UNIVERSIDAD DE GRANADA

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR 3 E INMUNOLOGÍA

"MECHANISMS OF INDUCTION OF APOPTOSIS IN HEMATOPOPIETIC CELLS BY SULPHATED BACTERIAL EXOPOLISACCHARIDES"

TESIS DOCTORAL PRESENTADA POR

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Granada, Enero 2008

Editor: Editorial de la Universidad de Granada
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D.L.: Gr. 591- 2008
ISBN: 978-84-338-4916-8

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Introduction

1. **Halophiles**

In microbial world, halophiles are extremophiles (bacteria and other micro-organisms) that thrive in environments with very high concentrations of salt (at least 2 M, approximately ten times the salt level of ocean water). Normal ocean water has a saline/salt level of 30 percent. Some environments that halophiles live in are the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and saltines (crackers). The name “Halophiles” comes from Greek for "salt-loving" (halo=salt and phile=loving). Some halophiles are classified into the Archaea kingdom, but there are bacterial halophiles as well.

Halophiles are categorized as debile, moderate and extreme by the extent of their halophilism.

- No halophiles

Its optimal growth takes place at a concentration of NaCl inferior to 0.2 M (1%p/v). Bacteria able to grow in the absence of salt as well as in the presence of relatively high salt concentrations are designated halotolerant (or extremely halotolerant if growth extends above 2.5 M).

- Debile halophiles

Also called marine bacteria. Its optimal growth takes place at a concentration of NaCl of 0.2-0.5 M (1-3% p/v).

- Moderate

They grow in optimal form at concentrations of NaCl between 0.5 and 2.5 M (3-15% p/v).

- Extreme halophiles,

They have an optimal growth between 2.5 and 5.2 M of NaCl. The majority of halophiles are classified as moderate or debile. It is very difficult to encounter an extreme halophile.

Compared to the extensive literature on the physiology, biochemistry, and ecology of the Archaea, the halophilic bacteria have been relatively little studied. However, halophilic bacteria have the potential for exciting and promising applications. Not only do many of them produce compounds of industrial interest (enzymes, polymers, and osmoprotectans), but also they possess useful physiological properties which can facilitate their exploitation for commercial purposes. First, most of them can grow at high salt concentrations (Eisenberg, 1995), minimizing the risk of contamination. Second, they are easy to grow, and their nutritional requirements are simple: the majority can use a large range of compounds as their carbon and energy source (Kushner and Kamekura, 1988). A number of studies carried out, especially during the last decade, have increased our current knowledge about different aspects of halophilic bacteria, such as their physiology, ecology, taxonomy or phylogenetic relationships with other microorganisms, and, to a lesser extent, their genetics and industrial applications of their special activities. These studies show that each halophile has distinct characteristics, and it is expected that the identification of these unique properties of each strain will contribute to the development of novel biotechnological uses for these organisms.

Some novel groups of *Halomonas* have been identified in Southern Spain over the last few years- *Halomonas maura* was isolated from soils of Salinas de Sabinar, Murcia, and San Pedro del Pinatar, Murcia (Bouchotroch et al., 2001). *Halomonas anticariensis*

FP34 (Martinez-Canovas et al., 2004e; Martinez-Canovas et al., 2004c; Martinez-Canovas et al., 2004b; Martinez-Canovas et al., 2004d; Martinez-Canovas et al., 2004a) was isolated from soils of Laguna Redonda, Málaga.

2. **Exopolysaccharides**

Polysaccharides are made up of multiple monosaccharides joined together by glycosidic linkages. They are therefore very large, often branched, molecules that tend to be amorphous, insoluble in water, and have no sweet taste. Polysaccharides have a general formula of $C_n(H_2O)_n$ where n is usually a large number between 200 and 2500. The general formula can also be represented as $(C_6H_{12}O_6)_n$ where n=40-3000.

In the microbial environment, bacteria produce a wide variety of exopolysaccharide (EPS) that are used by these microorganisms to cope in various ways with adverse external environments. Bacterial EPS are highly heterogeneous polymers containing a number of distinct monosaccharides and non-carbohydrate substituents, many of which are strain-specific (Sutherland, 1985; Whitfield, 1988). External environment of bacteria can be extreme or non-extreme, but the EPS of extracellular matrix of bacteria provide them protection and improve their surviving condition in the environment. Specificity of EPS makes them important in bacterial infections of animals and plants because these components may support the bacteria to evade host defences or to adhere to appropriate surfaces and generate their surviving condition. They are essential for the establishment of bacterial biofilms in a variety of environments (Costerton et al., 1995; Leigh and Coplin, 1992; Moxon and Kroll, 1990).

From past many years there has been an increased scientific understanding of EPS biological specificity and its subsequent relation to the body's immune system. Not only EPS of microbes have biological importance for immune system, but EPS of plants and animals also have very useful biological properties which can be investigated and utilized in the biotechnology industry for their possible application in controlling the immune system. As we know, an EPS rich extracellular matrix surrounds all plant cells that is called primary cell wall. Obviously primary cell wall of plant has a structural function and influences plant cell expansion, morphogenesis and differentiation. But EPS present as a component of plant cell walls also play a role of signal molecules in defence mechanisms. In recent years, EPS of plant origin have emerged as an important class of bioactive natural products. They are reported to have blood anticoagulant (Shanmugam et al., 2001; Siddhanta et al., 1999), anti-tumour, anti-mutagenic, anti-complementary, immunomodulating, hypoglycemic, antiviral, hypolipidemic and anti-inflammatory activities (Srivastava and Mustafa, 1989; Srivastava, 1989).

EPS are also very important for some animal bodies. They are made available to body through the food. As an example, chitin makes up exoskeletons of small insects, spiders, animals, etc. and it consists of N-acetylglucosamine that has bioactive nature. This way, there are many body components which are made of polysaccharides and the properties of the polysaccharides provide them different functional properties. A powerful blood anticoagulant activity has been detected in various animal glycosaminoglycans/proteoglycans. Heparin is the most widely known and therapeutically used glycosaminoglycan, comprising various sulphated, alternating, 1,4-linked residues of uronic acid and D-glucosamine (Casu, 1985; Lane and Caso, 1989). Heparan sulphate and dermatan sulphate are other endogenous sulphated EPS which

play a physiological anticoagulant role (Lane et al., 1992; Teien et al., 1976). Chemically-related polysaccharides have also been identified in numerous living organisms. However, there is a lack of knowledge of the correlation between the molecular structure of polysaccharides, which can today be altered in an almost unlimited ways, and their material properties. Furthermore, there is a lack of technology for the controlled conversion of these polysaccharides for industrial exploitation.

Thus, many EPS can be extracted from plants, animals and microbes and their bioactive properties can be utilized for their biotechnological exploitation or the development of new therapeutic drugs.

3. **Physical and Chemical Properties of EPS**

Previous studies have precisely identified the physical and biochemical properties of the EPS used in this study (Mata et al., 2006); Mata et al, unpublished results).

Table 1. Composition of EPS B100, N12 and FP34 (Mata et al, unpublished results)

EPS	**Chemical Composition (%p/p)**								
	CH	Prot	URO	Acet	Pir	Hex	Ashes	Sulphat	Phosphat
H.maura B100	33.2	1.65	4.1	0.25	2.7	2.4	16.5	7.9	0.25
H. maura N12	30.4	1.05	1.65	1.95	2.15	2.8	18.5	2.45	0.35
H.anticariensis FP34	30	3.5	2.4	0.75	2.3	2.4	24.5	2.25	0.4

EPS	MW (Da)	**Monosaccharides (%p/p)**								
		Glu	Man	Gal	Ram	Fuc	Ara	Xil	AGal	AGlc
H.maura B100	$3.75x10^5$	44.5	15	40.5	ND	ND	ND	ND	ND	ND
H.maura N12	$2.5x10^5$	48.82	25.47	ND	ND	25.69	ND	ND	ND	ND
H.anticariensis FP34	$5.3x10^4$	14.6	59.95	ND	1.15	ND	ND	1.35	22.95	ND

*CH: Carbohydrates; Prot: Protein; URO: Uronic acid; Acet: Acetyl rests; Pir: Piruvic; Hex: Hexamine; ND: Not detected; MW: Molecular weight; Glu: Glucose; Man: Manose; Gal: Galactose; Ram: Ramnose; Fuc: Fucose; Ara: Arabinose; Xil: Xilose; AGal: Galacturonic acid; AGlc: Glucouronic acid

The interactions of every EPS with the environment, including those with other microbes and host organisms, its infectiousness, the exchange of nutrients and waste products, and the resistance to external stresses as caused by mechanical, chemical, thermal, and osmotic factors determine largely the physical and chemical properties of EPS of the extracellular matrix. At a molecular level, the physical properties of EPS are greatly influenced by the ionic content of the surrounding bulk aqueous phase and by water molecules. The physic-chemical properties of EPS will differ significantly from

those of purified components and will also be substantially affected by the ionic strength of the surrounding medium and the nature of the cationic species (Allison and Matthews, 1992). Moreover, EPS also vary in their water solubility, some being highly soluble in water or dilute salt solutions whilst others are virtually insoluble in water (Sutherland, 1985). Thus, polysaccharides may be hydrophilic but can also have hydrophobic properties (Neu et al., 1992; Neu and Marshall, 1990). Most EPS in solution undergo a change from order to disorder on heating or on removal of ions. Such changes may be reflected by increasing solubility and eventual dissolution or sloughing from an EPS (Parolis et al., 1996). The biological properties such as blood anticoagulant, anti-tumour, anti-mutagenic, anti-complementary, immunomodulating, hypoglycemic, antiviral, hypolipidemic and anti-inflammatory activities (Srivastava and Mustafa, 1989; Srivastava, 1989) of EPS are of interest for the development of new therapeutic drugs. Thus, the precise knowledge of the chemical composition and physical, chemical and biological properties of EPS are of the utmost importance to design the strategies for developing these new potentially therapeutic drugs.

4. Exopolysaccharides and Immunology

As it has been already mentioned in this introduction, the peculiar properties of EPSs form the basis for a wide range of applications in food, pharmaceutical, petroleum, and other industries. It has been shown that EPSs isolated from bacteria have interesting immunomodulatory properties. A simple demonstration of these properties is evident with an EPS purified from culture supernatants of *Paenibacillus jamilae* CP-7, a gram-positive bacillus isolated from compost prepared with olive mill wastewaters that has shown interesting immunomodulatory properties (Ruiz-Bravo et al., 2001). It was

demonstrated that administration of EPS to BALB/c mice by the intraperitoneal route had a low level of acute toxicity. There was suppression of the proliferative responses of splenocytes to B-cell and T-cell mitogens but a significant enhancement in the production of gamma interferon and granulocyte-macrophage colony-stimulating factor by unstimulated and lipopolysaccharide-stimulated splenocytes *in vitro*, and there was also an increase in the levels of resistance to the intracellular pathogen *Listeria monocytogenes* in mice due to the immunomodulatory effects of EPS from *P. jamilae* (Ruiz-Bravo et al., 2001). These results support the potential pharmacological activity of EPSs. In recent years, research has focused on the mechanisms of action of EPSs (Yan et al., 1999b), as well as on the discovery of new ones (Tian et al., 1999b). Until now, several EPSs have been discovered which are known to possess immunological activities with potential pharmacological applications as biological response modifiers (BRMs). The wide spectrum of BRMs currently available have anti-viral, anti-bacterial, anti-fungal, anti-parasitic, or anti-tumor activities as well as other therapeutic properties which may play critical immunological roles in protecting human health (Leung et al., 2006). BRMs are defined as agents with the property of altering the existing immune response after binding with specific cell surface receptors. BRMs can be cytokines, produced endogenously by immune cells or derivatives of bacteria, fungi, brown algae, *Aloe vera* and photosynthetic plants. Such exogeneous derivatives (exogeneous BRMs) can be nucleic acid (CpG), lipid (lipotechoic acid), protein or polysaccharide in nature, but polysaccharide BRMs have the widest occurrence in nature. A number of EPSs are able to induce resistance to bacterial infections in experimental models (Tzianabos et al., 1998), and some of them have been evaluated in clinical trials (Babineau et al., 1994). Lentinan and other fungal glucans, yeast mannan fractions, and a number of bacterial

EPSs have been identified and have been found to have the ability to favor tumor rejection (Whistler et al., 1976).

Investigators have focused to find the biomolecules that provoke a strong immune response while doing no damage to immune system. EPS are considered to be T-cell-independent antigens that do not elicit cell-mediated immune responses (Tzianabos, 2000). Recently, certain polysaccharides of microbial origin have also been shown to act as potent immunomodulators with specific activity for both T cells and antigen-presenting cells, such as monocytes and macrophages. Till now, very few EPS immunomodulators have been identified by researchers. Most of the studies for identification of EPS have been anecdotal in nature. Relatively very few EPS have been examined in detail where both structure-function and mechanism of action studies have been performed. Recent investigations have led to a more detailed understanding of structural aspects of EPS that govern biologic function and regulation of cytokine networks thus influencing host immune responses. Particular structure-function relationships have been identified and polysaccharide-specific receptors have been discovered that should provide a base for the development of compounds with novel activities. There are many bacterial EPSs which have mitogenic activities, such as the component of the capsular complex of *Bacteroides fragilis*, polysaccharide A that possesses mitogenic activity for T lymphocytes (Brubaker et al., 1999), inducing the production of interleukin-2 (IL-2) by $CD4^+$ T cells (Tzianabos et al., 1999). Not only bacteria, a number of fungi and yeasts (*Saccharomyces cerevisiae*) (Patchen et al., 1998) also produce EPS with immunomodulatory activities. Some fungi and yeasts produce β-(1,3)-glucans that differed in molecular weight and the number of 1-6 linkages and have important biologic activities (Tzianabos, 2000). A soluble derivative of β-(1,3)-glucan

has the ability to prime granulocytes and macrophages for enhanced cytokine release (Sherwood et al., 1987; Soltys and Quinn, 1999), reactive nitrogen intermediate production (Cleary et al., 1999), and bactericidal capacity (Wakshull et al., 1999) . EPS do not affect only the level of cytokine release but they also affect the levels of other molecules involved in the immune response. An EPS from *Pseudomonas aeruginosa*, mannuronan, enhances natural cytotoxicity by increasing Fas ligand expression in NK cells (Halaas et al., 1998) and is also able to significantly promote hematopoiesis (Halaas et al., 1997). EPS purified from spent culture fluid of three *Capnocytophaga* species has shown its ability to activate the human complement system (Bolton and Dyer, 1986). Thus, the potential usefulness of EPSs due to their distinct biological properties in the treatment of infectious diseases has been clearly demonstrated.

5. **Apoptosis**

As we know, every cell has a time to live and a time to die. A cell can either die by injurious agents (e.g. mechanical damage, exposure to toxic chemicals) or be induced to commit suicide. Programmed cell death (PCD) is an interesting and very important cellular phenomena of life. PCD is as intrinsic for cells as mitosis and is involved in both degenerative and developmental processes of organs and organisms. It is distinguished from death by necrosis, an uncontrolled cell death, by the absence of an associated inflammatory response and lysis of cells that may induce serious health problems (Ellis et al., 1991). PCD comprises several subtypes, as revealed by electron microscopy. Apoptosis, or type I programmed cell death, is characterized by condensation of cytoplasm, preservation of organelles and disintegration of cytoskeletal elements in early stages. Autophagic cell death, or type II programmed cell death, occurs

in physiological and disease states and exhibits extensive autophagic degradation of Golgi apparatus, polyribosomes and endoplasmatic reticulum, which precedes nuclear destruction (Bursch et al., 2000b; Bursch et al., 2000a; Hughes and Rusten, 2007). Intermediate and microfilaments are largely preserved; presumably the cytoskeleton is required for autophagocytosis (Bursch et al., 2000b; Bursch et al., 2000a). Briefly, the autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes (Klionsky and Emr, 2000).

The term Apoptosis is taken from Greek (apo- from, ptosis- falling) and it means "dropping off" of petals or leaves from plants or trees. Apoptosis was first identified by John F. Kerr in 1965 as a distinct death mechanism (Kerr, 1965), and by 1972 the term "apoptosis" had already been coined by Kerr and co-workers (Kerr et al., 1972). Furthermore, this seminal study recognized by electron microscopy the three main mechanisms of cell death: apoptosis, necrosis and autophagy (Kerr et al., 1972), although recent studies suggest the possibility of new mechanisms of PCD (O'Connell and Stenson-Cox, 2007).

Apoptosis involves biochemical events in a controlled, regulated fashion leading to a characteristic cell morphology and death in response to a variety of stimuli. In apoptotic process, a cell is executed in such a way as to safely dispose of cell corpses and fragments. This way, we can say apoptosis is a process in which cells play an active role in their own death. Apoptotic cells display distinctive morphology during the apoptotic process. Mainly, apoptosis is defined by the orchestrated collapse of a cell characterised by membrane blebbing, cell shrinkage resulting into loss of surface contact as cell separates from its neighbours, and loss of specialised membrane structures e.g.

microvilli and desmosomes; cell organelles become tightly packed making the cytoplasm appears dense; condensation of chromatin to the periphery of the nucleus forming crescents; nucleolus disintegration; loss of nuclear pores; nuclear fragmentation (karyorrhexis); and fragmentation of DNA followed by rapid engulfment of the apoptotic bodies containing cytoplasm, organelles and nuclear fragments by neighbouring cells or macrophages.

Table 2. Characteristic pattern of morphological, biochemical and molecular changes of apoptosis

Morphological Changes	Functional/Biochemical Changes
• Cell shrinkage • Cell shape change • Condensation of cytoplasm • Nuclear envelope changes • Nuclear fragmentation • Loss of cell surface structures • Apoptotic bodies • Cell detachment • Phagocytosis of remains	• Free calcium ion rise • Bcl-2/Bax interaction • Cell dehydration • Loss of mitochondrial membrane potential • Proteolysis • Phosphatidylserine externalisation • Lamin B proteolysis • DNA denaturation • Intranucleosomal cleavage • Protein cross-linking

Apoptosis is known as a genetically regulated form of cell death. Mainly apoptosis would be used to:

- "Sculpt" an organism during development such as during embryo development, metamorphosis and tissue atrophy
- Regulate the total number of cells.
- Defend and remove unwanted or dangerous cells like tumor cells, virally infected cells, or immune cells that recognize self (which could lead to autoimmune disease).

Apoptosis plays a central role not only in maintaining health but also in the pathogenesis of numerous diseases. Today, it is clear that this fundamental biological concept can be utilized for curing many important diseases such as AIDS, cancer or auto-immunity, and therefore has deep impacts on pharmaceutical markets and interests of research and development. In short, apoptosis is implicated in biological processes ranging from embryogenesis to ageing, from normal tissue homoeostasis to many human diseases, and it has therefore become one of the most relevant fields of biomedical research (Renehan et al., 2001).

Apoptosis is associated with controlling different types of diseases in two major ways. It has been already mentioned how increased apoptosis is characteristic of AIDS (Holm et al., 2004; Holm and Gabuzda, 2005), in which T helper cell numbers plummet and part of the dramatic decline in these cells is caused by increased apoptosis of that leads T helper cells into committing suicide; neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Leuner et al., 2007) and amyotrophic lateral sclerosis (Bedlack et al., 2007); ischaemic injury after myocardial infarction, stroke and infectious diseases such as hepatitis; and graft versus host disease (Renehan et al., 2001). In contrast, decreased apoptosis is a feature of many other diseases such as cancer, in which tumor cells lose their ability to undergo apoptosis (Renehan et al., 2001). Investigation of cancer has already provided us with explanations about some causes of generation of cancer and how defects in apoptotic pathways allow cells with genetic abnormalities to survive (Sjostrom and Bergh, 2001). Experiments of the role of apoptosis in neurodegeneration have suggested how pro-apoptotic agents, such as caspases, might be new targets for therapeutic approaches (Gibson, 2001). There are many existing treatments which act through apoptosis, such as non-steroidal anti-

inflammatory drugs and anticancer treatments (Chan et al., 1998; Lu et al., 1995; Pasricha et al., 1995; Piazza et al., 1995; Shiff et al., 1995; Shiff et al., 1996)

Apoptosis normally involves two stages:

1. ***Initiation*:** The first stage of apoptosis is the decision to activate the pathway by initiators. Initiators of apoptosis include deprivation of survival signals such as cytokines (Evans et al., 2003), growth factors (Gulli et al., 1996), hormones (Takebayashi et al., 1996); ligands for "death receptors" such as Fas and tumour necrosis factor receptors (Wallach et al., 1999), DNA damage (via p53) (Strasser et al., 1994), toxic agents (Schumacher et al., 2006), gamma (Kitada et al., 1996) and ultraviolet irradiation (Naik et al., 2007), anticancer drugs (Cummings et al., 2004) etc.
2. ***Activation of effector mechanisms:*** Through a variety of apoptotic pathways, these stimuli in turn generate a characteristic regulated pattern of gene expression also resulting into "suicide" of the cell (Williams and Smith, 1993). In general, caspases act as the central effectors or executioners of apoptosis but there are several molecular regulators of caspases and other caspase-independent effectors of apoptosis (Hail, Jr. et al., 2006). Through a variety of apoptotic pathways, these stimuli in turn generate a characteristic regulated pattern of gene expression also resulting into "suicide" of the cell (Williams and Smith, 1993).

5.1. **Caspases**

The execution of the death signal takes place through a common pathway that involves the activation of a series of proteases called caspases. The finding of Robert Horvitz and

colleagues in 1993 that the *ced-3* gene was required for the cell death that took place during the development of the nematode *C. elegans* (Yuan et al., 1993) became the basis for the importance of caspases in apoptosis. The discovery of caspases as having a role in regulation of apoptosis led to an explosion of interest in these enzymes, which has resulted in what is probably a fairly accurate view of the fundamental mechanisms of apoptosis employed to kill cells. Horvitz and his colleague Junying Yuan (Yuan et al., 1993) reported that the protein encoded by the *ced-3* gene was a cysteine protease with similar properties and homologous in sequence to the mammalian interleukin-1-beta converting enzyme (ICE) (now known as caspase-1), a protein released by certain immune cells during proteolytic activation of the precursor to interleukin-1 and which can promote inflammation. It was the only known caspase at that time but these publications initiated a successful search of mammalian ICE homologues that should govern cell death.

Table 3. Milestones in caspase research

Year	Discovery
1989	• Identification of pro- interleukin-1-beta processing activity (Black et al., 1989; Kostura et al., 1989)
1992	• Purification and cloning of caspase-1 (Cerretti et al., 1992; Thornberry et al., 1992) • First viral caspase inhibitor (CrmA) identified (Ray et al., 1992)
1993	• CED-3 found to be closely related to Caspase-1 (Yuan et al., 1993)
1994	• Solution of first caspase three dimensional structure (Walker et al., 1994; Wilson et al., 1994) • Identification of first mammalian caspase homologue (caspase-2) (Wang et al., 1994) • Identification of first apoptotic caspase substrate (PARP) (Lazebnik et al., 1994)
1995	• First caspase knockout mouse (caspase-1) (Kuida et al., 1995; Li et al., 1995) • Caspase-3 implicated as a central mediator of apoptosis (Nicholson et al., 1995b; Tewari et al., 1995) • Granzyme B found to activate caspase in CTL-mediated death (Darmon et al., 1995)
1996	• Caspase-3 knockout mouse described; demonstration that caspases are essential for mammalian apoptosis (Kuida et al., 1996) • Fas/FADD/Caspase-8 signaling pathway identified (Boldin et al., 1996; Muzio et al., 1996)
1997	• Apaf1/caspase-9 signaling pathway identified (Li et al., 1997) • Identification of first mammalian caspase inhibitor (XIAP) (Deveraux et al., 1997) • Role of caspases in DNA degradation elucidated (DFF/CAD-ICAD) (Enari et al., 1998; Liu et al., 1997) • Identification of first decoy caspase (Irmler et al., 1997; Shu et al., 1997)
1998	• Caspase mediated cleavage of Bid established as a link between FAS and mitochondrial signalling pathways (Li et al., 1998; Luo et al., 1998)
1999	• First identification of caspase mutation in a human genetic disease (caspase-10) (Wang et al., 1999)

Caspases are critical for apoptosis, inflammation, cytokine maturation and cell growth and differentiation (Denault and Salvesen, 2002; Denault and Salvesen, 2008; Wang and Lenardo, 2000). They constitute a family of intracellular cysteine proteases which are

found in the cell as inactive pro-forms or zymogens that must undergo limited proteolysis for activation (Wang and Lenardo, 2000). Caspases are among the most selective proteases, with a stringent specificity for cleavage after aspartic acid. Their catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide QACRG (Alnemri et al., 1996; Nicholson et al., 1995a). Hence, they are known as **caspases** (**c**ysteine-dependent **asp**artate-specific prote**ases**). In general, three amino acid residues that lie upstream of the aspartate residue in the substrate determine the specificity of recognition by individual caspases (Nicholson and Thornberry, 1997; Thornberry et al., 1997; Thornberry, 1997). So far, 7 different caspases have been identified in Drosophila, and 15 different members of the caspase-family have been described in mammals, which are named in the order of their discovery. All of them are expressed in humans except for caspase-11, which is solely found in mice (Denault and Salvesen, 2002; Denault and Salvesen, 2008; Richardson and Kumar, 2002), caspase-13, that is a bovine gene, and caspase-15 which is expressed in pig, dog and cattle.

Caspase-1 as well as caspases-4, -5, -11, and –12 appear to be mainly involved in the proteolytic maturation of pro-inflammatory cytokines such as pro-IL-1ß and pro-IL-18 and their contribution to the execution of apoptosis remains questionable (Denault and Salvesen, 2002; Denault and Salvesen, 2008). Caspase-3, -9, -8, and additionally caspases-2, -6, -7, and –10 have been recognized to play an important role in the apoptotic signalling machinery (Earnshaw et al., 1999). The proapoptotic caspases can be divided into the group of initiator caspases including procaspases-2, -8, -9 and –10, which are the first to be activated, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short

prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2. Each caspase contains a consensus sequence for an initiator caspase, so that they cleave each other in an activation cascade mechanism, similar to the coagulation protease cascade. Therefore, caspases are activated in an ordered cascade with active initiators caspases cleaving and activating the effector caspases, which in turn degrade different cellular proteins such as kinases, cytoskeletal proteins and DNA repair proteins that finally lead to destruction of "housekeeping" cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (Mancini et al., 1998; Thornberry and Lazebnik, 1998) and cell removal by phagocytosis.

Cell survival or death is determined by a balance between the pro-life (anti-apoptotic) and pro-suicide (apoptotic) molecular signals. Apoptosis--the regulated destruction of a cell--is a complicated process. The decision to die cannot be taken lightly, and the activities of many genes influence a cell's likelihood of activating its self-destruction programme (Hengartner, 2000). Normally the molecular machinery required for apoptosis lays dormant in the cell, and it just requires appropriate activation. Usually, molecular events in apoptosis follow two pathways in accordance with signals involved in activating apoptosis:

1. Extrinsic pathway
2. Intrinsic pathway

5.2. **Extrinsic pathway of apoptosis**

The extrinsic pathway of apoptosis is induced by extra cellular signals (Ghobrial et al., 2005). These signals are trimeric death ligands that may be integral membrane proteins on the surface of a second cell (e.g., Fas/CD95/Apo-1 ligand) or soluble extracellular proteins (e.g., tumor necrosis factor-α) (Ashkenazi and Dixit, 1998). Binding of death ligands (FasL/CD95L, TRAIL/Apo-2L, Apo-3L and TNF-α) induces trimerization of their corresponding death domain-containing receptors (CD95/Fas, DR4, DR5, Apo-3/DR3 and TNFR1) which then recruit adaptors such as FADD (Fas-associated death domain protein) and TRADD (TNFR-associated death domain protein) and activate caspases (Ashkenazi, 2002; Ashkenazi and Dixit, 1998; Debatin and Krammer, 2004; Fulda and Debatin, 2006; Rowinsky, 2005; Siegel et al., 2000; Zapata et al., 2001).

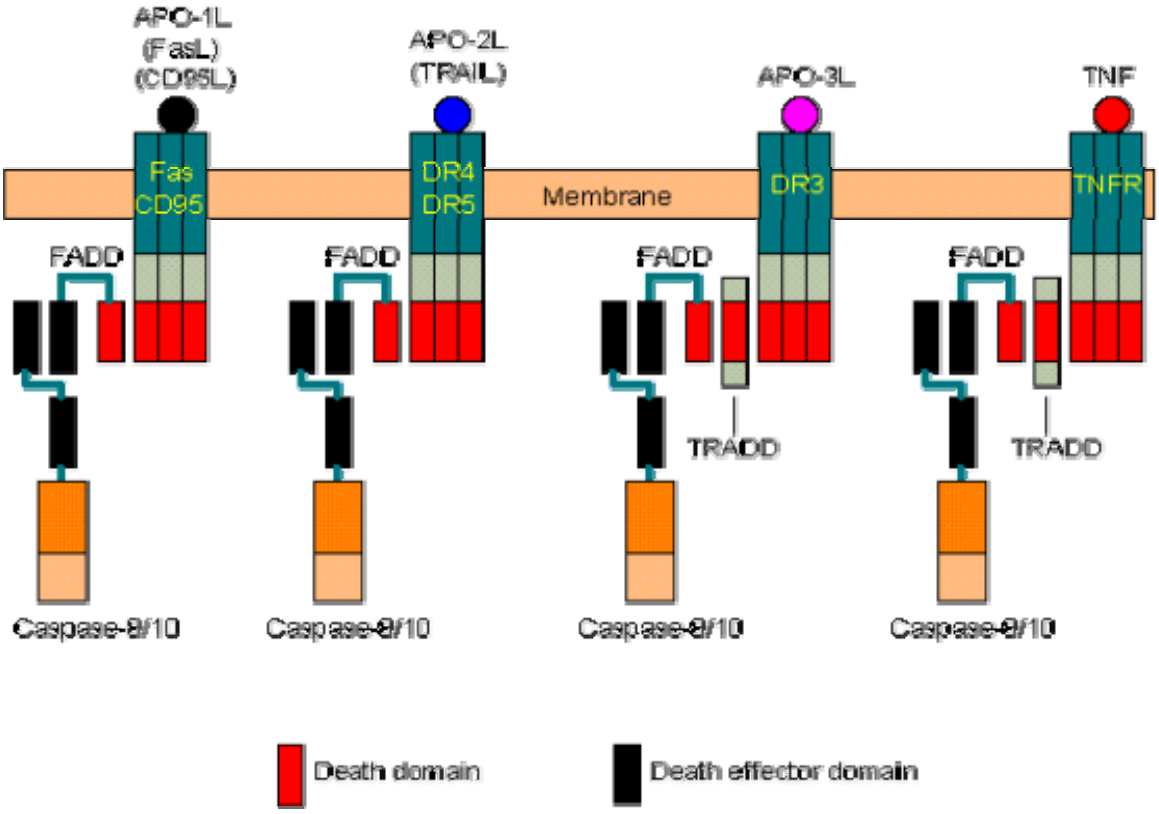

Figure 1. Coupling of ligands to their corresponding receptors and of caspase-8 or -10 to death receptors.

After binding of extracellular pro-apoptotic ligands, the intracellular domains of pro-apoptotic receptors, known as 'death domains', bind to the cytoplasmic adaptor proteins FADD and/or TRADD via homophilic interactions, which in turn interact with initiator caspases-8 and –10 through a death effector domain thus leading to the assembly of the death-inducing signaling complex (DISC) (Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995; Wang et al., 2001). TRADD is involved only in the coupling between caspases and DR3 or TNFR. When bound to the DISC, several procaspase-8 and -10 molecules are in close proximity and they are assumed to activate each other by autoproteolysis (Denault and Salvesen, 2002; Denault and Salvesen, 2008). This way, caspases-8 and -10 undergo self processing, releasing active enzyme molecules into the cytosol, where they activate effector caspases (Ashkenazi, 2002; Ghobrial et al., 2005; Thornberry and Lazebnik, 1998).

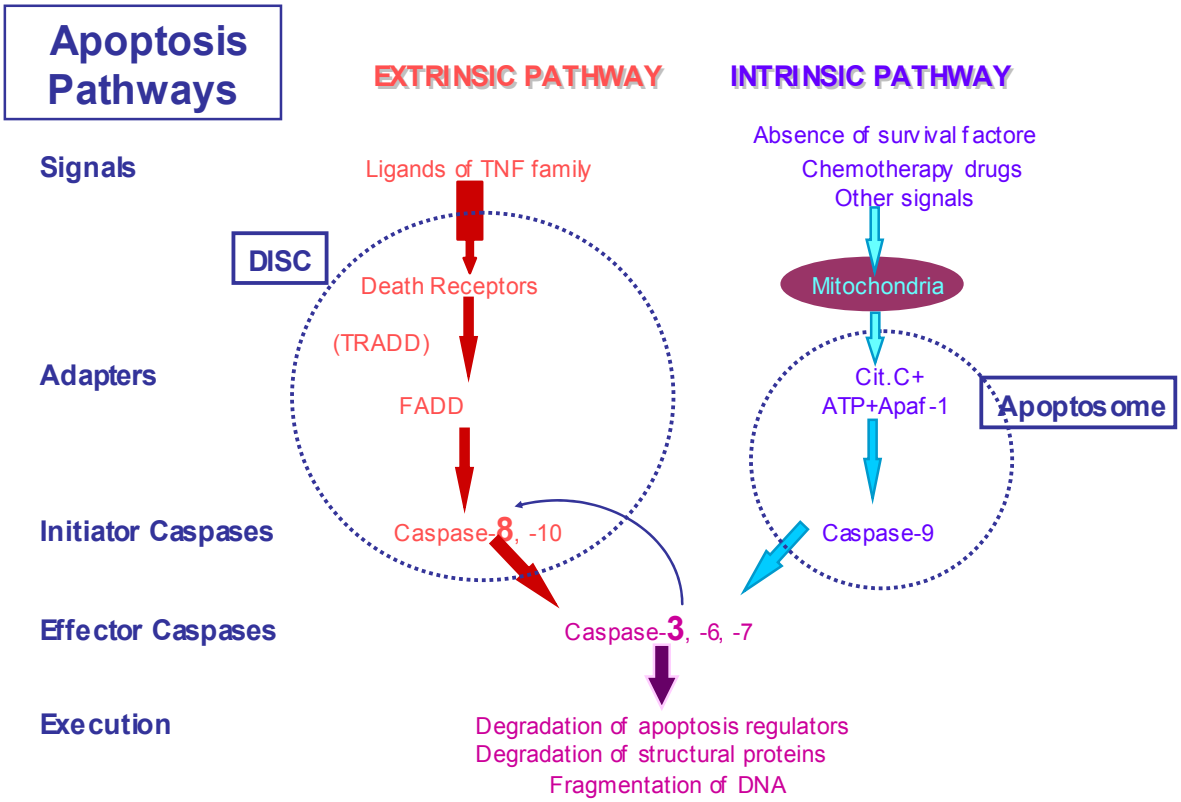

Figure 2. Apoptotic pathways: Extrinsic and Intrinsic. The induction of extrinsic pathway leads to the assembly of DISC. Self processing of initiator caspases-8 and –10 in DISC leads to release of active enzyme molecules into the cytosol to activate caspases -3, -6 and -7 which are also activated by the intrinsic pathway. The induction of intrinsic pathway leads to the destabilization of the mitochondrial membrane and release of mitochondrial apoptotic factors into the cytosol, which in turn cause apoptosome formation to activate caspase proteolytic cascade for leading to cell death ultimately.

5.3. **Intrinsic pathway of apoptosis**

The second pathway of apoptosis is the intrinsic or mitochondrial pathway. The intrinsic pathway was discovered in 1995 in the laboratory of Guido Kroemer (Hirsch et al., 1997; Kroemer et al., 1995; Marchetti et al., 1996; Zamzami et al., 1995b; Zamzami et al., 1995a; Zamzami et al., 1996a; Zamzami et al., 1996b) and it is initiated in response to DNA damage, hypoxia, loss of cell survival factors, binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, oxidative damage from free radicals or other types of severe cell stress that lead to the release of intracellular apoptotic signals resulting ultimately in cell suicide. The reception of stress signals leads to the destabilization of the mitochondrial membrane, activation of pro-apoptotic proteins of the Bcl-2 family and then release of mitochondrial apoptotic factors into the cytosol. Once released into the cytosol, these mitochondrial proteins activate both caspase-dependent and -independent cell death pathways. After release from damaged mitochondria into the cytosol, a mitochondrial apoptotic protein, cytochrome *c*, binds with Apaf1 and caspase-9 to form the apoptosome, which then activates the caspase proteolytic cascade, (Hockenbery et al., 1990). Other released mitochondrial proteins include the pro-apoptotic proteins Smac/DIABLO and Omi/HtrA-2, which are antagonists of the inhibitor of apoptosis proteins (IAPs), apoptosis-inducing factor (AIF) and endonuclease G, both of which contribute to apoptotic nuclear DNA damage in a caspase-independent way (Saelens et al., 2004).

5.3.1. **Mitochondria**

Mitochondria are important organelles of the cells with two membranes -smooth outer membrane and inner folded or shelved (cristae) membrane that provide surface for generating adenosine triphosphate (ATP)-; different shapes varying from rod-shaped to round depending on the cell type; and that occur in different numbers in correlation with the cell's level of metabolic activity as mitochondria provide the energy that a cell needs to move, divide, produce secretory products, contract etc. Therefore, they are considered the power generators of the cell, converting oxygen and nutrients into ATP. At one hand, mitochondria play very important role in aerobic respiration for generating the energy for cell survival, but on the other hand, they have critical role in suicide of the cell also because change in the structure and function of mitochondria deeply affect the surviving machinery thus leading to apoptosis. Therefore, researchers have focused their attention on the mitochondria for exploring the biochemical changes of mitochondria during apoptosis.

Basic knowledge of apoptosis regulators provides the basis for novel therapeutic strategies aimed at promoting cell death in a number of pathological disorders including cancer or enhancing susceptibility to apoptotic inducers (Fernandez-Luna, 2007). Thus, if mitochondria are pivotal in controlling cell life and death, understanding the changes in regulation of biochemical events in mitochondria are very important for making novel therapeutic strategies. In general, these mechanisms are already known, and their effects may be interrelated:

1. Disruption of oxidative phosphorylation and electron transport, caused by irradiation and certain second messengers such as ceramide, which lead to disruption of the production of adenosine triphosphate (ATP).

2. Disruption of mitochondrial transmembrane reduction-oxidation (redox) potential ($\Delta\Psi m$) after exposure to different types of apoptosis-inducing stimuli (Marchetti et al., 1996; Zamzami et al., 1995a) that may lead to the generation of reactive oxygen species (**ROS**).
3. Damage of mitochondrial and nuclear DNA by oxidants (Ames, 1989) or radiation (UV, X-rays, gamma irradiation) (Takai et al., 2006).
4. Increases in intracellular calcium ions through signal transduction.
5. Release of proteins from intermembrane space that trigger activation of the caspase family Membrane permeabilization leads to the release of apoptogenic proteins: cytochrome *c*, apoptosis-inducing factor (AIF) (Susin et al., 1999), Smac/Diablo (Verhagen et al., 2000), HtrA2/Omi (Verhagen et al., 2002), and endonuclease G (Li et al., 2001).

Mitochondrial death decision is centred on various processes. At a first level, numerous physiological and some pathological stimuli trigger an increase in mitochondrial membrane permeability: inner membrane permeabilization, such as that promoted by the mitochondrial permeability transition pore formed across inner membranes when Ca2+ reaches a critical threshold; and mitochondrial outer membrane permeabilization, in which the pro-apoptotic proteins Bax, and Bak play active roles (Belizario et al., 2007) in the molecular composition of membrane pores leading to permeabilization (see below). It has been reported that the mitochondrial permeability transition (MPT) pore - a dynamic multiprotein complex that spans both the outer and inner mitochondrial membranes – may be involved in the apoptotic events. It has been found to minimally consist of:

- An outer membrane protein known as VDAC (voltage-dependent anion channel).
- Adenine nucleotide translocator (ANT), a mitochondrial inner membrane protein involved in the ADP/ATP exchange.
- Several auxiliary proteins (e.g. matrix cyclophilin D).

In mammalian apoptosis, the MPT pore can mediate mitochondrial outer membrane permeabilization (MOMP), which is suspected to be responsible for the release of apoptogenic factors, including cytochrome *c* (Pereira et al., 2007). Most of research reports consider that the MPT complex can function as a sensor for stress and damage, probably at sites where the inner and outer membranes are in contact, as well as for certain signals connected to receptors. Other transporting pores and channels, including the ceramide channel as well as a non-specific outer membrane rupture may also be potential pathways for the release of factors through the outer membrane and to dissipate the electrochemical gradient of the inner membrane. However, the structure of MPT pore and its involvement in apoptosis are still highly controversial (Lawen, 2007) because many researchers believe that opening of MPT pore would lead to matrix swelling, subsequent rupture of the outer membrane, and an unspecific release of intermembrane proteins into the cytosol (Kinnally and Antonsson, 2007), while many others believe that MPT complex opening is a consequence of apoptosis and this channel play a principal role in necrosis, not apoptosis (Kinnally and Antonsson, 2007). In agreement with a critical role for MPT in apoptosis, it has been demonstrated that inhibition of transition pore by pharmacological intervention on mitochondrial structures or mitochondrial expression of the apoptosis-inhibitory oncoprotein Bcl-2 (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985) or Bcl-x_L (Boise et

al., 1993) prevents cell death. Moreover, bioenergetic catastrophe within the cells starts due to collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, and outflow of matrix calcium and glutathione.

Mitochondrial cytochrome *c* (Liu et al., 1996; Yang et al., 1997) is a heme protein localized in the mitochondrial intermembrane space and is part of the mitochondrial electron transport chain. It acts as a water soluble mobile carrier of electrons in mitochondrial oxidative phosphorylation, shuttling electrons through cytochrome *c* oxidase or complex IV. Release of cytochrome *c* from mitochondria results into inhibition of electron transfer in mitochondrial electron transport chain. That causes the accumulation of reducing equivalents in the middle portion of the electron transfer chain, and direct one-electron transfer to O_2 to produce superoxide (Turrens, 1997). Thus production of ROS, and the resulting cellular redox change, can be part of the mitochondrial signal transduction pathway during apoptosis. Yet, the mechanisms of ROS generation, and its relationship with the well studied caspase activation pathways, have not been resolved. This way, the fact that mitochondrial events control cell death has major implications for the development of cytoprotective and cytotoxic drugs.

5.3.2. **The Bcl-2 family**

The Bcl-2 family of proteins comprises well characterized regulators of apoptosis that act upstream of caspase activation in the intrinsic pathway (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000). It consists of the following three distinct subfamilies: 1) anti-apoptotic members, such as Bcl-2 and Bcl-x_L, with sequence homology at Bcl-2

homology 1 (BH1), BH2, and BH3 domains, and in most cases BH4 domain as well; 2) pro-apoptotic members, such as Bax and Bak, with sequence homology at BH1, BH2, and BH3; and 3) pro-apoptotic proteins that only share homology at the BH3 domain (BH3-only proteins), such as Bid, Bik, and Bim (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000). It has been shown that, in addition to BH1 and BH2, the BH4 domain is required for anti-apoptotic activity of Bcl-2 and Bcl-x_L and that the BH3 domain of the pro-apoptotic members is essential and, itself, sufficient for pro-apoptotic activity (Adams and Cory, 1998; Green and Reed, 1998; Tsujimoto and Shimizu, 2000).

As it has been previously mentioned, both Bcl-2/Bcl-x_L and Bax/Bak interact with VDAC to change the membrane permeability (Shimizu et al., 2000). The anti-apoptotic Bcl-2 and Bcl-x_L close the VDAC channel. In addition to the prevention of release of mitochondrial apoptogenic factors to the cytoplasm, Bcl-2/Bcl-x_L have also been shown to inhibit caspase-9 activation via forming complexes, comprising Bcl-2/Bcl-x_L-Apaf-1-procaspase-9 (Pan et al., 1998). The pro-apoptotic Bax and Bak proteins undergo conformational changes upon commitment to apoptosis and they can contribute to open the VDAC channel for the release of cytochrome *c*. However, the most likely mechanism for MOMP is the formation of a Bax/Bak-lipid pore with no further requirement for proteins in the inner or in the outer mitochondrial membranes(Martinou and Green, 2001). Although a Bax/Bak-independent, serine protease(s)-dependent, mechanism has been recently described for the released of cytochrome *c* in response to certain stimuli (Mizuta et al., 2007), most authors agree that MOMP is a process entirely controlled by the Bcl-2 family of proteins and their regulators. The anti-apoptotic Bcl-2 family members bind and sequester Bax and Bak inhibiting their activation. In contrast, the BH3-only proteins act upstream of Bax and Bak activating them in two different

ways. Some BH3-only proteins may directly bind to Bax and Bak to promote their activation while others bind to their pro-survival relatives, such as bcl-2 and Bcl-x_L, thereby preventing them from inhibiting Bax and Bak (Adams and Cory, 1998; Green and Reed, 1998; Tsujimoto and Shimizu, 2000).

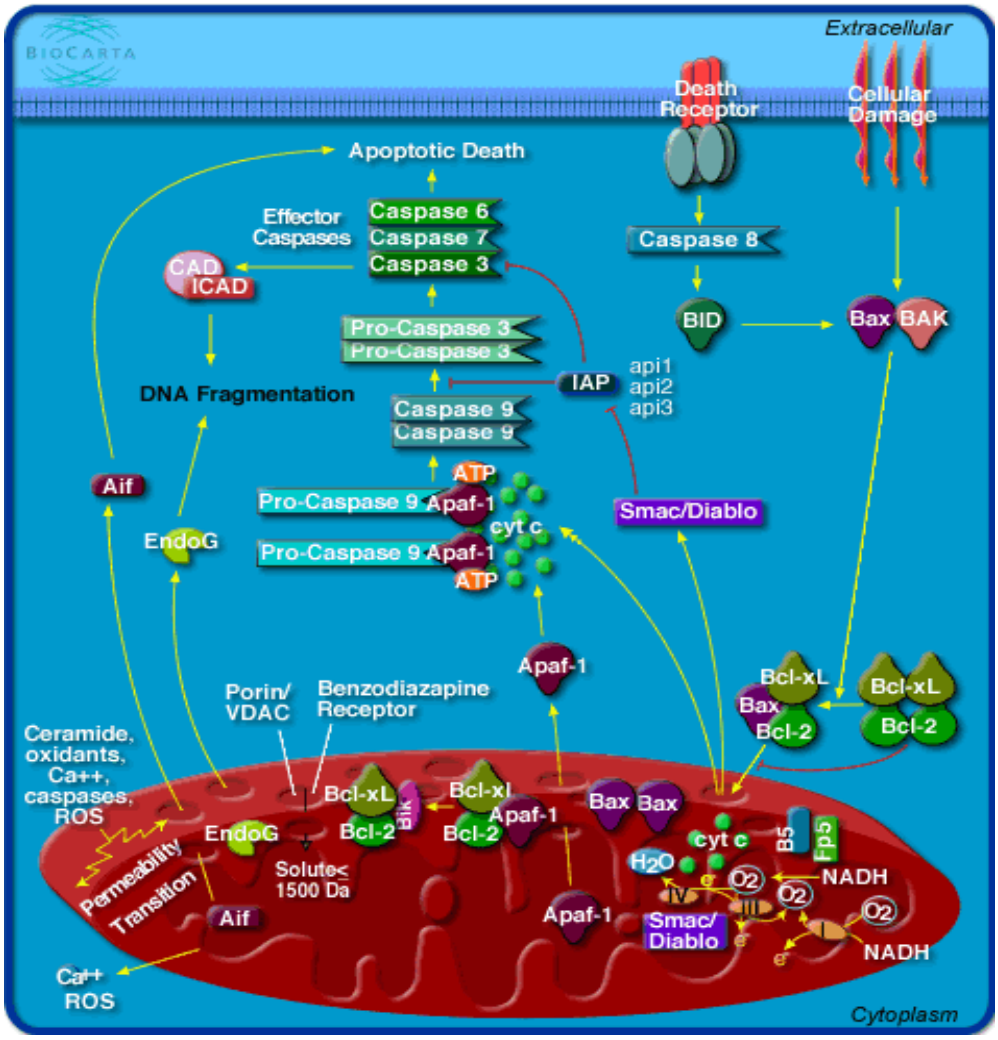

Figure 3.- Role of Mitochondria in cell death. Macauley, J. Taken from http://www.biocarta.com/pathfiles/h_mitochondriaPathway.asp

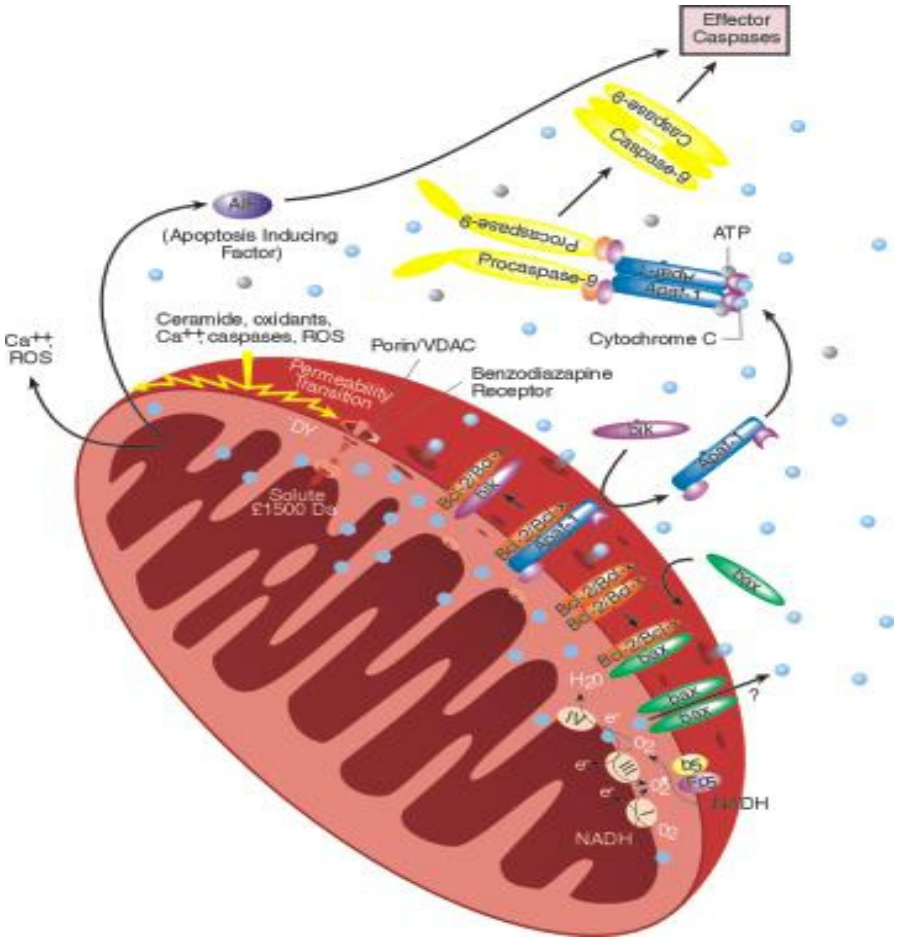

Figure 4. Mitochondrial Apoptotic Pathway. The induction of intrinsic pathway leads to the alteration in mitochondrial transmembrane permeability and reduction-oxidation (redox) potential. Changes in transmembrane permeability result into the release of cytochrome *c* which binds Apaf-1, caspase-9 and ATP for formation of apoptosome. Alteration of transmembrane redox potential results into production of ROS.
Figure taken from http://www.rndsystems.com/DAM_public/5218.jpg

6. **Cross-talk between Apoptotic Pathways**

The existence of at least two well distinguishable cell death–signalling pathways (extrinsic and intrinsic) and the identification of the major molecular components of these pathways provide a framework for predicting the circumstances under which each one becomes engaged to initiate apoptosis. In recent years, it has become clear that there is a cross talk between the extrinsic and intrinsic pathways, but controversy surrounds

the degree to which cross-talk occurs between these two pathways *in vivo*. At a molecular level, this appears to occur via the proteolysis of Bid, a BH3 domain-containing protein known to interact with both Bcl-2 and Bax. When Bid is truncated by caspase-8 (derived from the extrinsic pathway), the COOH-terminal part (tBid) translocates to mitochondria where it triggers cytochrome *c* release leading to activation of the intrinsic pathway (Luo et al., 1998). Hence, the identification of Bid as the 'go-between,' transmitting signals from the extrinsic to the intrinsic pathway, provides a molecular basis for the cross-talk between the two pathways (Roy and Nicholson, 2000). The balance between proapoptotic factors (Bid, Bad, Bim, Bax, Bak) and antiapoptotic factors (Bcl-2, Bcl-xl, Bcl-w, Mcl-1) determines cytochrome *c* release and the fate of the cell (Liou et al., 2003). This balance depends not only on the respective levels of expression of these factors but also on their post-translational modifications and interactions (Liou et al., 2003). In principle, signals from the extrinsic pathway may require the assistance of the intrinsic pathway, i.e., when the signal strength is weak. Some cell types produce insufficient amounts of active caspase-8 at the receptor level, therefore, the induction of apoptosis via the extrinsic pathway requires additional cleavage of the proapoptotic Bcl-2 family member Bid through caspase-8, which activates the intrinsic pathway and subsequently apoptosis (type II cell-mechanism) (Barnhart et al., 2003; Luo et al., 1998).

Objectives

The biological importance of bacterial EPS has been extensively reviewed in the introduction of this work, being evident that these peculiar molecules have an extraordinary potential for the development of new applications both in biotechnology and drug development. Some sulphated EPS modulate the proliferation of different cell lines, and the presence of sulphate groups in EPS may critically influence the biological activity of the molecule. Consistent with this observation, preliminary studies carried out in our group have shown that hyperoversulphated forms of EPS of *Halomonas maura* (B100 and N12) and EPS of *Halomonas anticariensis* (FP34) cause a profound inhibition of cell proliferation in certain haematopoietic tumor cell lines (unpublished data). Although it has been demonstrated that this anti-proliferative effect is linked to the presence of sulphate groups in EPS B100, N12 and FP34, the mechanisms behind this activity and the significance of the sulphate groups are still undiscovered.

In this work, we have investigated the immunological importance of EPS of halophilic bacteria *Halomonas maura* and *Halomonas anticariensis*. The specific aims of this research work were:

1. To study if the anti-proliferative effect of the above indicated EPS is due to inhibition of cell division, the induction of necrosis or the induction of apoptosis.
2. To identify the characteristics of the apoptosis induced by EPS, the significance of the sulphate groups in the induction of apoptosis and the degree of susceptibility of cells from different lineages.
3. To study the effect of EPS on primary cells.
4. To dissect the molecular and biochemical pathways leading to the induction of apoptosis by EPS, including their intracellular mediator and effector molecules.

Materials and Methods

1. Reagents and antibodies

Native and oversulphated forms of exopolysaccharides B100, N12 and FP34 were provided by Dr. Emilia Quesada from the Department of Microbiology, Faculty of Pharmacy, University of Granada, and stored at room temperature. A stock of 5 mg/ml of each exopolysaccharide was prepared in sterile PBS and stored at -20 ºC. From this stock, dilutions of 0.5 mg/ml, 0.05 mg/ml and 0.005 mg/ml were used for the experiments. Native EPS were oversulphated according to the fucan sulfation method previously described (Nishino and Nagumo, 1992). Briefly, 0.5 g of EPS were dissolved in 50 ml of dimethyl formamide and stirred for 2 hours at room temperature, followed by an additional incubation of 2 hours at 45ºC. A complex of pyridine-SO_3 was added dropwise in excess and incubated again for 2 hours at 45ºC. The reaction was stopped by adding 20 ml of water, the pH adjusted to 9.0 and dialyzed against water. The sample was finally subject to lyophilization. Native EPS were alternatively desulphated by a salvolysis process in dimethyl sulfoxide (Haroun-Bouhedja et al., 2000).

Human recombinant TRAIL was prepared as described previously (MacFarlane et al., 1997). General caspase inhibitor Z-VAD-FMK, caspase-9 inhibitor Z-LEHD-FMK and monoclonal anti-human caspase-9 antibody were provided by R&D system (Minneapolis, MN). Anti-human caspase-8 monoclonal antibody was purchased from Alexis Biochemicals (San Diego, CA). Anti-human caspase-3 polyclonal antibody was purchased from Stressgen bioreagents corp. (Victoria, CA). Mouse anti-human CD28 antibody was from eBioscience (San Diego, CA). The fluorescent cationic lipophilic dye 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was obtained from Molecular

Probes/Invitrogen (USA). Phytohemaglutinin (PHA), oxidation-sensitive fluorescent probe 5, 6-carboxy-2', 7'-dichlorofluorescein-diacetate (DCFH-DA) and N-acetyl cystein (NAC) were purchased from Sigma Aldrich (St. Louis, MO).

2. Cells and cell culture

Following informed consent, blood samples were obtained from healthy donors and leukaemia patients of the Servicio de Análisis Clínicos, Hospital Virgen de las Nieves and Centro Regional de Transfusión Sanguínea, Granada, and collected into citrate tubes. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Histopaque (Sigma) density gradient centrifugation and adherent monocytes were depleted by culture on plastic dishes for 1 h at 37 ºC. Resting T lymphocytes were resuspended in RPMI 1640 medium (BioWhittaker Inc.) containing 10% fetal bovine serum (FBS) (Gibco-BRL, Middlesex, UK), 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Hoffman-LaRoche, Nutley, NJ, USA) and incubated at 37 ºC in a humidified 5% CO_2, 95% air incubator. For activation, resting T cells were cultured at 2×10^6 cells/ml with 5 µg/ml PHA and 1 µg/ml anti-CD28 for 20 h. After washing, cells were incubated in complete medium supplemented with 25 U/ml IL-2 (kindly supplied by the National Institutes of Health AIDS reagent program, Rockville, MD, USA) for an additional 5 or 9 days.

The cell lines Jurkat, CEM, MOLT-4 and HPB-ALL (derived from acute lymphoblastic leukemia), Raji, Daudi and Namalwa (from Burkitt's lymphoma), HL-60 (from human promyelocytic leukemia), K562 and HEL (from human erythroleukemia), U937 (from human promonocytic leukemia), SAM31 (Epstein-Barr virus transformed human B cells), ITA (Herpesvirus Saimiri-immortalized human T cells) and SKBr3, EVSA-T and

MCF-7 (derived from a human breast carcinoma), were all maintained in culture in RPMI 1640 medium containing 10% FBS, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37 ºC in a humidified 5% CO_2, 95% air incubator, with the exception of Namalwa cells that were maintained with 5% FBS. BW5147 cells (mouse thymoma) were incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS, 2-mercaptoethanol (0.5 μM), penicillin and streptomycin at 37ºC in a humidified 5% CO_2, 95% air incubator. DSC cells (human decidual stromal cells) were maintained in Opti-MEM (Invitrogen, Paisley, UK) with 100 U/ml penicillin, 50 μg/ml gentamicin and 3% FBS. For all adherent cell lines, 10% trypsin was used for collecting cells and maintaining cell culture.

HPB-ALL, Namalwa and Daudi cell lines were kindly provided by Dr. Jaime Sancho (Instituto de Parasitología y Biomedicina, Granada, Spain). EVSA-T and MCF-7 cell lines were provided by Dr. Mariano Ruiz de Almodóvar (Facultad de Medicina, Universidad de Granada, Spain). DSC cells were donated by Dr. Enrique García Olivares (Facultad de Medicina, Universidad de Granada, Spain). SKBR3 cells were provided by Dr. Abelardo López Rivas (CABIMER, Sevilla, Spain). Jurkat cells overexpressing human Bcl-x_L protein were kindly donated by Dr. Jacint Boix (Departamento de Ciencias Médicas Básicas, Universidad de Lleida, Spain) and maintained as parental Jurkat cells.

3. Analysis of cell cycle and determination of apoptotic cells

Cells (200.000 to 500.000 cells/well depending on cell type) were cultured in 12-well cell culture plates for treatments. After the indicated times, cells were collected,

centrifuged and supernatants were discarded. The cells were washed with PBS, fixed with 70% cold ethanol for 5 min at 4 ºC and then incubated with DNA extraction buffer (0.2 M Na_2HPO_4 and 0.004 M citric acid) at 37 ºC for 10 min. Finally, cells were resuspended in 200 µl PBS containing 100 µg/ml RNase and 40 µg/ml propidium iodide and incubated at 37 ºC for 30 min in the dark. Quantitative analysis of cells with sub-G1 DNA content was carried out in a FACScan cytometer using the Cell Quest software (BD Biosciences).

4. Immunoblot detection of proteins

For detection of cytosolic proteins, cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl pH 8.0, 1% NP-40 and protease inhibitors for 30 min. Samples were then centrifuged for 10 min at 13000 rpm, supernatants were collected and protein levels were determined by the Bradford colorimetric assay (Sigma). After adding 6x loading buffer (0.1% bromophenol blue, 60% glycerol, 30% β-mercaptoethanol, 12% SDS, 375 mM Tris-Cl pH 6.8) to each sample, proteins of cytosolic supernatants were resolved on SDS-PAGE minigels in reducing conditions and electrotransferred to Hybond-P polyvinylidene difluoride membrane (PVDF) (Amersham, Buckinghamshire, UK) by the semi-dry transference system Trans-Blot SD (BioRad) at a constant current (50 mA) for 1 h. Membranes were blocked with 5% non-fat milk in PBS 0.1% Tween 20 (PBS/Tween) for 1 h, washed with PBS/Tween and incubated for either 1 h at room temperature or overnight at 4 ºC with anti-caspase-9, anti-caspase-8 or anti-caspase-3 antibodies at 1µg/ml, 1:500 and 1:2000 dilutions, respectively, in PBS-Tween containing 1% non-fat milk. After washing 3 times for 5 min with PBS-Tween, membranes were incubated with the appropiate horseradish

peroxidase (HRP)-labelled goat anti-mouse or anti-rabbit antibody (1:10,000 dilution) for 1 h at room temperature and washed again. The blots were developed by enhanced chemiluminescence (ECL, Amersham) and exposed to film for autoradiography (Amersham). Loading controls were carried out by rehybridization of stripped membranes with an anti-human β-actin monoclonal antibody (Sigma).

5. Flow cytometric analysis of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was measured by using $DiOC_6$ dye because of its high capacity to bind mitochondrial membranes. Fluorescence of $DiOC_6$ is oxidation-independent and correlates with mitochondrial transmembrane potential. Briefly, cells (200.000 cells/well) were treated as indicated, collected and washed with PBS. Supernatants were then removed and cells were resuspended in 250 μl of PBS. After adding 40 nM of $DIOC_6$ to each sample, they were incubated for 15 min in dark at room temperature. $DiOC_6$ green fluorescence was measured by using the FL1 channel in FACScan cytometer and data were analyzed by using Cell Quest software.

6. Flow cytometric analysis of ROS production

Intracellular ROS levels were measured by flow cytometry in cells loaded with the redox-sensitive dye DCFH-DA (5, 6-carboxy-2,7-dichlorofluorescein-diacetate). The nonfluorescent DCFH-DA readily diffuses into the cells, where it is hydrolysed to the polar derivative DCFH. Then, it is oxidized in the presence of free radicals to the highly fluorescent DCF, which is trapped inside the cell because of its poor permeability.

After stimulation, 250.000 cells/well were incubated in the dark for 30 min at 37 ºC with 10 μM DCFH-DA, washed and resuspended in PBS. Fluorescence of DCF was recorded on the FL-1 channel of FACScan flow cytometer, and data were analyzed with the Cell Quest program.

Results

Oversulphated B100 EPS induce apoptosis in haematopoietic cell lines

Previous results demonstrated that sulphatated EPS B100, N12 and FP34 caused a dose-dependent inhibition of proliferation in Jurkat and CEM leukemic T-cell lines (Mata, J. A. et al, unpublished data). Whether this effect in cell proliferation was only due to inhibition of cell cycle or there was also an effect on cell viability had not been studied. Therefore, we started to investigate the effect of native and oversulphated forms of EPS B100, N12 and FP34 on the viaiblity of three different leukemic T-cell lines, Jurkat, MOLT-4 and CEM, as described in materials and methods. Cells were treated with 500, 50 and 5 μg/ml of oversulphated and native forms of the three EPS for 24 hours. Cells were subsequently stained with propidium iodide for determining the percentage of sub-G1 apoptotic cells by flow cytometry.

We found that the native form of EPS B100 had no apoptotic effect in any of the T cell lines studied, whereas its oversulphated form strongly induced apoptosis. Flow cytometry analysis showed that 70.22%, 50.69% and 16.85% of Jurkat cells were apoptotic after treatment with the three doses indicated above of the oversulphated EPS B100 (figure1); 82.22%, 77.40% and 74.53% of apoptosis was induced in MOLT-4 (figure 2) and 32.245%, 18.5% and 13.25% of apoptosis was induced in CEM (figure 3) upon treatment with doses of 500, 50 and 5 μg/ml of oversulphated B100, respectively. These results indicate that the induction of apoptosis by oversulphated B100 in leukemic T-cell lines is a dose-dependent and cell line-dependent effect. Of the three cell lines, MOLT-4 is the most sensitive to the induction of apoptosis by oversulphated form of B100 while CEM is the less sensitive cell line. There was no induction of apoptosis by any of the doses of native and oversulphated forms of N12 and FP34 in any

of the three T cell lines studied (figures 1, 2, 3 and data not shown). Thus, although a modest anti-proliferative effect is seen in these cells after treatment with native and oversulphated forms of B100, N12 and FP34, only oversulphated B100 has the ability to induce apoptosis in leukemic T-cell lines.

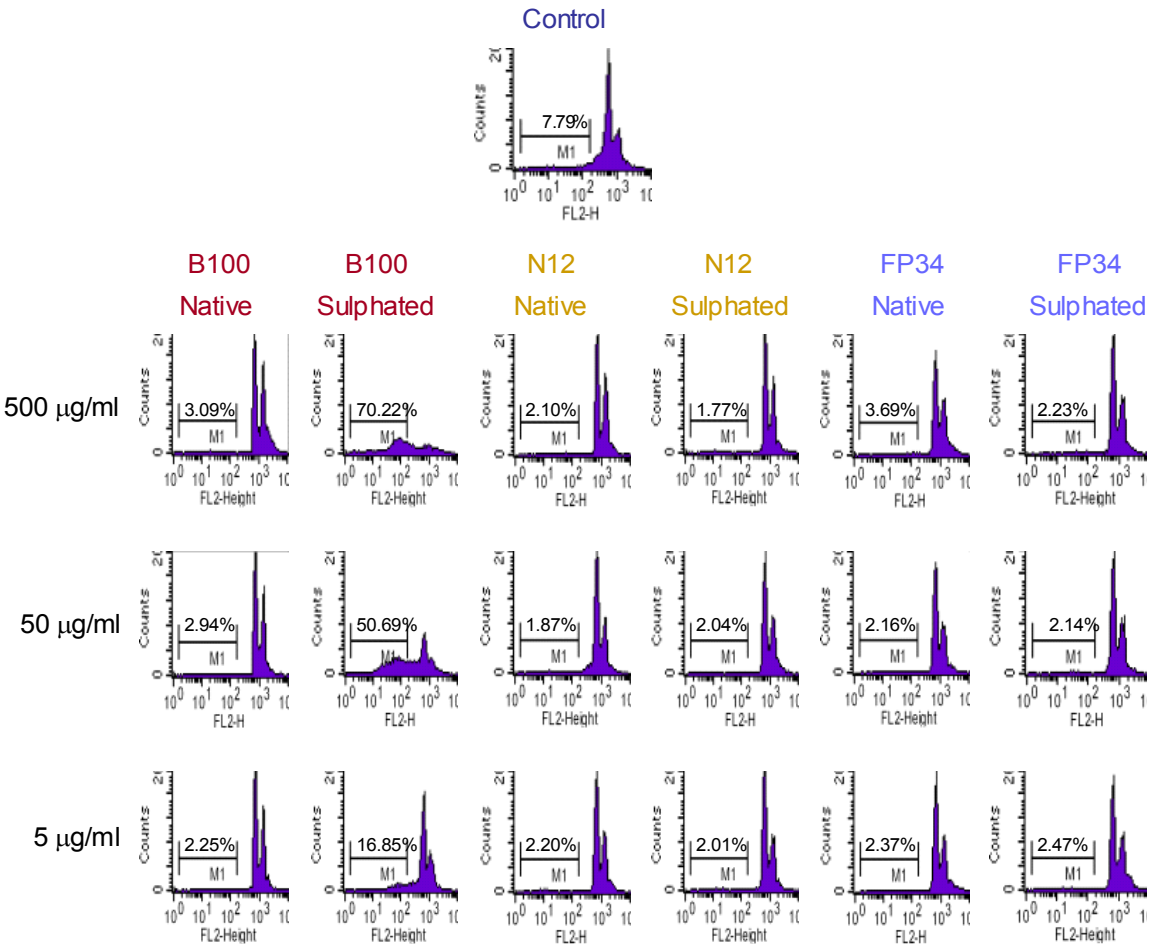

Figure 1. Induction of apoptosis in Jurkat cells by EPS. Jurkat cells (T cell leukemia) were treated for 24 h with different doses (500, 50 and 5 µg/ml) of native or oversulphated EPS B100, N12 and FP34. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. A representative experiment out of three is shown. Control cells were untreated.

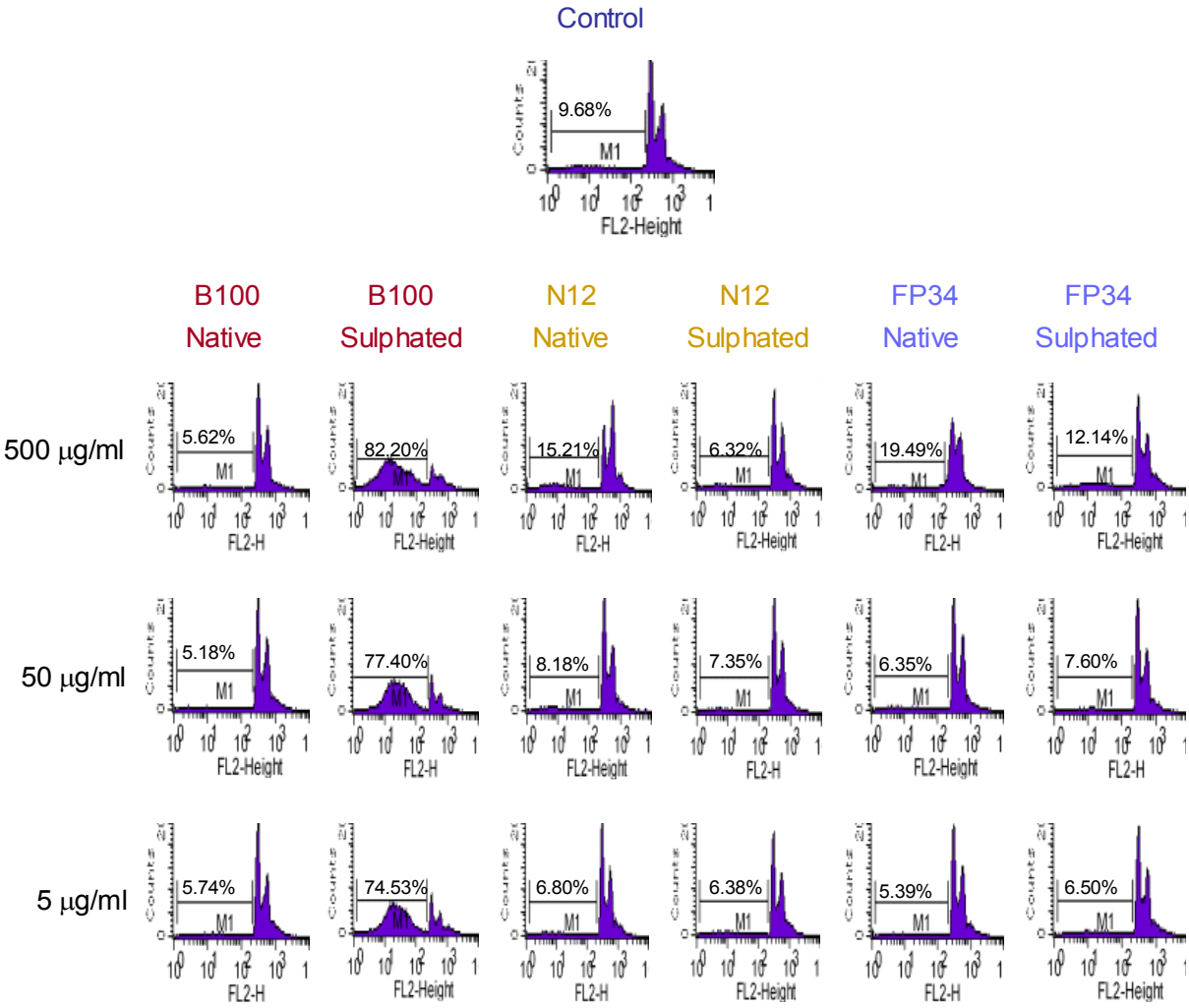

Figure 2. Induction of apoptosis in MOLT-4 cells by EPS. MOLT-4 (T cells leukemia) were treated for 24 h with different doses (500, 50 and 5 μg/ml) of native or oversulphated EPS B100, N12 and FP34. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. A representative experiment out of three is shown. Control cells were untreated.

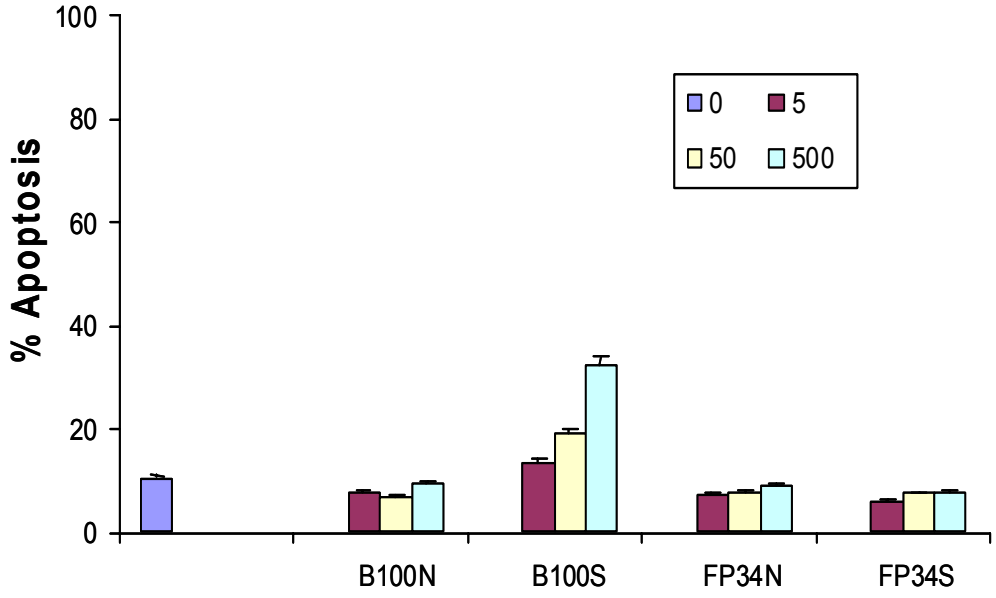

Figure 3. Induction of apoptosis in CEM cells by EPS. CEM cells were treated without or with different doses (500, 50 and 5 µg/ml) of native B100 (B100N), oversulphated B100 (B100S), native FP34 (FP34N) and oversulphated FP34 (FP34S) for 24 h. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Bars represent Mean ±SD from three independent experiments.

We analyzed the time-course of the induction of apoptosis by oversulphated EPS B100 in MOLT cells. The analysis of data showed 4.76%, 12.45%, 13.86% and 28.86% of apoptosis induced by oversulphated B100 upon 2, 4, 6 and 12 h of treatment, respectively (figure 4). Thus, the induction of apoptosis in MOLT-4 cells by oversulphated B100 starts at 4 hours, and at 12 hours it is highly significant.

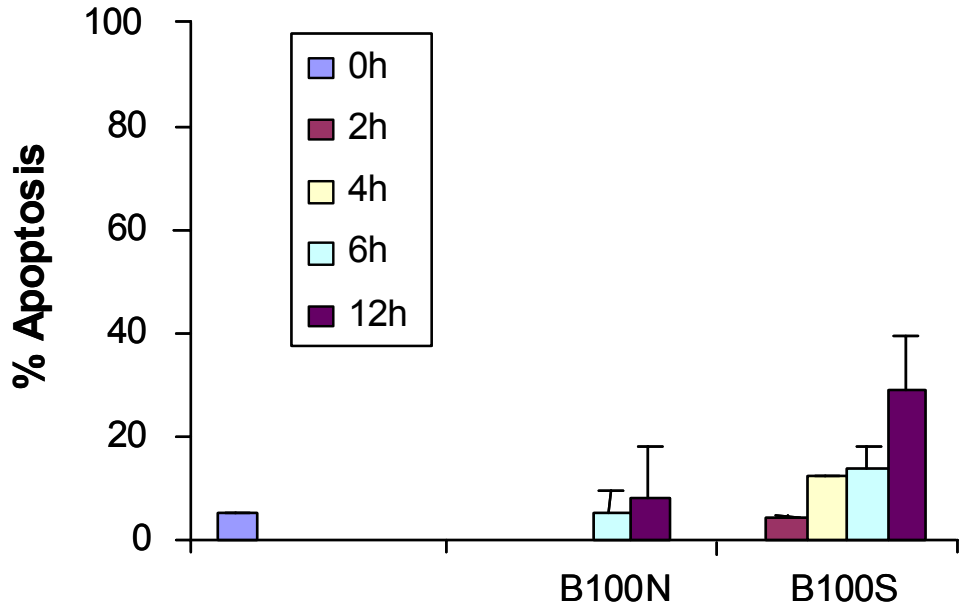

Figure 4. Time-course of the induction of apoptosis in MOLT-4 cells by oversulphated B100. MOLT-4 cells were incubated with or without 50 µg/ml of oversulphated B100 (B100S) or native B100 (B100N) for 2, 4, 6 and 12 h. Sub-G1 apoptotic cells were analyzed by flow cytometry. Bars represent Mean ±SD from three independent experiments.

In order to study whether the pro-apoptotic activity of oversulphated B100 is specific for leukemic T-cells, we analysed the sensitivity of other haematopoietic tumor cell lines. First, we determined the effect of the different native and oversulphated EPS in the human Burkitt´s lymphoma B-cell line Raji. It has been described that these cells are resistant to nuclear apoptosis induced by various stimuli, and neither mitochondrial activation nor activation of caspase-3 led to DNA fragmentation (Hirokawa et al., 2002).

Results shown in figure 5 indicate that there was no induction of apoptosis in Raji cells, neither by oversulphated nor by native forms of B100, N12 and FP34.

We then analyzed the effect of EPS in two other Burkitt´s lymphoma B cell lines, Namalwa and Daudi; the EBV-transformed SAM31 B cells; the promyelocytic leukaemia cell line HL-60; the promonocytic leukaemia cell line U937; the erythroleukemia cell lines, K562 and HEL; the herpesvirus saimiri immortalized T cells ITA; the T cell leukaemia cell line HPB-ALL; and the mouse thymoma cell line BW5147. After treating the cells for 24 hours with the oversulphated EPS B100 at the doses of 500, 50 and 5 μg/ml, the flow cytometry data analysis showed 33.58%, 43.5%, 24.78% of apoptosis in SAM31; 28.53%, 21.94% and 7.62% of apoptosis in HL-60; 30.95%, 12.77% and 5.28% of apoptosis in HPB-ALL; and 6.04%, 18.53% and 6.04% of apoptosis in K562 (figures 6, 7, 8 and 9). These results showed that oversulphated B100 induces moderate apoptosis in SAM31, HL-60, HPB-ALL and K562 cell lines, similar to that found in CEM cells. As previously observed, no apoptosis was detected in response to treatment with the native form of B100, N12 and FP34 or the oversulphated form of N12 and FP34 (figures 6, 7 and data not shown).

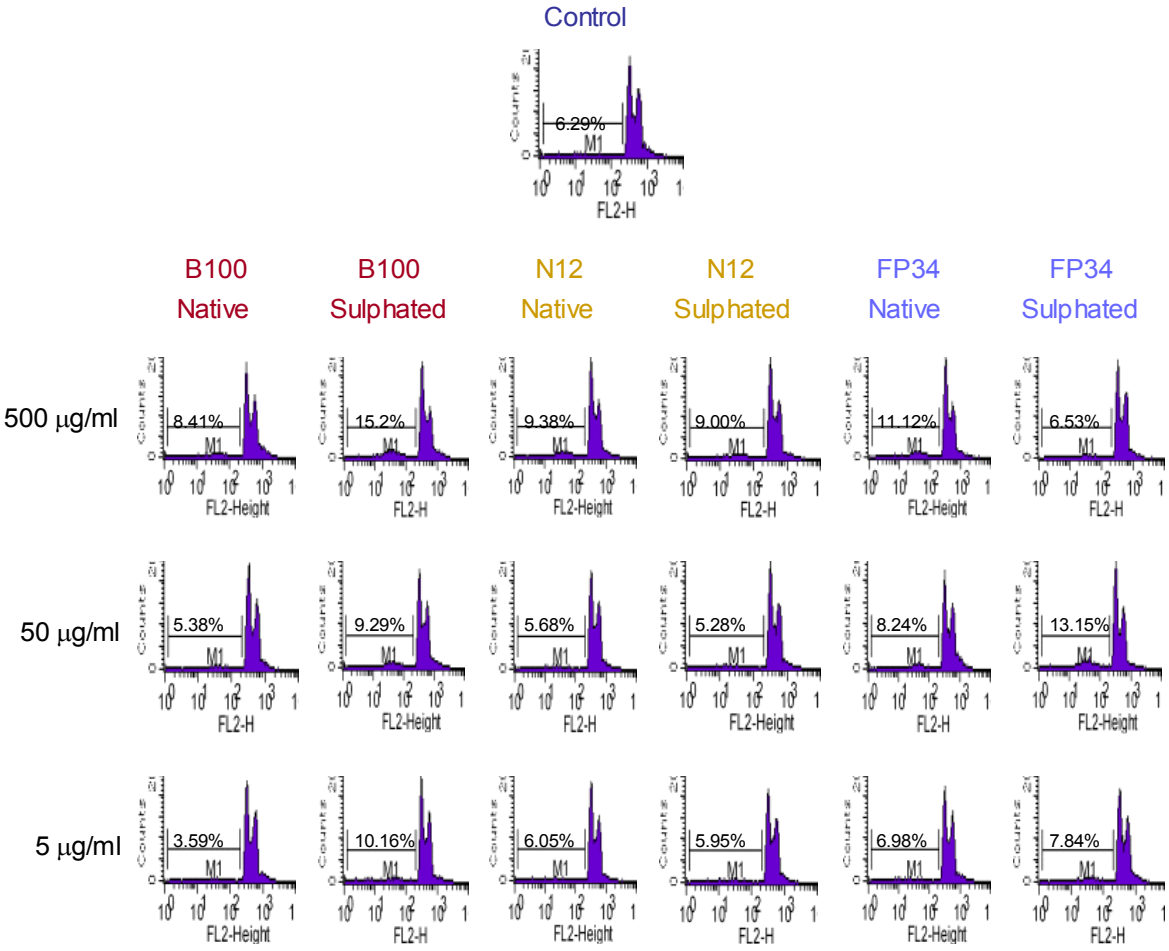

Figure 5. Induction of apoptosis in Raji cells by EPS. Raji cells were treated without or with different doses (500, 50 and 5 μg/ml) of native B100, oversulphated B100, native N12, oversulphated N12, native FP34 and oversulphated FP34 for 24 h. The percentage of sub-G1 apoptotic cells was analyzed by flow cytometry. Results of one representative experiment out of three are shown.

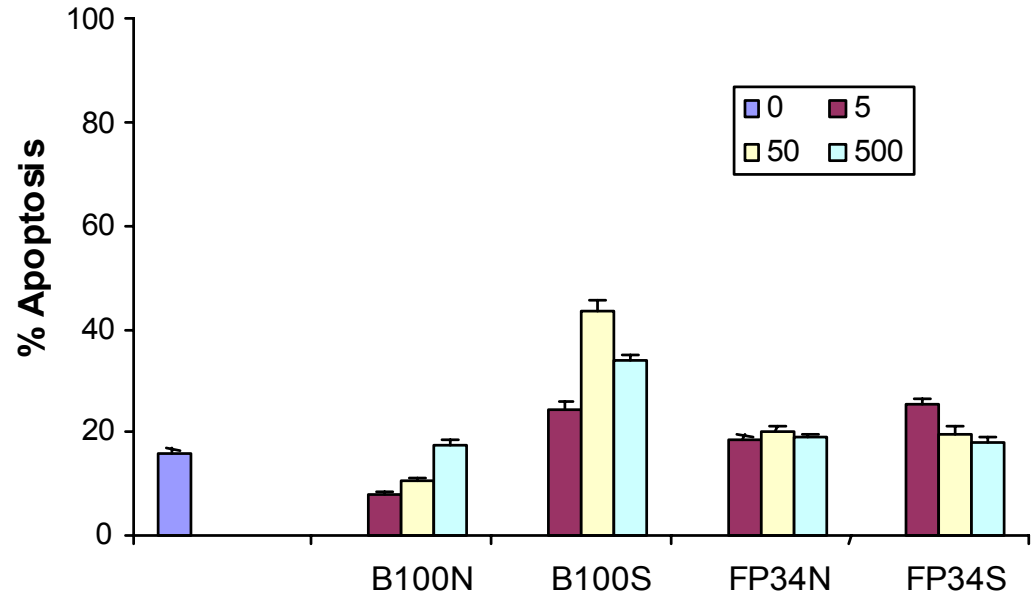

Figure 6. Induction of apoptosis in SAM31 cells by EPS. SAM31 cells were treated without or with different doses (500, 50 and 5 µg/ml) of native (N) or oversulphated (S) EPS B100 and FP34 for 24 h. Sub-G1 apoptotic cells were analyzed by flow cytometry. Bars represent Mean±SD from three independent experiments.

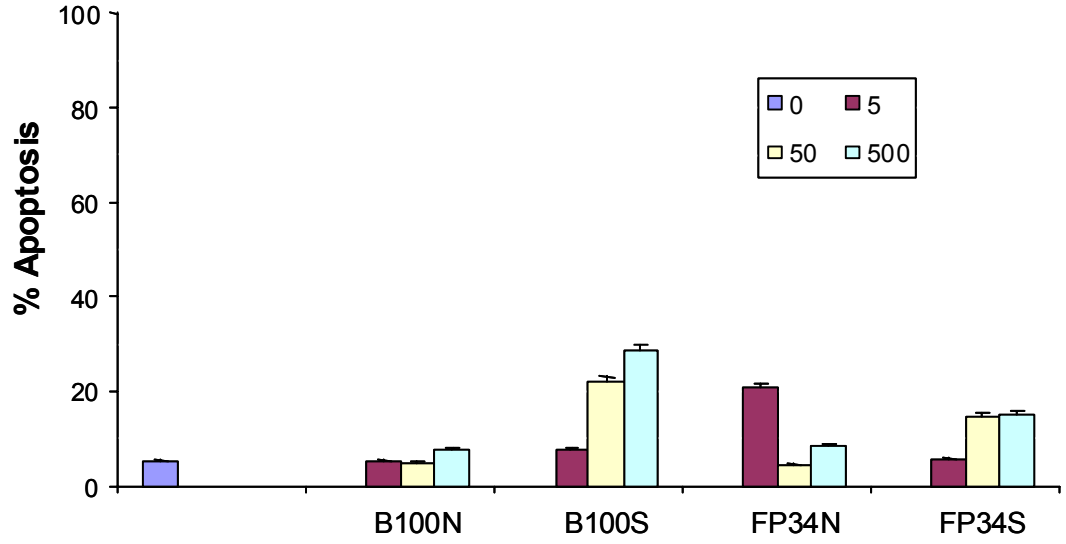

Figure 7. Induction of apoptosis in HL-60 cells by EPS. HL-60 cells were treated without or with different doses (500, 50 and 5 µg/ml) of native (N) or oversulphated (S) EPS B100 and FP34 for 24 h. Sub-G1 apoptotic cells were analyzed by flow cytometry. Bars represent Mean±SD from three independent experiments.

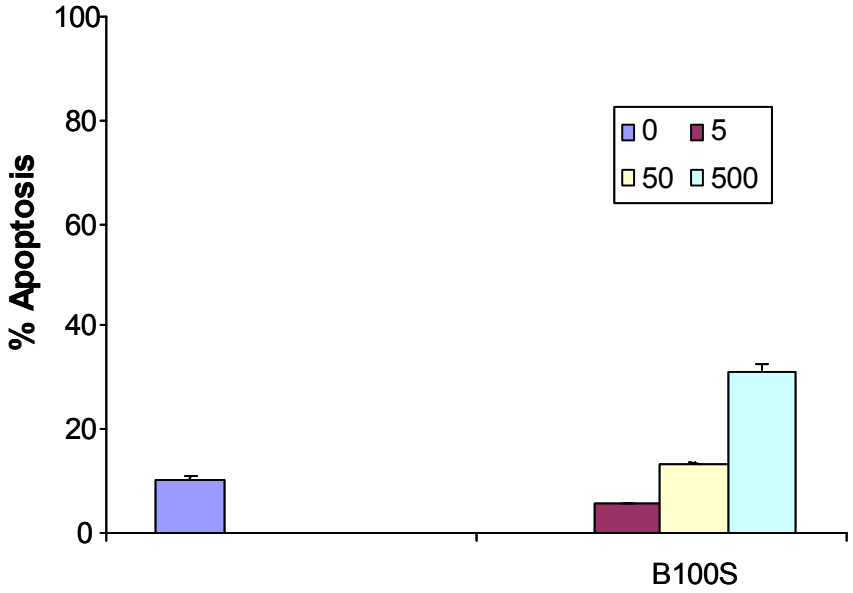

Figure 8. Induction of apoptosis in HPB-ALL cells by oversulphated B100. HPB-ALL cells were treated without or with different doses (500, 50 and 5 µg/ml) of oversulphated B100 (B100S) for 24 h. The percentage of sub-G1 apoptotic cells was analyzed by flow cytometry Bars represent Mean±SD from three independent experiments.

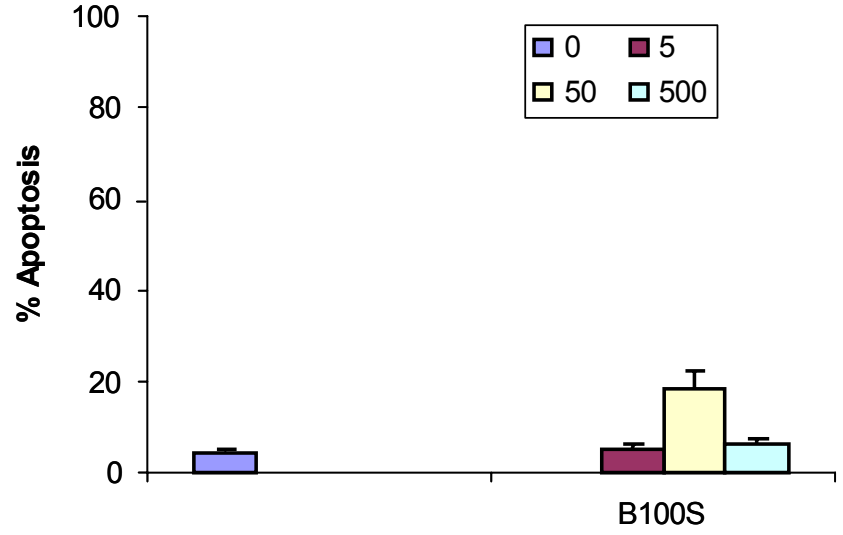

Figure 9. Induction of apoptosis in K562 cells by oversulphated B100. K562 cells were incubated without or with different doses (500, 50 and 5 µg/ml) of oversulphated B100 (B100S) for 24 h and the percentage of apoptotic cells was determined by flow cytometry analysis. Bars represent Mean±SD from three independent experiments.

In contrast to the cell lines described above, we observed no effect of oversulphated B100 on the viability of HEL, U937, ITA, BW5147, Daudi and Namalwa cells (figure

10). The oversulphated forms of N12 and FP34 and the native forms of the three EPS did not induce apoptosis in these cell lines either (data not shown).

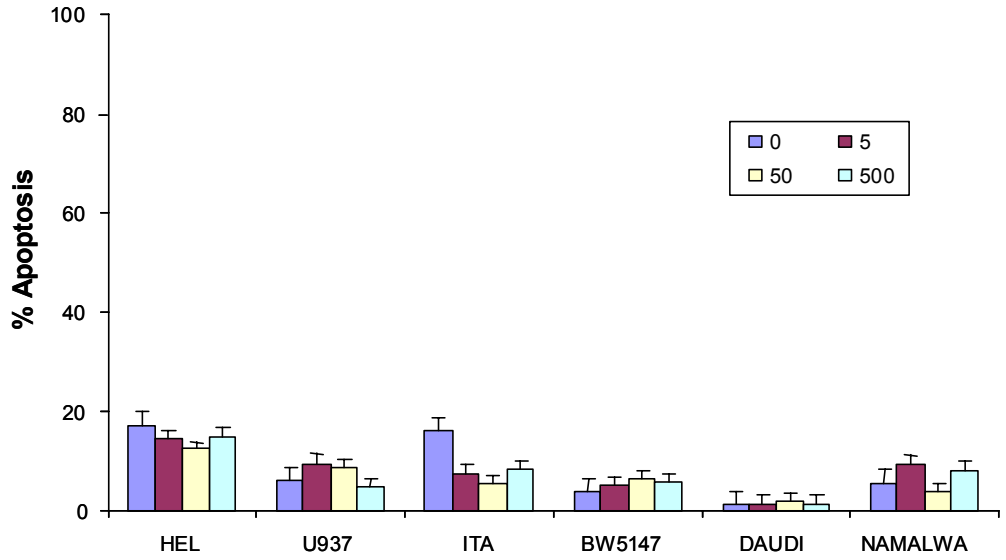

Figure 10. Induction of apoptosis in different haematopoietic cell lines by oversulphated B100. The indicated cell lines were treated with different doses (500, 50 and 5 µg/ml) of oversulphated B100 for 24 hours. Sub-G1 apoptotic cells were analyzed by flow cytometry. Bars represent Mean±SD from three independent experiments.

Our earlier experiments were based on 24 hours treatments of cells with different doses of oversulphated B100. Considering the possibility of a delay in the induction of apoptosis in the less sensitive cell lines, we analyzed the effect of native and oversulphated B100 at 48 hours in the moderately sensitive CEM and HL-60 cells and in the resistant Raji cells. As shown in figure 11, there was a modest increase in the induction of apoptosis in CEM cells, compared to that observed at 24 hours (figure 3), in response to all doses of oversulphated B100. In contrast, the percentage of apoptotic HL60 cells was similar to that observed at 24 hours (figure 7) and Raji cells remained resistant to oversulphated B100 after 48 hours treatment

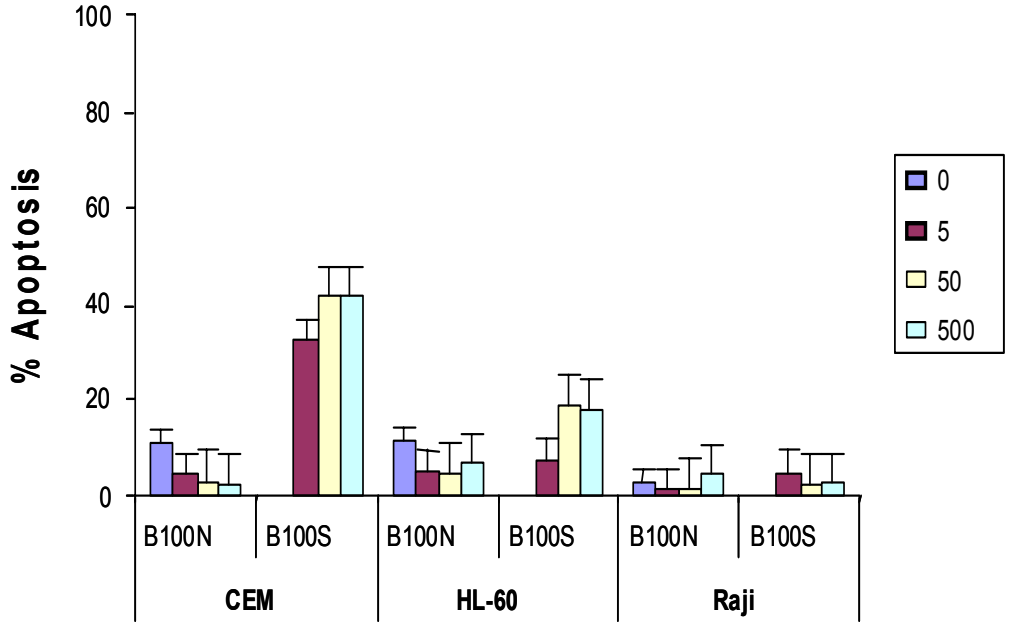

Figure 11. Induction of apoptosis in CEM, HL-60 and Raji cells by oversulphated B100 at 48 h incubation. CEM, HL60 and Raji cells were treated with different doses (500, 50 and 5 μg/ml) of oversulphated B100 (B100S) or native B100 (B100N) for 48 h and the percentage of apoptotic cells was determined by flow cytometry. Bars represent Mean±SD from three independent experiments.

Overall, these results indicate that oversulphated B100 EPS, but not native B100, induces apoptosis in human leukemic T cell lines such as Jurkat, MOLT-4, CEM and HPB-ALL, although the sensitivity of these cell lines is variable. B100S also modestly induces apoptosis in other hematopoietic tumor cells. In contrast, oversulphated and native form of N12 and FP34 EPS are unable to induce apoptosis although they have been demonstrated to show anti-proliferative activity against leukemic cell lines (Mata et al., unpublished results).

Induction of apoptosis by oversulphated B100 in non-haematopoietic cell lines

To determine the possible effect of oversulphated B100 in non-haematopoietic cells, we analyzed the induction of apoptosis in four human breast cancer cell lines, i.e. SKBr3,

EVSA-T, MCF-7 and MDA-MB231, and in one primary human decidual stromal cell line, DSC. We observed no effect in MDA and EVSA-T cell lines and a very mild effect in DSC cells (figure 12). In contrast, data analysis showed 19.4%, 26.82% and 19.4% of apoptosis in MCF-7 cells in response to 500, 50 and 5 µg/ml of oversulphated B100, respectively (figure 12). Moreover, SKBr3 cells were also moderately sensitive to high doses of this EPS. There were not any effect of native form of B100 and native and oversulphated forms of N12 and FP34 on these cells (data not shown).

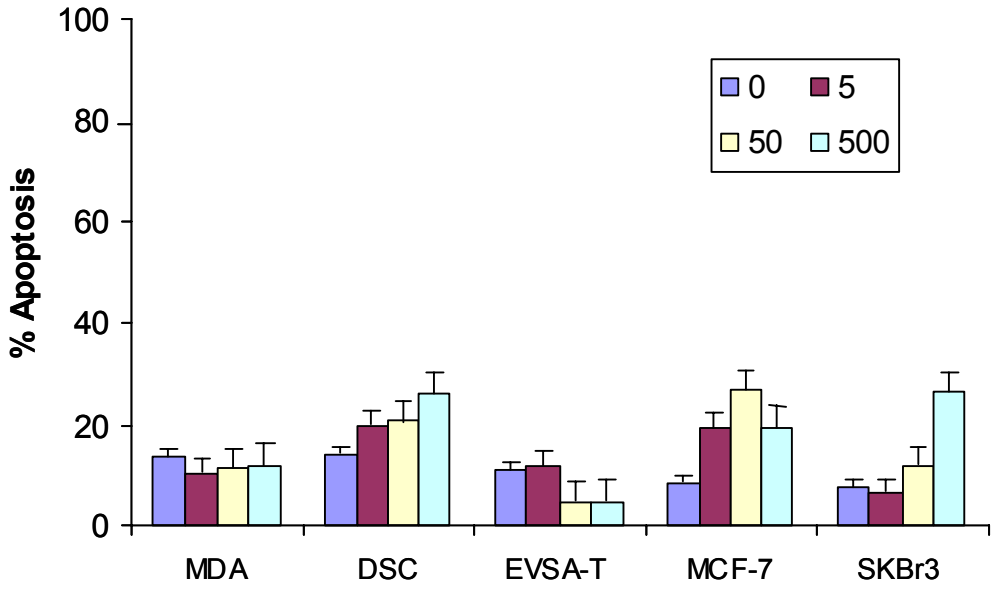

Figure 12. Induction of apoptosis by oversulphated B100 in breast tumor cells and primary decidual stromal cells. MDA-MB231, DSC, EVSA-T, MCF-7 and SKBr3 cells were incubated for 24 h with diferent doses (500, 50 and 5 µg/ml) of oversulphated B100. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Bars represent Mean±SD from three independent experiments.

Induction of apoptosis by oversulphated B100 in primary normal lymphocytes

The fact that there was a deep induction of apoptosis in some T cell lines led us to investigate the effect of oversulphated B100 on primary lymphocytes of healthy individuals. To this end, we treated resting isolated T lymphocytes, as well as T

lymphocytes activated for 6 and 10 days, with different doses of oversulphated and native B100 and in the presence or in the absence of IL-2. Only the higher dose of oversulphated B100 was able to slightly induce apoptosis in resting T lymphocytes both in the presence and in the absence of IL-2 (20.25% and 18.3%, respectively) (figure 13). In contrast, there was no induction of apoptosis by oversulphated B100 in activated T lymphocytes, neither at 6-day nor at 10-day activation (figure 13). Native B100 had also no effect in normal T lymphocytes. These results clearly show a different behaviour between activated and resting lymphocytes of normal individuals in response to treatment with oversulphated B100 EPS, being resting lymphocytes moderately sensitive to this EPS.

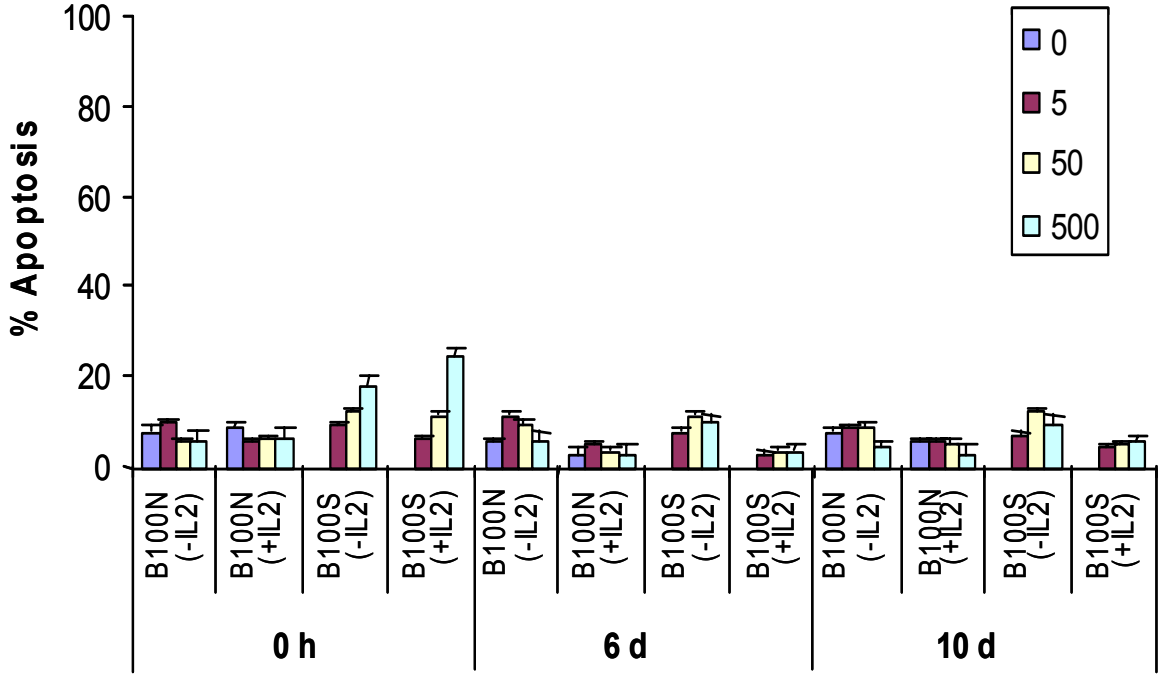

Figure 13. Induction of apoptosis in resting and activated primary T lymphocytes by oversulphated B100. Resting and activated (6 days and 10 days after activation) T lymphocytes were treated with different doses (500, 50 and 5 µg/ml) of native (B100N) and oversulphated (B100S) B100 with or without IL-2 for 24 h. The percentage of apoptotic cells was determined by flow cytometry. Bars represent Mean±SD from three independent experiments.

Reproducibility and temperature stability of the oversulphated B100 EPS

To verify that the induction of apoptosis observed in response to oversulphated B100 was reproducible and not an artefact of the stock solution of this EPS used in the initial experiments, we analysed whether independently produced and purified batches of oversulphated B100 had the same effect on the viability of the human leukemic T cell line Jurkat. Therefore, Jurkat cells were treated with different doses of oversulphated B100 of different batches (B100S-1 and B100S-2). HEL cells were used as a negative control since earlier experiments showed that they were resistant to the induction of apoptosis by oversulphated B100. Doses of 500, 50 and 5 µg/ml of B100S-1 batch induced 59.43%, 55.92% and 18.88% of apoptosis, respectively, while 70.22%, 50.69% and 16.85% of apoptosis was observed in response to B100S-2 batch (figure 14). No induction of apoptosis was detected in HEL cells with either batch.

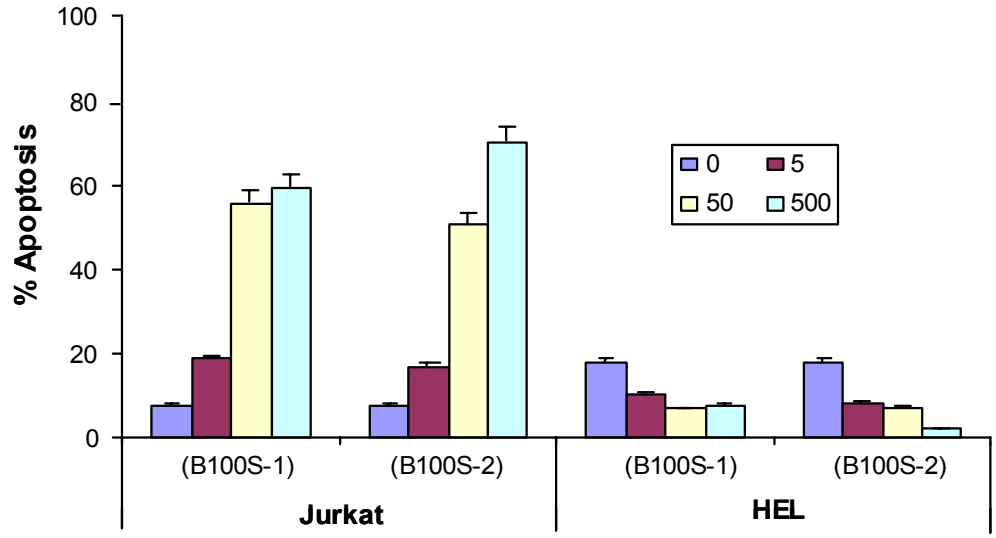

Figure 14. Induction of apoptosis by independent batches of oversulphated B100. Jurkat and HEL cells were treated with different doses (500, 50 and 5 µg/ml) of two different batches (B100S-1, B100S-2) of oversulphated B100 for 24 h. The percentage of sub-G1 apoptotic cells was determined by flow cytometry. Bars represent Mean±SD from three independent experiments.

We also checked the effect of the storage temperature on the activity of oversulphated B100. Jurkat cells were treated with 500 µg/ml and 50 µg/ml of oversulphated B100 stored at –20 ºC, 4 ºC and 37 ºC. The analysis of results showed that oversulphated B100 stored at different temperatures had similar ability to induce apoptosis in Jurkat cells (figure 15).

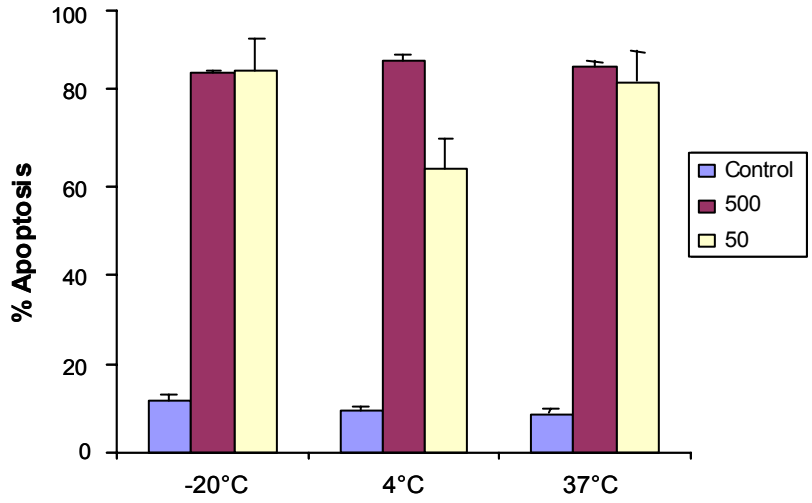

Figure 15. Induction of apoptosis by oversulphated B100 stored at different temperatures. Jurkat cells were incubated with different doses (500 and 50 µg/ml) of oversulphated B100 stored at the indicated temperatures for 24 h, and the percentage of sub-G1 apoptotic cells was determined. Bars represent Mean±SD from three independent experiments.

Apoptosis induced by oversulphated B100 in leukemic T cells is caspase-dependent

Induction of apoptosis in a cell results into transduction of signals through the intrinsic, the extrinsic or both interconnected pathways. Although one of them may prevail, depending on the apoptotic stimulus or the cell type examined, in most cases factors of both pathways act in concert, mutually facilitating and amplifying each other's effects. The study of the activation of caspase cascade can therefore provide information about

the apoptotic pathway involved in oversulphated B100 induced apoptosis in T leukemic cell lines.

For detecting the activation of caspase-3 and caspase-8 by oversulphated B100, we treated Jurkat cells with 500, 50 and 5 µg/ml of native and oversulphated forms of B100 for 6 hours. Immunoblot assay clearly showed cleaved subunits of caspase-3 and caspase-8 at doses of 50 and 500 µg/ml of oversulphated B100 (figure 16). No activation of caspases was observed in cells treated with the native form of B100 (figure 16).

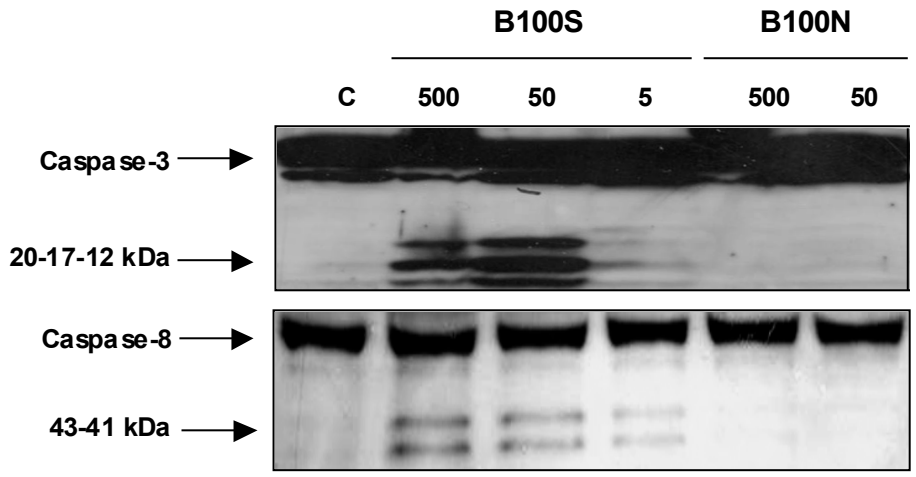

Figure 16. Activation of caspases in response to treatment with oversulphated B100. Jurkat cells were incubated without (C) or with different doses (500, 50 and 50 µg/ml) of native (B100N) and oversulphated (B100S) B100 for 6 hours. After treatment, caspase-3 and caspase-8 processing was assayed by immunoblot. Bands correponding to cleaved products of caspase-3 (20, 17 and 12 kDa) and caspase-8 (43-41 kDa) are indicated. The results shown are representative of two independent experiments.

A time-course of caspase-3, caspase-8 and caspase-9 activation may provide useful information to better understand the cascade of caspases activation upon induction of apoptosis by oversulphated B100. Jurkat cells were treated for 4, 6 and 8 hours with a dose of 50 µg/ml of oversulphated B100. The immunoblot detection of caspases clearly showed that activation of caspase-9 started at 4 hours while only a slight activation of caspase-3 was seen at this time and activation of caspase-8 started at 6 hours (figure 17). No caspase activation was observed upon 8 hours treatment with the native form of B100 (figure 17). These results suggest that caspase-9 is the initiator caspase in the pathway of apoptosis induction by oversulphated B100 and caspase-8 is possibly being activated downstream of effector caspases, such as caspase-3, as part of an amplification loop.

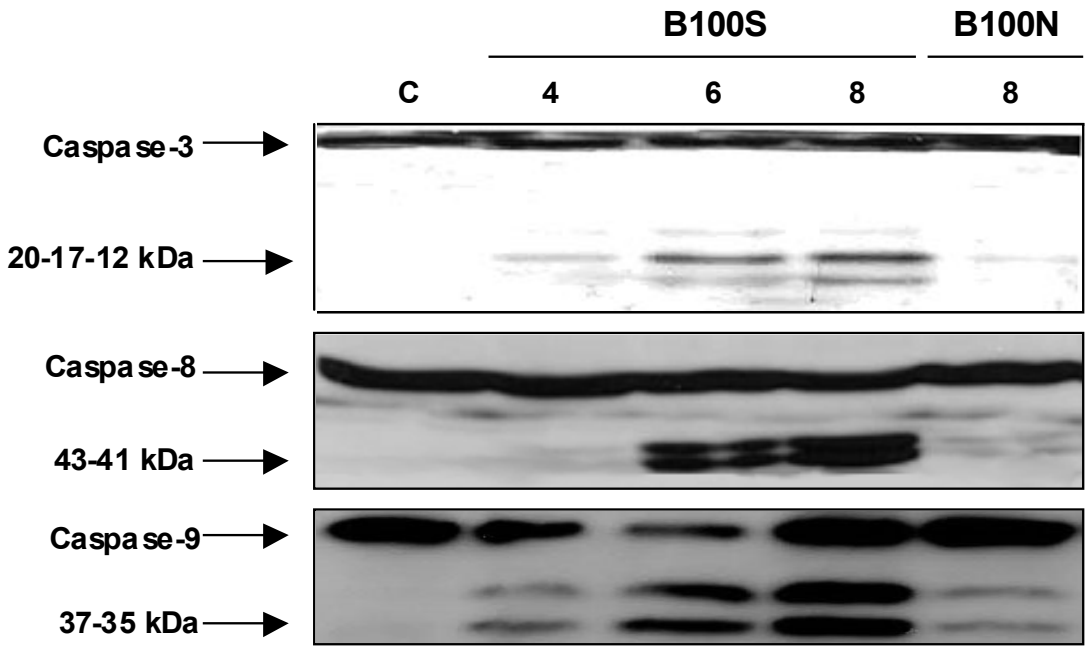

Figure 17. Time course of caspases activation in cells treated with oversulphated B100. Jurkat cells were incubated without (C) or with 50 µg/ml of oversulphated B100 (B100S) for 4, 6 and 8 hours, and with native B100 (B100N) for 8 hours. Activation of caspases was determined by Western blot analysis. Bands correponding to cleaved products of caspase-3 (20, 17 and 12 kDa), caspase-8 (43-41 kDa) and caspase-9 (37-35 kDa) are indicated. The results shown are representative of two independent experiments.

An issue that has received increasing attention during the last years is the existence of caspase-independent death programs. If apoptosis is blocked with caspase inhibitors, other cell death pathways may be initiated. To further prove that oversulphated B100 induces apoptosis in a caspase-dependent manner and that caspase-9 is activated upstream of caspase-3 and -8, we analysed the effect of two inhibitors of caspases, the general caspase inhibitor Z-VAD-FMK and the specific caspase-9 inhibitor Z-LEHD-FMK, in the induction of apoptosis and the activation of caspases by oversulphated B100. These caspase inhibitors, which design is based on the optimal peptide recognition motifs described for each caspase, irreversibly bind to the active site of the corresponding caspase (Cryns and Yuan, 1998; Garcia-Calvo et al., 1998; Talanian et al., 1997c).

Jurkat cells were preincubated for 1 hour with the caspases inhibitor before treatment with oversulphated B100 for 12 hours. Flow cytometric analysis data in figure 18 show that both caspase inhibitors, Z-VAD and LEHD, completely prevented cell death induced by oversulphated B100. Tumor necrosis factor apoptosis ligand (TRAIL) is a type II transmembrane protein displaying expression in a broad range of tissues and exhibiting a high grade of homology with the cytotoxic Fas ligand (Boehrer et al., 2006). Employing the extrinsic pathway, TRAIL-induced apoptosis is dependent on the activation of caspases. Therefore, we analysed the effects of caspase inhibitors on the induction of apoptosis by TRAIL in Jurkat cells, as a positive control of the functionality of these inhibitors (figure 18).

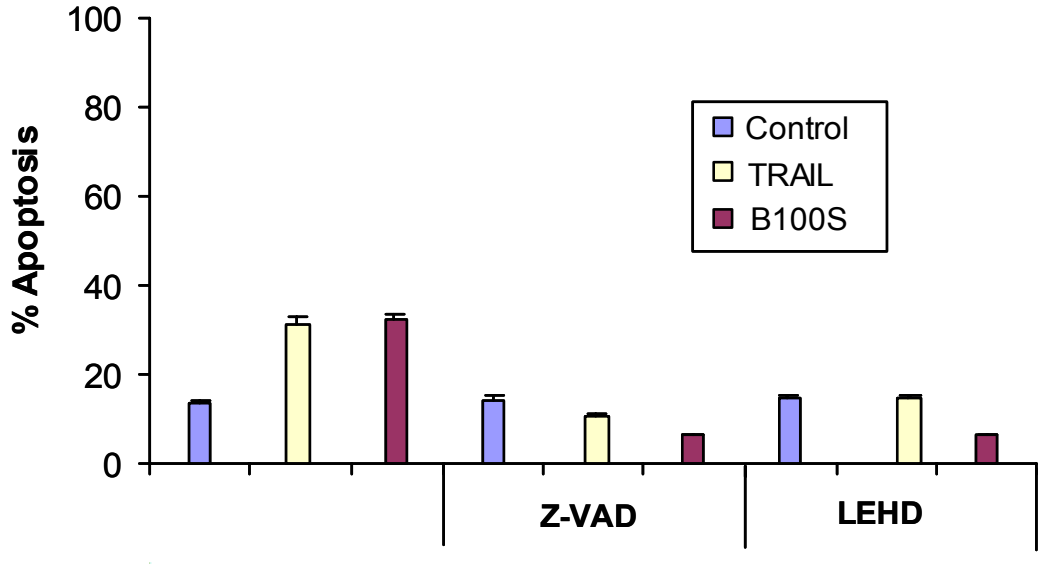

Figure 18. Effect of caspase inhibitors on the induction of apoptosis by oversulphated B100. Jurkat cells were preincubated for 1 h with 50μM Z-VAD-FMK or Z-LEHD-FMK before incubation with 50 μg/ml oversulphated B100 for 12 h. As a positive control, cells were incubated with 100 ng/ml of TRAIL. Apoptosis was assessed by analysis of the percentage of cells with sub-G_1 content. Bars represent Mean±SD from three independent experiments.

Immunoblot detection of caspase-8 and caspase-9 also showed inhibition by Z-VAD-FMK and Z-LEHD-FMK of the activation of both caspases in response to oversulphated B100 (figure 19). These results not only confirm that apoptosis induced by oversulphated B100 is caspase-dependent but also that initiator caspase-9 plays a critical role in this apoptotic pathway.

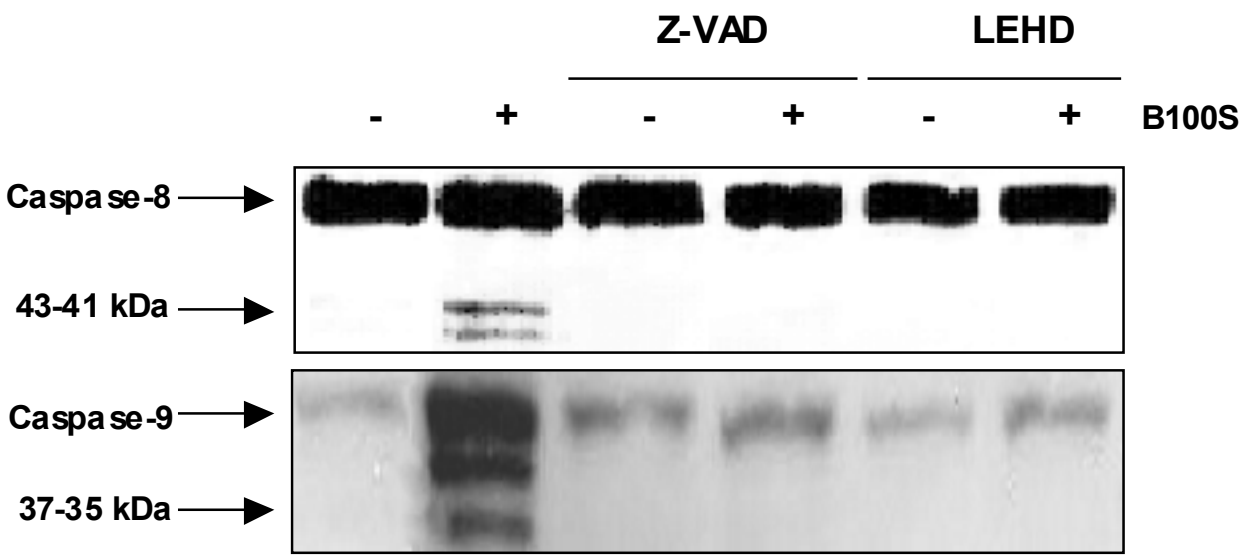

Figure 19. Effect of caspase inhibitors on the activation of caspases in response to oversulphated B100. Jurkat cells were preincubated with 50μM Z-VAD-FMK or Z-LEHD-FMK before treatment with or without 50 μg/ml oversulphated B100 for 12 h. Activation of caspase-8 and caspase-9 were analyzed by Western-blot. Bands corresponding to cleaved products of caspase-8 (43-41 kDa) and caspase-9 (37-35 kDa) are indicated. The results shown are representative of two independent experiments.

Mitochondrial alterations associated with oversulphated B100-induces apoptosis in leukemic T cells

The results obtained in relation with the activation of the caspase cascade suggest that oversulphated B100 is acting through the mitochondrial pathway where caspase-9 is the first to be activated. Therefore, we decided to analyse mitochondrial alterations such as loss of mitochondrial transmembrane potential induced by oversulphated B100 as it may serve as a checkpoint for full commitment to apoptosis.

Analysis of mitochondrial membrane depolarization in MOLT-4 cells treated with different concentrations of oversulphated and native B100 showed that, only doses of 50

and 5 μg/ml of oversulphated B100 were able to induce the loss of mitochondrial membrane potential while no effect was observed with lower doses of this compound or with native B100 (figure 20). Cells were also treated with 500 μg/ml of oversulphated B100 but they were completely damaged, therefore, we were unable to analyze alteration of mitochondrial membrane potential at this concentration (data not shown). It has been shown that TRAIL-mediated apoptosis is accompanied by a loss of mitochondrial membrane potential. We therefore used this death ligand as a positive control in our experiments.

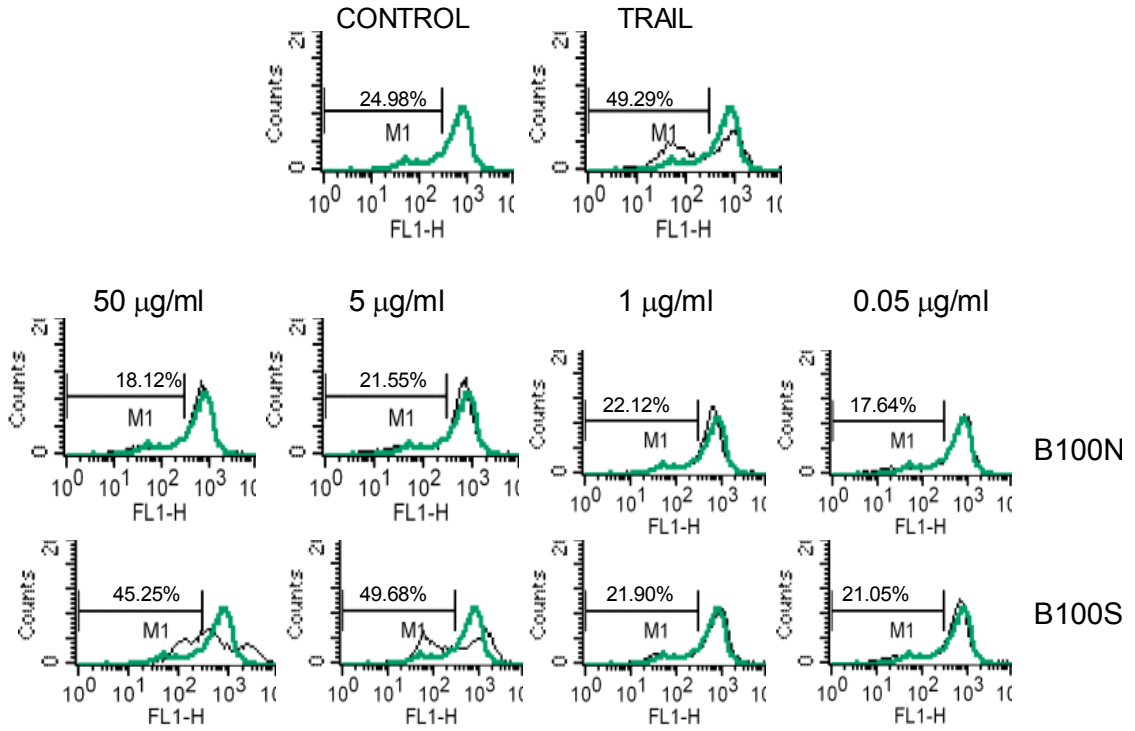

Figure 20. Alteration of mitochondrial membrane potential in reponse to treatment with oversulphated B100. MOLT-4 cells were treated with different doses (50, 5, 1 and 0.05 μg/ml) of oversulphated B100 (B100S) and native B100 (B100N) for 24 h. Depolarization of mitochondrial membrane was analyzed by flow cytometry. As a positive control, cells were treated with 100 ng/ml TRAIL. Data shown are representative of two independent experiments.

The results about the loss of mitochondrial membrane potential were in agreement with those obtained by analysis of the percentage of apoptotic cells in response to treatment

of cells with the same doses of native and oversulphated B100 (figure 21). We used concentrations of 1 µg/ml and 0.05 µg/ml of oversulphated B100 in this set of experiments to establish the lowest dose able to induce apoptosis and alter mitochondrial potential and to verify that both events are correlated. This was further confirmed by the results obtained with the death ligand TRAIL used as a positive control. The percentage of apoptotic cells in response to TRAIL was similar to the percentage of apoptotic cells upon treatment with 5 µg/ml of oversulphated B100 (figure 21) and the same was true for the values of loss of mitochondrial membrane potential (figure 20).

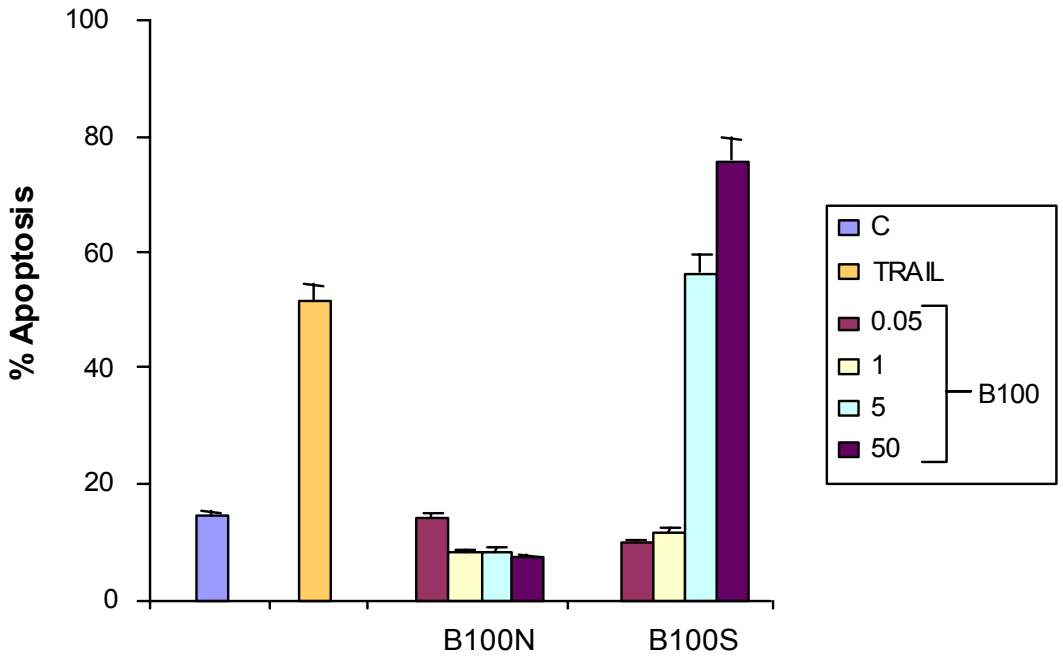

Figure 21. Induction of apoptosis in response to treatment with different doses of oversulphated B100. MOLT-4 cells were treated with 50, 5, 1 and 0.05 µg/ml of oversulphated B100 (B100S) and native B100 (B100N) for 24 h. As a positive control, cells were treated with 100 ng/ml TRAIL. The percentage of sub-G1 apoptotic cells was determined. Bars represent Mean±SD from three independent experiments.

Together with the mitochondrial membrane depolarization, production of reactive oxygen species (ROS) in the mitochondria seem to play an important role in the

induction of apoptosis under both physiologic and pathologic conditions (Simon et al., 2000), contributing to the dismantling of the cell (Ricci et al., 2003). Thus, we analyzed the time course of ROS production in cells undergoing apoptosis in response to treatment with oversulphated B100. The analysis of results showed that accumulation of ROS started at 6 hours and it increased with time (figure 22). No increase in the production of ROS was observed in cells treated with native B100. Therefore, that increase of ROS levels in cells treated with oversulphated B100 may be an important step in the apoptotic route induced by this EPS.

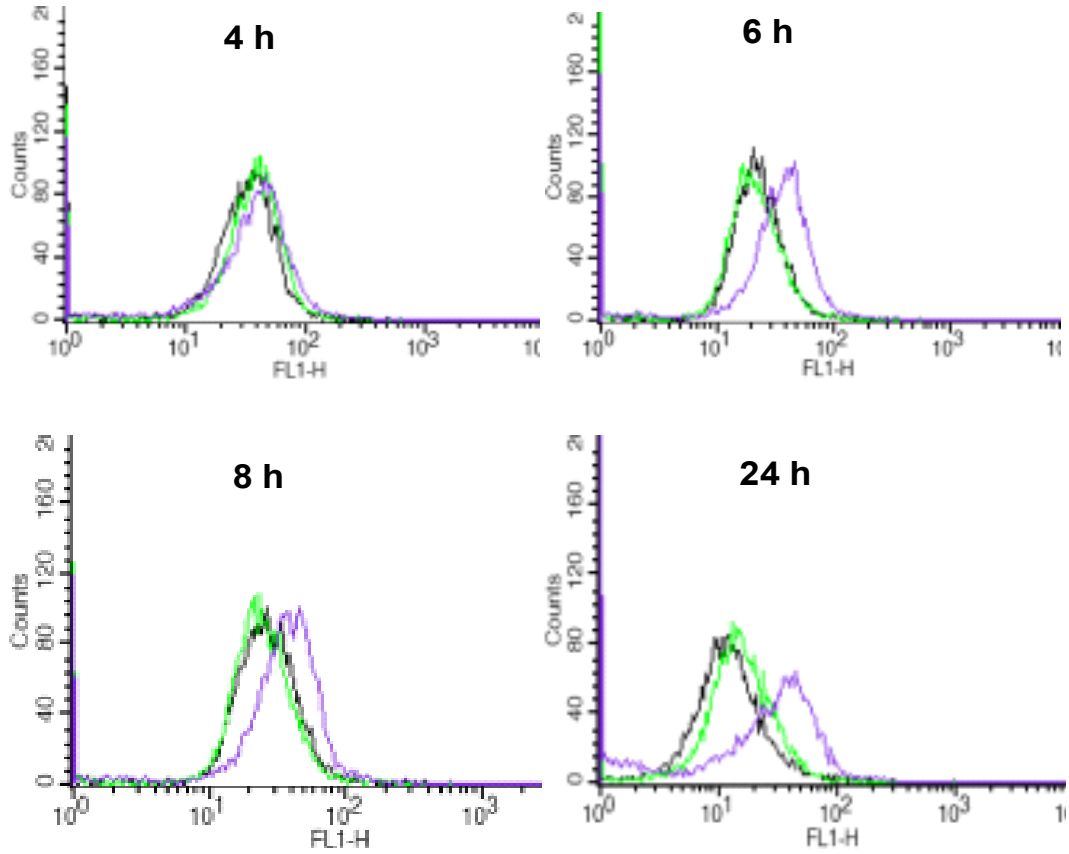

Figure 22. Production of ROS in response to treatment with oversulphated B100 EPS. MOLT-4 cells were treated without (black line) or with 50 µg/ml of oversulphated B100 (violet line) or native B100 (green line) for 4, 6, 8 and 24 h. Production of ROS was determined by flow cytometry. Data shown are representative of three independent experiments.

The question arising from the observed early ROS accumulation after treatment with oversulphated B100 is whether intracellular oxidation is the responsible for the induction of apoptosis by this EPS. N-Acetyl Cystein (NAC) is a widely used thiol-

containing antioxidant that is precursor of reduced glutathione (GSH). GSH scavenges ROS in cells by interacting with OH- and H_2O_2, thus affecting ROS mediated signalling pathways. We analyzed the induction of apoptosis by oversulphated B100 in cells preincubated with NAC at a concentration previously established (Goldsmit et al., 2001; Oh and Lim, 2006). While 51.75% of apoptosis was detected upon 24 hours treatment with oversulphated B100 in the absence of NAC, 37.71% of apoptosis was observed when cells were treated in the presence of this antioxidant agent (figure 23), that is, NAC partially inhibited apoptosis induced by oversulphated B100. We also determined the production of ROS in cells preincubated with NAC before being stimulated with 50 µg/ml of oversulphated B100 for 4, 6, 8 and 24 hours. There was a partial inhibition of ROS production at 6 hours by NAC, however, no inhibition was observed at 8 and 24 hours (figure 24).

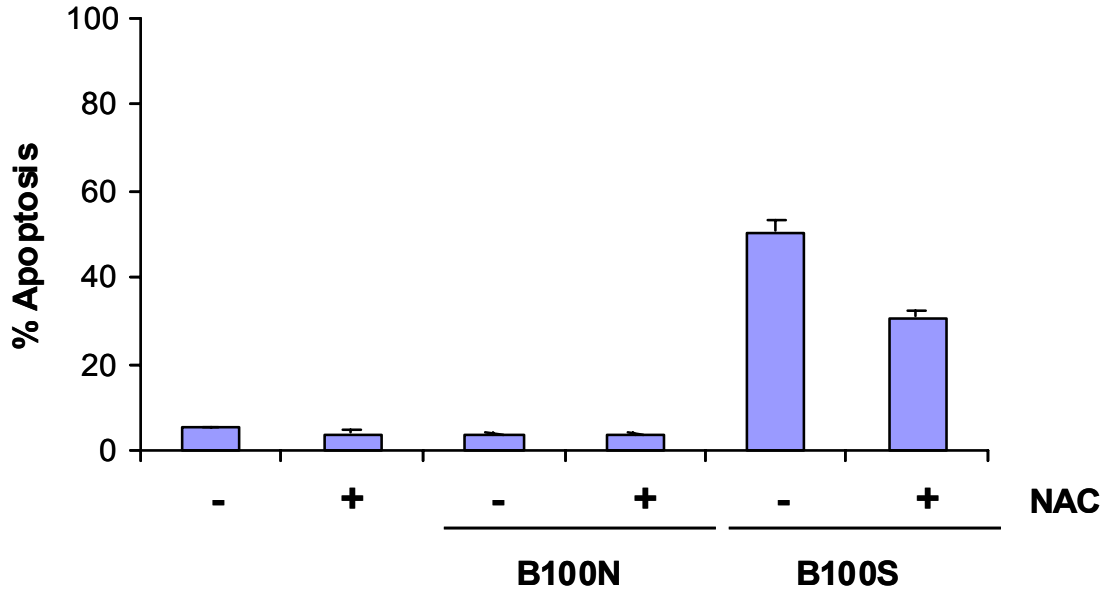

Figure 23. Effect of NAC on the induction of apoptosis by oversulphated B100. MOLT-4 cells were preincubated for 1 h with 10mM NAC before being treated with 50 µg/ml of oversulphated B100 (B100S) or native B100 (B100N) for 24 h. The percentage of apoptotic cells was determined by flow cytometry. Error bars show SD from three independent experiments.

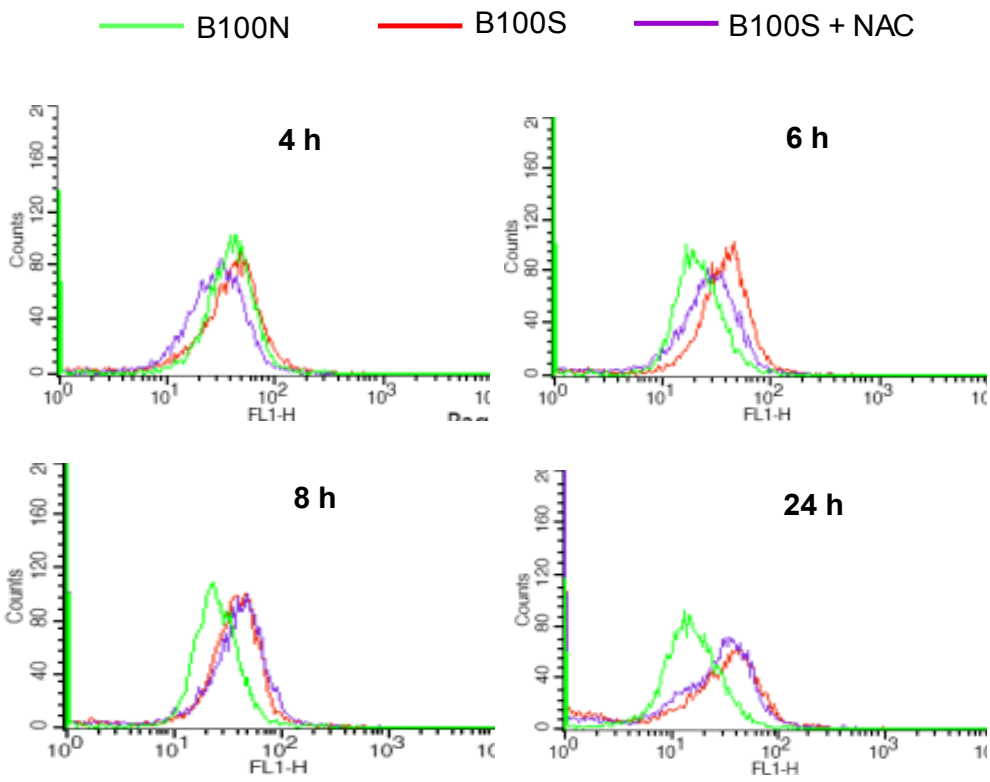

Figure 24. Inhibition by NAC of ROS production in cells treated with oversulphated B100. MOLT-4 cells were treated with 50 µg/ml of oversulphated B100 or native B100 for 4, 6, 8 and 24 h after being preincubated in the absence or in the presence of 10mM NAC for 1 h. Production of ROS was determined by flow cytometry. Data shown are representative of three independent experiments.

These results suggest that production of ROS may be a critical step in the induction of apoptosis by this EPS. NAC seems to be able to block the initial intracellular oxidation induced by oversulphated B100 but is inefficient at latter times and thus, cells undergo partial apoptosis in the presence of NAC.

Overexpression of Bcl-x_L causes partial protection of apoptosis induced by oversulphated B100

Bcl-x_L and certain other members of the Bcl-2 family are powerful inhibitors of mitochondrial alteration and thus may prevent the activation of mitochondria-dependent pathways of apoptosis. To investigate the effect of Bcl-x_L protein on oversulphated B100-induced apoptosis in leukemic T cells, we treated Jurkat cells overexpressing Bcl-x_L and Jurkat cells transfected with the empty vector pcDNA3-neo with different concentrations of oversulphated B100 for 24 hours. As shown in figure 25, no induction of apoptosis was observed in Jurkat Bcl-x_L upon treatment with 50 µg/ml of oversulphated B100. With the higher dose of 500 µg/ml there was a clear, although not complete, inhibition of cell death in Bcl-x_L-overexpressing Jurkat cells as compared to mock-transfected cells. These results indicate that overexpression of Bcl-x_L in Jurkat cells may at least partially inhibit the induction of apoptosis by oversulphated B100 and further demonstrate the involvement of mitochondria in this apoptotic pathway.

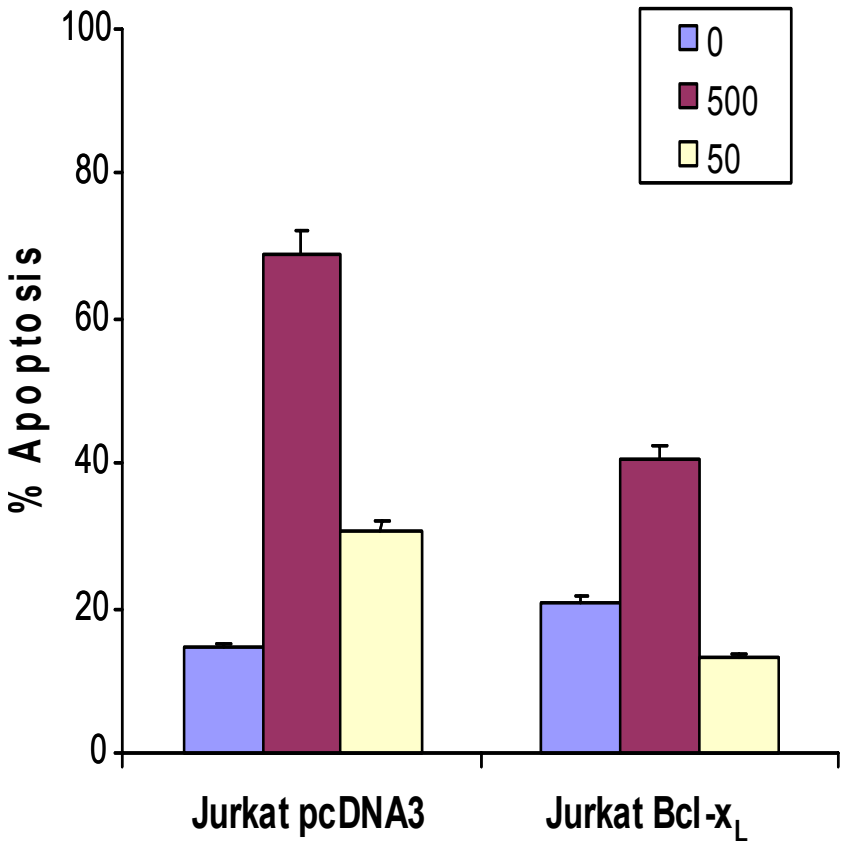

Figure 25. Induction of apoptosis by oversulphated B100 in Bclx-$_L$ overexpressing Jurkat cells. Jurkat cells transfected with the empty vector pcDNA3-neo (Jurkat pcDNA3) or with pcDNA3-Bcl-x_L (Jurkat Bcl-$x_{L)}$ were treated with different doses (500 and 50 μg/ml) of oversulphated B100 for 24 h. Sub-G1 apoptotic cells were analyzed by flow cytometry. Bars represent Mean±SD from three independent experiments.

Oversulphated B100 induces apoptosis in primary leukemic T cells

Our previous data obtained by using leukemia cell lines encouraged us to investigate the effect of oversulphated B100 in primary leukemic cells. Blood samples from two different patients with acute myeloid leukaemia (AML) and acute T lymphocytic leukaemia (T-ALL), respectively, were collected and lymphocytes were treated with oversulphated B100. There was no induction of apoptosis in the myeloid leukaemia cells (data not shown). In contrast, a weak effect was observed with the dose of 500

µg/ml of oversulphated B100 in the acute lymphocytic leukaemia cells (figure 26) suggesting the possibility of utilizing the oversulphated B100 EPS in the treatment of this type of leukaemia. Native B100 had no effect either on AML cells or on ALL cells (data not shown).

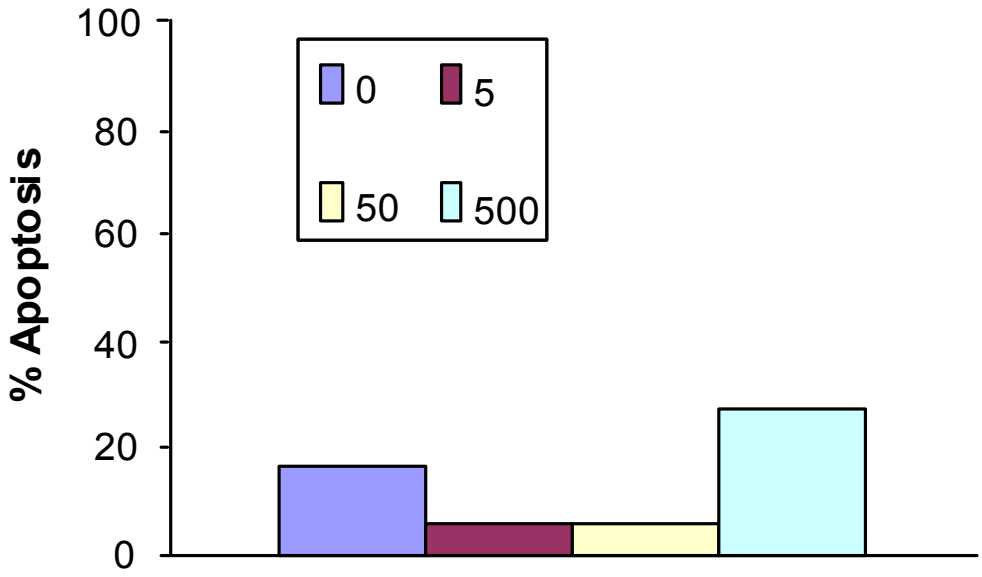

Figure 26. Induction of apoptosis by oversulphated B100 in primary T-Acute lymphocytic leukaemia cells. Freshly isolated T-ALL cells from a patient were treated with different doses (500, 50 and 5 µg/ml) of oversulphated B100 for 24 h and the percentage of apoptotic cells was determined by flow cytometry.

Discussion

Halophiles thrive in environments with very high concentrations of salt (at least 2 M, approximately ten times the salt level of ocean water). Although halophilic bacteria have been relatively little studied, they have the potential for exciting and promising applications due their unique properties. Biologists are currently focusing in further recognizing those special activities of halophiles because of their potential roles for the development of a wide range of applications in food, pharmaceutical, petroleum, and other industries (Shatwell et al., 1990). Some special activities of the EPS produced by the extracellular matrix of bacteria are important for coping with the different external environmental conditions, and thus, help bacteria in surviving in animal, plants and other extreme conditions. Among the peculiar biological properties of EPS are the activities as blood anticoagulant, anti-tumour, anti-mutagenic, anti-complementary, immunomodulating, hypoglycemic, antiviral, hypolipidemic and anti-inflammatory (Srivastava, 1989) anti-bacterial, anti-fungal, anti-parasitic, etc., which play very important roles in the modulation of the immune system. It has been therefore evident an increasing interest in the investigation of the physical, chemical and biological properties and mechanisms of action of EPS (Yan et al., 1999a), as well as on the discovery of new ones (Tian et al., 1999a). However, there is a lack of knowledge for the controlled conversion of EPS for industrial exploitation. In this way, our investigation for exploring the immunobioproperties of EPSs of *Halomonas maura* (B100, N12) and *Halomonas anticariensis* (FP34) is an innovative approach for utilizing the immunobioproperties of these EPSs for therapeutic purposes.

Oversulphated EPS are rare among those of microbial origin, and the mechanisms of their actions remain obscure. We therefore started to investigate the biological effects

that the native and oversulphated forms of B100, N12 and FP34 have on a panel of haematopoitic and non-haematopoitic cell lines. Our results showed that irrespective of their modulatory effects, the native form of B100 and the native and oversulphated forms of N12 and FP34 did not induce significant apoptosis in any of the haematopoitic and non-haematopietic cell lines, thus, excluding the possibility of utilizing them in therapeutic approaches based on the modulation of apoptosis. Remarkably, we observed a dose-dependent strong apoptosis induced by the oversulphated form of B100 in Jurkat and MOLT-4 cells and a moderate effect in other cells of both haematopoitic (CEM, SAM31, HL-60, HPB-ALL and K562 cell lines) and non-haematopoitic lineages (SKBr-3 and MCF-7) cells. Oversulphated B100 has therefore some specific immunobioproperties to which a number of cell lines have different sensitivities. Our observations that many cell lines of haematopoietic origin (HEL, U937, ITA, BW5147, Daudi and Namalwa) and non-haematopoietic cells (MDA, DSC and EVSA-T cells) are completely resistant to apoptosis induced by oversulphated B100 are also consistent with a susceptibility gradient in different cell lines to the pro-apoptotic activity of oversulphated B100 EPS. The optimal dose for the induction of apoptosis in both Jurkat and MOLT-4 cells is 50 μg/ml of oversulphated B100, as revealed by analysis of cell cycle and caspases cleavage detected by Western Blot. The optimal effect achieved at this low dose and the remarkable biological stability of the EPS suggests the possibility that the EPS sB100 will have a vigorous therapeutic potential *in vivo*. Furthermore, the apoptosis induced by EPS sB100 is a very rapid event as it appeared to start at 4 hours and a significant percentage of apoptotic cells were detected after only 12 hours treatment.

The conclusion that there was a deep induction of apoptosis in T cell lines led us to investigate the effect of oversulphated B100 on lymphocytes of normal individuals. The flow cytometric analysis of the cell cycle showed that oversulphated B100 induces very weak apoptosis in unstimulated fresh lymphocytes, but there was not any induction of apoptosis observed at 2, 6 and 10 days after activation of lymphocytes of normal individuals. This result was strong evidence supporting the conclusion that oversulphated B100 primarily induces apoptosis in tumor cells (Jurkat, MOLT) whereas their unstransformed counterparts are poor targets for the EPS.

Apoptotic cell death is essential for normal B-cell development and for shaping the B-cell repertoire, and therefore, investigating the effects of oversulphated B100 in inducing apoptosis in B cells allowed us to compare the different sensitivities of T and B cells to this apoptosis inductor. Our results showed that there was not any induction of apoptosis in Raji cells by oversulphated B100. This finding was not surprising since earlier studies reported that the human B lymphoma Raji cells are resistant to nuclear apoptosis induced by various stimuli, whereas HL-60 and Jurkat cells are apoptosis-sensitive cell lines (Kawabata et al., 1999). Moreover, none of the Burkitt´s lymphoma B cell lines analyzed showed sensitivity to the induction of apoptosis by oversulphated B100 indicating that this type of tumor cells are not suitable candidates for treatment with the EPS.

Apoptosis or type I programmed cell death involves biochemical events in a controlled, regulated fashion leading to a characteristic cell morphology and death in response to a variety of stimuli. Induction of apoptosis results into transduction of signals through either the intrinsic or extrinsic pathways or even through interconnection of both.

Although one of them may prevail, depending on the apoptotic stimulus or the cell type examined, in most cases they act in concert, mutually facilitating and amplifying each other's effects. HPB-ALL cells are normally resistant to CD95-mediated apoptosis (Russo et al., 2003) but oversulphated B100 caused a moderate induction of apoptosis in these cells. These resultsindicate that oversulphated B100 is able to induce apoptosis in cell lines resistant to the induction of apoptosis via the extrinsic pathway.

Both extrinsic and intrinsic pathways of apoptosis lead to the execution of a death signal through activation of a caspase cascade. Caspase-3 is a downstream caspase of the caspase cascade that is common to both apoptotic pathways. Activation of caspase-3 cleaves the inhibitor of the caspase-activated deoxyribonuclease, and it becomes active leading to nuclear apoptosis. Immunoblot observation of the activation of caspases in Jurkat cells clearly showed the cleaved subunit of caspase-3 at 6 hours, supporting that the apoptosis induced by oversulphated B100 involves the induction of a caspase-dependent apoptotic pathway. Moreover, a general caspase inhibitor (Z-VAD-FMK) (Cryns and Yuan, 1998; Garcia-Calvo et al., 1998; Talanian et al., 1997b) caused not only the inhibition of activation of all caspases analyzed but also the inhibition of oversulphated B100-induced apoptosis.

Caspase-8 is the initiator caspase of the extrinsic pathway while caspase-9 is the most apical caspase in the intrinsic pathway. Our results demonstrate a clear activation of caspase-9 and a slight cleavage of caspase-3 is already observed at 4 hours after treatment with oversulphated B100 whereas cleavage of caspase-8 become evident only after 6 hours of exposure to the EPS. These data suggest that caspase-9 is the initiator caspase in the pathway of apoptosis induced by oversulphated B100. Caspase-8 is likely

being activated downstream of effector caspases such as caspase-3, as part of an amplification loop. This possibility was further supported by experiments using the specific caspase-9 inhibitor (Z-LEHD-FMK) (Cryns and Yuan, 1998; Garcia-Calvo et al., 1998; Talanian et al., 1997a), which prevented not only the activation of caspase-9 but also that of caspases-3 and –8 as well as the induction of apoptosis by oversulphated B100. Induction of apoptosis by oversulphated B100, therefore, involves an interconnection of both apoptotic pathways, although the intrinsic apoptotic pathway clearly prevails.

The early stage of the intrinsic apoptotic pathway, i.e. the stage which precedes nuclear disintegration, is characterized by the breakdown of the mitochondrial transmembrane potential ($\Delta\Psi m$) (Cossarizza et al., 1994; Inai et al., 1997; Zamzami et al., 1996a) resulting into the release of several apoptotic factors, including the apoptosis-inducing factor (AIF), cytochrome *c* and other pro-apoptotic factors from the mitochondrial intermembrane space, thus inducing caspase activation and potentiating cell death. We have showed the loss of mitochondrial membrane potencial in MOLT-4 cells in response to doses of oversulphated B100 that are able to induce apoptosis, also demonstrating the involvement of the intrinsic apoptotic pathway in the induction of apoptosis by this EPS.

The Bcl-2 family of proteins are well characterized regulators of apoptosis that act upstream of caspase activation (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000). Pro-apoptotic family members Bax and Bak may oligomerize and interact with VDAC to change mitochondrial membrane permeability and allow the release of apoptogenic factors into the cytosol. On the other hand, the anti-apoptotic Bcl-2 and Bcl-x_L proteins

bind and sequester Bax and Bak leading to the blockage of the induction of apoptosis. Partial inhibition of apoptosis induced by oversulphated B100 in Jurkat cells overexpressing Bcl-x_L further confirms the involvement of the mitochondrial apoptotic signals in the mechanism of cell death induced by oversulphated B100 EPS.

Apart from this pivotal role of mitochondria during the execution phase of apoptosis, it appears that reactive oxygen species (ROS) produced by the mitochondria can be involved in cell death (Fleury et al., 2002). Mitochondria rapidly lose their transmembrane potential and generate reactive oxygen species (ROS), both of which are likely to contribute to the dismantling of the cell (Ricci et al., 2003). Treatment of cells with oversulphated B100 resulted into accumulation of ROS at 6 hours and it was maintained for at least 24 hours. The involvement of ROS in the induction of apoptosis by oversulphated B100 is suggested by the fact that it was partially prevented by NAC antioxidant.This antioxidant inhibited ROS production at 6 hours but it was not able to block it at any time point after 8 hours of treatmen with oversulphated B100, which may explain the partial inhibitory effect against the induction of apoptosis by this EPS.

The high susceptibility of two leukemic T cell lines to oversulphated B100 suggests that this EPS may be an appropriate therapeutic strategy for the treatment of this type of tumors.

In agreement with that, we have observed a modest induction of apoptosis in primary cells from a T-ALL patient in response to oversulphated B100 while there was not any induction of apoptosis in cells from another patient with AML. This is a preliminary result that requires more experiments with patient samples before concluding that oversulphated B100 may be a new tool suitable for inducing apoptosis in primary

leukemic cells, therefore opening the prospect for treatment of patients with T-cell leukaemia. In addition, experiments aiming to evaluate the toxicity and the efficacy of the oversulphated B100 EPS treatment of T cell leukemias in animal models are currently being planned.

Conclusions

1.- The oversulphated, but not the native, form of the *Halomonas maura* EPS B100 is a potent inducer of apoptosis. This proapoptotic activity is not observed with any of the native or oversulphated forms of the *Halomonas maura* EPS N12 or the EPS FP34 from *Halomona anticariensis.*

2.- The proapoptotic effect of the oversulphated form of EPS B100 shows a gradient of sensitivity within a panel of hematopoietic and non-hematopoietic cells, being leukemic T cell lines the best targets for the EPS. Primary and non-tumor T lymphocytes are resistant to induction of apoptosis by oversulphated B100.

3.- The apoptosis induced by the oversulphated form of B100 involves an early activation of the caspase cascade. The apical caspase in this cascade is caspase-9.

4.- The orderly activation of caspases, the alteration of mitochondrial membrane potential and the production of Reactive Oxygen Species are evidence supporting that the apoptosis induced by the oversulphated B100 EPS is triggered mainly through the mitochondrial or intrinsic pathway of opoptosis.

5.- The potent proapoptotic effect of oversulphated B100 on leukemic T cell lines as well as the effect observed on peripheral cells from a patient with T-ALL opens the possibility for therapeutic applications of the oversulphated B100 EPS in human T cell leukemias.

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