THE UNIVERSITY OF CALGARY

Characterization of the T Lymphocyte Response to *Pseudomonas aeruginosa* Exoenzyme S

by

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ABSTRACT

Exoenzyme S was shown to induce proliferation of immunologically naive lymphocytes suggesting that it stimulates T cells as a mitogen. The proliferative response was dependent on accessory cells, independent of HLA-DR, and did not oligoclonally stimulate T cells based on their Vβ expression. The early T cell activation marker, CD69, was detected at levels intermediate between a panel of superantigens and mitogens, but did not precede detectable expression of other delayed markers of activation such as CD25 and CD71. Proliferative responses to exoenzyme S were significantly lower, and the kinetics more delayed, when compared to proliferative responses to other mitogens. Further studies revealed that T cell responses to exoenzyme S was similar in healthy adults and CF patients and shown to induce significant apoptosis in vitro. Together, these data implicate exoenzyme S as an important contributor to the pulmonary inflammation associated with chronic lung infection by *Pseudomonas aeruginosa.*

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Dedication

I dedicate this work to my mom and dad. As a token of gratitude for their love and support.

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AC - accessory cell

AET - 2-aminoethylisothiouronium bromide

APC - antigen presenting cell

ADD - 7-amino actinomycin

ADP - adenosine diphosphate

AICD - activation induced cell death

ANOVA - analysis of variance

AP-1 - activating protein-1

BCR - B cell receptor

BRMP - biological response modifiers program

BSA - bovine serum albumin

COPD - chronic obstructive pulmonary disease

CF - cystic fibrosis

CFTR - cystic fibrosis transmembrane conductance regulator

CMI - cell-mediated immunity

DAG - diacylglycerol

DNA - deoxyribonucleic acid

ExoS/DG1 - exoenzyme S from *Pseudomonas aeruginosa* strain DG1

Exo S - exoenzyme S

FAS - factor activating exoenzyme S

FBMC - fetal blood mononuclear cell

FEV_1 - forced expiratory volume in one second

FITC - fluorescein-isothyocyanate

GTP - guanine diphosphate

HBSS - Hank's balanced salt solution

ICE - interleukin-1 converting enzyme

IL - interleukin

INF-γ - interferon-gamma

IP_3 - inositol 1,4,5-triphosphate

ITAM - immunoreceptor tyrosine-based activation motif

LPS - lipopolysaccharide

MHC - major histocompatibility molecule

NAD - nicotinamide adenine dinucleotide

PBS - phosphate buffered saline

PBMC - peripheral blood mononuclear cell

PE - phycoerythrin

PerCP - peridinin chloryphyll

PHA - phytohemaglutinin

PI - propidium iodide

PI-PLC-γ1 - phophatidylinositol phopholipase C-γ1

PKC - protein kinase C

$PtdInsP_2$ - phospholipid phophatidylinositol 4,5-bisphophate

PTK - protein tyrosine kinase

rExoS - recombinant exoenzyme S

SE - *Staphylococcal* enterotoxin

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SRBC - sheep red blood cell

TBS - Tris buffered saline

TCR - T cell receptor

TEMED - N,N,N',N'-tetramethyl ethylene diamine

TNF-α - tumor necrosis factor- alpha

TSST -1 - toxic shock syndrome toxin

YOP - *Yersinia* outer proteins

If I have ever made any valuable discoveries, it has been owing more to patient attention, than to any other talent.

Isaac Newton

I have never let my schooling interfere with my education.

Mark Twain

Consider the postage stamp: its usefulness consists in the ability to stick to one thing till it gets there.

Josh Billings

1.0 INTRODUCTION

1.1 Cystic Fibrosis

1.1.1 Defect, Disease and Infection

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that occurs in an estimated 1 in 2,500 Caucasian births in Canada and around the world [Corey and Farewell, 1996]. It is the most common cause of pulmonary insufficiency in the first three decades of life [Davis, 1991]. The main cause of death is respiratory insufficiency due to chronic obstructive pulmonary disease (COPD) [Davis, 1991]. Fortunately, advanced symptomatic treatment of the disease in Canada has increased mean survival age from early infancy in the 1940's to over 30 years in the 1990's [Corey and Farewell, 1996]. Since Tsui *et al.* identified the gene responsible for cystic fibrosis in 1989, researchers have quickly turned their focus to delivering a normal copy of the gene through the advancement of gene therapy [Zabner *et al.*, 1993], [Yei *et al.*, 1994], [Knowles *et al.*, 1995]. The most common gene therapy uses adenovirus as the vector, however low viral infection rates and induction of dose-dependent upper and lower airway inflammation limits the use of doses necessary to correct the electrophysiological defect [Crystal *et al.*, 1994], [Knowles *et al.*, 1995]. Other gene therapy delivery methods have showed even less promise [Caplen *et al.*, 1995], [Marshall *et al.*, 1995]. For these reasons effective symptomatic management of the disease is still the single most important determinant for long term survival.

The major defect in patients with CF is an inability of the cystic fibrosis transmembrane conductance regulator (CFTR) protein to regulate chloride ion transport in respiratory epithelium [Drumm *et al.*, 1990], [Rich *et al.*, 1990]. Over 400 different mutations to the CFTR gene are known including missense mutations, premature stop codons, and frameshift mutations; the most common mutation of the CFTR gene (ΔF508) results in a significant impairment of expression and hence a functional defect [Davis *et al.*, 1996]. As a result, the impairment of chloride movement directly or indirectly causes the

increased absorption of sodium ions resulting in the epithelia to produce large quantities of viscid mucus [Davis *et al.*, 1996]. By its very nature the mucus becomes thick and difficult to remove compromising the mucociliary clearance of the host. Soon after birth, CF patients acquire chronic bacterial infection which leads to a self-sustaining pulmonary state of inflammation for the remainder of life [Fick *et al.*, 1984], [Berger *et al.*, 1989], [Tosi *et al.*, 1990]. Together, the infection and resultant inflammation destroys the airways, impairs gas exchange, and ultimately results in death [Konstan and Berger, 1993]. Although respiratory infection with *Staphylococcus aureus* and *Haemophilus influenzae* occur in CF patients, chronic *Pseudomonas aeruginosa* bronchiolitis and bronchitis are the major source of morbidity and mortality in these patients. Ultimately, *P. aeruginosa* infection occurs in virtually all patients [Konstan and Berger, 1993] (Fig. 1).

1.2 *Pseudomonas aeruginosa*

1.2.1 Colonization of the Cystic Fibrosis Lung

It is estimated that over 90% of adult CF patients are colonized with *P. aeruginosa* [Laraya-Cuasay *et al.*, 1976], [Hoiby, 1982]. The precise mechanism by which electrolyte defects lead to infection and inflammation has not been established, however a theory explaining the predilection of *Pseudomonas* sp. to the CF lung has recently been identified. It has been demonstrated that epithelial cells derived from CF patients have increased levels of asialo-GM1, a surface molecule that has been shown to bind *P. aeruginosa* [Saiman *et al.*, 1993]. The increased binding correlates with decreased sialic acid residues on the surface of these cells implying that *P. aeruginosa* binds more effectively to asialo proteins (specifically asialo-GM1) than it does to sialylated forms [Saiman *et al.*, 1993]. These studies however, have been challenged primarily due to the inability to detect differences in binding between epithelial cells from CF and non-CF patients [Plotkowski *et al.*, 1992], [Cervin *et al.*, 1994]. More recently, it has been shown that epithelial cells lacking CFTR

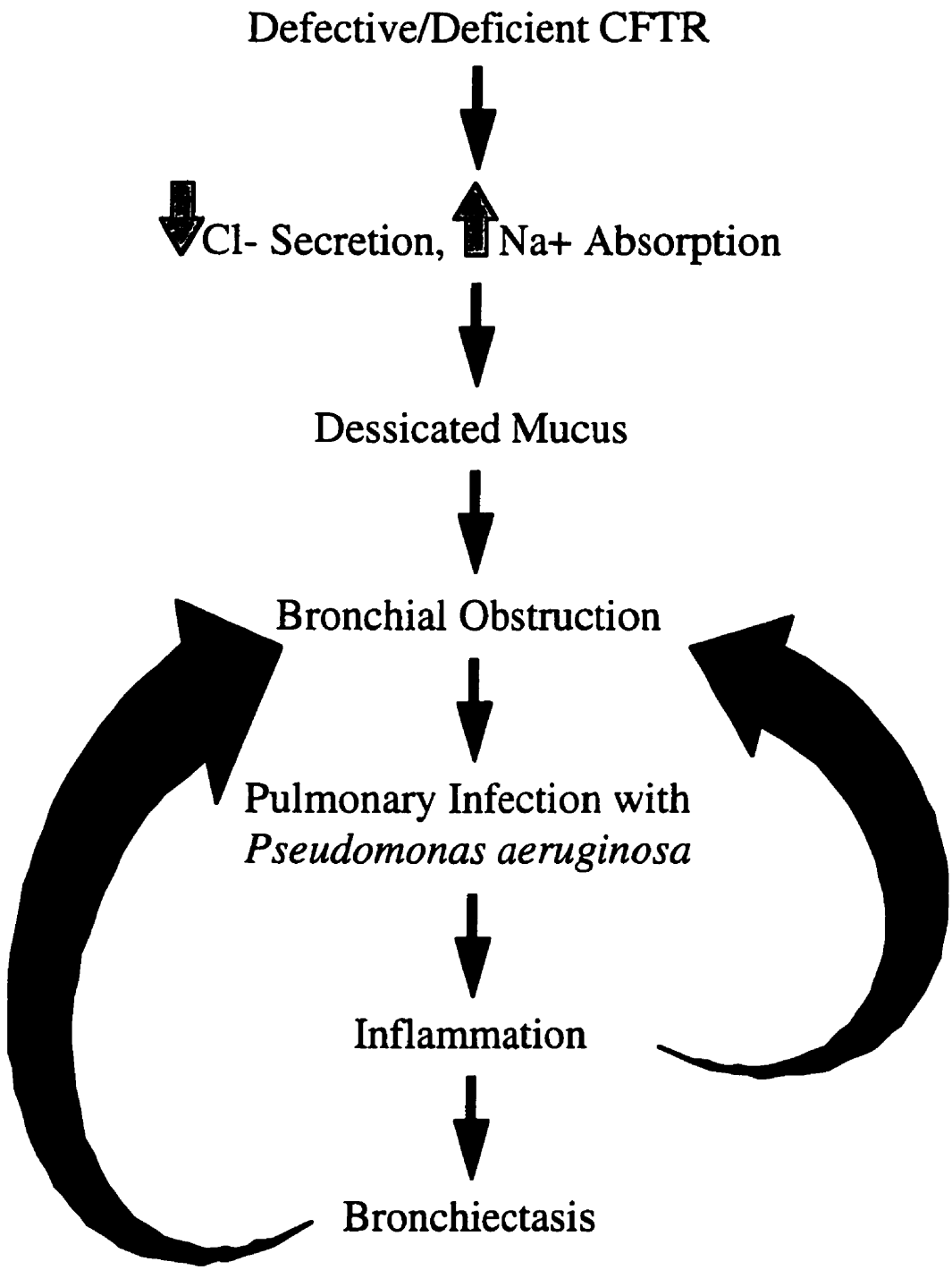


Fig. 1 Pathophysiological cascade of pulmonary disease in CF (modified from Davis *et al.*. Am. J. Respir. Crit. Care Med. 1996. 154: 1229-1256).

expression are incapable of ingesting *Pseudomonas aeruginosa in vitro* [Pier *et al.*, 1996a], [Pier *et al.*, 1996b]. Binding assays using isogenic mutants suggested that the outer-core oligosaccharide portion of the LPS as the principal ligand mediating uptake [Pier *et al.*, 1996a]. The defect was specific for *P. aeruginosa* and implicates epithelial cell ingestion and desquamation as an important innate defense mechanism. Although the predilection for *Pseudomonas aeruginosa* to preferentially colonize CF airways is not completely understood, the relationship between colonization and subsequent infection as they relate to CF pathogenesis is the focus of extensive research.

Although there remains some debate as to whether the pulmonary inflammation seen in CF is solely a result of infection or whether there may be an inherent immune defect (prompted mainly by studies that showed signs of inflammation in CF infants in the absence of infection [Armstrong *et al.*, 1995], [Khan *et al.*, 1995]), it has certainly been well established that accelerated decline in pulmonary function increases upon acquisition of mucoid phenotypes of *Pseudomonas* [Henry *et al.*, 1992], [Demko *et al.*, 1995]. In fact, a likely scenario explaining the clinical manifestations characteristic of CF has been suggested [Pier *et al.*, 1996b]. Initially, impaired mucociliary clearance and defective uptake by epithelial cells (lacking functional CFTR) of non-mucoid, smooth LPS isolates promotes initial colonization by *P. aeruginosa.* Following colonization, the bacterium changes its surface phenotype to express incomplete LPS (further protecting itself from ingestion) and produces numerous virulence factors directly damaging the airway. Finally, a vigorous and ineffective immune response ensues, contributing significantly to the respiratory damage typical of CF patients.

1.2.2 Virulence Factors

Despite being equipped with numerous virulence factors, *P. aeruginosa* is primarily an opportunistic human pathogen infecting only the immunocompromised [Buret and Cripps, 1993]. Factors such as pili and mucoid exopolysaccharide (MEP/alginate) have

both been shown to mediate adherence to buccal epithelial cells [Woods *et al.*, 1980], [Hata and Fick, 1991]. Other studies have suggested a role for alginate in the scavenging of reactive oxide intermediates [Simpson *et al.*, 1989], the impairment of both opsonic and non-opsonic phagocytosis [Marrie *et al.*, 1979], [Hahn and Kaufmann, 1981], and the general decrease in leukocyte function [Mai *et al.*, 1993]. Other enzymes such as elastase and alkaline protease are associated with inactivation of innate, humoral and cellular components of the immune response to *P. aeruginosa* [Kharazmi, 1989]. Suppression via these enzymes is mediated through proteolytic digestion and inactivation of: complement proteins [Schultz and Miller, 1974], cytokines such as interleukin-2 (IL-2) [Theander *et al.*, 1988], interferon-γ (IFN-γ) [Horvat *et al.*, 1989], tumor necrosis factor-α (TNF-α) [Parmely *et al.*, 1990] and immunoglobulin proteins [Fick *et al.*, 1985]. There are yet other factors such as leukocidin and exotoxin A which are cytotoxic to neutrophils and macrophages respectively [Scharmann *et al.*, 1976], [Pollack and Anderson, 1978]. Exotoxin A has also been shown to directly affect T cell function [Holt and Misfeldt, 1986]. Exoenzyme S is another virulence factor closely related to exotoxin A, however, insight into its role in immune deviation, activation, or suppression has remained, for the most part, undefined.

1.2.3 Exoenzyme S

1.2.3.1 Biochemistry

Two decades ago, exoenzyme S was first identified as an ADP-ribosyltransferase distinct from exotoxin A [Iglewski *et al.*, 1978]. Two proteins of different molecular mass co-purified with enzymatic activity. Under reducing conditions in SDS-PAGE gels, two immunologically related forms of exoenzyme S were isolated; the 53 kDa form (now termed Exo T or Exo 53) and the 49 kDa form (Exo S or Exo 49) [Nicas and Iglewski, 1984]. It has now been established that these two closely related forms of exoenzyme S (75% amino acid identity) are encoded by separate genes [Kulich *et al.*, 1995]. ADP-

ribosylation by exoenzyme S is dependent on the presence of the ubiquitous factor activating exoenzyme S (FAS) [Coburn and Gill, 1991]. Sequence analysis assigned FAS to be part of the widely conserved 14-3-3 family of proteins, which interestingly, are found only in eukaryotes and are primarily involved in cellular signal transduction [Fu *et al.*, 1993]. The primary *in vitro* targets for ADP-ribosylation by exoenzyme S are vimentin [Coburn *et al.*, 1989], an intermediate filament, and a number of low molecular weight GTP-binding proteins of the ras superfamily [Coburn *et al.*, 1989], [Coburn and Gill, 1991]. It should be noted that the *in vivo* targets of exoenzyme S have not yet been identified.

Despite the significant homology between the two forms of exoenzyme S, the rate of ADP-ribosylation by Exo T is only 0.2% of the rate by Exo S [Yahr *et al.*, 1996]. Deletion mapping experiments demonstrated that exoenzyme S possesses two functional domains. The amino-terminal domain consists of 99 amino acids and is required for aggregation and secretion while the carboxyl terminal domain encodes 222 amino acids responsible for enzymatic activity [Knight *et al.*, 1995]. However, studies with exoenzyme S to date have failed to identify a putative 'B' domain of a typical A:B bacterial toxin.

1.2.3.2 Regulon

Studies that characterized the exoenzyme S locus have shed light on the regulation of its production. Complementation analysis of *Pseudomonas aeruginosa* strain 388 *exs*::Tn1 mutants (exoenzyme S deficient) with the plasmid pDF100 resulted in restoration of exoenzyme S production and secretion [Frank and Iglewski, 1991]. Further mapping revealed the exoenzyme S regulon contained the structural gene for Exo S and Exo T as well as the trans-regulatory locus encoding several open reading frames [Frank and Iglewski, 1991]. The trans-regulatory locus consists of *exsA*, *exsB*, *exsC*, and *exsD*. ExsA appears to be the central regulatory factor for the production of exoenzyme S (Exo S and Exo T) [Goranson *et al.*, 1997], for the production of other exoproducts such as Exo

U [Finck-Barbancon *et al.*, 1997], and for a number of extracellular proteins thought to be involved primarily in the translocation and secretion of exoenzyme S [Goranson *et al.*, 1997].

Sequence analysis of the proteins encoded within the exoenzyme S regulon showed homology to exoproducts encoded by the *Yersinia* YOP virulon [Frank and Iglewski, 1991]. Similarly, exoenzyme S has also been shown to be secreted by a type III secretion system [Yahr *et al.*, 1996], [Frithz-Lindsten *et al.*, 1997]. This was demonstrated clearly in cytotoxicity assays which confirmed that both intact ExsA [Sawa *et al.*, 1997] and bacteria/host cell contact were needed to mediate delivery of exoenzyme S into sensitive eukaryotic targets [Apodaca *et al.*, 1995] [Olson *et al.*, 1997]. More recently however, exoenzyme S has been shown to act as an ecto-ADP-ribosyltransferase extending its physiological importance beyond the described type III delivery system [Knight and Barbieri, 1997].

1.2.3.3 Role of in Lung Disease

Much work has been done regarding the importance of exoenzyme S as a virulence factor in both acute and chronic *Pseudomonas aeruginosa* infections [Nicas and Iglewski, 1984], [Nicas *et al.*, 1985a], [Nicas *et al.*, 1985b], [Woods *et al.*, 1985], [Woods *et al.*, 1986], [Woods *et al.*, 1988], [Woods *et al.*, 1989], [Kudoh *et al.*, 1994]. Greater than 90% of sputum samples from CF patients contain exoenzyme S-producing isolates [Woods *et al.*, 1986]. Moreover, increases in pathology and mortality rates have been associated with exoenzyme S producing strains compared to non-producers in the rat model of chronic *P. aeruginosa* lung infections [Woods and Sokol, 1985], indicating that exoenzyme S plays a prominent role. Most noticeably, increased exoenzyme S production in the lungs of CF patients chronically colonized with *P. aeruginosa* was accompanied by acute deterioration of pulmonary function [Grimwood *et al.*, 1993]. Other CF studies have shown that exoenzyme S levels (but not bacterial load) fall following antibiotic treatment of purulent

exacerbations correlating with clinical amelioration [Grimwood *et al.*, 1989], [Grimwood *et al.*, 1993]. Together, these studies demonstrate that exoenzyme S must be considered a principal virulence factor capable of causing significant morbidity in CF patients.

1.2.4 Host Defense to

A number of different immune cells are involved in effective host defense to *Pseudomonas aeruginosa.* Central to this are alveolar macrophages and polymorphonuclear neutrophils. Much work has been done demonstrating the importance of these leukocytes in the phagocytic killing of *Pseudomonas aeruginosa* [Speert *et al.*, 1984], [Rosales and Brown, 1991], [Sundaram *et al.*, 1991], [Buret and Cripps, 1993]. Clearly however, our knowledge of the inflammatory process in response to *Pseudomonas aeruginosa* is incomplete as strategies aimed at controlling it have had limited success. In an effort to increase our knowledge, studies have examined the contribution of other immune cells in host defense to this bacterium. Researchers have begun focusing more attention to the role of T lymphocytes for many reasons. Firstly, the number of T lymphocytes in the lung can increase several-fold in the course of immune reactions against inhaled antigens [Weissman *et al.*, 1994] suggesting they play an important role in host defense in the lung. Secondly, a major role of these T cells is to produce lymphokines capable of activating alveolar macrophages, natural killer cells and polymorphonuclear cells for efficient killing of infectious agents [Platsoucas, 1982], [Brummer and Stevens, 1984], [Murray *et al.*, 1985]. These studies have demonstrated the importance of a functional arm of cellular immunity in pulmonary defense.

The importance of cell mediated immunity (CMI) in pulmonary defense to *P. aeruginosa* has previously been well established. The role of cellular immunity as a mechanism of host defense to *P. aeruginosa* has come from studies showing that T cells proliferate to whole organisms *in vitro* [Porwoll *et al.*, 1983], [Markham *et al.*, 1985]. Other researchers have also demonstrated that T lymphocytes play an important *in vivo* role

in host defense to infection with *P. aeruginosa* as it has been shown that adoptive transfer of T lymphocytes from immune animals can confer protection to non-immune recipients, even in the absence of granulocytes [Powderly *et al.*., 1986]. A study from Markham and colleagues demonstrated that splenic T cells isolated from immune mice are capable of significant killing *in vitro* upon reexposure to *P. aeruginosa* [Markham *et al.*, 1984]. Although macrophages were necessary for T cell-mediated *in vitro* killing, they neither functioned as antigen presenting cells nor as phagocytic cells. The killing ability of the T cells was non-specific as it was shown that T cells harvested from the spleens of mice immunized with *P. aeruginosa* were able to kill *E. coli* and *S. aureus in vitro* with the same efficiency [Markham *et al.*, 1984]. Interestingly, the presence of macrophages was not necessary during the *in vitro* exposure to the bacteria, but a prior interaction with immune T cells was obligatory for optimal bactericidal affect. In a subsequent study, it was demonstrated that the ability of macrophages to enhance T cell killing of *Pseudomonas aeruginosa* could be replaced by exposure to recombinant IL-1 [Markham *et al.*, 1985]. To date however, the lymphokine responsible for the direct T cell-mediated killing of *Pseudomonas aeruginosa* has not been identified. Although, T cell production of interferon-γ has been shown to possess anti-viral capabilities [Morris *et al.*, 1982], [Leist *et al.*, 1989], the notion that T cells produce bactericidal lymphokines is relatively novel. Therefore, in addition to the well characterized function of T cells as immunoregulators (via the release of cytokines), T cells must also be recognized for their direct ability to kill extracellular bacteria such as *Pseudomonas aeruginosa.*

Although no primary immune deficiency appears to be associated with CF [Davis, 1985], [Pier, 1985], patients invariably fail to clear the organism from the lungs. In this regard, studies have shown that ineffective *in vitro* lymphocyte responses to *P. aeruginosa* correlate with advanced pulmonary disease in CF patients [Sorensen *et al.*, 1977], [Sorensen *et al.*, 1978], [Sorensen *et al.*, 1979], [Sorensen *et al.*, 1981], suggesting that secondary defects in cellular immunity may be contributing to the pathogenicity of

Pseudomonas aeruginosa infection. Unfortunately, strategies to prevent or control infection are in part limited because the mechanisms responsible for the unsuccessful eradication of the bacteria are still poorly understood. Therefore, the possibility that a failure of local T cell immunity may account for persistent pulmonary infection in CF is of considerable interest and must be thoroughly investigated. The purpose of these studies was to characterize the T lymphocyte response to exoenzyme S in order to shed light on the potential physiological contribution of this exoproduct to the pathogenicity of *Pseudomonas aeruginosa* chronic lung infection.

1.3 T Cells

1.3.1 T Cell Activation and Proliferation

Activation of resting T cells is central to establishing their regulatory and effector functions. Normally, activation of quiescent T cells by antigen (Ag) results in their differentiation and transition from Go/G1 to S phase of the cell cycle. Each T cell expresses a single α/β (or γ/δ) heterodimeric T cell receptor (TCR) and hence every T cell has only one antigenic specificity. The physiological consequence of this is that recognition of a particular foreign antigen activates only a small subset of the greater than 10^{16} T cell clones [Davis and Bjorkman, 1988]. Therefore, soon after activation and differentiation, the percentage of responding antigen-specific T cells increases and allows for a more efficient clearance of the antigen.

The T cell receptor, unlike surface immunoglobulin on B cells (BCR), can only recognize Ag presented by antigen presenting cells (APC) in the context of major histocompatability molecules (MHC) [Katz *et al.*, 1973], [Zinkernagel and Doherty, 1979]. Therefore, T lymphocyte recognition of foreign antigen is a result of the complex interaction between TCR, antigen, and MHC. However, efficient activation resulting in effector T cell functions (i.e. T cell help for B cells, cytokine secretion, or cytotoxicity) requires multiple receptor-ligand interactions occurring between the T cell and APC [van

Seventer *et al.*, 1991], [Mondino and Jenkins, 1994], [Wingren *et al.*, 1995]. It should be noted that the requirement of these interactions for proper T cell activation is not empirical; studies have shown that different antigens, antigen presenting cells, and for that matter, different effector functions require some, but not all of these signals [Springer *et al.*, 1987], [van Seventer *et al.*, 1990], [Lucas *et al.*, 1995], [Bachmann *et al.*, 1996], [Kundig *et al.*, 1996], [Bachmann *et al.*, 1997]. Furthermore, the absence of one or more costimulatory molecules necessary for the response may deliver a negative signal to the T cell resulting in clonal unresponsiveness (anergy) [Schwartz, 1990].

T cell recognition of antigen in the context of MHC and in the presence of proper costimulatory molecules delivers a cascade of intracellular signal transduction events which typically culminates in cytokine production and proliferation [Crabtree, 1989]. The T cell receptor is associated with, and signals via, proteins of the CD3 complex (consisting of a $\gamma\varepsilon$ heterodimer, a $\delta\varepsilon$ heterodimer and a $\zeta\zeta$ homodimer) [Clevers *et al.*, 1988], [Alarcon *et al.*, 1991]. Four main and interrelated events occur after antigen presentation to T cells; 1) phosphorylation of membrane proteins by protein tyrosine kinases (PTK), 2) phophatidylinositol membrane hydrolysis, 3) phosphorylation of cytosolic proteins by protein kinase C (PKC), and 4) increases in cytoplasmic Ca^{2+}.

One of the earliest events which occurs immediately after TCR stimulation is the translocation and docking of $p56^{lck}$ from the intracellular domain of the CD4 or CD8 molecule to one of the three immunoreceptor tyrosine-based activation motifs (ITAMs) found on the cytoplasmic component of the ζ chains of the TCR/CD3 complex [DeFranco, 1995]. This allows the docking of $p59^{fyn}$ (also a PTK) via its src-homology domain which recognizes the newly phosphorylated tyrosine residues on the ζ chain. After binding, it too is phosphorylated and activated. Presumably, this allows for the direct or indirect docking, phophorylation and activation of the third PTK, ZAP-70. In a still poorly defined mechanism, ZAP-70 may couple the TCR to more distal intracellular signalling events such as the activation of phophatidylinositol phopholipase C-γ1 (PI-PLC-γ1), perhaps through a

G-protein intermediate [Peter *et al.*, 1992], [Sancho *et al.*, 1993]. PI-PLC-γ1 catalyzes the hydrolysis of the phospholipid phophatidylinositol 4,5-bisphophate ($PtdInsP_2$) which results in two breakdown products; inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The former is important in the release of intracellular stores of sequestered Ca^{2+} while the latter activates PKC. Elevated calcium favors its association with calmodulin which is necessary for the activation of the phosphatase, calcineurin. Ultimately, calcineurin phosphorylates cytoplasmic nuclear factor activating T cells ($NFAT_c$) which translocates into the nucleus and acts as a co-transcriptional activator of early gene expression. In addition to inducing the release of the transcriptional factor NF-κB from its repressor and its subsequent nuclear translocation, another immediate downstream target of PKC is the G-protein Ras, which has been shown to activate a number of signal-transducing effectors [Izquierdo *et al.*, 1992]. One of the best characterized effectors is Raf-1 [Avruch *et al.*, 1994] although others have also been identified [Deng and Michael, 1994], [Rodriguez-Viciana *et al.*, 1994], [Yan *et al.*, 1994]. After GTP binding and activation in the cytoplasm, Raf-1 has been shown to induce transcription of the cellular protooncogene *c-fos* [Kortenjann *et al.*, 1994]. The activating protein-1 (AP-1) transcription factor is composed of the homodimer fos/fos or of the heterodimer fos/jun [Curran and Franza, 1988] and in tandem with NFAT and NF-κB bind to their respective consensus sequences in the IL-2 promoter and induce IL-2 gene transcription [Crabtree, 1989]. IL-2 is a powerful T and B cell growth factor which can work in an autocrine and paracrine fashion, bind to surface IL-2 receptors, and promote growth and differentiation in response to antigenic stimulation [Greene, 1986]. It should be noted that the requirement for transcriptional factors is specific for the gene in question and therefore only certain genes are transcribed while others are not. The determination of which genes are transcribed therefore depends on the transcriptional factors available, and more specifically on the signalling pathway(s) used to initiate the production/activation of these factors.

Other stimuli that have a unique mechanism of T cell activation different from typical antigens, are also capable of inducing T cell proliferation and differentiation and are termed mitogens and superantigens. Mitogens are typically plant lectins which polyclonally induce massive T lymphocyte proliferation of naive T cells [Nowell, 1960]. The exact mechanism of T cell activation is not understood, however is thought to be the summation of binding of these lectins to a number of distinct molecules in addition to the TCR [Mueller *et al.*, 1989]. The response requires accessory cells but does not require major histocompatability complex (MHC) molecules [Sopori *et al.*, 1987], [Halvorsen *et al.*, 1988]. Superantigens on the other hand, are produced by some bacteria and viruses and are a unique type of mitogen because they stimulate T cells based on the expression of the Vβ chain of the TCR [Kappler *et al.*, 1987]. Superantigens induce T cell proliferation by crosslinking MHC class II molecules of accessory cells with the Vβ chain of the TCR [Dellabona *et al.*, 1990]. Because of these mechanisms, the frequency of responding T cells to mitogens and superantigens is approximately 1:10 and 1:20 respectively, compared to $1:10^{4}$-10^{5} for recall antigens [Vose and Bonnard, 1983], [Licastro *et al.*, 1993], [Scherer *et al.*, 1993]. The physiological consequences of mitogenic activation of T cells (by mitogens or superantigens) are significant. Recently, the possibility that bacterial and viral mitogens contribute to host evasion *in vivo* has been proposed. Unlike antigens that are generally capable of generating an effective immune response leading to clearance of the organism, mitogens induce a transient state of hyperactivation resulting in the release of a number of cytokines which promote a non-specific inflammatory state [Miethke *et al.*, 1993], [Scherer *et al.*, 1993]. After this initial stage, lymphocytes are rendered unresponsive (anergic) [Ignatowicz *et al.*, 1992], or are signaled to undergo apoptosis [Jenkinson *et al.*, 1989], [Damle *et al.*, 1993], [McCormack *et al.*, 1993], effectively reducing their effector functions. Mitogens therefore, tend to induce an overall impairment of T cell function and cell mediated immunity.

It has previously been shown that greater than 90% of adult volunteers had a proliferative response to exoenzyme S [Mody *et al.*, 1995] suggesting that exoenzyme S may be a mitogen. The possibility that exoenzyme S may be stimulating T cells as a mitogen has numerous implications with respect to the pathogenesis of *Pseudomonas aeruginosa* infection in CF. Firstly, a local and non-specific inflammatory response resulting from hypersecretion of lymphokines, such as interferon-γ or the colony stimulating factors from exoenzyme S-activated T cells in the pulmonary compartment may hyperactivate other immune cells including alveolar macrophages and neutrophils as well as alveolar epithelium. In turn, these cells may also contribute to the inflammation by the secretion of proinflammatory cytokines, reactive oxide intermediates, and chemokines. Together, an exaggerated and harmful inflammatory response ensues which may, in a feedback mechanism, lead to a cycle of further recruitment and activation of other lymphocytes, monocytes, and neutrophils from circulation. This scenario may contribute to the chronic pulmonary inflammation characteristic of CF patients. In this regard, it has recently been shown that PAOexsA::Omega (an exoenzyme S mutant) induced significantly less IL-8 (neutrophil chemokine) compared to the wildtype strain in a bioassay, suggesting that partial production of this proinflammatory cytokine can be directly attributable to exoenzyme S [Tang *et al.*, 1996]. It has previously been shown that the high morbidity and mortality rates related to *P. aeruginosa* infection seen in CF patients is in large part be due to lung damage caused by substances released by neutrophils during activation and phagocytosis [Berger *et al.*, 1991]. On the other hand, endobronchial inflammation induced by *P. aeruginosa* infection varies in resistant and susceptible strains of mice [Morissette *et al.*, 1995]. More precisely, the lack of recruitment of inflammatory cells into the airways is seen selectively in susceptible strains of mice and correlates with increased bacterial burden and mortality rates. Therefore, there seems to be a delicate balance between the initiation of a controlled inflammatory response capable of competent clearing of the airspace and one which is deregulated, non-specific and results in chronic inflammation and tissue

destruction. Thus, the notion that T cells are important orchestrators of immune responses and serve as a link between the specific and innate arms of the immune system, underlies the importance of understanding their role in the pulmonary inflammation seen in CF patients.

Another functional consequence of mitogenic activation of recruited T cells by exoenzyme S may be to induce T cell apoptosis or induce a state which renders them anergic. This leads to a functional inactivation of recruited T cells. This inability of T cells to exert their effector functions may also contribute to the impaired ability of these patients to clear *Pseudomonas aeruginosa* from their lungs.

1.3.2 CD69 Expression

'T cell activation' is an ambiguous term unless one defines its meaning. That is, activation may refer to any one of a number of events a T cell may undergo including: tyrosine phosphorylation, G-protein activation, cytoplasmic alkalinization, Ca^{2+} flux, protooncogene expression, cytokine secretion, upregulation of surface molecules, blast transformation (proliferation), and apoptosis to name a few. Depending on the activation marker being investigated and its relative proximity to the initial stimulus, one can infer the relative state of activation of the T cell. In other words, if a T cell underwent blast transformation in response to a given stimulus, virtually all other events in the cascade would have also had to occur, as they are upstream and contingent for T cell proliferation [Crabtree, 1989]. Normally, such a T cell would m*eet al.*l the definitions for 'T cell activation'. On the other hand, certain stimuli may only induce a few, but not all activation events; typically, signals most proximal to the stimulus event would remain intact while latter, more distal events such as proliferation may be absent [Mueller *et al.*, 1989], [Paine *et al.*, 1991], [Rabinowitz *et al.*, 1996]. Alternatively, negative signals may be transduced leading to T cell anergy [Schwartz, 1990].

One of the earliest cell surface molecules expressed by T cells following activation is CD69 [Cebrián *et al.*, 1988]. It is expressed as a 60 kDa orphan receptor which contains an external C-type lectin domain capable of binding many different sugar moieties [Ziegler *et al.*, 1994]. Transcripts are detectable after 30 minutes and decline by 4-6 hours, while surface expression is detectable by 2 hours and decreases with a $T_{1/2}$ of 24 hours if the stimulus is withdrawn [Testi *et al.*, 1989]. The key signalling event is Ras activation and more specifically, activation of the downstream effector Raf-1 [Taylor-Fishwick and Siegel, 1995]. Interestingly, CD69 transcription has recently been shown to be dependent on AP-1 which is activated by Raf-1 [Castellanos *et al.*, 1997]. This is the first description which identifies the link between TCR stimulation and upregulation of CD69. Alternatively, CD69 transcription can be induced after culture with cytokines such as TNF-α through an NF-κB-dependent mechanism [López-Cabrera *et al.*, 1995]. However, the kinetics of TNF-α-induced expression of CD69 is delayed (appearing at 16 hrs.), and is dependent on high doses of TNF-α exposure (50 ng/ml) [López-Cabrera *et al.*, 1995]. Nonetheless, this clearly demonstrates a redundancy in the regulation of CD69; different stimuli and therefore different signal transduction pathways lead to the activation of distinct transcriptional regulators, each capable of binding to their respective consensus sequences in the CD69 promoter. CD69 expression has been shown to be a prerequisite for proliferation under most circumstances [Risso *et al.*, 1991], [Caruso *et al.*, 1997] and has also been correlated with T cell apoptosis [Kishimoto *et al.*, 1995], [Wu *et al.*, 1997] consistent with the fact that CD69 expression can be induced via any number of 'private' signal transduction pathways.

1.3.3 T Cell Apoptosis

As mentioned previously, mitogens and superantigens are capable of inducing T cell apoptosis [Jenkinson *et al.*, 1989], [Damle *et al.*, 1993], [McCormack *et al.*, 1993]. Apoptosis is a form of cell death which is distinct and mutually exclusive from necrosis

[Darzynkiewicz *et al.*, 1997]. It is an active and physiological mode of cell death, in which the cell itself designs and executes the program of its own demise. Events associated with necrosis are mitochondrial swelling, plasma membrane rupture, and release of cytoplasmic constituents into the extracellular milieu [Majno and Joris, 1995]. Unlike necrosis (thought of as 'cell murder'), apoptosis (also called 'cell suicide') can be identified by cell shrinkage, membrane phospholipid translocation, condensation of nuclear chromatin, DNA degradation into multiples of 180 base pairs, and the formation of apoptotic bodies, although not all characteristics need to be present [Darzynkiewicz *et al.*, 1997]. In late stage apoptosis, the plasma membrane becomes ruffled and begins to bleb in a way more pronounced than for necrosis. The cell begins to break up into apoptotic bodies which are packets of cytosol and DNA surrounded by intact plasma membrane [Cohen, 1993]. Importantly, these bodies are sealed and maintain their osmotic gradients and hence no spilling of noxious intracellular constituents is allowed, which is one of the main differences between necrosis and apoptosis [Cohen, 1993]. The apoptotic process can be divided into three functionally distinct phases: initiation, effector, and degradation [Kroemer *et al.*, 1997]. During the initiation phase, cells receive their death inducing signals which are specific for the lethal stimulus, while during the effector phase, activation of endogenous proteases (caspases) occurs and the 'decision to die' is taken. During the degradation phase, an increase in overall entropy occurs due to substantial protein degradation and DNA fragmentation.

The most physiologically important form of T cell apoptosis is activation-induced cell death (AICD) [Kabelitz *et al.*, 1993], which is triggered through TCR/CD3 stimulation. In its several forms it is central to the development, regulation and function of the immune system and is particularly important in the generation and maintenance of immune tolerance [Smith *et al.*, 1989], [Liu and Janeway, 1990], [Russell *et al.*, 1991], [D'Adamio *et al.*, 1993]. AICD is necessary for clonal downsizing and therefore is thought to be important in the downregulation of the immune response to antigenic stimulation [Crispe, 1994].

Importantly, there is an ever increasing body of literature demonstrating the capabilities of bacteria (or their products) to induce apoptosis in a number of different immune cells [Zychlinsky *et al.*, 1992], [Kochi and Collier, 1993], [Wahl *et al.*, 1993], [Chen *et al.*, 1996], [Monack *et al.*, 1996], [Rogers *et al.*, 1996] [Monack *et al.*, 1997]. Therefore, the deregulation of apoptosis can be exploited by bacteria and should be considered as a novel and important mechanism of host evasion.

Importantly, with respect to the pathogenesis of *P. aeruginosa* infection in CF, there is evidence to suggest that recruited T cells may be predisposed to apoptosis compared with circulating lymphocytes. Although most of the T cells in the lung are thought to be recruited from the circulation, lung and blood T cells differ in a number of important respects. Firstly, virtually all lung T cells express the memory phenotype (i.e. CD45RO), while approximately equal numbers of T cells express either memory or naive (i.e. CD45RA) phenotypes in the circulation [Dominique *et al.*, 1990], [Saltini *et al.*, 1990], [Strickland *et al.*, 1993]. Other, more recent markers of activation, such as HLA-DR and VLA-1, are also preferentially expressed on lung T cells [Davidson *et al.*, 1985], [Yamaguchi *et al.*, 1990]. Down-modulation of the TCR-CD3 complex, known to be associated with recent activation, is also a characteristic of T cells isolated from the lung [Yamaguchi *et al.*, 1990]. Together, these results suggest that lung T cells possess an activated phenotype compared to peripheral T cells. Importantly, these phenotypic differences between lung and blood T cells have been associated with a number of *in vitro* differences in T cell function. Compared to circulating T cells, lung T cells have been shown to respond poorly to some proliferative stimuli [Holt *et al.*, 1988], [Lecossier *et al.*, 1988] and have also been shown to produce skewed patterns of cytokine secretion [Holt *et al.*, 1988], [Garlepp *et al.*, 1992]. Because activated T cells are hypersensitive to the induction of AICD [Kabelitz *et al.*, 1993], [Crispe, 1994], it may also be true that lung T cells may be more prone to apoptotic death induced by certain stimuli than would T cells from peripheral circulation. Indeed, Herry and colleagues have recently demonstrated that

lavage T cells isolated from healthy adults showed a significantly greater loss of viability following *in vitro* culture compared to peripheral T cells, and that this loss was attributable to increased induction of apoptosis [Herry *et al.*, 1996]. The possibility then exists that T cells which are recruited to the lung in individuals with CF and exposed to *P. aeruginosa* and its exoproducts may have a predisposition to undergo AICD, assuming that T cell exposure to these exoproducts can induce heightened T cell activation.

1.4 Summary

Previous studies from our laboratory reported that a purified preparation of exoenzyme S induces T cells to proliferate in over 95% of adults [Mody *et al.*, 1995]. The high frequency of adult responders suggests that exoenzyme S may be stimulating T cells as a mitogen or superantigen. If exoenzyme S were a mitogen or a superantigen, it might have quite different clinical implications for the CF patient than if it were an antigen. As a result, the large percentage of lymphocytes could initiate an inflammatory reaction rather than an antigen specific immune response. This could promote two interrelated events; firstly, it may contribute to the chronic and deleterious state of inflammation seen in the lungs of CF patients through the release of proinflammatory cytokines, and secondly, it may impair host clearance of the organism via induction of T cell anergy or apoptosis.

1.5 Specific Aims

The **objective of this research** was to determine the mechanism of T cell activation by exoenzyme S. This study contains three **specific aims**:

1) To determine whether exoenzyme S activates T cells as an antigen, superantigen, or as a mitogen (sections 3.1 - 3.3)

2) To investigate one of the consequences of activation of T cells by exoenzyme S (healthy adults - section 3.4.1; CF patients - section 3.4.2)

3) To examine whether rExoS from *Pseudomonas aeruginosa* strain 388 and ExoS/DG1 induce a similar mode of T cell activation (section 3.5)

2.0 METHODS AND MATERIALS

2.1.1 Purification of Exoenzyme S from *Pseudomonas aeruginosa* Strain DG1

Purification was performed as previously described [Woods and Que, 1987]. Briefly, *P. aeruginosa* DG1 was grown in aerated S media containing 1 g NH_4Cl, 3 g Na_2HPO_4, 5 g NaCl, 0.1 g $MgSO_4$, and 27 g sodium succinate (per liter of distilled water) for 18 hours at 32°C. Cultures were then centrifuged at 4°C for 20 minutes at 10,000 x g. Ammonium sulfate was added slowly to the culture supernatant to 60% saturation and incubated overnight at 4°C. Supernatants were then centrifuged at 15,000 x g for 30 minutes at 4°C to obtain the precipitated protein. The precipitate was dissolved in 100 ml of 0.05 M Tris hydrochloride buffer (pH 8.0) and dialyzed overnight at 4°C against 6 liters of the same buffer. The dialyzed material was then applied to a DEAE-Sephacel column (Pharmacia) previously equilibrated in Tris buffer. Elution was performed using a linear gradient of 0.01 to 1.01 M NaCl in Tris buffer. Protein containing fractions were collected by measuring the absorption at 280 nm. The fractions eluting at 0.4 to 0.5 M NaCl were then pooled and additional NaCl was added to make the final concentration 1 M. This solution underwent acetone precipitation (acetone previously cooled to -20°C) in a ice-salt bath, never allowing the solution temperature to rise above 3°C. When the acetone concentration reached 33%, the solution was allowed to cool to 0°C and equilibrated for 15 minutes. The acetone solution was centrifuged at 5,000 x g for 20 minutes at 0°C and the precipitate was redissolved in a small volume of Tris buffer and dialyzed overnight at 4°C in 6 liters of the same buffer. The dialyzed material was finally applied to a G-100 gel filtration column previously equilibrated in Tris buffer and protein containing fractions were detected by measuring the A_{280}. Endotoxin levels were measured using the *Limulus* amebocyte lysate kit (Associates of Cape Cod; Woods Hole, Maryland) as per manufacturer's protocol and determined to be less than 0.2 μg per μg of exoenzyme S. This

concentration of LPS does not cause T lymphocyte activation (CD69) at 4 hours or proliferation (^{3}H-TdR incorporation).

2.1.2 Purification of Recombinant Exoenzyme S from *Pseudomonas aeruginosa* Strain 388

Recombinant exoenzyme S was purified from *P. aeruginosa* strain PA103 inserted with the pUCP*exos* expression vector as previously described [Knight *et al.*, 1995] and was a kind gift from Dr. Frank and Dr. J. Barbieri (U. of Wisconsin). The former preparation will be referred to as exoenzyme S (Exo S/DG1) and the latter as recombinant exoenzyme S (rExo S).

2.1.3 Antibodies

The monoclonal antibody, 9-49-9, was raised against exoenzyme S purified from *P. aeruginosa* DG1 [Woods and Que, 1987]. Briefly, purified exoenzyme S was dialyzed against 0.1 M Tris buffer (pH 8.0) containing 1% formalin and 0.2 M l-lysine (Calbiochem-Behring, La Jolle, California) at 37°C for 72 hours. This toxoid preparation was again dialyzed against 0.2 M L-lysine-saline solution (0.85% NaCl, pH 6.3) at 4°C for 48 hours and used as the immunogen for mAb production. BALB/c mice were injected four times intraperitoneally with 50 μg/ml exoenzyme S toxoid on days 0, 7, 14, and 21. Three days after the final injection, spleens were removed and splenocytes fused with NS-1 cells (non secreting myeloma cell line; 10:1) using 50% (wt/wt) polyethylene glycol. Fused cells were then transferred into microtiter plates at a density of 6×10^4 in HAT medium for 14 days. The tissue culture supernatants were then tested for the presence of monoclonal antibody to exoenzyme S by ELISA. Hybridomas producing exoenzyme S mAbs were transferred to HT medium and cloned by limiting dilution. 2×10^6 cells were injected into pristane primed mice for ascites tumor induction and ascites fluid collected.

2.1.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970). Proteins were separated using a discontinuous gel system; 10 or 15% acrylamide resolving gels and 4% stacking gels were utilized. Gels were prepared from stock solutions of 30% acrylamide plus 0.8% N,N, 19-methylene-bisacrylamide, water, the catalyst N,N,N',N'-tetramethyl ethylene diamine (TEMED) and the initiator ammonium persulfate which was made fresh each time. Proteins were reduced in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue) and boiled for 5 minutes. Samples were then loaded in the gels and subjected to electrophoresis using the Mini-Protean II vertical electrophoresis cell system (Bio-Rad). The running buffer was made (25mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.3) from five-fold stock solution. Samples were run at room temperature at a constant voltage of 120V and visualized using Coomassie brilliant blue stain.

2.1.5 Immunoblotting

Samples which were run on SDS-PAGE gels and not stained with Coomassie were electrophoretically transferred to methanol activated immobilon P paper (Millipore, Bedford, Massachusetts) at 10V overnight at 4°C. Again, the Mini-Protean II vertical electrophoresis cell system was used. Towbin's transfer buffer (25 mM Tris-base, 192 mM glycine, 0.1% SDS, 20% methanol) was used for overnight transfer. Transfer was verified by visualization of prestained molecular weight markers (Sigma). The immobilon was blocked using 0.5% milk in Tris buffered saline (TBS - 0.9% NaCl, 20 mM Tris-HCl, pH 7.5) for 30 minutes at 32°C or up to an hour at room temperature and washed 3-5 times (5 minutes each) in TBS before detection. The immobilon was incubated for 45 minutes at 32°C in the presence of the monoclonal antibody 9-49-9 previously diluted at 1:1000. Excess antibody was washed off as before with TBS. Peroxidase-conjugated polyvalent

goat anti-mouse (Sigma) was used at 1:2000 as the secondary antibody and incubated as above. Chloronapthol (Biorad, Richmond, California) in methanol (30mg in 10 mls) was mixed with H_2O_2 in TBS (300µl in 50 mls) and used as the chromogenic substrate.

2.1.6 ADP-ribosyltransferase Assay

The sugar transfer capacity of exoenzyme S preparations was determined using a previously described method [Iglewski and Sadoff, 1979]. Briefly, 10 µl of exoenzyme S was mixed with 25 µl reaction buffer (50 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 40 mM DTT), and 5 µl of C^{14} NAD in triplicate 5 ml tubes. As a source of FAS, 25 µl wheat germ extract was added. The reaction took place at 24°C for 20-30 minutes at which point the reaction was stopped by addition of 100 µl 10% TCA. Tube contents were filtered through a millipore apparatus and washed with two volumes of 5% TCA. Counts per minute were determined by liquid scintillation and is a reflection of the amount of ADP-ribose (radiolabelled) that is transfered to TCA precipitable proteins from NAD. Tubes containing reaction buffer and radiolabelled NAD were used to determine background counts.

2.1.7 Cellular Isolation

2.1.7.1 Peripheral Blood Mononuclear Cells and Fetal Blood Mononuclear Cells

Peripheral blood was obtained by venipuncture from healthy adults. PBMC were isolated by centrifugation (800 x g, 20 min.) over Ficoll-Hypaque density gradient (C-six Diagnostics Inc., Mequon, Wisconsin). Mononuclear cells were harvested and washed three times in Hank's balanced salt solution (HBSS) (Gibco, Burlington, Ontario). Blood was also collected from the umbilical vein of fresh human placenta and fetal blood mononuclear cells (FBMC) were isolated in similar manner as PBMC. Residual red blood cells were removed by a 3-5 minute lysis treatment (0.15 M NH_4Cl, 0.01 M $NaHCO_3$, 0.001 M EDTA). Viable cells were then counted by trypan blue exclusion as visualized by light microscopy. Cells were resuspended in medium containing RPMI 1640 (Gibco), 5%

human AB serum (BioWittaker, Walkersville, Maryland), 100 units/ml of penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (all from Gibco).

2.1.7.2 T Lymphocytes and Monocytes

To isolate T cells, PBMC were plated in petri plates (Corning Glass Works, New York, New York) in the presence of RPMI for 1 hour at 37°C and non-adherent populations were collected by rinsing twice in medium. The non-adherent cells were then rosetted to 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC; Cedarlane, Hornby, Ontario) as previously described [Kanof, 1996] with minor modifications. Briefly, 20 ml AET solution was added to 5 ml washed SRBC for 15 minutes at 37°C. AET-treated SRBC was made to 5% by addition of 20% HS-RPMI. AET-SRBC was added to non-adherent cells (<10 x 10^6 cells/ml) at a 1:2 ratio. The suspension was incubated at 37°C for 10 minutes, centrifuged (500 x g, 10 min.), and refrigerated overnight. Rosette positive cells were finally passed through a nylon wool column [Julius *et al.*, 1973] and non-adherent cells were collected. T cells isolated in this fashion were typically >95% $CD3^+$ as analyzed by flow cytometry. Cells that were adherent to plastic after a 1 hour incubation and irradiated (3000 rads) were used as a source of accessory cells.

To obtain $CD45RA^+$ and $CD45RO^+$ enriched cells, non-adherent cells were depleted of CD45RO or CD45RA cells by immunomagnetic separation. Briefly, non-adherent cells were incubated with either anti-CD45RA (L48) or anti-CD45RO (UCHL-1) antibody (Becton Dickinson) for 30 minutes at 4°C under gentle agitation. The cells were then centrifuged (800 x g, 10 min.) and the supernatant discarded. The cells were washed three times in PBS containing 2% fetal calf serum (Gibco). M-450 conjugated goat anti-mouse antibody (Dynabeads, Dynal, Oslo, Norway) was added at bead to target cell ratio of 3:1 for 10 minutes at 4°C under gentle agitation. The labelled cells were removed using a

magnet (Dynal). The enriched populations contained <3% contaminating cells of the reciprocal subset as analyzed by flow cytometry. Irradiated adherent cells (3000 rads; 1 x 10^5) were added (1:1) to the enriched cells as a source of accessory cells.

2.1.8 T Lymphocyte Activation

1 x 10^6 cells were washed 3 times in cold wash PBS (PBS with 1% FCS, 0.1% NaN_3) and mixed with a saturating amount of the appropriate monoclonal antibody. Fluorescein-isothyocyanate (FITC)-conjugated anti-CD25 (anti-IL-2 receptor) (Becton Dickinson, San Jose, California) or FITC-conjugated anti-CD71 (anti-transferrin receptor) (Becton Dickinson) was added in conjunction with peridinin chlorophyll (PerCP)-conjugated anti-CD3 (Becton Dickinson). An antibody cocktail containing phycoerythrin (PE)-conjugated CD69/CD3 PerCP (Becton Dickinson) was also used to measure early T cell activation. Appropriate FITC-conjugated or PE-conjugated isotype controls (all from Becton Dickinson) were used in conjunction with CD3-PerCP in all experiments. After 4 hours cultured cells were labelled with anti-CD69-PE/anti-CD3-PerCP (Becton Dickinson). T cells were gated on their side scatter versus CD3-PerCP profile and then analyzed for the expression of the activation markers CD69, CD25, or CD71. Positive cells were defined as those with a fluorescent intensity beyond the margin established on the negative controls. The percentage of cells above this threshold was subtracted from percentage of positive cells in experimental groups. In some experiments, FITC-conjugated anti-Vβ mAbs (Vβ-2, -3, -5a, -5b, -5c, -6, -8, -12, -13; all from T Cell Diagnostics) or FITC-conjugated anti-CD4 or anti-CD8 (Becton Dickinson) were used in conjunction with CD69-PE/CD3-PerCP for simultaneous three color immunolabelling. FITC-conjugated and PE-conjugated isotype matched antibodies (IgG_1-FITC/IgG_1-PE/CD3-PerCP; Becton Dickinson) were used as control antibodies. The percentage of cells within each Vβ subset that expressed CD69 was determined at 4 hours. Net values were calculated by subtracting the percentage of positive cells in the unstimulated group from the values of the experimental groups. After 7 days of

culture, the total number of cells in each Vβ subset was also determined. Fluorescent analysis was performed using Lysis II software on a FACScan fluorocytometer (Becton Dickinson). The percentage of activated $CD4^+$ and $CD8^+$ T cells was determined by two-color dot plot analysis. Activated T cell subsets were determined by dividing the number of double positive cells (quadrant 2) by the sum of double positives (quadrant 2) and single positives (quadrant 4) (i.e. 100 x $[CD4^+/CD69^+]/CD4^+$ or 100 x $[CD8^+/CD69^+]/CD8^+$).

2.1.9 IL-2 Bioassay

Culture supernatants were harvested at 24 hour intervals up to 7 days incubation and IL-2 production measured using either of the IL-2 dependent cell lines, CTLL-2 (ATCC TIB 214) or HT-2. 100 ng/ml anti-TAC monoclonal (Boehringer Mannheim) was added to cultures 24 hours prior to collection of supernatants. A standard curve was constructed using serial dilutions of purified hIL-2 (Pharmingen, San Diego, California) where 1 U was defined by using the BRMP (Biological Response Modifiers Program) standard. After 24 hours incubation, cells will be pulsed with 1 μCi ^{3}H-thymidine (ICN) for an additional 24 hours, harvested on glass filters, and counts per minute determined by liquid scintillation counting.

2.2.0 Lymphocyte Proliferation Assay

Exoenzyme S (10 - 0.001 μg/ml) was added to 2 x 10^5 PBMC (or FBMC) and incubated for 7 days (predetermined optimal day for proliferation, [Mody *et al.*, 1995]) in 96-well round bottom plates (Nunc, Roskilde, Denmark). Eighteen hours before the end of incubation, 1 μCi of ^{3}H-TdR was added. Cells were harvested on glass filters, and counts per minute (CPM) determined using a liquid scintillation counter. Values shown are the mean of quadruplicate wells ± standard error of the mean (SEM) in CPM (10^3). As controls, the superantigens (*Staphylococcal* enterotoxins -A, -B, -C2, -E and TSST-1; Toxin Technologies; Sarasota, Florida) and the mitogenic lectins (concanavalin A,

phytohemagglutinin; Sigma, St. Louis, Missouri) were all used at 1μg/ml and harvested on day 3. Tetanus toxoid (10^{-2} Lf units; Connaught, Willowdale, Ontario) was used as a recall antigen control and harvested on day 7.

For major histocompatability (MHC) molecule blocking experiments, anti-DR mAb (L243, Becton Dickinson) was washed three times in PBS before use to remove azide. PBMC (2×10^5) were pretreated for 1 hour with 3 μg/ml anti-DR or isotype matched control (Becton Dickinson) and then stimulated with or without 1 μg/ml exoenzyme S for 7 days. As a positive control, PBMC were stimulated with TSST-1 and used to ensure that anti-DR can block class II-mediated responses.

2.2.1 Apoptosis Assays

2.2.1.1 Propidium Iodide Staining of DNA

Propidium iodide staining for DNA quantification was performed as previously described (Douglas *et al.*, 1995). Camptothecin (Aldrich, Milwaukee, Wisconsin) was used at 1 μM as a positive control to induce apoptosis [Darzynkiewicz *et al.*, 1997]. Briefly, cell cultures (2×10^6) were harvested after incubation with the appropriate stimulus, washed twice in wash PBS (10 minutes, 400 x g) and fixed with the slow addition of 2 ml 75% ethanol (-20°C). Cells were incubated for at least 1 hour at -20°C, centrifuged and washed as before. RNAse (1 mg/ml) (Type I-A, Sigma, St. Louis, Missouri) and propidium iodide (50 μg/ml) (Sigma) was added 15 minutes prior to analysis. Propidium iodide intensity was analyzed using the Lysis II program on a FACScan fluorocytometer (Becton Dickinson).

2.2.1.2 DNA Fragmentation by Agarose Gel Electrophoresis

Cultures were harvested and resuspended at a concentration of 10^8 per ml and DNA isolation was performed using the TACS Apoptotic DNA laddering kit (Trevigen, Gaithersburg, Maryland) following the manufacturer's protocol. Briefly, cells underwent

lysis and phenol-based DNA extraction. Extracts were centrifuged, the aqueous layer collected and DNA was precipitated using a 1:10 volume of sodium acetate and 2-propanol at -20°C. DNA samples were run in a 1.5 % Trevigel at constant voltage for 60 minutes in a horizontal gel apparatus (Gibco BRL).

2.2.1.3 Detection of Phosphatidylserine Upregulation

PBMC (8×10^5) were harvested after incubation with appropriate stimuli and stained as before with FITC-conjugated anti-CD3. After washing twice in wash buffer, 2×10^5 FITC-labelled cells were resuspended in 150 µl of binding buffer (25 mM Ca^{2+} in HEPES buffered saline). 10 µl of PE-conjugated annexin V (R&D) and 10 µl of 5 µg/ml 7-ADD (Sigma) were added for 15 minutes and incubated in the dark. Before FACS analysis an additional 350 µl of binding buffer was added. All samples were analyzed within 30 minutes. T lymphocytes were gated on their side scatter versus CD3-FITC profile and then analyzed for fluorescence in FL2 (annexin) and FL3 (7-ADD). Healthy cells are double negative, cells undergoing early events associated with apoptosis will be FL2 positive and FL3 negative, and cells in later stages of apoptosis will be FL2 and FL3 positive.

2.2.2 Statistics

Values are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed by linear regression, paired analysis of variance (ANOVA - SPSS, SPSS Inc., Chicago, Illinois), two-sample, two-tailed, paired Student's *t* test, or repeated measures of ANOVA based on the pair-wise Laird-Ware mixed model (Stataquest; Stata Corporation, College Station, Texas). $P<0.05$ were considered significant.

3.0 RESULTS

3.1 Exoenzyme S as a T Cell Mitogen

The measurement of mitotic activity by ^{3}H-TdR incorporation into newly synthesized DNA reflects cell cycle transition from Go or G1 to S phase and is one of the most frequently used assays for T cell proliferation. Previously, these experiments performed in our laboratory have shown that exoenzyme S is capable of inducing lymphocytes to proliferate [Mody *et al.*, 1995]. It was established in those experiments that the optimal response to exoenzyme S occurred on day 7, at 10 μg/ml and 4 x 10^5 PBMC per microtiter well [Mody *et al.*, 1995]. In my experiments however, there was no observable difference in the magnitude of the response when using either 1 μg/ml exoenzyme S and 2 x 10^5 PBMC or 10 μg/ml exoenzyme S and 4 x 10^5 PBMC per microtiter well (stimulation index; 1 μg/ml, 2 x 10^5 PBMC = 10.53; 10 μg/ml, 4 x 10^5 PBMC = 9.66). Therefore, in all subsequent experiments 1 μg/ml of exoenzyme S was used in conjunction with 2 x 10^5 PBMC per microtiter well unless otherwise stated. It should be noted that human proliferative responses to exoenzyme S (or any stimulus for that matter) *in vitro* are quite heterogenous in nature and therefore a significant portion of experiments had marginal or insignificant stimulation indeces in response to exoenzyme S. The data presented herein will, in part, help explain the reason for this apparent inconsistency.

Although T lymphocytes were previously shown to be the predominant cell type that was being stimulated by exoenzyme S [Mody *et al.*, 1995], the mechanism of action was not investigated. In order to more precisely determine whether exoenzyme S was stimulating T cells as a mitogen, the ability to induce immunologically naive lymphocytes (fetal blood mononuclear cells and adult CD45RA$^+$ T cells) to proliferate was determined. Exoenzyme S was capable of stimulating fetal cord lymphocytes to proliferate. FBMC showed a dose dependent proliferation to exoenzyme S between 0.5 - 10 μg/ml while tetanus toxoid (a recall antigen control) did not induce significant proliferation (Fig. 2).

This result suggests that exoenzyme S is capable of stimulating naive T lymphocytes to proliferate.

CD45RA is an isoform of the CD45 molecule that is expressed by naive T cells while CD45RO is expressed by memory T cells [Prince *et al.*, 1992]. To determine whether T cells bearing the CD45RA isoform are capable of proliferating to exoenzyme S, non-adherent adult cells were depleted of either $CD45RA^+$ or $CD45RO^+$ cells. CD45RA-enriched, and CD45RO-enriched cells were stimulated with exoenzyme S in the presence of irradiated adherent cells as a source of accessory cells. Exoenzyme S was capable of inducing both CD45RA-enriched and CD45RO-enriched populations to proliferate (Fig. 3A). This demonstrates that T cells expressing the phenotype of naive and memory cells proliferate to exoenzyme S. Conversely, the recall antigen tetanus toxoid could only induce CD45RO-enriched cells to proliferate, while the T cell mitogen phytohemaglutinin (PHA) induced significant proliferation in both CD45RA- and CD45RO-enriched cultures (Fig. 3B). Taken together, these results are most consistent with exoenzyme S stimulating T cells as a mitogen.

The ability of exoenzyme S to induce early activation in both $CD45RA^+$ and $CD45RO^+$ T cells was also shown in a preliminary experiment. The ratio of activated (cells that expressed CD69) CD45RA cells to CD45RO cells stimulated with exoenzyme S was similar to PHA-stimulated cultures (Fig. 4), while tetanus toxoid predominantly activated cells enriched for the memory phenotype. In short, exoenzyme S is capable of inducing naive lymphocytes to proliferate; although statistically significant, the degree of proliferation was consistently lower than control mitogens. Therefore, the ability of exoenzyme S to activate T cells, regardless of their CD45 isotype expression, is consistent with its ability to induce both populations into cell cycle.

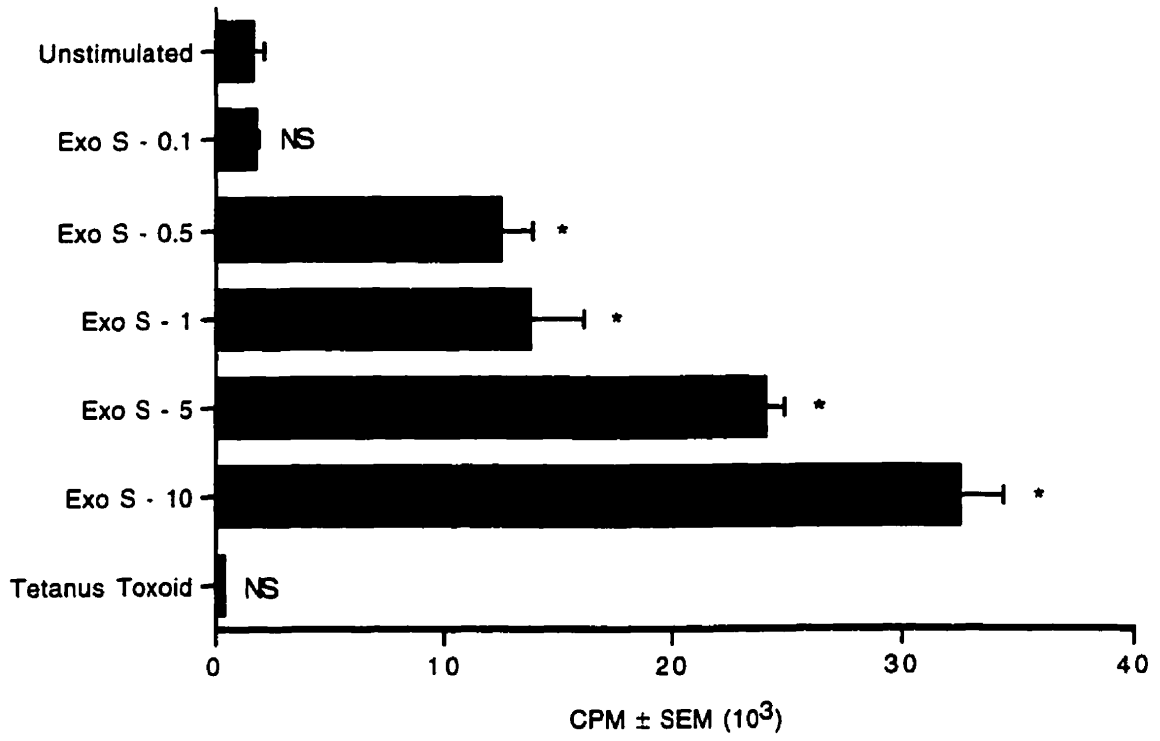


Fig. 2 Fetal blood mononuclear cells proliferate in response to exoenzyme S. FBMC (2×10^5) were cultured with ten-fold dilutions of exoenzyme S (0.1 - 10 μg/ml) or 10^{-2} Lf units of tetanus toxoid for 7 days. The experiment was performed twice with similar results. *, $P < 0.05$ calculated by ANOVA compared to unstimulated group; NS, non-significant difference compared to unstimulated group.

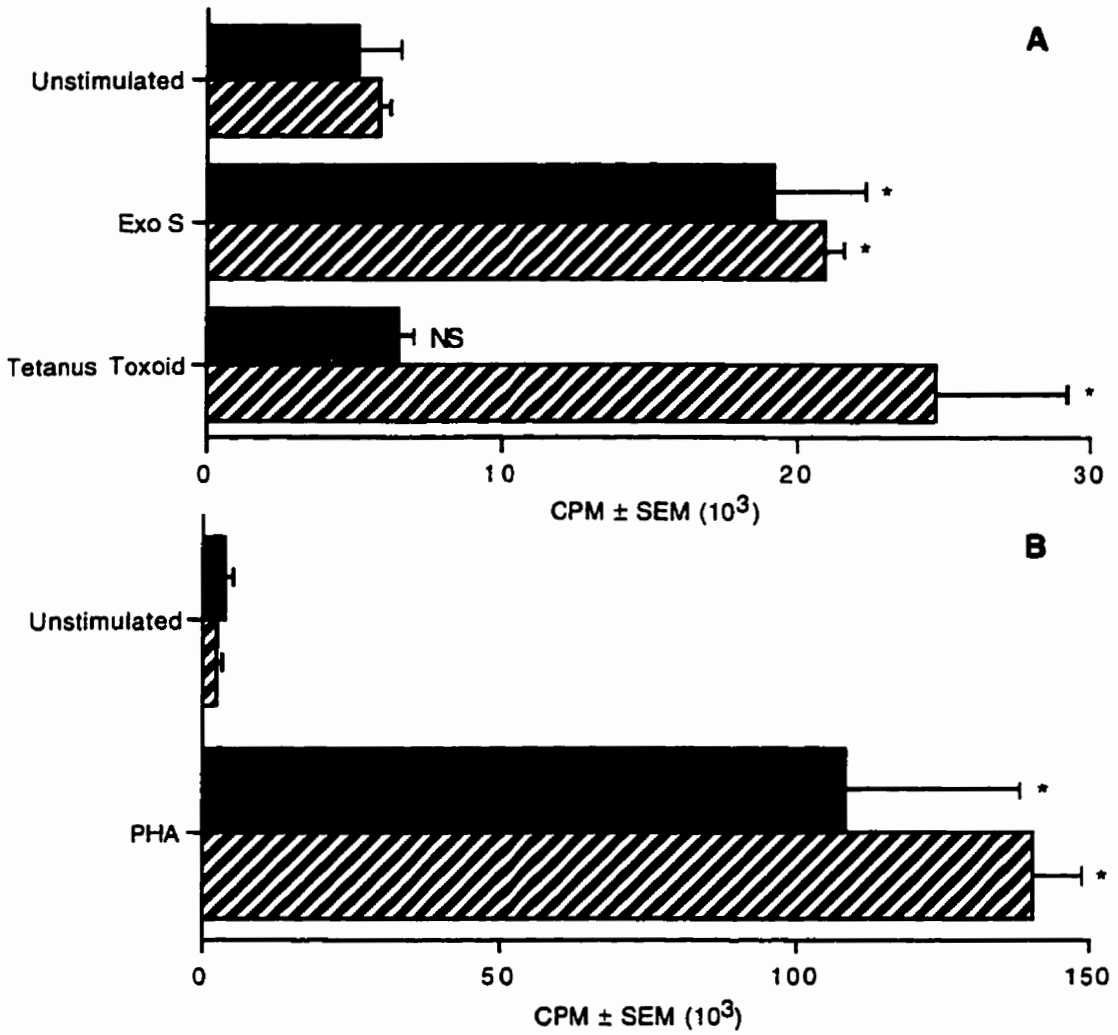


Fig. 3 Proliferation of T cell subsets in response to exoenzyme S. CD45RA-enriched (solid bars), and CD45RO-enriched (striped bars) T cells were cultured with irradiated accessory cells in the presence of 1μg/ml exoenzyme S (Exo S), 10^{-2} Lf units tetanus toxoid (Panel B) or 1μg/ml phytohemaglutinin (PHA - Panel B). Cultures stimulated with PHA were harvested on day 3 and cultures stimulated with exoenzyme S or tetanus toxoid were harvested on day 7. The experiment was repeated three times with similar results. *, $P < 0.05$ calculated by ANOVA compared with corresponding unstimulated group.

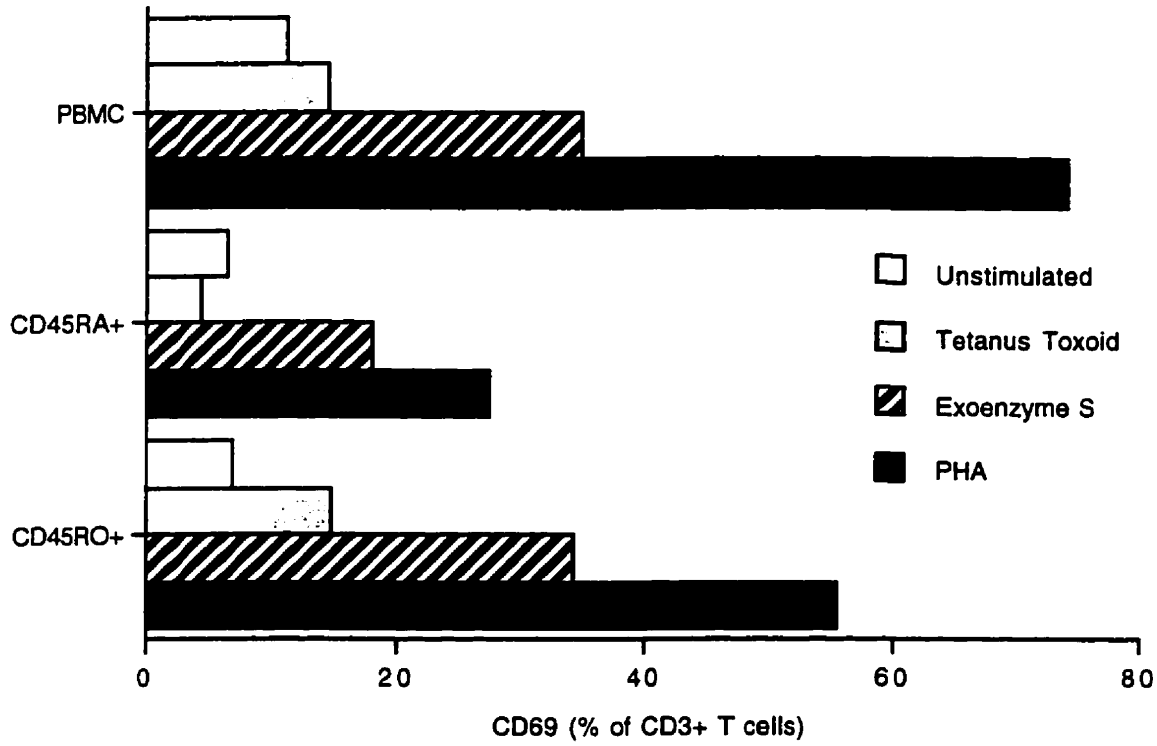


Fig. 4 Activation of T cell subsets in response to exoenzyme S. PBMC, CD45RA-enriched, and CD45RO-enriched T cells were stimulated with 1μg/ml exoenzyme S (Exo S), 10^{-2} Lf units tetanus toxoid (Panel B) or 1μg/ml phytohemaglutinin (PHA - Panel B) for 4 hours. Samples were harvested and labelled with anti-CD69-PE/ anti-CD3-PerCP. The percentage of CD3 cells in each group expressing CD69 was determined. This experiment was repeated with a similar result.

To further determine whether exoenzyme S was activating T cells as an antigen or a mitogen, the requirement for accessory cells and presentation by major histocompatability (MHC) molecules was determined. T cells can be stimulated to proliferate by a variety of mechanisms which have different accessory cell requirements. Mitogens require accessory cells to provide costimulatory molecules that are necessary for T cell stimulation [Sopori *et al.*, 1987]. In contrast, recall antigens must be taken up by accessory cells (in this case termed 'antigen presenting cells'), processed internally, and presented in the context of MHC to T cells [Brodsky and Guagliardi, 1991]. Lastly, pharmacological treatment of purified T cells with a phorbol ester and calcium ionophore induces significant lymphocyte proliferation even in the absence of accessory cells [Truneh *et al.*, 1985]. Experiments were therefore designed to determine whether the proliferative response was dependent on accessory cells (as is the case for antigens or mitogens) or whether exoenzyme S is capable of bypassing the cell surface molecules that are required for physiologic T cell responses (i.e. pharmacological agents). T cells were stimulated with or without irradiated accessory cells. Exoenzyme S could not induce the T cell enriched population to proliferate, but addition of irradiated accessory cells reconstituted the response (Fig. 5A) suggesting that the proliferative response of T cells to exoenzyme S is dependent on accessory cells. To ensure that T cells were depleted of accessory cells, concanavalin A was used. T cells were incapable of proliferating to concanavalin A unless irradiated accessory cells were added suggesting a high degree of purity was attained (Fig. 5B; [Sopori *et al.*, 1987]). Together this data implies that exoenzyme S does not activate T cells non-specifically as a pharmacological agent, but rather induces T cell proliferation in a manner that is dependent on the presence of accessory cells.

The previous experiments were able to show that accessory cells are required for T cell proliferation to exoenzyme S. In order to determine whether MHC molecules were involved in the presentation of exoenzyme S, we pretreated PBMC with anti-DR or isotype

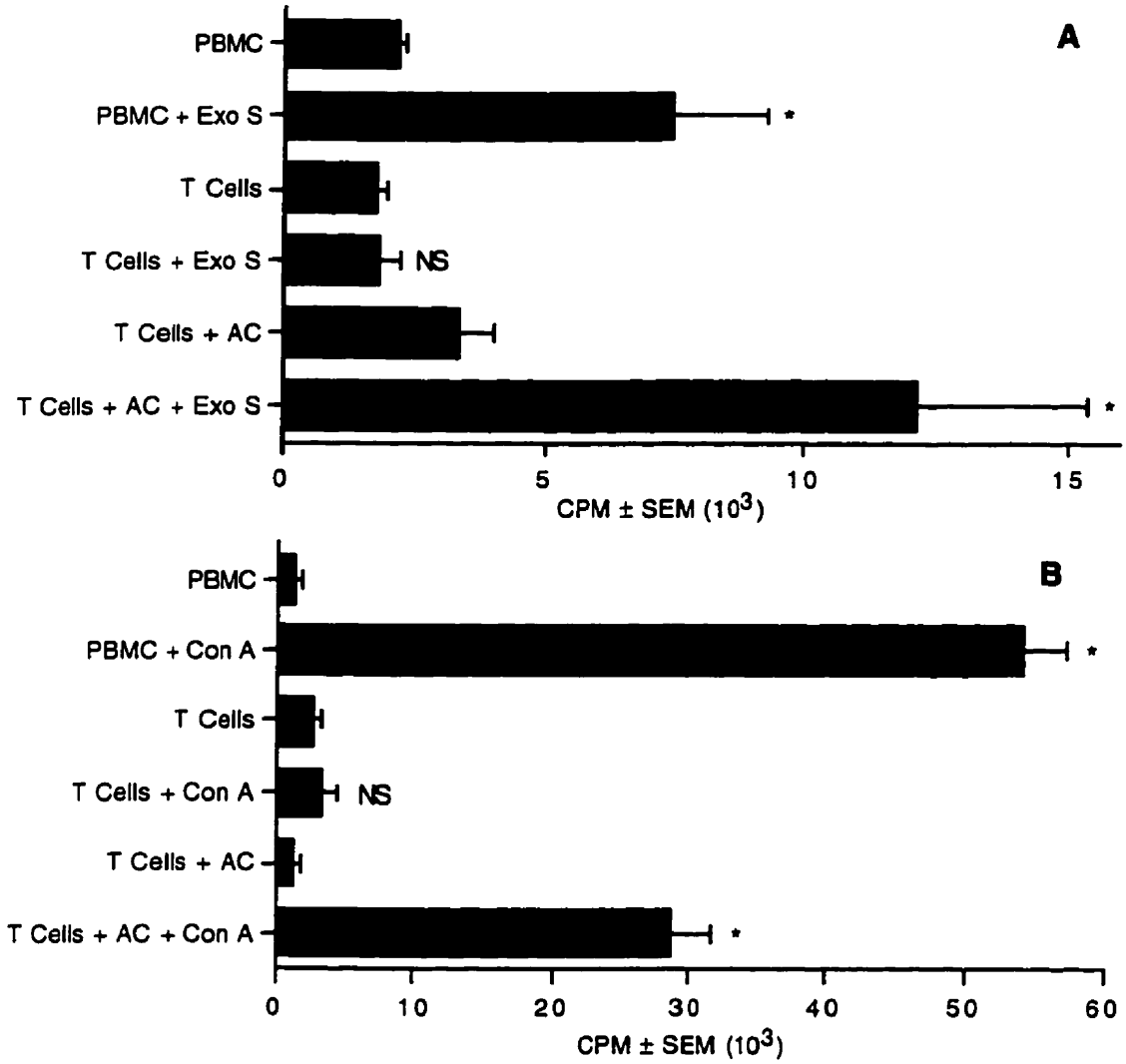


Fig. 5 Antigen presenting cells are required for T cell proliferation to exoenzyme S. PBMC or purified T cells with and without accessory cells were incubated with 1 μg/ml exoenzyme S (Exo S - Panel A) or 10 μg/ml concanavalin A (Con A - Panel B). Cultures stimulated with Con A were harvested on day 3 while cultures stimulated with exoenzyme S were harvested on day 7. The experiment was repeated three times with similar results.*, $P < 0.05$ calculated by ANOVA compared with corresponding unstimulated group; NS, non-significant difference compared with the corresponding unstimulated group.

control mAb for 1 hour before the addition of stimulus and determined the proliferative response by ^{3}H-TdR incorporation. Treatment with anti-DR mAb was used to block MHC presentation as it has been shown that the vast majority superantigens are presented preferentially by HLA-DR [Hermann *et al.*, 1989], [Herman *et al.*, 1990], [Mollick *et al.*, 1991], [Uchiyama *et al.*, 1994]. HLA-DR or isotype control did not significantly abrogate the proliferative response to exoenzyme S (Fig. 6A) suggesting that MHC is not directly involved in the presentation of exoenzyme S to T cells. In control experiments, we confirmed that anti-DR antibody blocked the response to the superantigen TSST-1, but not concanavalin A (Fig. 6B; [See *et al.*, 1992]). In this regard, T cell stimulation by exoenzyme S more closely resembles that induced by concanavalin A than it does TSST-1 in that it demonstrates stimulation by exoenzyme S is independent of MHC.

It should be noted that our data does not exclude the unlikely possibility that exoenzyme S may possess a binding site that allows crosslinking of the TCR with MHC that is not interfered by the anti-MHC antibody we used. Therefore, we performed experiments to determine whether there was oligoclonal activation or oligoclonal proliferation of Vβ subsets. In contrast to mitogens, superantigens oligoclonally activate T cells based on the Vβ elements of the T cell receptor [Dellabona *et al.*, 1990]. Experiments were performed to determine whether exoenzyme S was capable of preferentially activating T cells based on their TCR Vβ expression. Exoenzyme S activated T cells bearing all of the 9 Vβ elements analyzed (Fig. 7A), while *Staphylococcal* enterotoxin E (SEE), a control superantigen known to stimulate Vβ8^{+} T cells, caused a preferential activation of T cells bearing Vβ8 and failed to activate those bearing Vβ12 (Fig. 7B). Additionally, after 7 days of stimulation with exoenzyme S, there was no apparent increase or decrease in the percentage of T cells bearing any of the 9 Vβ elements analyzed (Fig. 8A). By contrast, SEE caused a significant increase in the percentage of T cells bearing Vβ8 and a concomitant decrease in the percentage of cells bearing Vβ12 (Fig. 8B). Therefore,

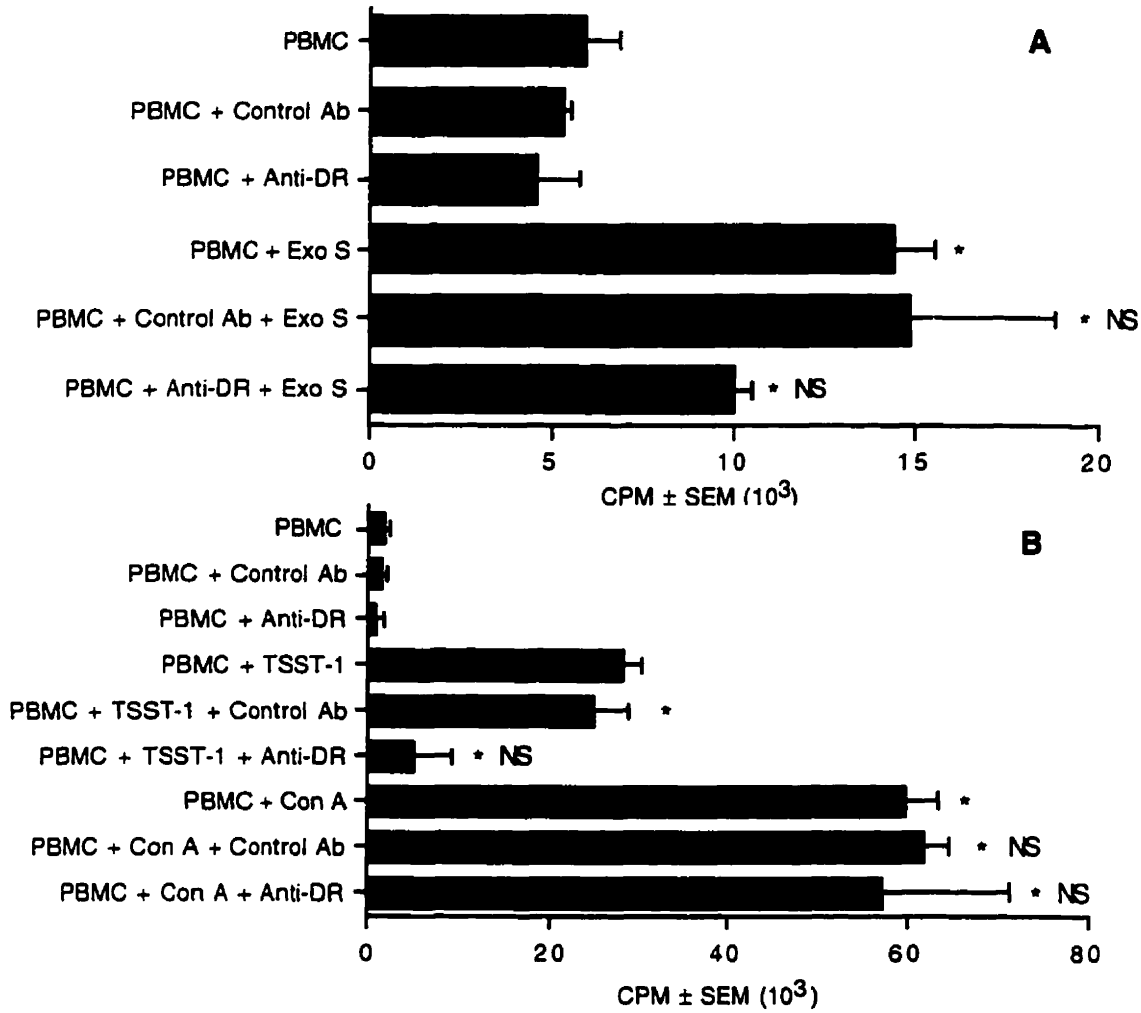


Fig. 6 HLA-DR is not necessary for the proliferative response to exoenzyme S. PBMC were pretreated with anti-DR mAb, isotype control or left untreated for 1 hour and then stimulated with 0.1 μg/ml toxic shock syndrome toxin-1 (TSST-1), or 10 μg/ml Concanavalin A (Con A - Panel A) for 3 days or 1 μg/ml exoenzyme S (Exo S - Panel B) for 7 days. This experiment was performed 3 times with similar results.*, $P < 0.05$ calculated by ANOVA compared to corresponding unstimulated group; NS, non-significant difference compared to stimulated PBMC.

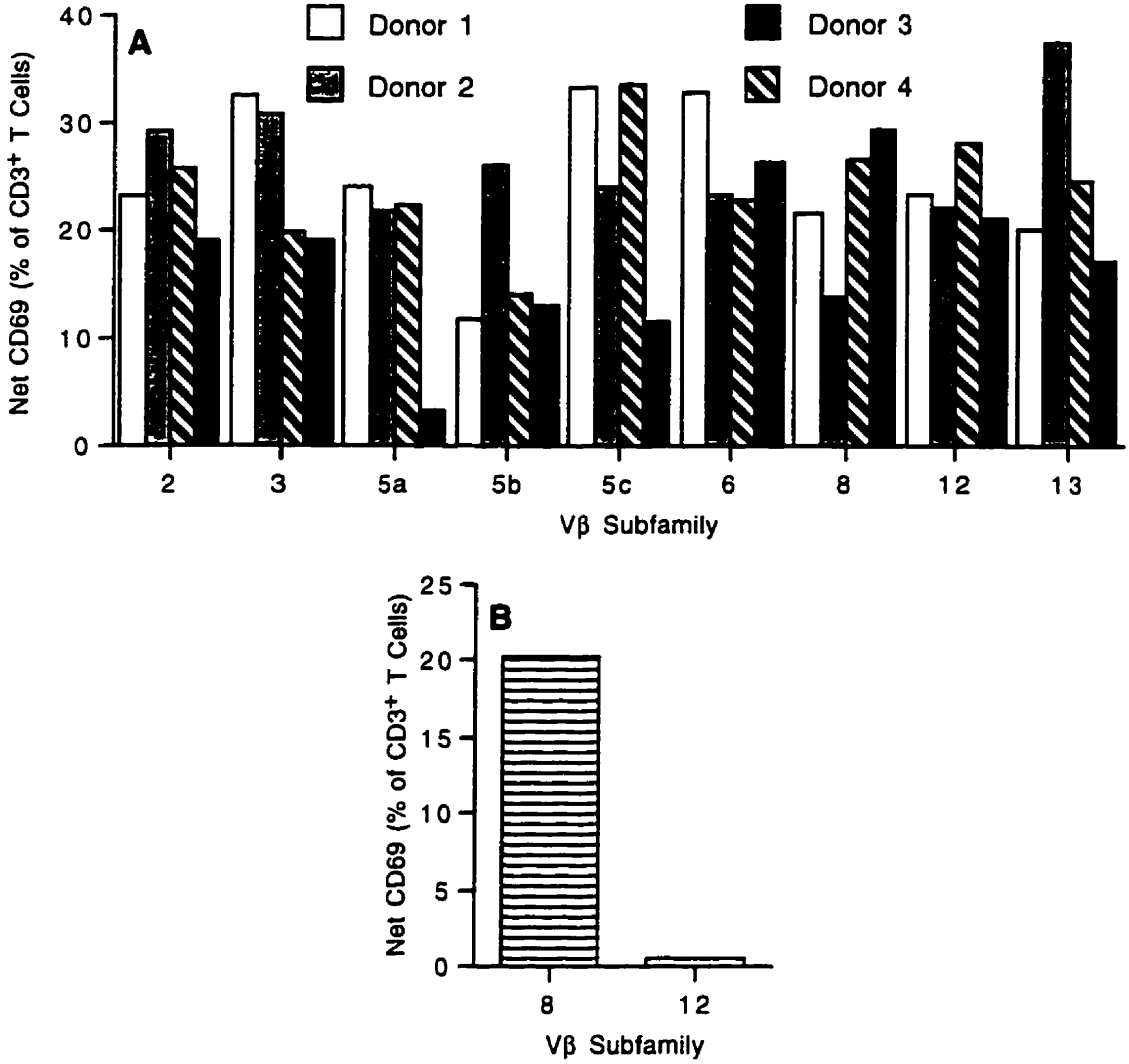
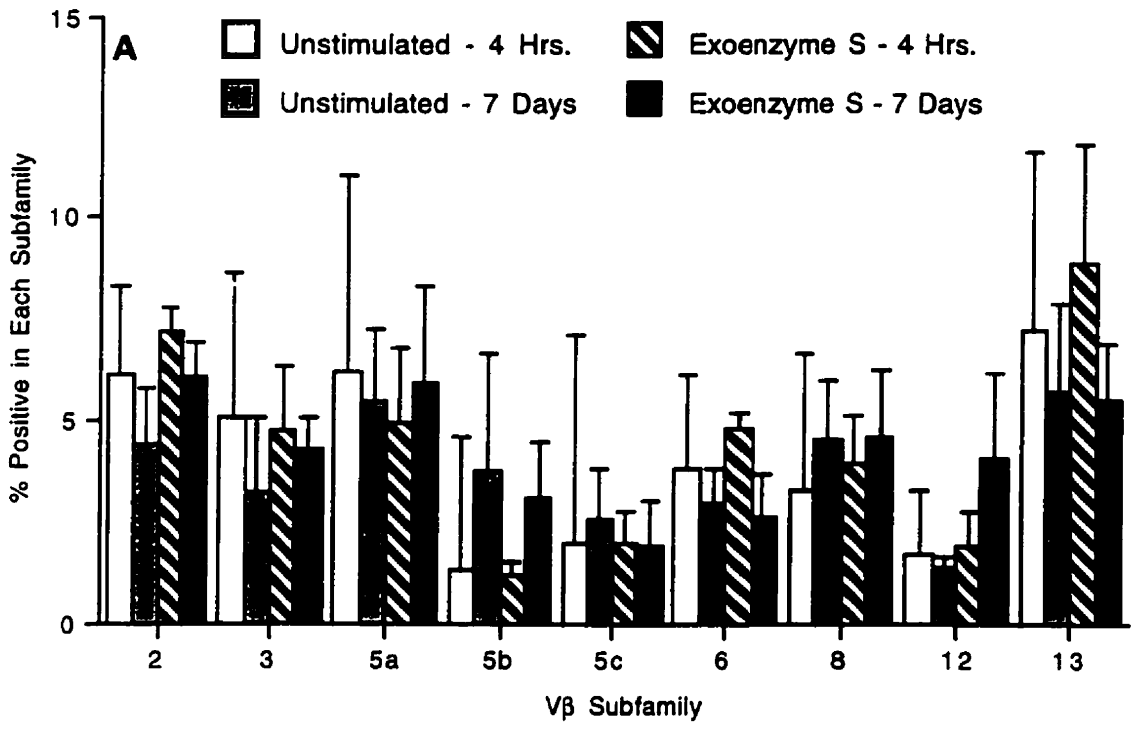


Fig. 7 Vβ-specific activation (Panel A and B) of human T lymphocytes. PBMC (2 x 10^5) were stimulated with 1 μg/ml exoenzyme S (Panel A) or *Staphylococcal* enterotoxin -E (SEE - Panel B) for 4 hours. Cells were labelled with anti-CD69-PE/anti-CD3-PerCP and one of the FITC-conjugated Vβ-specific mAbs. After gating on CD3, the net percentage of cells within each Vβ family that expressed CD69 was determined by subtracting the CD69 expression of unstimulated cultures from exoenzyme S- (Panel A) or SEE (Panel B) stimulated cultures. The mean ± SEM of four separate experiments are shown.


A
Unstimulated - 4 Hrs.
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% Positive in Each Subfamily
15
10
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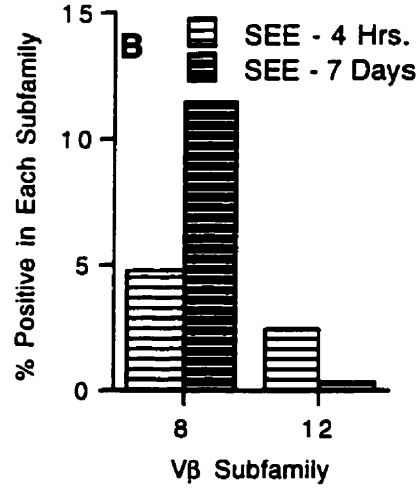
B
SEE - 4 Hrs.
SEE - 7 Days
% Positive in Each Subfamily
15
10
5
0
8
12
Vβ Subfamily

Fig. 8 Vβ-specific expansion of human T lymphocytes. PBMC (2×10^5) were stimulated with 1 μg/ml exoenzyme S (Panel A) or *Staphylococcal* enterotoxin -E (SEE - Panel B) for 4 hours. Co-cultures were also stimulated for 7 days and 3 days respectively. After 3 days (SEE) and 7 days (exoenzyme S), cultured cells were labelled with anti-Vβ-specific (FITC) mAb and anti-CD3 (PerCP). Vβ expression is shown as a percentage of cells gated on CD3 for unstimulated and exoenzyme S-stimulated (Panel A) as well as for SEE-stimulated cultures (Panel B).

exoenzyme S induced polyclonal, not oligoclonal activation and proliferation in PBMC cultures, suggesting it is not a superantigen.

3.2 Characterization of T Cell Activation by Exoenzyme S

There are a number of important pieces of evidence which suggest that exoenzyme S stimulates T cells as a mitogen: i) exoenzyme S induces fetal lymphocytes to proliferate; ii) $CD45RA^{+}$ lymphocytes express CD69 and proliferate in response to exoenzyme S; iii) the proliferative response to exoenzyme S is dependent on accessory cells, but independent of HLA-DR. Nonetheless, proliferative responses to exoenzyme S were significantly lower in magnitude, and the kinetics more delayed, when compared to proliferative responses to other mitogens. To better characterize this apparent discrepancy, earlier markers of T cell activation were evaluated in subsequent experiments.

CD69 is an inducible surface molecule found on T cells following stimulation; mRNA transcripts can be detected after 30 minutes and surface expression can be detected after 2 hours and declines with a half-life of 24 hours if the stimulus is withdrawn [Testi *et al.*, 1989], [Ziegler *et al.*, 1994]. Although under certain conditions, CD69 expression levels on T lymphocytes can be shown to be comparable to proliferative responses measured by ^{3}H-TdR uptake, this is not always the case [Maino *et al.*, 1995], [Caruso *et al.*, 1997].

A time course for expression of CD69 by PBMC stimulated with exoenzyme S shows that elevated expression of this marker can be seen as early as 2 hours and decline to background levels between 2-3 days (Fig. 9). Peak levels are expressed between 2-8 hours. Typically, CD69 expression induced by SEB is more delayed and does not surpass levels induced by exoenzyme S until 8-16 hours. Because significant surface expression of CD69 on T lymphocytes occurs early, it therefore reflects the cellular and biochemical events germane to the initial stages of activation by exoenzyme S and reduces non-specific

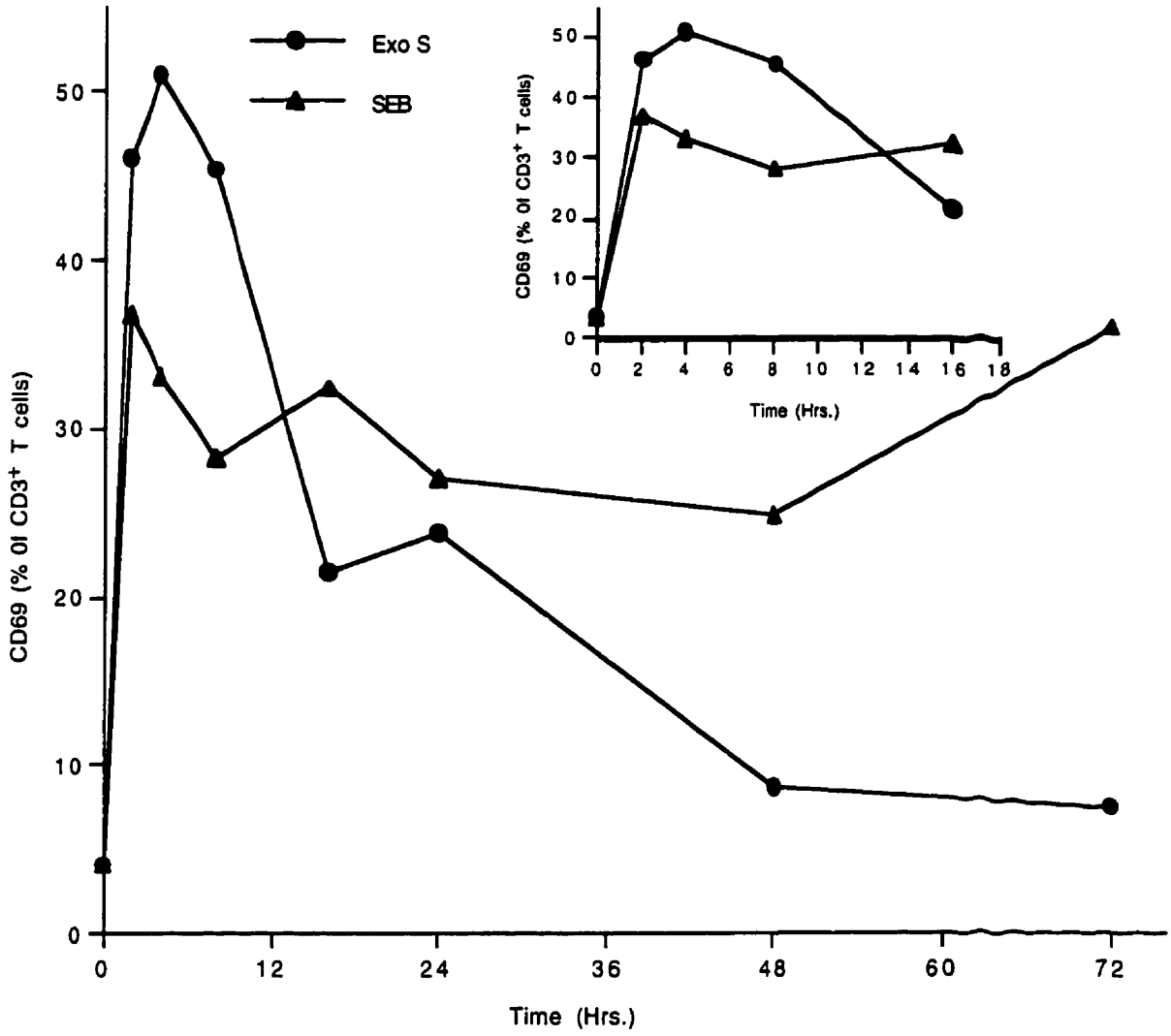

Fig. 9 Induction of CD69 expression on peripheral T lymphocytes. PBMC cultures were stimulated with 1 μg/ml of *Staphylococcal* enterotoxins -B or exoenzyme S and at various times in culture were harvested and labelled with anti-CD69-PE/ anti-CD3-PerCP. Inset demonstrates the differences in kinetic expression of CD69 between the stimuli during early timepoints. The percentage of CD3 cells expressing CD69 was determined for each group at the indicted times. This experiment was repeated three times with similar results.

bystander activation. For this reason, CD69 levels were analyzed at 4 hours for all subsequent experiments unless otherwise stated.

$CD4^+$ T cells are known as helper cells because they secrete a number of lymphokines which are necessary for the activation and subsequent effector functions of a number of different inflammatory leukocytes [Brummer and Stevens, 1984], [Murray *et al.*, 1985], [Hunninghake *et al.*, 1987], [Rollins, 1997]. Because of this characteristic, they are of obvious importance as orchestrators of the inflammatory process. $CD8^+$ T cells, on the other hand, are known as cytotoxic cells because of their predisposition to kill specific targets [Wagner *et al.*, 1982], [Cerottini and Brunner, 1989]. Preferential activation of either $CD4^+$ (T_h) or $CD8^+$ (T_c) T cells would have functional outcomes and would imply a preferential induction of either T_h to secrete cytokines or T_c to promote cytotoxic activity respectively [Reinherz *et al.*, 1979], [Reinherz and Schlossman, 1980], [Moretta *et al.*, 1984]. Preferential activation of T cell subsets has previously been demonstrated for a number of stimuli. Superantigens such as SEA and SEB have been shown to preferentially activate $CD4^+$ over $CD8^+$ T cells [Kawabe and Ochi, 1990], [Abe *et al.*, 1991]. Other stimuli such as *Candida albicans* antigen preferentially activate $CD8^+$ T cells [Maino *et al.*, 1995], while the mitogen, concanavalin A, activates both subsets to the same extent [Biselli *et al.*, 1992]. Experiments were therefore designed to determine whether there was preferential activation of either T cell subset after exoenzyme S stimulation. The expression of CD69 was analyzed on both $CD4^+/CD3^+$ or $CD8^+/CD3^+$ T cell subsets as calculated in materials and methods. Exoenzyme S preferentially activated $CD4^+$ over $CD8^+$ T cells at a ratio of 2:1, which was similar to both SEB and SEE (Fig. 10). PHA induced 1.5 times more $CD4^+$ T cells to express CD69 than $CD8^+$ T cells, while tetanus toxoid did not show preferential activation of either subset. This suggests that exoenzyme S, like some mitogens and superantigens, activates $CD4^+$ T cells to a greater extent than $CD8^+$ T cells, and this preferentially activation may favor the production and secretion of inflammatory cytokines. In this regard, current studies in the laboratory are

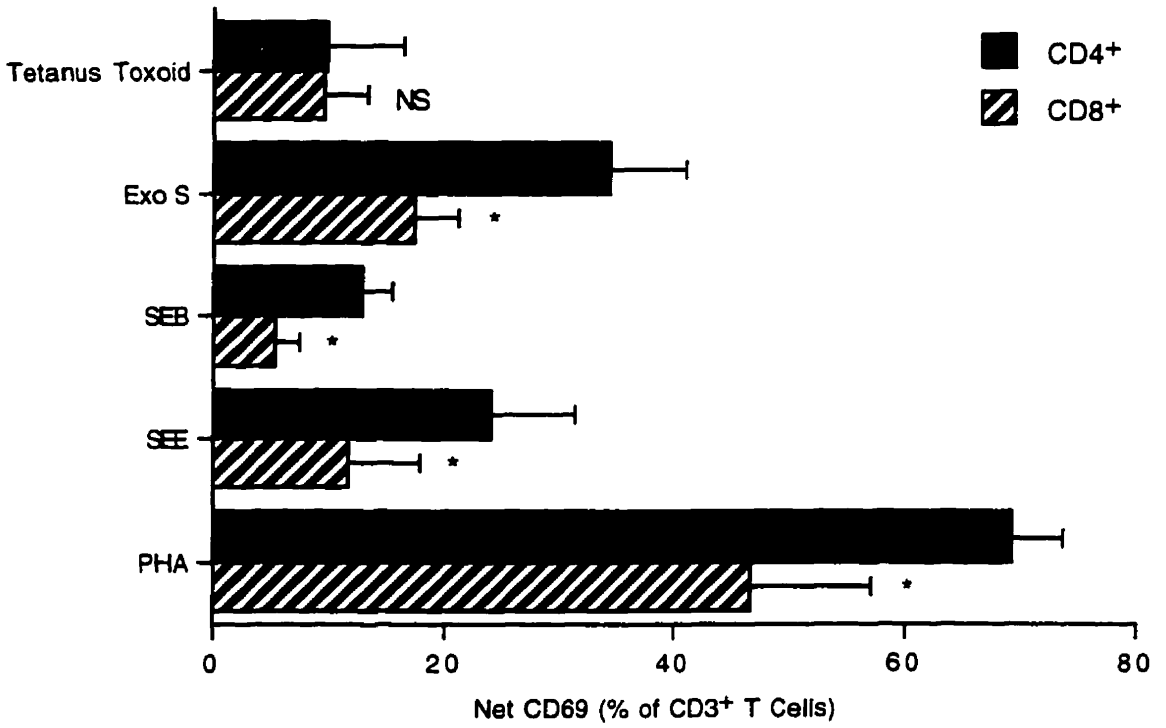

Fig. 10 T cell subset activation by exoenzyme S. PBMC cultures were stimulated with 10^{-2} Lf units tetanus toxoid, 1 μg/ml of *Staphylococcal* enterotoxins -B, -E, phytohemaglutinin (PHA), or exoenzyme S for 4 hours. Samples were then harvested and labelled with FITC-conjugated anti-CD4 or anti-CD8 and anti-CD69-PE/anti-CD3-PerCP. The net percentage of $CD4^+CD3^+$ or $CD8^+CD3^+$ cells that expressed CD69 was determined as before. *, $P < 0.05$ using a paired student's t test compared to corresponding $CD4^+$ group; NS, non-significant difference compared to corresponding $CD4^+$ group (n=4 SEE; n=5 for tetanus toxoid, SEB, and PHA; n=6 for exoenzyme S).

focused on screening for transcription of proinflammatory cytokines and chemokines synthesized by T cells after PBMC stimulation by exoenzyme S.

Because of the apparent discrepancy between the magnitude of early T cell activation and subsequent proliferation, the following experiments were performed in order to compare the percentage of T cells that become activated by exoenzyme S to the percentage of T cells activated by antigens, superantigens, and mitogenic lectins within the same donors. While the kinetics for induction and duration of CD69 expression by exoenzyme S, superantigens, and other mitogens are different (as seen for SEB in Fig. 9), these stimuli consistently induced significant T cell activation at 4 hours. Exoenzyme S stimulated a large percentage of peripheral T cells to express CD69 (Fig. 11). The percentage of T cells activated by exoenzyme S was 10-17% greater than that induced by any of the *Staphylococcal* superantigens tested and 25% greater than that induced by concanavalin A. However, less T cells were activated by exoenzyme S than by PHA or anti-CD2. The recall antigen, tetanus toxoid, induced minimal T cell activation over background (unstimulated mean = 11.78% ± 2.13%, n=19; tetanus toxoid mean = 16.64% ± 5.71%, n=4). Overall, the magnitude of early (4 hours) T cell activation induced by exoenzyme S is greater than a panel of superantigens and some mitogens. This implies that the initial number of T cells that are able to be directly activated by exoenzyme S is on a similar scale as for other well characterized T cell mitogens. However, T cells stimulated with exoenzyme S do not seem to be able to sustain this level of activation during later stages of culture.

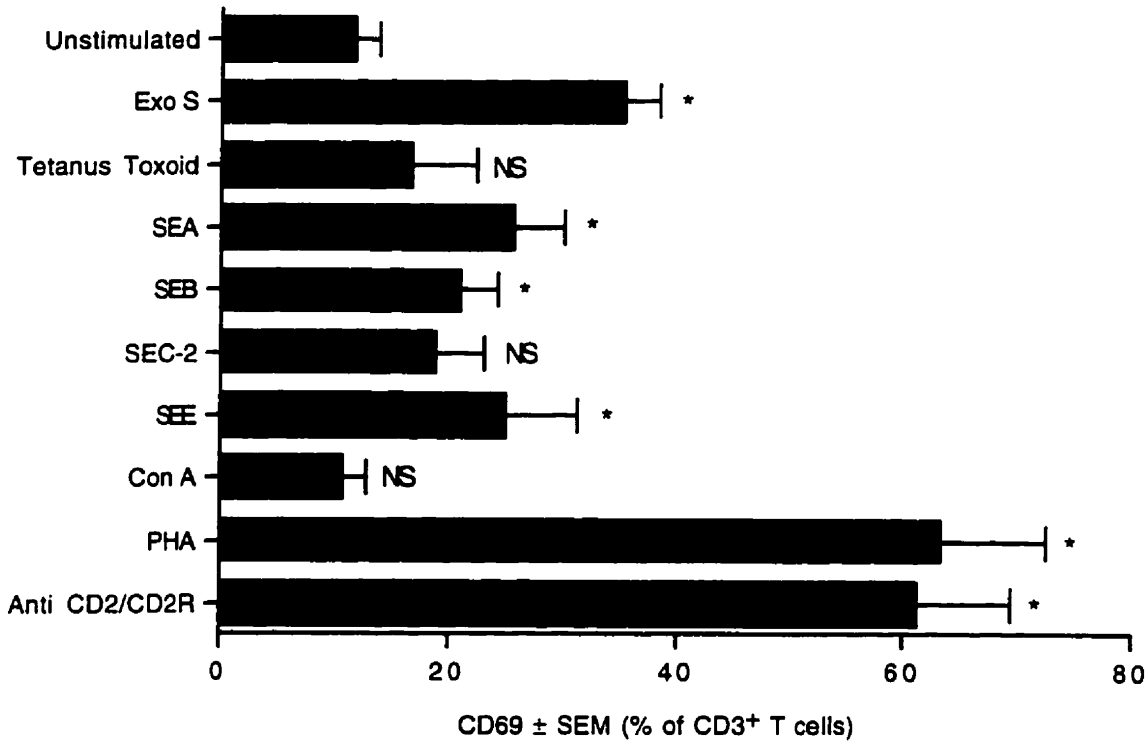

Fig. 11 Comparison of CD69 expression on peripheral T lymphocytes by various stimuli. PBMC were cultured with 1 μg/ml exoenzyme S, tetanus toxoid, 1 μg/ml of the *Staphylococcal* enterotoxins -A, -B, -C-2, -E, concanavalin A (Con A), phytohemaglutinin (PHA), or 10 μl of anti-CD2/CD2R for 4 hours. Samples were harvested and labelled with anti-CD69-PE/ anti-CD3-PerCP. The percentage of CD3 cells expressing CD69 was determined. *, $P < 0.05$ using repeated measures of ANOVA based on the Laird-Ware mixed model compared to unstimulated group; NS, non-significant difference compared to unstimulated group (n=19 for unstimulated and exoenzyme S, n=13 for SEB, n=8 for PHA, n=5 for SEE, n=4 for tetanus, n=3 for SEA, SEC-2 and anti-CD2/CD2R).

3.3 Induction of Activation Arrest and Apoptosis

3.3.1 Healthy Adult Donors

The competency of exoenzyme S to mediate significant early activation in peripheral T cells has been established (Fig. 11). However, comparison of this early level of activation to subsequent levels of proliferation, measured by ^{3}H-TdR uptake and visual counting, suggests a discrepancy in the magnitude of early versus late responses. Cultures stimulated with PHA and *Staphylococcal* enterotoxins induced a significant proliferative response (stimulation index from 37 to 22; PHA>SEC-2>SEE>SEB>TSST-1>SEA), while exoenzyme S and tetanus toxoid induced little proliferation (Fig. 12). In contrast to proliferative levels, CD69 expression was consistently greater in cultures stimulated with exoenzyme S compared to all other stimuli tested with the exception of PHA. In this regard, exoenzyme S may be inducing a state of T cell anergy as seen for other mitogens [Kawabe and Ochi, 1990], [MacDonald *et al.*, 1991], [Heeg *et al.*, 1993], [Bemer *et al.*, 1995].

The previous experiment suggests that initial T cell signalling by exoenzyme S is intact while the signal for latter events such as proliferation become perturbed or arrested. As mentioned previously, there have been a number of investigations that demonstrate the ability of mitogens to induce lymphocyte unresponsiveness [Ignatowicz *et al.*, 1992], or apoptosis [Jenkinson *et al.*, 1989], [Damle *et al.*, 1993], [McCormack *et al.*, 1993]. Perhaps the degree of anergy or apoptosis induced by exoenzyme S is greater than for most other mitogens and this could explain the relatively delayed kinetics and modest degree of proliferation that was seen in exoenzyme S-stimulated cultures compared to that seen for other well characterized mitogens.

Anergy is a form of T cell unresponsiveness that can be induced by antigens, mitogens, or superantigens [Weigle, 1973], [Kawabe and Ochi, 1990], [MacDonald *et al.*, 1991], [Heeg *et al.*, 1993], [Bemer *et al.*, 1995]. Regardless of the mechanism of stimulation, the universal cause for its induction is a lack of IL-2 responsiveness upon TCR

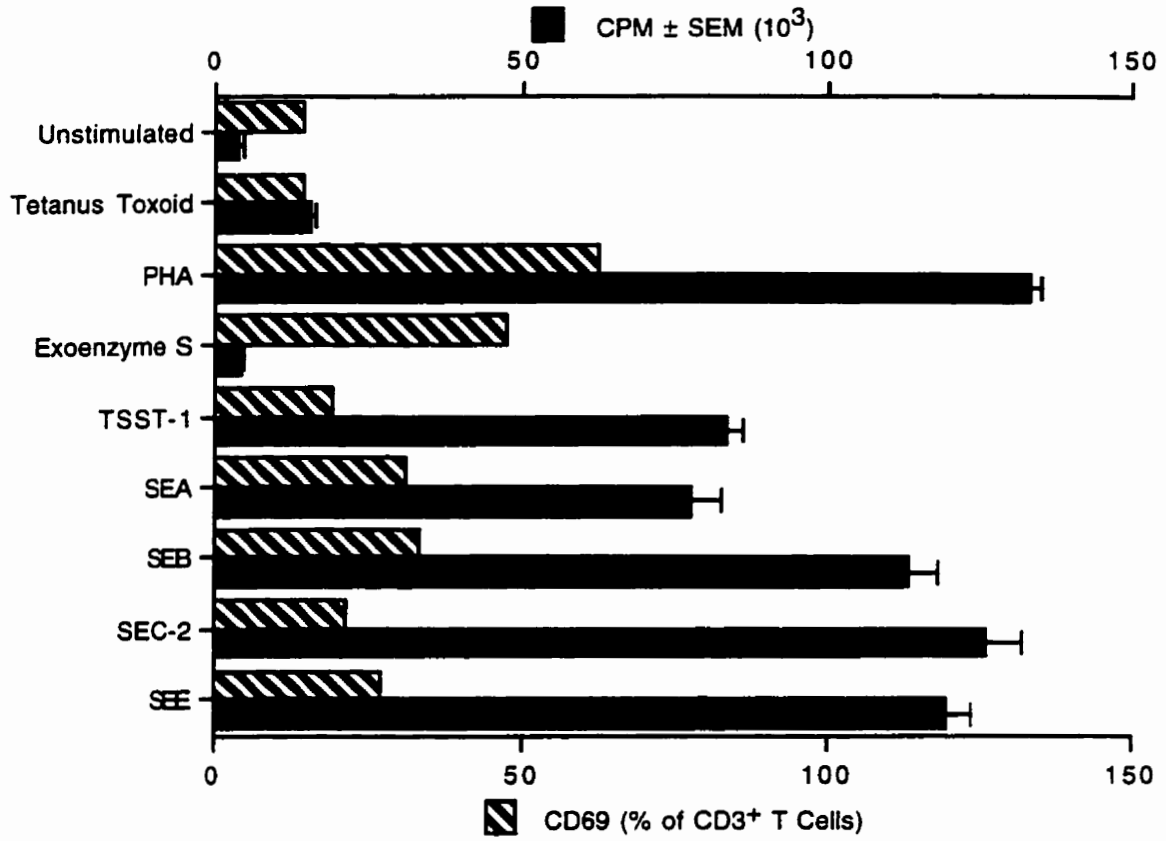

Fig. 12 Correlation between early T cell activation and subsequent proliferation induced by a panel of stimuli. Induction of CD69 was used as a measure of early T cell activation and ^{3}H-TdR incorporation was used to measure subsequent proliferation of co-cultures, calculated by subtracting the proliferative response in unstimulated cultures from experimental groups. Day 3 proliferation is shown for the superantigens (Staphylococcal enterotoxin -A, -B, -C2, -E, and TSST-1) and the T cell mitogen (phytohemagglutinin) while day 7 proliferation is shown for exoenzyme S and tetanus. All stimuli were used at 1 μg/ml, except for tetanus (10^{-2} Lf units). Induction of CD69 expression was measured after 4 hours in culture with the respective stimuli. Labelled cells were gated on CD3 and analyzed for CD69 expression. Values were calculated by subtracting the isotype control values from the experimental groups. *, $P < 0.05$ calculated by ANOVA compared to unstimulated group; NS, non-significant response compared to unstimulated group. This experiment was performed four times with similar results.

signalling [Schwartz, 1990]. IL- 2 is a T cell-derived cytokine that can stimulate proliferation in an autocrine and paracrine fashion [Greene, 1986]. Therefore, a possible mechanism for the incapacity of PBMC to proliferate to exoenzyme S may be due to a lack of IL-2 production.

It has long been established that various stimuli (eg. antigens, superantigens, mitogens) induce secretion of IL-2 and expression of IL-2 receptors (IL-2Rα, Tac, CD25) prior to T cell proliferation. Therefore, proliferation of T cells is induced primarily in an autocrine fashion, in which the responding T cells secrete their own growth-promoting factors and also express cell surface receptors for these cytokines. IL-2 is the principle autocrine growth factor for T cells. Therefore, the measurement of IL-2 secretion and CD25 expression (in addition to ^{3}H-thymidine incorporation) allows the added benefit of looking at a more proximal sign of T cell stimulation.

In order to determine whether IL-2 was being produced in cultures stimulated with exoenzyme S, supernatants were collected at different time intervals and quantitated in a bioassay. Exoenzyme S (0.1 - 10 μg/ml) was incapable of inducing significant levels of IL-2 (Fig. 13). This suggests that a lack of IL-2 secretion may be limiting in exoenzyme S-stimulated cultures and therefore exogenous addition of the growth factor would be expected to restore this defect. Suprisingly, the weak proliferative response in exoenzyme S cultures could not be augmented by the addition of IL-2 at the beginning of culture (Fig. 14), indicating that exoenzyme S was not inducing anergy. In these experiments, exogenous addition of IL-2 did not augment the proliferative response to exoenzyme S suggesting that CD25 is not upregulated during stimulation with exoenzyme S.

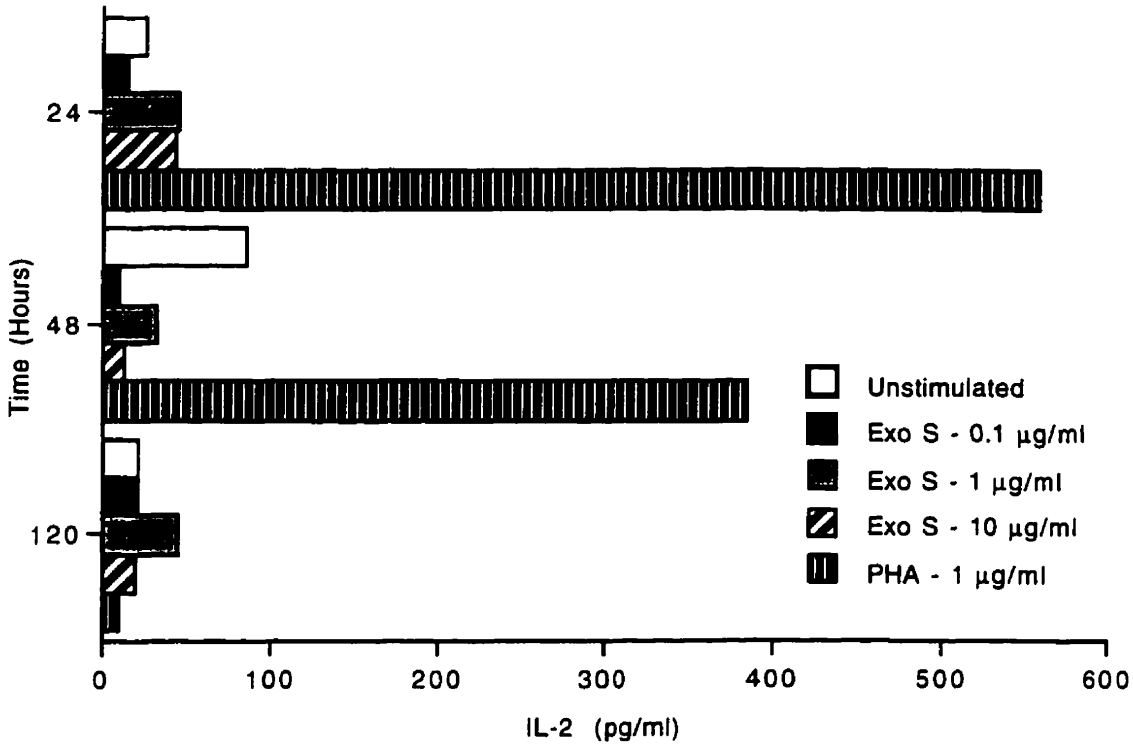

Fig. 13 Exoenzyme S does not induce IL-2 secretion. PBMC (2×10^5) were stimulated with ten-fold dilutions of exoenzyme S (Exo S - 0.1-10 µg/ml) or 1 µg/ml phytohemaglutinin (PHA) for various times in culture. Twenty-four hours before the indicated times, 50 µl of supernatants was replaced with fresh media containing 100 ng/ml anti-TAC and frozen at -70°C until analyzed by HT-2 assay as previously described.

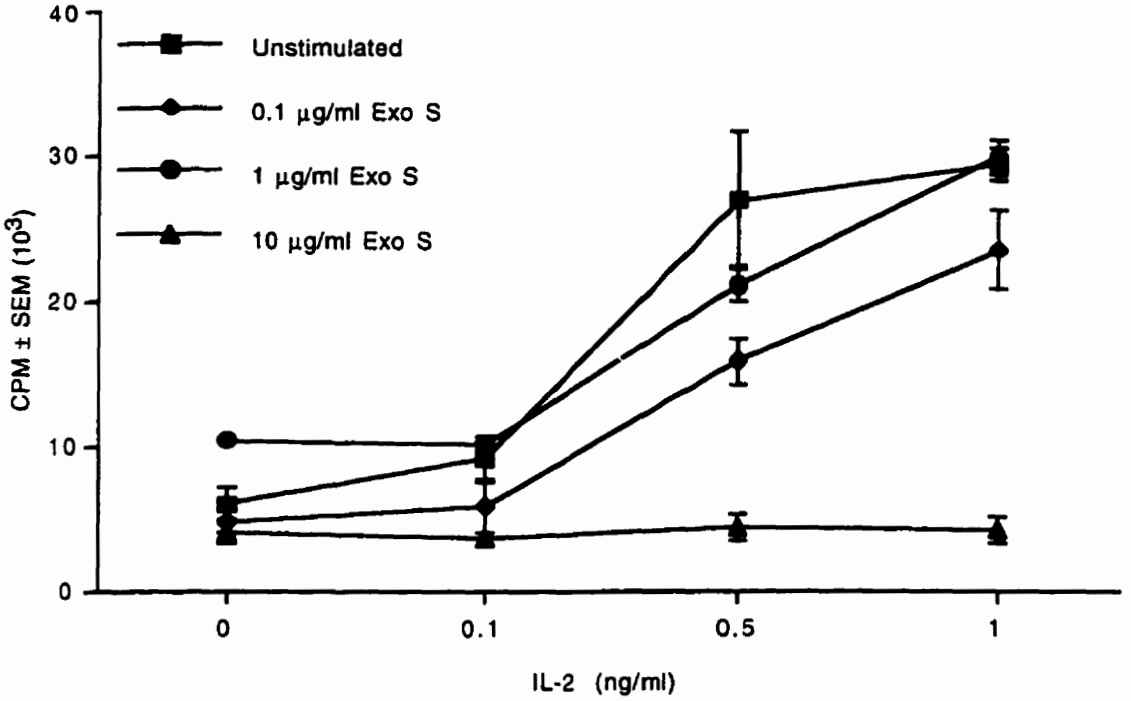

Fig. 14 Interleukin-2 does not augment the proliferative response to exoenzyme S. PBMC (2×10^5), in the absence or presence of different concentrations of IL-2 (0.1, 0.5, 1ng/ml), were stimulated with ten-fold dilutions of exoenzyme S (Exo S - 0.1-10 µg/ml) for 7 days and analyzed for ^{3}H-TdR incorporation (unstimulated - square, exoenzyme S: 0.1 µg/ml - diamond, 1 µg/ml - circle, 10 µg/ml - triangle). This experiment was repeated with a similar result.

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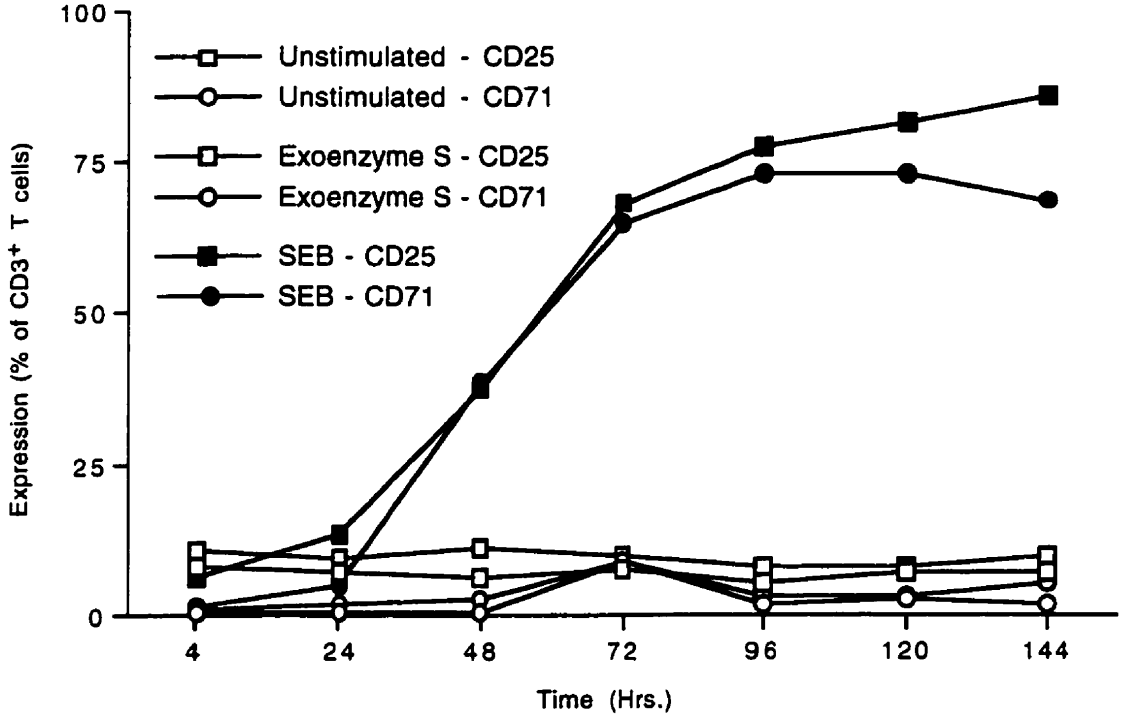


Fig. 15 Surface expression of the T cell activation markers IL-2 receptor (CD25) and transferrin receptor (CD71) in response to exoenzyme S. PBMC (2 x 10^5) were left unstimulated (open symbols), or stimulated with 1 μg/ml exoenzyme S (grey symbols) or 1 μg/ml *Staphylococcal* enterotoxin -B (black symbols). At the indicated times, cells were harvested and labelled with an antibody cocktail consisting of CD3-PerCP and either CD25-FITC or CD71-FITC. Samples were gated on CD3 and analyzed for expression of CD25 (squares) or CD71 (circles). Values are shown as a percentage of T cells expressing the indicated marker and were calculated by subtracting isotype control values from experimental groups. Shown is a representative experiment performed three times.

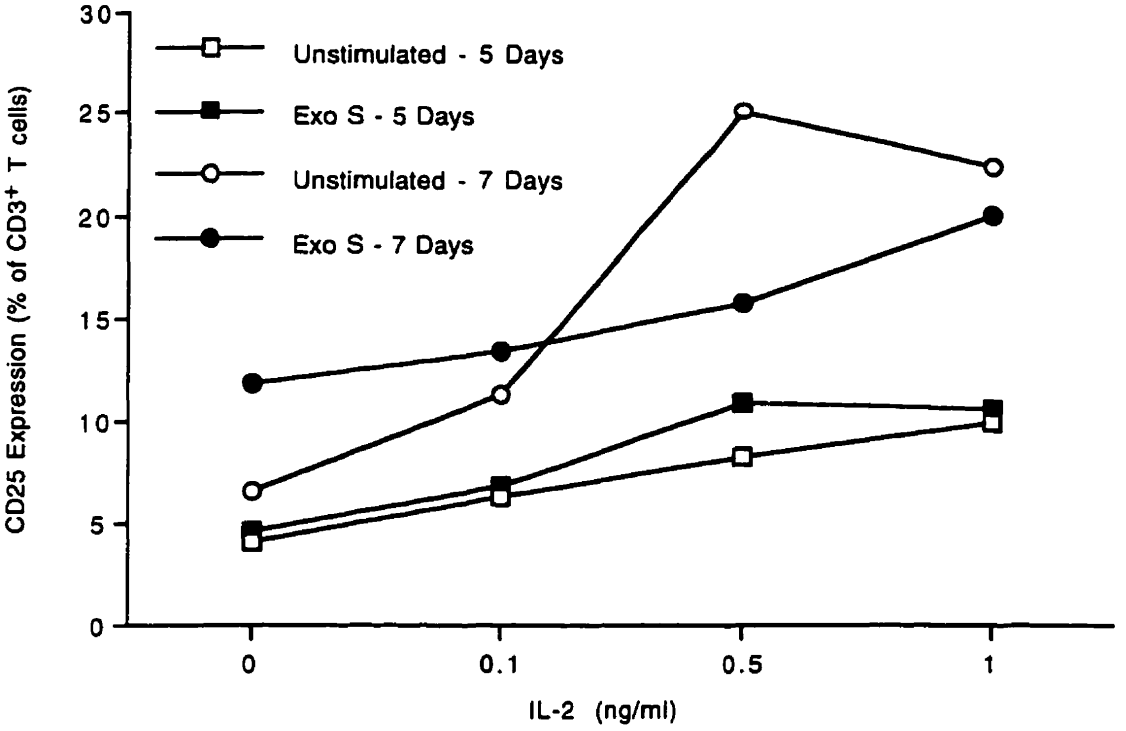


Fig. 16 Interleukin-2 does not induce of IL-2 receptor (CD25) expression on T cells in response to exoenzyme S. PBMC (2 x 10^5), in the absence or presence of different concentrations of IL-2 (0.1, 0.5, 1ng/ml), were left unstimulated (open symbols), or stimulated with 1 μg/ml exoenzyme S (solid symbols).At the indicated times (Day 5 - squares; Day 7 - circles), cells were harvested and labelled with an antibody cocktail consisting of CD3-PerCP and either CD25-FITC. Samples were gated on CD3 and analyzed for expression of CD25. Values are shown as a percentage of T cells expressing the indicated marker and were calculated by subtracting isotype control values from experimental groups. Shown is a representative experiment performed twice.

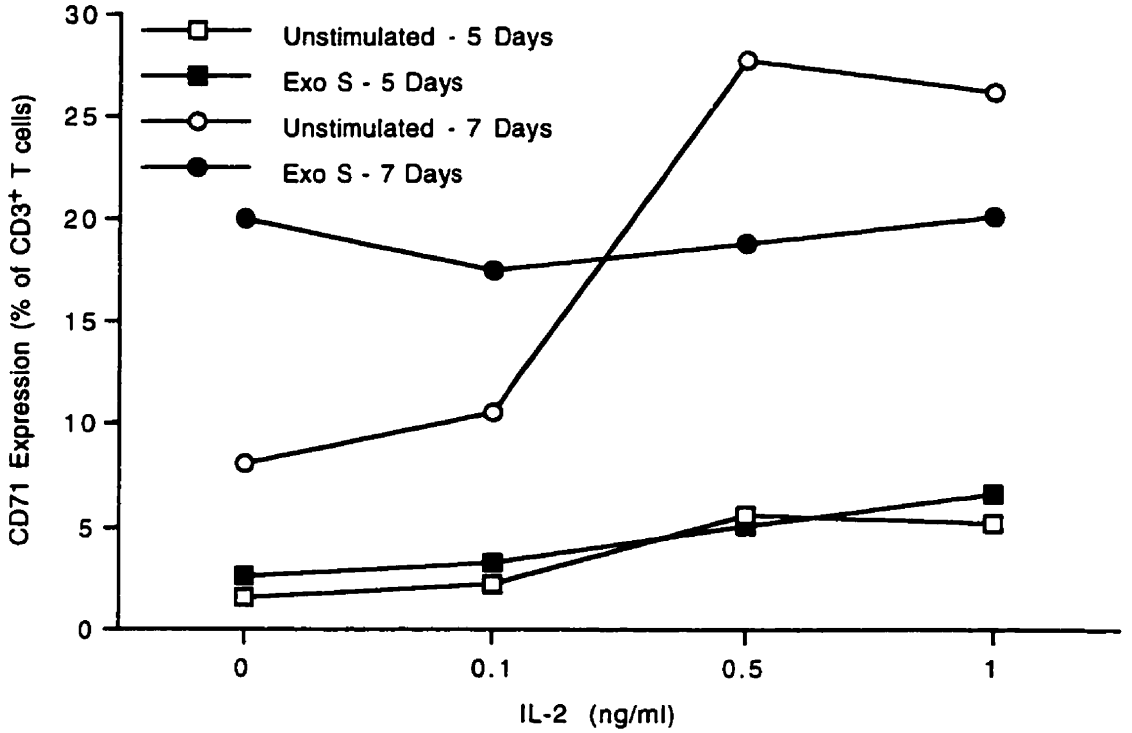


Fig. 17 Interleukin-2 does not induce of transferrin receptor (CD71) expression on T cells in response to exoenzyme S. PBMC (2 x 10^5), in the absence or presence of different concentrations of IL-2 (0.1, 0.5, 1ng/ml), were left unstimulated (open symbols), or stimulated with 1 μg/ml exoenzyme S (solid symbols).At the indicated times (Day 5 - squares; Day 7 - circles), cells were harvested and labelled with an antibody cocktail consisting of CD3-PerCP and either CD71-FITC. Samples were gated on CD3 and analyzed for expression of CD71. Values are shown as a percentage of T cells expressing the indicated marker and were calculated by subtracting isotype control values from experimental groups. Shown is a representative experiment performed twice.

as clonal exhaustion and proliferation are not distinguishable characteristics in these cultures. It was therefore important to look at apoptosis as a possible mechanism by which exoenzyme S-stimulated cells were hyporesponsive during longer cultures.

In order to evaluate whether exoenzyme S was capable of inducing unresponsiveness by an apoptotic mechanism DNA content was quantitated by propidium iodide staining at various timepoints. Although cultures stimulated with exoenzyme S became hypodiploid at a rate slightly slower than that induced by the positive control camptothecin, maximal apoptosis occurred on day 7 for both (exoenzyme S = 59.36 ± 11.43%; camptothecin = 59.58 ±7.69%; Fig. 18). Tetanus toxoid on the other hand, induced only marginal levels of apoptosis above the unstimulated background (tetanus toxoid = 28.18 ± 3.42%; unstimulated = 21.99 ± 4.42%). This suggests that cells were dying the longer they were in culture with exoenzyme S. In retrospect, this finding was consistent with previous results in that IL-2 was not capable of augmenting the proliferative response to exoenzyme S (Fig. 14). However, higher concentrations of exoenzyme S actually showed a reduction of proliferation compared to unstimulated groups despite exogenous addition of IL-2.

To confirm the hypodiploidy we saw using propidium iodide staining was due to programmed cell death, we analyzed co-cultures for the distinctive DNA fragmentation associated with end-stage apoptosis. Electrophoresis of DNA isolated from PBMC cultured for 5 days in the presence of 10 μg/ml exoenzyme S showed the distinctive laddering pattern associated with apoptosis as did cultures incubated with 1μM camptothecin (lane B and lane C respectively; Fig. 19). PBMC stimulated with tetanus toxoid did not show laddering up to 7 days in culture (data not shown).

Staining with propidium iodide allowed for distinction between different stages of the apoptotic cycle. Cells actively undergoing apoptosis (apoptotic) and cells in late stage apoptosis which have begun to bleb off plasma membrane containing partially degraded

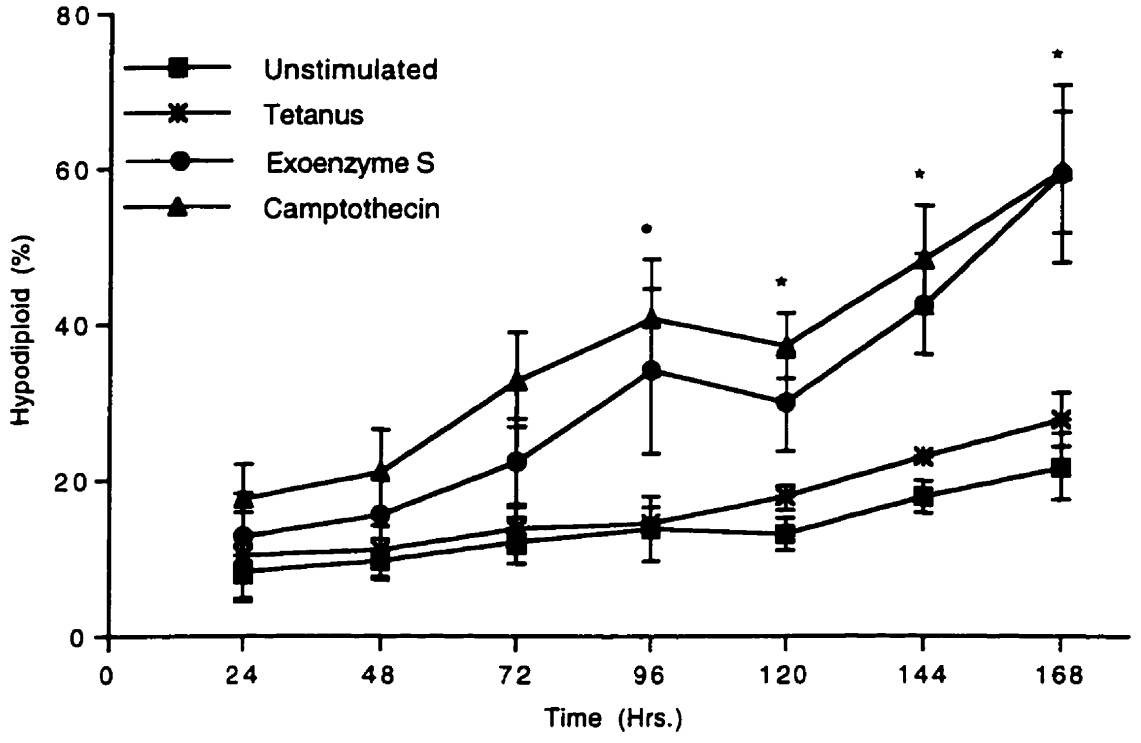


Fig. 18 Induction of apoptosis by exoenzyme S. PBMC (2 x 10^5) were either left unstimulated (squares), cultured with 10 μg/ml exoenzyme S (circles), 1 μM camptothecin (triangles), or 10 μg/ml tetanus toxoid (star) for various times in culture. Cells were harvested at the indicted times and analyzed for DNA content using propidium iodine staining and flow cytometry. This experiment was performed three times. Values are shown as the mean percentage of apoptotic cells ± SEM. *, P <0.05 calculated by ANOVA compared to unstimulated group; NS, non-significant response compared to unstimulated group, n=3.

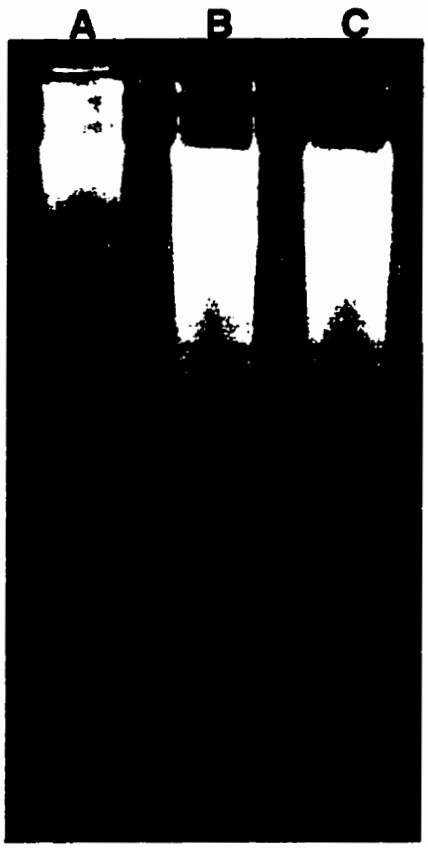
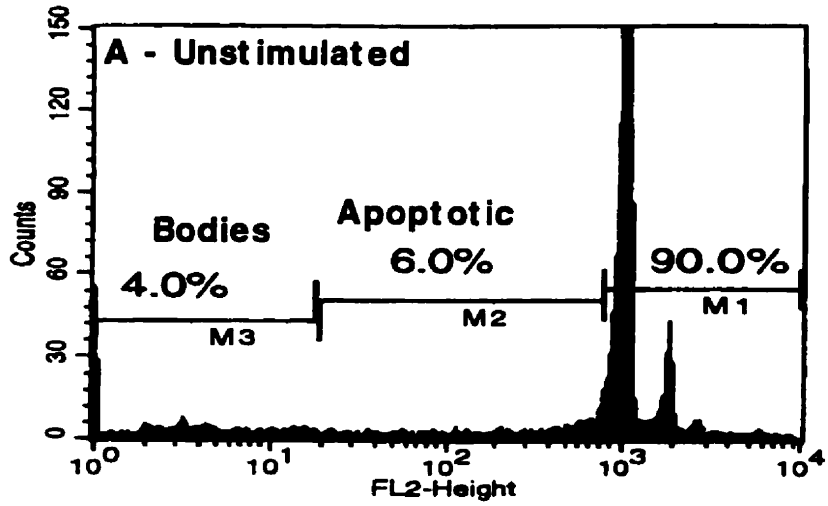
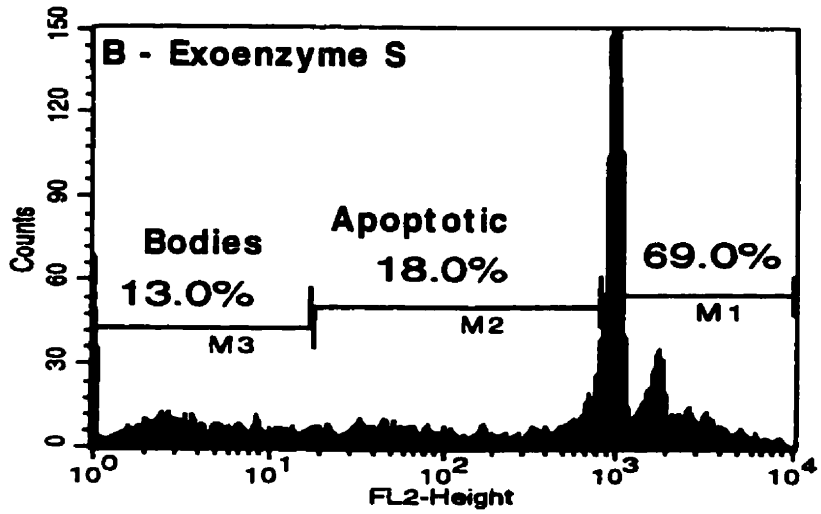


Fig. 19 DNA fragmentation of induced by exoenzyme S. PBMC (2 x 10^5) were either left unstimulated (lane A), cultured with 10 μg/ml exoenzyme S (lane B), or 1 μM camptothecin (lane C). After 5 days in culture, cells were harvested, pooled to a concentration of 10^8 per ml, and subjected to a phenol based extraction.

DNA (apoptotic bodies) constitute the total hypodiploid population (Fig. 20). In order to determine whether T lymphocytes were undergoing death, two different techniques were used. PBMC populations were labelled with FITC-conjugated anti-CD3 in conjunction with either PI staining for DNA quantitation or with PE-conjugated annexin and the vital dye 7-AAD. There was a substantial degree of apoptosis in $CD3^+$ T lymphocytes detected by DNA quantitation (unstimulated = 25.26%; exoenzyme S = 64.94%; camptothecin = 69.76%; Fig.21). Accordingly, similar levels of apoptosis were detected by quantitation of phophatidylserine translocation on $CD3^+$ T cells (unstimulated = 39.37%; exoenzyme S = 67.73%, Fig. 22). Therefore, exoenzyme S is capable of inducing significant T cell apoptosis *in vitro*.


A - Unstimulated
Bodies
4.0%
M3
Apoptotic
6.0%
M2
90.0%
M1
Counts
FL2-Height


B - Exoenzyme S
Bodies
13.0%
M3
Apoptotic
18.0%
M2
69.0%
M1
Counts
FL2-Height

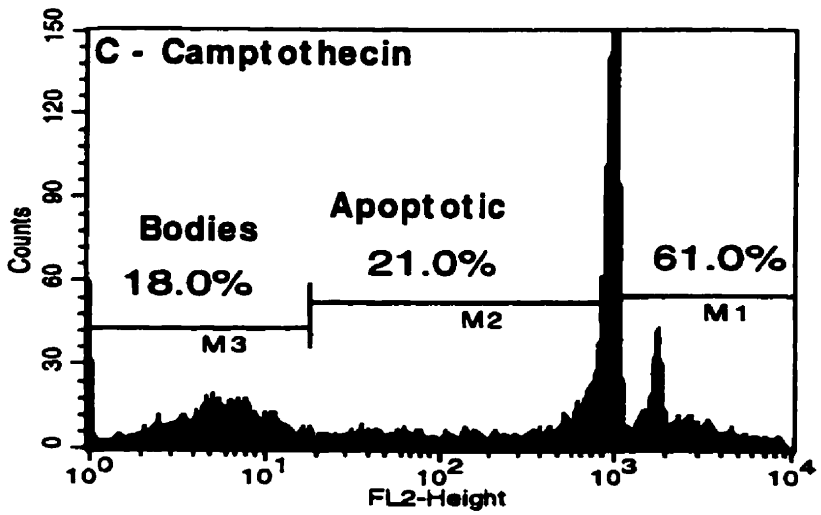

C - Camptothecin
Bodies
18.0%
M3
Apoptotic
21.0%
M2
61.0%
M1
Counts
FL2-Height

Fig. 20 Analysis of the different stages of apoptosis induced by exoenzyme S. PBMC (2×10^5) were either left unstimulated (panel A), cultured with 10 μg/ml exoenzyme S (panel B), or 1 μM camptothecin (panel C). Cultures were harvested after 5 days and analyzed for DNA content using propidium iodine staining. Cells in different stages of the death pathway are separated with margins and the percent of cells in each stage is indicated. This experiment was repeated twice with similar results.

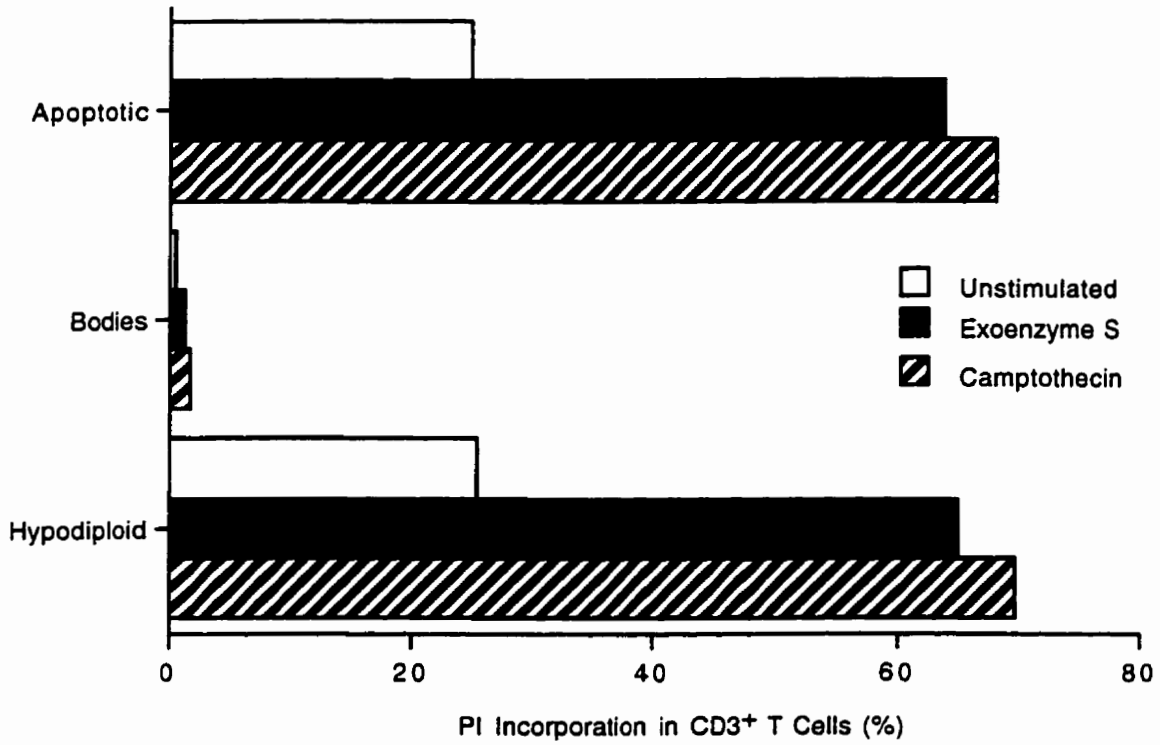


Fig. 21 Analysis of apoptosis by exoenzyme S in T cells. PBMC (2×10^5) were either left unstimulated (open bars), cultured with 10 μg/ml exoenzyme S (solid bars), or 1 μM camptothecin (striped bars). Cultures were harvested after 5 days, stained with FITC-conjugated anti-CD3 and analyzed for DNA content using propidium iodine staining. Cells were gated on CD3 and analyzed for DNA content. This experiment was performed twice.

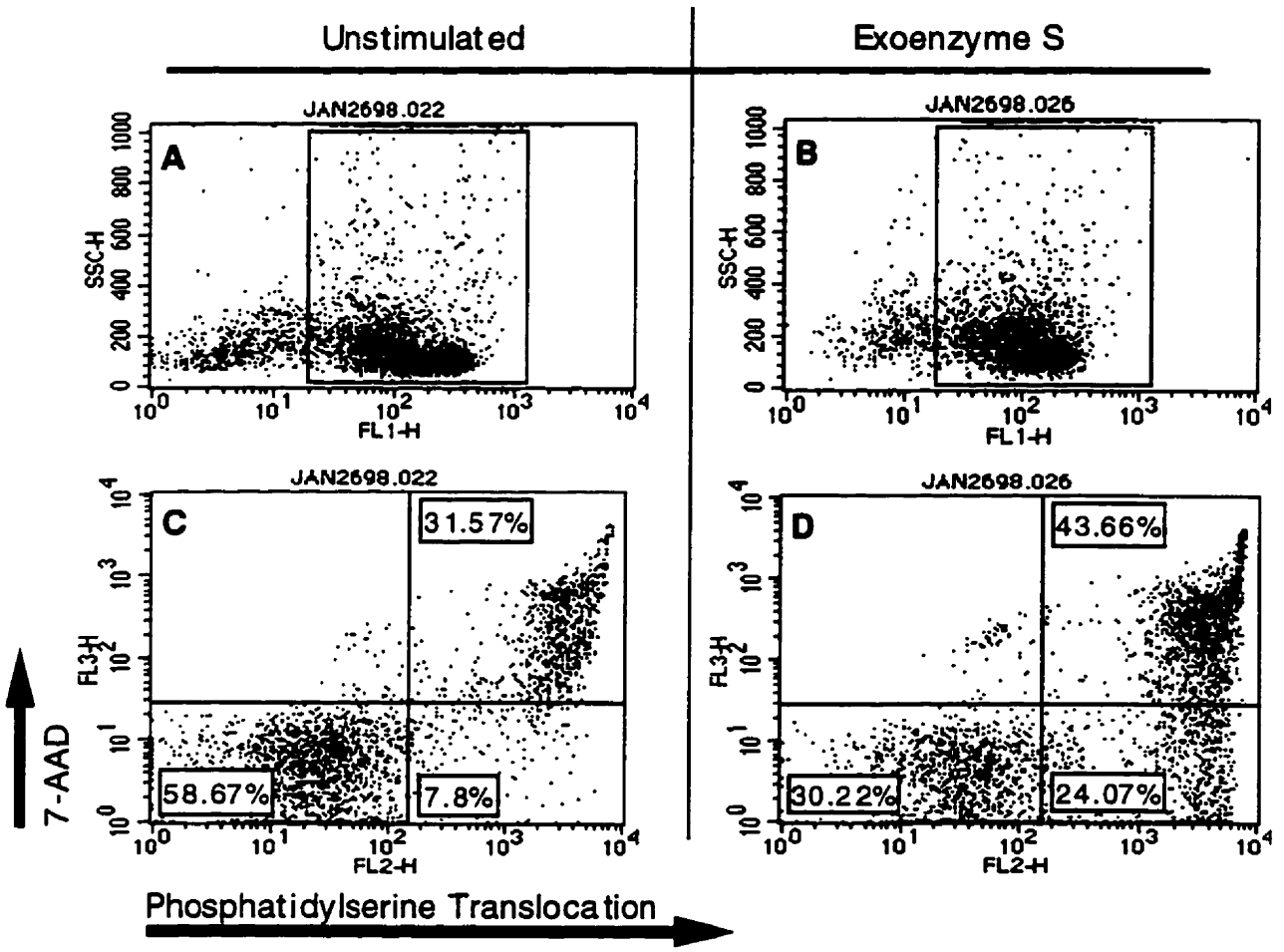


Fig. 22 Dot plot analysis of apoptosis by exoenzyme S in T cells. PBMC (2×10^5) were either left unstimulated (Panel A & C) or stimulated with 10 μg/ml exoenzyme S (Panel B &D) for 5 days and labelled with FITC-conjugated anti-CD3 (FL1). DNA content was analyzed by using PE-conjugated annexin V (FL2) and 7-AAD (FL3) (Panel C & D) to measure phosphatidylserine translocation and membrane permeability respectively after electronic gate setting on CD3 versus side scatter characteristics (Panel A &B) to identify T cells. This experiment was performed twice.

3.3.2 Cystic Fibrosis Donors

Due to practical issues it was first necessary to develop and optimize assays using healthy adults as donors. Although no researchers have been able to demonstrate any primary immune defect in CF patients [Davis, 1985], [Pier, 1985], the possibility remained that patients having been chronically colonized with *Pseudomonas aeruginosa* and potentially exposed to exoenzyme S may have an altered immune response compared to normals. Indeed, other investigators have demonstrated secondary immune defects in T cells isolated from CF patients. Impairments of *in vitro* proliferative responses to whole organism as well as a decrease in the T cell production of IL-10 have both been shown in CF patients [Sorensen *et al.*, 1977], [Moss *et al.*, 1996]. It was anticipated therefore, that peripheral T cells isolated from CF patients (with positive *Pseudomonas aeruginosa* cultures) may be chronically exposed to exoproducts and as a consequence, would have altered responses compared to normals. Having previously established a relevant mechanism for exoenzyme S to induce T cell activation arrest and apoptosis in PBMC isolated from healthy donors, it was necessary to extend these findings using PBMC isolated from adult CF patients. CD69 expression levels were again determined on $CD3^+$ T cells after 4 hours culture with exoenzyme S and compared to levels induced by SEB. Both SEB and exoenzyme S were capable of inducing significant T cell activation in PBMC isolated from cystic fibrosis patients (Fig. 23A; unstimulated = 7.09% ± 2.61%; exoenzyme S = 28.58% ± 7.32%; SEB = 19.59% ± 2.44%, n=4) and normal control (Fig. 23B; unstimulated = 5.18% exoenzyme S = 31.9%; SEB = 13.51%). Suprisingly, magnitudes of T cell activation (CD69 expression) did not differ between CF patients and normal controls.

In order to determine whether there was a discrepancy between the levels of T cell activation and subsequent proliferation, co-cultures stimulated with exoenzyme S were analyzed for CD69 at 4 hours and for ^{3}H-TdR uptake at 7 days. Early levels of activation did not correlate with subsequent levels of proliferation for either CF donors (Fig. 24A) or

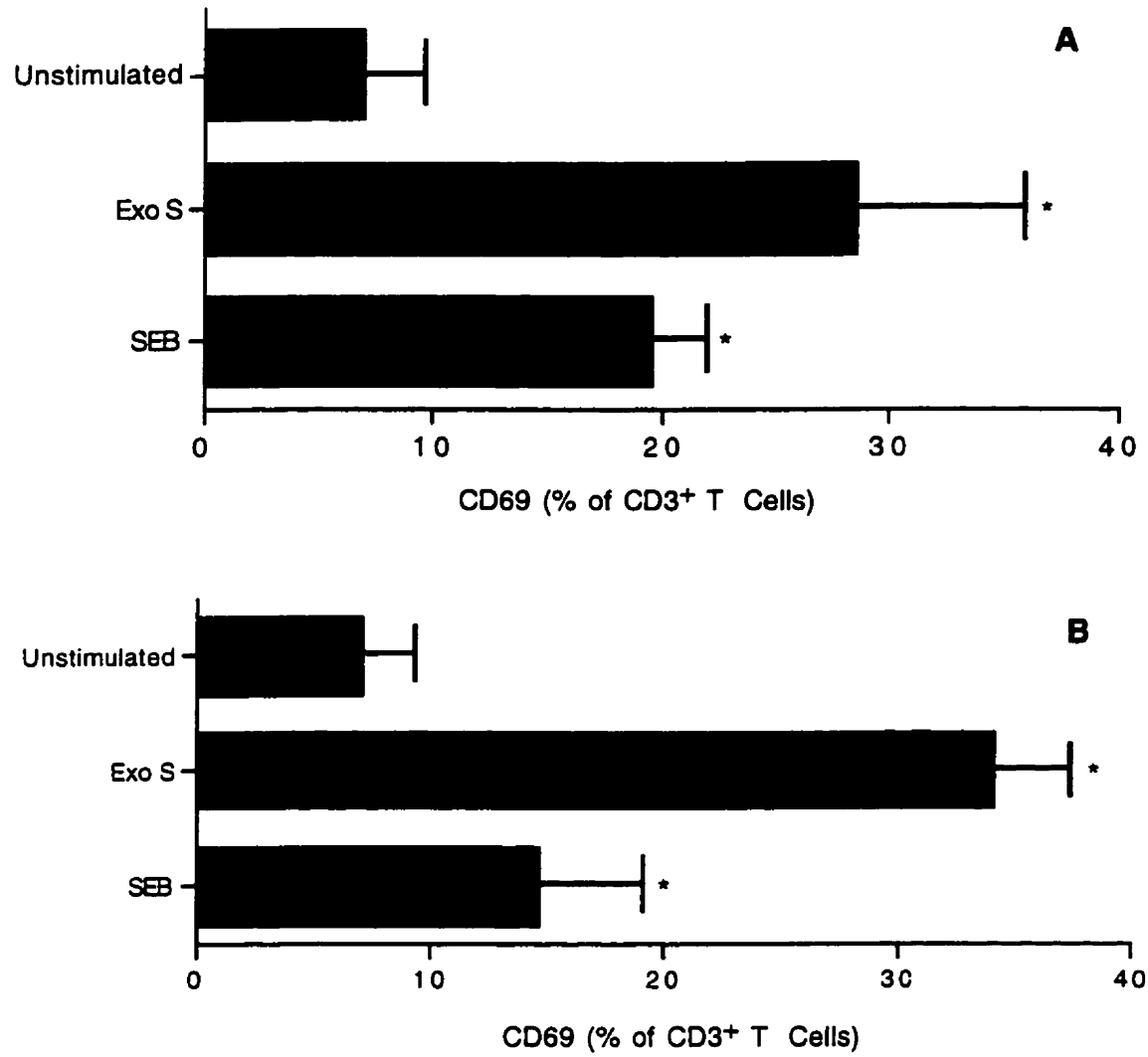
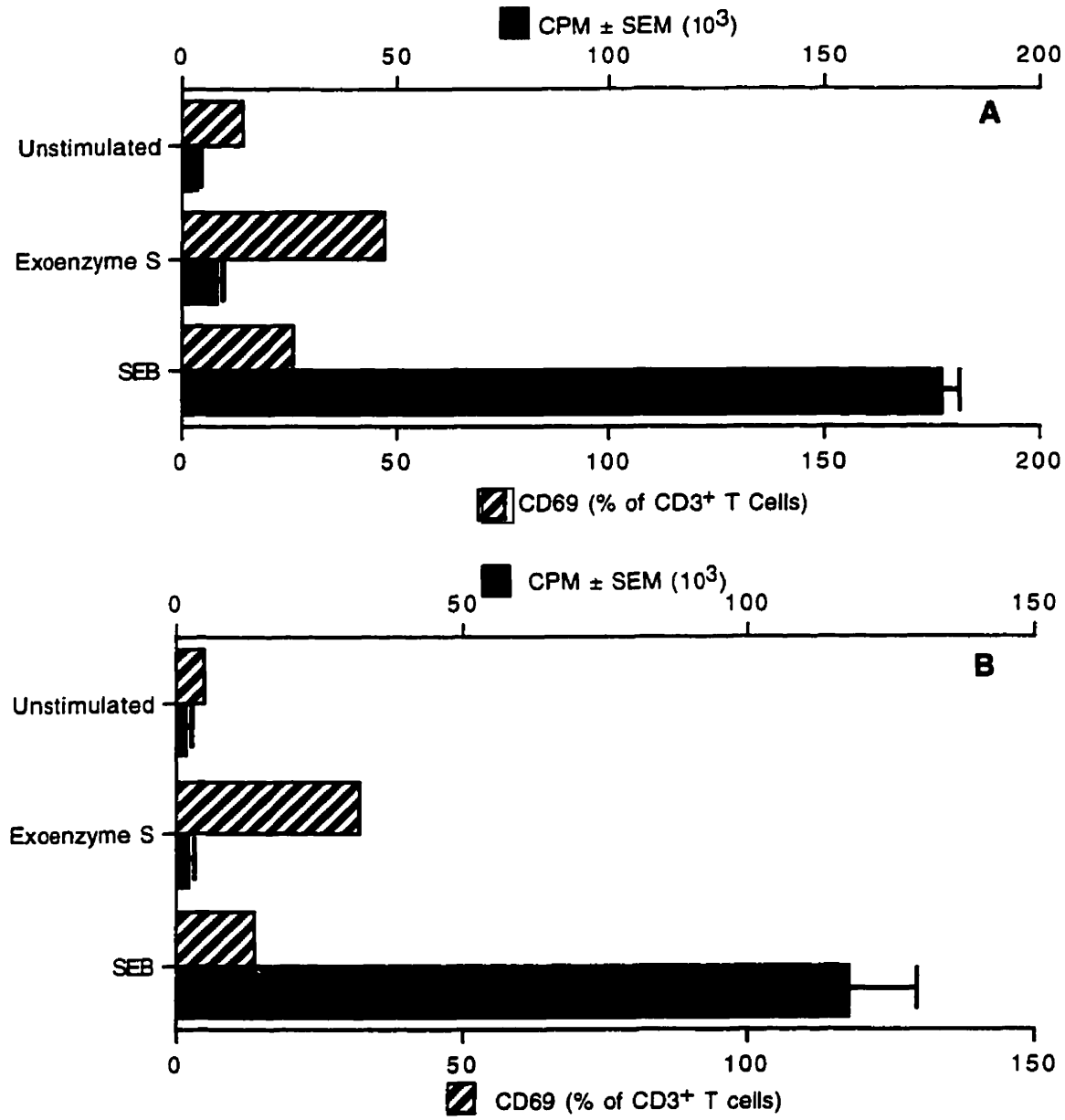


Fig. 23 Comparison of CD69 expression on peripheral T lymphocytes between CF patients and normal controls. PBMC (CF - panel A; normal - panel B) were left unstimulated or cultured with 1 μg/ml exoenzyme S or*Staphylococcal* enterotoxins -B (SEB) for 4 hours. Samples were harvested and labelled with anti-CD69-PE/ anti-CD3-PerCP. The percentage of CD3 cells expressing CD69 was determined. *, $P < 0.05$ using student's t test compared to corresponding unstimulated group.


CPM ± SEM (10^3)
0
50
100
150
200
A
Unstimulated
Exoenzyme S
SEB
CD69 (% of CD3+ T Cells)
CPM ± SEM (10^3)
0
50
100
150
B
Unstimulated
Exoenzyme S
SEB
CD69 (% of CD3+ T cells)

a healthy control (Fig. 24B), reinforcing the previous findings that early T cell activation is intact, but incapable of inducing significant levels of 'full activation' in the form of proliferation. Thus, despite the fact that these CF patients were chronically colonized with *P. aeruginosa*, it appears that the responses from these patients to exoenzyme S is not any different than what is seen from normal controls.

As before, other markers of T cell activation such as IL-2 production, CD25 and CD71 expression were measured on $CD3^+$ T cells in response to exoenzyme S. IL-2 levels were not produced above background in response to exoenzyme S, unlike SEB which induced significant levels (Fig. 25). Further, exoenzyme S could not induce significant expression of CD25 nor CD71 (Fig. 26). The positive control, SEB, did however induce significant expression of both markers. Again, similar to data from healthy subjects, this implies that the mechanism for decreased proliferative responses to exoenzyme S is distinct from anergy. Importantly, no observable differences in the magnitude of the responses (expression of CD69, CD25, CD71, or production of IL-2) to either exoenzyme S or SEB was noticed between CF patients and healthy controls. These series of experiments demonstrate that the responses to superantigens or exoenzyme S by PBMC cultures from CF patients are not different than those obtained from healthy adult donors suggesting a similar T cell activation mechanism is being utilized.

Recruited T lymphocytes from the lungs of CF patients may be more susceptible to apoptotic death induced by exoenzyme S when compared to healthy donors because their T cells may be preconditioned by a constitutive elevated state of activation. To determine whether PBMC were undergoing apoptosis in response to exoenzyme S, cultures were harvested at 5 and 7 days and analyzed for DNA content. As seen in healthy controls, PBMC isolated from CF patients showed an increase in both the percentage of cells actively undergoing apoptosis (apoptotic) and in cells already having undergone apoptosis (bodies) (Fig. 27 and Fig. 28). Preliminary experiments also suggested that the degree of apoptosis induced by exoenzyme S *in vitro* correlated inversely with the patient's pulmonary

function. That is, the lower the pulmonary function score, determined by a one second forced expiratory volume (FEV_1), the greater the magnitude of apoptosis in response to exoenzyme S *in vitro* (Fig. 29). Interestingly, this correlation was specific only for exoenzyme S, as co-cultures induced with camptothecin did not show this effect (exoenzyme S: R^2=0.9, $p<0.01$; camptothecin: R^2=0.79, $p<0.11$). It is conceded that the number of patients is too small to analyze appropriately and therefore this correlation is not free of scrutiny. However, it is interesting to speculate that exoenzyme S-induced T cell activation may transduce more of a 'death signal' in potentially activated T cells (assuming patients with decreased pulmonary function have a higher antigen load) compared to relatively quiescent cells isolated from asymptomatic individuals. Alternatively, this may simply indicate that T lymphocytes isolated from CF patients with exacerbated disease may be more susceptible to this form of apoptotic signalling.

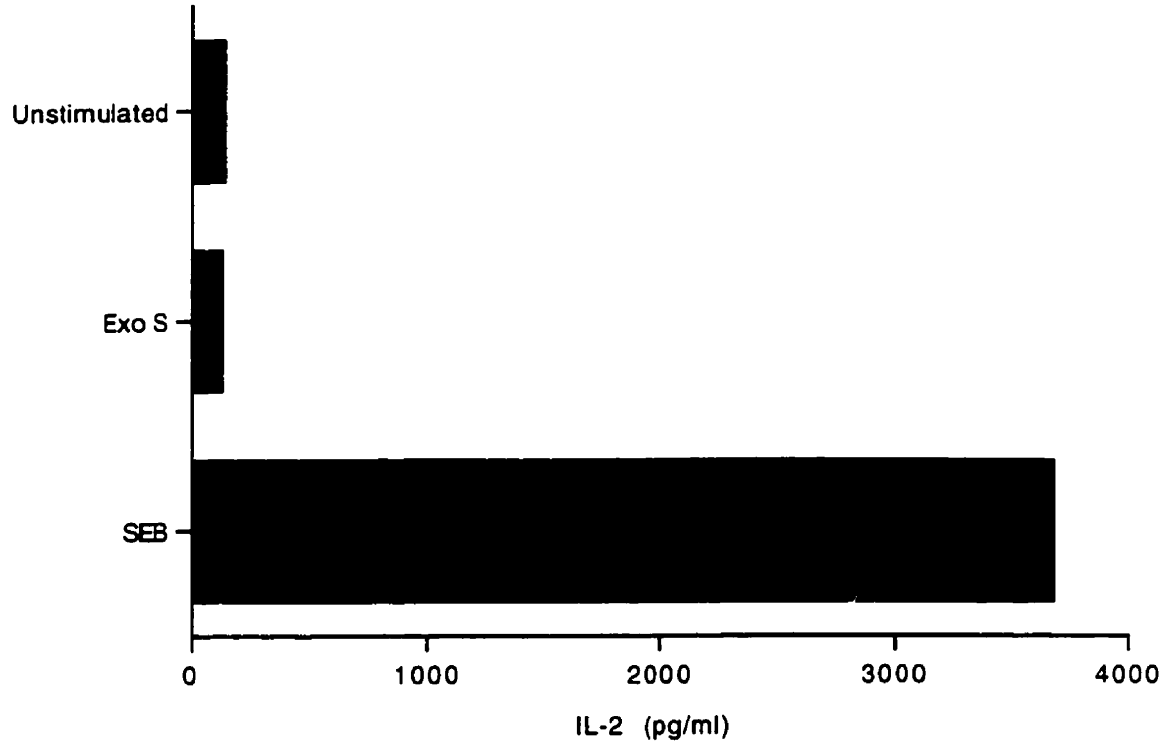
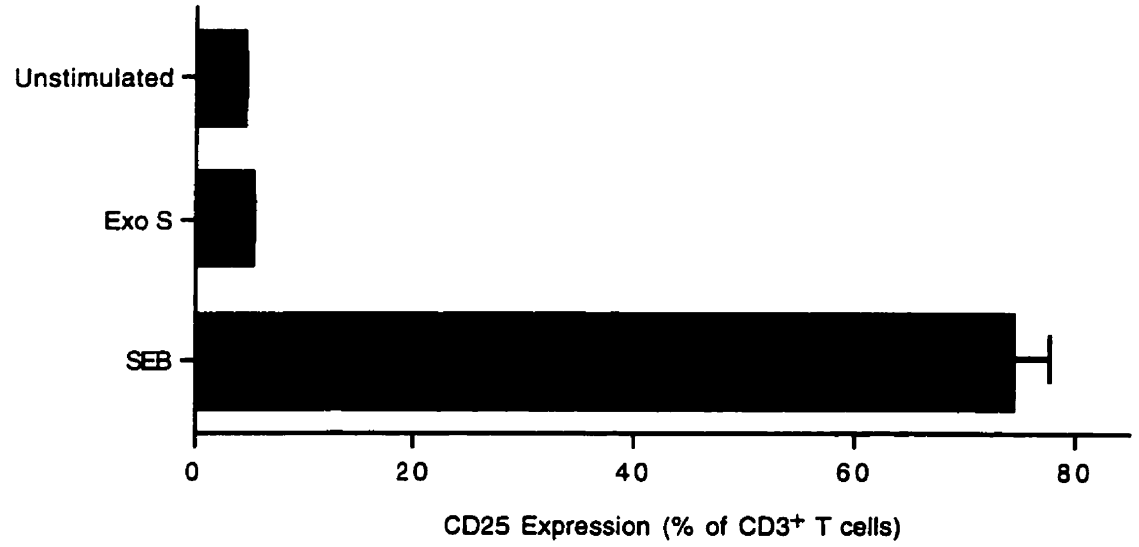


Fig. 25 Exoenzyme S induced IL-2 secretion from PBMC isolated from CF patients. PBMC (2×10^5) were stimulated with 1 μg/ml of exoenzyme S or*Staphylococcal* enterotoxins -B (SEB) for 5 days. Twenty-four hours prior to harvest, 50 μl of supernatants was replaced with fresh media containing 100 ng/ml anti-TAC and frozen at -70°C until analyzed by HT-2 assay as previously described. This experiment was repeated twice with similar results.


Unstimulated
Exo S
SEB
0
20
40
60
80
CD25 Expression (% of CD3+ T cells)

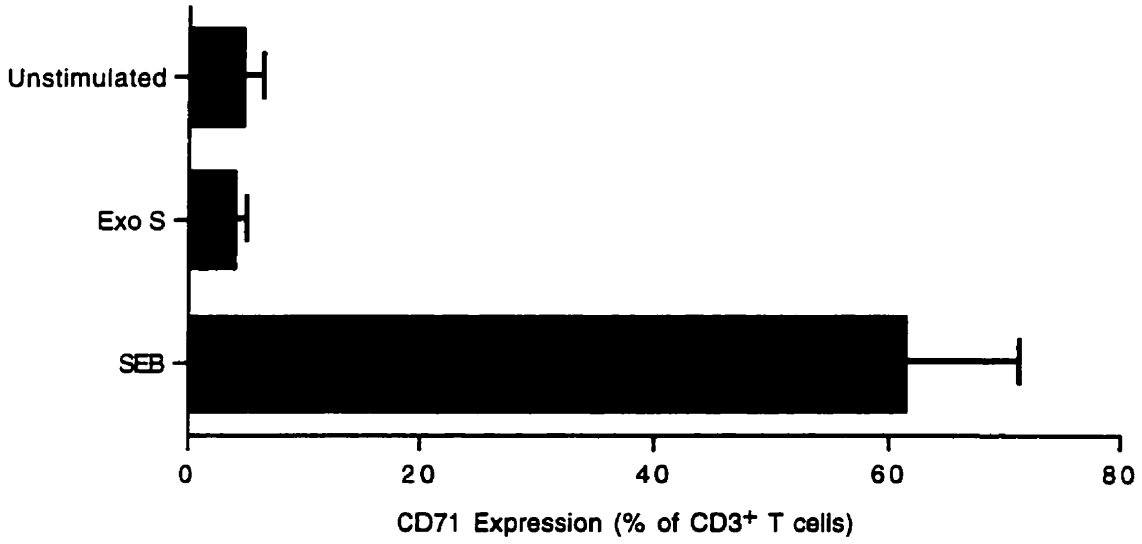

Unstimulated
Exo S
SEB
0
20
40
60
80
CD71 Expression (% of CD3+ T cells)

Fig. 26 Surface expression of the IL-2 receptor (CD25) and transferrin receptor (CD71) on PBMC isolated from CF patients in response to exoenzyme S. PBMC (2×10^5) were left unstimulated, or stimulated with 1 μg/ml exoenzyme S or*Staphylococcal* enterotoxin -B (SEB). After 5 days, cells were harvested and labelled with an antibody cocktail consisting of CD3-PerCP and either CD25-FITC or CD71-FITC. Samples were gated on CD3 and analyzed for expression of CD25 (panel A) or CD71 (panel B). Values are shown as a percentage of T cells expressing the indicated marker and were calculated by subtracting isotype control values from experimental groups. *, $P < 0.05$ using student's t test compared to corresponding unstimulated group; NS, non-significant response compared to corresponding unstimulated group, n=4.

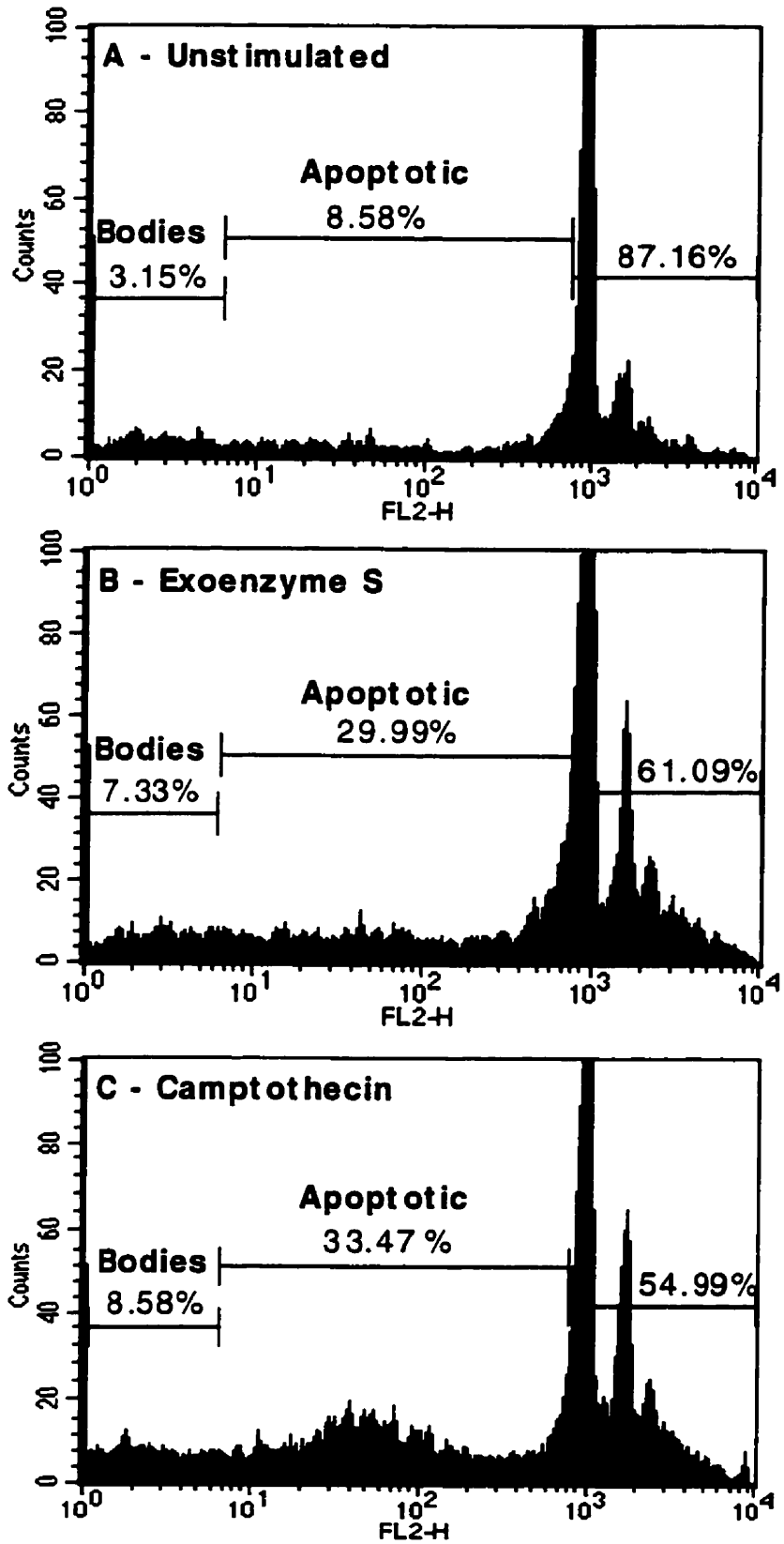

A - Unstimulated
Apoptotic
8.58%
Bodies
3.15%
87.16%
Counts
FL2-H
B - Exoenzyme S
Apoptotic
29.99%
Bodies
7.33%
61.09%
Counts
FL2-H
C - Camptothecin
Apoptotic
33.47 %
Bodies
8.58%
54.99%
Counts
FL2-H

Fig. 27 Analysis of the different stages of apoptosis induced in PBMC isolated from a CF patient. PBMC (2×10^5) were either left unstimulated (panel A), cultured with 10 μg/ml exoenzyme S (panel B), or 1 μM camptothecin (panel C). Cultures were harvested after 5 days and analyzed for DNA content using propidium iodine staining. Cells in different stages of the death pathway are separated with margins and the percent of cells in each stage is indicated. This experiment was repeated twice with similar results.

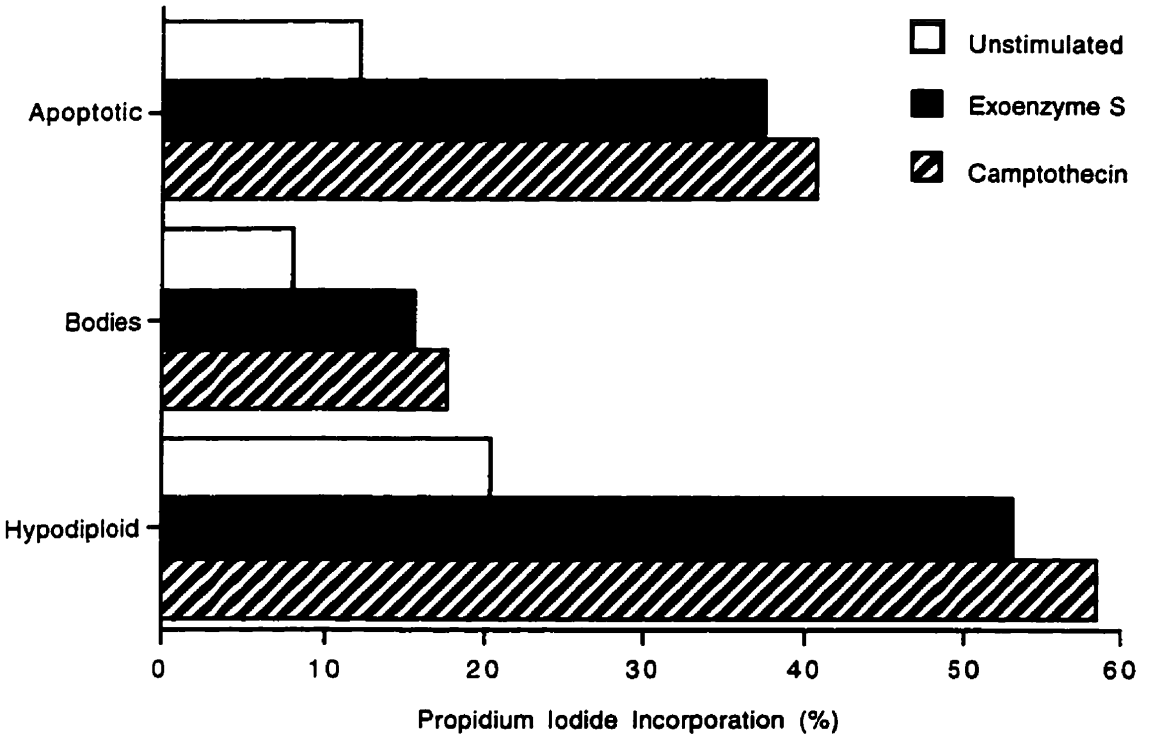


Fig. 28 Analysis of apoptosis by exoenzyme S in T cells. PBMC (2×10^5) were either left unstimulated (open bars), treated with 10 μg/ml exoenzyme S (solid bars), or 1 μM camptothecin (striped bars). Cultures were harvested after 5 days, stained with propidium iodine and analyzed for DNA content using flow cytometry. This is one representative experiment of three.

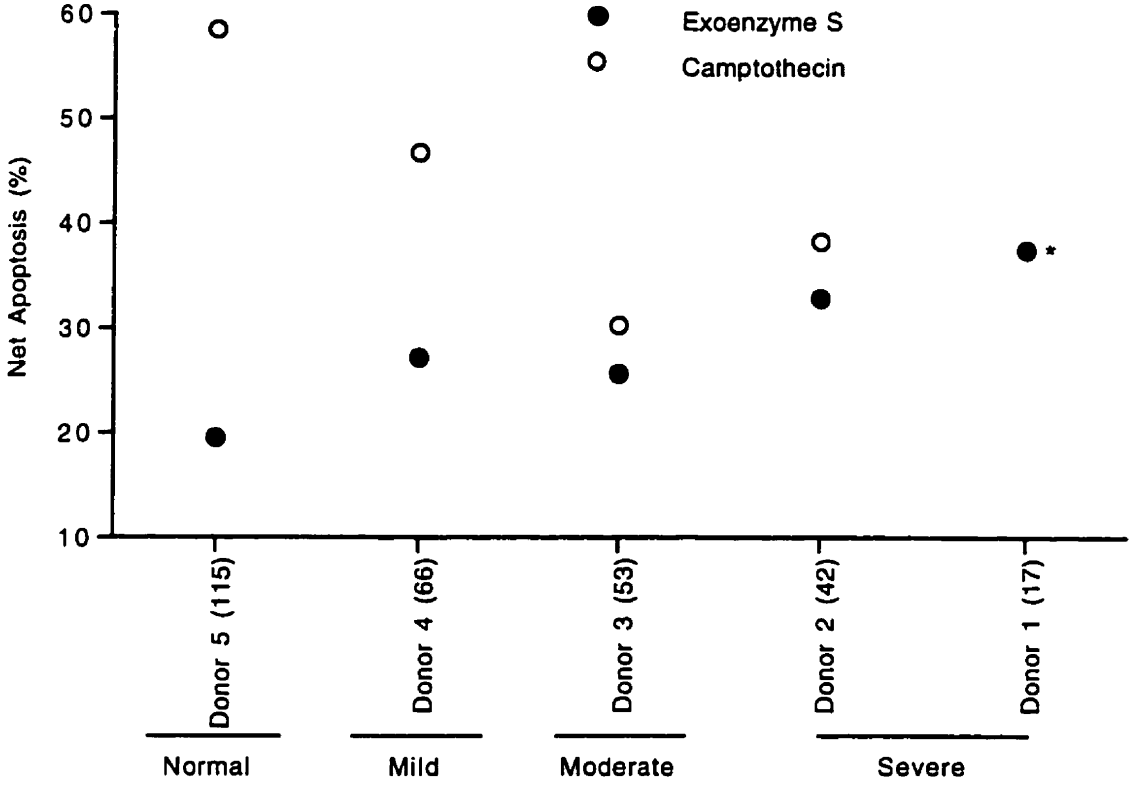


Fig. 29 Inverse correlation between apoptosis and pulmonary function. PBMC (2 x 10^5) were either left unstimulated, treated with 10 μg/ml exoenzyme S, or 1 μM camptothecin. Apoptotic values are seen as the increase in hypodiploid DNA calculated by subtracting the degree of hypodiploidy in unstimulated populations from that seen in either experimental group. Hypodiploid values are displayed as a percent of total cells. Cultures were harvested after 5 days, stained with propidium iodine and analyzed for DNA content using flow cytometry. Five donors are shown with their respective FEV_1 scores in parenthesis. *, $P < 0.01$ calculated by regressional statistics, $R^2=0.9$ for exoenzyme S ; $P < 0.11$, $R^2=0.79$ for camptothecin.

3.4 Comparison of the *In vitro* Responses to Exoenzyme S from *Pseudomonas aeruginosa* Strain DG1 and Recombinant Exoenzyme S from Strain 388

There has been much debate about the true nature and purity of exoenzyme S purified from strain DG1(ExoS/DG1) by the method of Woods [Goranson and Frank, 1996]. Much of the controversy surrounds the fact that ExoS/DG1 is purified in an enzymatically inactive state [Woods and Que, 1987]. Recently, the gene coding for the 49 kDa form of exoenzyme S (*exoS*) from strain 388 has been sequenced and cloned into both *Escherichia coli* BL21(DE3) and *Pseudomonas aeruginosa* PA103 [Knight *et al.*, 1995]. It was therefore important for our laboratory to determine whether there were significant differences in the lymphocyte responses to ExoS/DG1 and rExoS. Joseph Barbieri and Dara Frank (both from the Medical College of Wisconsin) graciously donated rExoS purified from *Pseudomonas aeruginosa* PA103 in order to carry out these studies. Initially, the enzymatic activity was determined for both preparations using a standard ADP-ribosyl transferase assay in which [adenine-^{14}C]NAD^{+} is used as the donor of ADP-ribose [Woods and Que, 1987]. Compared to rExoS, the specific activity of ExoS/DG1 was less than 1% (Table 1), indicating that these two preparations are vastly different in their ADP-ribosyl transferase activity. This data confirms the previous association.

In order to determine whether rExoS could activate T cells as demonstrated for ExoS/DG1, activation assays were performed as previously described. PBMC cultured for 4 hours with rExoS induced an increase in the percentage of T cells that expressed CD69. In response to rExoS, 26.2% of T cells that were previously negative for CD69 became CD69^{+}, while 29.2% of T cells expressed CD69 in response to ExoS/DG1 (Figure 30). The mean fluorescent intensity increased from 18 to 65 in response to rExoS, and to 61 in response to ExoS/DG1. Thus, the frequency and level of T cells that were activated in response to rExoS and ExoS/DG1 was similar.

Table 1. Comparison of ADP-ribosyl transferase activity between ExoS/DG1 and rExoS.

Preparation	Specific Activity[1]
Blank	4.2
Exo/DG1	1344
rExo	221,627

[1] Specific activity was calculated as CPM per mg of preparation per minute of reaction time.

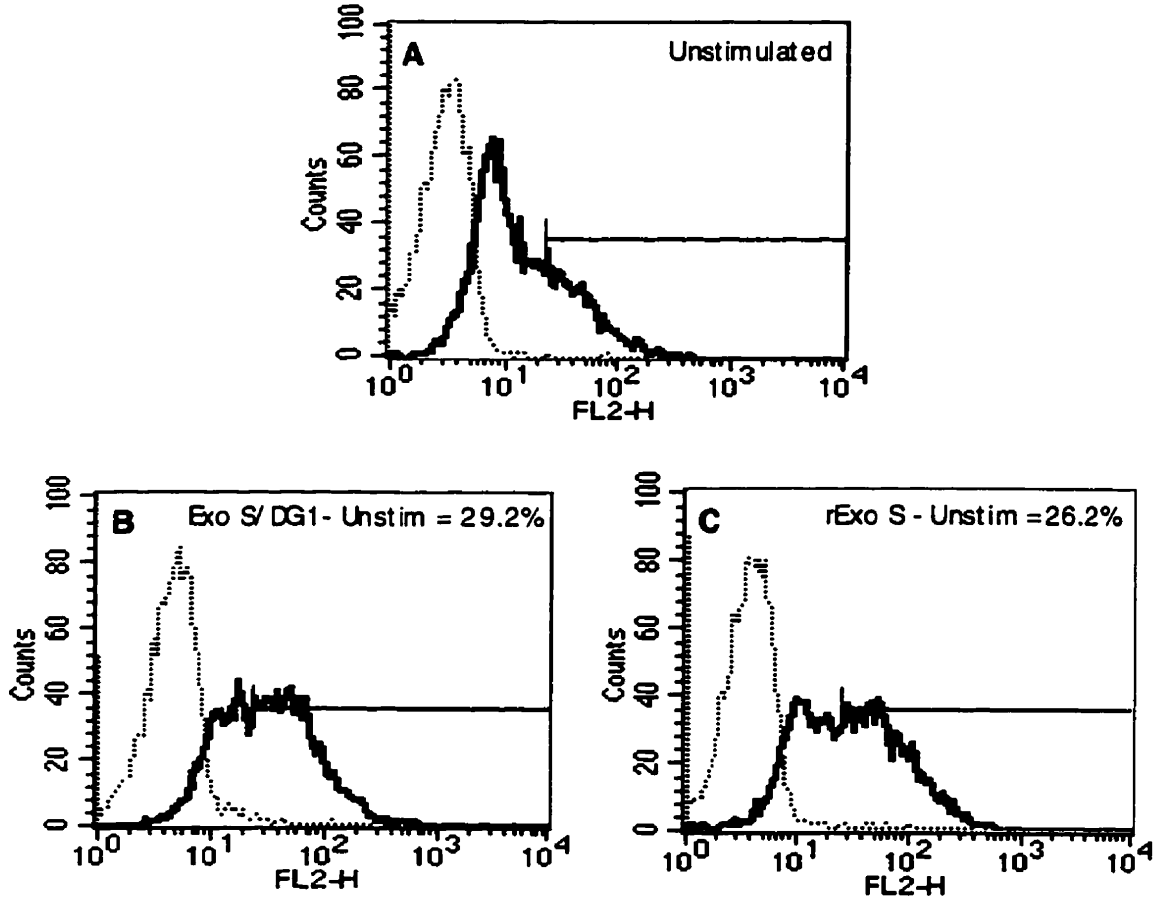


Fig. 30. T cell activation by ExoS/DG1 and rExoS. PBMC were unstimulated (panel A), stimulated with 1 μg/ml ExoS/DG1 (panel B), or stimulated with 1 μg/ml of rExoS (panel C) for 4 hours and analyzed for CD69 expression (solid line). Each cell population was also labeled with an isotype-matched antibody (dotted lines). The experiment was repeated three times with similar results.

To determine whether rExoS induces lymphocyte proliferation, PBMC were stimulated with rExoS or ExoS/DG1 and ^{3}H-TdR incorporation was assessed as a measure of new DNA synthesis. Significant ^{3}H-TdR incorporation occurred at concentrations of 1.0 μg/ml with both preparations (Table 2), and the stimulation index for rExoS and ExoS/DG1 was similar. At 1.0 μg/ml, the stimulation index for ExoS/DG1 and rExoS was 4.2 ± 1.1 and 3.5 ± 0.4 respectively. Thus, the magnitude of proliferation is similar in response to both preparations of exoenzyme S.

It has already been shown that T cell stimulation by ExoS/DG1 induces a large and transient surface expression of CD69 followed by a relatively modest level of cellular proliferation (Fig. 12). In order to demonstrate that rExoS induced similar levels of initial activation and subsequent proliferation, co-cultures were stimulated with either ExoS/DG1 or rExoS and analyzed for CD69 expression and ^{3}H-TdR incorporation. rExoS does induce a similar activation/proliferation profile as ExoS/DG1 (Fig 31; CD69: rExoS = 27.52%; ExoS/DG1 = 31.96; Net CPM: rExoS = 5.66 ; ExoS/DG1 = 7.76).

The previous studies showed that the activation and proliferation induced by rExoS and ExoS/DG1 was similar. To determine whether the region of ExoS/DG1 that was responsible for T cell activation was shared by rExoS, a panel of mAbs raised against ExoS/DG1 were screened for their ability to neutralize CD69 expression. The mAb 9-49-9, which showed the greatest neutralization capacity against ExoS/DG1 was used to neutralize the stimulatory capacity induced by rExoS (Table 3). PBMC were stimulated with rExoS or ExoS/DG1 in the presence of mAb 9-49-9 or control ascites for four hours and analyzed for expression of CD69. mAb 9-49-9 significantly reduced T cell activation by rExoS and ExoS/DG1, suggesting that these preparations share the stimulatory epitope. The mAb 9-49-9 did not block T cell activation induced by other T cell stimuli such phytohemagglutinin suggesting that the neutralization was specific for exoenzyme S.

Table 2. Proliferation of PBMC in response to rExoS and ExoS/DG1

	Stimulation Index (CPM ± SEM X 10^3)[1]	
Experiment	rExoS	ExoS/DG1
1	4.11 (22.6)*	2.09 (11.5)
2	2.96 (7.7)*	4.50 (11.7)*
3	3.17 (1.9)	6.17 (3.7)*

[1]PBMC (2 x 10^5/well) were stimulated with 1.0 μg/ml of rExoS or ExoS/DG1. Stimulation index calculated by dividing proliferative responses of experimental groups by unstimulated values. * $p<0.05$ compared to unstimulated PBMC as determined by ANOVA.49-9 did not block T cell activation induced by phytohemagglutinin suggesting that the neutralization was specific for exoenzyme S.

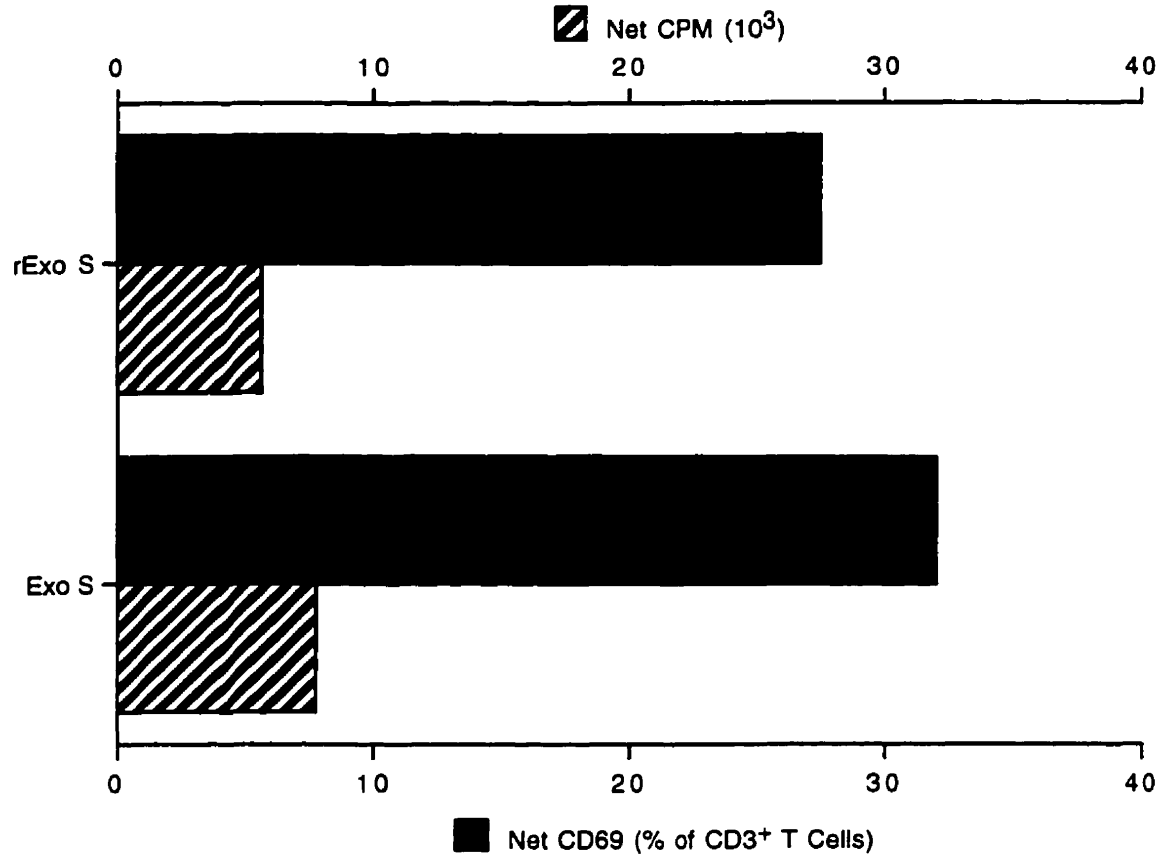


Fig. 31 Correlation between early T cell activation and subsequent proliferation for ExoS/DG1 and rExoS. Induction of CD69 was used as a measure of early T cell activation and ^{3}H-TdR incorporation was used to measure subsequent proliferation of co-cultures. Day 7 proliferation is shown for both preparations and was calculated by subtracting the proliferative response in unstimulated cultures from both experimental groups (both at 1 μg/ml). Induction of CD69 expression was measured after 4 hours in culture. Labelled cells were gated on CD3 and analyzed for CD69 expression. Values were calculated by subtracting the isotype control values from the experimental groups. *, P <0.05 calculated by ANOVA compared to unstimulated group; NS, non-significant response compared to unstimulated group. This experiment was performed four times with similar results.

Table 3 mAb 9-49-9 inhibits T cell activation induced by ExoS/DG1 and rExoS

	% of CD3 cells that newly express CD69 (% inhibition)[1]					
	rExo S		Exo S/DG1		PHA	
Experiment	Control Ab	9-49-9	Control Ab	9-49-9	Control Ab	9-49-9
1	16.3	10.3 (37)	20.3	15.1 (26)	83.16	84.81 (0)
2	14.2	6.5 (54)	23.4	4.0 (83)	n/d	n/d

[1]T cell activation is shown as the percentage of $CD3^+$ cells that newly expressed CD69 after stimulation with 1 μg/ml rExoS or ExoS/DG1 in the presence of 10% v/v control Ab (ascites) or mAb raised against ExoS/DG1 (9-49-9).

n/d - not determined.

To demonstrate the specificity of binding between 9-49-9 and rExoS, immunoblotting was performed with rExoS. Binding of mAb 9-49-9 to ExoS/DG1 was confirmed in a dot blot and also reacted strongly to a 49 kDa band in the preparation of rExoS (Fig. 32). The mAb 9-49-9 was not binding in a non-specifically since it did not bind to the irrelevant protein (bovine serum albumin). This suggests that the epitope responsible for T lymphocyte activation is shared by rExoS and ExoS/DG1 and can be blocked by 9-49-9.

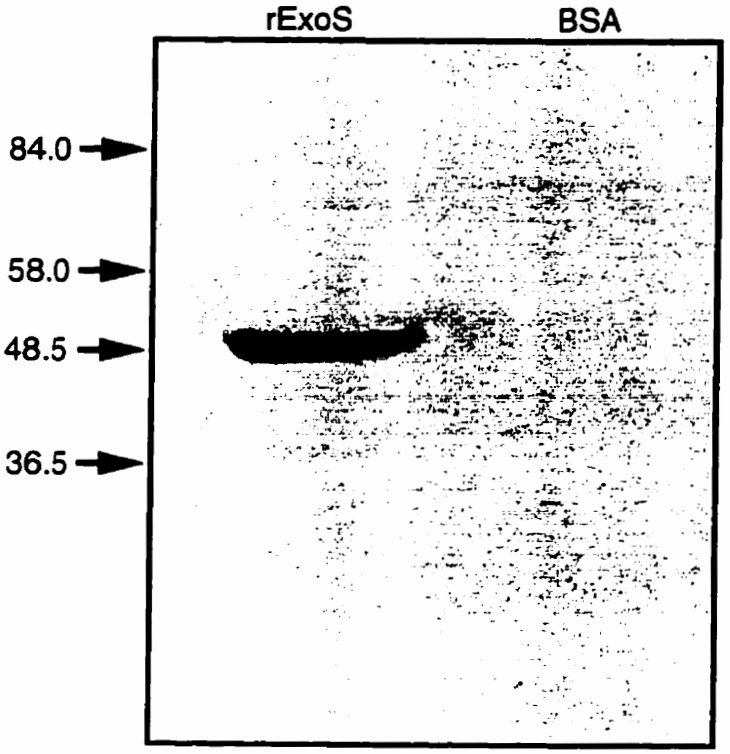


Fig. 32. Immunoblot of rExoS from *P. aeruginosa* strain PA103 inserted with the pUCP*exoS* vector. rExoS (3 μg) and BSA (10 μg)was loaded into a 15% SDS-PAGE gel, electrophorectically transferred to immobilon P and probed with 9-49-9 mAb (1:1000).

4.0 DISCUSSION

4.1 T Cell Activation by Exoenzyme S

There are a number of important pieces of evidence which suggest that exoenzyme S stimulates T cells as a mitogen: i) exoenzyme S induces fetal lymphocytes to proliferate; ii) $CD45RA^+$ lymphocytes express CD69 and proliferate in response to exoenzyme S; iii) the level of CD69 expression induced by exoenzyme S is greater than that induced by a panel of superantigens and antigens; iv) the proliferative response to exoenzyme S is dependent on accessory cells, but independent of HLA-DR.

Greater than 90-95% of fetal lymphocytes express the CD45RA isoform of the CD45 molecule [Hayward *et al.*, 1989], [Clement *et al.*, 1990]. The expression of this molecule is restricted to a unique pool of lymphocytes thought to have never encountered antigen and are thus termed immunologically naive [Prince *et al.*, 1992]. Activation and induction of T cell proliferation in this immunologically naive population of lymphocytes is therefore a characteristic of T cell mitogens [Stiehm *et al.*, 1979], [Hanzel *et al.*, 1980]. Therefore, the ability of exoenzyme S to induce proliferation in fetal lymphocytes suggests it is stimulating T cells as a mitogen. Further, tetanus toxoid could not induce proliferation in fetal T cells consistent with the fact that immunologically naive cells cannot respond to recall antigens without prior priming. Despite this evidence, there remained the unlikely possibility that a portion of the fetal lymphocytes may have already encountered antigen (i.e. fetal recognition of *Pseudomonas* antigens after a maternal bacteremia) and may be reacting to exoenzyme S. To resolve this issue, magnetic separation of immunologically naive and memory T cells from adult volunteers was performed and analyzed for their ability to undergo activation and proliferation in response to exoenzyme S. Magnetic separation resulted in greater than 97% purity in both subsets. Both $CD45RA^+$ and $CD45RO^+$ T cells underwent activation and proliferation in response to exoenzyme S and PHA, although the responses from the $CD45RA^+$ population to these stimuli was consistently lower than in the memory population. The negative control, tetanus toxoid,

was only capable of inducing CD69 expression and proliferation in the memory phenotype. Therefore, it has been demonstrated that these assays are able to discriminate between stimulation by antigens and mitogens and that T cell stimulation by exoenzyme S most closely resembles the latter.

The magnitude of CD69 expression induced by exoenzyme S after 4 hours culture is impressive. CD69 levels, after stimulation by exoenzyme S, were consistently greater than that induced by a panel of antigens and superantigens. Only anti-CD2 and PHA were able to induce a greater response at this time point. It was also noted that CD69 expression induced by exoenzyme S stimulation decreases at a faster rate compared to levels induced by superantigens or PHA. In fact, superantigen-induced CD69 expression surpassess exoenzyme S-induced levels usually after 16-24 hours in culture and is the result of the inability of exoenzyme S-treated T cells to sustain activation (CD69 expression). Although expression of CD69, as a marker of activation, represents one of the earliest molecules induced on the cell surface of stimulated T lymphocytes [Cebrián *et al.*, 1988], [Testi *et al.*, 1989], [Ziegler *et al.*, 1994], expression induced by exoenzyme S does not correlate with a subsequent commitment to enter cell cycle, as has also been found in other systems [Maino *et al.*, 1995], [Caruso *et al.*, 1997]. Indeed, the possibility exists that exoenzyme S, as a mechanism of host evasion, may stimulate a large portion of T cells to become activated and after a certain 'activation state' (or threshold) is reached, it modulates intracellular signalling pathways in responding T cells, promoting their subsequent death. Nevertheless, it may be possible for exoenzyme S to stimulate a large portion of T cells to secrete a number of different cytokines, thereby indirectly extending its effects beyond those seen in T cells alone.

It has been demonstrated that exoenzyme S preferentially activates $CD4^+$ T cells over $CD8^+$ T cells, a phenomenon that was common to the mitogenic lectins and superantigens tested. Previous studies have demonstrated that the superantigen-mediated interaction between the TCR and MHC class II is in some cases stabilized by the CD4

molecule and therefore a greater number of responding $CD4^+$ T cells are activated, although both subtypes respond [Kawabe and Ochi, 1990], [Abe *et al.*, 1991]. Although PHA preferentially induced CD69 expression on $CD4^+$ T cells more efficiently than $CD8^+$ T cells, the difference was not as pronounced as for exoenzyme S or the superantigens. PHA can utilize the CD4 molecule to stimulate T cells [Mann *et al.*, 1987], and this may explain the preferential expansion of CD4 cells. In contrast, expression of CD69 following stimulation with the recall antigen, tetanus toxoid, did not show preferential activation of either subset. No data has been presented to suggest that the T cell response (activation or proliferation) to exoenzyme S utilizes the CD4 molecule, however we cannot exclude this as a possible explanation for the preferential activation of this T cell subset. Certainly, the fact that $CD4^+$ T cells are preferentially activated by exoenzyme S suggests two potentially interrelated events. Firstly, it lends credence to the possibility that exoenzyme S may be modulating cytokine production by T_h cells. Secondly, it suggests that there may be preferential signalling for death in these cells, assuming CD69 expression and subsequent apoptosis are related events.

In this study, it was also shown that the proliferative response to exoenzyme S is dependent on the presence of accessory cells and that adherent cells (i.e. monocytes) were capable of delivering competent, but perhaps suboptimal, costimulatory signals. Additionally, it has previously been shown that B cell proliferation is dependent on the presence of activated T cells [Mody *et al.*, 1995], and that the 53 kDa form of exoenzyme S (Exo T) strain 388 does not induce ^{3}H-TdR incorporation in fibroblasts or epidermoid cells [Olson *et al.*, 1997]. Together, the data suggests that the mitogenic activity of exoenzyme S is specific for T lymphocytes.

A number of bacterial mitogens stimulate T cells as superantigens. Superantigens bind and crosslink MHC II on the accessory cell with a restricted repertoire of Vβ elements of the T cell receptor resulting in stimulation of T cells bearing these Vβ elements, but not others. Since it was determined that exoenzyme S is a bacterial mitogen, it was of great

interest to identify whether it possessed characteristics of a superantigen. There are two pieces of evidence that suggest exoenzyme S is not a superantigen. First, antibodies to anti-DR block the response to superantigens such as TSST-1 [See *et al.*, 1992], but did not block the response to exoenzyme S. It should be noted that our data does not exclude the possibility that exoenzyme S may possess a binding site that allows crosslinking of the TCR with MHC that is not interfered by the anti-MHC antibody that was used. Therefore, experiments were performed to determine whether there was oligoclonal activation or oligoclonal proliferation of Vβ subsets. It was found that although exoenzyme S activated some subfamilies more or less than others for certain donors, these findings were not the same for all donors. Further, increases in Vβ-specific activation did not correlate with increases in Vβ-specific proliferation within donors. On the other hand, the positive control SEE showed preferential activation and expansion of Vβ8 but not Vβ12. In short, exoenzyme S stimulation of T cells did not correlate with activation of any specific subset tested. Rather, it caused polyclonal activation and expansion of Vβ subsets tested. Thus, these data are most consistent with exoenzyme S being a mitogen, but not a superantigen.

The inability of exoenzyme S to induce other markers of T cell activation (i.e. expression of CD25 and CD71) was not expected. As previously demonstrated by other investigators, cells that are induced to express significant levels of CD69 by antigens, mitogens, or superantigens typically also express significant levels of CD25 and CD71, although the kinetics for their expression is usually more delayed [Caruso *et al.*, 1997]. Accordingly, the finding that exoenzyme S induces only CD69 without subsequent CD25 or CD71 expression suggests that the activation signal needed for CD25 and CD71 may be becoming arrested upstream of their regulators. That is, exoenzyme S may be incapable of signalling a 'full blossoming' of T cell activation which is why later markers of activation are absent and earlier events remain intact. During the hierarchical continuum of T cell activation, exoenzyme S may be able to induce phosphorylation of CD3 ζ chains, initial calcium flux, and acid release within the first minutes of activation as all events can be

triggered in the absence of significant T cell proliferation [Rabinowitz *et al.*, 1996]. Alternatively, the initial activation signal may be deviated to a different pathway. Another possible mechanism may be that the duration of the stimulatory event is not long enough and may be triggering incomplete signalling. In support of this, it has been shown that stimuli that induce partial T cell activation tend to have lower TCR affinities and faster dissociation rates than ligands that trigger full T cell activation [Al-Ramadi *et al.*, 1995], [Margulies, 1996]. This theoretical model suggests that the continuous spectrum of T cell responses reflects the continuous range of dissociation rates of the stimulus [Rabinowitz *et al.*, 1996]. Although the suggested mechanisms by which the T cells fail to undergo full activation may be different, ultimately the physiological outcome is similar.

The ability for T cells to proliferate requires two distinct signals as initially proposed by Bretscher and Cohn in 1970 [Bretscher and Cohn, 1970]. The theory, in its simplest terms, states that responding T cells require cognate antigen recognition via its TCR (signal 1) and another signal delivered by costimulatory molecules (signal 2). It was also suggested that signal 1 in the absence of signal 2 would lead to T cell 'paralysis' [Bretscher and Cohn, 1970]. Indeed, it has been shown by a number of investigators that TCR stimulation in the presence of incompetent accessory cells (cells not bearing appropriate costimulatory molecules) can lead to lymphocyte paralysis (anergy, in more modern terminology) [Mueller *et al.*, 1989], [Perez *et al.*, 1997]. The term 'clonal anergy' refers to the incapacity of activated T cells to produce IL-2, thereby preventing the cell to respond to subsequent antigenic (or mitogenic) challenge and from helping other immune cells to proliferate and differentiate [Schwartz, 1990]. This state has been shown to be the result of inappropriate costimulation and is typically reversible by the exogenous addition of IL-2 [Beverly *et al.*, 1992]. In this regard, TCR stimulation by antigens, superantigens, and mitogens all require costimulatory signals to induce proliferation [Sopori *et al.*, 1987], [Licastro *et al.*, 1993]; only the combination of phorbol esters with calcium ionophores can induce full T cell activation (i.e. proliferation) in the absence of accessory cells [Truneh *et*

al., 1985]. This study has established that PBMC cultures stimulated with exoenzyme S do not produce significant levels of IL-2 as detected by bioassay. Further, the initial stimulation event by exoenzyme S does not induce measurable increases in CD25 expression. Appropriately, the lack of CD25 expression is also consistent with the finding that exogenous addition of IL-2 did not reverse the arrested state. In short, the current level of understanding suggests that exoenzyme S, in the strictest sense of the term, does not induce anergy, although it is acknowledged that more detailed studies to address this issue remain to be performed.

Previous observations demonstrated that the T cell response to exoenzyme S had characteristics of a mitogen, but that it also had characteristics of a recall antigen [Mody *et al.*, 1995]. Consistent with this, the results herein showed that exoenzyme S could activate naive lymphocytes and induce their proliferation, however the magnitude of proliferation was significantly less than all mitogens and superantigens tested. More specifically, the mitogenic response induced by exoenzyme S is defined by relatively slow proliferation kinetics (day 7) and a low magnitude of proliferation (SI<5). Subsequent analysis has shown that the relative discrepancy between significant early activation and marginal subsequent proliferation can be explained, at least in part, by the induction of an apoptotic signaling pathway.

4.2 Apoptosis as a Mechanism of Host Evasion

Apoptosis has long been shown to be an important mechanism for the maintenance of both central and peripheral tolerance [Singer and Abbas, 1994]. The deregulation of which has also been shown to result in autoimmune disease. Mice incapable of undergoing apoptosis due to deficiencies in Fas (*lpr*) or Fas ligand (*gld*) underlie the importance of apoptosis as a homeostatic mechanism of the immune system, as such animals suffer from generalized lymphadenopathies and lymphoproliferative disorders [Davidson *et al.*, 1986]. For a number of other diseases, the modulation of apoptosis has been shown to be

important in the ensuing pathology [Barr and Tomei, 1994]. In AIDS, the envelope protein gp120 is thought to signal death by crosslinking of the CD4 molecule. This form of activation induced cell death is thought to be an important mechanism in the depletion of T cells and a subsequent rapid decline in function of the immune system in patients with disease [Pantaleo and Fauci, 1995]. On the other hand, infection of B cells with Epstein-Barr virus, the etiological agent of Burkitt's lymphoma, can lead to synthesis of a viral protein with great homology to the human Bcl-2 protein [Henderson, 1993]. Bcl-2 is a protooncogene product which has been shown to be a suppressor of apoptotic death and thus virally infected B cells, with high levels of cytoplasmic Bcl-2, are more susceptible to a number of different malignancies [Henderson, 1993].

Apoptosis is thought of primarily as a non-inflammatory mode of cell death because professional phagocytes identify targeted cells by specific surface receptors and engulf them before cellular lysis and release of cytoplasmic constituents can occur [Savill *et al.*, 1993]. Recently however, apoptosis induced by certain bacteria has also been shown to produce a proinflammatory state [Zychlinsky and Sansonetti, 1997]. The best studied bacteria has been *Shigella*. After ingestion, Shigella reaches the colon, where it is taken up by M cells, and is transferred to the basolateral surface where it comes in contact with resident macrophages [Wassef *et al.*, 1989]. *Shigella* is engulfed by the macrophage population, but escapes the endocytic vesicles within minutes and once free in the cytoplasm, induces apoptosis [Zychlinsky *et al.*, 1996]. It has now been shown that IpaB (invasion plasmid antigen B) is the sole requirement for induction of apoptosis in macrophages and that the mechanism of this induction is via direct cleavage of caspase-1 (also known as interleukin-1 converting enzyme; ICE) [Chen *et al.*, 1996]. IpaB cleaves the pro-ICE into its active form and in turn, ICE cleaves and activates IL-1β which is then released [Zychlinsky *et al.*, 1994]. In this manner, *Shigella* appears to accomplish two important tasks: i) it kills the most effective bactericidal cell and ii) it promotes an inflammatory response that causes tissue damage and further bacterial invasion [Zychlinsky

and Sansonetti, 1997]. Therefore, there are at least three distinct mechanisms by which bacterial apoptosis may contribute to disease: i) cellular deletion of responding leukocytes; ii) inhibition of apoptosis in infected cells; and iii) initiation of inflammation [Zychlinsky and Sansonetti, 1997].

It has clearly been demonstrated that purified preparations of exoenzyme S can cause significant levels of apoptosis in PBMC cultures. This is not the first report of *P. aeruginosa* exoproducts causing apoptosis; in 1993 it was reported that exotoxin A induced apoptosis in cultured U937 cells [Kochi and Collier, 1993]. However, the mechanism of induction was shown to be a non-specific result of inhibition of protein synthesis [Kochi and Collier, 1993]. Clearly, the mechanism by which exoenzyme S induces apoptosis is different as the preparation used in these experiments (ExoS/DG1) is enzymatically inactive. This is the first report of a *Pseudomonas* exoproduct to activate a cell death pathway. The apoptosis induced by exoenzyme S showed both early (phosphatidylserine translocation) and late (DNA fragmentation) features characteristic of this death [Darzynkiewicz *et al.*, 1997]. Phenotypic analysis determined that freshly isolated T cells do undergo apoptosis in response to exoenzyme S, even though this population has been shown by others to be relatively resistant to death [Boehme and Lenardo, 1993]. Preliminary experiments suggest that death may also be occurring in monocyte populations although the paucity of this cell type did not allow for accurate calculations. Exoenzyme S may also be able to induce apoptosis of B cells, however the high degree of spontaneous apoptosis in this population [Oyaizu *et al.*, 1997], did not allow for precise or reproducible results. Therefore, the only PBMC population that consistently underwent apoptosis in both assays in response to exoenzyme S was $CD3^+$ T cells. Although measurable levels of exoenzyme S-induced apoptosis does not occur until day 3 of culture, certainly the initiation phase of the apoptotic pathway may be signaled immediately and once this path is taken, it may not be reversible. The induction of apoptosis by exoenzyme S is not contingent on previous proliferation as has been shown to occur for bacterial superantigens

[MacDonald *et al.*, 1991], although it is most probable that both proliferation and apoptosis are occurring in culture. Therefore, the proliferative response that is measured on day 7 by uptake of ^{3}H-TdR is, in reality, the net effect of these two events. Indeed, given the fact that cells undergoing apoptosis increase throughout the length of the assay, the proliferative levels could potentially resemble those of other mitogens if the apoptotic pathway utilized by exoenzyme S could be disassociated or prevented.

Although apoptosis of cultures induced by exoenzyme S is initiated after the expression of CD69 occurs, these two events may be associated. Despite the fact that there is no correlation between levels of activation and subsequent levels of apoptosis in response to exoenzyme S, the possibility remains that exoenzyme S may be able to induce sufficient levels of CD69 (that in turn, signal for subsequent death) in all cultures. Interestingly, crosslinking of surface CD69 has been shown to induce apoptosis in eosinophils [Walsh *et al.*, 1996]. More importantly, CD69 expression on freshly isolated T cells has been shown to correlate with subsequent apoptosis [Wu *et al.*, 1997]. Indeed, the higher degree of resistance to apoptosis in freshly isolated peripheral T cells is suggested to be a result of their resting state [Boehme and Lenardo, 1993]. In this regard, the degree of T cell apoptosis induced by exoenzyme S in the lung may indeed be higher than the *in vitro* results obtained in this study as, by definition, recruited T cells in the lungs are already in an activated state [Davidson *et al.*, 1985], [Dominique *et al.*, 1990], [Saltini *et al.*, 1990], [Yamaguchi *et al.*, 1990], [Strickland *et al.*, 1993].

Suprisingly, this study demonstrated that T cell responses to exoenzyme S between CF patients and normal controls were similar. Previous studies by others have shown that PBMC responses to a variety of mitogens are the same for CF patients and normal controls [Sorensen *et al.*, 1977], suggesting that peripheral T cell capabilities are not altered. However, it is interesting to speculate whether lung T cell responses from CF patients would have altered proliferative or apoptotic capabilities, assuming T cell stimulation occurs under significantly different activation states [Saltini *et al.*, 1990], [Strickland *et al.*,

1993]. In other words, the relative activation state of the T cell (and the local environment it is in) may define its predilection to inflammation or apoptosis. With this view, it is not suprising that peripheral T cell responses to exoenzyme S did not differ between CF patients and healthy controls. Indeed, future studies using exoenzyme S-stimulated lung T cells from CF patients and healthy controls may help clarify this issue.

The switch that determines whether T cells undergo activation induced death or proliferation is of great interest, but unfortunately remains poorly understood. It is interesting to speculate however, that exoenzyme S may induce CD69 expression and subsequent apoptosis through a common pathway. Supporting this possibility is evidence which shows that induction of the transcriptional regulator AP-1 occurs in apoptotic cells [Sikora *et al.*, 1997]. Yet other regulators, namely NF-kB, are also involved in the regulation of activation induced apoptosis in T lymphocytes [Ivanov *et al.*, 1997]. As stated previously, induction of CD69 expression on T cells has been shown to be signaled via these regulators [López-Cabrera *et al.*, 1995], [Castellanos *et al.*, 1997], raising support for the possibility that early activation and subsequent death signals induced by exoenzyme S may be one in the same.

4.3 Hypothetical Model of Exoenzyme S-Induced Pathogenesis

The outcome of the T cell response to mitogens, superantigens, and antigens is quite different. Although T cell responses *in vivo* are complex, in general, mitogens and superantigens are both capable of inducing a non-specific inflammatory response and can also promote a subsequent state of unresponsiveness [MacDonald *et al.*, 1991], [Ignatowicz *et al.*, 1992], or apoptosis [Jenkinson *et al.*, 1989], [Webb *et al.*, 1990], [MacDonald *et al.*, 1991], [Damle *et al.*, 1993], [McCormack *et al.*, 1993]. In general, antigens stimulate T lymphocytes for B cell help, for delayed hypersensitivity and for production of memory cells. Thus, recall antigens are capable of stimulating an effective immunologic response resulting in enhanced host defense, while stimulation by mitogens

and superantigens result in inflammation, and potentially, impaired host defense [Andersson *et al.*, 1992], [Kotzin *et al.*, 1993], [Uchiyama *et al.*, 1994],

Cystic fibrosis can be characterized as a persistent, self-sustaining state of pulmonary inflammation that ultimately destroys the airways, impedes gas exchange and leads to death [Konstan and Berger, 1993]. T lymphocyte activation has been implicated in the onset of pulmonary inflammation in CF patients [Azzawi *et al.*, 1992], [Dagli *et al.*, 1992]. With as much as 10% of bronchoalveolar lavage fluid (BAL) cells being lymphocytes and the majority of these being T cells [Hunninghake *et al.*, 1979], the potential contribution to pulmonary inflammation by these cells remains quite high. It is proposed that initial T cell activation induced by exoenzyme S may result in the release of proinflammatory cytokines, thereby contributing to the chronic state of inflammation by activating a large percentage of naive T cells as they are recruited to the lung (Fig. 33). Certainly, the overproduction of T cell derived cytokines could contribute to respiratory exacerbations of disease in CF patients. Therefore, the T cell derived mediators could promote a chronic state of inflammation and impede host clearance of the organism in CF patients.

During an infection with *Pseudomonas aeruginosa*, T cells are recruited to sites of inflammation from hilar lymph nodes. In order for T cells to enter the lungs, expression of adhesion molecules (i.e. β2 integrins) on the surface of both recruited T cells and pulmonary vascular endothelium is required [Albelda and Buck, 1990], [Dustin and Springer, 1991]. Once recruited, local exposure of T cells to exoenzyme S may result in the release of cytokines that amplify an inflammatory response by recruiting and activating other T cells and non-specific effector cells such as monocytes and neutrophils [Brummer and Stevens, 1984], [Murray *et al.*, 1985], [Hunninghake *et al.*, 1987], [Rollins, 1997]. The importance of T cell activation in the pathology of CF is supported by the fact that peripheral T cell activation (soluble CD25 secretion) has been shown to precede clinical evidence of inflammation [Dagli *et al.*, 1992]. Further, the evidence that supports a

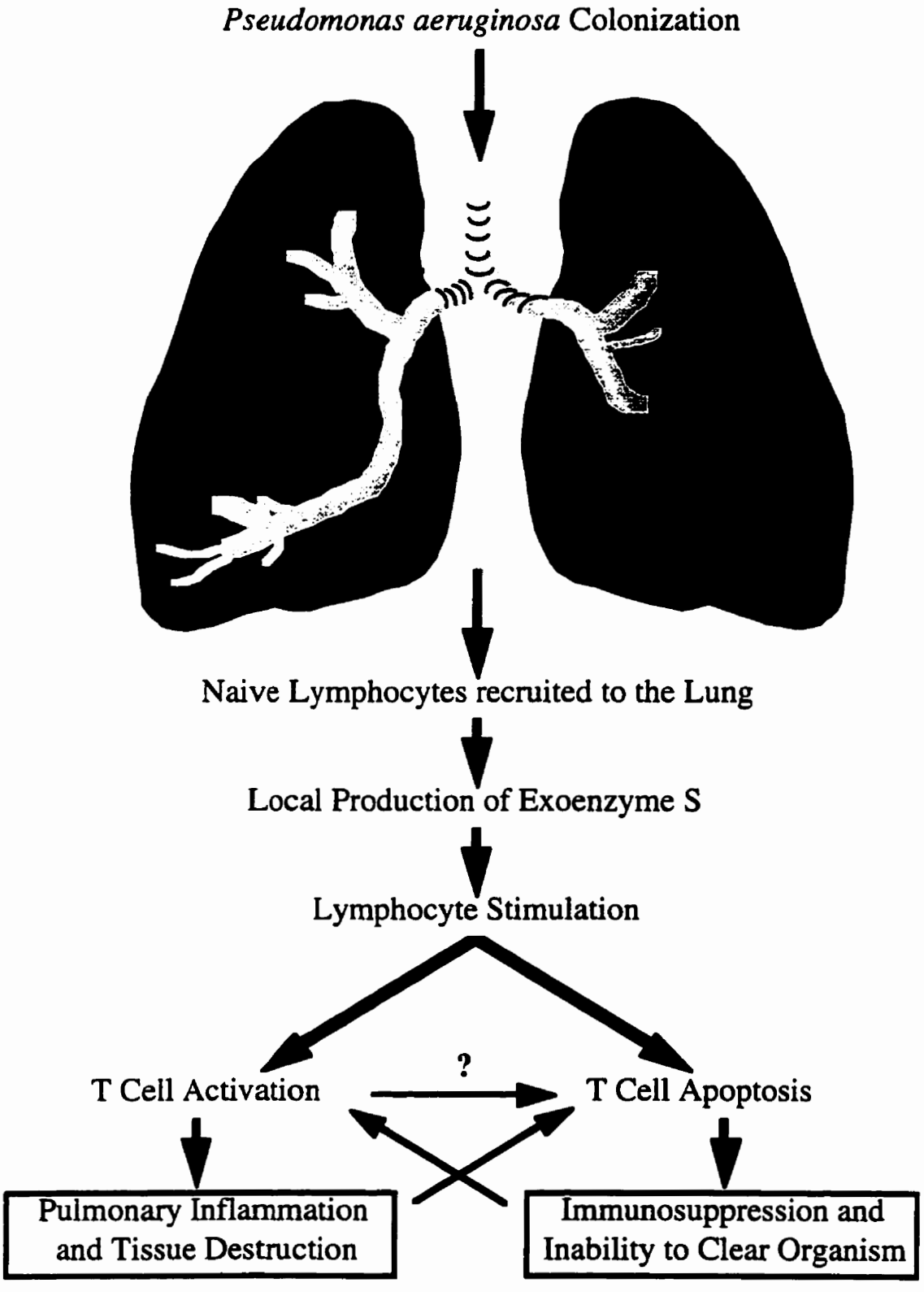


Fig. 32 Model of lymphocyte-mediated CF pathogenesis in response to exoenzyme S.

proinflammatory release of cytokines in the CF lung is substantial. Early studies showed that levels of TNF-α, IL-1α, IL-1β, and IL-6 were elevated in sputum samples from CF patients infected with *Pseudomonas aeruginosa* [Kronborg *et al.*, 1993] and that there is an increase in circulating TNF-α in the weeks prior to death [Elborn *et al.*, 1993]. More recently, Bonfield and colleagues demonstrated that TNF-α, IL-1β, IL-6 and IL-8 were all significantly increased while IL-10 was decreased in CF bronchoalveolar lavage fluid (BAL) compared to healthy controls [Bonfield *et al.*, 1995]. Subsequent studies showed that bronchial epithelial cells from CF patients downregulate the production of the anti-inflammatory cytokine IL-10, which is constitutively produced by these cells in healthy control subjects [Bonfield *et al.*, 1995]. To this end, exoenzyme S-stimulated T cells may regulate the release of monocyte derived proinflammatory cytokines, perhaps through the release of IFN-γ [Murray *et al.*, 1985]. Understanding the mechanism of T cell activation by exoenzyme S will help direct future therapies. This study suggests that strategies aimed at downregulating the T cell response to exoenzyme S would benefit CF patients. These strategies could potentially subvert a large, but ineffective inflammatory response reducing pulmonary damage.

4.4 ExoS/DG1 and rExoS Possess Similar Ability to Activate T Lymphocytes

P. aeruginosa strain 388 and strain DG1 (the respective strains from which rExo S and ExoS/DG1 are purified) have been shown to cause significantly greater cellular toxicity and dissemination of infection compared to isogenic mutants when instilled using the rat agar bead model [Nicas *et al.*, 1985b], [Woods and Sokol, 1985]. *In vitro*, mutation of the exoenzyme S gene abrogates cellular cytotoxicity of strain 388 [Olson *et al.*, 1997] and ExoS/DG1 produces cellular cytotoxicity [Woods *et al.*, 1988]. Although one of the most important properties of exoenzyme S is its ability to ADP-ribosylate target proteins [Iglewski *et al.*, 1978], cellular cytotoxicity does not require ADP-ribosyl transferase activity for either preparation [Woods *et al.*, 1988], [Kudoh *et al.*, 1994], Despite their

similarities, there are differences between rExoS and ExoS/DG1. rExoS produces a single band at 49 kDa, while ExoS/DG1 runs at 50 kDa. Both preparations initially undergo anion exchange chromatography; however, rExoS is purified via ultrafiltration (XM300), while ExoS/DG1 is subject to acetone precipitation and gel filtration (G-100). Cellular toxicity for rExoS requires bacterial contact with target cells [Olson *et al.*, 1997], while ExoS/DG1 does not [Woods *et al.*, 1988]. This study has shown that rExoS possesses ADP-ribosyl transferase activity while ExoS/DG1 does not, consistent with previous findings [Woods and Que, 1987], [Kulich *et al.*, 1995]. It was because of these differences that it was important to determine whether both preparations activated lymphocytes suggesting that the region in ExoS/DG1 that was responsible for lymphocyte activation and proliferation was shared by rExoS. In this regard, both preparations induced significant levels of CD69, and relatively modest levels of proliferation. Further, evidence for a shared immunostimulatory epitope between preparations is suggested by the ability of the monoclonal antibody 9-49-9 (raised against ExoS/DG1) to significantly neutralize CD69 expression induced by either preparation. It was shown that the interaction between 9-49-9 and rExoS was specific as determined by immunoblotting. To summarize, the data herein show that although differences exist between ExoS/DG1 and rExoS, there remains significant physiological similarity between them, at least in terms of their ability to activate T cells. It is interesting to speculate whether all members of the family of related proteins (ExoS, ExoT and ExoU) might also cause T cell activation; indeed, it is possible that ExoS/DG1 is functionally more similar to one of these related proteins. Further testing will be required to determine whether all members of the gene cluster activate lymphocytes.

4.5 Future Directions

Determining whether T cells are capable of inducing proinflammatory cytokine secretion is of obvious clinical importance in the treatment of CF. In this regard it is interesting to speculate whether induction of CD69 expression induced by exoenzyme S (and other members of the gene cluster such as Exo T and Exo U) is associated with cytokine secretion and apoptosis. Preliminary studies performed by other members of the laboratory have indeed shown transcript upregulation of a number of proinflammatory cytokines from PBMC stimulated with exoenzyme S *in vitro*. Depending on the secretion patterns of biologically active cytokines, complimentary killing assays that would use exoenzyme S to prime T cells to kill *Pseudomonas aeruginosa in vitro* would add to our limited knowledge on how to clear the organism *in vivo*. In the event that synchronicity exists between early T cell activation and subsequent signalling for apoptosis, the cellular transduction pathways which are being signaled by exoenzyme S should be explored in a sequential manner beginning with transducers most proximal to the initial event. These experiments are intended to detect gene products which may be involved in the arrestation of T cell activation and execution of the death pathway. To date, the use of specific aldehyde or chloromethylketone groups *in vivo* as therapeutic inhibitors of apoptosis is restricted due to their toxic nature, however their use in *in vitro* assays should also provide insight into which caspases are becoming activated in response to exoenzyme S. Because of the differences in activation states between peripheral T cells and lung T cells, future experiments should compare their *in vitro* responses (early and late activation, proliferation, apoptosis) to exoenzyme S within individual donors (normals, CF patients, and patients with idiopathic bronchiectasis). This proposed research will contribute to our understanding of the role of exoenzyme S and other exoproducts from *Pseudomonas aeruginosa* in the pathogenesis of CF in hopes of developing appropriate therapeutic strategies.

4.6 Summary

Exoenzyme S from *Pseudomonas aeruginosa* strain DG1 purified by the method of Woods [Woods and Que, 1987] is immunologically similar to recombinant exoenzyme S purified by the method of Kulich [Kulich *et al.*, 1995]. Both preparations have been shown to induce freshly isolated human peripheral T cells to express similar levels of CD69 and with similar kinetics. The induction of significant T cell activation induced by these preparations however, does not translate into similarly high levels of subsequent proliferation determined by ^{3}H-TdR incorporation. The relatedness of these two preparations was demonstrated by the ability of monoclonal antibodies (9-49-9) raised against ExoS/DG1 to neutralize the *in vitro* induction of T cell activation (CD69 expression) by either ExoS/DG1 or rExoS. The specificity of the interaction between mAb 9-49-9 and ExoS/DG1 and between mAb 9-49-9 and rExoS was visualized by dotblot and immunoblot respectively. This data clearly demonstrates that these preparations are immunologically similar in these assays. Further, the ability of mAb 9-49-9 to specifically recognize rExoS and neutralize its activity *in vitro* suggests that both preparations share a common stimulatory epitope.

Exoenzyme S (ExoS/DG1) was shown to stimulate fetal and adult CD45RA^{+} (immunologically naive) T cells to proliferate. T cell proliferation was shown to be dependent on the presence of accessory cells, but independent of HLA-DR. The Vβ (variable region of the β chain of the TCR) repertoire of responding T cells was also evaluated to determine whether exoenzyme S was stimulating T cells as a superantigen. T cell stimulation by exoenzyme S was shown to be polyclonal, rather than oligoclonal, in terms of Vβ TCR usage. Together, this data suggests that exoenzyme S stimulates T cells as a mitogen, but not a superantigen. However, some uncommon characteristics for mitogens were observed such as relatively slow kinetics (day 7 instead of day 3) and significantly lowered magnitudes for proliferation. This prompted further characterization of the T cell response to exoenzyme S.

leads to the possibility that it contributes to the local immune paralysis by destroying potential effector T cells .

REFERENCES

Abe, J., Forrester, J., Nakahara, T., Lafferty, J.A., Kotzin, B.L., and D.Y. Leung. 1991. Selective stimulation of human T cells with *streptococcal* erythrogenic toxins A and B. J. Immunol. 146:3747-3750.

Al-Ramadi, B.K., Jelonek, M.T., Boyd, L.F., Margulies, D.H., and A.L.M. Bothwell. 1995. Lack of strict correlation of functional sensitization with the apparent affinity of MHC-peptide complexes for the TCR. J. Immunol. 155:662-673.

Albelda, S.M. and C.A. Buck. 1990. Integrins and other cell adhesion molecules. FASEB J. 4:2868-2880.

Alarcon, B., Ley, S.C., Sanchez-Madrid, F., Blumberg, R.S., Ju, S.T., Fresno, M., and C. Terhorst. 1991. The CD3-gamma and CD3-delta subunits of the T cell antigen receptor can be expressed within distinct functional TCR/CD3 complexes. EMBO J. 10:903-912.

Andersson, J., Nagy, S., Bjork, L., Abrams, J., Holm, S., and U. Andersson. 1992. Bacterial Toxin-induced cytokine production studied at the single-cell level. Immunol. Rev. 127:69-96.

Apodaca, G., Bomsel, M., Lindstedt, R., Engel, J., Frank, D., Mostov, K.E., and J. Weiner-Kronish. 1995. Characterization of *Pseudomonas aeruginosa*-induced MDCK cell injury: glycosylation defective host cells are resistant to bacterial killing. Infect. Immun. 63:1541-1551.

Armstrong, D.S., Grimwood, K., Carzino, R., Carlin, J. B., Olinsky, A., and P.D. Phelan. 1995. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. B. M. J. 310:1571-1572.

Avruch, J., Zhang, X., and J.M. Kyriakis. 1994. Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem. Sci. USA 19:279-283.

Azzawi, M., Johnston, P.W., Majumdar, S., Kay, A.B., and P.K. Jeffrey. 1992. T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. Am. Rev. Respir. Dis. 145:1477-1482.

Bachmann, M.F., E. Sebzda, T.M. Kundig, A. Shahinian, and D.E. Speiser. 1996. T cell responses are governed by avidity and costimulatory thresholds. Eur. J. Immunol. 26:2017-2022.

Bachmann, M.F., Mckall-Faienza, K., Schmits, R., Bouchard, D., Beach, J., Speiser, D.E., Mak, T.W., and P.S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. Immunity 7:549-557.

Barr, P.J. and L.D. Tomei. 1994. Apoptosis and its role in human disease. Biotechnol. 12:487-493.

Bemer, V., Rovira, P. and P. Truffa-Bachi. 1995. T cell activation, anergy, and immunomodulation by molecules of viral, fungal, and vegetal origin. Res. Immunol. 146:249-262.

Berger, M., Sorensen, R.U., Tosi, M.F., Dearborn, D.G., and G. Doring. 1989. Complement receptor expression on neutrophils at an inflammatory site, the *Pseudomonas*-infected lung in cystic fibrosis. J. Clin. Invest. 84:1302-1313.

Beverly, B., Kang, S.M., Lenardo, M.J., and R.H. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. Inter. Immunol. 4:661-671.

Biselli, R., Matricardi, P.M., D'Amelio, R., and A. Fattorossi. 1992. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. Scand. J. Immunol. 35:439-447.

Boehme, S.A. and M.J. Lenardo. 1993. Propriocidal apoptosis of mature T-lymphocytes occurs at S phase of the cell cycle. Eur. J. Immunol. 23:1552-1560.

Bonfield, T.L., Panuska, J.R., Konstan, M.W., Hilliard, K.A., Hilliard, J.B., Ghnaim, H., and M. Berger. 1995. Inflammatory cytokines in cystic fibrosis lungs. Am. J. Respir. Crit. Care Med. 152:2111-2118.

Bretscher, P. and M. Cohn. 1970. A theory of self-nonself discrimination. Science 169:1042-1049.

Brodsky, F.M. and L.E. Guagliardi. 1991. The cell biology of antigen processing and presentation. Ann. Rev. Immunol. 9:707-744.

Brummer, E. and D.A. Stevens. 1984. Activation of murine polymorphonuclear neutrophils for fungicidal activity with supernatants from antigen-stimulated immune spleen cell cultures. Infect. Immun. 45:447-452.

Buret, A. and A.W. Cripps. 1993. The immunoevasive activities of *Pseudomonas aeruginosa* . Relevance for cystic fibrosis. Am. Rev. Respir. Dis. 148:793-805.

Caplen, N.J., Alton, E.F.W., Middleton, P.G., Dorin, J.P., Stevenson, B. I., Gao, X., Durham, S.R., Jeffrey, P.K., Hodson, M.E., Contelle, C., Huang, L., Portcons, D.J., Williamson, R., and D.M. Geddes. 1995. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nature Med. 1:39-46.

Caruso, A., Licenziati, S., Corulli, M., Canaris, A.D., Francesco, M.A.D., Fiorentini, S., Peroni, L., Fallacara, F., Dima, F., Balsari, A., and A. Turano. 1997. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry 27:71-76.

Castellanos, M. C., Munoz, C., Montoya, M. C., Lara-Pezzi, E., Lopez-Cabrera, M., and M.O. Landazuri. 1997. Expression of the leukocyte early activation antigen CD69 is regulated by the transcription factor AP-1. J. Immunol. 159:5463-5473.

Cebrián, M., Yagüe, E., Rincón, M., López-Botet, M., Landázuri, M. O. D., and F. Sánchez-Madrid. 1988. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. J. Exp. Med 168:1621-1637.

Cerottini, J.-C., and K.T. Brunner. 1989. The cellular basis of T cell memory. Ann. Rev. Immunol. 7:77-89.

Cervin, M.A., Simpson, D. A., Smith, A.L., and S. Lory. 1994. Differences in eukaryotic cell binding of *Pseudomonas*. Microb. Pathog. 17:291-299.

Chen, Y., Smith, M.R., Thirumalai, K., and A. Zychlinsky. 1996. A bacterial invasin induces macrophage apoptosis by binding directly to ice. EMBO J. 15:3853-3860.

Clement, L.T., Vink, P.E., and G.E. Bradley. 1990. Novel immunoregulatory functions of phenotypically distinct subpopulations of $CD4^+$ cells in the human neonate. J. Immunol 145:102:108.

Clevers, H., Alarcon, B., Wileman, T., and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. Ann. Rev. Immunol. 6:629-662.

Coburn, J., R.T. Wyatt, B.L. Iglewski, and D.M. Gill. 1989. Several GTP-binding proteins, including $p21^{c\text{-}H\text{-}ras}$ are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. J. Biol. Chem. 264-9004-9008.

Coburn, J. and D.M. Gill. 1991. ADP-ribosylation of $p21^{ras}$ and related proteins by *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. 159:4259-4262.

Cohen, J.J. 1993. Apoptosis. Immunol. Today. 14:126-130.

Corey, M. and V. Farewell. 1996. Determinants of Mortality from Cystic Fibrosis in Canada, 1970-1989. Am. J. Epidemiol. 143:1007-1017.

Crabtree, G.R. 1989. Contingent Regulatory Events in T Lymphocyte Activation. Science 243:355-361.

Crispe, I.N. 1994. Fatal interactions: Fas-induced apoptosis of mature T cells. Immunity 1:347-349.

Crystal, R.G., McElvaney, N.G., Rsenfeld, M.A., Chu, C.S., Mastrangeli, A., Hay, J.G., Brody, S.L., Jaffe, H.A., Eissa, N.T., and C. Danel. 1994. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nature Genetics 8:42-51.

Curran, T. and B.R. Franza Jr. 1988. Fos and jun: the AP-1 connection. Cell 55:395-397.

D'Adamio, L., Awad, K.M., and E.L. Reinherz. 1993. Thymic and peripheral apoptosis of antigen-specific T cells might cooperate in establishing self tolerance. Eur. J. immunol. 23:747-753.

Dagli, E., Warner, J.A., Besley, C.R., and J.O. Warner. 1992. Raised serum soluble interleukin-2 receptor concentrations in cystic fibrosis patients with and without evidence of lung disease. Arch. Dis. Child. 67:479-481.

Damle, N.K., Leytze, G., Klussman, K., and J.A. Ledbetter 1993. Activation with superantigens induces programmed death in antigen-primed $CD4^+$ class II^+ major histocompatibility complex T lymphocytes via a CD11a/CD18-dependent mechanism. Eur. J. Immunol. 23:1513-1522.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and F. Traganos. 1997. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis. Cytometry 27:1-20.

Davidson, B.L., Faust, F., Pessano, S., Daniele, R.P., and G. Rovera. 1985. Diffrentiation and activation phenotypes of lung T lymphocytes differ from those of circulating T lymphocytes. J. Clin. Invest. 76:60-65.

Davidson, W.F., Dumont, F.J., Bedigian, H.G., Fowlkes, B.J., and H.C. Morse III 1986. Phenotypic, functional, and molecular genetic comparisons of the abnormal lymphoid cells of C3H-lpr and C3H-gld/gld mice. J. Immunol. 136:4075-4084.

Davis, M.M. and P.J. Bjorkman. 1988. T cell receptor antigen genes and T cell recognition. Nature 334:395-402.

Davis, P.B. 1985. Pathophysiology of pulmonary disease in cystic fibrosis. Semin. Respir. Med. 6:261-270.

Davis, P.B. 1991. Cystic fibrosis: a major cause of obstructive airways disease in the young, pp. 297-307. *In* Neff, K. (ed.), Chronic Obstructive Pulmonary Disease, ed. W.B. Saunders Co., Philadelphia.

Davis, P.B., M. Drumm, and M.W. Konstan. 1996. Cystic Fibrosis. Am. J. Respir. Crit. Care Med. 154:1229-1256.

DeFranco, A.L. 1995. Transmembrane signaling by antigen receptors of B and T lymphocytes. Curr. Opin. Cell Biol. 7:163-175.

Dellabona, P., Peccoud, J., Kappler, J., Marrack, P., Benoist, C., and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. Cell 62:1115-1121.

Demko, C.A., Byard, P.J., and P.B. Davis. 1995. Gender differences in cystic fibrosis: *Pseudomonas aeruginosa* infection. J. Clin. Epidemiol. 48:1041-1049.

Deng, T. and K. Michael. 1994. c-Fos transcriptional activity stimulated by H-ras-activated protein kinase distinct from JNK and ERK. Nature 371:171-175.

Dominique, S., Bouchonnet, F., Smiejan, J.-M., and A.J. Hance. 1990. Expression of surface antigens "naive" and previously activated lymphocytes in bronchoalveolar lavage fluid. Thorax 45:391-396.

Douglas, R.S., A.D. Tarshis, C.H. Pletcher Jr., P.C. Nowell, and J.S. Moore. 1995. A simplified method for the coordinate examination of apoptosis and surface phenotype of murine lymphocytes. J. Immunol. Meth. 188:219-228.

Drumm, M.L., Pope, H.A., Cliff, W.H., Rommens, J.M., Marvin, S.A., Tsui, L.C., Collins, F.S., Frizzell, R.A., and J.M. Wilson. 1990. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell 62:1227-1233.

Dustin, M.D. and T.A. Springer. 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Ann. Rev. Immunol. 9:27-66.

Effros, R.B. and G. Pawelec. 1997. Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion. Immunol. Today 18:450-454.

Elborn, J.S., Cordon, S.M., Parker, D., Delamere, F.M., and D.J. Shale. 1993. The host inflammatory response prior to death in patients with cystic fibrosis and chronic *Pseudomonas aeruginosa*. Respir. Med. 87:603-607.

Fick, R.B. Jr., Nagel, G.P., Squier, S.U., Wood, R.E., Gee, J.B.L., and H.Y. Reynolds. 1984. Proteins of the cystic fibrosis respiratory tract. Fragmented immunoglobulin G opsonic antibody causing defective opsonophagocytosis. J. Clin. Invest. 74:236-248.

Fick, R.B.J., Baltimore, R.S., Squier, S.U., and H.Y. Reynolds. 1985. IgG proteolytic activity of *Pseudomonas aeruginosa* in cystic fibrosis. J. Infect. Dis. 151:589-598.

Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Weiner-Kronish, J.P., Fleiszig, S.M.J., Wu, C., Mende-Mueller, L., and D.W. Frank. 1997. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Molec. Microbiol. 25:547-557.

Frank, D.W. and B.H. Iglewski. 1991. Cloning and sequence analysis of a *trans*-regulatory locus required for exoenzyme S synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. 173:6460-6468.

Frithz-Lindsten, E., Du, Y., Rosqvist, R., and A. Forsberg. 1997. Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. Mol. Microbiol. 25:1125-1139.

Fu, H., Coburn, J. and R.J. Collier. 1993. The eukaryotic host factor that activates exoenzyme of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. Proc. Natl. Acad. Sci. USA 90:2320-2324.

Garlepp, M.J., Rose, A.H., Bowman, R.V., Mavaddat, N., Dench, J., Holt, B.J., Baron-Hay, M., Holt, P.G., and B.W.S. Robinson. 1992. A clonal analysis of lung T cells derived by brochoalveolar lavage of healthy individuals. Immunol. 77:31-37.

Goranson, J., and D.W. Frank. 1996. Genetic analysis of exoenzyme S by *Pseudomonas aeruginosa*. FEMS Microb. Let. 135:149-155.

Goranson, J., A.K. Hovey, and D.W. Frank. 1997. Functional analysis of *exsC* and *exsB* in the regulation of exoenzyme S production by *Pseudomonas aeruginosa*. J. Bacteriol. 179:1646-1654.

Greene, W. 1986. The human interleukin-2 receptor. Ann. Rev. Immunol. 4:69-95.

Grimwood, K., To, M., Rabin, H.R., and D.E. Woods. 1989. Inhibition of *Pseudomonas aeruginosa* exoenzyme expression by subinhibitory antibiotic concentrations. Antimicrob. Agents Chemother. 33:41-47.

Grimwood, K., To, M., Semple, R.A., Rabin, H.R., Sokol, P.A., and D.E. Woods. 1993. Elevated exoenzyme expression by *Pseudomonas aeruginosa* is correlated with exacerbations of lung disease in cystic fibrosis. Pediatr. Pulmonol. 15:135-139.

Hahn, H. and S.H.E. Kaufmann. 1981. The role fo cell-mediated immunity in bacterial infections. Rev. Infect. Dis. 3:1221-1250.

Halvorsen, R., Leivestad, T., Gaudernack, G., and E. Thorsby. 1988. Role of accessory cells in the activation of pure T cells via the T cell recptor-CD3 complex or with phytohemagglutinin. Scand. J. Immunol. 27:555-563.

Hanzel, Z.T., Levin, S., Dolphin, Z., Schlesinger, M., Hahn, T., Altman, Y., Schecter, B., Shenyour, A., and N. Trainin. 1980. Immune competence in newborn lymphocytes. Pediatrics 65:491-496.

Hata, J.S. and R.B. Fick. 1991. Airway adherence of *Pseudomonas aeruginosa*: mucoexopolysaccharide binding to human and bovine airway proteins. J. Lab. Clin. Med. 117:410-422.

Hayward, A.R., Lee, J., and P.C. Beverley. 1989. Ontogeny of expression of UCHL1 antigen on TCR-1$^+$ (CD4/8) and TCRδ^+ T cells. Eur J. Immunol 19:771-773.

Heeg, K., S. Bendigs, T. Miethke, and H. Wagner. 1993. Induction of unresponsiveness to the superantigen staphylococcal enterotoxin B: cyclosporin A resistant split unresponsiveness unfolds in vivo without preceeding clonal expansion. Int. Immunol. 5:929-937.

Henderson, S. 1993. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci 90:8479-8483.

Henry, R.L., Mellis, C.M., and L. Petrovic. 1992. Mucoid Pseudomonas aeruginosa is a marker of poor survival in cystic fibrosis. Pediatr. Pulmonol. 12:158-161.

Herman, A., G. Croteau, R. Sekaly, J. Kappler, and P. Marrack. 1990. HLA-DR alleles differ in their ability to present *Staphylococcal* enterotoxins to T cells. J. Exp. Med. 172:709-717.

Hermann, T., Accolla, R.S., and H.R. MacDonald. 1989. Different *Staphylococcal* enterotoxins bind preferentially to distinct major histocompatibility complex class II isotypes. Eur. J. Immunol. 19:2171-2174.

Herry, I., Bonay, M., Bouchonnet, F., Schuller, M., Lecossier, D., Tazi, A., Lynch, D., and A. Hance. 1996. Extensive apoptosis of lung T-Lymphocytes maintained in vitro. Am. J. Respir. Cell Mol. Biol. 15:339-347.

Hoiby, N. 1982. Microbiology of lung infections in cystic fibrosis patients. Acta. Pediatr. Scand. Supplement 301:33-54.

Holt, P.G., Kees, U.R., Shon-Hegrad, M.A., Rose, A., Ford, J., Bilyk, N., Bowman, R., and B.W.S. Robinson. 1988. Limiting dilution analysis of T cells extracted from solid human lung tissue: comparison of precursor frequencies for proliferative responses and lymphokine production between lung and blood T cells from individual donors. Immunol. 64:649-654.

Holt, P.S. and M.L. Misfeldt. 1986. Variables which affect suppression of the immune response induced by *Pseudomonas aeruginosa*. Infect. Immun. 52:96-100.

Horvat, R.T., Clabaugh, M., Duval-Jobe, C., and M.J. Parmely. 1989. Inactivation of human gamma interferon by *Pseudomonas aeruginosa* proteases: elastase augments the

effects of alkaline protease despite the presence of a_2-macroglobulin. Infect. Immun. 57:1668-1674.

Hunninghake, G.W., Gadek, J.E., Kawanami, O., Ferrans, V.J., and R.G. Crystal. 1979. Inflammatory and immune processes in the human lung in health and disease. Am. J. Path. 97:149-206.

Hunninghake, G.W., Glazier, A.J., Monick, M.M., and C.A. Dinarello. 1987. Interleukin-1 is a chemotactic factor for T-lymphocytes. Am. Rev. Resp. Dis. 135:66-71.

Iglewski, B.H. and J.C. Sadoff. 1979. Toxin inhibitors of protein synthesis: production, purification and assay of *Pseudomonas aeruginosa* toxin A. Methods Enzymol. 60:780-793.

Iglewski, B.H., Sadoff, J.C., Bjorn, M.J., and E.S. Maxwell. 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from exotoxin A. Proc. Natl. Acad. Sci. USA 75:3211-3215.

Ignatowicz, L., Kappler, J., and P. Marrack. 1992. The effects of chronic infection with a superantigen-producing virus. J. Exp. Med. 175:917-923.

Ivanov, V.N., Lee, R.K., Podack, E.R., and T.R. Malek. 1997. Regulation of Fas-dependent activation induced T cell apoptosis by cAMP signaling: a potential role for transcription factor NF-κB. Oncogene 14:2455-2464.

Izquierdo, M., Downward, J., Graves, J.D., and D.A. Cantrell. 1992. Role of protein kinase C in T-cell antigen receptor regulation of $p21^{ras}$: evidence that two $p21^{ras}$ regulatory pathways coexist in T cells. Mol. Cell Biol. 12:3305-3312.

Jenkinson, E.J., Kingston, E., Smith, C.A., Williams, G.T., and J.J. Owen. 1989. Antigen-induced apoptosis in developing T cells: a mechanism for negative selection of the T cell receptor repertoire. Eur. J. Immunol. 19:2175-2177.

Kabelitz, D., Pohl, T., and K. Pechhold. 1993. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. Immunol. Today 14:338-339.

Kanof, M.E. 1996. Isolation of T cells using rosetting procedures, pp. 7.2.3. *In* J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober. (ed.) Current protocols in immunology. John Wiley and Sons, Inc., New York, New York.

Kappler, J., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, N., and P. Marrack. 1987. A T cell receptor Vβ segment that imparts reactivity to a class II major histocompatibility complex product. Cell 49:263-271.

Katz, D.H., Hamaoka, T., and B. Benacerraf. 1973. Cell interactions between histocompatible T and B lymphocytes. II Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. J. Exp. Med. 137:1405-1418.

Kawabe, Y. and A. Ochi. 1990. Selective anergy of $V\beta8^{+}$ T cells in *Staphylococcus* enterotoxin B-primed mice. J. Exp. Med. 172:1065-1070.

Khan, T.Z., Wagener, J.S., Bost, T., Martinez, J., Accurso, F.J., and Riches, D.W. 1995. Early pulmonary inflammation in infants with cystic fibrosis. Am. J. Respir. Crit. Care Med. 151:1075-1082.

Kharazmi, A. 1989. Interactions of *Pseudomonas aeruginosa* proteases with the cells of the immune system, pp. 42-49. *In* Hoiby, N., Pedersen, S.S., Shand, G.H., Doring, G., and I.A. Holder (ed.), *Pseudomonas aeruginosa* infection, ed. Karger, Basel.

Kishimoto, H., Surh, C.D., and J. Sprent. 1995. Upregulation of surface markers on dying thymocytes. J. Exp. Med 181:649-655.

Knight, D.A., Finck-Barbancon, V., Kulich, S., and J.T. Barbieri. 1995. Functional Domains of *Pseudomonas aeruginosa* Exoenzyme S. Infect. Immun. 63:3182-3186.

Knight, D.A. and J.T. Barbieri. 1997. Ecto-ADP-Ribosyltransferase activity of *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. 65:3304-3309.

Knowles, M.R., Hohneker, K.W., Zhou, Z.Q., Olsen, J.C., Noah, T.L., Hu, P.C., Leigh, M.W., Engelhardt, J.F., Edwards, L.J., Jones, K.R., Grossman, M., Wilson, J.M., Johnson, L.G., and R.C. Boucher. 1995. A controlled study of adenoviral-vector-medicated gene transfer in the nasal epithelium of patients with cystic fibrosis. N. Engl. J. Med. 333:823-831.

Kochi, S.K. and R.J. Collier. 1993. DNA fragmentation and cytolysis in U937 cells treated with diptheria toxin or other inhibitors of protein synthesis. Exp. Cell Res. 208:296-302.

Konstan, M.W. and M. Berger. 1993. Infection and inflammation of the lung in cystic fibrosis, pp. 219-276. *In* P.B. Davis (ed.), Cystic Fibrosis, 1st ed. Marcel Dekker Inc., New York, New York.

Kortenjann, M., Thomae, O., and P.E. Shaw. 1994. Inhibition of v-*raf*-dependent c-*fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. Molecular and Cellular Biology 14:4815-4824.

Kotzin, B., Leung, D.Y.M., Kappler, J., and P. Marrack. 1993. Superantigens and their potential role in human disease. Adv. Immunol. 54:99-165.

Kroemer, G., Zamzami, N., and S. A. Susin. 1997. Mitochondrial control of apoptosis. Immunol. Today 18:44-51.

Kronborg, G., Hansen, M.B., Svenson, M., Fomsgaard, A., Hoiby, N., and K. Bendtzen. 1993. Cytokines in sputum and serum patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection as markers of destructive inflammation in the lungs. Ped. Pulmonol. 15:292-297.

Kudoh, I., Weiner-Kronish, J.P., Hashimoto, S., Pittet, J., and D. Frank. 1994. Exoproduct Secretions of *Pseudomonas aeruginosa* strains influence severity of alveolar epithelial injury. Am. J. Physiol. 267:L551-L556.

Kulich, S., D.W. Frank, and J.T. Barbieri. 1995. Expression of recombinant exoenzyme S of *Pseudomonas aeruginosa*. Infect. Immun. 63:1-8.

Kundig, T.M., A. Shahinian, K. Kawai, H.-W. Mittrucker, E. Sebzda, M.F. Bachmann, T.W. Mak, and P.S. Ohashi. 1996. Duration of TCR stimulation determines costimulatory requirements. Immunity. 5:41-52

Laraya-Cuasay, L.R., Cundy, K.R., and N.N. Huang. 1976. Pseudomonas carrier rates of patients with cystic fibrosis and members of their families. J. Pediatrics. 89:23-26.

Lecossier, D., Valeyre, D., Loiseau, A., Battesti, J.P., Soler, P., and A.J. Hance. 1988. T-lymphocytes recovered by bronchoalveolar lavage from normal subjects and patients with sarcoidosis are refractory to proliferative signals. Am. Rev. Respir. Dis 137:592-599.

Leist, T.P., Eppler, M., and R.M. Zinkernagel. 1989. Enhanced virus replication and inhibition of lymphocytic chorimeningitis virus disease in anti-IFN gamma-treated mice. J. Virol. 63:2813-2819.

Licastro, F., Davis, E.J., and M.C. Morini. 1993. Lectins and superantigens: membrane interactions of these compounds with T lymphocytes affect immune responses. Int. J. Biochem. 25:845-852.

Liu, Y. and C.A. Janeway Jr. 1990. Interferon γ plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. J. Exp. Med. 172:1735-1739.

López-Cabrera, M., Munoz, E., Blazquez, M.V., Ursa, M.A., Santis, A.G., and F. Sanchez-Madrid. 1995. Transcriptional regulation of the gene encoding the human C-type lectin leukocyte receptor AIM/CD69 and functional characterization of its tumor necrosis-a-responsive elements. J. Biol. Chem. 1995:21545.

Lucas, P.J., Negishi, I., Nakayama, K., Fields, L.E., and D.Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. J. Immunol. 154:5757-5768.

MacDonald, H. R., Baschieri, S., and R. K. Lees. 1991. Clonal expression precedes anergy and death of Vβ8^{+} peripheral T cells responding to *staphylococcal* enterotoxin B in vivo. Eur. J. Immunol 21:1963-1966.

Mai, G.T., Seow, W.K., Pier, G.B., McCormack, J.G., and Y.H. Thong. 1993. Suppression of lymphocyte and neutrophil functions by *Pseudomonas aeruginosa* mucoid exopolysaccharide (alginate): reversal by physiochemical, alginase, and specific antibody treatments. Infect. Immun. 61:559-564.

Maino, V.C., Suni, M.A., and J. Ruitenberg. 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 20:127-133.

Majno, G. and I. Joris. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. Am. J. Pathol. 146:3-16.

Mann, D.L., Lasane, F., Popovic, M., Arthur, L.O., Robey, W.G., Blattner, W.A., and M.J. Newman. 1987. HTLV-III large envelope protein (gp120) suppresses PHA-induced lymphocyte blastogenesis. J. Immunol. 138:2640-2644.

Margulies, D.H. 1996. An affinity for learning. Nature 381:558-559.

Markham, R.B., Goellner, J., and G.B. Pier. 1984. *In vitro* T cell-mediated killing of *Pseudomonas aeruginosa.* I. evidence that a lymphokine mediates killing. J. Immunol. 133:962-968.

Markham, R.B., Pier, G.B., Goellner, J.L., and S.B. Mizel. 1985. In vitro T cell mediated killing of *Pseudomonas aeruginosa* II. The role of macrophages and T cell subsets in T cell Killing. J. Immunol. 134:4112-4117.

Marrie, T.J., Harding, G.K.M., and A.R. Ronald. 1979. Influence of mucoid antibody coating of *Pseudomonas aeruginosa.* J. Infect. Dis. 139:357-361.

Marshall, J., Lee, E., Nietupski, J., Siegel, C., Nichols, M., Rafter, P., Cherry, M., Kaplan, J. M., George, J.A.S., Garlick, D., Jiang, C., Yew, N.S., Scheule, R.K., Harris, D., Smith, A.E., and S. H. Cheng. 1995. Development and use of novel synthetic cationic lipids with enhanced gene transfer activity. Ped. Pulmonol. Suppl. 12:216.

McCormack, J.E., J.E. Callahan, J. Kappler, and P.C. Marrack. 1993. Profound deletion of mature T cells in vitro by chronic exposure to exogenous superantigen. J. Immunol. 150:3785-3792.

Miethke, T., C. Wahl, D. Regele, H. Gaus, K. Heeg, and H. Wagner. 1993. Superantigen mediated shock: a cytokine release syndrome. Immunobiol. 189:270-284.

Mody, C.H., Buser, D.E., Syme, R.M., and D.E. Woods. 1995. *Pseudomonas aeruginosa* exoenzyme S induces proliferation of human T lymphocytes. Infect. Immun. 63:1800-1805.

Mollick, J.A., Chintagumpala, M., Cook, R.G., and R.R. Rich. 1991. *Staphylococcal* exotoxin activation of T cells. Role of exotoxin-MHC class II binding affinity and class II isotype. J. Immunol. 146:463-468.

Monack, D.M., Raupach, B., Hromockyj, A.E., and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA 93:9833-9838.

Monack, D.M., Mecsas, J., Ghori, N., and S. Falkow. 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. Proc. Natl. Acad. Sci. USA 94:10385-10390.

Mondino, A. and M.K. Jenkins. 1994. Surface proteins involved in T cell costimulation. J. Leuk. Biol. 55:805-815.

Moretta, A., Pantaleo, G., Maggi, E., and Mingari, M.C. 1984. Recent advances in the phenotypic and functional analysis of human T cells. Sem. Hematol. 21:257-279.

Morissette, C., E. Skamene, and F. Gervais. 1995. Endobronchial inflammation following *Pseudomonas aeruginosa* infection in resistant and susceptible strains of mice. Infect. Immun. 63:1718-1724.

Morris, A.G., Lin, Y.-L., and B.A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T-cell line meets its correct influenza-infected target cell. Nature 295:150-152.

Moss, R.B., Bocian, R.C., Hsu, Y.P., Dong, Y.J., Kemna, M., and T. Wei. 1996. Reduced IL-10 secretion by $CD4^+$ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR. Clin. Exp. Immunol. 106:374-388.

Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional inactivation. Ann. Rev. Immunol. 7:445-480.

Murray, H.W., Spitalny, G.L., and C.F. Nathan. 1985. Activation of mouse peritoneal macrophages in vitro and in vivo with interferon-gamma. J. Immunol. 134:1619-1622.

Nicas, T.I. and B.H. Iglewski. 1984. Isolation and characterization of transposon-induced mutants of *Pseudomonas aeruginosa* deficient in production of exoenzyme S. Infect. Immun. 45:470-474.

Nicas, T.I., Bradley, J., Lochner, J.E., and B.H. Iglewski. 1985a. The role of exoenzyme S in infections with *Pseudomonas aeruginosa*. J. Infect. Dis. 4:716-721.

Nicas, T.I., Frank, D.W., Stenzel, P., Lile, J.D., and B.H. Iglewski. 1985b. Role of exoenzyme S in chronic *Pseudomonas aeruginosa* lung infections. Eur. J. Clin. Microbiol. 4:175-179.

Nowell, P.C. 1960. Phytohemaglutinin: an initiator of mitosis in cultures of normal human leukocytes. Cancer Res. 20:462-466.

Oyaizu, N., Adachi, Y., Hashimoto, F., McCloskey, T.W., Hosaka, N., Kayagaki, N., Yagita, H., and S. Pahwa. 1997. Monocytes express Fas ligand upon CD4 Cross-linking

and induce $CD4^+$ T cells apoptosis. A possible Mechanism of bystander cell death in HIV Infection. J. Immunol. 158:2456-2463.

Olson, J. C., McGuffie, E.M., and D.W. Frank. 1997. Effects of Differential Expression of the 49-Kilodalton Exoenzyme S by *Pseudomonas aeruginosa* on Cultured Eukaryotic Cells. Infect. Immun. 65:248-256.

Paine, R.I., Mody, C.H., Chavis, A., Spahr, M.A., Turka, L.A., and G.B. Toews. 1991. Alveolar epithelial cells block lymphocyte proliferation *in vitro* without inhibiting activation. Am. J. Respir. Cell. Mol Biol. 5:221-229.

Pantaleo, G. and A.S. Fauci. 1995. New concepts in the immunopathogenesis of HIV infection. Ann. Rev. Immunol. 13:487-512.

Park, W., Weyand, C.M., Schmidt, D., and J.J. Goronzy. 1997. Co-stimulatory pathways controlling activation and peripheral tolerance of human $CD4^+CD28^-$ T cells. Eur. J. Immunol. 27:1082-1090.

Parmely, M., Gale, A., Clabaugh, M., Horvat, R., and W.-W. Zhou. 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. Infect. Immun. 58:3009-3014.

Perez, V.L., Parijs, L.V., Biuckians, A., Zheng, X.X., and T. B. Strom. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity 6:411-417.

Peter, M.E., Hall, C., Ruhlmann, A., Sancho, J., and C. Terhorst. 1992. The T cell receptor ζ chain contains a GTP-GDP binding site. EMBO J. 11:933-941.

Pier, G.B. 1985. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. 151: 575-580.

Pier, G.B., M. Grout, T.S. Zaidi, and J.B. Goldberg. 1996a. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science. 271:64-67.

Pier, G.B., M. Grout, T.S. Zaidi, and J.B. Goldberg. 1996b. How mutant CFTR may contribute to *Pseudomonas aeruginosa* infection in cystic fibrosis. Am. J. Respir. Crit. Care Med. 154:S175-S182.

Platsoucas, C.D. 1982. Augmentation of human natural killer cytoxicity by alpha-interferon and inducers of gamma-interferon: analysis by monoclonal antibodies. pp. 355-360.In: Herberman, R.B. (ed) NK cells and other natural effector cells. Academic Press, New York, New York.

Plotkowski, M.C., Chevillard, M., Pierrot, D., Altemayer, D., and E. Puchelle. 1992. Epithelial cells from cystic fibrosis patients do not possess specific *Pseudomonas aeruginosa*-adhesive properties. J. Med. Microbiol. 36:104-111.

Pollack, M. and S.E.J. Anderson. 1978. Toxicity of *Pseudomonas aeruginosa* exotoxin A for human macrophages. Infect. Immun. 19:1092-1096.

Porwoll, J. M., Gebel, H.M., Rodey, G.E., and R.B. Markham. 1983. In vitro response of human T cells to *Pseudomonas aeruginosa*. Infect. Immun 40:670-674.

Powderly, W.G., Pier, G.B., and R.B. Markham. 1986. T-lymphocyte-mediated protection against *Pseudomonas aeruginosa* infection in granulocytopenic mice. J. Clin. Invest. 78:375-380.

Prince, H.E., York, J., and E.R. Jensen. 1992. Phenotypic comparison of the three populations of human lymphocytes defined by CD45RO and CD45RA expression. Cell. Immunol. 145:254-262.

Rabinowitz, J.D., Beeson, C., Wulfing, C., Tate, K., Allen, P.M., Davis, M.M., and H.M. McConnell. 1996. Altered T cell receptor ligands trigger a subset of early T cell signals. Immunity 5:125-135.

Reinherz, E.L., Kung, P.C., Goldstein, G., and S.F. Schlossman. 1979. Separation of functional subsets of human T cells by monclonal antibody. Proc. Natl. Acad. Sci. USA 76:4061-4065.

Reinherz, E.L. and S.F. Schlossman. 1980. The differentiation and function of human T lymphocytes: a review. Cell 19:821-827.

Rich, D.P., Anderson, M.P., Gregory, R.J., Cheng, S.H., Paul, S., Jefferson, D., McCann, J.D., Klinger, K.W., Smith, A.E., and M. J. Welsh. 1990. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature 347:358-363.

Risso, A., Smilovich, D., Capra, M.C., Baldissarro, I., Yu, G., Bargellesi, A., and M.E. Cosulich. 1991. CD69 in resting and activated T lymphocytes. Its association with a GTP

binding protein and biochemical requirements for its expression. J. Immunol. 146:4105-4114.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., and J. Downward. 1994. Phosphatidylinositol-3-OH kinase as a direct target of ras. Nature 370:527-532.

Rogers, H.W., Callery, M.P., Deck, B., and E.R. Unanue. 1996. *Listeria monocytogenes* induces apoptosis of infected hepatocytes. J. Immunol. 156:679-684.

Rollins, B. J. 1997. Chemokines. Blood 90:909-928.

Rosales, C. and E.J. Brown. 1991. Two mechansims for IgG Fc-receptor-mediated phagocytosis by human neutrophils. J. Immunol. 146:3937-3944.

Russell, J.H., White, C.L., Loh, D.Y., and P. Meleedy-Rey. 1991. Receptor-stimulated death pathway is opened by antigen in mature T cells. Proc. Natl. Acad. Sci. USA 88:2151-2155.

Saiman, L. and A. Prince. 1993. Pseudomonas aeruginosa pili bind to asialo GM1, which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. 92:1875-1880.

Saltini, C., M. Kirby, B.C. Trapnell, N. Tamura, and R.G. Crystal. 1990. Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression on the epithelial surface of the human lung. J. Exp. Med. 171:1123-1140.

Sancho, J., Peter, M.E., Franco, R., Danielian, S., Kang, J.S., Fagard, R., Woods, J., Reed, J. C., Kamoun, M., and C. Terhorst. 1993. Coupling of GTP-binding to the T cell receptor (TCR) z-chain with TCR-mediated signal transduction. J. Immunol. 150:3230-3242.

Savill, J., Fadok, V., Henson, P., and C. Haslett. 1993. Phagocytic recognition of cells undergoing apoptosis. Immmunol. Today 14:131-136.

Sawa, T., Corry, D.B., Gropper, M.A., Ohara, M. , Kurahashi, K., and J.P.Weiner-Kronish. 1997. IL-10 improves lung injury and survival in *Pseudomonas aeruginosa* pneumonia. J. Immunol. 159: 2858-2866

Scharmann, W., Jacob, F., and J. Porstendorfer. 1976. The cytotoxic action of leucocidin from *Pseudomonas aeruginosa* on human polymorphonuclear leukocytes. J. Gen. Microbiol. 93:303-308.

Scherer, M.T., Ignatowicz, L., Winslow, G.M., Kappler, J.W., and P.C. Marrack. 1993. Superantigens: Bacterial and viral proteins that manipulate the immune response. Annu. Rev. Cell. Biol. 9:101-128.

Schultz, D.R. and K.D. Miller. 1974. Elastase of *Pseudomonas aeruginosa*: inactivation of complement components and complement-derived chemotactic and phagocytic factors. Infect. Immun. 10:128-135.

Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. Science 248:1349-1356.

See, R.H., Krystal, G., and A.W. Chow. 1992. Receptors for toxic shock syndrome toxin-1 and *Staphylococcal* enterotoxin A on human blood monocytes. Can. J. Microbiol. 38:937-944.

Sikora, E., Bielak-Zmijewska, A., Piwocka, K., and J. Skierski. 1997. Inhibition of proliferation and apoptosis of human and rat T lymphocytes by curcumin, a curry pigment. Biochem. Pharmacol. 54:899-907.

Simpson, J.A., Smith, S.E., and R.T. Dean. 1989. Scavenging by alginate of free radicals released by macrophages. Free Radical Biol. Med. 6:347-353.

Singer, G.G. and A.K. Abbas. 1994. The Fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. Immunity 1:365-371.

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., and J.J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induced death by apoptosis in mature T cells in thymic cultures. Nature 337:181-184.

Sopori, M.L., Hurt, Y.L., Cherian, S., Kaplan, A.M., and T. Diamantstein. 1987. Differential requirement for accessory cells in polyclonal T-cell activation. Cell. Immunol. 105:174-186.

Sorensen, R.U., Stern, R.C., and S.H. Polmar. 1977. Cellular immunity to bacteria: impairment of in vitro lymphocyte responses to *Pseudomonas aeruginosa* in cystic fibrosis patients. Infect. Immun. 18:735-740.

Sorensen, R.U., Stern, R.C., Chase, P.A., and S.H. Polmar. 1978. Lymphocyte responsiveness to *Pseudomonas aeruginosa* in cystic fibrosis: relationship to status of pulmonary disease in sibling pairs. J. Pediatr. 93:201-205.

Sorensen, R.U., Stern, R.C., Chase, P.A., and S.H. Polmar. 1979. Defective cellular immunity to gram-negative bacteria in cystic fibrosis patients. Infect. Immun. 23:398-402.

Sorensen, R.U., Stern, R.C., Chase, P.A., and S.H. Polmar. 1981. Changes in lymphocyte reactivity to *Pseudomonas aeruginosa* in hospitalized patients with cystic fibrosis. Am. Rev. Resp. Dis. 123:37-41.

Speert, D.P., Eftekhar, F., and M.L. Puterman. 1984. Nonopsonic phagocytosis of strains of *Pseudomonas aeruginosa* from cystic fibrosis patients. Infect. Immun. 43:1006-1011.

Springer, T.A., Dustin, M.L., Kishimoto, T.K., and S.D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2 and LFA-3 molecules: cell adhesion receptors for the immune system. Ann. Rev. Immunol. 5:223-252.

Stiehm, E.R., Winter, H.S., and Y.J. Bryson. 1979. Cellular immunity in the human newborn. Pediatrics 64:814-821.

Strickland, D.H., Thepen, T., Dees, U.R., Kraal, G., and P.G. Holt. 1993. Regulation of T cell function in lung tissue by pulmonary alveolar macrophages. Immunol. 80:266-272.

Sundaram, U., Knickelbein, R.G., and J.W. Dobbins. 1991. Mechanism of intestinal secretion. J. Clin. Invest. 87:743-746.

Tang, H.B., E. DiMango, R. Bryan, M. Gambello, B.H. Iglewski, J.B. Goldberg, and A. Prince. 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to the pathogenesis of pneumonia in a neonatal mouse model of infection. Infect. Immun. 64:37-43.

Taylor-Fishwick, D.A. and J.N. Siegel. 1995. Raf-1 provides a dominant but not exclusive signal for the induction of CD69 expression on T cells. Eur. J. Immunol. 25:3215-3221.

Testi, R., Phillips, J.H., and L.L. Lanier. 1989. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor signalling. J. Immunol. 142:1854-1860.

Theander, T.G., Kharazmi, A., Pedersen, B.K., Christensen, L.D., Tvede, N., Poulsen, L.K., Odum, N., Stevenson, M., and K. Bendtzen. 1988. Inhibition of human lymphocytes proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases. Infect. Immun. 56:1673-1677.

Tosi, M.F., Zakem, H., and M. Berger. 1990. Neutrophil elastase cleaves C3bi on opsonized *Pseudomonas* as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. J. Clin. Invest. 86:300-308.

Truneh, A., Albert, F., Golstein, P., and A.M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 313:318-320.

Uchiyama, T., Yan, X., Imanishi, K., and J. Yagi. 1994. Bacterial superantigens - mechanism of T cell activation by the superantigens and their role in the pathogenesis of infectious disease. Microbiol. Immunol. 38:245-256.

van Seventer, G.A., Schimizu, Y., Horgan, K.J., and S. Shaw. 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. J. Immunol. 144:4579-4586.

van Seventer, G.A., Schimizu, Y., and S. Shaw. 1991. Roles of multiple accessory molecules in T-cell activation. Curr. Opin. Immunol. 3:294-303.

Vose, B.M. and G.D. Bonnard. 1983. Limiting dilution analysis of the frequency of human T cells and large granular lymphocytes proliferating in response to interleukin 2. I. The effect of lectin on the proliferative frequency and cytotoxic activity of cultured lymphoid cells. J. Immunol. 130:687-693.

Wagner, H., C. Hardt, B.T. Rouse, M. Rollinghof, P. Scherich, and K. Pfizenmaier. 1982. Dissection of the proliferative and differentiation signals controlling murine CTL responses. J. Exp. Med. 155:1876-1881.

Wahl, S., T. Miethke, K. Heeg, and H. Wagner. 1993. Clonal deletion as direct consequence of an in vivo T cell response to bacterial superantigen. Eur. J. Immunol. 23:1197-1200.

Walsh, G.M., Williamson, M.L., Symon, F.A., Willars, G.B., and A.J. Wardlaw. 1996. Ligation of CD69 Induces apoptosis and cell death in human eosinophils cultured with granulocyte-macrophage colony-stimulating factor. Blood 87:2815-2851.

Wassef, J.S., Keren, D.F., and J.L Mailloux. 1989. Role of M cells in initial antigen uptake and ulcer formation in the rabbit intestinal loop model of Shigellosis. Infect. Immun. 57:858-863.

Webb, S., Morris, C., and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 63:1249-1256.

Weigle, W.O. 1973. Immunological unresponsiveness. Adv. Immunol. 16:61-122.

Weissman, D.N., Bice, D.E., Crowell, R.E., and M.R. Shuyler. 1994. Intrapulmonary antigen deposition in the human lung: local responses. Am. J. Respir. Cell Mol. Biol. 11:607-614.

Wingren, A.G., Parra, E., Varga, M., Kalland, T., Sjogren, H., Hedlund, G., and M. Dohlsten. 1995. T cell activation pathways: B7, LFA-3, and ICAM-1 shape unique T cell profiles. Crit. Rev. Immunol. 15:235-253.

Woods, D.E., Straus, D.C. Jr., W.G.J., Berry, V.K., and J.A. Bass. 1980. Role of pili in adherence of *P. aeruginosa* to mammalian buccal epithelial cells. Infect. Immunol. 29:1146-1151.

Woods, D.E. and P.A. Sokol. 1985. Use of transposon mutants to assess the role of exoenzyme S in chronic pulmonary disease due to *Pseudomonas aeruginosa*. Eur. J. Clin. Microbiol. 4:163-169.

Woods, D.E., Schaffer, M.S., Rabin, H.R., Campbell, G.D., and P.A. Sokol. 1986. Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. J. Clin. Microbiol. 24:260-264.

Woods, D.E. and J.U. Que. 1987. Purification of *Pseudomonas aeruginosa* Exoenzyme S. Infect. Immun. 55:579-586.

Woods, D.E., Hwang, W.S., Shahrabadi, M.S., and J.U. Que. 1988. Alteration of pulmonary structure by *Pseudomonas aeruginosa* exoenzyme S. J. Med. Microbiol. 26:133-141.

Woods, D.E., To, M., and P.A. Sokol. 1989. *Pseudomonas aeruginosa* Exoenzyme S as a Pathogenic Determinant in Respiratory Infections. Antibiot. Chemother. 42:27-35.

Wu, M.X., Ao, Z., Daley, J.F., and S.F. Schlossman. 1997. Induction and detection of apoptosis in human periphery blood T-cells. J. Immunol. Meth. 206:153-162.

Yahr, T., Barbieri, J.T., and D.W. Frank. 1996. Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from *Pseudomonas aeruginosa*. J. Bacter. 178: 1412-1419.

Yamaguchi, E., Okazaki, N., Itoh, A., Abe, S., and Y. Kawakami. 1990. Modulation of accessory molecules on lung T cells. Chest 97:1393-1400.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zona, L.I., Woodget, J.R., and D.J. Templeton. 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature 372:798-800.

Yei, S., Mittereder, N., Tang, K., O'Sullivan, C., and B.C. Trapnell. 1994. Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration to the lung. Gene Ther. 1:192-200.

Zabner, J., Couture, L. A., Gregory, R.J., Graham, S.M., Smith, A.E., and M.J. Welsh. 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transfer deficit in nasal epithelia of patients with cystic fibrosis. Cell 75:207-216.

Ziegler, S.F., Ramsdell, F., and M.R. Alderson. 1994. The activation antigen CD69. Stem Cells 12:456-465.

Zinkernagel, R. M. and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the role of polymorphic major transplantation antigens determining T-cell restriction specificity. Adv. Immunol. 27:51-177.

Zychlinsky, A., Prevost, M.C., and P.J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature 358:167-168.

Zychlinsky, A., Fitting, A., Cavaillon, J.-M., and P.J. Sansonetti. 1994. Interleukin-1 is released by murine macrophages during apoptosis induced by *Shigella flexneri*. J. Clin. Invest. 94:1328-1332.

Zychlinsky, A., Thirumalai, K., Arondel, J., Cantey, J.R., Aliprantis, A.O., and P.J. Sansonetti. 1996. In vivo apoptosis in *Shigella flexneri* infections. Infect. Immun. 64:5357-5365.

Zychlinsky, A. and P. J. Sansonetti. 1997. Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death. Trends Microbiol. 5:201-204.

Presentations and Publications

I. Peer Reviewed Literature

1. **Bruno, T.F.**; Buser, D.E.; Syme, R.M.; Woods, D.E.; Mody, C.H. *Pseudomonas aeruginosa* exoenzyme S is a mitogen, but not a superantigen for human T lymphocytes. Infection and Immunity (in press).

2. **Bruno, T.F.**; Woods, D.E.; Mody, C.H. Recombinant *Pseudomonas* exoenzyme S and exoenzyme S from *Pseudomonas aeruginosa* strain DG1 stimulate T lymphocytes to proliferate. (in preparation).

3. **Bruno, T.F.**; Woods, D.E.; Mody, C.H. *Pseudomonas aeruginosa* exoenzyme S induces T cell apoptosis. (in preparation).

4. Barclay N.G.; Spurell, J.C.L.; **Bruno, T.F.**; Woods, D.E.; Mody, C.H. A model for the proliferation of murine splenocytes in response to stimulation by *P. aeruginosa* exoenzyme S. (in preparation).

ii. Non-Peer Reviewed Literature

1. Epelman, S.; **Bruno, T.F.**; Wodds, D.E.; Mody C.H *Pseudomonas aeruginosa* exoenzyme S induces expression of inflammatory cytokines from PBMC. Presented at the American Society for Microbiology Conference, Atlanta, Ga., May 1998.

2. **Bruno, T.F.**; Woods, D.E.; Rabin, H.R.; Mody, C.H. *Pseudomonas aeruginosa* exoenzyme S induces apoptosis in T lymphoctres isolated from cystic fibrosis patients. Presented at the American Thoracic Society International Conference, San Francisco, Ca.; May 1997.

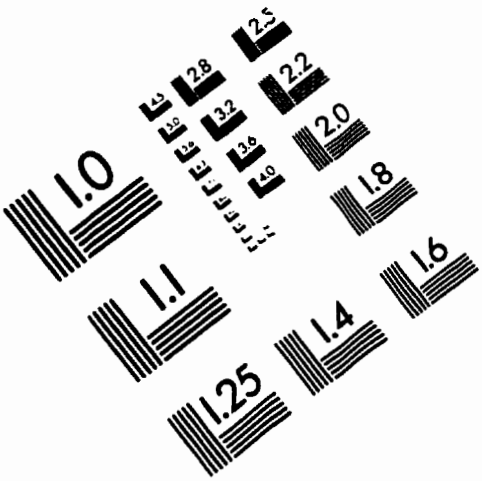
3. **Bruno, T.F.**; Woods, D.E.; Mody, C.H. Significant T cell activation by exoenzyme S preceeds unresponsiveness. Presented at the American Association of Immunologists Conference. New Orleans, LA.; June 1996.

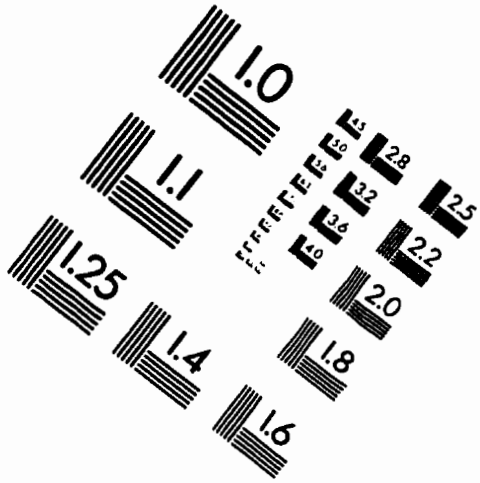
4. **Bruno, T.F.**; Woods, D.E.; Mody, C.H. Exoenzyme S arrests T cell activation prior to CD25 (IL-2R) expression and IL-2 secretion. Presented at the UA-UC International Conference on Infectious Diseases. Kananaskis Village, Alberta; May 1996.

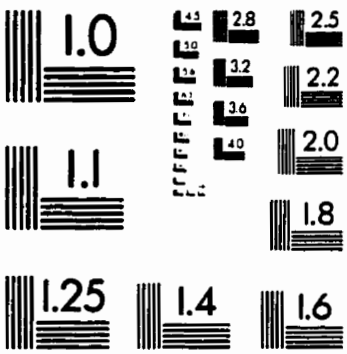
5. Barclay, N.G.; **Bruno, T.F.**; Woods, D.E.; Mody, C.H. *Pseudomonas aeruginosa* exoenzyme S is mitogenic for murine splenocytes. Presented at the UA-UC International Conference on Infectious Diseases. Kananaskis Village, Alberta; May 1996.

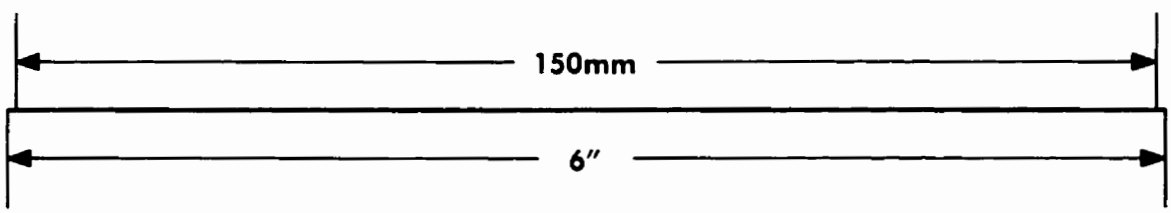
6. **Bruno, T.F.**; Woods, D.E.; Mody , C.H. Exoenzyme S promotes massive T cell activation without inducing subsequent proliferation. Presented at the Alberta Respiratory Disease Symposium. Jasper, Alberta; October, 1995.

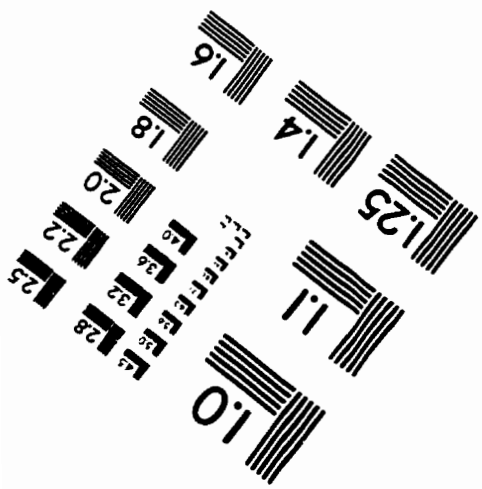
IMAGE EVALUATION
TEST TARGET (QA-3)

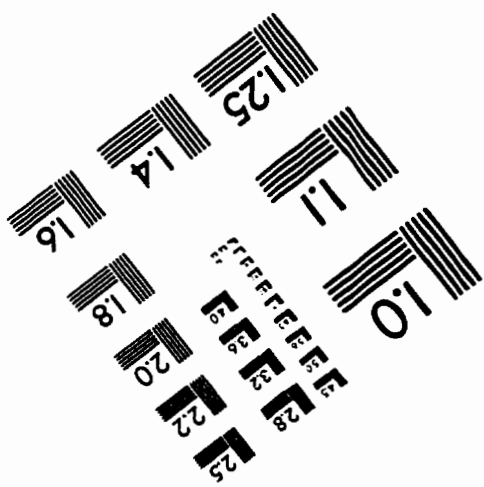












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