Isolation of a Novel Antiproliferative Cytokine From a Human Macrophage-like Cell Line

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By
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ISOLATION OF A NOVEL ANTIPROLIFERATIVE CYTOKINE FROM A HUMAN MACROPHAGE-LIKE CELL LINE

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I dedicate this thesis to all victims of cancer.

Abstract:

U937 cells can be induced to produce a soluble cytostatic mediator of cell growth. The production, characterization and purification of U937 derived growth inhibitory factor(s) (UGIF) are studied. The UGIF does not exhibit cytotoxic activity even at high doses, rather the growth regulation observed is cytostatic, and may relate to an induction of differentiation of affected cells. Effects of the UGIF are seen on all tumor cell lines examined.

The UGIF has been purified using anion exchange chromatography, gel permeation exclusion chromatography, and reverse phase HPLC. High Pressure Liquid Chromatography indicates the activity can be retained on DEAE, or C_{18} columns, and that it has a molecular weight greater than 300kDal. UGIF has a specific activity of >500,000 units/mg protein.

Much physico-chemical data is known about UGIF. The activity in crude supernatant concentrates is stable to heat treatment, surviving 5 minutes at 100 degrees celcius. It is also stable to pH extremes, between pH2- pH12. The material is filterable through .22u filters.

UGIF is not due to Tumor Necrosis Factor (TNF-) alpha or beta, Transforming Growth Factor (TGF-) beta one(TGF-b1) or beta two(TGF-b2), Interleukin six (IL-6), Interferon (IFN) or Interleukin one (IL-1). However, impure UGIF supernatants do contain low levels of TNF-alpha. Material purified by sequential

DEAE and C_{18} columns contains no detectable TNF activity despite having significant antiproliferative activity. UGIF cannot be shown to have any synergistic effects with TNF.

Neutralization experiments performed on highly purified material indicate that the UGIF activity is not neutralizable by anti-IL-6, anti-TNF, anti-TGFb1, or by a combination of anti-TNF and anti-TGFb1. The inhibitory activity of UGIF is therefore not a consequence of these cytokines, or of a synergistic effect related to them. The antiproliferative factor UGIF appears distinct from other known cytokines.

Introduction:

Cytokines are soluble mediators of cellular responses. The responses to cytokines can range from growth inhibitory effects to highly growth stimulatory effects. Cytokines can also have antiviral, differentiative, or chemotactic effects associated with them.

The regulation of cell growth is an important process to organisms with defined body sizes and morphologies. Organogenesis is one of many carefully regulated growth processes. The role of soluble mediators as growth regulatory molecules is gaining importance in the area of proliferation biology. Understanding the biology of growth regulatory molecules may influence research in the fields of Oncology, Immunology, and Developmental biology.

To examine the biology of regulatory cytokines a large amount of purified material is required. As the majority of cytokines are highly active at very low concentrations, this often means a great deal of crude material is required to purify the cytokine of interest.

Some sources of cytokines are primary cell cultures, normal tissues, and normal lymphocyte cells. However, it is often a problem to get a reasonable quantity of a cytokine from normal cells. Another problem with using normal cells as a source of cytokines is that obtaining large numbers of normal cells can be a significant obstacle.

The heterogeneity of normal cell populations complicate the production of cytokines even further. Heterogeneity can result in interactions between different cell types which down regulate, or reduce the production of a cytokine of interest. For example, in isolated peripheral blood lymphocytes there are T cells, B cells, macrophages and several other types of cells, all of which are capable of producing cytokines, which can interfere with each others production, and are certain to add a degree of complexity to bioassay and purification protocols. While there are techniques available for enriching for particular cell types, these are never 100% effective and the problem of heterogeneity still exists.

An effective approach to produce cytokines is to use a tumor cell line. This gives the advantages of having an unlimited number of cells all of the same specific type and lineage, and allows for standardization of production conditions.

Growth regulatory factors can be derived from the human Histiocytic Lymphoma cell line U937. This tumor cell line was originally derived from a patient with true Histiocytic Lymphoma.[1] The present study undertakes the characterization of some growth regulatory factors produced by this cell line. These activities are designated U937 Derived Growth Inhibitory Factors (UGIF).

Characterization of UGIF with respect to several known mediators reveals that these cells produce unique cytostatic regulators of mammalian cell growth. These materials act without killing the cells they affect and thus cause little damage to

surrounding cells by the toxic products which would be released by killing.

Activities like UGIF which inhibit tumor cell growth are of great interest as potential new therapeutic agents in the treatment of cancer. Such activities may also prove to be very useful in examining the biology of cell growth. Production, characterization and partial purification of UGIF is detailed in this thesis.

Literature Review:

Many different cell types are capable of producing cytokines of one type or another. One cell of great interest to immunologists is the macrophage. These cells have long been known to produce a virtual arsenal of regulatory molecules ranging from Tumor Necrosis Factor and Arachidonic acid to highly toxic oxygen radicals. The role of the macrophage in vivo is several fold including an essential role in antigen presentation, phagocytosis, and may have a role in immune regulation of tumor cell growth. While macrophages can be shown to have tumoricidal effects, these effects could be a manifestation of some other more immunologically relevant process, such as ridding the body of highly activated proliferating cells of the immune system to control normal immune responses. Exhaustive study of macrophage cells has revealed that they have a number of far reaching functions. The following table illustrates some of their functions;

Table of Macrophage Functions:

Function	Mechanism
Microbicidal activity	-Oxygen dependant, H_2O_2, O', OH', halogens -Oxygen independent, lysosome, acid
Tissue damage	-H_2O_2, Acid hydrolases, C3a
Tissue reorganization	-Cytokines, elastase, collagenase
Lymphocyte activation	-Ag presentation, IL-1 production
Inflammation/Fever	-IL-1,Prostaglandins, secreted mediators
Tumoricidal activity	-TNF, proteases, H_2O_2, cytotoxic factors

The macrophage is one of the key cells of the immune system and manufactures a wide range of soluble mediators which affect other cells. Some of the most important regulatory cytokines known to be produced by macrophages are described in the table below:

Cytokines produced by macrophage cells:

Cytokine	Molecular Wt.	Sources	Biological Activities
TNFa	17000	Monocytes, macrophage,	Cytotoxic
TNFb	20000	Lymphocytes	Cytotoxic
TGFb1	25000	Most cells	Stimulatory/Inhibitory
TGFb2	25000	Most cells	Stimulatory/Inhibitory
IL-1	17000	Monocytes,other cells	Stimulatory/Inhibitory
IL-6	26000	Many cells	Stimulatory/Inhibitory
gamma IFN	20 and 25 Kda	Most cells	Antiviral, Antiproliferative

The macrophage cell is known to produce several distinct immunoregulatory activities.[2 3 4 5] Many tumor cell lines have been shown to be capable of producing cytokines characteristic of their normal cellular counterparts.[6 7 8] Activities such as Tumor Necrosis Factor[9 10], Oncostatin M[11], gamma Interferon[12], Interleukin-One[13] , Transforming Growth Factor beta[59] , and Interleukin six [14] can be shown to be produced by monocytic cells.

The U937 cell line is characterized as a histiocytic lymphoma cell based on morphological and cytochemical properties[15 16], and has been shown to posses a number of properties similar to human monocytes.[17] While some activities derived from U937

cells are well characterized, there are others which remain to be fully described[18 19 20].

Interleukin-1 is a cytokine with many distinct functions[21]. Virtually all monocytes/macrophages produce IL-1[22 23]. It can exhibit antiproliferative effects on certain cells and must be considered when discussing monocyte derived antiproliferative factors[24 25]. IL-1 occurs in two forms in most species, IL-1 alpha and beta. It is the 17000 MW beta form which predominates in humans, although both forms appear to have much the same function. There are many assays for IL-1 including, radioimmuno assays'(RIA), in vivo endogenous pyrogen assays, and assorted coculture assays which typically rely on the induction of IL-2 production by IL-1 responsive T-cells.[7] In certain situations IL-1 has been shown to act in synergy with TNF. TNF and IL-1 have been shown to synergistically enhance the proliferation of osteoblast cells[26]. The possibility exists that this combination may also have a synergistic effect on the inhibition of growth of other cell types. The most relevant action of IL-1 in this discussion is that it is known to influence tumor cell growth in more than one way.

IL-1 can induce monocyte mediated tumoricidal activity, cytotoxic T-cell responses, or it can act directly on certain tumor cells. The augmentation of differentiation and inhibition of growth of myeloid leukemia cells by IL-1 has been described in the murine system by Onozaki et. al. Purified IL-1 has been shown by this group[27] to be cytocidal or cytostatic for some tumor cells. Bertoglio et.al. has shown that IL-1 can be cytocidal for melanoma

tumor cells while being a growth factor for an astrocytoma line.[28] At least some of the growth inhibitory actions of IL-1 can be ascribed to an induction of differentiation as is the case for the mouse cell line M1[29].

Cytotoxic lymphocytes and macrophages/monocytes are capable of producing pore-forming proteins (perforins), related to the ninth component of complement.[30] The perforins kill cells by punching holes, or pores in plasma membranes. Natural Killer cells (NK) and cytotoxic T lymphocytes lyse target cells through this mechanism. Perforins are not closely related to Lymphotoxin (LT) or Tumor Necrosis Factor (TNF), but act in a similar way to lyse target cells. [31]

Tumor Necrosis Factor can be cytostatic, cytotoxic, ineffective, or may even stimulate growth when tested on human tumor cell lines in vitro.[32 33 34 35] TNF can be induced by lipopolysaccaride, virus, or PMA stimulation of monocytic cell lines, including HL-60 and U937 [36 37]. TNF was first purified from the HL-60 cell line by Dr. B Aggarwal and W. Kuhr.[38]

Tumor Necrosis factor exists in at least two related forms, TNF alpha (17,000 mw), and TNF beta (Lymphotoxin 20,000 mw). The activities are functionally identical as defined by the standard TNF assay[5]. The two factors share strong (40%) homology at the amino acid level[39].

The effects of TNF are varied depending on the cells being examined and the presence or absence of other interacting mediators. TNF exerts it's effects on myeloid leukemia cells by inducing a terminal differentiation and thus inhibiting

proliferation, rather than by a cytotoxic means[40]. TNF has also been shown to suppress the growth of erythroid progenitor cells in a cytostatic manner[41]. The action of TNF on HL-60 promyelocytes is at the level of c-myc translation[42]. HL-60, ML-3 and U937 myeloid cell lines, can all be induced to differentiate in response to TNF[43]. Treatment of fibroblasts with TNF or IL-1 induces the production of IL-6 which has multiple functions itself. The synergistic cytotoxicity of TNF and gamma interferon (IFN) is documented[44]. IFN can augment the cytotoxicity of TNF and even induce TNF sensitivity in cells normally resistant to it's cytotoxic effects.

Transforming Growth Factor Beta (TGFb1 and b2) is another multifunctional cytokine, having either stimulatory (transforming) or inhibitory effects on specific target cells. These cytokines are part of a structurally related family of molecules belonging to the Transforming Growth Factor Family. Members of the family are listed in the table below;

Members of the Transforming Growth Factor Family;[45]

Member	*Protein*	*Receptor*
TGF-alpha	<10000 mw	c-erbB product 180kda
TGF-beta1 and 2	25000mw dimer	high affinity TGFb receptors
Inhibin	56000+multiple forms	unique Inhibin receptor
Oncostatin M	18000 mw	unavailable
Mullerian Inhibitory Substance	70000 mw dimer	EGF receptor
EGF(Epidermal Growth Factor)	53 aa peptide	c-erbB product180kda receptor

TGFb exists as a 25 Kda mol wt dimer cleaved from a larger inactive precursor molecule by proteases at acidic pH. The dimer structure is made up of either two related, or two identical 112 amino acid subunits TGFb1, b2, b12.

TGF beta is known to have antagonistic effects towards the actions of TGF alpha and EGF. Of the members of the TGF family, it is only the TGF betas, and recently Oncostatin M that are known for their growth inhibitory actions.

TGFb induces differentiation of immature epithelial cells and inhibits differentiation of 3T3 cells in vitro.[46] Normal human prokeratinocytes can have their growth reversibly inhibited by TGFb. [47] A similar action of TGFb is to potently inhibit the proliferation of murine megakaryocytes in vitro, [48], and to antagonize the stimulatory effect of IL-3 on their growth. Another effect of TGFb is that of inducing the production of extracellular matrix components, collagen and fibronectin, from human fibroblasts [49]. Induction of differentiation seems to play an important role as one of the main modes by which TGFb works.The multifunctionality of TGFb , and the presence of it's mRNA in virtually all cells make it an important molecule to bear in mind in any evaluation of an apparently new growth regulatory activity[50].

The most recent addition to the Transforming Growth Factor Family is Oncostatin M. Information released at a recent Gordon Conference in California argues that this cytokine should now be considered a member of the TGF family.

Oncostatin M has been isolated from PMA stimulated U937 supernatants [14]. Oncostatin is stable in the pH range of 2-11, but is sensitive to heat treatment @ 90'C for one hour. The molecular weight of Oncostatin is approx. 18000. Like the other transforming growth factors Oncostatin can be activated by treatment with 1M acetic acid.

Oncostatin M is similar to the TGFb1 and b2 in that it can be inhibitory for certain cells while stimulating the growth of others. The A375 target cells inhibited by Oncostatin are the same targets used to assay the growth inhibitory effects of TGFb1 and b2.[2] While A375 cells can be inhibited by Oncostatin, this factor actually increases the proliferation of normal human fibroblasts by 20-30%. It is not surprising that this cytokine has turned out to be another member of the TGF family.

The Interferon family of cytokines have antiviral and antiproliferative effects on cells.[51] Many studies indicate that interferon can have growth regulatory effects on both normal and tumor cells[52]. The inhibitory effects of interferon on cells are not known to be mediated by a cytotoxic mechanism. Certain cell lines are sensitive to inhibition by interferon, while others are not. Interferon has been shown to inhibit protein synthesis, polyribosome formation, and to inhibit DNA synthesis.[59] While IFN is recognized as an antiviral agent, it also has the inhibitory actions which may prove to be more important than the antiviral activity associated with it. However, a common assay for the presence of interferon is to look at the ability of a sample to protect a cell line from viral cytopathic effect. Thus if there is no

antiviral activity detected in a sample, it suggests that there is no interferon activity present in that sample[53]. An alternative approach to prove there is no interferon present in a sample with antiproliferative activity, is to attempt to neutralize the activity with neutralizing antibodies.

Interferon beta was the first of the interferons to be purified[54]. The interferons are glycoproteins, and the extent of glycosylation of a particular interferon can vary, giving a range of sizes for each. The size of human IFN alpha is from 16000 to 27000 daltons, human IFN gamma has a molecular weight range of 40000 to 60000 on gel filtration, and human IFN beta has an approximate molecular weight of 20000 daltons. The interferons all have specific activities in the order of tens of millions of units per milligram of protein. A good review of the purification and biological effects of the interferons is given by Pestka et. al. 1987[61].

Interleukins are part of an artificial classification of cytokine molecules with no clear cut criteria for inclusion of a given cytokine, and exclusion of another from the group. The terminology is very misleading and should perhaps be dropped from common use.

One of the most recent additions to the Interleukin family is Interleukin-Six (IL-6). However, the relatedness to other interleukins implied by such a grouping is not borne out by either structural or functional data, and one should not necessarily make such connections between the Interleukins. Nonetheless, IL-6

appears to be the accepted term for this new multifunctional cytokine.

This particular cytokine has been well characterized by several different groups. IL-6 was actually discovered on three distinct occasions, and given several different names. The activity was initially described as having interferon like antiviral effects[55], which coprecipitated with IFN-B, and was designated IFN-B2. It was soon discovered that the antiviral activity was not a consequence of IL-6 at all [56], and the factor was renamed 26Kda protein. Other researchers investigating a hybridoma growth factor (HGF) are also credited with discovering IL-6. Astaldi's lab discovered that there was a factor in the supernatants of human epithelial cells which could be used to replace feeder cells for the growth of mouse hybridomas[57]. The third discovery of IL-6 was by a group studying B Cell Differentiation Factors (BCDF's). The BCDF activity described as BSF-2 has been shown to be identical to IL-6 by amino acid sequencing[58]. As the three factors all turn out to be the same, and much work has been done characterizing each, the properties of IL-6 are quite well described.

IL-6 is an approx 26Kda glycoprotein, which is produced by many different cell types including; normal fibroblasts, endothelial cells, monocytes, T and B cell lines, and sarcoma or carcinoma derived cell lines. The cytokine can be induced by using a variety of means including; IL-1(alpha and beta), cycloheximide (inhibitor of protein synthesis), TNF, dsRNA, and viruses. Certain cells like T-cell hybridoma-d4 and HTLV transformed T-cells will produce IL-6 spontaneously. IL-6 can stop the growth of B-cells

by inducing a terminal differentiation and inducing Ig production. IL-6 can support and induce the growth of hybridomas and plasmacytomas, as well as mouse thymocytes. It can induce the secretion of acute phase proteins by hepatocytes.[59] Il-6 can be shown to induce a terminal differentiation of B-cells, and such molecules should be kept in mind in any discussion of cytostatic mediators.

It is particularly interesting that IL-6 can be shown to be carried by alpha-two-macroglobulin, giving it an extremely high apparent molecular weight (in the range of several hundred thousand to millions of daltons)[60]. The complex bound IL-6 retains it's biological activity, despite being a bound molecule. It's resistance to the action of protease is greatly enhanced over free IL-6. This means there is a precidence for bioactive cytokines being carried by a much larger molecule. There are several examples of peptides being carried by high molecular weight proteins and as part of large lipoprotein aggregates(eg. arachidonic acid, Nerve Growth Factor, and Platelet Derived Growth Factor)[61 62].

It may be significant that high molecular weight proteins can act as carriers for cytokines. The stability of small molecules can be drastically altered by being bound to large carriers. Treatments like boiling, protease digestion, and reduction may give misleading results if the activity in question is being carried. If the carrier is biologically inert, and it has a high affinity for the active small molecule, purification may be extremely difficult, and amino acid sequencing could be difficult due to the copurification. Other

complications of carrier molecules would include the presence of breakdown products, which could give rise to multiple species of active complexes of cytokine and carrier. Identification of carrier molecules may lead to engineering of new ways of stabilizing and delivering recombinant cytokines to their intended site of action.

In many cases alpha-two-Macroglobulin can be shown to act as a carrier for cytokines. In some situations cytokines are carried as inactive molecules, while others can exert their biological effects while part of the complex. A latent form of Transforming Growth Factor beta has been shown to be carried as part of an alpha-2-M complex. At least some of the TGFb appears to be covalently linked to the alpha-2-M. [63] When these ligand carrier units are boiled or reduced they do not dissociate readily due to the covalent bonds holding them together. If the TGFb-alpha-2-M complex had a measurable activity it might present the paradox of a very stable molecule with a very high molecular weight. This is a clear example that cytokines may appear to be much larger than they actually are when in a purified form. Another cytokine which can be shown to bind to alpha-2-M is Nerve Growth Factor (NGF).[64]

In the case of NGF it has been shown that NGF binds to Alpha-2-M in a two to one ratio. When unlabelled alpha-2-M is mixed with radioactivelly labelled NGF, an interesting result is observed. Radiolabelled NGF actually migrates as a very large molecule in both gel filtration and reducing SDS-PAGE. This seems to indicate that NGF also can bind covalently to Alpha-2-M. It is unfortunate that these studies did not attempt to measure bioactivity of the

complexes. However, Alpha-2-M has also been shown to bind covalently to Platelet Derived Growth Factor (PDGF), and the PDGF retains its mitogenic activity while part of the complex.[65]

In the case of PDGF complexes the covalent binding appears to be mediated through disulphide bonds as the complex can be destroyed by reduction. The complex is pH stable (.1M NaOH-1M acetic acid), and stable to treatment with 1% SDS, the PDGF combines in a two to one ratio with Alpha-2-M. The apparent molecular weight of the complex is 780,000 daltons. In this study the stabilty of the complex is evaluated by radioactive means, but the retention of biological activity after such treatments is not discussed.

There are a variety of biologically active cytokines which are well known, and a number that are poorly understood. Several distinct factors appear to have similar regulatory effects on target cells, others have completely opposite effects on the same targets. There is evidence that many cytokines act in synergy with other cytokines to produce large net effects not observed for the individual cytokine. Cytokines can even be carried as components of large macromolecular aggregates , and still exert a biological function. It is clear that the question of isolating and purifying a novel cytokine from a cell known to produce several factors can become an enourmous task. It is the production, characterization and partial purification of one such novel factor, from the U937 cell line, which is undertaken in this thesis.

Materials and Methods:

A. Media:

All cell lines were maintained in RPMI1640(Cellgro, Fisher Scientific) growth media, and supplemented with heat inactivated (56°C for 1/2 hour) fetal calf serum (FCS (Gibco)). Media for the production of UGIF consisted of RPMI1640 supplemented with .156mg/ml lactalbumin hydrolysate(Sigma). UGIF assay media consisted of RPMI 1640, 10% FCS supplemented with 100U/ml penicillin/100ug/ml streptomycin. U937 production stock and target cells were grown continuously in RPMI with 5% FCS. K562 target cells were maintained in RPMI with 10% FCS. A375 target cells were maintained in RPMI, 10% FCS. L929 cells were maintained in RPMI with 3% FCS.

B. Cell Lines:

The following human cell lines were originally purchased from American Type Culture Collection (ATCC).

1. A375 cells of melanoma lineage(10% FCS).[66]
2. U937 cells of histiocytic lymphoma lineage(5% FCS).[1]
3. K562 cells of myelogenous leukemic origin.(10% FCS).[67]
4. L929 mouse fibroblast cells were originally a gift from Dr. A. Greenberg at the Manitoba Cancer Institute(3%FCS).[68]

5. Peripheral Blood Lymphocytes (PBL) were isolated by the method of Boyum[69], using Histopaque (Sigma) density gradient centrifugation of normal donors blood. The cells were grown in RPMI 1640 (10% FCS), and stimulated with either Phytohemagglutinin-P (PHA) 1/1000 v/v(Difco), or Pokeweed Mitogen (PWM) 1/100 v/v(Gibco).

C. Mycoplasma:

U937 producer cultures were tested on several occasions for the presence of mycoplasma contamination by culture, and by the Hoescht DNA stain technique.[70] All assays for this organism were negative.

D. Production of UGIF:

1. Standard Method.

U937 cells are maintained in 850ml Falcon roller bottles, the cells are harvested by centrifugation , counted, and resuspended at 1.5 x 10^6/ml in serum-free RPMI1640 containing 20ng/ml 12-O-Tetradecanoylphorbol-13acetate (PMA/TPA, Sigma). Stimulated U937 cells are plated in Falcon Integrid Tissue Culture 150 cm diam. plates, and incubated overnight at 37°C in an atmosphere of 5% carbon dioxide. By four hours post stimulation the cells become adherent, and anchor/attach to the plastic in a monolayer. At 24hr. after the initial stimulation the cells are washed extensively with serum-free RPMI or Hanks balanced saline solution (HBSS). The washed monolayers of stimulated U937's are then plated in 15 ml of production media and incubated another 24 hours.

Supernatant is collected from the monolayers and replaced with fresh production media. Collected supernatants are filtered through nitrocellulose filter (.45u), dialyzed extensively against water (Spectrapor2 dialysis bags), and then concentrated by lyophilization for further analysis.

2. Large Scale.

The large scale production of UGIF makes use of the cell growth data which suggests that the inhibitory activity in supernatant builds up to a plateau level in a four-hour time period. However, repeat harvesting and replenishing the cultures with fresh media allows several rounds of production from the same cells. All procedures are carried out as above, except that a Millipore peristaltic pump is used to rapidly remove the supernatants from the plates every four hours. This is a two person procedure and should not be attempted by a sole researcher as it is critical not to leave the U937 monolayers exposed for any longer than is absolutely necessary when removing the supernatant and adding fresh media. One person does the harvesting via pump, while the other adds media and shuttles cells to and from the incubator. It is also important when producing on a large scale to ensure that the cells are kept in the incubator as much as possible during the procedure, for this reason the maximum number of integrid plates ever out of the incubator at one time is twenty five. In my hands this number has been found to be highest manageable number of plates.

E. Proliferation Assay:

1. K562 and U937 Standard 24 Hour Assay:

Samples are serially diluted in RPMI containing 10% FCS and 100 units per ml penicillin, 100ug/ml streptomycin(Gibco), then 50ul of sample is applied to 50ul of target cells at a density of 2500/well in flat bottomed 96 well plates (Falcon). The assay is incubated for 24 hours and pulse labelled for another six hours with 0.2uCi tritiated thymidine (5 Ci/mmole, Amersham Co., Oakville Ont.). Cells are collected onto glass filter disks using an automated cell harvester (Cambridge Technology, Cambridge, Mass.) and the incorporated radioactivity is assessed by liquid scintillation counting.

2. K562 and U937 increased sensitivity assay:

Samples and plates are handled as in the standard assay, except that the assay is incubated 48 hours rather than the standard twenty four. This technique takes a day longer, but can increase assay sensitivity up to four fold.

3. A375 proliferation assay[22]:

Performed as in the standard assay above except that serial doubling dilutions are made in 100ul volumes and applied to 100ul of 3000 target cells per well, the assay is incubated four days, and then pulse labelled with thymidine as above for 24 hours prior to harvesting. This assay is performed to determine if cells known to be responsive to the effects of TGFb and Oncostatin M are affected by the UGIF.

4. Peripheral Blood Lymphocyte (PBL) proliferation assay:

PHA-PBL: Samples are serially diluted in 100ul volumes of assay media supplemented with either PHA at 1/1000, or PWM at 1/100 dilution. PBL's are suspended in assay media containing mitogen and aliquoted in 100ul volumes at a density of 10,000 cells/well. PHA-PBL assays are incubated three days prior to harvesting the cells. PW-PBL assays are incubated five days prior to harvesting. In both cases 0.2 uCi tritiated thymidine is added for the final 24h of culture.

5. Calculation of units of activity;

The units of UGIF activity are calculated as follows;

Percent Inhibition =

[1- (average cpm of Sn treated cells/ untreated control cpm)]x 100

Units of Activity =1/highest dilution showing 50% Inhibition

Unless otherwise stated, the number of units of activity in a sample are based on the use of K562 cells as the targets.

F. Concentration of UGIF:

UGIF containing supernatants are first filtered (.45u) to remove any cell debris. The cell-free supernatant is then pumped through a tangential flow concentrator (Minitan system, Millipore) using membrane stacks with a 10000MW cut off. This provides a typical concentration of 10 fold. Both retentate and ultrafiltrate are assayed for UGIF to ensure integrity of the membranes. After tangential flow concentration the concentrated

supernatant is extensively dialyzed against water, frozen in lyophilization flasks and then lyophilized (Labconco lyophilizer and flasks). Dialysate and retentate are both assayed for the inhibitory activity. Lyophilized material is resuspended in double distilled water or column buffer and used for further analysis.

G. Protein Assay:

Estimates of the concentration of protein present in samples are required to be able to avoid overloading columns and gels used in the purification procedure. The bulk of the protein determinations required for this work are performed on supernatant concentrates containing Lactalbumin hydrolysate (LH). As LH concentrates were found not to bind stain by the methods of Lowry, or Biorad protein assays, the method of assay used is to read the absorbances of unstained solutions directly at 280nm. Standard curves consist of serially diluted LH in pure water, read at 280nm on a spectrophotometer (Gilson). Similarly, samples are diluted out in water and the absorbances are related to protein concentration directly from the standard curve. LH is used in place of BSA for four main reasons; 1) LH is inexpensive, 2) LH is dialysable, 3)LH is small, 4) LH supports U937 cells during UGIF production.

H. Purification of UGIF:

Size; UGIF concentrates containing more than 2500 units of activity/ml (>10 mg) are loaded in a volume of 1.5ml on a 1.5 x 250 cm ACA-44 (LKB) column and run in 25mM Hepes buffer, pH 7.2. One millilitre fractions are collected using an LKB Multirack

automated fraction collector, and the biological activity is assayed on U937, K562, A375, and L929 cells. Absorbance at 275 nm was monitored in line, genertaing a protein profile for each sample run on the column. Fractions were assayed on U937, K562, A375, and L929 cell lines. Active fractions from sepharose ACA44 separation are pooled, dialyzed, lyophilized, reconstituted, and loaded on an HPLC column for further analysis.

Ion Exchange; The analytical TSK DEAE-5PW column (6ml gel volume LKB Ultropac) is also run in 25mM Hepes buffer, at pH 8.0, and developed with a linear gradient of 0-1M NaCl in Hepes buffer, pH8.0. The gradient was generated by an LKB 2150 HPLC pump controlled by an LKB 2152 HPLC controller. Prior to loading sample test runs of the column are run to verify that the actual salt gradient of collected fractions match the recorded gradient (recorded on a LKB 2210 2 channel recorder) for each fraction. This meant that the recorder tracing was precalibrated to reflect the actual fractions collected with respect to the salt gradient. One ml samples are filtered (.22u), and loaded in a 2ml stainless steel sample loop, then injected into the column at t=0, a flow rate of .5ml/min is typically used in these experiments, and the absorbance range is 0-.5 O.D (monitored on an LKB 2138 Uvicord S). Unless otherwise stated the gradient is run from zero to one molar NaCl in a thirty minute interval. Fractions diluted and assayed directly on the K562 readout line immediately after the fractionation.

For larger volumes, UGIF containing concentrates are loaded on a MEMSEP 1010 DEAE cartridge (Phenomenex Inc.). Identical

buffers are used for this column as for the TSK DEAE column. However, the flow rate of 3ml/min is employed and the recorder was set for 0-1.0 O.D.@275 nm. The MEMSEP is loaded using a series of four to six 1ml (up to 30mg per injection) sample injections prior to initiating the salt gradient(0-.5M Nacl in 45 min, followed by a 15min. strip of the column with 1M NaCl.). The latter allows loading ACA-44 purified material without an intermediate concentration step. Using this method typical amounts of activity loaded ranged from 500 to 1500 units of UGIF activity per run, however an approximate loss of 60-80% of applied activity usually occurs after running crude concentrates through the column. Fractions eluted from the column are assayed immediately to provide an activity profile for the column. In fractionations where the running buffer could be shown to have inhibitory activity on the target cells, extensive dialysis against RPMI was carried out to remove the buffer effects.

SW-3000 Molecular Weight Sizing:

This column voids molecules with molecular weights greater than 300Kda. The LKB TSK G-SW3000 (600mm column length) is run using 25mM HEPES/.2M NaCl pH7.2 as running buffer. UGIF sequentially purified from ACA-44, and phenyl column is further purified using an analytical gel filtration column. Due to the scarcity of such purified UGIF and the limited information that the procedure yeilds, this experiment is performed only twice. Samples are loaded in 250ul volumes =.09 mg=75 units of UGIF), fractions are collected in .5ml volumes. The column is run twice and corresponding fractions from both runs are pooled,

concentrated 100x via speedvac, and assayed on K562 targets. 40% of applied activity can be recovered from this column. This procedure is used to help determine the molecular weight of UGIF, not to act as an additonal purification step.

Hydrophobicity; MEMSEP retained active UGIF peaks (from 300-1000units/mg specific activity) are separated on a Vydac C_{18} (1.5mm x 25 cm analytical) column by loading .5ml of a .22u filter sterilized (Millipore) sample in 20mM HEPES at pH 8.5 and eluting at .5ml/min, with a linear 30 minute gradient of 0-100% n-propanol in 20mM HEPES. Due to losses in earlier steps in the purification procedure, a maximum of three hundred units of DEAE purified activity was applied to the C_{18} column in these studies. Fractions collected from the C_{18} column are lyophilized to remove the propanol prior to assay, then resuspended in double distilled water, or if a large amount of activity is applied initially to the column, the fractions can be assayed directly. (The collected fractions are not typically filter sterilized at this point as this can lead to a partial loss of recovered activity. Antibiotics are included in the assay system to prevent microbial growth.) Controls for buffer effects on the assay system are examined by testing serial two fold dilutions of 100% n-propanol on the K562 target cells.

I. Physico chemical characterizations of UGIF.

1. Heat treatments:

Samples (0.1ml) in RPMI are placed in microfuge tubes (Eppendorf) and immersed in a water bath at 100'C for varying times. Buffer alone is also incubated as a control for

possible buffer effects. Unheated sample is used as a control for the heated samples. In all heat treatments samples are cooled to room temperature and assayed immediately on U937 or K562 cells.

2. pH stability:

Samples (0.1ml) in RPMI are either acidified or made basic by the addition of HCL or NaOH, incubated at room temp one hour, then neutralized and assayed. In some experiments acidification is done by direct addition of acid, followed by a one hour incubation and extensive dialysis to remove residual acid. In other experiments the acidification is performed by dialysis against acid, followed by extensive dialysis against RPMI to reestablish neutral pH. Where dialysis is employed, both dialysate and retentates are assayed for activity to control for dialysis bag integrity. As a control for residual acid, base, or salt effects, RPMI is subjected to the same conditions as the sample and assayed for any effects on the proliferation of the targets.

3. Protease stability:

Proteinase K (1mg/ml; BRL) was incubated with either C_{18} purified sample (80u/ml) or BSA (5mg/ml) for 24hr at 37°C then assayed, (the enzyme was shown to be active by the digestion of BSA, seen on SDS PAGE.) Controls for effects of enzyme alone on target cells were done simultaneously. The enzyme was found not to affect target cells at dilutions which would contain significant amounts of antiproliferative activity associated with UGIF samples.

4. SDS treatment:

Stock SDS (10%) was diluted 1/100 directly into C_{18} purified UGIF, and incubated four hours at 25°C, then assayed directly on target cells, SDS alone did not affect target cells proliferation at .005%. However, higher concentrations of SDS did have toxic effects on the targets, and this limits the assay range to relativelly high dilutions, so that the interfering SDS effects can be diluted out.

J. Cytokine Assays:

1. Tumor Necrosis Factor/ Lymphotoxin Assay:

This cytotoxicity assay is performed by the method of Granger et.al.[71] L929 target cells are harvested by trypsinization of stock cultures, and plated in 100ul aliquots in micrtiter wells containing 10%FCS at a density of 3 x 10^4 cells/well, incubated overnight (at 37°C in an atmosphere of 5% CO_2 incubator) prior to addition of samples. The next day doubling dilutions of sample are made gently in the wells containing the monolayers. Then 20ul of actinomycin D (5ug/ml; Sigma) is added per well, incubated overnight and stained for 15 min. with a .5% crystal violet methanol:water (1:4 v/v) solution, washed extensively (by submerging entire plate in a basin of cold water and shaking vigorously) and dried prior to reading. TNF positive wells are scored for lysis of the monolayer visually. TNF reference stock (RPMI1788 Sn) are assayed on the same plate as a positive

control. The endpoint is the highest dilution showing 50% lysis of the L929 monolayer.

2. Interleukin-1 Assay:

The IL-1 assay is done by the method of Conlon.[72] This assay is performed on UGIF concentrates using the LBRM conversion assay[73]. Briefly, the assay is a coculture of LBRM cells which respond to IL-1 by producing IL-2 (LBRM's), with IL-2 dependant CTLL-2 cells, the growth of the CTLL-2's is proportional to the amount of IL-2 released by the LBRM's in response to IL-1. Proliferation of the CTLL-2 cells is measured by the incorporation of tritiated thymidine, the cells are harvested onto glass disks, and then the incorporated isotope is counted on a liquid scintillation counting machine. The endpoint is the highest dilution showing 50% maximal stimulation of growth observed with controls grown in IL-2 containing media. This assay was performed by Patty Sauder of the Rheumatic Diseases Research Laboratory at The University of Manitoba.

3. Interferon Assay:

The interferon assays were performed by Loretta Sukhu of the Rheumatic Disease Research Laboratory according to the method of McManus[74] Briefly, this activity is assayed for by the ability of a sample to protect a monolayer of A549 (Human bronchial carcinoma cell line; ATCC) cells from the cytopathic effect of Encephalo myocarditis virus. Protection is scored by

staining the A549 monolayer with crystal violet, and observing protection as intact monolayers, or unprotected as clearing of the monolayer. The endpoint of the assay is the reciprocal of the dilution producing 50% protection of the A549 monolayer from viral mediated lysis. Standardized recombinant gamma interferon is used as a positive control and activity reference in each assay.

K. Purified or Recombinant Cytokine Effects.

The effects of purified or recombinant cytokines are examined on the U937 and K562 cells used typically as the readout lines for UGIF. This is done so that any possible interfering effects of other well characterized activities on the targets can be anticipated. rTNF and rLT used in these studies were donated by Dr. B. Aggarwahl of Genentech corp. Purified TGFb1 and TGFb2 were supplied as a gift from Dr. L. Ellingsworth of Collagen Corp. Serial two fold dilutions of 1ug/ml stock solutions of each of TGFb1 and b2 are examined in all proliferation assays described above. To control for buffer effects possible with the TGFb preparations, 0.1% BSA in Tris-HCl buffer is examined for effects on the targets. As a positive control the TGFb preparations examined on UGIF targets are simultaneously assayed on A375 cells known to be growth inhibited by TGFb. Preparations not affecting A375 cells are considered inactive, and the experiments are repeated with fresh material.

Serial two fold dilutions of each of rTNF, and rLT are examined at various starting concentrations ranging from 1.5 million

units/ml to 500 units/ml, on K562 and U937 targets as well as L929 cells to verify the level of applied activity.

L. Neutralization Experiments:

1. TNF and TGFb Neutralizations:

Neutralizing antibodies against both rTNF and rLT, were provided by Dr. B. Aggarwal (Genentech corp.). Neutralizing antibodies against purified transforming growth factors beta one and two were donated by Dr. L. Ellingsworth @ Collagen corp, and used for these studies. By examining the effects of purified cytokines (for example TNF, TGFb1, or IFN) at different doses on the target cells, an approximation of the maximum amount of cytokine present in a UGIF preparation (assuming that a particular cytokine was solely responsible for the inhibition seen) can be made.

A large excess of specific antibody (100 fold or greater excess in most cases) was incubated with the UGIF (partially purified by chromatography on ACA-44) at room temperature (22°) for two hours, the samples were then serially diluted and assayed simultaneously for inhibitory activity on each of the target cell lines L929, K562, A375, and U937. In all cases positive controls for the action of recombinant cytokines on the appropriate targets as described earlier were included, as were controls for the effects of antibodies alone in assay buffer.

The activities of the antibodies were confirmed in each assay by adding an excess of antibody to a known amount of purified cytokine and assaying for residual biological activity.

3. Neutralization of C18 Purified UGIF:

These experiments are carried out on C18 purified UGIF preparations (80 units/ml). Antibodies against TGFb1, TNF, or a combination of antibodies against TGFb1 and TNF are used in an attempt to neutralize the UGIF activity. Six thousand neutralizing units of anti-TNF are used either alone or in combination with anti-TGFb1 (100ng neutralizing quantity), which is also used alone. The C_{18} purified material alone or incubated with antibody(ies) is assayed on each of A375, U937, K562, and on PHA-PBL, as are positive controls for these cytokines. Assays for TNF are also performed simultaneously on this material. Further neutralization studies are conducted using 1000 neutralizing units of anti-Interleukin six antibodies, these also fail to neutralize the UGIF activity.

Results:

(I) Assay and Production:

(A) Typical Dose Response:

Stimulation of the U937 cell line with 20ng/ml PMA results in the production of unique factor(s) with antiproliferative activity (UGIF). Crude concentrates of the material (normally 300-600 fold) inhibit the proliferation of tumor cells in a dose dependent manner (Figure 1.) and effects can be seen in dilutions as high as 2000 fold. Exposure of U937 and K562 cell lines to >8 units of UGIF results in 95-99% inhibition of proliferation within 24 hours of exposure.

(B) Production:

(i) Problem of Low Levels and Feedback:

Production of UGIF appears to be autoregulatory, exhibiting classical signs of feedback inhibition of production. Unstimulated cells in production media produce no activity in a 72hr time frame, whereas stimulated cells do, showing that the cells must be stimulated to produce the antiproliferative activity. The actual levels of activity produced can range from undetectable to 16

units per ml of culture supernatant. Since initial experiments in which there was often less than 2 units of growth inhibitory activity per ml of Supernatant (Sn), the assay has now developed to the point that most supernatants contain 8-16 units of UGIF activity per ml. A method of producing UGIF activity in serum free media is used exclusively in the production of UGIF. As high levels of activity in the starting material are desirable for the purification of a cytokine, we examined ways of potentially increasing the output of the cells.

(ii) Kinetics:

Kinetics experiments demonstrate that feedback inhibition of UGIF production does occur (see Fig.2). Kinetics of production show that the total amount of activity producable by a given number of cells can be increased by removing an inhibitory activity which builds up in the supernatants and shuts down production. The kinetics of production of UGIF demonstrates that the feedback is observable by eight hours post-stimulation. Levels of UGIF activity do not increase after this time. Exchanges of Sn with fresh media allow a greater total level of activity to be produced by a culture than is attainable with a simple accumulation of activity over the same time. By removing the UGIF containing supernatant at 4 hour intervals and replacing it with fresh media, the total amount of activity produced by given set of cells can be significantly increased. Although a peak of activity is apparent at 8hrs in the figure, supernatants should be harvested at 4hr intervals to maintain the most rapid production of UGIF activity. While this method can result in increased

production of UGIF, to make full use of it's potential is very labor intensive. Alternative methods of increasing production would be a more desirable strategy.

(iii) Search for UGIF Producing Clones:

Clones of U937 were examined for spontaneous, and high level UGIF producers. Limiting Dilution Analysis was used to generate over 2000 clones from the U937 ATCC stock cultures. Each clone was expanded, and the supernatant from each culture was assayed either directly for the presence of UGIF, or PMA stimulated and examined for UGIF production. There were no spontaneous or significantly high UGIF producers found by this technique. Stimulated clone supernatants were able to produce UGIF activity at a level of 2-8 units/ml. This is within the normal variation of UGIF production from parental cultures.

(iv) Development of Resistant Lines:

U937 cells resistant to the effects of UGIF have been developed in an attempt to break the apparent feedback shutdown of UGIF production. By exposing U937 ATCC cells to gradually increasing doses of UGIF in their growth media over a period of several months, highly resistant cells (> 8 fold more resistant than controls, by 50% end point) can be induced. It is also interesting to note that resistant cells have a lower maximal response than control cells even at the lowest dilution of UGIF concentrates. The resistant cells do not produce UGIF under normal stimulation conditions, where unselected cells do. However, UGIF resistant cells may provide a useful tool for

examining the biology of UGIF and it's relationship to other cytokines.

The UGIF resistant cells show an intriguing inverse correlation between the increase in resistance to UGIF, and an extreme increase in sensitivity to inhibitory effects of TGFb (fig.3 and fig.4), n=3.

Figure 3 indicates that selected cells are less sensitive to UGIF mediated growth inhibition than the parental cells, and that the resistant cells show an increase in their sensitivity to $TGFb_2$ mediated growth inhibition. Note that TGFb2 at 25ng/ml shows only a 40% inhibition of growth on parental U937 cells, but inhibits the growth of UGIF resistant cells by 75% at the same dose. The 100% inhibition level was determined independantly for the U937 parental cells and the resistant U937 cells. Although the increase in sensitivity to $TGFb_2$ inhibition seen in figure 3 is striking, figure 4 demonstrates that the increase in sensitivity to antiproliferative effects of selected cells also applies to $TGFb_1$.

Figure 4 demonstrates the sharp contrast between selected U937 and parental responses to $TGFb_1$. Although this figure does not show the downward flex of the curve for $TGFb_1$, other experiments have taken the dilutions further, and the curve does titer out, indicating that this is a true dose-response of highly sensitive cells.

Resistant U937 cells have clearly not become resistant to all forms of cytokine mediated growth inhibition. The data suggest that growth inhibition by TGFb and UGIF are mediated through different mechanisms. The resistant phenotype is not stable in the

population and is lost shortly after removing the selective pressure (UGIF).

(v) Attempt at Superinduction:

Attempts were made to superinduce the U937 cells using a cycloheximide and Actinomycin D protocol. Low level activity was produced by the superinduction, but the superinducing agents could not be easily removed from the supernatant. As a consequence the inducing agents were carried over into the UGIF assay and interferes with the assay on the targets. In particular, Actinomycin D was found to have far reaching inhibitory effects of it's own on target cells. The low levels of activity produced using this method, combined with the problem of carry over resulted in the termination of this method of inducing UGIF.

(vi) Inhibition is Not a Consequence of Residual PMA:

Observations that stimulating agents can be carried into the assay of cell supernatants made us prudent about the role PMA may have in UGIF activity. There is much evidence that PMA is not responsible for UGIF mediated growth inhibition. Firstly, the levels of PMA used for stimulation are quite low (20ng/ml). Then the producer cells are washed extensivelly (two times) prior to the incubation required for supernatant production. This procedure should greatly deplete the culture of residual PMA.

Experiments have been done where mock supernatants have been produced by leaving the PMA in the collected supernatant. PMA either with or without U937 cells in UGIF production media

is left 24 hrs in the culture dishes, then harvested without a wash, filtered , and assayed on UGIF targets. At t=24 hours the maximum amount of PMA present in a 1/2 dilution of stimulated supernatant should be 10ng/ml. However, these mock supernatants do not contain UGIF like activity. PMA is known to be an unstable molecule, and it is not surprising that there is no inhibitory activity due to PMA after a 24hr incubation at 37'C.

PMA suspended in UGIF production media is not as stable as UGIF. Data indicate that while fresh PMA can inhibit the proliferation of target cells, this activity becomes greatly reduced (8 fold reduction in titer) after heating for a short period of time (2min, 100°C). UGIF antiproliferative activity does not show a decrease in activity after the same heat exposure (Figure 4a.). PMA is known to be heat sensitive, highly unstable and easily oxidizable[75], it is also sensitive to acid and alkali.[76] UGIF is not destroyed by exposure to strong acid or base, and is insensitive to heat (table A, figure 4a). The stability data argue strongly against PMA being responsible for UGIF activity.

Another fact which argues against PMA involvement is the Kinetics of production data (figure 2.) One would expect the highest levels of residual PMA to be present at the earliest time in the culture period. However, the earliest time point is consistantly the lowest level of UGIF activity.

A further distinction between UGIF and PMA is their molecular weights. The molecular weight of PMA is approx. 365 daltons, which is inconsistent with UGIF's mol. wt. of > 300 Kda.

Further data supporting UGIF as being a U937 derived cytokine is that identical stimulation protocols on other cell types (SKW and JURKAT) do not result in UGIF production. If the phenomenon was solely due to residual PMA, one would expect it to occur for virtually any cell type, and it does not.

While most data indicate that UGIF is not due to PMA, the possibility cannot be ruled out that PMA may be acting in conjunction with some U937 derived molecules to give the inhibitory effects observed. The only way that PMA can be completely ruled out as having any role in UGIF is by examining either a cloned molecule, or a homogeneous preparation, which first requires purification of the activity.

(vii) Mycoplasma Detection:

U937 producer cells and target cell cultures have been assayed repeatedly for the presence of mycoplasma both by culture and by DNA detection, and have always been negative for the presence of such organisms. There is no suggestion that any of the data reported in this thesis is a consequence of mycoplasma contamination.

(C) Target Cell Range:

Crude UGIF was assayed on many different target cells to find the most sensitive target possible (table B). UGIF exerts inhibitory effects on a broad range of target cells. It inhibits the growth of all tumor cell lines examined to varying degrees. K562 and NS1 cells are the most sensitive lines that have been examined. While both

K562 and NS1 cells appear to be equally sensitive to UGIF, we chose to use K562 as our standard target cell because much of our data had already been generated using this line by the time NS1 came to our attention. A further argument for the use of K562 over NS1 as targets is that K562 is of human origin whereas NS1 is of Murine origin. This means that K562 is more likely to provide the more relevant model of a human tumor cell.

To contrast the effects of UGIF on tumor cells with non-cancerous cells we chose to examine it's effects on Peripheral Blood Lymphocytes as well as tumor cell lines. PHA-PBL are the least sensitive to the effects of UGIF, requiring almost 300 times higher doses to be affected than the tumor cell targets (table B).

(D) Assay Development:

The assay for UGIF has evolved considerably as a consequence of the search for the most sensitive assay available for this activity. The original assay for UGIF was a 48 hour assay using 1×10^5 SKW or Jurkat cells/well. Several experiments were required before the currently used protocol was developed. Various assay times were evaluated, as were the numbers and types of cells in each assay. A forty eight hour assay using 2.5×10^3 K562 (erythrocytic leukemia) cells/well has been determined to be the most sensitive means of detecting UGIF activity in tritium uptake experiments.

(II) Purification:

(A) Starting Material:

The current level of UGIF activity in crude supernatants (4-16u/ml) is acceptable for a starting material for purification. The decision to proceed with experiments designed to mass produce, purify and characterize UGIF was made when it became clear that there was no apparent method available to drastically increase production levels of UGIF.

Concentration procedures have been developed which allow a rapid concentration of Supernatant. UGIF is very stable and survives lyophilization with almost 100% recovery. Culture Sn of PMA treated U937 were concentrated by a combination of tangential flow concentration (>10 fold), followed by lyophilization is used to make most concentrates (final concentrates of 300-600 fold). Several hundred fold concentrates are used as starting material for subsequent purification steps.

(B) ACA-44 Purification:

Molecular seive chromatography on ACA-44 columns reveals that UGIF has an apparent molecular weight of greater than 135 kda (Void of column), n=5. A nondenaturing buffer (Hepes 25mM, .15M Nacl) is used to run the column. Individual fractions from each run of the column are assayed for effects on the proliferation of K562 and U937 cells. The typical total recovery of applied activity attained with ACA-44 purification is approximately 40%.

As U937 cells are known to produce TNF, fractions are also examined on L929 cells for cytotoxic effects. Fractionation of

crude UGIF concentrates indicates that TNF activity is present in starting materials, but less than ten percent of total TNF activity copurifies with the UGIF(see figure 5).

Figure 5 demonstates a complete set of experiments in which a crude UGIF concentrate is fractionated on an ACA-44 column, and the fractions are assayed for UGIF and TNF activity. This particular fractionation was done as follows; 500ul of crude UGIF was loaded (1900 units), and developed with 25mM Hepes, .15M NaCl pH 7.2, 2ml fractions were collected, and absorbance was monitored @275nm with an absorbance range of 0-1.0 O.D. This figure shows that the bulk of the TNF activity (L929 activity) elutes later (fraction 50) than the bulk of the UGIF activity (void volume, fraction 37). This means that UGIF can be seperated from most contaminating TNF on the basis of size. Most importantly, this figure strongly indicates that UGIF is not a mere consequence of TNF activity.

However, as some TNF is seen in the ACA-44 void, we examined the effects of adding neutralizing antibody to the active fraction. The anti-TNF antibody was able to inhibit the TNF activity in the void fraction, but resulted in only a slight reduction of UGIF activity on U937 cells. This is compatible with the idea that U937 cells are sensitive to the effects of Tumor Necrosis Factor. The Neutralizing antibody did not affect the UGIF effects on K562 cells, suggesting that these cells are not sensitive to TNF (table C, figures 6 and 7)

Table C presents the actual data graphed in figures 6 and 7. Figure 6 demonstrates the sensitivity of U937 targets to TNF

inhibition, but also shows that there is more than just TNF activity inhibiting the growth of these cells. When all the TNF activity in fraction 37 is neutralized there is a drop of 30% in magnitude of inhibition of the first dilution on U937 cells. The data in table C show that this is a significant effect. Figure 7 shows the effects of neutralizing the TNF present in fraction 37, and then assaying this on K562 cells. There is no decrease in the level of inhibitory activity seen by this cell line on addition of antibody. However, when anti-TNF is added there is an apparent increase in inhibition at the low end of the curve for K562. The reason for this apparent increase is unknown at this time. The most important result these three figures demonstrate is that neutralization of TNF in purified materials does not neutralize UGIF activity (as defined earlier using K562 cells for the definition of units of activity.)

Active UGIF containing fractions purified by ACA-44 are assayed in the A549 interferon assay for antiviral activity and do not demonstrate any IFN activity associated with them. This is compatible with results using crude supernatant concentrates where IFN activity is never observed.

When ACA 44 voided UGIF is examined on an (.5mmx 600mm TSK-SW 3000) analytical molecular weight sizing HPLC column, the activity has an apparent molecular weight of >300kda (fig.8.) This means that the UGIF activity is too large to be resloved by the column. The maximum amount of protein suggested for loading this column is 1mg, we load the column at levels very close to this (.09 mg=75units.). 40% of the total activity applied to this column is recovered in the void volume.

The running conditions for this fractionation are as follows; 250ul (75units) of sample is loaded into a 1 ml injection loop, .5ml fractions are collected and the absorbance is monitored in line @275nm with a range o 0-.1 O.D. The buffer used to develop this column is Hepes (25mM) .2 M NaCL pH 7.2 at a flow rate of 1ml/min. In this experiment a total of 60 units of activity was recovered from the 150 units applied (2 runs of column). The starting material for this column has already been through ACA-44, and ion exchange purification steps to reduce the amount of protein in the sample.

The recovered activity is examined only for UGIF activity because of low amounts of material recovered. The active fractions are not monitored for other cytokines due to the limited amount of SW3000 purified material. The SW3000 approach was not pursued because of the low capacity of the column and the yields attainable with it. The important result obtained using this column is that it demonstrates that the apparent molecular weight of UGIF is greater than 300 kDal.

(C) Ion Exchange Chromatography:

The starting materials contain low levels of activity, therefore large volumes of supernatant are required to obtain sufficient activity for fractionation. This means that there is a large amount of protein present in the concentrates. As sizing columns lack the capacity needed to fractionate such concentrates, ion exchange columns were used.

The first attempt to use ion exchange chromatography employed a CM-TSK column. This cation exchange column was run using Hepes (.2M), pH8.0 and used a gradient of 0-1M NaCl in 10 min. for development. 1.5ml of ACA-44 purified sample containing 3000 units of activity (6.75 mg by Biorad protein assay) was applied to this column. All the recoverable activity from this column passed through prior to the initiation of the gradient, 1600 units were recovered. Most of the other U.V. absorbing protein also passed through the column and was collected along with the UGIF activity in the void volume. As this column gave very poor resolution, and resulted in a near 50% loss of activity, the CM column chromatography technique was not pursued. As UGIF did not bind to a cation exchange column, we suspected that it might bind to an oppositely charged matrix (anion exchange).

High Pressure Liquid Chromatography analysis of UGIF on TSK-DEAE-5PW columns indicates that the activity is retained in a narrow range of salt concentration (.29-.40M NaCl)(see fig.9,10,11).

Figure 9 shows the results of DEAE-5PW purification of post ACA-44 and CM-5PW sequentially purified UGIF. The conditions for this fractionation are as follows; 1600 units of UGIF activity were injected in a 2ml volume, the running buffer was 20mM Tris pH8.0, the gradient was run from 0 to 1M Nacl in 10 minutes at a flow rate of 1ml/min, 1ml fractions were collected. Absorbance was monitored @275nm in line with a range of 0-.5 O.D. Activity was recovered (192 units of 1600 applied) in the salt range of

.31-.39 M NaCl. This range accounts for a relatively small amount of the protein seen in the absorbance curve. However, the recovery was very low, 12% of applied activity. It is important to note that this experiment provides the first evidence that UGIF can bind to ion exchange columns.

Figure 10 illustrates the effect of fractionating crude UGIF concentrates directly on the analytical DEAE column. This column is run as follows; .5ml of crude UGIF concentrate (128 units) is run in Tris (20mM, pH 8.0), a 32 minute gradient of 0-1 M NaCl is run at a rate of 1ml/min, and 1 ml fractions are collected. Absorbance is monitored in line @ 275nm, with a range of 0-.5 O.D. Several interesting points are made clear by this figure. The figure demonstrates that while UGIF does not bind to the column when large amounts of crude material is applied, the total protein associated with the activity is greatly reduced. There is no UGIF activity detectable in any other fraction than the void. This result seems to be contradictory to the results seen in figure 9. However, when the activity in the void is run a second time on the DEAE column, binding does occur (figure 11).

Figure 11 demonstrates the results of running DEAE voided UGIF a second time on the same column (after the previously bound proteins have been stripped from it). The way this column is run is as follows; 1 ml of DEAE peak activity (560 units) (pooled from several runs) is loaded, and run in 25mM Hepes pH 8.0, using the same conditions as above. The figure shows that UGIF activity can be found both in the void, and as a bound peak eluting at .4-.5 M NaCl. This corresponds to fractions 24-27, and

contains the majority of the recovered activity. Of 560 units of activity applied 120 were recovered. Only 21% of applied activity is recovered, with >90% of it in the retained peak. The retained activity is followed through subsequent purifications on C18 columns. Voided activity contains some TNF, whereas the retained peak does not, thus the voided activity is not followed any further. It is not surprising to find TNF in the void as this material has not been purified prior to applying it to the DEAE column, and we know from previous experiments that TNF is present in crude concentrates.

Individual fractions are assayed on K562 and U937 cell lines to monitor the elution of UGIF. DEAE chromatography allows a fifty fold purification of the UGIF (12u/mg starting material to 600u/mg post DEAE material). However, there is a typical loss of 80% of applied activity every time DEAE columns are employed. (Actual losses can be much higher if Tris buffers are used as the column buffer. The most successful conditions for recovering activity is to use a HEPES buffer.)

It is interesting to note the apparent overload problem which occurs with DEAE analytical columns. If impure concentrates are loaded directly on the column, all detectable UGIF activity elutes in the void volume. However, if the voided activity is rechromatographed on the same column, the activity will bind and elute in the same salt range as is observed for partially purified material. The apparent overload was verified as an actual overload when an experiment showed that the concentration of Lactalbumin Hydrolysate could not be accurately estimated in the

Biorad protein assay. This meant that we were making underestimates of the amount of protein being applied to the columns. The analytical DEAE column we were using was rated for a maximum capacity of 10mg of protein per run, we were using at least 30 mg/run, overloading the column by at least 300%. The solution to the overload problem came from using higher capacity DEAE columns.

A major advance in the purification is the use of the MEMSEP1010 anion exchange column. This column has a 200mg capacity, it provides a useful, rapid first step in the purification protocol(see fig.12).

Figure 12 shows a typical MEMSEP fractionation of a crude UGIF concentrate. The fractionation is performed as follows; 2.75 ml (10,000 units, 40mg) of crude concentrate is loaded in 20mM Hepes with .15 M NaCl, and run at a flow rate of 3ml/min. A 42 minute gradient of .15-.50 M NaCl is run to develop the column, followed by a strip of 0-1M NaCl to clean the column. A minimum of 1080 units of activity are recovered, 720 units in the retained peak, and 360 units in the voided peak of activity. This gives a minimum recovery of 11% of applied activity, but may actually be more because of the dilutions (1/20) used to screen the fractions in the assay. Similar to the other DEAE fractionations, TNF is found only in the void of this column, and the retained peak is free of TNF activity. The retained peak of activity is used for subsequent purifications using the C18 column. The important point that this figure illustrates is that UGIF can be loaded directly onto a large

scale ion exchange column as a first step in purification, and a significant amount of activity can be recovered.

Crude concentrates can be loaded directly on the cartridge, and the column can be carefully controlled by using an HPLC control system. Fractionation using the MEMSEP results in inhibitory activity being detected in the void and as a retained peak in the same salt range as is observed for analytical columns.

DEAE purified material containing 320u/ml of UGIF contain <2u/ml of L929 activity, arguing strongly against the involvement of TNF in UGIF mediated antiproliferative activity. However, while the retained UGIF is free of TNF, the void of the MEMSEP has been shown to contain considerable TNF activity (beyond the 2000 unit end point of the assay.). When voided and retained peaks are recombined in varying proportions and examined for potential synergistic interactions, there is no synergy observed. Due to the contamination of the voided material with TNF, only the retained peak of UGIF activity is followed in further purifications. Analysis of retained UGIF in the Interferon assay reveals that there is no antiviral activity present.

Retention on DEAE columns indicates the activity is either itself negatively charged, or is carried in a negatively charged aggregate at pH8.0. CM columns (cation exchange) do not retain the activity at all.

(D) Reversed Phase Chromatography:

A further step in the purification of UGIF is the application of DEAE isolated material to a reversed phase HPLC column. A

strongly hydrophobic C18 column is used in this work as it gives good resolution and does not destroy UGIF activity. Good recovery (approx 50%) of applied activity can be attained using reversed phase. Only the DEAE retained UGIF peak is followed because this peak is known to be free of TNF activity.

A single peak of inhibitory activity can be isolated from a Vydac C_{18} column. Repeated runs of the column indicate that UGIF elutes between 50-60% 1-n-propanol in a gradient of 0-100% (Fig.13, 14.)

Figure 13 represents a fractionation of DEAE purfied UGIF on a C18 column. The fractionation was done as follows; 1.8ml of post-Memsep concentrated pool (1000 units). was run in 25mM Hepes buffer pH8.5, a flow rate of 200ul/min was used and 1 ml fractions were collected. An 80 minute gradient of 0-100% 1-propanol was used to elute the bound material. Absorbance was not monitored during this run of the column due to a recorder malfunction. A single peak of inhibitory activity elutes from the column in fractions 22 and 23 (approx. 60% propanol).

Figure 14 shows a second run of UGIF on the C18 column. The way this run was performed is as follows; .5ml of C18 purified UGIF (50units) is run in 25mM Hepes pH 8.5 at a flow rate of .2ml/min, 1ml fractions are collected. A 155 min. gradient of 1-propanol (0-90%) is used to elute the bound material. The absorbance @275 nm is monitored in line over the range of 0-.02 O.D. A single bound peak of U.V. absorbing material elutes from the column at approx. 50% propanol. A single peak of UGIF activity also elutes from the column. The eluted peak of activity

corresponds exactly with the peak of UV absorbance. This suggests strongly that there is at least some protein associated with the purified UGIF. When assayed in the Biorad protein assay the isolated UGIF activity peak fell below the limits of sensitivity of the assay.

Assays of C18 purified UGIF indicate that there is no TNF, or antiviral activity associated with it. Furthermore, Neutralizing antibodies against TGFb do not affect the titer of the fractions, indicating that the purified UGIF is not a consequence of this cytokine.

(E) Purity Analysis:

Analysis of 6.4 units of C18 purified UGIF on silver stained SDS-PAGE gels fails to produce any bands. This leads to an estimation that the specific activity of UGIF is greater than 500,000 units per mg of protein (almost a 55,000 fold purification over the raw material.)

The purification procedure has taken a starting activity of 12units/mg protein in crude raw supernatants to a level of 300-600 units/mg in DEAE purified material to >640000units/mg in the most highly purified samples (sequential DEAE and C_{18} purification). This specific activity is estimated as the amount of protein present after C_{18} purification was undetectable, and gave no bands on silver stained[77] SDS-PAGE (7% gel by the method of Laemmli[78]). When 6.4 units of purified activity was applied to the gel on two different occasions there were no detectable bands after silver staining. This means that 6.4 units of activity should

have less than 10ng protein associated with it if one accepts 10ng as approximately the limit of sensitivity of silver stained gels[79] (the actual sensitivity may be higher). If one assumes there is at most 10-100ng protein present, indicated by silver stain sensitivity, then the specific activity of the sample=6.4/10ng= .6-6x10^5 units/mg. This is at least a 53300 fold purification.

(III) Characterization of Partially Purified UGIF:

(A) Physico Chemical Characterization:

The inhibitory activity of crude UGIF is stable to heat and pH extremes, withstanding 100'C for 5 min. and surviving pH 2-12 treatment for 24 hr.

When UGIF activity is suspended in 0.1% SDS and serial two-fold dilutions are examined on targets, the inhibitory activity is left intact (Fig. 15.) Serial dilutions of 0.1% SDS suspended in RPMI are examined as controls for SDS effects on targets. While SDS alone can inhibit target proliferation (50% Inhibition at a 1/16 dilution), the UGIF inhibition can be diluted out past any effects attributable to SDS alone(SDS with UGIF gives 50% inhibition at 1/512 dilution.) The experiment was performed partially to see if UGIF could survive the SDS which would be present in preparative SDS-PAGE. As UGIF is not destroyed by SDS, it is reasonable to conclude that preperative electrophoresis may be a viable step in future purifications of UGIF.

Exposure of ACA44 purified UGIF to RNase(250 units), DNase(250 units), lipase(3750 units), and trypsin(dilute 50mg/ml

stock in saline to 6.25mg/ml final) show that only trypsin can reduce the titer of UGIF preparations (fig.16).

Figure 16 demonstrates the sensitivity of UGIF to several digestive enzymes. The experiment was done as follows; 32 units of UGIF activity were incubated overnight at 37°C with each of DNase, RNase, and Trypsin. At 24hr the digested samples, and controls for enzyme effects were assayed on K562 targets. Only the trypsin decreased the UGIF activity at all (loss of 1 dilution of activity).Trypsin digestion of crude concentrates can reduce the amount of UGIF activity by about 50% (n=3), higher concentrations of trypsin used for longer times do not increase this level of destruction.

UGIF purified sequentially from DEAE and C18 columns retains the observed physico-chemical properties observed for impure materials, stable to heat, pH extremes, and remains intact after protease digestion. (table A). Molecular weight sizing columns indicate the active material has a very high molecular wt. >300,000(figure 8).

(B) Antibody Neutralizations:

Effects of neutralizing antibodies on UGIF activity are examined to determine that the UGIF activity is not a consequence of one or more known cytokines. As a means of evaluating whether a specific cytokine might be responsible for the inhibition observed with UGIF, purified or recombinant cytokines are examined for their effects on UGIF target cells. When assays for known

cytokines are available, UGIF containing samples are evaluated for the presence of such cytokines.

Initially only crude UGIF concentrates were treated with anti-TGFb to see if the antiproliferative activity could be interfered with by this antibody. The antibody did not neutralize any of the UGIF activity in crude preparations. Our next step was to examine purified material for the presence of TGFb. In the experiments where TGFb1 and b2 were assayed for at Collagen Corp., the assays were performed on ACA44 purified UGIF. Their results indicate that there is no TGFb detectable in the samples. This is compatible our other data which demonstrates that anti-TGFb antibodies do not abrogate UGIF activity. Antibodies against TGFb1 or TGFb2 do not reduce the activity of UGIF, either in ACA44 purified, or in C18 purified forms.

Antibodies against Oncostatin M were unavailable commercially at the time of this work, and Oncogen would not supply them on our request. Therefore anti-Oncostatin experiments were not performed. However, Oncogen corp offered to assay our supernatants for their factor, and did so. A sample of the same ACA44 purified UGIF as was assayed for TGFb, was sent to Oncogen Corp to be tested for the presence of Oncostatin M. The results of the Oncostatin M assay were not given as numerical data, rather we were informed that the UGIF supernatants contained only "Negligable" amounts. As the reagents required to replicate the assay are unavailable we are unable to verify their results.

Neutralization of Interleukin-1 was carried out on crude UGIF preparations. Interleukin-1 was assayed for it's presence in ACA44 purified preparations and in crude supernatants. Limited availability of neutralizing anti-IL-1 antibodies meant that these were used only on ACA44 purified and crude UGIF preparations. Purified IL-1 was not available at the time of these experiments and was not examined on UGIF targets. IL-1 was not detected in UGIF preparations, and anti-IL-1 could not neutralize the antiproliferative activity of UGIF.

Some TNF activity in crude UGIF concentrates can be neutralized using anti-TNF alpha. Early experiments demonstrated that TNF could be inhibitory for U937 targets, so neutralizations were performed to rule out TNF as being responsible for UGIF activity. In crude U937 supernatants and in crude concentrates, TNF alpha is detected along with UGIF. However, the TNF activity can be completely abrogated by antibody while UGIF cannot. Anti-TNF alpha, and not beta can neutralize up to 50% of the inhibitory activity of crude supernatants seen by U937 targets. The antibodies do not abrogate the inhibitory activity seen by K562 cells. In C18 purified preparations containing 80 units of UGIF activity, there is no detectable TNF activity. Furthermore, anti-TNF antibodies (6000 Neutralizing units) do not reduce the titer of UGIF in C18 purified preparations.

Antibodies against gamma IFN have been shown to leave UGIF activity in ACA44 purified material unaffected. Gamma IFN has been assayed for in supernatants and concentrates at all levels of purity, and has never been detected. The effects of purified IFN

on UGIF target cells have not been examined due to limited availability of purified material.

The results of these studies are summarized in table D.

(C) Contrast With Purified Cytokines:

Experiments were performed to examine the response of UGIF target cells to several distinct growth regulatory cytokines. One experiment was to examine seven different cytokines individually on U937 and K562 target cells simultaneously. This allows a comparison of the effects of any of the seven cytokines with each other on each target cell line. It also allows a direct comparison of the effects of each cytokine between the two cell ines (i.e. IL-6 effects on U937 vs Il-6 effects on K562). The relationship of UGIF with respect to each of TGFb1, TGFb2, BCGF, rIL-2, rTNF, and rIL-6 are detailed below, and each section will refer to table E where these results are listed.

The relationship of UGIF to other cytokines shows that both TNF and TGFb1 can inhibit the growth of UGIF targets K562 and or U937 to an extent (see table E). However, the use of neutralizing antibodies against these cytokines fails to abrogate the UGIF activity associated with highly purified preparations (see table C, table F).

The UGIF exhibits a B-cell growth factor (BCGF), or IL-6-like activity on BCGF dependent cells. Neutralizations of UGIF using high levels of anti-IL-6(500 neutralizing units), fail to affect the UGIF activity on K562 (see figure 18) or U937 cells (figure19).

When commercial BCGF is examined on the UGIF targets there is no inhibitory activity associated with it (see table E). UGIF behaves very differently on target cells than does TNF alpha or beta, and it is not neutralizable by anti-TNF neutralizing antibodies (see Table E, fig.17.)

Figure 17 demonstrates the treatment of C_{18} purified UGIF with neutralizing antibodies against TNF, $TGFb_1$, and a combination of anti-TNF and anti-$TGFb_1$ antibodies. Sufficient anti-TGFb1 was used to neutralize up to 100ng of pure TGFb1. 6000 neutralizing units of anti-TNF was added either alone, or in combination with the anti-TGFb to 80 units/ml UGIF activity.
Early data demonstrates that some of the inhibitory activity was in fact due to the presence of TNF alpha, and could be neutralized partially by anti-TNF antibodies. Anti TNF and anti-LT are used on the crude and ACA-44 purified UGIF samples at a level of 20,000 neutralizing units/ml. These levels of antibody are in vast excess of the amount of detectable, or anticipated cytokine in the UGIF preparations. The major component of UGIF inhibition is unaffected by neutralizing anti-TNF antibodies.

UGIF does not affect target cells in the same way as TGF(b1 and b2)(see fig.3 and fig.4). Assays of UGIF concentrates by the manufacturers of TGFb(Collagen corp) indicate it is not present.

Neutralizing antibodies against TGFb fail to neutralize the effects of sequentially purified UGIF from DEAE and C18 columns (see table F, figure 17.) Table F demonstrates that anti-TGFb1 does not neutralize UGIF mediated antiproliferative activity. The most pure UGIF preparation (C18) available was examined and found to

be insensitive to the effects of the antibody. A further step in the experiment was to add anti-TNF neutralizing antibodies in combination with the anti TGFb. The combination of these two antibodies still failed to abrogate the UGIF inhibitory effects on K562 and U937 targets. Figure 17 demonstrates graphically that UGIF activity is not only left intact when exposed to anti-TGF, and anti-TNF, but it actually seems to increase in potency.

The apparent increase in UGIF activity (fig. 17.) when C18 purified material is treated with antibodies may be due to several factors. It may be that the apparent increase is actually due to the neutralization of small amounts of TNF or TGFb which may be present, and may be slightly growth stimulatory when present in undetectably small amounts. It is intriguing that the highest level of growth inhibition is observed when both anti TNF and anti TGFb are used.

A375 target cells are highly sensitive to the effects of TGFb and are used as the readout line for the presence of this factor in the neutralization experiments. However, A375 cells have been reported to also be sensitive to the inhibitory effects of TNF[48], and both factors must be considered with respect to potential effects on the cell line.

Anti-TGFb1 is used on UGIF samples at a level sufficient to neutralize 100ng of purified TGFb. Even at the highest levels of neutralizing antibody examined, the UGIF activity is not inhibited. Cells selected for resistance to UGIF do not become resistant to TGFb(see figure 4). The combination of anti-TNF and anti-TGF does not abrogate the inhibitory effects of UGIF.

UGIF Sn's have been repeatedly assayed for antiviral activity and do not contain any activity relating to the antiviral effects of the IFN family.

The LBRM assay for Interleukin-1 indicates that this is not present in UGIF-Sn's. Although macrophage and monocytic cells are known to produce IL-1, we did not observe this cytokine in UGIF supernatants. Both crude preparations and ACA44 purified materials were assayed and found to be negative for IL-1.

Assays done at Oncogen corp. indicate that the UGIF is not due to the presence of Oncostatin-M. UGIF also has an apparent mol.wt much greater than would be expected for Oncostatin(Onco-M@18-26k).

Assays for the presence of Macrophage Activating Factor were performed by Amgen corp. and the UGIF-Sn's did not contain detectable levels of this differentiation inducing factor in their system.

(D) Mode of Action:

The mode of action of UGIF appears to be through cytostasis rather than cytotoxicity. When assays of viability (trypan blue exclusion, data not shown) of target cells are performed in parallel with thymidine incorporation assays, it is clear that even in dilutions of UGIF having a 99.9% inhibition of growth, the cells are >90% viable.

UGIF effects on target cells are reversible up to a point, after which the inhibition appears to become irreversible. Cells exposed to cytostatic levels of UGIF for periods of time greater than two

weeks appear to lose the capacity to proliferate, but do not die from its effects. Other data emphasize the importance of a highly inhibitory dose to cause an irreversible inhibition.

Reversal of inhibition of tumor cell growth is possible only if the inhibitory dose of UGIF is 80% or less, and the cells are exposed to it for no longer than 2 weeks (at which time they are placed in normal growth media). Cytostasis becomes irreversible if cells are exposed to a higher initial dose, or maintained in UGIF containing media for periods longer than this. However, cells that recover from UGIF treatment do not gain a resistance to it's antiproliferative effects, indicating an actual recovery occurs rather than a mere selection for a resistant phenotype of cell that may reside at a low frequency in the population.

The ability of UGIF to induce the production of IgG from JR-2D3 cells (data not shown) indicates that UGIF can act by means of induction of differentiation. A further piece of evidence for UGIF acting as a differentiation inducing agent is the visual scoring of K562 target cells. K562 cells normally grow as fairly uniform cells in suspension, but when treated with UGIF, some flatten out tight against the culture bottom, while others become very elongate and look very much like macrophage cells. Similar differentiated phenotypes are observed for U937 cells (figure 23) exposed to UGIF.

(E) Cell Line Specificity:

Many cell lines were examined for their sensitivities to highly purified UGIF in an attempt to reevaluate the relative sensitivities of the targets to UGIF (See figures 21, 22, and 23.)

Figure 21 demonstrates the effects of DEAE purified UGIF on U937, K562, and on both PHA-PBL and PW-PBL. Note that there are differential effects on normal mitogen stimulated PBL and tumor cell lines. Both K562 and U937 cells are highly sensitive to the inhibitory effects of purified UGIF. PHA stimulated PBL proliferation is relativelly unaffected by the UGIF, while PW-PBL exhibit an apparent stimulation of growth at high UGIF concentrations. The effects of UGIF on normal PBL's titer out very quickly compared to the far reaching inhibitory effects on the tumor cell lines.

Figure 22 demonstrates the effects of C_{18} purified UGIF on several tumor cell lines. This figure shows that K562 cells are the most sensitive to UGIF effects. The pattern of sensitivities seen is similar to the sensitivity pattern observed for crude UGIF concentrates (table A).

Figure 23 shows the effects of the most highly purified UGIf preparation (C18) on tumor cell lines and normal mitogen stimulated PBL's. C18 purified UGIF has little effect on normal PHA-PBL, and still shows a stimulatory effect for PW-PBL at the highest concentration. The figure shows that even the most highly purified UGIF only affects normal mitogen stimulated PBL at doses very much higher than those required to observe effects on the K562 readout line.

The same relative sensitivities are seen for the most highly purified UGIF, as for the crude concentrates (K562 is most sensitive, PHA-PBL are least sensitive.) It is almost surprising that the target cell specificity does not change when UGIF is highly purified. During the course of purification many molecules could influence the growth of different cells in distinct ways, including TNF have been removed from the UGIF preparation. By retaining the same spectrum of target cells in the purified form as in crude preparations, the likelyhood that UGIF is due to a distinct entity is increased.

The antiproliferative activity UGIF appears to be unique from other known cytokines. Further in depth analysis of this material is required. Present results indicate that UGIF is a unique highly stable, high molecular weight, cytokine with antiproliferative activity on a wide range of target cells.

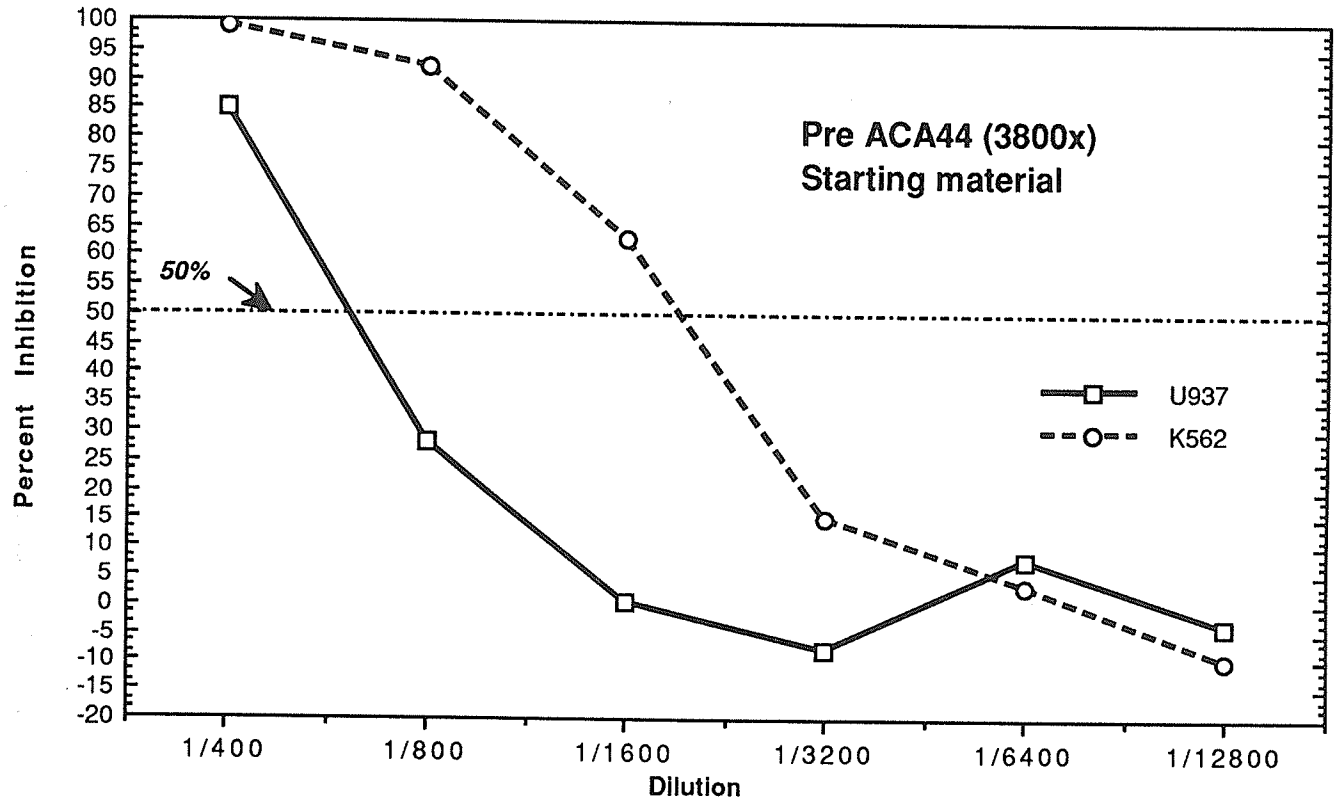


Figure 1.

Effects of a Crude UGIF Concentrate (600 fold) on the Two Readout Cell Lines U937 and K562. This is a typical dose-response curve for these cell lines when exposed to a UGIF concentrate. Note that K562 is more sensitive to inhibition than U937. Maximum inhibition of proliferation is very potent (98-99%), but quickly reaches the endpoint of the assay once the >90% inhibition level is departed from.

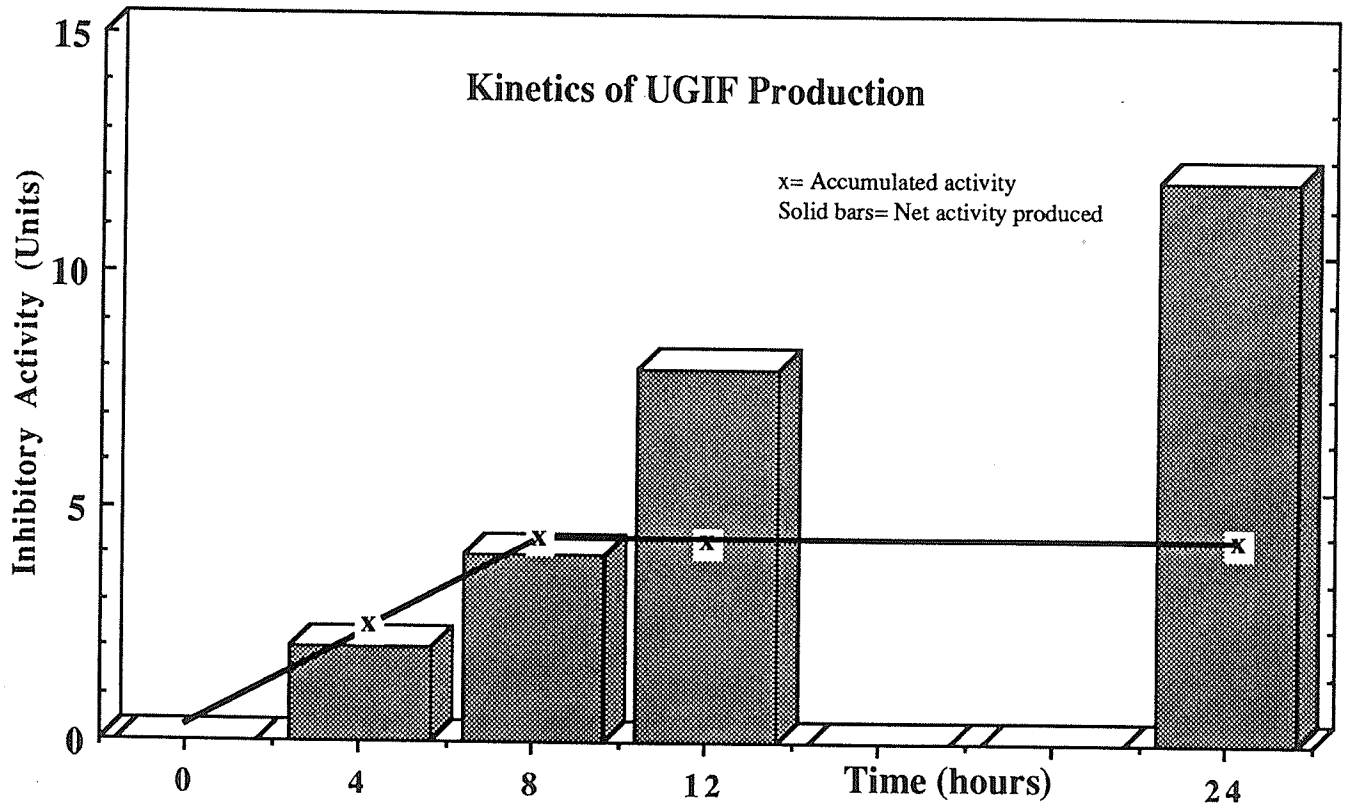


Figure 2.

Kinetics of UGIF Production.

Bar graph demonstrates the increased total activity attainable when Sn is replenished with fresh media.

Line graph demonstrates the feedback phenomena observed in cultures where media is not replenished.

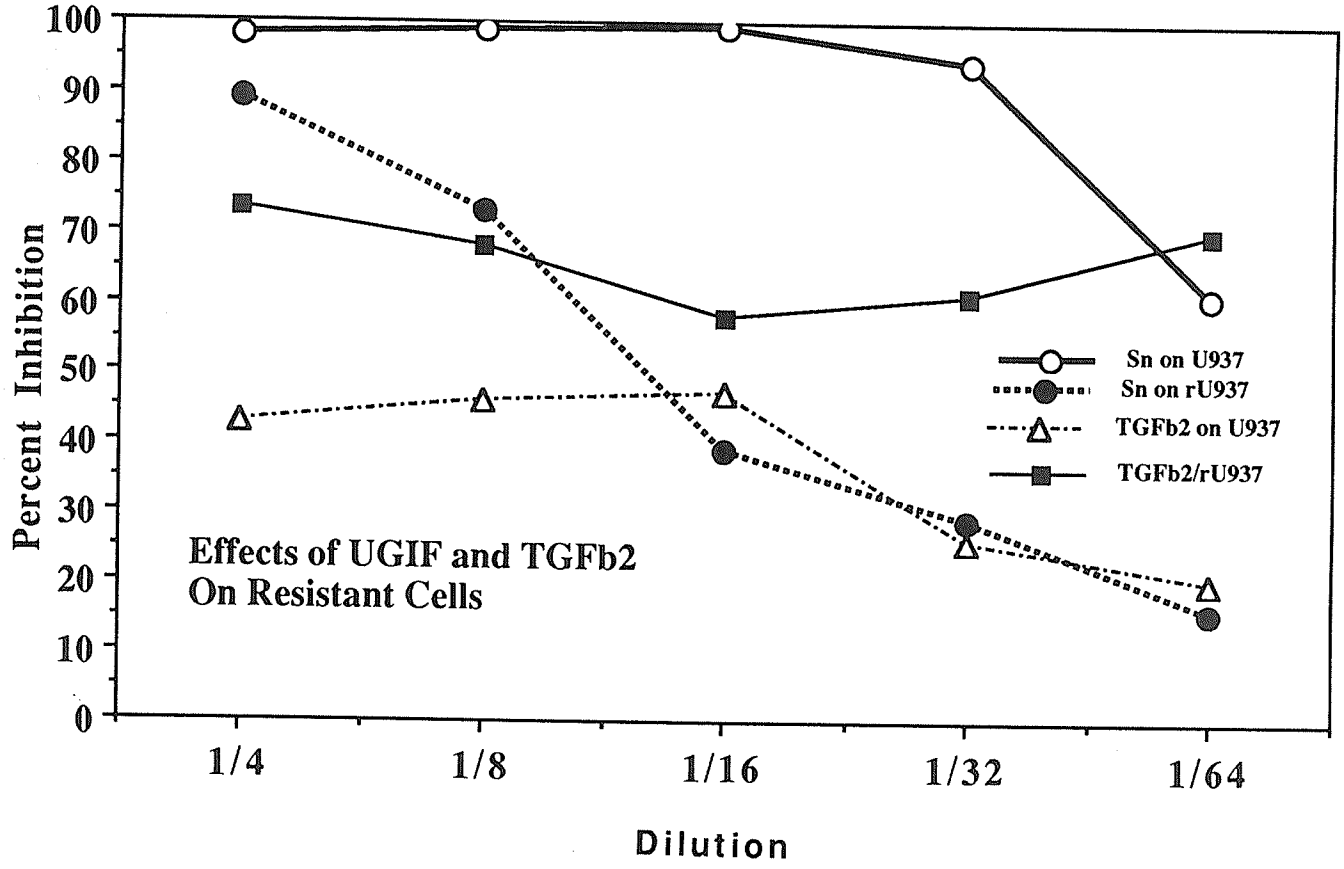
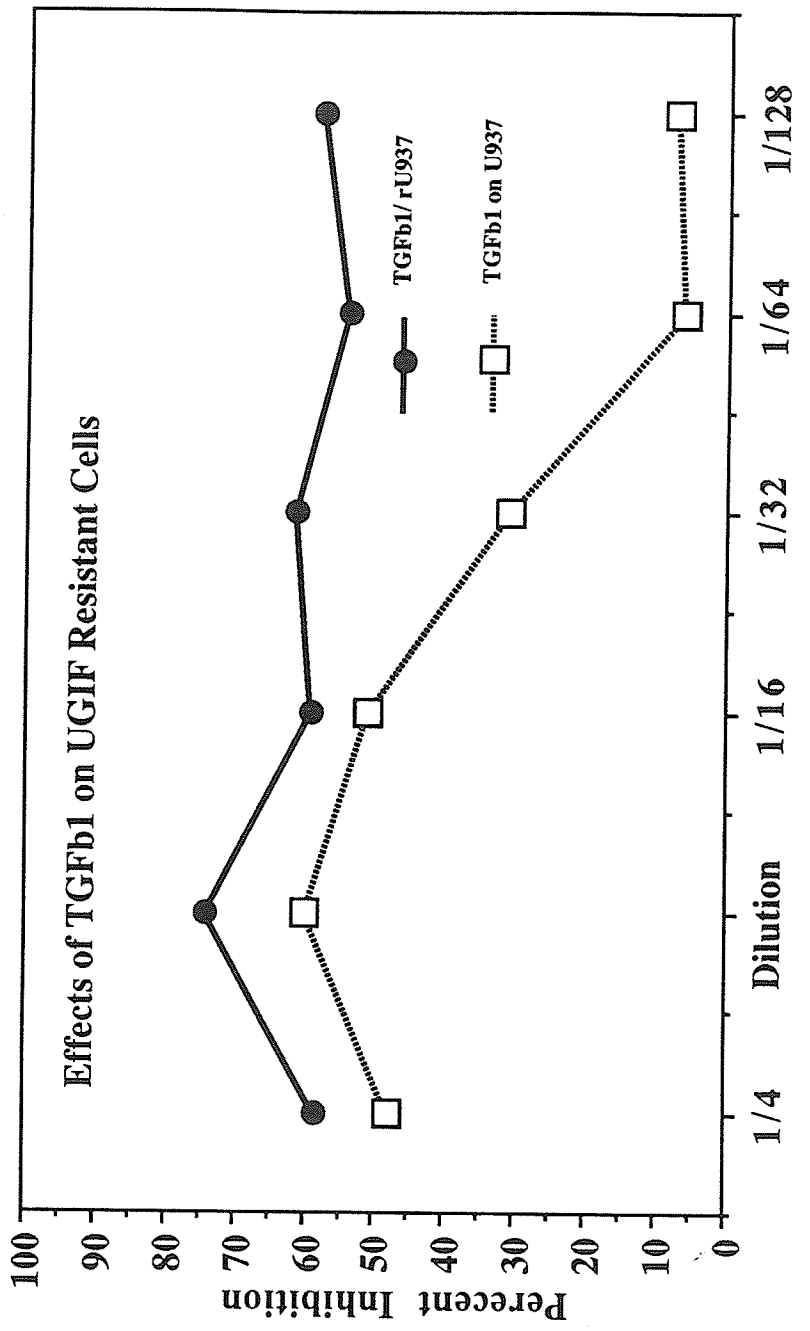


Figure 3.

The Effects of UGIF on Resistant and Unselected U937 Cells.
rU937= cells selected for resistance to UGIF.
U937= unselected parental cells.
Dilutions of TGFb are of a 100ng/ml stock.

Figure 4.



Effects of TGFb1 on UGIF resistant U937 cells.
rU937= U937 cells selected for resistance to UGIF.
U937= U937 unselected parental U937 cells.

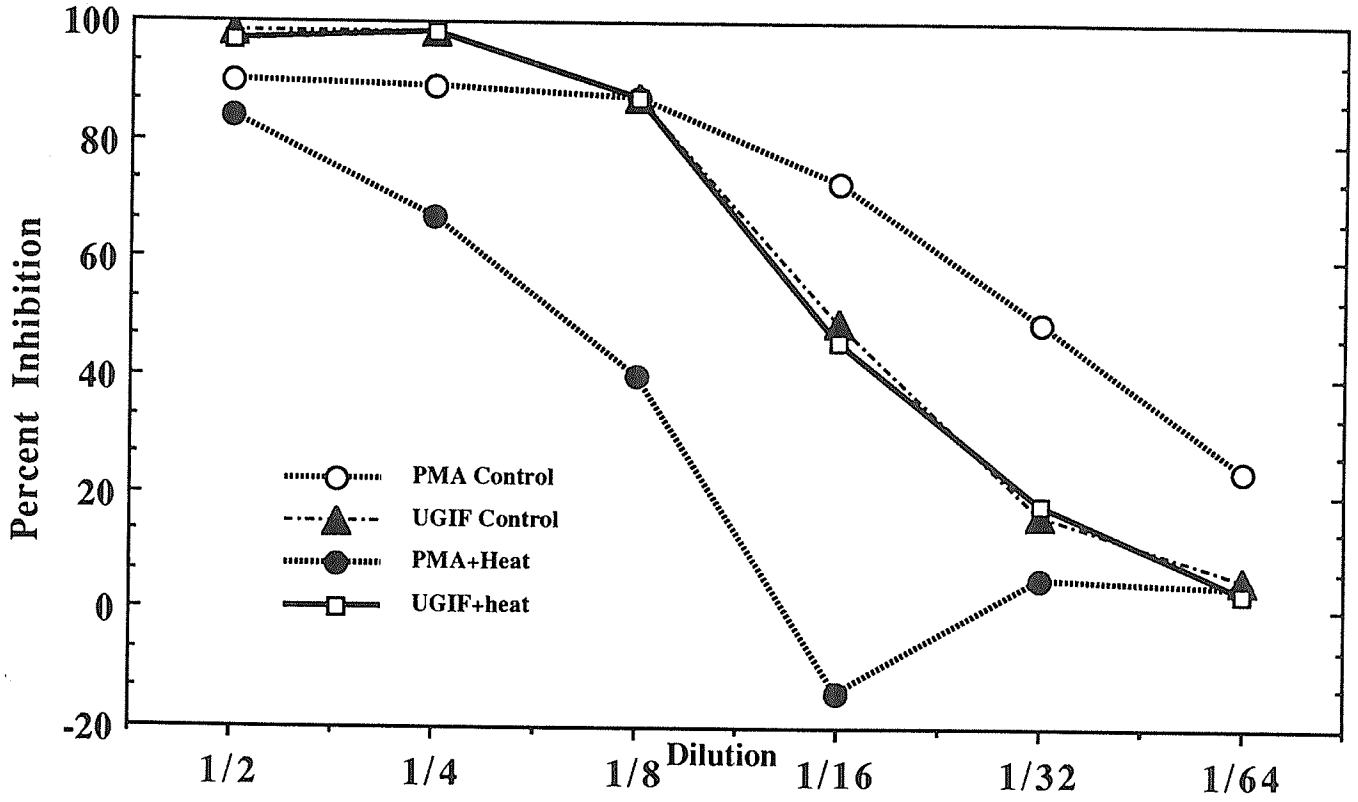
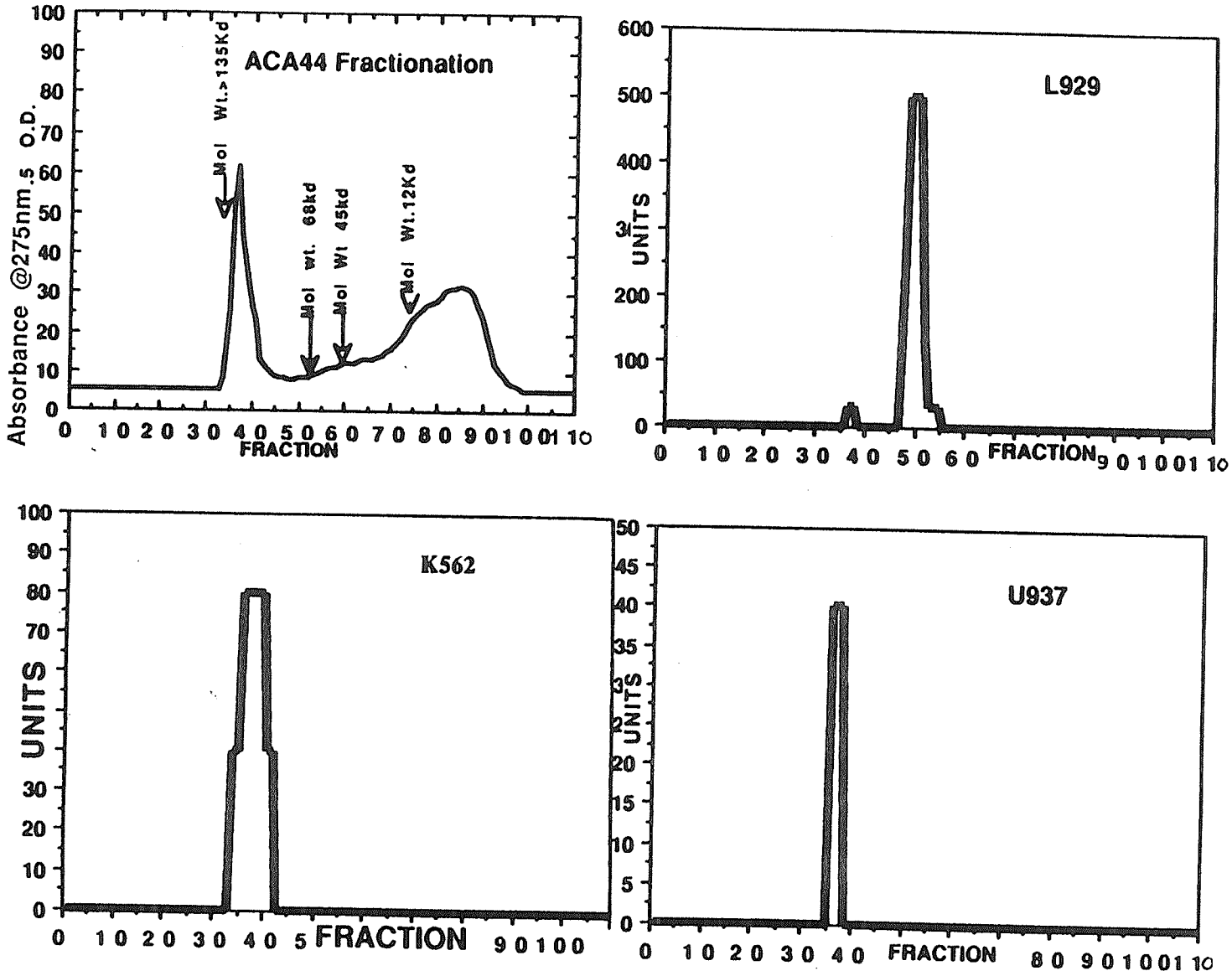


Figure 4a.

The Influence of Heat on UGIF and PMA

PMA Stock (40ng/ml)
UGIF Concentrate (1/50)

Molecular Weight Sizing of UGIF



ACA-44 Fractionation of Crude UGIF Isolates UGIF From Most TNF Activity. Note that L929 activity does elute in two peaks, a major peak(500units in fraction 50) isolated from the UGIF activity, and as a minor peak (32 units) contaminating the UGIF activity found in fraction 37.

The conditions used in this fractionation are as follows, The ACA-44 column is run in the 4 degree room. 1.5ml of crude UGIF concentrate was loaded in Hepes (25mM, .15M NaCl) pH 7.2, 2ml fractions are collected overnight, and absorbance is monitored @275nm in line over the range of 0-.5 O.D. Collected fractions are assayed on each of the target cell lines ablove.

Figure 5.

Stability of C18 Purified UGIF

Table A.

TREATMENT	*UNITS INTACT*
C18- UGIF Untreated	80
Acid (HCl pH 2.0, 1hr)	40
Base (NaOH pH 12.0, 1hr)	40
100 C 2 Min	80
100 C 5 Min	160
.1% SDS @22°C 4 Hr.	80
Proteinase K (1mg/ml)	80
Anti TNF alpha	80
Anti TGFb1	80
Anti TNF+ Anti TGFb1	160

C18 purified material has <2U TNF/ml by L929 assay.
C18 purified material does not affect PHA-PBL @ a 1/20 dilution.
Starting material on K562= 6400U/ml
Starting material on PHA-PBL= 20 U/ml =320x less sensitive.

Table B.

Relative Sensitivities of Target Cells to UGIF Concentrates;

Line	Lineage	No. of units required for 50% inhibition
K562	Myelogenous leukemia *	1
U937	Histiocytic lymphoma *	4
SKW6.4	B cell EBV*	16
A549	Bronchial carcinoma*	32
Jurkat	T leukemia*	16
COLO 205	Adenocarcinoma*	16
NS1	Myeloma+	1
Cess	B lymphoblast*	128
A375	Melanoma*	64
CTL-L2	T cell line+	16
PHA-PBL	Mitogen stim. lymphocytes*	256

Cells from different sources compared for relative sensitivity to UGIF. K562 is arbitrarily assigned 1 unit. Other cells require a several fold increase in the UGIF concentration required to obtain 50% inhibition of growth.

*= Human origin
+= Murine origin

Table C.

Experiment: TNF Neutralization of ACA-44 Purified UGIF Peak

U937 Targets

Fraction 37 alone			*Fraction 37+6000Nu anti-TNF*	
Dil'n	Avg.(c.p.m.)+/-s.d.	%Inh	Avg.(c.p.m.)+/-s.d.	%Inh.
1/8	4927+/-840	90	19876+/-56	60
1/16	31496+/-722	37	37382+/-513	26
1/32	44709+/-276	11	40873+/-552	19
1/64	47951+/-1295	5	45954+/-631	8
1/128	47378+/-408	6	48301+/-545	4
1/256	48798+/-3489	3	49653+/-709	1

Control(n=6)=50195+/-1923
6000Nu anti-TNF did not affect U937 proliferation at 1/8.

K562 Targets

Fraction 37 alone			*Fraction 37+6000Nu anti-TNF*	
Dil'n	Avg.(c.p.m.)+/-s.d.	%Inh.	Avg.(c.p.m.)+/-s.d.	%Inh.
1/8	442+/-57	99	682+/-234	99
1/16	2033+/-85	96	3346+/-210	93
1/32	8818+/-433	80	7200+/-21	84
1/64	18289+/-832	59	20533+/-72	54
1/128	30683+/-392	32	25348+/-694	44
1/256	40659+/-589	9	30606+/-325	32

Control(n=6)=44837+/-3235
6000Nu anti-TNF alpha did not affect K562 proliferation at 1/8.

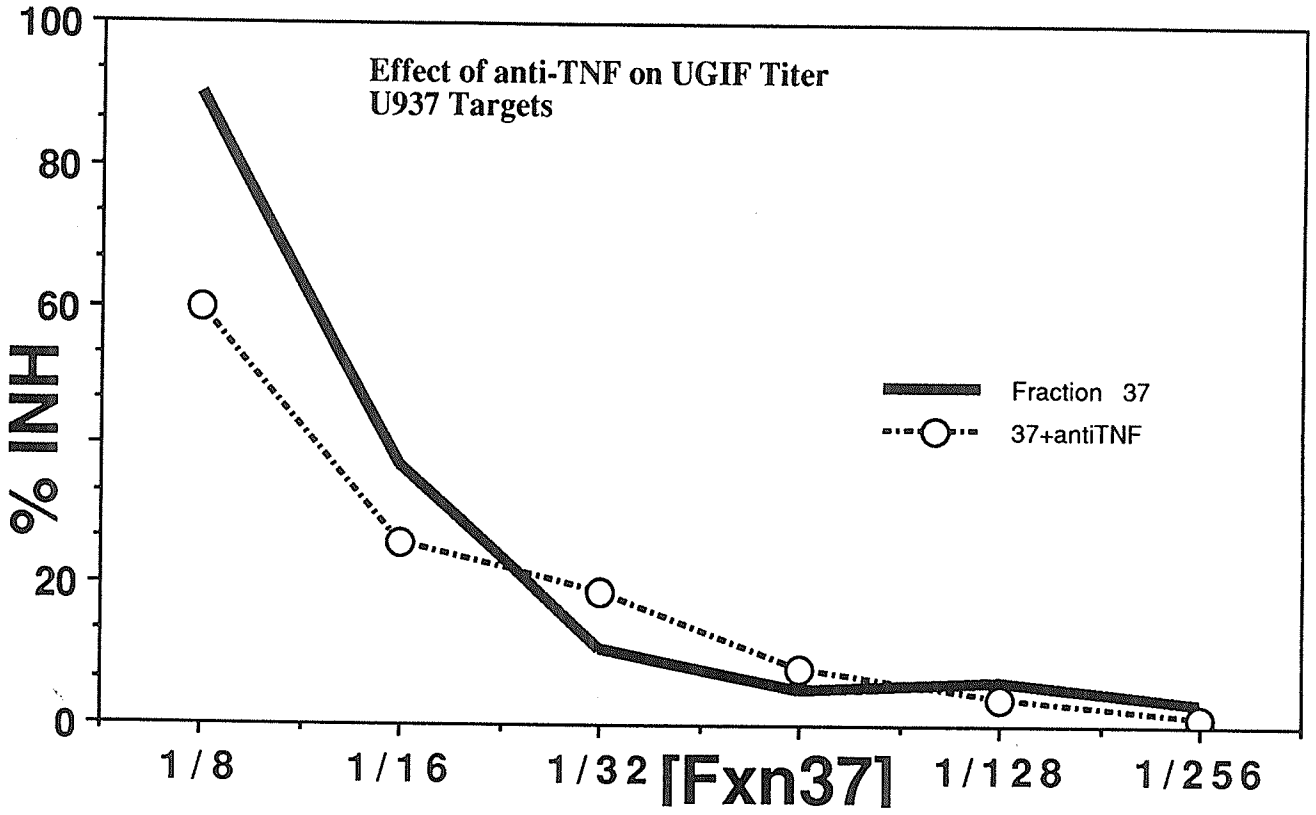


Figure 6.

Attempt to Neutralize ACA-44 Purified UGIF With Anti-TNF Antibodies. Neutralization of TNF activity present in the ACA-44 purified UGIF (64 standard UGIF units/ml premixed with 6000 neutralizing units/ml of anti-TNF) only slightly decreases the effects of the UGIF containing fraction on U937 targets.

Fraction 37= peak UGIF containing fraction from ACA-44 purification.

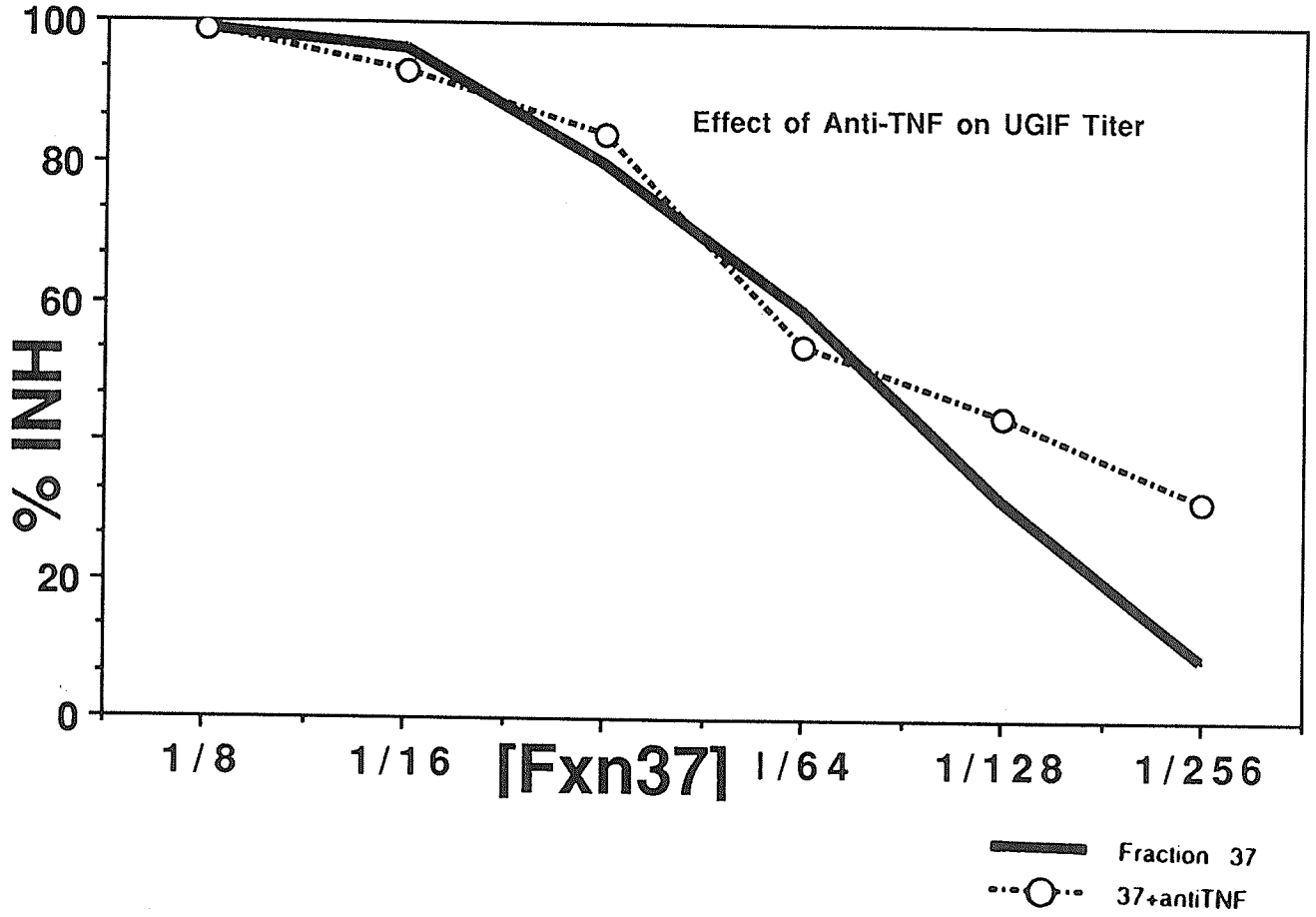


Figure 7.

Attempt to Neutralize ACA-44 Purified UGIF With Anti-TNF Antibodies. Neutralization of TNF activity present in the ACA-44 purified UGIF (64 standard UGIF units/ml premixed with 6000 neutralizing units of anti-TNF for 1 hr.) does not decrease the inhibitory activity of UGIF on K562 cells.

Fraction 37= peak UGIF containing fraction from ACA-44 purification.

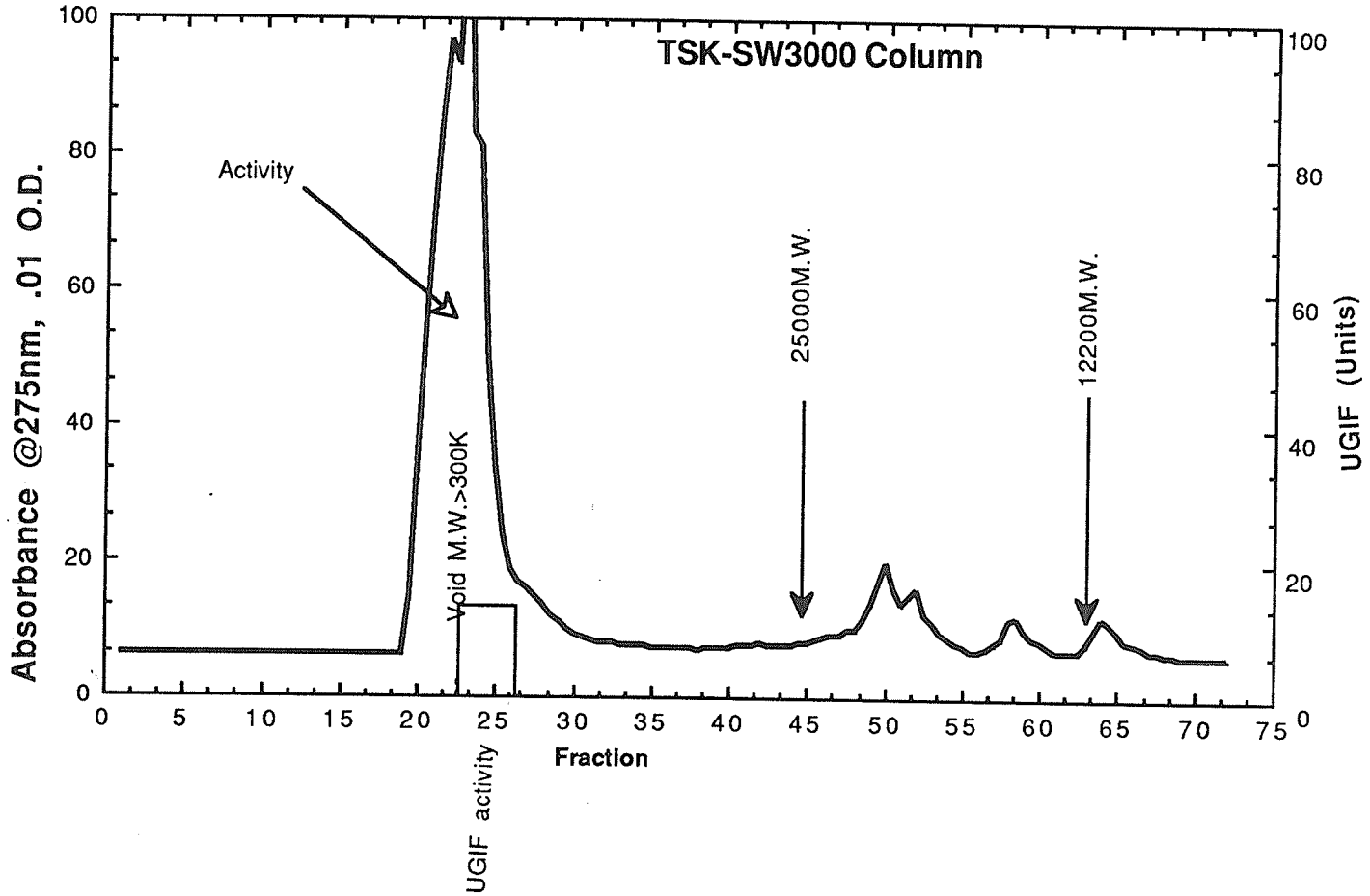


Figure 8.

Gel Permeation Exclusion Chromatography of Partially Purified UGIF. The exclusion limit of this SW-3000 column is about 300kDal. UGIF activity is detected only in the void volume, indicating that UGIF has an apparent molecular weight of>300kDal. The conditions used to run this column are as follows; 250ul (75 units) of sample is loaded in a 1ml injection loop, .5ml fractions are collected, and the absorbance is monitored @275nm in line, with a range of 0-0.1 O.D.. The running buffer is Hepes (25mM, .2M NaCl) pH 7.2, and runs at a flow rate of 1ml/min.

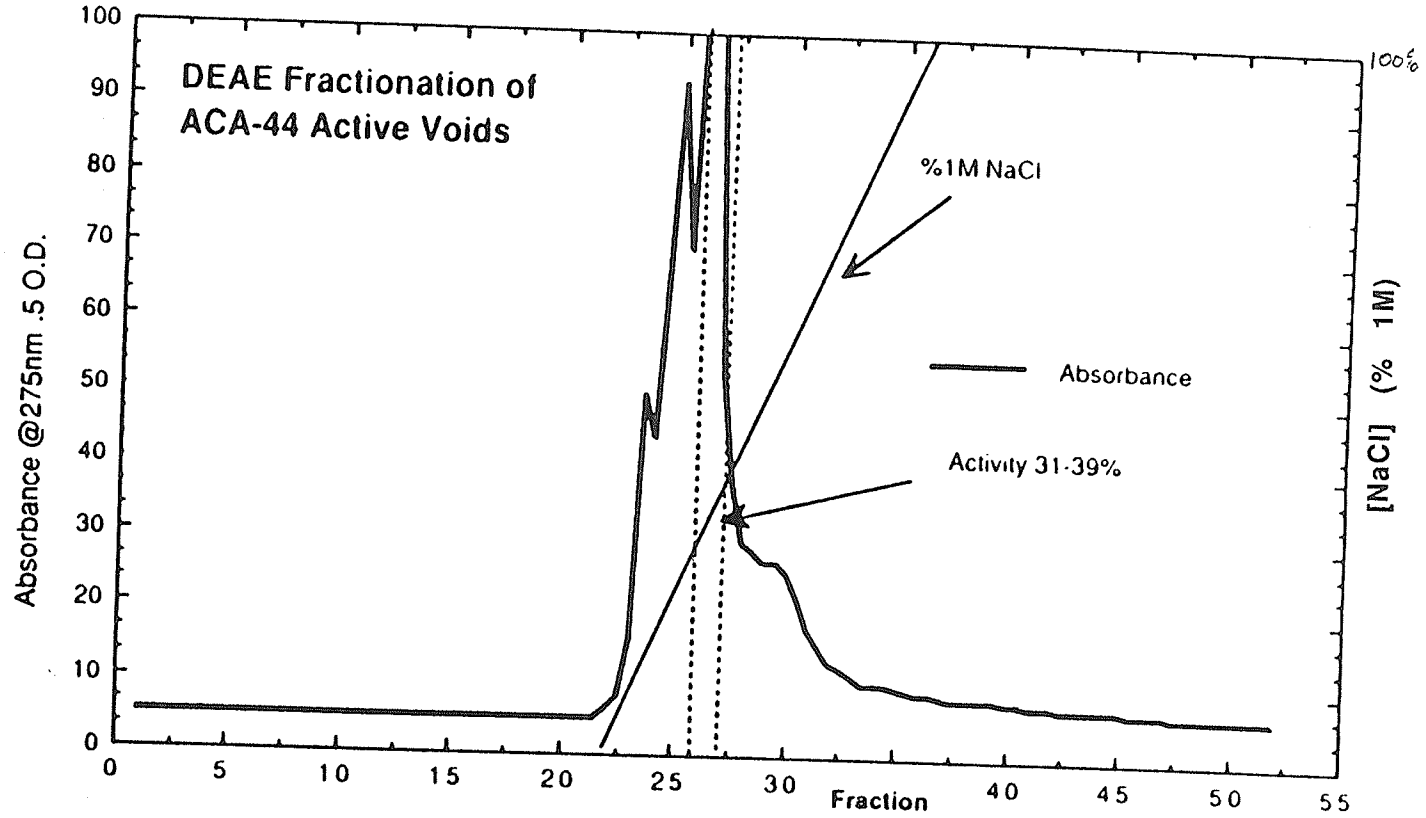


Figure 9.

Anion Exchange Chromatography of Partially Purified UGIF.

The conditions for the run of this column are as follows; 1600 units of partially purified UGIF activity were injected in a 2ml volume, in Tris (20mM) pH 8.0. A 10 min. gradient of 0-1M NaCl was run at a flow rate of 1ml/min. Absorbance was monitored @275nm in line with a range of 0-.5 O.D., 1ml fractions were collected and assayed on U937 and K562 cell lines. The activity was found to elute as a sharp peak between .31-.39 M NaCl on the gradient. Of 1600 units applied, 192 were recovered giving a yeild of only 12%.

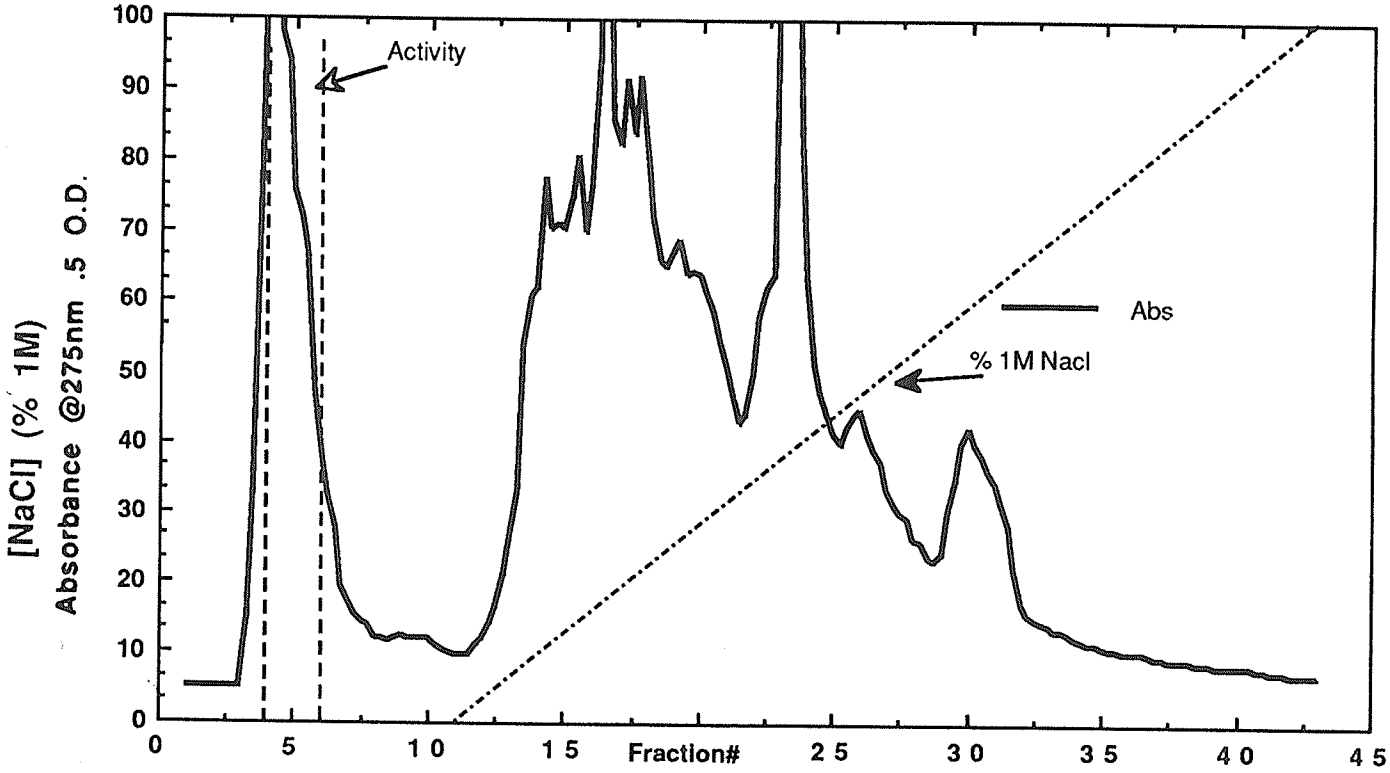


Figure 10.

Analytical DEAE Fractionation of Crude UGIF Concentrate. When the DEAE column is run as a first step in the purification protocol, the UGIF activity is only detected in the void of the column. Of 128 units applied, 96 units were recovered in the void, approx. 75% recovery. (represents at least 8-10 fold purification). Run Conditions; .5ml of crude concentrate is loaded in Tris (20mM, pH8.0), a 32 min. gradient of 0-1M NaCl is run at a rate of 1ml/min, and 1ml fractions are collected. Absorbance is monitored in line @275nm.

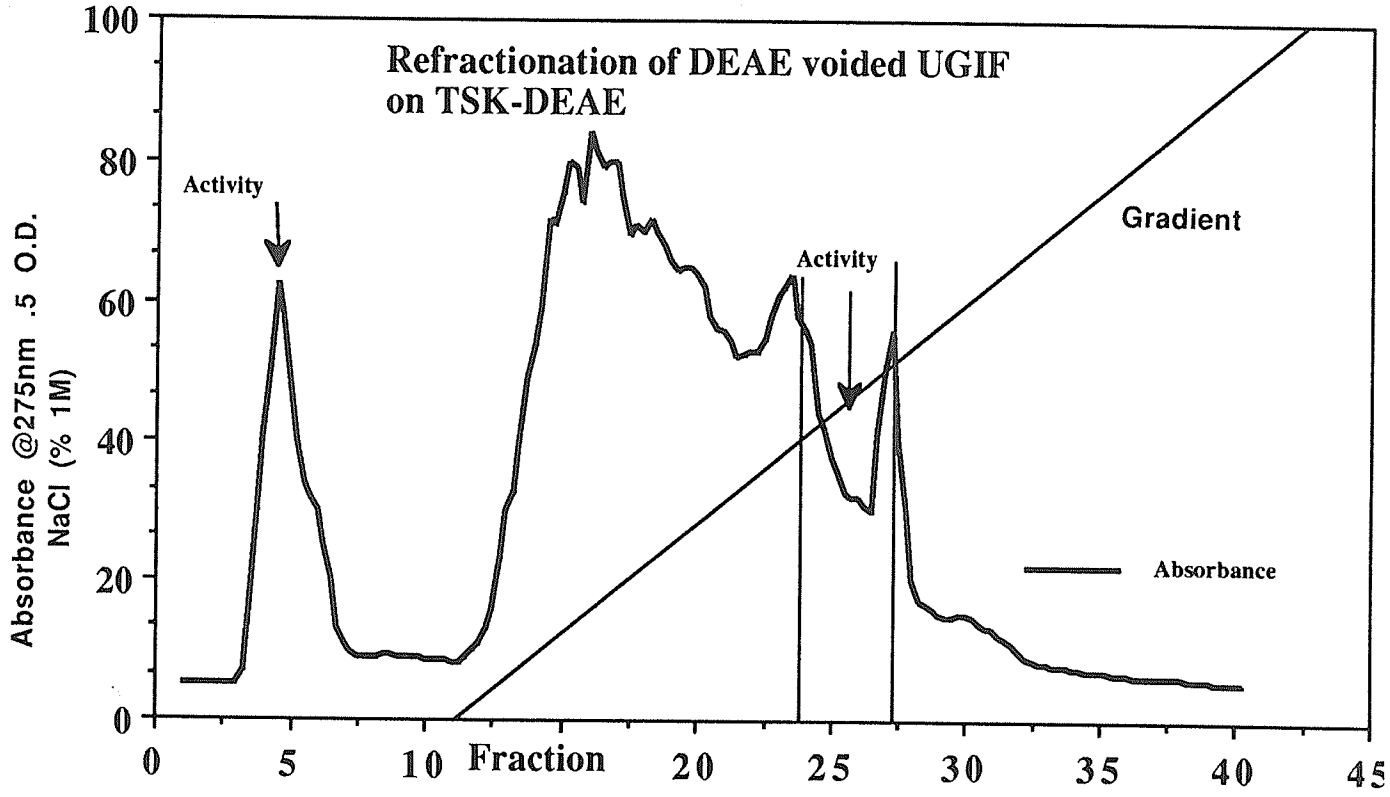


Figure 11.

Refractionation of DEAE voided UGIF. The major peak of activity elutes at .4-.5M NaCl. Note that activity can be found both in the void and as a retained peak.

The conditions for the run of this column are as follows; 1ml of DEAE purified UGIF (560 units) is loaded in Hepes (25mM) pH 8.0, a linear 32 minute gradient of 0-1M NaCl is used to develop the column, at a flow rate of 1ml/min, and 1ml fractions are collected. The absorbance is monitored @275nm in line with a range of 0-.5 O.D.

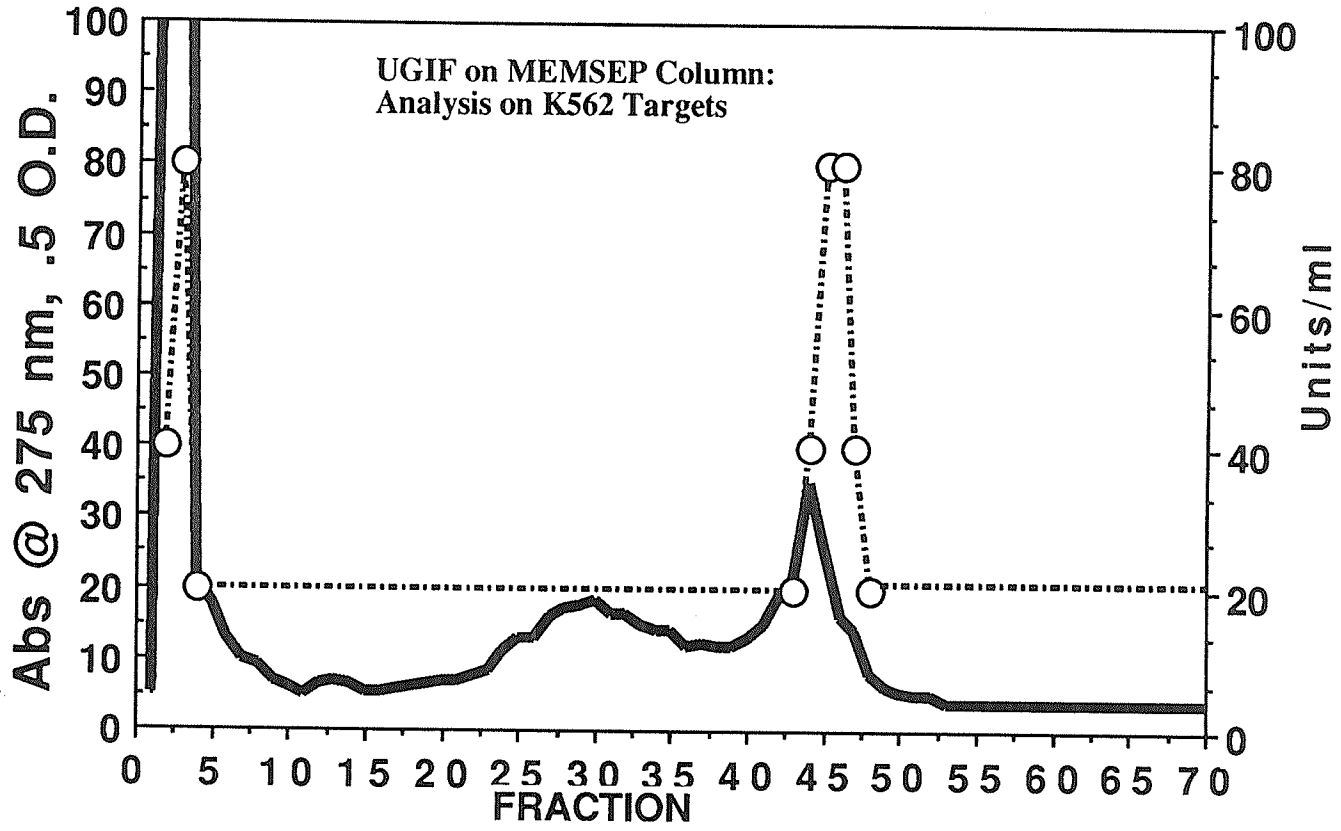


Figure 12.

Large Scale DEAE Fractionation of UGIF Using MEMSEP 1010 Column. There is TNF activity detected in the void of this column, but none is detected with the retained peak of UGIF.

The conditions for the run of this column are as follows; 10,000 units of crude UGIF activity (2.75ml, 40mg) is loaded in Hepes (20mM, .15M NaCl) pH 8.0, and run at flow rate of 3ml/min. A 42 min. gradient of .15-.50M NaCl is used to develop the column. There are two peaks of UGIF activity, one in the void volume, and one eluting at approx. .4M NaCl. Retained peak of UGIF is followed through reversed phase chromatography.

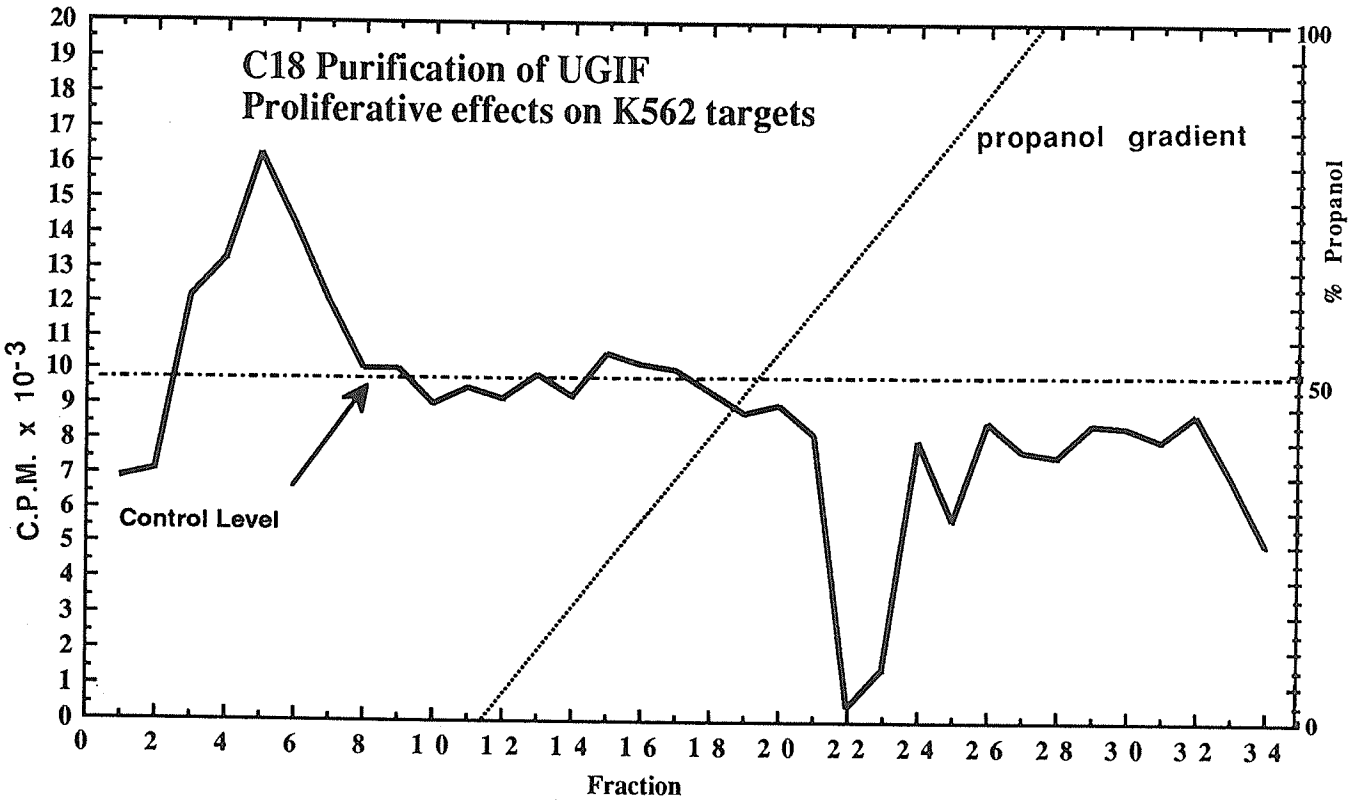
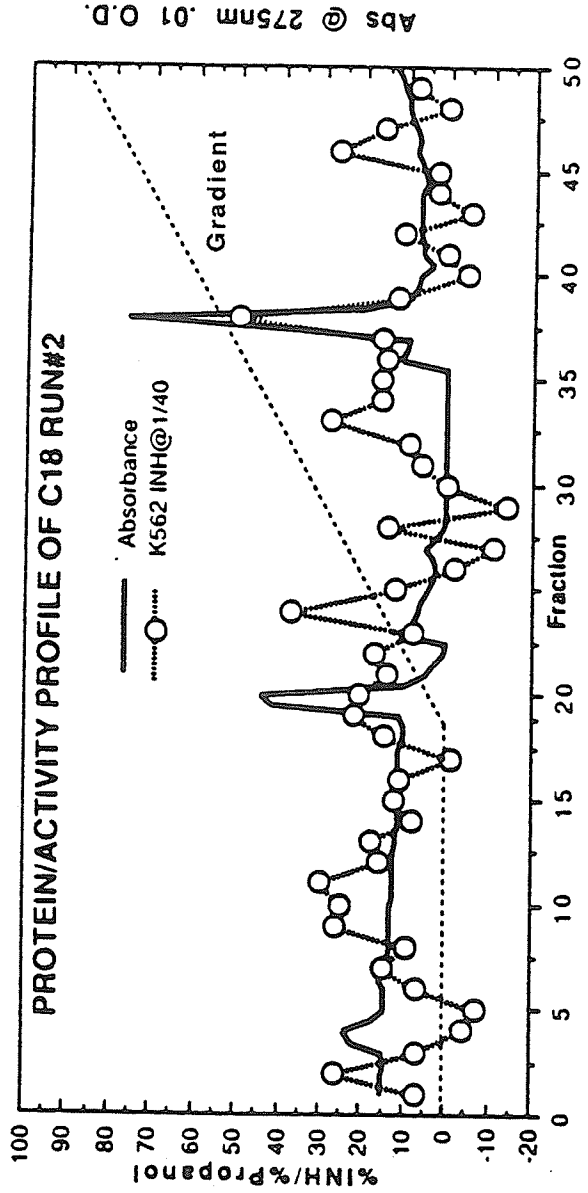


Figure 13.

C18 Purification of DEAE Retained UGIF. Effects of fractions on K562 proliferation are shown with respect to their position in the propanol gradient. A single peak of inhibitory activity elutes in fractions 22 and 23 (approx. 60% propanol).

The conditions used in this run are as follows; 1.8ml of post-DEAE (1000 units) UGIF was run in 25mM Hepes buffer pH8.5, a flow rate of 200ul/min was used, and 1ml fractions were collected. An 80min. gradient of 0-100% n-propanol was used to elute bound material.

Figure 14.



Refractionation of C_{18} Purified UGIF.

By running C_{18} purified UGIF on the C_{18} column a second consecutive time, one sharp peak of absorbance is seen to correspond with the UGIF activity. UGIF activity elutes at 50% propanol. Attempts to visualize C18 purified UGIF on SDS PAGE silver stained gels result in a complete lack of bands. Conditions used for this experiment are detailed in the text (pg 57).

Figure 15.

Stability of UGIF in the presence of SDS. Dilutions of .1% SDS suspended alone in RPMI, or mixed with UGIF are assayed on K562 target cells. SDS fails to destroy the UGIF's inhibitory activity.

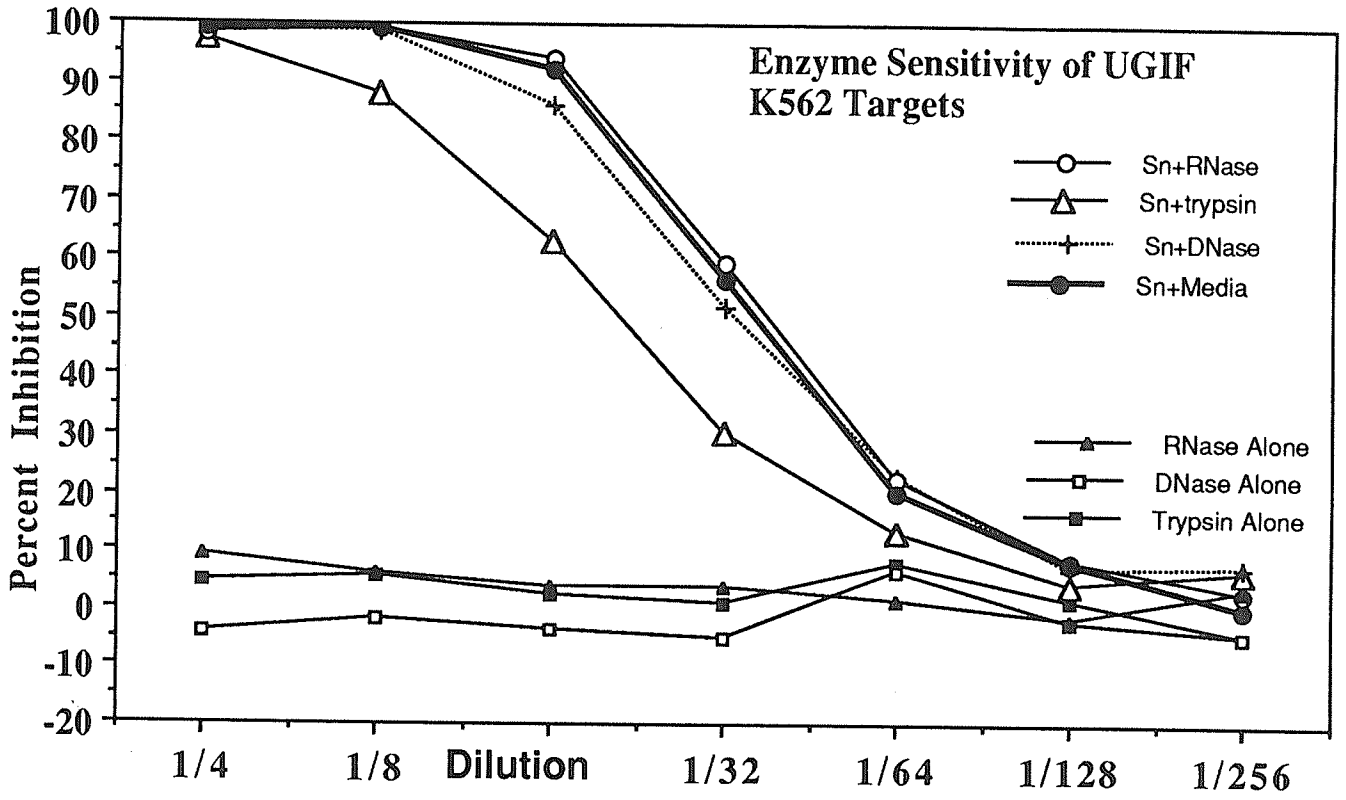


Figure 16.

The Influence of Enzymes on UGIF Activity. The sensitivity of UGIF activity (32 unit/ml) to digestion by DNase (250 units), RNase (250 units), and trypsin is examined. The nucleases were unable to affect the UGIF titer, however, trypsin reduced the activity by 50%.

Table D.

Relationship of UGIF to Other Cytokines

Cytokine	Detected in Sn	Antibody Neutralized UGIF Activity	Effect on Targets
TNF alpha **	No	No influence	Inhibitory
TNF beta**	No	No influence	None
IL-1	No	No influence	None
BCGF	similar effects on readout line	Not done	None
IL-6	Not Done	No influence	Inhib.K562,Stim U937
IL-2	No	No influence	None
IFN gamma	No	No influence	Not done
TGFb1*	No	No influence	Inhibitory
TGFb2*	No	Not done	Inhibitory
Oncostatin M	Negligable***	Not done	Not done

*Kindly provided by L. Ellingsworth at Collagen Corp.
**Kindly provided by B.B. Aggarwal, Genentech Inc.
***Kindly assayed at Oncogen Corp.

Table E.

Effect of Cytokines on K562 targets:

***Results are recorded as percent inhibition over untreated controls.*

Dilution	TGFb1 (200ng/ml)	TGFb2 (200ng/ml)	BCGF (100%)	rIL-2 (2000u/ml)	rTNF (2000u/ml)	rIL-6 (2000u/ml)	UGIF **
1/8	51%	30%	24%	20%	50%	13%	98%
1/16	32%	19%	4%	13%	26%	23%	93%
1/32	29%	20%	9%	7%	31%	18%	73%
1/64	38%	23%	14%	12%	27%	28%	60%
1/128	27%	30%	-2%	18%	30%	28%	46%
1/256	17%	29%	8%	17%	16%	34%	26%

Effect of Cytokines on U937 targets:

Dilution	TGFb1 (200ng/ml)	TGFb2 (200ng/ml)	BCGF (100%)	rIL-2 (2000u/ml)	rTNF (2000u/ml)	rIL-6 (2000u/ml)	UGIF **
1/8	18%	2%	7%	-1%	44%	-4%	98%
1/16	8%	11%	4%	-7%	36%	-10%	81%
1/32	10%	10%	4%	-7%	33%	-8%	43%
1/64	10%	10%	-1%	-4%	28%	-3%	24%
1/128	5%	8%	-9%	-6%	23%	-6%	14%
1/256	11%	12%	-7%	-10%	10%	-5%	5%

***DEAE purified UGIF was shown to contain 80u/ml in previous K562 assays.*

Table F.

Experiment: Attempt to Neutralize C18 Purified UGIF

K562 Targets

Dil'n	C18 Peak alone Avg.(c.p.m.)+/-s.d.	%Inh.	C18 Peak +anti TNF Avg.(c.p.m.)+/-s.d.	%Inh.	C18 Peak+anti TGFb1 Avg.(c.p.m.)+/-s.d.	%Inh.
1/20	346+/-15	98.3	277+/-32	98.6	280+/-8	98.5
1/40	1138+/-46	94.3	1025+/-107	95	808+/-168	96
1/80	7160+/-554	64	4841+/-145	76	4611+/-1056	77
1/160	13857+/-331	31	10847+/-734	45.6	11307+/-2323	43
1/320	21682+/-512	-9	17261+/-757	13.4	19266+/-1604	3

Control(n=8)=19924+/-2655

U937 Targets

Dil'n	C18 Peak Alone Avg.(c.p.m.)+/-s.d.	%Inh.	C18 Peak +anti TNF Avg.(c.p.m.)+/-s.d.	%Inh.	C18 Peak+anti TGFb1 Avg.(c.p.m.)+/-s.d.	%Inh.
1/20	1945+/-156	70	1114+/-23	83	1469+/-59	77.5
1/40	3864+/-306	41	2350+/-279	64	2208+/-743	66
1/80	5179+/-110	20	4674+/665	28	4191+/-486	36
1/160	5469+/-138	16	6535+/-445	-1	5774+/-550	11
1/320	5315+/-30	18	5565+/-199	15	5972+/-262	15

Control(n=8)=6506+/-519

Effect of anti-TNF+anti TGFb1 on C18 Purified UGIF

Dil'n	U937 Targets Avg.(c.p.m.)+/-s.d.	%Inh.	K562 Targets Avg.(c.p.m.)+/-s.d.	%Inh.
1/20	1135+/-73	83	249+/-102	98.6
1/40	2455+/-57	62	1025+/-107	95
1/80	4862+/-99	25	4841+/-145	76
1/160	6533+/-244	-.41	10847+/-734	46
1/320	5985+/-144	8	17261+/-757	13

Controls as above.
Antibodies alone had no effect on the targets over the same dilution range used above.

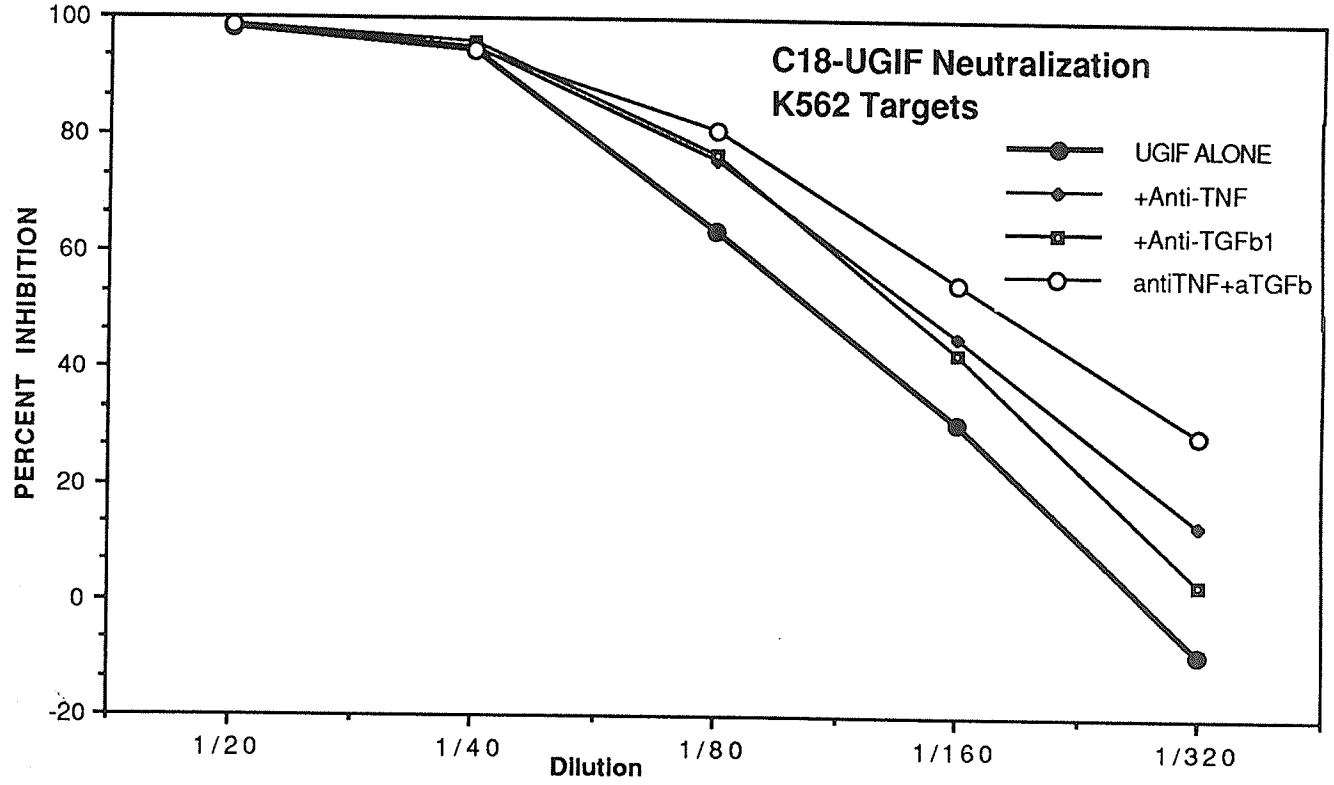


Figure 17.

Attempt to Neutralize C18 Purified UGIF. Sufficient anti-TGFb1 was added to neutralize 100ng of purified TGFb1. 6000 neutralizing units of anti-TNF was added either alone or in combination with the anti TGFb1.

UGIF Alone= 80u/ml untreated C18 purified UGIF, +Anti-TNF= 80u/ml UGIF premixed with 6000Nu anti-TNF, +anti-TGFb1=80u/ml UGIF premixed with anti-TGFb1, Anti-TNF+anti-TGFb=80u/ml UGIF premixed with both antibodies,

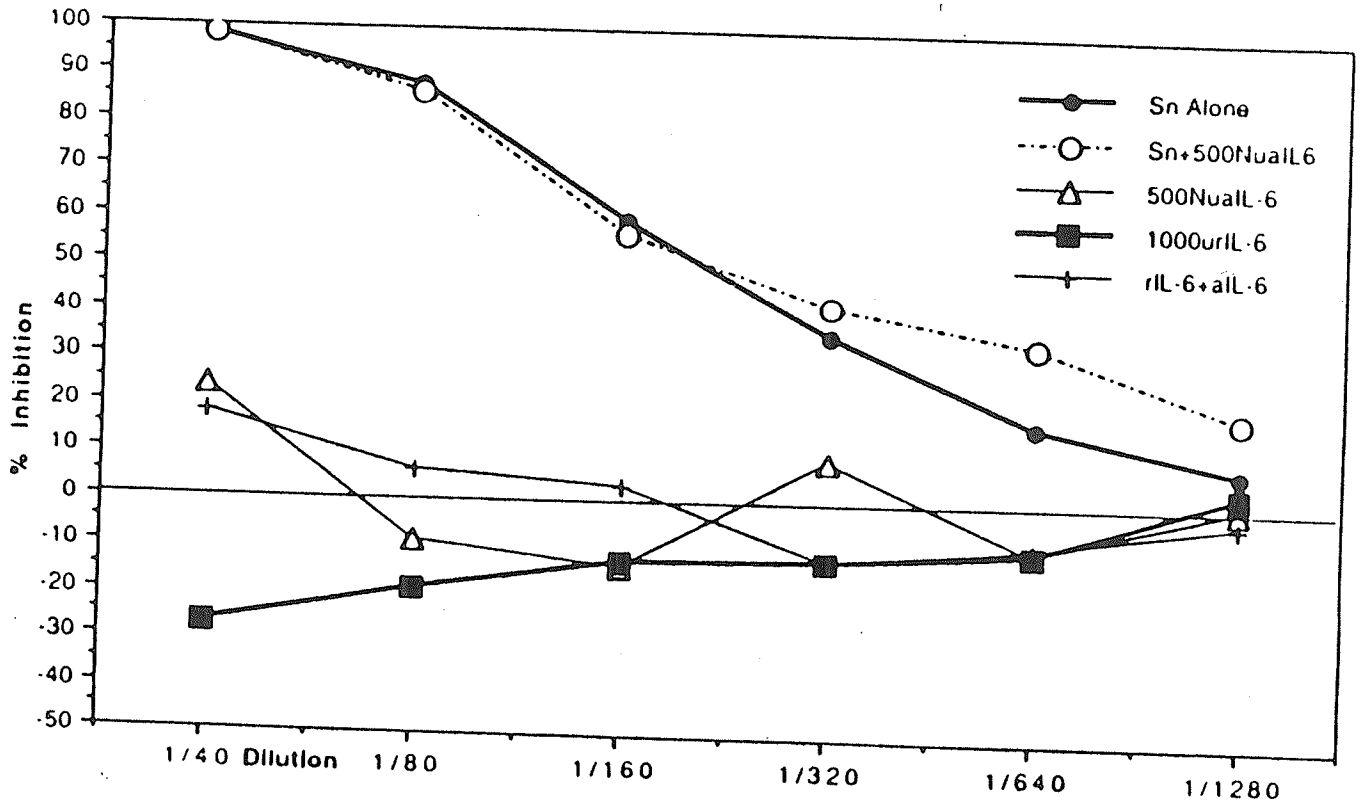


Figure 18.

Relationship of IL-6 to UGIF: K562 Targets. UGIF does not appear to be related to the effects of IL-6, and cannot be neutralized by antibodies against this cytokine. Recombinant IL-6 does not affect the proliferation of K562 target cells at the levels tested.

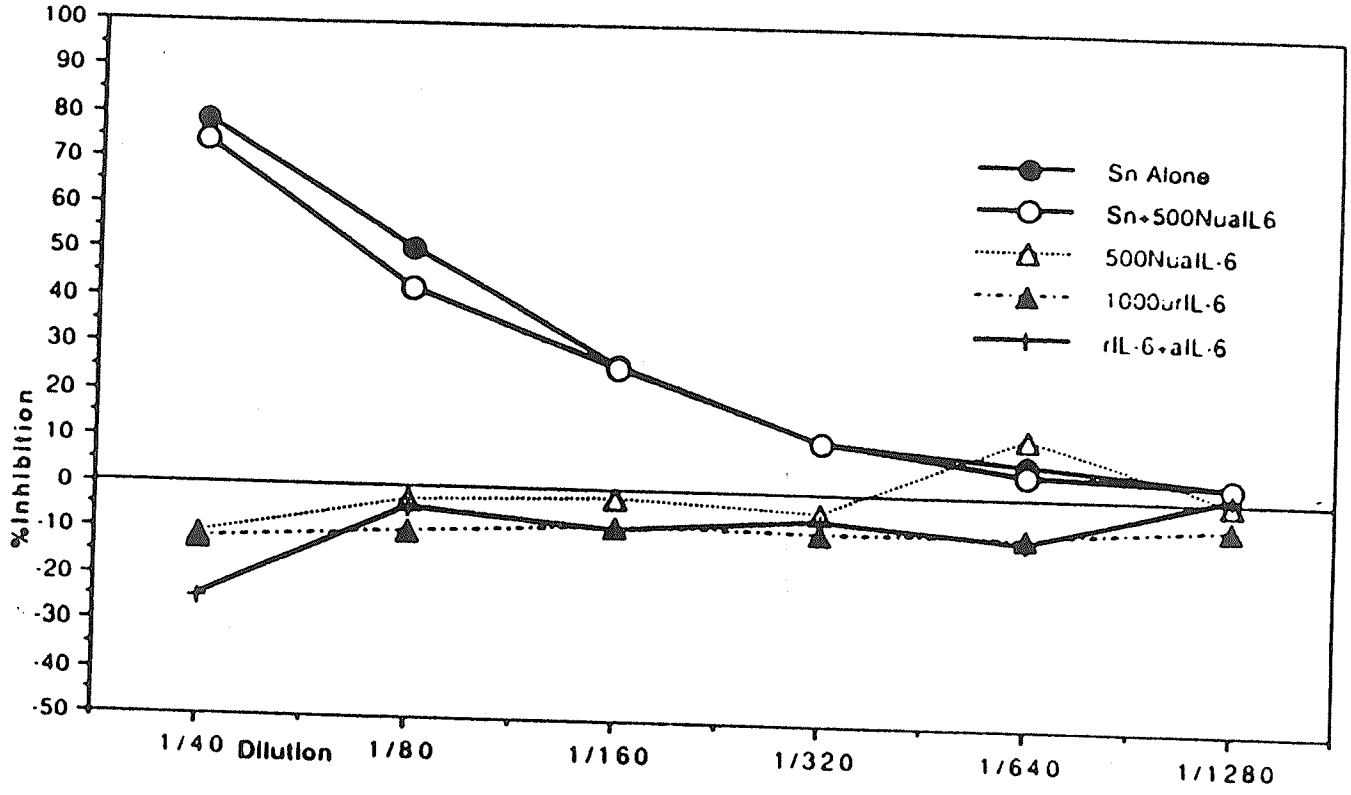
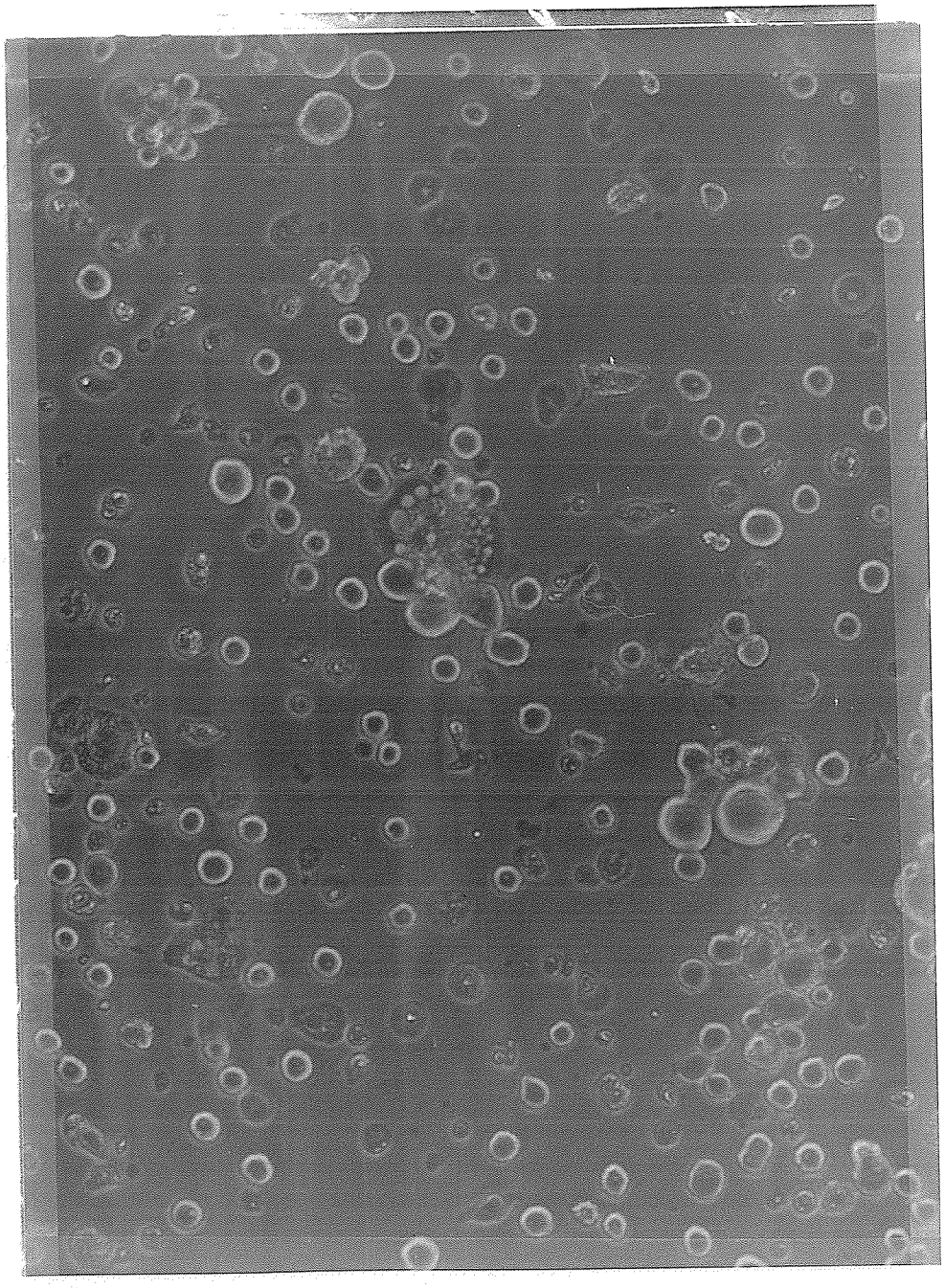


Figure 19.

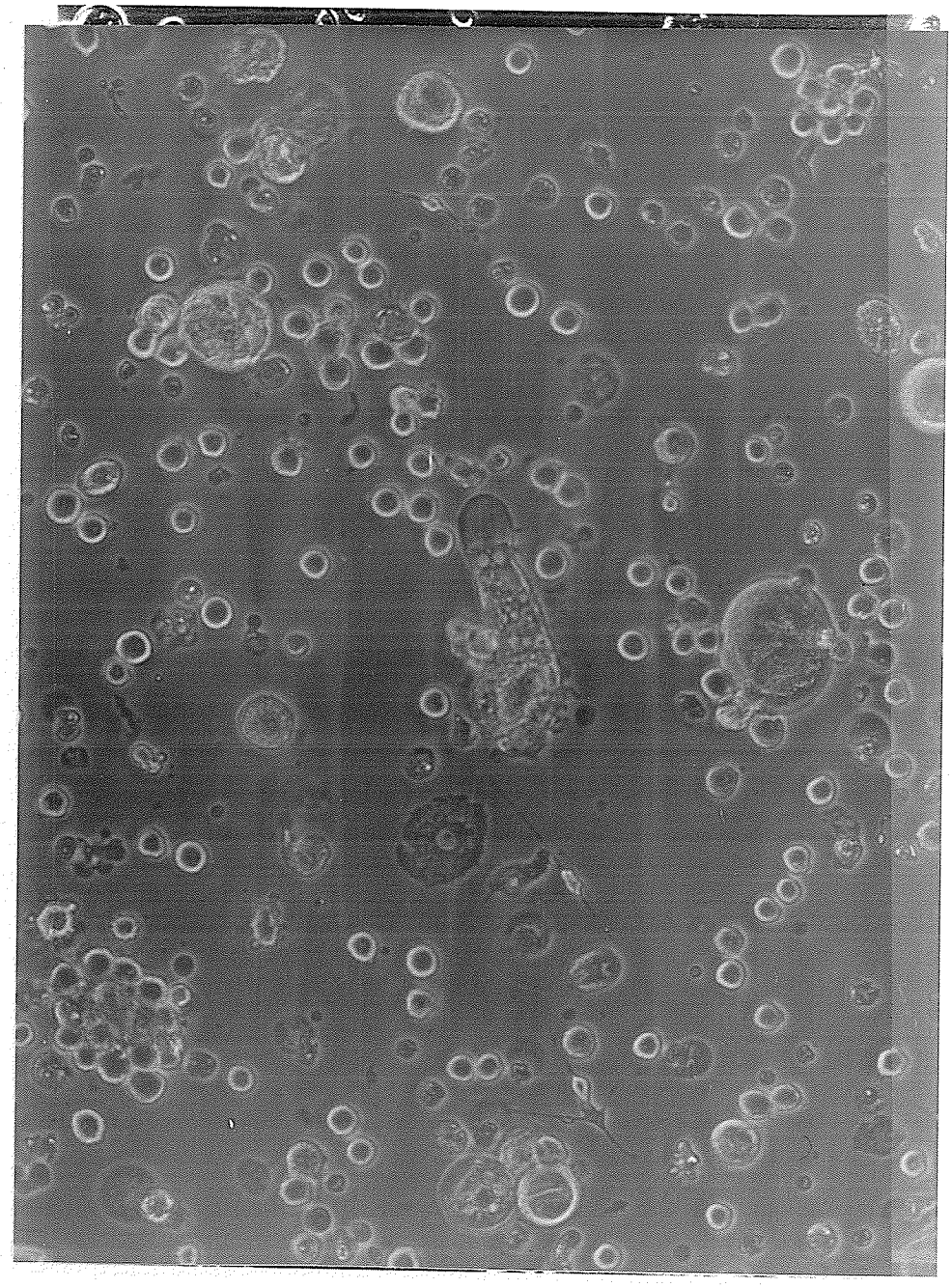
Relationship of IL-6 to UGIF: U937 Targets. UGIF does not appear to be related to the effects of IL-6, and cannot be neutralized by antibodies against this cytokine. Recombinant IL-6 does not affect the proliferation of U937 target cells at the levels tested.

Figure 20.



Differentiative effects of UGIF on U937 targets. This is a photo taken 24 hours after placing U937 ATCC cells into a flask containing UGIF (approx. 50% inhibitory dose.) Note the large granulated cells which appear to have undergone or are undergoing a differentiation. The cells treated with UGIF are very different from untreated controls. Untreated cells contain no large granulated cells.

Figure 20a



Differentiative effects of UGIF on U937 targets. This is a photo taken 24 hours after placing U937 ATCC cells into a flask containing UGIF (approx. 50% inhibitory dose.) Note the large granulated cells which appear to have undergone or are undergoing a differentiation. The cells treated with UGIF are very different from untreated controls. Untreated cells contain no large granulated cells.

Figure 20b

Differentiative effects of UGIF on U937 targets. This is a photo taken of UGIF resistant cells which are growing in the presence of UGIF (approx. 90% inhibitory level for unselected cells). Note that these cells are much more uniform in size and appearence than unselected cells treated with UGIF (figure 20a and 20b.) These cells appear much like untreated control cultures with the exception that untreated U937 cells are more uniform, and contain no large granulated cells. Selected cells appear to be more resistant to the differentiative effects of UGIF than unselected cells.

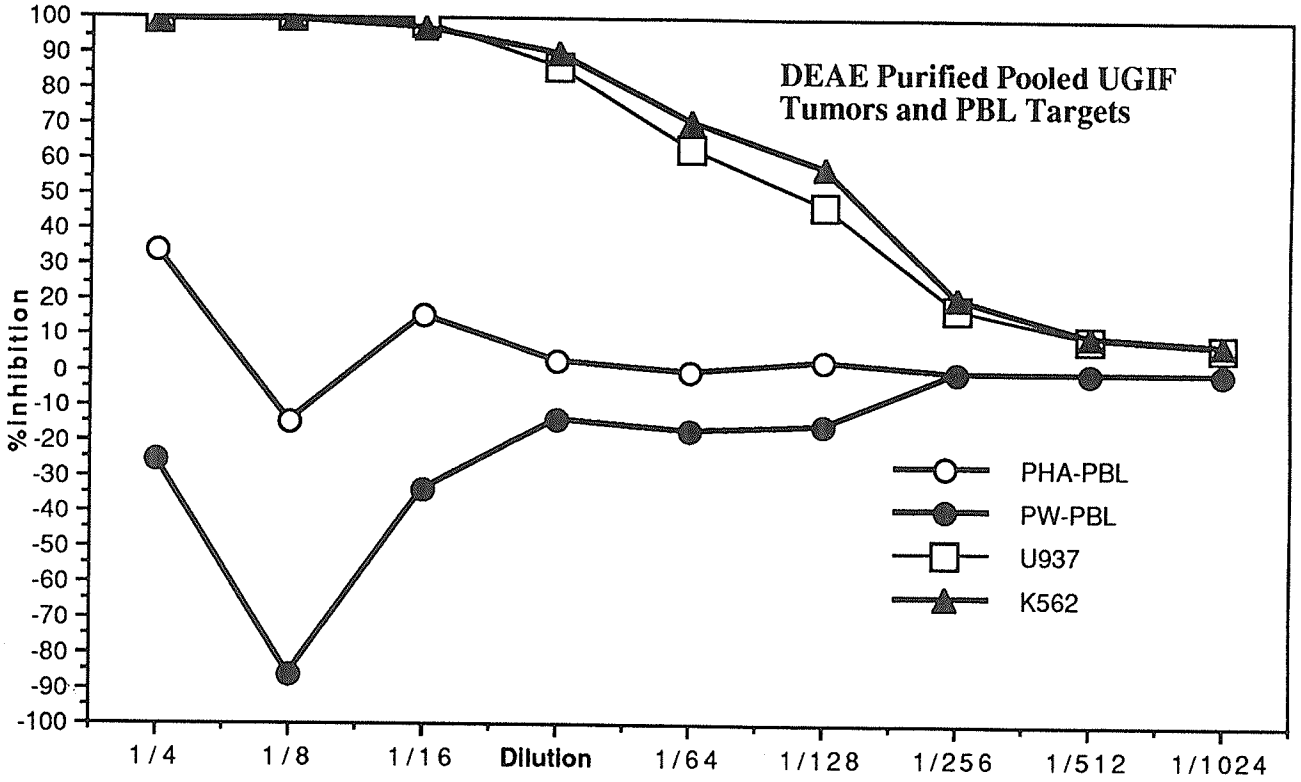


Figure 21.

Target Cell Specificity of DEAE Purified UGIF. Both U937 and K562 are highly sensitive to the inhibitory effects of purified UGIF. PHA stimulated PBL are relativelly unaffected by the UGIF while pokeweed mitogen stimulated PBL show a stimulated growth rate at high concentrations of UGIF. Effects of UGIF on normal PBL's titer out very quickly compared to the far reaching effects on the tumor cell lines.

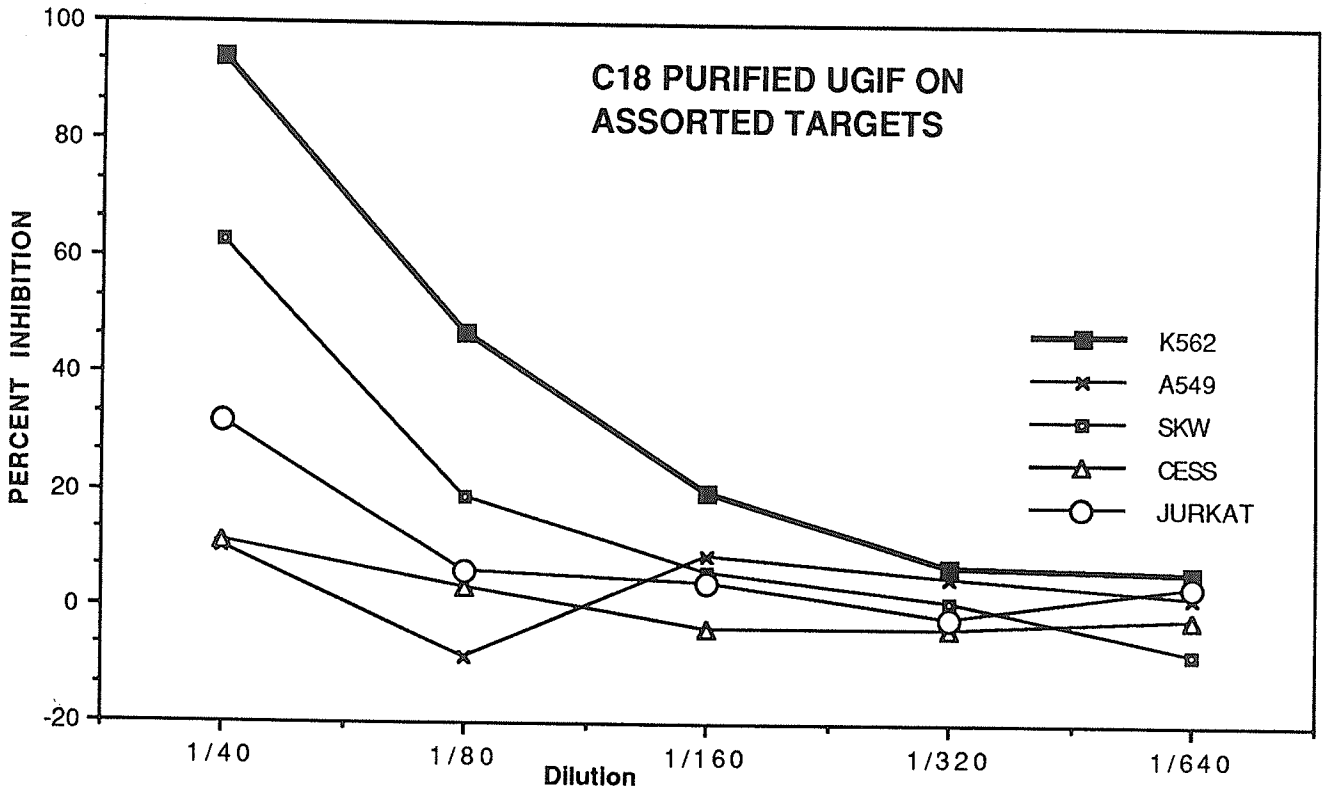


Figure 22.

Target Cell Sensitivity to Highly Purified (C_{18}) UGIF. The effects of purified UGIF on several tumor cell lines are shown. K562 cells are the most sensitive to UGIF inhibition. The pattern of sensitivities seen is similar to the pattern of senstivities observed for crude UGIF concentrates (table A).

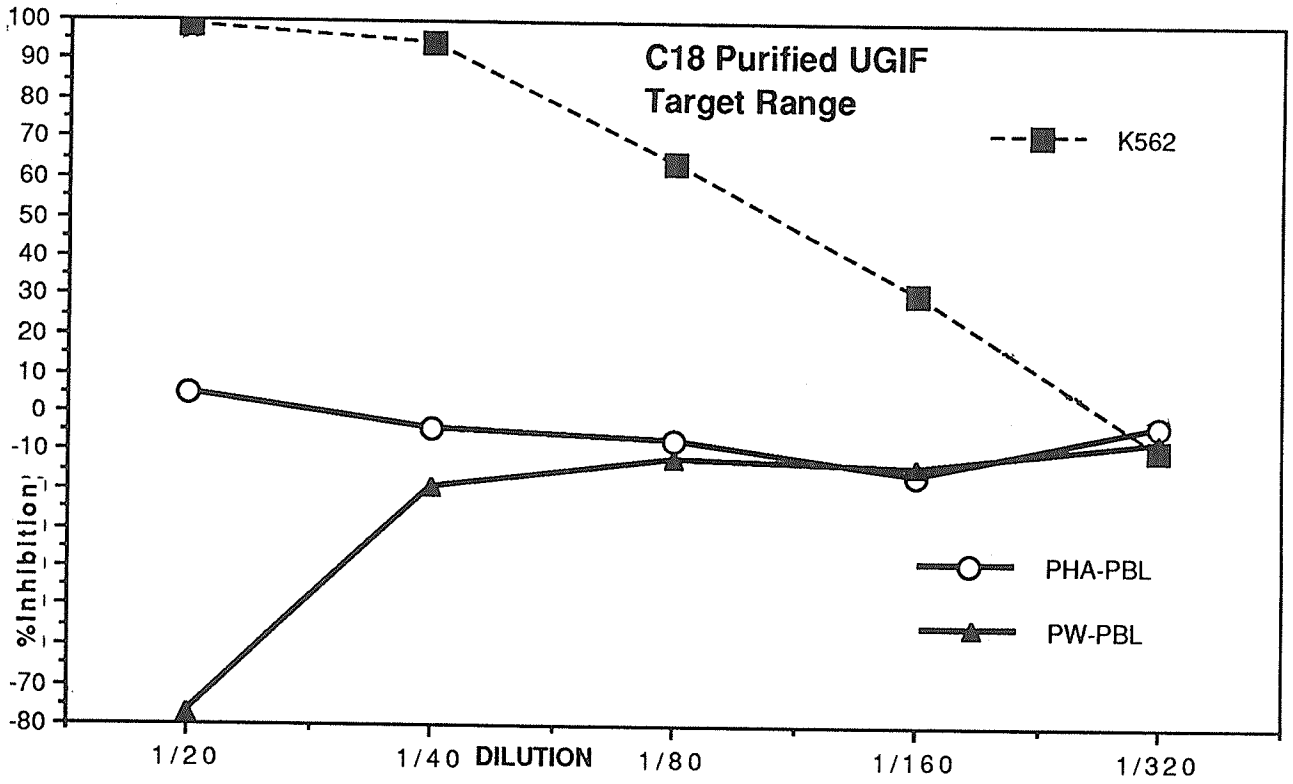


Figure 23.

Dose Response of K562 Tumor Cells and Normal PBL to C_{18} Purified UGIF. Highly purified UGIF has little effect on normal mitogen stimulated PBL. The figure demonstrates that even the most highly purified UGIF preparations only affect normal mitogen stimulated PBL proliferation at doses much higher than those required to observe effects on the K562 readout line.

Discussion:

The data presented indicates that PMA stimulation of U937 cells results in the production of a unique mediator of cell growth, UGIF. A number of distinct cytokines have been previously isolated from U937 cells, and other similar monocyte lines. As molecules like Tumor Necrosis Factor, Interleukin six, Oncostatin M, Transforming Growth Factor, Interferon, and Interleukin One all have at least some inhibitory activity on certain cells, we considered the possibility that UGIF activity may be due to one of these activities. Many techniques were employed to demonstrate that UGIF is distinct from other cytokines.

UGIF has an apparent molecular weight of >300kda, which is not compatible with most known cytokines, however the stability data suggests that at least the active form may be much smaller than this. Apparent molecular weight is insufficient criteria to judge whether an unknown activity can share identity with a known cytokine. Other more specific methods are employed to demonstrate that UGIF is a novel cytokine.

The data never give any indication of impure supernatant concentrates, or highly purified UGIF having any antiviral effects in the A549 interferon assay. The lack of antiviral activity suggests that the inhibition is not due to IFN. Antibodies against gamma IFN fail to affect the titer of UGIF, indicating that IFN does not mediate the antiproliferative effects seen with UGIF.

Purified IFN was examined by another lab on K562 target cells (the standard UGIF readout line) and found to have no effect on their proliferation. Even when large amounts of IFN alpha (5000 units) and IFN gamma (5000 units) have been applied to K562 cells (Ortaldo et al 1986 unpublished work) there is little effect on proliferation, suggesting further that UGIF is not due to the inhibitory effects of IFN.

Tumor Necrosis Factor is known to be present in crude U937 supernatants, but is not responsible for all the antiproliferative activity seen. The use of neutralizing antibodies against TNF allows us to block TNF lytic effects and still observe UGIF effects. Up to 50% of the antiproliferative effects of crude U937 supernatants are neutralizable by anti-TNF-alpha, the antibodies do not neutralize any of the inhibitory effects of DEAE, or C_{18} purified material (Figure 19.).

Gel filtration (ACA44) data indicate that the bulk of the TNF activity does not copurify with the bulk of the UGIF activity (Figure 7.). The small amount of TNF which does copurify through ACA-44 sizing is neutralizable by anti-TNF, while the UGIF remains active (Figure 17.). TNF is not detectable in the UGIF containing peaks after DEAE separations, suggesting further that UGIF is unique from the action of TNF.

Pure recombinant TNF is only poorly inhibitory for the standard target cells used to assay UGIF. High levels of inhibition (>60%) are not observed rTNF. Our experience has been that the amount of inhibition by TNF reaches a maximum level of approx. 50%, (table E.) but does not increase even over the range of

250u/ml to >1 millions units of TNF per ml of culture. Recombinant TNF does not mimic UGIF action at any concentration of TNF tested.

The cytokine Interleukin One is not detectable in concentrates containing high levels of UGIF. Neutralizing antibodies against IL-1 do not reduce the titer of UGIF in crude preparations (data not shown). Further evidence that UGIF is distinct from IL-1 is that UGIF appears to be far more stable to heat treatment than is compatible with IL-1. From the antibody data, and absence of detectable levels of IL-1 in crude and purified UGIF samples, we conclude that UGIF is distinct from IL-1.

Transforming Growth Factor beta is not detectable in UGIF concentrates in assays performed at Collagen Corp. However, TGFb1 and b2 have low level growth inhibitory effects on UGIF target cells. The inhibition observed with purified TGFb is at most a low level plateau which never approaches the near 100% inhibition seen with UGIF (Figure3, Figure 4.). The effects of purified TGFb do not mimic the effects of purified UGIF on K562 or U937 targets.

The possibility that low levels of TGFb could act in synergy with some other cytokine to give much higher levels of inhibition had to be examined. Antibodies able to neutralize the effects of TGFb1 cannot neutralize or reduce the inhibitory effects of UGIF on U937, K562, or A375 cells. Transforming Growth Factor beta does not seem to be involved in the antiproliferative activity of UGIF.

As both TNF and TGFb are known to have inhibitory effects on cells used in the assay for UGIF, the presence of these cytokines was ruled out as having any role in UGIF action. A set of experiments was performed on the most highly purified UGIF available to address this question. When combinations of anti TNF and anti TGF are used, the inhibitory activity of C_{18} purified UGIF is not reduced, indicating that UGIF is not due to a synergy between these molecules (Figure 17.). Further evidence that UGIF is not a consequence of TGFb comes from experiments where it was shown that U937 cells resistant to the effects of UGIF became exquisitely sensitive to the inhibitory effects of $TGFb_1$ and $TGFb_2$ (Figure 3, Figure 4.).

Interleukin six is another cytokine which had to be addressed. Using both semi-purified and highly (C_{18}) purified UGIF, attempts were made to neutralize the antiproliferative activity with anti-IL-6. UGIF activity is unaffected by the neutralizing antibody (Figure 19.). The effects of purified IL-6 on U937 and K562 target cells are also examined. The effect of high levels of IL-6 is slightly stimulatory, and does not inhibit the growth of U937 cells at any concentration examined. Effects on K562 cells are slightly inhibitory, but are far less than levels of inhibition observed with UGIF (Table E.) UGIF does not appear to be a consequence of IL-6.

Oncostatin M is a cytokine which could be related to UGIF, but we are unable to directly address this question at present as neither purified material, nor antibody are available for testing. Oncogen corp. assayed our UGIF material and reported only negligible amounts of Oncostatin M were present. The molecular

weight of UGIF, is very much higher than that reported for Oncostatin. UGIF does not become activated by acid treatment whereas Oncostatin and TGFb do. Based on the limited data available, UGIF appears to be distinct from Oncostatin M.

Extensive analysis of UGIF and it's biological relationship to other cytokines reveals a lack of identity with any of the cytokines examined. Purified cytokines do not mimic the effects of UGIF on standard target cells. Antibodies against TGFb, TNF-alpha, TNF-beta, IL-6, IL-1, IL-2, and IFN-gamma all fail to abrogate the antiproliferative activity of UGIF. Further disparity from other cytokines is apparent when the physico-chemical properties of UGIF are examined.

Physico chemical characterization of UGIF indicates that it is a highly stable activity with an apparent molecular weight of >300kda by SW3000 sizing (Figure 8.).

The UGIF activity is partially destroyed by extremes of pH, losing 50% of it's antiproliferative activity after a one hour exposure to either strong acid or strong base. Exposure of the UGIF to .1% SDS does not reduce it's activity (Figure 15.).

The C_{18} purified activity withstands 100°C for 5 minutes, and is not destroyed by a 24hr digestion with a nonspecific protease. That data may lead one to suspect that UGIF is not a protein. However, trypsin treatment does decrease the activity by about 50%, suggesting that protein is at least responsible for a component of the UGIF. The data suggest that UGIF is a large, and very stable activity (Table B.).

The stability data is more compatible with a very small molecule, or peptide than a huge >300kda moiety. A number of explanations could account for the observed stability and large size. A plausible explanation would be that the activity itself is actually a very small molecule and it only appears large because it is associated with a large carrier molecule.

Another explanation could be that UGIF is in fact very large, but does not have to be in the intact large form to affect target cells. It could be that a large bioactive molecule can be broken down to smaller active compnents, which would make it appear as if the original activity was unaffected by harsh treatments. As purifications continue and the characterization of UGIF advances, the answer to the apparent discrepancy between size and stability should become clear.

The precedent for a large protein molecule acting as a carrier for bioactive cytokines exists in the form of alpha-2-macroglobulin (Alpha-2). Alpha-2 can actually create a cage of protein surrounding the molecule it carries, making the carried molecule highly resistant to proteases. The carrier Alpha-2 is known to carry some cytokines in a ratio of two to one. It is possible that UGIF is carried by such a molecule which could account for it's resistance to protease, and high molecular weight. It might also explain why UGIF activity appears to increase after 5 min. of boiling (Table B.). One might envision that UGIF is carried in the two to one ratio, with one molecule completely protected by Alpha-2, the other partially exposed and responsible for the antiproliferative activity. Perhaps boiling for this period of time

causes the release of a protected molecule of UGIF, effectively doubling the concentration of active UGIF. While there is no data to support this being the case, it is an intriguing possibility. Alpha-2 is not the only means of increasing the apparent molecular weight of a molecule, lipid-protein complexes can have the same effect.

There are documented cases of extremely large lipid-protein complexes which may be capable of mediating inhibitory activities of eicosanoids. However, the stability of the activity in extremes of pH, heat, and SDS argue against the possibility of UGIF being due to a large lipid complex. The possibility exists that UGIF is being carried by a much larger molecule as there is no data at present to suggest otherwise. Further studies are required to answer this question. Reguardless of UGIF's apparent molecular weight, the antiproliferative activity associated with it is of great interest.

The cytostatic activity of UGIF acts on a wide range of target cells with tumor cell lines being far more sensitive to the effects than normal PHA stimulated PBLs (Table B.). This is itself a most interesting effect as the proliferation of PBL's have been shown to be more sensitive to certain antiproliferative signals than tumor cell lines[16]. An activity able to stop the growth of tumor cells without killing them would mean toxic products of killed cells would not be released into the microenvironment where such products could cause secondary effects on other cells.

The lack of effect on normal peripheral blood lymphocytes at doses of UGIF which completely inhibit tumor cells suggests that

this activity may have a natural role in preventing the growth of tumors in the body. However, UGIF may have a much more important role in regulation of proliferation of cells involved in the immune response. Once a cell mediated response is initiated, and it's job is done, there is a need for a mechanism to control the proliferation of cells actively involved in the immune response. UGIF may be one of several soluble mediators involved in the essential control of the immune response. It may be that UGIF acts both as an immunoregulator, and as a natural defense against tumor cell growth.

While tumor cells seem to be affected by UGIF in a preferential manner, it may be that all rapidly growing cells will be stopped by it's action. A molecule with the ability to halt the growth of virtually any cell must have it's production under tight control. A defect in the control of production could have disasterous effects on many cell types. UGIF production is very tightly regulated. It is the regulation of UGIF production by U937 cells which presents the most difficult problem in studying UGIF.

The low levels of UGIF produced by U937's, and the rapid plateau of accumulated activity in supernatants (Figure 2.) provides a problem. As a great deal of activity is lost in each purification step, much starting material is required to allow purification to homogeneity. Many of the questions about UGIF could be quickly answered if sufficient starting material could be generated.

The initial approach to the problem of low activity was to attempt to increase the amount produced, and to increase the

assay sensitivity to the effects of UGIF. Several agents were explored as an alternative to PMA stimulation. Calcium ionophore A23187 did not induce UGIF, Actinomycin D interfered with the assay system, PHA, PokeWeed Mitogen, and Wheat Germ Agglutinin were all explored. The results demonstrate that PMA is the best stimulus for UGIF production.

Attempts were undertaken to break the apparent feedback inhibiton of production by developing producer cells resistant to UGIF effects. While these experiments were successful in producing a cell type resistant to UGIF inhibition (Figure 3.), the resistant U937 cells did not show any increase in production of UGIF.

Clones from the parental U937 population were examined in hopes of finding either a sponaneous UGIF producer, or a high level UGIF producer. These studies failed to produce any cells that were better at producing UGIF than the parental line.

While attempting to increase the levels of UGIF produced by U937 cells, other experiments designed to increase the assay sensitivity were under way. Several different cell types were examined to find the line most sensitive to UGIF inhibition (Table B.). It was discovered that K562 cells were 16 times as sensitive to UGIF as the original UGIF targets (SKW). Further experiments with the assay system resulted in an increase of 2-4 fold in the K562 assay sensitivity. The most sensitive assay for UGIF is the 48hr (2500 cell/well) K562 assay. The new assay system meant that raw supernatants consistantly contained between 4-16 units of activity per ml. After attempts failed to increase the production

levels of UGIF, it was decided that the purification should proceed. Large scale production of U937 supernatants was undertaken to generate starting materials for the purification.

The first step in the purification protocol is gel permeation exclusion chromatography on an ACA-44 column. This step reveals that UGIF has an apparent molecular weight of >135 kda (Figure 5.). ACA-44 chromatography results in a 60-80% loss of applied activity. A second consecutive sizing using the HPLC SW-3000 column indicates a mol. wt. of >300kda. The physico chemical characterization of ACA-44 purified material indicates that it is very stable despite this apparent high mol. wt.

Further purification of UGIF is effected using anion exchange chromatography. The use of a MEMSEP 1010 DEAE column allows a fast first purification step. DEAE purification results in a fifty fold increase in specific activity (to 600u/mg), and a 80% loss of total activity applied to the column. While the loss is great, the increased specific activity is sufficient to allow another purification step using an analytical reversed phase HPLC column.

The use of a reverse phase column presents a powerful purification step. While a further (approx. 50%) loss of applied activity occurs in this step, the eluted UGIF has a high specific activity (>500,000 units/mg). The purified material has a specific activity well in the range of the specific activities observed for many cytokines. A total purification of almost 55,000 fold over the raw material can be performed using our protocol.

UGIF purified by reversed phase column shows no bands on silver stained SDS-PAGE gels. This may be due to the material

actually being present in a quantity too low to be detected by this technique. Another explanation for the absence of bands is that UGIF is actually too large to enter a 7% acrylamide gel, and that it is lost in the stacking gel. A further and possibility is that UGIF activity is not mediated by protein.

There are a number of reasons to suspect that UGIF is not due to protein. If UGIF is due to a polypeptide, especially a large polypeptide, one has difficulty explaining why it appears so resistant to the effects of proteases. An explanation might be that UGIF is protected from digestion by existing as a complex with lipoprotein or lipids, and that it simply doesn't have sensitive sites exposed to protease on the molecular level. Alpha-2 macroglobulin is known to increase the stability of cytokines that it carries by as much as 10 fold, with respect to protease.

Another possibility is that UGIF is part of a nucleic acid-protein complex, and the nucleic acid it associates with protects it from protease attack. Digestion experiments using nucleases suggest that UGIF is not due to nucleic acid alone, although these molecules can certainly have the same apparent size as UGIF.

Experiments done with porcine lipase to attempt to digest lipid would indicate that the activity is not mediated solely by lipids. While complex sugars can have very high molecular weights, it is unlikely that these could withstand the physicochemical extremes that UGIF does.

Perhaps the strongest arguement that UGIF is due in part at least to protein is that there is a corresponding protein peak in every fractionation and purification of UGIF activity. Even in the

second run of the C18 column, there is a sharp peak of U.V. absorbance corresponding exactly with a sharp peak of UGIF activity. This leads one to suspect that the observed protein peak may be responsible for the activity present in the fraction. If UGIF was not due to protein one might not expect this constant association with peaks of protein during the purification.

It is important to realize that even if UGIF is not a protein, the inhibitory activity may still be biologically relevant. To answer the question of what UGIF is, much more raw material will have to be produced and processed into purified form, than can be undertaken in this thesis.

The target specifictiy of UGIF is essentially the same after purification as is seen for crude UGIF concentrates. One may expect that if UGIF is due to an interaction between different cytokines that at least some of the components would be lost during purification, altering the target specificity of the residual activity. However, the spectrum of targets affected by purified UGIF doesn't change after purification, neither do the observed physical properties. This argues that UGIF is a distinct entity with an inhibitory activity unique from other cytokines, detectable in raw material.

The biological activity of UGIF is mostly an antiproliferative one. The mode of action of UGIF appears to be mainly through an induction of cytostasis with a corresponding cessation of DNA synthesis which is reflected in the Thymidine incorporation assay. Other molecules like the Perforins are known to stop cell growth by punching holes in the cells, killing them (cytotoxicity). The

exact mechanism of UGIF mediated growth inhibiton is unknown, but there is some evidence that an induction of differentiation of affected cells may be involved.

Treatment of K562 cells with inhibitory doses of UGIF results in an induction of morphological changes. K562 cells become very flattened, and extend long processes, and begin to resemble activated macrophage cells. U937 cells become adherent and develop extensive granules in their cytoplasm. Cultures of UGIF inhibited U937 cells contain several giant cells with clusters of smaller cells surrounding them. In both cases the morphology is very different from untreated control cells. An induction of differentiation appears to be occuring in these cell types, based on visual observations. It would be interesting to make use of fluorescence activate cell sorting (FACS) to verify and follow the apparent induction of differentiation over a time course.

Further evidence for differentiation induction by UGIF comes from a B-cell model in the Rheumatic Disease Research Lab. The cell line JR-2D3 is a pre-B cell line which responds to differentiation inducing stimuli by producing immunoglobulin (Ig). When UGIF is assayed on these cells, it greatly induces the production of Ig (10 fold increase over background) which is a direct indication of induction of differentiation of JR-2D3 cells. Interleukin six also causes an induction of Ig production in these cells. Despite this similarity, IL-6 and UGIF appear to be unique entities.

Peripheral blood lymphocytes are relativelly resistant to the effects of UGIF. Only at very high concentrations is any effect seen

on mitogen stimulated PBL. However, there are effects. PHA-PBL can be mildly inhibited at high concentrations, while Pokeweed mitogen stimulated PBL can actually have their growth stimulated (Figure 23.). It is important to realize that the effects of UGIF on PBL's are only seen at concentrations far higher than what is needed to totally inhibit tumor cell targets.

The fact that PBL are not very sensitive to UGIF may be explained in the following ways: Firstly, there are a number of different cells present in isolated PBL, which are capable of producing their own cytokines. The cytokines produced in response to mitogen or UGIF exposure may induce a resistance to UGIF. Secondly, PBLs may have a type of compensation mechanism which nullifies UGIF activity, perhaps an enzymatic destruction of UGIF in the PBL cell occurs. A third possible explanation for the apparent selective action of UGIF on tumor targets is that UGIF acts through an actual antigen or receptor which is greatly up regulated on tumor cells, but present in only low quantities on normal PBL cells. The true explanation must await further studies on the biology of UGIF, which must in turn await production of purified reagents.

There are many experiments to be done with purified UGIF. Amino acid sequencing (if it is a protein) could provide the final proof that UGIF is a unique cytokine. Once sequenced, UGIF could be cloned and recombinant material could be made available for further studies. The production of antibodies against highly purified UGIF would provide a very useful reagent for the study of the biology of this material.

Examining the effects of UGIF on tumor bearing animals would be an intriguing proposition. However, the availability of UGIF for such experiments is extremely limited due to the low levels produced by current techniques.

We still face the problem of low activity, but now have some important biochemical parameters to use in future purification schemes. It is of interest to note that the study of UGIF is hampered most by the availability of raw material.

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