May 1987

Purification and Characterization of an Inhibitor of Thymidine Uptake From Culture Supernatants of Human Tonsil Lymphocytes

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PURIFICATION AND CHARACTERIZATION OF AN INHIBITOR OF THYMIDINE UPTAKE FROM CULTURE SUPERNATANTS OF HUMAN TONSIL LYMPHOCYTES

East Tennessee State University PH.D. 1987

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PURIFICATION AND CHARACTERIZATION OF AN INHIBITOR OF THYMIDINE UPTAKE FROM CULTURE SUPERNATANTS OF HUMAN TONSIL LYMPHOCYTES

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Abdolreza Zarnegar

May, 1987

APPROVAL

This is to certify that the Graduate Committee of

Abdolreza Zarnegar

met on the

twentieth day of February, 1987.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Dean of the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science with emphasis in Biochemistry.

Chairman, Graduate Committee

Signed on behalf of
the Graduate Council

Dean, School of Graduate Studies

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF AN INHIBITOR OF THYMIDINE UPTAKE FROM CULTURE SUPERNATANTS OF HUMAN TONSIL LYMPHOCYTES

by

Abdolreza Zarnegar

Lymphocytes from human tonsils were cultured in the absence of serum for 3 days. In the presence of the concentrated culture supernatant the proliferative response of PBL to con A, as measured by the uptake of ^{3}H-tdr, was significantly reduced. The suppressor substance was referred to as SMAL (suppressor of mitogen activated lymphocytes). The estimated molecular weight of SMAL under nondenaturing conditions was 100,000-300,000. SMAL also suppressed the incorporation of ^{3}H-tdr by a variety of mouse and human tumor cell lines. The activity of SMAL was sensitive to pronase and heating at 100°C for 30 minutes but insensitive to RNase. Treatment with DNase, however, enhanced the activity of SMAL. SMAL activity was also destroyed by treatment with 5% TCA, 0.4 M HCl or 60% acetonitrile, but resistant to 6 M urea or dialysis against pH 2 buffer for 24 hours. SMAL activity was precipitated in 40-80% ammonium sulfate saturation. When applied to a phenyl-sepharose column no activity was recovered.

SMAL was not produced by heat-killed tonsil lymphocytes or lymphocytes-treated with cycloheximide. Maximal production occurred in the first 24 hours of culture, and progressively less was produced in subsequent 24-hour intervals. Both T- and B lymphocyte-enriched culture supernatants contained SMAL. SMAL adhered strongly to DEAE-celulose, but less than two-fold purification was achieved. Using QMA-Accell anion exchange medium, a 5-fold purification of SMAL with higher specific activity was obtained with HPLC. Activity of SMAL was recovered after native polyacrylamide gel electrophoresis by electroblotting to DEAE-cellulose paper followed by eluting the bound materials with salt. Two active components, one corresponding to a large and/or less negatively charged molecule and another corresponding to a small and/or highly acidic molecule, were recovered.

HPLC-purified SMAL at relatively low doses inhibited the uptake and phosphorylation of ^{3}H-tdr, without significant effect on cell proliferation. The inhibition of ^{3}H-tdr uptake was favored over that of ^{3}H-udr or ^{3}H-adr, and this effect was reversible. At relatively high doses of HPLC-purified SMAL, the growth of mouse thymoma EL-4 and human T cell leukemia CEM-CM$_3$ cell lines was inhibited.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the faculty of the Department of Biochemistry for their instructions and friendship. I would like also to express my gratitude to the members of my committee, Drs. E. M. Rasch, M. L. Ernst-Fonberg, W. C. Champney, and A. Hougland for their critical comments.

To Dr. F. P. Inman, the major advisor for this project, I give my sincere appreciation for his guidance, patience, encouragement, and above all his friendship.

Special thanks to Dr. E. M. Rasch for her guidance and assistance in determining the DNA content of EL-4 nuclei and preparation of autoradiographs. The assistance of Dr. W. Campbell in determination of the antiviral activity of SMAL is greatly appreciated.

My thanks to all the graduate students of the Department of Biochemistry for making my graduate years memorable and pleasurable.

I wish to express my loving appreciation to my parents for their continuous support and encouragement. They have sacrificed a lot to open the doors of higher education for me.

Finally, a heartfelt appreciation to Mrs. R. Cox for her diligence in the typing of this manuscript.

OBJECTIVES

The detailed biological and biochemical analysis of regulatory factors (lymphokines) has been precluded by the isolation of only small quantity of biological active materials. Many assay systems used for the demonstration of activating or inhibitory properties have been carried out using non-selected cell populations. This has complicated the detailed analysis of the target cells and the mode of action of factors.

It was shown previously (Hodge and Inman, 1982) that culture fluids from human tonsil lymphocytes inhibited the incorporation of ^{3}H-tdr by con A-activated human peripheral blood lymphocytes (PBL). This standard proliferation assay (con A stimulation of PBL) had several disadvantages. These included heterogeneity of the target cells, variation in response to con A among lymphocytes of different blood donors, and long period that was required to activate PBL. Therefore, one objective of this work was to develop a more sensitive and reliable assay for the suppressive-active substance in the culture supernatants. Once such an assay was developed it would facilitate investigation of some of the biological and biochemical properties of the suppressor factor.

Another problem with the previous study was that the culture supernatant used were heterogenous and contained many different proteins, and it was unclear which component was responsible for the suppressor activity. The next objective was to purify further the suppressor factor and demonstrate that the purified material(s) could also exhibit the same activity as that of the crude preparations. The

mechanism of action of the suppressor factor on human PBL also was not clear. One reason was that the target cells (PBL) consisted of a mixture of mononuclear cells namely T and B lymphocytes, and macrophages each of which are capable of producing helper and/or suppressor factors. This in turn could make the analysis of the mode of action of the suppressor factor difficult. The final objective was to investigate the modes by which the suppressor factor exerted its effects.

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ABBREVIATIONS

adr	Adenosine
BSF	B cell stimulatory factor
cAMP	Cyclic adenosine monophosphate
con A	Concanavalin A
CPE	Cytopathic effect
cpm	Counts per minute
d.	Dalton
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EtBr	Ethidium bromide
5-Fudr	5-Fluorodeoxyuridine
FBS	Fetal bovine serum
HEPES	[N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid]
HPGRT	Hypoxantine guanine phosphoribosyl transferase
HPLC	High performance liquid chromatography
HSV II	Herpes simplex virus type II
IDS	Inhibitor of DNA synthesis
IL-2	Interleukin-2
INF	Interferon
LT	Lymphotoxin
MASH	Multiple automated cell harvester
MLC	Mixed lymphocyte culture
Mr	Relative molecular mass
NBTI	Nitrobenzylthioinosine

PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PGFS	Polypeptide growth factors
PHA	Phytohemagglutinin
PIF	Proliferation inhibitor factor
PPD	Purified protein derivative of tuberculin
QMA	Quarternary methylamine
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SF	Suppressor factor
SIRS	Soluble immune response suppressor
SISS	Soluble immune suppressor substance
SMAL	Suppressor of mitogen activated lymphocyte
STIF	Stimulated rat T cell-derived inhibitor factor of DNA synthesis
TCA	Trichloroacetic acid
tdr	Thymidine
TK	Thymidine kinase
TL	Tonsilar lymphocytes
TNF	Tumor necrosis factor
Tris	[Hydroxymethyl]aminomethane
udr	Uridine

CHAPTER 1

Introduction

The immune response is the result of a complex network of interactions among different lymphocytes and accessory cells and is largely mediated or regulated by the effect of soluble factors (lymphokines and cytokines) (Adorini, 1982) on various cells. The word lymphokine was first proposed by Dumonde et al. (1969) and is synonymous with the following terms: lymphocyte mediator, soluble lymphocyte mediator, lymphocyte activation product, soluble lymphocyte product, mediator of cellular immunity. Lymphokines are defined by a number of characteristics that are briefly discussed below. They are soluble substances produced by lymphoid cells cultured in vitro for relatively brief intervals. Lymphokines are not immunoglobulins and do not possess structural similarity to any known immunoglobulin molecules. They are generally not preformed cellular components and are, therefore, not present, in substantial amounts, in resting lymphocytes. Most lymphokines are glycoproteins of a M.W. larger than 10,000. Each lymphokine was discovered, defined, and assayed by a specific biological activity on a target cell, in most cases under in vitro conditions. A number of lymphokines appear to affect their target cells by reacting with specific membrane receptors, although such receptors have not been demonstrated for most lymphokines. For the most part, lymphokines do not appear to be enzymes. They affect a wide variety of cells including lymphocytes, macrophages, neutrophils, basophils, eosinophils, polymorphonuclear leukocytes, thymocytes, and bone marrow

cells, fibroblasts, lymphoid cell lines, osteoclasts and endothelial cells. There is hardly any area of the immune response in which lymphokine action has not been demonstrated or inferred, including (1) attraction and trapping of circulating inflammatory cells; (2) regulation of lymphocyte traffic through lymphoid organs; (3) regulation of vascular permeability; (4) promotion of cell division and induction of cell maturation for a variety of cells, including T and B lymphocytes, macrophages and hematopoietic stem cells; (5) inhibition of cell proliferation and cytotoxicity for a variety of animal cells as well as bacteria and yeasts; and (6) cooperation between T- and B-lymphocytes or between macrophages and lymphocytes during the development of an immune response.

In attempting to define more precisely the biological role of a lymphokine and to characterize and isolate such a substance, one is repeatedly stumbling over a number of difficulties, which held the whole field in disrepute for a number of years. These are: (a) the fact that lymphokines are usually produced by a mixture of cell types and may often require cooperation (e.g. among lymphocytes and macrophages) in order to be effectively produced; (b) that, correspondingly, any culture supernatant from activated cells usually contains several different lymphokines; and (c) that the target cell populations used for assessing lymphokine biological activity are themselves frequently heterogenous and capable of producing lymphokines having an enhancing or inhibiting effect on the biological assay under study. Although more than 90 lymphokine activities have been described (Waksman, 1979) only a few of

them have been characterized as to their chemical structures, biologic activities, metabolic regulation, and functional mechanisms.

In most cases, the limitation is due to the unavailability of substantial quantities of particular lymphokines. Recently, by using improved methods of lymphokine production and purification it has been shown that the biological activity of some lymphokines can be attributed to single molecular entities which act on defined cell populations (Gillis and Inman, 1985).

A comprehensive review of all the lymphokines is beyond the scope of this dissertation; instead the reader is referred to other sources for further discussion of this extensive topic (Cohen et al., 1979; Hadden and Stewart, 1981; Pick, 1981; Altman and Katz, 1980; Taussig, 1980; Gillis and Inman, 1985; Lachman and Maizel, 1983). The present review will describe one of the best characterized lymphokines (IL-2), which is the prototype of helper factors.

Morgan et al. in 1976 observed that addition of tissue culture medium conditioned by mitogen-stimulated human peripheral blood lymphocytes to human leukemic blood or bone marrow cells resulted in the in vitro proliferation of these cells. Surface phenotype and functional data confirmed that the cells replicating in the presence of such conditioned media were normal T lymphocytes (Ruscetti et al., 1977). Gillis and Smith (1977) were then successful in developing methods which have allowed for the indefinite in vitro proliferation of murine cytotoxic T lymphocytes. Growth of these effector T-cell populations was dependent on the presence of murine or rat spleen cell conditioned medium. Subsequent experiments led to the development of similar

methods for the long-term culture of human cytotoxic T cell lines (Gillis et al., 1978), as well as adaptation of protocols for in vitro maintenance of cloned cytotoxic and helper T-cell populations (Gillis et al., 1979; Watson, 1979; Schreier et al., 1980). Purified IL-2 has been shown to trigger the proliferative expansion of activated T cell clones, enhance thymocyte mitogenesis, and induce alloantigen specific thymocytes and nude spleen cytolytic T cell reactivity (Watson et al., 1979; Gillis et al., 1980; Gillis and Watson, 1980).

The biological and biochemical characterization of this lymphokine has been enhanced because of a reliable and reproducible bioassay. The assay system is based on the original observation of Gillis and Smith (1977) that cytotoxic T lymphocyte lines (CTLL) depend upon the presence of IL-2 for proliferation. Because murine CTLL cells proliferate in response to IL-2 activity present in medium conditioned by mouse, rat, or human lymphocytes, IL-2 microassay have been invaluable in purifying and characterizing the IL-2 from all three species.

Production of large quantities of IL-2 by a human T-leukemia cell line, named Jurkat (Gillis and Watson, 1980), provided the first key to the purification and elucidation of the factor's structure. The second breakthrough was the cloning of a cDNA corresponding to IL-2 mRNA by Taniguchi et al. (1983) and Shuichiro et al. (1983) and production of recombinant IL-2 by Rosenberg and Grimm (1984). Wang et al. (1984) were able to mutagenize specifically the IL-2 gene by site directed mutagenesis and obtain useful data concerning the structure and function of IL-2. Nucleotide sequence analysis of the IL-2 gene revealed that the encoded IL-2 protein had three cysteines located at amino acid

residue 58, 105, and 125 of the mature protein. Since substitution of these cysteines by serine via site specific mutagenesis at either position 58 or 105 of the IL-2 protein substantially reduced the biological activity, the authors concluded that the cysteines at these positions were necessary for maintenance of the biologically active conformation and may, therefore, be linked by a disulfide bridge. The modified IL-2 protein containing a substitution at position 125 retained its full biological activity suggesting that the cysteine at this position was not involved in a disulfide bridge and that a free sulfhydryl group at that position was not necessary for receptor binding. Seigel et al. (1984) were able to localize the IL-2 gene on human chromosomes. Using a molecular clone of human IL-2 and DNA extracted from a panel of somatic cell hybrids the IL-2 structural gene was identified on human chromosome 4.

Recently, the IL-2 receptor was identified and characterized using monoclonal antibody against the receptor protein. Also three groups succeeded in cloning cDNA's for the human IL-2 receptor protein (for review see Waldmann, 1986). These findings provide a basis for further investigations of the biology, chemistry and molecular genetics of other lymphokines.

A rather large number of lymphokines have also been implicated as suppressors of cellular proliferation and DNA synthesis of immunocompetent cells. Larsson and Blomgren (1978) demonstrated the existence of a factor produced by PHA-stimulated PBL which induced macrophages to become suppressive to antigen-stimulated PBL. Green et al. (1970) isolated a suppressor factor from PHA-stimulated human PBL

that inhibited the growth of a variety of transformed cell lines. Williams et al. (1978) described a factor produced by con A-stimulated T cell-enriched lymphocytes that could suppress the mixed lymphocyte culture response without loss of viability. The activity was sensitive to chymotrypsin treatment, non-dialyzable, partially labile at 56°C for 30 min, and sensitive to pH 2 after 24 hr. Shou et al. (1980) showed that supernatants from unstimulated normal human PBL contained factor(s) that inhibited the con A-activation of human PBL. Greene et al. (1981) identified two soluble immune suppressor substances (SISS) in PHA or con A-stimulated human PBL culture supernatants. SISS-T was shown to inhibit the proliferative response of T cells to mitogens and antigens. This factor had an apparent M.W. of 30,000-40,000. The other factor SISS-B inhibited polyclonal B cell activation and had a M.W. of 80,000. Both factors were sensitive to heat (56°C for 30 min). Another soluble suppressor factor (SSF) produced by Pokweed mitogen-stimulated PBL was found by Hoffman et al. (1980). It inhibited MLC and the generation of killer cells.

An inhibitor of DNA synthesis (IDS) was suggested by Jegasothy et al. (1981). IDS was produced by con A-stimulated human PBL and was glycoprotein in nature. The presence of the carbohydrate moiety was shown to be necessary for its biological activity. The M.W. of the monomer IDS was estimated to be 20,000 but it was usually found as dimers, trimers, and tetramers in solution. It was sensitive to trypsin and periodate but stable to heat (60°C for 30 min) and was not cytotoxic for target cells.

Cell extracts from lysed PBL were shown by Tibbetts et al. (1975) to contain a suppressor factor which inhibited both the PHA-stimulation of PBL, and the growth of a transformed B cell line. The suppressor was trypsin sensitive, stable to both treatment with DNase or exposure to pH values between 5.4-8.5, and had an apparent M.W. of greater than 100,000. Wolf et al. (1978) demonstrated that a subpopulation of T lymphocytes also produced a suppressor factor that inhibited the activation of human PBL by con A. The factor had an apparent M.W. greater than 100,000 and was stable to heating at 60°C for 15 min.

Culture supernatants from transformed cell lines have also been used as a source of soluble suppressor factors. Vesole et al. (1979) identified a soluble inhibitor of PHA-stimulated lymphocytes from either human T or B cell lines. The suppressor activity co-eluted from a sephadex G-100 with albumin. Green et al. (1974) isolated a suppressor of mitogen-stimulated PBL from crowded lymphoblastoid culture supernatant fluids. The factor was nondialyzable and had an apparent M.W. of 40,000 to 70,000. The suppressor activity was heat stable at 56°C for 30 min but was destroyed at 80°C for 30 min. It was also sensitive to pronase and to pH below 2.4.

Another suppressor factor named stimulated rat T-cell derived inhibitory factor (STIF) was isolated from the culture supernatant of con A-stimulated rat spleen cells (Chiba et al., 1985a). STIF could inhibit the DNA synthesis of mouse bone marrow cells as well as a variety of normal and neoplastic cells from the mouse, rat, and human. STIF was a protein with an apparent M.W. of 45,000-50,000 as estimated

by gel filtration. It apparently could inhibit the production of T cell growth factor (IL-2) as well as proliferation of IL-2-dependent T cells (Chiba et al., 1985b).

Kramer et al. (1982) isolated a 10,000 M.W. suppressor factor (SF) from culture supernatant of alloantigen-activated mouse spleen cells. SF was shown to inhibit the proliferative response of T cells to alloantigen and the release of IL-2 from producer T cells.

Suppressor cell induction factor (SIF) was isolated from MLC culture supernatant by Kasakura et al. (1983) which had the ability to induce suppressor function in human T cells. These T cells in turn could inhibit the proliferation of the responder cells in MLC. SIF had a M.W. of 18,000 to 29,000 as estimated by gel filtration column chromatography and did not bind to DEAE-cellulose (Kasakura et al., 1983).

Proliferation Inhibitor Factor (PIF) reportedly was produced by unstimulated or by mitogen-activated leukocytes (Badger et al., 1971). This factor was trypsin sensitive, heat stable at 85°C for 30 min, and nondializable. PIF and lymphotoxin subsequently were shown to be very similar or identical molecules (Jeffes and Granger, 1975).

Another soluble suppressor factor, named soluble suppressor of immune response (SIRS), was isolated from mouse T cells activated with con A or interferon (Rich and Pierce, 1974; Aune and Pierce, 1982; Schnaper et al., 1984). It inhibited cell division in a variety of normal and transformed cell lines and blocked antibody production by B cells <u>in vitro</u>. SIRS was stable at 56°C for 60 min, but was destroyed within 10 min at 70°C and by dialysis against pH 2 buffer. SIRS

activity was resistant to DNase and RNase, but sensitive to trypsin, chymotrypsin, and neuraminidase. Studies by Aune and Pierce (1981a,b) established that SIRS must be activated by H_2O_2 to exert its biological effects. SIRS action was blocked by sulfhydryl reagents, catalase, and ascorbic acid. It appeared that SIRS mediated inhibition of cell division by causing oxidation of sulfhydryl groups of cellular proteins. It was demonstrated that SIRS could disrupt the normal array of intracellular microtubules as well as inhibition of tublin polymerization in a cell free system. SIRS was purified by reverse phase HPLC and was found to have a M.W. of 14,000-21,000. mRNA for SIRS was also isolated and characterized by Nowowiejski-Wieder et al. (1984) using a cell free translation system. RNA coding SIRS activity was recovered in two fractions: one corresponded to a 25S fragment, the other was a 21S fragment. Further analysis of SIRS showed that it existed in three different isoforms as revealed by differential elution from reverse phase HPLC or by isoelectrofocusing (Webb et al., 1985).

Recently Schnaper and Aune, (1986) demonstrated that purified SIRS could also suppress the immune response of mice to sheep erythrocytes <u>in vivo</u>.

Medoff et al. (1986) isolated a suppressor factor from ascites fluid of cancer patients. The factor could reduce the incorporation of ^{3}H-tdr by con A-stimulated normal human PBL. The suppressor was precipitated at 0 to 35% ammonium sulfate saturation. Sephadex G-200 revealed that aggregation of the factor occurred in isotonic buffer, however, aggregation was reduced in the presence of 8 M urea. Purification of this factor was achieved by precipitation with 5% TCA.

The suppressor factor remained soluble in TCA and demonstrated a 95% increase in the specific activity. The suppressor activity was stable to heat (70°C for 30 min), pH 2 and pH 10 for 24 hr. Delipidation by chloroform-methanol extraction, or proteolytic enzyme digestion did not affect the suppressor activity. However, periodate oxidation irreversibly destroyed suppressor activity which suggested the importance of a carbohydrate moiety present in the suppressor molecule.

It was shown by Rabin et al. (1986) that gamma interferon could inhibit the activation of resting B-cells by B-cell stimulatory factor (BSF). Interferon blocked both the volume enlargement and preparation for DNA synthesis caused by B-cell stimulatory factor, although it had little effect on B cells already stimulated by BSF. This suggested a mutual regulatory interaction between the interferon and BSF both of which are T cell derived products.

Sayers et al. (1986) demonstrated that supernatants from the coculture of human PBL and the natural killer cell-susceptible cell line K562 were cytostatic against a variety of tumor cell lines. This factor was different from other known factors such as interferon, lymphotoxin or tumor necrosis factor. The physicochemical properties of this factor has not been reported yet.

Fujiwara et al. (1987) reported that U937 cells, a human macrophage-like cell line, spontaneously produced a factor which inhibited blastogenic responses of human blood T lymphocytes stimulated with PHA or tuberculin-purified protein derivative (PPD). This suppressor factor was precipitable by 33 to 67% saturated ammonium sulfate and was inactivated at pH 2 or pH 11. The apparent M.W. of the

suppressor was between 67,000 and 130,000 as was estimated by Sephacryl S-200 gel filtration chromatogrpahy. The U937 suppressor factor inhibited the production of IL-2 by normal as well as the leukemic human T cell line, Jurkat, when were stimulated with PHA. It inferfered also with the expression of IL-2 receptor on stimulated blood T lymphocytes. However, this factor did not inhibit spontaneous proliferation of the human leukemic T cell lines Jurkat or CCRF-CEM at low doses.

In recent years, much interest also has been centered on proteins isolated from activated leukocytes that possess cytotoxic or cytostatic activities against tumor cells in vivo and in vitro. For example, interferons (IFN) have been reported to have growth inhibitory activity against a variety of cell lines in vitro (Gresser and Tovey, 1978; Rubin and Gupta, 1980; Tyring et al., 1982; Blalock et al., 1980). Another anticellular agent is lymphotoxin (LT) which was initially derived from culture supernatants of antigen or mitogen-activated lymphocytes (Granger et al., 1969; Hiserodt et al., 1976). Lymphotoxin was reported to have tumoricidal activity against a range of tumor cells in vitro and anticarcinogenic activity against cells undergoing neoplastic transformation (Rosenberg et al., 1973; Sawada et al., 1976; Ransom et al., 1983). In 1975, Carswell et al. described a molecule with properties similar to LT which they called tumor necrosis factor (TNF). In common with LT, TNF displayed cytostatic and/or cytocidal activity against several tranformed cell lines in vitro, but normal cells were unaffected by its action (Carswell et al., 1975; Mathews and Watkins, 1978; Helson et al., 1975).

Because only very small amounts of LT or TNF were available, little was known of their biochemical properties. Indeed, a variety of M.W. had been reported probably due to differing molecular forms of the molecules. Recently, however, Aggarwal et al. (1985a,b), have succeeded in purifying monomeric LT and TNF to homogeneity. LT obtained from a lymphoblastoid cell line and had a M.W. of 18,644, whereas, TNF prepared from a promyelocytic leukemia cell line had an apparent M.W. of 17,000. Although strong similarities in amino acid composition were noted, antibodies were developed to each molecule which could distinguish between the two. Successful cloning of LT and TNF genes into E. coli has further demonstrated strong similarities in amino acid composition between the two (about 30% amino acid sequence homology) (Gray et al., 1984; Pennica et al., 1984).

As documented by the preceding literature review, there are large numbers of suppressor proteins produced by lymphocytes which also affect lymphocyte proliferation. The precise molecular mechanism by which these factors inhibit cell proliferation is not completely known. Therefore an in depth understanding of the modes of action of growth inhibitors may help to answer some of the many questions regarding the control of cell proliferation, which is a fundamental problem in biological science.

Hodge and Inman (1982) reported that culture supernatants of human tonsilar lymphocytes contained a high M.W. suppressor factor that could suppress the proliferative response of PBL to con A as measured by the reduced incorporation of ^{3}H-tdr. The use of PBL as target cells was, however, lengthy and inconvenient. This made the assay less

attractive for monitoring further purification and characterization of the suppressor factor. The objectives of the present work were to purify the suppressive protein produced by TL and to determine how it suppresses the proliferation of target EL-4 cells. In this paper, the progress toward purification and characterization of the suppressor protein is reported.

CHAPTER 2

Material and Methods

Preparation of Tonsil Lymphocyte Culture Supernatants. Palatine tonsils from patients undergoing elective surgery for recurrent tonsilitis were placed immediately in ice-cold transport medium consisting of RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (25 mM), and Fungisone (2 µg/ml) for transport from the hospital to the laboratory. Tonsil lymphocytes (TL) were obtained essentially as described by Hodge and Inman (1982) and adjusted to the desired cell density (1-12 x 10^6/ml) in TL culture medium (RPMI-1640 supplemented as above but without Fungisone). These primary cultures were incubated in tissue culture flasks for 1-3 days in 95% air-5% CO_2 at 37°C.

The cultures were terminated by centrifugation at 400 x g for 10 min, and the supernatants to be assayed on human PBL were lyophilized. The powder then was dissolved in a volume (typically 30-50 ml) of deionized water and centrifuged for 1 hr at 100,000 x g. The supernatant was dialyzed in 0.05 M NH_4HCO_3 solution adjusted to pH 7.2 with 1 M HCl, and lyophilized. The powder was stored at -70°C or rehydrated and used immediately. When EL-4 cells were used as targets the culture supernatants were concentrated 40-fold by Diaflo ultrafiltration at 4°C with nitrogen using XM-100 or YM-100 membranes. The membranes had a retention selectivity of 100,000. The retentates were washed 3 times by diluting them 30-fold with culture medium and

concentrating them again. The washed retentates were centrifuged at 100,000 x g for 30 min at 5°C. They were used soon or were stored at -70°C.

Tumor Cells. All cultures were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 25 mM HEPES, penicillin, streptomycin, and 10% FBS. The following cell lines were used: EL-4 (a mouse thymoma cell line), P388D (a mouse macrophage cell line), CEM-CM_3 (a human T cell leukemia), MRC (a human embryonic lung fibroblast cell line), HEP-2 (a human epithelial carcinoma cell line), and MDCK (a dog epithelial cell line). Cells were maintained by transferring them to fresh medium every 3 to 4 days. This resulted in a starting cell density of about 2×10^5 cells per ml.

Assay for Suppression of ^{3}H-tdr Uptake by Activated PBL. The lyophilized TL culture supernatant was dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.0076 M K_2HPO_4, 0.0024 M KH_2PO_4, adjusted to pH 7.4 with 1 M NaOH), and dialyzed in PBL medium [TL culture medium plus gentamycin sulfate (50 µg/ml)]. The assay on con A-activated PBL was done as described previously by Hodge and Inman (1982). In brief, 0.1 ml PBL (4×10^6 cells/ml) was pipetted into each well of the Costar Tissue Culture Cluster followed by 0.1 ml of the reconstituted TL culture supernatant, usually at a 40-fold concentration. 0.025 ml of 100 µg/ml stock solution of con A was added, and the cultures were incubated for 72 hr. Controls included the response of the PBL to con A in the absence of TL culture supernatant as well as PBL incubated only in PBL medium with serum. To each well was added 1 µCi of ^{3}H-tdr and incubation was continued another 14 hr. The cells were then harvested

on GF/C filter papers using a MASH-II multiple harvester (Microbiological Associates). The filters were dried and placed in plastic mini-vials containing 4 ml Scintisol (Isolab), and radioactivity was determined in a Beckman 3155T Scintillation Counter. Suppression was calculated by the the formula:

$$\% \text{ Suppression} = \left(1 - \frac{\text{cpm experimental}}{\text{cpm con A-stimulated control}}\right) \times 100$$

Molecular Sieve Chromatography. Concentrated TL culture supernatants were filtered through a column (1.6 cm x 90 cm) of Fractogel TSK-HW 55S equilibrated with 0.05 M Tris HCl, 0.5 M NaCl buffer, pH 8. The column was eluted at about 19 ml/hr and 2-ml fractions from the column were collected. Calibration proteins included ferritin (440,000 d.), catalase (232,000 d.), aldolase (158,000 d.), and myoglobin (17,000 d.). Fractions were pooled to encompass peaks and other regions detected at wavelength of 280 nm. The pools were concentrated on a YM-10 ultrafilter (Amicon) to an appropriate volume which was loaded originally on the column, washed with 0.05 M Tris, pH 8, dialyzed in PBS and then in PBL medium. The pools were filter-sterilized (0.22 micron) and assayed using EL-4 cells as targets (see below).

Ultrafiltration of TL Culture Supernatants. TL culture supernatants were filtered through various Amicon ultrafilters using N_2 for pressurization. The ultrafilters included XM-300, XM-100 or YM-100, XM-50, YM-10 and YM-2. The retentates were washed with RPMI-1640 three times and filter-sterilized prior to assay for SMAL. Retentates of

various samples not used for assay were washed with a buffer appropriate for their intended use.

Analytical Gel Electrophoresis. Electrophoresis of 30-100 µg of 20 mM mercaptoethanol-reduced and 0.1% SDS-denatured protein samples was done in 10% polyacrylamide gels 1.5 mm thick as described by Laemmli (1970) using a Bio-Rad Mini-vertical Model 360 slab gel electrophoresis apparatus (8 cm x 8 cm). An LKB 2103 power supplier provided 15 mA during stacking and 25 mA constant current after the tracking dye entered the separating gel. The gels were stained with Coomassie Brilliant Blue dye (1.2 g/l) in isopropanol:glacial acetic acid:water (1:1:8). Destaining was done using the same solvent. Silver staining was done by a modification of the method of Switzer et al. (1979). The gels were loaded with 10 µg protein from XM-100 or YM-100 washed retentates of TL culture supernatants, or with 200 ng purified SMAL. After electrophoresis the gels were fixed in methanol:water:glacial acetic acid (5:5:1) for at least 2 hr, were then washed with deionized water for 15 min followed by staining for 45 min in a solution of 5.9 mM $AgNO_3$, 31 mM NH_4OH and 2 mM NaOH. After washing the gels three times with deionized water (5 min each time), proteins were visualized by developing the gels in a solution containing 0.05% citric acid and 0.02% formaldehyde. Molecular weight standards (Pharmacia) included phosphorylase b (94,000 d.), bovine serum albumin (67,000 d.), ovalbumin (43,000 d.), carbonic anhydrase (30,000 d.), trypsin inhibitor (20,100 d.), and alpha-lactalbumin (14,400 d.). For detection of DNA, 1% agarose or 10% polyacrylamide gels were loaded with 20 µl of 40-50-fold concentrated (XM-100 retentate) TL culture supernatant or

5 ng purified SMAL calculated as protein. After electrophoresis (100 V, 38 mA for 50 min), the gels were stained for 30 min in ethidium bromide (0.5 µg/ml), and examined under UV light (Maniatis et al., 1982).

Elution of SMAL from Native Polyacrylamide Gel Electrophoresis by Electroblotting to DEAE-cellulose Paper. TL culture supernatant concentrated on a YM-100 membrane was treated with DNase, dialyzed against 0.05 Tris buffer pH 7 and subjected to 10% PAGE electrophoresis under native conditions. A Bio-Rad mini-vertical model 360 slab gel (1.5 mm thick) electrophoresis apparatus (8 cm x 8 cm) was used. A total of 250 µl (250 µg) of TL culture supernatant was applied to 5 wells of the stacking gel. After electrophoresis (about 150 min at 20 mA) the gel was placed against a sheet of DEAE-cellulose paper and electroblotted for 5 hr at 200 mA using a Hoeffer Scientific Instrument TE Transphor Electrophoresis unit. The buffer used for electroblotting contained 25 mM Tris, and 192 mM glycine HCl, adjusted to a pH of 8.3. After electroblotting, the DEAE-cellulose paper was cut to strips 1 cm wide, which were placed in 15 ml of 1 M NaCl at 4°C overnight. The substances eluted by the solvent were first dialyzed against PBS, followed by RPMI-1640 and then assayed for inhibition of ^{3}H-tdr uptake using EL-4 or CEM-CM_3 cell lines as target cells.

Protein Determination. Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Bioassay for Suppression of ^{3}H-tdr Uptake by EL-4 Cell. Assays were done in triplicate similar to those described above. Each well received 0.1 ml of the actively growing cell suspension adjusted to

4-6 x 10^5 cells/ml and 0.1 ml of filter-sterilized TL culture supernatant or other samples which had been equilibrated with RPMI-1640 by ultrafiltration or dialysis. The plates were incubated for a minimum of 22 hr before the addition of 1 μCi of ^{3}H-tdr (6 Ci/mmole) to each well. The plates were incubated another 2 hr, after which the cells were harvested as described in the assay for suppression of activated PBL. Controls consisted of cells cultured with medium in the absence of TL culture supernatant. Suppression of the uptake and incorporation of radioactivity was calculated using the formula given above.

Assignment of SMAL Unit. The quantity of SMAL was estimated using serially diluted TL culture supernatants or other samples which were subjected to bioassay for the suppression of ^{3}H-tdr uptake by EL-4 cells as described above. Data were plotted on probability papers as function of % suppression vs. reciprocal of the dilution. The reciprocal of dilution that resulted in 50% suppression was used as the number of units/0.1 ml of the tested sample. The amount of SMAL that results in 50% inhibition of ^{3}H-tdr incorporation is assigned one unit.

Inhibition of Production of SMAL by Cycloheximide and Heat. Suspensions of TL were prepared at 10 x 10^6 cells/ml and were divided into experimental and control groups. The experimental groups were either treated with cycloheximide (100 μg/ml) during culture or were killed by heating to 56°C for 1.5 hr before culture. The control were comprised of normal untreated TL cells. Each group then was cultured for four days in TL culture medium.

Fractionation of T and B Lymphocytes. T enriched- and B enriched-lymphocyte fractions were prepared from TL on a discontinuous gradient of Percoll according to Gutierrez et al. (1979). Each of the two fractions was then washed once with TL culture medium, sedimented at 400 x g for 10 min, and resuspended at a cell density of 10 x 10^6/ml in TL culture medium. The cells were grown four days and the culture supernatants were assayed for the amounts of SMAL units as described above.

The spontaneous proliferation of the TL and the T enriched- and B enriched-lymphocyte fractions was measured by suspending 4 x 10^6 cells in TL culture medium (1 ml total volume) in tissue culture tubes. The cells were pulsed with 4 µCi ^{3}H-tdr (6 Ci/mmol) for 24 hr. The cells were pelleted at 400 x g for 10 min, resuspended in 5% TCA allowed to stand for 15 min, and harvested on GF/C filter paper. The paper was washed five times with 5% TCA and finally with 70% ethanol, and air dried. ^{3}H-tdr cpm were determined as described above.

Effect of Various Treatments on the Activity of SMAL. XM-100 retentates of TL culture supernatants were treated in various ways to determine treatment affects on the activity of SMAL. Retentates were examined for thermal stability by heating 1 ml of 40-fold concentrated supernatant in a capped pyrex culture tube in a water bath at 56°C, 70°C, or 100°C for 1 hr. The samples were dialyzed against TL culture medium, filter-sterilized, and assayed for suppressive activity. The control was the unheated supernatant fluid treated similarly. XM-100 retentates washed with PBS were dialyzed overnight in 0.2 M glycine-HCl, pH 2, or made 0.4 M in HCl, 10% in TCA, 6 M in urea, or 60% in

acetonitrile. The acids caused precipitation, so the precipitates and their supernatants were assayed. The samples were dialyzed first in PBS, then TL culture medium, and assayed as above. XM-100 retentates in RPMI-1640 were digested with pronase (45 units/ml; 70 units/mg; Calbiochem), deoxyribonuclease I (130 µg/ml; 2621 units/mg; Sigma) or ribonuclease T_1 (14 mg/ml; 300,000 units/mg; Millipore, Inc.) for 2.5-3.0 hr at 37°C. After digestion, the samples were washed on an YM-100 ultrafilter several times, adjusted to the correct volume, and assayed for percent suppression compared to the untreated control. As a control for the assay, the same amount of pronase, DNase, or RNase were added to EL-4 cells.

Solid NH_4SO_4 was added to 5-10 ml of 100-fold concentrated XM-100 retentate in PBS at 5°C. The precipitates which formed at 0%-40%, 40%-80%, and 80%-100% saturation were recovered at 4°C in centrifuge at 20,000 x g for 30 min. Precipitates were dissolved in PBS, dialyzed against large volumes of PBS and finally TL culture medium. Dialysate were then subjected to bioassay for percent suppression of ^{3}H-tdr uptake by EL-4 cells.

DEAE-anion Exchange Chromatography. DE-52 (Whatman) was washed, equilibrated in start buffer (0.05 M Tris-HCl, pH 8), and packed under 25 cm pressure in a K 9/15 column (Pharmacia). One to two ml of XM-100 retentate of TL culture supernatant (2-6 mg protein) equilibrated in start buffer were loaded on the column. The column was flushed with two volumes (20 ml) start buffer before commencing a linear gradient with 1 M NaCl in start buffer using a gradient former. The flow rate was 10 ml/hr. Conductivity of the fractions (2 ml/tube) was determined with a

Radiometer CDM 2 meter, while the optical density of each fraction was determined at 280 nm. Fractions were pooled as shown in Figure 11, dialyzed against PBS, concentrated to the volume loaded on the column by using a YM-10 ultrafilter, and then analyzed.

Absorbance of Ion-Exchange Pooled Fractions. Pools 1, 2, and 3 resulting from DEAE chromatography and purified SMAL from HPLC (see below) were scanned for their absorbance of light between 240 and 300 nm using a Beckman Model 35 Spectrophotometer attached to a recorder. The samples were in start buffer or TL culture medium.

Ion-exchange HPLC. Two to eight mg of protein (2 ml) of XM-100 retentates of TL culture supernatants which were treated with deoxyribonuclease I as described above were loaded onto a QMA-Accell anion exchange column (Waters Associates) previously equilibrated with start buffer. The column was attached to two Waters Model 510 HPLC pumps using a flow rate of 2 ml/min. After a 25 min isocratic elution, a linear gradient of 0 to 1 M NaCl in start buffer commenced and was run for 45 min using a Waters Automatic Gradient Controller. The NaCl was held at 1 M for an additional 25 min of the 95 min total running time. The optical density of the eluate was monitored at 280 nm by a Waters Lambda Max LC Spectrophotometer attached to a Data Module set at a chart speed of 0.2 cm/min. Fractions collected (2 ml) were pooled, concentrated on a YM-10 ultrafilter and analyzed by SDS-PAGE or subjected to bioassay for the suppression of ^{3}H-tdr by EL-4 cells.

Assay for the Uptake of ^{3}H-tdr, ^{3}H-udr, and ^{3}H-adr. Actively growing EL-4 cells (0.1 ml at 6 x 10^{5} cells/ml) were added to triplicate

wells of 96-well tissue culture plates (Costar with flat bottom) followed by 0.1 ml of SMAL (experimental) or RPMI-1640 (control). Plates were incubated for 24 hr at 37°C and then pulsed with either 1 μCi of ^{3}H-tdr (6 Ci/mmol), ^{3}H-udr (15 Ci/mmol), or ^{3}H-adr (50 Ci/mmol). At different time intervals samples were harvested on GF/C filter papers using a MASH-II multiple harvester (Microbiological Associates). The filters were dried and placed in plastic mini-vials containing four ml Scintisol, and radioactivity was determined in a Beckman 3155T scintillation counter.

Incorporation of ^{3}H-tdr into Acid-insoluble and Acid-soluble Materials. EL-4 cells were cultured in the presence and absence of SMAL as described above followed by pulsing with 1 μCi of ^{3}H-tdr for an additional 2 hr. The cell suspensions were transferred to 1.5 ml Eppendorf tubes and washed three times with 1 ml of cold RPMI-1640 (30 second 13,000 x g). Duplicate samples of the cell pellet were resuspended in 200 μl of cold 5% TCA followed by centrifugation at 13,000 x g for 5 min. Fifty microliter aliquots of the supernatants (TCA soluble) were added to plastic mini-vials containing four ml of scintisol. The TCA-precipitable material was resuspended in 5% TCA and harvested on GF/C filters. Filters were washed five times with 5% TCA followed by a final rinse with 70% ethanol. Radioactivity was determined as described above.

To study the effect of increasing concentration of ^{3}H-tdr on the incorporation of ^{3}H-tdr by control and SMAL-treated cells, actively growing cells were cultured in the presence and absence of SMAL for 24 hr as described above. They were then pulsed for 2 hr with

increasing concentrations of ^{3}H-tdr (up to 6 μM) and the extent of incorporation was determined.

Effect of 5-Fluorodeoxyuridine on the Growth of EL-4 Cells in the Presence and Absence of SMAL. Actively growing EL-4 cells were cultured in the presence and absence of SMAL for 24 hr. Different concentrations of 5-Fudr were then added to each well and after an additional 48 hr incubation the number of cells were determined using a hemocytometer. Control cultures contained cells that were grown in the absence of SMAL and 5-Fudr. For calculating the percent of growth inhibition, the original cell concentration of the experimental cultures was subtracted from its final cell number and was applied to the following formula.

$$\% \text{ Growth Inhibition} = \left(1 - \frac{\text{Cell number experimental}}{\text{Cell number control}} \right) \times 100$$

Binding of ^{3}H-NBTI to EL-4 Cells. EL-4 cells were grown in the presence and absence of SMAL for 24 hr and then were pulsed with 1 μCi of ^{3}H-nitrobenzylthioinosine (^{3}H-NBTI) (20 Ci/mmol) (Moravek Biochemical, Brea, Ca) for and additional 30 min at 37°C or 25°C. The cells were harvested on GF/C filter papers and washed 5 times with ice-cold buffer (0.05 M Tris HCl pH 7.5) and radioactivity was determined as above. Non-specific binding of ^{3}H-NBTI to cells was calculated by including in the experiment cells that received a high concentration of unlabeled NBTI (50 μM) prior to the addition of labeled ^{3}H-NBTI and subtracting these values from those obtained in the presence of ^{3}H-NBTI only.

Thymidine Kinase Assay. EL-4 cells were grown in the presence or absence of HPLC-purified SMAL for 24 hr (10 ml total volume). Cells were counted and then harvested by centrifugation at 500 x g for 15 min at 5°C. The pellet was washed once with ice cold RPMI-1640, resuspended in 1 ml of buffer containing 0.05 M Tris pH 7.5 and 5 mM $MgCl_2$ and were then sonicated (3 pulses of 10 second) with Branson cell disruptor Model 185. Cell debris was removed by centrifugation at 500 x g for 15 min followed by a second centrifugation at 100,000 x g for 1 hr. The supernatants obtained after the second centrifugation were used immediately for TK assay. Enzyme activities were determined by following the phosphorylation of labeled ^{3}H-tdr according to the procedure of Madhav et al. (1980). The reaction mixture contained cell extracts (70 µg of total protein) in 0.05 M Tris HCl pH 7.5, 5 mM $MgCl_2$, 10 mM ATP and 1 µCi ^{3}H-tdr (6 Ci/mmol) in a total volume of 0.1 ml. After 30 min incubation at 37°C, 25 to 50 µl aliquots of the duplicate samples were spotted on 2.5 cm discs of DEAE-cellulose paper (DE-81). The discs were placed into individual beakers containing 30 ml of 90% ethanol, were washed five times (10 min each) with 30 ml ethanol, dried and placed in plastic mini vials containing 4 ml of Scintisol Radioactivity was determined by a liquid scintillation counter.

Determination of DNA Content and Autoradiography of SMAL-treated EL-4 Cells. Logarithmically-growing EL-4 cells (4 x 10^5 cells/ml) were cultured in the presence and absence of SMAL for 24 hr. Small aliquot (0.1 ml) of cell suspensions were removed and placed in tubes containing 5 ml of fixative solution consisting of methanol, and acetic acid (3:1). Cells were washed twice in this solution. The cell pellets were

resuspended in 200 µl of the fixative and smeared across the microscope slides. After the slides were air dried, they were stained with Feulgen reagent for DNA. A Vickers-M86 scanning and integrating microdensitometer (Vickers Instruments Inc., Malden, MA 02148) interfaced to a Sol system IIIA microcomputer was used to determine net absorbance of the Feulgen-DNA complex in individual nuclei. All measurements were performed as described by Lee and Rasch (1984). Approximately 200 nuclei were scanned for each slide.

For autoradiography, the remaining cell suspension were pulsed for 1 hr with 4 µCi/ml of ^{3}H-tdr (6 Ci/mmol). Samples taken at the end of 1 hr were considered to be zero time samples. The rest of the cells were washed with RPMI-1640 three times resuspended in fresh medium containing 10% FBS and incubated at 37°C. These cell suspensions were sampled at various subsequent time intervals to prepare microscope slides as described above.

Slides were then dipped into Kodak nuclear track emulsion type NTB-2 in the dark and were exposed at 4°C in a light proof container for 24 hr. Slides were developed in Kodak D19 developing solution and stained with Giemsa. The background was less than 3 grains per nucleus, and nuclei with 5 or more grains were regarded as labeled. About 1000 nuclei per each slide were counted under the microscope to determine the percentage of labeled nuclei.

Determination of Cytopathic Effect of HSV II on MRC and HEP-2 Cell Line in the Presence or Absence of SMAL. Actively growing MRC or HEP-2 cell lines were cultured in 96-well microtiter plates with flat bottoms

at concentration of 2 x 10^5 cells/ml in RPMI-1640 in the presence of 10% FBS. Serial dilutions of SMAL were added to each well, and the plates were incubated for 24 hr at 37°C. At the end of 24 hr, serial dilutions of a clinically isolated HSV II (obtained from the Department of Microbiology, Quillen-Dishner College of Medicine, ETSU) were added to the cells and the plates were incubated for several days at 37°C. At 24 hr intervals, the cells were observed for cytopathic effect (CPE) using an inverted microscope. The extent of CPE was recorded on a scale from + 4, meaning extensive damage and rounding up of the cell monolayer, to a negative sign (-) meaning no cellular damage.

CHAPTER 3

Results

Tonsil Lymphocyte Culture Supernatant Suppresses the Uptake of ^{3}H-tdr by Con A-Stimulated Lymphocytes and EL-4 Cells. Human tonsil lymphocytes were cultured in medium for 24 hr. The supernatant culture fluid was dialyzed and lyophilized, and then tested for its effect on the incorporation of ^{3}H-tdr by con A-stimulated PBL or EL-4 cells. The data are presented in Table 1. The uptake of ^{3}H-tdr by con A-stimulated human PBL and by xenogeneic mouse thymoma EL-4 cells was considerably reduced by tonsil lymphocyte culture supernatant. The extent of inhibition ranged from 94% to 98% for PBL and 76% to 94% for EL-4 cells. This suppressor of mitogen activated lymphocytes was referred to by its acronym, SMAL.

Determination of Growth Curve for EL-4 Cells. EL-4 cells were cultured for 5 days in antibiotic supplemented RPMI-1640 containing 10% FBS at initial cell density of 2×10^{5}/ml. At different time intervals small aliquots of cell suspension were removed, and cell density was determined using a hemocytometer. As it is shown in Figure 1, the doubling time for EL-4 cells was about 18 hr. This information was necessary for using EL-4 cells as targets for bioassay of TL culture supernatant.

Kinetics of Inhibiton of ^{3}H-tdr Uptake by EL-4 Cells Treated with SMAL. The relationship of exposure time of EL-4 cells to SMAL and the suppression of ^{3}H-tdr uptake was investigated. Cells were incubated

TABLE 1. Inhibition of the Uptake of ^{3}H-tdr by Con A-Stimulated Human PBL and Mouse EL-4 Cells in the Presence of TL Culture Supernatant (SMAL)

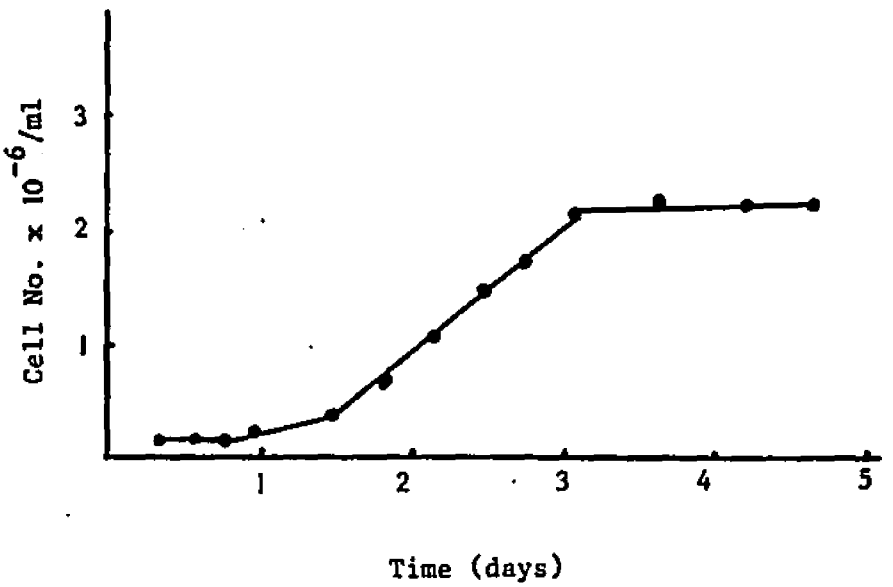
	Uptake of ^{3}H-tdr (mean cpm x 10^{-3} ± SD)			
	PBL		EL-4	
Exp. No.	Control[a]	Experimental	Control[b]	Experimental
I	37 ± 1.2	1.2 ± 0.1(98)[c]	402 ± 9	23.3 ± 1.7 (94)
II	40.4 ± 3.5	3.5 ± 0.2(91)	178 ± 17	12.4 ± 1.4 (76)

[a] PBL were cultured in TL medium containing an optimal dose of con A (control) or TL culture supernatant (experimental).

[b] EL-4 cells were cultured in TL medium (control) or TL culture supernatant (experimental).

[c] % suppression

Figure 1. Growth Curve of EL-4 Cells. Cells were cultured at an initial density of 2×10^5/ml in TL medium supplemented with 10% FBS. At different time intervals aliquots of all cell suspensions were removed to determine the cell concentration by a hemocytometer.


Cell No. x 10^{-6}/ml
3
2
1
1
2
3
4
5
Time (days)

with SMAL and the uptake of ^{3}H-tdr at various times was determined. As it is shown in Figure 2 a minimum of 6 hr exposure of EL-4 cells to SMAL was necessary in order to detect substantial inhibition of ^{3}H-tdr uptake. Suppression, however, reached to its maximum by 24 hr.

Fractionation of Tonsil Lymphocyte Culture Supernatant by Ultrafiltration. Tonsil lymphocyte culture supernatant was subjected to serial fractionation using Amicon Diaflo ultrafilter membranes with M.W. cut-offs of 300 kd. (XM-300), 100 kd. (XM-100), 50 kd. (XM-50), 10 kd. (YM-10), and 2 kd. (YM-2). The retentate from each membrane fraction then was tested for the inhibition of ^{3}H-tdr, ^{3}H-udr or ^{3}H-Leu uptake by EL-4 cells. Only retentates of XM-100 membrane could inhibit the uptake of ^{3}H-tdr by EL-4 cells (65%). No inhibition of ^{3}H-Leu uptake was noticed with an XM-100 retentate, but the uptake of ^{3}H-udr was also suppressed (35%) (Table 2).

Assignment of SMAL Unit. EL-4 cells were cultured in the presence of varying concentrations of TL supernatant for 22 hr and were pulsed with ^{3}H-tdr for 2 hr. The inhibition of the ^{3}H-tdr incorporation in the cells was dependent on the dose of TL culture supernatant (Fig. 3A). The data were subjected to a Probit analysis. When percent inhibition of the ^{3}H-tdr incorporation was plotted against reciprocal of serial dilution of a sample, the activity of SMAL could be defined by the reciprocal of dilution of the sample that resulted in a value of 50% inhibition (Fig. 3B). Therefore, one unit is that amount of SMAL which results in 50% suppression of ^{3}H-tdr uptake by EL-4 cells. Based on this assay, typical TL culture supernatants usually contained 2-10 units/ml of SMAL.

Figure 2. Time Course Effect of SMAL on the uptake of ^{3}H-tdr into EL-4 Cells. EL-4 cells (4×10^{4} per well) taken from mid-log phase of growth were placed in the wells of a microtiter tissue culture plate essentially as described for the assay for suppression of EL-4 cells, except that ^{3}H-tdr was added 2 hr before each harvest time point.

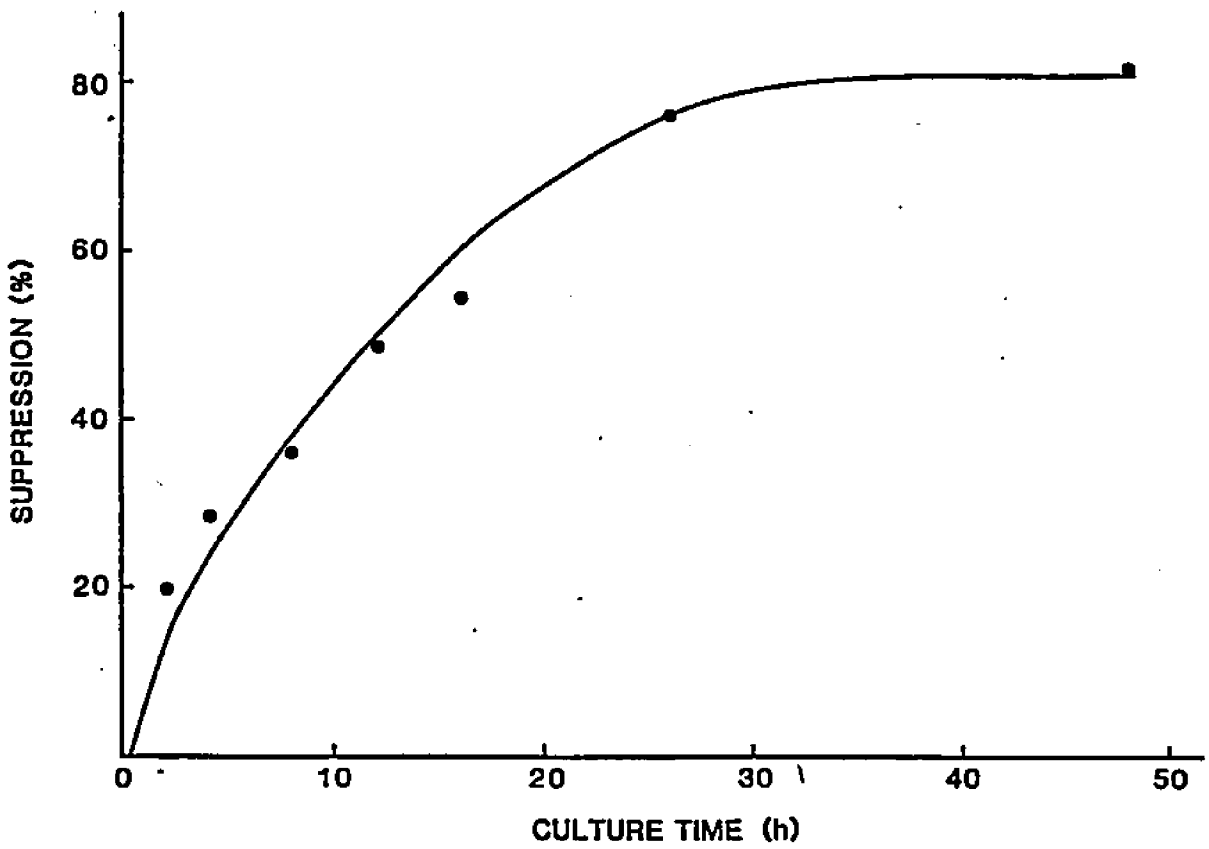

80
60
40
20
SUPPRESSION (%)
0
10
20
30
40
50
CULTURE TIME (h)

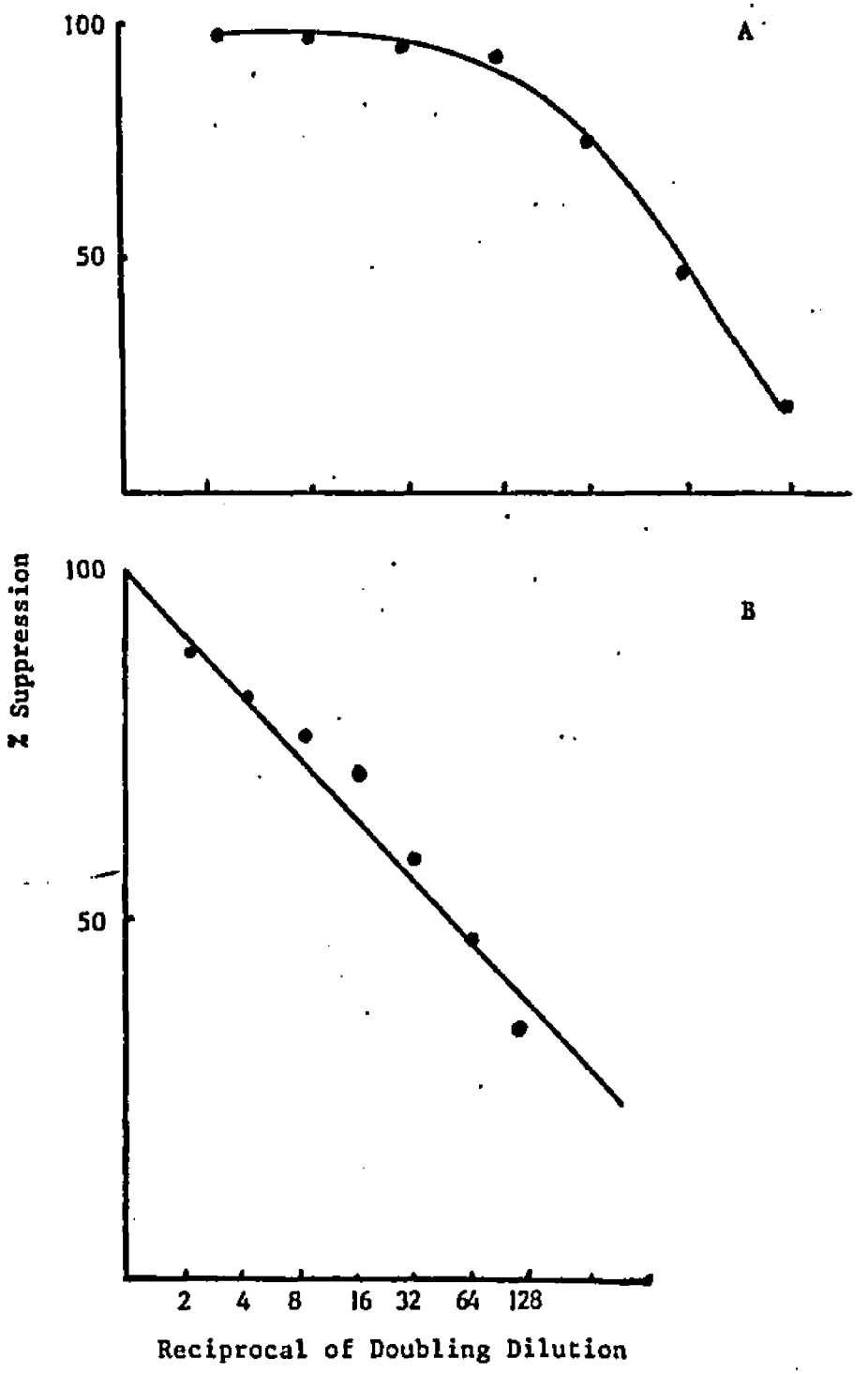
TABLE 2. Fractionation of TL Culture Supernatant by Diaflo Ultrafiltration

	Membrane Type					
	XM-300	XM-100	XM-50	YM-10	YM-2	YM-2Filtrate
Labeled Substance Incorporated	Inhibition of Incorporation (%)					
^{3}H-tdr	0	65	0	0	10	0
^{3}H-udr	ND	35	0	0	0	0
^{3}H-Leu	ND	0	ND[a]	ND	ND	ND

TL culture supernatant was subjected to serial ultrafiltration starting with the largest pore-size membrane. After each retentate was concentrated to 40-fold, it was subjected to bioassay using EL-4 cells as target.

[a] Not Done

Figure 3. Assignment of SMAL Unit. Figure 3A, activity of serial dilutions of the TL culture supernatants were assayed against EL-4 cells as described in Materials and Methods. Figure 3B, Probit plotting of the data in Figure 3A provided units of SMAL activity. One unit is the amount of SMAL which causes 50% inhibition of incorporation of ^{3}H-tdr.


A
100
50
B
100
50
% Suppression
2
4
8
16
32
64
128
Reciprocal of Doubling Dilution

Characteristics of the Production of SMAL. Four types of experiments were performed to investigate certain characteristics of the production of SMAL by TL. Suspensions of TL were divided into a control group and two experimental groups. The experimental cultures were treated with cycloheximide during culture or were heat killed before being cultured. The culture supernatants were collected and assayed for SMAL. There was no activity in the culture fluid of the heat-killed cells or the cells cultured with cycloheximide (Fig. 4). The untreated TL produced about 200 units of SMAL/100 ml of culture supernatant.

For the second experiment, TL were cultured at 4 X 10^6 cells/ml for 24 hr. The supernatant was collected and the cells were resuspended in fresh medium. After another 24 hr culture, the process was repeated, and with the collection of the third 24 hr culture supernatant, the cells were discarded. The XM-100 retentates of the three culture supernatants were assayed against EL-4 cells. As shown in Figure 5 maximal production of SMAL occurred during the first 24 hr period, and the production diminished during each subsequent 24 hr culture period.

TL were cultured at several cell densities for 24 hr in the third experiment. The production of SMAL in these respective culture supernatants increased with increasing cell densities (Fig. 6). At cell densities of 2 X 10^6 cells/ml or less, however, there was no detectable suppressive activity. In the fourth experiment TL at 10 X 10^6 cells per ml were cultured 1, 3, or 5 days. The units of SMAL in their supernatants increased with the culture time (Table 3) as predicted by Figure 5; 70% of the amount of SMAL found after 5 days of culture was produced within the first 24 hr of culture.

Figure 4. Production of SMAL by Heat-killed or Cycloheximide-treated TL. TL were cultured with cycloheximide (100 µg/ml), or were placed in TL Culture medium after being killed by heating to 56°C, or were cultured untreated (none). The supernatants of the three cultures were assayed for units of SMAL.

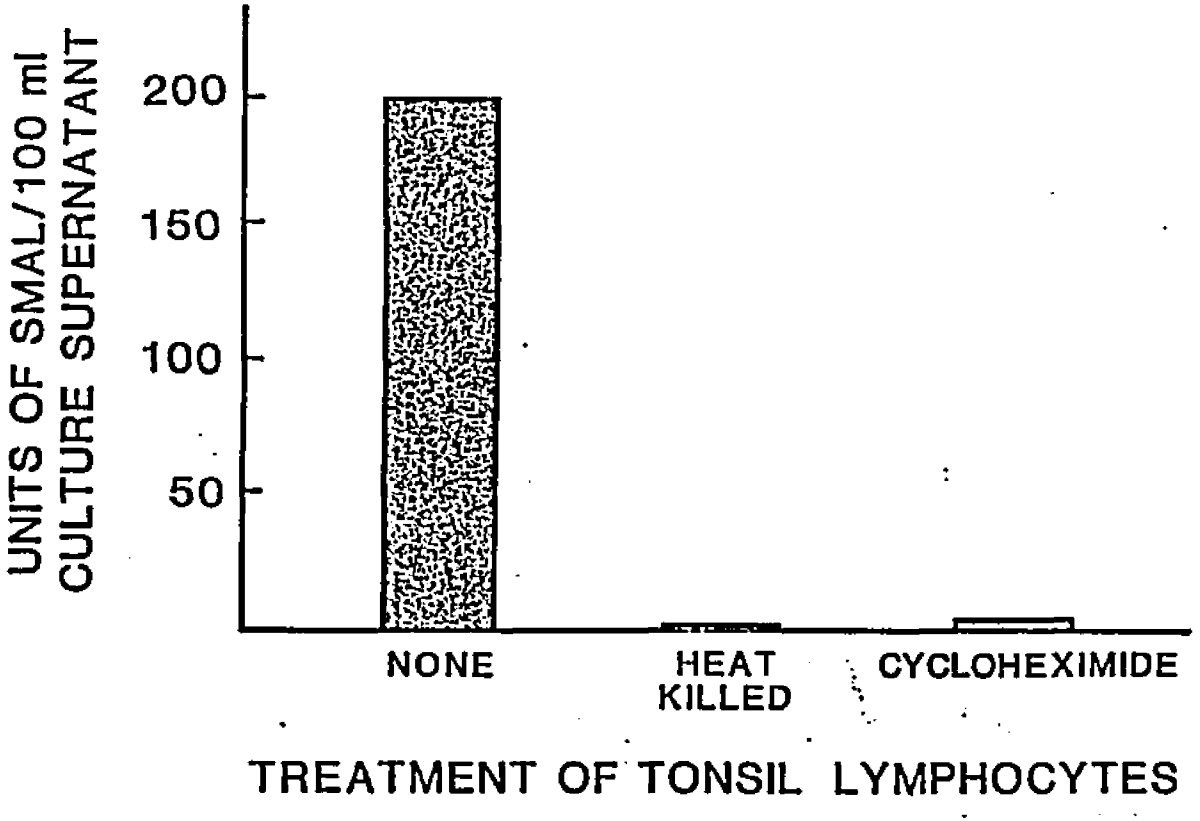

UNITS OF SMAL/100 ml CULTURE SUPERNATANT
200
150
100
50
NONE
HEAT KILLED
CYCLOHEXIMIDE
TREATMENT OF TONSIL LYMPHOCYTES

Figure 5. Kinetics of the Appearance of SMAL in the culture Supernatants of TL. The cells were cultured at 4 x 10^6 cells/ml for three days, but the culture supernatants were collected every 24 hr and fresh TL culture medium was added to the cells. The supernatants were assayed for units of SMAL.

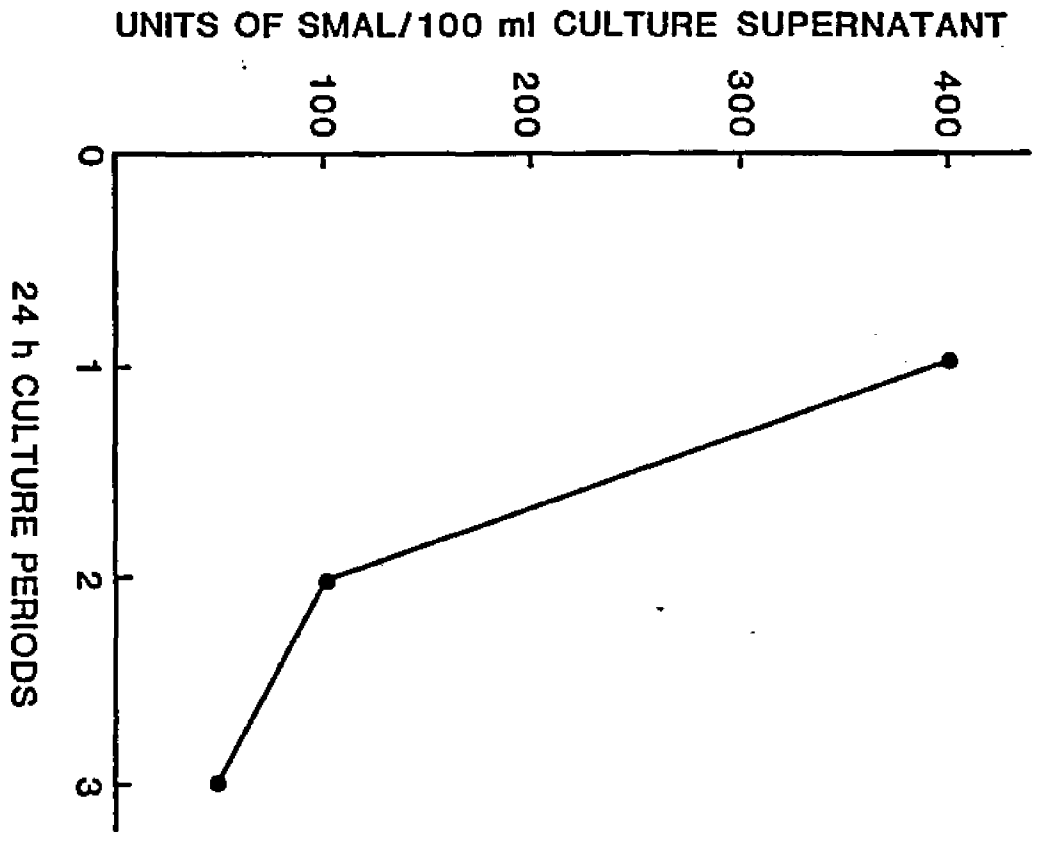

UNITS OF SMAL/100 ml CULTURE SUPERNATANT
100
200
300
400
0
1
2
3
24 h CULTURE PERIODS

Figure 6. Production of SMAL as a Function of TL Cell Density. TL were cultured at different cell densities as indicated. The culture supernatants were harvested and assayed for units of SMAL at the end of the culture period.

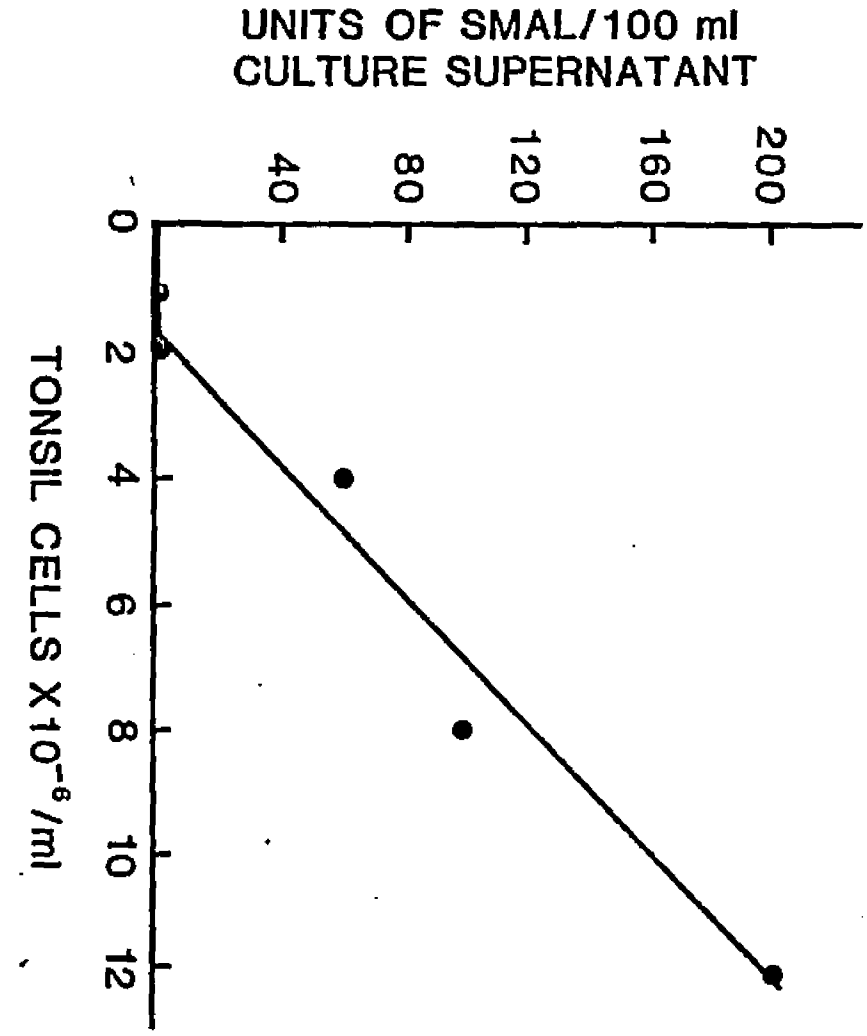

UNITS OF SMAL/100 ml
CULTURE SUPERNATANT
40
80
120
160
200
0
2
4
6
8
10
12
TONSIL CELLS X10⁻⁶/ml

TABLE 3. Accumulation of SMAL

During Uninterrupted Culture[a]

Length of Culture (days)	SMAL (units/100 ml Supernatant)[b]
1	700
3	825
5	1000

[a] TL were cultured 1, 3, or 5 days at a beginning density of 10×10^6/ml. At the end of culture, the supernatants were assayed against EL-4 cells.

[b] Units of each time point are accumulative.

Culture Supernatants from Tonsil T and B Lymphocyte-enriched Fractions Contained SMAL. TL and the T lymphocyte- and B lymphocyte-rich fractions were cultured in small tubes for 24 hr and their incorporation of ^{3}H-tdr was determined. It was clearly evident that tonsils contained lymphocytes which synthesized DNA spontaneously (Table 4) as compared to unstimulated PBL which did not incorporate ^{3}H-tdr. Several experiments of this kind showed that the extent of thymidine incorporation was quite variable, however, and thus the extent of spontaneous DNA synthesis by TL was unpredictable. The data in Table 4 also indicated that the T and B lymphocyte-enriched fractions incorporated ^{3}H-tdr. The B lymphocyte fractions incorporated over twice as much ^{3}H-tdr as the T lymphocyte fractions. The ratio of B lymphocyte to T lymphocyte cpm seemed to hold regardless of the ability of the unfractionated TL to incorporate ^{3}H-tdr.

In other experiments, the T lymphocyte- and B lymphocyte-rich fractions were cultured at 10 x 10^{6} cells/ml for 4 days. The culture supernatants were then collected and assayed for SMAL. The XM-100 retentates of each culture supernatants inhibited the incorporation of ^{3}H-tdr by EL-4 cells up to 85%-90% (Fig. 7). The supernatant of the cultured T lymphocyte fraction contained 1200 units of SMAL/100 ml, but that of the B lymphocyte fraction contained 6400 units/100 ml.

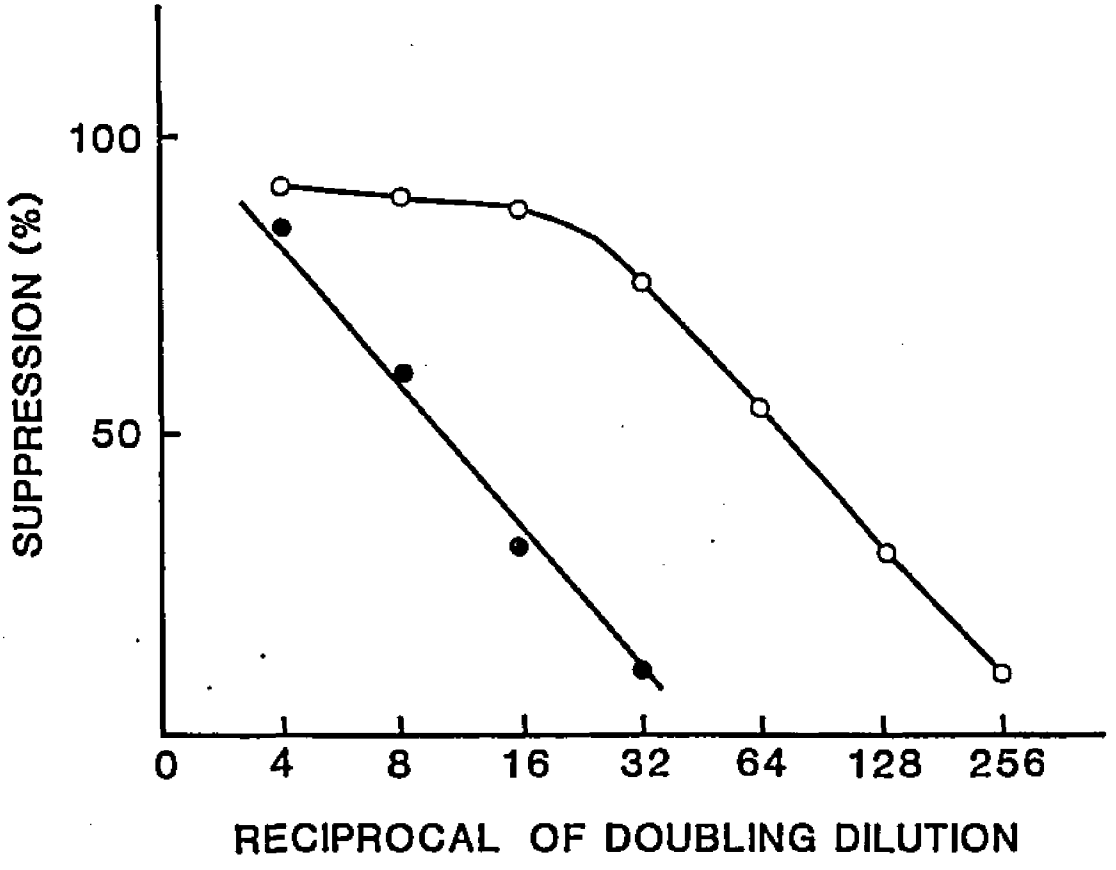
Presence of SMAL-like Activity in the Tonsil Lymphocyte Sonicate. Tonsil lymphocytes prepared as described previously were resuspended at 5 x 10^{7} cells/ml in RPMI-1640 and were lysed by sonication. After centrifugation at 100,000 x g the supernatants were dialyzed on an

TABLE 4. Spontaneous Proliferation of Tonsil T and B Enriched Lymphocytes

	^{3}H-tdr Incorporation cpm[a]		
Experiment No.	Unseparated TL	T Lymphocytes	B Lymphocytes
I	52,200	42,000	107,000
II	55,600	41,000	100,500
III	11,000	11,000	26,000

[a] Several experiments indicated that unstimulated PBL did not incorporate ^{3}H-tdr and had a mean cpm of 1000.

Figure 7. Production of SMAL by T Cell-enriched and B Cell-enriched Tonsil Lymphocyte Preparations. Tonsil lymphocytes were separated to B and T cell-enriched populations by Percoll density gradient centrifugation as described in Materials and Methods. The cultures were incubated for four days and the culture fluids were collected and assayed for SMAL. •— T-cell culture fluids, o— B-cell culture fluid.


100
50
SUPPRESSION (%)
0
4
8
16
32
64
128
256
RECIPROCAL OF DOUBLING DILUTION

XM-100 membrane and subjected to bioassay using EL-4 cells. The percent of inhibition of ^{3}H-tdr incorporation by TL sonicates ranged from 86 to 92% in different experiments (Table 5).

Effect of Various Treatments on the Activity of SMAL. XM-100 retentates of culture supernatants were subjected to various kinds of treatment, and the residual activity of suppression of incorporation of ^{3}H-tdr by EL-4 cells was measured. Heating the retentate to 56°C for 1 hr reduced its activity only 15%, but heating to 70°C reduced it by 70%. The activity was totally abolished upon heating to 100°C (Table 6). Treatment of retentates with glycine (pH 2), HCl or TCA caused precipitation. When the precipitates and their supernatants were assayed, about 85% of the activity of SMAL was recovered in the glycine supernatant, but no activity was recovered in any of the other supernatants or precipitates. Making the active XM-100 retentate of a culture supernatant 60% in acetonitrile also resulted in complete loss of activity, but 6 M urea had no measurable affect (Table 6). The activity of SMAL was sensitive to pronase treatment, while RNase had no apparent effect. Treatment with DNase, however, enhanced the activity by 1.5-fold (Table 6).

Precipitation of SMAL Activity by Ammonium Sulfate. TL supernatant concentrated on XM-100 membrane was precipitated in a stepwise manner by ammonium sulfate. The majority of activity of SMAL was present in the 40 to 80% ammonium sulfate saturation precipitate (Table 7). The recovery of SMAL activity was very poor, however, being less than 25% of the original activity (Table 8).

TABLE 5. Tonsil Lymphocyte Sonicate Inhibits The Uptake of ^{3}H-tdr by EL-4 Cells

	Uptake of ^{3}H-tdr (mean cpm x 10^{-3} ± SD)	
Experiment No.	Control[a]	Experimental[b]
I	97 ± 4	9 ± 4.8 (91)[c]
II	131 ± 13	19 ± 0.18 (86)
III	92 ± 6	7 ± 3 (92)

[a] Control EL-4 cells were cultured in the presence of culture medium only.

[b] Experimental EL-4 cells were cultured in the presence of medium and TL sonicate that had been washed on a XM-100 membrane with culture medium.

[c] Figures in parentheses represent % suppression of ^{3}H-tdr uptake.

TABLE 6. Stability of SMAL Activity to Different Treatment

Treatment	% of Control Activity
56°C 1 hour	83
70°C 1 hour	30
100°C 1 hour	0
Pronase	0
DNase	149
RNase	102
glycine (pH 2)	85
HCl (0.4 M)	0
TCA (10%)	0
Urea (6 M)	100
Acetonitrile (60%)	0

TABLE 7. Fractionation of SMAL Activity

by Ammonium Sulfate Precipitation

	Fractions		
Experiment No.	0-40[a]	40-80	80-100
1	20[b]	40	25
2	40	87	40
3	ND[c]	53	0
4	13	40	28
5	ND	73	ND

[a] % Ammonium sulfate saturation

[b] % Suppression of ^{3}H-tdr incorporation

[c] Not done

TABLE 8. Purification of SMAL by Ammonium Sulfate Saturation Precipitation

Step	Protein mg[a]/ml	Activity Units/ml	SA[b] Units/mg protein	Purification Fold	Recovery %
Crude	1.8	160	89	1.0	100
40-80%	0.75	40	53	0.6	25

[a] Solid ammonium sulfate was added to a solution of TL culture supernatant (100-fold concentrated) under constant agitation and the mixture was kept for 4 hr at 4°C. The material precipitated between 0 to 40% was pelleted by centrifugation at 12,000 x g for 20 min, resuspended in PBS and dialyzed against PBS. To the remainder of the solution solid ammonium sulfate was added and the precipitation process was repeated. Each fraction then was dialyzed against RPMI-1640 and subjected to bioassay for SMAL against EL-4 cells.

[b] Specific activity.

Partial Purification of SMAL by Molecular Sieve Chromatography. It was determined earlier that the suppressive activity in TL culture supernatants eluted from columns of Sephadex G-100 at V_o (Hodge and Inman, 1982). Other attempts were made to resolve SMAL by molecular sieve chromatography using Bio-Gel A-0.5 m and Fractogel TSK 55S. The fraction most active in suppressing incorporation of ^{3}H-tdr into EL-4 cells eluted at V_o using the A-0.5 m medium, but various amounts of suppressive activity were detected in pooled fractions throughout the profile with subsequent uses of the same column. In contrast, when culture supernatants were filtered through Fractogel, SMAL activity on EL-4 cells (see below) was associated with pooled fractions whose peak M.W. were about 630,000, 148,000, and 16,000 (Fig. 8 and 9).

Since the TSK 55S elution profile indicated that SMAL could have a M.W. as low as 16,000, the volumes of culture supernatants were reduced by filtration through a YM-10 ultrafilter. This ultrafilter should have retained the SMAL. The retentates were diluted and reconcentrated on a YM-100 ultrafilter which retains proteins of M.W. about 100,000. The original YM-10 retentates and the YM-100 retentates were examined by native PAGE. The patterns were very similar and clearly showed protein heterogeneity in the retentates (Fig. 10). The units of SMAL recovered in the YM-100 retentate were 95% of those in the YM-10 retentate. The reduction of culture supernatant volumes was much more rapid using XM-100 or YM-100 ultrafilters than YM-10 ultrafilters with no differences in protein content or SMAL activity. Therefore, we routinely used the larger pore ultrafilters for this purpose. The retention of SMAL by the XM-100 ultrafilters was consistent with the

Figure 8. Calibration of Fractogel TSK 55S Molecular Sieve Column. The column was equilibrated and packed in 0.05 M Tris HCl and 0.5 M NaCl pH 8 at flow rate of 19 ml/hr. The column then was calibrated with the following standards. A. Myoglobin (17,200), B. Aldolase (158,000), C. Catalase (232,000), and D. Ferritin (440,000). Column size (1.5 x 90 cm). Fractions of 2 ml were collected.

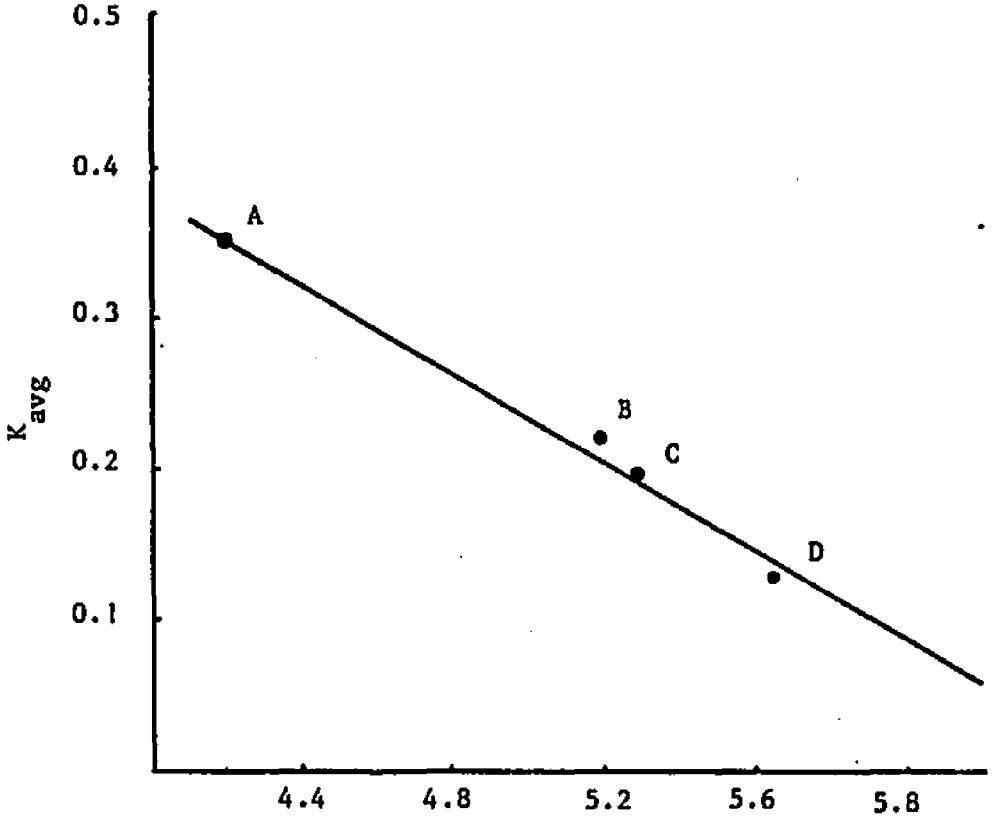

0.5
0.4
0.3
0.2
0.1
K_{avg}
A
B
C
D
4.4
4.8
5.2
5.6
5.8
Log Molecular Weight

Figure 9. Fractionation of SMAL by TSK 55S Molecular Sieve Column Chromatography. An aliquot of 1 ml of TL culture supernatant (concentrated to 100-fold on XM-100 membrane) containing 2 mg of protein was applied to the column. Fractions of 2 ml were collected, pooled as shown and concentrated on YM-10 membrane. After dialysis against RPMI-1640 they were subjected to assay for SMAL. Bars represent pooled fractions having SMAL activity.

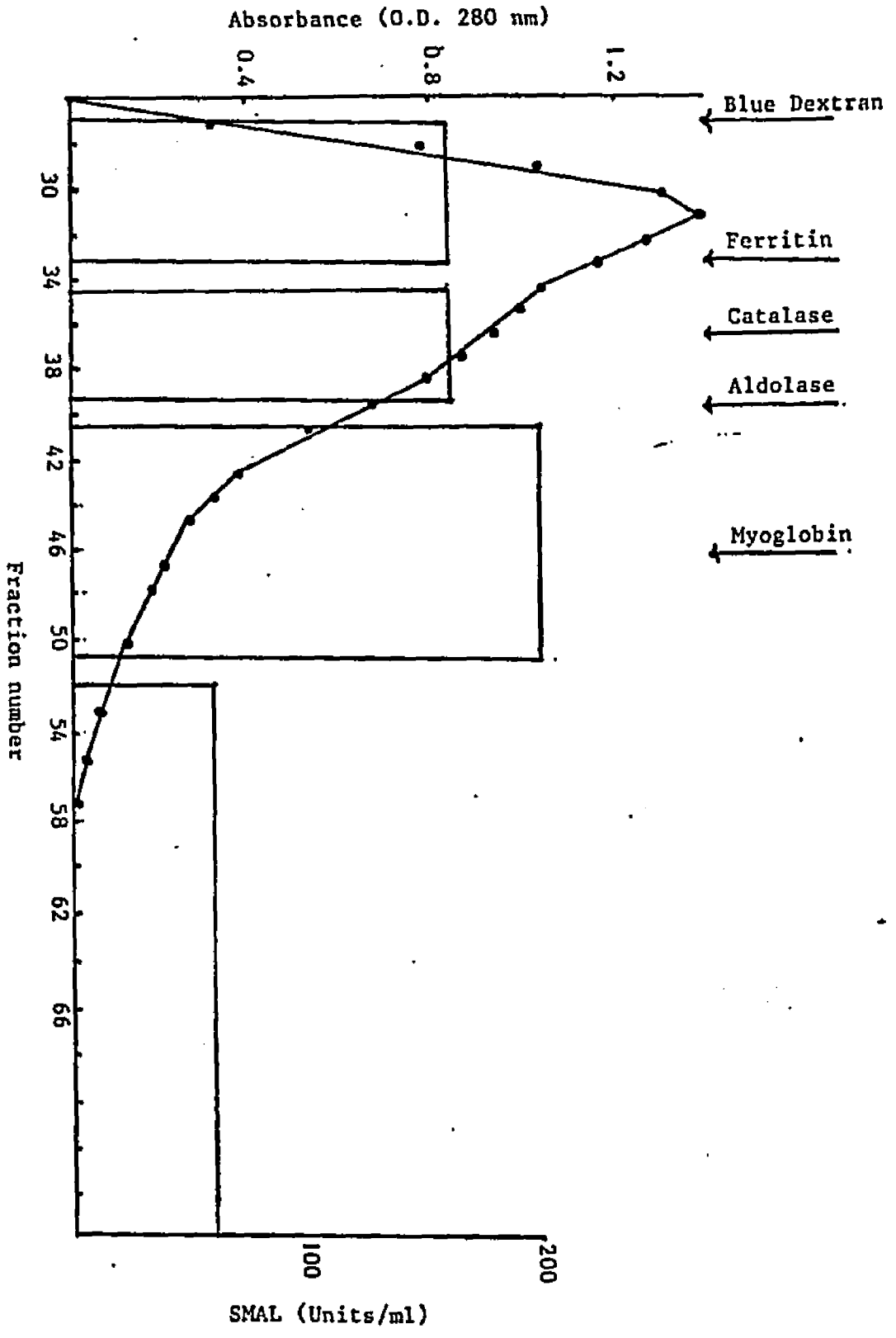
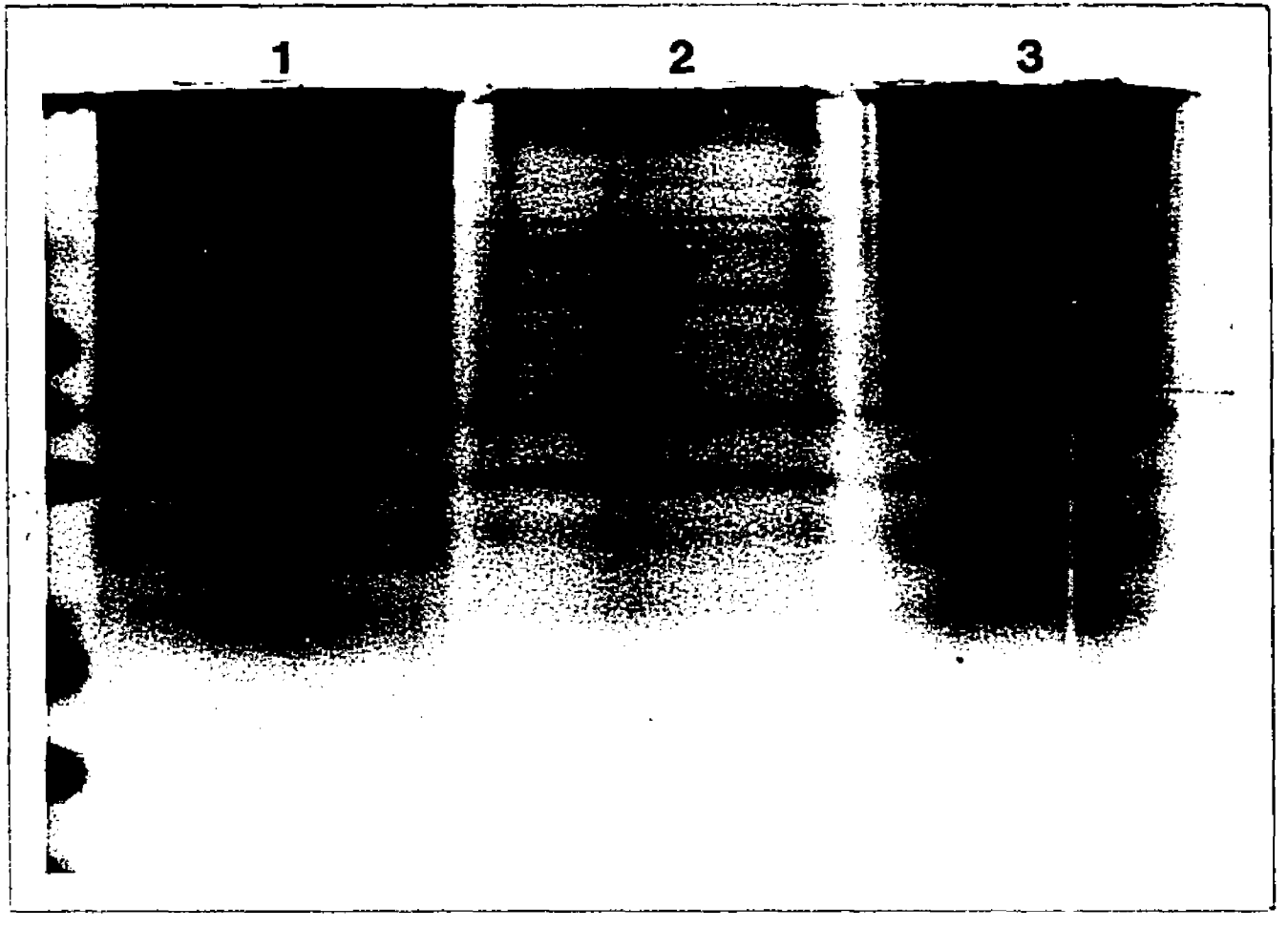

Absorbance (O.D. 280 nm)
0.4
0.8
1.2
Blue Dextran
Ferritin
Catalase
Aldolase
Myoglobin
30
34
38
42
46
50
54
58
62
66
Fraction number
100
200
SMAL (Units/ml)

Figure 10. Native Polyacrylamide Gel Electrophoresis of TL Supernatants Concentrated on YM-10, or YM-100 Diaflo Membranes. TL supernatant was concentrated by ultrafiltration using Amicon membranes YM-10 or YM-100. A portion of YM-100 retentate was treated with DNase. Fractions were subjected to 10% polyacrylamide gel electrophoresis under native conditions and stained with Coomassie blue. Lane 1, YM-10 retentate; lane 2, YM-100 retentate DNase treated; and lane 3, YM-100 retentate.


1
2
3

the data obtained by molecular sieve chromatography for a M.W. in excess of 100,000.

Purification by Anion Exchange Chromatography. YM-100 retentates were applied to a DE-52 column. The elution profile showed some material (Fig. 11, pool 1) did not adhere, but there were two optical density peaks of adhering components that were detected after their elution by salt. Fractions 3-12 were pooled, and fractions with conductivities of 2-14 mMhO and 18-30 mMhO which eluted after the salt gradient commenced were pooled as indicated on Figure 11. Neither pool 1 nor pool 2 assayed positive for SMAL. The activity of SMAL, however, was almost always associated with pool 3 and based on numerous experiments like this, SMAL activity usually eluted between 18-30 mMho. Six mg of protein containing 1120 units of SMAL in 1 ml of a YM-100 retentate of culture supernatant were loaded on the column (Fig. 11 and Table 9). Pool 3 (Fig. 11) contained 0.9 mg protein and 288 units of SMAL. Its specific activity was 320 units SMAL/mg (Table 9). Only 15% of the loaded protein eluted in pool 3, but 26% of the total activity of SMAL was recovered in this pool. This constituted 1.7-fold purification of the units of SMAL (Table 9).

The YM-100 retentate (Table 9) and pool 3 (Fig. 11) were subjected to SDS-PAGE and then stained with silver. The protein content of the retentate was very heterogeneous with stained bands which were distributed throughout the gel (Fig. 12, lane 2). Pool 3, on the other hand, was much less heterogeneous, and the smallest component was about Mr 25,000 (Fig. 12, lane 3). The most heavily stained component of both samples was about Mr 43,000. Since both samples were retentates of

Figure 11. Elution Profile of TL Culture Supernatant Fractionated on a Column of DEAE-cellulose. TL culture supernatants were concentrated on YM-100 membranes, applied to a DE-52 column and eluted with a linear gradient of salt ranging from 5 to 35 mMHO (solid line). Pooled fractions (2 ml per tube) were assayed for SMAL as described in Materials and Methods. Pool 1, fractions 3-12; pool 2, fractions 17-24; pool 3, fractions 28-38. Shaded area denotes SMAL activity per ml of pool 3 after it was reduced in volume to equal that of retentate loaded.

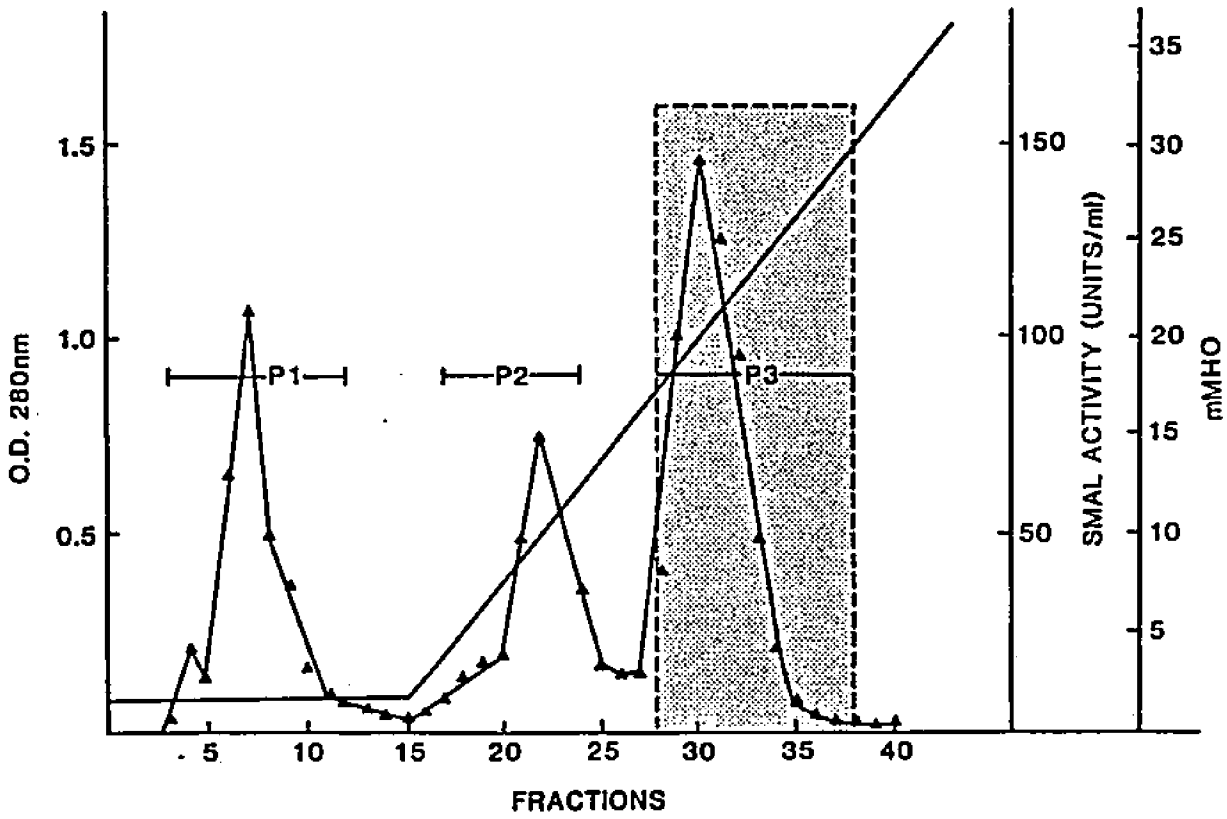

O.D. 280nm
1.5
1.0
0.5
P1
P2
P3
5
10
15
20
25
30
35
40
FRACTIONS
150
100
50
SMAL ACTIVITY (UNITS/ml)
35
30
25
20
15
10
5
mMHO

TABLE 9. Purification of SMAL by DEAE-Cellulose Ion Exchange Chromatography

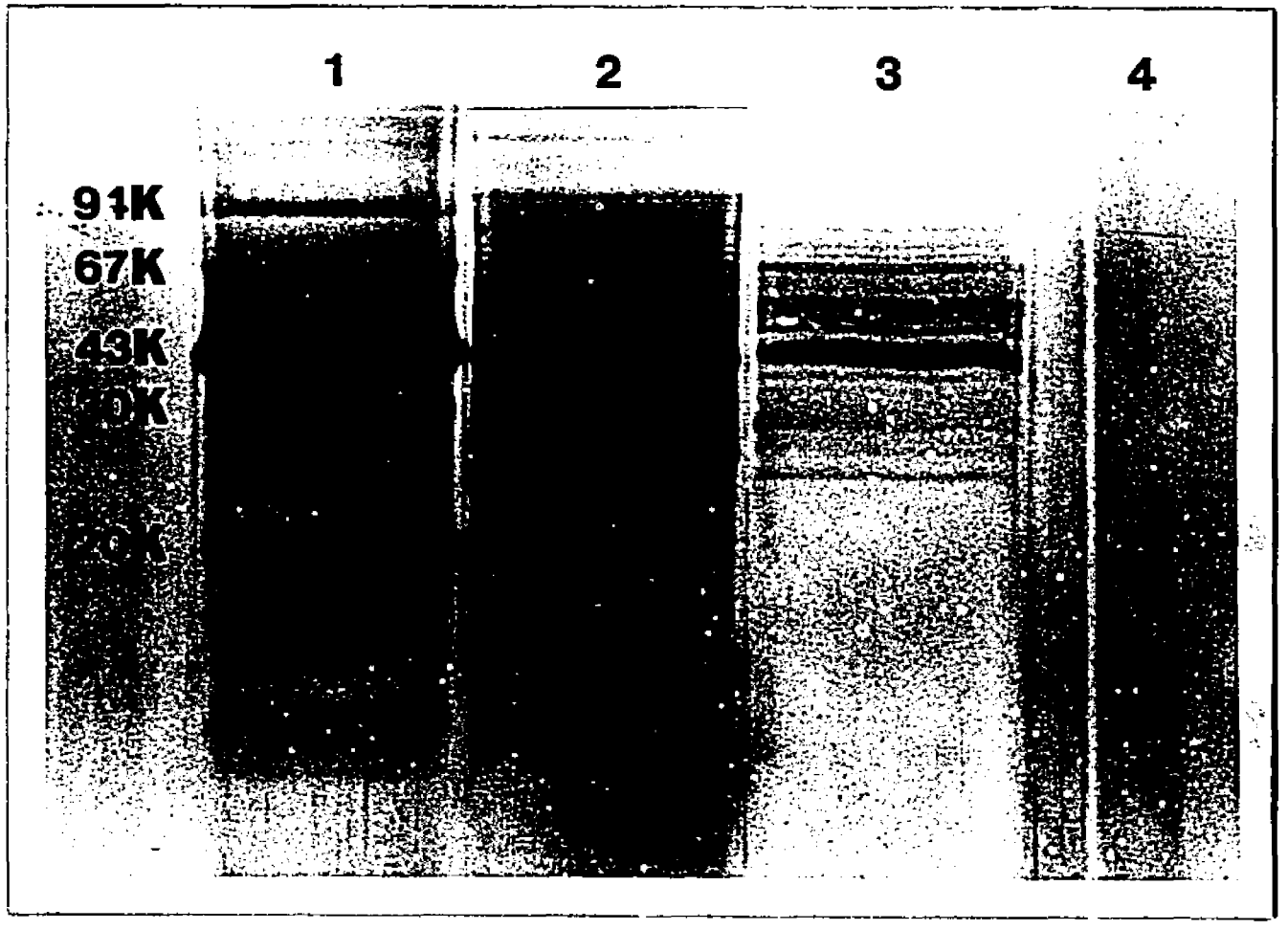
Step	Volume ml	Total Protein (mg)	Activity Units/ml	S.A. Units/mg	Total Activity Units	Purification fold	Recovery %
Crude	1330	6.65	0.9	180	1197	1	100
YM-100[a]	10	6	112	186	1120	1.03	94
DEAE-Cellulose[b]	1.8	0.936	160	308	288	1.7	24

[a] Tonsil lymphocytes culture supernatant was concentrated 133-fold by ultrafiltration using a YM-100 membrane.

[b] Pool 3 from DEAE-cellulose anion exchange column.

S.A. Specific Activity

Figure 12. SDS-PAGE Analysis of a YM-100 Retentate and Pool 3 from Anion Exchange Column. 50 µl of 130-fold concentrated YM-100 retentate (30 µg total proteins) and 50 µl (30 µg) of pool 3 (Fig. 11) were loaded in lanes 2 and 3, respectively, electrophoresed and stained with silver. Lane 1 contained M.W. markers whose weights are indicated in the figure. Lane 4 was loaded with buffer.


1
2
3
4
91K
67K
43K

an ultrafilter with a Mr 100,000 exclusion, it was likely that in the absence of denaturing substances, the proteins were polymerized.

The absorbance spectra of the three pools (cf. Fig. 11) was obtained. Pool 1 had an absorption maximum at 280 nm, but it was a broad absorbance extending down to about 262 nm (Fig. 13). Pool 2 absorbed relatively strongly at about 275 nm but its absorbance range also was broad. Pool 3 had an absorbance maximum at 258 nm, and it also absorbed strongly at 280 nm. These data indicated the presence of both proteins and nucleic acids in pool 3 and in the XM-100 retentates of cultured supernatants.

Detection of DNA in the Tonsil Lymphocyte Culture Supernatant by Ethidium Bromide Staining. The high absorbance at 260 nm of crude TL culture supernatant and pool 3 from DEAE-cellulose column indicated the presence of nucleic acid in these preparations (Fig. 13).

It was necessary to determine the nature of 260 nm-absorbing material in the concentrated crude TL culture supernatant. To do so, the effects of various chemical and enzymatic treatments were investigated. As shown in Figure 14, the nucleic acid stainable with EtBr was sensitive to 0.45 M HCl, DNase, micrococcal nuclease, and TCA (lanes 3, 5, 6 and 8) but insensitive to RNase, and NaOH hydrolysis (Fig. 14, lanes 4, and 7), respectively. These results indicated that XM-100 retentates of crude TL culture supernatants contained DNA.

It was also necessary to find out if DNA per se had any suppressive activity on the uptake of ^{3}H-tdr by EL-4 cells. Calf thymus DNA (up to 2 mg/ml) was added to EL-4 cells for 24 hr after which the cells were assayed for ^{3}H-tdr uptake. No inhibition of ^{3}H-tdr uptake was noted.

Figure 13. Absorbance Spectra of Pooled Fractions. Pools 1, 2, and 3 from a typical ion exchange column fractionation of a YM-100 retentate of TL culture supernatant (Fig. 11) were scanned for their absorbance of light between 240 nm and 300 nm.

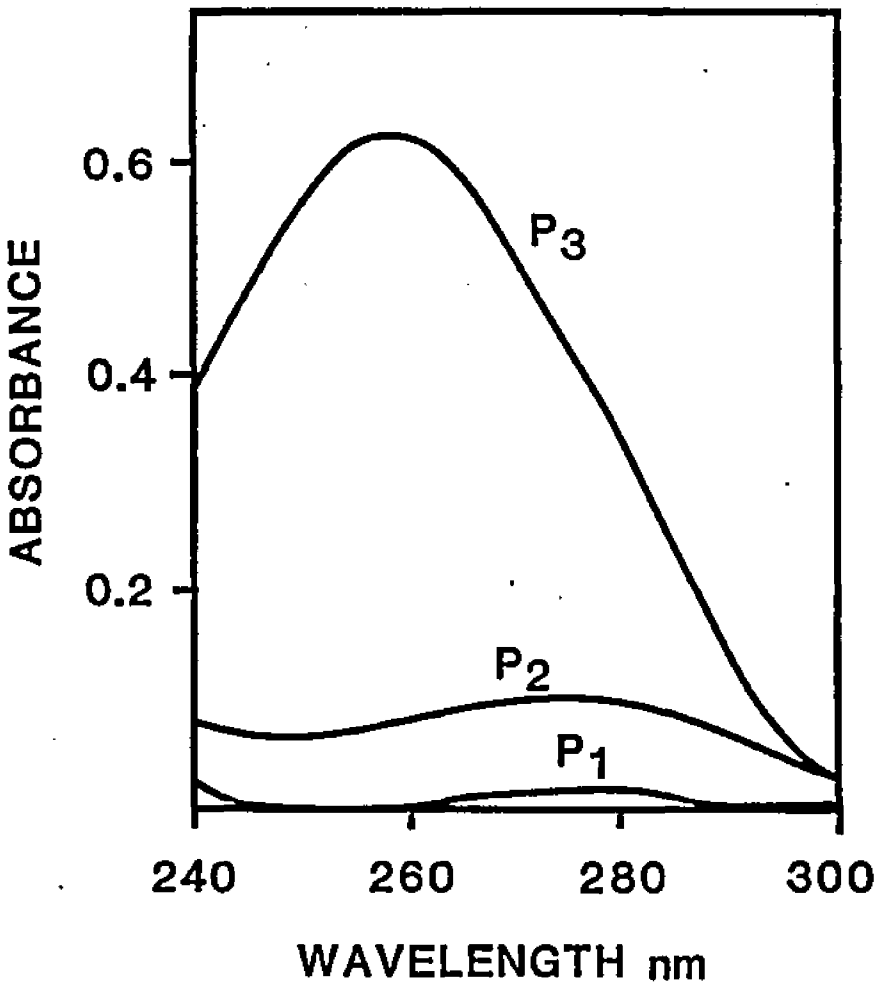
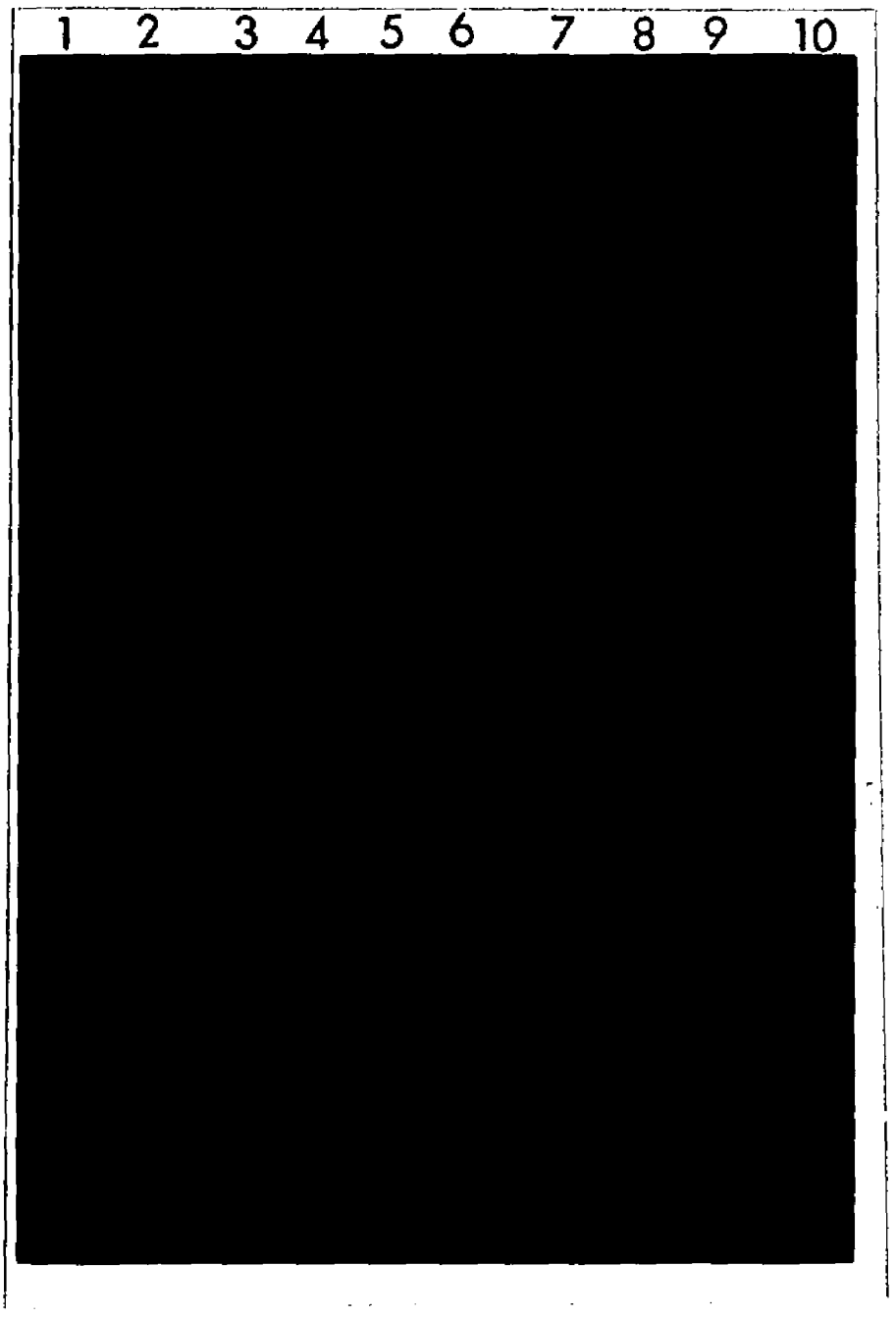

ABSORBANCE
0.6
0.4
0.2
P3
P2
P1
240
260
280
300
WAVELENGTH nm

Figure 14. Presence of DNA in the TL Culture Supernatant. XM-100 retentate of TL culture supernatant was treated with DNase, RNase, TCA, HCl, NaOH, and micrococcal nuclease (lanes 3-8) and after dialysis was subjected to agarose gel electrophoresis. The gel was stained with ethidium bromide and excited with fluorescent light for visualization of stained material. Lane 1 contained sample buffer, lane 2 contained untreated XM-100 retentate, lanes 3 through 8 contained XM-100 retentate treated with HCl, RNase, DNase, micrococcal nuclease NAOH, and TCA respectively, lane 9 and 10 contained standard DNA plasmic (PBR) and yeast tRNA, respectively.


1 2 3 4 5 6 7 8 9 10

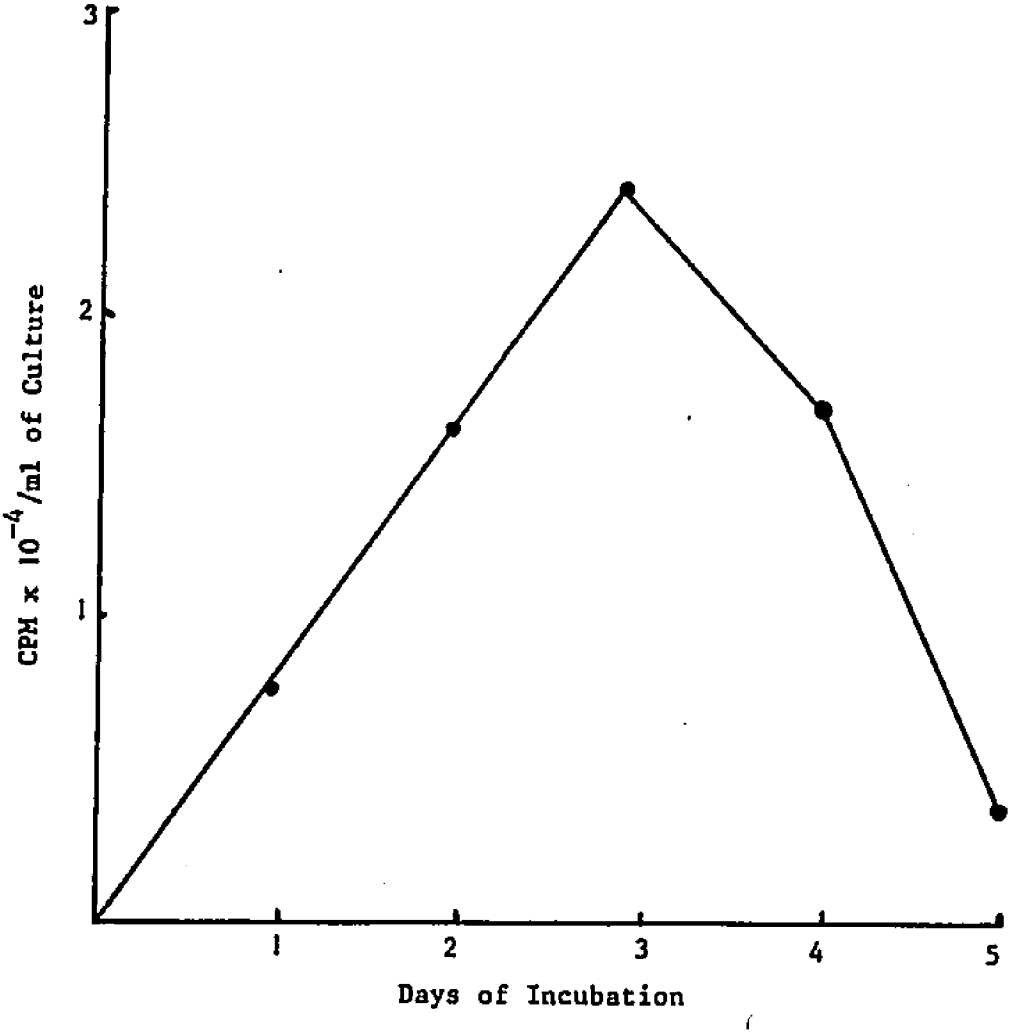
These data indicated that purified calf thymus DNA even at high concentration did not inhibit the uptake of ^{3}H-tdr by EL-4 cells.

Digestion of an XM-100 retentate with pronase caused complete loss of SMAL activity, but treatment with ribonuclease had no effect. Deoxyribonuclease treatment, however, significantly increased the suppressive activity (Table 6). This observation and the spectral absorbance data suggested SMAL might be associated with DNA.

Kinetics of Appearance of DNA in the Culture Supernatant of Tonsil Lymphocytes. Tonsil lymphocytes were cultured in the presence of ^{3}H-tdr for 24 hr. The supernatant was collected and the cells were washed and placed in fresh medium in the absence of ^{3}H-tdr and incubated up to 5 days with changing the medium every 24 hr. Figure 15 shows the appearance of TCA-precipitable ^{3}H-tdr in the culture supernatant of tonsil lymphocytes as a function of time. The maximum amount of newly synthesized DNA was released into the culture fluid on day 3 of culture and subsequently reduced by day 4 and 5 of culturing.

^{3}H-tdr-Labeled DNA from TL Culture Fluid was not Taken up by EL-4 Cells. Since tonsil lymphocyte culture supernatants contained substantial amounts of DNA, it was possible that during the incubation of these supernatants with EL-4 cells the DNA was hydrolyzed and its nucleotides were converted to nucleosides by phosphatases. The presence of unlabeled tdr drived from DNA, therefore, could have resulted in a substantial reduction in the specific radioactivity of the labeled ^{3}H-tdr used in the bioassay for SMAL, and the resulting data would have been interpreted as indicating inhibition of ^{3}H-tdr incorporation.

Figure 15. Kinetics of Appearance of Newly Synthesized DNA in the Culture Supernatant of TL. TL were cultured in the presence of 4 μCi/ml of ^{3}H-tdr for 24 hr. Culture supernatant was collected and the cells were washed and resuspended in fresh culture medium free of ^{3}H-tdr. Cells were incubated up to 5 days, with changing the culture medium every 24 hr. A portion of each culture supernatant was precipitated by 10% TCA, collected on GF/C filter paper and the amount of radioactivity incorporated to DNA was measured by a liquid scintillation counter.


3
2
1
CPM x 10^{-4}/ml of Culture
1
2
3
4
5
Days of Incubation

In order to investigate this possibility, tonsil lymphocytes were cultured in the presence of ^{3}H-tdr for 24 hr. The culture supernatant was collected and tested for TCA-precipitable DNA (Fig. 15). This supernatant was then concentrated to 40-fold and washed with TL medium on a Diaflo ultrafiltration membrane (XM-100). This ^{3}H-tdr labeled TL Supernatant was added to EL-4 cells for 24 hr and the cells were tested for the uptake of ^{3}H-tdr derived from labeled DNA. The results in Table 10 demonstrated that EL-4 cells did not take up any of the ^{3}H-tdr present in the labeled DNA.

Preparative Polyacrylamide Gel Electrophoresis of XM-100 Retentate Culture Supernatant. In order to determine which component separated by native polyacrylamide gel electrophoresis (Fig. 10) was responsible for the bioactivity of SMAL, concentrated (130-fold) tonsil lymphocyte culture supernatants treated with DNase were subjected to gel electrophoresis under native conditions. A total of 1.1 ml of the concentrated TL culture supernatant was applied to 11 polyacrylamide tube gels (0.1 ml/tube). After electrophoresis one of the gels was stained with Coomassie blue and each of the other 10 were cut into 1-cm long pieces. Corresponding pieces were pooled, crushed to very small sizes (using a fixed 80-mesh screen) and eluted in PBS at 4°C overnight. Each fraction corresponding to a specific segment of the gels was then dialyzed, concentrated to 1 ml and subjected to bioassay for SMAL activity. There was no demonstrable biological activity in any of the fractions. This experiment was repeated, but replacing the tube gel with a vertical slab gel. After electrophoresis of TL supernatant the gel was electrobloted to a sheet of DEAE-cellulose paper.

TABLE 10. EL-4 Cells did not take up ^{3}H-tdr Labeled DNA Released by Tonsil Lymphocytes

	Mean CPM ± S.D.	
	^{3}H-tdr-labeled TL culture fluid (XM100 Retentate)	
Experiment No.	Control[a]	Experimental[b]
I	96 ± 19	70 ± 35
II	63 ± 17	72 ± 15

[a] Consisted of ^{3}H-tdr-labeled tonsil lymphocyte culture fluid incubated in the absence of EL-4 cells.

[b] Consisted of ^{3}H-tdr-labeled tonsil lymphocyte culture fluid (6,000 TCA-precipitable cpm) in the presence of 0.2 ml of EL-4 cells at 4 x 10^{5} cells/ml.

The DEAE-cellulose paper then was cut into 6 pieces corresponding to 1 cm-length segments of the gel and each was placed into a beaker containing 1 M NaCl to elute the bound material. Each fraction then was dialyzed, concentrated by ultrafiltration and subjected to bioassay for suppressor activity. No suppressor activity could be recovered from these fractions when EL-4 cells were used as target cells. However, when CEM-CM_3 cell line were used as target cells, two regions of activity were recovered corresponding to 2 cm and 5.5 cm gel slices (Fig. 16B). As a background control, an identical gel was loaded with sample buffer and treated as described for the experimental gel. As can be seen in Figure 16A, there was no detectable suppressive activity associated with any of the control gel slices.

Purification of SMAL by HPLC. XM-100 retentates of TL culture supernatants treated with DNase were chromatographed on a QMA-Accell column attached to HPLC equipment. A typical elution profile is shown in Figure 17A. Some material did not adhere to the column. Two peaks of protein eluted in the salt gradient with retention times of 56.89 min and 70.26 min. Fractions were pooled as indicated on Figure 17A, dialyzed, and washed on an YM-10 ultrafilter. SMAL was associated only with the pooled fractions with retention time of 65 to 90 min. The pool was chromatographed again on the QMA-Accell column and the elution profile is shown in Figure 17B. Almost all the protein eluted in the salt gradient with a retention time of 70.45 min. This material was collected as shown on Figure 17B, and concentrated on a YM-10 ultrafilter. It comprised 0.16 mg protein and 320 units of purified SMAL with a specific activity of 2000 units/mg (Table 11).

Figure 16. Preparative Native Polyacrylamide Gel Electrophoresis of SMAL from YM-100 Retentate Treated with DNase. TL culture supernatant was concentrated on YM-100 membrane treated with DNase dialyzed and subjected to 10% polyacrylamide gel electrophoresis under native condition. The gel then was electroblotted to a sheet of DEAE-cellulose paper. The DEAE-cellulose paper was cut to 1 cm pieces. The materials bound to this paper were eluted with 1 M NaCl. Each fraction was dialyzed against PBS then TL medium and subjected to bioassay using CEM-CM_3 human T cell line as target cell. A. is control gel loaded with buffer and B. is experimental gel loaded with SMAL.

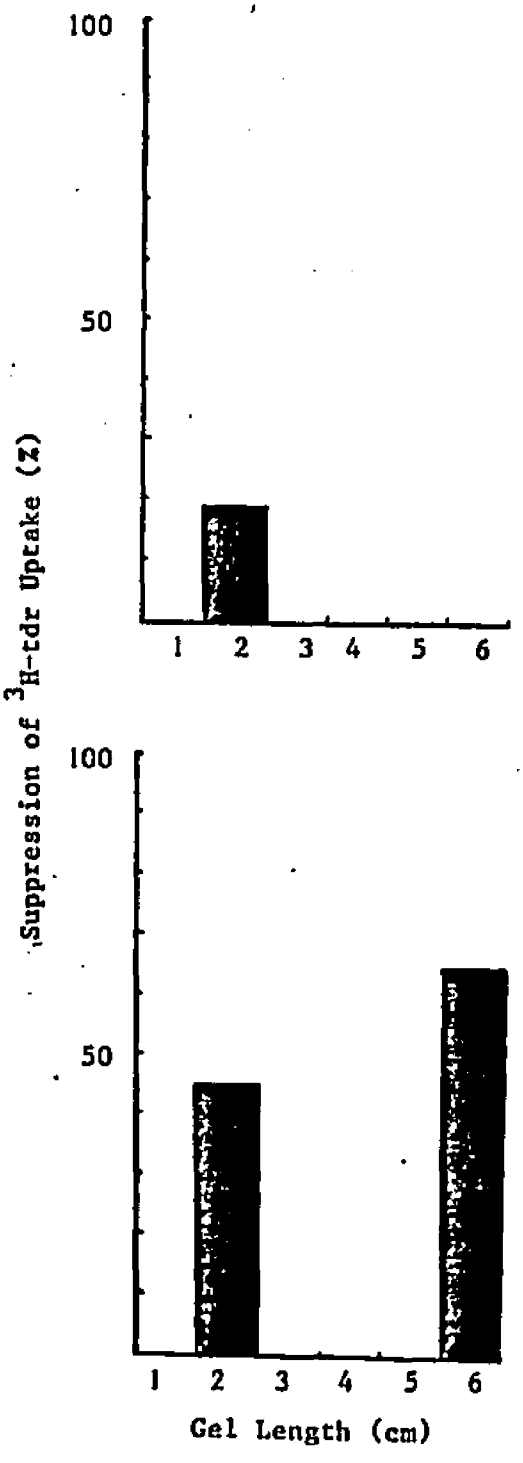

A
100
50
1 2 3 4 5 6
B
100
50
1 2 3 4 5 6
Suppression of ^{3}H-tdr Uptake (%)
Gel Length (cm)

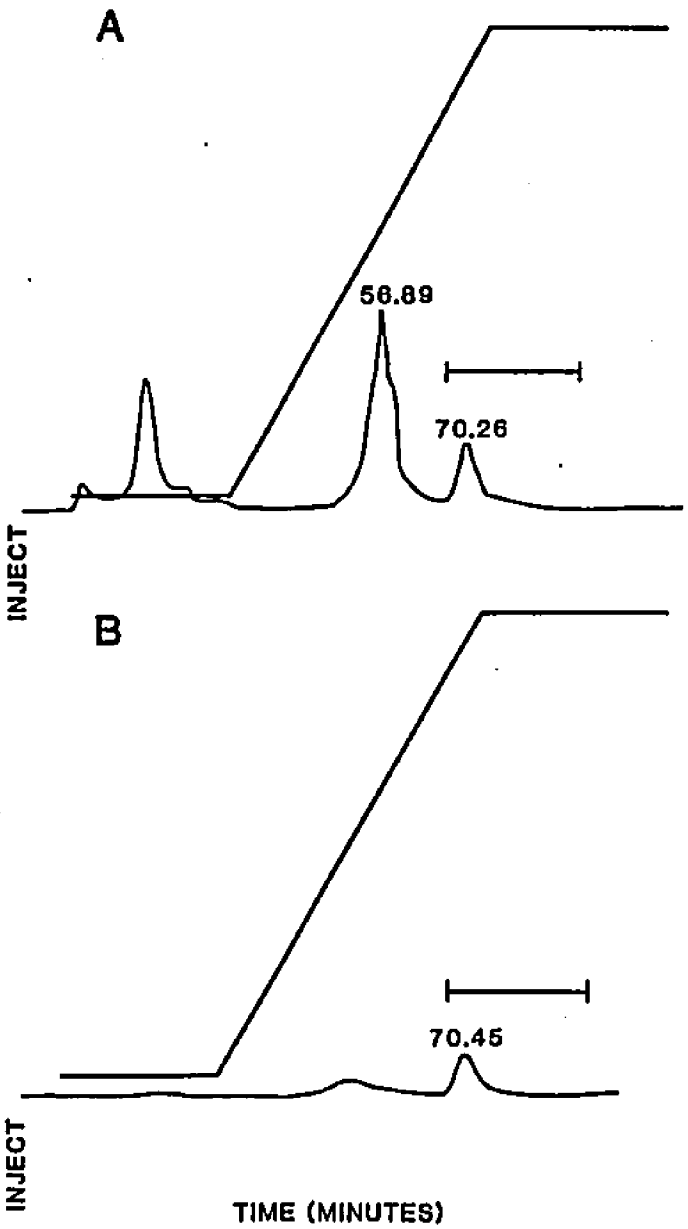
TABLE 11. Purification of SMAL by QMA-Accell Anion Exchange Column Attached to HPLC Equipment

Purification Step	Total Volume ml	Total Protein mg	Total Activity units	S.A.[a] units/mg	Purification fold	Recovery %
XM-100	8	8	3200	400	1	100
QMA-Accell[b] HPLC	16	0.16	320	2000	5	10

[a] Specific activity.

[b] Pool 3 from QMA-Accell column (Fig. 17B).

Figure 17. Purification of SMAL by HPLC. A QMA-accell anion exchange column was operated with HPLC equipment. A. sample chromatographed was an XM-100 retentate of TL culture supernatant treated with deoxyribonuclease. Fractions indicated on the figure (17A) were pooled and concentrated on a YM-10 membrane. B. The pool (above) was rechromatographed under the same conditions, fractions were pooled as indicated (⊢——⊣) and assayed for SMAL activity against EL-4 cells. The numbers are retention time. The salt gradient is described by the rising solid line. Peaks are absorption at 280 nm.


A
56.89
70.26
INJECT
B
70.45
INJECT
TIME (MINUTES)

The purification was 5-fold. Although 98% of the protein originally in the culture supernatant was removed, 10% of the activity was recovered (Table 11). This highly purified SMAL did not bind ethidium bromide (Fig. 18, lane 4) and had no peak of absorbance at 258 nm.

Samples of SMAL recovered from the first HPLC purification (Fig. 17A) and purified SMAL from the second passage (Fig. 17B) were subjected to SDS-PAGE and the gels were stained with silver. SMAL from several preparations of the first purification comprised 4-8 components (Fig. 19, lane 1) and purified SMAL from several preparations exhibited only 3-4 bands (Fig. 19, lane 2). In both samples of SMAL, the most heavily stained components were Mr 43,000, and 30,000. The smallest component we detected was the Mr 30,000 one. It was not possible to purify SMAL to homogeneity or to determine which component was responsible for the suppression of the incorporation of ^{3}H-tdr by EL-4 cells.

Modes of Action of SMAL. In the experiment reported in Table 12, the incorporation of ^{3}H-tdr by EL-4 cells in the presence of XM-100 retentate of TL supernatant (SMAL) was inhibited by 87%. Since the viabilities of these and the control cells was 98%, SMAL appeared to inhibit the incorporation of ^{3}H-tdr into EL-4 cells by a noncytotoxic mechanism. When the numbers of cells in the control and experimental cultures were determined, there was no noticeable difference (Table 12).

Distribution of Nuclear DNA in SMAL-treated Cells. EL-4 cells were cultured in the presence of XM-100 retentate of TL culture supernatant (SMAL) for 24 hr. At the end of this time period, the cells will be

Figure 18. Absence of DNA in SMAL Purified by HPLC. Samples of YM-100 retentate of culture supernatant (lane 1), YM-100 retentate treated with DNase (lane 2), pool 3 from DEAE-cellulose column (Fig. 11) (lane 3), and SMAL purified by HPLC (lane 4) were subjected to 10% polyacrylamide gel electrophoresis. The gel was then stained with Ethidium bromide and exited with fluorescent light for visualization of stained materials (lane 5 is calf thymus DNA).

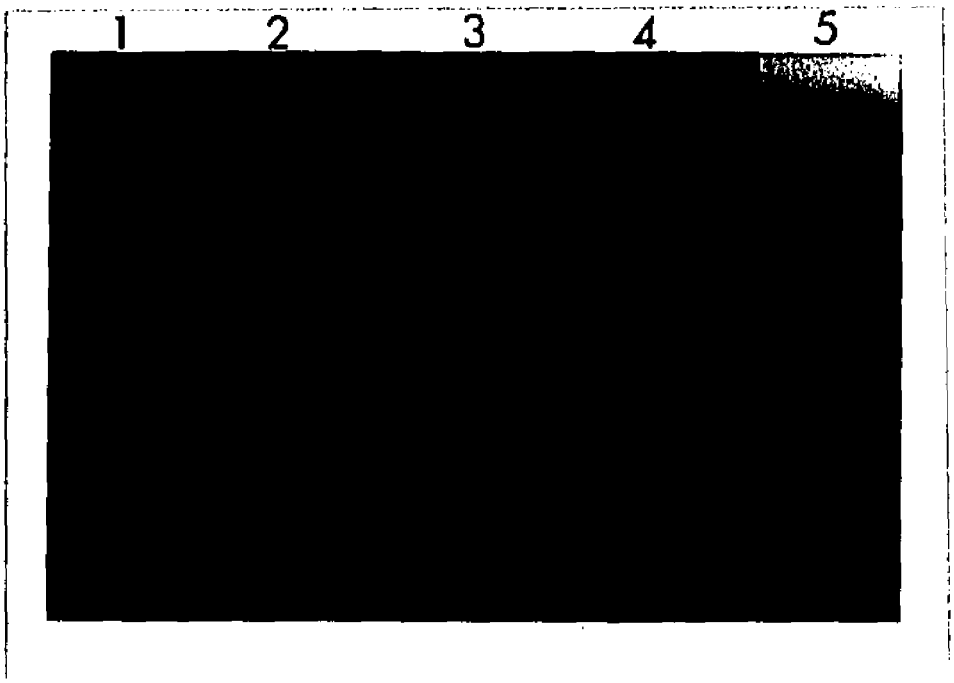

1
2
3
4
5

Figure 19. SDS-PAGE Analysis of Pooled Fractions from HPLC. A YM-100 retentate treated with DNase was chromatographed on an anion exchange column (QMA-Accell) attached to the HPLC system. Pooled fractions from the first and the second purifications by HPLC were analyzed by SDS-PAGE followed by staining with silver. Lane 1, pooled fractions from the first purification (Fig. 17A). Lane 2, purified SMAL from the second purification by HPLC (Fig. 17B). The positions of M.W. markers are on the left. Lane 3 was loaded with buffers only.

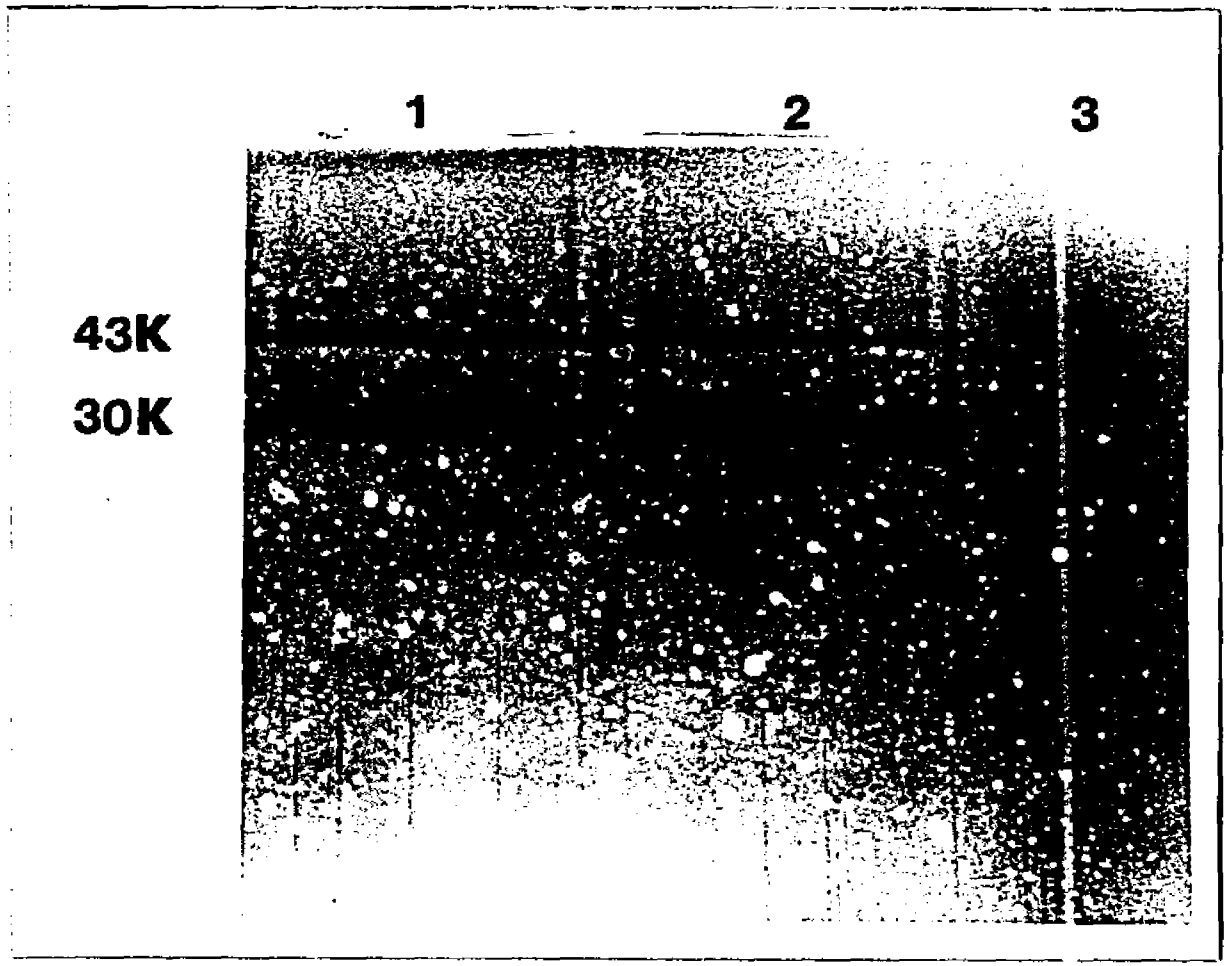

1
2
3
43K
30K

TABLE 12. Effect of SMAL on the Viability and Proliferation of EL-4 Cells

Exp. No.	Mean Cell Number x 10^{-5} ± SD		Mean CPM x 10^{-3} ± SD	
	Control	Experimental	Control	Experimental
I	8.0 ± 0.3	7.9 ± 0.15 (98)[a]	229 ± 39	31 ± 5 (87)[b]
II	8.3 ± 0.58	7.2 ± 0.5 (98)	226 ± 24	105 ± 3 (60)
III	8.0 ± 0.35	8.0 ± 0.2 (90)	110 ± 8	65 ± 4 (40)

[a] Percent viability of EL-4 cells as determined by Trypan Blue dye exclusion.

[b] Percent suppression of ^{3}H-tdr incorporation.

Cell number was determined by counting the cells in a hemocytometer.

highly suppressed for their incorporation of ^{3}H-tdr given as a 2-hr pulse. The cells were stained with the Feulgen reagent for DNA and the levels of DNA in individual nuclei were determined. There was no difference in the distribution of nuclear DNA values between control and SMAL-treated cells (Fig. 20). The presence of interclass DNA values (Fig. 20B) suggested that DNA synthesis per se was not inhibited by SMAL. The data also showed that some cells contained amounts of DNA that were roughly even geometric multiples of the 2c and 4c nuclear DNA class levels, indicating the presence of cells with hyper tetraploid and even octaploid nuclei. Also, metaphase configurations were seen that contained 80 or more chromosomes rather than the diploid number of 40-42 chromosomes expected for EL-4 cells (Gehring et al., 1985).

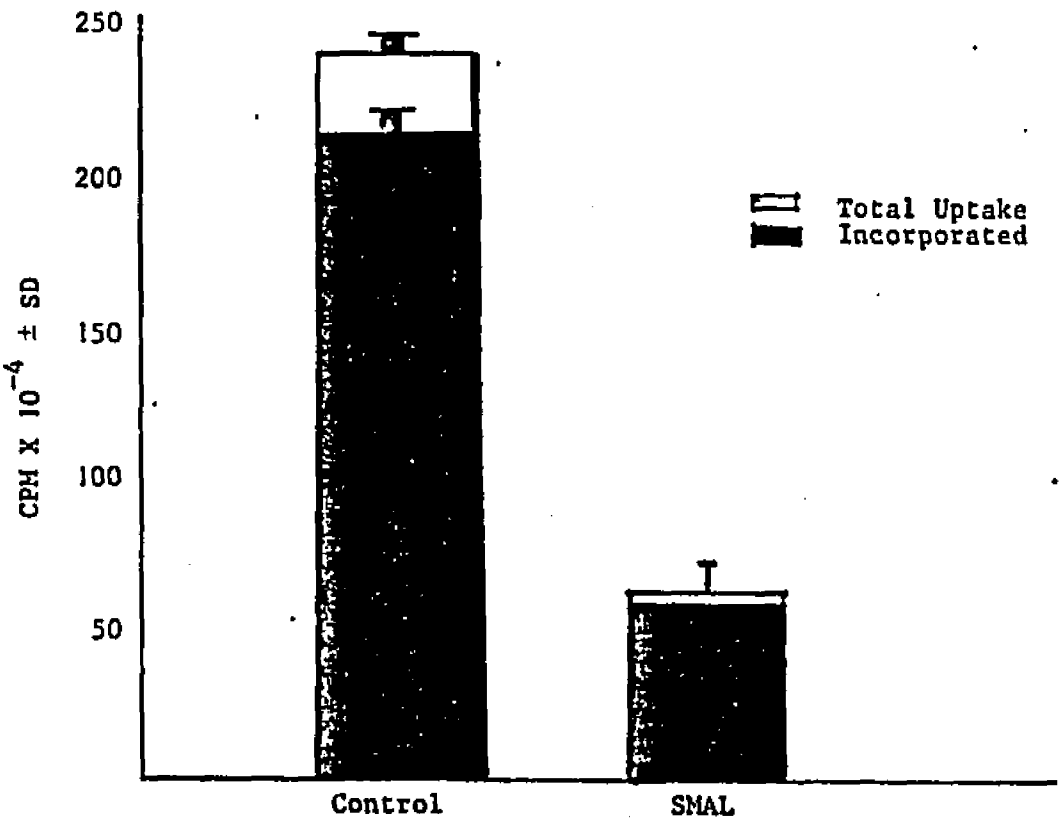
SMAL Inhibits the Uptake of ^{3}H-tdr. Actively growing EL-4 cells were cultured for 24 hr in the presence of XM-100 retentate of TL supernatant containing 24 units SMAL/ml or in the absence of TL supernatant. The cell numbers were determined and found to be identical for both populations. During this period the cell density increased from 3 x 10^{5}/ml to 8 x 10^{5}/ml. The cultures were pulsed with ^{3}H-tdr for 2 hr, and after washing the cells the radioactivity in the TCA-soluble and TCA-precipitable fractions was determined. As depicted in Figure 21, the total uptake of ^{3}H-tdr by SMAL-treated cells was much less (74%) than that of the control cells. The ratio of ^{3}H-tdr incorporation into TCA-precipitable material to total uptake was, however, similar for the control and experimental samples.

The extent of suppression of ^{3}H-tdr incorporation in the presence of increasing concentrations of ^{3}H-tdr was determined. A double

Figure 20. DNA Levels in Nuclei from Control (A) and SMAL-treated (B) EL-4 Cells. EL-4 cells were cultured in the presence or absence of XM-100 retentate of TL culture supernatant (SMAL) for 24 hr. Cells were then fixed with methanol, acetic acid (3:1 v/v) and microscope slides carrying air-dried droplets of fixed cells were stained with the Feulgen reagent for DNA. The amount of DNA-Feulgen staining per nucleus was measured by using a Vickers M-86 scanning and integrating microdensitometer.


A
B
Frequency
Log 10 DNA Feulgen per Nucleus

Figure 21. Determination of Total Uptake and Incorporated ^{3}H-tdr by SMAL-treated EL-4 Cells. Actively growing EL-4 cells were cultured in the presence or absence of SMAL (XM-100 retentate of TL culture supernatant) for 24 hr and pulsed with ^{3}H-tdr for an additional 2 hr. The radioactivity incorporated into TCA insoluble materials and total uptake of ^{3}H-tdr was determined as described in the Materials and Methods.



The Uptake and Incorporation of ^{3}H-Thymidine into EL-4 Cells in the Presence and Absence of SMAL

reciprocal plot of the data in which 1/[S] is the reciprocal of the ^{3}H-tdr concentration and 1/V is the reciprocal of the amount of ^{3}H-tdr that was incorporated (p mol per 2 hr) at increasing concentrations of ^{3}H-tdr up to 6 μM, is shown as Figure 22. There was no change in the extent of suppression of ^{3}H-tdr incorporation by SMAL, indicating that SMAL did not directly compete with the nucleoside.

The Affect of SMAL (XM-100 Retentate of TL Culture Supernatant) on EL-4 Cells was Reversible. The possibility of adsorption of SMAL to EL-4 cells was investigated by measuring the loss of subsequent suppressive activity in supernatants from cells cultured in known amounts of SMAL. It was observed that 50% of the original activity was removed by 6 x 10^{5} cells cultured with either 80 or 700 units/ml of SMAL for 24 hr (Table 13).

It was then of interest to determine if the suppression of incorporation of ^{3}H-tdr was permanent. Cells were cultured for 22 hr in the presence of SMAL, then washed, placed in fresh medium, and assayed for their incorporation of ^{3}H-tdr. The results are given in Table 14. More than 50% of the original suppression remained when these cells were cultured for the last 2 hr in the absence of SMAL. Cells were also cultured for 24 hr in the presence of SMAL followed by 24 hr in its absence. These cells no longer were suppressed (Table 14).

The reversibility of the effect of SMAL was also demonstrated by a different approach. Cells were cultured with SMAL for 24 hr followed by a 1 hr pulse with ^{3}H-tdr. The cells were then washed and cultured in fresh medium. At different time intervals, aliquots of cell suspension were removed, and slides were prepared for autoradiography. As shown in

Figure 22. Effect of Increasing Concentration of ^{3}H-tdr on the Incorporation of this Nucleoside by EL-4 Cells in the Presence or Absence of SMAL (XM-100 retentate of TL culture supernatant). EL-4 cells were cultured in the presence or absence of XM-100 retentate of TL culture supernatant for 24 hr and pulsed with different concentrations of ^{3}H-tdr. After an additional 2 hr of incubation, the incorporation of ^{3}H-tdr was measured with a liquid scintillation counter. The figure is a double reciprocal plot of data 1/s is μM concentration of ^{3}H-tdr, 1/v is the amount of thymidine (p mol) incorporated to 1 x 10^{5} cells in 2 hr of pulse.

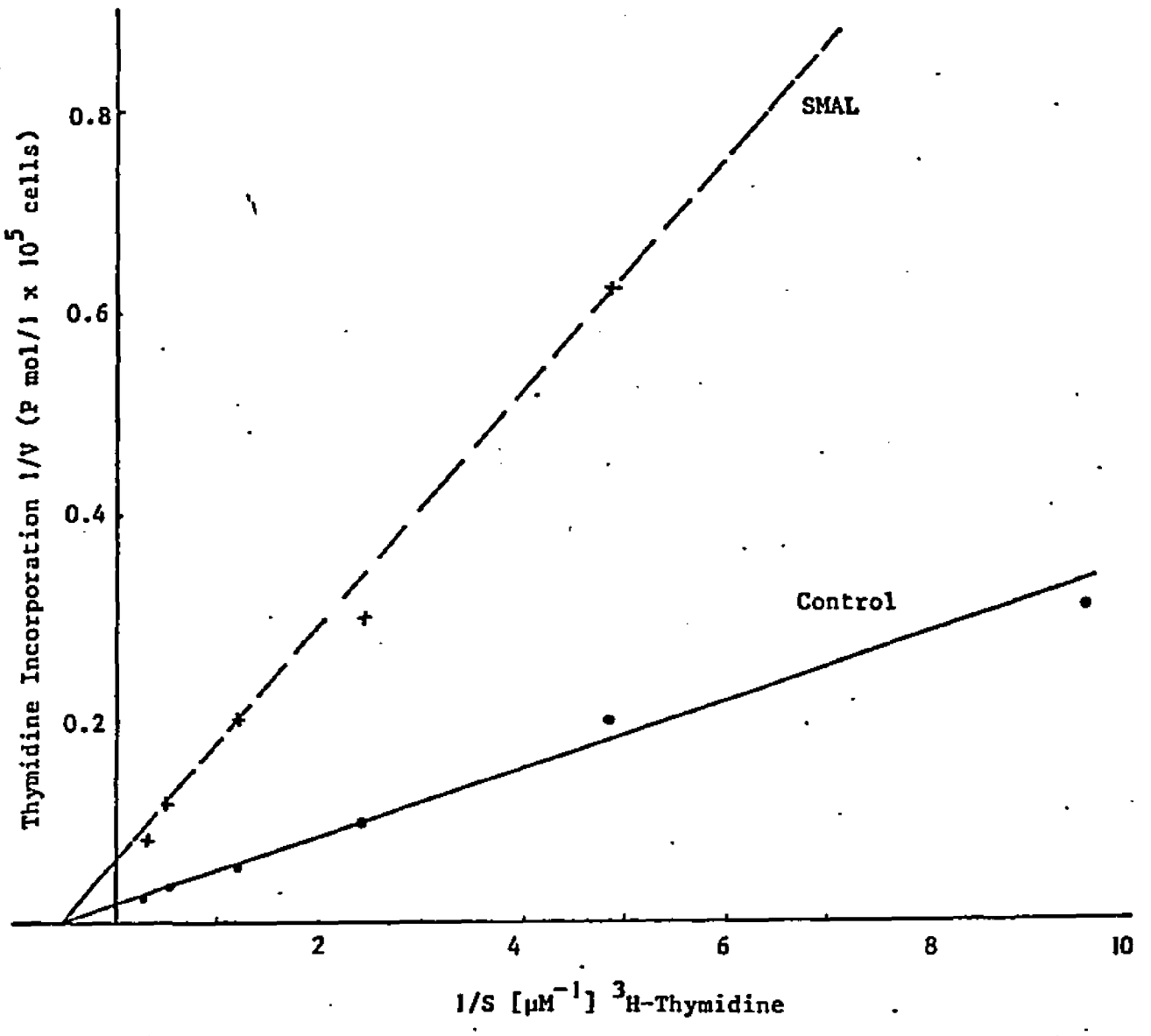

SMAL
Control
0.8
0.6
0.4
0.2
2
4
6
8
10
Thymidine Incorporation 1/V (p mol/l x 10^5 cells)
1/S [μM^{-1}] ^{3}H-Thymidine

TABLE 13. Removal of Bioactivity of SMAL
(XM-100 retentate of TL culture supernatant)
Incubated with EL-4 Cells

Experiment No.	SMAL Concentration (units/ml)	
	Control[a]	Experimental[b] (% loss)
I	80	40 (50)
II	700	350 (50)

[a] SMAL was incubated in the absence of EL-4 cells for 24 hr.

[b] SMAL was incubated in the presence of 6×10^5 EL-4 cells/ml for 24 hr. The supernatants then were collected and assayed for SMAL activity as described in Materials and Methods.

TABLE 14. Reversibility of Suppression of Incorporation of ^{3}H-tdr by EL-4 Cells Treated with SMAL

Culture Time		Suppression
SMAL Present[a] (hr)	SMAL Absent[b] (hr)	(% ± SD)[e]
24[c]	0	100
22	2	60 ± 19
48[d]	0	100
24	24	6 ± 9

[a] 4 x 10^{5} EL-4 cells were cultured in 1 ml of XM-100 retentate (36-fold).

[b] Cells were sedimented at 400 x g for 10 min., washed 3 times with 5 ml growth medium, and resuspended to 1 ml in growth medium containing 10% FBS. ^{3}H-Tdr (4 µCi) was added immediately, and the cells were cultured for the indicated time.

[c] ^{3}H-Tdr (4 µCi) was added after 22 hr, and cultured continued for 2 more hr.

[d] ^{3}H-Tdr (4 µCi) was added after 46 hr, and culture continued for 2 more hr.

[e] Data represent three experiments.

Table 15, at zero time, cells treated with SMAL had less than 0.1% labeled nuclei, whereas 56% of the untreated cells had heavily labeled nuclei (Fig. 23). After 4 hr of incubation in the absence of SMAL and ^{3}H-tdr, there was a considerable increase in the percentage of labeled nuclei of treated cells (12.4%). It was concluded that in the presence of SMAL the uptake of ^{3}H-tdr by the cells was impaired, and the incorporation of ^{3}H-tdr into nuclear DNA of the ^{3}H-tdr which did get into the cells was inhibited. When SMAL was removed, however, the cells recovered their ability to incorporate into the nuclei that portion of ^{3}H-tdr inside them. This was demonstrable by the rise in the number of labeled nuclei.

Differential Inhibition of Nucleoside Uptake and Incorporation by SMAL-treated EL-4 Cells. The effect of SMAL (XM-100 retentate of TL culture supernatant) on the incorporation of different nucleosides was investigated. Cells were cultured in the presence or absence of SMAL for 24 hr. They were then pulsed with ^{3}H-tdr, ^{3}H-adr or ^{3}H-udr and incorporation was determined at different time intervals (Fig. 24). Labeling of the cells with ^{3}H-tdr was strongly inhibited (90%) in the presence of SMAL at the end of 6 hr pulse. The extent of inhibition for the other two nucleosides was much less pronounced, being 30% for ^{3}H-adr and 19% for ^{3}H-udr.

Resistance of SMAL-Treated EL-4 Cells to 5-Fluorodeoxyuridine. 5-Fudr is a cytotoxic analog of thymidine. Once inside the cell, it is phosphorylated to 5-Fudr-monophosphate by the enzyme thymidine kinase. It is the phosphorylated form of 5-Fudr that is cytotoxic because it

TABLE 15. Determination of Labeling Indicies of EL-4 Cell Nuclei Treated with SMAL

	% of Labeled Nuclei[a]	
Time Post Pulse (hr)	SMAL Absent	SMAL Present
0	56.0	0.1
4	57.0	12.4
13	14.4	5.6
26	11.4	6.5

[a] Slides from population of EL-4 cells grown in the presence or absence of SMAL (XM-100 retentate of TL culture supernatant) were prepared for autoradiography as described in Materials and Methods. After 24 hr exposure the slides were developed and stained with Giemsa. About 1000 nuclei per preparation were counted to determine the percentage of labeled nuclei using an Olympus microscope.

Figure 23. EL-4 Cell Nuclei Labeled with ^{3}H-tdr. EL-4 cells were cultured in the presence of ^{3}H-tdr (4 μCi/ml) for 1 hr. They were washed, fixed and microscopic slides were prepared for autoradiography as described in Materials and Methods. Some labeled and unlabeled nuclei are shown with arrows.

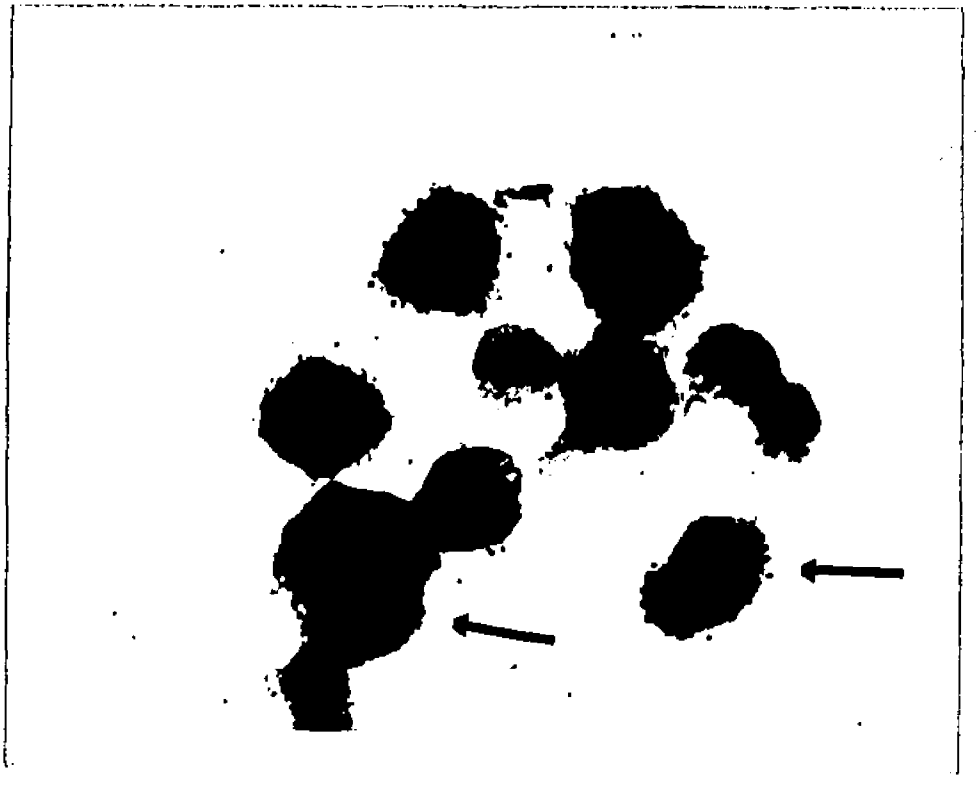
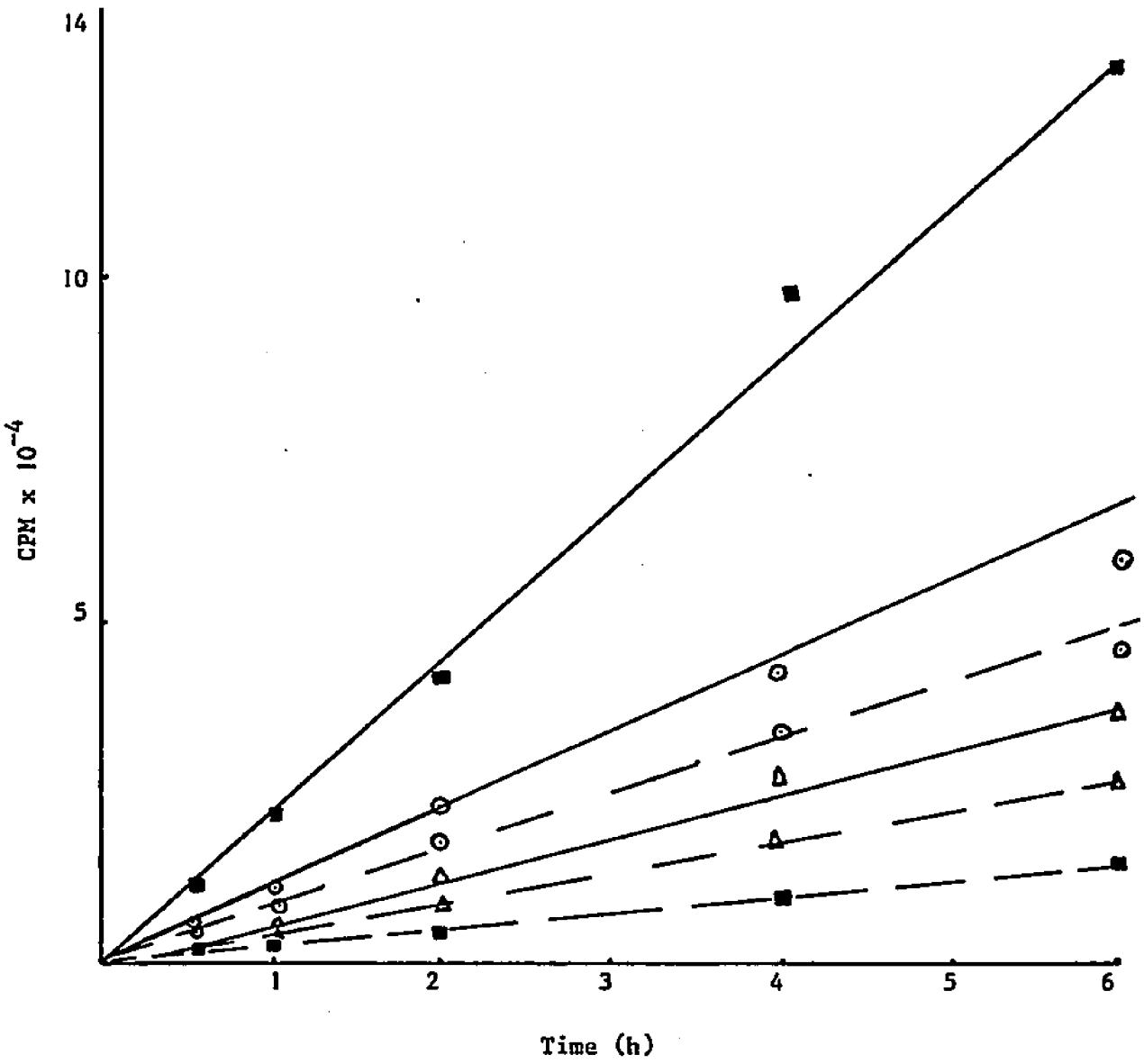


Figure 24. Differential Inhibition of Nucleosides Uptake by SMAL. Actively growing EL-4 cells were cultured in the presence or absence of SMAL (XM-100 retentate of TL culture supernatant) for 24 hr. The cells were then pulsed with ^{3}H-tdr, ^{3}H-udr, or ^{3}H-adr as indicated. At the end of 6 hr pulse the cells were harvested and the extent of nucleoside uptake was determined by liuqid scintillation counter as described in Materials and Methods. ■—— ^{3}H-tdr, ●—— ^{3}H-adr, Δ—— ^{3}H-udr, Control (solid lines), SMAL-treated (broken lines).


14
10
5
CPM x 10^{-4}
1
2
3
4
5
6
Time (h)

binds irreversibly to thymidylate synthetase and by doing so blocks synthesis of dTmp. In the presence of SMAL the cytotoxic effect of 5-Fudr should be reduced by inhibiting its entry, preventing its subsequent phosphorylation or both. Cells were pretreated with SMAL (XM-100 retentate of TL culture supernatant) for 24 hr and then the cultures were given different concentrations of 5-Fudr. After 72 hr the percentages of growth with respect to untreated cells were determined. As shown in Figure 25, the SMAL-treated cells required more than 26 times 5-Fudr to reduce their growth by 50% (260 nM vs. 10 nM), as compared to the cells cultured in the absence of SMAL. These results were consistent with the others which showed uptake of ^{3}H-Udr was reduced in cells suppressed by SMAL, but the huge reduction in cytotoxicity of 5-Fudr suggested that phosphorylation of that nucleoside analog was inhibited also.

Since these results indicated that the uptake of thymidine was reduced in SMAL-treated cells, the possibility that SMAL bound directly to the nucleoside transporter site on the cell membrane to block the transport of the nucleoside was investigated. Nitrobenzylthioinosine (NBTI) is a potent inhibitor of nucleoside transport. It binds to the nucleoside transport sites on the membrane. If SMAL was inhibiting the nucleoside uptake by binding to these sites, then the binding of ^{3}H-NBTI to the cells treated with SMAL should be reduced. Actively growing EL-4 cells were cultured in the presence or absence of SMAL (XM-100 retentate of TL culture supernatant) for 24 hr. ^{3}H-NBTI was added and its binding to the cells was determined. The data (Table 16) showed that there was no reduction in the binding of ^{3}H-NBTI to the cells in the presence of

Figure 25. Resistance of SMAL-treated Cells to Cytotoxic Effect of 5-Fluorodeoxyuridine. EL-4 cells were incubated in the presence —●— or absence —●— of SMAL (XM-100 retentate of TL culture supernatant) for 24 hr and then received different concentrations of 5-Fudr as indicated. After 72 hr, the number of cells were determined with a hemocytometer. The percent of growth inhibition was determined by comparison to the control cells that did not receive 5-Fudr or SMAL.

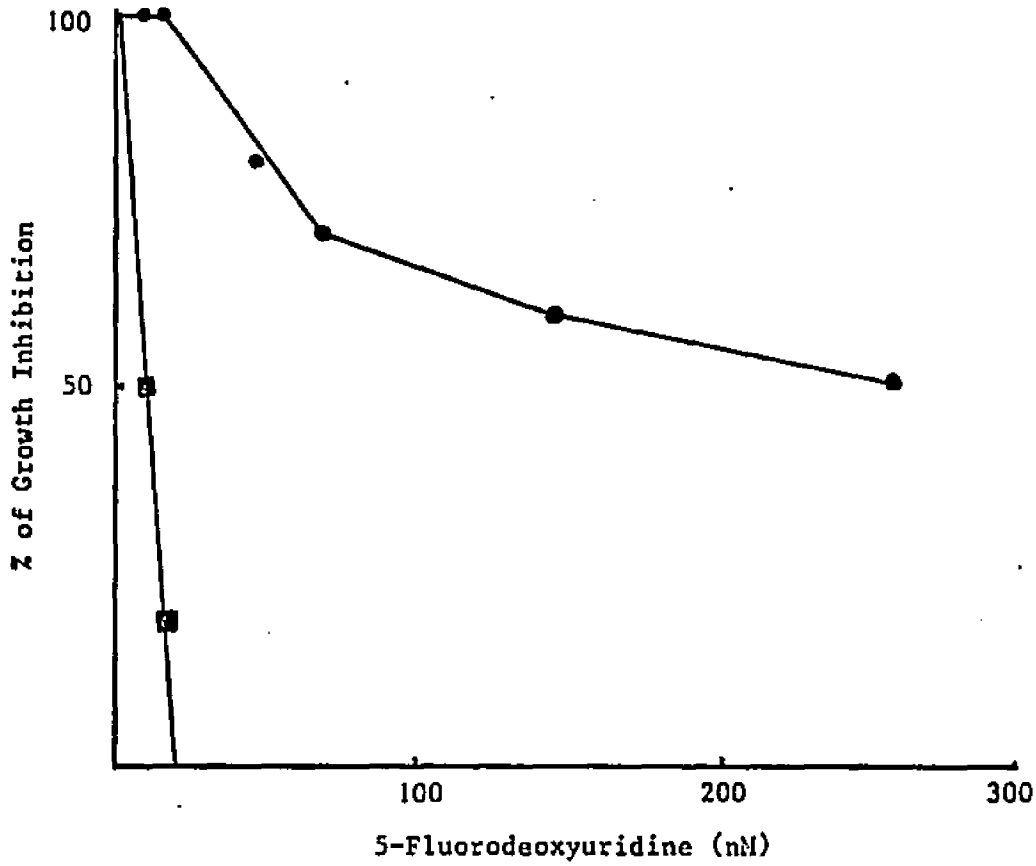

100
50
% of Growth Inhibition
100
200
300
5-Fluorodeoxyuridine (nM)

TABLE 16. Binding of ^{3}H-NBTI to SMAL-treated EL-4 Cells

Labeled Substance	Mean cpm ± SD	
	Control	Experimental
^{3}H-tdr	54,188 ± 3,600	470 ± 170
^{3}H-NBTI[a]	1,254 ± 139	1,328 ± 40
^{3}H-NBTI[b]	1,269 ± 54	1,229 ± 35
^{3}H-NBTI[c]	1,443 ± 331	1,381 ± 196

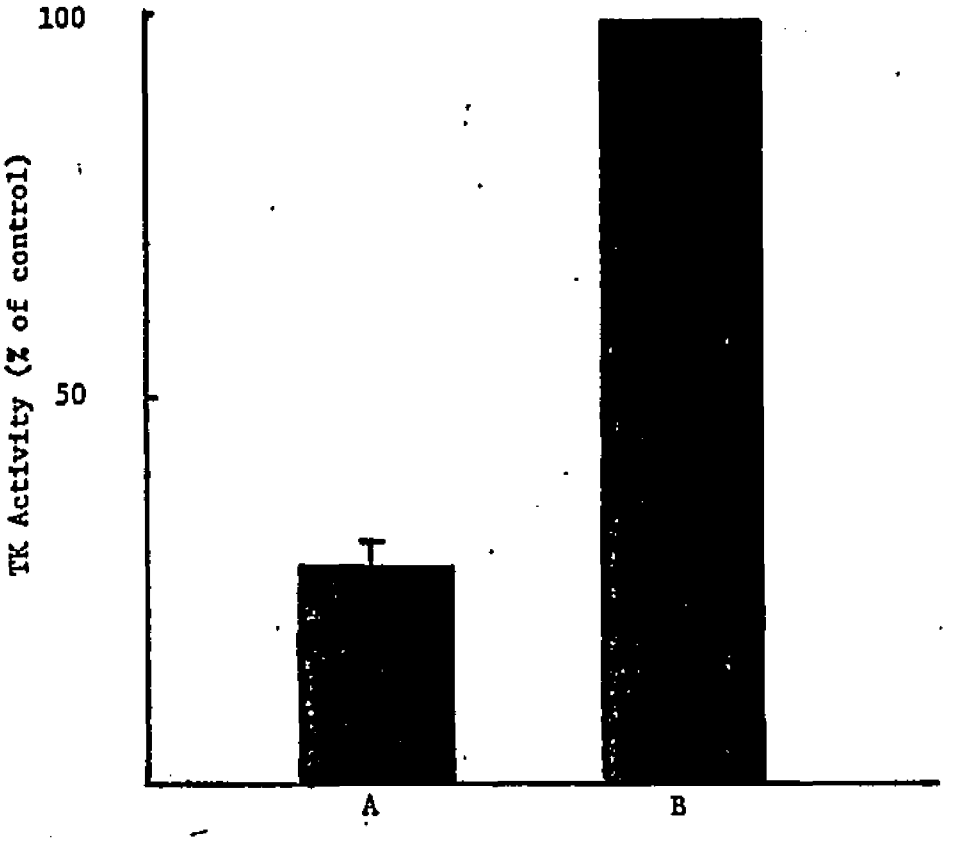
[a, b, c] Represent 3 different experiments.

[d] Actively growing EL-4 cells were incubated in the absence (control) or presence (experimental) of SMAL (XM-100 retentate of TL culture supernatant) for 24 hr. Cells were then pulsed with 1 µCi of ^{3}H-NBTI or ^{3}H-tdr and harvested on GF/C filter paper using a MASH-II multiple harvester as described in Materials and Methods.

SMAL despite vast inhibition in the total uptake of ^{3}H-tdr caused by SMAL. Therefore, it seems likely that SMAL inhibited the uptake of ^{3}H-tdr via a mechanism other than binding to the nucleoside transporter where ^{3}H-NBTI binds.

Reduction of Thymidine Kinase Activity in the Cell Extract of EL-4 Cells Grown in the Presence of HPLC-Purified SMAL. As mentioned above, one effect of SMAL on ^{3}H-tdr might be a reduction in the activity of cellular thymidine kinase. To test this possibility, actively growing EL-4 cells were cultured in the presence or absence of HPLC-purified SMAL for 24 hr. Cell extracts were then prepared and the activity of thymidine kinase in these extracts was determined. Data representative of several experiments are shown in Figure 26. The phosphorylation of thymidine by thymidine kinase in the extract of EL-4 cells grown in the presence of SMAL (HPLC-purified) was inhibited substantially (Fig. 26A). The inhibition ranged from 48% to 70% in different experiments. The observed extent of inhibition of the enzyme, however, was not as marked as the suppression of incorporation of ^{3}H-tdr into intact cells which ranged up to 99%. For example, in the experiment for Figure 26 the same amount of purified SMAL which led to 70% inhibition of thymidine kinase in the treated-cell extract also caused 93% suppression of incoporation of ^{3}H-tdr into the intact cells. This might be due to the fact that in intact cells a combination of transport and phosphorylation was being measured. It was also found that SMAL did not directly inhibit thymidine kinase. When the same amount of purified SMAL was mixed with the control cell extract, the activity of the enzyme was unaffected (Fig. 26B). Mixing lysates from control and SMAL-treated cells

Figure 26. Reduced Thymidine Kinase Activity in the Extracts of SMAL-treated EL-4 Cells (A) and Lack of Direct Inhibition of Thymidine Kinase by SMAL (B). Actively growing EL-4 cells were incubated in the presence or absence of SMAL (HPLC-purified) for 24 hr. Equal number of cells were lysed by sonication and the activity of thymidine kinase was determined (A). The same amount of purified SMAL was added directly to the lysate of untreated cells and the mixture was assayed for thymidine kinase activity as described (B).



Reduced thymidine kinase activity in the extract of SMAL-treated EL-4 cells (A) and lack of direct inhibition of thymidine kinase by SMAL (B).

revealed that the reduction in TK activity was not due to the presence of a difusable secondary inhibitor in SMAL-treated cell extract (Fig. 27).

Effect of SMAL on the Incorporation of ^{3}H-tdr by Different Cell Lines. The incorporation of ^{3}H-tdr by different cell lines in the presence of SMAL was investigated. SMAL (XM-100 retentate of TL culture supernatant) was added to the following lines: EL-4 mouse thymoma, P338D mouse macrophage, CEM-CM_3 human T cell leukemia, and MDCK dog kidney epithelial cell line. After 24 hr of incubation they were pulsed with ^{3}H-tdr for 2 hr and the amounts of radioactivity taken up by these cells was determined. Results are shown in Figure 28. The most sensitive of the cell lines to the inhibition by SMAL was CEM-CM3 and the least was MDCK. At protein concentration of 15 µg per ml, 85% inhibition of ^{3}H-tdr incorporation was obtained by CEM-CM_3. At the same protein concentration only 18% inhibition was noted for MDCK. Percent inhibition for P388D and EL-4 cells were 70 and 40 respectively (Fig. 28).

SMAL (HPLC-Purified) at High Concentration was Cytostatic for EL-4 and CEM-CM_3 Cells Lines. When EL-4 or CEM-CM_3 cells were incubated in the presence of different concentrations of SMAL for 48 hr, the incorporation of ^{3}H-tdr was inhibited at low concentrations of SMAL whereas cell growth was not affected. For example, at 20 units of SMAL/ml more than 90% inhibition of ^{3}H-tdr incorporation was noticed (Fig. 29), but no considerable inhibition of cell proliferation was demonstrable by this concentration of SMAL (Fig. 30). At higher

Figure 27. Thymidine Kinase Activity in Mixtures of Untreated and SMAL-treated EL-4 Cell Lysates. EL-4 cells were incubated in the presence or absence of SMAL (HPLC-purified) for 24 hr. Equal numbers of cells were lysed by sonication and lysates were mixed to different proportions as indicated in the figure and assayed for thymidine kinase activity as described in Materials and Methods.

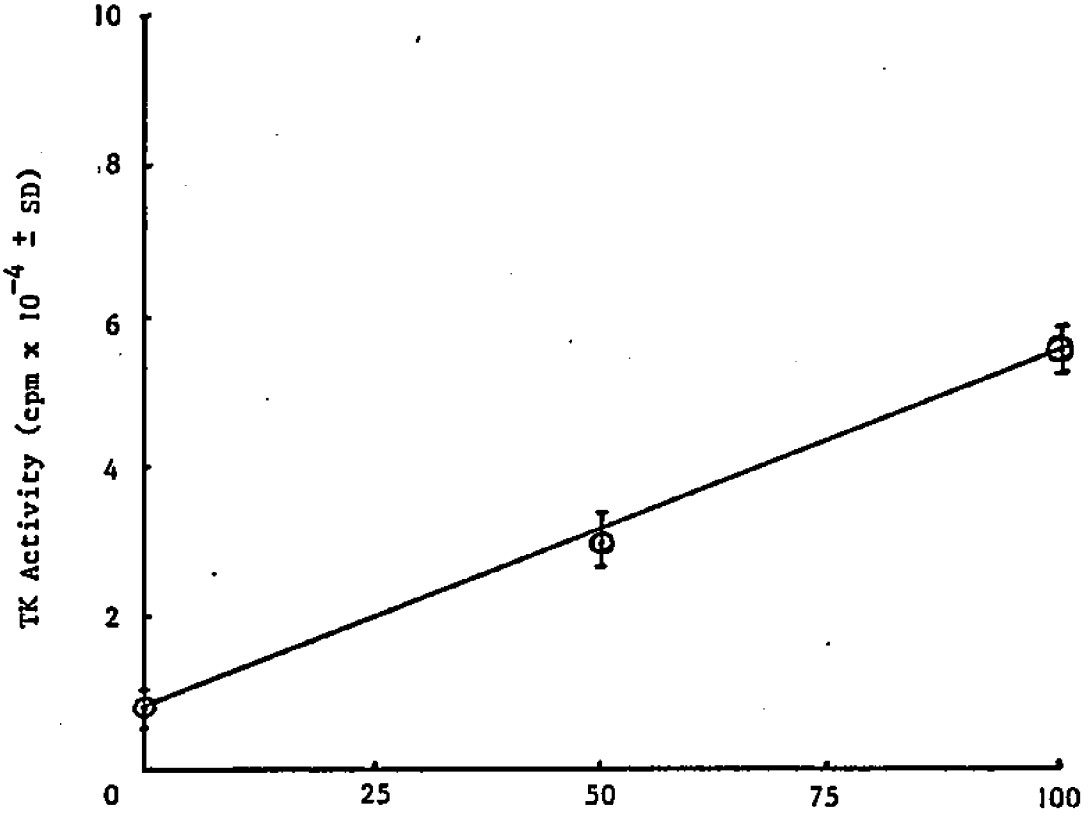

10
8
6
4
2
0
25
50
75
100
TK Activity (cpm x 10^{-4} ± SD)
Percent of Control Lysate in Total Lysate Volume

Figure 28. Inhibition of ^{3}H-tdr Incorporation by Different Cell Lines. Actively growing EL-4, CEM-CM_3, P388D, and MDCK cell lines were incubated in 96-well tissue culture plate in the presence or absence of SMAL (XM-100 retentate of TL culture supernatants) for 24 hr. The cells were pulsed with 1 μCi of ^{3}H-tdr and the extent of ^{3}H-tdr incorporation into TCA-insoluble materials was determined by addition of cold TCA to final concentration of 10%. Cells were harvested with a MASH-II multiple harvester and radioactivity was determined by liquid scintillation counting. ⊙— CEM-CM_3, ■— P388D, ●— EL-4, and ▲— MDCK cell line.

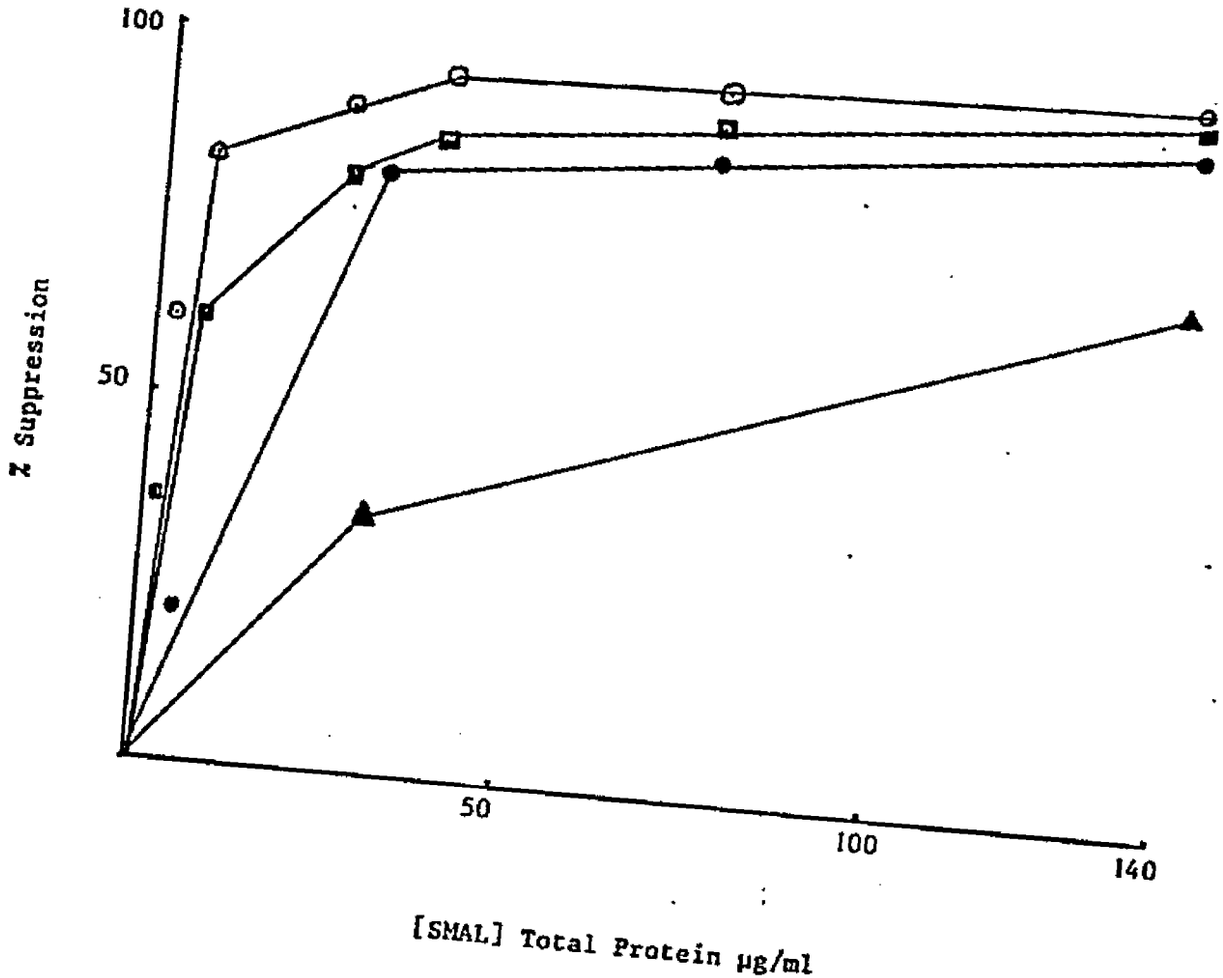

100
50
% Suppression
50
100
140
[SMAL] Total Protein µg/ml

Figure 29. Inhibition of ^{3}H-tdr Uptake by EL-4 and CEM-CM_3 Cell Lines at Low Doses of SMAL (HPLC-purified). Actively growing EL-4 or CEM-CM_3 cells were cultured in the presence of different concentrations of SMAL up to 30 units/ml. The cells were pulsed with ^{3}H-tdr for 2 hr and the extent of suppression of ^{3}H-tdr uptake was determined as described in Materials and Methods. ●— EL-4 cells; X--- CEM-CM_3 cells.

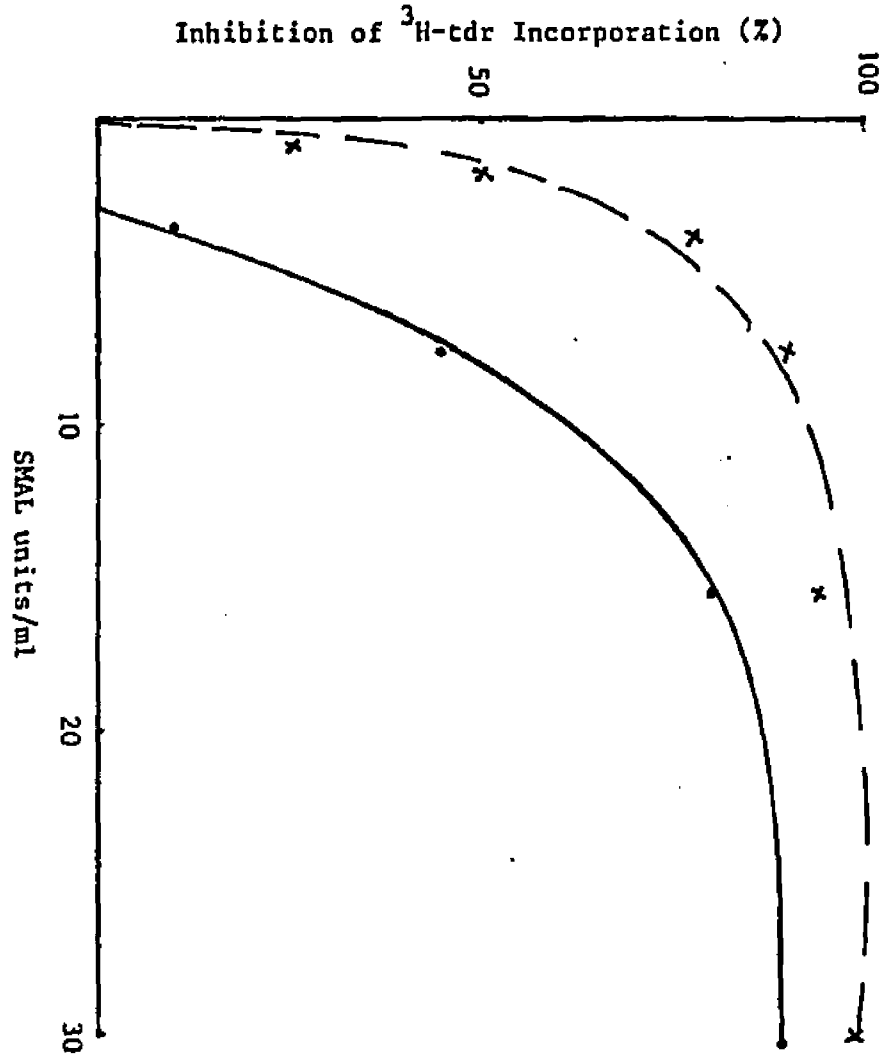
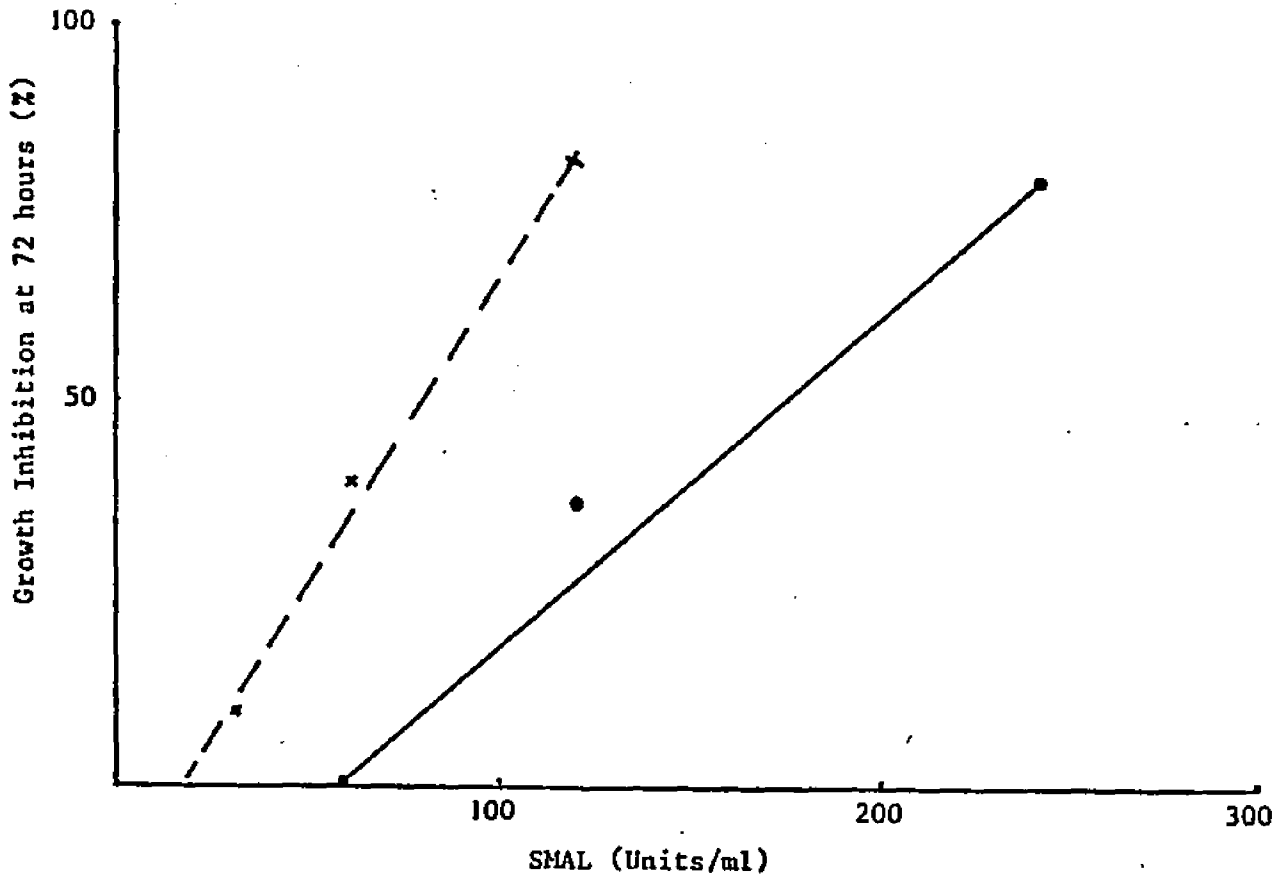

Inhibition of ^{3}H-tdr Incorporation (%)
50
100
10
20
30
SMAL units/ml

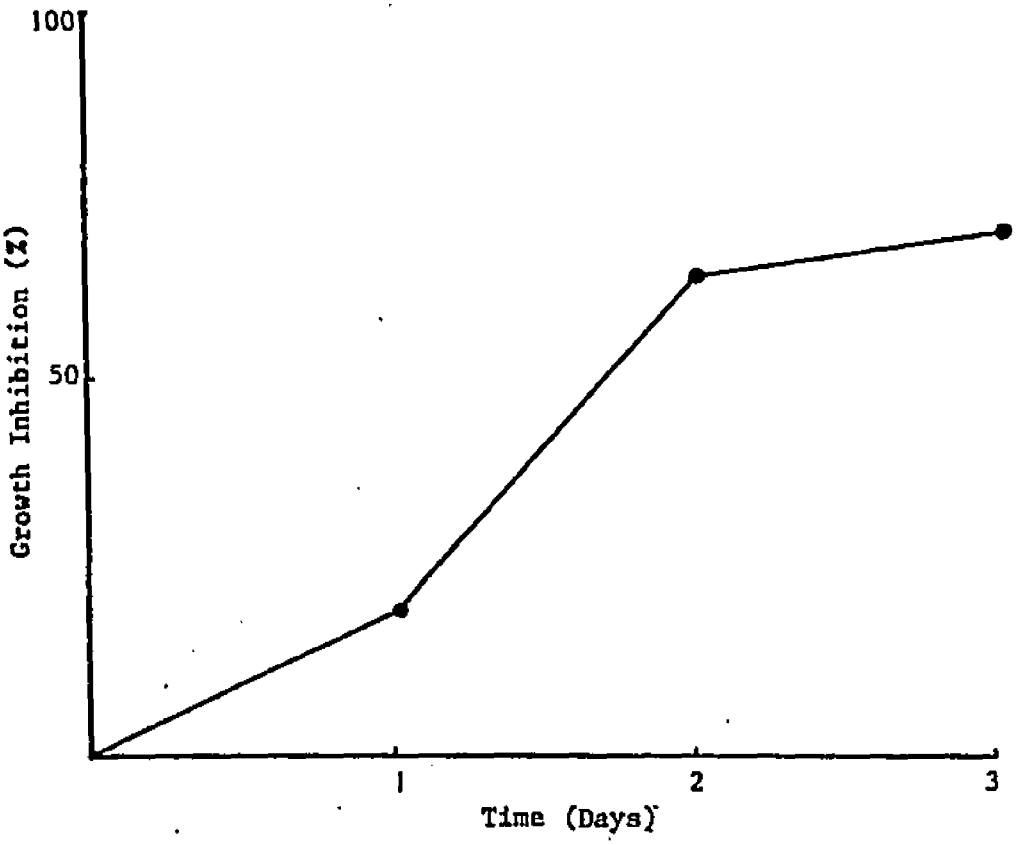
Figure 30. Inhibition of Cell Growth at Higher Concentrations of SMAL (HPLC purified). Actively growing EL-4 or CEM-CM_3 cells were cultured at initial concentration of 2 x 10^5 cells/ml in the presence of different concentration of HPLC-purified SMAL for 72 hr. The number of cells were then determined with a hemocytometer and the extent of growth inhibition was determined as described in Materials and Methods. ●— EL-4 cells; X--- CEM-CM_3 cells.


100
50
Growth Inhibition at 72 hours (%)
100
200
300
SMAL (Units/ml)

concentrations of SMAL (240 units of SMAL/ml) up to 80% inhibition of cell growth was obtained (Fig. 30). By using the inhibition of thymidine incorporation as an assay for SMAL, this system allowed detection of low concentrations of SMAL. Comparison of the time-course of the impairment in DNA labeling by SMAL and of the inhibition of cell proliferation further revealed that the former effect appeared earlier after treatment of the target cells with SMAL than did the suppression of cell proliferation (Fig. 2 and 31). It is clear that in the presence of 240 units of SMAL/ml, the extent of the inhibition of cell growth became maximal within 3 days (Fig. 31), whereas, inhibition of thymidine incorporation was demonstrated within 6-8 hr and reached its maximum within 24 hr (Fig. 2). As mentioned earlier, the CEM-CM_3 cell line appeared to be more sensitive to the effects of SMAL than was the EL-4 cell line.

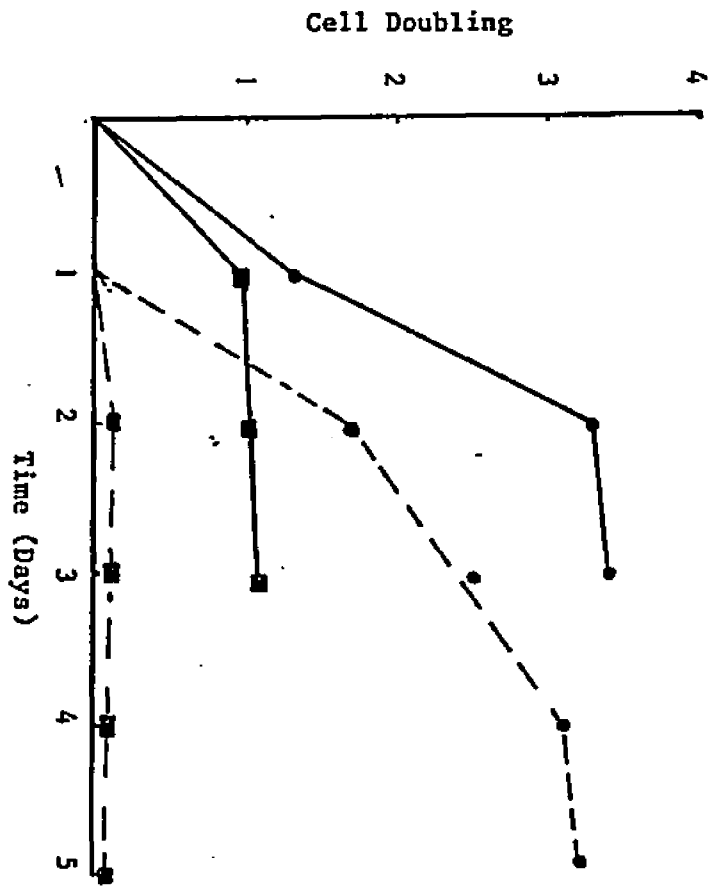
When EL-4 cells taken from the log phase of cell growth were incubated with 300 units of SMAL/ml, they could go through almost one round of cell doubling within the first 24 hr of the culture period. During the next 48 hr, however, there was no further cell proliferation. On the contrary, stationary phase EL-4 cells cultured with the same amount of SMAL did not go through any cell division during the 5-day incubation time (Fig. 32). This might be due to the fact that logorithmically growing EL-4 cells might have produced enough of an unknown substance needed for their first round of cell doubling. The stationary cells on the other hand presumably were arrested in G_0 of the cell cycle and did not produce any of this substance. Although this substance might be thymidine kinase, EL-4 cells could grow in

Figure 31. Time Course Effect of SMAL (HPLC-purified) on the Growth of EL-4 Cells. Actively growing EL-4 Cells were Cultured in the Presence of 240 Units/ml of SMAL up to 3 Days. At 24 hr intervals the cell density was determined by counting the cells in a hemocytometer. The extent of inhibition of cell growth was determined as described in the legends of Figure 30.



Time Course Effect of SMAL (240 units/ml) on the Growth of EL-4 Cells

Figure 32. Effect of High Dose of SMAL (HPLC-purified) on the Stationary and Log Phase EL-4 Cells. EL-4 cells taken from stationary phase or log phase of cell growth (Fig. 1) were cultured in the presence of SMAL (300 units/ml) for 5 days. At 24-hr intervals, the cell density was determined and the extent of inhibition of cell growth was determined as described in Figure legend 30. ●— controls, —■ SMAL-treated cells, solid lines log phase cells, broken lines stationary phase cells.


Cell Doubling
1
2
3
4
Time (Days)
1
2
3
4
5

nucleoside-free medium and were not dependent on the exogenous thymidine. Therefore, this substance might be of a different nature or have an unrecognized function.

SMAL (XM-100 Retentate of TL Culture Supernatant) Did Not Have Antiviral Effect. Since some of the properties of SMAL were similar to that of interferon it was of interest to see if SMAL had any antiviral activity. To investigate this, human embryonic lung fibroblast cell line MRC and human epithelial carcinoma cell line HEP-2 were incubated with SMAL for 24 hr. These cultures were then infected with different concentrations of clinically isolated HSV II. The extent of cytopathic effect of HSV II was determined daily for three days using an inverted microscope. As shown in Table 17, the extent of cytopathic effect of HSV II was similar in control and SMAL-treated cells at the range of SMAL concentrations of 7.5 to 120 units/ml. It is interesting to note that SMAL at concentration of 10 units/ml inhibited the incorporation of ^{3}H-tdr by MRC and HEP-2 cell lines by 70% and 80% respectively (Fig. 33).

These results indicated that SMAL was distinct from interferon which was shown to have antiviral activity (Rose et al., 1986).

TABLE 17. SMAL did not Inhibit the Cytopathic Effect of Herpes Simplex Virus II on the Human Embryonic Lung Fibroblast MRC Cell Line or HEP-2 Cell Line

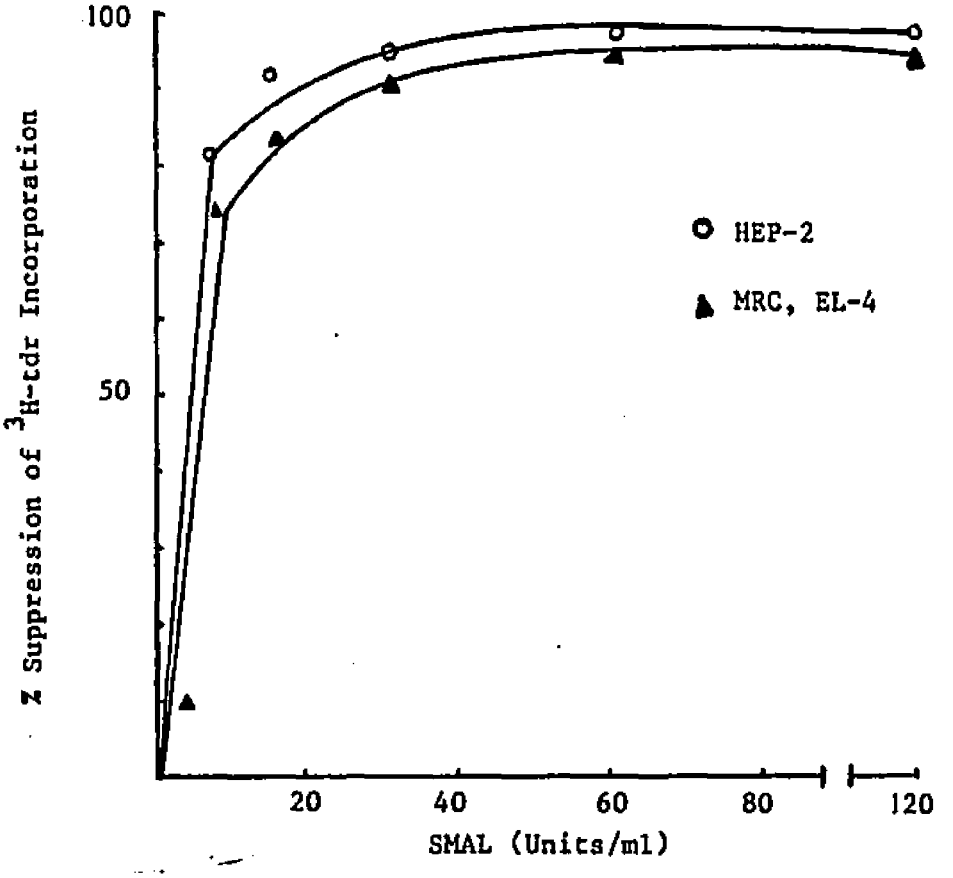
Virus[a] Dilution	Control[b]	SMAL Units/ml				
		120	60	30	15	7.5
	Cytopathic Effect at 72 hours					
virus	-[c]	-	-	-	-	-
dilution						
2×10	+4	+4	+4	+4	+4	+4
2×10^3	+4	+4	+4	+4	+4	+4
2×10^4	+3	+3	+3	+3	+3	+3
2×10^5	+2	+2	+2	+2	+2	+2
2×10^7	+2	+2	+2	+2	+2	+2

[a] Serial dilutions of a stock solution of HSV II were added to MRC or HEP-2 cell lines that had previously been treated with SMAL (XM-100 retentate of TL culture supernatant) for 24 hr. After 72 hr the extent of cytopathic effect of virus were determined as described in Materials and Methods.

[b] Control cells did not receive any SMAL.

[c] Negative sign means no cytopathic effect. Positive sign and the numbers 1 to 4 denotes increasing extent of cytopathic effect.

Figure 33. Inhibition of ^{3}H-tdr Incorporation by SMAL-Treated MRC and HEP-2 Cell Line. Human embryonic lung fibroblast MRC and human epithelial carcinoma HEP-2 cell lines were cultured at cell density of 2×10^{5}/ml in 96-well tissue culture plate in the presence or absence of SMAL (XM-100 retentate of TL culture supernatant) for 48 hr. The cells were then pulsed with 1 µCi of ^{3}H-tdr and after 2 hr were harvested on a GF/C filter paper with a MASH-II multiple harvester after addition of TCA to final concentration of 10%. Percent suppression was determined as described in Materials and Methods.


100
50
% Suppression of ^{3}H-tdr Incorporation
HEP-2
MRC, EL-4
20
40
60
80
120
SMAL (Units/ml)

CHAPTER 4

Discussion

The fact that immune systems are regulated by a variety of soluble suppressor factors is well documented. Some of these are: Inhibitor of DNA synthesis (Namba and Waksman, 1975; Jegasothy and Battles 1979); soluble immune response suppressor (Aunne et al., 1983; Tadakuma and Pierce, 1978); nonspecific suppressor factor (Aunne and Pierce, 1981b); soluble suppressor (Thomas et al., 1975); soluble immune suppressor of T cell proliferation (Greene et al., 1981); inhibitor for DNA polymerase (Lee and Lucas, 1977); leukocyte inhibitory factor (Rocklin et al., 1982); lymphotoxin (Granger et al., 1978); and stimulated rat T-cell derived inhibitory factor for cellular DNA synthesis (Chiba et al., 1985a,b).

Data were presented here that showed the supernatant fluid from cultured human tonsil lymphocytes could suppress the incorporation of ^{3}H-tdr into con A-activated human PBL, confirming the report of Hodge and Inman (1982). The substance responsible for this suppression was referred to as SMAL.

The most important conclusions drawn from this work were: A) SMAL, also, suppressed the incorporation of ^{3}H-tdr into mouse EL-4 thymoma cell line, human CEM-CM_3 T cell leukemia cell line, and mouse P388D macrophage cell line; B) proliferation and viability of target cells were not affected when the cells were exposed to relatively low concentrations of SMAL when they were in early log phase of cell growth; C) at relatively high concentrations of SMAL (240 units/ml) target cell

proliferation was also impaired; D) the inhibition of thymidine incorporation began within 6 hr and became extensive within 24 hr; however, the inhibition of cell proliferation at high doses of SMAL was very slow during the first 24 hr of culture, and peaked within the next 48 hr; E) the inhibition of thymidine uptake and thymidine incorporation was heavily favored over uridine and adenosine, and the uptake of ^{3}H-udr and ^{3}H-adr was not affected to the same extent; F) the reduction in the uptake and incorporation of ^{3}H-tdr was due in part to both an impairment of thymidine transport and a decrease in the activity of thymidine kinase, G) SMAL was produced by living TL capable of synthesizing protein, and H) SMAL was a protein which could be purified by anion-exchange chromatography using HPLC.

The M.W. of SMAL could not be established. When filtered through Fractogel TSK 55S, the suppressive activity attributed to SMAL was associated with component pools of Mr 630,000, 148,000, and 16,000. This suggested that SMAL may be a polymer whose smallest M.W. was about 15,000-16,000. When filtered through XM-300 ultrafilters, however, no activity was associated with the retentate. In contrast, all of the activity was retained by XM-100 or YM-100 ultrafilters. Alternatively the poor resolution of SMAL by gel filtration could be due to a direct or indirect binding of SMAL to the gel matrix. The material which was applied to the column was TL culture fluid which had been concentrated by ultrafiltration on a XM-100 Diaflo membrane. This solution, which was later shown to contain DNA, was not treated with DNase prior to application. Several lines of evidence suggested that SMAL was associated with DNA, thus the nonspecific interactions of DNA with the

gel matrix could indirectly result in the retention of SMAL activity on the gel. The activity of SMAL was also retained by XM-100 after treatment with DNase. These facts indicated the M.W. of SMAL was between 100,000 and 300,000 and the Mr 15,000 unit comprised only a minor part of the total activity. Under the denaturing conditions of SDS-PAGE, the M.W. of the components of HPLC-purified SMAL were less than 100,000. Constituents of Mr 30,000 and 43,000 stained prominently. The 30,000 component was present but was not as noticeable in pool 3 from the DE-52 column. In both of these preparations, however, a Mr 43,000 component was prominent.

The response to con A of PBL drawn from various people, or even from the same person on different days, varied. This observation, the inconvenience and cost of working with donors, and the difficulty of verifying inhibition of proliferation by counting con A-treated PBL led to considering the use of a target cell other than human PBL. The EL-4 cell line was one which was available, and the cells proliferated without the necessity of activating them with con A.

The same preparations of SMAL which suppressed incorporation of thymidine into PBL also suppressed its incorporation into the xenogeneic murine lymphocytes EL-4 cell line and macrophage P388D cell line as well as human T cell leukemia CEM-CM_3 cell line. We chose to use EL-4 cells as target because of its rapid growth rate and ease of culturing and maintenance. SMAL was not cytotoxic to its target cells, and it did not inhibit proliferation of them if the cells were exposed to low doses (24 units/ml) of SMAL and when they were actively growing. The data presented showed that even though the uptake and incorporation of

exogenous thymidine were inhibited, the rate of DNA synthesis and cell proliferation were not affected at low concentrations of the suppressor. This effect could be demonstrated within 6 hr of treatment of the EL-4 cells with SMAL and gradually increased to its maximum at about 24 hr for the population of cells. The use of EL-4 cells allowed one to revise the original assay procedure of Hodge and Inman (1982) and discontinue the use of PBL. The revised assay was more reliable and more convenient than the earlier assay and took considerably less time.

SMAL was produced by living lymphocytes. Neither heat-killed TL nor those whose protein synthesis was disrupted by cycloheximide released SMAL into culture medium. When tonsil lymphocytes were sonicated before culturing a substantial amount of SMAL-like activity was detected in the sonicates. This indicated that SMAL was present in the cells in vivo as expected and was not an artifactual product of in vitro culture.

The suppressor was produced in a linear relationship to increasing cell density. Most of it was released into the culture supernatants during the first 24 hr culture, and progressively less was produced during subsequent 24 hr periods. SMAL was accumulated during uninterrupted culture time. The TL which were cultured had been activated in vivo to various extents as measured by their spontaneous incorporation of ^{3}H-tdr. Both the T lymphocyte- and the B lymphocyte-enriched fractions were active in this respect. It was also revealed that both T and B lymphocyte-enriched fractions produced SMAL and that B lymphocyte culture supernatants contained about five times more SMAL than T lymphocyte culture supernatants. The Percoll density

gradient used to separate tonsil lymphocytes into B and T lymphocytes results only in enriched populations of T and B lymphocyte fractions. According to Gutierrez et al. (1979), this procedure would yield a B cell population consisting of 73% B cells (identified by the presence of surface Immunoglobulin G), 11% T cells (identified by formation of rosettes with sheep erythrorytes), and remaining being mainly monocytes. The T cell fractions contained 78% T cells, 4% B cells, and the rest were negative for surface immunoglobulin G and rosette formation. Assuming that my T and B cell-enriched preparations were similar to those of Gutierrez et al (1979), the data suggested that both T and B cells synthesized SMAL.

SMAL could be precipitated by ammonium sulfate in a rather broad range of 40-80% ammonium sulfate saturation, but the recovery was poor. Purification of SMAL by phenyl-sepharose column chromatography was unsuccessful and resulted in loss of biological activity.

When concentrated TL culture superantant fluids were chromatographed on a DE-52 anion exchange column, SMAL was a component of material which adhered rather strongly. Its elution in 0.4 M-0.6 M NaCl achieved less than 2-fold purification. It was associated with DNA, but pronase destroyed the activity indicating the active moiety was the protein. The DNA in the XM-100 or YM-100 retentates of culture supernatants was digested by treatment with deoxyribonuclease. The retentates then were chromatographed by HPLC. SMAL still adhered strongly to the anion-exchanger and was reproducibly eluted with salt. This resulted in 5-fold purification of high specific activity which comprised only 3-4 components ranging from approximately

Mr 30,000-50,000 under denaturing conditions. This purified SMAL did not bind EtBr and did not adsorb strongly at 260 nm indicating the absence of DNA. There are numerous reports indicating that cultured lymphocytes activated by mitogens or antigens release newly synthesized DNA into the culture medium (Sasvari et al., 1984; Banfalvi, 1984; Anker et al., 1976; Staub and Antoni, 1978; Adams and Gahan, 1982; Boldt et al., 1977; Anker et al., 1975; Rogers, 1976; Rogers and Rucinsky, 1982).

It was demonstrated by Staub and Antoni (1978) that the main part of newly synthesized DNA was preferentially released in vitro both by non-stimulated and PHA-stimulated tonsil lymphocytes. Although a number of hypotheses have been put forward to explain the role of excreted DNA, its function remains obscure. It was thought originally that released DNA might be related to gene amplification (Rogers et al., 1972), but other evidence suggested that the released DNA was involved in the immune response (Rogers and Kerstin, 1981).

The results presented in this paper also demonstrated that cultured human TL synthesised and released DNA into the culture medium, and that SMAL was most probably associated and copurified with it on a DEAE-cellulose column. Since the bioactivity of SMAL was not lost upon treatment with deoxyribonuclease, it is plausible that DNA served as a carrier for SMAL. When DNase-treated XM-100 retentates were subjected to polyacrylamide gel electrophoresis under native conditions, two peaks of SMAL activity were recovered from the gels by electroblotting to DEAE-cellulose paper. The bound materials were then eluted by salts. SMAL activity was associated with two regions of the gel. The first region corresponded to the upper part of the gel indicating that this

material was large and/or less negatively charged. The second region of activity resided at the bottom of the gel, representing a smaller and/or highly negatively charged molecule. It has not been possible to purify SMAL to homogeneity or to attribute its activity to any single band on SDS-PAGE due to the lack of sufficient material and loss of bioactivity of SMAL during the purification.

A major problem inherent in the isolation and purification of most lymphokines has been the lack of substantial quantities of starting material for biochemical analysis. It was thought that tonsils would provide a large and readily available source of SMAL. However, during the course of this work, it became apparent that large volumes of tonsil culture supernatants (100 liters) would be needed for purification and biochemical characterization of SMAL. Lack of a regular supply of tonsils as well as inconvenience of preparing tonsil lymphocyte cultures prompted us to consider establishing a somatic cell hybrid (that can produce SMAL) between human tonsil lymphocytes and human T cell leukemia cell line CEM-CM_3.

Several investigators have produced hybridomas that can potentially provide an invaluable resource for a variety of lymphokines (Kouttab et al., 1985; Okada et al., 1981; Irigoyen et al., 1981; Theodore et al., 1986). In the present study, three different fusions were made between TL and CEM-CM_3 cells but no stable hybrid could be established.

Now, let us discuss some of the effects of SMAL on the EL-4 cells. As mentioned earlier, SMAL appeared to have two distinct effects on the cells depending on the concentration of SMAL. At low doses, SMAL only impaired the uptake and incorporation of exogenous thymidine without any

effect on the synthesis of DNA or cell division. At higher concentrations, however, treatment of EL-4 or CEM-CM_3 cell lines with SMAL resulted in the inhibition of cell growth. First, the effect of low doses of SMAL will be described, then its antiproliferative properties will be discussed.

The effect of SMAL was specific in impairing the uptake and incorporation of thymidine. In contrast, there was only marginal effect on the uptake and incorporation of ^{3}H-udr or ^{3}H-adr. Membrane transport of nucleosides in eukaryotic cells occurs by non-concentrative facilitated diffusion processes; phosphorylation of the substrate traps it inside the cells (Oliver and Peterson, 1971; Cass and Paterson, 1972; Aronow and Ullman, 1985). The nucleoside transporter in man and pig is a polypeptide with M.W. of 45,000-65,000 (Wu et al., 1983). It is known that nitrobenzylthioinosine (NBTI) is an irreversible high affinity inhibitor of nucleoside transport (Young et al., 1984). It is also known that NBTI binds to the transporter protein as was demonstrated by covalent affinity labeling of the protein with photoactivated NBTI (Young et al., 1983).

Although SMAL inhibited the uptake of ^{3}H-tdr, it apparently did not affect the binding of ^{3}H-NBTI to the intact EL-4 cells membrane. Thus, it was possible that SMAL was inhibiting the transport of thymidine by binding to a site on the plasma membrane of cells distinct from that of the NBTI binding site. The possibility of SMAL binding to EL-4 cells was demonstrated by the loss of activity of SMAL in supernatants of culture which were done in the presence of known amounts of the factor. Whether this loss of activity was due to the inactivation of SMAL during

the incubation period with cells or an actual indication of interaction of SMAL with a receptor was not clear. The fact that SMAL had to be incubated with the cells a minimum of 6-8 hr in order to detect a considerable inhibition of ^{3}H-tdr uptake might indicate a slow interaction and/or entry into the cells. The observed change in membrane transport of thymidine may reflect an effect of SMAL on the cell surface which could in turn cause an inhibition of ^{3}H-tdr uptake and inhibition of thymidine kinase. In any event, it was clear that the effect of SMAL on the uptake of ^{3}H-tdr was reversible, suggesting perhaps low affinity binding or endocytosis of SMAL. Whatever this effect was, it did not inhibit uridine or adenosine uptake to the same extent. These nucleosides may utilize a different system distinct from that required for thymidine uptake (Plageman and Erbe, 1972; 1974).

Extract from SMAL-treated cells contained reduced thymidine kinase activity as compared to untreated control cells. There are several reports correlating thymidine kinase activity with cell growth (Mei-Heui and Weber, 1983; Baserga, 1981; Bradshaw, 1983; Cheng et al., 1977; Claycomb, 1979; Enggan et al., 1983). However, in spite of the decreased thymidine kinase activity in EL-4 cells after 24 hr of treatment with SMAL, there was no reduction in the proportion of cells in S Phase and no effect on cell proliferation.

Since EL-4 cells propagated in culture medium free of exogenous thymidine, SMAL-treated cells could satisfy their growth requirement for nucleotides by utilizing their de novo pathway of nucleotide biosynthesis. The possibility that nucleosides were derived from serum added to the culture was very low because these cells were able to grow

in the presence of serum as low as 1%. The obvious question of how SMAL from outside the cells could effect thymidine kinase, a cytosolic enzyme, remains for further investigations. Coppock and Paradee (1985) have shown that the induction of thymidine kinase activity after the stimulation of quiescent cells with serum was regulated by a labile protein. This protein in turn caused an increase in the level of thymidine kinase mRNA in late G_1 and early S phase. It is, therefore, possible that SMAL has some effect on this labile protein.

There are also reports showing that thymidine kinase can exist in different enzyme variants in Physarium polycephalum. Some of these enzymes can be phosphorylated at the beginning of the S phase (Brobner and Loidl, 1984). The phosphorylated thymidine kinase differs from the non-phosphorylated form with respect to inhibition by dTTP and Km value for thymidine. The phosphorylated forms are less inhibited by dTTP and are present in S phase of the cell cycle (Brobner and Loidl, 1984). Therefore, it is possible that SMAL is interfering with the phosphorylation of thymidine kinase in EL-4 cells and by doing so, reducing the enzyme activity.

When SMAL was directly added to the cell extracts which then were assayed for thymidine kinase activity, there was no effect on the activity of this enzyme. Also, when extracts from SMAL-treated and untreated EL-4 cells were mixed, no inhibition of TK activity was noticed. These data excluded the possibility of the presence of diffusable inhibitors in the extracts of SMAL-treated cells. The data were insufficient, however, to conclude whether the reduction in thymidine kinase activity was due to the actual inhibition of the enzyme

or a result of reduction in the amount of enzyme. The effect of SMAL on the level of transcription or translation of thymidine kinase remains to be explored.

It was shown by several investigators that interferons (INF) also have effects on the uptake and phosphorylation of thymidine by human or mouse lymphoma cell lines (Gewert et al., 1981; 1983; Lundblad and Lundgren, 1982; Gewert et al., 1984). These effects were very similar to that of SMAL on EL-4 cells. When SMAL was tested for antiviral effect, no inhibition of the cytopathic effect of herpes simplex virus on human MRC and HEP-2 cell lines was noted. But, SMAL inhibited the incorporation of ^{3}H-tdr by these cell lines. In addition, the activity of SMAL was not destroyed at pH 2, whereas, that of INF was (Valle et al., 1975). These observations and the apparently smaller M.W. of INF (Ware and Granger, 1979; Rubenstein, 1982; Wiranowska-Stewart, 1981) indicated that SMAL and INF probably were not identical molecules.

There were also reports that show D-glucosamine has inhibitory effect on the utilization of exogenous thymidine. Friedman et al. (1977) reported that in glial tumor cells, the amino sugar caused a decreased rate of utilization of exogenous ^{3}H-tdr for cellular DNA synthesis. Tesorier et al. (1984) also observed that D-glucosamine markedly inhibited the incorporation of ^{3}H-dtr into TCA-precipitable materials. This effect was due to the inhibition of thymidine transport and thymidine kinase activity in HeLa cells.

Klein et al. (1979) observed that an extract from rat kidney could inhibit thymidine kinase activity and thymidine incorporation in 3T3 mouse fibroblasts. This inhibitor was partially purified, and its

activity was detected after treatment with heat, pronase, DNase, and RNAse. However, it was lost if the pronase or trypsin treated material was subsequently dialyzed. The inhibitory activity was apparently related to the carbohydrate moiety of a mucoprotein.

Ashkenazi and Ashkenazi (1976) demonstrated that a DNA extract prepared from normal human diploid fibroblast cell culture inhibited the uptake and incorporation of ^{3}H-tdr into HeLa cells, while the uptake of ^{3}H-udr was stimulated. This inhibitor was also heat and pronase stable and seemed to be related to nucleic acids. Biochemical and biophysical properties of SMAL compared to these inhibitors are different. SMAL is heat and pronase sensitive, RNase or DNase stable, and has a M.W. of at least 100,000 but smaller than 300,000, and is an acidic protein.

As mentioned earlier, SMAL at higher doses (240 units/ml) was cytostatic for its target cells. The inhibition of cell proliferation by SMAL was a dose dependent phenomenon. When SMAL was added to EL-4 or CEM-CM_3 cells at 100 units of SMAL/ml, the growth of CEM-CM_3 was inhibited by 80%, but more than 240 units of SMAL was required in order to inhibit the growth of EL-4 cells by 80%.

CEM-CM_3 cells appeared to be more sensitive to the inhibitory effect of SMAL on both the uptake of ^{3}H-tdr and cell proliferation. It was known that CEM-CM_3 cell line lacked a functional HGPRT (Hypoxantine-guanine phosphoriboxyl transferase). This enzyme is a key enzyme in the salvage pathway of purine nucleosides. Since SMAL appeared to inhibit thymidine kinase, a key enzyme in the salvage pathway of pyrimidine nucleosides, the higher sensitivity of CEM-CM_3 might be due to the combined effect of a deficiency in HGPRT and

SMAL-inhibited thymidine kinase. The inhibition of cell growth by SMAL was a slow process. Within the first 24 hr of incubation of EL-4 cells with SMAL cells only 20-30% inhibition of cell proliferation was noted. Suppression was maximal with 3 days of incubation (80%). In contrast, the cytostatic effect of SMAL was pronounced during the first 24 hr of culture time if target cells were taken from the stationary phase of growth rather than from the log phase. This might be due to the fact that logarithmically growing EL-4 cells have produced enough of an unknown substance (i.e. thymidine kinase) needed for their first round of cell doubling. The stationary phase cells presumably were docked in G_0 of cell cycle and did not produce any of this substance.

Whether the inhibition of cell growth was due to the impairment of thymidine uptake and thymidine kinase is not clear. As pointed out earlier EL-4 cells were routinely cultured in nucleoside-free medium. Therefore, it appears that inhibition of cell growth is very unlikely to be due to thymidine starvation caused by SMAL. Gewert et al. (1984) demonstrated that neither an intact nucleoside transport system nor fully active thymidine kinase were essential for the expression of the antiproliferative effect of interferon which inhibits both thymidine transport and thymidine kinase. The primary action of SMAL may be at DNA level and thymidine metabolism could be altered as a consequence of this effect. Alternatively, disruption of the metabolism of thymidine may lead to impairment of DNA synthesis.

There were several reports of lymphocytes and tumor cells which when cultured, produced supernatants that inhibited target lymphocyte proliferation (Rich et al., 1978; Wolf et al., 1978; Shou et al., 1980;

Garner et al., 1980; Renk et al., 1980; Lederman et al., 1981; Greene et al., 1981; Kramer and Koszinovski, 1982; Chiba et al., 1985b). Most of these cited inhibition of the incorporation of ^{3}H-tdr as evidence for suppression of proliferation. In the case of the action of SMAL, such a conclusion based on similar evidence would have been incorrect when relatively low doses of SMAL were used.

According to current information on SMAL, it had biochemical and biological effects characteristics which were both similar and dissimilar to those of some of the other factors. Tumor necrosis factor (TNF), for example, was cytostatic or cytolytic for several transformed cell lines (Carswell et al., 1975; Ruff and Gifford, 1981a) and cytolytic for mouse L-929 fibroblast (Ruff and Gifford, 1981b), but it did not affect normal cells. TNF was made by macrophages (Männel, 1980), but SMAL is produced by macrophage depleted lymphocytes. Under nondenaturing conditions, the reported M.W. of TNF derived from human monocytes or HL-60 cells varied from 34,000-40,000 (Matthews, 1981; Nissen-Meyer and Hammerstrom, 1982; Aggarwal et al., 1985b) and that from immune rabbit serum was 52,000-55,000 (Ruff and Gifford, 1980). Purified TNF, however, had a M.W. of 17,000, suggesting native TNF is a dimer or trimer.

Lymphotoxin (LT) is a lymphokine which, like SMAL, is made by tonsil cells. When assayed on mouse L-929 fibroblasts, LT is cytolytic within 24 hr (Spofford et al., 1974) whereas SMAL is not cytolytic for lymphocytes. LT elutes from DEAE-cellulose with 0.1 M NaCl (Aggarwal et al., 1984), well below the requirement for elution of SMAL. Under nondenaturing conditions, the M.W. of LT derived from a lymphoblastoid

cell line is 60,000, but when its weight is estimated by SDS-PAGE, it is 20,000, which closely agrees with the weight calculated from its amino acid composition (Aggarwal, 1985a). In contrast, the smallest component in purified SMAL preparations had a M.W. of about 30,000.

Another recently recognized tumor cell proliferation inhibiting lymphokine which has some characteristics similar to SMAL is leukoregulin. Its M.W. is 135,000 under nondenaturing conditions, and 32,000 when determined by SDS-PAGE (Ransom et al., 1985; Sayers et al., 1986). It is made by T cells and null cells from human PBL, a B-lymphoblastoid cell line and human B cell mouse myeloma when stimulated with PHA. It elutes from DEAE-cellulose in 0.1 M NaCl which is less than the concentration required to elute SMAL.

Tonsils are lymphatic organs which are comprised of similar percentages of T and B lymphocytes (Sesterhan et al., 1977; Brochier, 1978) and very small number of macrophages. Their cells produce varieties of lymphokines such as lymphotoxin (Granger, 1978), colony stimulating factor (Melamed, 1978), and IL-2 (Smith and Ruscetti, 1981). Since proliferative response of tonsil lymphocytes in some humans is evident by frequent episodes of tissue swelling, it is likely that SMAL might be engaged in some immunoregulatory activities.

It will be appropriate to briefly review what is known about the mechanism of action of polypeptide growth factors and mitogens and their role in stimulating growth. This information might be useful to identify possible mechanisms by which SMAL inhibits target cell proliferation. The fundamental importance of polypeptide growth factors in stimulating growth and maintaining viability in a broad variety of

cell types has become a generally accepted principal of developmental biology. In the past several years, major progress occurred in three areas: identification and isolation of a large number of polypeptide growth factors; structural analysis of several of these, including mRNA and gene sequences; and receptor characterization for several polypeptide growth factors (PGFS).

Among the well characterized PGFS that stimulate the growth of T lymphocytes is Interleukin 2, formerly known as T cell growth factor. IL-2 is a 15,000 M.W. glycoprotein secreted by T cells in response to mitogenic or antigenic stimulation (Morgan et al., 1976). The biological role of IL-2 as an immunostimulant intimately involved in the control of T cell proliferation has been firmly established by numerous investigations (Lachman and Maizel, 1983).

The availability of large quantities of IL-2 produced by the human T-leukemia cell line Jurkat (Gillis and Watson, 1981) provided the first key to the purification and elucidation of the factors structure.

The second breakthrough was the cloning and sequencing of a cDNA corresponding to IL-2 mRNA by Taniguchi et al. (1983) and Shuichiro et al. (1983). Furthermore the gene encoding IL-2 has been expressed in eukariotic and bacterial cells (Rosenberg and Grimm, 1984). The IL-2 receptor has also been purified and characterized (Kuo and Robb, 1986). It was shown by Gaulton and Eardley (1986) that binding of IL-2 to IL-2 receptor on Con A-stimulated cells caused rapid phosphorylation of IL-2 receptor and other membrane proteins. Despite the considerable advances noted, little is known regarding the mechanism by which binding of IL-2 to its receptor induces cellular proliferation.

The following events happen, in general, after binding of any mitogen or polypeptide growth factor to its receptor. The receptor undergoes rapid phosphorylation, redistribution in the plane of the membrane and endocytosis (James and Bradshaw, 1984). The binding of the growth factor promotes the generation of early signal in the membrane and cytosol. Within minutes, the mitogenic signal is propagated into the nucleus. These events eventually lead to synthesis of DNA and cell division. Some growth factor receptors has been shown to have tyrosine specific protein kinase activity (Nishimura et al., 1982; Cooper et al., 1982; Pike et al., 1983). Also the products of retroviral oncogenes exhibit tyrosine specific protein kinase activity. Therefore, it is possible that increased tyrosine phosphorylation is involved in the triggering of mitogenesis (Hunter, 1985). After binding, the mitogen or polypeptide growth factor stimulates a complex set of early events in the membrane, cytosol, and nucleus. These events are: an increase in the fluxes of Na^+, K^+, and H^+ across the plasma membrane (Smith and Rosengurt, 1978).

In addition to monovalent ion fluxes, there is a marked increase in the infflux of Ca^{++} (Lopez-Rivas et al., 1983). The mobilization of Ca^{++} may be mediated by inositoltriphosphate (Berridge and Irvine, 1984).

Inositoltriphosphate is formed as a result of increased hydrolysis of phosphatidylinositol 4,5 bisphosphate in the plasma membrane, and this process also generates diacylglycerol. Diacylglycerol is a potent activator of purified protein kinase C or protein kinase C in intact cells. This enzyme is a major receptor for tumor promoters of the phorbol ester family (Nishizuka, 1984).

Activation of this enzyme directly or indirectly leads to increased activity of the Na^+/H^+ antiport system, which in turn increases intracellular pH and stimulates the Na^+/K^+ pump activity (Vara and Rosengurt, 1985). Polypeptide growth factors like platelet-derived growth factor (PDGF) also elicit a striking release of arachidonate which is partly derived from diacylglycerol. Arachidonate is converted to many biologically active metabolites including E-type prostaglandins. This in turn leaves the cells and stimulates the synthesis of cAMP via its own receptor. It was shown that cAMP constitutes a growth promoting signal for 3T3 mouse fibroblasts (Rosengurt et al., 1981). In addition to these events that occur in plasma membranes and cytosols, polypeptide growth factors also rapidly and transiently induce the expression of the cellular oncogenes c-fos and c-myc. These oncogenes encode nuclear proteins that might have a role in mitogenesis (Rosengurt, 1986).

Any of the events described above that finally lead to DNA synthesis and cell division might be a potential target for the antiproliferative effect of SMAL. SMAL might prevent the binding of a polypeptide growth factor (in the case of EL-4 or CEM-CM_3 cells, IL-2) to its specific receptor, or SMAL might inhibit the tyrosin specific protein kinase activity of the receptor, and/or inhibit protein kinase C. The exact mechanism of action of SMAL on the inhibition of cell growth and inhibition of uptake and incorporation of exogenous thymidine remains open for future investigations. Further information on the molecular mechanism of action of SMAL might be useful to elucidate some of the unknown events in the signal transduction for mitogenesis and cell growth.

In summary, we have demonstrated in these studies that SMAL is an acidic protein made by tonsil lymphocytes which has the ability to suppress the incorporation of exogenous thymidine into con A-stimulated normal human PBL, human leukemia T cell line CEM-CM_3, normal human embryonic lung fibroblast MRC, human epidermal carcinoma HEP-2, mouse thymoma EL-4, and mouse macrophage cell line P388D. SMAL is thermostable at 56°C, but thermolabile at 70°C. It is stable in glycine pH 2, but destroyed in HCl and TCA. SMAL is also destroyed by proteolytic enzymes. Its M.W. under nondenaturing conditions is in excess of 100,000, but it may be a polymer whose smallest M.W. may be 30,000. The activity noted in the heterogenous YM-100 or XM-100 retentates of TL culture supernatants is the same as that expressed by purified SMAL, i.e. suppression of incorporation of ^3H-tdr and inhibition of proliferation of target cells.

Future research on SMAL should focus in the following areas:

1. Production of large quantities of SMAL for further purification and characterization by either establishing a hybridoma between tonsil lymphocytes and an appropriate partner like CEM-CM_3 line, or screening a variety tumor cell lines for constitutive release of SMAL.
2. Replacement of EL-4 cells by CEM-CM_3 cell line in the bioassay for SMAL. Because these cells are more sensitive to the effect of SMAL.

3. Production of monoclonal antibody against HPLC-purified SMAL. This antibody can be used to prepare immunoadsorbent affinity columns for purification of SMAL, and for development of ELISA or RIA for detection of SMAL.
4. Identification, purification and characterization of receptor(s) for SMAL using radiolabeled highly purified SMAL and following the fate of SMAL after binding to its receptor(s).
5. Determination of effect of SMAL on protein phosphorylation, specifically tyrosine-specific protein kinase activity and protein kinase C activity.
6. Partial determination of N terminal amino acid sequence of highly purified SMAL which may lead to the synthesis of an oligonucleotide probe, and utilization of this probe for identification, isolation, and cloning of the gene(s) for SMAL.
7. Information derived from the above experiments might be helpful in unraveling the apparently complicated mechanism of control of cell proliferation and cell growth.
8. The antiproliferative effect of SMAL should be investigated in vivo, that is to test if SMAL inhibits tumor formation in experimental animals like mice. SMAL might have some antitumor potential.

REFERENCES

Adams, D. H. and P. B. Gahan. 1982. Stimulated and non-stimulated rat spleen cells release different DNA-complexes. Differentiation, Springer-Verlag. 22:47.

Adorini, L. 1982. Biochemical and functional analysis of antigen-specific products obtained from virus-transformed, lysosome specific suppressor T cell lines. The Lymphokines 5:411.

Aggarwal, B. B., W. J. Henzel, B. Moffat, W. J. Kohr, and R. N. Harkins. 1985a. Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line. J. Biol. Chem. 260:2334.

Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spenar, W. J. Henzel, T. S. Bringman, G. E. Nedwin, D. V. Goeddel, and R.N. Harkins. 1985b. Human tumor necrosis factor. Production, purification, and characterization. J. Biol. Chem. 260:2345.

Aggarwal, B. B., B. Moffat, and R. N. Harkins. 1984. Human lymphotoxin. Production by a lymphoblastoid cell line, purification, and initial characterization. J. Biol. Chem. 259:686.

Altman, A., and D. Katz. 1980. Production and isolation of helper and suppressor factors. J. Immun. Meth. 38:9.

Anker, P., M. Stroun, and P. A. Maurice. 1975. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. Cancer Res. 35:2375.

Anker, P., M. Stroun, and P. A. Maurice. 1976. Spontaneous extracellular synthesis of DNA released by human blood lymphocytes. Cancer Res. 36:2832.

Aronow, B., and B. Ullman. 1985. Thymidine incorporation in nucleoside transport-deficient lymphoma cells. J. Biol. Chem. 260(30):16274.

Ashkenazi, E. R., and I. E. Ashkenazi. 1976. Incorporation and uptake of thymidine and uridine by Hela cells: Effect of DNA extract prepared from normal human fibroblasts. In Vitro 12(6):442.

Aune, T. M., and W. C. Pierce. 1981a. Conversion of soluble immune response suppressor factor by peroxide. Proc. Natl. Acad. Sci. USA. 78:5099.

Aune, T. M., and C. W. Pierce. 1981b. Identification and initial characterization of a nonspecific suppressor factor produced by (SIRS)-treated macrophages. J. Immunol. 127:1828.

Aune, T. M., and C. W. Pierce. 1982. Activation of a suppressor T cell pathway by interferon. Proc. Natl. Acad. Sci. USA 79:3803.

Aune, T. M., D. R. Webb, and C. W. Pierce. 1983. Purification and initial characterization of the lymphokine, soluble immune response suppressor. J. Immunol. 131:2848.

Badger, A. M., S. R. Cooperband, and J. A. Green. 1971. Effect of proliferation inhibitory factor on the clonal growth of target cells. J. Immunol. 197:1259.

Banfalvi, G., K. Szyfter, and F. Antoni. 1984. Analysis of 5'-ends of short DNA fragments excreted by phytohemagglutinin stimulated lymphocytes. Biochem. Biophys. Res. Comm. 118:140.

Baserga, R. 1981. The cell cycle. New England J. of Med. 304:453.

Berridge, M. J., and R. F. Irvine. 1984. Inosine triphosphate, a novel second messenger in cellular transduction. Nature 312:315.

Blalock, J. E., J. A. Georgiades, and N. F. Johnson. 1980. Purified human interferon has more potent anticellular activity than fibroblast interferon. Cell. Immunol. 49:390.

Boldt, D. H., R. P. MacDermott, S. F. Speckart, and G. S. Nash. 1977. Excretion of DNA by purified human lymphocyte subpopulations. J. Immunol. 118:1495.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248.20.

Bradshaw, H. D., Jr. 1983. Molecular cloning and cell cycle specific regulation of a functional human thymidine kinase gene. Proc. Natl. Acad. Sci. USA 80:5588.

Brobner, P., and P. Loidl. 1984. Thymdiine kinase a novel affinity chromatography of the enzyme and its regulations by phosphorylation in physarum polycephalum. J. Biol. Chem. 259:8212.

Brochier, J., C. L. T. Samarut, and J. P. Revillard. 1978. Studies of human T and B lymphocytes with heterologous antisera. Immunology 35:827.

Carswell, E. A., L. J. Old, R. I. Kassel, S. Green, N. Fiore, and B. Williams. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U.S.A. 72:3666.

Cass, C. E. and A. R. P. Paterson. 1972. Mediated transport of nucleosides in human erythrocytes. J. Biol. Chem. 247:3341.

Cheng, T. C., K. C. Chadha, and R. G. Hughes, Jr. 1977. Biochemical and immunological characterization of thymdiine kinase of thymidine kinaselen Hela cells biochemically transformed by Herpes simplex virus. Inf. Immun. 16:486.

Chiba, K., T. Nishimura, and Y. Hashimoto. 1985a. Stimulated rat T cell-derived inhibitory factor for cellular DNA synthesis (STIF). I. Isolation and characterization. J. Immunol. 134:1019.

Chiba, K., T. Nishimura, and Y. Hashimoto. 1985b. Stimulated rat T cell-derived inhibitory factor for cellular DNA synthesis (STIF). III. Effect on cell proliferation and immune response. J. Immunol. 134:3172.

Claycomb, W. C. 1979. DNA synthesis and DNA enzymes in terminally differentiating cardiac muscle cells. Exper. cell Res. 118:111.

Cohen, S., E. Pick, J. J. Oppenheim. 1979. Biology of the Lymphokines. Acad. Press, New York.

Cooper, J. A., D. F. Bowen-Pope, E. Raines, R. Ross, and T. Hunter. 1982. Similar effect of platelet-drived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. Cell 31:263.

Coppock, D. L., and A. B. Paradee. 1985. Regulation of thymidine kinase activity in the cell cycle by a labile protein. J. Cell. Phys. 124:269.

Dumonde, D. C., R. A. Wolstencroft, G. S. Panayi, M. Mathew, J. Morley, and W. T. Hawson. 1969. Lymphokines: non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature 224:38.

Enggan, T., J. L. Brumley, and M. B. Van der Weyden. 1983. Human thymidine kinase. J. Biol. Chem. 258:7000.

Friedman, S. J., C. D. Trotter, T. Kimball, and P. J. Skehan. 1977. The inhibition of thymidine metabolism in tumor cells treated with D-glucosamine. Cancer Res. 37:1141.

Fujiwara, H., Z. Toossie, K. Ohnishi, K. Edmonds, and J. J. Ellner. 1987. Spontaneous production of a suppressor factor by a human macrophage-like cell line U937. II. Suppression of antigen- and mitogen-induced blastogenesis, IL-2 production and IL-2 receptor expression in T lymphocytes. J. Immunol. 138:197.

Garner, W. D., J. L. Farmer, and M. D. Prager. 1980. Human plasma-derived immunosuppressive factors having anti-lymphoma activity. Cancer Immunol. Immunother. 10:7.

Gaulton, G. N., and D. D. Eardley. 1986. Interleukin 2-dependent phosphorylation of Interleukin 2 receptors and other T cell membrane proteins. J. Immunol. 136:2470.

Gehring, U., B. Segnitz, B. Foellmer, and U. Francke. 1985. Assignment of the human gene for the glycocorticoid receptor to chromosome 5. Proc. Natl. Acad. Sci. USA 82:3751.

Gewert, D. R., S. Cohen, B. R. G. Williams. 1984. The effect of interferon on cells deficient in nucleoside transport or lacking thymidine kinase activity. Biochem. Biophys. Res. Commun. 118:124.

Gewert, D. R., G. Moore, and M. J. Clemens. 1983. Inhibition of cell division by interferon: The relationship between changes in utilization of thymidine for DNA synthesis and control of proliferations in daudicelles. Biochem. J. 214:983.

Gewert, D. R., S. Shah, and M. J. Clemens. 1981. Inhibition of cell division by interferons: Changes in transport and intracellular metabolism of thymidine in human lymphoblastoid cells. Eur. J. Biochem. 116:487.

Gillis, S., P. E. Baker, F. W. Ruscetti, and K. A. Smith. 1978. Long term culture of human antigen-specific cytotoxic T-cell lines. J. Exp. Med. 148:1093.

Gillis, S., P. E. Baker, N. A. Union, and K. A. Smith. 1979. The in vitro generation and sustained culture of nude mouse cytolytic T-lymphocytes. J. Exp. Med. 149:1460.

Gillis, S., and F. P. Inman. 1985. The Interleukins. Cont. Topics Mol. Immunol. Vol. 10:1.

Gillis, S., and K. A. Smith. 1977. Long term culture of tumor-specific cytotoxic T cells. Nature 268:154.

Gillis, S., K. A. Smith, and J. Watson. 1980. Biochemical and biological characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. J. Immunol. 124:1954.

Gillis, S. and J. Watson. 1980. Biochemical and biological characterization of lymphocytes regulatory molecules. V. Identification of an Interleukin 2 producer human leukemic T cell line. J. Exp. Med. 152:1709.

Granger, G. 1978. The human lymphotoxin system. Physical and chemical heterogeneity of lymphotoxin molecules released by mitogen activated human lymphocytes. Cell. Immun. 38:388.

Granger, G. A., S. J. Shacks, T. W. Williams, and W. P. Kolb. 1969. Lymphocyte in vitro cytotoxity: Specific release of lymphotoxin-like molecules from tuberculin-sensitive lymphoid cells. Nature 221:1155.

Granger, G. A., R. S. Yamamoto, D. S. Fair, and J. C. Hiserodt. 1978. The human lymphotoxin system. I. Physiochemical heterogeneity of LT released by human lymphocytes. Cell. Immunol. 38:388.

Gray, P. W.., B. B. Aggarwal, C. L. Benton, T. S. Bringman, W. J. Hanzel, J. A. Jarrett, and G. E. Nedwin. 1984. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumor necrosis activity. Nature 312:721.

Green, J. A., S. R. Cooperband, J. A. Rustein, and S. Kilbrick. 1970. Inhibition of target cell proliferation by supernatant from cultured human PBL. J. Immunol. 105:48.

Green, S. S., R. Witsal, and K. W. Sell. 1974. Mitogen-stimulated lymphocyte inhibition by supernatants of crowded lymphocytoblasts. Transplantation 18:496.

Greene, W. C., T. A. Fleisher, and T. A. Waldman. 1981. Soluble suppressor supernatants elaborated by concanavalin A-activated human mononuclear cells. I. Characterization of a soluble suppressor of T cell proliferation. J. Immunol. 126:1185.

Gresser, I., and M. G. Tovey. 1978. Antitumor effect of interferon. Biochem. Biophys. Acta 516:231.

Gutierrez, C., R. R. Bernake, and J. Vegd. 1979. Purification of human T and B cells by a discontinuous density gradient of Percoll. J. Immunol. Meth. 29:57.

Hadden, J., W. Stewart. 1981. The Lymphokines. Human Press, New Jersey.

Helson, L., S. Green, E. Carswell, and L. J. Old. 1975. Effect of tumor necrosis factor on melanoma cells. Nature 258:731.

Hiserodt, J. C., A. M. Pricine, and C. A. Granger. 1976. Evidence of Multiple cytotoxic molecules secreted by mitogen-activated human lymphoid cells. Cell. Immunol. 24:277.

Hodge, T. W. and F. P. Inman. 1982. Characterization of a suppressor of mitogen-activated lymphocytes. J. Biol. Resp. Modif. 1:163.

Hoffman, F., C. F. Spina, N. Rana, and J. L. Fahey. 1980. Soluble suppressor factor produced by PWM-stimulated human PBL. Cell. Immunol. 55:20.

Hunter, T. 1985. Protein tyrosine kinase. Annu. Rev. of Biochem. 54:897.

Irigoyen, O., P. V. Rizzolo, Y. Thomas, L. Rogozinski, and L. Chess. 1981. Generation of functional human T-cell hybrids. J. Exp. Med. 154:1827.

James, R., and R. A. Bradshaw. 1984. Polypeptide growth factors. Annu. Rev. of Biochem. 53:259.

Jeffes, E. W. B., and G. A. Granger. 1975. Relationship of cloning inhibition factor, lymphotoxin, and proliferation inhibition factor release by mitogen-activated human lymphocytes. J. Immunol. 114:64.

Jegasothy, B. V., and D. R. Battles. 1979. Immuno suppressive lymphocyte factors. I. Purification of inhibitor of DNA synthesis to homogeneity. J. Exp. Med. 150:622.

Jegasothy, B., and D. B. Battles. 1981. Immunosuppressive lymphocyte factors. III. Complete purification and partial characterization of a human inhibitor of DNA synthesis. Mol. Immunol. 18:395.

Kasakura, S., M. Taguchi, T. Murachi, H. Uchino, and M. Hanaoka. 1983. A new mediator (suppressor cell induction factor) activating T cell mediated suppression. Characterization of suppressor cells, kinetics of their generation, and mechanism of their action. J. Immunol. 131:2307.

Klein, K., M. L. Coetzee, R. Madhav, and P. Ove. 1979. Inhibition of tritiated thymidine incorporation in cultured cells by rat kidney extract. J. Nat. Cancer Inst. 62:1557.

Kouttab, N., N. Tannir, S. Pathak, A. Berger, C. G Sahasrabuddhe, and A. L. Maizel. 1985. Establishment of stable human T-T hybridonas. J. Immunol. Meth. 77:165.

Kramer, M., and U. Koszinowski. 1982. T cell-specific suppressor factors with regulatory influence on IL-2 production and function. J. Immunol. 128:784.

Kuo, L.-M., and R. J. Robb. 1986. Structure-function relationships for the IL-2-receptor system. I. Localization of a receptor binding site on IL-2. J. Immunol. 137:1538.

Lachman, L. B., and A. L. Maizel. 1983. Human immunoregulatory molecules: Interleukin 1, Interleukin 2, and B-cell growth factor. Cont. Topics Mol. Immun. 9:147.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature. 227:680.

Larsson, E. L., and H. Blomgren. 1978. Evidence that non-specific inhibitors are induced by soluble products of activated human lymphocytes. Int. Arch. Allergy. Appl. Immunol. 57:333.

Lederman, M. M., J. J. Ellner, and H. M. Rodman. 1981. Generation of a soluble immune response suppressor factor (IRSF) by unstimulated leukocytes of healthy subjects. Clin. Exp. Immunol. 45:191.

Lee, G. M., and E. M. Rasch. 1984. Cytophotometric comparisons of DNA levels in neuronal and glial cells of the cerebellum: A comparative study. Cell Biochem. and Function 2:225.

Lee, S. C., and Z. J. Lucas. 1977. Regulatory factors produced by lymphocytes. II. Inhibition of cellular DNA synthesis is associated with a factor inhibiting DNA polymerase activity. J. Immunol. 118:88.

Lopez-Rivas, A., and E. Rosengurt. 1983. Serum rapidly mobilizes calcium from an intracellular pool in quiescent fibroblastic cells. Biochem. Biophys. Res. Commun. 114:240.

Lundblad, D., and E. Lundgren. 1982. Changes in DNA-polymerase and thymidine kinase activity during interferon treatment. Biochem. Biophys. Res. Commun. 105:1569.

Madhav, R., M. L. Coetzee, and P. Ove. 1980. Purification of thymidine kinase by affinity chromatography with an enzyme inhibitor as the ligand. Arch. Biochem. Biophys. 200:99.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory, p. 161.

Männel, D. N., R. N. Moore, and S. E. Mergenhagen. 1980. Macrophages as a source of tumoricidal activity (tumor necrotizing factor). Infect. Immun. 30:523.

Matthews, N. 1981. Production of a anti-tumor cytotoxin by human monocytes. Immunol. 44:135.

Matthews, N., and J. F. Watkins. 1978. Tumor necrosis factor from the rabbit. I. Mode of action, specificity, and physiochemical properties. Br. J. Cancer 38:302.

Medoff, J. R., V. D. Clack, and J. K. Roche. 1986. Characterization of an immunosuppressive factor from malignant ascites that resembles a factor induced in vitro by carcinoembryonic antigen. J. Immunol. 137:2057.

Mei-Huei, T. L., and G. Weber. 1983. Increased concentration of thymidine kinase in rat hepatomas. Biochem. Biophys. Res. Commun. 111:280.

Melamed, J. 1978. Mitogenic induction of macrophage and neutrophil proliferation in human tonsils. Clin. Immunol. Immunopath. 11:406.

Morgan, D. A., F. Ruscetti, and R. Gallo. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrow. Science 193:1007.

Namba, R., and B. H. Waksman. 1975. Regulatory substances produced by lymphocytes. I. Inhibitor of DNA synthesis in the rat. Inflammation 1:5.

Nishimura, J., J. S. Huang, and T. F. Deuel. 1982. Platelet-drived growth factor stimulates tyrosine-specific protein kinase activity in Swiss mouse 3T3 cell membranes. Proc. Natl. Acad. Sci. USA 79:4303.

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 208:593.

Nissen-Meyer, J., and J. Hammerström. 1982. Physicochemical characterization of cytostatic factors released from human monocytes. Infect. Immun. 38:67.

Nowowiejeski-Wieder, I., T. M. Aune, C. W. Pierce, and D. R. Webb. 1984. Cell free translation of the lymphokine soluble immune response suppressor (SIRS) and characterization of its mRNA. J. Immunol. 132:556.

Okada, M., N. Yoshimura, T. Kaieda, Y. Yamamura, and T. Kishimoto. 1981. Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc. Natl. Acad. Sci. USA 78:7717.

Oliver, J. M., and A. R. P. Paterson. 1971. Nucleoside transport. A mediated protein in human erythrocytes. Canadian J. Biochem. 49:262.

Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, B. B. Aggarwal, M. A. Palladino, and D. V. Groeddel. 1984. Human tumor necrosis factor: Precursor structure, expression, and homology to LT. Nature 312:724.

Pick, E. 1981. Lymphokines: A forum for immunoregulatory cell products. Vol. 1 and 2. Academic Press, New York.

Pike, L. J., D. F. Bowen-Pope, R. Russ, and E. G. Krebs. 1983. Characterization of platelet-derived growth factor-stimulated phosphorylation in cell membranes. J. Biol. Chem. 258:9383.

Plagemann, P. G., and J. Erbe. 1972. Thymidine transport by cultured novikof hepatoma cells and uptake by simple diffusion and relationship to incorporation into DNA. J. Cell. Biol. 55:161.

Plagemann, P. G., and E. Erbe. 1974. The deoxyribonucleoside transport systems of cultured novikoff rat hepatoma cells. J. Cell. Phys. 83:337.

Rabin, E. M., J. J. Mond, J. Ohara, and W. E. Paul. 1986. Interferon- inhibits the action of B cell stimulatory factor (BSF)-1 on resting B cells. J. Immunol. 137:1573.

Ransom, J., C. H. Evans, A. E. Jones, and J. A. DiPaolo. 1983. Control of the carcinogenic poetntial of technetium by immunologic hormone lymphotoxin. Cancer Immunol. Immunother. 15:126.

Ransom, J., J. H. Evans, R. P. McCabe, N. Pomato, J. A. Heinbaugh, M. Chin, and M. G. Hanna. 1985. Leukoregulin, a direct-acting anticancer immunological hormone that is distinct from lymphotoxin and interferon. Cancer Res. 45:851.

Renk, C. M., R. K. Gupta, and D. L. Morton. 1980. Immunosuppressive factors from human breast carcinoma cell lines that affect initiation of lymphocyte proliferation. Cancer Immunol. Immunother. 9:55.

Rich, R. R., and D. L. Kastner. 1978. Suppressor factor activity in cell-mediated immune responses. J. Retic. Soc. 24:417.

Rich, R. R., and C. W. Pierce. 1974. Biological expression of lymphocyte activation. III. Suppression of plaque-forming cell responses in vitro by supernatan fluids from con A-activated spleen cell cultures. J. Immunol. 112:1360.

Rocklin, R. E., A. Blidy, J. Butler, and J. Minowada. 1982. Partial characterization of a lymphoid cell line product with leukocyte inhibitory factor activity. J. Immunol. 127:534

Rogers, J. C. 1976. Characterization of DNA excreted from phytohemagglutinin-stimulated lymphocytes. J. Exp. Med. 143:1249.

Rogers, J. C., D. Boldt, S. Kornfeld, S. A. Skinner, and C. R. Valeri. 1972. Excretion of DNA by lymphocytes stimulated with PHA or antigen. Proc. Natl. Acad. Sci. USA. 69:1685.

Rogers, J. C., and J. W. Kerstin. 1981. Capping of DNA on PHA-stimulated human lymphoblasts. J. Immunol. 126:703

Rogers, J. C. and T. E. Rucinsky. 1982. Unstable high molecular weight inverted repetitive DNA in human lymphocytes. Nucleic Acids Res. 10:5483.

Rose, R. M., A. S. Wasserman, W. Y. Weiser, and H. G. Remold. 1986. An acidic lymphokine distinct from Interferon- inhibits the replication of herpes simplex virus in human pulmonary macrophages. Cell. Immunol. 97:397.

Rosenberg, S. A., and E. A. Grimm. 1984. Biological activity of recombinant Interleukin-2 produced in E. coli. Science 223:1412.

Rosenberg, S. A., M. Henrichon, J. A. Cyone, and J. A. David. 1973. In vitro studies of LT produced in response to antigen stimulation of lymphocytes. J. Immunol. 110: 1623.

Rosengurt, E. 1986. Early signals in the mitogenic response. Science. 234:161.

Rosengurt, E., A. Legg, G. Strang, and N. Luck. 1981. Cyclic AMP. A mitogenic signal for Swiss 3T3 cells. Proc. Natl. Acad. Sci. USA 78:4932.

Rubenstein, M. 1982. The structure of human interferons. Biochem. Biophys. Acta. 695:5.

Rubin, B. Y., and S. L. Gupta. 1980. Differential efficacies of human type I and type II interferon as antiviral and antiproliferative agents. Proc. Natl. Acad. Sci. USA 77:5928.

Ruff, M. R., and G. E. Gifford. 1980. Purification and physico-chemical characterization of rabbit tumor necrosis factor. J. Immunol. 125:1671.

Ruff, M. R., and G. E. Gifford. 1981a. Tumor necrosis factor in Lymphokine Reports. Vol. 2, pp. 235-275. Edited by E. Pick. Academic Press, N.Y.

Ruff, M. R., and G. E. Gifford. 1981b. Rabbit tumor necrosis factor: mechanism of action. Infect. Immun. 31:380.

Ruscetti, F., D. A. Morgan, and R. Gallo. 1977. Functional and morphological characteristics of human T cells continuously grown in vitro. J. Immunol. 119:131.

Sasvari-Szekely, M., M. Staub, J. Taljanidisz, G. Szabo, Jr., J. Takacs, and F. Antoni. 1984. Cultured tonsillar lymphocytes excrete [^{3}H] thymidine labeled DNA and revert to resting state. Biochem. Biophys. Res. Commun. 125:1110.

Sawada, J., K. Shiori, and T. Osowa. 1976. Cytotoxic activity of purified lymphotoxin against various cell lines. Jpn. J. Exp. Med. 4:263.

Sayers, T. J., J. H. Ransom, A. C. Denn, III, R. B. Herberman, and J. J. Ortaldo. 1986. Analysis of a cytostatic lymphokine produced by incubation of lymphocytes with tumor cells: Relationship to leukoregulin and distinction from recombinant lymphotoxin, recombinant tumor necrosis factor, and natural killer cytotoxic factor. J. Immunol. 137:385.

Schnaper. H. W., and T. M. Aune. 1986. Suppression of immune responses to SRBC by the lymphokine (SIRS) in vivo. J. Immunol. 137:863.

Schnaper, H. W., C. W. Pierce, and T. M. Aune. 1984. Identification and initial characterization of con A and interferon induced human suppressor factors: Evidence for a human equivalent of murine soluble immune response suppressor (SIRS). J. Immunol. 132:2429

Schreier, M. H., N. N. Iscov, R. Tees, L. Aarden, and H. V. Boehmer. 1980. Clones of killer and helper T cells: Growth requirements, specificity and retention of function in long-term culture. Immunological Rev. 51:315.

Seigel, L. J., F. Wong-Stall, R. C. Gallo, W. G. Nash, and S. J. O'Brien. 1984. Gene for T-cell growth factor: Location on human chromosome 4 g and feline chromosome B1. Science 223:175.

Sesterhan, K. G., R. F. Krueger, and C. Uhlmann. 1977. Percent distribution of T and B cells in tonsils of children, juveniles, and adults. Arch. Oto-Rhino-Laryng 218:37-41.

Shou, L., S. Schwartz, R. Cood, R. Peng, and C. L. Chen. 1980. A human soluble suppressor factor affecting lymphocyte responses in vitro. Proc. Natl. Acad. Sci. USA 77:6096.

Shuichiro, M., N. Nishino, K. Obaru, S. Mita, K. Fujimoto, and K. Onoue. 1983. Cloning of Interleukin-2 mRNA from human tonsils. Biochem. Biophys. Res. Commun. 115:1040.

Smith, J. B., and E. Rosengurt. 1978. Serum stumulates the Na^+, K^+ pump in quiescent fibroblasts by increasing N^+ entry. Proc. Natl. Acad. Sci. USA 75:5560.

Smith, K. A., and F. Ruscetti. 1981. T-cell growth factor and the culture of cloned functional T cells. Adv. Immunol. 31:137.

Spofford, B. T., R. A. Daynes, and G. A. Granger. 1974. Cell mediated immunity in vitro: a highly sensitive assay for lymphotoxin. J. Immunol. 112:2111.

Staub, M., and F. Antoni. 1978. Excretion of newly synthesized DNA by tonsil lymphocytes. Nucleic Acids Res. 5:3071.

Switzer, R. C., C. R. Merril, and S. Shifrin. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. 98:231.

Tadakuma, T., and C. W. Pierce. 1978. Mode of action of a soluble immune response suppressor produced by con A-stimulated spleen cells. J. Immunol. 120:481.

Taniguchi, T., H., Matsui, T. Fujita, C. Takaoka, N. Kashimo, R. Yochimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human IL-2. Nature 302:305.

Taussig, M. 1980. Antigen specific T cell factors. Clin. Immunol. and Immunopath. 9:23.

Tesoriere, G., R. Tesoriere, R. Vento, M. Gluliano, and G. Cantoro. 1984. The inhibitory effect of D. glucosamine on thymidine kinase in chick embryo retinas and Hela cells. Experientia 40:705-707.

Theodore, A. C., D. M. Center, W. W. Cruikshank, and D. J. Beer. 1986. A human T-T-cell hybridoma-derived lymphocyte chemoattractant factor. Cell. Immunol. 98:411.

Thomas, D. W., W. K. Roberts, and D. W. Talmadge. 1975. Regulation of the immune response: Production of a soluble suppressor by immune spleen cells in vitro. J. Immunol. 114:1616.

Tibbetts, L., P. R. Glade, and P. S. Papageorgiou. 1975. Presence of a reversible inhibitor for human lymphoid cell DNA synthesis in extracts of human PBL. Cell. Immunol. 18:384.

Tyring, S., G. R. Klimpel, and S. Baron. 1982. Direct cytolysis by partially purified interferon. Int. J. Cancer 30:59.

Valle, M. J., G. W. Jordan, S. Haahr, and T. C. Merigan. 1975. Characteristics of immune interferon produced by human lymphocyte cultures compared to other human interferons. J. Immunol. 115:230.

Vara, F., and Rosengurt. 1985. Stimulation of Na^+/H^+ antiport activity by epidermal growth factor and insulin occurs without activation of protein kinase C. Biochem. Biophys. Res. Commun. 130:646.

Vesole, D., J. M. Goust, J. Fett, P. Arnaud, and H. Fudenberg. 1979. An inhibitor of DNA synthesis produced by established lymphoid cell lines. Clin. Immunol. Immunopath. 14:489

Waksman, B. H. 1979. Role of different lymphokines in regulating immunological reactivity. In subcellular factors in immunity. Ed. by: H. Friedman. New York Academy of Sciences, New York, p. 299.

Waldman, T. A. 1986. The structure, function, and expression of IL-2 receptors on normal and malignant lymphocytes. Science 232:727.

Wang, A., L. Shi-Da, and D. F. Mark. 1984. Site specific mutagenesis of the human Interleukin-2 gene: Structure-function analysis of the cystein residues. Science 224:1431.

Ware, C. F., and G. A. Granger. 1979. A physicochemical and immunologic comparison of the cell growth inhibitory activity of human lymphotoxins and interferons in vitro. J. Immunol. 122:1763.

Watson, J. 1979. Continuous proliferation of murine antigen specific helper T-lymphocytes in culture. J. Exp. Med. 150:1510.

Watson, J. D., S. Gillis, J. Marbrook, D. Mochizuki, and A. K. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. J. Exp. Med. 150:849.

Webb, D. R., K. Mason, G. Semenuk, T. M. Aune, and C. W. Pierce. 1985. Purification and analysis of soluble immune response suppressor. J. Immunol. 135:3238.

Williams, R., and S. Korsmeyer. 1978. Studies of human lymphocyte interactions with emphasis on soluble suppressor activity. Clin. Immunol. Immunopath. 9:335.

Wiranowska-Stewart, M., and W. E. Stewart. 1981. Interferons. Types alpha, beta and gamma in the Lymphokines: Biochemistry and Biological Activity. pp. 133-148. Edited by J. W. Hadden and W. S. Stewart. Humana Press, Clifton, N.J.

Wolf, R. L., H. Whitsed, F. S. Rosen, and E. Merler. 1978. A soluble inhibitor of B and T cell proliferation and antibody synthesis produced by dividing human T cells. Cell Immunol. 36:231.

Wu, J.-S. R., F. Y. P. Kwong, S. M. Jarvis, and J. D. Young. 1983. Identification of the erythrocyte nucleoside transporter as a band 4.5 polypeptide. J. Biol. Chem. 258:13745.

Young, J. D., S. M. Jarvis, J. A. Belt, W. P. Gati, and A. R. P. Peterson. 1984. Identification of the nucleoside transporter in cultured mouse lymphoma cells. J. Biol. Chem. 259:8363.

Young, J. D., S. M. Jarvis, M. J. Robins, and A. R. P. Peterson. 1983. Photoaffinity labeling of the human erythrocyte nucleoside transporter by nitrobenzylthiodinosine. J. Biol. Chem. 258:2202.

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Zarnegar, A., and F. P. Inman. 1987. Characterization and purification of an inhibitor of pyrimidine salvate pathway produced by human lymphocytes. Manuscript in preparation.

Zarnegar, A., and F. P. Inman. 1985. Identification of a factor from human lymphocytes that inhibits incorporation of thymidine into EL-4 cell line. Presented at Southeastern Immunology Conference, Atlanta, Georgia and at the Research Forum at East Tennessee State Univeristy.

A. Zarnegar and R. N. Kinsely. 1982. Removal of bacteriophases of E. coli and S. feacium from water by soil. M. S. Thesis.