PARTIAL CHARACTERIZATION AND PURIFICATION OF A MURINE SUPPRESSOR-INDUCER FACTOR SECRETED BY A NATURAL SUPPRESSOR CELL LINE

and

CHARACTERIZATION OF THE BINDING OF ANTISERA RAISED TO MURINE ANTIGEN-SPECIFIC SUPPRESSIVE MATERIAL IN HUMAN PERIPHERAL LEUKOCYTES

BY

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B.Sc., University of Toronto, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
Microbiology

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ABSTRACT

A large amount of effort has gone into the elucidation of the mechanism of suppression of the immune system. This level of immunoregulation has been demonstrated to be mediated by both antigen-nonspecific and antigen-specific protein factors elicited by leukocytes.

In this work, two different modes of immunosuppression were investigated. First, an attempt was made to purifiy an antigen-nonspecific protein factor, SIF (Suppressor Inducer Factor) secreted by the Natural Suppressor cell line M1-A5. M1-A5 culture supernatants were subjected to ion exchange chromatogrphy (IEC) and fast protein liquid chromatography (FPLC). Bioactivity of eluted fractions was determined by the plaque forming cell assay and followed through the purification. Reducing SDS-PAGE of selected fractions suggested that bands with Mr's of >110 KD and/or 55 KD were mediating the suppressive activity. In addition, an assay was developed to further investigate the mode of action of SIF.

Second, the binding of two antisera raised to components associated with murine antigen-specific suppression was studied using human peripheral leucocytes and several human tumour cell lines. Anti-p80 and anti-p30 binding was found to be variable (within a range) and to involve two populations of human mononuclear cells. Subsequently, it was found that all $CD3^+$ (T cells), $CD19^+$ (B cells) and neutophils expressed both the p80 and p30 determinants.

Four human leukemic cell lines were found to express varying levels of the p80 and p30 determinants. Cell lysates from each of the cell lines were subjected to Western blot analyses using anti-p30. The results showed that anti-p30 binds to a major band of 42 KD and minor bands of 60 and 80 KD in all lysates. In addition, a 25 KD band was observed in RAJI and CEM-CM3 lysates only. Thus, it appears that HuT 78 cells sythesize but are unable to express the p30-containing, 42 KD molecule on the cell surface.

No firm conclusions can be made with respect to the biochemical nature or mechanisism of action of either of the two suppressor factors studied in this work. Research into the mechanisms of suppression of the immune system is complex and multi-faceted, and it seems that for now, there will remain a gap in our overall understanding of immune regulation.

TABLE OF CONTENTS

LIST OF TABLES

SECTION 1:

SECTION 2:

LIST OF FIGURES

SECTION 1:

SECTION 2:

ACKNOWLEDGEMENTS

First I would like to thank Dr. Julia Levy and all of the people in her laboroatory for their continual support and useful discussions regarding these experiments. Special thanks to Janice North and Doug Carlow for their help and advice in the cell surface studies, and to Dr. Barbara Pope and her laboratory for screening fractions by PFC Assay in Halifax.

On a more personal note, I would like to pay tribute to my friends and family in Ontario who always manage to offer support and encouragement over the miles in trying times. And finally, thanks to D. Mitchell, for his friendship and constant faith in my abilities.

MAIN INTRODUCTION

AN OVERVIEW OF IMMUNE FUNCTION

The immune system has been functionally divided into the innate immune system and the adaptive immune system. In most cases, innate immunity comprises the first line of defense against invading microorganisms, however, if these defenses are overwhelmed or unable to function, the adaptive immune system is activated to fight the infection.

The two distinguishing features of the adaptive immune system are specificity and memory, both of which are mediated by T and B lymphocytes. Activated B cells secrete bifunctional molecules called immunoglobulins (Ig) or antibodies (Ab) that bind to specific antigen (via the Fab domain), to Fc receptors on phagocytes and fix complement (via the Fc domain). T cells can be further subdivided into cytotoxic/ suppressor T lymphocytes (CTL/Ts) and helper/inducer T lymphocytes (Th). Upon activation, T cells secrete protein factors (lymphokines) some of which act nonspecifically and others that bind specific antigen. In concert, the products released by antigen activated lymphoid and accessory cells (such as antigen-presenting cells which present antigen to T and B lymphocytes) regulate both the level and type of response generated to a specific antigen.

REGULATION OF IMMUNE RESPONSES BY T LYMPHOCYTES

Although a great deal of effort has resulted in a widely accepted mechanisim of induction of immune responses, relatively little has been learned about the manner in which one particular response is chosen over another, eg. cell mediated (CTL, or NK cells) versus humoral (Abs), or the way in which reponses are suppressed in normal and pathological states.

Most immune responses are the result of interactions between subsets of lymphocytes with regulatory and/or effector functions. T lymphocytes, in addition to their cytolytic functions (CTL), participate in immune response regulation by either inducing (Th), amplifiying (Th and Ts) or diminishing (Ts) specific immune cell responses.

Analysis of human and murine T lymphocyte subpopulations has been facilitated by the availability of monoclonal antibodies that define subset-specific cell surface determinants. Use of these reagents coupled with flow cytometric techniques has allowed the detection,

quantitation and isolation of subsets of cells expressing one, two and sometimes three specific cell surface markers. The most important division of the human T cell population has been into $CD4^+$ and $CD8^+$ subsets (rev. in Reinherz *et al.*, 1980), the murine homologs of which were originally designated L3T4 and Ly2 respectively (Dialynas *et al.*, 1983).

By *in vitro* analyses the $CD8^+$ subset was found to mediate most cytotoxic (CTL) and suppressor (Ts) functions and the $CD4^+$ subset was shown to provide helper/ inducer effects required for the differentiation of B cells into Ig-secreting cells and also for the maturation of $CD8^+$ CTL and $CD8^+$ Ts (Reinherz *et al.*, 1980). Although the correlation of CD4 /CD8 expression with biologic function has been useful, there exists a more consistent association with the class of major histocompatibility complex, or MHC (HLA in humans and H-2 in mouse) alloantigens used for antigen recognition (Englemen *et al.*, 1981). Thus $CD8^+$ T cells respond to antigen in association with Class I MHC proteins (A, B and C in humans and K, D and Qa in mouse) and $CD4^+$ cells respond to antigen in association with Class II MHC proteins (DP, DQ and DR in humans and I-A and I-E in mouse).

Characterization of the polypeptide hormones or lymphokines (LK) secreted by activated helper T cells (Th) and antigen-presenting cells (APC) has done much to advance our understanding of the inductive mechanisms for both T and B cell mediated immunity. Upon activation by antigen expressed in the context of class II MHC proteins, $CD4^+$ T cells synthesize and secrete a variety of growth-promoting and differentiation-inducing lymphokines such as IL-2, IL-4, IFN-γ and BCGF, whereas activated $CD8^+$ CTL cells secrete products which are ultimately cytotoxic to their targets and $CD8^+$ Ts secrete product(s) that have yet to be clearly characterized.

IMMUNOSUPPRESSION

The topic of immunosuppression is currently controversial (Moller, 1988 for eg.). Although it has been repeatedly demonstrated *in vivo* and *in vitro*, and in many different systems, that suppression of the immune system exists, it is curious that the mechanism(s) remain elusive. If taken together, all of the published work on immunosuppression suggest that it is a very heterogeneous phenomenon. Reports have shown that it involves dispartate molecules and cells ranging from antigen-specific suppressor factors and antigen-specific T cells to common cytokines and nonspecific

protein factors elicited from non-lymphoid hemopoietic cells (rev. in Teillaud and Fridman, 1989). Briefly, the major problems encountered in studies on suppression have been in cloning suppressor cell lines, isolating and characterizing suppressor factors and identifying distinct cell surface determinants to further define their population.

AN OVERVIEW OF RESEARCH TO BE PRESENTED

In this work, different aspects of two distinct yet related types of suppression were studied. First, an attempt was made to purify and partly characterize an antigen-nonspecific suppressor inducer factor (SIF) secreted by a third population LGL cell line (M1-A5) derived from the spleen of a tumor-bearing mouse. Second, two antisera (anti-p80 and anti-p30) raised against reduced components of a partially characterized murine antigen-specific suppressor factor (Fd11F) were used to determine their reactivity with normal human peripheral lymphocytes and several lymphoid leukemic cell lines and cell lysates.

As the work to be presented involves research within two different areas of immuno-suppression this thesis has been divided into two main sections entitled "Antigen Nonspecific Suppression" (Section 1) and "Antigen-Specific Suppression" (Section 2).

SECTION 1: ANTIGEN-NONSPECIFIC SUPPRESSION

INTRODUCTION

AN OVERVIEW OF ANTIGEN-NONSPECIFIC, OR NATURAL SUPPRESSION.

Natural suppression (NS) is defined as the ability of an LGL-like population of null cells to suppress immune functions in an antigen nonspecific, MHC nonrestricted manner (Saffran and Singhal, 1990 and Maier *et al*, 1986). The cell surface phenotype of NS cells derived from various locations was found to be similar to that reported for cloned natural killer (NK) cells (Thy-1$^+$, Ly-5$^+$, asialo-GM1$^+$, sIg$^-$, Lyt-1$^-$, Lyt-2$^-$, L3T4$^-$, Ia$^-$, MAC-1$^-$) (Brooks *et al*, 1982 and Schwadron *et al.*, 1989), yet cloned NS cells are unable to kill NK cell targets such as YAC-1 (Hertel-Wulff *et al*, 1984). A recent report attempting to sort out the relationship between NK and NS cells was only able confirm that these two cell types are related yet distinct (Saffran and Singhal, 1990). To further confuse the origin of NS cells, several cloned NS cell lines have been shown to harbor rearranged and expressed T cell receptor (TCR) genes. Thus even though the cell surface phenotype of NS cells is distinct from that of any mature T cell characterized to date, it is now assumed by some groups that NS cells are of the T cell lineage (Hertel-Wulff *et al.*, 1987).

SITES OF NS CELL ACTIVITY

NS activity was observed first in murine (Singhal *et al*, 1972 and Duwe and Singhal, 1979) and later, human (Bains *et al*, 1982 and Mortari *et al*, 1986) bone marrow (BM). BM-derived natural NS cells were reported to inhibit the *in vitro* generation of IgM Ab responses (Mortari *et al.*, 1986 for eg.), mitogen activation (Maier *et al*, 1985) and MLR's (Sugiura *et al*, 1988 for eg.). *In vivo*, BM-derived NS cells were able to prevent graft versus host disease (GVHD) during the generation of murine-mixed allogeneic chimeras (Sykes *et al*, 1988).

Cells with the functional and phenotypic characteristics of NS cells have been found in a number of locations and resulting from a variety treatments (rev. in. Maier *et al.*, 1989) Definite sites in addition to bone marrow (BM), include fetal and newborn

lymphoid tissues, and spleens after total lymphoid irradiation (TLI), cyclophosphamide (CY) treatment and during chronic graft versus host disease (GVHD). NS cell activity has also been reported to be associated with processes such as pregnancy, tumor progression and treatment with radioactive Strontium (^{89}Sr).

All of the locations in which NS cell activity has been observed have two things in common. The first is that they are all sites of hematopoiesis. For example, fetal and newborn liver and spleen, as well as BM throughout life, are hematopoietic organs, and after TLI, spared progenitors from BM auto-transfuse the spleen which subsequently becomes a site of rapid cell turnover. Also, during GVHD in irradiated recipients, there is considerable proliferation of donor cells in the host spleen and after ^{89}Sr treatment which detroys BM, the spleen takes over as the primary hematopoietic organ. Finally, some tumours cause increased proliferation of certain stem cell populations which may correlate with the presence of NS cells. Thus, one function of NS cells may be to regulate cell growth in hematopoietic organs - possibly by suppression of growth factor production or target cell activation (Soderberg, 1985).

The second characteristic which most of the sites of NS cell activity have in common is that they are locations in which the induction of tolerance can occur. It is known that the fetal/newborn period is the point in development where self-tolerance is established and aquired tolerance can be induced. (Billingham *et al.*, 1953 and Streilein, 1979). Also, it has been suggested that BM is a site throughout life where tolerance can be induced (Hurme and Sihvola, 1985 for eg.). During pregnancy, there exits a transient state of tolerance between the mother (host) and fetus (graft) and immunosuppressive and/or cytoreductive treatments (such as TLI or CY) produce environments conducive to tolerance induction. In contrast, reasons for the state of tolerance that has been observed in some tumor-bearing animals have not been offered.

POSSIBLE MODE OF ACTION OF NS CELLS

NS cells have been reported to be stimulated by T cell lymphokines such as IFNγ (Maier *et al.*, 1985b and 1986 and Holda *et al.*, 1986), thus it can be theorized that T cells activated (in the appropriate environment) by self or foreign antigens release lymphokines which stimulate NS cells. NS cells in turn, may mediate the suppression of the development of mature T cell clones directed against that antigen. Some experiments have shown that Ts (T suppressor) cells are refractory to NS cell-mediated down

regulation, whereas Th (T helper) cells are responsive (Okada *et al.*, 1982). This would prevent sensitization to the antigen (Th) and ensure nonresponsiveness during future antigenic challenges by allowing the expansion of antigen-specific Ts clones.

ANTIGEN-NONSPECIFIC SUPPRESSOR FACTORS

A few groups have described the existence and purification of antigen-nonspecific immunosuppressive factors released from NS or other cell types. Two examples of purified nonspecific suppressor factors are SIRS (Soluble Immune Response Suppressor) and IBF (Immunoglobulin Binding Factor). Both are released by murine activated T cells (SIRS specifically by Ly $1^{-}2^{+}$ T cells) and nonspecifically inhibit *in vitro* antibody production to both T-dependent and T-independent antigens. SIRS also inhibits *in vitro* B cell proliferation but does not inhibit CTL induction or MLR responses (Aune and Pierce, 1984). In addition, SIRS has been shown to have a molecular weight between 48 and 67 KD and require macrophage oxidation for activation. IBF has been demonstrated to consist of FcR's shed from the surface of alloantigen-activated, FcR^{+} T cells (Lowy, *et al.*, 1983). This factor (chains of 38 and 18 KD) mediates suppression by binding to the Fc portion of IgG and blocking complement activation.

There have also been examples of NS cell activity shown to be mediated by a secreted product. One example is the NS factor described by Hertel-Wulff and Strober (1988). A cloned NS cell line was isolated from the spleens of mice given TLI. Upon activation, these cells released a protein factor between 50 and 100 KD which was able to suppress the murine MLR. This NS factor was shown to have a mode of action which lacked antigen specificity and MHC restriction. In addition, it did not appear to affect B cell proliferation (the factor was unable to suppress mitogenic B cell stimulation).

Although there have been reports on a diverse array of antigen-nonspecific suppressor factors, there has yet to be agreement on the molecular nature and function of these cytokines in the suppression of immune responses. Few believe that natural suppression is a phenomenon acting in isolation. Rather, it is thought that this level of immune regulation acts in concert with that of antigen-specific suppression mediated by Ts cell circuits and their soluble products.

NS CELL ACTIVITY IN RESPONSE TO TUMORS

NS cell activity has been shown on several occasions to be associated with a decreased immune response to tumours. TLI and GVHD-induced NS cells have been demonstrated to inhibit the *in vitro* generation of tumour-specific CTL (cytotoxic T lymphocytes) (Okada and Strober, 1982 for eg.). In addition, natural killer (NK) cells, which are thought to be important in limiting cancer metastases *in vivo*, seem to exist in a reciprocal relationship with NS cells (Maier *et al.*, 1990). Thus, NS cells may suppress NK cell activity, or in light of their cytological and phenotypic similarities, may represent a different stage of differentiation of the NK cell lineage.

Although antigen-specific suppressor circuits have been described in tumor-bearing animals (Schatten *et al.*, 1984 and Green *et al.*, 1983), less is known about antigen-nonspecific or natural suppression (NS). Several cell types such as B cell blasts, tumor-activated T cells and non-T cells from tumor-bearing mice have the ability to induce NS cells and it is postulated that this results in an additional level of control superimposed on antigen-specific circuits (rev. in Pope, 1985).

SUPPRESSOR INDUCER CELLS

As part of an ongoing study of suppressor T cell circuits associated with the growth of M-1 (methylcholanthrene-induced) fibrosarcomas (Pope *et al.*, 1976 and 1978), Pope described the existence of suppressor inducer cells that secrete soluble factor(s) capable of recruiting and activating suppressor effector T cells from unprimed splenocytes. The activated effector cells were found to be Thy-1^+, Lyt 1^+2^+ and had the ability to suppress the anti-IgM response to a T cell-dependent antigen (SRBC). As the antibody response to a T cell independent antigen (DNP-LPS), and that mediated by *Nu/Nu* spleen cells (activated by exogenously added growth factors), were not suppressed, Pope suggested that the mode of action of activated Ts might be inhibition of the production of lymphokines by Th cells. This theory has since been supported by others working in different suppressor systems and with Ca^{++} flux studies (Malkovsky *et al.*, 1982 for eg. & Utsunomiya *et al.*, 1990).

ISOLATION OF THE NS CELL LINE M1-A5

Almawi and Pope in 1985, reported the isolation of an antigen-nonspecific suppressor inducer cell line (M1-A5). M1-A5 was generated from the spleen of a mouse bearing an advanced M-1 fibrosarcoma. When 2 x 10^4 M1-A5 cells were cocultured with 10^6 normal spleen cells (NSC), and 5 x 10^5 sheep red blood cells (SRBC) for 5 days, subsequent challenge with SRBC resulted in a 99% suppression of antibody synthesis (as assessed by the plaque forming cell (PFC) assay of Cunningham and Szenberg, 1968). This effect was shown to titrate with decreasing numbers of M1-A5 cells cocultured.

M1-A5 cells displayed a similar morphology (large granular lymphocyte or LGL) and cell surface phenotype (Thy-1$^-$, Lyt-1$^-$2$^-$, sIg$^-$, MAC-1$^-$, Ly-5$^+$ and asialo-GM1$^+$) to natural suppressor (and NK) cells. In addidtion, M1-A5 was able to lyse the natural cytotoxic (NC) cell target WEHI-164, and to a small extent cultured M-1 fibrosarcoma cells, but not the NK cell target YAC-1. Tritiated thymidine uptake experiments showed that M1-A5 cells required IL-3, but not IL-1, IL-2 or GM-CSF to proliferate. Cell-free supernatant (shown to be nonsuppressive) from the murine, IL-3-producing, promyelocytic cell line WEHI-3B (Lee *et al.*, 1982), was used to support the growth of M1-A5 cells cultured *in vitro*.

IN VITRO BIOACTIVITY OF M1-A5 CELLS

In order to investigate whether the mechanism of suppression by M1-A5 cells involved direct cellular contact or the secretion of soluble suppressor factor(s), Marbrook chambers were used. M1-A5 cells (in the outer chamber) were separated from NSC (in the inner chamber) by dialysis tubing (mol. cutoff of 12 KD). After 20 hours, potentially activated NSC were then cocultured with SRBC and assayed for antibody producing cells as above. It was found that 2.5 x 10^6 M1-A5 cells incubated in the outer chamber were able, via the secretion of a factor of less than or equal to 12 KD, to suppress the PFC response of 10^6 NSC by approximately 50% (Almawi and Pope, 1985). Further, increasing numbers of M1-A5 cells activating the same number of NSC resulted in a greater decrease in PFC response.

Additional experiments showed that a minimum preincubation of 8 hours was needed for M1-A5 cells to activate NSC in Marbrook vessels. Longer incubation periods resulted in only a slight enhancement of suppression. Also, incubating NSC for a minimum of 3

hours in 50% dialyzed M1-A5 supernatant (SN) resulted in 50% suppresssion of the PFC response, an effect which titrated through to 90% SN (Fig. 1a and Discussion). Finally, the suppressor inducer factor (named SIF) was shown to be protein in nature as a loss of bioactivity was observed upon heat, acid, base and trypsin treatments (Almawi and Pope, 1985).

In 1987, Pope further characterized the range, potency and kinetics of SIF activity. She found that M1-A5 cells and S/N were able to 1)suppress the generation of CTL's, 2)inhibit T cell proliferation in reponse to alloantigens (MLR) and 3)suppress the generation of antibody producing cells (PFC). However, neither M1-A5 nor SIF were able to suppress the proliferative T cell response to Concanavilin A (Con A). Suppression of the MLR, the PFC response and the generation of CTL was only observed when M1-A5 cells or SN was added early (in the first day of culture for MLR and PFC and at the start of culure for CTL). In the same report, the mechanisim of M1-A5-mediated suppression was shown to differ from another IL-3 dependent cell line FDC-P1. FDC-P1 was directly suppressive in all of the assays but the spent SN from these cells was completely inactive.

Almawi and Pope (1987) found that addition of acetylsalicylic acid (ASA) or ibuprofen (which blocks prostaglandin synthesis) to M1-A5 cells in culture effectively abolished the ability of M1-A5 cells and SN to induce suppression. Exogenously added prostaglandins PGE_1, E_2 and I_2 (but not $F_2\alpha$ or D_2), were found to restore the ability of M1-A5 cells to mediate suppression but could not replace the suppressor inducer activity of SN from ASA or ibuprofen treated cultures. Further, Almawi *et al.* (1987a) found that cyclic AMP was the second messenger in the PGE-mediated modulation of SIF secretion.

BIOCHEMICAL PURIFICATION OF MI-A5 DERIVED SUPPRESSOR INDUCER FACTOR (SIF)

Almawi *et al.*, (1987b) initially purified 100 µg of SIF from 6 liters of M1-A5 cell free SN by Sephadex G-100 size fractionation and ion exchange chromatography. Significant levels of bioactivity were found in peaks eluting around M_r 70 KD, termed SIFα, and M_r 5.5 KD, termed SIFβ. When SIF α and β were subjected to ion exchange chromatography, SIFβ was shown to bind to the cationic exhanger QAE-Sephadex and elute in a single peak, whereas SIFα activity was found in the void volume in addition to the eluate of a DEAE-Sephacel column. Reducing SDS-PAGE of the bioactive QAE fraction (SIFβ) showed one protein band with a M_r of approximately 6KD. Overall, the purifi-

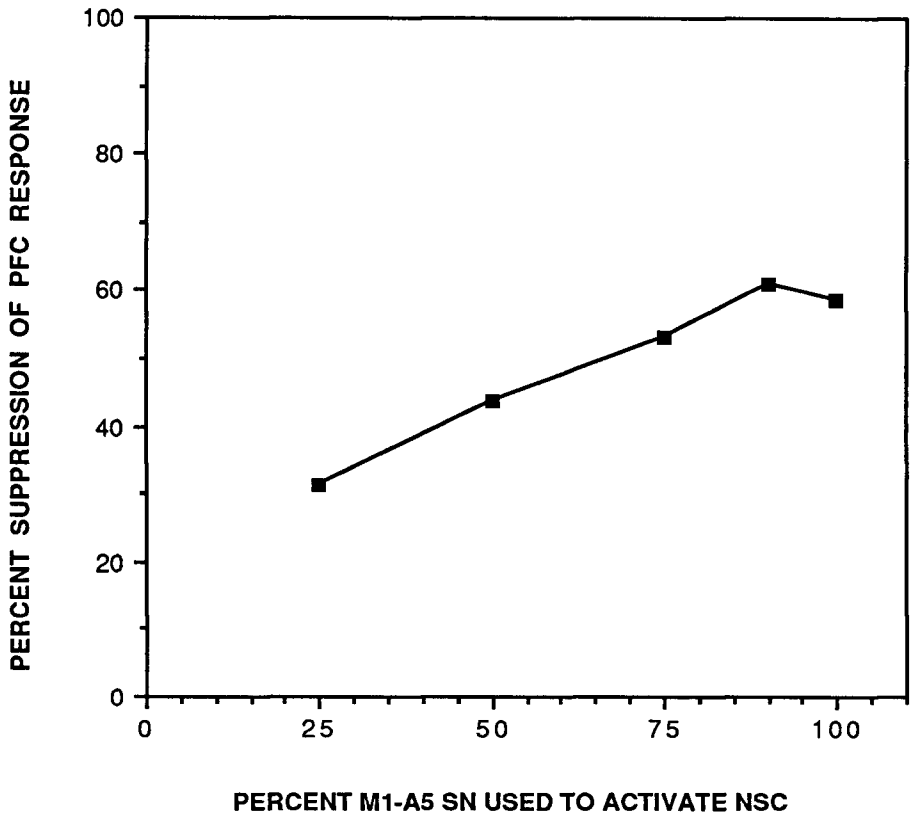


FIGURE 1a: **INDUCTION OF SUPPRESSION IN NORMAL SPLEEN CELL PREPARATIONS BY M1-A5 SUPERNATANT.** Taken from Pope *et al.*, 1987. 5 X 10^5 NSC were incubated with increasing amounts of dialyzed (Mol. wt. cutoff-12 KD) M1-A5 supernatant for at least 4 hours, cultured *in vitro* with 1 X 10^6 untreated NSC and SRBC for 5 days and assayed for the number of plaque forming cells per culture by the method of Cunningham and Szenberg (1968). Percent suppression was calculated from cultures in which NSC had been incubated with RPMI medium instead of supernatant. Each point represents the mean of three independent tests.

cation resulted in a 10^4 fold increase in activity for SIFβ and only a 4 fold increase in activity for SIFα. In the same report, SIFα and β were demonstrated to have a genetically nonrestricted mode of action and the equal ability to suppress the antibody response of unprimed splenocytes to SRBC *in vivo* (Almawi *et al.*, 1988a).

Also in 1988 (b), Almawi *et al.* reported on the large scale purification of SIFα and β from 15 litres of M1-A5 SN using subsequent steps of Sephadex G-100 gel filtration, cationic (α) and anionic (β) exchange chromatography and two rounds of reverse phase high performance liquid chromatography (RP-HPLC). Fractions were screened by a modified PFC assay which resulted in an immense increase in sensitivity (Fig. 1b and Discussion). Overall, 40 μg of SIFβ with a 3×10^4 fold increase in activity and 5 μg of SIFα with a 1.6×10^3 fold increase in activity were purified. Final purity was assesed by the migration of the factors as a single band on SDS-polyacrylamide gels and elution from the HPLC column as sharp, single peaks.

SECTION 1: SUMMARY OF WORK TO BE PRESENTED

The above purifcation process was modified (see below) and set up in our laboratory with the hope of obtaining enough purified SIFβ to get the protein sequence from an internal peptide (in the previous purification, the N-terminus was shown to be blocked) and to begin the production of a monoclonal antibody in rat. Simultaneously, an *in vitro* assay already in use in the laboratory to study suppression of the antibody response to the antigen ferredoxin was adapted for studying SIF action.

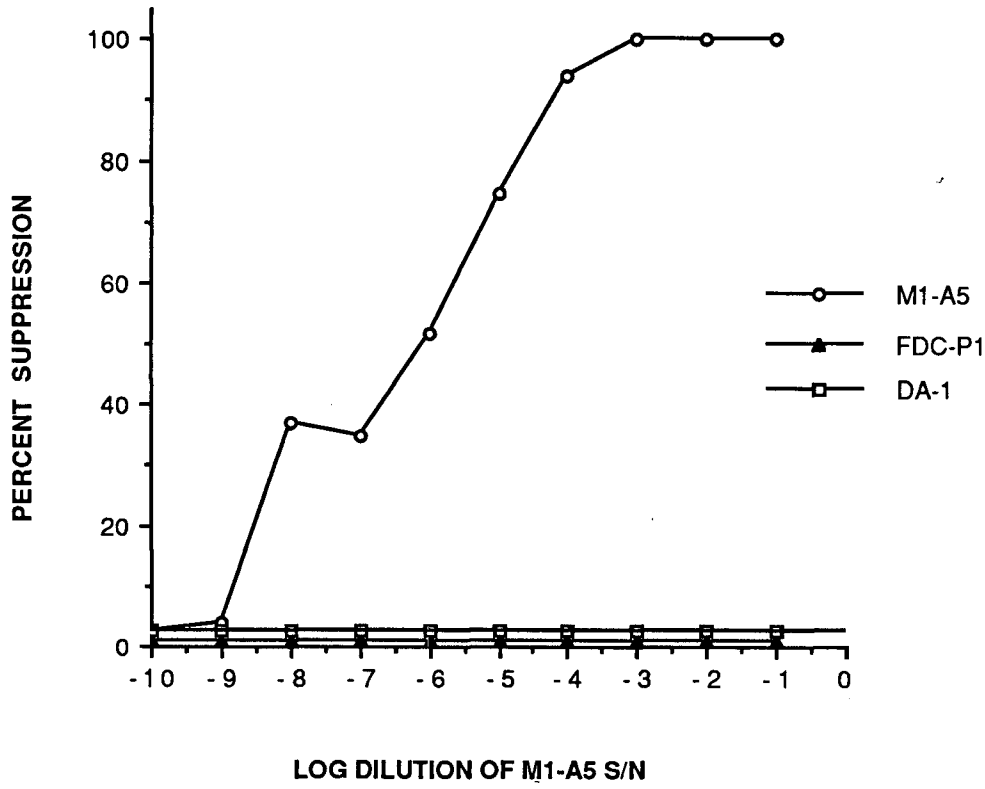


FIGURE 1b: **SUPPRESSION OF THE PLAQUE-FORMING CELL ASSAY BY M1-A5 SUPERNATANT.** Taken from Almawi *et al.*, 1988b. Naive spleen cells were cultured *in vitro* with SRBC and log dilutions of M1-A5 supernatant for five days and then assayed for the number of plaque forming cells per culture as above Percent suppression was calculated from cultures in which RPMI media was added instead of suppressor sample. Each point represents the average of three independent experiments.

SECTION 1: MATERIALS AND METHODS

CELL LINES: M1-A5 and WEHI-3B cells were obtained from the laboratory of Dr. B. Pope (Dalhousie Univeristy, Halifax, Nova Scotia) and were grown in Flow RPMI 1640 supplemented with 1% and 5% heat inactivated fetal bovine serum respectively in a humidified, 37°C, 5% CO_2 incubator. M1-A5 growth media was supplemented with 20% WEHI-3B cell-free supernatant as a source of IL-3. Both cell lines were grown in large (175cm^2) Nunc tissue culture flasks; M1-A5 upright and WEHI-3B flat.

HARVESTING SUPERNATANTS: When cultured WEHI-3B cells reached a density of 5 x 10^5 cells per ml, the cell suspension was centrifuged and the supernatant filtered (0.2 µm filter) for addition to the M1-A5 growth media. Excess supernatant was occasionally frozen at -70°C for future use. When the M1-A5 cells reached a density of 5 x 10^5 - 1 x 10^6 cells per ml, the supernatant was aseptically removed from the cells resting on the bottom with a 25 ml pipette. Cells were harvested, washed three times with sterile PBS, resupended at 5 x 10^5 cells per ml in serum-free RPMI and incubated at 37°C, 5% CO_2 overnight. The next day cell free supernatant was harvested and frozen at -70°C until approximately 3.0 litres was collected.

BIOCHEMICAL PURIFICATION OF SIFα AND SIFβ FROM M1-A5 SN: M1-A5 serum free SN was thawed at room temperature and applied via gravity feed to DEAE-Sephacel and S-Sepharose ion exchange columns (Pharmacia) set up in tandem and equilibrated with PBS at 4°C. Columns were washed separately with an equal volume of cold PBS and eluted with 150 ml continuous salt gradients (0.15M to 0.95M NaCl) at 0.5-0.7 ml/min. 10 ml fractions were collected with a Pharmacia Frac 100 fraction collector and the amount of protein in each determined by Micro-Lowry Assay. The fractions were dialyzed at 4°C against PBS and tested for bioactivity by the Plaque Forming Cell (PFC) Assay (B. Pope, Halfax). Active fractions were pooled, concentrated on a bed of sucrose (Fisher Spectra Por dialysis tubing, MWt. Cutoff of 3500 Dal) and back dialyzed in 50mM NH_4HCO_3 (2L x 12 hours twice). Pooled fractions were lyophilized, and resuspended in PBS. The pooled, concentrated, bioactive fractions were next subjected to FPLC at room temperature. Approximately 4.5 mg of protein was applied to an FPLC Superose 12 HR column (Pharmacia) per run. The column was eluted with 30.0 ml of PBS and 0.5 ml fractions were collected and pooled from three to four

successive runs. Fractions were frozen at -70°C and assayed for bioactivity by PFC (Dr. Pope).

SODIUM DODECYL SULFATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE): The content and purity of selected fractions obtained from FPLC was assessed using 12% (w/v) acrylamide (Bio-Rad) gels in a mini-protean II slab gel apparatus (Bio-Rad). Samples were dialyzed against 50 mM ammonium bicarbonate, lyophilized and approximately 2.0 µg was resuspended in 10-15 µl of SDS sample buffer (10% glycerol, 2% SDS, 6.25 mM Tris-HCl buffer and bromphenol blue). Material was reduced by the addition of dithiothreitol (DTT) to 10 mM, and heating to 100°C for 4-5 minutes, and then cooled to room temperature for loading. Separated proteins in the gel were fixed in 50% methanol/10% glacial acetic acid/40% dH_20 for 30 min. and rehydrated by incubation in 10% methanol/10% glacial acetic acid/80% dH_20 (in a microwave for 1 min.) followed by 100% dH_20 for 5 minutes at room temperature. The gel was next treated with 33 µl of 100 mM DTT in 100 ml dH_20 for 1 min. in a microwave and then 1 ml of 10% silver nitrate in 99 ml dH20 for 15-20 min. at room temperature. Silver-binding material was visualized by the addition of 40-50 ml of developing reagent (40 ml of 15% Na_2C0_3/60 ml of H_20 and 200 µl of 37% formaldehyde) for a variable period of time. Developing was stopped by the addition of 3-4 ml of glacial acetic acid. Gels were stored in dH_20 and subsequently dried on a Bio-Rad slab gel dryer.

SCREENING FOR SIF BIOACTIVITY -THE MODIFIED SUPPRESSOR CELL ASSAY (Dr. B. Pope, Halifax, N.S.): Fresh sheep red blood cells (SRBC) were collected aseptically in Alsever's solution and stored in aliquots at 4°C for up to one week. Before use SRBC's were washed 2-3 times (or until supernatant was clear) with PBS. Fresh guinea pig serum was used as a source of complement after being adsorbed with 1 ml of packed SRBC per 10 ml serum for 30 minutes on ice. The SRBC were removed by centrifugation and the serum was stored in aliquots and frozen at -70°C. Spleens from 6-8 week old BALB/c or DBA/2J mice were removed aseptically, pressed through a metal screen into 10 ml of serum-free RPMI, washed once (10 min. x 400 g), and resuspended in 5 ml of RPMI per spleen. After a viability count, cells were diluted to 1.2×10^7 per ml in RPMI. Suppressor samples were added to the wells of flat-bottom, 96-well microtiter trays (Dynatech-Immulon 2) in duplicate or triplicate and brought to 100 µl with

medium. 100 µl of medium alone was added to positive control wells. 100 µl of the normal spleen cell (NSC) suspension (1.2 x 10^6 cells) and 50 µl of a mixture of 50% FBS, 25% RPMI 1640, 12.5% 2 mM 2-mercaptoethanol and 12.5% SRBC (at 4 x 10^7 cells per ml) were added to bring the final volume per well to 250 µl. The cell mixture was then incubated at 37°C, 5% CO_2 for 5 days and assessed by PFC assay (see below).

PLAQUE FORMING CELL (PFC) ASSAY: (Cunningham and Szenberg, 1968) SRBC-primed SC (from above) were harvested from wells with a pasteur pipette and duplicate or triplicate tests pooled. After centrifugation (10 min x 400g) the pellet was resuspended in 300 or 450 µl (150 µl per well) of PBS. 25 µl of this mixture was diluted with medium to give a volume of 150 µl. 50 µl of a 25% suspension of washed, packed SRBC in RPMI + 5% FBS and 50 µl of guinea pig complement diluted 1:1 in RPMI were added to give a final volume of 250 µl. 65 µl of this final solution was added to previously prepared Cunningham slides which were then sealed with melted vaseline to prevent evaporation. Slides were incubated at 37°C for 1 hour and plaques counted against the light of an X-ray box. The number of PFC per culture was determined by multiplying the number of PFC per chamber (mean of 2-3 chambers) by the appropriate dilution factors.

ANTIBODY CULTURE (Ab C) ASSAY:

6-8 week old, female DBA/2J mice were primed subcutaneously (s.c.) with 50 µg of kehole limpet hemocyanin (KLH, Calbiochem) emulsified in an equal volume of complete Fruend's Adjuvant (CFA). After 21 days, animals were boosted s.c. with 50 µg KLH emulsified in incomplete Freund's adjuvant. Serum anti-KLH levels were evaluated by ELISA as follows; Dynatech-Immulon 2, 96-well microtiter plates were coated with 100 ng KLH per well in sodium bicarbonate buffer (left at 4°C, overnight). Serum obtained from tail vein blood of primed animals was added at various dilitions in duplicate to the KLH-coated wells and incubated for 1-1.5 hours at 37°C. Plates were developed using goat anti-mouse antibody conjugated to alkaline phosphatase (37°C x 1 hour, Jackson Labs) and paranitrophenyl-phosphate (Sigma) substrate (37°C x 15-30 min), and read on a Titertek Microplate Reader at 405 nm. The spleens of animals with the highest serum levels of anti-KLH antibodies were aseptically harvested and pressed through a sterile metal screen with the aid of the plunger from a 3 ml syringe into 10 ml of DME with 10% FBS. Splenocytes were pelleted, resupended in 10 ml medium and divided into two 5 ml portions. One portion recieved a 250 µg per spleen per ml *in vitro* pulse of KLH in

medium and the other an equal volume of media alone. Suspensions were incubated for 2 hours at 37°C with 5% CO_2 and periodic tapping to resuspend settled out cells. After two washes with medium and a viability count, cells were resuspended in RPMI 1640 with 10% FBS and 5 x 10^{-5} M 2-mercaptoethanol at a density of 2.5 x 10^6 cells per ml. 2 ml (5 x 10^6 cells) of each suspension (KLH-pulsed and media-pulsed) were added to sterile Nunc 24-well culture plates. An aliquot of M1-A5 serum-free supernatant was added to both the KLH-pulsed and medium-pulsed wells. An equal volume of medium alone was added to cells in duplicate and constituted positive controls. Plates were incubated for 7 days in a humidified, 37°C, 5% CO_2 incubator. On day 7, 1.5 ml of culture supernatant from each well was removed and stored in 0.02% sodium azide at 4°C, or frozen at -20°C. The level of anti-KLH antibody produced was evaluated by testing 100 µl of each supernatant in a KLH ELISA (as described above).

To test the relevance of time of factor addition to the assay, serum-free supernatant was incubated with KLH-primed splenocytes for 6 hours at 37°C before the KLH or medium pulse.

***MYCOPLASMA* TESTING AND CELL LINE CURING:**

Cells in the laboratory were periodically tested for *Mycoplasma* infection by cytochemical analysis using the flourescent dye 4-6-diamine-2-phenyl indole dihydrochloride or DAPI (Boeringher Mannheim). Dr. Pope's laboratory, in addition to the DAPI method, occasionally sent cell lines to the Victoria General Hospital Diagnostic Laboratory (Halifax, Nova Scotia) for their more sensitive *Mycoplasma* detection system. The cell pellet from a 150 ml culture of cells was washed, resuspended in *Mycoplasma* growth media and after an appropriate incubation period, assayed for the presence of the organism by plating out samples of supernatant.

Three M1-A5 subcultures were cured of *Mycoplasma* using the BM-Cycline (Boeringher Mannheim) antibiotic kit. Samples of treated cultures were sent to the General Hospital to determine the effecincy of the procedure by the method described above.

SECTION 1: RESULTS

CULTURING WEHI-3B AND M1-A5 CELL LINES:

M1-A5 cells have been shown to require the hemopoietic growth factor Interleukin-3 (IL-3)(Pope *et al.*, 1986). The mouse promyelocytic cell line WEHI-3B (Lee *et al.*, 1982) constitutively secretes IL-3; thus, filtered WEHI supernatant was used to support the growth of M1-A5 cells *in vitro*. During the large scale culturing of M1-A5, it was observed that if the WEHI cells were allowed to reach a density exceeding 1 x 10^6 per ml they began to secrete a substance which initially inhibited cell growth and finally caused autolysis and lysis of the M1-A5 cells. This was not due to exhaustion of the nutrient supply in the growth medium, as seeding WEHI cells into fresh medium at too high a density (5 x 10^5 cells per ml) did not produce a plateau in the exponential decline of the growth curve. Fig. 2 shows several growth curves for the WEHI-3B cell line. Each curve represents a different seeding density at day 0. The culture which was started with 5 x 10^5 cells per ml immediately began an exponential decrease in cell number with virtually no viable cells remaining after 3 days. The culture seeded at 1 x 10^5 cells per ml gave a growth curve which increased to 7 x 10^5 cells per ml (day 2), plateaued (day 2-4) then exponentially decreased. Those cultures seeded at 1 x 10^4 and 5 x 10^4 cells per ml both displayed normal exponential increases in cell number to 7 x 10^5 per ml then declined as that observed with the culture seeded at 5 x 10^5 cells per ml. From these curves, and assessing the response of the M1-A5 cells to various samples of WEHI-3B supernatant (data not shown), it was determined that the optimal conditions for harvesting WEHI supernatant was from cultures seeded at 1 or 5 x 10^4 cells per ml and allowed to reach a density of 5 x 10^5 cells per ml.

SIF ACTIVITY IN THE ANTIBODY CULTURE ASSAY:

The Antibody Culture (Ab C) Assay has been in use in this laboratory for several years to screen for suppressive ability of the antigen-specific, murine suppressor factor Fd11F. Fd11F is specific for the nominal antigen ferredoxin, thus the mouse immunization schedule and Ab C Assay have been developed to optimize production of anti-Fd antibodies by Fd-immune splenocytes (R. Chu, 1989 for eg.). Since SIF has previously

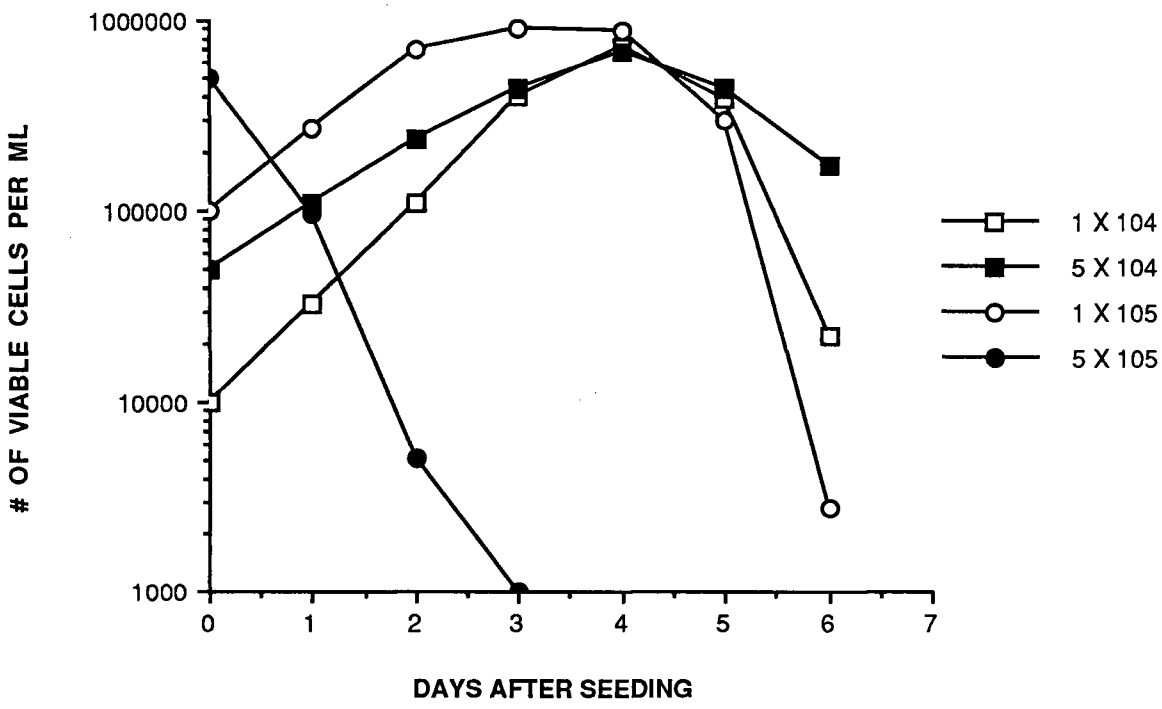


FIGURE 2: GROWTH CURVES FOR WEHI-3B CELLS SEEDED AT DIFFERENT DENSITIES. WEHI-3B cells were cultured in RPMI supplemented with 10% FCS in a humidified, 37°C, 5% CO_2 incubator. Cell viability was assessed and quantitated by light microscopy using a hemocytometer and Eosin Y stain every 24 +/- 2 hours. The number of cells per ml plotted at each 24 hour time interval represents the average of four values.

been shown to have a mode of action that is antigen-dependent yet nonspecific, the molecule kehole limpet hemocyanin (KLH) was chosen (for it's availability and immunogenicity) as the antigen to adapt the Ab C Assay to study SIF activity. KLH-primed mouse splenocytes were pulsed *in vitro* with KLH or medium alone and subsequently cultured for 7 days with serum-free M1-A5 or control medium. Anti-KLH levels were evalulated by ELISA using KLH-coated plates. As can been seen in Fig. 3, M1-A5 serum-free supernatant suppresses 11% of the anti-KLH response at a dilution of 10^{-8} and 93% at a dilution of one in 4. From this suppression curve, an ED_{50} of 10^{-2} was obtained. Thus, the sensitivity for detecting SIF activity in the antibody culture assay is 100 times more sensitive than that obtained by Dr. Pope in the original suppressor assay (Fig. 1a) and 10,000 fold less sensitive than the straight PFC assay used to screen for SIF activity in the biochemical purification (Fig. 1b). Although the suppressive effect does titrate in all three of the assays, the shape of the curves differ significantly (FIG.'s 1a, 1b & 3).

PREINCUBATION OF KLH-IMMUNE SPENOCYTES WITH M1-A5 SN

To investigate whether the SIF-induced suppression of the KLH Ab response was dependent on the incubation time of factor with splenocytes prior to antigen pulse, KLH-immune splenocytes were divided into two equal portions. One portion was preincubated with suppressor sample for 6 hours prior to the *in vitro* KLH pulse and the other was given the antigen-pulse directly and then cultured with SIF sample. The 6 hour preincubation time was chosen because Pope *et al.* (1987) have shown that a minimum of 8 hours is required for washed M1-A5 cells to synthesize and secrete enough SIF to activate the suppressor population of NSC in Marbrook vessels and that SIF already in SN only needs 4 hours. Since in this experiment the SIF sample is already in the supernatant, 6 hours was chosen to ensure more than enough time for induction of suppression in the assay. After 7 days, anti-KLH levels were compared for both groups of cultured splenocytes. From the results presented in Fig. 4, the ED_{50} of the supernatant not preincubated was 5×10^{-3} whereas the maximum level of suppression achieved with the preincubated sample was 22% at the lowest dilution (0% at all other dilutions). Since the positive control anti-KLH levels from cells preincubated and not preincubated with M1-A5 supernatant (OD_{405} of 0.82 vs. 0.89 in the KLH ELISA respectively) differed by only 8%, the 6 hour preincubation step is not significantly affecting the functional capability of KLH-immune splenocytes.

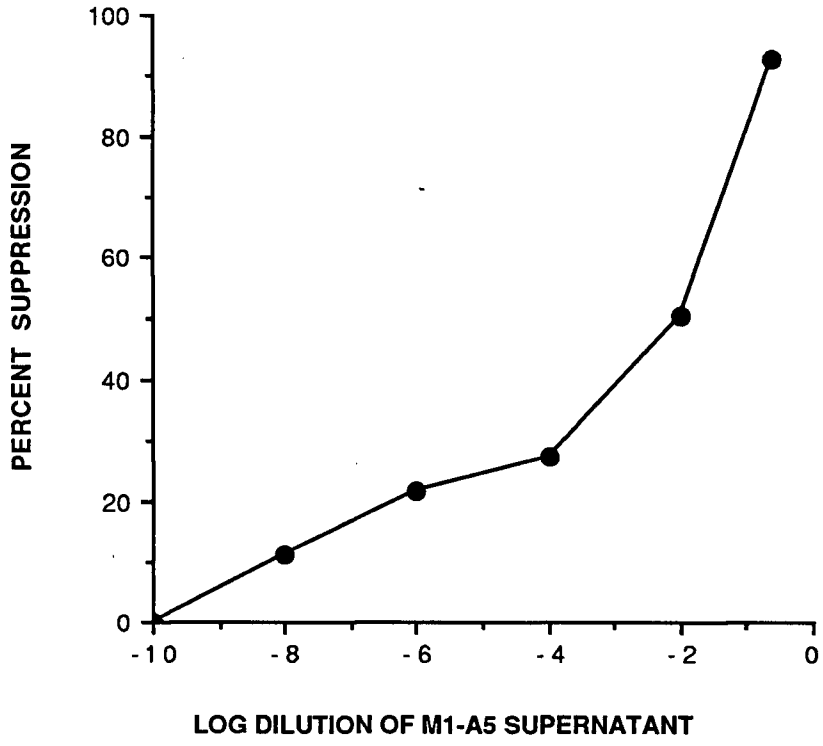


FIGURE 3: SUPPRESSION OF KLH ANTIBODY PRODUCTION BY M1-A5 SUPERNATANT. The spleens of KLH-immune mice were harvested, mashed through a metal screen into DME/10% FCS media, pulsed with KLH for 2 hours and washed. Activated splenocytes were incubated in duplicate with various dilutions of M1-A5 supernatant for 7 days at which point culture supernatants were collected and the amount of anti-KLH antibodies in each quantitated by KLH-ELISA (each dilution tested in triplicate). Percent suppression was calculated from cultures pulsed with antigen but incubated with DME media instead of supernatant. Each point represents the average of the values obtained in two separate experiments.

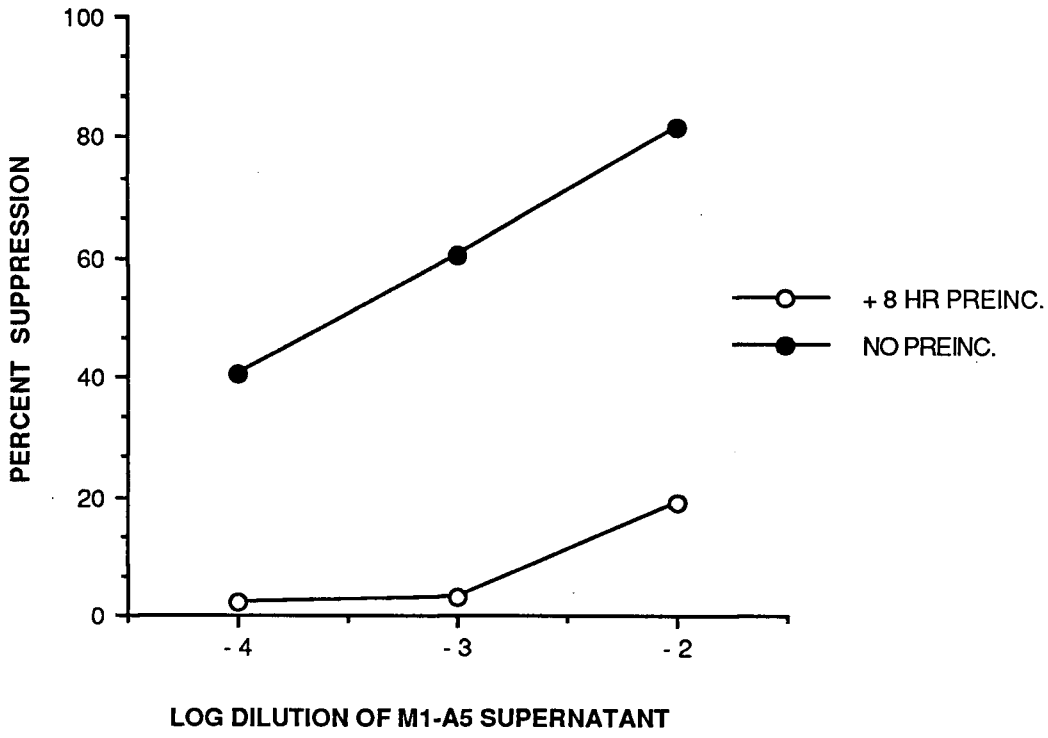


FIGURE 4: THE EFFECT OF PREINCUBATION OF M1-A5 SUPERNATANT WITH KLH-IMMUNE SPLENOCYTES ON THE KLH ANTIBODY CULTURE ASSAY. KLH-immune splenocytes were harvested and split into two portions. One portion was preincubated with M1-A5 supernatant for 8 hours prior to the KLH pulse and the other was pulsed, washed and put into culture with M1-A5 supernatant directly. All cultures were set up in duplicate. After 7 days, culture supernatants were analzyed as in FIG. 3. Percent suppression of preincubated SC was calculated from cultures in which SC were preincubated with DME media instead of supernatant and that of untreated SC, as in FIG. 3. Each point represents the results of one experiment.

BIOCHEMICAL PURIFICATION OF SUPPRESSOR INDUCER FACTOR (SIF) FROM M1-A5 SERUM-FREE SUPERNATANT:

The previously described SIF purification process (Almawi *et al.*, 1988b) was set up in our lab in an attempt to obtain enough purified SIFβ from M1-A5 cell culture SN's to synthesize a monoclonal antibody in rat. Several modifications were made to this procedure. First, the initial Sephadex G-100 size separation (gel exclusion chromatography) was omitted and serum-free M1-A5 SN was applied directly to the ion exchange columns. The rationale for this change was the high binding capacity and resilience of modern ion exchange column matrices and charged groups. The second alteration replaced three rounds of reverse-phase high pressure liquid chromatography (RP-HPLC) with one round of FPLC. This change was made because purified SIFβ was found to be blocked at the N terminus when sent for protein sequencing -presumably due to the harsh conditions imposed by the acetonitrile elutions. FPLC fractions were eluted with PBS.

M1-A5 cells grown in 75% RPMI 1640, 5% FBS, 20% WEHI-3B cell-free SN (as a source of IL-3) were harvested, washed with PBS and incubated at a density of 5 x 10^6 cells/ml for 16-20 hours in serum free RPMI 1640. Once 2.5-3.0 litres of cell- and serum free M1-A5 supernatant had been collected it was passed over anionic and cationic ion exchange columns set up in tandem. The anionic (upper) exchange column consisted of DEAE-Sephacel and had been previously shown to bind SIFβ but not SIFα (W. Almawi, unpublished). The flowthrough from this column was directed over the cationic (lower) exchange column containing S-Sepharose which was known to bind SIFα. Columns were washed separately with PBS and eluted with 150 ml 0.15M to 0.95M NaCl continuous salt gradients.

Ten ml fractions were collected and tested for protein content by Micro-Lowry Assay. One large protein peak eluting betweeen 0.31M and 0.57M NaCl (Fractions #4-7) was obtained in the first round with a shoulder at 0.63M (Fraction #8) and two smaller peaks eluting between 0.31-0.42M (Fraction #4) and 0.42-0.57M NaCl (Fractions #6,7) were obtained in the second round of DEAE-Sephacel chromatography. One very large protein peak eluted from the S-Sepharose column between 0.42 and 0.84M NaCl (Fractions #6-12) with a shoulder at 0.36 (Fraction #5). The second set of S-Sepharose fractions unfortunately were lost.

Technical difficulties were experienced in attempts to set up the PFC Assay practised in the laboratory of Dr. B. Pope at Dalhousie University. In order to continue with the

purification process as speedily as possible, aliquots of the 10 ml fractions eluted from ion exhange columns were dialyzed against PBS and sent to Dr. Pope's laboratory where they were screened for bioactivity by PFC assay. The elution profiles, and bioassy results for both the DEAE-Sephacel (SIFβ) and S-Sepharose (SIFα) runs are shown in Fig. 5a, b, and c.

In Fig. 5a, it can be seen that the highest levels of bioactivity are found within the main protein peak (Fractions #4-7) but not in the shoulder (Fraction #8). In Fig.5b, most of the bioactivity is found in Fractions #3-7, with lower amounts of protein in Fraction #5 and especially #3. Also in the second round, significant levels of suppression (greater than 40%) were observed in Fractions #9 and 11 (56% and 54% respectively). These fractions however, contained much lower amounts of protein (0.55 and 0.10 mg). In Fig. 5c it can be seen that there are no clear peaks of suppressive activity in the fractions eluted from the cationic exchange column. The protein fraction eluting at 0.73M Na^+ (Fraction #11) suppressed the PFC response of NSC by 44% and contained a significant amount of protein (3.9 mg). Fraction #15 gave the same level of bioactivity (44%)yet contained trace amounts of protein (0.004 mg).

The previously documented SIF purification protocol (Almawi *et al.*, 1987) has shown SIFβ to be 2500 times more active as SIFα. Given this, plus the virtual lack of significant levels of bioactivity in the S-Sepharose fractions obtained in this work, only the bioactive fractions obtained from the DEAE-Sephacel (anionic exhanger) column were taken to the next step of the purification procedure.

Anionic exchange fractions comprising the peak suppressive activity (Round 1, Fraction #4-7, and Round 2, Fraction #3-7 and 9-11) were pooled (to become D1, D2a and D2b respectively), concentrated on a bed of sucrose, back-dialyzed against 50 mM NH_4HCO_3 and lyophilized. Pellets were resuspended in PBS and subjected to FPLC using a Superose 12 HR column (approximately 4.5 mg/run). The column was eluted with PBS at room temperature (30 ml in 60 minutes, collecting 0.5 ml fractions). Given that each FPLC run produced identical profiles (data not shown), fractions from successive runs were pooled. As with the dialyzed ion exchange fractions, an aliquot of each FPLC fraction was sent to Dr. Pope in Halifax to be screened for bioactivity by PFC assay. Representative FPLC elution profiles are shown in Fig. 6 and the corresponding bioassay results are given in Table I. In all three sets of FPLC, the highest levels of SIF activity were found in the earliest eluting fractions, with lower levels scattered throughout the profile.

FIGURE 5: ELUTION AND BIOACTIVITY PROFILES FROM ION EXCHANGE CHROMATOGRAPHY OF M1-A5 SUPERNATANT. Results from two separate rounds of DEAE-Sephacel chromatography (**a** - Round 1 and **b** - Round 2) and one round of S-Sepharose chromatography (**c**) are presented. Three litres of serum-free M1-A5 SN was applied via gravity feed to DEAE-Sephacel (anionic exchanger) and S-Sepharose (cationic exchanger) columns set up in tandem and equilibriated with PBS at 4°C. Columns were washed and eluted separately with a 150 ml continuous salt gradient from 0.15 M (PBS) to 0.95 M NaCl (a 1M solution in PBS). 10 ml fractions were collected, dialyzed against PBS and screened for bioactivity by PFC assay (Dr. Pope - various dilutions were cultured with naive spenocytes and SRBC for 5 days). Percent suppression was calculated from cultures incubated with PBS instead of eluted fractions, and each value given represents the average from three wells and two slide chambers. The amount of protein in each well was determined by Micro Lowry assay.

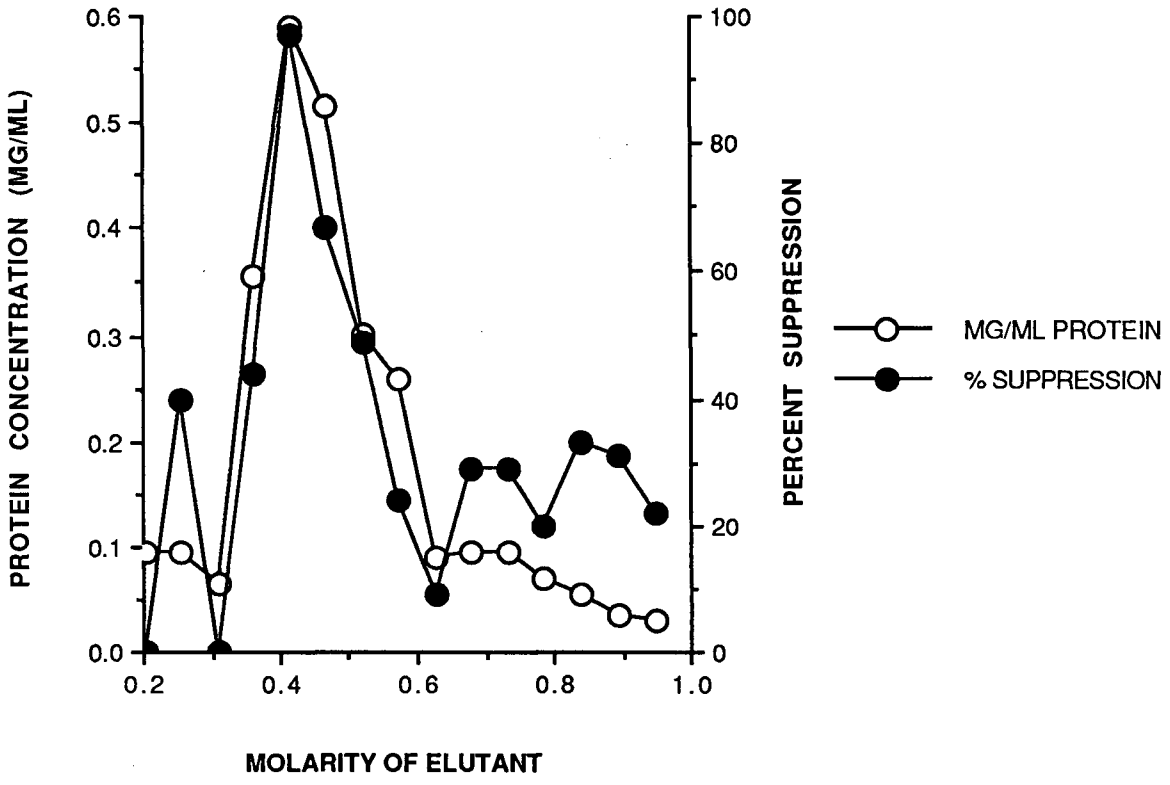


FIGURE 5a: ELUTION AND BIOACTIVITY PROFILE FOR ROUND 1 OF DEAE-SEPHACEL CHROMATOGRAPHY (D1).

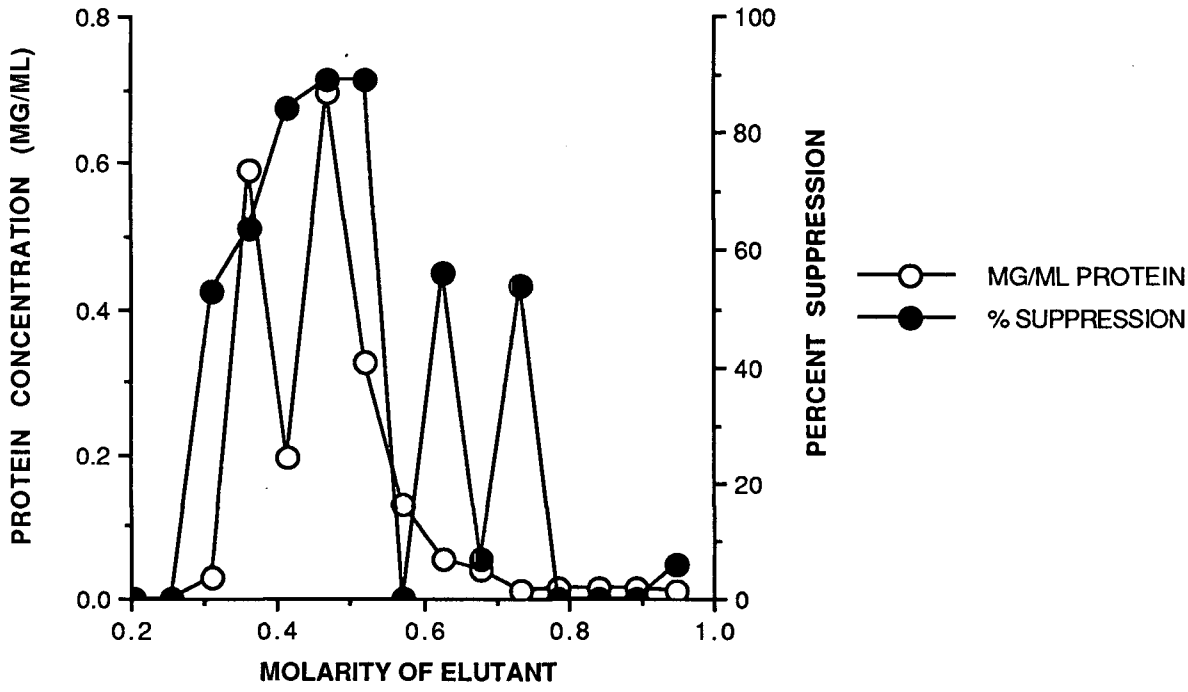


FIGURE 5b: ELUTION AND BIOACTIVITY PROFILE FOR ROUND 2 OF DEAE-SEPHACEL CHROMATOGRAPHY (D2).

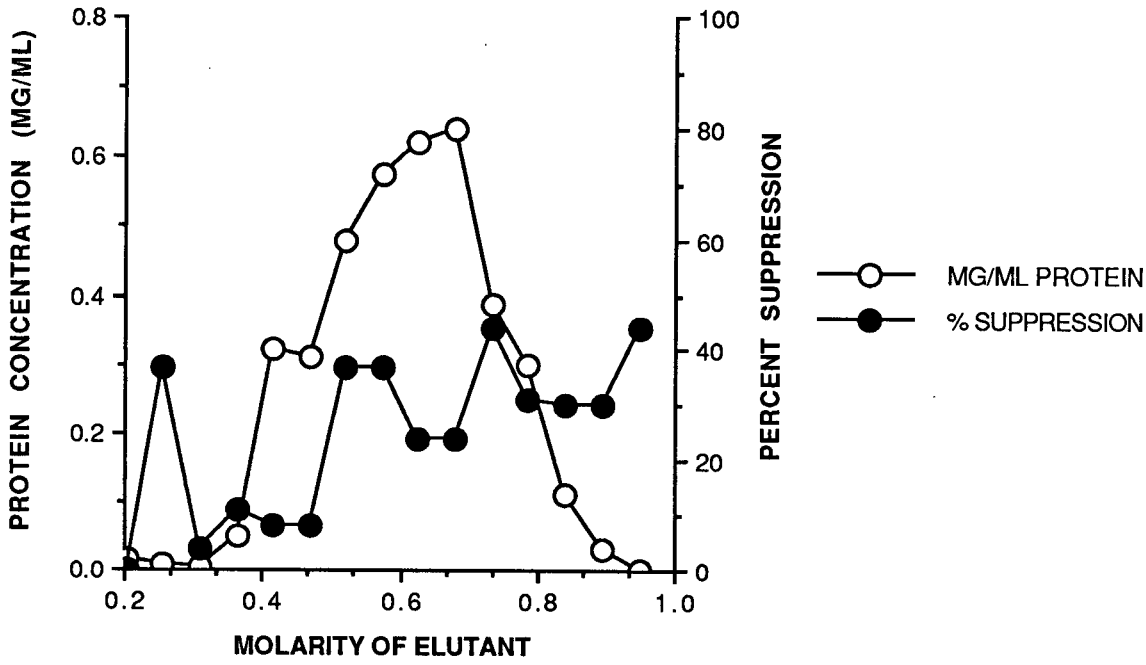


FIGURE 5c: ELUTION AND BIOACTIVITY PROFILE FROM S-SEPHAROSE CHROMATOGRAPHY.

FIGURE 6: **SAMPLE FPLC ELUTION PROFILES OF POOLED, BIOACTIVE ION EXCHANGE FRACTIONS.** DEAE-eluted fractions #4-7, Round 1 (D1) , #3-7, Round 2 (D2a) and #9-11, Round 2 (D2b) were each pooled and subjected to FPLC using a Superose 12 HR column. D1: (**a**), D2a: (**b**) and D2b: (**c**). Approximately 4.0 mg of protein was applied to the column per run. Protein was eluted with 30 mls of PBS over a 60 minute time interval and 0.5 ml fractions were collected and pooled from subsequent runs (in a and b).

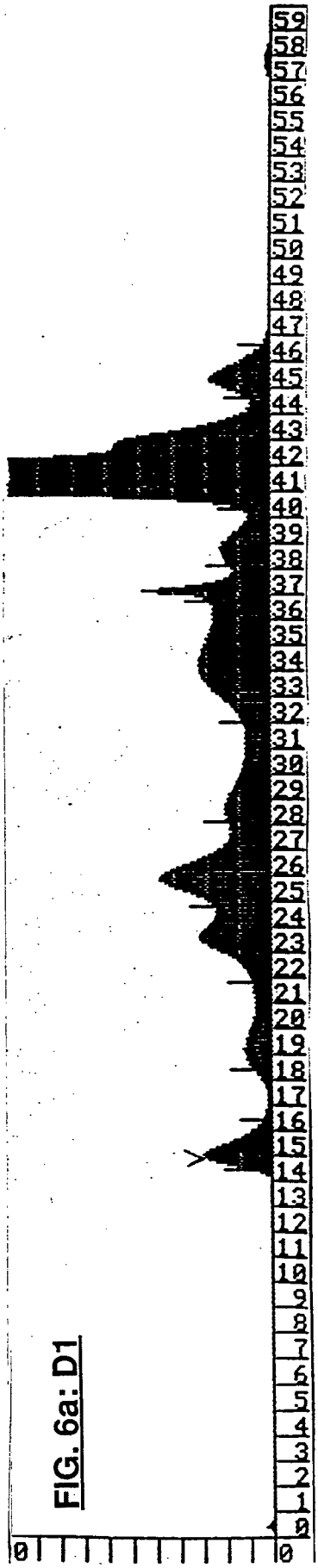


FIG. 6a: D1

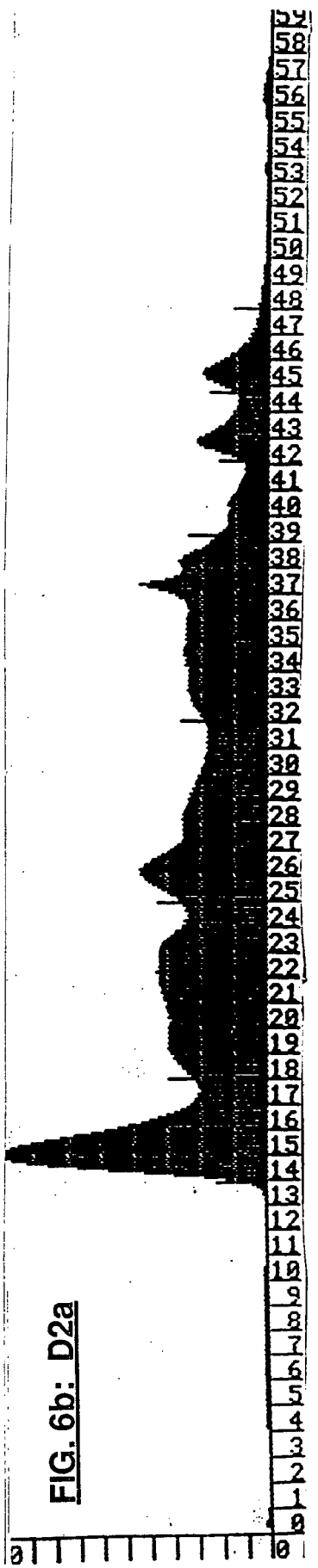


FIG. 6b: D2a

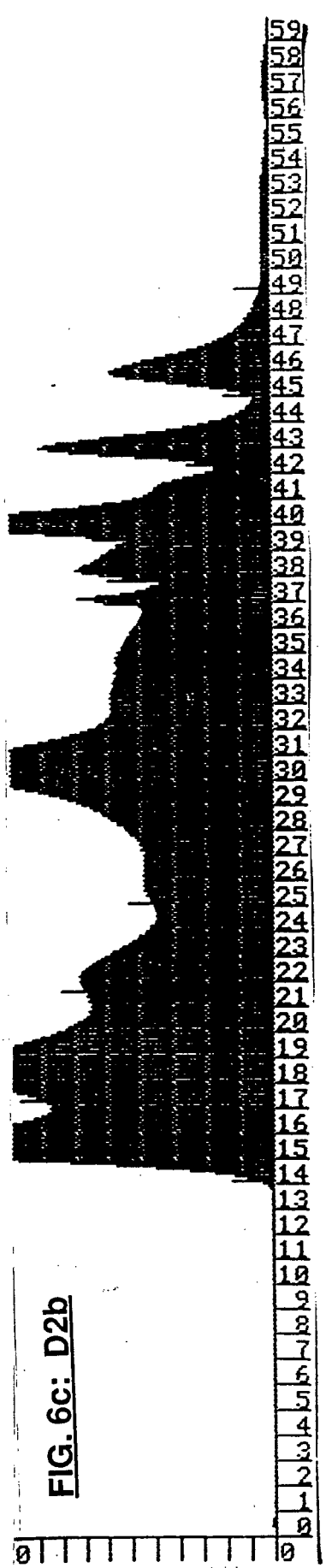


FIG. 6c: D2b

TABLE I: SUPPRESSION OF THE PFC RESPONSE BY FPLC FRACTIONS: D1 represents pooled DEAE fractions #4-7 from round 1, D2a represents pooled DEAE fractons #3-7 from round 2 and D2b represents #9-11 from round 2. Eluted FPLC fractions comprising distinct peaks were pooled and an aliquot of each frozen at -70°C and sent to Halifax to be screened by PFC assay. Percent suppression is given for samples diluted 1 in 20 in the assay and was caluculated from the number of PFC's obtained in PBS versus suppressor sample-treated cultures. Each value represents the average of three values (#PFC's) from duplicate assays.

D1

FRACTION #	% SUPPRESSION
14, 15	79
18-20	28
22-24	0
25, 26	0
28, 29	5
33-35	55
36, 37	15
38, 39	0
40-43	0
44-46	6
57, 58	21

D2a

FRACTION #	% SUPPRESSION
14-16	97
18-20	55
21-23	0
25, 26	0
27-29	45
32-35	29
36, 37	0
38-41	0
42, 43	0
45-46	24
55-57	44

D2b

FRACTION#	% SUPPRESSION
14-16	92
17-19	79
20-23	25
24-27	49
28-31	36
32-35	44
36, 37	16
38	78
39-41	62
42-44	55
45-48	3

REDUCING SDS-PAGE OF POOLED FPLC FRACTIONS:

Eluted FPLC fractions comprising distinct protein peaks were pooled, dialyzed against 50 mM ammonium bicarbonate, lyophilized and subjected to reducing SDS-PAGE. A representative gel is presented in Fig. 7. There are several bands in common between lanes containing bioactive material. As was mentioned, the earliest eluting peak in all three FPLC runs (Fractions 14 and 15 from D1 and 14-16 from D2a and D2b) contained the highest levels of bioactivity. The proteins contained within D2b (14-16) appear to be mostly degraded and thus cannot be analyzed. The first peaks from D1 and D2a however, both can be seen to contain bands with M_r's of >110 KD, 70 KD, 55 KD and 40 KD. Of these four bands, the 55 KD and possibly the 110 KD bands are unique to lanes containing bioactive material. FPLC peak D1(25, 26), was shown to have zero bioactivity, yet contain larger amounts of many different proteins. For this reason, it is difficult to confirm the presence or absence of the 70 KD and 55 KD bands. However, it is evident that this peak does contain the 40 KD, as well as a small amount of >110 KD protein (see discussion). Peak D2a(18-20), was able to suppress the PFC response by 55% (Table I). This peak is seen to also contain the >110 KD, the 70 KD and the 40 KD protein bands, but the 55 KD band is not evident. Peak D2a(27-29) is shown to be comprised of large amounts of different proteins, and had some bioactivity (45%). This peak does not show bands with Mr's of >110 KD, or 70 KD but does contain the the 50 KD protein. The remaining lanes contain material with zero bioactivity and none of the above discussed four bands are present.

In summary, although the suppressor activity in FPLC-eluted material cannot be definitely assigned to any one protein, bands with Mr's of >110 KD and 55 KD were only observed in lanes containing bioactive material (except the small amount of >110 KD that can be seen in D1(25, 26), see discussion).

MYCOPLASMA INFECTION OF THE M1-A5 CELL LINE

As the above described purification process was being carried out, M1-A5 cells started to exhibit a different growth pattern, dysplasia and increased (over 50%) cell death. The first observed change was a greatly decreased doubling time coincident with an increase in cytoplasmic granularity. Eventually cells in culture began to crenate slightly and occassionally formed large multi-nucleated syncitia. Simultaneously, the characteristic

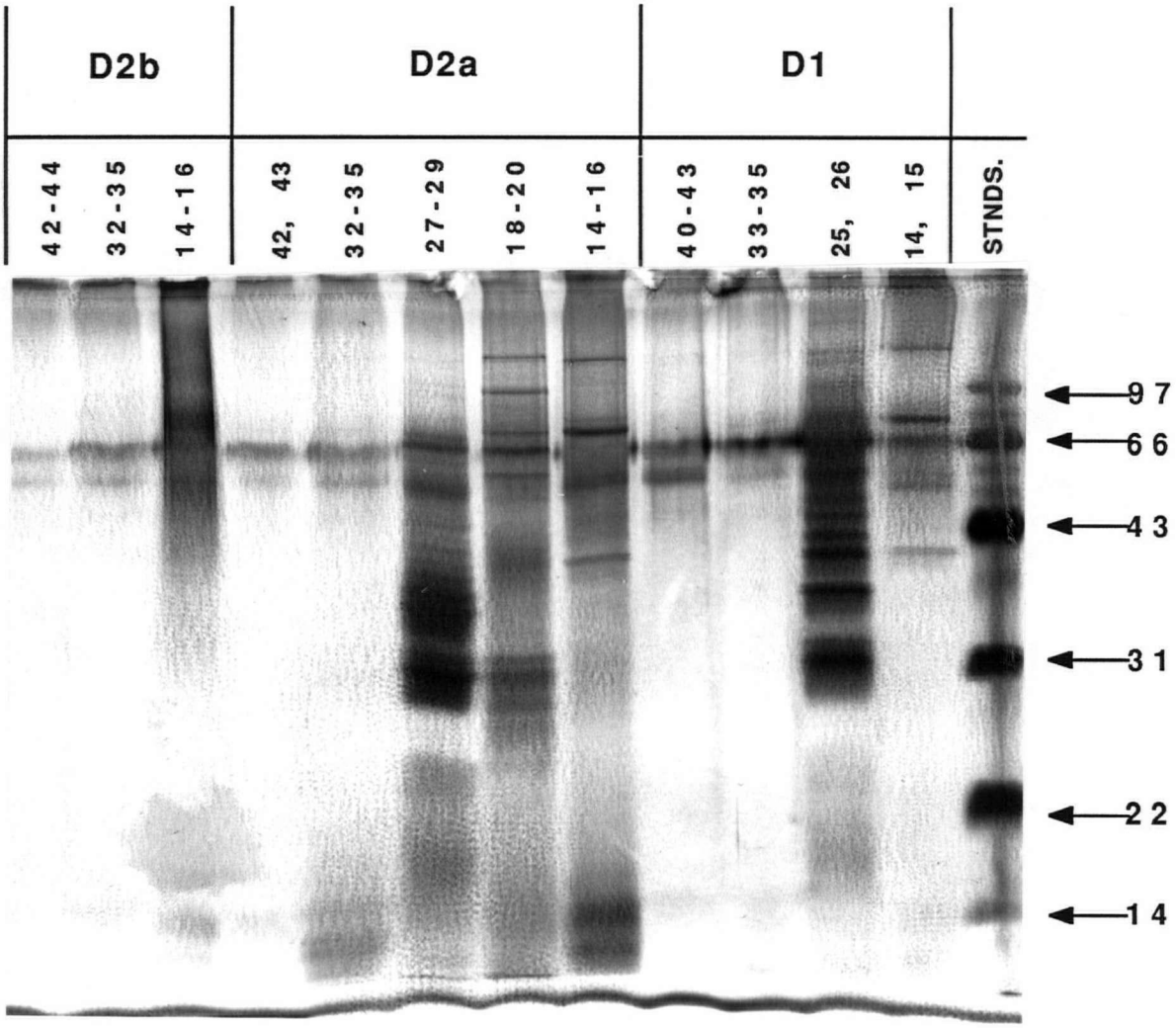


FIGURE 7: SDS-PAGE OF SELECTED FRACTIONS OBTAINED FROM FPLC. Eluted fractions comprising protein peaks were pooled, dialyzed against 50 mM ammonium bicarbonate, lyophilized and subjected to reducing (10 mM DTT for 5 minutes at 100°C), SDS-PAGE using 10% acrylamide. Protein bands were visualized by staining with 0.1% $AgNO_3$. D1, D2a and D2b represent pooled, bioactive, DEAE-Sephacel fractions #4-7 from round 1, #3-7 from round 2 and #9-11 from round 2 respectively that were subjected to FPLC.

suppression of the serum-free culture supernatant became irregular in both the KLH Antibody Culture and the PFC Assays. Shortly thereafter, Dr. Pope's laboratory communicated to us that the M1-A5 cell line had come back positive for an animal strain of *Mycoplasma*. Several attempts were made in Halifax to cure the cell stocks (which also proved to test positive) of the *Mycoplasma* infection.

MYCOPLASMA TESTING AND ANTIBIOTIC CURING OF M1-A5 CELLS:

When the M1-A5 cell line was originally isolated in the lab. of Dr. B. Pope, cells were tested for *Mycoplasma* both in the lab. (by DAPI immunofluorescent staining) and at a nearby hospital (*Mycoplasma* culturing) on a regular basis. All results had been negative. Here at UBC, our lab. also does routine testing of all cell lines using the DAPI method. Our M1-A5 stock tested negative three times by these methods. Once Dr. Pope received word from the hospital labs that her current and frozen stocks were contaminated, an aliquot of our stock was sent and also found to be positive. The circumstances of this seemingly sudden pan-contamination are curious, as the hospital diagnostic technologists assured Dr. Pope that there had been no change in the sensitivity of the detection method in the time that she had been sending cells there for testing.

Dr. Pope's lab. undertook attempts to cure M1-A5 of *Mycoplasma* with the commercially available antibiotic kit from Boeringher Mannheim. The treatment was successful, however, coincident with losing the *Mycoplasma*, cell supernatants lost the ability to suppress the PFC response. Three independently cured M1-A5 cultures gave the same result. Given our inability to prove that the previously observed suppression was caused by products other than those which were *Mycoplasma*-derived, Dr. Pope decided to discard the M1-A5 system and begin the process of isolating a new natural suppressor cell line. In light of her decision, and the *Mycoplasma* problem, our lab decided to follow her lead and leave this project.

SECTION 1: DISCUSSION.

Experiments investigating the mode of action of SIF have been interpreted to suggest that it acts by inducing suppressor cells present in normal murine splenocyte preparations in an antigen nonspecific fashion (hence the name Suppressor Inducer Factor - SIF). In the original suppressor cell assay of Almawi and Pope (1985) and Pope (1987), normal splenocytes exposed to M1-A5 supernatant in Marbrook vessels were co-cultured with twice the number of untreated normal spleen cells (NSC) and SRBC. After 5 days, the cell mixture was tested for its' ability to launch a B cell response against SRBC (by PFC Assay). Pope explains the observed suppression by presuming that SIF (in the M1-A5 SN) induces a suppressor cell population(s) or network which in turn inhibits the secondary immune response of the spleen cells to SRBC. The level of suppression obtained in these experiments was from 55-67%, depending on the number of M1-A5 cells or concentration of the M1-A5 supernatant (see Fig. 1a) in the outer chamber of the Marbrook vessel in the activation step. Considering that one third of the cells in the suppressor assay culture are SIF-treated and two thirds are untreated, the fact that the level of suppression obtained by PFC Assay is greater than 33% (ie. one third) suggests that SIF does not act directly on effector cells (B or T cells). Partly in support of this is Pope's assertion that blastogenesis is not affected by SIF treatment because (a) normal cell numbers were obtained in control and suppressor tests and (b) SIF treatment had no effect on splenocytes ability to respond to Con A (Pope, 1987). However, Con A has no effect on B cell blastogenesis, thus this observation does not rule out the possibility of abnormal B cell proliferation, IgH gene class-switiching and/or inhibition of antibody production. It would have been useful to have tested the ability of SIF to suppress the splenocyte response to LPS, which selectively activates B cells or PWM which activates both T and B cells. It is possible that SIF somehow alters the functional ability of B cells directly. Some mechanisms might be inhibition of the B cell response to T cell-derived B cell growth factors such as BCGF and IL-3.

Experiments by Almawi and Pope (1985) and Pope (1987) have suggested that SIF-induced suppresssor cells act via the release of suppressor factor(s). It was observed that SIF-activated NSC (incubated in the inner chamber of a Marbrook vessel) secreted a substance(s) (less than 12 KD) which was able to suppress the PFC response of untreated NSC incubated in the outer chamber. Two attempts have been made to purify enough SIF material to obtain a protein sequence, begin production of a monoclonal antibody and study

its mode of action. In this work, a third attempt was made to purify SIFβ, and a new assay was adapted to study suppression of antibody responses by SIF.

For the results presented in Fig. 1b and this purification work, the suppressor assay discussed above was simplified in order to screen suppressor samples faster and with more ease. Instead of preactivation of NSC in Marbrook vessels, just a straight PFC assay was done. SIF sample was added directly to a mixture of NSC and SRBC and incubated for 5 days before assaying for the presence of antibody producing cells. This change in assay procedure should not alter the normal action of SIF, but as expected, did increase the sensitivity of the assay. However, this increase was unexpectedly large in magnitude (see dilution of SN that gives 50% suppression in Fig. 1a versus 1b). Some of this difference in sensitivity can be explained by examining each protocol. In the basic PFC assay used by Almawi *et al* (1988b) to screen for SIF activity, SIF is added to all cells in culture for the duration of the five day incubation with antigen. Thus, the entire culture is 'activated' and SIF has the potential to continue 'inducing' for as long as its activity is stable in serum-containing media. In Pope's suppressor assay (Pope *et al.*, 1987), one third of the NSC preparation is treated, washed and then co-cultured with the remaining two thirds of the NSC's (untreated). Thus any observed suppression of the 2° Ab response to SRBC 5 days later must be mediated by the suppressor cell population within the initially activated NCS preparation. It would have been interesting to observe the effect of activating increasing numbers of NSC (and co-culturing these with decreasing numbers of untreated cells) on the magnitude of suppression.

In an attempt to study the nature of SIF action further, KLH-immune splenocytes were activated by antigen and subsequently cultured for 7 days with various dilutions of M1-A5 serum-free SN in the Ab C assay. Anti-KLH titers were determined by KLH-ELISA. From Fig. 4, the ED_{50} of M1-A5 SN in this assay was found to be 10^{-2}. Thus this assay is more than 100 times more sensitive than the original suppressor assay of Pope *et al.* and 10,000 times less sensitive than the PFC assay used to screen for SIF activity.

Both the Antibody Culture (Ab C) and the Plaque Forming Cell (PFC) Assay measure the ability of primed murine splenocytes to respond to furthur antigenic challenge by quantitating the B cell response. However, these two tests also have significant differences. In the PFC assay, suppression of the secondary response to antigen is measured after SIF (or activated SC) is introduced in the priming reaction and in the Ab C Assay, suppression of the tertiary response to antigen is measured with no interference in the primary or secondary response (occurs *in vivo*). It would appear from this and the results presented in Fig.'s 1 & 3, that SIF, whatever it's exact mode of action, is much

more efficient at effecting suppression of the B cell primary responses (ie. priming) than secondary or tertiary responses. If the priming reaction is inhibited *in vitro* in the PFC assay, suppression of the response to subsequent challenge with antigen would be observed. Alternately, it was possible that a longer incubation period was required for SIF and unactivated (but immune) splenocytes in order to see an optimal effect. SIF is added to culture directly after the *in vitro* KLH boost in the Ab C Assay. Given that a tertiary response is being elicited, this protocol may not have allowed sufficient time for SIF to induce the suppressor cell population and/or for this population to act on target cells before too much stimulation or activation of immune splenocytes had occurred.

In order to determine whether the time of additon of SIF or M1-A5 supernatant to splenocytes in the Ab C assay was a factor in the magnitude of the resultant inhibition, an experiment was done in which one half a preparation of KLH-immune splenocytes was preincubated for six hours with SN and then pulsed with KLH and cultured, and the other half pulsed and cultured with the SN immediately. The results (Fig. 4) clearly indicate that the preincubation step abolished most of the inhibition of the anti-KLH response. This loss of suppresssion with preincubation would suggest that some antigen must be present in order for SIF to act. It would have been interesting to know whether the injection of purified, bioactive M1-A5 material with KLH into syngeneic mice in the priming stage of the immunization schedule would have any effect in the resultant anti-KLH levels in this assay. It is also reasonable to question the stability of SIF in serum-containing media. In the PFC assay, with antigen present, SIF may act early to induce the suppressor population and thereafter be inactive in the culture. If this were the case, in the preincubated Ab C assay, SIF may have been unable to act on the immune SC preparation without antigen present. The factor may then have been degraded or rendered inactive by serum components before the *in vitro* antigen pulse.

There are also differences between the Ab C and PFC Assays that are inherent in their modes of quantitation that would most likely give different results regardless of the mode of action of the factor being tested. In the PFC Assay, one activated antibody-producing cell forms 1 plaque, thus an accurate measure of the magnitude of the B cell response is obtained by quantifying the number of plaques produced in each test. The directness of this assay results in increased sensitivity. In the Ab C Assay, the relative amount of specific antibody produced (not antibody-producing cells) in each test culture is determined by sandwich ELISA. Thus, this assay measures the magnitude of the B cell response in a more indirect manner, resulting in decreased sensitivity. In addition, there are many more steps in which variability can be introduced, from the immunizations through to the

antigen-coating and developing of ELISA test plates. It would be very difficult to quantitate what proportion of the large difference in sensitivity observed in this work (Fig. 1b versus Fig. 3) is inherent in the protocols of these assays versus a reflection of the mode of action of SIF.

M1-A5 cell cultures were expanded in order to begin the purification of SIF from spent cell SN. During this time, it was observed that if the WEHI-3B cells were allowed to overgrow slightly before harvesting the SN for addition to M1-A5 growth media, they released a product which was autolytic and potent. This factor, when added (at 1 in 5 dilution) to M1-A5 media subsequently caused complete death of the culture within 2 days. When WEHI cells were seeded into fresh media, the remaining live cells did not recover. From several WEHI-3B growth curves representing cultures seeded at increasing cell densities (Fig. 2), it was found that the optimal cell concentration to harvest SN to support the growth of M1-A5 cells was 5 X 10^5 cells per ml from cultures seeded at 1-5 X 10^4 cells per ml.

The previously described process for purifying SIF α and β from serum-free culture supernatants involved size fractionation on a Sephadex G-100 column followed by ion exchange chromatography and two rounds of RP-HPLC of bioactive fractions (Almawi *et al.*, 1988b). The size and composition of the bioactive HPLC fraction(s) was determined by SDS-PAGE using a 5-15% acrylamide gradient. When the SIFβ preparation was sent out of the lab. for sequencing, it was reported that the amino terminus of the peptide was blocked. This was thought to be caused by the harsh conditions of acetonitrile elution during the two rounds of RP-HPLC and unfortunately there was not enough purified material to effect the sequencing of separated tryptic peptides.

Due to the high binding capacity of the more contemporary ion exchange column matricies, it was decided that the size fractionation step of the purification process could be omitted and serum-free culture supernatants were added directly to DEAE-Sephacel and S-Sepharose columns set up in tandem. Eluted, bioactive fractions were pooled in peaks and subjected to FPLC, and eluted bioactive FPLC fractions comprising distinct peaks were pooled and a sample from each subjected to SDS-PAGE to determine the composition and level of purity. The lack of distinct peaks and significant levels of bioactivity obtained in the S-Sepharose eluate and the fact that purified SIFα has been shown to be much less active than SIFβ prompted the decision to continue with the purification of SIFβ only.

The bioactivity eluted with the main protein peak in both rounds of DEAE-Sephacel chromatography (D1 and D2a). Suppression was also seen in two fractions eluting at higher molarities in the second round (D2b) but these contained lower amounts of protein.

Peaks of bioactivity were pooled and subjected to FPLC using a Superose 12 HR column. The highest levels of bioactivity were found to reside in the earliest eluting peaks in all three rounds (D1, D2a and D2b, see Fig. 6 and Table I) of FPLC. This was suprising given that the molecular weight of purified SIFβ has been determined to be 6 KD (Almawi *et al.*, 1987 and 1988b). The bioactive component of this protein peak was possibly in the form of large multimers, or alternately, associated with another protein.

Material within bioactive and inactive FPLC-eluted peaks were subjected to reducing SDS-PAGE (Fig. 7). The first peak from D1 and D2a had several protein bands in common (Mr's of >110 KD, 70 KD, 55 KD and 40 KD). Of these, there is only the suggestion that the 55 KD, and possibly the >110 KD bands are unique to lanes containing suppressive activity. Peak D1(25, 26), although inactive in the PFC assay at a 1 in 20 dilution, was found to contain many different size proteins. Thus, eventhough there is a small amount of the >110KD band in this lane, if this is the protein which mediated the observed suppression in other peaks, it's activity may have been diluted out or altered in this peak by another component.

Although the protein concentration and bioactivity of the serum-free M1-A5 supernatant was not determined at the start of this purification, 17.60 mg (Fractions #4-7) of pooled bioactive material was obtained in the first round of anion exchange (DEAE) chromatography and 18.35 mg (Fractions #3-7) plus 1.05 mg (Fractions #9-11) were obtained in the second round.

The average ED_{50} of D2a was 4.74 µg, that is, approximately 4.74 µg of the pooled sample gives 50% inhibition of the antibody response of murine NSC to SRBC (as determined by PFC Assay). Although much less protein was obtained in D2b (1.05 mg), this pool gave an average ED_{50} of 0.340 µg, indicating a 14-fold higher level of activity in this sample. A similar titration of bioactivity was not done in the screening of the first set of DEAE fractions, thus the ED_{50} of D1 cannot be determined for comparison. However, if one compares the amount of suppression obtained at a similar dilution (1 in 20) in D1 and D2a, and knowing the protein concentration of each, the activity (expressed as percent suppression per µg protein) can be determined.

The recovery of protein from the FPLC Superose 12 HR column (Pharmacia) has been documented as being from 80-85% (Pharmacia Superose 12 HR product file). For these purposes, the lowest value was chosen (80%). As the FPLC monitor integrates the area beneath each eluted protein peak (elution time or volume versus absorbance at 280 nm) and presents this value as percent of protein loaded onto the column, the protein concentration of each peak can be determined. Unfortunately, limiting dilutions of each

FPLC fraction were not tested by the PFC Assay. Thus, the increase in activity from the bioactive ion exchange fractions to the FPLC fractions cannot be determined by standard methods (ie. comparing ED_{50}'s or units of activity per amount protein). Instead, as described above, the percent suppression per μg protein (determined from a sample diluted 1 in 20 in the assay) was used to determine which protein peaks contained the highest levels of activity.and compare activity between steps of the purification. To further complicate the analysis, in 2 of the 3 most active FPLC peaks (Peak #1 in all three runs), the level of activity had not even begun to titrate at the 1 in 20 dilution (the only common dilution between all samples in the purification) tested, thus no accurate quantitation could be made.

In FPLC profile D1, 112 μg of protein eluted in the first peak. Of this amount, 11.20 μg (a 1 in 5 dilution in the PFC assay) and 5.60 μg (a 1 in 10 dilution) suppressed the PFC response by 95 and 90% respectively. When 2.80 μg (a 1 in 20 dilution) was tested, 79% suppression was observed. As the bioactivity had begun to titrate, Peak 1 of D1 can be said to have an activity of 28.2% per μg protein. Peak #1 from D2a contained much higher amount of protein (700 μg). 70 μg (1 in 5 dilution in the PFC assay), 35 μg (1 in 10) and 17.5 μg (1 in 20) all gave from 97 to 100% suppression. The bioactivity contained in this sample had not begun to titrate at the dilutions tested and thus a calculation of percent suppression per μg protein would be meaningless. If the relative amounts of protein tested from Peak #1 in D1 and D2a at the 1 in 20 dilution are compared (2.8 versus 17.5 μg), it is not suprising that a decrease in the amount of suppression was not observed. In the third FPLC run (D2b), 1.05 mg of protein was separated. Coincidently, approximately the same amount of protein eluted in the first peak of this run (115 μg) as in the first run (112 μg) eventhough greater than 4 times the amount of protein was loaded in the latter. 1 in 5 (11.5 μg) and 1 in 10 (5.75 μg) dilutions of the material in this peak both gave 98% suppression of the PFC response, whereas the 1 in 20 dilution (2.87 μg) gave 92%. If this 6% decrease in activity with one doubling dilution was the start of a titration, the activity of this first Peak would be 32.1 percent per μg protein, which compares well with the value of 28.2 obtained from 2.8 μg of Peak #1 in D1. In addition, peak D2b(17-19) and D2b(38) were found to have activities of 20.4 and 53.7 percent per μg protein respectively. Lower levels of suppression were also observed scatterred throughout all three elution profiles (Table I)

During the purification process presented in this work, M1-A5 stocks and cultures were found to be infected with an animal strain of *Mycoplasma.* Since curing cultures of the infection resulted in a complete loss of suppressive activity in three separate

attempts, the origin of the moiety mediating the above discussed bioactivity was brought into question. Dr. Pope maintains that the characteristics of suppression by M1-A5 SN became irregular a few months before the above determination was made.

It is quite possible that the problems encountered in the culturing of M1-A5 cells in this lab (slow doubling time, large amount of cell death etc.) are the direct result of the *Mycoplasma* infection. In addition, it is also possible, though less likely, that all of the suppression observed in the M1-A5 system has been mediated by products of *Mycoplasma* origin. Thus the purification presented in this work may have been guided by following the bioactivity levels of a *Mycoplasma*-derived product.

SECTION 1: CONCLUSIONS

Given the antigen-nonspecific, and MHC nonrestricted mode of action of the partially characterized Suppressor Inducer Factor (SIF) secreted by the isolated NS cell line M1-A5, an attempt was made to purify the β form to homogeneity for biological study, amino acid sequencing and the synthesis of a monoclonal antibody. Following a modified version of the previously described SIF purification protocol, an attempt was made to purify SIFβ using ion exchange chromatography, and FPLC. Although, the level of bioactivity was followed through these two steps, in the final analysis of FPLC eluted proteins by SDS-PAGE and PFC assay, suppressive ability could not be definitively assigned to any one protein band (although there are possibilities which could have been further investigated) and high levels of activity (percent suppression per μg protein) were found to be scattered throughout the profiles. Whether this reflects a mixture of M1-A5 and Mycoplasma derived products was not determined.

In addition, an *in vitro* assay was adapted for studying suppression by M1-A5 SN. M1-A5 SN in the Ab C assay gave a suppression curve from which an ED_{50} of 10^{-2} was obtained. In addition, it was found that preincubating SN with immune SC prior to antigen pulse and culture virtually abolished all suppressive ability. These results were discussed and compared with data obtained in earlier work using different assays. It can be concluded that suppression by M1-A5 SN requires the presence of antigen, yet is most pronounced when present during the generation of specific T cell clones and memory cells (ie priming or generation of the primary response).

The origin and nature of the soluble product(s) studied in this work remain unknown, as this line of research has been discontinued.

SECTION 2: ANTIGEN-SPECIFIC IMMUNOSUPPRESSION

INTRODUCTION

Active investigation into the phenomenon of antigen-specific suppression began in the mid-seventies with several groups reporting the existence of antigen-specific Ts cells and soluble "suppressive" factors. For example, spleen and thymus extracts from mice primed with hapten-carrier conjugates were shown to contain a suppressive factor which bound specific antigen, reacted with anti-MHC serum and had a MHC-restricted mode of action (Kapp *et al.*, 1974 and 1976 and Takemori and Tada, 1975). This restriction, which mapped to the I region of the mouse MHC, and was shown to be distinct from that mediated by I-A and I-E products, was named I-J (Murphy *et al.*, and Tada *et al.*, 1976).

Shortly thereafter, Cantor and Gershon (1979) postulated the existence of distinct subsets of Ts cells which could be distinguished according to their expression of various cell surface markers and individual roles in effecting suppression. Yamauchi *et al.* (1981a & b) confirmed that suppression involved several distinct Ts cell types. Further, they showed that this suppression was mediated by the release of soluble factors (TsF's) which functioned to activate the specific Ts subsets in a sequential manner. This, and other studies has led to the general acceptance of the existence of antigen-specific Ts cell circuits. In the most widely accepted pathway (rev. in Germain and Benacerraf, 1981) of antigen-specific suppression, a Ts1 (suppressor inducer) cell stimulated by antigen releases TsF-1, an antigen- or idiotype-specific suppressor factor that activates a Ts2 (transducer or regulatory) cell. It in turn releases TsF-2, which is thought to be anti-idiotypic, and cause activation of the Ts3 cell (effector). The Ts3 cell is then thought to act (directly or via antigen-/idiotype-specific TsF-3 secretion) on Th cells to ultimately cause down-regulation of B cell and CTL function.

ATTEMPTS TO CORRELATE Ts CELL SURFACE PHENOTYPE WITH FUNCTION

Initial cell surface characterization of the components of murine suppressor T cell circuits described $Ly1^+2^-$, $I\text{-}J^+$ cells that functioned as inducers and activated $Ly1^+2^+$ regulatory cells. These cells in turn stimulated $Ly1^-2^+$, $I\text{-}J^+$ effector cells which were

distinct from the $Ly1^-2^+$, $I\text{-}J^-$ CTL population (Cantor and Gershon, 1979). In support of this work Yamauchi *et al.* (1981a and b) reported that $Ly1^+2^-$ (Ts1) cells released a factor (TsF-1) which bound antigen and could stimulate $Ly2^+$ cells. They showed that the final step in the path was mediated by $Ly1^-2^+$ (Ts3) cells via the release of effector TsF (or TsF-3). Other groups have reported similar T cell circuits bridged by the release of antigen-binding TsF's (Taniguchi and Tokuhisa, 1980, Hausman *et al.*, 1986 and rev. in Tada, 1984).

Flow cytometric studies of human Ts1 suppressor inducer cells using various monoclonal antibodies combined with biological assays demonstrated that $CD4^+$ T cells could be separated into three distinct subpopulations: (1)$CD4^+$ $Leu8^+$ $CD29^-$(4B4) $CD45R^+$(2H4), (2)$CD4^+$ $Leu8^+$ $CD29^+$ $CD45R^-$ and (3)$CD4^+$ $Leu8^-$ $CD29^+$ $CD45R^-$ (Mohagheghpour *et al.*, 1983, and Damle *et al.*, 1987). All three subpopulations, upon stimulation with alloantigen proliferated and produced IL-2. However, only the latter two subsets were able to respond to soluble antigenic stimuli and only the second group was capable of inducing $CD8^+$ Ts (Damle *et al.*, 1985, 1986 and 1987). The first subset was subsequently found to represent a circulating pool of naive (have not yet interacted with specific antigen) T lymphocytes and not a distinct $CD4^+$ sublineage as originally assumed. Expression of the CD29 and CD45R markers was shown to reflect different stages of immunological differentiation from naive ($CD29^-CD45R^+$) to memory ($CD29^+CD45R^-$) $CD4^+$ cells (Sauders *et al.*, 1988). Although the expression of the Leu8 determinant did not seem to be affected by the state of activation or differentiation of the cell, this marker was later further characterized and found to be expressed on 75% of peripheral T cells as well as some B cells, monocytes and neutrophils (Rich *et al.*, 1986). The authors however still maintain that the $Leu8^+$ and $Leu8^-$ subsets of $CD4^+$ $CD29^+CD45R^-$ T cells mediate suppressor-inducer and helper functions respectively (Kansas *et al.*, 1985 for eg.).

Despite the fact that both CTL and Ts3 (suppressor effector) cells are $CD8^+$, the antigen-mediated activation of each is known to occur by a different mechanism. $CD8^+$ $CD28^+$ CTL are stimulated by direct contact with activated APC or altered self (ie viral or allogenic determinants expressed on autologous cells) and ultimately are cytotoxic to their target, whereas $CD8^+CD28^-$ Ts are activated by indirect contact (via soluble TsF) with antigen-stimulated $CD4^+$ suppressor-inducer cells (Damle et al., 1984 and 1985).

PURIFICATION OF T CELL-DERIVED SUPPRESSOR FACTORS (TsF's)

Only very small quantities of TsF were detected in the extracts from murine lymphoid organs in early suppression studies, and it eventually became critical to purify TsF's to homogeneity to further characterize their nature. Since biochemical purifications require large amounts of starting material, attempts were made to isolate Ts cell clones and make Ts cell hybridomas, that would constitutively synthesize and secrete TsF into their growth media.

Some groups attempted to obtain Ts cell clones by adding exogenous IL-2 to *in vitro* cultures enriched for Ts cells (Nakauchi *et al.*, 1984 for eg.) and others fused enriched Ts cell preparations with the thymoma BW5147 to obtain Ts cell hybridomas (Kapp *et al.*, 1980 for eg.). Using these approaches several groups (Taniguchi *et al.*, 1979 and 1980, Kitamara *et al.* and Nakauchi *et al.*, 1984, Kapp *et al.*, 1980 , Krupen *et al.*, 1982, Turck *et al.*, 1986, Saito and Taniguchi, 1984, Webb *et al.*, 1989, Fresno *et al.*, 1981 and Hausman *et al.*, 1986 for eg.) reported on the purification and characterization of antigen-specific TsF's, yet to date none of the genes encoding such factors have been cloned. There is no firm consensus with respect to size (from 19 to 90 KD M_r), structure (single versus two-chain and disulfide bonded versus noncovalent association) serology (reactivities with I-J antisera and anti-TCR reagents) or mode of action (MHC restricted or nonrestricted) of TsF's (Steele *et al.*, 1987a).

There is however, tentative agreement as to the nature of TsF-1 (suppressor-inducer factor). TsF-1 is thought to be composed of at least two molecules, a T cell derived antigen binding molecule (T-ABM) and an antigen-nonspecific accessory molecule which bears I-J determinants. TsF-1's are: (1)antigen specific (2)display Igh but not MHC restriction (3)function during the inductive but not effector phase of the suppression response (4)suppress both delayed type hypersensitivity (DTH) and plaque forming cell (PFC) responses and (5)bind to antigen and isotype-specific anti-TsF antibodies (from Kuchroo *et al*, 1990).

One group has isolated (in the form of Ts cell hybridomas) all three orders of Ts cells and TsF's that participate in suppressing the immune response in the NP (dinitrophenyl) hapten system (Hausman *et al.*, 1986 and Kuchroo *et al.*, 1990). The NP-TsF-1 was found to be idiotypic (binds to NP), express I-J determinants and have a nonrestricted mode of action. The NP-TsF-2 also expressed I-J determinants, but had a restricted mode of action and was anti-idiotypic (binds to anti-NP Abs). Finally, NP-TsF-3, like TsF-1, was found to be idiotypic (binds to NP), and $I\text{-}J^+$. With respect to biological function,

TsF-1 could suppress the contact sensitivity (CS) to NP if it was administered to the animal at the time of antigen-priming, TsF-2 could suppress the same response at any time in previously primed animals, and TsF-3, which was postulated as being primed simultaneouly with Th cells, had the ability to not only suppress the CS response to NP but also to other antigens (bystander suppresssion) (Hausman *et al.*, 1986). As mentioned above, other Ts/TsF pathways are reviewed by Tada (1984).

Very recently, Kuchroo et al. (1990) working in the NP system has made Ts hybridomas using a new fusion partner (BW100) that is functionally TCR$\alpha\beta^-$. These should help to characterize and clarify the nature of Ts antigen-receptor interactions.

MOLECULAR INTERACTIONS IN Ts-MEDIATED SUPPRESSION

Although fairly well characterized in B cells, CTL and Th, the nature of the receptor-ligand interaction that mediates Ts activity is poorly understood. Work from several laboratories (Zheng *et al*, 1988 and 1989 and Fairchild *et al*, 1988 for eg.) have finally confirmed that Ts cells interact with antigen via the standard TCR$\alpha\beta$ complex. Early studies on Ts hybridomas indicated that there was no CD3/TCR cell surface expression nor functional TCRα or β RNA transcripts in Ts - suggesting that these cells utilize some other form of antigen receptor. A possible reason for the conflicting reports and discussions in regard to this matter was offered initially by Weiner *et al*, who in 1988 found that 1-3% of Ts hybridoma cells in culture expressed the CD3 marker. Upon cloning these cells, as well as several $CD3^-$ cells from the same culture, it was found that only the $CD3^+$ cells were capable of sythesizing detectable amounts of factor. The CD3 molecules were subsequently shown to be associated with a functional TCR$\alpha\beta$. The above has been repeated with the same results on another apparently $CD3^-$ hybridoma (Kutchroo *et al*, 1988). In this latter study, it was also shown that modulation of the CD3/TCR complex from the cell surface effectively removed antigen binding by the cell suggesting that no other antigen-binding moiety resided on the Ts cell surface. Thus it is possible that as Ts hybridomas are maintained for extended periods in culture, fewer cells express TCR (possibly due to chromosome loss). This may in turn correlate with a decrease in TsF production and be a contributing factor in the difficulties that have been experienced in their biochemical purification.

The effector leg of antigen-specific suppression circuits are thought to act by blocking the activation of B cells and CTL by inhibiting the ability of $CD4^+$ helper/ inducer cells to

promote their growth and differentiation (Rich *et al*, 1986). Recent Ca^{++} flux studies have suggested that the critical event in this final step of the suppression pathway is the inhibition of early Th membrane signal transduction (Utsunomiya *et al*, 1990).

As was mentioned previously, the genetically restricted mode of action of some of the described TsF's was found to map to the I region of the mouse MHC (Murphy *et al.*, 1976), yet the expressed determinant appeared to be distinct from I-A and I-E. Subsequently, the entire I region was analyzed at the molecular level and the gene(s) encoding I-J was not found (Kronenberg, 1983). Thus the question of: "What is I-J?" still remains. One group has suggested that I-J is a receptor for I-A products and another that I-J is actually a TCR$\alpha\beta$ determinant (Green and Zheng, 1989). Whatever I-J is, it has been demonstrated to play an important role in Ts systems in some strains of mice, and thus may be involved in the control of suppression of immune responses.

The finding that Ts cells do express conventional TCR$\alpha\beta$ has raised the question of whether there is any relationship between Ts TCR and antigen-specific TsF's. Work by several groups (rev. in Green and Zheng, 1989) has suggested that TCR genes, especially α, play an imporant role in mediating the fine antigen-specificity of TsF's. Since there are important differences between TCR and TsF's, if this theory turns out to be true, Green concedes that the mechanism of TsF generation will likely involve alternate RNA splicing or post-translational modification of TCR-gene products. It is interesting that alternate splicing of TCR RNA has already been observed by different groups (Behlke and Loh, 1986 for eg.).

THE P815 TUMOR, Ts CELLS AND THE MAb B16G

Several groups have studied the nature of Ts cells in tumor-bearing animals (Fisher and Kripke, 1982, Granstein *et al.*, 1984 and Mills and North, 1985 for eg.). Working with the murine mastocytoma P815, Takei *et al.* (1976) observed that the presence of Ts cells in DBA/2 tumor-bearing animals correlated with an increase in tumor growth. In 1978, Takei *et al.* demonstrated the presence of a soluble factor (TsF) in the thymocytes of tumor-bearing DBA/2 mice that was able to suppress the *in vitro* generation of CTL to P815 tumor cells.

The above TsF was affinity-purified over a P815 membrane extract column and used to synthesize the TsF-specific monoclonal antibody B16G (Maier *et al.*, 1983). Injection of B16G into mice slowed the growth of not only the P815 tumor, but also the chemically

induced syngeneic fibrosarcoma M-1. B16G was also found to be nonspeci-fically immunoenhancing and to selectively remove a subset of regulatory T cells from a thymocyte preparation. In light of the above observations, it was postulated that B16G recogizes an invariant epitope on TsF's.

ISOLATION OF A10, A Ts CELL HYBRIDOMA

In order to further study and characterize the nature of TsF's, P815-stimulated thymocytes were fused with the thymoma BW5147 and the antigen-specific hybridoma A10 was isolated (Steele *et al.*, 1985). A soluble factor (A10F) released by A10 cells was bound by P815 and B16G affinity columns and was able to accelerate the growth of the P815 tumor, but not other tumors (M-1 or L1210). Affinity enriched A10F appeared as 3 distinct protein bands of 140, 80 and 45 KD when subjected to reducing SDS-PAGE (Chan *et al.*, 1988 for eg.). Of these, only the 140 and 80 KD proteins had suppressive ability. The 80KD band was thought to dimerize (to the 140KD band) and a minor protein, appearing occasionally at 30KD was assumed to be a 80KD breakdown product. Protein sequence data from the N-terminus of the 140 and 80 KD bands showed identity whereas that from the 30KD band was distinct. As the antigen to which A10F was specific was uncharacterized, it was difficult to further characterize the biochemical and biological nature of this TsF *in vitro.*

Fd11: A Ts HYBRIDOMA SPECIFIC FOR THE ANTIGEN FERREDOXIN (Fd)

The immune respone to the antigen ferredoxin (Fd) has been an important line of investigation in this lab (rev. in Chu, 1989). Fd is a prokaryotic protein of an electron-carrier chain and has been well characterized biochemically and structurally (Jensen, 1974 and Graves *et al.*, 1985) For these reasons and previous work demonstrating the existence of Fd-specific Ts cell circuits in mice classified as nonresponders to Fd (Weaver *et al.*, 1983 and Singhai *et al.*, 1984), this antigen was chosen for use in the synthesis of a new Ts hybridoma (Fd11). Fd11, like A10, was found to secrete a factor (Fd11F) which bound to Fd and B16G affinity columns, and was able to suppress the *in vitro* and *in vivo* antibody response of Fd immune splenocytes (Steele *et al.*, 1987 and Chu, 1989). Using a Ca^{++}-sensitive reagent (Quin-2), Steele showed that the addition of Fd activates Fd11 cells in culture.

To purify Fd11F to homogeneity, Fd11 cell-free SN was subjected to steps of B16G-affinity chromatography, followed by fast protein liquid chromatography (FPLC) using a Superose 12 HR column (Chu, 1989). Material eluting in the void volume (Mol. wt. >150 KD) of the Superose column was found by ELISA to bind Fd and by *in vitro* Ab Culture Assay to have suppressive ability. Reducing SDS-PAGE of the bioactive FPLC eluate gave protein bands of approximately 80 and 30 KD, as well as some minor bands. However, some 80 KD material was also seen on SDS gels of FPLC eluates from a control cell line (BW5147, the fusion partner for Fd11) SN.

In an attempt to clarify which component(s) of the reduced eluate were responsible for the antigen-binding and suppressive activity, the same FPLC eluates were run out on preparative SDS gels. Protein eluted from gel slices was tested for binding to Fd (by ELISA) and bioactivity (by *in vitro* Ab Culture). Antigen binding was observed in 80, 30 and 16 KD bands from the Fd11F preparation whereas none was observed from the BW5147 preparation. None of the gel slice eluates showed consistent immunosuppressive ability. The 80, 30 and 16 KD components of the above Fd11F preparation were shown to react with an antisera raised to the 30 KD moiety eluted from a gel slice from an B16G-affinity enriched Fd11F preparation (see below). From these results, the author asserts that the both the 80 and 30 KD components of the Fd11 preparation are components of Fd11F.

P80 AND P30: ANTISERA MADE TO COMPONENTS OF AFFINITY-ENRICHED Fd11F

Preparative gels were run on B16G affinity-enriched Fd11F and the 30 KD band excised. Eluted 30 KD protein was used to prime (0.1-2.0 µg in 50% CFA) and repeatedly boost NZW rabbits (North *et al.*, 1988). The anti-p30 titer of the immunized rabbit serum was evaluated by Fd11F ELISA. Normal rabbit serum for use as a control for nonspecific binding was obtained from the blood of rabbits prior to immunization. Anti-p80 was made by the same procedure starting with the 80 KD band from the same Fd11F preparation. This antiserum was shown to have a high background (nonspecific binding) and thus results obtained with this reagent were not reported.

Using flow cytometry, anti-p30 was shown to bind to the surface of Fd11and A10 Ts hybridomas (although with less intensity to the latter), but not to several other Th and Tc hybridomas nor the surface of the thymoma BW5147 (North *et al.*, 1988). The p30

marker was found to be down regulated on the surface of Fd11 cells in response to treatment with phorbol esters. In five different strains of mice, anti-p30 was shown to react with approximately 4% of thymic T cells and 10% of splenic T cells (purified over nylon wool).

To further characterize the population of murine $p30^+$ cells, nylon wool-purified T cells were double labelled with either anti-L3T4 or Ly 2 monoclonals and anti-p30 serum and analyzed on a Coulter EPICS machine. North showed that twice as many $L3T4^+$ cells were $p30^+$ as Ly 2^+ cells. Also, after complement mediated lysis of L3T4 and Ly 2 positive cells, the remaining double negative population was enriched for p30 binding (30% positive).

North *et al.* also tested the ability of anti-p30 to block the suppressive effect of Fd11F in the *in vitro* Ab Culture Assay. It was demonstrated that anti-p30 alone had the ability to enhance the anti-Fd Ab response, and Fd11F alone had the ability to suppress the response (as had been shown previously). Further, the enhancing effect of anti-p30 was shown to be dominant over the suppressive effect of Fd11F when both were added to the same culture.

Although anti-p30 was tested and found to not react with cells from some non-lymphoid tissues (brain, liver, kidney) and bone marrow, it was not tested on B cells or immune cells of the myeloid lineage.

SUMMARY OF WORK TO BE PRESENTED

Using anti-p30 and anti-p80, the population of human periheral blood mononuclear cells staining positive was characterized with respect to other cell surface markers. In addition, cell surface studies and Western blot analyses were done on several $p80^+$ and /or $p30^+$ lymphoid leukemic cell lines.

SECTION 2: MATERIALS & METHODS

ANTISERA: P30 and P80 antisera were prepared as described in North *et al.*, 1987. Briefly, Fd11F was affinity-purified using the monoclonal antibody B16G from spent Fd11 (a Ts cell hybridoma-R. Chu, 1989 for eg.) culture supernatants. Eluted protein, when subjected to reducing SDS-PAGE gives two distinct bands at 80 and 30 KD. NZW rabbits were primed and boosted subcutanesously with 0.1-2.0 µg of protein eluted from 80 and 30 KD preparative gel slices. Serum antibody titers were evaluated by ELISA using Fd11F-coated plates. Sera was stored frozen at -70°C or in 0.02% Sodium Azide at 4°C. Normal rabbit serum samples were harvested preimmunization and used as controls for studies using the matched immune sera.

ANTIBODIES: Phycoerythrin (PE) conjugated anti-Leu 2a/2b (CD 8), anti-Leu 3a (CD 4), anti-Leu 4 (CD 3) and anti-Leu 12 (CD 19) and fluoroscein isothiocyanin (FITC)-conjugated anti-TCRαβ-1 were purchased from Becton Dickinson (Mississauga, Ont.) and used at 1 in 30 final dilution. Goat anti-rabbit Ig-FITC (Fab_2) was obtained from Cappel through Cooper Biomedical (Malvern, PA) and used at 1 in 50 dilution. All antibodies were stored at 4°C in the dark.

HARVESTING HUMAN PERIPHERAL MONONUCLEAR CELLS FROM NORMAL WHOLE BLOOD: Blood was collected into 10.0 ml sterile tubes containing Lithium heparin (Becton Dickinson), pooled and diluted to twice the initial volume with PBS at 37°C. 10.0 ml aliquots of the mixture was carefully layered onto 3.0 ml of room temperature Ficoll Hypaque (Pharmacia, density = 1.077 grams per ml), in 15.0 ml polyethylene tubes. Tubes were spun at 3.2 g for 10 minutes or until good separation of serum, mononuclear cells, and granulocytes/erythrocytes was effected. The mononuclear layer (between serum and erythrocytes) was harvested with a pasteur pipette and washed twice with 12.0 ml of warm PBS (3.2 g for 7 minutes). Pellets were pooled and washed a final time and the cells resuspended in 1.0-2.5 ml of PBS. Cell number and viability was assessed with a hemocytometer.

STAINING OF PERIPHERAL BLOOD MONONUCLEAR (PBMN) CELLS WITH ANTISERA & MAbs: An appropriate dilution of antibody/antisera was added to1.0-2.0 x 10^6 MN cells in 5.0 ml polyethylene tubes and brought to 200 µl with cold PBS. All

incubations were on ice for 30-40 minutes in the dark and all washes were with 4.5 ml of cold PBS and 3.2 g x 7 minutes. Serum binding was visualized by a 1 in 50 dilution of Goat anti-rabbit Ig-FITC. The washed pellet was resuspended in 300 µl cold PBS. If samples were to be analyzed (FACScan) or sorted (FACStar)the same day, stained mixtures were stored on ice and in the dark. If samples were to be analyzed the following day by EPICS (Coulter EPICS C-Flow Cytometer, Coulter Electronics, Hialeah, FL), they were fixed in 1-1.5 ml of 1% paraformaldehyde and stored in the dark at 4°C.

ISOLATION OF CD3, CD4 and CD8 POSITIVE PBMN CELLS FOR STAINING WITH ANTISERA: MN cells from 40 ml of fresh blood were harvested and washed as described above. All incubations were for 20-30 minutes on ice and in the dark and between steps cells were washed twice with 4.5 mls cold PBS. 15 x 10^7 cells were incubated at 1.0 x 10^7 cells per ml with 1)1 in 20 dilution of NRS, 2)1 in 50 dilution of Goat anti-rabbit Ig-FITC 3)1 in 20 NRS again. Stained cells were then split into three equal portions and treated with 1 in 30 dilutions of anti-CD3-PE, anti-CD4-PE and anti-CD8-PE respectively. Cells were sorted by FACStar and live $CD3^+$ (1st), $CD4^+$ (2nd) and $CD8^+$ (3rd) cells were collected on ice in FCS. CD3, CD4 and CD8 positive cells were pelleted, washed and split into five ($CD3^+$), four ($CD4^+$) or three ($CD8^+$) equal portions in round-bottom Microtiter plates. Each population was screened for binding with anti-p80, anti-p30, and NRS (at 1 in 20 dilutons) The goat anti-rabbit Ig-FITC secondary Ab was preadsorbed with normal mouse serum (1:1, 15 min. on ice) to remove any cross-reactivity with the murine, anti-human PE conjugated reagents already on the cell surface. Samples were analyzed by FACScan.

SCREENING $CD19^+$ CELLS FOR P80/P30 REACTIVITY:

MN cells were isolated and pretreated with a 1 in 15 dilution of NHS and then incubated with the following reagents: 1)NRS, anti-p80 or anti-p30 at 1 in 20, 2)Goat anti-rabbit-FITC at 1 in 50 (preadsorbed with NHS), 3)1:1 NMS/NRS at 1 in 20 and 4)anti-CD19-PE at 1 in 10. All incubations were for 20-30 minutes on ice and in the dark. Between steps cells were washed with 2 x 4.5 mls of cold PBS and samples were analyzed by FACScan.

ISOLATION OF GRANULOCYTES AND SCREENING FOR P80/P30 REACTIVITY:

Red blood cells (RBC's) in pellets from two tubes containing ficoll-hypaque-separated whole blood were lysed by repeated incubations in 3 volumes of Tris ammonium cholride

for 10 minutes at 37°C. Between treatments cells were washed with warm PBS and the amount of lysis evaluated by visual inspection of the WBC pellet. Once a pellet was obtained that was relatively free of RBC, cells were pretreated with 1 in 15 normal human serum (at 10^7 cells/ml to block FcR's) followed by NRS, anti-p80 or anti-p30 at 1 in 20 and then goat anti-rabbit Ig-FITC at 1 in 50. Cells were washed between steps and analyzed as above.

CELL SURFACE STUDIES WITH HUMAN LYMPHOCYTIC CELL LINES:

CELLS: The human cell lines MOLT-4, HuT 78, CEM-CM3 and RAJI were all obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI (Flow) supplemented with 10% FCS (Gibco) in a humidified, 37°C, 5% CO_2 incubator.

STAINING: Cultured cells in the logarithmic phase were harvested and washed three times with cold PBS. 1.0-1.5 x 10^6 cells were stained per test and analyzed by EPICS-C flow cytometry as described above. In the initial double label studies, PE-conjugated reagents were added first and the antisera/NRS second.

WESTERN BLOT ANALYSIS OF LEUKEMIC CELL LYSATES:

LYSATES: Cultured cells in the logarithmic phase were harvested, washed three times with cold (0°C) PBS, resuspended in 1.0 ml of lysis buffer (20 mM Tris HCl-pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.5% sodium deoxycholate in ddH_20) and vortexed. The resultant aggregate of cells and cell debris was partially broken up by repeated passage through an 18, followed by a 22 gauge syringe. The mixture was incubated at 4°C for 30 minutes, centrifuged for 20 minutes (15 K) and the protein content of the supernatant determined by Micro Lowry Assay. 10 and 20 µg of each lysate were subjected to reducing SDS-PAGE as is described in Section 1 Materials and Methods.

WESTERN BLOTS: 35, 50 and 80 µg of lysate from each of MOLT-4, HuT 78, CEM-CM3 and RAJI were subjected to reducing (10 mM DTT/100°C x 5 minutes) SDS-PAGE (12% acrylamide). Separated proteins were electro-blotted onto pre-wetted nitrocellulose filters with Towbin Transfer Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% v/v methanol, pH 8.3). Gels were stained with silver nitrate to determine the relative efficiency of protein transfer. Nitrocellulose blots were washed in NET buffer (15 mM NaCl, 0.5 mM EDTA, 5 mM Tris HCl-pH 7.4, 0.025% gelatin, 0.005% Triton X-100) and incubated overnight with gentle shaking in a 1 in 500 dilution of anti-p30 or normal rabbit serum in NET buffer (50 ml final volume). The next morning, blots were washed with 4 x 50 mls of NET buffer and incubated with a 1 in 500 dilution of sheep anti-rabbit

Ig with shaking for 1.5-2.0 hours. After 4 x 50 ml washes with NET buffer, 10 ml per blot of I^{125}-protein A was added to 30-40 ml of NET buffer and incubated for 2 hours with shaking. After 5 x 50 mls washes with NET buffer, filters were air-dried and put with X-ray film (X-omat, Kodak) at -70°C for 3-5 days.

SECTION 2: RESULTS

ANTI-P80 AND ANTI-P30 REACTIVITY WITH MURINE CELLS

Fd11F, a T cell-derived suppressor factor purified from Fd11 (a T_S cell hybridoma) cell supernatants over a B16G-affinity column gives distinct and unique protein bands at 80, 30, and occasionally 45-50 KD with reducing SDS polyacrylamide gel electrophoresis (PAGE). Chu (1989) has shown antigen-binding and suppressive activity associated with the 80 and 30 KD bands. Subsequently, North *et al* (1988) eluted the 80 and 30 KD components from preparative gel slices and raised antisera in rabbits. Anti-p30 was shown to bind to the surface of 4% of murine thymocytes and 10% of splenic T cells. In addition, both $CD4^+$ and $CD8^+$ T subsets contained $p30^+$ populations and $CD4^-CD8^-$ cells showed enhanced p30 reactivity ($30\%^+$). Finally, anti-p30 was demonstrated to abrogate the suppressive effect of affinity-purified Fd11F *in vitro*.

SCREENING HUMAN PBMN CELLS FOR ANTI-P80 AND ANTI-P30 BINDING.

An initial investigation was undertaken to determine the reactivity of anti-p80 and anti-p30 sera with normal human peripheral blood mononuclear (PBMN) cells. This involved collecting blood from four individuals and isolating and staining the mononuclear (MN) cells with normal rabbit serum (a control for serum components nonspecifically binding to cell surface proteins), anti-p80 and anti-p30. Binding to the cell surface was detected by the addition of goat anti-rabbit Ig-FITC and flow cytometry.

Initial studies of this type invariably demonstrated the existence of a small population of anti-p80/30 intensely staining cells and more variably, a larger population (usually the remainder of the cell population, ie. 65-70%) of weakly staining cells (Fig. 8 for eg.). Given the values obtained for the percent p30 positive T cells in the mouse system, this smaller, intensely-staining population may have represented a specific suppressor-related subset of human MN cells. The range of mononuclear cells intensely staining with anti-p80 serum was found to be 5.6% to 29.5%, whereas the range with anti-p30 serum was lower, 3.1% to 9.8% with both sets of data corrected for nonspecific binding of normal rabbit serum at the same dilution (Table II). It is interesting that both of the individuals who showed significantly higher percentages of high and low affinity anti-p80

FIGURE 8: **SAMPLE PROFILE OF NORMAL HUMAN MONONUCLEAR CELLS (a) STAINED WITH NORMAL RABBIT SERUM (b), ANTI-p80 (c) AND ANTI-p30 (d).** Mononuclear cells were separated from fresh whole blood over ficoll-hypaque and incubated with 1 in 20 dilutions in PBS of NRS, anti-p80 or anti-p30. Cell surface binding was detected by goat anti-rabbit Ig-FITC (1 in 50 in PBS) and samples were analyzed in an EPICS-C flow cytometer gated for lymphocytes.

Fig.8a:

Flow Cytometer Gating.

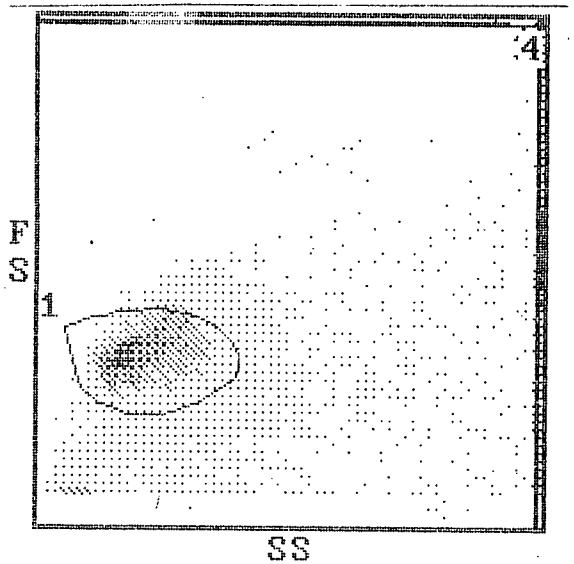
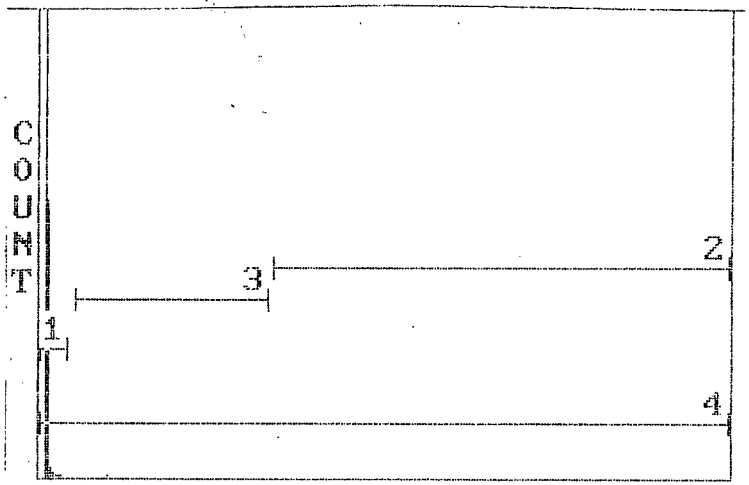


Fig. 8b:

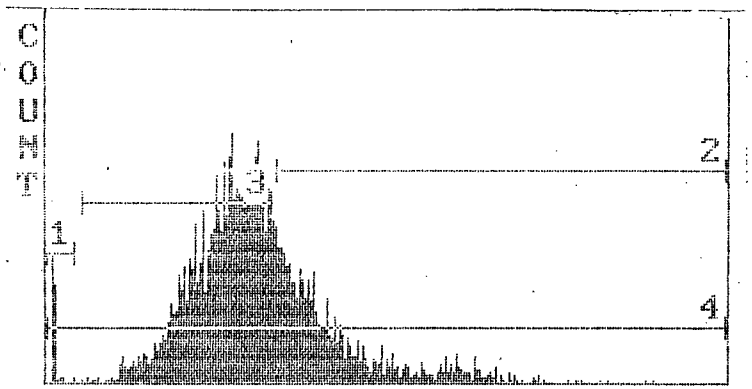
NRS + GαRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	4727	89.7	1.067	1.051	70.6
2	10.43	1023.	93	1.8	17.11	2.01	0.67
3	1.454	10.14	426	8.1	3.076	1.836	4.18
4	1.024	1023.	5270	100.0	1.225	1.686	70.6

Fig. 8c:

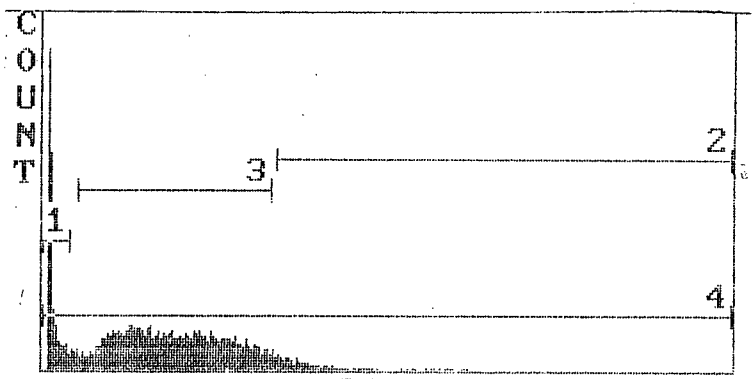
αp80 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	96	2.4	1.073	1.061	76.7
2	10.43	1023.	1205	30.3	19.78	2.01	27.6
3	1.454	10.14	2620	65.8	5.848	1.469	18.5
4	1.024	1023.	3981	100.0	8.181	2.270	18.5

Fig. 8d:

αp30 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	2387	22.2	1.103	1.093	79.2
2	10.43	1023.	1116	10.4	23.28	2.37	45.3
3	1.454	10.14	7098	65.9	3.695	1.700	62.3
4	1.024	1023.	10770	100.0	3.396	2.680	79.2

TABLE II: SCREENING OF HUMAN PERIPHERAL LYMPHOCYTES FOR anti-p80/anti-p30 BINDING: Mononuclear cells were harvested from the fresh whole blood of several healthy donors over ficoll- hypaque. Cells were incubated with 1 in 20 dilutions of NRS, anti-p80 or anti-p30 followed by GαRIg-FITC at 1 in 50. See Fig. 8 for details. Statistics were obtained from the amount of binding above that of the negative control (NRS).

DONOR#	ANTIBODY	% POSITIVE[a]
1	αp80	5.6
2	"	6.6
3	"	27.4
4	"	10.5
5	"	29.5
1	αp30	3.5
2	"	3.6
3	"	8.6
4	"	3.1
5	"	9.8

[a]All fluorescence values corrected for binding of normal rabbit serum.

and anti-p30 binding are atopic.

Upon repeated testing of the same individual (data not shown), the percentage of p80 and p30 intensely staining cells was shown to vary, sometimes by as much as 10%. Thus the results indicate that two different populations of anti-p80 and anti-p30 positive cells exist in the mononuclear fraction of human blood. Each of these populations express the p80 and p30 markers at different densities or, express a different form of these proteins for which the specific Abs in the sera have more or less affinity. In addition, there is variability in anti-p80 and anti-p30 binding between individuals and at different times within the same individual.

EXPRESSION OF THE P80 AND P30 DETERMINANTS BY HUMAN T LYMPHOCYTES.

In order to begin characterizing these populations of human anti-p80/anti-p30 positive mononuclear cells, CD 3, CD 4 and CD 8 positive cells were isolated from PBMN cells pretreated with NRS and goat anti-rabbit Ig-FITC by cell sorting and stained with each of anti-p80, anti-p30, NRS and goat anti-rabbit Ig-FITC alone (preadsorbed with normal mouse serum). The resulting FACScan profiles are presented in Fig. 9 where it can be seen that $CD3^+$ cells display a 1 log increase in anti-p80 binding and a slightly smaller increase in anti-p30 binding over NRS. Thus T cells express lower levels of anti-p30 than anti-p80, or the anti-p30 sera has a lower affinity for T cells than the anti-p80 sera. A similar result was obtained for the CD 4^+ and CD 8^+ subsets (data not shown). In addition, there is a small population of $CD3^+$ cells (2.1% with anti-p80 and 1.5% with anti-p30) which stain at a much higher intensity (1.5-2 logs increased binding over background) than the rest of the preparation. A slightly larger population showed this same level of binding in the human screen discussed above. Whether these two intensely staining populations represent the same cell subset (potentially of T cell origin) with a high affinity for the antisera, or artefactual binding is not known. The antisera profiles of $CD4^+$ cells gave 4.5 and 3.5% intense staining with anti-p80 and anti-p30 respectively whereas less than 1% of $CD8^+$ positive cells stained at this level with both antisera (data not shown).

FIGURE 9: **ISOLATED HUMAN CD3$^+$ CELLS BIND ANTI-P80 AND ANTI-P30.** Human mononuclear cells were isolated as in Fig. 8, and treated with 1)NRS (1 in 20), 2)GαRIg-FITC (1 in 50), 3)NRS (1 in 20) and 4)αCD3-PE (1 in 30). All incubations were for 20-30 minutes on ice and in the dark. Cells were washed with 4.5 mls of ice cold PBS between each step. Stained cells were sorted by FACStar and CD3$^+$ cells were collected in FCS. CD3$^+$ cells were then washed and screened for reactivity with NRS (**b**), anti-p80 (**c**) and anti-p30 (**d**) as has been described. GαRIg-FITC (preadsorbed with NMS) was used at 1 in 50 dilution and samples were anlysed by FACScan. GαRIg-FITC alone (**a**).

Fig. 9a:

Flow Cytometer Gating.

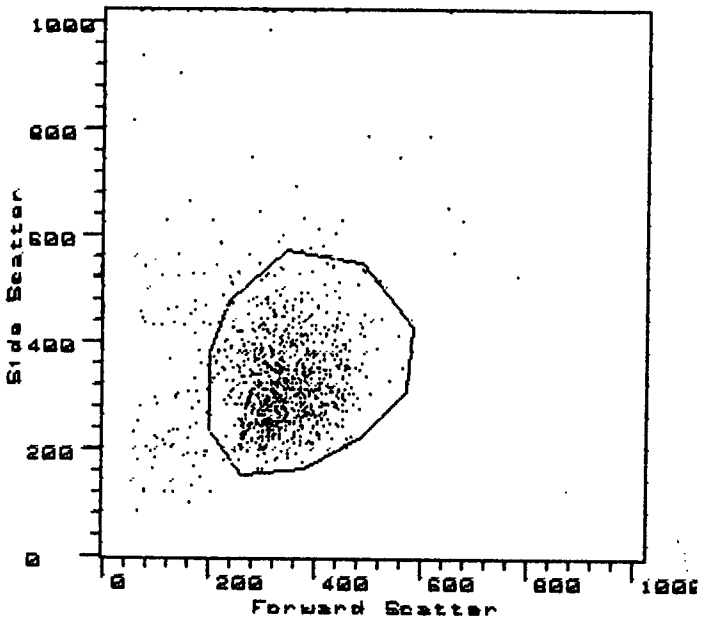


Fig. 9b:

NRS + GαRIg-FITC.

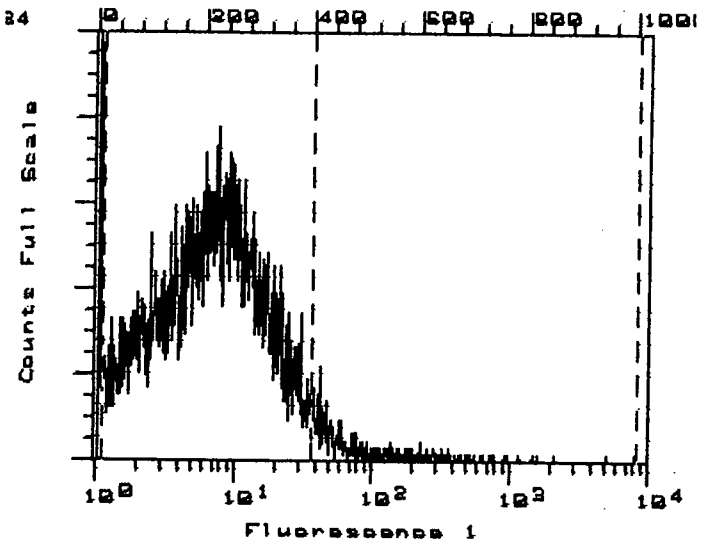


Fig. 9c:

αp80 + GaRIg-FITC.

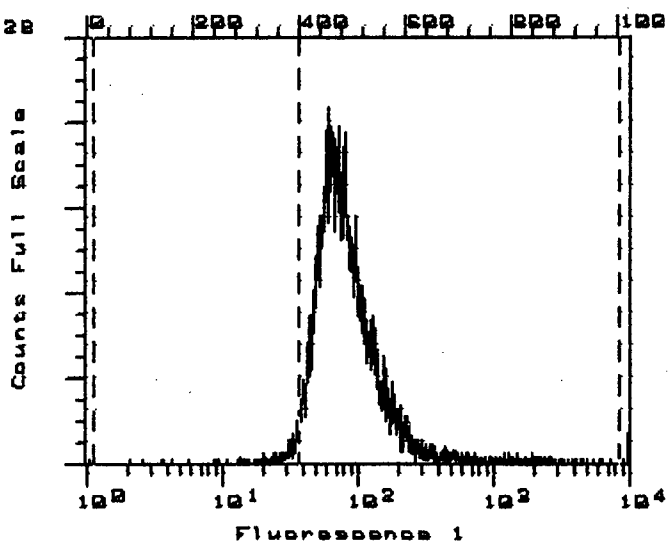
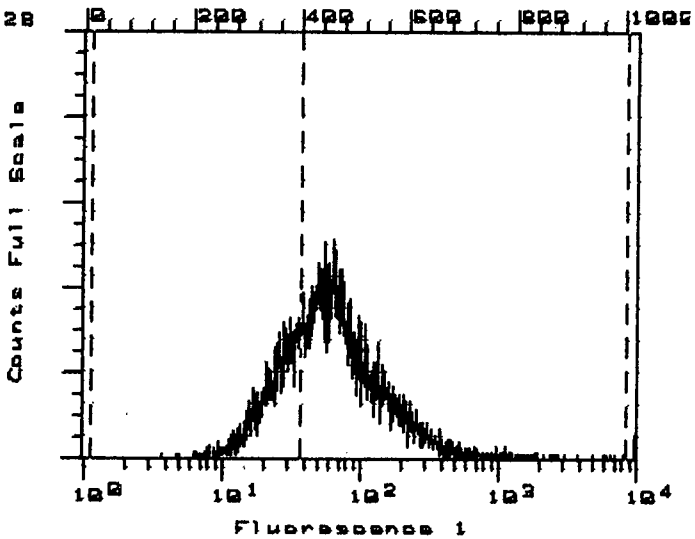


Fig. 9d:

αp30 + GaRIg-FITC.



THE BINDING OF ANTI-P80 AND ANTI-P30 TO THE SURFACE OF HUMAN B LYMPHOCYTES.

Since the data indicated that human $CD3^+$ MN cells bound anti-p80 and anti-p30 (to a lesser extent), it was of interest to investigate whether peripheral B cells showed any reactivity with these reagents. PBMN cells were double labelled with NRS, anti-p80 or anti-p30 (followed by goat anti-rabbit Ig-FITC) and anti-CD19-PE. Fig. 10 shows two different populations of MN cells which are binding the CD19-PE antibody. From a subsequent experiment, it was determined that the more intensely staining cells were specifically binding anti-CD19 and thus it was concluded that the weaker staining population was nonspecific or artefactual. The $CD19^+$ cells show approximately the same level of antisera binding (1 log increase over NRS with anti-p80) as the $CD3^+$ cells with lower levels of anti-p30 binding (log intensity was taken from the centre of each treated $CD19^+$ population as with NRS and anti-p30 samples staining was more variable). This experiment suggests that peripheral B lymphocytes also express the p80 and p30 determinants. A B cell subpopulation staining with an intensity greater than that observed for the main population was not observed in this experiment.

EXPRESSION OF THE P80 AND P30 DETERMINANTS BY HUMAN GRANULOCYTES.

Since the p80 and p30 antisera were shown to bind to peripheral T and B lymphocytes, an experiment was done to investigate whether cells of the myeloid lineage also expressed this marker. Granulocytes were obtained by repeated Tris ammonium chloride treatment of RBC pellets from a sample of whole blood separated over Ficoll-hypaque. The resultant WBC pellet was washed and pretreated first with normal human serum (NHS) to block FcR's and second with normal rabbit serum (NRS) to adsorb out any nonspecific binding to cells by serum components. A portion of the cells stained with anti-CD19-PE and anti-CD3-PE showed that the preparation contained very few contaminating lymphocytes. The remaining cells were screened for NRS, anti-p80, and anti-p30 binding. As can be seen in Fig. 11, even though the Goat anti-rabbit Ig-FITC secondary Ab (preadsorbed with NHS) is binding to the human Abs on the surface of the granulocytes, addition of NRS (negative control) does not shift the peak. However, addition of anti-p80 or anti-p30 (followed by GαRIg-FITC) does shift the entire peak to the right by one log. In this experiment, equal levels of binding were obtained with both antisera. Thus it appears that

.FIGURE 10: DOUBLE LABELLING OF HUMAN MONONUCLEAR CELLS WITH ANTI-CD19 AND ANTI-P80/ANTI-P30. Human mononuclear cells were isolated as in Fig. 8, and pretreated with a 1 in 15 dilution of NHS. Cells were washed and incubated with reagents in the following order. 1)NRS (**b**), anti-p80 (**c**) or anti-p30 (**d**) at 1 in 20 2)goat anti-rabbit Ig-FITC at 1 in 50 3)NMS and NRS each at 1 in 20 and 4)anti-CD19-PE. All incubations were from 20-30 minutes on ice and in the dark. Cells were washed twice with 4.5 mls of ice cold PBS between steps and stained populations were analyzed by FACScan with the gating set for lymphocytes (**a**). Fluorescence 2 (y-axis) represents binding of anti-CD19-PE (red) and Fluorescence 1 (x-axis) represents binding of goat anti-rabbit Ig-FITC (green).

Fig.10a:

GαRIg-FITC only.

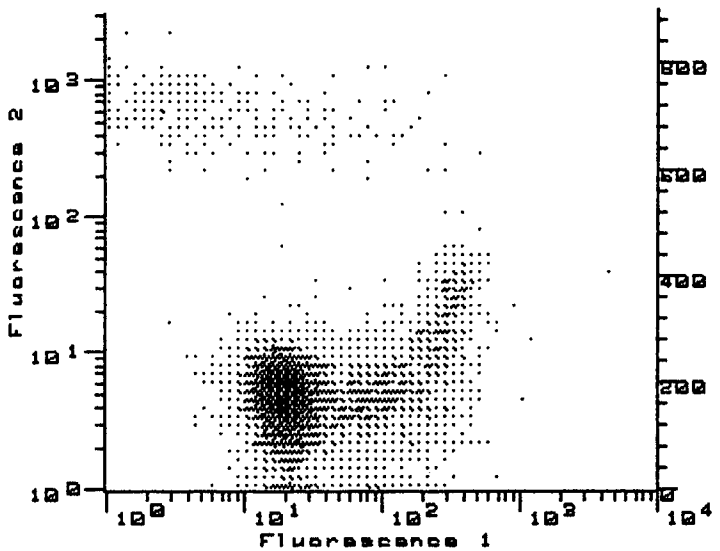


Fig.10b:

NRS + GαRIg-FITC.

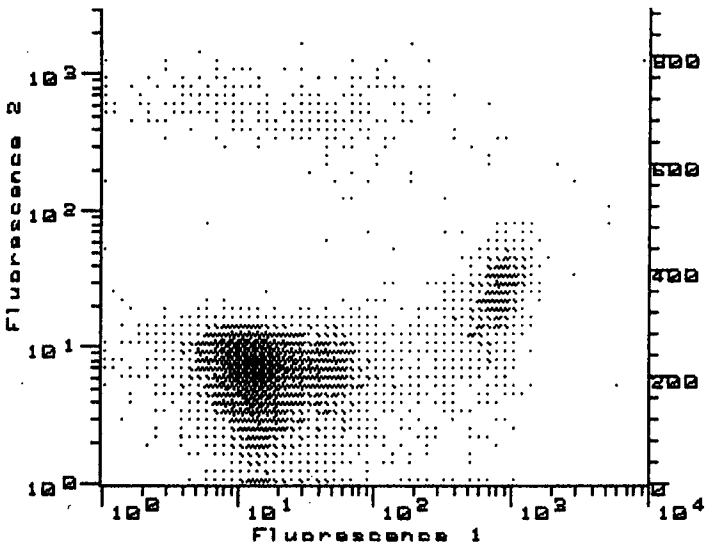


Fig.10c:

αp80 + GαRIg-FITC.

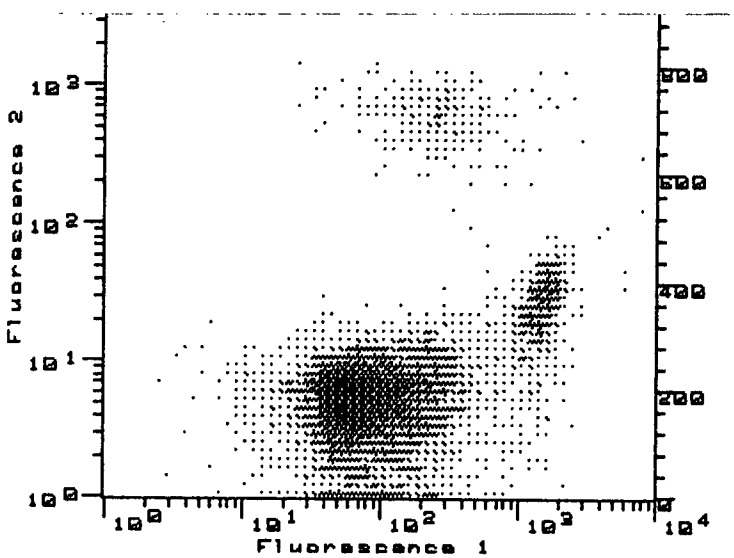


Fig.10d:

αp30 + GαRIg-FITC.

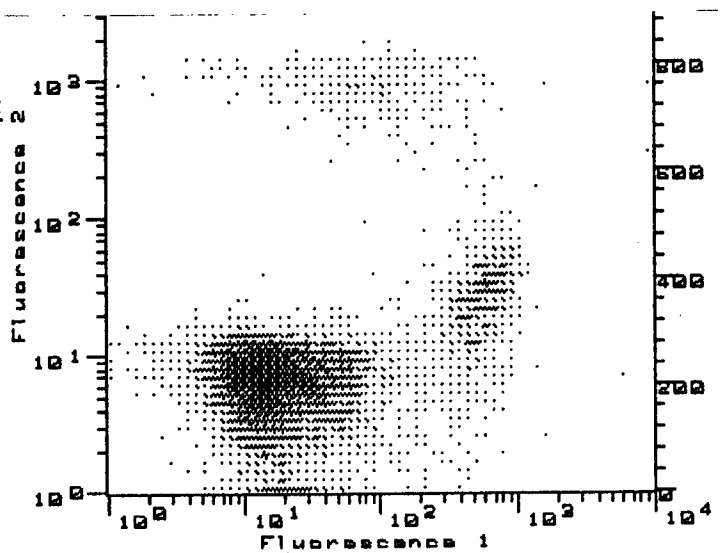


FIGURE 11: **CELL SURFACE ANALYSIS OF HUMAN GRANULOCYTES USING ANTI-P80 AND ANTI-P30.** Human granulocytes were isolated from red blood cells in the pellets of a ficoll-hypaque separated whole blood by repeated 10 minute incubations (at 37°C) with Tris ammonium chloride. The degree of remaining RBC's was assessed by pelleting cells between incubations. Isolated granulocytes were washed and screened for contaminating lymphocytes with αCD3-PE and αCD19-PE. Cells were pretreated with a 1 in 15 dilution of NHS and then incubated with NRS (**b**), anti-p80 (**c**) or anti-p30 (**d**) at 1 in 20 dilutions. followed by GαRIg-FITC (preadsorbed with NHS) at 1 in 50. GαRIg-FITC alone (**a**). Samples were analyzed by a FACScan gated for granulocytes..

Fig.11a:

GαRIg-FITC only.

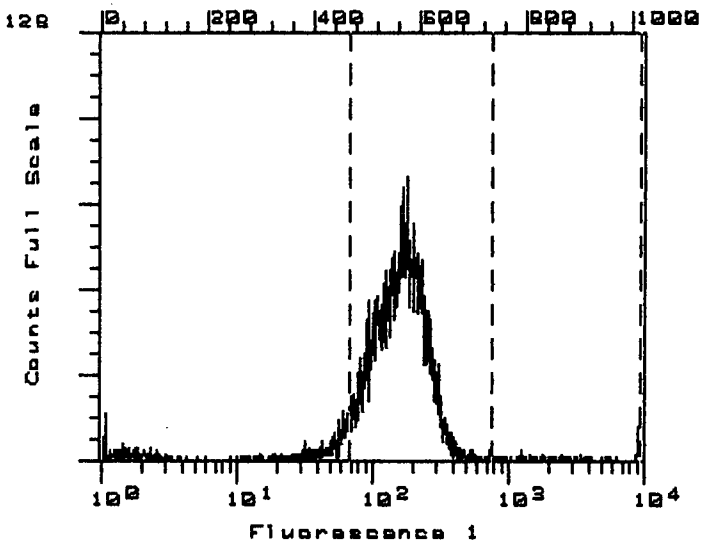


Fig.11b:

NRS + GαRIg-FITC.

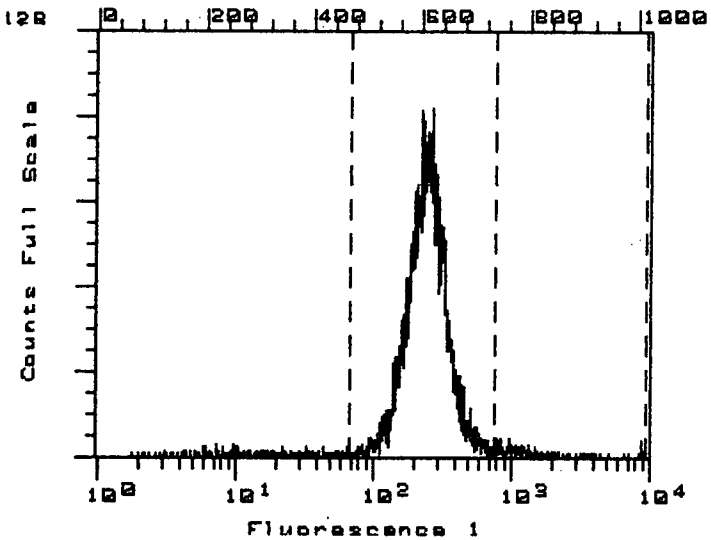


Fig.11c:

αp80 + GαRIg-FITC.

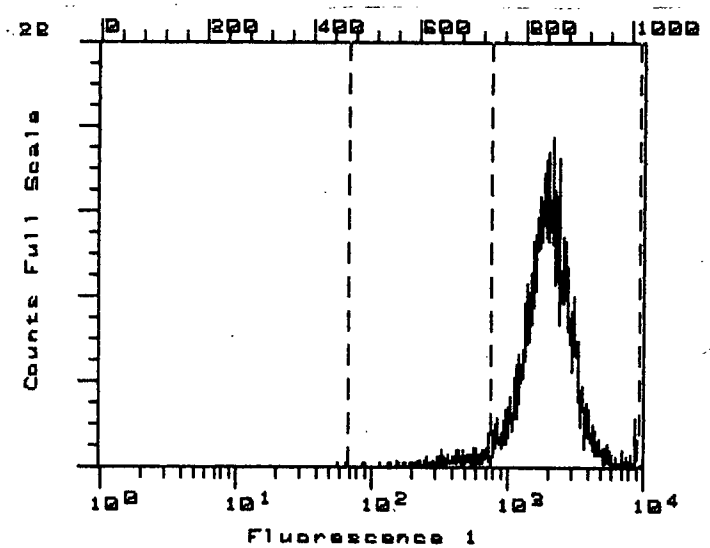
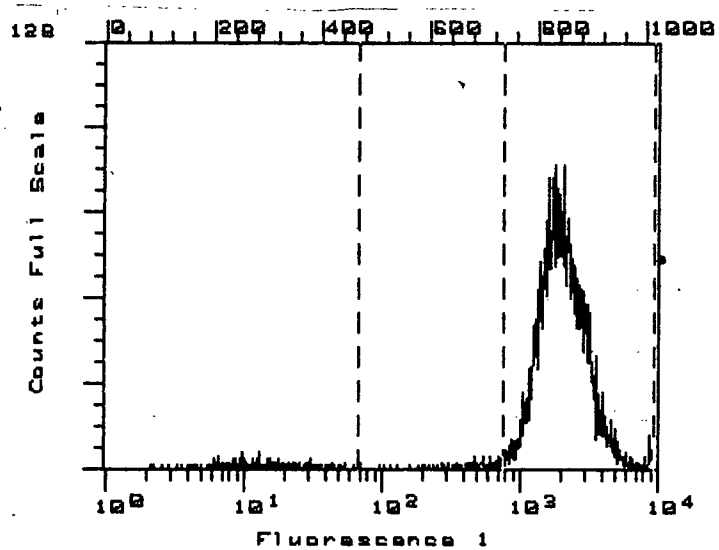


Fig.11d:

αp30 + GαRIg-FITC.



neutrophils (the main cell type in the granulocyte population), in addition to lymphocytes express the p80 and the p30 cell surface markers.

SCREENING OF HUMAN LYMPHOCYTIC CELL LINES WITH ANTI-P80 AND ANTI-P30.

To furthur study the binding characteristics of anti-p80 and anti-p30, several human lymphocytic cell lines (obtained from the ATCC) were tested with limiting dilutions of both antisera. A sample anti-p80/anti-p30/NRS EPICS-C profile, showing the 1 in 20 dilution from the titration series is shown for each of the cell lines in Fig. 12a-d and the titration curves for MOLT-4, CEM-CM3 (both isolated from human T cell leukemias), HuT 78 (isolated from a patient with Sezary Syndrome) and RAJI (isolated from a Burkitt's Lymphoma) with anti-p80 and anti-p30 are shown in Fig. 13a and 13b respectively. Since 1 in 20 is the dilution of sera used throughout the cell surface studies, the percent of cells staining at this dilution was used to evaluate which lines were anti-p80$^+$ and anti-p30$^+$. HuT 78 is the only cell line which shows strong binding (between 90 and 100% of cells were positive-even at 1 in 80 dilution) of the anti-p80 antiserum. MOLT-4, CEM-CM3 and RAJI lines all gave between 20 and 50% binding of anti-p80 at 1 in 20. Conversely, with anti-p30, 90-100% of both MOLT-4 and CEM-CM3 cells, 70-80% of RAJI cells, and less than 10% of HuT 78 cells stained positve. These cell lines were also tested for other T and B cell markers, the results of which are presented in Table III.

WESTERN BLOT ANALYSIS OF HUMAN CELL LINES.

In an attempt to characterize the marker to which the p30 antiserum was binding, Western Blot analyses of leukemic cell lysates were undertaken. Since HuT 78 did not bind the anti-p30 serum at any dilution and anti-p80 bound to all of the cell lines to varying extents, to avoid ambiguity, anti-p30 was chosen over anti-p80 in the analysis, and HuT 78 cell lysates were used as a negative control.

Cell lysates were subjected to reducing SDS-PAGE (Fig. 14) to show that the protein concentration was approximately equal in all lysates and that good separation of bands was being achieved. 35, 50 and 80 µg of each cell lysate were separated and blotted onto

FIGURE 12: **ANTI-P80 AND ANTI-P30 REACTIVITY WITH SEVERAL HUMAN LEUKEMIC CELL LINES.** MOLT-4 (**a**), CEM-CM3(**b**), HuT 78(**c**) and RAJI (**d**) cells in the logarithmic phase were harvested, washed with cold PBS and incubated with NRS (**ii**), anti-p80 (**iii**) or anti-p30 (**iv**) at 1 in 20 dilutions followed by GαRIg-FITC at 1 in 50. Incubations were for 30-40 minutes on ice and in the dark. Cells were washed twice with 4.5 mls of ice cold PBS between steps and analyzed by EPICS-C. The gated cell populaton is shown in (**i**).

Fig.12a (i):

Flow Cytometer Gating.

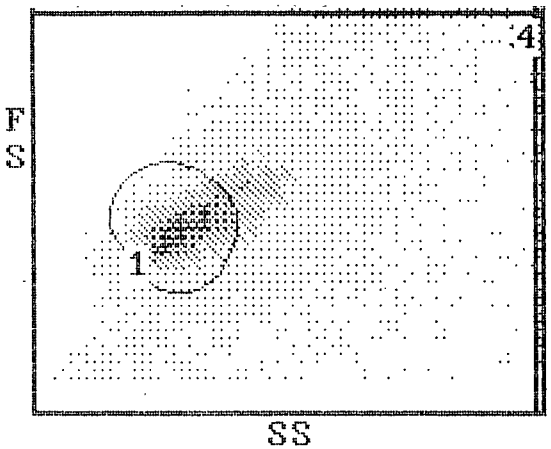
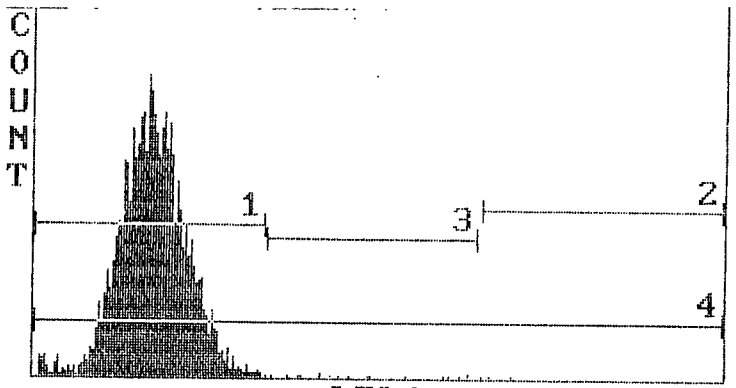


Fig.12a (ii):

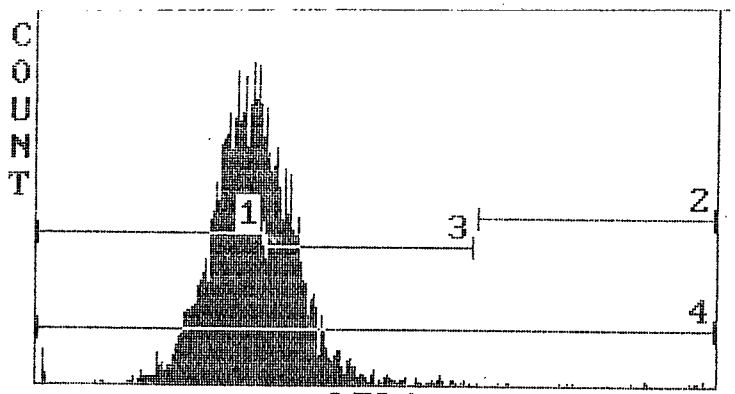
NRS + GαRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	10.42	2939	99.3	3.250	1.440	22.0
2	90.28	1023.	2	0.1	106.2	1.1	0.25
3	10.43	87.82	17	0.6	28.12	1.99	0.40
4	1.024	1023.	2959	100.0	3.302	1.521	22.0

Fig.12a (iii):

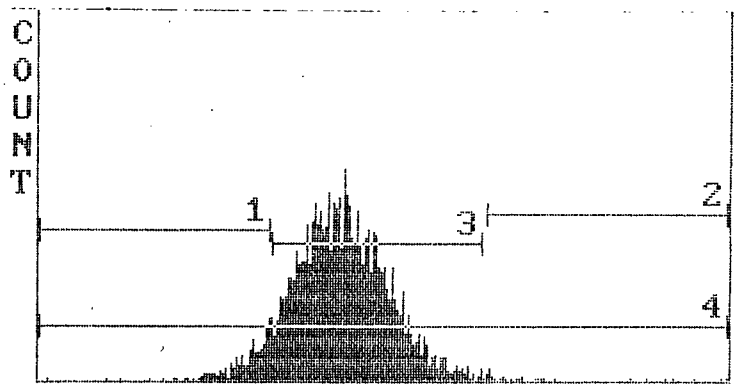
αp80 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	10.42	2618	65.4	6.983	1.392	16.4
2	90.28	1023.	19	0.5	453.2	2.2	
3	10.43	87.82	1368	34.1	14.34	1.43	16.7
4	1.024	1023.	4006	100.0	9.112	1.759	16.4

Fig.12a (iv):

αp30 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	10.42	237	8.2	7.885	1.365	30.2
2	90.28	1023.	33	1.1	208.6	2.6	0.53
3	10.43	87.82	2623	90.6	21.80	1.56	13.0
4	1.024	1023.	2896	100.0	20.61	1.80	13.0

Fig.12b (i):

Flow Cytometer Gating.

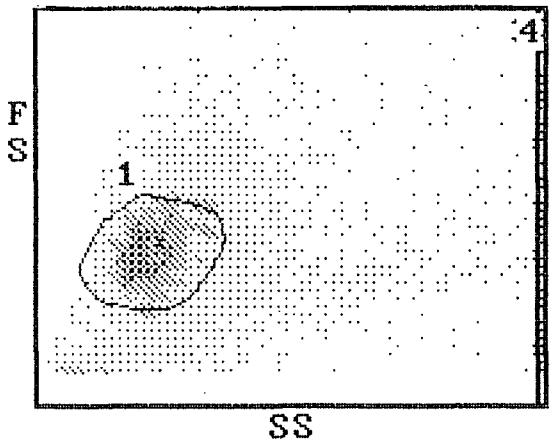
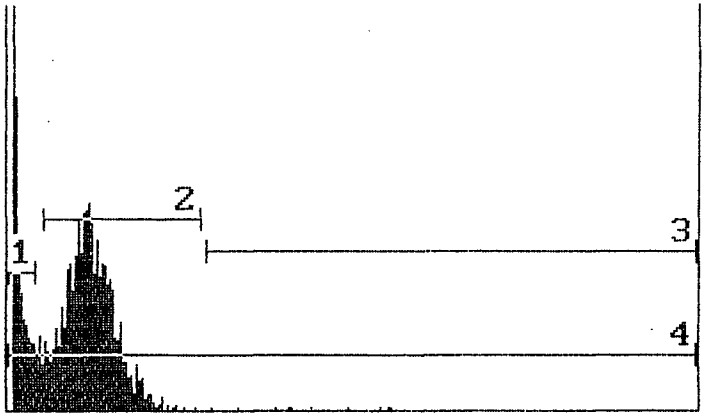


Fig.12b (ii):

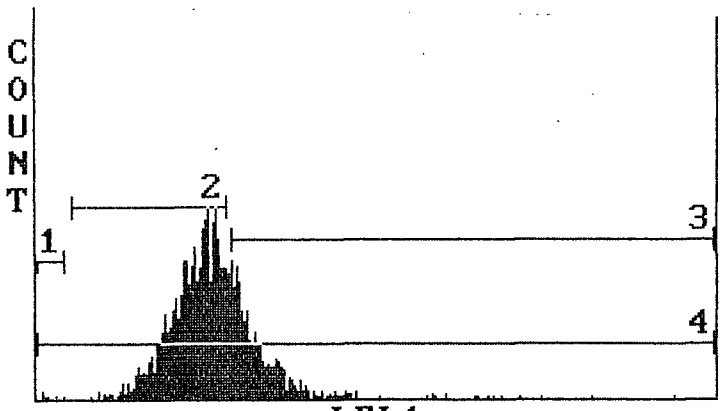
NRS + GαRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	550	26.2	1.126	1.102	88.9
2	1.454	7.141	1494	71.2	2.288	1.303	26.3
3	7.341	1023.	10	0.5	20.86	1.87	0.57
4	1.024	1023.	2099	100.0	1.900	1.547	88.9

Fig.12b (iii):

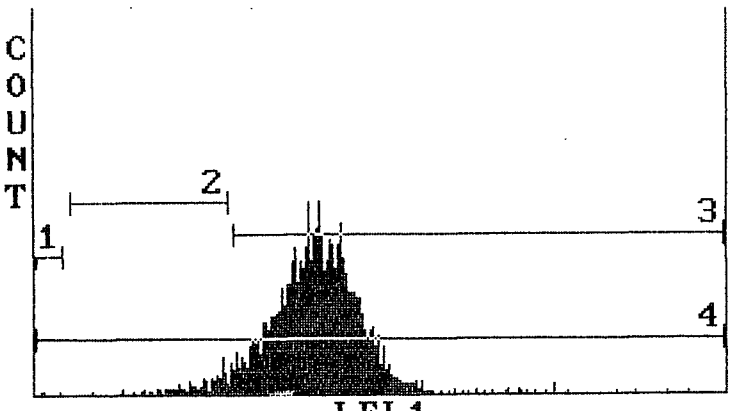
αp80 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	7	0.3	1.119	1.106	53.1
2	1.454	7.141	1404	69.9	4.834	1.328	15.3
3	7.341	1023.	546	27.2	10.43	1.78	17.6
4	1.024	1023.	2009	100.0	5.988	1.700	15.3

Fig.12b (iv):

αp30 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	3	0.1	1.130	1.113	84.9
2	1.454	7.141	122	5.4	5.183	1.352	1.33
3	7.341	1023.	2142	94.1	17.26	1.62	11.3
4	1.024	1023.	2276	100.0	16.07	1.75	11.3

Fig.12c (i):

Flow Cytometer Gating.

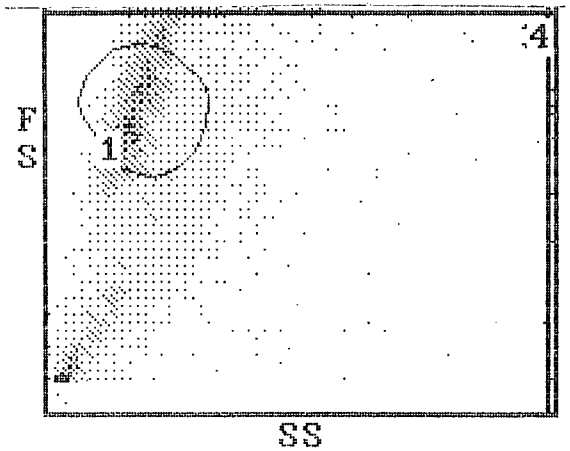
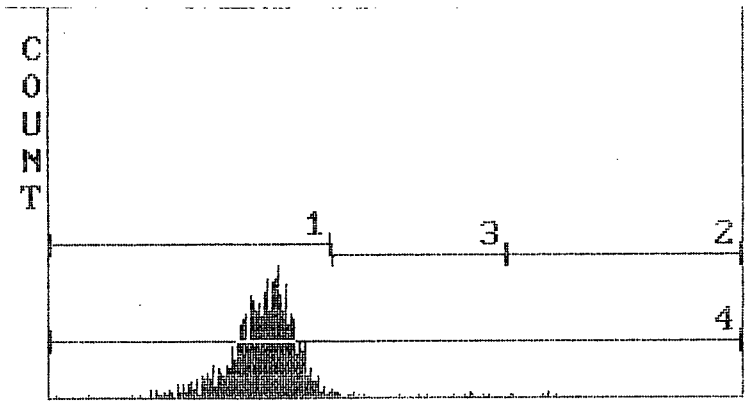


Fig.12c (ii):

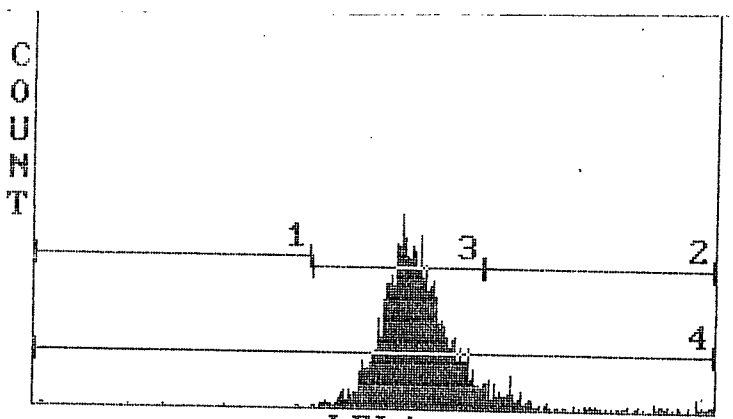
NRS + GαRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	16.93	1061	97.3	8.141	1.427	8.77
2	97.90	1023.	8	0.7	212.7	2.5	0.23
3	16.95	97.83	21	1.9	31.93	1.82	2.04
4	1.024	1023.	1090	100.0	8.561	1.656	8.77

Fig.12c (iii):

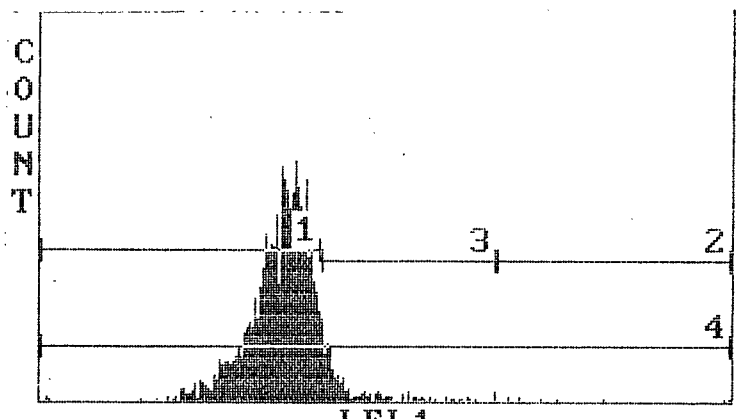
αp80 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	16.93	8	0.4	4.703	2.957	84.9
2	97.90	1023.	211	10.8	248.1	2.5	
3	16.95	97.83	1728	88.8	46.25	1.40	6.47
4	1.024	1023.	1947	100.0	54.97	2.04	6.47

Fig.12c (iv):

αp30 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	16.93	1917	90.7	10.58	1.39	5.92
2	97.90	1023.	12	0.6	315.2	2.7	
3	16.95	97.83	184	8.7	23.96	1.56	17.4
4	1.024	1023.	2113	100.0	11.59	1.64	5.92

Fig.12d (i):

Flow Cytometer Gating.

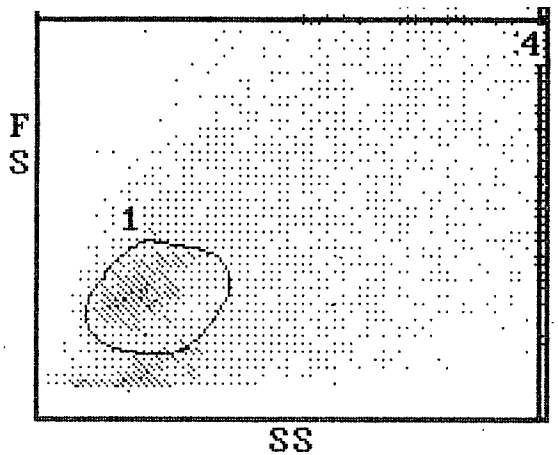
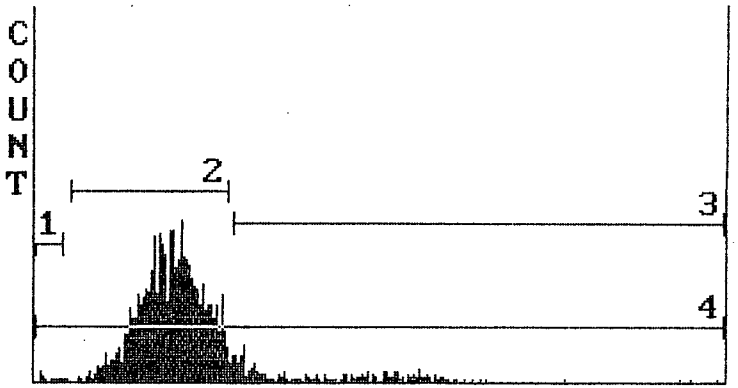


Fig.12d (ii):

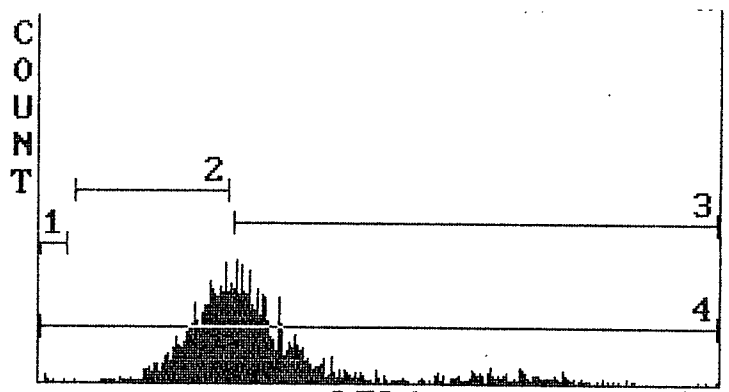
NRS + GαRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	13	0.8	1.150	1.117	63.7
2	1.454	7.141	1491	86.5	3.854	1.389	9.40
3	7.341	1023.	208	12.1	21.46	2.94	1.44
4	1.024	1023.	1723	100.0	4.713	2.140	9.40

Fig.12d (iii):

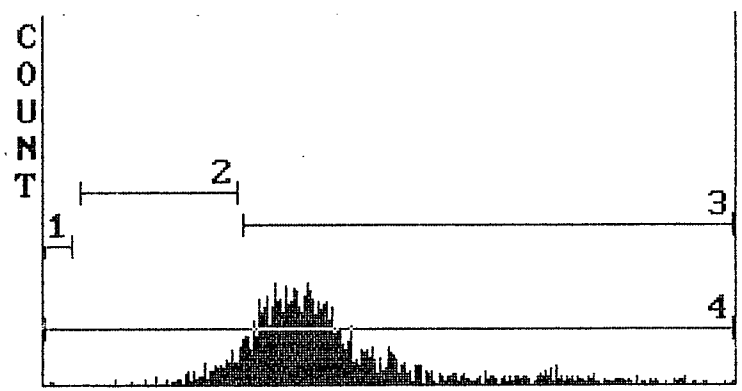
αp80 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	6	0.4	1.105	1.097	53.1
2	1.454	7.141	707	42.0	5.080	1.319	15.9
3	7.341	1023.	935	55.5	16.66	2.86	14.9
4	1.024	1023.	1684	100.0	9.833	2.744	14.9

Fig.12d (iv):

αp30 + GaRIg-FITC.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	1.377	2	0.1	1.066	1.038	84.9
2	1.454	7.141	145	8.7	5.307	1.322	29.7
3	7.341	1023.	1510	90.2	19.83	2.67	14.6
4	1.024	1023.	1674	100.0	17.45	2.77	14.6

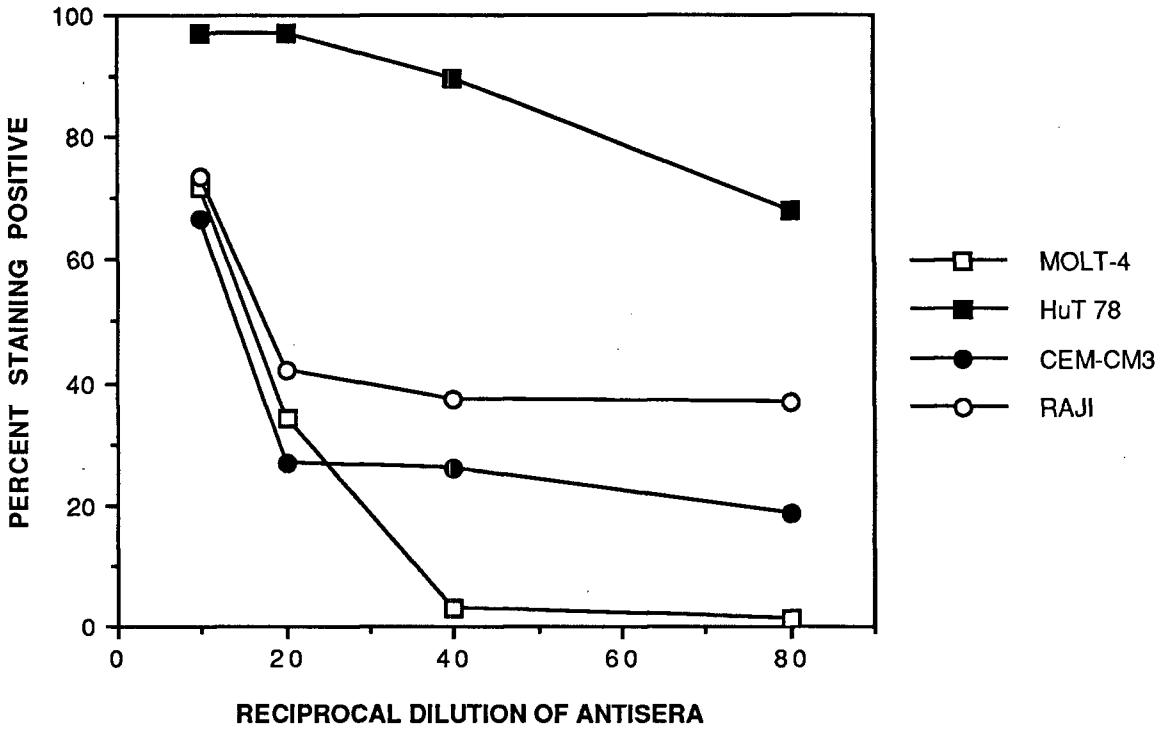


FIGURE 13a: **TITRATION OF ANTI-P80 ON HUMAN LEUKEMIC CELL LINES.**

FIGURE 13: **TITRATION OF ANTI-P80 (a) AND ANTI-P30 (b) ON THE SURFACE OF MOLT-4, CEM-CM3, HuT 78 AND RAJI HUMAN CELL LINES.** Cells were harvested and treated as in FIG. 12. Cells were incubated with doubling dilutions (from 1 in 10 through to 1 in 80) of NRS, anti-p80 or anti-p30. GαRIg-FITC was used at 1 in 50. and incubations were for 30-40 minutes in the dark and on ice. Cells were washed between steps with 2 x 4.5 mls cold PBS and analyzed by EPICS-C. Plotted points have been corrected for NRS binding at the same dilution.

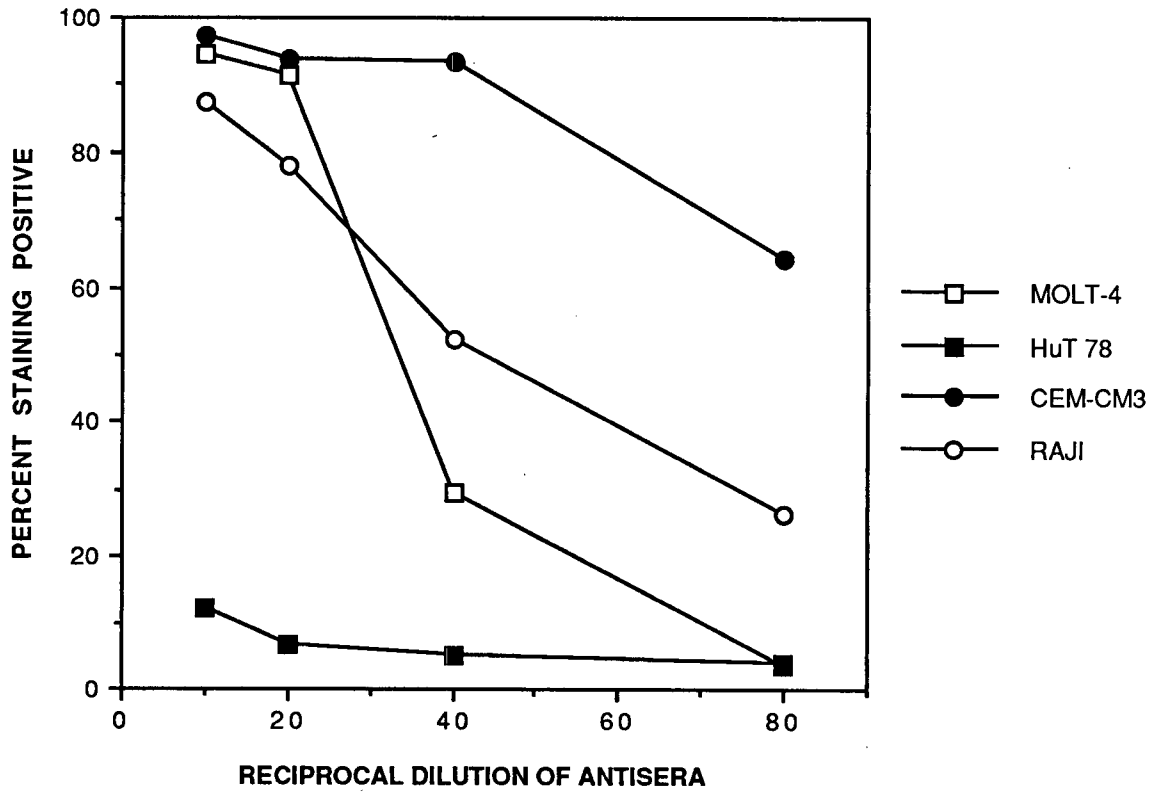


FIGURE 13b: TITRATION OF ANTI-P30 ON HUMAN LEUKEMIC CELL LINES.

TABLE III: BINDING OF αp30/αp80, AND CELL SURFACE CHARACTERIZATION OF HUMAN LYMPHOCYTIC CELL LINES. Cells in the logarithmic phase were harvested, washed and incubated with rabbit antiserum followed by goat anti-rabbit Ig-FITC or directly conjugated monoclonal antibodies specific for various cell surface markers. See Fig. 13 for details. Values given as percent positive (%+) FITC is the amount of binding above control serum in green fluorescence and %+ PE, the percent positive with red fluorescence (phycoerythrin).

CELL LINE	ANTIBODY	DILUTION	%+(FITC/PE)
MOLT-4	αp80	1/10	94.3
	"	1/20	91.3
	"	1/40	29.7
	"	1/80	3.7
	αp30	1/10	71.8
	"	1/20	34.2
	"	1/40	3.0
	"	1/80	1.1
	B16G	1/10	0.0
	WT-31	1/20	0.0
	αCD3	1/30	2.1
	αCD8	1/30	85.1
	αCD4	1/30	59.0
HuT 78	αp80	1/10	97.0
	"	1/20	97.0
	"	1/40	89.4
	"	1/80	68.0
	αp30	1/10	12.4
	"	1/20	6.7
	"	1/40	5.0
	"	1/80	3.8
	WT-31	1/16	91.5
	αCD3	1/30	96.2
	αCD8	1/30	33.4
	αCD4	1/30	98.2

CON'T...

TABLE III CON'T.

CELL LINE	ANTIBODY	DILUTION	%+(FITC/PE)
CEM-CM3	αp80	1/10	66.7
	"	1/20	26.7
	"	1/40	26.0
	"	1/80	18.5
	αp30	1/10	97.3
	"	1/20	93.6
	"	1/40	93.5
	"	1/80	64.3
	WT-31	1/20	4.2
	αCD3	1/30	18.8
	αCD8	1/30	2.0
	αCD4	1/30	90.9
RAJI	αp80	1/10	73.7
	"	1/20	42.2
	"	1/40	37.1
	"	1/80	36.6
	αp30	1/10	87.3
	"	1/20	78.1
	"	1/40	52.4
	"	1/80	26.4
	αCD19	1/30	89.5
	WT-31	1/20	2.8
	αCD8	1/30	3.2
	αCD4	1/30	3.5

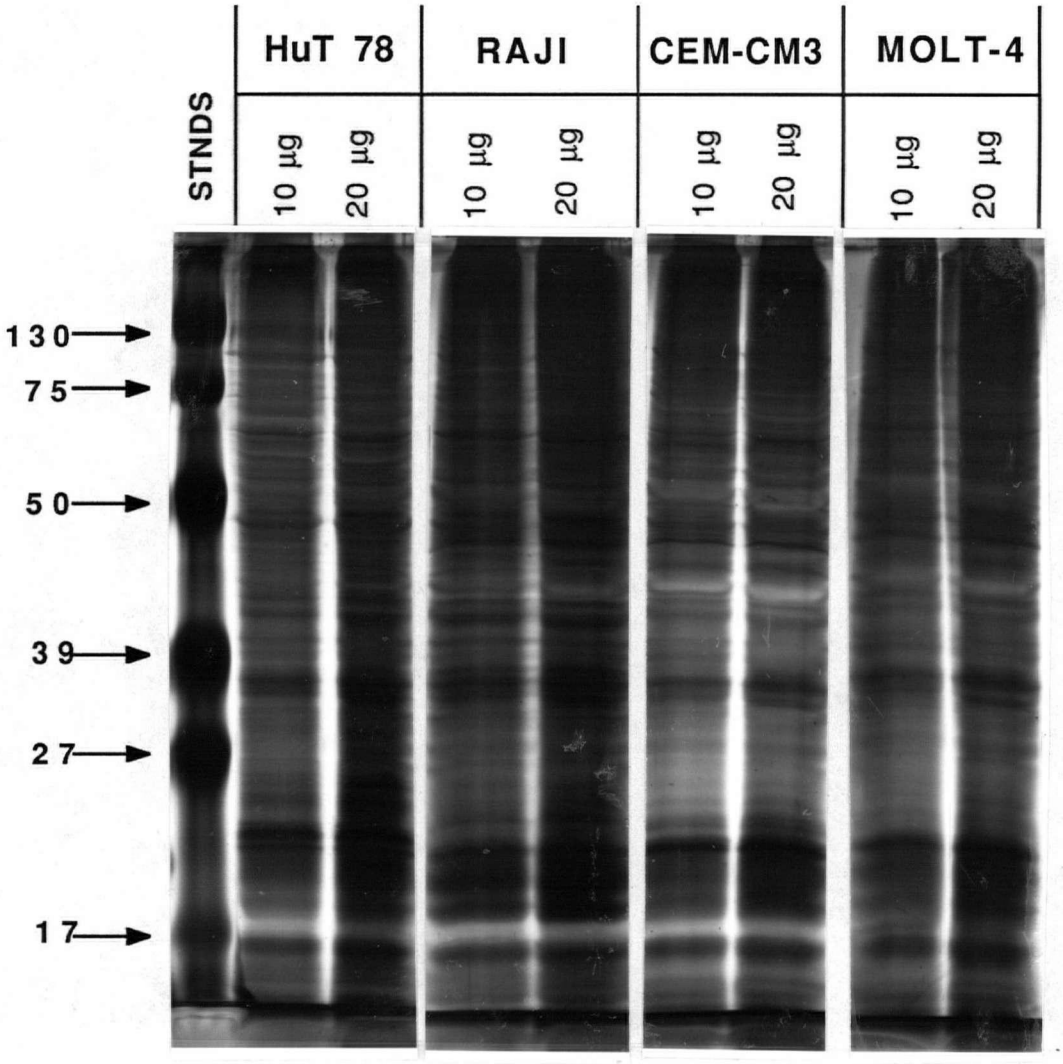


FIGURE 14: SDS-PAGE OF HUMAN CELL LINE LYSATES. The protein content in each of the cell lysates was determined by Micro Lowry Assay. 10 and 20 µg of each lysate was reduced (10 mM DTT/100°C x 5 min.) and loaded onto a 12% acrylamide mini-gel with prestained protein standards (Bio Rad). Proteins were separated and stained with 0.1% silver nitrate.

nitrocellulose. Blots were incubated with a 1 in 500 dilution of anti-p30 or NRS (followed by sheep anti-rabbit Ig) or sheep anti-rabbit Ig alone. Reactive protein bands were visualized by incubating blots with Protein A coupled I^{125} and autoradiography at -70°C.

In Fig. 15, photos of an autorad obtained from I^{125}-labelled blots containing 80 µg of each cell lysate are shown. It can be seen that anti-p30, but not NRS (or SαRIg, data not shown) binds to one main protein band with an M_r of approximately 42KD in all cell lysates and to a more minor band with an M_r of 25 KD in RAJI and CEM-CM3 lysates only. Two distinct but weaker bands were observed at 60 and 80 KD in all cell lines (the 80 KD band is not visible in the enlarged photo in MOLT-4 or RAJI lanes). Also, in each lysate, high molecular weight material which did not enter the gel to a significant extent was shown to bind anti-p30. The 42 KD band was not visible on blots containing 35 µg of HuT 78 lysate whereas it was clearly visible with the same amount of lysate from other cell lines (data not shown). Although anti-p30 did react with a protein of 25 KD in two of the cell lines, it did not bind with any material around 30 KD.

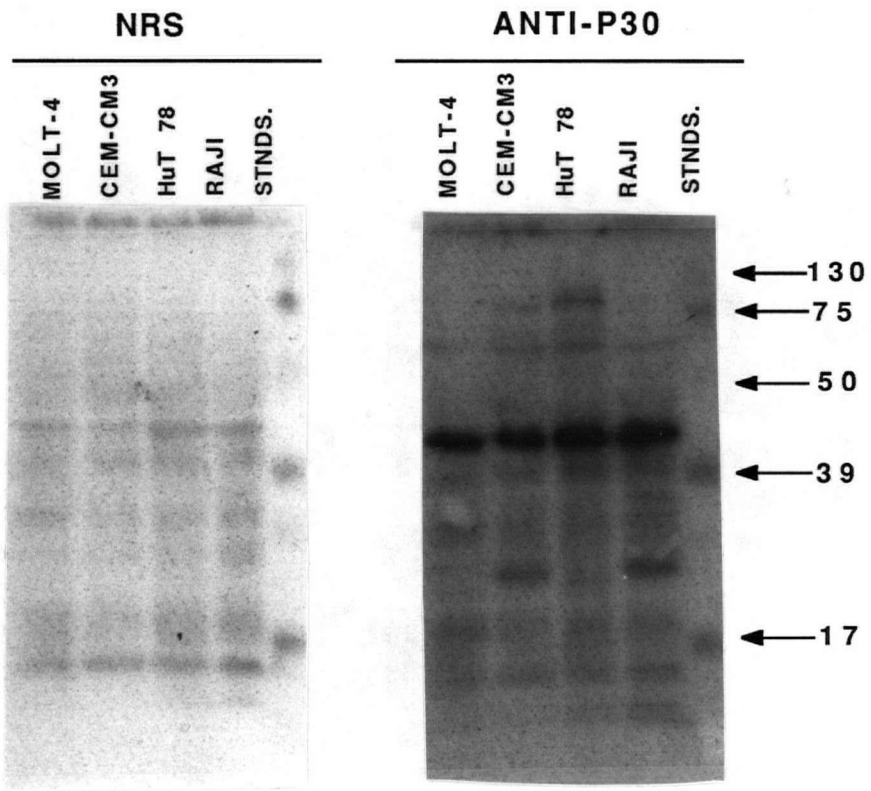


FIGURE 15: WESTERN BLOTS OF HUMAN CELL LINE LYSATES WITH ANTI-P30. Reducing SDS gels containing 80 µg of each cell line lysate were run, blotted onto nitrocellulose and incubated with a 1 in 500 dilution in NET buffer of NRS or anti-p30. Binding was assessed by the addition of sheep anti-rabbit Ig (1 in 500) followed by protein A-conjugated ^{125}I and autoradiography for 5 days at -70°C.

SECTION 2: DISCUSSION

Given the observation that antisera raised to reduced components of the affinity-enriched T suppressor factor Fd11F binds to distinct populations of murine T lymphocytes, these reagents were tested for reactivity with human lymphoid and myeloid peripheral blood cells. Initial studies showed two positive populations of PBMN cells, one strongly positive and one weakly positive (Fig. 8). Further, the percentage of intensely staining cells (from 5.6% to 29.5% with anti-p80 and from 3.1% to 9.8% with anti-p30) was found to be variable both between individuals and at different time points within the same individual (Table II).

The observed variability may be a result of harsh *in vitro* conditions imposed on the cells, or reflect a characteristic of the p80/p30 positive population(s). For example, lymphocytes from different individuals may vary in their sensitivity to ficoll-hypaque, being centrifuged and pipetted repeatedly or being incubated on ice. Potentially, any of these treatments could cause cell surface changes that might result in increased (most likely) or decreased binding of antisera. Alternately, it has been observed that the number of peripheral B lymphocytes varies both between individuals and on a daily basis within the same individual (Clark, P. *et al.*, 1986 for eg.), thus it seemed conceivable that the observed variability was a naturally occurring phemonenon.

After several attempts to double label MN cell preparations with anti-T cell marker Abs and anti-p80/p30 failed due to high amounts of nonspecific binding, a different method was used which involved collecting cells that bound anti-CD3-PE but not NRS (visualized by GαRIg-FITC) by cell sorting, thus eliminating from the preparation those cells which bound serum components, and staining with NRS, anti-p80 and anti-p30. The results (Fig. 9) indicated that all of the $CD3^+$ (as well as $CD4^+$ and $CD8^+$) cells expressed the p80 marker(s) and most (at least 75%) of the $CD3^+$ cells expressed p30 determinant(s). Thus peripheral T cells express lower levels of p30 than p80, or alternately the p30 antisera has a lower affinity than the p80 antisera for T cells. Since the range of peripheral T cells (65-70%) present in MN cell preparations corresponded well with the number of cells found in the weakly staining peak in the initial screening experiments, and given the difficulties experienced with background in trying to double label this population, it seemed probable that this peak, which was originally assumed to reflect nonspecific binding of the immune serum, actually represented T cells. In this experiment it was also found that a subpopulation of T cells (2.1% with anti-p80 and

1.5% with anti-p30) bound the antisera to a much greater extent-possibly correlating with the intensely staining population observed in the human screening study.

It was of interest to investigate whether the p80 and p30 determinants were expressed on all MN cells of the lymphoid lineage or only on the T cell subset. Since there are much lower numbers of peripheral B cells than peripheral T cells and the cell surface characteristics of B cells predispose them to binding antibody (FcR^+), an attempt was not made to isolate $CD19^+$ cells from MN cells by flow cytometry. Instead, this population was double labelled directly with antisera (and GαRIg-FITC) and anti-CD19-PE. Although there was some artefactual or nonspecific red fluorescence evident in these profiles (Fig. 10), once the more intensely staining population was shown to be the one binding anti-CD19-PE by a subsequent experiment, it was concluded that B lymphocytes bound anti-p80 and anti-p30 at levels similar to that observed with T lymphocytes (ie. 1 log increase in binding over NRS with anti-p80 and slightly less of an increase with anti-p30).

Given that cells of the human lymphoid lineage had been shown to express both the p80 and p30 markers, cells of the myeloid lineage were next investigated. Granulocytes were harvested, pretreated with NHS to block free FcR's and screened for anti-p80 and anti-p30 binding. The gated population displayed binding to the goat anti-rabbit Ig-FITC secondary, indicating that this anti-rodent Ig reagent also recognized human Ig bound to the granulocyte cell surface. Aside from this, it was still clearly evident that this cell population was $p80^+$ and $p30^+$ as a full log higher binding over NRS was observed with both immune sera (Fig. 11). Treatment of granulocytes with anti-CD3 and anti-CD19 showed that very few lymphocytes contaminated the cell preparation.

The finding that neutrophils, the major cell type of the myeloid lineage bind anti-p80, anti-p30 and goat anti-rabbit Ig-FITC, raised the possibility that the variability in intensely staining cells in initial experiments was due to varying numbers of contaminating granulocytes. What appeared to be intensly staining cells may have been a reflection of the binding of antisera on top of the already high amount of binding with goat anti-rabbit Ig. Eventhough MN cell preparations were not pretreated with NHS, neutrophil FcR's are known to exist in constant equilibrium with serum Ig, and thus it is conceivable that these cells were effectively sIg^+ as in the granulocyte experiment. In retrospect, it would have been useful to have consistently tested MN cell preparations with a granulocyte- or neutrophil-specific MAb to determine the degree of contamination. Also, if this were the case, the same intensely staining population should have been observed with the NRS treated MN cells although at a relatively lower level (since GαRIg-

FITC is used as the secondary Ab).

The origin of the cell population which stains intensely with anti-p80 and anti-p30 sera remains uncertain. From the data presented, it appears that the larger, more weakly staining population of MN cells observed in the initial screenings is comprised of T cells and most likely B cells (both bind approximately the same levels of antisera). Since the T cell population also contained small subsets of intensely $p80^+$(2.1%) and $p30^+$(1.5%) cells, these may represent the true p80 and/or p30 positive population, thus suggesting that lower levels of binding represent cross-reactivity with another related or unrelated human cell surface protein or alternately, that the immune sera contain antibodies to a ubiquitous leucocyte marker in addition to the Fd11F-associated products. This latter explaination is plausible, given the finding that the bands excised from preparative gels have been shown to contain more than one protein species by isoelectric focussing (IEF, R. Chu, 1989). The observation that all of the profiles obtained with anti-p80 and anti-p30 were very similar in levels of binding and range of activity, suggest that p80 and p30 are of a common origin, ie. are epitopes on the same protein or molecular complex.

Prior to the discovery that cells other than T cells expressed p80 and p30, a study was undertaken in an attempt to characterize the molecular size of the cell surface marker(s) being recognized. Four leukemic cell lines were ordered from the ATCC; MOLT-4, isolated from a mature T lymphocytic leukemia, CEM-CM3 isolated from an acute T lymphoblastic leukemia, HuT 78, from a patient with Sezary Syndrome (a cutaneous T cell lymphoma) and RAJI, from a Burkitt's Lymphoma (B cell). 90% of HuT 78 cells were shown to bind anti-p80 even at low dilution (1 in 80). In contrast, the other three cell lines all displayed much lower levels of reactivity. Conversely, MOLT-4, CEM-CM3 and RAJI all bound anti-p30, whereas HuT 78 cells were effectively $p30^-$ (as assessed at a 1 in 20 dilution). Given the clarity of this latter result with anti-p30, this reagent was chosen to screen Western blots of cell lysates obtained from each of these cell lines.

The same amount of each cell lysate was analyzed by Western blot analysis using anti-p30/NRS, sheep anti-rabbit Ig and Protein A-conjugated ^{125}I. Anti-p30 was shown to bind to one major band with an approximate M_r of 42 KD in all cell lysates and to a minor band of 25 KD in RAJI and CEM-CM3 lysates only. In addition, weaker binding to bands of 60 KD, 80 KD and high molecular weight material was observed in all lysates. The blot containing 35 µg of each lysate suggested that the 42 KD band was missing in HuT 78 cells, however it is present in equal intensity to that observed in the lysates from the other cell lines on 50 and 80 µg blots (Fig. 15).

Only the 25 KD band is absent in HuT 78 lysates. However, this band is also missing

in MOLT-4 lysates. The cell surface data obtained with anti-p30 (Fig. 13b) show that CEM-CM3 express the highest levels of the p30 determinant(s) whereas HuT 78 cells express virtually none. At the lowest dilution tested (1 in 80), it can be seen that very few MOLT-4 cells (6%) bind anti-p30 whereas close to 30% of RAJI cells do (approx. 70% of CEM cells are positive at this dilution). Given the fact that a dilution of 1 in 500 of anti-p30 was used in the Western blot analysis, the cell surface data do correlate with the Western blot data. It would be more convincing if there were a visible increase in intensity of this band in the CEM-CM3 lane (expresses higher levels of p30) versus the RAJI lane. Also, as was mentioned, the 42 KD, not the 25 KD band is the major band visible on anti-p30 blots.

If the 42 KD band contains the protein of interest, in light of the anti-p30 cell surface data, it can be concluded that HuT 78 cells synthesize, but do not express the p30 determinant(s). If the molecule bearing p30 is normally expressed on the cell surface in a complex with one or more other molecules, deletion or mutation of one molecule may completely abolish cell surface expression of the other. A well-documented example of this is the CD3/TCR complex. In fact, the MOLT-4 cell line utilized in this study has a TCRαC gene deletion and is cell surface CD3$^-$ as well as TCR$\alpha\beta^-$. Leukemic cell lines are known to harbour substantial mutations and chromosomal aberrations. In fact, HuT 78 has been shown to have a translocation of 2q34 to 3' of *c-myc* on chromosome 8. An additional point of interest regarding HuT 78 cells is that they grow very slowly in culture. The ATCC recomends the addition of rIL-2 to cultures to increase the doubling time. It is possible that the molecule bearing the p30 determinant is synthesized by HuT 78 cells but not expressed on the cell surface unless the cell is activated. It would be interesting to determine the reactivity of HuT 78 cells grown in cultures with exogenously added IL-2 with anti-p30. The lack of HuT 78 p30 surface expression may also be due to a defect in intracellular processing or transport.

The observation that anti-p30 did not bind to any 30 KD material in lysates is not that surprising given cross-species variation and the above mentioned uncertainty of the content of the priming antigen preparation in the production of the antisera. In humans, the 25 KD band may contain the the murine equivalent of the p30 determinant. Alternately, the 25 KD band may represent nonspecific binding of the immune serum and the 42 KD band may contain the protein of interest. It is possible that the material to which anti-p30 was raised against was an antigen-binding breakdown product of Fd11F. Since Fd11F is secreted and/or cell surface, it is likely that very little would be found in lysates obtained from healthy p30$^+$ cells. If the 42 KD band represents specific anti-p30

binding, evidence for this would have been the presence of a distinct, or much more intense band in HuT 78 lysates (given the conclusion that these cells are unable to express or secrete the $p30^+$ product, it would have to be broken down intracellularly). On the other hand, if the 25 KD band is of significance, then HuT 78 cells synthesize low levels of $p30^+$ protein (similar to MOLT-4 cells), or none at all. Other Western blot analyses done in this laboratory on Fd11 and A10 (Ts hybridomas) lysates have also shown a lack of reactivity of anti-p30 with a 30 KD protein band (M. MacDonald, unpublished). The binding of anti-p30 to 80 and 42 KD proteins does correspond somewhat with data obtained in the murine system, in that reduced Fd11F affinity-enriched preparations have been shown to be comprised of bands with M_r's of 80 KD, 45 KD (more variable) and 30 KD (R. Chu, 1989 for eg.).

The binding of anti-p30 to an 80 KD, 60 KD and possibly 25 KD bands in cell lysates may represent cross-reactivity with cellular proteins. Given that the 80 KD band present in affinity-enriched murine Fd11F preparations has been shown to harbour antigen-binding and suppressive capabilities, it may be a coincidence that a protein of approximately the same size is cross reacting with the anti-p30 serum. It is however possible, that the human equivalent of Fd11F is expressed on the cell surface as a multimer of 42, 80 and/or 60 KD components.

Only limited comparisons can be drawn between the work presented here and the anti-p30 murine cell surface labelling studies of North *et al.* (1988). The staining protocol used in the one- and two-colour analysis of murine thymocytes was very similar to that used in the initial screening of human MN cells with anti-p30/anti-p80 and profiles with similar characteristics were obtained. However, the protocol used in this work to further characterize the $p80/p30^+$ human population which involved removing NRS-reactive cells with goat-anti-rabbit Ig-FITC and collecting $CD3^+$ cells in a cell sorter before screening for reactivity with the antisera has shown that all human peripheral T cells bind anti-p80 and anti-p30 (to a lesser extent). Also, although North *et al.* screened several other murine tissues with anti-p30, B lymphocytes and cells of the myeloid lineage were not tested. An important difference between these two sets of experiments is that murine T cells were obtained from the spleen and thymus, whereas human leucocytes were obtained from peripheral blood. This may be significant given the fact that cells of the immune system express specific receptors or adhesion molecules when in ciruclation and in response to injury or infection (Springer, T., 1990). This is addressed further (in light of recent findings) below.

The lower levels of antisera binding to whole MN cell preparations versus that

obtained with isolated T cells is a paradox. In retrospect, it would seem that a better control rabbit serum than that obtained from pre-immunization bleeds would have been immune serum (raised against a molecule known not to be expressed by the test population). It appears that one of the components in the immune sera sticks and masks the p80 and p30 determinants in whole MN cell preparations, giving lower levels of binding and making statistical and two colour analysis very difficult. However, binding of anti-p80 and anti-p30 to cell lines was reproducible and always at significant levels over that of NRS. This may be due to higher levels of p80 and p30 cell surface expression on postive tumour cell lines.

An alternate solution to this paradox may relate to the state of cell activation as was briefly discussed for HuT 78 lysates. Since anti-CD3 antibodies are known to stimulate T cell blastogenesis (rev. in Altman *et al.*, 1990), the pan-T reactivity of isolated T cells may be due to activation-induced up-regulation of p80/p30 expression. Eventhough CD4 and CD8 molecules are known to be associated with T cell signal transduction and activation, the fact that all sorted $CD4^+$ and $CD8^+$ cells pretreated as above also expressed p80/p30 argues against this theory somewhat. In addition, the p30/p80 determinants were also found to be expressed by resting cells of the myeloid lineage (unless neutrophils are activated by *in vitro* manipulation).

Anti-p80 and anti-p30 were raised against antigen-binding moieties present in Fd11F preparations affinity-enriched from a Ts1 hybridoma (Fd11) culture supernatant using the TsF framework antibody B16G. This information, considering the fact that only a proportion of peripheral blood $CD4^+$ T lymphocytes function as suppressor-inducer cells, raises the question of the nature and function of the p80/p30 determinant in the human (and possibly the murine) immune system. Any model which postulates the function of the p80/p30 protein must take into account its possibly ubiquitous expression by peripheral white blood cells (monocytes, basophils, eosinophils and mast cells were not specifically tested).

A recent review by T. Springer (1990) contains a discussion of a family of immune system adhesion molecules named 'selectins'. Selectins have varied functions in adhesion yet all appear to be involved with the regulation of leucocyte binding to endothelium at the sites of inflammation. One of the human molecules within this family, LAM1 (for leucocyte adhesion molecule 1), has been shown to bear the epitopes recognized by antibodies raised to MEL-14 (a murine lymphocyte homing receptor) and Leu 8 (a fairly ubiquitous cell surface marker of circulating white blood cells which has also been shown to define suppressor-inducer subpopulations in the human $CD4^+$ T cell subset - see

Introduction). It is interesting that upon immunoprecipitation, the Leu 8 marker was originally described as having a molecular weight of 80 KD. The LAM1 molecule has recently been immuno-precipitated from peripheral T cells and found to be 74 KD under reducing conditions, whereas LAM1 precipitated from neutrophils was 95 KD (Tedder *et al.*, 1990). Selectins have an N-terminal, Ca^{++}-dependent, lectin-like domain, an epidermal growth factor-like domain and short consensus repeats similar to that observed for C3/C4 complement binding proteins (Springer, 1990).

There are several similarities between LAM1 and Fd11F preparations in addition to molecular size. First, PMA stimulation of LAM1-expressing peripheral thymocytes results in rapid modulation of this marker from the cell surface as was observed for Fd11 cells using anti-p30. Further, lymphocytes and neutrophils have been demonstrated to actively shed LAM1 into the culture media upon activation. In light of these similarities, the percent $p30^+$ T cells obtained by North *et al.*, using murine, spleen- and thumus-derived T cells versus the virtual pan-reactivity of anti-p30 with human, peripheral T lymphocytes could be significant and relate to the presence of Leu 8 on the surface of cells in the periphery and it's absence on the surface of cells out of ciruclation. It would be interesting to test cells from human lymphoid organs and from murine peripheral blood with anti-p80 and anti-p30 as well as anti-Leu 8 or LAM1. Although specific antigen-binding and suppressive ability have obviously not been assayed for selectins, the possibility exists that Fd11F, and even suppression of the immune system in general somehow involves solublized adhesion molecules which bind to target receptors and block cell to cell interactions. Antigen-specificity potentially could be obtained as Green believes, by utilizing alternately spliced TCR transcripts. Idiotype-bearing molecules may even associate with adhesion molecules intracellularly and be secreted upon activation by antigen.

Whatever the function of the molecule(s) bearing the p80 and p30 determinants is in humans, it must relate to it's near ubiquitous expression on peripheral white blood cells and it's origin from a preparation enriched by binding to a TsF-framework-specific monoclonal antibody. Finally, it should be noted that the possibility exists that the human cell surface and cell lysate binding of these antisera represent interspecies cross-reactivity with related or even unreleated (human tissues other than those from the hematopoietic system were not tested in this work) cell surface protein(s).

SECTION 2: CONCLUSIONS

Antisera raised in rabbits against two distinct components of an affinity enriched antigen-specific, murine TsF were used to screen subsets of human peripheral white blood cells. Anti-p80 and anti-p30 were both shown to bind to the surface of two distinct populations of mononuclear cells on the basis of different levels of binding. With anti-p80, on average 65% of the preparation showed lower levels of binding and 15% gave higher levels. With anti-p30, approximately 50% of the MN population showed weak binding and 6% gave strong binding. An experiment which isolated the T lymphocyte population from nonspecific binding to NRS and non T cells was able to clearly show that this subset of PBMN cells expresses both the p80 and p30 cell surface determinants (although the level of binding was slightly lower with the latter). These antisera were also shown to bind in a similar manner (same increase in binding over background) to peripheral B lymphocytes as to peripheral T lymphocytes. Finally, it was shown that human neutrophils express the p80 and p30 determinants at equal levels, even though the goat anti-rabbit Ig-FITC displayed significant binding to human Abs (bound to FcR's) on the granulocyte cell surface.

In an attempt to characterize the determinant(s) to which these antisera were binding in humans, several human, leukemic cell lines were obtained and screened for reactivity. MOLT-4, CEM-CM3, HuT 78 and RAJI all bound anti-p80 to varying extents. All of these lines expressed high levels of p30 at a dilution of 1 in 20 except HuT 78, which was effectively $p30^-$. With Hut 78 as a negative control, a Western blot analysis of cell lysates was undertaken with anti-p30 and these four cell lines. Anti-p30 was found to bind to a major band of 42 KD, to minor bands at 60 and 80 KD and to high molecular weight material in all cell lysates. In addition a band of 25 KD was observed in CEM-CM3 and RAJI lysates only.

If the minor bands are ignored, the cell suface and Western blot data suggest that HuT 78 cells synthesize but do not express the p30-expressing 42 KD protein. This could be due to an intracellular processing or transport defect or a mutation resulting in the lack of association with a component of a multimeric complex. However, since HuT 78 cells are $p30^-$, and the 25 KD band is close in size to 30 KD and absent in HuT 78 lysates, the 42 KD band may represent strong interspecies cross-reactivity with Abs contained within the anti-p30 serum, and the 25 KD band may represent specific binding to a suppression-related protein.

Finally, given what seems to be ubiquitous expression of the p80 and p30 markers on peripheral leucocytes, it is interesting that very recently, a molecule (Leu 8) of 80 KD, that is expressed on most peripheral white blood cells, and known to be associated with the suppressor-inducer subset of $CD4^+$ T cells, has recently been identified as LAM1 a new member of the 'selectin' family of adhesion molecules of the immune system.

CLOSING DISCUSSION

The work presented in this thesis has involved different aspects of two different modes of immunosuppression. Section 1 dealt with the further characterization and partial purification of an antigen-nonspecific suppressor inducer factor (SIF) secreted by an LGL, third population cell line. In addition, the mode of action of SIF was investigated and discussed for different assay systems. This work was discontinued shortly after the discovery was made that the M1-A5 cell line was infected with *Mycoplasma* and that the suppressive phenomena could not be separated from the presence of the organism. Section 2 utilized antisera raised to components of a murine, antigen-specific suppressor factor secreted by a Ts cell hybridoma to investigate their reactivity with normal, human, peripheral leucocytes and several human, lymphoid leukemic cell lines. In addition, an attempt was made to characterize the protein expressing the p80 and p30 epitopes using cell line lysates and Western blot analysis.

Although many difficulties were experienced in obtaining the results presented in this work, similar problems have been encountered by many other workers in the field of immunosuppression. Most of the assays required to characterize bioactivity of suppressor factors are variable and dependent on many different elements acting in concert and often *in vitro.* In addition, suppressor cells are extremely difficult to clone and purified suppressor factors seem to be an unbelievably diverse group of molecules to mediate the fine level of control that has been shown to exist in the immune system.

Putting the technical difficulties of this field of research aside, it is still curious that with the current advanced state of technology, so little about the basic mechanism of suppression of the immune system is actually understood. There seems to be no other way to increase our knowledge in this area but to keep 'following leads'. I believe that once the general mechanism of immune down-regulation has been clearly laid out, important and exciting advances in this field will not be far behind.

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