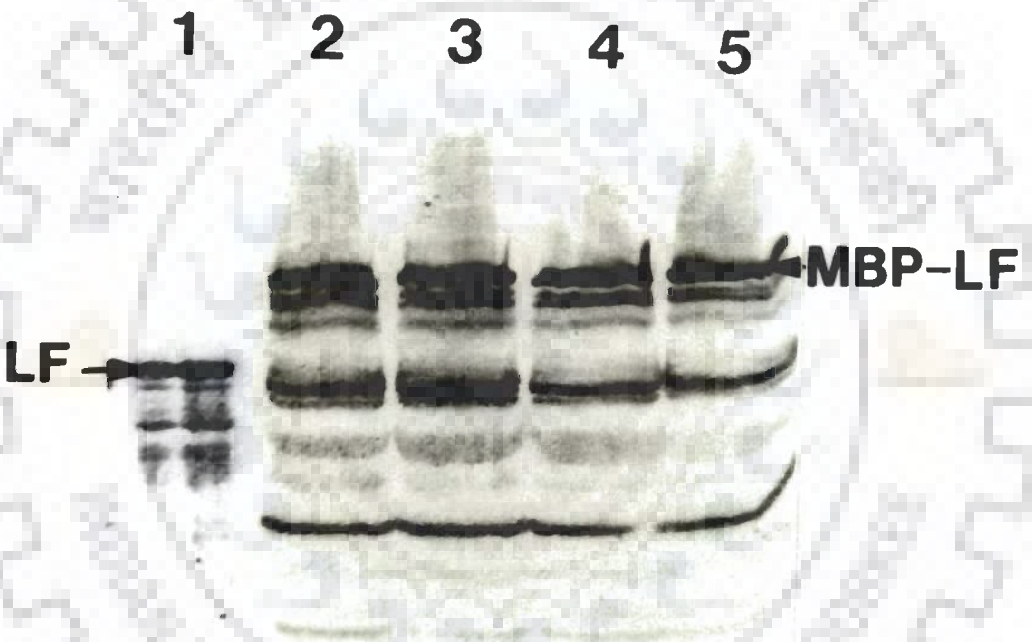
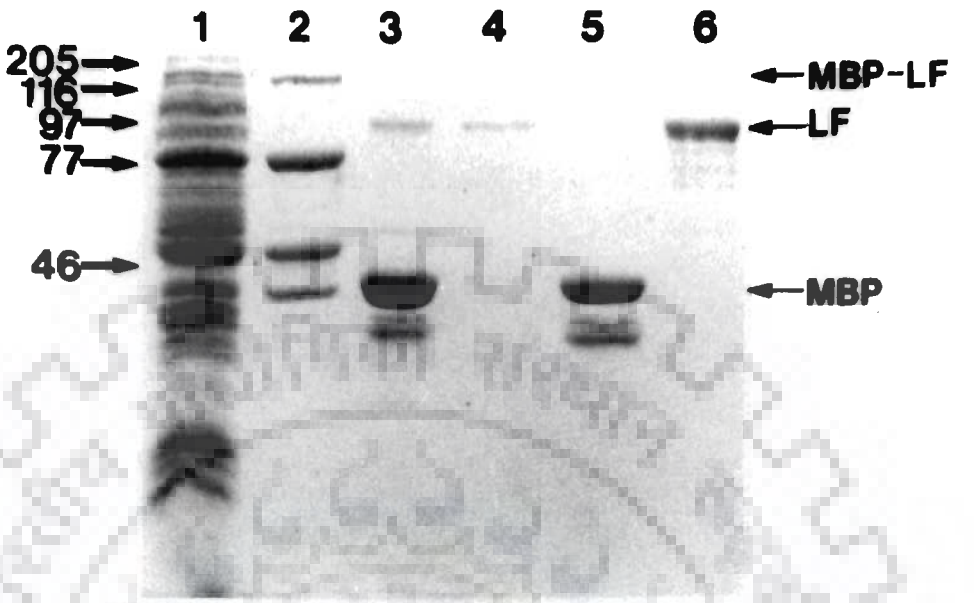
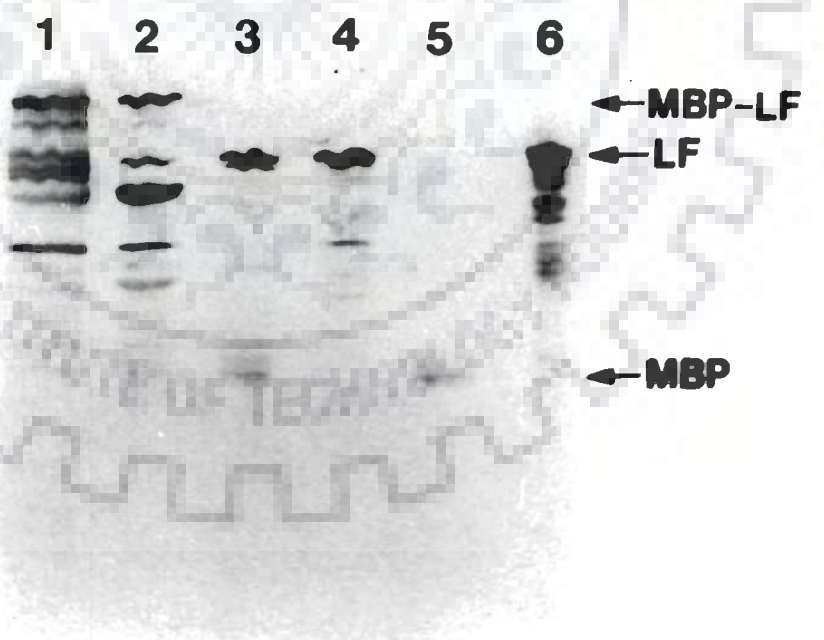


Figure 4 : Electrophoretic analysis of *Escherichia coli* expressed LF protein. Proteins were separated on 10% SDS-PAGE and (A) stained with Coomassie Brilliant Blue; (B) Western blot using polyclonal LF antibodies raised in rabbit. Lane 1, *E. coli* cells expressing LF; lane 2, MBP-LF fusion protein; lane 3, MBP-LF cleaved with factor Xa; lane 4, LF after passing through Sephadex G-100 column; lane 5, degraded proteins of LF obtained from Sephadex G- 100 column; lane 6, LF purified from *B. anthracis*. Numbers at the left side of the lanes denote the position of molecular weight in kDa.

ELECTROPHORETIC ANALYSIS OF *ESCHERICHIA COLI* EXPRESSED LF PROTEIN



A



B

Figure 5 : Biological activity of LF purified from *B. anthracis* and *Escherichia coli.* J774A.1 cells were incubated with PA (0.5 μg/ml) in combination with varying concentration of LF in DMEM for three hours at 37^{o}C. Cell viability was determined by MTT. o , LF purified from *B. anthracis*; ● , LF purified from *E. coli.*

BIOLOGICAL ACTIVITY OF LF PURIFIED FROM *B. ANTHRACIS* AND *ESCHERICHIA COLI*

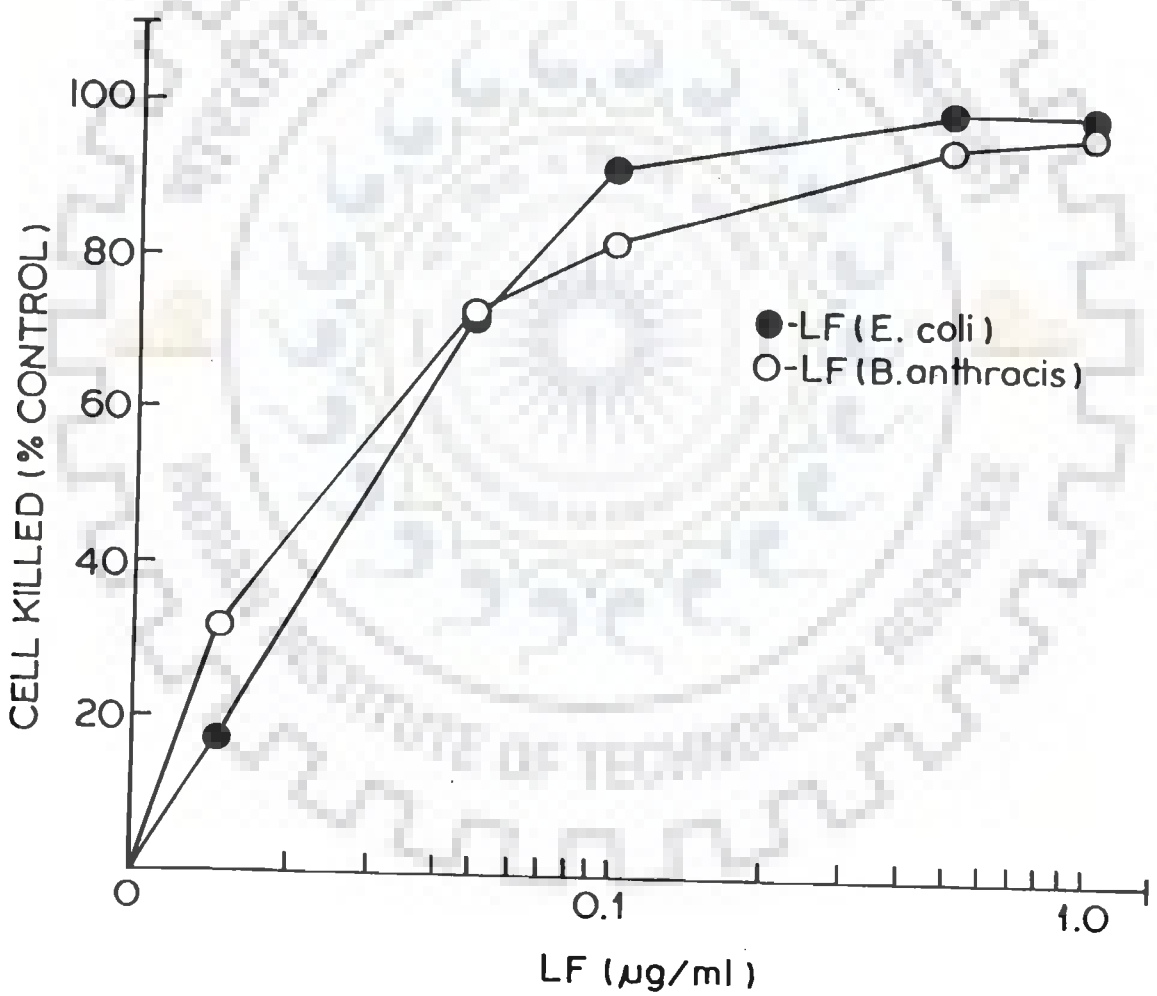
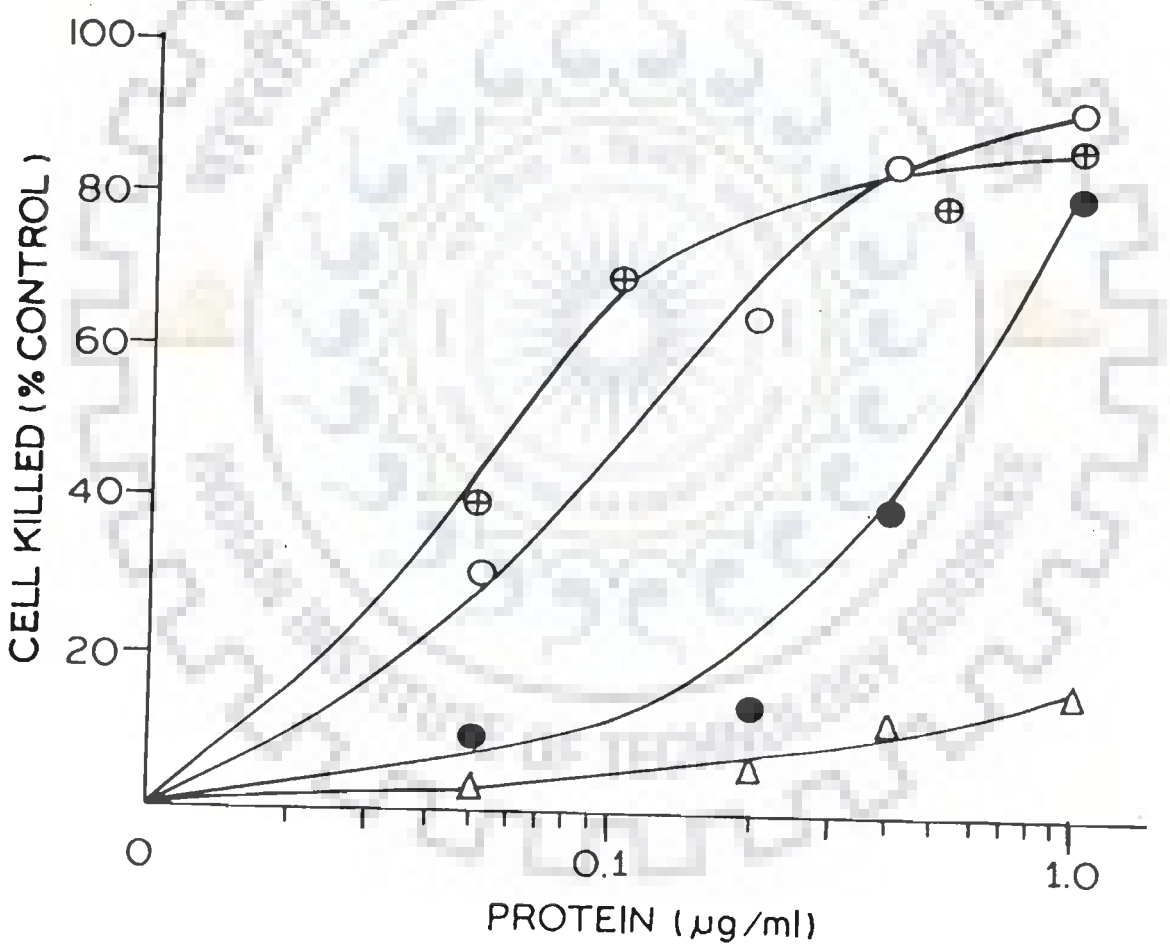


Figure 6 : Toxicity of LF after internalization through virosomes. Cells were incubated with virosomes containing toxin proteins in DMEM for three hours at 37°C. Cell viability was determined by MTT assay. ⊕ , PA and LF added in DMEM; o , Virosome containing PA and LF; ● , Virosome containing LF alone; ▲,Virosome containing PA alone.

TOXICITY OF LF AFTER INTERNALIZATION THROUGH VIROSOMES



virosomes. LF induced 80% killing of J774A.1 cells at a protein concentration of 1 μg/ml. Incubation of cells with virosomes without any protein or containing PA alone had no significant effect on cell viability. LF or PA proteins added alone in the media were also not toxic to the cells. The data in figure 6 shows that 0.1 μg of PA and LF each were required for 50% lysis of J774A.1 cells. However, when LF was delivered through virosomes 0.5 μg of LF produced 50% lysis of J774A.1 cells at 37^{o}C in the specified period of incubation (Fig. 6). Thus, the relative potency of LF was atleast five fold lower when delivered through virosomes than the PA receptor mediated process.

INVOLVEMENT OF GOLGI IN THE CYTOTOXICITY OF LETHAL TOXIN

Anthrax lethal toxin acts in a binary fashion, PA binds to the receptor and facilitates translocation of LF in the cytosol. Many protein toxins pass through endoplasmic reticulum and golgi before reaching their cellular target (Phelam, 1991; Donta et al., 1993; Nambier et al., 1993). Intervention in the vesicular trafficking of these toxin proteins abolishes the toxic activity suggesting that intracellular trafficking through certain organelles are important phenomenon in the process of cytotoxicity. Brafeldin A disrupts the protein distribution of golgi and ER, thus affecting the normal functioning of the vesicles leading to inhibition of activation/processing in the cytosol. To find out a possible involvement of golgi and ER in the cytotoxic activity of anthrax lethal toxin, macrophage cells (J774A.1) were preincubated with brefeldin (100 ng/ml) and then treated with lethal toxin. Lethal toxin induced 80% killing of cells within 3 hours at 37^{o}C. Preincubation of cells with brefeldin had no effect on LT induced cytotoxicity. Incubation of brefeldin in combination with LT was not protective to cells (Fig. 7).

PROTEOLYTIC ACTIVATION OF LF

Several bacterial and plant protein toxins require proteolytic activation for expression of their toxic activity. The biological activity of a number of toxins,

including PA of anthrax, pseudomonas exotoxin A, cholera, diphtheria and tetanus toxin are activated by furin and other cellular proteases (Gorden et al 1995). Diphtheria toxin and pseudomonas exotoxin A are cleaved at the sequence $RVRR^{193}$ and $RQPR^{279}$ respectively for translocation of the ADP-ribosyltransferase domain into the cytosol (Cieplak et al.,1988; Ogata et al.,1992). In anthrax lethal toxin, proteolytic cleavage of PA_{83} to form PA_{63} is prerequisite for LF binding, oligomerization and channel formation. Several reports have indicated that LF is transferred to the cytosol through a channel formed by PA_{63} heptamer (Milne et al., 1994; Singh et al., 1994; Zhao et al., 1995). Whether LF requires proteolytic activation for translocation or expression of its activity in the cytosol has not been studied. To answer these questions, iodinated LF (0.1 μg/ml) was incubated with J774A.1 cells for varying time period at 37^{o}C and the cell associated LF was analysed by gel electrophoresis for proteolytic cleavage. Figure 8 shows that LF remains intact even after two hours of internalization into J774A.1 cells (lane 2-6). These results indicate that LF is not proteolytically cleaved for expression of its biological activity.

SUBCELLULAR DISTRIBUTION OF LETHAL FACTOR

PA binds and translocates LF in the cytosol. Proteolytic activation of PA is an obligatory step for LF binding and subsequent internalization of lethal factor in the cytosol. Anthrax toxin contains a second catalytic molecule EF, which is also endocytosed into the cell in a similar manner as LF. Studies on EF have shown that EF is an adenylate cyclase which requires calmodulin and divalent cation Ca^{++} for its activity (Leppla, 1982). Similarity in the processes of internalization of both ET and LT suggested that cytosol could be an essential environment for the activity of LF like EF in cells. To study the intracellular routing of LF in macrophages iodinated LF (0.1 μg/ml) was added in combination with PA (0.5 μg/ml) on the macrophage (J774A.1) cells. Cells were incubated for different time periods (15 min to 220 min at 37^{o}C) and scrapped in sucrose solution to homogenize in a motor driven homogenizer (10 strokes of 5 seconds each).

Figure 7 : Effect of Brefeldin A on the activity of anthrax lethal toxin. J774A.1 cells were incubated with PA (0.5 μg/ml) and LF (0.1-2 μg/ml) for three hours at 37^{o}C. Brefeldin A (0.1 μg/ml) was preincubated or added in combination with lethal toxin. o , Lethal toxin alone; ▲ , Preincubated with brefeldin A and ● , Brefeldin A and toxin added together.

EFFECT OF BREFELDIN A ON THE ACTIVITY OF ANTHRAX LETHAL TOXIN

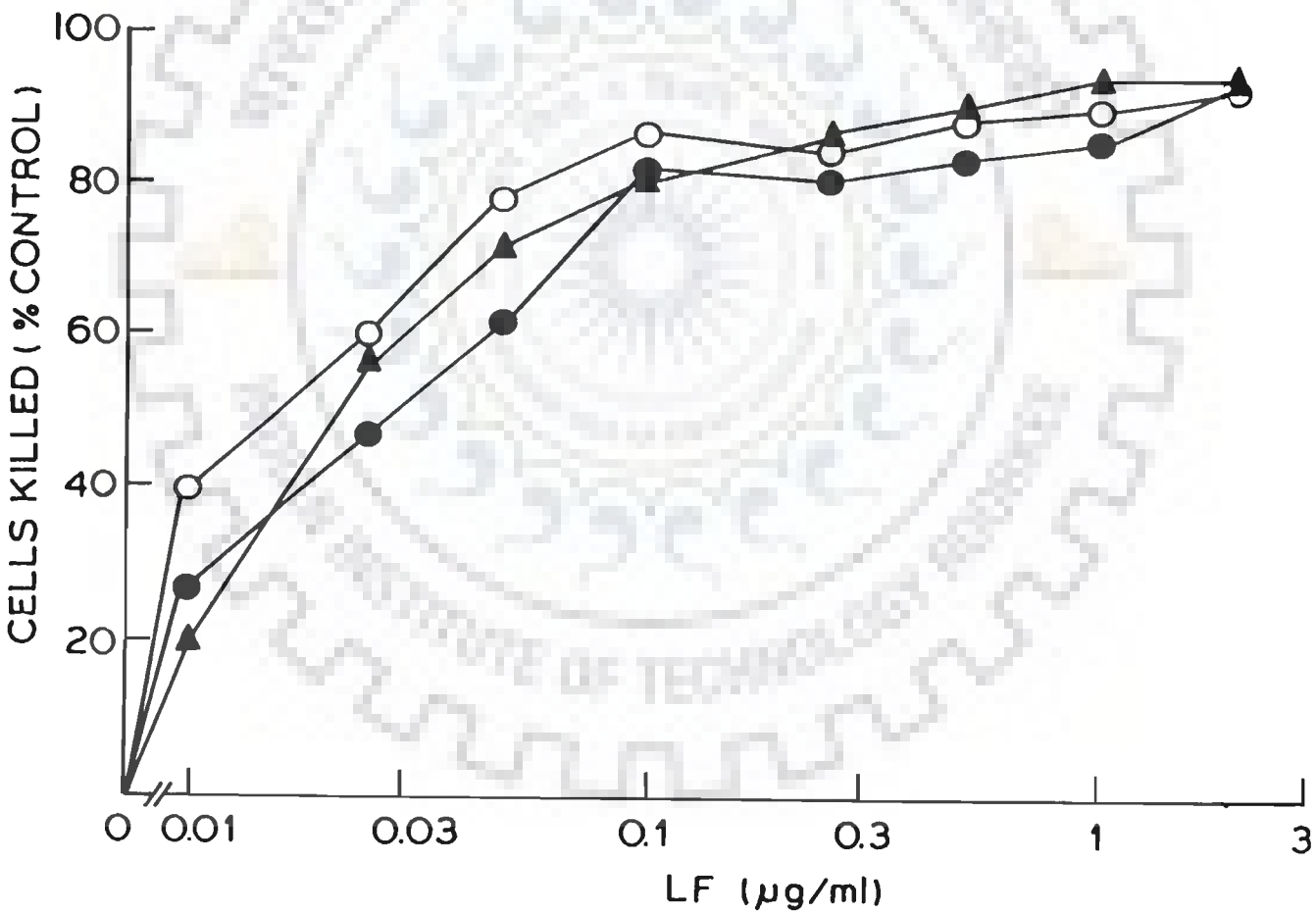


Figure 8 : Electrophoretic analysis of macrophage cell-associated LF. J774A.1 cells were incubated with ^{125}I-LF (0.1 μg/ml) in combination with PA (0.5 μg/ml) at 37^{o}C. At the end of incubation cells were washed with DMEM to remove free LF and solubilized in SDS-sample buffer. Proteins were separated in 10% SDS-PAGE, dried and autoradiographed. Lane 1, ^{125}I-LF standard; lane 2-6, ^{125}I-LF and PA incubated on cells for different time period (lane 2, 0 min.; lane 3, 30 min.; lane 4, 60 min.; lane 5, 90 min.; lane 6, 120 min.)

ELECTROPHORETIC ANALYSIS OF CELL ASSOCIATED LF

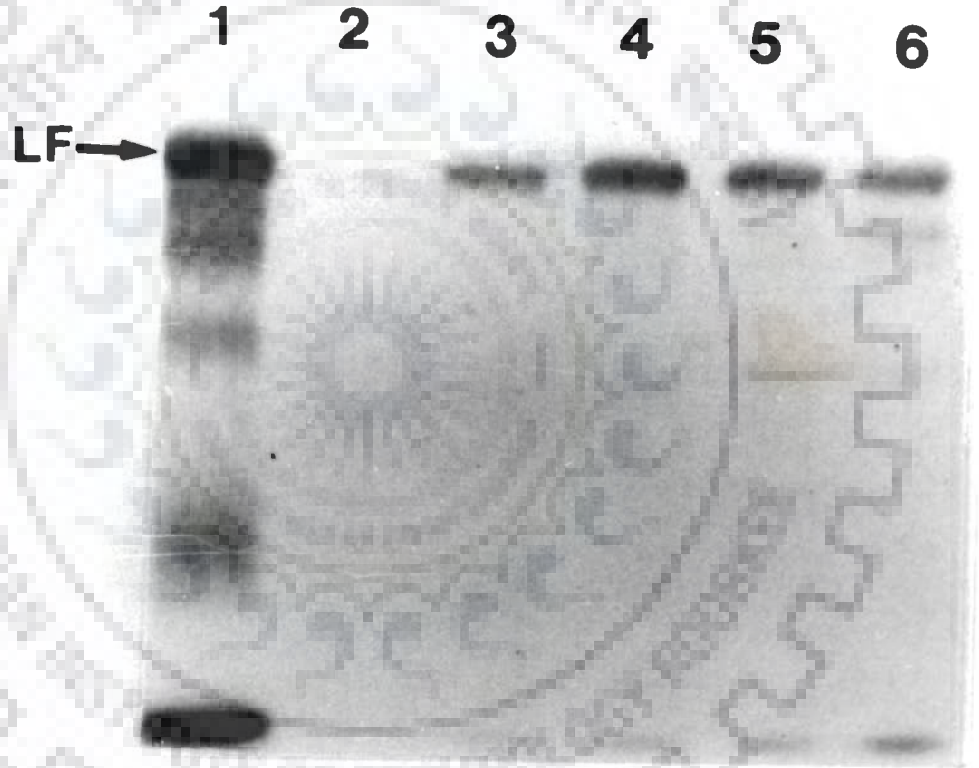


Table 1 : Sub-cellular Distribution of LF in Macrophage Cells.

Time (min)	Percent ^{125}I-LF				
	Cytosolic	Microsomal	Mitochondrial	Lysosomal	Nuclear
15	63.9	5.9	3.7	15.1	11.4
45	53.5	9.8	4.7	22.9	9.1
90	47.9	8.9	8.3	28.3	6.6
120	40.6	7.1	11.0	29.4	11.9
180	59.4	5.1	8.4	19.1	8.1
220	71.4	5.4	3.4	13.4	6.3

Sub-cellular fractionation of macrophage cells treated with lethal toxin. J774A.1 cells incubated with ^{125}I-LF (0.1 μg/ml) and PA (0.5 μg/ml) for various time periods. Cells were washed with DMEM to remove free proteins and scrapped out in 250 mM sucrose solution containing 20 mM Tris-Cl, pH 7.4 and 1 mM EDTA. Cells were homogenized and subjected to differential centrifugation to isolate nucleus, mitochondria, lysosome, microsome and cytosol. Amount of LF associated with the organelle fractions was determined in a γ-counter. Each value is the average of two individual flasks and the percent count expressed as compared to the total count in the cell.

Differential centrifugation was used to separate nucleus, mitochondria, lysosome, microsome and cytosolic fractions (Bijsterbosch and Van Berkel, 1992). Each fraction was counted for thepresence of iodinated LF (Table 1). The results indicate that a substantial amount of LF is present in the cytosol (Table 1). The concentration of cytosolic LF remains unchanged during the experiment. The amount of LF associated with microsome, mitochondria and nuclear fractions was very less in comparison to cytosol (Table 1). A small increase in LF with time was observed in lysosomal fraction, which again decreased after 120 min of incubation (Table 1). These results indicate that LF is associated mainly with lysosome and cytosol.

EFFECT OF LETHAL TOXIN ON CELLULAR PROTEIN SYNTHESIS

Several bacterial and plant protein toxins cause cell death by their ability to inhibit protein synthesis. To study the possibility of mammalian protein synthesis inhibition by anthrax lethal toxin, J774A.1 cells were incubated with radioactive leucine (5 μCi/ml) along with the lethal toxin. In the experiment cell viability was determined simultaneously to correlate the toxicity with the protein synthesis inhibition activity of the LT. Incorporation of leucine into proteins and oxidation of MTT was monitored at various time points. Figure 9 shows the effect of anthrax lethal toxin on cellular protein synthesis and its role in cell death. Incubation of J774A.1 cells with lethal toxin (0.5 μg/ml each PA and LF) had no significant effect on protein synthesis upto 120 minutes. However, the incorporation of ^{14}C- leucine started decreasing in lethal toxin treated cells after 120 minutes of incubation. In the same experiment, lethal toxin was not toxic upto 90 minutes, as observed by oxidation of MTT. The toxic effect of lethal toxin started after 90 minutes of incubation and increased with time (Fig. 9). An inhibition of 50% in the rate of protein synthesis was observed at 225 minutes whereas, 50% cytotoxicity was recorded in 160 minutes suggesting that inhibition of protein synthesis is not the primary event in the action of anthrax lethal toxin.

INHIBITION OF HOST CELL PROTEIN SYNTHESIS PROTECTS AGAINST LETHAL TOXIN

Several bacterial toxin proteins require intracellular processing like proteolytic activation and thus they need other proteins for the expression of their activity. Many toxins have special sequences which permit routing through particular organelle. For example pseudomonas exotoxin carries REDLK at its COOH-terminal region which helps the toxin to pass through the endoplasmic reticulum and has been shown to be essential for its toxicity (Chaudhary et al., 1990; Saraswathy et al., 1991). The trafficking or processing of lethal toxin may involve several cellular proteins. The involvement of cellular protein(s) for the toxicity of lethal toxin was studied by blocking the cellular protein synthesis and then exposing the cells to anthrax lethal toxin. Figure 10 shows the role of mammalian protein synthesis in the lysis of J774A.1 cells by anthrax lethal toxin. Incubation of J774A.1 cells with cycloheximide (0.5 μg/ml) and anthrax lethal toxin (0.5 μg/ml each) together for three hours did not provide any protection to cells although an 83% inhibition of cellular protein synthesis was observed (Fig 10 D). Preincubation of cells for 6 hours with cycloheximide (0.5 μg/ml) followed by incubation with LT (0.5 μg/ml PA and LF each) without cycloheximide provided only 16% protection to the cells (Fig. 10 B). However, when cells were incubated for 6 hours with cycloheximide and then exposed to the lethal toxin in the presence of cycloheximide (0.5 μg/ml) 56% protection was observed at the level of 83% inhibition of cellular protein synthesis. In a similar set of experiments no protection to the cells was observed when lethal toxin was added to the cells already incubated for 6 hours in DMEM containing cycloheximide followed by one hour incubation in DMEM without cycloheximide. In such cells the level of inhibition of protein synthesis was observed to be 19% before toxin challenge. Thus, it can be suggested that the protection conferred to the cells by the inhibition of protein synthesis could be due to the involvement of macrophage protein(s) in the toxicity of anthrax lethal toxin. To rule out the possibility of

Figure 9 : Inhibition of protein synthesis by lethal toxin. Cells were incubated with lethal toxin (0.5 μg/ml each PA and LF) for various time periods. Protein synthesis (o) by ^{14}C-leucine and cell viability (●) by MTT assay were determined.

INHIBITION OF PROTEIN SYNTHESIS BY LETHAL TOXIN

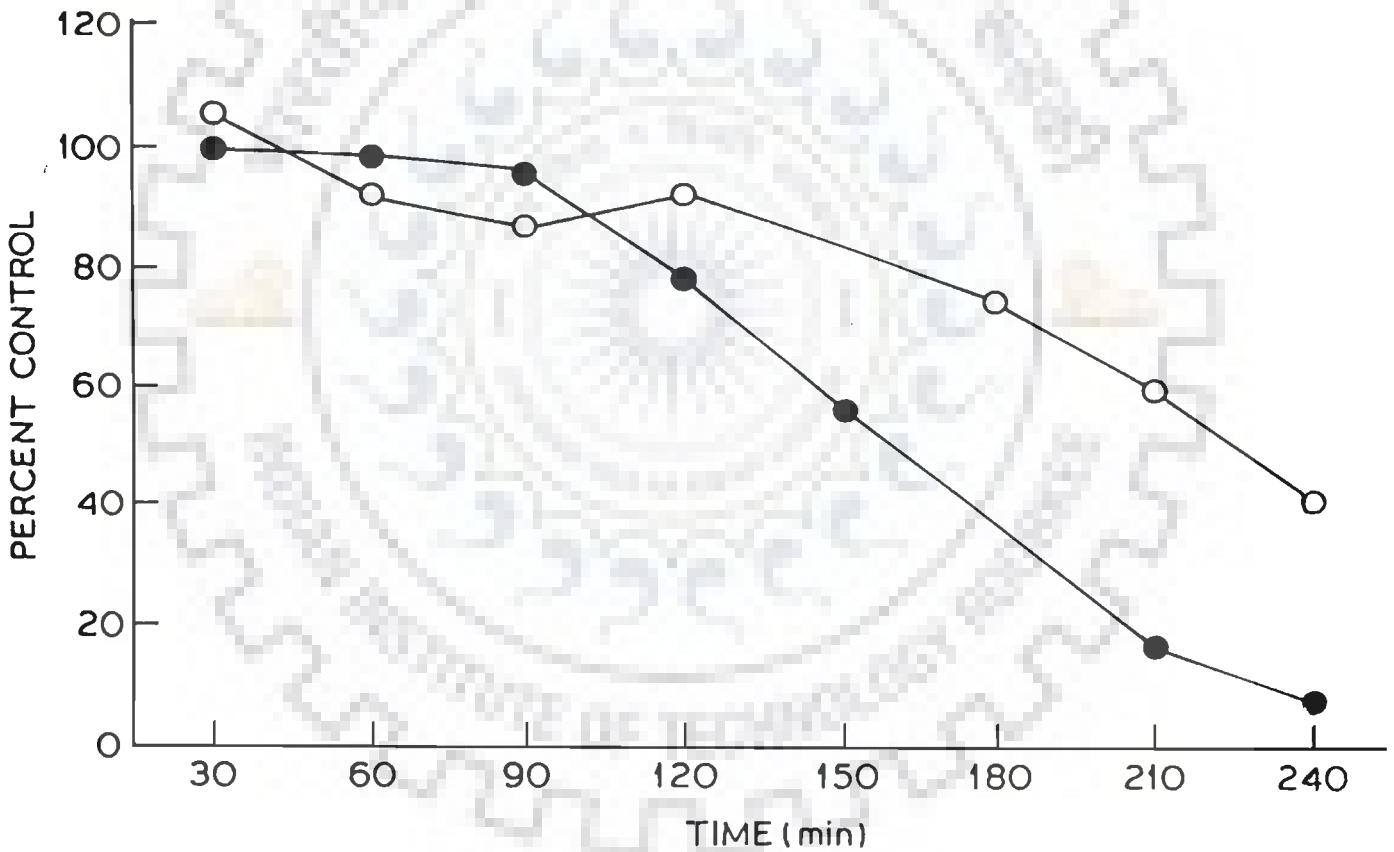


Figure 10 : Effect of cycloheximide on the toxicity induced by lethal toxin. Cells were incubated with cycloheximide (0.5 μg/ml) for different time period. The role of protein synthesis (▭) was monitered by the incorporation of ^{14}C-leucine into the proteins for 2.5 hours and cell viability (▬) was determined by MTT assay. (A) Cells incubated with cycloheximide and lethal toxin for three hours. (B) Cells were preincubated with cycloheximide for six hours, then lethal toxin was added and incubated for three hours (cycloheximide was absent in last three hours). (C) Cells were preincubated with cycloheximide for six hours and then lethal toxin added together with cycloheximide for three hours. (D) cells were preincubated with cycloheximide for six hours followed by one hour incubation with DMEM (cycloheximide absent) and then lethal toxin was added for six hours without cycloheximide.

EFFECT OF CYCLOHEXIMIDE ON THE TOXICITY INDUCED BY LETHAL TOXIN

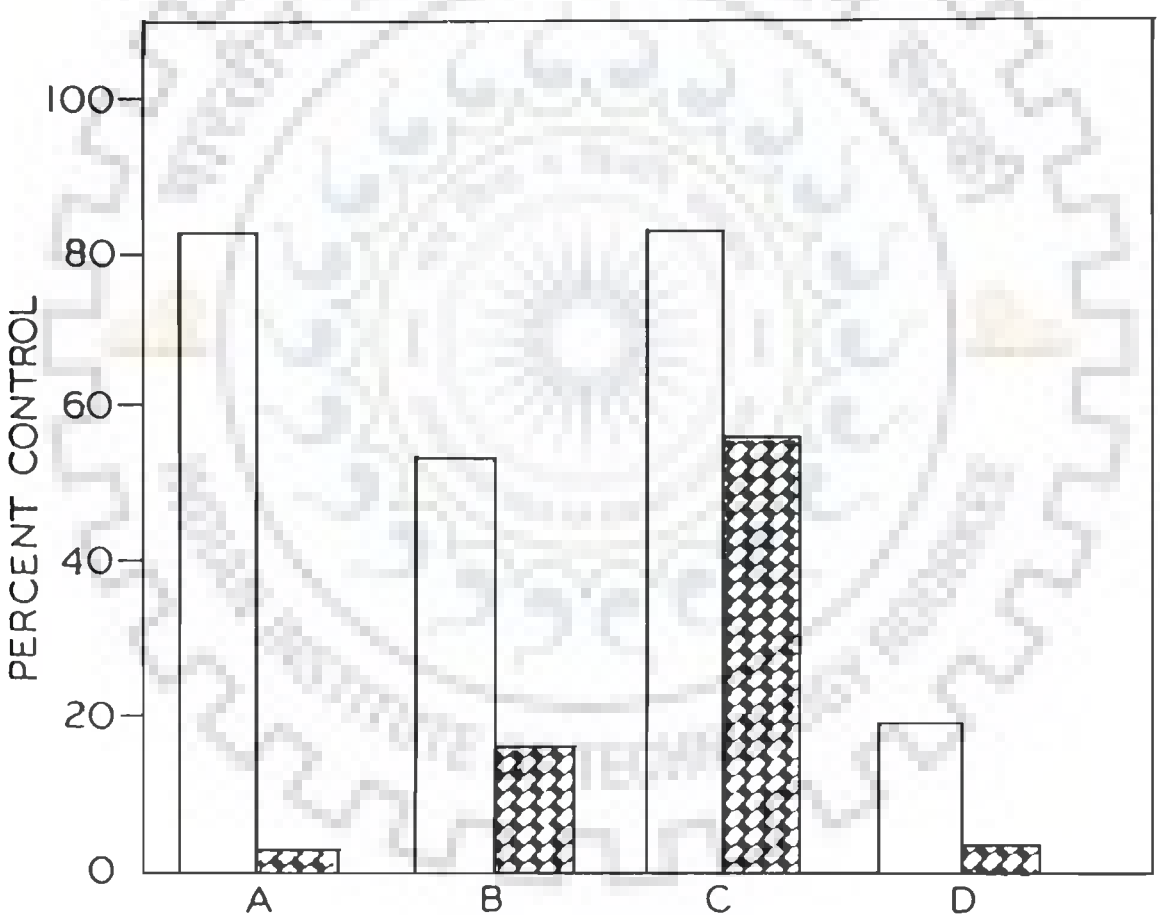


Table 2 : Effect of cycloheximide on the binding of PA to cell receptors

Treatment	PA bound (cpm/mg protein)
None	20659 ± 522
Cycloheximide	19979 ± 1572

Cells were cultured in 6-well plates in DMEM containing 10% fetal calf serum and cycloheximide (0.5 μg/ml) for six hours. ^{125}I-PA (0.5 μg/ml) in DMEM was added and incubated at 37^{o}C for two hours. Cells were washed four times with DMEM and solubilized in 0.1 M NaOH. Protein concentration was determined using BCA reagent. Each value is average ± SE of four individual wells. Non-specific binding was determined by competing with 100 fold nonradioactive PA and was less than 20%.

decreased binding of PA on the macrophage cells, J774A.1 cells were incubated with ^{125}I-PA (0.5 μg/ml) after the preincubation of cells with cycloheximide (0.5 μg/ml). Table 2 shows the effect of cycloheximide on the binding of PA to cell surface receptors. Cycloheximide had no significant effect on the binding of PA to the receptors suggesting that cycloheximide inhibited some cellular protein(s) other than the PA receptors to make macrophages resistant to lethal toxin.

GENERATION OF INOSITOL PHOSPHATE BY LETHAL TOXIN

Calcium is required at several steps in the intoxication of cells by anthrax lethal toxin (Bhatnagar et al., 1989). Inositol- 1,4,5-triphosphate (IP_3) has been implicated to mobilize stored calcium and to promote an influx of external calcium in conjuction with Inositol-1,3,4,5-tetrakisphosphate (IP_4). The influx of Ca^{++} in the cells increases after 90 min of toxin treatment (Bhatnagar et al., 1989). To study the generation of IP_3 by anthrax lethal toxin, J774A.1 cells were labeled with ^{3}H- Inositol. The labeled cells were incubated with LT for 30 and 90 minutes at 37^{o}C. At the end of incubation cells were processed for extraction of inositol phosphate. Incubation of cells with lethal toxin (0.001-0.1 μg/ml) had no effect on the level of inositol phosphates upto 30 minutes (Fig. 11). The amount of inositol phosphates increased after 90 minutes of incubation with toxin (Fig. 11). The increase in inositol phosphate was dependent on the concentration of lethal toxin. These results suggest that lethal toxin induces release of inositol phosphate which in turn may lead to increased influx of Ca^{++} in the cell.

EFFECT OF pH ON LF AND PAn (TRYPSIN NICKED PA)

Protective antigen is cleaved by trypsin or by cell surface proteases to produce 63 kDa carboxy-terminal and 20 kDa amino- terminal fragments (Leppla et al., 1988). Cleavage of PA results in the formation of oligomeric form of PA_{63}. The oligomerization process is enhanced by acidic pH. The effect of pH on trypsin nicked PA (PAn) and LF was studied in non-denaturing conditions by gel electrophoresis. Electrophoretic analysis of PAn showed two expected bands of

63 and 20 kDa protein (Fig. 12, lane 1). Acidification of PAn resulted in the appearance of two prominent slow moving bands (Fig. 12, lane 2-4). The amount of protein present in the upper band was four times higher than the lower band as determined by densitometric analysis. Purified LF appeared as single band (Fig. 12, lane 5). The electrophoretic mobility of LF was not affected by change in pH (Fig. 12, lane 6- 8).

EFFECT OF pH ON THE PAn-LF COMPLEX

Acidification is required for insertion of PA_{63} into lipid membrane (Blaustein et al., 1989) and translocation of LF into cytosol (Zhao et al 1995). The changes in PA-LF complex induced by acidification was studied by mixing the LF with PAn at various pH values and then analysing on native gradient gel (8-25%, Pharmacia). LF combined with PAn at neutral, basic and acidic pH conditions and resolved as a single band on the gel (Fig. 13, lane 5-7). However, the electrophoretic mobility of the complex showed a shift at acidic pH (Fig. 13, lane 7). A similar shift in the mobility of the complex was observed when PAn was allowed to combine with LF at pH 7.0 and then changed to pH 5.0 (Fig. 13, lane 8). The above results clearly demonstrated that acidic pH causes a shift in the mobility of the PA-LF complex. However, these experiments failed to point that PA, LF or both the proteins cause the shift in the mobility of the complex. The stability of PAn-LF complex at acidic pH was also not defined by these experiment. To resolve these questions iodinated PA and LF were used to study the effect of pH on the mobility PAn-LF complex.

Acidic pH induces formation of heptameric oligomer of PAn or PA_{63} in solution as well as on cells (Milne et al., 1994). The PA_{63} heptamers are very stable and SDS resistant (Milne et al., 1994, Singh et al., 1994). The complex of PAn and LF is not SDS resistant as the individual proteins dissociate apart after SDS- treatment and can be visualized as individual bands on gel. To study the effect of pH on PAn-LF complex, radiolabeled LF was combined with PAn (unlabeled) at several pH values. The samples were analysed on native (8-25%)

Figure 11 : Lethal toxin induced production of inositol phosphates in macrophage cells. J774A.1 cells incubated with (4 μCi/ml) ^{3}H- Inositol for 12 hours at 37^{o}C. Cells were induced in DMEM containing PA (0.1 μg/ml) and varying concentrations of LF (0.001-0.1 μg/ml). Total inositol phosphate was extracted. The radioactivity was counted in a β−counter. ▭ , Control without any toxin; ▭ , Inositol phosphates with 0.001 μg/ml of LF; ▭ , Inositol phosphate with 0.01 μg/ml of LF, ▬ , Inositol phosphate with 0.1 μg/ml of LF.

LETHAL TOXIN INDUCED PRODUCTION OF INOSITOL PHOSPHATES IN MACROPHAGE CELLS

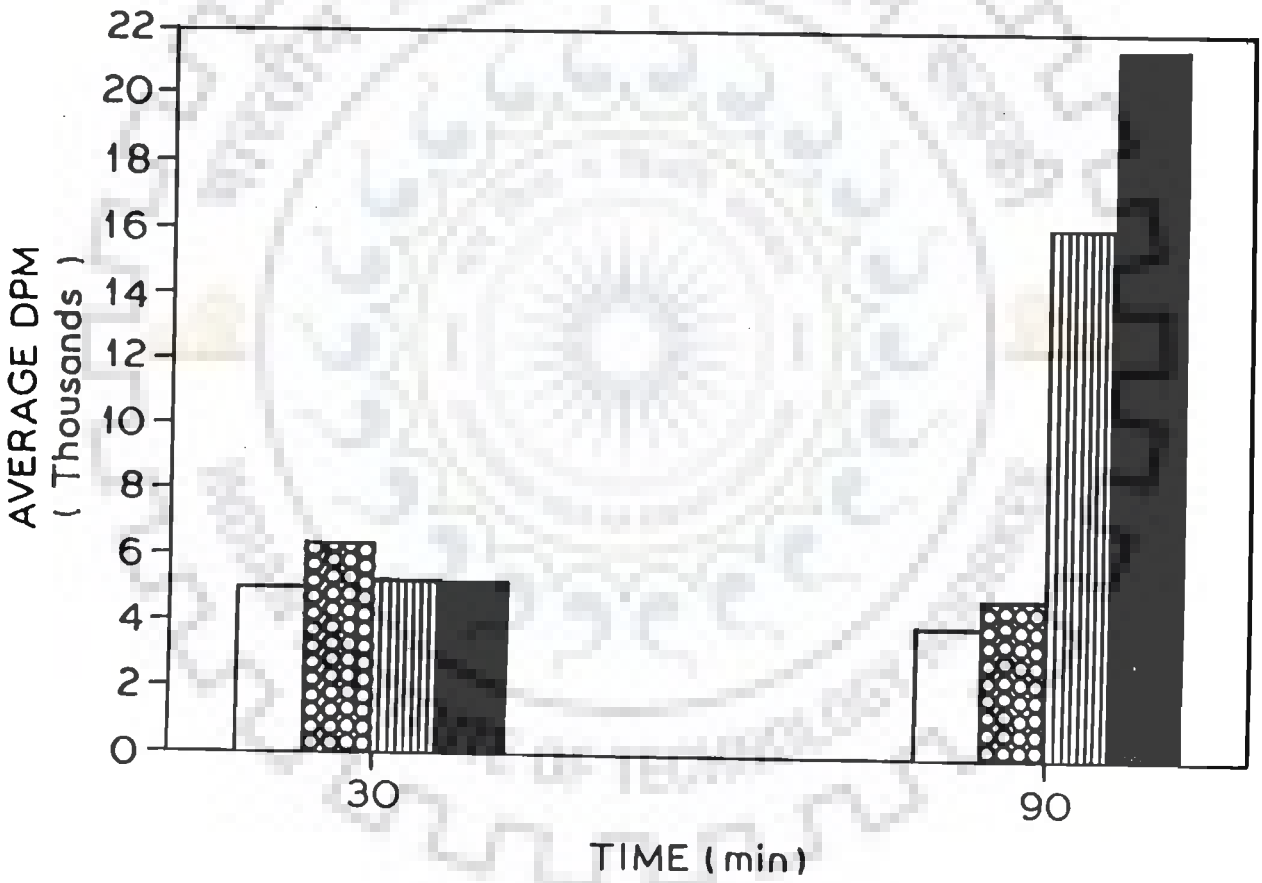


Figure 12 : Effect of pH on the electrophoretic mobility of PAn and LF. PA protein was cleaved with trypsin (2.5 ng/ μg of protein) at room temperature. pH of the samples was turned acidic by dilute HCl and electrophoretic mobility was determined on native gradient (8-25%) gel. Gel was stained with Coomassie Brilliant Blue. lane 1-4, PA(trypsin nicked); lane 5-8, LF at pH 7.2, 6.0, 5.5, 4.5. respectively.

EFFECT OF pH ON THE ELECTROPHORETIC MOBILITY OF PAn AND LF

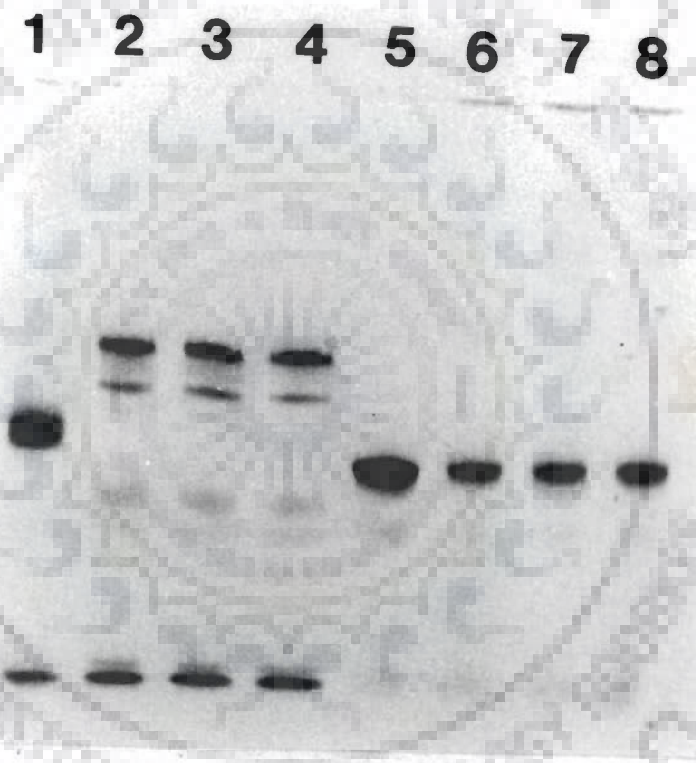
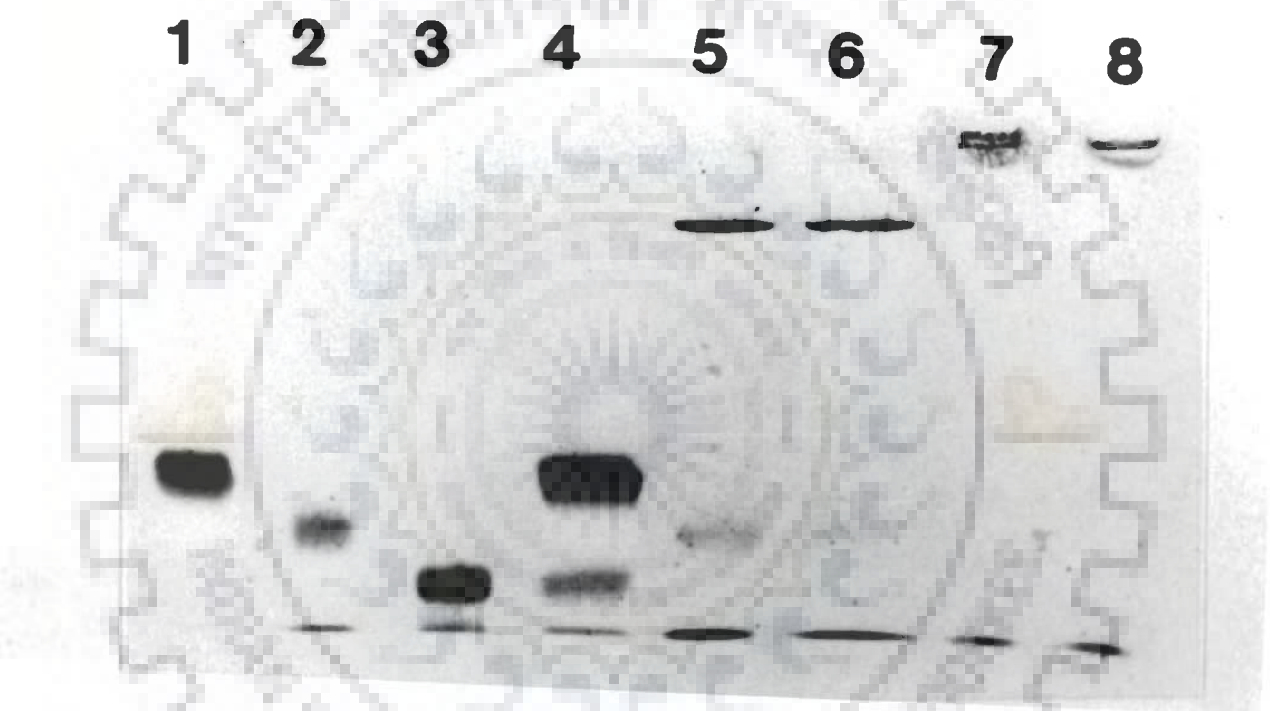


Figure 13 : Effect of pH on binding of LF to PAn in solution. PA was trypsinized and incubated with equimolar concentration of LF for 20 minutes at room temperature. The samples were analysed on non- denaturing gradient (8-25%) gel. Gel was stained with Coomassie Brilliant Blue. Lane 1, PA; lane 2, PAn; lane 3, LF; lane 4, PA and LF; lane 5, PAn-LF pH 7.2; lane 6, PAn-LF pH 9.6; PAn-LF pH 5.0; lane 8, PAn-LF initially bound at pH 7.2 and then turned to 5.0.

EFFECT OF pH ON BINDING OF LF TO PAn IN SOLUTION



gradient gel. Autoradiogram of the gel showed that LF remains associated with PA monomer and oligomers at acidic pH values (Fig. 14 A, lane 1-4). All the LF added to the PAn was bound to form PAn-LF complex without leaving any LF free.

Identical experiment using ^{125}I-PA and LF caused a shift in the mobility of high molecular complex at acidic pH (Fig. 14 B, lane 2-4). The high molecular protein bands (Fig. 14 B, lane 1- 4) on the autoradiogram were correspondingly identical to bands observed in lane 1-4, where radiolabeled LF was used in the experiment (Fig. 14 A). The results indicate that acidic pH does not inhibit formation of PAn-LF complex.

To provide more evidence that binding of LF to PAn was not affected by pH, PAn and ^{125}I-LF were combined at various pH conditions. The solution containing PAn-^{125}I-LF was added to the RAW264.7 cells and incubated for 2 hours at 37^{o}C. PA receptors on the surface of the macrophage cells bind to PAn-LF complex. At the end of the experiment free proteins were washed with DMEM and cells were solubilized in SDS-sample buffer and analysed by 10% SDS- PAGE. Autoradiogram (Fig. 15, lane 2-5) showed no difference in the band intensity of LF suggesting that pH does not inhibit the binding of LF to PA in solution.

CIRCULAR DICHROISM

The CD spectrum of a protein reflects the overall picture of the molecule. Prediction of secondary structure from amino acid sequence combined with analysis of CD spectra have proved to be useful in elucidating the structure of proteins that have not been crystallized (Manavalan and Johnson, 1983).

The CD spectrum of PA protein (150 μg/ml) in 10 mM Tris buffer, pH 7.2 containing 1mM $CaCl_2$ showed a negative band with extremum at 205 nm (Molar ellipticity $[\theta]_{205}$ = -7200 deg cm^2 dmole^{-1}) (Fig. 16 A). Proteolytic cleavage of PA was performed in the cuvette by adding trypsin (300 ng) into the solution.

Trypsinization of PA resulted in decreased molar ellipticity at 205 nm to -5600 deg cm^2 $dmole^{-1}$, without any change in the band extremum (Fig. 16 A). The analysis of CD spectra showed that PA has a substantial fraction of *b*-structures (sheet and plates). The fractions of *a* helix were less and the pattern of spectra matches very well to the spectra of β-class of proteins (Manavalan and Johnson, 1983). Cleavage with trypsin resulted in decrease of the fractions of β-sheet and simultaneous increase in the fraction of unordered structure. Such changes in the secondary conformation could be essential for inter-protein interaction like binding of LF to PA in solution.

CD spectra of native LF (120 μg/ml) in 10 mM Tris buffer, pH 7.2 containing 1 mM $CaCl_2$ showed a negative band ($[\theta]_{207}$ = -6600 deg cm^2 $dmol^{-1}$) at 207 nm with a shoulder band at 218 nm (Fig 16 B). The spectra typically fits the pattern reported for alpha and Beta group of proteins (Manavalan and Johnson, 1983). The analysis of secondary structure of LF showed that β-content is more than the α-helix. However, the helical content in the secondary structure of LF was more than that in PA or PAn.

ANS BINDING STUDY

Proteolytic activation is an essential process for biological activity of several protein toxins. Such proteolytic processing of the proteins like PA of anthrax (Leppla et al., 1988) and aerolysin (van der Goot et al., 1992) has been found to bring a change in the structural conformation of the protein. ANS and TNS are the hydrophobic fluorescent markers used for such studies. In case of PA of anthrax toxin, it has been reported that proteolytic cleavage results in higher relative fluorescence than native PA. The fluorescence further increases with decrease in pH. Conformational changes in the protein structure due to change in the environment (pH of the solution) or proteolytic cleavage exposes the burried hydrophobic areas of the protein. Since LF bound to PA is endocytosed and gets exposed to the acidic pH during the process of translocation into the cytosol, the LF may undergo a similar change in its structure.

EFFECT OF pH ON THE ELECTROPHORETIC MOBILITY OF PAn-LF COMPLEX

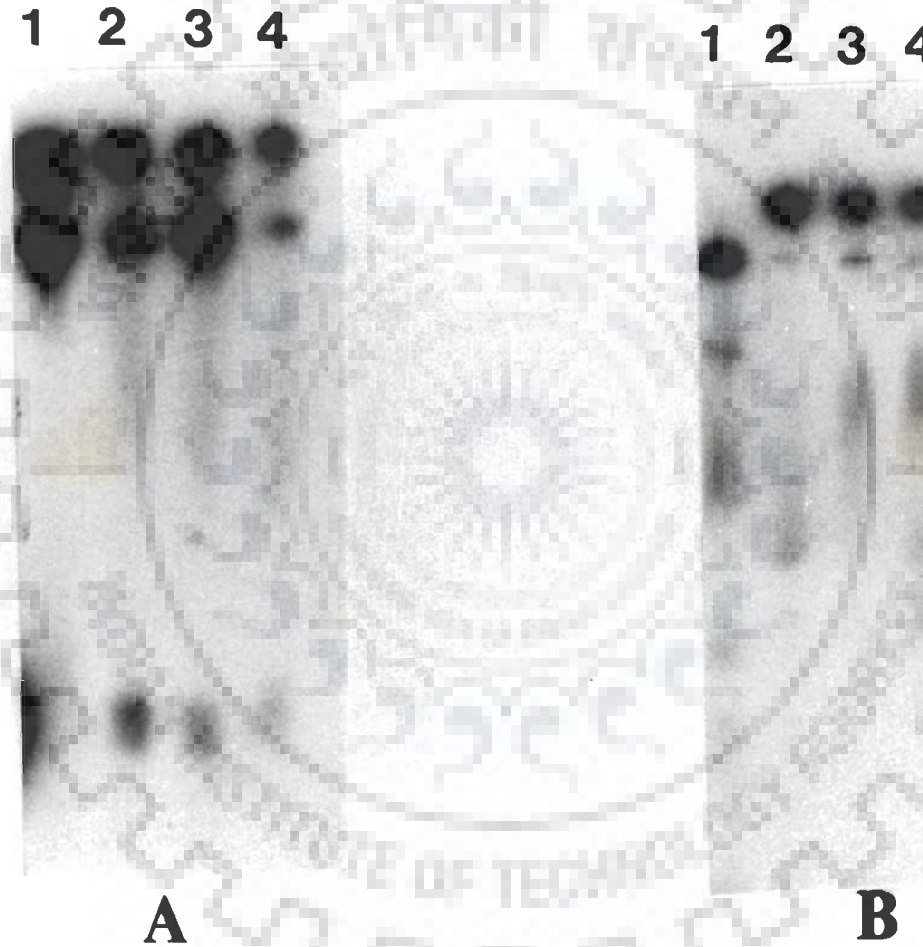


Figure 15 : Effect of pH on the receptor binding activity of PAn-LF complex. Macrophage cells (RAW264.7) were incubated at 37^{o}C with iodinated PA and LF or PAn-LF complex for two hours. At the end of the experiment unbound proteins were removed by washing with DMEM. Cells were solubilized in SDS-sample buffer and analysed by 10% SDS-PAGE. Gel was stained, destained, dried and autoradiographed. Lane 1, ^{125}I-PA + LF; lane 2, PA + ^{125}I-LF; lane 3, PAn-^{125}I-LF, pH 7.2; lane 4, PAn-^{125}I-LF pH 5.0; lane 5, (PAn, pH 5.0)-^{125}I-LF

EFFECT OF pH ON THE RECEPTOR BINDING ACTIVITY OF PAn-LF COMPLEX

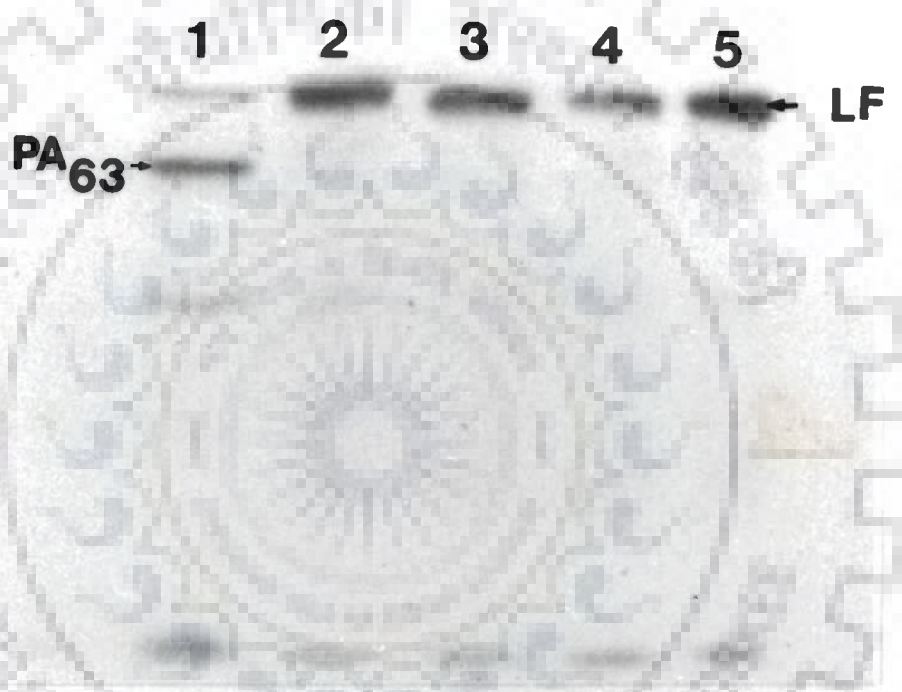
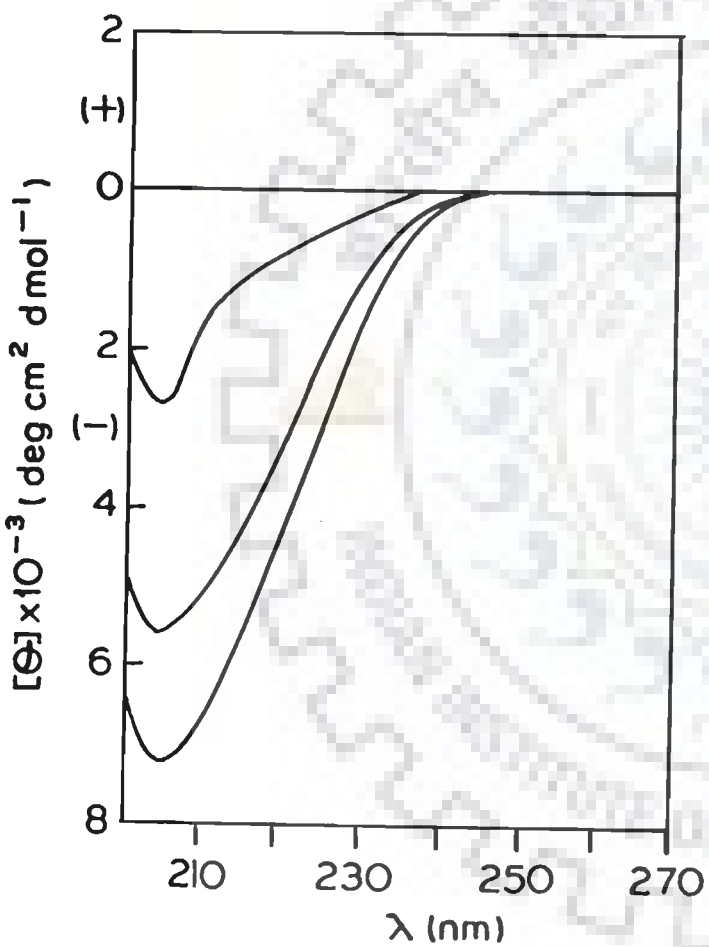
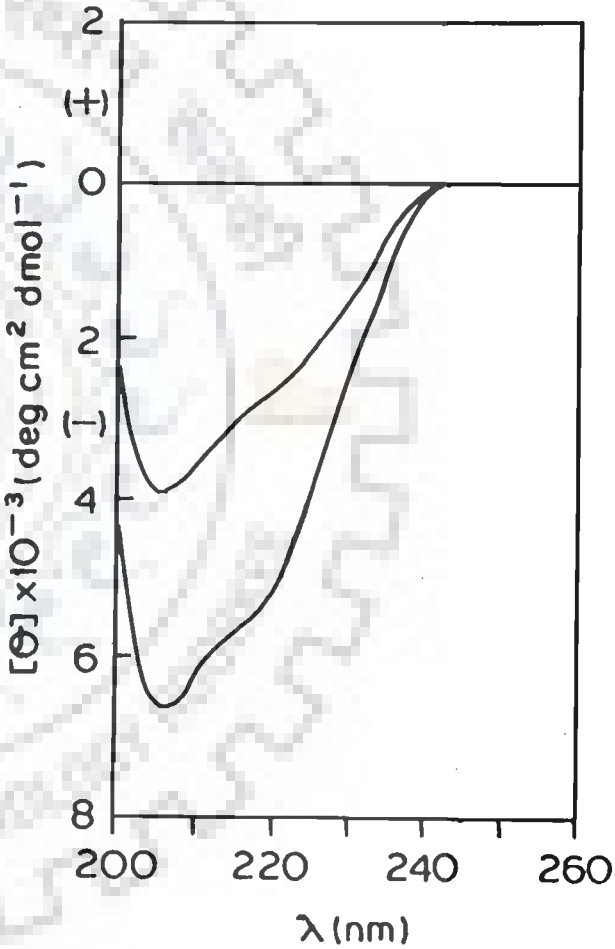


Figure 16 : Circular dichroism spectra of PA and LF. CD spectra of (A) PA and (B) LF in 10 mM Tris buffer, pH 7.2

CIRCULAR DICHROISM SPECTRA OF PA AND LF



A



B

To study the pH induced changes in LF, fluorescence studies were performed. LF (10 μg/ml) was incubated with ANS (5 μg/ml) in 10 mM Tris acetate buffer, pH 6.8. The pH of the solution was made acidic (pH 4.0) by dilute HCl and fluorescence spectra were recorded in the wave length region, 400-540 nm (Fig. 17). The emission spectra of ANS bound to LF at acidic pH (4.0) showed 20% increased fluorescence. The result suggests that acidic pH induces conformational changes on LF exposing hydrophobic regions on the protein.

STOICHIOMETRY OF BINDING OF LF TO PA IN SOLUTION

Proteolytic cleavage of PA at the furin site, $RKKR^{167}$, either in solution (Leppla et al 1991) or on the cell surface (Klimpel et al 1992) leads to the exposure of a high affinity binding site for LF or EF on the 63 kDa carboxyl-terminal fragment. Little is known about the stoichiometry of binding of LF/EF to PA_{63}. The interaction of PA_{63} and LF in solution was studied by gel electrophoresis under non-denaturing conditions. The commercially available phast gel (4-15%, Pharmacia) was soaked with a pH 8.8 buffer containing CHAPS. Agarose buffer strips were prepared containing alanine solution that produces a stacking effect. In this electrophoresis system, native PA and LF have a high mobility (Fig. 18A , lane 1 and 3). PA was cleaved with low concentration of trypsin and then incubated at pH 9.0 with LF to allow complex formation. Trypsin cleaved PA, in the absence of LF, formed a diffuse and slow moving band (Fig. 18A, lane 2). The stoichiometry of binding was estimated by adding increasing amount of LF to a fixed amount of PA_{63}. No free LF was observed when the amount of LF was one-half or equal to the amount of PA, indicating that all the LF was associated with the slowly migrating PA_{63} band (Fig. 18A, lane 4 and 5). When LF was increased to twice the mass of PA, half of the LF migrated as free LF (Fig. 18A, lane 6).

A more precise measurement of the capacity of PA_{63} to bind LF was made by adding amounts of LF that were nearly equal to the amount of PA_{63}

(Fig. 18 B). In this case small amounts of free LF first become clearly evident in lane 3, which contained 297 ng LF and 270 ng of trypsin nicked PA. Because the molecular weights of LF and full size PA are 90,200 and 82, 700, respectively, this lane contained LF and PA_{63} at a mass ratio of 1.1:1.0 and a molar ratio of 1.0:1.0. As the amount of LF increased further, the band of free LF increased proportionately indicating that the slower-migrating PA_{63} oligomer was saturated with LF (Fig. 18B, lane 4-6).

Gel electrophoresis of mixtures containing limiting amounts of LF with purified PA_{63} are shown in figure 19. Incubation of PA_{63} oligomer with limiting amounts of LF caused formation of PA_{63}-LF complexes with varying number of LF. The prominent band observed in all samples with LF:PA ratio between 0.4-2.0 (Fig. 19) may be a complex having a equal numbers of PA_{63} and LF molecules, presumably seven of each. These lanes also contain two faint bands having lower mobility than the putative 7:7 complex. The data from the denaturing gels suggest that the PA_{63} oligomer can bind variable numbers of LF molecules up to a maximum of seven in the heptameric complex.

STOICHIOMETRY OF PA AND LF BINDING TO CELLS

For LF to be delivered to the cytosol of the target cells, it must bind to the receptor-bound, proteolytically-activated form of PA, PA_{63}. It is well established that proteolytic activation is essential for oligomerization and LF binding (Leppla, 1991; Singh et al., 1989; Milne et al., 1994). To determine the stoichiometry of LF and PA binding on the surface of cells, RAW264.7 cells were incubated with 0.1 μg/ml ^{125}I-PA alone or combined with 0.5 μg/ml ^{125}I-LF for 12 hours at 4^{o}C or 2 hours at 37^{o}C, and cell associated radioactivity was measured (Table 3). The amounts of LF bound to the cells were determined by subtracting the radioacivity on wells containing PA alone. The result showed an equimolar binding of PA and LF to the cells at 4^{o}C. A similar analysis done for 2 hours at 37^{o}C showed 4 fold greater binding than at 4^{o}C without any change in their molar ratio.

Figure 17 : Effect of pH on the fluorescence of ANS incubated with LF. LF was diluted to 10 μg/ml with 5 μg/ml ANS in 10 mM Tris acetate buffer, pH 4.0-6.8. The fluorescence emission spectrum of the sample was read at 480-540 nm (excitation wave length was 380 nm).

EFFECT OF pH ON THE FLUORESCENCE OF ANS INCUBATED WITH LF

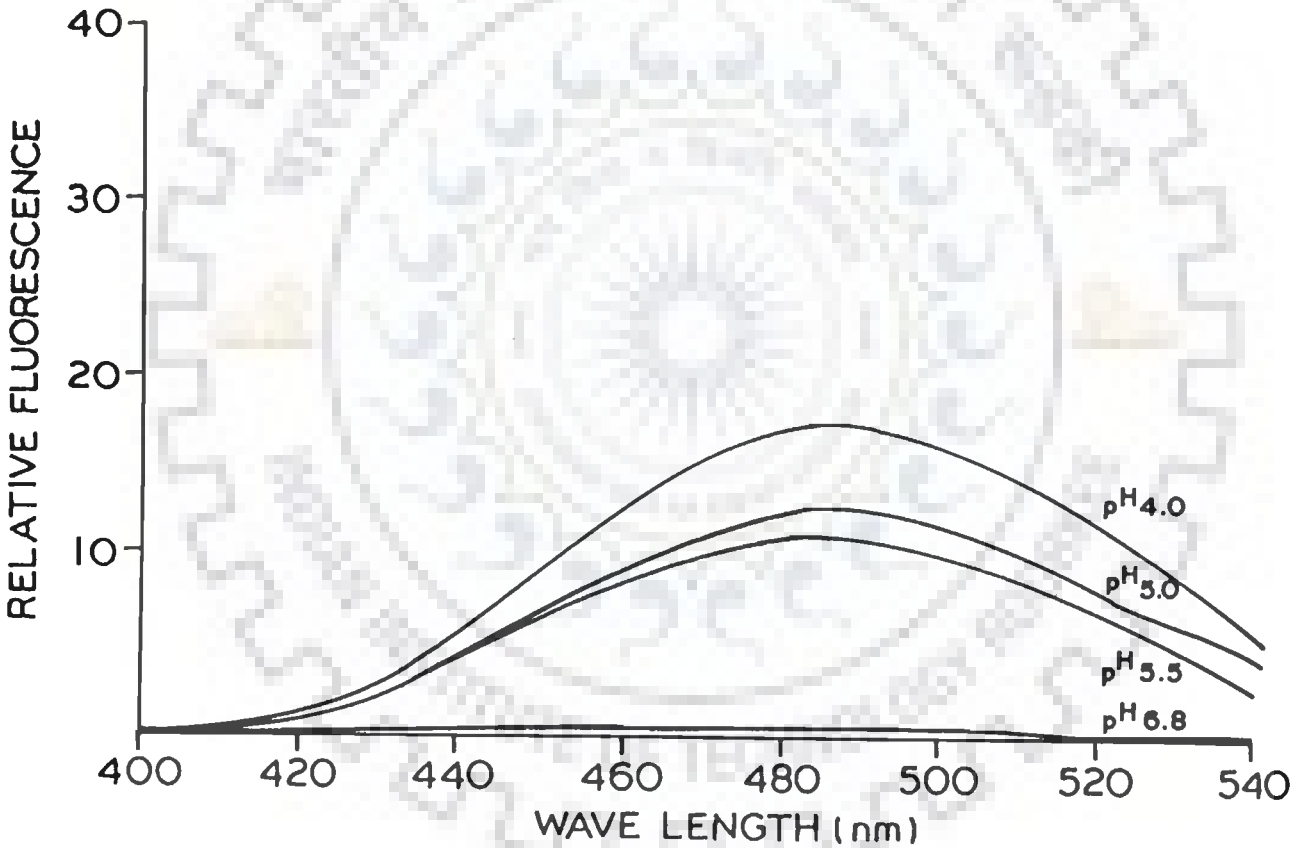


Figure 18 : Stoichiometry of PA and LF binding in solution. Increasing amounts of LF were incubated with PA that had been nicked with trypsin for 30 min at room temperature. Samples were separated on 4-15% gradient gel (Phast) under non-denaturing conditions and stained with Coomassie Blue. (A) Lane 1, 270 ng of PA (unnicked); lane 2, 270 ng of PA nicked with trypsin; lane 3, 270 ng of LF; lane 4-6, 270 ng of trypsin-nicked PA and 135, 270, and 540 ng of LF. (B): lane 1-6, 270 ng trypsin-nicked PA and increasing amounts of LF (243, 270, 297, 337, 378, and 405 ng). The mass ratio of LF:PA is indicated above each lane.

STOICHIOMETRY OF PA AND LF BINDING IN SOLUTION

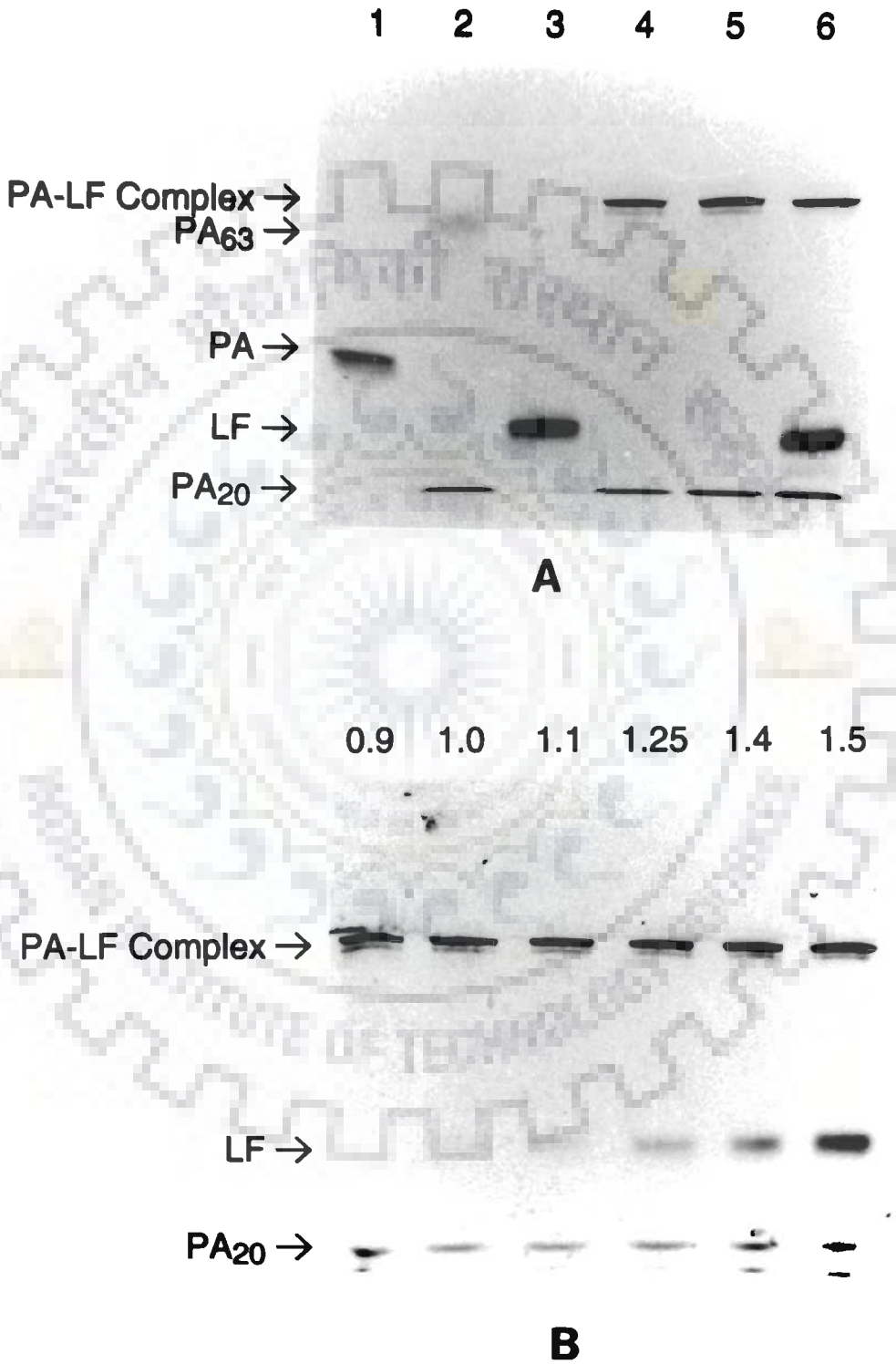


Figure 19 : Determination of multimeric forms of PA-LF complex. Purified PA_{63} (468 ng) was incubated with various amounts of LF for 30 minutes at room temperature. Samples were separated on 4-15% non- denaturing Phast gradient gel. Lanes 1-8, increasing amounts of LF (0, 47, 94, 187, 374, 515, 562, and 936 ng). The mass ratio of LF:PA is indicated above each lane.

DETERMINATION OF MULTIMERIC FORMS OF PA-LF COMPLEX

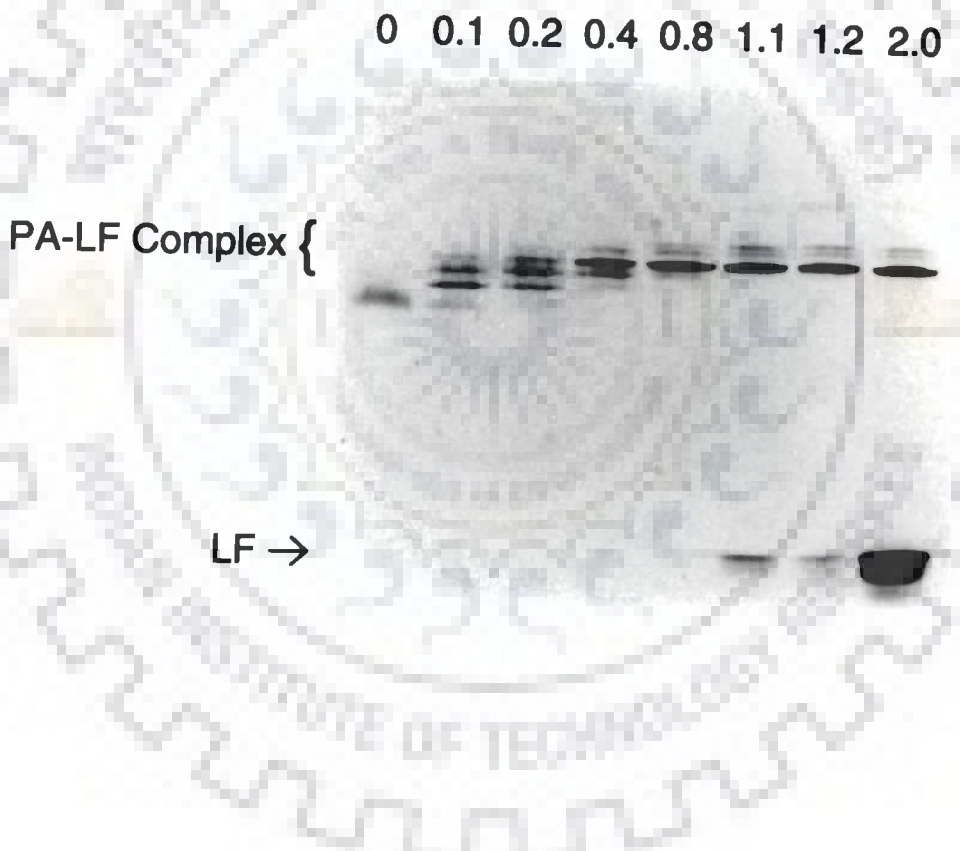


Table 3 : Binding of PA and LF on RAW264.7 cells.

Temp ^{o}C	ng toxin/mg cell protein		
	PA	LF	PA/LF Ratio
4	2.25 $\pm$ 0.35	2.22 $\pm$ 0.19	1: 0.98
37	9.15 $\pm$ 0.32	8.32 $\pm$ 0.27	1: 0.91

RAW264.7 cells cultured in 12-well plates were incubated with 0.1 μg/ml ^{125}I-PA and 0.5 μg/ml ^{125}I-LF at 4^{o}C for 12 hours or at 37^{o}C for 2 hours. Cells were washed four times and solubilized in 0.1 M NaOH. Non- specific binding was determined by incubating the cells with nonradioactive PA or LF at 100-fold excess over labeled protein. The specific cpm/well and percent nonspecific radioactivity at 4^{o}C were: PA, 1545 cpm (10.6%) and LF, 1797 cpm (8.9%); and at 37^{o}C: PA, 6381 cpm (9.0%), and LF 4515 cpm (3.47%).

DISCUSSION

DISCUSSION

EXPRESSION AND PURIFICATION OF LETHAL FACTOR

Anthrax toxin consists of three distinct proteins, protective antigen, edema factor and lethal factor. To understand the role of each component in the activity of toxin and its use in diagnostic systems highly purified proteins are required. Anthrax toxin is usually purified from culture supernatant of *B. anthracis* (Leppla, 1988). Fish et al (1968) described the purification of PA, EF and LF with 78%, 30% and 11% yield, respectively. Higher yield of the toxin component has been reported using sequential chromatography on DEAE-cellulose and hydroxyapatite (Leppla, 1982, 1984; Leppla et al., 1988). Because of the infectious nature of the organism, purification of the toxin components from *B. anthracis* requires BL3 level laboratory facility. The purified toxin components from *B. anthracis* were contaminated with small amount of other components (Leppla, 1984). To overcome these problems genes for each component have been cloned in *E. coli* and *B. subtilis* (Vodkin and Leppla, 1983; Ivins and Welkos, 1986; Singh et al., 1989a). In most of the earlier studies efforts were made to express and purify protective antigen component of the toxin.

In this study LF was expressed and purified from *E. coli*. The LF gene was subcloned in a vector pMAL designed for expression of fusion proteins. Using this vector, LF was expressed as fusion protein to COOH-terminus of maltose binding protein, a 42 kDa protein, having affinity for amylose. The expressed MBP-LF protein (130 kDa) was soluble and affinity purified from cell lysate using an amylose column. MBP-LF protein was very sensitive to *E. coli* proteases, possibly due to its large size (Fig 4A and B).

Earlier attempts to express and purify LF from *E. coli* and *B. subtilis* were less efficient than the other *B. anthracis* genes (Robertson and Leppla, 1986; Quinn et al., 1991). In this study the LF was expressed and purified from pMAL vector. The expression of LF from pMAL was less in comparison to PA in other

systems. Hence, there is a need to develop an expression vector for LF as has been achieved in the case of PA.

To use *E. coli* expressed LF in diagnosis or in studying its mechanism of action, it is critical that LF purified from *E. coli* behave like that of *B. anthracis*. In macrophage lysis assay, LF from *E. coli* was as toxic as that of *B. anthracis* (Fig. 5). Since LF does not have any cysteine which could form different intermolecular or intramolecular species, LF purified from *E. coli* was expected to be similar to LF purified from *B. anthracis*.

INTERNALIZATION AND INTRACELLULAR TRAFFICKING OF LF

Recent studies have indicated that in the process of translocation, PA_{63} oligomerizes to form a channel through which LF is delivered in the cytosol (Leppla, 1991; Milne et al., 1994; Singh et al., 1994; Zhao et al 1995). No biochemical methods are known for measuring productive internalization of the LF or its complex with PA_{63}. Thus it is not clear if the lethal effect produced is by LF alone or its complex with PA_{63}. Introduction of LF by osmotic lysis of pinocytic vesicles lyses the J774A.1 cells but the process required very high concentration of LF suggesting that PA_{63} may be facilitating the killing process (Singh et al., 1989b). In the present study LF was introduced directly into cytosol through Sendai virus envelopes (virosome). Introduction of LF in the cytosol induced concentration dependent lysis of cells (Fig. 6). The virosome mediated delivery also required five fold more LF for killing cells than the receptor mediated process. Reconstituted Sendai virus envelopes containing fusion protein and hemagglutinin neuraminidase have been shown to be an efficient method for delivering water soluble molecules into the cytosol (Loyter et al., 1991). This process is mediated by binding of hemagglutinin neuraminidase protein of virosomes to sialoglycoconjugates present on the cells. Although the potency of LF was less in virosome mediated delivery in comparison to PA mediated delivery, it was still fifty fold more over the process of delivery through osmotic lysis of pinocytic vesicles. The low potency of LF could be due to its

different processing when delivered directly into cytosol.

In the receptor mediated process of cytotoxicity by anthrax lethal toxin, the complex of PA_{63} with LF is internalized into eukaryotic cells by endocytosis (Leppla et al., 1988). Acidification of endosomes then causes translocation of LF to the cytosol (Friedlander, 1986). Lethal toxin is considered to be the cause of death in animals. However, it is not known if after translocation LF remains in the cytosol or becomes associated with other cellular organelles for its activity. Studies on the distribution of ^{125}I-LF in the sub-cellular organellesindicated that significant amount of LF was associated with lysosome and cytosol fractions (Table 1). Only small amount of LF was associated with other cellular organelles, suggesting that LF is translocated to cytosol from endocytic vesicles.

Processing of several bacterial toxins in the target cell has been shown to be an important phenomenon for their biological activity. These processing include mostly proteolytic cleavage by eukaryotic proteases. Several bacterial toxins like cholera, tetanus and botulinum neurotoxin are proteolytically activated during translocation into cytosol (Gorden et al., 1995). In anthrax lethal toxin proteolytic cleavage of PA to form PA_{63} is a prerequisite for LF binding, oligomerization and channel formation (Leppla et al., 1988; Singh et al., 1989a; Novak et al., 1992; Milne et al., 1994). However, proteolytic activation of LF for its translocation and activity has not been studied. To answer this question, iodinated LF was incubated with RAW264.7 cells at 37^{o}C and cell associated LF was analysed by SDS-PAGE. No difference in the electrophoretic mobility of native and cell associated LF was observed suggesting that proteolytic activation of LF is not required for the expression of its biological activity (Fig. 8). The SDS-PAGE analysis of cell associated LF proteins could not detect a modification of LF or cleavage which removes small number of amino acids from the protein.

There are some bacterial and plant toxins like pseudomonas, ricin, and cholera toxin which require intact golgi for their processing (Donta et al., 1993). A fungal antibiotic brefeldin A disrupts golgi network and inhibits the activity of those toxins whose processing occurs in golgi (Nambiar et al., 1993). Brefeldin

had no effect on lethal toxin induced cytotoxicity (Fig. 7). The result suggest that LF is translocated from acidified endosome to the cytosol without passing through golgi. Similar observations have been reported for LF fusion proteins to the enzymatic units of pseudomonas exotoxin, diphtheria toxin and tetanus where LF translocates protein into cytosol (Arora et al., 1994). Sequence analysis of LF indicates that LF has no known sequence which could direct the protein to pass through the cellular organelles.

REQUIREMENT OF PROTEIN SYNTHESIS FOR THE ACTION OF LETHAL TOXIN

Protein synthesis inhibition is the major mechanism of cell cytolysis for several plant and bacterial protein toxins (Honjo et al., 1968; Hwang et al., 1987). To explore the possibility of lethal toxin inhibiting protein synthesis in cells, incorporation of radioactive leucine in TCA precipitable proteins was monitered in the presence of lethal toxin. Incubation of cells with LT had no significant effect on protein synthesis upto 120 minutes, Whereas, the toxic effect of lethal toxin started after 90 minutes. An inhibition of 50% in rate of protein synthesis was observed at 225 minutes whereas 50% cytotoxicity was recorded within 160 minutes suggesting that protein synthesis inhibition is not the primary event in the action of lethal toxin (Fig. 9). The killing of cells through inhibition of protein synthesis normally requires several hours whereas the killing of cells by anthrax lethal toxin is much faster and at high concentration it kills the cells in less than three hours. Thus, the cessation of protein synthesis is not the cause of cell lysis by anthrax lethal toxin.

Intracellular processing and trafficking through cell organelle is an essential step in the action of several bacterial toxins. These processes require cellular proteins (Ogata et al., 1990). The involvement of cellular protein(s) in the toxicity of anthrax lethal toxin was studied by blocking the protein synthesis for various time periods and exposing the cells to lethal toxin. The results indicate that cycloheximide treated cells were resistant to the cytolytic activity of lethal toxin

(Fig. 10).A similar report has been made by Bhatnagar and Friedlander (1994), where they have shown that cells can be protected from LT by blocking the protein synthesis with puromycin. Puromycin and cycloheximide act at different steps of translation in inhibiting the protein synthesis of eukaryotic cells (Haaselkorn and Rothman-Denes, 1973; Pestka, 1971). Since these inhibitors work in a generalized way to inhibit the protein synthesis, it is very difficult to predict the possible protein(s) involved in the cytotoxicity of lethal toxin. However, Bhatnagar and Friedlander (1994) have suggested that the calcium influx is inhibited by these inhibitors. The rapid uptake of calcium, which is usually observed in toxin treated cells, is inhibited by treatment with cycloheximide and puromycin thereby producing protection from lethal toxin. Cycloheximide had no effect on the binding of PA to the cell surface receptor (Table 2) suggesting that the protection of cells was not due to the reduced toxin binding on the cell.

The incubation of cells for 6 hours with cycloheximide inhibited the protein synthesis and decreased the level(s) of several cytoplasmic proteins. The increased protection after 6 hours of preincubation with cycloheximide could be due to the reduced level(s) of specific protein(s) required for cytotoxicity. Incubation of cells with cycloheximide for longer time may completely remove the protein(s) involved in cytotoxicity but were toxic to J774A.1 cells. Cellular protein(s) may be important for LF processing or may get induced by lethal factor through unknown mechanism to lyse the cells. Support to this hypothesis comes from a report in which anthrax lethal toxin has been shown to induce production of ROI, IL-1 and TNF (Hanna et al., 1993; 1994) which leads to lysis of macrophage cells.

LETHAL TOXIN INDUCED GENERATION OF INOSITOL PHOSPHATES

Calcium ion is required for the toxic activity of lethal toxin (Bhatnagar et al., 1989). The calcium requiring step is subsequent to the binding and endocytosis of PA-LF into the macrophage cells. Onset of such event occurs later to the amine blockage step in the process of toxicity of anthrax lethal toxin (Bhatnagar et al., 1989). Calmodulin dependency of lethal toxin also suggests

that calcium ion has some role in producing the toxic effect of lethal toxin (Bhatnagar et al., 1989). Calcium has previously been shown to be required for the action of diphtheria toxin (Sandvig et al., 1982), pseudomonas exotoxin A (FitzGerald et al., 1982), pseudomonas leukocidin (Hirayama et al., 1984) and shiga toxin (Sandvig and Brown, 1987). In eukaryotic cells a low concentration of cytosolic calcium (10^{-6} to 10^{-8}M) is maintained (Fiskum and Lehninger, 1982; Penniston, 1983) despite a very high extracellular calcium concentration (10^{-3}M). Such calcium ion gradient is maintained by energy- dependent pumping of calcium from the cytosol to mitochondria, endoplasmic reticulum and extracellular medium (Fiskum and Lehninger, 1982; Penniston, 1983). It has been reported that there is an uncontrolled calcium influx into the cell after 90 minutes of incubation with lethal toxin. The influx of calcium is one of the reasons of the cytolysis by lethal toxin (Bhatnagar et al., 1989). The cytosolic calcium concentration is regulated by biological membranes through several pathways. Several mediators including inositol phosphates have been shown to act as second messengers regulating calcium related changes in the cell (Berridge, 1993). Rapid increase in the intracellular calcium concentration could be due to the generation of toxin induced inositol phosphates. To study the involvement of inositol phosphates in influx of calcium during the process of cytotoxicity by LT, cells were assayed for toxin induced generation of inositol phosphates. Lethal toxin induced generation of five fold inositol phosphates than control cells after 90 minutes of incubation (Fig. 11). Influx of calcium occurs after 90 minutes and reaches to maximum concentration after 150 minutes followed by the lysis of cells at 180 minutes of the incubation with lethal toxin. The five fold increase in the total IP level following toxin treatment could be due to the activation of phospholipases which in turn degrade the cellular phospholipid precursors to produce IP_3 and diacylglycerol. IP_3 can induce the release of calcium from internal reserves as well as influx from external media (Berridge, 1993). The concentration dependent increase in total IP_3 in the cell which preceded the influx of calcium indicated that calcium influx could be due to the generation of inositol phosphates.

EFFECT OF pH ON THE INTERACTION OF LF AND PA

Proteolytic cleavage of PA is an essential step in the activity of lethal toxin. Both trypsin and furin, cleave PA precisely at RKKR^{167} exposing a sitefor binding to LF. Subsequently, PA-LF gets endocytosed and pass through an acidic vesicle to deliver LF in the cytosol. To study the effect of pH on the electrophoretic mobility and binding of LF to PAn, PAn was incubated with LF at several pH values and analysesd on a gradient (8-25%) native gel.

No interaction between PA_{83} and LF was observed when resolved as separate protein bands on the gel. It confirmed the fact that amino-terminal 20 kDa of PA has to be removed for its interaction with LF. Trypsin-nicked PA bound LF at all pH values tested, and migrated as a retarded protein band on the native gel. Acidic pH had no effect on the binding of LF to PAn but retarded in the mobility of the PAn-LF complex (Fig. 13, lane 7-8 Fig. 14A, lane 1-3 and Fig. 14B, lane 2-4) as compared to the PAn-LF band at the alkaline and neutral pH (Fig 13, lane 5-6; Fig. 14A, lane 4 and Fig. 14B, lane 1). The change in mobility could be due to any or both the proteins of the complex. PAn electrophoressed in the identical conditions also showed shift in mobility at acidic pH values. Acidic pH induces oligomerization of PA_{63} (Milne et al., 1994). The retarded mobility was observed only in the 63 kDa band without any effect on the 20 kDa protein of PA. It is very difficult to predict the molecular weight of the protein in the native gel electrophoresis because the relative migration in native gel is affected by the net charge, shape and size of the protein. The relative migration of different protein fragments of PA suggested that PA_{63} (the slow migrating fragment) was retarded further at acidic pH values which can be visualized from the absence of any corresponding band in the lane 1 (Fig. 12). Since PA_{63} forms stable oligomers, the bands observed could be the result of oligomers of PA formed at acidic pH. The oligomers of PAn resolved into two bands. The slowest migrating oligomer of PA contains 64%, whereas the faster migrating band contains approximately 20% of the PA protein (densitometric

analysis). It has been reported that PA_{63} oligomerizes to form approximately 70% heptamers and 30% other oligomers at acidic pH (Milne et al., 1994). In these experiments PA protein was cleaved enzymatically by trypsin. The cleavage of PA may be at other sites to generate PA molecule different from PA_{63}. Such PA proteins may not behave like PA_{63} to form heptameric rings which has been reported as a stable oligomeric form of PA_{63}, required in channel formation and translocation of LF in the cytosol. The elecrophoretic mobility of LF was not affected by pH.

Using radioiodinated PA electrophoresis of PAn-LF complex at different pH values also showed the identical shift in the electrophoretic mobility of the complex (PAn-LF) (Fig. 14B, lane 1-4). These complexes also resolved into two bands which may be heptamer and other multimeric form of PAn-LF complex (lane 2-4).

Experiments with ^{125}I-LF showed an identical electrophoretic pattern of the PAn-LF complex at acidic pH values (Fig. 14A, lane 1-4). The intensity of the two protein bands of PAn-LF complex was different from that observed with ^{125}I-PA. The change in the intensity could be due to uneven distribution of ^{125}I-LF in the two bands. However, no LF was released from the complex by decreasing the pH.

These experiments suggested that pH plays an important role in oligomerization of PA but has no effect on binding of LF to PA. However these experiments do not indicate that PAn-LF complex has exposed receptor and LF binding site as present in native PA.

To investigate that PAn-LF complex has free receptor binding site as present in native PA, iodinated LF was incubated with trypsin nicked PA at basic and acidic pH. The complex of PAn-LF formed at different pH was added to RAW264.7 cells and incubated for 2 hours at 37^{o}C. The presence of equal amount of LF indicated that the LF binding region on PAn is exposed at both acidic and basic pH conditions (Fig. 15, lane 2-5). LF carries the catalytic

domain at the COOH-terminus which is well apart from the PA binding region present at amino-terminus 1-254 amino acids of the protein. These experiments suggest that LF remains bound to PAn at acidic pH values. The experiments do not indicate how LF is released in the cytosol.

Acidic pH has been reported to induce exposure of hydrophobic domains on PA protein. Proteolytic activation also causes exposure of hydrophobic domains on the protein as shown by enhanced fluorescence of protein bound TNS, a hydrophobic fluorescent marker (Uderfriend et al., 1967). Aerolysin and PA proteins show enhanced fluorescence after trypsinization (Koehler and Collier, 1991). In case of PA, acidic pH enhances the fluorescence of TNS suggesting exposure of hydrophobic areas on the protein. It has been reported that hydrophobic areas help in interaction of the protein with the lipid membranes and help in explaining the formation of channel by PAn at acidic pH for translocating of LF in the cytosol. The experiment with ANS (hydrophobic marker) showed an 20% increase in the intensity of fluorescence emission after binding of LF at acidic pH values (Fig. 17). The increased fluorescence can be attributed to the effect of pH on exposure of hydrophobic areas on LF protein. These changes may lead to a conformation by LF which can easily be translocated through the lumen of the heptameric channel of PA. Zhao et al (1995) have suggested that LF gets translocated through the channel of PA_{63} and addition of LF (1-254 residues) blocked the Rb^+ efflux which otherwise occured normally due to the channel forming activity of PA in macrophage cells.

SPECTROSCOPIC STUDIES OF PA AND LF

To study the secondary structure of PA and LF proteins, circular dichroism spectra of the individual proteins were recorded in Tris buffer, pH 7.2. The analysis of the CD spectrum showed that the protein belongs to B-class of protein. The decrease in the molar ellipticity of its negative band (Fig. 16A) by trypsinization suggest that cleavage causes disruption of the ordered structure of PA.

In the process of cytotoxicity PA-LF complex is endocytosed and passes through the acidic vesicles to translocate LF into the cytosol. To study the possible effect of pH in the secondary structure of PA, CD spectra were recorded at low pH values. The acidification of PA resulted in a significant decrease in the molar ellipticity values, probably due to the protonation of the amino acids at low pH environments and subsequent changes in the ionic interactions. Low pH induced randomness in the secondary structure of PA, as shown in the CD spectra (Fig. 16A) suggesting that acidification renders the protein to be more amphiphilic for its interaction with the membranes as well as other proteins like LF in the cell. The other channel forming proteins like porin and α-haemolysin are also known to have a maximum percentage of β-sheets in their secondary structure (Eisele and Rosenbusch, 1990; Tobkes et al., 1985).

In contrast, LF appeared to be a protein having both α helices and β-sheets in its secondary structure. The decrease in the molar ellipticity suggests that acidification leads to a decrease in the alpha helical content of the protein. Analysis of the secondary structure indicates that acidification induced an increase in the fraction of the unordered structure. Such increase in randomness of LF could be facilitating the interprotein interactions and translocation into the cytosol.

STOICHIOMETRY OF PA AND LF BINDING

Protein toxins work in A-B model to get internalized in the eukaryotic cells for expression of their activity. The entire process includes several events which can be attributed to the individual molecules of the toxin. In the biological system, A and B molecules combine at a specific stoichiometry to produce the optimum effect in the cells. Cholera toxin consists of pentameric B unit and a single A chain (Kaper et al., 1995). Similarly PA, the B unit of anthrax lethal toxin forms heptamers to achieve thermodynamically stable structure (Milne et al., 1994) required to form membrane spanning channel for LF translocation. LF binds to PA on the surface before the endocytosis and translocation in the cell.

However, the entire series of events is not understood sequentially including the stoichiometry of binding between PA and LF. The stoichiometry of binding was estimated by adding increasing amount of LF to a fixed amount of PA_{63}. The result suggest that PA_{63} was able to bind approximately an equal weight of LF (Fig. 18 A and B). In solution, binding and gel shift assay showed that the proteins bind at an equimolar concentration of protein. Trypsin nicked PA as well as the purified PA_{63} both produced similar results suggesting that the stoichiometry of PA:LF is 1:1. Incubation of the PA_{63} oligomer with limiting amount of LF caused formation of PA_{63}-LF complexes with varying number of LF (Fig. 19). The prominent band observed in all samples may be a complex having equal number of PA_{63} and LF molecules, presumably seven of each. These results suggest that PA_{63} oligomers are able to bind variable number of LF. However the data does not indicate which of these PA-LF complexes are biologically active. It is possible that PA-LF complex with any number of LF molecule will be active and the potency may increase with increasing number of LF.

To determine whether stoichiometry of PA_{63} and LF binding remains the same on the cell surface, the cells were incubated with iodinated PA alone or in combination with iodinated LF for 12 hours at $4^{o}C$ or 2 hours at $37^{o}C$. The molar amounts of LF bound and internalized were nearly equal to that of PA (Table 3). These results suggest that nearly every molecule of PA binds and delivers a molecule of LF into the cytosol.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Anthrax is a toxinogenic disease of both human and animals. It is caused by a Gram positive bacteria known as *Bacillus anthracis*. The virulence of *B. anthracis* is attributed to two factors, poly-D-glutamic acid capsule and a protein exotoxin. The exotoxin consists of three proteins protective antigen (PA), lethal factor (LF) and edema factor (EF). These toxin proteins are individually non-pathogenic but when introduced in combination produce distinct pathological symptoms in cell as well as in animals. PA binds to the receptor on eukaryotic cells and translocates the other two components (LF and EF) into the cytosol. Combination of PA and LF is lethal to animals and macrophage cells and is known as lethal toxin. PA together with EF forms edema toxin which increases cyclic-AMP levels in the cells and causes edema in animals. EF has been shown to be acalcium and calmodulin dependent adenylate cyclase. LF has been proposed to have zinc dependent metalloprotease activity, but the substrate or the cellular target for LF has not been identified. The present work entitled "Molecular and Biochemical studies on Anthrax Lethal Toxin" was aimed to explore the molecular events associated with the action of lethal toxin.

The work has been divided into three parts:

* Expression and purification of lethal factor from *Escherichia coli.*
* Biochemical studies to understand the mechanisms involved in the cytotoxic activity of lethal toxin and,
* Biophysical studies on anthrax lethal toxin.

LF gene was cloned in pMAL-P vector and expressed as a fusion protein, MBP-LF, in E. coli (BL-21 λ DE3). The fusion protein was purified by affinity chromatography using an amylose column, which binds to MBP of the fusion protein. Presence of factor Xa site between MBP and LF allowed the cleavage and separation of LF from MBP. The final purification was performed on the Sephadex G-100 column to separate LF from the degraded products. The yield

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of purified LF from this procedure was 200 μg/litre. The purified protein was compared for its biological activity with LF purified from *B. anthracis*. The LF purified from *E. coli* was similar to the LF purified from *B. anthracis* in its ability to lyse a macrophage cell line (J774A.1).

Several bacterial toxins require intracellular processing and activation for its biological activity. It is well established that PA is proteolytically activated to expose a site for binding of LF and its translocation into cytosol. The complete mechanism of internalization process and the role of individual components in toxicity is not yet understood. To delineate the action of individual proteins, reconstituted sendai virus envelope was used to deliver the toxin components (PA, LF and PA+LF) directly into the cytosol. Virosomes containing no protein or PA alone were not toxic to cells. However, virosome containing LF or PA+LF together were toxic to macrophage cells. The results suggest that LF is toxic component of lethal toxin.

Some bacterial toxins like cholera and E. coli labile toxin require intact golgi for their biological activity. To study if golgi has any role in the activity of anthrax lethal toxin, macrophage cells were preincubated with brefeldin A (disrupts golgi) and challenged with lethal toxin. Brefeldin treatment which failed to confer any protection on the macrophage cells against the lethal toxicity suggests that golgi apparatus is not required for the cytotoxic process of lethal toxin.

A number of toxins including PA of anthrax, pseudomonas exotoxin A, cholera, diphtheria and tetanus toxin are activated by furin and other cellular proteases for their biological activity. To study the requirement of proteolytic activation of LF besides PA in lethal toxin, RAW264.7 cells were incubated with ^{125}I-LF and PA for varying time periods at 37^{o}C. Analysis of cell associated LF on SDS-PAGE showed no difference in the electrophoretic migration of the LF protein suggesting that LF is not proteolytically cleaved while expressing its biological activity in the cell.

During the process of cytotoxicity, PA-LF complex is endocytosed and passes through the acidic vesicle to reach the cytosolic target in the cell. The intracellular target of LF and its distribution in different organelles is not known. To study the distribution of LF in cell organelles, cells (J774A.1) were incubated with ^{125}I-LF together with PA for different time periods. At the end of the incubation, cells were homogenized and subjected to differential centrifugation to isolate different sub-cellular organelles like: nucleus, mitochondria, lysosome and microsome. The results indicate that LF is associated mainly with lysosome and cytosol.

Bacterial protein toxins kill the mammalian cells by interfering withthe normal metabolism of the cell. Inhibition of eukaryotic protein synthesis is one of those processes reported in the mechanism of action of many bacterial toxins like pseudomonas exotoxin and diphtheria toxin. To study if lethal toxin has protein synthesis inhibitory activity, macrophage cells were treated with lethal toxin and then the rate of protein synthesis was determined. The protein synthesis was not inhibited by lethal toxin suggesting that inhibition of protein synthesis is not a primary event in the lethal toxin induced cytotoxicity. On the contrary, when macrophagal protein synthesis was inhibited by cycloheximide (0.5 μg/ml), the cells became resistant to toxin challenge indicating that the lethal toxin requires some protein factors for expression of its cytotoxic activity.

Lethal toxin induces an influx of Ca^{++} before the lysis of macrophages. Inositol-1, 4, 5-triphosphate has been implicated in mobilizing stored calcium and in promoting an influx of external calcium in conjuction with inositol-1, 3, 4, 5-tetrakisphosphate in cells. To determine LT induced generation of inositol phosphates, ^{3}H-inositol labeled macrophage cells were incubated with lethal toxin for 30 and 90 min at 37^{o}C. After 90 min of post incubation with toxin, cells showed five fold increase in the level of inositol phosphate. These results suggest that lethal toxin induces generation of inositol phosphate which in turn may lead to increase in influx of Ca^{++} causing cell lysis.

The proteolytic activation of PA on the surface of the eukaryotic cells to

form PA_{63} is prerequisite for interaction with LF. It is well established that at acidic pH PA_{63} forms a heptameric structure, a thermodynamically stable form of oligomer which channelizes the membrane translocation of LF. Oligomer formation was not observed with LF at acidic pH as it was recorded with PA_{63}. Trypsin-nicked PA (PAn) and LF combined at different pH values to form complexes suggesting that their interaction is pH independent. Spectrofluorometric analysis with ANS, a hydrophobic marker, bound to LF showed an enhanced fluorescence at acidic pH. These results suggest that acidic pH exposes hydrophobic areas on LF, which could facilitate its translocation into the cytosol.

To elucidate the pH induced structural changes in the toxin proteins, circulating dichroism (CD) studies of PA and LF were carried out in 10 mM Tris buffer, pH 7.2. Analysis of the CD spectra suggests that PA is a β-class protein whichshows an increase in the randomness in its secondary structure when subjected to acidic pH. On the contrary, LF can be catagorized as $\alpha+\beta$ - class of protein, showing an identical increase in the randomness in its secondary structure at lower pH. These results suggest that increased randomness of the proteins could be enhancing inter-protein interactions between PA and LF.

The components of the protein toxin maintain a particular symmetric configuration for its optimum activity in the cell. Such stoichiometric arrangement is different for different toxins. For example cholera toxin consists of pentameric B unit and a single A unit in the active complex. To study the stoichiometry of binding between the proteins in anthrax lethal toxin, PA and LF were combined at different molar ratio to form complexes. The analysis of the complex on native phast gel suggests that each PAn subunit in the heptamer can bind one LF molecule.

CONCLUSIONS

1. Lethal factor of anthrax toxin purified from *E. coli* is similar to LF purified from *B. anthracis* in its ability to lyse the macrophages.
2. LF is the toxic component of lethal toxin.
3. Golgi apparatus is not involved in the cytotoxicity of lethal toxin in cells.
4. Proteolytic activation is not required for expression of LF activity.
5. Lethal toxin does not inhibit cellular protein synthesis but requires host cell protein(s) for its cytotoxicity.
6. Lethal toxin increases inositol phosphates in cell, which inturn may cause Ca^{++} influx in the cytosol.
7. Binding of LF to PA is not pH dependent and the complex is stable at acidic pH.
8. Acidic pH induces exposure of hydrophobic areas on LF.
9. PA is a β-class protein, whereas LF has both $\alpha+\beta$ in its secondary structure.
10. The PA and LF bind in the stoichiometry ratio of 1:1 both on cells and in solution.

REFERENCES

REFERENCES

1. Aktories K, Barmann M, Ohishi I, Tsuyama S, Jacobs K H and Habermann E (1986). Botulinum C2 toxin ADP-ribosylates actin. Nature 322: 390-2

2. Arora N, Klimpel K R, Singh Y and Leppla S H (1992). Fusion of anthrax toxin lethal factor to the ADP-ribosylation domain of Pseudomonas exotoxin A are potent cytotoxins which are translocated to the cytosol of mammalian cells. J. Biol. Chem. 267: 15542-8

3. Arora N and Leppla S H (1993). Residues 1-254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. J. Biol. Chem. 268: 3334-41

4. Arora N, and Leppla S H (1994). Fusion of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. Infect. Immun. 62: 4955-61

5. Bail O (1904). Unterschungen uber naturliche and kunstliche Milzbrand imunitat. A. Bakteriol. Parasitenk. 37: 270

6. Bairoch, A and Cox, J A (1990). EF-hand motifs in inositol phosphate-specific phospholipase C. FEBS Lett. 269, 454-456

7. Bairoch A (1993). The PROSITE dictionary of sites and patterns in proteins, its current status. Nucl. Acid. Res. 21: 3097-103

8. Bagai S, Puri A, Blumenthal R and Sarkar D P (1993). Hemagglutinin-neuraminidase enhances F-protein mediated membrane fusion of reconstituted Sendai virus envelopes with cells, J. Virol. 67: 3312-3318

9. Bagai S and Sarkar D P (1994) Fusion-mediated microinjection of lysosome into Hep G_2 cells through hemagglutinin neuraminidase-depleted sendai virus envelopes. J. Biol. Chem. 269: 1966-1972

10. Beall F A, Taylor M J and Thorne C B (1962). Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. 83: 1274-80

11. Beaven M A, Moore J P, Smith G A, Hesketh T R and Metcalfe J C (1984). The calcium signal and phosphatidylinositol breakdown in 2H3 cells. J. Biol. Chem. 259: 7137-7142

12. Berridge M J, Dowson R M C, Peter Downes C, Heslop J P and Irvine R F (1983). Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. 212: 473-482

13. Berridge M J and Irvine R F (1989). Inositol phosphates and cell signalling. Nature, 341: 197-205

14. Berridge M J (1993) Inositol triphosphate and calcium signalling. Nature. 361: 315-325

15. Bhatnagar R, Singh Y, Leppla S H and Friedlander A M (1989). Calcium is required for the expression of anthrax lethal toxin activity in the macrophage like cell line J744A.1. Infect. Immun. 57: 2107-114

16. Bhatnagar R and Friedlander A M (1994). Protein synthesis is required for expression of anthrax lethal toxin cytotoxicity. Infect. Immun. 62: 2958-62

17. Bijsterbosch M K and Van Berkel J C (1992) Lactosylated high density lipoprotein: A potential carrier for the site- specific delivery of the drugs to parenchymal liver cells. Mol. Pharmacol. 41: 404-411

18. Blaustein R O, Koehler T M, Collier R J and Finkelstein A (1989). Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc. Natl. Acad. Sci. USA 86: 2209-13

19. Blaustein R O, Lea E J and Finkelstein A (1990). Voltage- dependent block of anthrax toxin channels in planar phospholipid bilayer membranes

by symmetric tetraalkylammonium ions. Single-channel analysis. J. Gen. Physiol. 96: 921-42

20. Brachman P S, Gold H, Plotkin S A, Fekety F R, Werrin M and Ingraham N R (1962). Field evaluation of a human anthrax vaccine. American J. Public Health 52: 632-646

21. Bragg T S and Robertson D L (1989). Nucleotide sequence and analysis of the lethal factor gene (lef) from Bacillus anthracis. Gene 81: 45-54

22. Brauell (1857) *Bacillus*, In: Veterinary Bacteriology and Virology by Merchant I A and Packer R A (ed) (1956). 5th edition, The Iowa State College Press, USA.

23. Chaudhary V K, Jinno Y, FitzGerald D J and Pastan I (1990). Pseudomonas exotoxin contains a specific sequence at carboxyl terminus that is required for cytotoxicity. Proc. Natl. Acad. sci. USA. 95: 907-

24. Cieplak W, Hasemann C and Eidels L (1988). Specific cleavage of diphtheria toxin by human urokinase. Biochem. Biophys. Res. Commun. 157: 747-754

25. Cohen S N, Chang A C Y and Hsu L (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of Escherichia coli by R-factor DNA. Proc. Natl. Acad. Sci. USA 69: 2110

26. Dalafond (1860) Bacillus, In: Veterinary Bacteriology and Virology by Merchant I A and Packer R A (ed) (1956). 5th edition, The Iowa State College Press, USA.

27. Davine C (1863). Comptes Rendus Hebdomadaires des Seances de I Academie des Sciences 57: 220 1863b ibid. 57:351; 1863c ibid 57:386; 1864 ibid 59: 393.

28. Donta S T, Beristain S and Tomocic T K (1993). Inhibition of heat-labile cholera and *Escherichia coli* entero toxin by brefeldin A. Infect. Immun. 61: 3282-3286

29. Ebedes H (1967). Medoquo 10: 99; 1981, A new look at anthrax. In: wildlife disease of the Pacific basin and other countries. M. E. Fowler (ed) Proc. of 4th Int. Conf. Wildlife Disease Assoc. (25-28th August) Sydney: 85-88

30. Eisele J and Rosenbusch J P (1990). In vitro folding and oligomerization of a membrane protein: transition of bacterial porin from random coil to native conformation. J. Biol. Chem. 265: 10217-20

31. Escuyer V and Collier R J (1991). Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. Infect. Immun. 59: 3381-3386

32. Ezzell J W Jr and Abshire T G (1992). Serum protease cleavage of *Bacillus anthracis* protective antigen. J. Gen. Microbiol. 138: 543-9

33. Fairweather N F, and Lyness V A (1986). The complete nucleotide sequence of tetanus toxin. Nucl. Acids Res. 14: 7809-12

34. Ferris C D and Snyder S H (1992). J. of Neurosci., 12, 1567-1574

35. Finkelstein A (1994). The channel formed in planar lipid bilayers by the protective antigen component of anthrax toxin. Toxicology 87: 29-41

36. Fiskum G and Lehninger A L (1982). Mitochondrial regulation of intracellular calcium. In: Calcium and cell function (ed. W Y Cheung) p. 39-80, Academic Press, Inc, NY.

37. Fish D C, Mahlandt B G, Dobbs J P and Lincoln R (1968). Purification and properties of in vitro produced anthrax toxin components, J. Bacteriol., 95: 907

38. FitzGerald D, Morris R E and Saelinger C B (1982). Essential role of calcium in cellular internalization of *Pseudomonas* toxin. Infect. Immun. 35: 715-720

39. Friedlander A M, Bhatnagar R, Leppla S H, Johnson L and Singh Y (1993). Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect. Immun. 61: 245-252

40. Friedlander A M (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. 261: 7123-26

41. Genetics Computer Group (1991). Program Manual for the GCG package, Version 7, April 1991. 575 Scince Drive; Madison, Wisconsin, USA.

42. Gill D M (1978). Seven toxin peptides that cross cell membranes. In Bacterial Toxins and Cell Membranes, Jeljaszewicz J and Wadstrom T (Eds.) Academic, New York, 291-332

43. Gladstone G P (1946). Immunity to anthrax. Protective antigen present in cell free culture filtrates. Br. J. Exp. Pathol. 27:394

44. Gorden V M, Leppla S H and Hewlett E L (1988). Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun. 56: 1066-1069

45. Gorden V M, Klimpel K R, Arora N, Henderson M A and Leppla S H (1995). Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional proteases. 63: 82-87

46. Goren H J, McMillin C R and Walton A G (1977). Poly (L-Lysyl-L-Alanyl-alpha-L-Glutamic acid) II. conformational studies. Biopolymers 16: 1527-40

47. Green B D, Battisti L, Koehler T M, Thorne C B and Ivins B E (1985). Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. 49: 291-7

48. Haaselkorn R and Rothman-Denes L B (1973). Protein synthesis. Annu. Rev. Biochem. 42: 397-438

49. Hambleton P, Carman J A and Melling J (1984). Anthrax: the disease in relation to vaccines. Vaccine 2: 125-32

50. Hanna P C, Kouchi S, and Collier R J (1992). Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. Mol. Biol. Cell 3: 1269-77

51. Hanna P C, Acosta D and Collier R J (1993). On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. USA 90: 10198-201

52. Hanna P C, Kruskal B A, Allen R, Ezekowitz B, Bloom B R and Collier R J (1994). Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol. Medicine 1: 7-18

53. Hase C C and Finkelstein R A (1993). Bacterial extracellular zinc-containing metalloproteases. Microbiol. Rev. 57: 823-37

54. Heemskerk D D and Thorne C B (1990). Genetic exchange and transposon mutagenesis in *Bacillus anthracis*. Salisbury Med. Bull., 68, Spec. Suppl. 63-7

55. Helenius A, Kartenbeck J, Simons K and Fries E (1980). On the entry of Semliki Forest virus in BHK-21 cells, J. Cell Biol. 84: 404

56. Higgins C F, Hiles I D, Salmond G P C, Gill D R, Downie J A, Evans I J, Holland I B, Gray L, Buckel S D, Bell A W and Hermodson M A (1986). A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature, 323: 448

57. Hirayama T and Kato I (1984). Mode of cytotoxic action of pseudomonas leukocidin on phosphatidylinositol metabolism and activation of lysosomal enzymes in rabbit leukocytes. Infect. Immun. 43: 21-27

58. Honjo T, Nishizuka Y, Hayashi O and Kato I (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. J. Biol. Chem. 243: 3553-3555

59. Hornung J M and Thorne C B (1991). Insertion mutations affecting pXO1-associated toxin production in *Bacillus anthracis*, Abstr. 91st Ann. Meet. Am. Soc. Microbiol. 98, p. 98, Abstr. D-121

60. Hwang J, FitzGerald D J P, Adhya S and Pastan I (1987). Functional domain of pseudomonas exotoxin identified by deletion analysis of the gene expression in E. coli. Cell 48: 129-136

61. Irvine R F (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates - a possible mechanism. FEBS Lett. 263: 5-9

62. Ivins B E and Welkos S L (1986). Cloning and expression of the *Bacillus anthracis* protective antigen in *Bacillus subtilis*. Infect. Immun. 54: 537-542

63. Ivins B E and Welkos S L (1988). Recent advances in the development of an improved human anthrax vaccine. Eur. J. Epidemiol., 4: 12-19

64. Ivins B E, Welkos S L, Kundson G B and Little S F (1990). Immunization against anthrax with aromatic compound- dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of Bacillus subtilis that produce anthrax protective antigen. Infect. Immun. 58: 303-308

65. Jongeneel C V, Bouvier J, and Bairoch A (1989). A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett. 242: 211-214

66. Kaper J B, Morris J G Jr and Levine M M (1995). Cholera: review. Clinical Microbiol. Rev. 8:48-86

67. Kasper R L and Robertson D L (1987). Purification and physical analysis of *Bacillus anthracis* plasmids pXO1 and pXO2. Biochem. Biophys. Res. Commun. 149: 362-8

68. Kawamura F and Doi R H (1984). Construction of a Bacillus subtilis double mutant deficient in extracellular alkaline and neutral proteases. J. Bacteriol. 160: 442-4

69. Keppie J, Harris-Smith P W, and Smith H (1963). The chemical basis of the virulence of *Bacillus anthracis*. IX. Its aggressins and their mode of action. Br. J. Exp. Pathol. 44: 446-53

70. King A C, and Cuatrecasas P (1982). Exposure of cells to an acidic environment reverses the inhibition by methylamine of the mutagenic response to epidermal growth factor, Biochem. Biophys. Res. Commun. 106: 479

71. Klimpel K R, Molloy S S, Thomas G and Leppla S H (1992). Anthrax toxin protective antigen is activated by a cell- surface protease with the sequence specificity and calalytic properties of furin. Proc. Natl. Acad. Sci. USA. 89: 10277-81

72. Klimpel K R, Arora N, and Leppla S H (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol. Microbiol. 13: 1093-1100

73. Koch R (1876). Beitrage zur Biologie der Pflanzen 2:277

74. Kochi S K, Martin I, Schiavo G, Mock M and Cabiaux V (1994). The effects of pH on the interaction of anthrax toxin lethal and edema factors with phospholipid vesicles. Biochemistry 33: 2604-9

75. Koehler T M and Collier R J (1991). Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. Mol. Microbiol. 5: 1501-6

76. Kubo M and Imanaka T (1988). Cloning and nucleotide sequence of the highly thermostable neutral protease gene from *Bacillus stearothermophilus*. J. gen. Microbiol. 134: 1883-92

77. Labruyere E, Mock M, Surewicz W K, Mantsch H H, Rose T, Munier H, Sarfati R S and Barzu O (1991). Structural and ligand-binding properties of a truncated form of a *Bacillus anthracis* adenylate cyclase and of a catalytically inactive variant in which glutamine substitutes for Lysine-346, Biochemistry. 30: 2619-2624

78. Laemmli U K (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) 227, 680

79. Larson D K, Calton G J, Little S F, Leppla S H and Burnett J W (1988). Separation of three exotoxin factors of Bacillus anthracis by sequential immunosorbent chromatography. Toxicon 26: 913-21

80. Leppla S H (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. (USA) 79: 3162- 3166

81. Leppla S H (1984). Bacillus anthracis calmodulin- dependent-adenylate cyclase: chemical and enzymatic properties and interaction with eukaryotic cells. In: Advances in Cyclic Nucleotide and Protein Phosphorylation Research. 17 (ed. P. Greengard) 189-198, Raven Press, NY.

82. Leppla S H, Friedlander A M and Cora E (1988). Proteolytic activation of anthrax toxin bound to cell receptors. In: Bacterial Protein Toxin (eds F Fehrenbach, J E Alouf, P Falmagne, W Goebel, J Jeljaszewicz, D Jurgen and R Rappouli) 111-112. Gustav Fischer, New York.

83. Leppla S H (1988). Production and purification of anthrax toxin. In: Methods Enzymol. 165: 103-16 (ed. S Harshman), Academic Press, Florida.

84. Leppla S H (1991). The anthrax toxin complex. In Source Book of Bacterial Protein Toxins, Alouf J E and Freer J H (Eds), Academic, London, 277-302

85. Leppla S H (1995). Anthrax Toxins: In Bacterial toxins and virulence factors in diseases (Eds.) Moss J, Iglewski B, Vaughan M, Tu A T, Hand Book of natural toxins Vol. 8, Marcel Dekker Inc. NY, 543-72

86. Little S F, Leppla S H and Cora E (1988). Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. Infect. Immun. 56: 1807-13

87. Little S F, Leppla S H and Friedlander A M (1990). Production and characterization of monoclonal antibodies against the lethal factor component of *Bacillus anthracis* lethal toxin. Infect. Immun. 58: 1606-13

88. Little S F and Lowe J R (1991). Location of receptor- binding region of protective antigen from *Bacillus anthracis*. Biochem. Biophys. Res. Commun. 180: 531-37

89. Lowe J R, Salter O and Avina J A (1990). Abstract of American Society of Microbiology, Abstract B320, Am. Soc. Microbiol. Washington, DC.

90. Loyter A, Citovsky V and Ballas N (1991). In: Membrane fusion (J Wilschut and D Hoekstra (ed.) Marcel Dekker Inc. NY, pp 731-749

91. Madshus I H, Olsnes S and Sandvig K (1984). Mechanism of entry into the cytosol of Poliovirus type 1: requirement for low pH, J. Cell. Biol. 98: 1194

92. Makino S, Uchida I, Terakado N, Sasakawa C and Yoshikawa M (1989). Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in *Bacillus anthracis*. J. Bactariol. 171: 722-730

93. Makino S, Sasakawa C, Terakado N and Yoshikawa M (1988). Cloning and CO_2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. Mol. Microbiol. 2: 371-6

94. Manavalan P and Johnson Jr C W (1983). Sensitivity of circular dichroism to protein tertiary structure class. Nature 305: 831-2

95. Mandel M and Higa A (1970). Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159

96. Marnell M H, Shia S-P, Stookey M and Drapper R K (1984). Evidence for penetration of diphtheria toxin to the cytosol through a prelysosomal membrane. Infect. Immun. 44: 145

97. Merchant I A and Packer R A (1956). Bacillus, In: Veterinary Bacteriology and Virology. 5th edition, The Iowa State College Press, USA.

100. Meselson M, Guillemin J, Huge-Jones M, Langmuir A, Popova I, Shelokov A, Yompolskaya O (1994). The Sverdlovsk Anthrax Outbreak of 1979. Science. 266: 1202-08

101. Middlebrook J L and Dorland R B (1984). Bacterial toxins: cellular mechanism of action. Microbiol. Rev. 48: 199

102. Mikesell P, Ivins B E, Ristroph J D and Dreier T M (1983). Evidence of plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. 39: 371-6

103. Miller C (1991). 1990: annus mirabilis of potassium channels. Science 252: 1092-6

104. Milne J C and Collier R J (1993). pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax toxin protective antigen. Mol. Microbiol. 10: 647-53

105. Milne J C, Furlong D, Hanna P C, Wall J S and Collier R J (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J. Biol. Chem. 269: 20607- 20612

106. Molloy S S, Bresnahan P A, leppla S H, Klimpel K R and Thomas G (1992). Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J. Biol. Chem. 267: 16396-402

107. Munier H, Blanco F, Precheur B, Diesis E, Nieto J L Craescu C T and Barzu O (1993). Characterization of a synthetic calmodulin-binding peptide derived from *Bacillus anthracis* adenylate cyclase. J. Biol. chem. 268: 1695-701

108. Morris R E and Saelinger C B (1986). Reduced temperature alters pseudomonas exotoxin A entry into the mouse LM cell. Infect. Immun. 52: 445

109. Novak J M, Stein M P, Little S F, Leppla S H and Friedlander A M (1992). Functional characterization of protease-treated *Bacillus anthracis* protective antigen. J. Biol. Chem. 267: 17186-93

110. Nambiar M P, Oda T, Chen C, Kuwazuru Y and Wu H C (1993). Involvemen of golgi region in the intracellular trafficking of cholera toxin, J. Cell Physiol. 154: 222-228

111. O'Brien I, Friedlander A, Dreier T, Ezzell J and Leppla S (1985). Effects of anthrax toxin components on human neutrophils. Infect. Immun. 47: 306-10

112. Ogata M, Chaudhary V K, Pastan I and FitzGerald D J (1990). Processing of pseudomonas exotoxin by a cellular protease results in the generation of a 37,000 Da toxin fragment that is translocated to the cytosol. J. Biol. Chem. 265: 20678-20685

113. Ogata M, Fryling C M, Pastan I and Fitz Gerald D J (1992). Cell mediated cleavage of Pseudomonas exotoxin between Arg^{279} and Gly^{280} generates the enzymatically active fragment which translocates to the cytosol. J. Biol. Chem. 267: 25396- 25401

114. Ohishi I, Iwasaki M and Sakaguchi G (1980). Purification and characterization of two components of botulinum C2 toxin. Infect. Immun. 30: 668-73

115. Ohishi I (1987). Activation of botulinum C2 toxin by trypsin. Infect. Immun. 55: 1461-65

116. Olsnes S and Sandvig K (1985). Entry of polypeptide toxins into animal cells, In: Endocytosis, Pastan I and Willingham M C (Eds.), Plenum press, New York.: 195

117. Pasteur L (1881). De I attenuation des virus et de leur retour a la virulence. C. R. Acad. Sci. Agric. Bulg. 92: 429-435

118. Pelham H R B (1991). Multiple targets for brefeldin A. Cell, 67: 449-451

119. Penniston J P (1983). Plasma membrane Ca^{++} ATPase as active pumps, : 99-149. In: Calcium and Cell Function (ed. W Y Cheung) Academic Press Inc., NY.

120. Perelle S, Gibert M, Boquet P and Popoff M R (1993). Characterization of *Clostridium perfringens* iota-toxin genes and expression in Escherichia coli. Infect. Immun. 61: 5147-5156

121. Pestka S (1971). Inhibitors of ribosome functions. Annu. Rev. Microbiol. 25: 487-562

122. Pezard C, Duflot E and Mock M (1993). Construction of *Bacillus anthracis* mutant strains producing a single toxin component. J. Gen. Microbiol. 139: 2459-63

123. Pezard C, Weber M, Sirard J, Berche P and Mock M (1995). Protective immunity induced by *Bacillus anthracis* toxin- deficient strains. Infect. Immun. 63: 1369-72

124. Pienaar U de V (1967). Epidemiology of anthrax in wild animals and the control of anthrax epizootics in the Kruger National Park, South Africa, Fed. Proc. 26: 1496-1502

125. Pollack M R and Richmond M H (1962). Low cysteine content of bacterial extracellular proteins: its possible physiological significance. Nature 194: 446-9

126. Quinn C P, Shone C C, Turnbull P C, and Melling J (1988). Purification of anthrax-toxin components by high- performance anion-exchange, gel-filtration and hydrophobic- interaction. Biochem. J. 252: 753-8

127. Quinn C P, Singh Y, Klimpel K R and Leppla S H (1991). Functional mapping of anthrax toxin lethal factor by in- frame insertion mutagenesis. J. Biol. Chem. 266: 20124-30

128. Rayer (1850) Bacillus, In: Veterinary Bacteriology and Virology by Merchant I A and Packer R A (ed) (1956). 5th edition, The Iowa State College Press, USA.

129. Ray and David (1850) Bacillus, In: Veterinary Bacteriology and Virology by Merchant I A and Packer R A (ed) (1956). 5th edition, The Iowa State College Press, USA.

130. Ristroph J D and Ivins B E (1983). Elaboration of *Bacillus anthracis* antigens in a new, defined culture media. Infect. Immun. 39: 483-6

131. Robertson D L and Leppla S H (1986). Molecular cloning and expression in *Escherichia coli* of the lethal factor gene of *Bacillus anthracis*, Gene (Amst.) 44: 71-78.

132. Robertson D L (1988). Relationships between the calmodulin-dependent adenylate cyclases produced by *Bacillus anthracis* and *Bordetella pertussis*. Biochem. Biophys. Res. Comm. 157: 1027-1032

133. Robertson D L, Tippetts M T and Leppla S H (1988). Nucleotide sequence of the *Bacillus anthracis* edema factor gene (cya): a calmodulin-dependent adenylate cyclase. Gene 73: 363-71

134. Robertson D L, Bragg T S, Simpson S, Kaspar R, Xie W and Tippetts M T (1990). Mapping and characterization of *Bacillus anthracis* plasmids pXO1 and pXO2. Salisbury Med. Bull. 68, Spec. Suppl., 55-8

135. Sandvig K and Olsnes S (1982). Entry of the toxic proteins abrin, modeccin, ricin and diphtheria toxin into cells. 1. Requirement for calcium. J. Biol. Chem. 257: 7495-7503

136. Sandvig K and Brown J E (1987). Ionic requirements for entry of shiga toxin from *Shigella dysenteriae* I into cells. Infect. Immun. 55: 298-303

137. Saraswathy S, Chaudhary V K, FitzGerald D J and Pastan I (1991). Increased cytotoxic activity of pseudomonas exotoxin and two chimeric toxins ending in KDEL. J. Biol. Chem. 266: 17376-17381

138. Sharma M, Swain P K, Chopra A, Chaudhary V K and Singh Y (1995). Expression and purification of anthrax toxin protective antigen from *Escherichia coli*, Protein Expression and Purification. 7: 33-38

139. Schiavo G, Poulain B, Rossetto O, Benfenati F, Tauc L and Montecucco C (1992a). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. EMBO J. 11: 3577-83

140. Schiavo G, Rossetto O, Santucci A, Dasgupta B R and Montecocco C (1992b). Botulinum neurotoxins are zinc proteins. J. Biol. Chem. 267: 23479-83

141. Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, Dasgupta B R and Montecucco C (1992c). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature 359: 832- 5

142. Schiavo G, Shone C C, Rossetto O, Alexander F C and Montecucco C (1993). Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol. Chem. 268: 11516-9

143. Singh Y, Chaudhary V K, and Leppla S H (1989a). A deleted variant of *Bacillus anthracis* protective antigen is non- toxic and blocks anthrax toxin action in vivo. J. Biol. Chem. 264: 19103-7

144. Singh Y, Leppla S H, Bhatnagar R, and Friedlander A M (1989b). Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and resistant cells. J. Biol. Chem. 264: 11099-11102

145. Singh Y, Klimpel K R, Quinn C P, Chaudhary V K and Leppla S H (1991). The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J. Biol. Chem. 266: 15493-97

146. Singh Y, Klimpel K R, Arora N, Sharma M and Leppla S H (1994). The chymotrypsin-sensitive site, FFD^{315}, in anthrax toxin protective antigen is required for translocation of lethal factor. J. Biol. Chem. 269: 29039-46

147. Smith H and Keppie J (1954). Observation on experimental anthrax: demonstration of a specific lethal factor produced in vivo by *Bacillus anthracis*. Nature 173: 869-870

148. Smith H, Tempest D W, Stanley J L, Harris-Smith P W and Gallop R C (1956). The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: their relationship to known immunizing agressins. Br. J. Exp. Pathol. 37: 263

149. Smith H and Stanley J L (1962). Purification of the third factor of anthrax toxin. J. Gen. Microbiol. 29: 517-21

150. Stanley J L and Smith H (1961). Purification of factor I and recognition of a third factor of anthrax toxin. J. Gen. Microbiol. 26: 49-66

151. Steiner D F, Smeekens S P, Ohagi S and Chan S J (1992). The new enzymology of precursor processing endoproteases, J. Biol. Chem. 267: 23435-8

152. Stephen J (1986). Anthrax toxin. In pharmacology of bacterial toxins, Dorner F and Drews J (eds) Pergamon, Oxford, 381-95

153. Sterne M (1937). Variation in *Bacillus anthracis* onderstepoort J. vet. Sci. Anim. Ind. 8: 271-349

154. Streb H, Irvine R F, Berridge M J and Schulz I (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1, 4, 5- triphosphate. Nature. 306: 67-69

155. Thorne C B, Molnar D M and Strange R E (1960). Production of toxin in-vitro by *Bacillus anthracis* and its separation into two components. J. Bacteriol. 79: 450-55

156. Thorne C B (1985). Genetics of Bacillus anthracis. In: Microbiology - 85 (eds Lieve L, Bonventre P F, Morello J A, Schlessinger S, Silver S D and Wu H C), 56 American Society for Microbiology, Washington, DC

157. Tobkes N, Wallace B A and Bayley H (1985). Secondary structure and assembly mechanism of an oligomeric channel protein. Biochemistry, 24: 1915-1920

158. Turnbull P (1990). Anthrax. In Topley and Wilson's Principles of Bacteriology, Virology and Immunity, Vol. 3, Bacterial Diseases, Smith G R and Easmon C R (Eds), Edward Arnold, Sevenoaks, Kent, UK, 364-77

159. Uchida I, Sekizaki T, Hashimoto K and Terakado N (1985). Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. J. Gen. Microbiol. 131: 363-7

160. Uchida I, Hashimoto K and Terakado N (1986). Virulence and immunogenicity in experimental animals of *Bacillus anthracis* strains harbouring or lacking 110 MDa and 60 MDa plasmids. J. Gen. Microbiol. 132: 557-9

161. Underfriend, Guroff and Zaltzman-Nirenberg (1967). Use of fluorescence as a probe. In Fluorescence theory, instrumentation and practice (ed. G G Guilbault) 362-563, Marcel Dekker Inc., NY.

162. Vallee B L, and Auld D S (1990). Active-site zinc ligands and activated H_2O of zinc enzymes. Proc. Natl. Acad. Sci. USA 87: 220-4

163. Vallee B L, and Auld D S (1990a). Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry 29: 5647-59

164. Van de Ven W J, Voorberg J, Fontijn R, Pannekoek H, van den Ouweland A M, van Duijnhoven H L, Roebroek A J and Siezen R J (1990b). Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes, Mol. Biol. Rep. 14: 265-75

165. Van der Goot F G, Lakey J, Pattus F, Kay C M, Sorokine O, Dorsselaer A V and Buckley J T (1992). Spectroscopic study of the activation and oligomerization of the channel- forming toxin aerolysin: identification of the site of proteolytic activation. Biochemistry 31: 8566-70

166. Vodkin M H and Leppla S H (1983). Cloning of the protective antigen gene of *Bacillus anthracis*. Cell 34: 693-7

167. Walev I, Palmer M, Valeva A, Weller U and Bhakdi S Binding (1995), Oligomerization, pore formation by streptolysin O in erythrocytes and fibroblast membranes: detection of nonlytic polymers. Infect Immun. 63: 1188-94

168. Watt V M and Yip C C (1989). Amino acid sequence deduced from a rat kidney cDNA suggests it encodes the Zn-peptidase aminopeptidase N. J. Biol. Chem. 264: 5480-7

169. Watson D W, Cromartie W J, Boom W L, Kegeles G, and Heckly R J (1947). Studies on infection with *Bacillus anthracis*. III. Chemical and immunological properties of the protective antigen in crude extracts of skin lesions of *B. anthracis*. J. Inf. Dis. 80:28

170. Welkos S L and Friedlander A M (1988). Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb. Pathogen. 5: 127-39

171. Welkos S L, Lowe J R, Eden-McCutchan F, Vodkin M, Leppla S H and Schmidt J J (1988). Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene 69: 287-300

172. Welkos S L (1991). Plasmid-associated virulence factors of non-toxigenic (pXO1-) *Bacillus anthracis*. Microb. Pathog. 10: 183-98

173. Wetmore D R, Wong S I and Roche R S (1992). The role of the pro-sequence in the processing and secretion of the thermolysin-like neutral protease from *Bacillus cereus*. Mol. Microbiol. 6: 1593-604

174. Wilkie M H and Ward M K (1967). Characterization of anthrax toxin. Fed. Proc. 26: 1527-1531

175. Wright G G, Hedberg M A, and Slein J B (1954). Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically defined, non protein medium. J. Immunol. 72: 263

176. Wu X C, Lee W, Tran L and Wong S L (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173: 4952-8

177. Xia Z G and Storm D R (1990). A-type ATP binding consensus sequences are critical for the catalytic activity of the calmodulin-sensitive adenylyl cyclase from *Bacillus anthracis*. J. Biol. Chem. 265: 6517-6520

178. Yanisch-Perron C, Vieira J and Messing J (1985). Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119

179. Zhao J, Milne J C and collier R J (1995). Effect of anthrax toxin's lethal factor on ion channels formed by the protective antigen. J. Biol. Chem. 270: 18626-30

APPENDICES

PREPARATION OF BACTERIAL CULTURE MEDIA

LB MEDIUM (Luria-bertani Medium)

Bacto-tryptone	10 g
Bacto- yeast extract	5 g
NaCl	10 g

Dissolved in 950 ml of deionozed water. After the solutes have dissolved completely, pH adjusted to 7.0 with 5 M NaOH. Final volume was made up to 1 liter with water. The media was sterilized by autoclaving for 30 minutes at 15 lb/sq pressure.

ANTIBIOTIC SOLUTIONS

Ampicillin 50 mg/ml stock solution in water and stored at -20^{o}C.

SOLUTIONS FOR PLASMID ISOLATION AND PURIFICATION

Lysozyme solution

4 mg of lysozyme was dissolved in 1 ml buffer containing 50 mM glucose, 25 mm Tris buffer, ph 8.0, 10 mM EDTA

Alkaline-SDS solution

1% SDS solution was prepared in 0.2 N NaOH

Potassium acetate solution

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

Tris-EDTA solution

To 98.2 ml of sterile Milli Q water, 1 ml of 1 M Tris buffer, pH 8.0 and 0.2 ml of 0.5 M of EDTA were added.

TAE (0.04 M Tris acetate, 1 mM EDTA) 50x stock solution

Dissolve Tris base 242 g in 700 ml Milli Q water and 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA, making the final volume to 1 litre.

Ligation buffer

500 mM Tris buffer ph 7.8
100 mM $MgCl_2$
100 mM DTT
10 mM ATP

BUFFERS

Phosphate buffer saline (pH 7.2)

Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
KCl	0.20 g
NaCl	8.00 g

Dissolved in 900 ml of Milli Q water and pH adjusted with 1 N HCl and final volume made to 1000 ml.

SDS-PAGE running buffer

Tris base	3.0 g
Glycine	14.4 g
SDS	1.0 g

Dissolved in 800 ml distilled water and final volume made upto 1 litre.

Electrophoresis transfer buffer

Tris base	3.0 g

Glycine	6.9 g
Methanol	150 ml

Dissolve Tris and glycine in 500 ml of distilled water, add methanol and finally adjust the volume upto 1 litre.

2X SDS-sample buffer (0.125 M Tris buffer, pH 6.8, 4% SDS, 20% glycerol, 10% β - ME)

Tris buffer pH 6.8	2.5 ml of 0.5 M stock
SDS	4.0 ml of 10% SDS stock solution
Glycerol	2.0 ml
β - ME	1.0 ml

Make the final volume to 10 ml with Milli Q water.

Solution A

8% Sodium carbonate
1% Sodium hydroxide
1.6% Sodium-potassium tartarate

Solution B

4% Bicinconinic acid in distillied water and adjust pH 7.0 by dropwise addition of NaOH.

Solution C

4% Cu SO_4 dissolve in distilled water (prefer fresh always).

PUBLICATIONS

1) **Prabodha K. Swain**, Neelam K. Sarkar, Manju Sharma, Seema Goel, Rajesh P. Singh and Yogendra Singh (1996) Internalization of *Bacillus anthracis* lethal toxin into mammalian cells. Ind. J. Biophys. Biochem. (In Press).

2) Yogendra Singh, Kurt R. Klimpel, **Prabodha K. Swain**, Seema Goel and Stephen H. Leppla (1996) Protectiveantigen based engineered vaccines. Salisbury Medical Bulletin Special Supplement (In Press)

3) Manju Sharma, **Prabodha K. Swain**, Arun P. Chopra, Vijay K. Chaudhary and Yogendra Singh (1996) Expression and purification of anthrax toxin protective antigen from *E. coli*. Protein Expression and Purification. 7: 33-38

4) Yogendra Singh, Kurt R. Klimpel, Seema Goel, **Prabodha K. Swain** and Stephen H. Leppla (1996) Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. (submitted to J. Biol. Chem.)

PAPERS PRESENTED

1) **Prabodha K. Swain**, Anthony Thomas, Manju sharma, Arun P. Chopra, R. P. Singh and Yogendra Singh (1996). The effect of pH on the internalization of anthrax lethal factor and protective antigen in solution in 4th International Symposium on Biochemical Roles of Eukaryotic Cell Surface Macromolecules at New Delhi (1996)

2) **Prabodha K. Swain**, Neelam K. Sarkar, S. Goel. Rajesh. P. Singh, Manju Sharma and Yogendra Singh (1994). On the Mechanism of Anthrax Lethal Toxin" in 16th International congress of Biochemistry and Molecular biology, New Delhi

3) **Prabodha K. Swain**, Neelam K. Sarkar, Seema Goel, Rajesh P. Singh and Yogendra Singh (1993).Translocation Mechanism of Anthrax Lethal Toxin: in 62nd Annual Society of Biological Chemists (India) Meeting at Madurai.

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Expression and Purification of Anthrax Toxin Protective Antigen from *Escherichia coli*

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Anthrax toxin consists of three separate proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF). PA binds to the receptor on mammalian cells and facilitates translocation of EF or LF into the cytosol. PA is the primary component of several anthrax vaccines. In this study we expressed and purified PA from *Escherichia coli*. The purification of PA from *E. coli* was possible after transporting the protein into the periplasmic space using the outer membrane protein A signal sequence. The purification involved sequential chromatography through hydroxyapatite, DEAE Sepharose CL-4B, followed by Sephadex G-100. The typical yield of purified PA from this procedure was 500 μg/liter. PA expressed and purified from *E. coli* was similar to the PA purified from *Bacillus anthracis* in its ability to lyse a macrophage cell line (J774A.1). The present results suggest that a signal sequence is required for the efficient translocation of PA into *E. coli* periplasmic space. © 1996 Academic Press, Inc.

The virulence of *Bacillus anthracis,* the causative agent of anthrax, depends on two factors, a poly-D-glutamic acid capsule and a three component protein exotoxin. The genes coding for the toxin and the enzymes responsible for capsule production are carried on *B. anthracis* plasmids pXOI and pXO2, respectively (1,2). The three proteins of the exotoxins are protective antigen (PA), lethal factor (LF), and edema factor (EF). These proteins are individually nontoxic, but in combination form two distinct toxins causing different pathogenic responses in animals and cultured cells (3,4). Protective antigen binds to a cell surface receptor and facilitates the internalization of EF or LF and their translocation into the cytosol (5,6). EF is a calmodulin-dependent adenylate cyclase which increases cAMP to nonphysiological concentrations in eukaryotic cells (5). LF is believed to be a zinc-dependent metalloprotease (7). Lethal toxin has been found to cause an early influx of sodium and efflux of potassium from J774A.1 cells, leading to ATP depletion resulting in lysis of cells (8). PA alone is nontoxic, an essential immunogen, and the primary component of the human vaccine against anthrax (9,10).

The PA gene has been cloned, sequenced (11), and expressed in *B. subtilis* (12), *Escherichia coli* (13), and viruses (14). The purification of PA from sonicates of *E. coli* was not successful due to low expression and extensive degradation by *E. coli* cellular proteases (15). In this study attempts have been made to transport the PA protein into the periplasmic space to reduce degradation. Most proteins translocated across bacterial membranes are synthesized as protein precursors, containing a 15–20 amino acid extension at the N-terminus called signal peptide (16). Apart from the signal peptide, the ultimate localization of the proteins among the inner membrane, periplasm, or outer membrane depends on structural information contained within the protein itself (17).

In this paper, we report that the addition of the signal sequence for the outer membrane protein A (OmpA) of *E. coli* at the 5′-end of the PA gene results in the efficient transport of the expressed PA protein to the periplasmic space. The accumulation of PA in the periplasm facilitated protein purification by protecting PA from degradation by isolating it from several cellular proteases. The deletion of 14 amino acids at the COOH-terminus resulted in the formation of inclusion bodies. Addition of the OmpA signal sequence could not enable the transport of the COOH-terminus deleted PA into the periplasm.

MATERIALS AND METHODS

Reagents. Restriction enzymes and chemicals were purchased from New England BioLabs (Beverley, MA)

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or Bethesda Research Labs (Gaithersburg, MD). The oligonucleotides were synthesized on a model 881 DNA synthesizer and purified using a oligonucleotide purification cartridge from Applied Biosystems. Polymerase chain reaction (PCR) was done using a DNA amplification kit from Perkin–Elmer–Cetus.

Media components for bacterial growth were from Difco Laboratories (Detroit, MI). J774A.1 cells were provided by Dr. P. C. Ghosh (South Campus, University of Delhi, India).

Plasmid construction. The signal sequence was added on a previously constructed plasmid pYS2 containing the gene for native PA (15). The fourteen amino acid COOH-terminal deleted mutant (pYS10) was generated by PCR amplification of native PA gene from pYS5 template (18). To clone the OmpA signal sequence, a *Bgl*II–*Nde*I cassette of 144 base pairs containing the signal sequence was isolated from pVEX115 f(+)T (19) and inserted into pYS2 or pYS10 upstream of the PA gene after deleting a 76-bp *Bgl*II–*Nde*I fragment. The resulting plasmids were designated pMS1 and pMS2 (pMS2 was similar to pMS1 except that pMS2 does not contain codons for the last 14 amino acids) (Fig. 1).

Expression and purification of PA. *E. coli* strain (BL21, λDE3) carrying plasmids pYS2, pMS1, pYS10, or pMS2 were grown at 37°C in LB broth with 100 μg/ml ampicillin at 250 rpm. When A_{600} reached 1.0, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 2 h of induction, cells were harvested by centrifugation at 4000*g* for 15 min. For the preparation of periplasm, cells from 1-liter culture were suspended in 200 ml of 20% sucrose solution (30 mM Tris pH 8.0, 1 mM EDTA, and 1 mM PMSF), incubated on ice for 10 min, and centrifuged at 4000*g* for 10 min. The pellet was resuspended gently in 50 ml of cold distilled water with 1 mM PMSF, kept on ice for 10 min, and harvested at 10,000*g* for 15 min. The supernatant containing periplasmic proteins was saved for purification of PA. The quality of periplasm was determined by assaying the activity of succinic dehydrogenase and β-lactamase (20,21).

A protein profile of total cell and periplasmic preparations were obtained on 10% SDS–PAGE. Gels were either stained with Coomassie brilliant blue or probed with polyclonal rabbit anti-PA sera after electroblotting onto a nitrocellulose membrane.

A 30% ammonium sulfate precipitation was carried out on the periplasmic preparation. The precipitate was pelleted at 15,000*g* for 20 min at 4°C. The supernatant was collected and proteins were precipitated for 2 h at 4°C by addition of ammonium sulfate to 70% saturation. The precipitate was pelleted by centrifugation at 15,000*g* for 60 min, suspended in 20 ml of buffer A (5 mM potassium phosphate, pH 7.0; 0.1 M NaCl; 0.05 mM 1,10 phenanthroline; 2 mM β-mercaptoethanol), and dialyzed against the same buffer overnight.

TABLE 1

Purification of PA from *Escherichia coli*

Fractions	Volume (ml)	Protein (mg/ml)	Activity (EC_{50})	Purification (fold)
Periplasm	50	1.1	8.0	1
Ammonium Sulfate	10	2.5	4.5	2
Hydroxyapatite	9	1.6	2.5	3
DEAE	3	0.7	0.34	24
Sephadex G-100	1	0.5	0.076	105

Note. J774A.1 cells were incubated with several concentration of PA containing proteins obtained after each column and LF (1.0 μg/ml) for 3 h at 37°C. After 3 h of incubation, cell viability was determined by MTT assay as described under Materials and Methods. Activity (EC_{50}) is the concentration of protein (μg/ml) required to kill 50% of the cells. Purification (fold) was determined by dividing the EC_{50} for periplasm with EC_{50} for fractions obtained from different columns.

The dialyzed sample was loaded on 5 ml hydroxyapatite column previously equilibrated with buffer A. The column was washed with 100 ml of buffer A. A linear gradient of 75 ml each of 0 and 0.5 M potassium phosphate, pH 7.0, was used to elute the proteins. Fractions of 5.0 ml were collected in tubes containing 20 μl of 400 mM EDTA. The fractions containing PA proteins were pooled and dialyzed against buffer B (0.01 M Tris–Cl; 0.025 M NaCl; 0.05 mM 1,10 phenanthroline; 2 mM β-mercaptoethanol; 1% glycerol; pH 8.0).

The dialyzed sample was passed through 15 ml DEAE-Sepharose CL-4B column equilibrated with buffer B. The proteins were eluted with a gradient of 100 ml each of 0 and 0.25 M NaCl in buffer B. The fractions (5 ml) containing PA protein were pooled and precipitated with ammonium sulfate (70% saturation). The precipitate was dissolved in 2 ml of buffer C (0.1 M Tris–Cl, pH 8.0; 1 mM EDTA; 50 mM NaCl) and dialyzed overnight against buffer C for the final purification step on a 90-ml Sephadex G-100 column. The purified PA protein was dialyzed against 5 mM Hepes buffer, pH 7.0, containing 50 mM NaCl and frozen at −70°C in aliquots.

Quantitation of PA. The fold purifications of PA at different column stages were determined by calculating the amount of protein required to kill 50% of the J774A.1 cells (EC_{50}) when incubated with LF (1.0 μg/ml) at 37°C (Table 1). The protein was measured by the method of Lowry *et al* (22).

Cell culture and cytotoxicity. A macrophage like cell line (J774A.1) was maintained in Dulbecco's modified

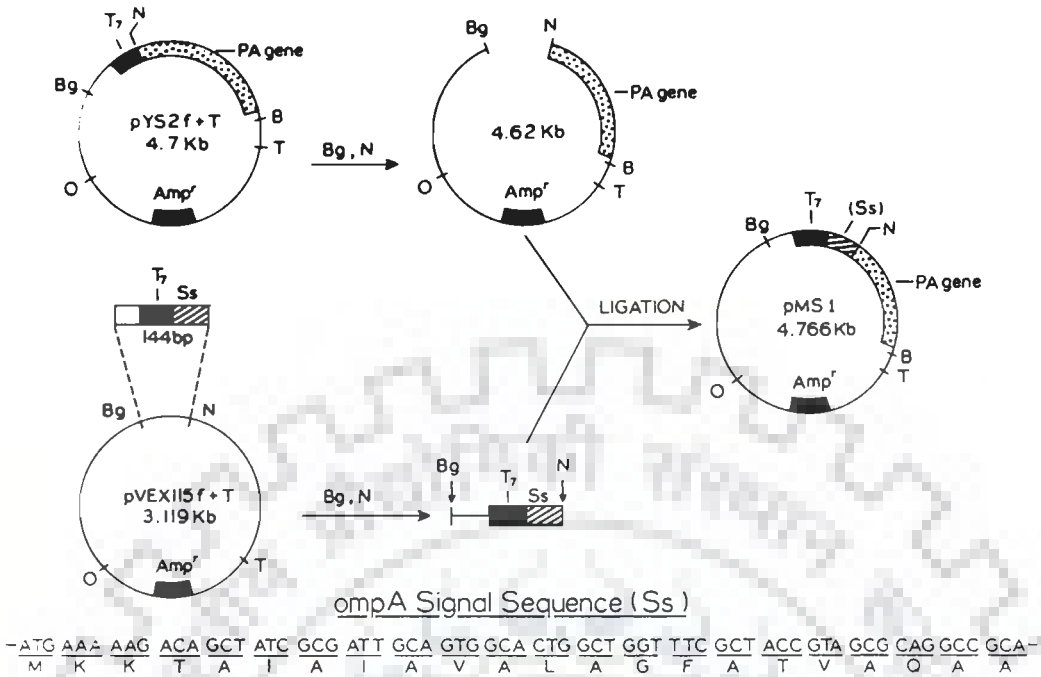

FIG. 1. Construction of plasmids used for expression of PA. Plasmid pMS1 contained the entire native sequence of PA gene. B, *Bam*HI; Bg, *Bgl*II; N, *Nde*I; O, pBR322 origin of replication; T7, T7promoter; T, transcription terminator. Plasmid pMS2 (not shown) was similar to pMS1 except that 42 bases coding for 14 COOH-terminus amino acids were deleted.

Eagle's medium (DMEM) containing 10% fetal bovine serum and 2 mM glutamine. The PA proteins purified from either *E. coli* or *B. anthracis* were added to the cells in combination with LF (1 μg/ml) and incubated for 3 h at 37°C. After 3 h the cell viability was determined using 3-(4-5-dimethylthiazol-2-*yl*)-2-5-diphenyltetrazolium bromide (MTT) dye assay. MTT dissolved in DMEM was added to cells to a final concentration of 0.5 mg/ml and the cells were incubated for 30 min at 37°C. The medium was replaced by 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl in 90% isopropyl alcohol, and vortexed. The absorption at 540 nm was read using a microplate reader (Nunc, GMBH).

RESULTS AND DISCUSSION

Anthrax toxin is usually purified from the culture supernatant of *B. anthracis* (23,24). The main objective of this study was to express and purify the PA of anthrax toxin from *E. coli*. The PA gene was previously subcloned into the *E. coli* expression vector pVC8f(+)T (15) using oligonucleotides. The resulting plasmid pYS2 has the PA gene under control of the bacteriophage T7 promoter. The plasmid pYS2 was transformed into BL21 strain of *E. coli* which produced native PA when induced with IPTG. The PA expressed in *E. coli* was not secreted into periplasmic spaces or into the medium. The presence of PA was determined by Western blot and by using the macrophage lethality assay. The purification of PA from sonicates of *E. coli* was not successful due to extensive degradation of PA and the presence of a large amount of *E. coli* protein in comparison to the PA protein (data not shown). To overcome this problem, an *E. coli* signal sequence (OmpA) was cloned at the 5′ end of the PA gene (Fig. 1) to produce plasmid pMS1. Addition of the signal sequence caused PA to accumulate in the periplasmic space (Fig. 2).

To purify PA from *E. coli*, the BL-21 strain carrying the plasmid pMS1 was grown to an O.D. of 1.0 and then induced with IPTG (1 mM) for 2 h. The cells were harvested from 1 liter culture after induction and periplasmic proteins were extracted in 50 ml of buffer. The periplasmic preparations were used to purify PA. The estimation of succinic dehydrogenase and β-lactamase in the total cell, spheroplast, and periplasm indicated that 20% of the succinic dehydrogenase and 85% of β-lactamase activity was present in the periplasmic fraction (data not presented). The periplasmic proteins were first precipitated with ammonium sulfate. It was noticed using iodinated PA that 95% of the PA precipitated at a concentration of 30–70% ammonium sulfate. The ammonium sulfate precipitation up to 30% removed some undesired proteins without loss of significant amount of PA. The supernatant was reprecipitated with 70% ammonium sulfate (final concentration).

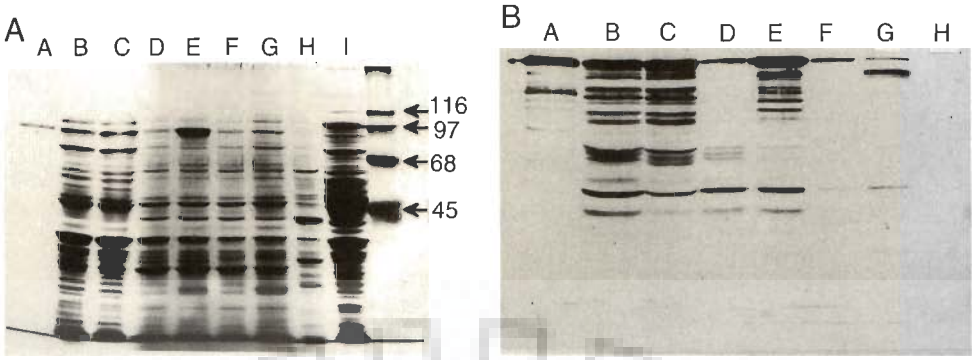

FIG. 2. Electrophoretic analysis of *E. coli*-expressed PA protein. Proteins were separated by 10% SDS–PAGE and stained with Coomassie blue (A) and Western blot of the *E. coli* proteins containing PA, developed with a rabbit polyclonal PA antibody (B). Lane A, PA purified from *B. anthracis;* Lane B, *E. coli* cells expressing PA without OmpA signal sequence; Lane C, *E. coli* cells expressing 14 amino acids deleted PA without signal sequence; Lane D, periplasmic proteins of cells expressing PA without signal sequence; Lane E, periplasmic proteins of *E. coli* expressing PA with OmpA signal sequence; Lane F, periplasmic proteins of cells expressing 14 amino acids deleted PA without signal sequence; Lane G, periplasmic proteins of cells expressing 14 amino acids deleted PA with signal sequence; Lane H, periplasmic preparation of *E. coli* cells carrying vector without any PA gene; Lane I, *E. coli* cells carrying vector without PA gene.

After solubilization and dialysis the precipitate was passed through a hydroxyapatite column and PA was eluted at 80–125 mM phosphate gradient. The fractions containing PA protein were pooled and passed through DEAE-Sepharose CL-4B. The protein was eluted at a gradient of 100–150 mM NaCl. This resulted in approximately 80% pure PA protein with few degraded PA proteins and small amounts of *E. coli* proteins. The final purification was done through Sephadex G-100 which removed all the low molecular weight proteins (Fig. 3). The purified PA was dialyzed against 5 mM Hepes buffer containing 50 mM NaCl and stored frozen at −70°C until further use. PA does not have any enzymatic activity and the method to quantitate PA in crude or semipurified sample is by macrophage lethality assay. The purity and biological activity at each column stage was determined by SDS–PAGE or by determining the concentration of protein (EC_{50}) required in combination with LF to kill 50% of the cells. The EC_{50} values obtained for each column fractions containing PA were used to calculate fold purification of PA after each column. By using this procedure PA was purified to homogeneity with a 105-fold purification over the periplasmic preparation.

This procedure allows PA to be purified using conventional chromatographic techniques. Furthermore the *E. coli* system is convenient to transform, grow, and maintain in the laboratory. One liter of culture yielded 500 μg of PA. The purity of the *E. coli*-expressed PA and its biological activity were compared with that of PA purified from *B. anthracis* using the macrophage lysis assay. The PA purified from *E. coli* showed comparable biological activity to PA of *B. anthracis* in the macrophage lysis assay (Fig. 4). The PA purified from

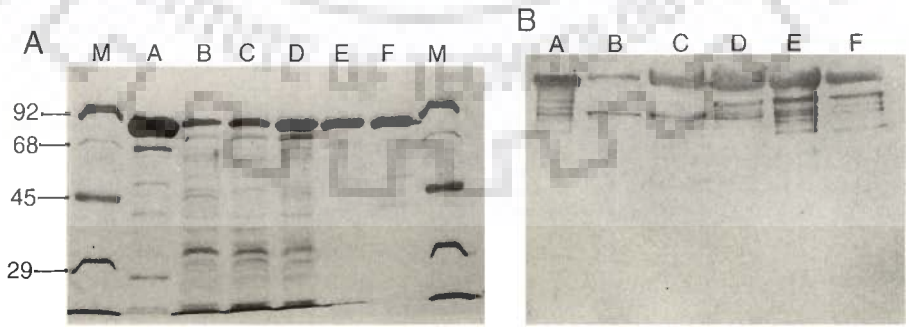

FIG. 3. Purification of PA from periplasmic preparation of *E. coli* cells expressing PA. The proteins were analyzed on 10% SDS–PAGE and stained with Coomassie blue (A) and Western blot of PA proteins, developed with polyclonal rabbit PA antibody (B). Lane M, molecular weight standard ($\times 10^3$); Lane A, PA protein purified from *B. anthracis;* Lane B, Periplasmic proteins of *E. coli;* Lane C, ammonium sulfate (70%) precipitated proteins of periplasmic preparation; Lane D, proteins after passing through hydroxyapatite column; Lane E, protein after passing through DEAE-Sepharose CL4B column; Lane F, PA protein after Sephadex G-100 column.

E. coli was equally sensitive to trypsin and chymotrypsin cleavage as that purified from *B. anthracis* (Data not presented). Since PA does not have any cysteine which could lead to the formation of different intermolecular or intramolecular species, PA purified from *E. coli* was expected to be similar to PA purified from *B. anthracis.*

In previous work we showed that deletion of fourteen amino acids at COOH-terminus eliminated the receptor binding activity of the PA making it nontoxic (18). Earlier attempts to purify mutant PA proteins lacking 12 or 14 amino acids at COOH terminus from *B. subtilis* supernatant were unsuccessful due to extensive degradation. These proteins were purified by an immunoadsorbant column using monoclonal antibody 10G4. However, a large portion of these PA proteins were proteolytically nicked (18). The deletion of fourteen amino acids from the carboxy terminus resulted in mostly insoluble protein and only a small fraction of the total PA produced was transported into the periplasm. In this study the OmpA signal sequence was also added to the 5′ end of a PA gene carrying the 14 codon truncation at the 3′ end. It was expected that the signal sequence would help the deleted PA to translocate into the periplasmic space, preventing degradation by cellular proteases. However, the addition of signal sequence to the 14 amino acid deleted PA did not improve the transport to the periplasm (Fig. 2).

The data presented here provide an easy and convenient method for expression and purification of PA protein and demonstrate that OmpA signal sequence transport native PA protein efficiently into the periplasmic space.

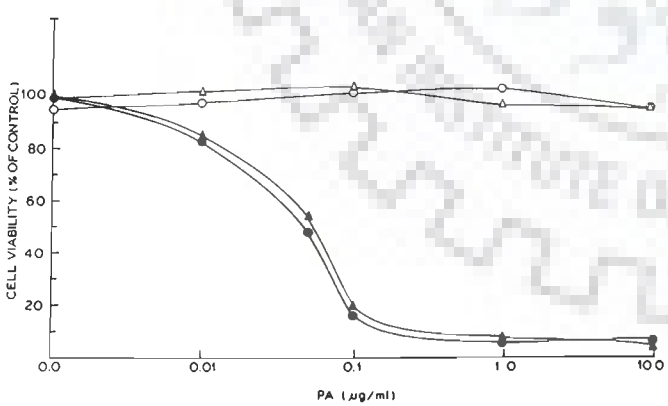

FIG. 4. Biological activity of PA proteins purified from *B. anthracis* and *E. coli.* J774A.1 cells were incubated with varying concentration of PA alone or in combination of LF (1 μg/ml) for 3 h at 37°C. Cell viability was determined by MTT assay as described under Materials and Methods. Open symbols represent PA alone and closed symbols for PA in combination of LF. ○, PA purified from *B. anthracis;* △, PA purified from *E. coli.*

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REFERENCES

1. Green, B. D., Battisti, L., Koehler, T. M., Thorne, C. B., and Ivins, B. E. (1985) Demonstration of a capsule plasmid in *Bacillus anthracis. Infect. Immun.* **49,** 291–297.
2. Mikesell, O. P., Ivins, B. E., Ristroph, J. D., and Dreier, T. M. (1983) Evidence for plasmid mediated toxin production in *Bacillus anthracis. Infect. Immun.* **39,** 371–376.
3. Friedlander, A. M. (1986) Macrophages are sensitive to anthrax lethal toxin through an acid dependent process. *J. Biol. Chem.* **261,** 7123–7126.
4. Stanley, J. L., and Smith, H. (1961) Purification of factor I and recognition of a third factor of anthrax toxin. *J. Gen. Microbiol.* **26,** 49–66.
5. Leppla, S. H. (1982) Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentration of eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **79,** 3162–3166.
6. Singh, Y., Klimpel, K. R., Arora, N., Sharma, M., and Leppla, S. H. (1994) The chymotrypsin sensitive site FFD (315), in anthrax toxin PA is required for translocation of lethal factor. *J. Biol. Chem.* **269,** 29039–29046.
7. Klimpel, K. R., Arora, N., and Leppla, S. H. (1994) Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol. Microbiol.* **13,** 1093–1100.
8. Hanna, P. C., Kochi, S., and Collier, R. J. (1992) Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophages like cells. *Mol. Biol. Cell.* **3,** 1269–1277.
9. Hambleton, P., Carman, J. A., and Melling, J. (1984) Anthrax: The disease in relation to vaccines. *Vaccine* **2,** 125–132.
10. Ivins, B. E., and Welkos, S. L. (1987) Recent advances in the development of an improved human anthrax vaccine. *Eur. J. Epidemiol.* **4,** 12–19.
11. Welkos, S. L., Lowe, J. R. Eden-McCutchan, F., Vodkin, M., Leppla, S. H., and Schmidt, J. J. (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis. Gene* **69,** 287–300.
12. Ivins, B. E., and Welkos, S. H. (1986) Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis. Infect. Immun.* **54,** 537–542.
13. Vodkin, M. H., and Leppla, S. H. (1983) Cloning of the protective antigen gene of *Bacillus anthracis. Cell* **34,** 693–697.
14. Iacono-Connors, L. C., Schmaljohn, C. S., and Dalrymple, J. M. (1990) Expression of the *Bacillus anthracis* protective antigen gene by baculovirus and vaccinia virus recombinants. *Infect. Immun.* **58,** 366–372.
15. Singh, Y., Chaudhary, V. K., and Leppla, S. H. (1989) A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action invivo. *J. Biol. Chem.* **264,** 19103–19107.
16. Silhavy, T. J., Benson, S. A., and Emr, S. D. (1983) Mechanisms of protein localization. *Microbiol. Rev.* **47,** 313–344.
17. Inovye, M., and Halegoua, S. (1979) Secretion and membrane

localization of proteins in *Escherichia coli. CRC Crit. Rev. Biochem.* **7,** 339–371.

18. Singh, Y., Klimpel, K. R., Quinn, C. P., Chaudhary, V. K., and Leppla, S. H. (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *J. Biol. Chem.* **266,** 15493–15497.
19. Ogata, M., Chaudhary, V. K., FitzGerald, D. J., and Pastan, I. (1989) Cytotoxic activity of a recombinant fusion protein between interleukin 4 and Pseudomonas exotoxin. *Proc. Natl. Acad. Sci. USA* **86,** 4215–4219.
20. Ells, A. H. (1959) A colorimetric method for the assay of soluble succinic dehydrogenase and pyridine nucleotide-linked dehydrogenases. *Arch. Biochem. Biophys.* **85,** 561–562.
21. Novick, R. P. (1962) Micro-iodometric assay of penicillinase. *Biochem. J.* **83,** 236–240.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
23. Leppla, S. H. (1988) Production and purification of anthrax toxin, *in* "Methods in Enzymology" (Harshman, S., Ed.), Vol. 165, pp. 103–116, Academic Press, San Diego.
24. Quinn, C. P., Shone, C. C., Turnbull, P. C. B., and Melling, J. (1988) Purification of anthrax toxin components by high performance anion-exchange, gel filtration and hydrophobic-interaction chromatography. *Biochem. J.* **252,** 753–758.