CHARACTERIZATION OF A POLYPEPTIDE FACTOR THAT INHIBITS THE GROWTH OF A HUMAN BREAST CANCER LINE *IN VITRO*

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INTRODUCTION

This thesis concerns a melanoma-derived growth regulatory factor that inhibited proliferation of several malignant human cell lines, and, in particular, a line designated UCT-BR-1, which was derived from a human breast cancer metastasis.

The work is presented in four chapters. Chapter 1 provides a review of the relevant literature at the time of writing; Chapters 2 and 3 describe the experimental work that was done; and in Chapter 4 I discuss the implications of my results for current and future work in growth factors.

Experimental results are presented as Charts (which may be Figures or Tables) and the methods and experimental protocols that I used are described in the Chart legends and not in the main text of the thesis.

The Appendix contains details of the tissue culture techniques and descriptions of the cell lines that were used. Sources of the various laboratory materials as well as the methods that were employed for the more routine procedures are also described in the appendix.

CONTENTS

CHARTS

ACKNOWLEDGEMENTS

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ABSTRACT

A transformed human cell line of neural crest origin has been adapted to grow under entirely serum-free conditions. The adapted line, termed Bowes/sf, offered a unique opportunity to study factors secreted by transformed cells in culture. Conditioned medium from the Bowes/sf line paradoxically inhibited the growth of a number of human epithelial cell lines following a latent period of several days. The human breast carcinoma UCT-BR-1 displayed the greatest sensitivity to this growth inhibitory effect.

Preliminary studies indicated that the growth inhibition was due to a trypsin-sensitive, partially heat-stable macromolecule produced by the Bowes/sf cells. This putative molecule was named MDGIF (melanoma-derived growth inhibitory factor). In order to facilitate the purification of the MDGIF a reliable assay needed to be developed. This was achieved by measuring the inhibitory effect on the incorporation of radiolabelled thymidine by UCT-BR-1 cells grown in 96-well microtiter plates.

MDGIF was shown to withstand repeated concentration followed by lyophilization and it retained activity following extraction into 1M acetic acid. A preliminary gel-filtration chromatography step gave an estimated molecular weight of between 10 000 and 60 000 daltons. Ion-exchange chromatography indicated that the MDGIF was positively charged while reverse-phase HPLC and phenyl-agarose chromatography demonstrated the high intrinsic hydrophobicity of the molecule. MDGIF that had been partially purified by hydrophobic interaction chromatography was found to contain small quantities of TGF-beta. This growth factor is known to inhibit cell proliferation in certain circumstances.

The final characterization involved a combination of acetic acid extraction of lyophilized concentrated conditioned medium, followed by gel-filtration chromatography. Concentration of the conditioned medium, followed by acid extraction, increased the specific activity (inhibitory units/mg protein) 8-fold and resulted in an overall four-fold increase in the total yield. The subsequent gel-filtration chromatography step demonstrated the presence of several molecular species. The material with an estimated Mr of 20 - 25 kilodaltons represented 0.75% of the total protein in the conditioned medium and had a specific activity 115-fold greater than the starting conditioned medium. This partially-purified MDGIF was chosen for further characterization. The partially purified MDGIF retained over

50% of its activity when heated at 70^{o}C C for one-and-a-half hours. Time-course experiments showed that the effects of partially purified MDGIF on UCT-BR-1 cells were still reversible for periods of up to 24 hours.

Reverse-phase HPLC failed to increase the specific activity of the MDGIF preparation. One possible explanation was that there were several interacting growth inhibitory factors that became separated from one another during the chromatography.

In summary, this thesis describes the characterization of an acid-stable growth inhibitory polypeptide present in medium conditioned by Bowes/sf cells. The exact molecular nature of this factor (or factors) remains unknown. The multifunctional TGF-β appeared to have been responsible for some of the observed inhibitory effect.

Numerous studies of cell growth factors now indicate that the final effect of any factor is dependent upon the type of cell used as well as the presence or absence of other factors. The Bowes/sf cell line which produces an inhibitory factor that affects epithelial, but not melanoma cells, remains an extremely useful system for the further study of autocrine and paracrine cell growth factors.

CHAPTER 1

LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

The turn of the century was remarkable for the many outstanding scientific discoveries that were made and contemporary biological science rests heavily upon the achievements of this splendid period. One of the most significant of these was undoubtedly the development of techniques for the long-term *in vitro* culture of avian and mammalian cells. Medicine, virology, oncology and immunology have been particular beneficiaries of the contribution of such pioneers in tissue culture as Rous (1), Harrison (2) and Carrel (3).

In most of these fields, tissue culture has been used mainly as a technique for studying phenomena related rather to the interests of the particular discipline than to the understanding of cellular function. With concurrent conceptual and technical developments in other fields, however, tissue culture has now come to provide the substrate for the study of the cell itself and "cell biology" is a substantive and expanding field of endeavour.

A good deal of recent research effort, in cell biology, has been spent in attempts to elucidate the mechanisms that regulate the rate at which eukaryotic cells proliferate. Early workers who used the technique of tissue culture noted that cells would not survive for any great length of time in culture without the addition of complex biological additives such as fetal calf serum, embryo extract or vegetable extracts. Our understanding of these phenomena was greatly enhanced by the work of such men as Eagle (4) who made a systematic study of medium requirements for cell growth in vitro, and, more recently, by the observations of Sato (5) who pioneered the development of completely defined artificial media.

Thanks largely to the work of these people, tissue culture has moved forward, since the beginning of the 1970's, into the scientific arena where cellular requirements for survival and proliferation are becoming identified and characterized.

During the course of these studies it has become apparent that growth of mammalian cells in culture requires the presence of complex macromolecular signals known as "growth factors" (6). These growth factors are not nutrients; they do not function as intermediates in metabolic pathways and therefore they neither provide a source of energy for the

cell nor do they ultimately become incorporated as structural components of the cell. Instead, they exert their effects on cells by interacting with specific cell-surface receptors. These receptors serve to convert this interaction into an intracellular signal. In other words, growth factors appear to act in a manner akin to the hormones of "classical endocrinology". Growth factors are therefore a heterogenous group of non-nutritive macromolecules that control the rate of cell division. They may elicit one of the following reactions:

(i) increase in the rate of cell division

(ii) decrease in the rate of cell division

(iii) a change in cell phenotype i.e. the appearance and behaviour of cells.

All of the growth factors that have been studied in detail are polypeptides. Many, but not all, of these molecules are unusually stable and can withstand extremes of temperature and pH. For example, epidermal growth factor (EGF) (6,7), platelet-derived growth factor (PDGF)(6,8), type alpha and beta transforming growth factors (TGFs)(9,10) and type alpha and beta interferons (IFN)(11,12,13) are characteristically stable in 1 M acetic acid at pH 2.5. All of these molecules are fairly heat stable as well. Platelet-derived growth factor (PDGF) can be boiled at 100°C for 10 minutes without

loss of activity (6). Type beta TGF withstands heating at 90°C, provided that the pH is kept at 2.5.

This physico-chemical stability has allowed workers to isolate growth factors by using high-resolution purification procedures that would damage more labile molecules; for example, reverse-phase high-performance liquid chromatography (RP-HPLC) has been an invaluable aid in the study of growth factors. Many of the growth factors are resistant to the denaturing effects of the volatile solvents used in RP-HPLC. Because these volatile solvents can be completely removed by lyophilization, RP-HPLC is the method of choice whenever sensitive biological assays need to be performed on chromatography fractions.

As regards the actual molecular structure, there is probably no common feature of note that these factors share. They vary in molecular weight from peptides of 6000 daltons to proteins of 30 000 daltons as shown in Table 1.

Like many polypeptides, growth factors appear to be synthesized intracellularly as high molecular weight precursors (14). Prior to secretion, these precursors are cleaved by proteases to form the mature growth factor polypeptide.

Contd./12

FACTOR	FACTOR IS DEFINED OPERATIONALLY ACCORDING TO THE FOLLOWING CHARACTERISTICS:	MR OF MATURE POLYPEPTIDE (DALTONS)	STRUCTURE OF MATURE POLYPEPTIDE	PRECURSOR POLYPEPTIDE	MRNA (KILO BASES)	SOURCE OF GROWTH FACTOR	GROWTH FACTOR RECEPTOR	ADDITIONAL INFORMATION
EGF	1. Enhances epidermal growth and keratinization in vivo. 2. Causes hyperplasia of rabbit corneal epithelium in vivo. 3. Mitogenic for a wide variety of epidermal and mesenchymal cells in vitro.	6045 (Murine)	1. Single chain polypeptide; 53 amino acids long. 2. 3 intrachain disulphide bonds. 3. Contains methionine - therefore is cleaved by cyanogen bromide	1217 amino acids +130 000 daltons (28) (29)	4.9 (29)	Male mouse sub-mandibular salivary gland is richest source - production is under the control of androgens. EGF is also present in human tissues - but there is no concentrated organ-source.	180 000 dalton transmembrane glycoprotein - with tyrosine kinase activity.	
TGF-α	1. Together with TGF-β, this factor causes transformation of rat NRK-49F cells in vitro. 2. Competes with EGF for binding to EGF receptor. 3. Does not cross-react with antibodies to EGF.	5616 (Rat)	1. Single chain polypeptide; 50 amino acids long. 2. 3 intrachain disulphide bonds with similar placement to EGF. 3. No methionine. Not cleaved by cyanogen bromide.	160 amino acids +18 000 daltons (69)	4.8-5.0	Produced by malignant tumours in vivo and transformed cell lines in vitro.	TGF-α and EGF appear to bind to the same receptor.	TGF-α and EGF are regarded as members of the same family of polypeptides.
TGF-β	1. Together with TGF-α (or EGF), this factor causes transformation of rat NKR-49F cells in vitro. 2. TGF-β alone transforms murine AKR-2B cells in vitro. 3. Does not compete with EGF for binding to the EGF receptor.	25 000 (Human)	Homodimer; two identical 12 500 dalton polypeptide chains linked by disulphide bonds. Cleavage of these bonds destroys biological activity.	391 amino acids. +44 000 daltons (71)	2.4	Many different cell types make TGF-β. The richest source is the blood platelet. Also produced in - kidney - placenta - transformed cells in vitro. - malignant tumours in vivo TGF-β MRNA present in all tissues except liver (71).	1. Receptor is distinct from EGF/TGF-α receptor. 2. +300 000 dalton glycoprotein; probably linked to form a complex of 600 000 daltons.	1. TGF-β not related to EGF or TGF-α. 2. TGF-β has both growth stimulatory and inhibitory properties. It is a bifunctional factor.

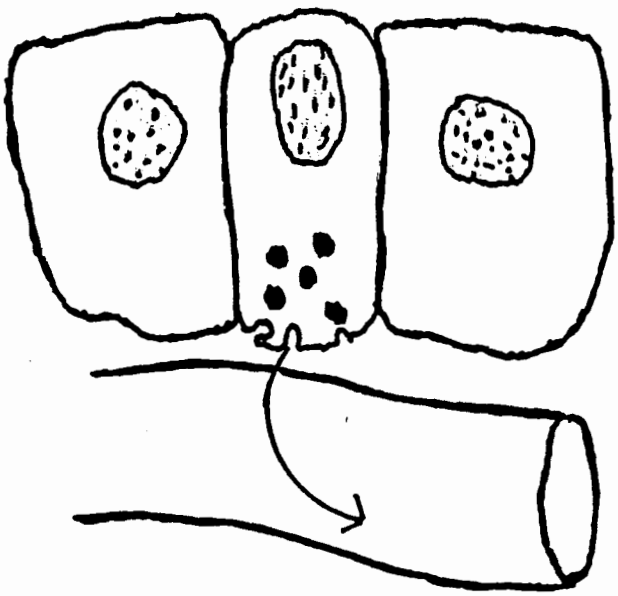
TABLE 1

FACTOR	FACTOR IS DEFINED OPERATIONALLY ACCORDING TO THE FOLLOWING CHARACTERISTICS:	MR OF MATURE POLYPEPTIDE (DALTONS)	STRUCTURE OF MATURE POLYPEPTIDE	PRECURSOR POLYPEPTIDE	MRNA (KILO BASES)	SOURCE OF GROWTH FACTOR	GROWTH FACTOR RECEPTOR	ADDITIONAL INFORMATION
PDGF	1. This factor is stored in platelet alpha granules; it is present in serum, but is absent from plasma. 2. PDGF is mitogenic for a number of mesenchymal cells in vivo. 3. PDGF induces a state of "competence" in resting cells.	Approx. 30 000 daltons (Human)	1. Glycoprotein 2. A heterodimer containing disulphide linked A and B chains. The A and B polypeptides are homologous, but are clearly distinct polypeptides. Both have a Mr of approximately 18 000 daltons.	B-chain precursor from endothelial cells is 241 amino acids long. 27281 daltons (124)	3.5 (endothelial cells) (124)	1. Blood platelets 2. Endothelial cells 3. Cells infected with simian sarcoma virus (SSV) 4. Immature aortic smooth muscle cells. 5. Certain transformed cells.	Approx. 180 000 daltons Possesses tyrosine kinase activity	1. PDGF is probably essential for normal wound healing. 2. The oncogene SIS codes for a PDGF-like molecule. 3. PDGF acts via protein kinase C and stimulates expression of the oncogene C-MYC
IGF	1. Synthesis of IGFs appears to depend on the presence of pituitary growth hormones. 2. IGFs are related, structurally, to insulin and they display a partial cross-reactivity with the insulin receptor; they do not cross-react with antibodies to insulin. 3. Generally, insulin is more potent in producing short-term metabolic effects; IGFs are more important in promoting growth.	Approx. 7400 daltons (Human)	IGF-I and II are described in humans. Both are single chain polypeptides; both of these bear homology to the A and B domain of proinsulin. There is no homology with the C domain. IGF-I is 70 amino acids long. IGF-II is 67 amino acids long. (125-129)	IGF-I 130 amino acids IGF-II 180 amino acids (125)	VARIABLE IGF-I 0.9-7.7 IGF-II 4.9-6.0 (129)	IGFs are probably synthesized by many tissues. mRNA has been detected in liver, heart, lung, kidney, pancreas, spleen, small intestine, colon, brain, pituitary.	IGFs possess distinct cell-surface receptors. These receptor proteins have tyrosine kinase activity.	IGFs are also called SOMATOMEDINS. In humans, the IGF-I gene maps to chromosome 12. The gene spans >35 kilobase pairs (kbp). The IGF-II gene is on chromosome 11 and is at least 15 kbp long. (129)

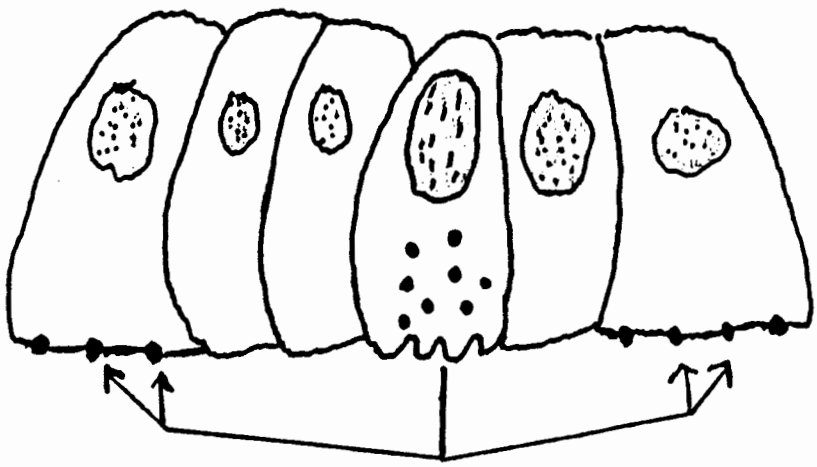
TABLE 1

A considerable amount of research has been directed at the growth factor receptor. These receptors are typically cell surface proteins. The growth factor binds specifically to this cell surface receptor and this binding normally occurs with a very high affinity; the dissociation constant (Kd) of the interaction is in the order of 10^{-10} to 10^{-12} moles per litre. Once the receptor and growth factor are bound together the entire complex may be internalized by receptor-mediated endocytosis. This process has been extensively studied in connection with internalization of low-density lipoproteins (15) and is now known to take place following the binding of EGF (7) and PDGF (16) to their respective receptors. After exposure to PDGF and EGF, target cells will express fewer receptors for the growth factor when compared with untreated cells. What appears to happen is that the target cell synthesises fewer new receptors or alternatively, there is less recycling of receptors following receptor-factor internalization. In this way, a "negative feedback" control is exerted over the number of receptors; the more growth factor present in the surrounding medium, then the less the number of expressed receptors. This phenomenon is termed receptor down-regulation. Many different cell-surface receptors are probably regulated by a similar mechanism.

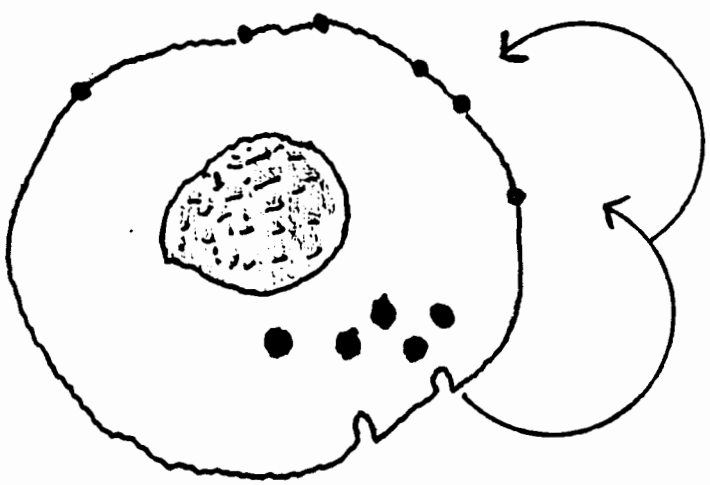
Figure 1 (After Sporn and Todaro (21))



Endocrine



Paracrine



Autocrine

A number of the well-studied growth factor receptors appear to possess intrinsic enzymatic activity which enables them to phosphorylate susceptible tyrosine residues on target proteins. These receptors are therefore classified as tyrosine kinases. The EGF receptor is a tyrosine kinase (17) as are the receptors for PDGF (8), insulin and insulin-like growth factor type I (IGF-I)(18,19). A distinct possibility exists that phosphorylation of proteins at tyrosine may play an important role in the control of cell growth. This hypothesis is supported by recent research which has demonstrated that many viral and cellular oncogenes are now known to code for enzymes which possess specific tyrosine kinase activities (19,10).

A pertinent question concerns the route(s) by which growth factors reach their respective target cells <u>in vivo</u>. Michael Sporn and George Todaro have presented a hypothesis which attempts to define three possible pathways (see Figure 1)(21). In the endocrine pathway, the growth factor is secreted into the circulatory system. In this manner, the factor is transported to its specific receptor which may be localized to a single target organ or, alternatively, may be distributed throughout many different organs. The central feature of the paracrine pathway is that the growth factor engages its receptor after diffusion through the interstitial fluid as shown in Figure 1. A requisite of this

proposal is that the cells that secrete the growth factor must lie in close proximity to the target cells. The autocrine pathway is a revolutionary concept which proposes that certain cells may synthesize and secrete growth factors for which they themselves have receptors. Such cells would be partially or even entirely autonomous with regard to their growth factor requirements. In their presentation of the autocrine hypothesis (21) Sporn and Todaro note that transformed cells in culture have reduced growth factor requirements when compared with non-transformed cells. A newer, modified autocrine hypothesis (22) allows for both stimulatory and inhibitory autocrine factors.

It is not immediately apparent how we might examine these ideas with cells cultivated <u>in vitro</u>. Clearly in cell-culture systems there is no circulation and there are neither "organ sources" nor are there "target tissues". One method to reconcile this problem would be to classify growth factors as being either exogenous or endogenous. Exogenous factors would be those supplied by the serum (typically, fetal calf serum) which supplements the culture medium in most cases. Exogenous factors could be regarded as analogous to endocrine or paracrine growth factors <u>in vivo</u>. On the other hand, endogenous factors are made by the cells themselves.

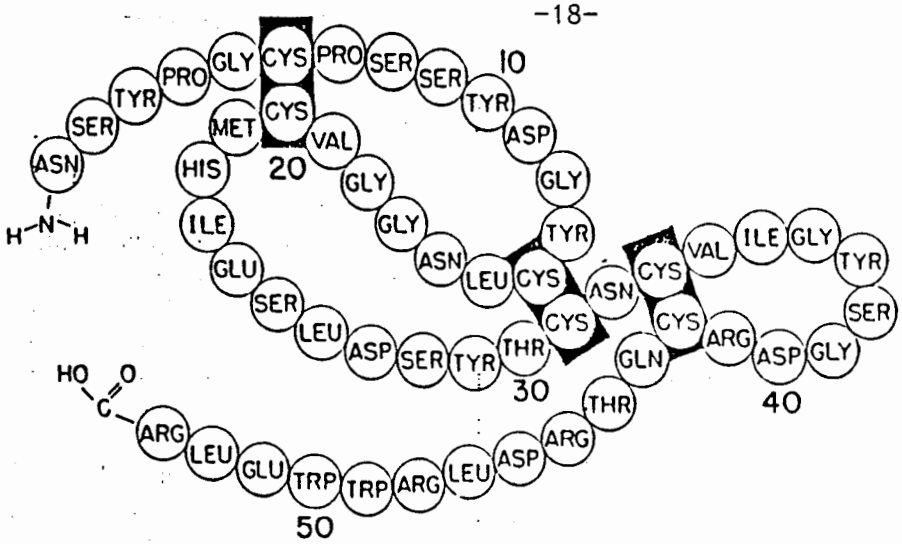
A number of workers have used serum-free cell culture systems to study endogenous factors produced by the cells in vitro, on the reasonable assumption that these, if they could be shown to be autostimulatory, may be the in vitro equivalents of autocrine factors. It should, however, be borne in mind that such factors, notwithstanding the homogeneity of the cell type in culture, may well be paracrine or endocrine in their in vivo action.

This is an appropriate point at which to define the term "conditioned medium", a term that refers specifically to cell culture medium, which may or may not be serum-free, in which cells have been cultured for a defined period. The main features of conditioned medium are that: (a) it contains "waste" metabolites produced by the cells; (b) some of its nutrients have been depleted; (c) it will contain endogenous growth factors. This last attribute has recommended the use of conditioned medium as a starting point for the purification of growth factors. In most instances it has been difficult to prove that the purified endogenous growth factor has a direct inhibitory or stimulatory effect upon the cells which synthesize it, although the matter is receiving considerable attention in the literature (22). Conditioned medium is certainly not the only source of growth factors. For example, growth

factors have also been isolated from extracts of cells in culture as well as from whole organ extracts.

I would now like to discuss in some detail the salient features of certain very important growth factors that have elicited much excitment in scientific circles. Before concluding this introduction, however, two significant points need to be made. Firstly, growth factors are best seen as being part of a broad family of regulatory polypeptides; this group would comprise all polypeptide hormones, neuropeptides, paracrine-acting gastrointestinal hormones and, of course, cell growth factors.

Secondly, although it is true that most of our knowledge of growth factors has been derived from highly artificial cell culture systems, it is nevertheless almost certain that these factors do play an essential part in the growth and development of normal tissues as well as in wound healing (8,23). There is also some evidence that autocrine pathways may operate normally in certain tissues during embryogenesis (24). These pathways would eventually be "switched off" as the embryo matured and would only be reactivated if a malignant state were to develop.



murine EGF (6)

Figure 2

EGF (Epidermal Growth Factor) is a 53 amino acid polypeptide with a molecular weight of 6045 daltons. It exists at a high concentration within the submandibular salivary gland of male mice. Despite its name, EGF is mitogenic for both epithelial as well as mesenchymal cells <u>in vitro</u>.

EGF has been identified in human tissues and body fluids. However, in humans, unlike mice, there is apparently no concentrated source of the growth factor.

EPIDERMAL GROWTH FACTOR (6,7)

Murine epidermal growth factor (EGF) is the form of this growth factor that has been studied most intensively. This is because EGF is present in very high concentrations in the male mouse submandibular salivary gland. The term EPIDERMAL originates from observations that subcutaneous injections of the factor into newborn mice would result in precocious opening of the eyelids and premature eruption of the incisors. Enhancement of epidermal growth and keratinization appeared to be responsible for these phenomena. Subsequently, it was demonstrated that EGF was mitogenic for corneal epithelial tissue in vivo; EGF was found to induce hyperplasia of the corneal epithelium if applied topically to experimentally wounded rabbit corneae. Fetal lambs treated with EGF in utero manifested a proliferative response of the lungs, trachea and oesophagus.

EGF is mitogenic for both epithelial as well as mesenchymal cells in vitro where, characteristically, it will allow cells to grow under "serum-starved" conditions. Thus, diploid human fibroblasts grow rapidly in EGF-supplemented medium containing only 1% serum. (A serum concentration of 10% is normally used to produce an optimal rate of cell growth). EGF has also been reported to increase the

saturation density achieved by certain cell types in culture. For example, Carpenter and Cohen have demonstrated that EGF treatment of human fibroblasts can result in the formation of orderly multiple cell layers, in contrast to the confluent monolayer which is the normal pattern for these cells (6).

Murine EGF (mEGF) is found in the highest concentration in the submandibular salivary glands of the mature male mouse where concentrations may be as high as 1 ug per mg of tissue (wet weight). Its accumulation in this gland appears to be under androgenic control since it has been shown that female mice have much lower submandibular gland EGF concentrations - approximately 70 ng (0.07 μg) per mg of tissue (wet weight). As one might expect, there is a marked difference in the EGF content of the saliva in male and female mice (1-2 μg/ml and 50 ng/ml respectively). Paradoxically, plasma concentrations of EGF in mice appear to be the same for both sexes - 1-2 ng/ml. Murine milk is reported to contain 300 ng/ml of EGF. There is no known explanation for the high salivary gland EGF in mice, especially the males. Recently, Okamoto and Oka (25) have published experiments that may shed some light on this phenomenon. Their work has suggested that submandibular gland EGF may be essential for normal milk production in lactating female mice.

EGF has also been identified in humans, but there does not appear to be a concentrated source of EGF in any tissue or organ. The concentration in human submandibular salivary gland (1-2 ng of EGF per gram of tissue (wet weight)) is only one millionth that found in the male mouse. The EGF concentration that is present in various human body fluids is as follows (6):

Plasma - 2ng/ml; Saliva - 12 ng/ml; Milk - 80 ng/ml; Urine - 100 ng/ml.

The daily urinary excretion of EGF, about 50-60 μg per 24 hours, is high relative to its low tissue concentration. Human urinary EGF is probably identical to a hormone that was previously identified as β-urogastrone, and characterized by its ability to inhibit gastric acid secretion.

Savage and Cohen (26) used acid extraction, followed by chromatography on Bio-Gel P10 and DEAE-cellulose ion-exchange chromatography to purify EGF from male mouse submandibular salivary gland. More recently, Burgess et al. (27) have purified mEGF on reverse-phase HPLC columns.

mEGF is a single-chain polypeptide comprising 53 amino acids. It has a relative molecular mass (Mr) of 6045 and a

pI (isoelectric point) of 4.6. The molecule contains 3 intrachain disulphide bonds and sedimentation studies suggest that it exists as a relatively compact globular peptide. Alanine, phenylalanine and lysine do not appear in mEGF. Its single methionine residue makes it susceptible to cleavage by cyanogen bromide.

The molecule is both heat and acid stable - attributes that greatly facilitate the purification of active growth factor under acid conditions. When isolated at neutral pH, mEGF is found to consist of a high molecular weight complex comprising two molecules of EGF (Mr 6045) non-covalently associated with two molecules of an EGF-binding protein (Mr 29 300). The intact complex, termed High Molecular Weight (HMW) EGF, has an Mr of ±74 000. HMW EGF is stable over a pH range of 5 to 8. It is of interest to note that the EGF-binding protein has proteolytic activity and functions as an arginine esterase. The C-terminal arginine of EGF is essential for binding to this esterase. It is believed that the EGF-binding arginine esterase may be involved in the proteolytic processing of precursor forms of EGF. As is the case with many other regulatory polypeptides, a "pre-prohormone" or precursor form of EGF has been identified (28,29). The molecule, having an Mr of ±130 000, is very large and contains 1217 amino acids. The EGF precursor is

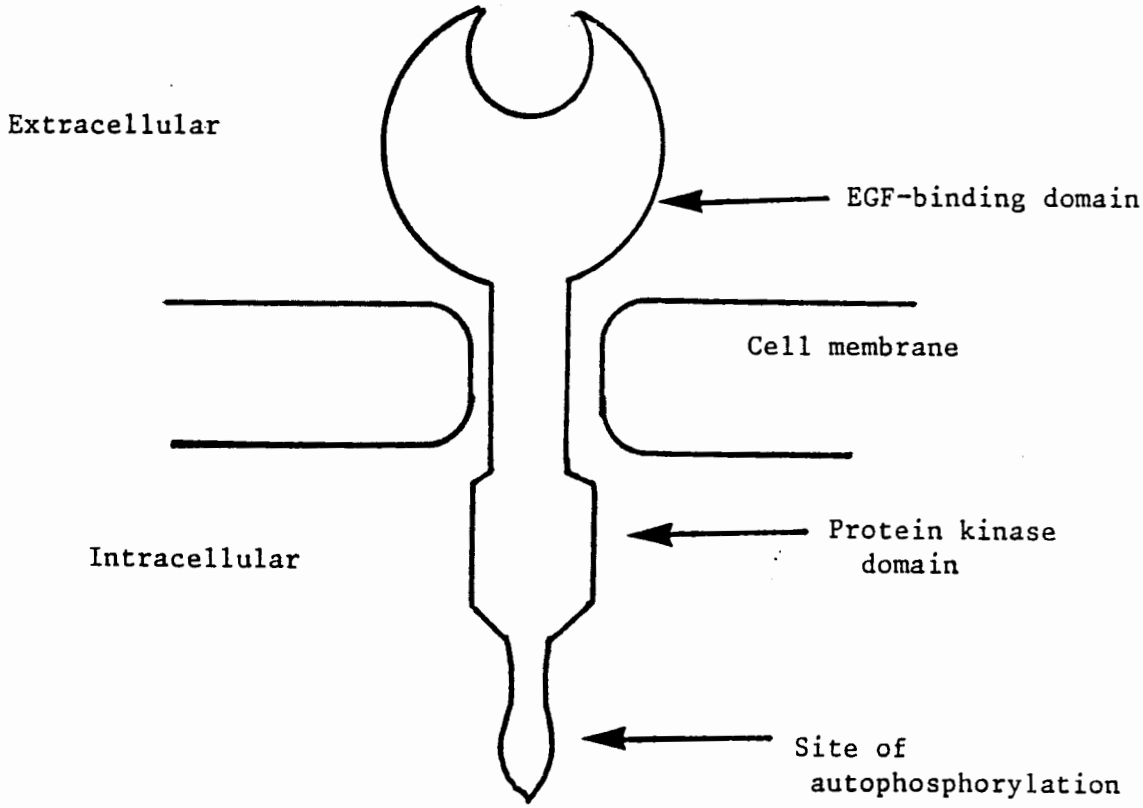


Figure 3

The receptor for EGF (Epidermal Growth Factor) is a 180 000 dalton transmembrane glycoprotein. The internal cytoplasmic domain of the receptor possesses tyrosine-specific kinase activity. In many respects, the EGF-receptor has come to represent a "prototype" for growth factor receptors.

therefore more than twenty times larger than the mature growth factor.

Human EGF (hEGF) appears to be structurally similar to mEGF, and has a Mr of ±6000. The murine and human polypeptides cross-react antigenically.

The EGF receptor is perhaps the best studied of all the known growth factor receptors (17,30,31,32). Human fibroblasts possess about 40 000 to 100 000 EGF receptors per cell, while the transformed cell line A431 (derived from an epidermoid carcinoma) has two to three million EGF receptors per cell. The equilibrium dissociation constant (Kd) for the interaction of EGF with its receptor is probably in the order of 2-4 x 10^{-10}M. The binding of EGF to its receptor appears to result in the specific uptake and internalization of the EGF-receptor complex. Following this process of "receptor-mediated endocytosis", the internalized EGF is degraded in lysosomes (7).

The EGF receptor is a 180 000 dalton transmembrane glycoprotein with intrinsic tyrosine-specific kinase activity (17,30,31,32). The extracellular portion of the receptor contains the EGF-binding domain; the protein kinase domain is found intracellularly (see diagram, Figure 3). The major

autophosphorylation site appears to be found on the C-terminal tail of the molecule.

In conclusion, EGF can be regarded as a "prototype model" for growth factors because it has been studied so extensively by many workers. However, much work still needs to be done to determine the true physiological role of EGF *in vivo*.

PLATELET-DERIVED GROWTH FACTOR (6,8)

Platelet-derived growth factor (PDGF) is a powerful mitogen released from the alpha-granules of platelets and its discovery stemmed from the observation that serum derived from clotted blood has superior mitogenic properties when compared with plasma. Balk and co-workers (33) in 1973 were among the first to suggest that serum might contain a mitogenic factor that was not present in plasma. By 1974, Ross et al. (34) and Kohler and Lipton (35) had identified the platelet as the source of the mitogenic factor. Ross used arterial smooth muscle cells for his assays. He showed that whereas these cells proliferated at a normal rate in whole blood-derived serum, they remained dormant in serum derived from cell and platelet-deprived plasma.

PDGF appears to be mitogenic for a number of cells of mesenchymal origin, including skin fibroblasts, mouse embryo 3T3 cells, smooth muscle cells and glial cells. It is powerfully chemo-attractant for neutrophils, monocytes, smooth muscle cells and fibroblasts and is able to activate neutrophils to generate and release active oxygen species.

These features of PDGF strongly suggest that it has a normal physiological role in wound healing. An attractive hypothesis is that PDGF may orchestrate the process of thrombus "organization" - the well recognized, orderly sequence of events by which a blood clot is replaced with fibrous tissue. PDGF may also act as an "atherosclerosis factor" that is released by platelets that aggregate at the site of endothelial damage. Arterial smooth muscle cells are thus induced to migrate through the internal elastic lamina and to proliferate in the subintima.

Stiles and co-workers (36,37) have highlighted the role played by PDGF during the normal cell cycle. PDGF renders the cells "competent" to leave the G0/G1 phase of the cell cycle. In Stiles' terminology, PDGF is a "competence" factor. It does not initiate DNA-synthesis on its own. Other growth factors (termed "progression" factors) are necessary for the movement of competent cells into the S-phase of the cell cycle. Without the prior action of

competence factors, progression factors acting alone are similarly ineffective. The insulin-like growth factors (IGFs) belong to the category of progression factors.

The structure of PDGF has been studied in detail in a number of laboratories (8,38,39). It is a faily large basic glycoprotein (Mr ±30 000 pI 10.2) that is found in two forms (8,38). PDGF-I is approximately 31 000 daltons and contains 7% carbohydrate. PDGF-II is approximately 28 000 daltons and contains 4% carbohydrate. The intact PDGF molecule comprises two disulphide-linked polypeptide chains, A and B. The A chain has an Mr of approximately 17 000 in PDGF-I and approximately 15 000 in PDGF-II. The B chain has an Mr of approximately 14 000 in both PDGF-I and -II. Separation of the intact molecule into the two individual chains destroys its biological activity.

Some of the best evidence for autocrine control of growth has been derived from studies of PDGF. It is now known, for example, that cells infected with the Simian Sarcoma Virus (SSV) secrete a PDGF-like molecule owing to the presence of the v-sis oncogene in the viral genome (40,41,42,43,44). This viral oncogene like its counterpart in mammalian cells, c-sis, is known to code for a molecule that has a PDGF-like structure and can bind to receptors for PDGF. Huang, Huang and Deuel (45) demonstrated that an

antiserum to PDGF was able to block DNA-synthesis in SSV-infected cells. The implication of this work is that the cells were being autostimulated by the PDGF-like oncogene product of the SSV. Garrett et al. (46) were able to show that while SSV-infected cells did not display PDGF receptors under normal conditions, treatment with suramin (a compound which prevents PDGF binding to its receptor) allowed the cells to express the PDGF receptor. These workers concluded that the virally-induced autocrine production of PDGF-like molecules had downregulated the PDGF receptors in the cells. Seifert et al. (24), showed that cultured aortic smooth muscle cells derived from very young rat pups secreted PDGF-like molecules. No such molecules were secreted by comparable cultures of smooth muscle cells isolated from adult rats. Clemmons et al. (47) had previously shown that fetal fibroblast cultures secreted a functional homologue of PDGF. Equivalent cultures from human donors (post-infancy) did not produce PDGF. The major implication of these studies is that autocrine secretion of growth factors may serve a physiological purpose during embryogenesis.

TRANSFORMING GROWTH FACTORS (9,10).

The transforming growth factors (TGFs) are a family of regulatory polypeptides that are operationally defined by their ability to induce malignant transformation of non-transformed indicator cells *in vitro* (9,10). The features of malignant transformation of cells in culture include:-

(a) loss of density-dependent inhibition of growth in monolayer culture,

(b) disruption of the normal growth pattern of the cells resulting in the formation of randomly criss-crossed multiple cell layers (48) and

(c) anchorage-independent growth i.e. the ability to form growing colonies of cells in soft-agar culture.

The indicator non-transformed cell line that responds so characteristically to the transforming growth factors is the NRK-49F rat kidney cell line (48,49). The parental NRK line was originally established by Duc-Nguyen et al. in 1966 (50) and the ability of clone 49F to respond so dramatically to transforming growth factors was first reported in the 1970's by Todaro and De Larco who undertook much of the pioneering work in this field (48,49,51,42,53).

Using the NRK-49F cell assay, Roberts and co-workers have identified at least two types of transforming growth factors; these are designated type alpha (TGF-α) and type beta (TGF-β)(9). In the NRK-49F indicator cell system, both types of TGFs must be present to induce the characteristic malignant transformation (9), whereas TGF-β alone is sufficient to initiate transformation of AKR-2B mouse embryo cells (54).

Types α and β TGF are very different from one another. TGF-α appears to be mainly associated with malignant cells and has been isolated from the conditioned media of many transformed cell lines in vitro, as well as from whole extracts of transformed cells (55). A high molecular weight form of TGF-α has been isolated from the urine of patients with disseminated malignancies (56). TGF-α has also been described as existing in mouse embryos (57) and it may possibly be present in the placenta (58). TGF-β is more ubiquitous than TGF-α. The type beta form has been isolated from a wide variety of both neoplastic and non-neoplastic tissues (59-66). The richest source of TGF-β is the blood platelet (64) and it is likely that this growth factor, like PDGF, functions in the wound healing response (23). TGF-β has also been isolated from human placenta (63) and from bovine kidney (65). Many transformed cells in culture have been shown to produce TGF-β (9,10,62,71).

TGF-α is characterized by its ability to compete with epidermal growth factor (EGF) for binding to the EGF receptor (9,10). This can be explained by the significant degree of amino acid sequence homology that exists between EGF and TGF-α as well as by the similar placement of intra-chain disulphide bonds in these two molecules. Rat TGF-α (or type I TGF) has been sequenced by Marquardt et al. (67). It is a 50 amino acid polypeptide of Mr 5616 and it has 33% and 44% homology with murine and human EGF sequences respectively (67,68). Type-α appears to be made intracellularly as a 160 amino acid precursor polypeptide (this would have an expected Mr of ±18 000) (69).

Although TGF-α and EGF belong to the same family of growth factors inasmuch as they have a similar structure and they share the same cell-surface receptor, the TGF-α molecule does not cross-react with antisera specific for EGF (10). Another distinguishing feature is the lack of the amino acid methionine in the TGF-α molecule (55,70). Methionine occurs in both murine and human EGF so that EGF is susceptible to cleavage with cyanogen bromide (70) whereas TGF-α is not.

TGF-β bears no structural similarity to EGF. TGF-β is a 25 000 dalton protein that comprises two 12 500 dalton polypeptide chains bound by disulphide bridges (62). If the disulphide bonds are cleaved, TGF-β loses all biological

activity. Available evidence indicates that TGF-β is a homodimer i.e. the two polypeptide chains are identical (71). The precursor form of each polypeptide chain comprises 391 amino acids (71) giving it an estimated Mr of 44 000. TGF-β has a unique cell surface receptor and does not bind to the EGF receptor (72,73,74). The TGF-β receptor appears to be a glycoprotein with a Mr of 280 000 - 330 000 daltons. This glycoprotein is probably linked via disulphide bonds to form a complex with a Mr of approximately 600 000 (10,73,74).

Both TGF-α and TGF-β are very stable molecules that are resistant to low pH and to organic solvents (55,62,70). The initial steps in TGF purification protocols thus typically involve the extraction of tissue or lyophilized conditioned medium with 1M acetic acid followed by gel-filtration chromatography under acid conditions (55,62,70). Reverse-phase HPLC has been used with such great success (75) for the purification of both TGF-α and TGF-β that it may fairly be said to have revolutionized the purification of these compounds.

TGF-β displays unique growth inhibitory properties (76) and it can function both as a stimulator or an inhibitor of cell division; the eventual cellular response depends on the surrounding milieu of growth factors as well as the cell

type that is being studied (76). This phenomenon will be dealt with once more in the section on growth inhibitory factors. The widespread distribtuion of TGF-β as well as its unique bifunctional growth-controlling properties imply that is may be important in the control of cell division in normal tissues.

Since tumour cells in culture may produce TGF-α and TGF-β, it has been suggested (10,22) that these growth factors might be involved in the autocrine control of tumour cell growth. Evidence also exists to indicate that a TGF-α-related compound is responsible for the common and distressing hypercalcemia that may accompany malignancies (77,78).

THE INSULIN-LIKE GROWTH FACTORS (79)

Human plasma is known to contain a family of potent growth factors that are structurally similar to insulin. These insulin-like growth factors (IGFs) are often referred to as "somatomedins" since there is evidence to indicate that pituitary growth hormone exerts a control over plasma IGF levels and that many of the biological effects of growth hormone may be mediated by the IGFs (79).

The two main IGFs in humans are IGF-I (Mr 7469, 70 amino acids long) and IGF-II (Mr 7471, 67 amino acids long). IGF-I is now known to be identical with the molecule previously designated "somatomedin C".

The regulation of the synthesis and release of IGF-I is more sensitive to growth hormone than is that of IGF-II and patients with acromegaly consequently have high IGF-I plasma levels whereas their IGF-II levels are normal (80). In growth hormone deficiency, however, both IGF-I and IGF-II levels are decreased. IGF-I is now believed to be important in post natal growth (80,81,82) in contrast to IGF-II which is implicated in intra-uterine growth (82,83,84).

IGFs show approximately 40% homology with the A and B domains of human proinsulin (84,85), and IGF-I and II have 62% amino acid sequence identity with each other. Although IGFs do not cross-react immunochemically with insulin, a significant degree of IGF binding to the insulin receptor does occur. Two subtypes of IGF receptor are described (79) - one preferentially binds IGF-I, whilst the other prefers IGF-II. The IGF-I receptor is structurally similar to the insulin receptor and like the latter, it possesses tyrosine kinase activity (18,19).

Unlike insulin, which is found free in the plasma, IGFs are bound to high molecular weight carrier proteins (79). These binding proteins may function to prevent IGFs from exerting acute metabolic effects such as hypoglycemia.

IGFs are important stimulatory growth factors. They are well known for their ability to promote radioactive sulphur incorporation into the proteoglycans of growing cartilage. The elegant work of Stiles et al. (37) has shown that the IGFs cooperate with other growth factors, especially PDGF, to allow progression through the cell cycle. Massagué et al. highlighted the importance of IGFs when they showed that the transforming activity of TGF-β was dependent on the presence of IGFs (86).

Since fibroblasts can produce IGFs in monolayer culture, it is possible that many tissues synthesize IGFs <u>in vivo</u>. There does not appear to be a concentrated organ-source of IGF.

GROWTH INHIBITORY FACTORS

In this review of growth factors, I have emphasized the growth-stimulating properties of these molecules. I shall devote the remainder of this chapter to growth inhibitory factors. These are of special relevance to this thesis which deals with the characterization of an inhibitory factor. Only those factors that have been well-characterized biochemically will be considered in the following discussion. This group includes the interferons, type-beta transforming growth factor, tumour necrosis factor and lymphotoxin. TGF-β, which I have previously discussed is unique because of its bifunctional stimulatory and inhibitory properties. This emphasizes the concept that stimulatory and inhibitory factors should be regarded as being part of the same family of growth regulatory polypeptides.

A considerable body of literature has now accumulated around the subject of growth factors and their relationships to the "chalones" - the putative growth regulatory substances of a previous era when definitive purification techniques for the characterization of such compounds were not available. These issues are discussed in several comprehensive reviews

(6,87) and little purpose would be served by repeating them here.

THE INTERFERON SYSTEM (11).

The interferons (11,12,13,88,89) are a related yet heterogenous group of proteins that were first identified as cellular secretory products whose synthesis and secretion were induced by exposure to viruses, bacteria or other foreign macromolecules.

The interferons are best known for their anti-viral activity i.e. their ability to inhibit viral replication in susceptible target cells, and as recently as 1980, an international group that met under the sponsorship of the National Institute of Allergy and Infectious Diseases and the World Health Organization (112) agreed that an interferon should be defined as a "protein which exerts virus nonspecific antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein". Pestka and Baron (88), however, as if recognizing the difficulties that may arise when compounds are named or classified exclusively on the basis of a single common function, have stated that "...it should not be surprising [when studying interferons] to find a homologous molecule with antiproliferative and other

activities, but essentially devoid of antiviral activity. If such a putative molecule shows extensive homology to interferon it should be considered an interferon." These comments of Pestka and Baron emphasize the need for molecular characterization of all biologically active molecules and also draw attention to the now well-known fact that interferons may inhibit cellular proliferation in vitro.

Both non-transformed cells, including embryonic cells, as well as transformed cells are sensitive to the anti-proliferative property of interferon (11,12,88, 89,90,91,92,93). Interferons have now been accepted as being "negative growth factors" (90). Implicit in this definition is the suggestion that interferons may have an important regulatory role in controlling growth. If this is so, then these molecules are the first well-characterized growth inhibitory factors.

Interferons almost certainly have multiple biological functions, and these can be listed as follows:-

(1) Inhibition of viral replication.
(2) Anti-proliferative effect (decreased rate of cell division).
(3) Regulation of cellular differentiaation.
(4) Immunomodulation.

Interferons have been studied most fully in the human and mouse systems and have been classified into three major groups - α, β and γ - according to their antigenic properties. A summary of the formal nomenclature is given below:

Formal Designation	Abbreviation	Alternative Name
Alpha interferon	IFN-α	Leukocyte interferon
Beta interferon	IFN-β	Fibroblast interferon
Gamma interferon	IFN-γ	Immune interferon

(11)

Alpha interferon is the major species produced by virus-stimulated leukocytes. The synthesis of IFN-α was once regarded as the exclusive function of B lymphocytes, macrophages and Null cells, but it has now been shown that it can also be produced by T lymphocytes (94). Fibroblasts infected with virus produce predominantly the beta type of interferon, although some IFN-α will also be made. On the other hand, fibroblasts that have been induced with synthetic double-stranded RNA (dsRNA) will produce exclusively IFN-β. Virus-infected lymphoblastoid cell lines are commonly used as a source of interferon. Here the

interferon is a mixture of the α and β types, with the beta form predominating. Gamma interferon (IFN-γ) is a recently characterized interferon that is produced by T lymphocytes and Natural Killer (NK) cells (94).

All three types of human interferon are polypeptides in the 18000-25000 dalton range (11,94). Human IFN-α appears to comprise a family of closely related polypeptides. There are at least 14 distinct subtypes of IFN-α in the human system, each coded for by a different gene (12). In contrast to this, there appears to be only one type of IFN-β (12) and one IFN-γ. (Two forms of IFN-γ, differing in molecular weight, have been described but this is believed to be a result of differences in post-translational processing (94)). Both IFN-β and IFN-γ are glycosylated whereas the IFN-alpha family is non-glycosylated (95). The carbohydrate moieties of the glycosylated interferons appear to be essential for the secretion of these molecules from the cell (95).

All IFNs (IFN-β and IFN-γ in particular) are hydrophobic proteins. IFN-alpha and IFN-β are stable at pH 2.0 whereas IFN-γ is not. In humans, the multiple genes of the α interferon family are clustered on chromosome 9 which also carries the single gene for IFN-β (94,96). The gene for IFN-γ has been localized to chromosome 12 (94,96).

At this stage, not much is known about the interferon receptor. One of the stumbling blocks has been the intrinsic hydrophobicity of the interferon molecule that causes considerable non-specific binding in equilibrium-binding studies (97). It is believed that there is probably more than one type of interferon receptor. In humans, at least one of the interferon receptors appears to be a product of a gene on chromosome 21 (11,12). In a recent paper, Thompson et al. (97) propose that there are distinct receptors for IFN-α and IFN-γ and that IFN-β can bind to both the α- and the γ-specific receptors.

Although the induction of interferon synthesis by mammalian cells is classically the result of a viral infection, there is a group of synthetic, non-viral interferon inducers - the ribopolynucleotides. Perhaps the best known member of this group is polyriboinosinic acid:polyribocytidylic acid (Poly IC).

It is important to appreciate that the interferons are true regulatory polypeptides. They are secreted by specific cell populations and they probably reach their target cells by the circulation or by local diffusion. Binding to specific cell-surface receptors is followed by synthesis of new mRNA and protein. Because of the latter process, interferons cannot exert their multiple effects immediately. It

typically takes several hours for the full effect to manifest (11).

Interferons exert wide-ranging effects upon the cell's biochemistry (12), a full discussion of which would be beyond the intended scope of this thesis. There is, however, no clear-cut mechanism known that would explain the anti-proliferative effect. A recent observation that may be of major relevance is that interferons appear to decrease the expression of a number of cellular oncogenes in transformed cell lines (98). For example, human IFN-β was shown to decrease the transcription of c-myc in Daudi cells (99)(Daudi is a lymphoblastoid cell line that is very sensitive to the growth inhibitory effects of interferon). Transformed mouse 3T3 cells, when treated with murine IFN-β showed a reduction in the m-RNA of the oncogene c-Ha-Ras (100). Whether these oncogene effects are primary (i.e. an immediate result of interferon action) or secondary to the slowing of cell division is an important question that is yet unanswered.

As regards the actual anti-proliferative (i.e. growth inhibitory) property of the interferons, there would appear to be some controversy as to whether this action is reversible. I think that most workers regard the action of interferon as being a non-toxic reversible form of growth

inhibition (11). Certain studies, however, have shown that interferons can exert both a cytostatic as well as a cytotoxic effect in vitro (91).

The technical approach that I adopted to study the growth inhibitor that I report in this thesis stemmed, in large part, from that that had been used to purify interferon. I shall, for this reason, discuss the question of interferon purification in some detail. On reviewing the literature (13,101-108), one cannnot help but echo the words of Robert Friedman (11) when he wrote that "almost every method of protein separation that has been discovered has been employed on the problem of interferon purification..." Fortunately, new developments and accumulated experience have brought order to the field and there are now certain specific methods that are widely employed. For example, much use has been made of the high intrinsic hydrophobicity of the interferons, especially that of IFN-β (109). Hydrophobic interactions are responsible for the binding of IFN-beta to immobilized amino acids (110), as well as to the polychromatic dye Cibacron Blue F3GA (111). The bound IFN-β can be released only when the polarity of the surrounding mobile phase has been sufficiently lowered. This is typically achieved by adding a high concentration of ethylene glycol, a molecule which is much less polar than water. IFN-α is less hydrophobic than IFN-β and as a result

will only bind to immobilized aromatic dipeptides such as L-tryptophyl L-tryptophan (110). Human IFN-α also interacts with Cibacron Blue F3GA, but in this case the binding appears to be primarily electrostatic and is not due to hydrophobic interaction (111). These above mentioned studies have led to improved rapid purification procedures for the interferons. For example, chromatographic methods using "Blue-Sepharose" (which contains Cibacron Blue F3GA) and "Phenyl-Sepharose" (106,108) have been published in recent years for the purification of IFN-β. In both of the purification procedures, ethylene glycol is used as the eluting agent.

Reverse-phase high-performance liquid chromatography (RP-HPLC) has also been used extensively for interferon purification (13). The purification of IFN-α by RP-HPLC was one of the first occasions on which this procedure was used to isolate a "large protein" (i.e. a polypeptide greater than ±12000 daltons)(13,104). RP-HPLC is still the method of choice for the purification of IFN-α, and is also used as a final step in the purification of IFN-β. The advantage of this procedure is that volatile solvents are used. These are easily removed by distillation and one is thus able to recover the purified interferon entirely salt-free.

TYPE BETA TRANSFORMING GROWTH FACTOR

It has been convincingly demonstrated by Roberts et al. (76) that type β transforming growth factor (TGF-β) is a bifunctional factor that can either stimulate or inhibit cellular division. The final outcome of the addition of TGF-β to cells in culture depends on the type of cell and on the presence or absence of associated growth factors in the culture medium. Evidence for this is as follows:

(a) TGF-β, in the presence of either EGF or TGF-α, stimulates growth of NRK-49F rat kidney fibroblasts in soft agar. This is the basis of the assay for transforming growth factors. The growth in soft agar of transformed cell lines, on the other hand, is inhibited by TGF-β. The human lung carcinoma line A-549 is especially sensitive to this inhibitory effect. In these experiments, TGF-β exerts its inhibitory action at concentrations which would normally promote growth of NRK-49F colonies.

(b) Roberts and her co-workers have transfected a cellular myc gene (c-myc) into Fischer rat fibroblasts. In the transfected cell line, now termed MYC-1, the myc gene is under the control of

the Simian virus 40 (SV-40) promoter region. The MYC-1 cells are very sensitive to the growth-promoting action of EGF, and can be stimulated by EGF, to form colonies in soft agar. This action is antagonized by TGF-β, showing that TGF-β inhibits the colony promoting effect that EGF exerts on MYC-1 cells.

PDGF alone has no growth controlling action on MYC-1 cells. However, TGF-β, in the presence of PDGF, causes significant stimulation of growth of MYC-1 cells. TGF-β can therefore function as a bifunctional growth factor in the same cell line.

(c) The inhibitory effects of TGF-β are not confined to cells growing in agar but are also seen with certain monolayer cell cultures. In other words, TGF-β can also inhibit both anchorage-dependent and anchorage-independent growth (76). Again, the lung carcinoma cell line A-549 is sensitive to this form of growth inhibition. In these experiments it was noted that TGF-β is cytostatic only; it had no effect on cell viability.

(d) Robert Holley and his fellow workers (113,114) have identified an auto-inhibitory growth factor present

in the conditioned medium of Green Monkey kidney (BSC-1) cells. Although this itself was a novel finding at the time, the real significance of the discovery was realized only when it was subsequently demonstrated that TGF-β and the BSC-1 inhibitory factor were identical (115).

There is therefore a considerable body of evidence to support the notion that type β transforming growth factor has both stimulatory and inhibitory properties. Roberts et al. have stated that "TGF-β is therefore one of the first peptide 'Chalones' or growth regulators that has been purified to homogeneity" (76).

LYMPHOTOXIN AND TUMOUR NECROSIS FACTOR

Lymphotoxin (LT) and tumour necrosis factor (TNF) are polypeptides secreted by activated lymphocytes and macrophages respectively (116,117). Both molecules appear to possess the unique property of being able to kill certain malignant cell types *in vitro* and *in vivo* without having any effect on normal cells (117). These observations have obvious therapeutic implications.

Both LT and TNF have been purified to homogeneity. The molecules have been sequenced and their respective genes

have been cloned (118-122). Recombinant forms of LT and TNF have been produced (119,120,122). These remarkable achievements are the result of the enormous advances in biotechnology that have occurred in the last ten years.

Lymphotoxin is a 25 000 dalton glycoprotein (119), while tumour necrosis factor is non-glycosylated and has an Mr of 17 000 (120,121). Although the two molecules are clearly distinct from one another, they have a significant degree of amino acid sequence homology; the latter might explain ther similar biological effects (121).

In all experiments, normal cells appear to be unaffected by either LT or TNF. The sensitivity of malignant cells to these factors varies widely. For example, some malignant cell lines (such as HeLa cells) are resistant to the factor (122). In other transformed cells, TNF has only a cytostatic action, while in many other lines, cell lysis occurs.

The normal physiological function of these factors is still a matter of debate. It appears likely that they are major participants in the response of the immune system to invading malignant cells. Playfair et al. (123) have, however, proposed that TNF functions to protect the host against infections with the parasite Plasmodium sp. and that

the tumour necrosis activity is not a physiological function of this molecule. Clearly, there is still much work to be done in this field.

CHAPTER 2

PRELIMINARY CHARACTERIZATION OF THE

INHIBITORY PHENOMENON

CHAPTER 2

INTRODUCTION

Bowes/sf is a transformed human cell line that has the unique ability to grow in the absence of added fetal calf serum. This cell line has been observed in the laboratory for a number of years and it has been shown that growth in vitro of the Bowes/sf cells requires neither polypeptide growth factors nor other additives such as transferrin, steroids or thyroid hormones. The most plausible explanation for this phenomenon is that the Bowes/sf cells produce autocrine stimulatory growth factors, thereby circumventing the need for serum supplementation.

To examine this possibility, the effect of Bowes/sf medium on the proliferation of serum-dependent cells was tested. Such cells, for the purpose of this discussion, can be termed "indicator" cell lines. All the indicator lines tested were transformed human epithelial lines that required fetal calf serum for cell viability, for attachment to the substratum and for proliferation. It was expected that the Bowes/sf conditioned medium would either stimulate the indicator cells or would have no effect.

Paradoxically, it was found the Bowes/sf medium inhibited the growth of a number of the indicator lines, even in the

presence of 10% fetal calf serum. This unexpected phenomenon was observed even when the conditioned medium was serially diluted several-fold making it unlikely that the growth inhibition was simply due to nutrient depletion of the medium by the Bowes/sf cells.

This chapter is an attempt to describe the phenomenon of growth inhibition and represents, therefore, a pragmatic characterization of the factor. Certain essential questions are addressed:

1. What effect does the Bowes/sf conditioned medium have on the kinetics of growth of serum-dependent human cells?

2. What effect does the Bowes/sf medium have on the morphology (by light microscopy) of the growing cells?

3. Is the inhibitory action of the Bowes/sf medium a consequence of some material <u>added</u> to the medium by the cells, or is it due to nutrient depletion?

4. If the inhibitor appears to be a secreted material, is it dialysable, or is it <u>retained</u> by dialysis membranes and by ultrafiltration membranes?

5. Could the inhibitory material be a growth regulatory polypeptide?

6. What is the heat and pH stability of the growth inhibitor?

These questions had to be addressed with an *in vitro* experimental system in which growing cells were used as the indicators. *In vitro* cell culture systems are notorious for the many variables they present so that it is difficult in the preliminary stages of work of this sort, to give attention to all of the questions that arise or to follow all of the many leads that tentative experiments suggest. In circumstances such as these it is generally preferable to define the primary phenomenon operationally and to confine attention to that phenomenon without being distracted by secondary questions that are best left until the descriptive work has been completed.

In this Chapter, therefore, I avoid consideration of the following issues.

(i) The effect of the Bowes/sf growth inhibitor on normal (as opposed to transformed) human epithelial cells.

(ii) The effect of the growth inhibitor on human cells of mesenchymal origin.

(iii) The action of the growth inhibitor on the Bowes/sf cells themselves.

(iv) The possible presence of growth stimulatory factors, the effects of which were hidden (or over-ridden) by the growth inhibitor.

PREPARATION OF BOWES/SF CONDITIONED MEDIUM

Most of the experiments were performed with serum-free medium collected after 24-48 hours of conditioning with Bowes/sf cells. The medium was clarified by centrifugation at low speed (400g, 10 minutes) and the supernatant was made up to 10%v/v with fresh fetal calf serum. The mixture was then sterilized by filtration through either a 0.45 or a 0.22 micron filter and was stored in glass bottles at 4°C for no longer than 4 days.

This medium could then be added to cells in culture to determine effects on cell growth and morphology. Dilutions of serum-supplemented conditioned medium were made with RPMI medium containing 10% fetal calf serum (RPMI-10).

Fetal calf serum was not added to conditioned medium if it was to be concentrated by ultrafiltration or used as the starting material for further purification.

RESULTS

BOWES/SF CONDITIONED MEDIUM INHIBITED THE GROWTH OF SEVERAL HUMAN CELL LINES

The effect of Bowes/sf conditioned medium on the growth and morphology of four human indicator lines was assessed as described in Chart 1. After the conditioned medium had been in contact with the cells for 9 days, several of the lines showed distinct changes in morphology. These changes included alterations in cell shape and an increased granularity of the cell cytoplasm. UCT-BR-1 (a breast carcinoma) and UCT-CA-2 (bronchial carcinoma) appeared to have undergone greater changes than UCT-SQ-1 (carcinoma of the lip). A431 (epidermal carcinoma, rich in EGF receptors) was unaffected.

After 13 days of exposure to the various dilutions of the Bowes/sf medium, the adherent cultures were fixed and stained. Dishes that contained Bowes/sf conditioned medium up to a dilution of 1:64 showed minimal patchy staining of the rings seeded with UCT-BR-1, UCT-SQ-1 and UCT-CA-2 when compared with

Contd./68

CHARTS 1, 2, 3

CHART 1

INHIBITION OF "INDICATOR" CELL GROWTH BY BOWES/SF CONDITIONED MEDIUM

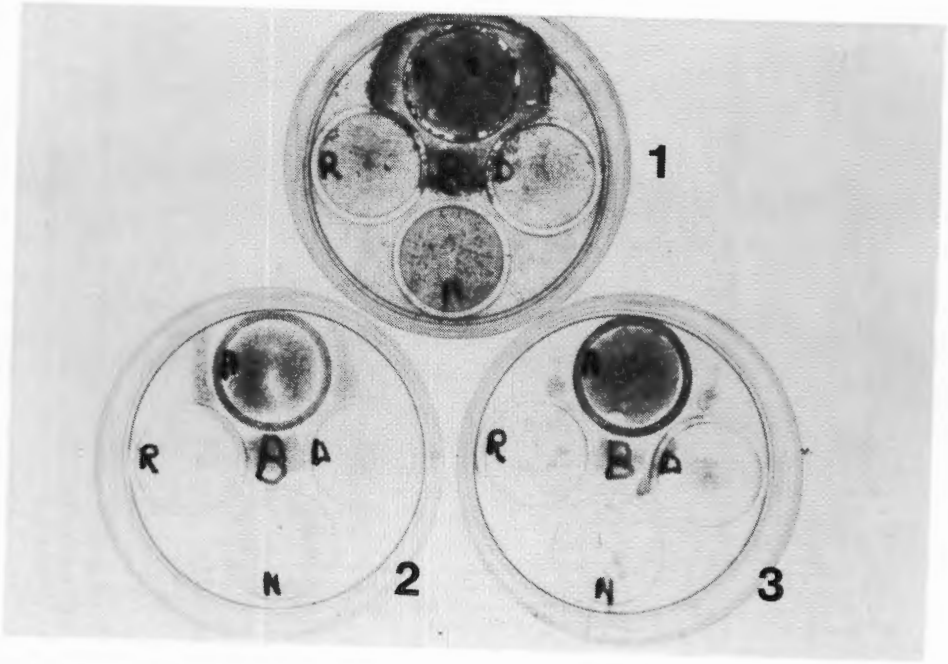
Bowes/sf conditioned medium inhibited the growth of three out of four human cell lines, viz UCT-BR-1, UCT-CA-2 AND UCT-SQ-1. Cell growth was assessed by examining the macroscopic stained pattern of the spreading monolayer.

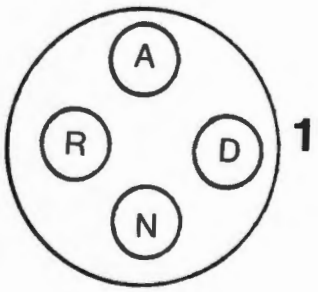
Indicator cells were seeded in eight replicate 35 mm petri dishes, each of which was divided into four shallow circular chambers by 4 internal rings. Each ring enclosed an area of approximately 0.95cm^2 (Greiner Cat. No. 627 170)

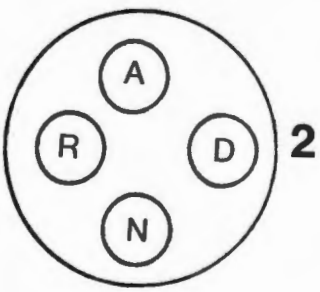
Four cell lines (A431, UCT-BR-1, UCT-SQ-1, UCT-CA-2) were used and each was seeded in an individual chamber so that each dish contained representative cultures of each of the lines. The seeding densities (cells per chamber) were as follows: A431, 3 x 10^4; UCT-BR-1, 4 x 10^4; UCT-SQ-1, 2 x 10^4; UCT-CA-2, 3 x 10^4. The cells, each in a separate volume of medium retained by the shallow rings, were allowed to settle and attach overnight at 37°C. The next day the individual seeding media were aspirated and the petri dishes were filled, each with a dilution of Bowes/sf conditioned medium supplemented with 10% FCS.

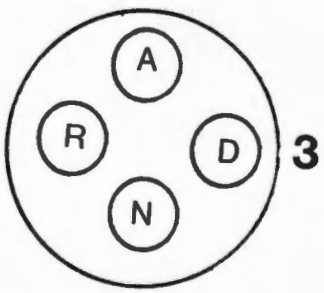
Two-fold serial dilutions of Bowes conditioned medium (from 1:1 to 1:64) were used for 7 of the replicates. The control dish (replicate 8) received RPMI-10.

CHART 1









1. Control, RPMI-10

2. Bowes/sf, diluted 1/32
3. Bowes/sf, diluted 1/64

A: A431
D: UCT-SQ-1
N: UCT-CA-2
R: UCT-BR-1

CHART 2

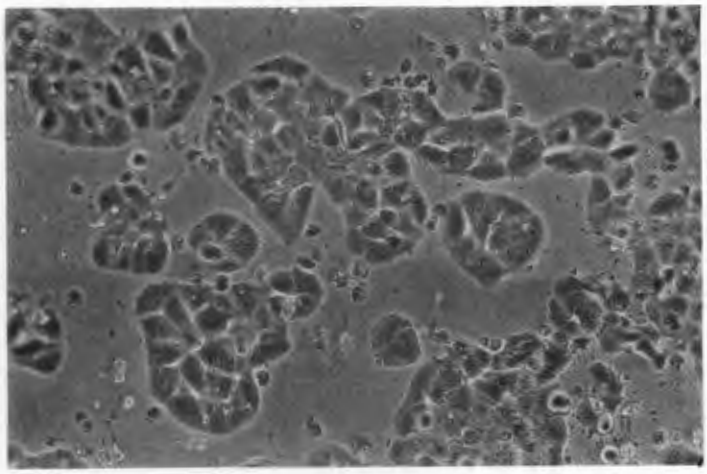
EFFECT OF BOWES/SF CONDITIONED MEDIUM ON CELLULAR MORPHOLOGY

UCT-BR-1, UCT-CA-2, UCT-SQ-1 and A431 cells were grown for 10 days in serial dilutions of Bowes/sf conditioned medium. Undiluted Bowes/sf medium inhibited the growth of all 4 cell lines but only UCT-BR-1 and UCT-CA-2 were affected by dilutions of the conditioned medium. UCT-BR-1 appeared to be the most sensitive indicator line. Cell growth was assessed by phase-contrast microscopy.

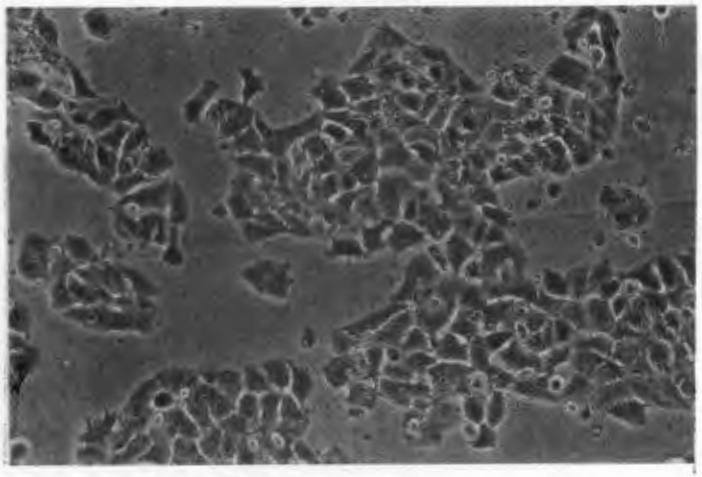
Cells were seeded in 24-well plates at the following densities, in RPMI-10. A431, 1.3 x 10^4; UCT-BR-1, 5.5 x 10^4; UCT-SQ-1, 5.5 x 10^4 and UCT-CA-2, 1 x 10^4. After an overnight incubation at 37°C, the seeding medium was aspirated, and 1.0 ml of serial dilutions of Bowes/sf conditioned medium supplemented with 10% FCS was added. All dilutions were made in RPMI-10. After 3 days of incubation at 37°C the medium was aspirated from all the wells and fresh dilutions of Bowes/sf conditioned medium or RPMI-10 were added. Thereafter a fresh set of conditioned medium dilutions was added daily.

The cells were assessed by daily observation with an inverted phase contrast microscope.

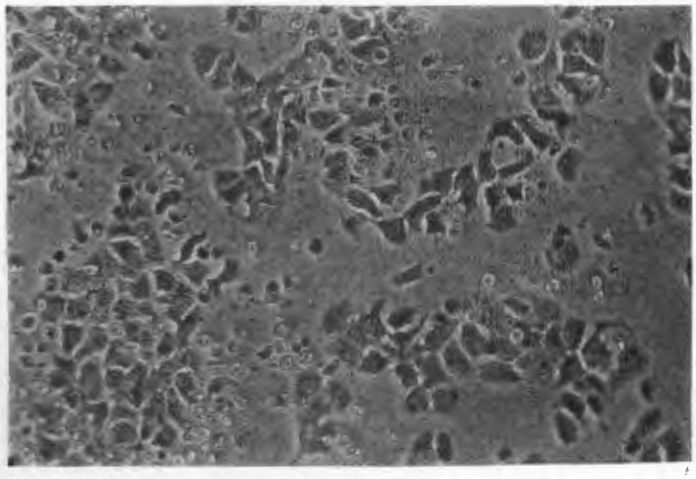
CHART 2
EFFECT OF BOWES/SF MEDIUM ON MORPHOLOGY OF INDICATOR CELLS



1. UCT-BR-1 cells in RPMI-10. 5 days after seeding.

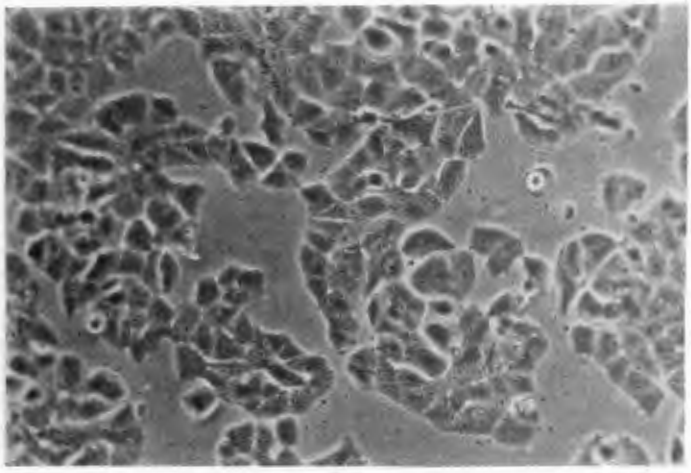


2. UCT-BR-1 cells in Bowes/sf conditioned medium diluted 1/16 in RPMI-10. 5 days after seeding.

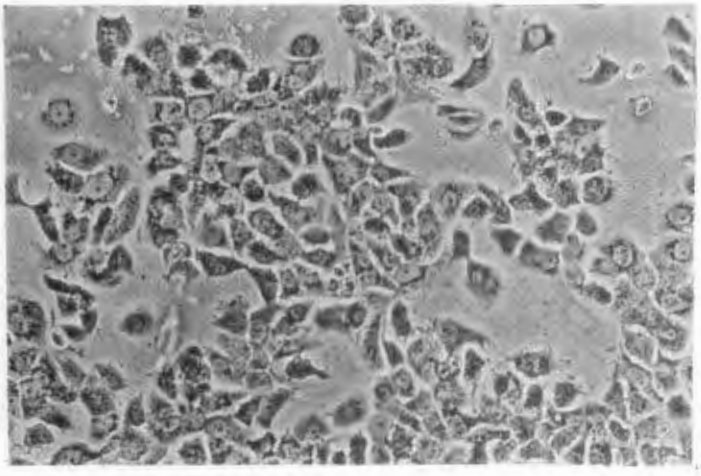


3. UCT-BR-1 cells grown in undiluted ("neat") Bowes/sf medium supplemented with 10% FCS. 5 days after seeding.

CHART 2

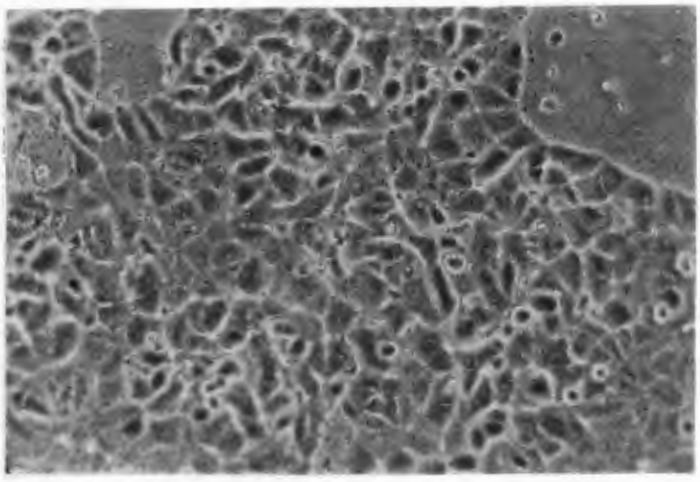


4. UCT-BR-1 cells in RPMI-10.
   8 days after seeding.

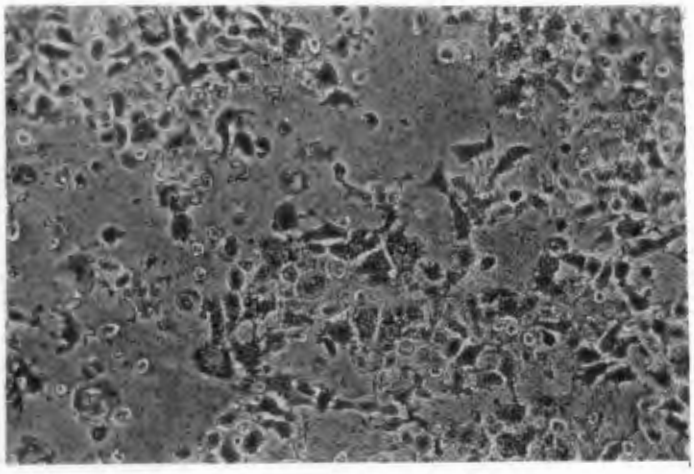


5. UCT-BR-1 cells in "neat" Bowes/sf medium
   supplemented with 10% FCS. 8 days after seeding.

CHART 2

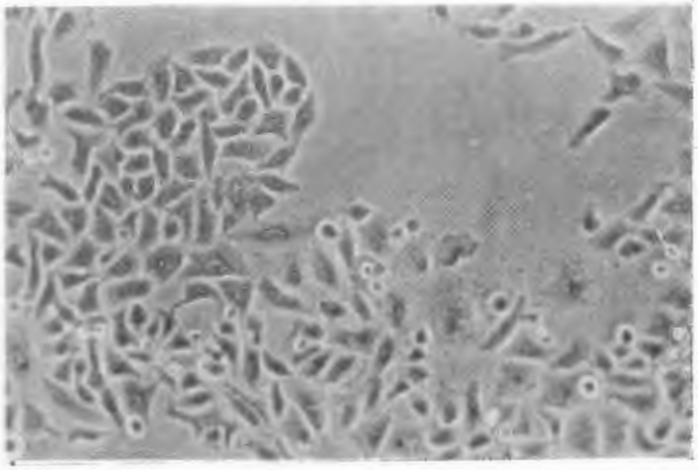


6. UCT-BR-1 cells in RPMI-10.
   10 days after seeding.

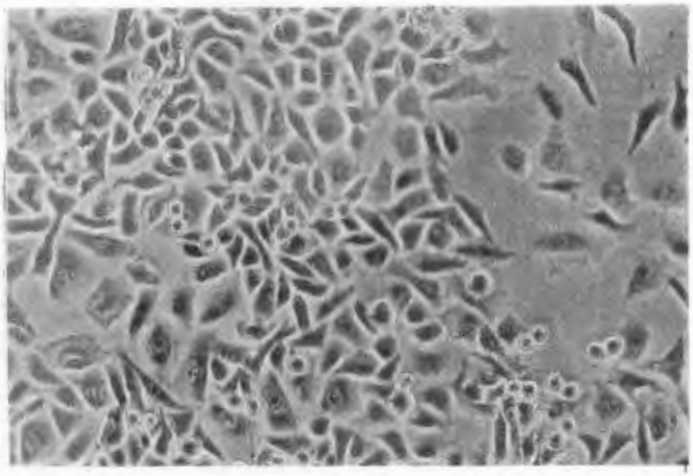


7. UCT-BR-1 cells in Bowes/sf medium diluted 1/16.
   10 days after seeding.

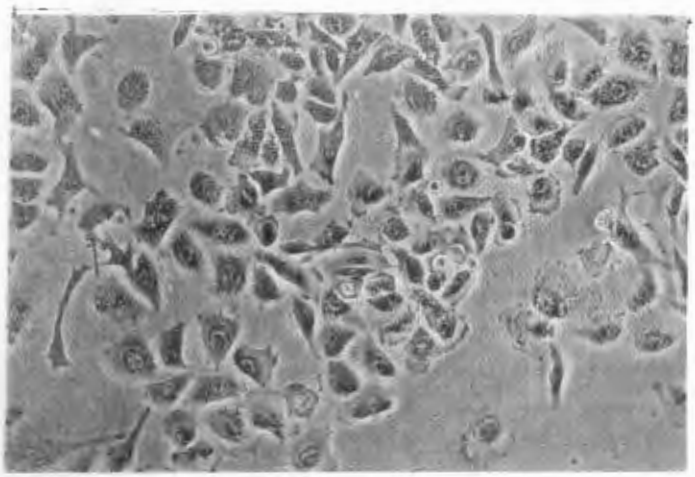
CHART 2



8. UCT-CA-2 cells in RPMI-10. 4 days after seeding.

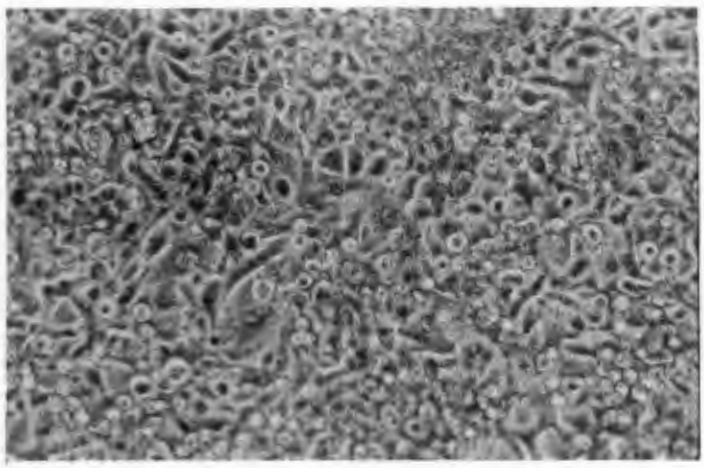


9. UCT-CA-2 cells grown in Bowes/sf medium diluted 1/4 with RPMI-10. 4 days after seeding.

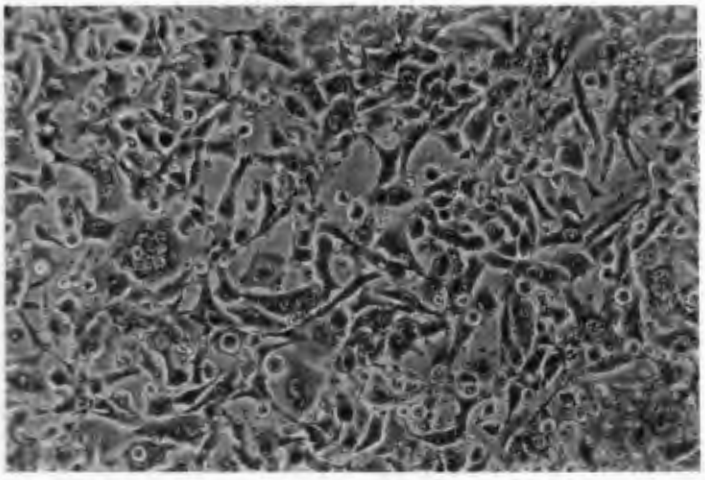


10. UCT-CA-2 cells grown in undiluted Bowes/sf medium supplemented with 10% FCS. 4 days after seeding.

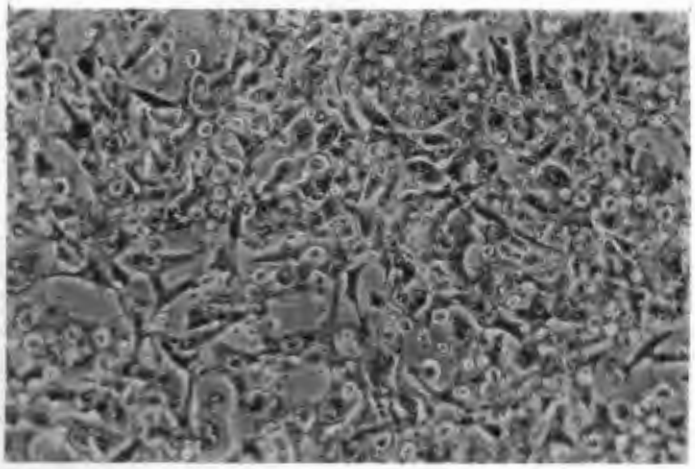
CHART 2



11. UCT-CA-2 cells in RPMI-10. 10 days after seeding.

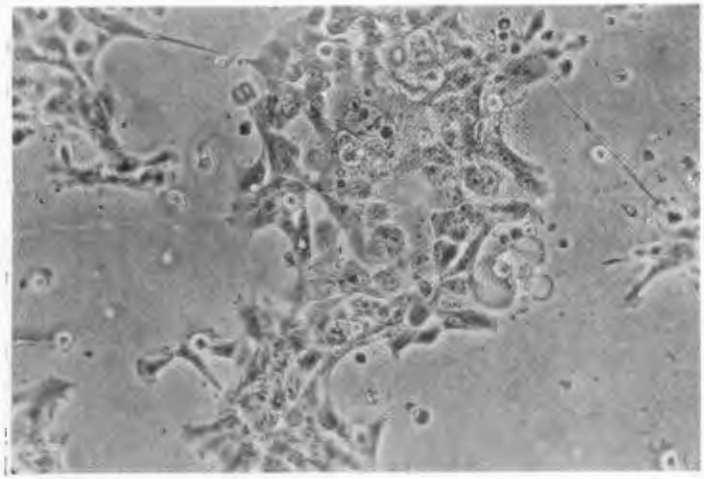


12. UCT-CA-2 cells in Bowes/sf medium diluted 1/16 with RPMI-10. 10 days after seeding.

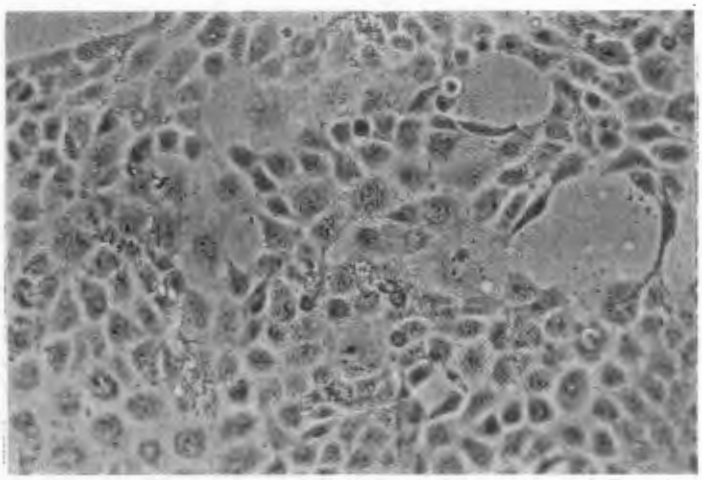


13. UCT-CA-2 cells in Bowes/sf medium diluted 1/2 with RPMI-10. 10 days after seeding.

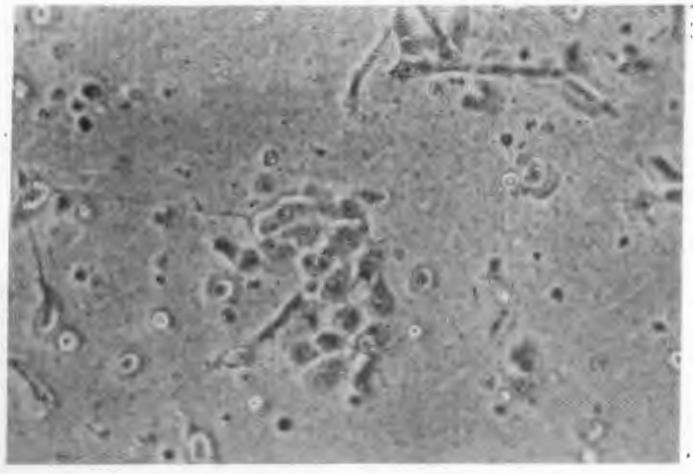
CHART 2



14. A431 cells in RPMI-10. 5 days after seeding.

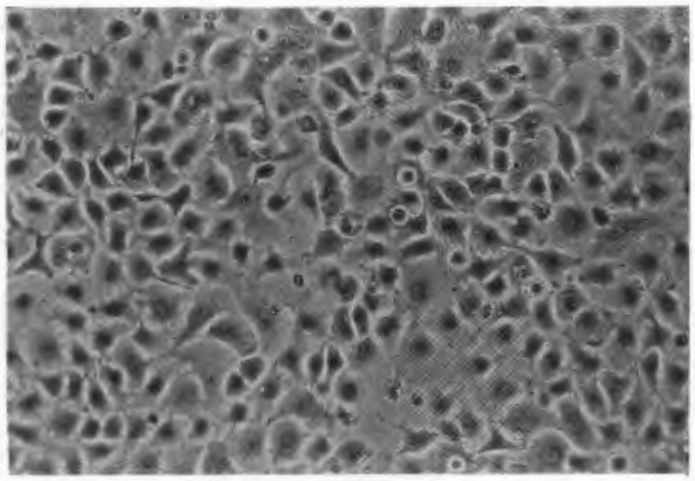


15. A431 cells in Bowes/sf diluted 1/4 with RPMI-10. 5 days after seeding.

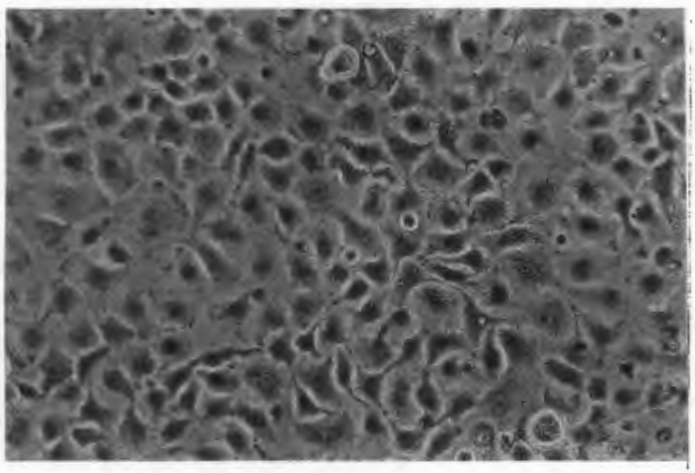


16. A431 cells in undiluted Bowes/sf medium supplemented with 10% FCS. 5 days after seeding.

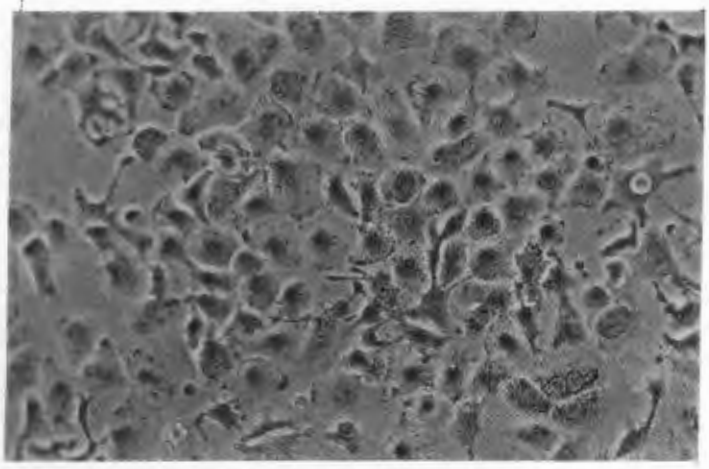
CHART 2



17. UCT-SQ-1 cells in RPMI-10. 5 days after seeding.



18. UCT-SQ-1 cells in Bowes/sf medium diluted 1/2 with RPMI-10. 5 days after seeding.



19. UCT-SQ-1 cells in undiluted Bowes/sf medium supplemented with 10% FCS. 5 days after seeding.

CHART 3

GROWTH OF UCT-BR-1 CELLS IN DILUTIONS OF BOWES/SF CONDITIONED MEDIUM

UCT-BR-1 cells were grown for 9 days in Bowes/sf conditioned medium. Cell growth was significantly inhibited by both 1:2 and 1:4 dilutions of Bowes/sf conditioned medium when assessed at day six and day nine.

UCT-BR-1 cells were seeded in 6-well tissue culture plates at 2.5×10^5 cells per well in RPMI-10. The following day the medium was replaced with the appropriate dilutions of the Bowes/sf harvest fluid. All dilutions were made with RPMI and were made up to 10% (v/v) with fresh fetal calf serum. Cells were fed on alternate days with fresh medium or with the appropriate dilution of Bowes/sf conditioned medium. At the individual time points, cells were released from the plates by trypsinization and were counted with a haemocytometer. Each time point was performed in duplicate.

CHART 3 CONTD.

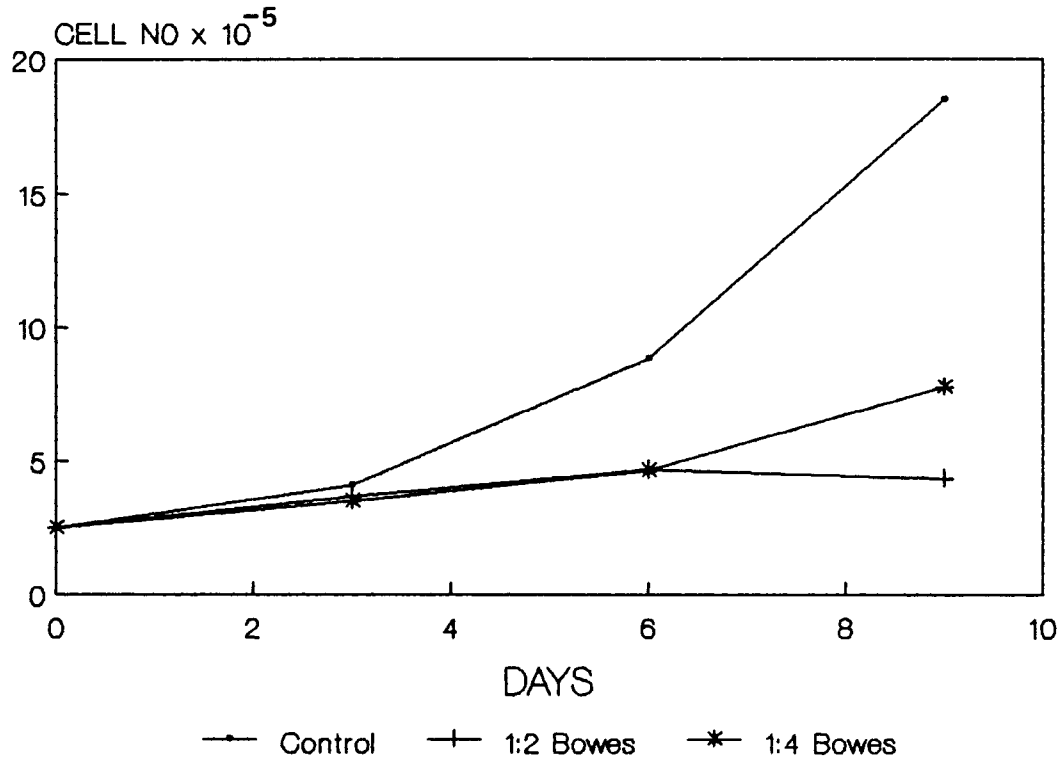
Values shown below are means ± S.E.M. of duplicate cell counts performed on days 3, 6 and 9 of culture. These cell counts need to be multiplied by 10^5 to obtain the true value per well of a 6-well plate. p-values were determined using the Student's t-test.

DAY	CELLS GROWN IN		
	RPMI-10 (CONTROL)	BOWES/SF DILUTED TO 1/2	BOWES/SF DILUTED TO 1/4
0	2.5[Plating density]	2.5[Plating density]	2.5[Plating density]
3	4.1±0.009	3.67±0.22 NS	3.53±0.2 NS
6	8.83±0.2	4.68±0.25 p<0.01	4.64±0.09 p<0.005
9	18.5±0	4.35±0.1 p<0.005	7.77±0.2 p<0.005

NS = Not significant

CHART 3

Growth of UCT-BR-1 in Bowes/sf medium



the staining of the control dish (Chart 1). The A431 cells appeared to have been affected only minimally by the Bowes/sf medium.

A photographic record (Chart 2) was produced in order to demonstrate the effect of serial dilutions of Bowes/sf (up to 1/16) on the indicator cells when present for periods of four to ten days. The indicator cells were the same four human lines used previously. All these cell lines were inhibited by the undiluted ("neat") Bowes/sf medium supplemented with 10% FCS. However, only UCT-BR-1 and UCT-CA-2 displayed obvious changes in the various dilutions of the Bowes/sf medium. After seven days, the UCT-BR-1 cells, in all the dilutions of the Bowes/sf medium showed pronounced changes when compared with cells grown in the control medium (RPMI-10). These changes included an increased cytoplasmic granularity and vacuolation as well as a loss of the normal smooth acinar pattern that is so characteristic of this cell line. The affected cells were no longer as refractile as control UCT-BR-1 CELLS grown in RPMI-10. *The human breast carcinoma line UCT-BR-1 was the most sensitive to the degenerative or growth inhibitory action of the Bowes/sf conditioned medium and was chosen as the indicator line for all the subsequent experiments.*

A growth study was performed on UCT-BR-1 indicator cells using two dilutions of Bowes/sf conditioned medium. (See Chart 3 for details). Growth of the UCT-BR-1 cells in Bowes/sf was

compared with control UCT-BR-1 cells grown in normal RPMI-10.

The Bowes/sf conditioned medium inhibited the proliferation of the indicator cells. This inhibition was highly significant after 6 and 9 days of culture.

The Bowes/sf medium also inhibited the incorporation of a radiolabelled DNA precursor into UCT-BR-1 cells. (see Chart 4). Uptake of tritiated thymidine (^{3}H-thymidine) into UCT-BR-1 cells was measured using an assay adapted from a standard laboratory protocol aimed at assessing lymphocyte proliferation in microtiter plates (137). The technical details of the assay are supplied in the thesis appendix. *Serial dilutions of Bowes/sf conditioned medium (to 1 in 8) were found to significantly inhibit ^{3}H-thymidine incorporation into UCT-BR-1 cells when compared with cells grown in control RPMI-10.* (Chart 4)

% inhibition of ^{3}H-thymidine uptake was defined as:

$$\frac{\text{(control cpm - test cpm)}}{\text{control cpm}} \times 100$$

The polypeptides of the interferon (IFN) family are known to be growth inhibitory to a number of different mammalian cells in vitro. (See Chapter 1 for the review of the literature). The microplate assay was shown to be sensitive enough to detect the inhibitory action of human lymphoblastoid IFN when serially diluted to 31 i.u./ml. (Chart 5). The inhibitory action of IFN could be blocked by a specific polyclonal antiserum to interferon (Chart 5) thus demonstrating that the

Contd./74

CHARTS 4, 5

CHART 4

EFFECT OF BOWES/SF CONDITIONED MEDIUM ON ^{3}H-THYMIDINE INCORPORATION BY PROLIFERATING UCT-BR-1 CELLS

UCT-BR-1 cells were grown in 96-well microtiter plates for 72 hours. Serial dilutions of Bowes/sf conditioned medium inhibited the incorporation of tritiated thymidine by these cells.

UCT-BR-1 cells were seeded at a density of 1.8×10^4 cells per well of a 96-well microtiter plate. After an overnight incubation at 37°, the seeding medium was replaced with 0.2 ml of the test medium. Twelve-fold replicates were performed for each dilution of the Bowes/sf conditioned medium. Incubation of the cells proceeded at 37°C. Eighteen hours prior to the termination of the assay, ^{3}H-thymidine was added to a final concentration of 1 μCi/ml. Following this, the cells were harvested as described in the thesis appendix.

Values shown below are the means ± S.E.M. of 12-fold replicate counts(cpm) performed in a 96-well microtiter plate.
p values were determined using Student's t-test.

RPMI-10 (CONTROL)		16414 ± 332	
Bowes/sf - undiluted		2468 ± 324	p<0.005
Bowes/sf - diluted	1/2	8881 ± 976	p<0.005
	1/4	12210 ± 497	p<0.005
	1/8	12815 ± 456	p<0.005
	1/16	15420 ± 806	not significant

CHART 4

UCT-BR-1 microplate assay

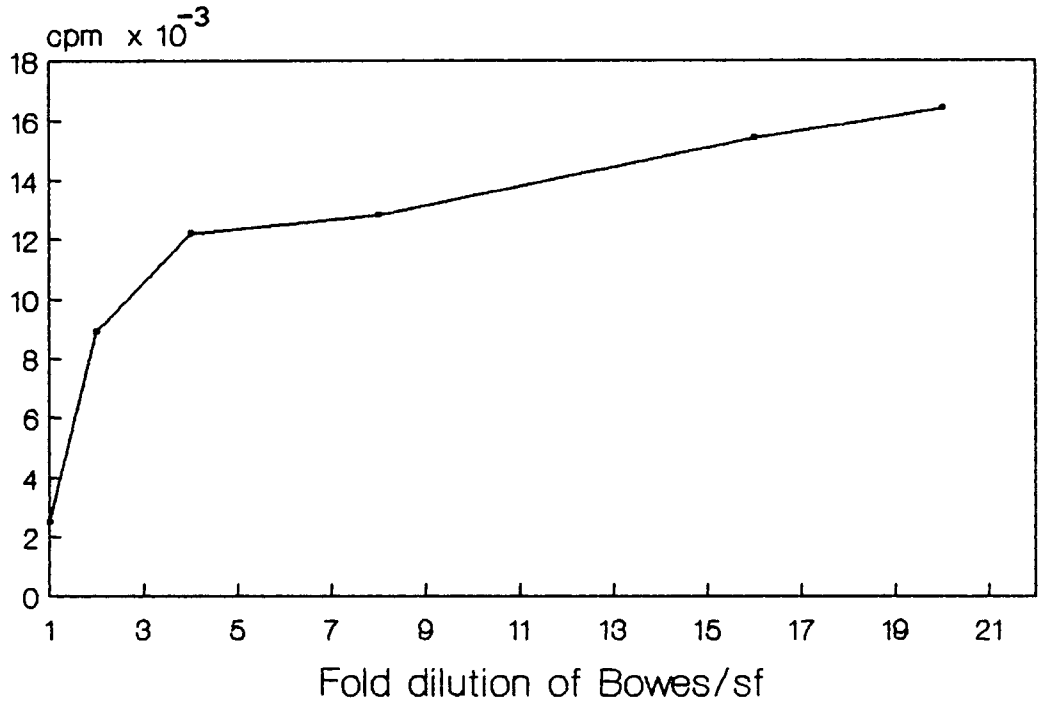


CHART 5

THE EFFECT OF HUMAN LYMPHOBLASTOID INTERFERON ON ^{3}H-THYMIDINE INCORPORATION BY UCT-BR-1 CELLS

Lymphoblastoid interferon inhibited the incorporation of ^{3}H-thymidine into UCT-BR-1 cells in a 72 hour microplate assay. This inhibitory action was blocked by a specific antiserum to interferon.

Human lymphoblastoid interferon (16 000 i.u./ml - see appendix for further details) was diluted to 1000 i.u./ml in RPMI-10 and was sterile filtered through a Millex-GV unit (Millipore). Serial 2-fold dilutions to 1/32 (i.e. 31 i.u./ml) were made in RPMI-10. These dilutions were assayed for their ability to inhibit ^{3}H-thymidine uptake by UCT-BR-1 cells in a mictotiter plate assay.

CHART 5 contd

INTERFERON CONC.(i.u./ml)	CPM	% INHIBITION
1000	5020	77
500	5687	74
250	7665	65
125	9718	56
62.5	10837	50
31.3	12419	43

Control 21872 ± 1130 cpm

All values are means of quadruplicate counts

A replicate set of microplate wells received an additional 10.0µl of the sterile 'desalted' interferon antibody preparation. This preparation contained antibodies to IFN-alpha and IFN-beta (see Appendix). A necessary control was the addition of anti-IFN to cells that had been fed with plain RPMI-10 only. The anti-IFN was added at time zero.

INTERFERON CONC.(i.u./ml)	CPM	% INHIBITION
1000	14697	20
500	15458	15.9
250	16279	11.4
125	15500	15.6
62.5	18522	-0.8
31.25	18938	-3.1

Control (Plus Anti-IFN) = 18370 ± 1553 cpm

inhibitory action of the IFN preparation was due to the IFN polypeptide and not due to some non-specific effect.

THE GROWTH INHIBITORY ACTION OF THE BOWES/SF CONDITIONED MEDIUM WAS DUE TO A MACROMOLECULE.

Serum-free Bowes/sf conditioned medium was concentrated either in a stirred pressure cell (using an ultrafiltration membrane with a 1000-dalton cutoff) or with a hollow fibre apparatus using a cartridge with a molecular weight limit of 5000 daltons. Both the ultrafiltrate and the retentate were made to 10% (v/v) with FCS and were tested at different dilutions on growing UCT-BR-1 cells. A photographic record of the effects of the retentate and ultrafiltrate on the indicator cells is presented in Chart 6.

The two methods of concentrating the medium produced very similar results. In either case, the retentate appeared to be profoundly inhibitory and produced substantial degenerative changes in the cells. Conversely, the ultrafiltrate had no effect on the microscopic appearance of the cells (Chart 6).

A growth study of the indicator cells confirmed the above results. UCT-BR-1 cells were grown for 200 hours in the presence of Bowes/sf conditioned medium preparations that had

been treated in a stirred pressure cell using a membrane with a limit of 1000 daltons. Both retentate and ultrafiltrate were tested. *Whereas the ultrafiltrate had no growth inhibitory action, the dilutions of the Bowes/sf retentate were inhibitory as shown in Chart 7.*

Further experiments (results not shown) demonstrated that the growth inhibitory factor could be concentrated using an ultrafiltration membrane with a 10 000 dalton molecular weight limit. Subsequent experiments were performed with this type of membrane because it allowed a more rapid concentration.

It was also demonstrated that the inhibitory activity could be retained by a dialysis membrane with a nominal molecular weight limit of approximately 12000 daltons. The technique of dialysis was employed in the experiments which demonstrated the trypsin-sensitivity of the inhibitor. These experiments will be described in a later section (see page 76 and Chart 9).

<u>The Bowes/sf growth inhibitor showed a partial but reversible susceptibility to heat denaturation.</u>

Bowes/sf conditioned medium was heated at 65°C for increasing periods of time up to twenty-four hours. The heated conditioned media were subsequently allowed to cool and were diluted with fresh RPMI and made to 10% (v/v) with fetal calf serum. These samples were tested on UCT-BR-1 cells in the

microplate assay and the effect was compared with that of unheated conditioned medium (see Chart 8).

Heating the Bowes/sf conditioned medium partially reduced its inhibitory capacity but failed to abolish it entirely. Partial loss of inhibitory activity was maximal at the two and the six hour time points. The thermal effect was clearly reversible since after 24 hours, the medium appeared to regain its full inhibitory activity.

The Bowes/sf growth inhibitor therefore appeared to be reversibly and partially susceptible to denaturation by heating at 65°C for periods up to 24 hours.

<u>The Bowes/sf growth inhibitor was sensitive to trypsin.</u>

Serum-free unconditioned RPMI medium or Bowes/sf conditioned medium was treated either with trypsin alone or with trypsin plus soybean trypsin inhibitor. (See Chart 9). Treatment was carried out at 37°C for 3 hours. The various media (including untreated RPMI and Bowes/sf medium) were dialysed against distilled water and were evaporated to dryness. Samples were reconstituted in RPMI-10 and were assayed for inhibitory activity in the UCT-BR-1 microtiterplate assay.

Contd./88

CHARTS 6, 7, 8, 9

CHART 6

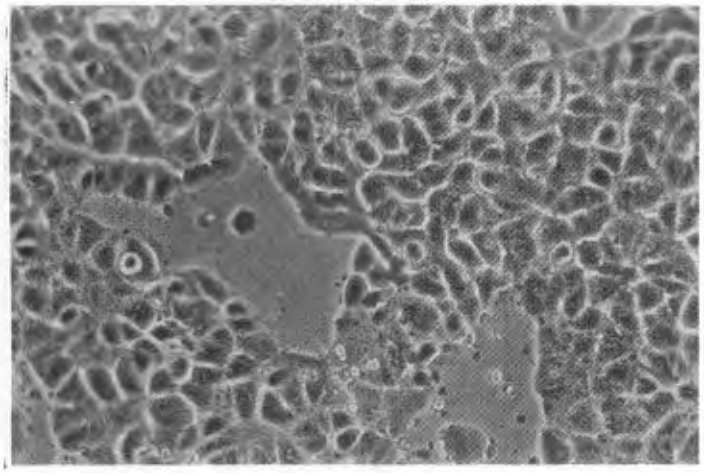
THE EFFECT OF ULTRAFILTRATION UPON THE ACTIVITY OF THE BOWES/SF CONDITIONED MEDIUM : A PHOTOGRAPHIC RECORD.

Bowes/sf conditioned medium was concentrated several-fold by ultrafiltration. Only the retentate was able to produce changes in the morphology of UCT-BR-1 suggestive of growth inhibition. The ultrafiltrate had no activity.

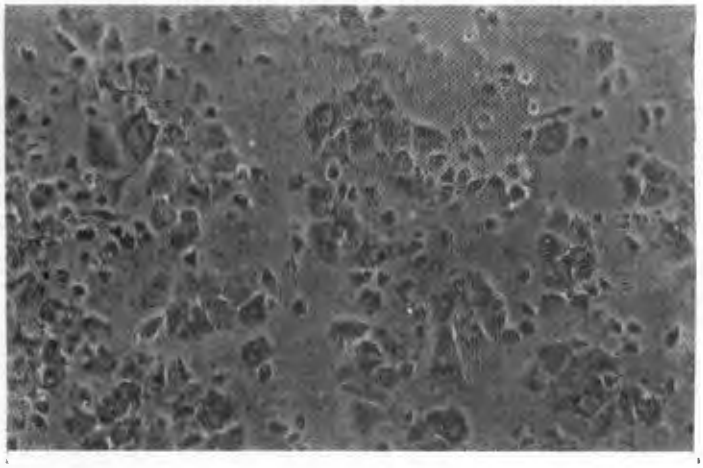
UCT-BR-1 cells were seeded in 24-well plates (Linbro; Flow Laboratories) at 5.5 x 10^4 cells per well. After an overnight incubation, the media to be tested were added.

Bowes/sf medium was concentrated either five-fold in an Amicon HIP5 hollow fibre cartridge with a M.W. limit of 5000 daltons, or twelve-fold in a stirred cell fitted with a 1000-dalton cutoff membrane (Amicon, type UM-2). Both the retentate and ultrafiltrate were made up to 10% (v/v) with FCS, and were then tested, at various dilutions, on the UCT-BR-1 cells.

CHART 6
EFFECT OF ULTRA FILTRATION ON BOWES/SF MEDIUM

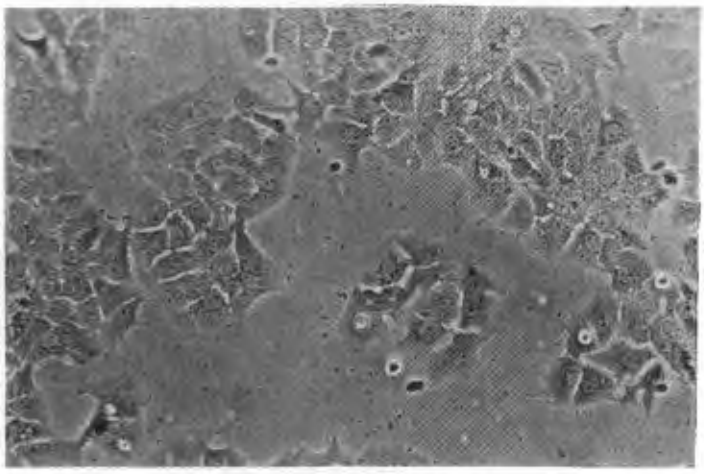


1. UCT-BR-1 cells in RPMI-10.

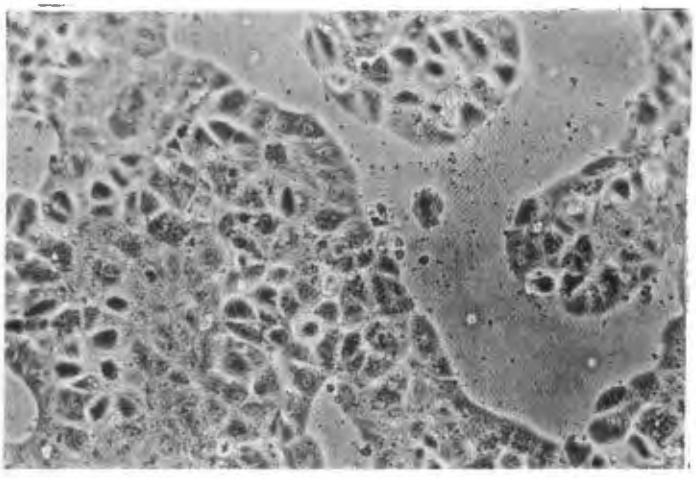


2. UCT-BR-1 cells in unfractionated Bowes/sf medium diluted 1/5 in RPMI-10.

CHART 6



3. Effect of retentate: UCT-BR-1 cells in Bowes/sf medium concentrated 5-fold, then diluted 1/5.



4. Effect of ultrafiltrate: UCT-BR-1 cells grown in Bowes/sf medium filtered through a 5000 dalton cutoff membrane. The ultrafiltrate has been supplemented with 10% FCS.

CHART 7

GROWTH OF UCT-BR-1 CELLS IN CONDITIONED MEDIUM RETENTATE AND IN ULTRAFILTRATE

Bowes/sf conditioned medium was concentrated by ultrafiltration. The retentate, and dilutions thereof, inhibited the growth of UCT-BR-1 cells. The ultrafiltrate was not growth inhibitory.

Ultrafiltration was performed on conditioned medium that had been ultracentrifuged (100000g; 40 mins) to remove aggregates and particulate matter. This was followed by 10-fold concentration in a stirred cell fitted with a 1000 dalton cut-off membrane. Both the retentate and ultrafiltrate were supplemented with FCS prior to testing, and were tested at several dilutions.

The growth of the indicator UCT-BR-1 cells was measured in three replicate 24-well plates. On alternate days, the cells were fed with fresh conditioned medium preparations. Time points were taken at 60, 132 and 204 hours. At each time point, all the wells of a plate were treated with trypsin and the cells were counted.

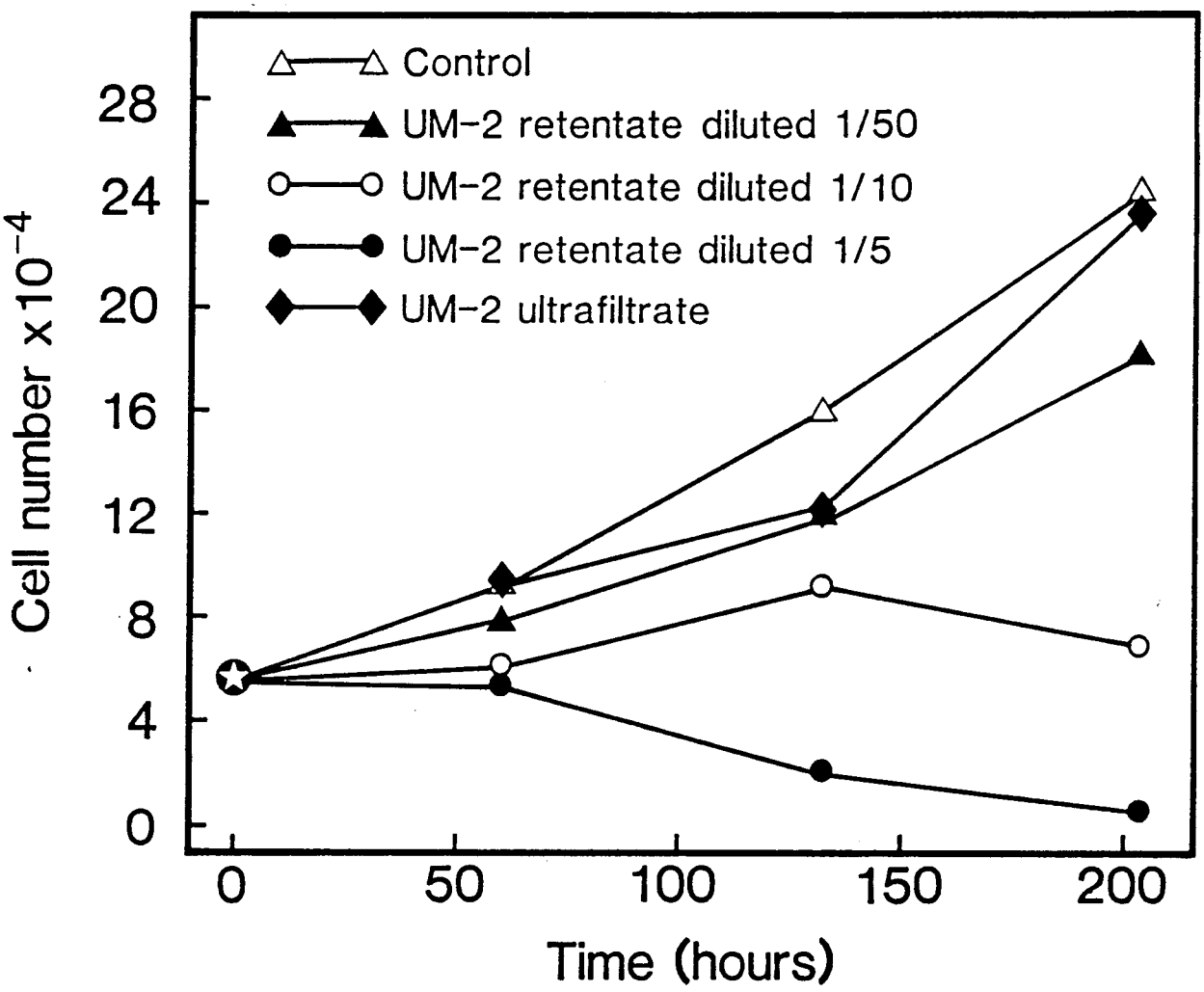

Control
UM-2 retentate diluted 1/50
UM-2 retentate diluted 1/10
UM-2 retentate diluted 1/5
UM-2 ultrafiltrate
Cell number x10⁻⁴
28
24
20
16
12
8
4
0
0
50
100
150
200
Time (hours)

CHART 7

CHART 8

THE EFFECT OF HEAT ON THE ACTIVITY OF BOWES/SF MEDIUM

Bowes/sf conditioned medium was heated at 65°C for increasing periods of time up to 24 hours. There was a substantial loss of inhibitory activity after 2 to 6 hours of heating. This was followed by a paradoxical recovery of activity at 24 hours. Nevertheless, at all times the heated conditioned medium displayed a significant inhibition when compared to control medium.

2.0 ml aliquots of serum-free 48-hour Bowes/sf conditioned medium were heated at 65°C for 30 minutes, 2 hours, 6 hours and 24 hours. Serum-free RPMI was also heated at 65°C for 24 hours. When heating was completed, all the media were diluted with an equal volume of RPMI and made to 10% (v/v) with fetal calf serum. This was followed by sterile filtration through a Millex-GV unit. These samples were tested for growth inhibitory activity on UCT-BR-1 cells in the standard microplate assay as described in the appendix. Statistical analysis using Student's t-test was carried out in order to compare (i) heated vs. unheated conditioned medium (ii) heated conditioned medium vs. control medium.

CHART 8 contd

MEDIUM	TREATMENT	MEAN ^{3}H-THYMIDINE UPTAKE ±S.E.M.	% INHIBITION (CONTROL = 0)		
RPMI CONTROL	NIL	20131±433	0		
RPMI	65°C, 24 hours	22567±512	-12		
				P1	P2
BOWES	UNHEATED	12943±394	36	-	p<0.005
"	30 min, 65°C	14703±272	27	p<0.025	p<0.005
"	2 hours, 65°C	17169±472	15	p<0.005	p<0.005
"	6 hours, 65°C	16709±322	17	p<0.005	p<0.005
"	24 hours, 65°C	13243±263	34	NS	p<0.005

P1 compares all relevant data with unheated Bowes/sf conditioned medium.

P2 compares all relevant data with the control RPMI-10.

NS = not significant.

CHART 8

Heating of Bowes/sf

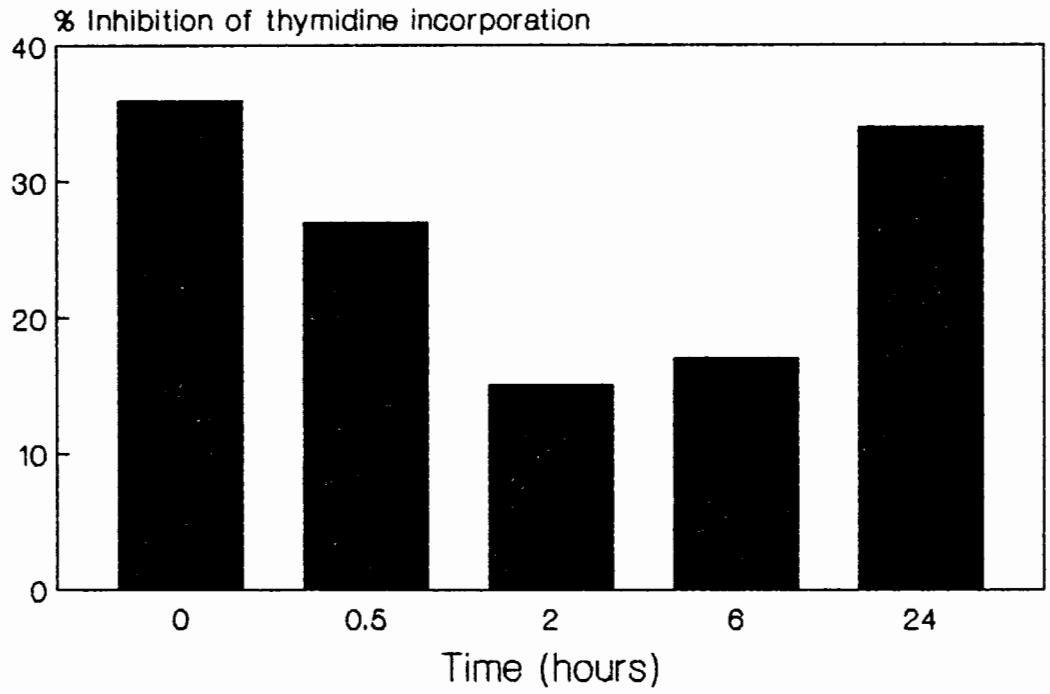


CHART 9

THE EFFECT OF TRYPSIN ON BOWES MEDIUM

The growth inhibitory effect of the Bowes/sf conditioned medium was destroyed by trypsin. This indicated that the growth inhibitor was a protein. The action of trypsin on the growth inhibitor was completely blocked by soybean trypsin inhibitor.

Trypsin (Sigma T 8642, TPCK-treated) and soybean trypsin inhibitor (SBTI, Sigma T-9003) were dissolved in PBS, pH 7.4, to give final concentrations of 1 mg/ml and 2 mg/ml respectively. The 10 ml of serum-free medium to be tested received either:

(i) No addition
(ii) 40 µg of trypsin alone,
(iii) 40 µg of trypsin together with 80µg of SBTI.

Both serum-free unconditioned medium (RPMI) and serum-free Bowes/sf conditioned medium were tested in this system.

The media were incubated at 37°C for 3 hours in a shaking water bath. At the end of the incubation, 80µg of SBTI was added to the media that had previously received 40µg of trypsin only.

The media were then dialysed against two changes of 100 volumes of sterile distilled water. Dialysis membranes with nominal molecular weight limits of 12000 daltons were used.

The dialysed media were evaporated to dryness in a vacuum centrifuge, and were reconstituted in 8.0 ml of medium plus serum (RPMI-10).

CHART 9 contd

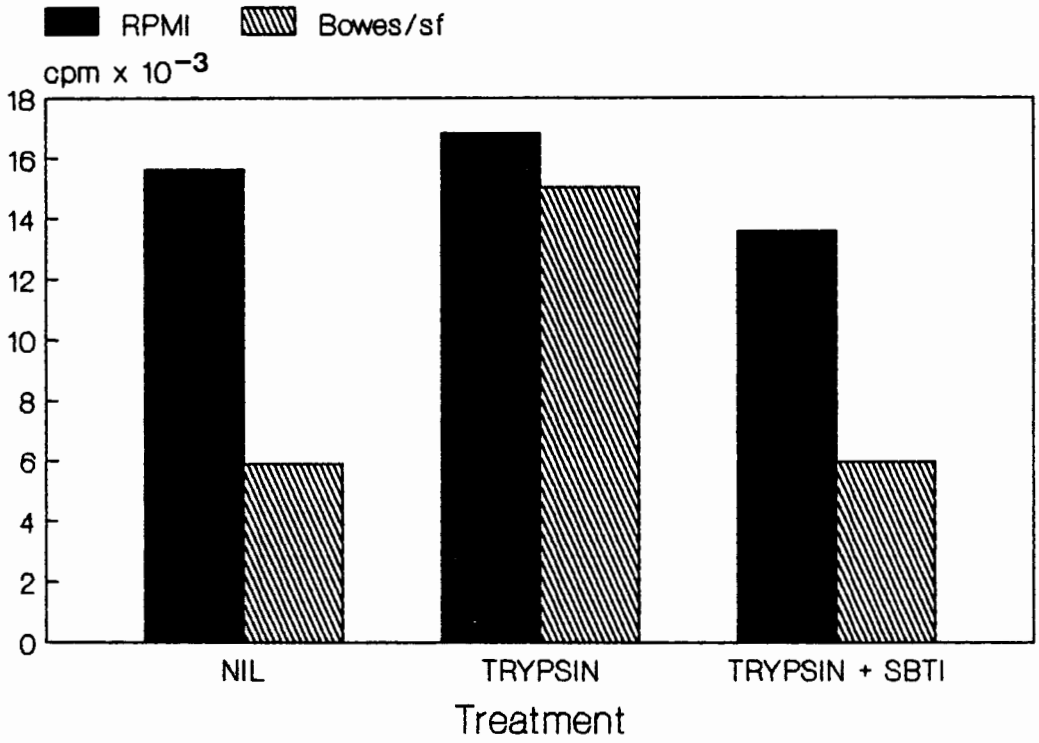
These samples were sterile filtered and were tested for growth inhibitory action on UCT-BR-1 indicator cells using the standard microtiter plate assay.

The results below display the ^{3}H-thymidine uptake (in cpm) by UCT-BR-1 cells in the presence of the test media. All results are shown ± S.E.M. and represent the means of quadruplicate counts. p values were obtained using Student's t-test. NS = Not significant.

	RPMI (unconditioned medium)	Bowes/sf conditioned medium	
	No treatment	*No treatment*	
Undiluted	15632±744	5895±830	p<0.005
2-fold diln.	16500±518	8401±461	p<0.005
	Trypsin, 37°C, 3hr	*Trypsin 37°, 3hr*	
Undiluted	16811±690	15050±580	NS
2-fold diln.	17448±426	17096±1018	NS
	Trypsin+SBTI 37°, 3hr	*Trypsin+SBTI 37°, 3 hr*	
Undiluted	13595±1394	5957±819	p<0.005
2-fold diln	15276±2067	7970±312	p<0.025

CHART 9

Trypsin susceptibility



The growth inhibitory activity of the Bowes/sf conditioned medium was abolished by the presence of trypsin (Chart 9). Furthermore, this effect of trypsin was blocked by soybean trypsin inhibitor. Neither trypsin nor the trypsin inhibitor had any effect on the unconditioned control RPMI medium.

The growth inhibitory action of the Bowes/sf medium appeared therefore to be due to the presence of a trypsin-sensitive polypeptide.

THE GROWTH INHIBITORY ACTIVITY OF THE BOWES/SF WAS RETAINED FOLLOWING CONCENTRATION OF THE CONDITIONED MEDIUM, REMOVAL OF SALTS AND LYOPHILIZATION.

Holley and co-workers (113) were able to identify a growth inhibitory factor in the serum-free conditioned medium of BSC-1 cells, a line derived from Green Monkey kidney. The attempted chromatographic purification of this factor was preceded by the many-fold concentration of the conditioned medium. An adaptation of this procedure (113) was used in order to assist in the characterization of the Bowes/sf growth inhibitor.

Bowes/sf conditioned medium was concentrated 140-fold in a stirred pressure cell fitted with a hydrophilic membrane that had a molecular weight limit of 10 000 daltons. Buffer salts

and material with a molecular weight of less than 10 000 were removed by a process of constant volume ultrafiltration. The retentate was lyophilized and was tested for growth inhibitory activity on UCT-BR-1 indicator cells as described in Chart 10. This lyophilisate (referred to as 'fraction A' in the chart) was found to retain significant growth inhibitory activity. Whereas the UCT-BR-1 cells in the control medium (RPMI-10) had multiplied approximately three-fold (from 10 to 32.6 x 10^5), the cells in the undiluted fraction A extract, as well as those grown in the extract that was diluted two-fold, had actually decreased relative to the original number of cells.

ANALYSIS BY GEL-FILTRATION CHROMATOGRAPHY

Bowes/sf conditioned medium was concentrated as described in the preceding paragraph and a crude lyophilisate was obtained (Fraction A). This was extracted into 1M acetic acid and chromatographed on a 79 ml bed volume gel filtration column (Bio-Gel P60) developed with 1M acetic acid. The gel had an exclusion limit of 60 000 daltons for globular proteins. Thirty fractions were obtained, each of which was tested both for growth inhibitory activity on UCT-BR-1 cells and for the ability to inhibit tritiated thymidine incorporation by these cells (Chart 11). In addition, a photographic record was made in order to record changes in cell morphology (Chart 11). Fractions 8, 9 and 10, corresponding to a Ve (elution volume)

Contd./102

CHARTS 10, 11

CHART 10

GROWTH OF UCT-BR-1 CELLS IN BOWES/SF FRACTION A

Fraction A was a crude lyophilisate prepared from Bowes/sf conditioned medium by concentration followed by a constant volume wash to remove buffer salts and low molecular weight components. Fraction A was found to inhibit the growth of UCT-BR-1 cells after seven days in culture.

Three and a half litres of Bowes/sf conditioned medium was concentrated to 25mls by ultrafiltration using a 400 ml stirred pressure cell fitted with a hydrophilic YM-10 membrane (see Appendix). When this was completed, Tris-Saline 'Ultrafiltration buffer' pH 7.5 (see Appendix) was added to the cell to dilute the conditioned medium concentrate to 400 mls. This was subsequently reconcentrated to 20 mls after which triple distilled water was added to the stirred cell to restore the volume to 400mls. Following several cycles of "diafiltration" the conditioned medium preparation, which had been, in effect, dialysed against distilled water, was lyophilized. 66 mg by weight of lyophilizate, termed "Fraction A", was obtained from a starting volume of three and a half litres of conditioned medium.

To determine growth inhibitory activity, 10.8 mg of Fraction A was added to 15 ml of serum-free RPMI medium and was allowed to stir overnight at 4°C. The material, or 'Fraction A extract', was clarified by centrifugation and a protein

CHART 10 contd

determination, by the method of Lowry (135), gave an estimate of 0.45 mg/ml in the undiluted extract. The latter was made to 10% (v/v) with fetal calf serum and was sterilized by filtration through a 0.45 micron filter (Millipore HAWP025). The extract was tested for growth inhibitory activity on UCT-BR-1 cells by the following procedure:

1. UCT-BR-1 cells were seeded in a 24-well plate (Linbro, Flow Laboratories) at 1 x 10^5 cells per well.
2. After an overnight incubation at 37°C the medium was aspirated and was replaced with 0.5 ml of either RPMI-10 (control) or Fraction A extract appropriately diluted in RPMI-10.
3. The plate was incubated at 37°C for seven days without change of medium.
4. At the end of this time the cells were released from the wells by trypsinization and were counted using a Coulter Counter.

CHART 10 contd

GROWTH OF UCT-BR-1 CELLS IN BOWES/SF FRACTION A

FRACTION A EXTRACT DILUTION		ESTIMATED FRACTION A CONTENT (µgPROTEIN PER ML)	CELL NUMBER
Undiluted		450	6.38
Diluted	1:2	230	8.82
	1:4	113	16.6
	1:8	56	19.5
	1:16	28	26.4
	1:32	14	35.4
Control medium		0	32.6

The cell counts are the averages of duplicate counts.

The cell number x 10^4 gives the cell count per well.

CHART 11

ANALYSIS OF BOWES/SF GROWTH INHIBITOR BY GEL-FILTRATION CHROMATOGRAPHY

Bowes/sf Fraction A, prepared from Bowes/sf conditioned medium, was extracted into acetic acid and was chromatographed on a Bio-Gel P60 column (exclusion limit for globular proteins is 60 000 daltons). Fractions 8, 9 and 10 were shown to be growth inhibitory to UCT-BR-1 indicator cells by three independent methods viz (i) Inhibition of cell growth (ii) Inhibition of ^{3}H-thymidine uptake (iii) Alterations in cellular morphology.

42 mg Bowes Fraction A was extracted into 10 mls of 1 M acetic acid by stirring overnight at 4°c. The following day, the extract was clarified by centrifugation at 4°C (1000g; 15 minutes). This extract was then concentrated to approximately 4 ml in an Amicon 8mc micro-ultrafiltration system fitted with a YM-10 membrane. The concentrated extract was chromatographed on a Bio-Gel P60 column (2.3 x 19 cm) in 1 M acetic acid. The column was run at 4°C with a flow rate of 20ml per minute. Column effluent was monitored at 280 nm using an ultraviolet spectrophotometer (Uvicord; LKB). 4ml fractions were collected. The fractions were each freeze-dried, reconstituted in 4mls of distilled water, and then re-lyophilized.

The lyophilized column fractions were each reconstituted in 2.0mls of tissue culture medium (RPMI-10). These fractions were then individually sterilized by filtration through a 0.45 micron filter (Millipore HAWP025), and were added to UCT-BR-1 cells seeded the previous day in 96-well plates (see Appendix for microplate assay details) or 24-well plates (Linbro, Flow Laboratories) at 1 x 10^{5} cells per well. After 96 hours in culture the cells in the 24-well plates were released by trypsinization and counted using a Coulter Counter.

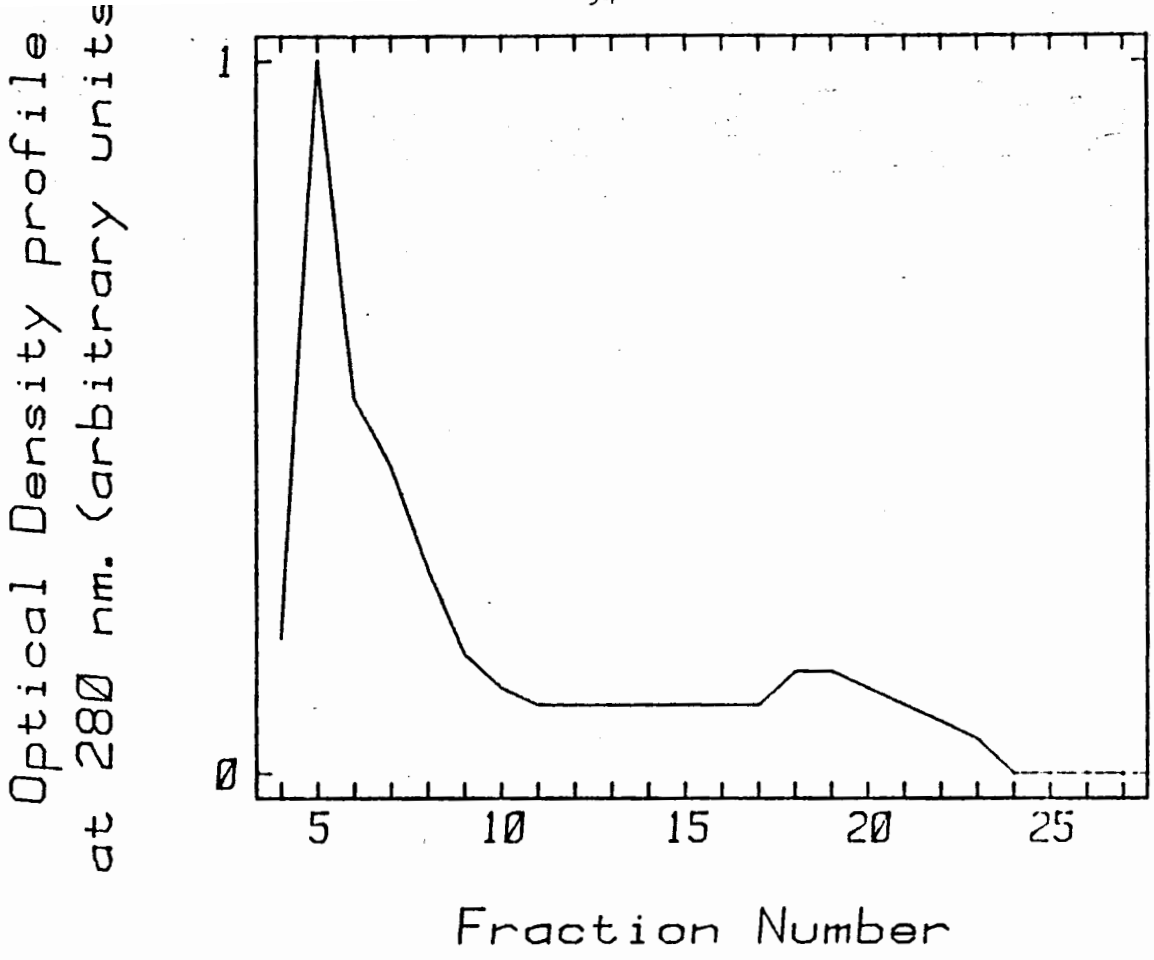


CHART 11

OD_{280} of Bio Gel P-60 Effluent

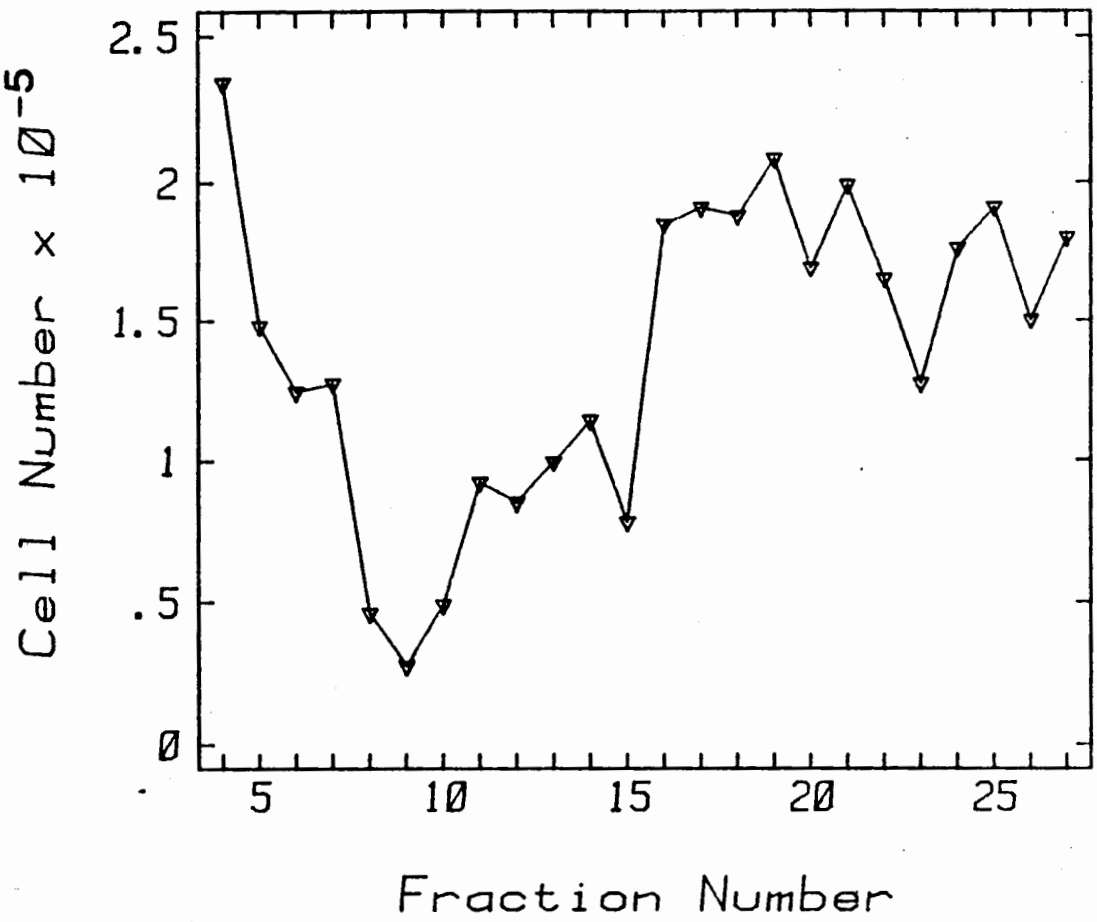


CHART 11
Showing inhibition of cell growth

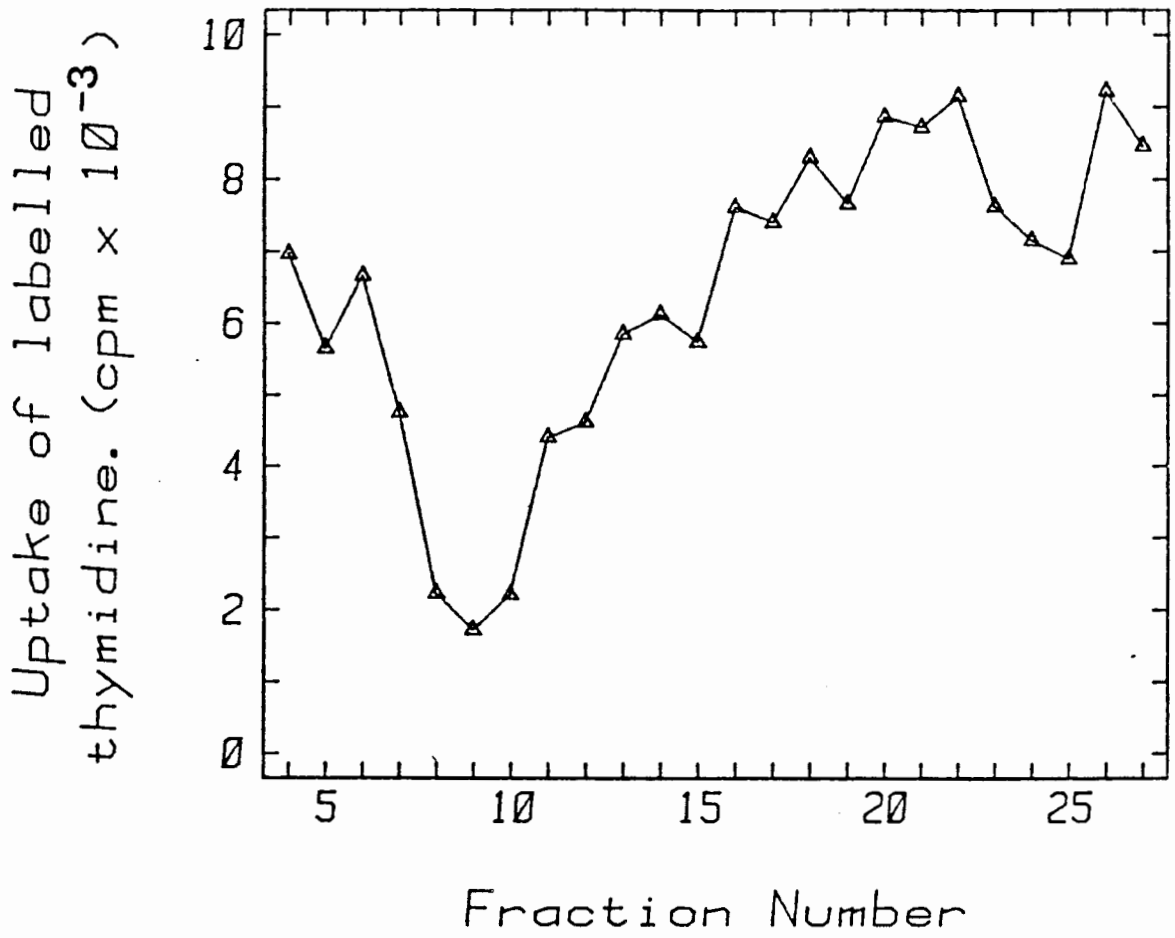
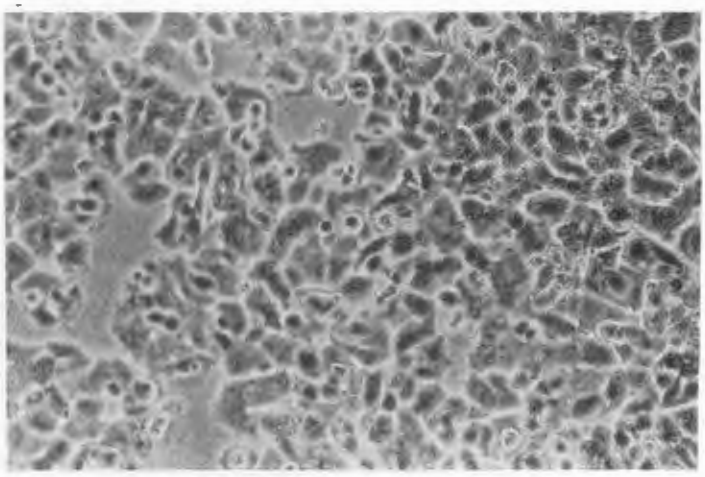
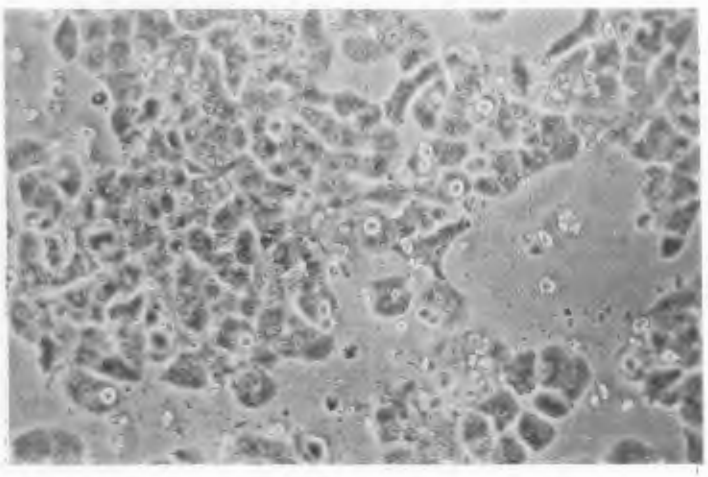


CHART 11
Showing inhibition of
^{3}H-Thymidine incorporation

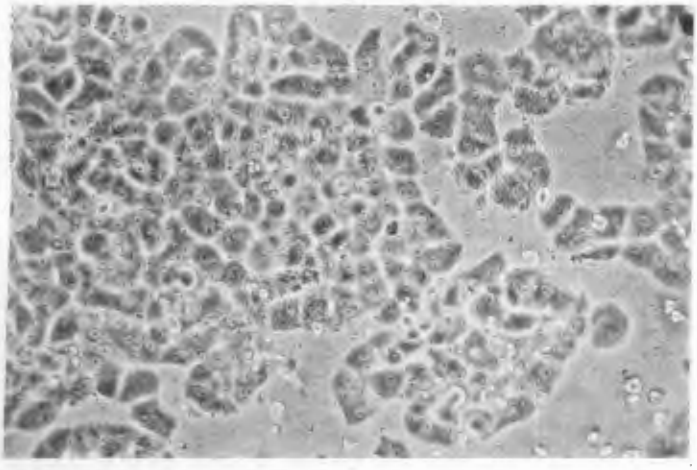
CHART 11
DEMONSTRATING THE MORPHOLOGICAL CHANGES INDUCED BY VARIOUS BIO-GEL P60 FRACTIONS



FRACTION 4

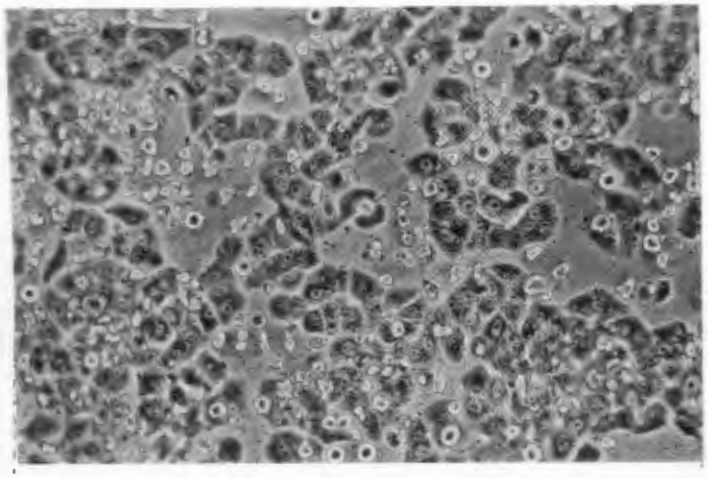


FRACTION 5

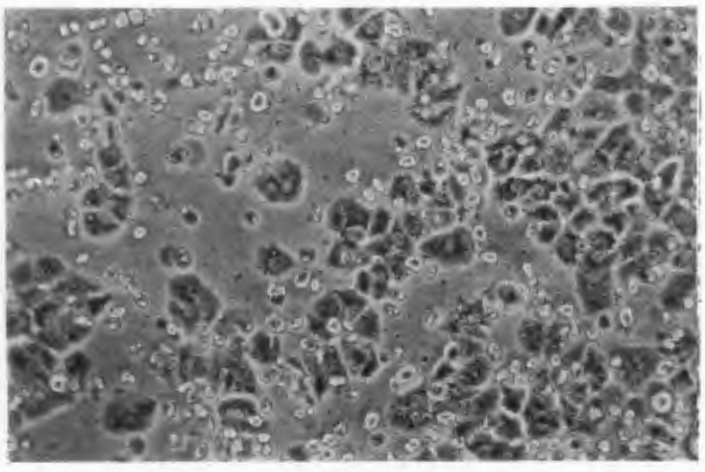


FRACTION 6

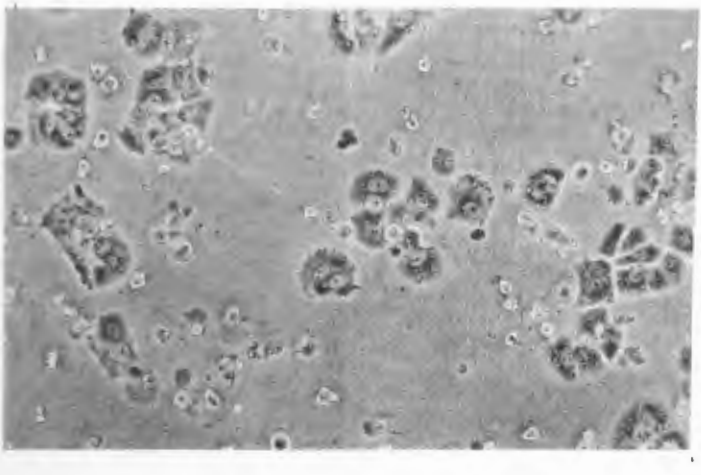
CHART 11



FRACTION 7

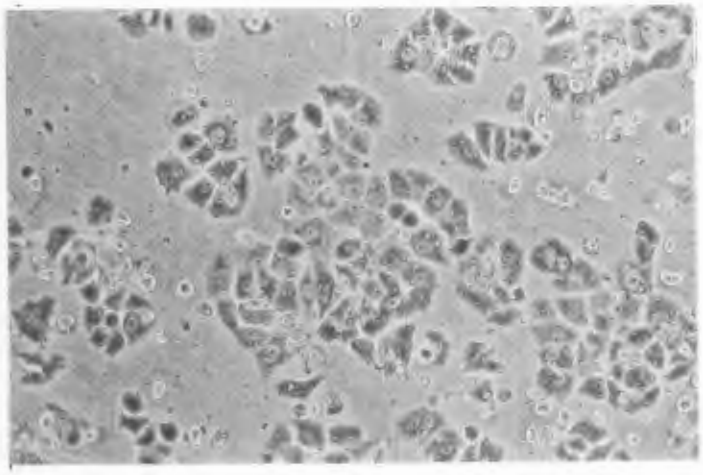


FRACTION 8

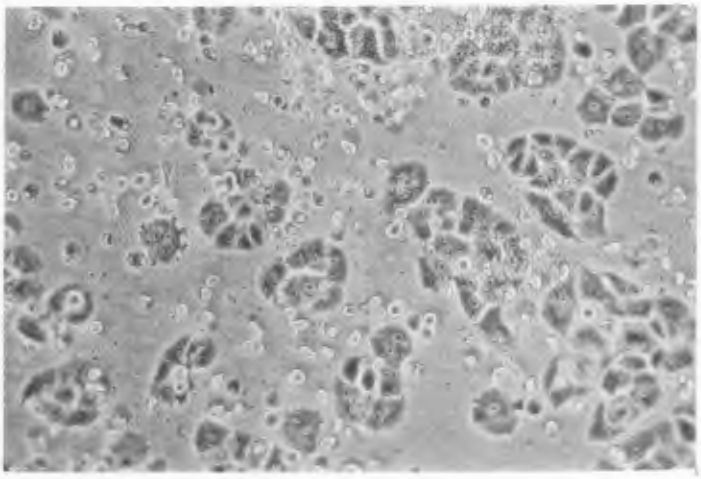


FRACTION 9

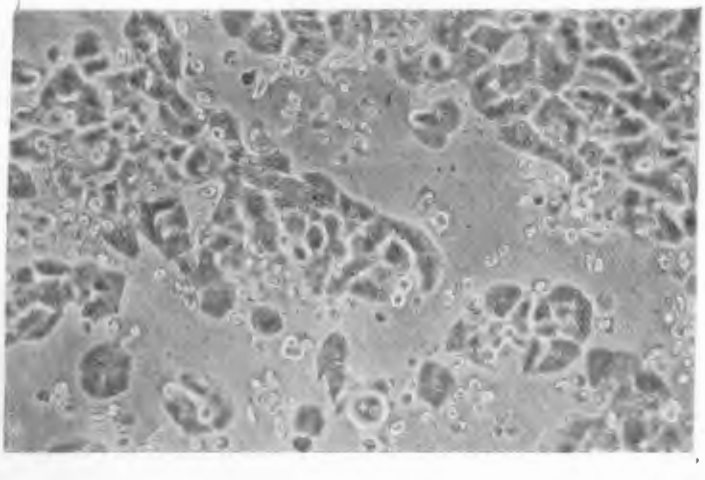
CHART 11



FRACTION 10

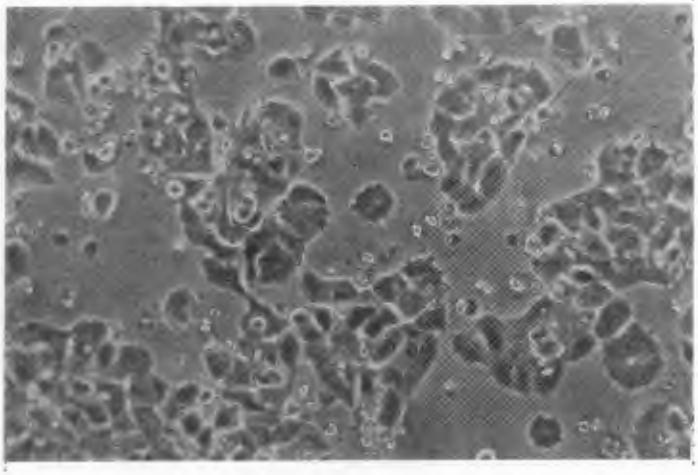


FRACTION 11

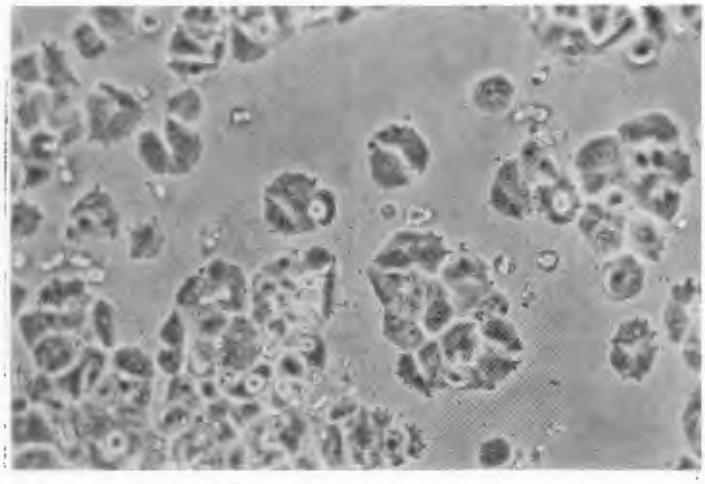


FRACTION 13

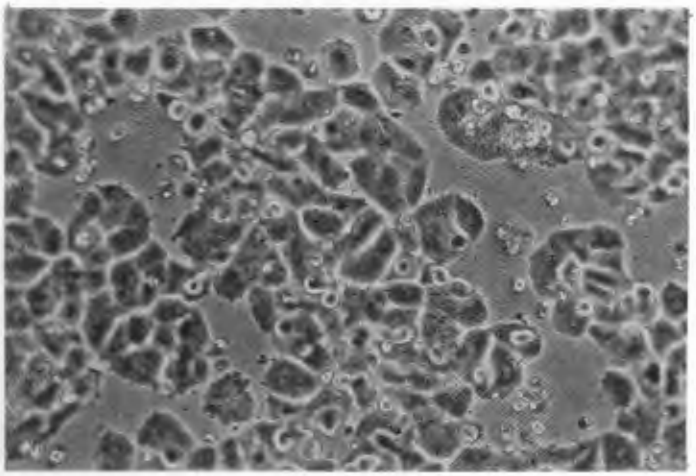
CHART 11



FRACTION 14

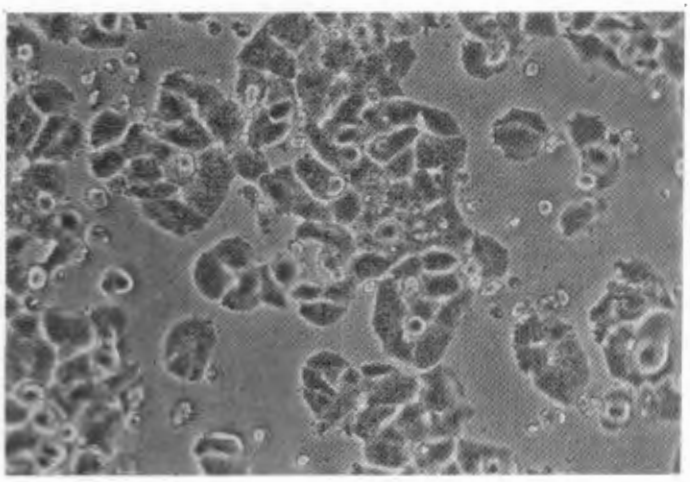


FRACTION 15



FRACTION 20

CHART 11



FRACTION 23

of 32-40 mls, inhibited UCT-BR-1 growth as well as the incorporation of ^{3}H-thymidine. Degenerative changes were apparent in the indicator cells grown in these fractions, especially fraction 9 which also produced the maximal inhibitory effect. Both the photographs and the growth studies showed that there was a gradation of inhibitory activity preceding and also following the major inhibitory fractions.

(N.B. A more definitive gel chromatography step using a calibrated column is described in the next chapter.)

DISCUSSION

In initial experiments, in which fixed and stained cellular monolayers were examined macroscopically, it was clearly shown that certain human epithelial cell lines failed to proliferate in the presence of diluted Bowes/sf conditioned medium supplemented with 10% fetal calf serum (Chart 1). Further experiments (Chart 2) demonstrated that one of the indicator cell lines, the human breast carcinoma line UCT-BR-1, was the most sensitive to the growth inhibitory actions of Bowes/sf. In all cell lines tested, growth inhibition and morphological changes became apparent only after a latent period of several days. An analysis of cell growth kinetics indicated that this latent period was probably about 3 days in length. After 6

days, the growth inhibitory action was quite apparent (Chart 3). Because of its sensitivity, UCT-BR-1 was chosen as the indicator line to be used for the growth inhibitor assay.

An assay that was originally designed to test the proliferation of human lymphocytes was adapted to measure the incorporation of ^{3}H-thymidine (a DNA precursor) by UCT-BR-1 cells.

Serial dilutions of Bowes/sf conditioned medium significantly limited the uptake of ^{3}H-thymidine by UCT-BR-1 cells (Chart 4). Because this assay was conducted in a 96-well microtitre plate it could be used to screen a large number of different samples with multiple replicates for inhibition of ^{3}H-thymidine incorporation. This technique clearly demonstrated the marked inhibitory action of an interferon preparation on ^{3}H-thymidine uptake by UCT-BR-1 cells (Chart 5). Interferon, as indicated in the literature review, classically has a growth inhibitory action on a number of human cells.

Growth inhibitory activity in Bowes/sf conditioned medium was retained by ultrafiltration membranes with nominal molecular weight limits of 1000, 5000 and 10000 daltons. There appeared to be little or no inhibitory activity in the ultrafiltrate (Charts 6 and 7). In these critical experiments, activities were assayed both by assessing cell proliferation as well as

by recording changes in cell morphology. The growth inhibitory activity, therefore, appeared to be a consequence of some macromolecular substance added to the medium by the Bowes/sf cells. In a somewhat different system, this inhibitory activity was also retained by a conventional dialysis membrane (Chart 9).

The Bowes/sf conditioned medium retained a significant degree of inhibitory activity when heated at 65°C for several hours, up to one day (Chart 8). Although there was some loss of activity after heating, the effect was reversible.

Treatment of the Bowes/sf medium with trypsin effectively abolished the inhibitory activity as assessed by the microtiter plate assay (Chart 9). This action of trypsin was blocked by the addition of a specific inhibitor of trypsin. This demonstrated that the Bowes/sf growth inhibitor was a polypeptide.

Finally, the growth inhibitory activity could be recovered after concentration, lyophilization, acetic acid extraction and gel-filtration chromatography under acid conditions (Charts 10 and 11). The material which eluted from the gel-filtration column inhibited both UCT-BR-1 growth, and the incorporation of ^{3}H-thymidine by UCT-BR-1 cells and furthermore produced the identical morphological changes seen in the presence of untreated Bowes/sf conditioned medium. A

detailed analysis of yield and % recovery was not undertaken at this preliminary stage, nor was the column calibrated in order to estimate the molecular weight of the inhibitor. Such studies will be described in the following chapter.

This pragmatic characterization of the growth inhibitory phenomenon demonstrated that the Bowes/sf cells secreted a polypeptide, that was responsible both for inhibiting the growth of UCT-BR-1 cells and for inhibiting ^{3}H-thymidine incorporation by these cells. This factor exerted its effect only after a latent period of several days. There was, however, no proof that a single molecular species was responsible for the observed phenomena. A number of different polypeptides may well have been involved. Indeed, the heating experiment indicated that there was partial loss of activity, and this may possibly have been a consequence of the inactivation of one or more inhibitors, with the remaining inhibitors being heat resistant. The last experiments of the series described in this chapter showed that in addition to possessing the abovementioned properties, at least one of the Bowes/sf growth inhibitors was recoverable after acid extraction and gel-filtration. Therefore, at least one of the inhibitors had features that were characteristic of a number of well-described paracrine and autocrine growth regulatory polypeptides including EGF, TGF-α, TGF-β, PDGF (see literature review in Chapter 1).

In the absence of specific antibodies or c-DNA probes to any of the abovementioned factors, it was decided to focus only on the characterization of the acid-stable Bowes/sf polypeptides. The chapter which follows is a more formal characterization of this factor (or factors).

Definition of specific activity

Before proceeding with the characterization of any biological activity it is important to define that activity in quantitative terms. I have therefore defined one unit of growth inhibitory activity as that amount that inhibited the incorporation of ^{3}H-thymidine by 50% in the standard UCT-BR-1 microtiter plate assay.

For the purposes of the characterization process, the specific activity will refer to the number of units of inhibitory activity per mg of protein. It appeared reasonable to base the specific activity on protein content since the preliminary experiments had shown the inhibitor to be a polypeptide.

CHAPTER 3

CHARACTERIZATION OF THE BOWES/SF

GROWTH INHIBITOR

CHAPTER 3

INTRODUCTION

Preliminary studies of the growth inhibitory activity secreted by the Bowes/sf cell line demonstrated that the active material was an acid-stable trypsin-sensitive polypeptide with a molecular weight (based on dialysis and ultrafiltration experiments) greater than 10 000 daltons. In this Chapter I describe my attempts to characterize the Bowes/sf growth inhibitor more definitively.

The procedure that I eventually adopted in my attempts to purify the factor was as follows. Serum-free harvest fluid was filtered to remove debris and concentrated approximately 300-fold by ultrafiltration, dialysed into low ionic strength buffer and lyophilized. The dry material was taken up in acetic acid and chromatographed successively on a Biogel P-60 gel-filtration column and on a C18 reverse-phase HPLC system.

Many preliminary experiments went into the development of this protocol; I present a brief summary of the relevant results in the section that follows the Introduction. Two of the runs were reasonably successful in that they went without hitch and gave results that were reproducible in the sense that the behaviour of the Bowes/sf growth inhibitor was qualitatively consistent in both of them. The quantitative results,

however, were less reproducible - a fact that is evident from the data that I give in the following two tables.

RUN ONE NOVEMBER 1984 - MARCH 1985

Step	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)	Yield (%)	Purification (fold)
Conditioned medium	100	600	60000	100	1
Concentration, Diafiltration*, Lyophilisation	50	270	13500	23	0.45
Bio-Gel P60	1	1785	1785	3	3
C_{18} RP-HPLC	~0.1	1200	120	0.2	2

*Buffer exchange by ultrafiltration.

RUN TWO JANUARY 1988 - APRIL 1988

Step	Total Protein (mg)	Specific Activity (units/mg)	Total Activity (units)	Yield (%)	Purification (fold)
Conditioned medium	100	769	76900	100	1
Concentration, Diafiltration*, Lyophilisation	50	6290	314500	409	8
Bio-Gel P-60	0.75	90416	67812	88	118
C_{18} RP-HPLC	0.07	11000	770	1	14

* Buffer exchange by ultrafiltration.

Before proceeding with a detailed account of the protocol and the results that I obtained, it is appropriate that I discuss some of the possible reasons for the differences in yield and final specific activity that came from the two runs.

Run 2 gave a higher recovery of purer inhibitor than did Run 1 and I attribute this to the following factors.

Firstly, when I came to perform Run 2 I was much more familiar with the system; I was better able to plan each stage and, as a result delays were minimized and labile stages kept to a minimum.

Secondly, I found that earlier passage UCT-BR-1 cells were much more sensitive to the Bowes/sf inhibitor i.e. there was intrinsic variation within the indicator cells themselves. I therefore used the earlier passage UCT-BR-1 as the indicator cells in Run 2.

In the pages that follow I shall consider the results of Run 2 as the more definitive data set and I shall present the data of Run 1 only to provide evidence of qualitative reproducibility in chromatographic behaviour.

The further characterization of the Bowes/sf inhibitor, as described in this chapter, can be divided into three phases.

Firstly, as described in the following section, certain preliminary chromatographic analyses were performed. These empirical experiments made use of a number of standard techniques which are commonly utilized in protein purification including ion-exchange chromatography, hydrophobic interaction chromatography and reverse-phase high-performance liquid chromatography (HPLC). Only the essential findings are discussed in this Chapter.

Secondly, a number of important questions were addressed. These included:-

(i) Was it possible to enhance the specific activity of the putative growth inhibitor?

(ii) What was the approximate molecular weight of the growth inhibitor?

(iii) Was there an indication that more than one inhibitor may be present?

(iv) Were there any clues from the published literature as to the possible identity of the inhibitor?

Most of the above questions were addressed within the biological limitations imposed by the UCT-BR-1 microplate

assay and this somewhat restricted the flexibility of the investigations. Ideally, the type of analysis outlined in this chapter requires either a specific antibody to the molecule of interest or, if the molecule is an enzyme, an assay that exploits a catalytic property.

Thirdly, some observations were made concerning the possible biological mechanisms of action of the growth inhibitor.

For the purposes of clarity and presentation, the putative Bowes/sf inhibitor is referred to as MDGIF ("Melanoma-Derived Growth Inhibitor") or more simply GI ("Growth Inhibitor").

RESULTS

PRELIMINARY CHROMATOGRAPHIC ANALYSES

The Bowes/sf growth inhibitory material (MDGIF) was found to adhere to cation exchange columns, indicating the major inhibitory fraction to be positively-charged (or basic) at neutral pH. This was demonstrated both with "conventional" ion-exchange resins and with high-performance ion-exchange liquid chromatography (HPLC). Ion-exchange chromatography was, however, not deemed to be a suitable means of

purification and characterization because of the problems associated with desalting the column fractions. Since the assay of the growth inhibitor depended on demonstrating inhibition of proliferation over 72 hours, it was critical to remove all buffer salts from the samples. In theory this may be achieved by (i) dialysis (ii) constant-volume ultrafiltration (iii) gel-filtration chromatography. In practice, these procedures were often associated with unacceptable losses of activity presumably because the fractions concerned had extremely low protein concentrations. Furthermore, the problem was compounded by the need to sterile-filter all samples prior to assay.

Although similar problems were encountered with attempts at hydrophobic-interaction chromatography, these experiments (using Phenyl-Sepharose$^{(R)}$) strongly suggested that the major growth inhibitor was very hydrophobic; the MDGIF bound to the Phenyl-Sepharose in a phosphate buffer containing 0.8M ammonium sulphate and eluted only when the ammonium sulphate was removed and when ethylene glycol (75% v/v) was added to the eluting buffer. Because the interferons (see literature review) are very hydrophobic, Bowes/sf conditioned medium was tested for the presence of interferon. I am grateful to Prof. B. Schoub,(National Institute of Virology, Sandringham, Transvaal) for performing the interferon immunoassay. No interferon immunoreactivity was detected.

The hydrophobic material that eluted from the Phenyl-Sepharose column was tested for type beta transforming growth factor (TGF-β) activity using the NRK-49F assay described in the literature review. (Courtesy of Dr. A.B. Roberts, National Cancer Institute, NIH, Bethesda, Md.). Active TGF-β was detected at ~ 6-10 ng per mg of MDGIF protein. TGF-β is known to be a bifunctional regulator of cell growth (76) and this raised the possibility that it alone might be responsible for the growth inhibitory activity of the Bowes/sf medium.

Purified human TGF-β (obtained courtesy of Dr. A.B. Roberts) was tested in the UCT-BR-1 72-hour microplate assay, using conditions identical to those employed in determining MDGIF activity (Methods and results are displayed in Chart 12). TGF-β was found to have a bell-shaped dose-response effect on ^{3}H-thymidine incorporation by UCT-BR-1 cells i.e.both low and high doses had a minimal effect on the indicator cells. Only concentrations in the range 0.78-25 ng/ml (0.031-1nM) resulted in a % inhibition that was greater than 20 (Chart 12). The maximum degree of inhibition was ~33%, significantly less than the inhibition obtained in many preliminary MDGIF assays. Therefore, although purified TGF-β was inhibitory in the microtiter plate assay, it was unable, at the doses used, to achieve the same degree of inhibition demonstrated by MDGIF. This strongly suggested that TGF-β alone was not responsible for the phenomenon described in the previous Chapter and that there must be at least one other polypeptide factor involved.

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CHART 12

CHART 12

THE EFFECT OF PURIFIED TGF-BETA ON UCT-BR-1 CELLS IN THE STANDARD MICROTITER PLATE ASSAY.

Purified human TGF-beta had a significant inhibitory action on UCT-BR-1 cells using the ^{3}H-thymidine uptake assay. Maximal inhibition was 33%, and this occurred in the 1-6 ng/ml (~100pM) range.

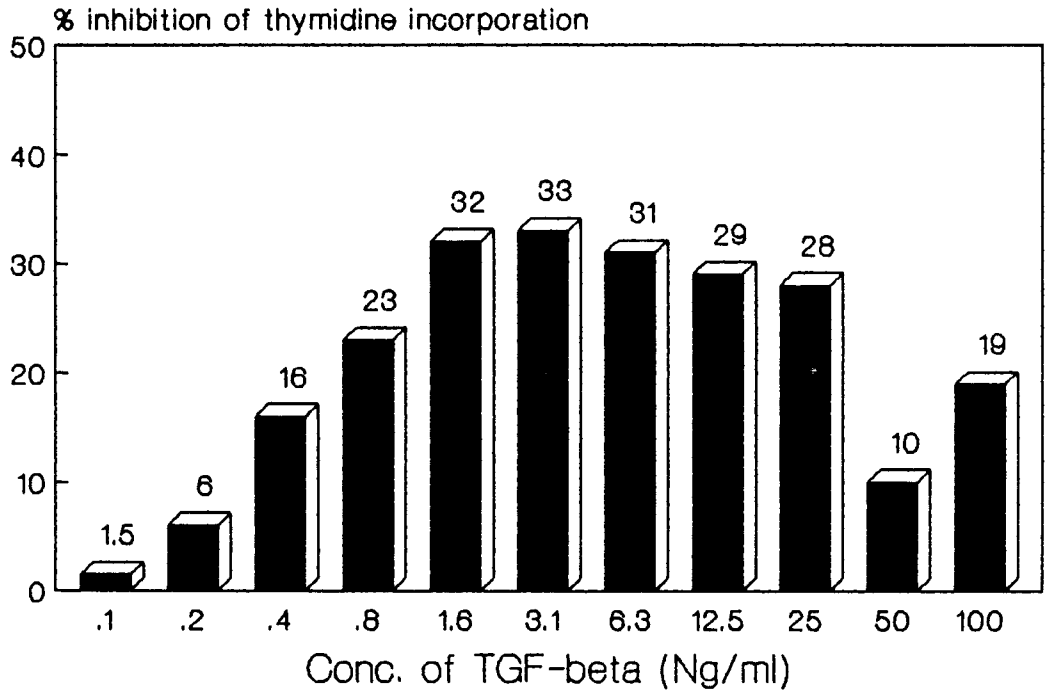
Concentration of TGF-β in the medium (Ng/ml)	Mean cpm (± SEM)	% Inhibition	p value
100	31384±1085	19	<0.05
50	31423±212	10	<0.05
25	27911±555	28	<0.01
12.5	27280±793	29	<0.01
6.25	26703±668	31	<0.025
3.125	25992±911	33	<0.005
1.56	26417±689	32	<0.005
0.78	29733±916	23	<0.025
0.39	32329±887	16	~0.05
0.2	36366±1221	6	NS
0.1	38022±1236	1.5	NS

Control : 38591 cpm ± 4774

NS = Not significant. p values determined by Student's t-test.

NB. TGF-beta concentration of 200 ng/ml or greater did not appear to produce any further inhibition.

CHART 12
Effect of TGF-beta



DETERMINATION OF SPECIFIC ACTIVITY

As outlined in the previous Chapter, one unit of MDGIF activity is that quantity of a particular MDGIF preparation that inhibits ^{3}H-thymidine incorporation into UCT-BR-1 cells (seeded in microtiter plate wells) by 50%, over a 72 hour period. Furthermore, it was decided that specific activities were to be defined in terms of units of activity per mg of protein. In order to do this, the following protocol was adopted:

Step One

(i) An aliquot of MDGIF preparation was lyophilized and was then reconstituted in a minimal volume of 4mM HCl.

(ii) This was subsequently diluted 10-fold in PBS, pH 7.4.

(iii) The protein concentration of this aliquot was determined by standard techniques, as described in the appendix.

Step Two

(i) An *identical aliquot* of the MDGIF preparation was lyophilized and was reconstituted in 4mM HCl containing bovine serum albumin (BSA) at 1 mg/ml. This HCl/BSA solution is referred to as "HB".

(ii) The HB sample was diluted 10-fold in sterile RPMI medium containing 10% fetal calf serum. In this manner, the amount of MDGIF protein in the sample could be quite accurately determined. The low pH of the HB was sufficient to sterilize the sample and this precluded filtration procedures.

(iii) Finally, the reconstituted material was serially diluted in RPMI-10 and each dilution was tested in quadruplicate in the standard microplate assay, at a final volume of 0.1ml per well.

(iv) The response in the UCT-BR-1 microtiter assay was defined in terms of % inhibition of ^{3}H-thymidine uptake relative to control UCT-BR-1 cells that had received only RPMI-10.

$$\% \text{ Inhibition} = \frac{\text{Control cpm} - \text{Test cpm}}{\text{Control cpm}} \times \frac{100}{1}$$

Step Three

(i) A dose-response curve was constructed in which the percentage inhibition of ^{3}H-thymidine incorporation was plotted as a function of the protein concentration of a particular MDGIF preparation. This relationship could be linearized by plotting the logit transform of the % inhibition (or of the thymidine incorporation expressed as a fraction of the control value) as a function of the logarithm of the protein concentration.

The logit transform of a value 'y' is defined as:

$$\text{logit } (y) = \log (y/100 - y).$$

(ii) One unit of activity was determined, either by interpolation or by extrapolation, as that point where the logit was equal to zero (i.e. y = 50% or 0.5).

The logit transformation is commonly used to linearize the dose-response curve of complement-mediated immune hemolysis (138).

FURTHER CHARACTERIZATION OF MDGIF

The MDGIF displayed the characteristics of molecule (or a number of distinct molecules) with a molecular weight(s) in excess of 10 000 daltons. This conclusion was based upon several ultrafiltration and dialysis experiments, as described in Chapter 2.

It therefore appeared reasonable to attempt to concentrate the Bowes/sf by membrane ultrafiltration. This approach had been adopted by Holley and co-workers in the characterization of the TGF-beta/BSC-1 growth inhibitory factor (113,114).

Accordingly, 6 to 7 liters of pooled Bowes/sf conditioned medium, which had been stored in the presence of 0.05% sodium azide, was concentrated to 700 mls using a Millipore (Minitan) concentrator fitted with a set of membranes, each of which had a molecular weight "cut-off" limit of 10 000 daltons. Further concentration was completed in a stirred pressure cell, and this was followed by constant volume ultrafiltration (commonly referred to as "diafiltration") to remove the sodium azide, phenol red and buffer salts. The material was then lyophilized.

Typically, six to seven liters of serum-free Bowes conditioned medium yielded 250-350 mg (by weight) after concentration, diafiltration and lyophilization. This material, which

represented the crude macromolecular retentate of the ultrafiltration process was termed "Fraction A" and was found to contain ~ 0.18 mg of protein per mg of dry material. (The method of Fraction A preparation is described in detail in Chart 13). Therefore, the yield of protein from 6-7 liters of conditioned medium was of the order of 50 mg. The protein concentration of unconcentrated Bowes/sf conditioned medium was previously estimated to be ~ 17 mg/litre. (This estimation was carried out by determining the protein content of dialysed 48-hour conditioned medium). Therefore 6-7 litres of medium should contain ~ 100 mg of protein. Clearly, there was loss of protein, amounting to about 50% of the starting material, in the concentration and lyophilization process. The protein content of the conditioned medium ultrafiltrate, previously shown to have no growth inhibitory activity, was very low - less than 2 mg per liter. Therefore, of the 50% loss of protein that occurred during the preparation of fraction A, less than 12% was lost in the ultrafiltrate whilst the remainder (i.e. 38% or more) probably represented non-specific and unavoidable adherence of protein to membranes and to other surfaces. The loss of protein by this latter mechanism had been anticipated and it was for this reason that the tangential ultrafiltration mechanism, that the "Minitan" system provided, was chosen in preference to other methods. Furthermore, hydrophilic ultrafiltration membranes were utilized at all times in order to further minimize losses.

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CHARTS 13, 14

CHART 13

PREPARATION OF 'FRACTION A'.

"Fraction A" represented a crude preparation of macromolecular material that contained the inhibitor present in the serum-free conditioned medium. It provided the first step in the attempt to further characterize the inhibitory factor.

The Bowes/sf conditioned medium, which had been collected and stored at 4°C in the presence of 0.05% NaN_3, was first concentrated about ten-fold on a Millipore *Minitan*(R) concentrator that is described in the Appendix. During any 'run', 6 to 7 litres of conditioned medium would be concentrated to about 700 to 800 mls.

Further concentration was carried out using a 400ml stirred pressure cell (Amicon) fitted with a YM-10 membrane. The ±800ml of conditioned medium from the *Minitan* was concentrated to give 20mls, making the total concentration factor 300-fold.

This was followed by a process of diafiltration that had been adapted from the method used by Holley et al. (113). These workers had employed this procedure for the purification of the BSC-1 inhibitor/TGF-beta. The protocol was as follows:

The 20 ml of conditioned medium was diluted to 400ml with tris-buffered saline or "ultrafiltration buffer" (Appendix). This was reconcentrated to ±20 ml and was then subsequently diluted five-fold to 100ml with sterile triple-distilled water. Re-concentration to 25mls followed. The ionic strength of the sample at this stage would have been

CHART 13 contd

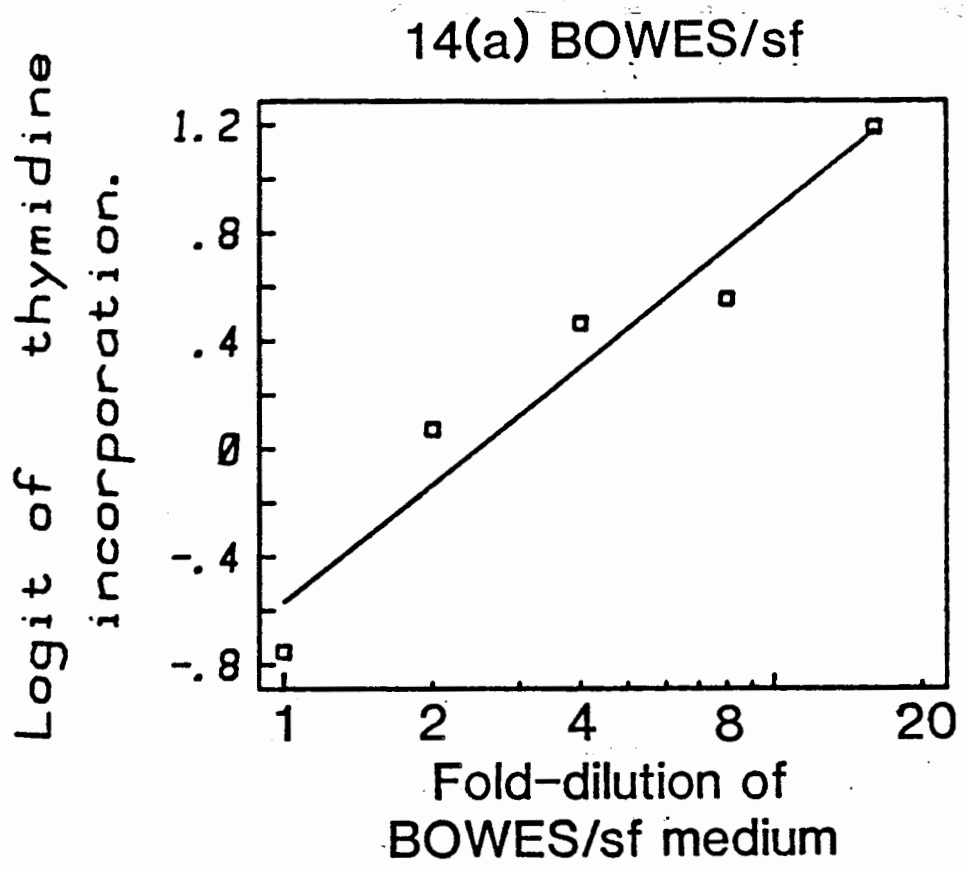
equivalent to 0.03M NaCl; the azide, phenol red, as well as the other RPMI components would have been reduced to 1/100 of their initial concentration.

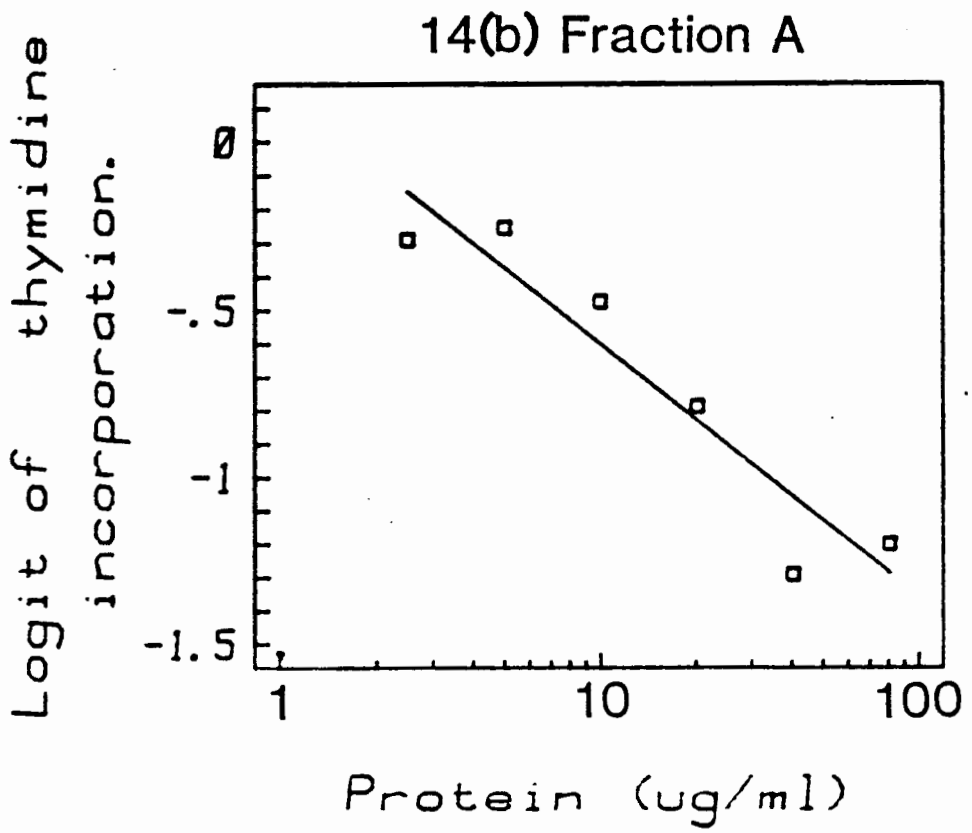
The 25ml of diafiltered material was then removed from the stirred pressure cell and immediately lyophilized. This material was termed "Fraction A". The ultrafiltration membrane used for the concentration process was also removed from the cell and washed overnight in 20 ml of 1M acetic acid to release adherent material.

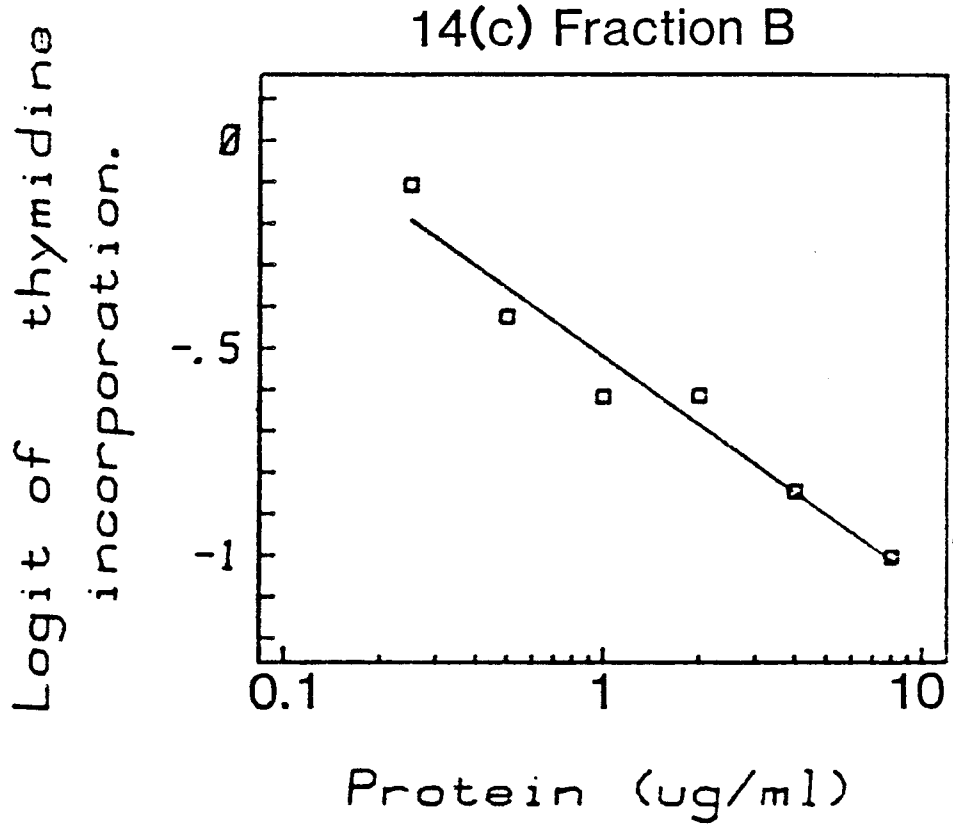
CHART 14

CALCULATION OF THE SPECIFIC ACTIVITIES OF VARIOUS MDGIF PREPARATIONS

Log/Logit Plots were used to transform the inhibitory dose-response curve of the Bowes/sf inhibitor into a linear function.






14(c) Fraction B
Logit of thymidine incorporation.
Ø
-.5
-1
0.1
1
10
Protein (ug/ml)

The specific activity of untreated Bowes/sf conditioned medium was determined from the dose-response curve previously displayed in Chapter 2 (Chart 4). A log/logit plot of this data indicated that 200 µl of a 2.46-fold dilution of Bowes/sf conditioned medium produced 50% inhibition of ^{3}H-thymidine incorporation by UCT-BR-1 cells. (This data is displayed in Chart 14a). Therefore it was calculated that 1 ml of Bowes/sf conditioned medium, representing ~ 16µg of protein, contained 12.3 units of inhibitory activity i.e. 1 mg contained 769 inhibitory units. Similarly it was possible to calculate the specific activity of the Fraction A protein by the previously described methods. (This data is displayed in Chart 14b). 1 mg of 'Fraction A' protein represented therefore, approximately 6290 inhibitory units.

Therefore, this characterization yielded the following data:

	Amount of Protein (mg)	Specific Activity (Inhibitory units/ mg Protein)	Yield (Total Units)
Untreated Bowes/sf Conditioned medium	100	769	76900
Bowes/sf "Fraction A" Preparation	50	6290	314500

This demonstrated that there was not only an approximate 8-fold enhancement of specific activity, there was in addition an unexpected four-fold increase in the total number of inhibitory units present.

In the purification of a biologically active macromolecule, one usually finds that the yield decreases with each consecutive stage of purification. However, this does not necessarily have to be the case and there are several plausible reasons why this might be so:

(A) Certain growth regulatory polypeptides e.g. TGF-β (140) are secreted mainly in an inactive or latent form and are known to undergo activation during the purification process. Activation of latent growth regulatory factors could occur either under acid conditions (140) or as a consequence of the actions of specific proteolytic enzymes.

(B) The crude conditioned Bowes/sf medium may have contained either growth stimulatory factors or factors which interfered with the mode of action of the growth inhibitor, and such factors might have been removed during the initial purification steps. In the case of the Bowes/sf conditioned medium, there was no evidence that the conditioned medium

ultrafiltrate possessed growth stimulatory activity. However, the UCT-BR-1 microplate assay, conducted in the presence of 10% FCS, was designed to detect growth inhibition and not stimulation. Therefore, the possible co-existence of growth inhibitors and growth stimulators could not be ruled out.

In the next stage of the characterization, the Bowes/sf growth inhibitory material was chromatographed on a gel-filtration column under acid conditions (Chart 15). Lyophilized MDGIF Fraction A was extracted overnight in 1M acetic acid and the extract was chromatographed on a Bio-Gel P-60 column in the presence of acetic acid. This column had previously been calibrated with several standard molecular weight markers (Chart 15). The fractions were analysed for growth inhibitory activity using the UCT-BR-1 microtiter plate assay. Two peaks of inhibitory activity were obtained (see Chart 15), one with a maximum at fractions 15 to 18 (peak 1) and the other with a maximum at fractions 26 to 30 (peak 2). The degree of overlap between these two peaks varied. On some occasions they were clearly separate, whilst on others, two broadly overlapping peaks were observed. (Chart 15 displays data from two individual gel-filtration chromatography runs.) According to the elution positions of the various molecular weight markers the maximum of inhibitory peak 2 suggested a molecule with a molecular weight of the order of 20-25000 daltons, whereas the maximum of peak 1 suggested material with a

Contd./132

CHART 15

CHART 15

Profile of inhibitory material eluting from Bio-Gel P60 gel filtration column. Fractions from peak 2 were pooled and lyophilized. This material was termed 'Fraction B'. The data from two individual chromatograms is displayed (Runs 1 and 2).

The lyophilisate of the material concentrated on the stirred pressure cell (Fraction A) was extracted overnight at 4°C in ~40ml of 1M acetic acid. The extract was clarified by centrifugation and was then combined with the 20ml of 1M acetic acid that had been used to wash the YM-10 ultrafiltration membrane. This gave some 50 to 60 ml of 1M acetic acid extract which was lyophilized and reconstituted in 15 mls of 1M acetic acid. This 15 ml of concentrated extract represented the starting material for Bio-Gel P60 chromatography.

The 15 ml of acetic acid - extracted material was chromatographed on a 89 x 2.6 cm (470 ml bed volume) column that had been equilibrated in 1M acetic acid. The column was run at room temperature using a gravity-induced flow rate of 10 mls per hour (1.88 mls/hour/cm^2). A Mariotte flask kept the flow rate constant. Prior to this analysis the column had been calibrated under identical conditions using standard molecular weight markers. These included (i) Blue dextran (ii) Albumin (67000 daltons) (iii) Ovalbumin (43000 daltons) (iv) Ribonuclease A (13700 daltons) and (v) Insulin (6000 daltons). The albumin was found to co-elute with the void volume marker viz. Blue dextran.

7.5 ml fractions were collected. 0.5 ml aliquots were removed from each fraction and lyophilized. These samples were subsequently reconstituted in 0.1 of 4mM HCl containing 1mg/ml BSA (Sigma) and were transferred to 0.9 ml of RPMI-10, for assay in the UCT-BR-1 microplate procedure.

CHART 15
Protein Profile of Bio-Gel P60

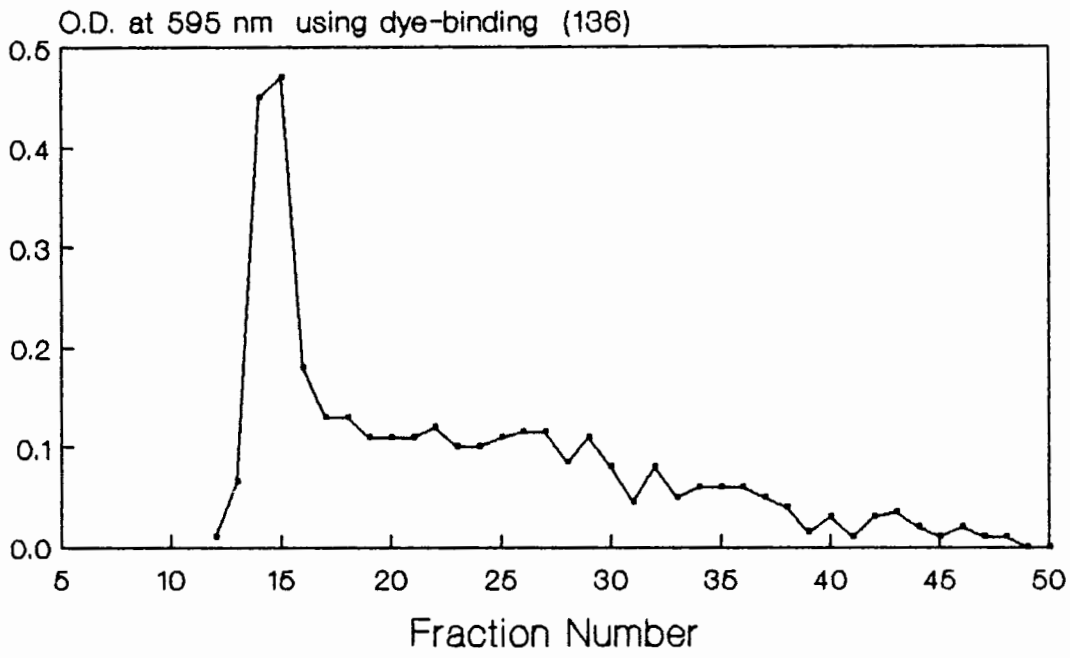


CHART 15
Bio-Gel P60 RUN 1

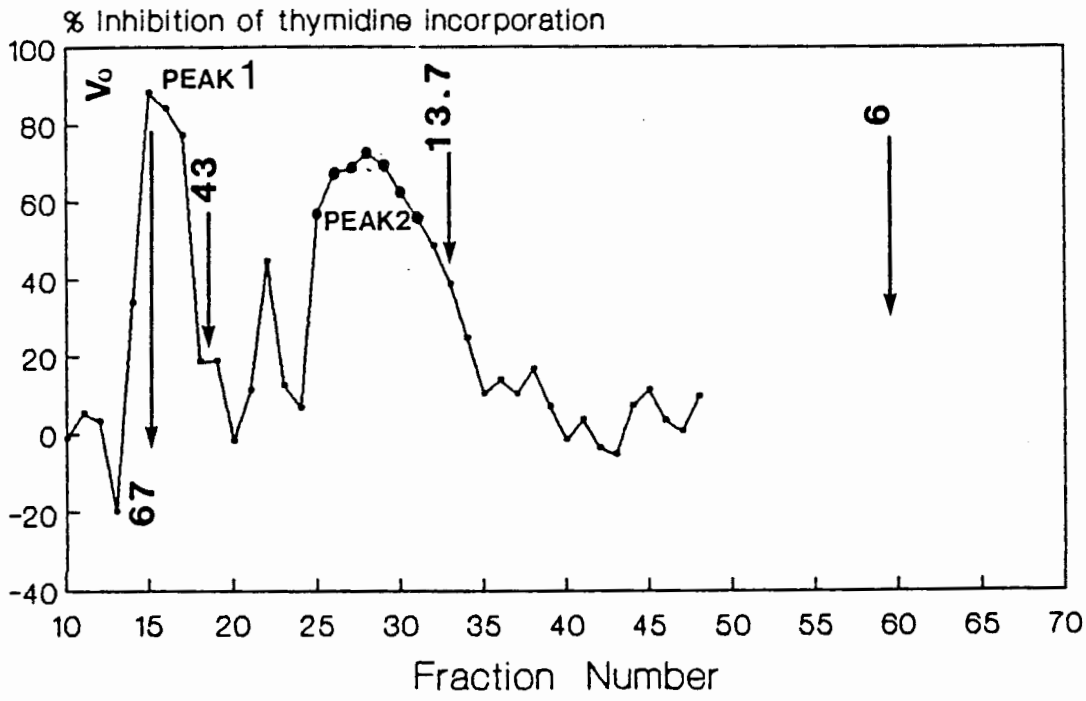
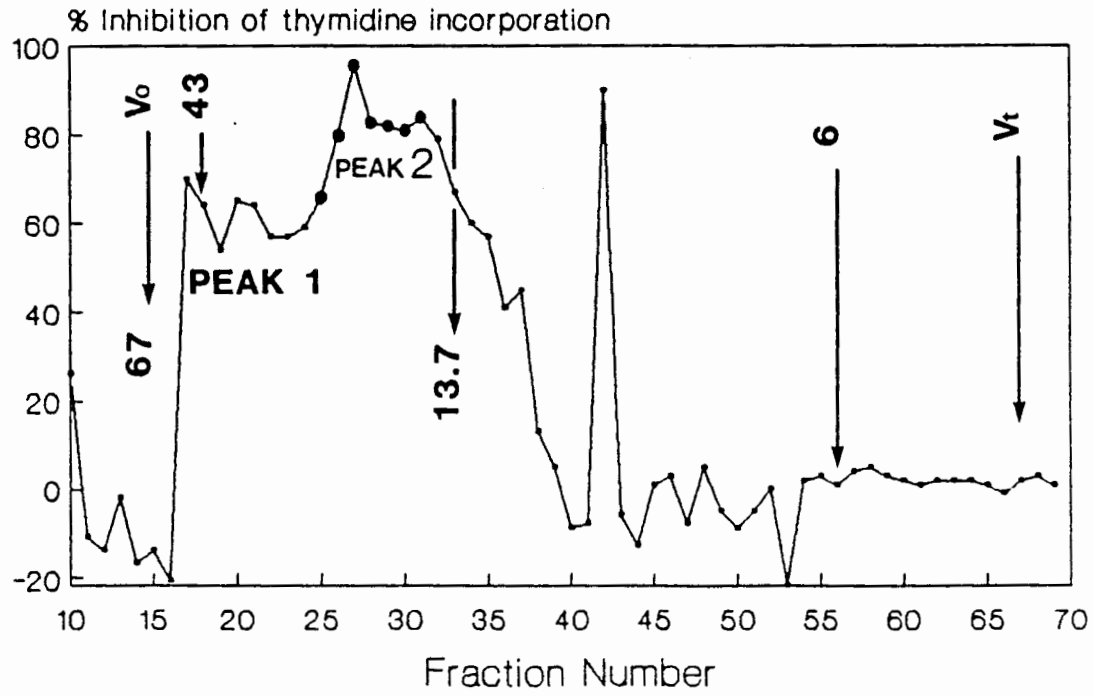


CHART 15
Bio-Gel P60 RUN 2



All molecular weights are given in kilodaltons. The void volume (V_o) corresponded to the elution volume of the 67 kd marker.

molecular weight equal to or in excess of 50000 daltons. It was conceivable that the material in peak 1 therefore represented dimeric or perhaps multimeric forms of the growth inhibitory molecule. For this reason, it was decided to further characterize only peak 2. Hence, these fractions were pooled and lyophilized. The material obtained by this procedure was termed "fraction B". The specific activity of fraction B was fourteen-fold greater than that of fraction A, as displayed below:

	AMOUNT OF PROTEIN (mg)	SPECIFIC ACTIVITY units/mg	YIELD UNITS	(%)
Untreated Conditioned Medium (Bowes/sf)	100	769	76900	(100)
Fraction A	50	6290	314500	(409)
Fraction B (Peak 2 from Gel-filtration column)	0.75	90416	67812	(88)

Note that in terms of yield, fraction B represented ~ 20% of the total number of inhibitory units in fraction A. The specific activity of fraction B was ~ 115-fold greater than that of the starting material, and 14-fold greater than that

of fraction A. The amount of protein represented by fraction B was ~ 0.75% of that present in the starting material.

Fraction B appeared to be relatively heat stable. Over 50% of the inhibitory activity was retained following a period of heating for 2 hours at 70°C in the presence of 1 M acetic acid, as shown in Chart 16 below:

CHART 16

	SPECIFIC ACTIVITY OF BOWES/SF FRACTION B
Unheated	110 000 units/mg (100%)
Heated at 70°C for 2 hrs	59 000 units/mg (54%)

An aliquot of Fraction B containing 20 µg of protein was heated at 70°C for 90 minutes. The sample was then lyophilized, as was a control 20 µg that had not been heated. Both the control and the heated material were tested in the UCT-BR-1 microtiterplate assay.

The next question that was addressed was that of the reversibility of the growth inhibitory effect. UCT-BR-1 cells were seeded in a microtiter plate in the presence of RPMI-10

medium containing a concentration of Fraction B that was able to inhibit ^{3}H-thymidine incorporation by 40-50%. At various times, the MDGIF was removed from the UCT-BR-1 cells and was replaced with fresh medium. These indicator cells were therefore in contact with the growth inhibitor for increasing periods of time ranging from 6 to 90 hours. The results of this assay are displayed below (Chart 17).

CHART 17

DURATION OF EXPOSURE TO MDGIF ELUTED FROM BIO-GEL P-60 COLUMN (FRACTION B)	% INHIBITION	
6 hours	2	NS*
24 "	15	p<0.005
55 "	55	p<0.005
70 "	70	p<0.005
90 "	70	p<0.005

Control counts were 22082 $\pm$ 1300

*NS = Not significant

p values were determined by Student's t-test.

This data demonstrated that the effects of the MDGIF on UCT-BR-1 cells were fully reversible after 6 hours and were almost completely reversible after 24 hours. The % inhibition after 24 hours incubation in MDGIF was minimal (15%) but was nevertheless statistically significant. However, the growth inhibitory effects were entirely irreversible after longer exposure periods.

Previous preliminary experiments that utilized hydrophobic-interaction chromatography (phenyl-sepharose) had indicated the active MDGIF to be a relatively hydrophobic molecule. Further investigations demonstrated that the growth inhibitory activity could be retained on reverse-phase HPLC columns and would elute in the presence of organic solvents such as acetonitrile. This behaviour was known to be a feature of many previously characterized growth factor polypeptides, and reverse-phase HPLC had subsequently proved to be an important tool in their purification (13,38,62). For these reasons, and because of the apparent stability of the active MDGIF, the use of reverse-phase HPLC as a possible means of purification of the growth inhibitor was investigated. Fraction B was diluted in a relatively polar solvent and was loaded onto a semipreparative reverse-phase HPLC column (see Chart 18). Following this step, a linear acetonitrile gradient was used to elute material from the column.

This gradient yielded the absorbence profile (at 280 nm) as well as the biological growth inhibitory profile that is displayed in Chart 18. One major peak of growth inhibitory activity was found. This comprised fractions 20, 21, 22, 23 and corresponded to material which eluted at ~ 62 to ~ 74% acetonitrile. No inhibitory activity was present in equivalent fractions from an identical blank gradient, run under the same conditions.

The fractions containing the growth inhibitory activity were pooled and lyophilized. This pool, designated fraction C, contained only ~ 0.07 mg of protein. However, the specific activity was no greater than that of fraction B and in most of the repeat analyses that were performed, the specific activity was much less than that of fraction B, being usually of the order of ~11000 units per mg protein. Furthermore, the inhibitory material from the HPLC appeared unstable; the specific activity of the lyophilized fraction C decreased significantly over fairly short time spans (2-3 weeks). Reverse-phase HPLC, using the conditions described above, therefore did not appear to be a suitable strategy for further characterization of the Bowes/sf growth inhibitor.

Contd./143

CHARTS 18, 19

CHART 18

HPLC ANALYSIS OF FRACTION B

Chromatography of the MDGIF on a C_{18} reverse-phase HPLC system demonstrated a major inhibitory peak eluting at ~65% acetonitrile. The data from two individual chromatograms is displayed (Runs 1 and 2).

The active fractions, consituting Fraction B, from the Bio-Gel P60 column were pooled and diluted 1 to 1 with distilled water. The final acetic acid concentration of the active pool therefore amounted to 0.5M. Acetonitrile (Waters HPLC grade) and tri-fluoroacetic acid (TFA) were added to give final concentrations of 5% (v/v) and 0.05% (v/v) respectively.

A Waters semi-preparative reverse-phase C18 (octadecylsilane) HPLC column was used. This column had an internal diameter of 7.8mm and a length of 30cm. The column was first equilibrated with Solvent A (0.5M acetic acid, 5% acetonitrile, 0.05% TFA, pH 2.2) for at least 1 hour. The sample (usually having a volume of 100ml or more) was loaded onto the column through the solvent inflow channel, at 1 ml/min. When loading was completed, the column was washed with solvent A at 1 ml/min for one hour. A linear acetonitrile gradient from 5% to 75% was then run at 1% per minute, using a flow rate of 1 ml per minute. Two solvents were used to create the gradient; these were Solvent A (the formulation of which is given above) and Solvent B, which was 100% acetonitrile containing 0.05% TFA.

CHART 18 contd

Whilst the acetonitrile gradient was being run, 3ml fractions were collected until the gradient was completed. This would happen at fraction 24. Thereafter a further 10 x 3.0 ml fractions were collected under isocratic conditions, during which the acetonitrile concentration was held constant at 75% (v/v).

For assay purposes, 0.5ml aliquots were removed from each fraction (1 to 34 inclusive) and lyophilized. The remaining fractions were diluted 1 to 1 with distilled water, in order to lower the final acetonitrile concentration, and then were stored at 4°C until the results of the assay were known. The active fractions were then pooled and lyophilised.

CHART 18

Elution of OD_{280}-absorbing material from RP-HPLC

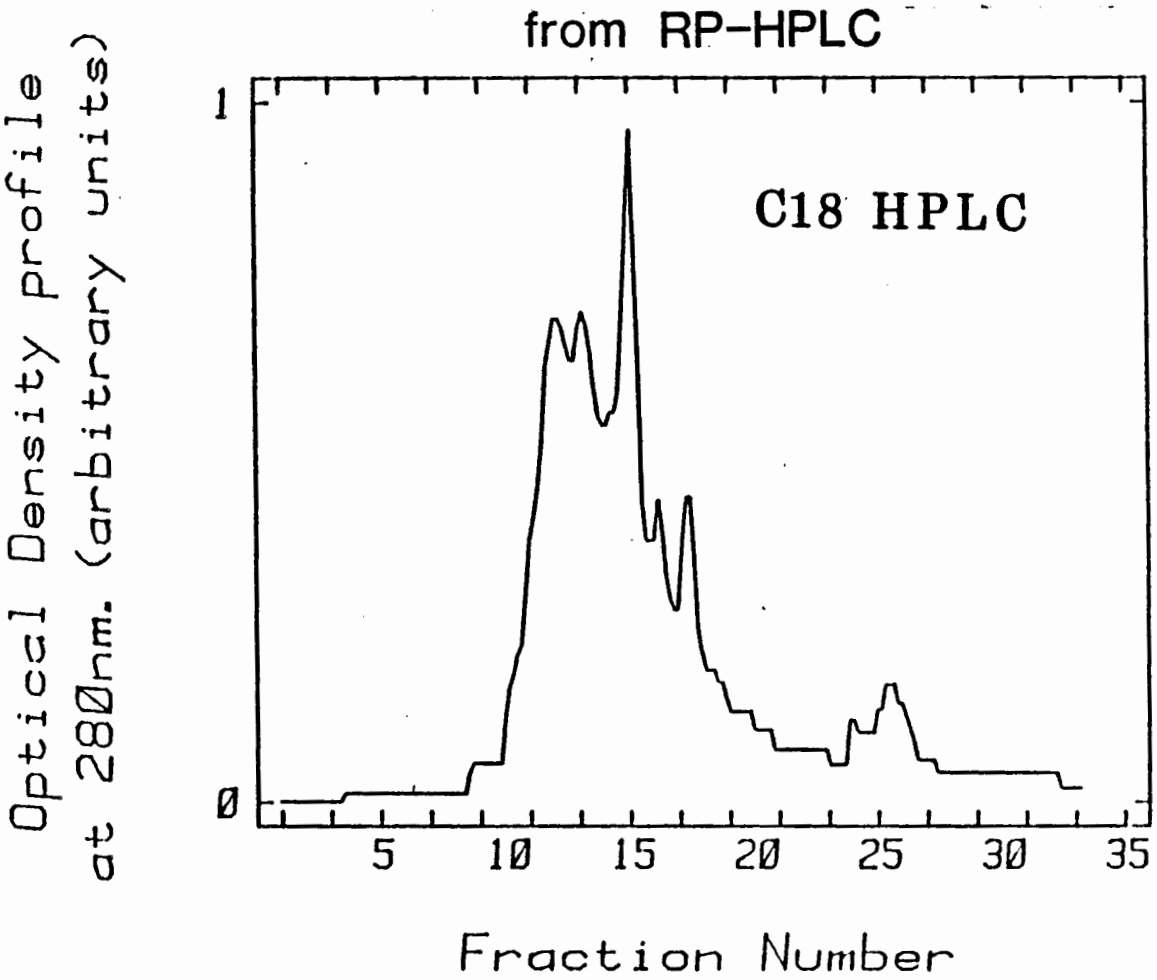


CHART 18
RP-HPLC RUN 1

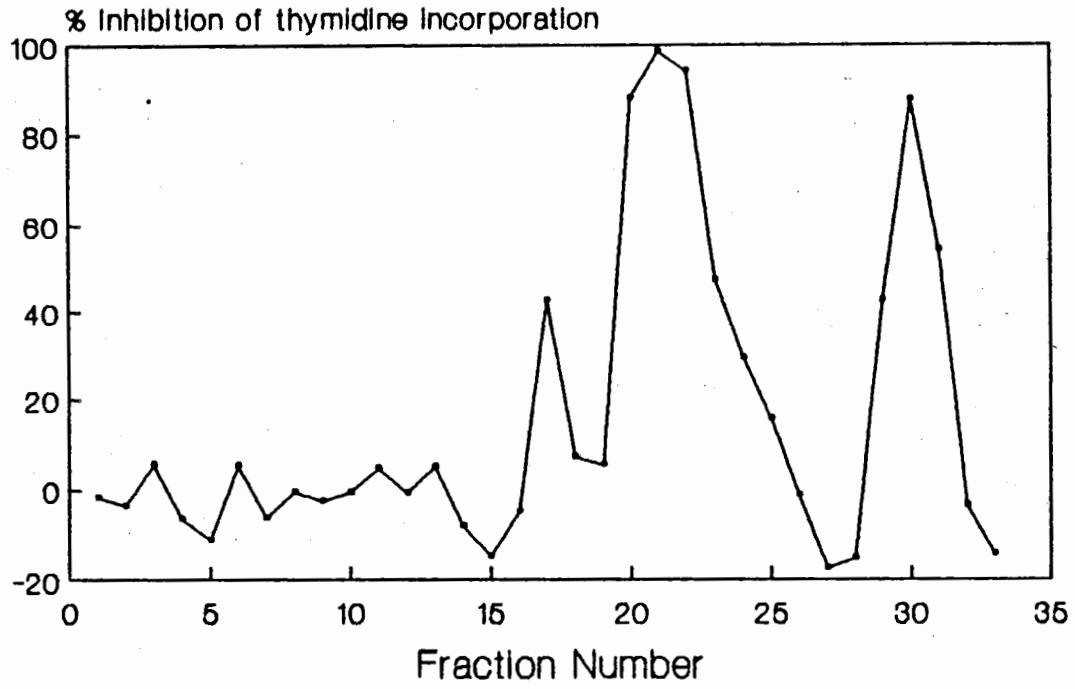


CHART 18
RP-HPLC RUN 2

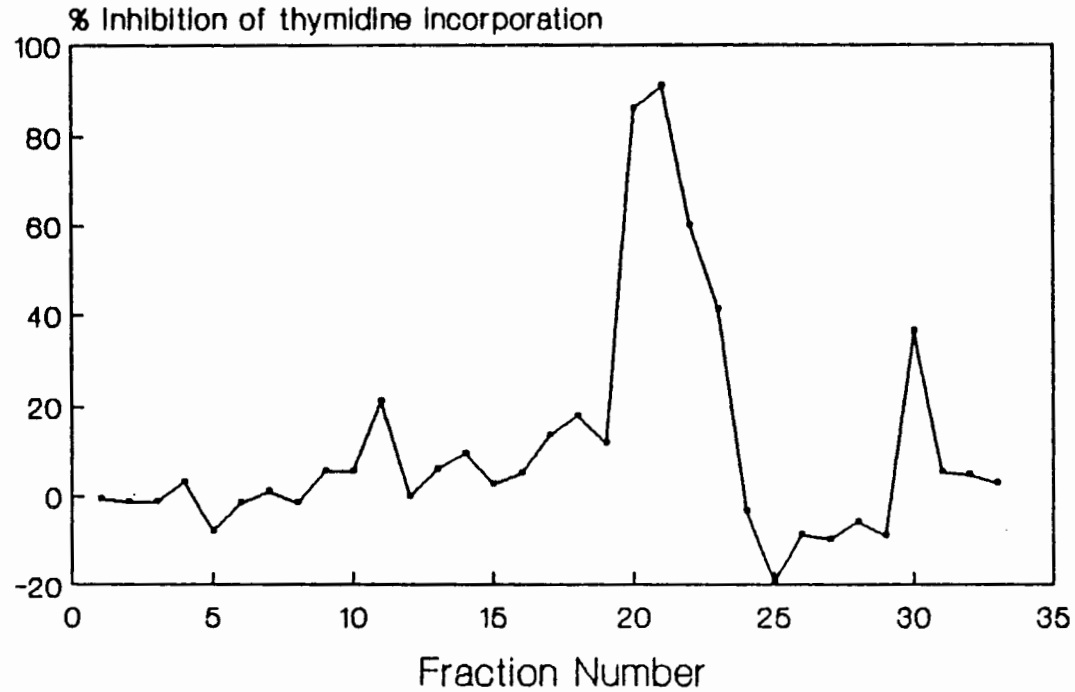


CHART 19

Autoradiograph of 15% SDS-PAGE of ^{125}I-labelled Fraction C from the RP-HPLC column. The sample was electrophoresed in two adjacent tracks.

^{125}I-fraction C was prepared using the iodogen procedure (see appendix). A specific activity of 4.4×10^6 cpm per μg of protein was achieved.

11 ng of ^{125}I-fraction C (48000 cpm) was mixed with 20μg of non-iodinated ("cold") Fraction C in a total volume of 10 μl. 16μl of SDS-sample buffer containing buffer containing beta-mercapto-ethanol (2-4%) and 2% SDS was added and the sample was boiled for 2 minutes.

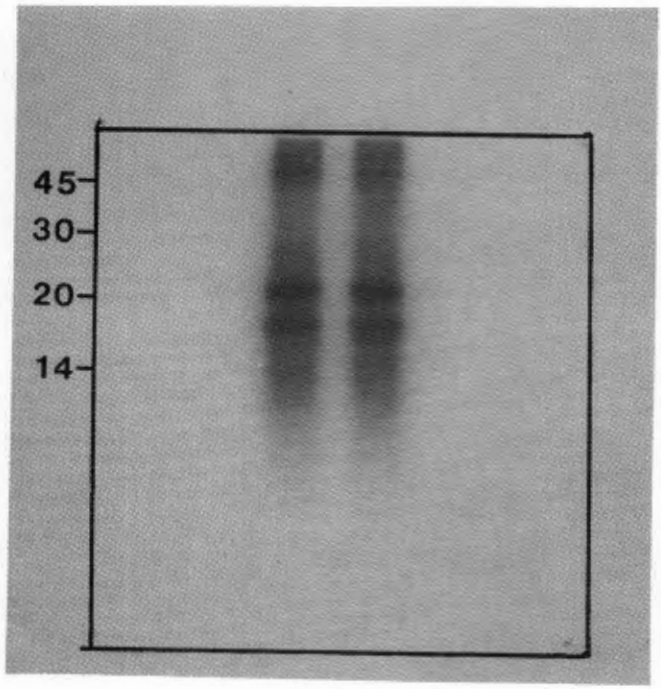
The sample was electrophoresed in 2 adjacent tracks on a SDS-polyacrylamide gel that utilized a 15% resolving gel and a 4% stacking gel. Low molecular weight markers (Pharmacia) were run in tracks adjacent to that of the sample.

The gel was stained with Coomassie Blue, and was then dried and subjected to autoradiography using Kodirex X-ray film (1 KD,5UT) at -20°C for seven days.

m.w. = Molecular Weight

kd. = Kilodaltons

CHART 19



15% SDS-PAGE

DISCUSSION

The experiments outlined in this Chapter demonstrated that the specific activity of the Bowes/sf growth inhibitor could be enhanced ~115-fold by processes involving membrane ultrafiltration and concentration as well as by acetic acid extraction and gel filtration chromatography. The significant increase in the specific activity was explicable by one of several possible processes: (a) enrichment of the growth inhibitory molecule (or molecules) in the preparation (b) activation of latent inhibitory materials (c) selective removal of factors which interfered with the growth inhibitory potential of the MDGIF. It was likely that more than one of these above processes contributed to the enhancement of the specific activity. If only the first process operated, one would not expect to have obtained an increase in the total number of inhibitory units (i.e. the yield) as occurred in the preparation of fraction A.

There was a substantial fall in total yield following the gel-filtration chromatography. This was most likely a result of a decision not to examine the apparently higher molecular weight forms of the growth inhibitor.

What of the identity of the inhibitor? Listed in point form, the major characteristics of the inhibitory material were as follows:

(i) It was a macromolecule with a molecular weight in excess of 10000 daltons.

(ii) It was both acid-soluble and acid-stable (in 1 M acetic acid pH 2.5).

(iii) It was fairly heat stable at 65°C in the environment of the untreated conditioned medium as well as in the presence of 1M acetic acid.

(iv) The molecule was sensitive to trypsin and therefore appeared to be a protein.

(v) Protein constituted about 18% of the dry weight of the material secreted by the Bowes/sf line.

(vi) When chromatographed on a gel-filtration column, the major growth inhibitory fractions eluted with an apparent molecular weight in the 20 - 25000 dalton range.

(vii) It was possible to enhance the specific activity of the growth inhibitor by a process of ultrafiltration/concentration, acid extraction and gel-filtration chromatography.

Analysis of the data suggested that activation of previously latent material might well play a role.

(viii) There was a latent period between the addition of the growth inhibitor and observed morphological changes and growth inhibitory effect. This latent period was of at least one to two days duration. Furthermore, the growth inhibitory effect was reversible if the inhibitor was present for less than 24 hours and was then replaced by fresh medium.

(ix) The inhibitor was not an interferon.

(x) Biologically active TGF-β was detected in fractions eluted from hydrophobic exchange columns. TGF-β is a 25000 dalton acid-stable growth regulatory polypeptide (76) with growth inhibitory effects, especially on breast tumour cells (139). Purified TGF-β had some growth inhibitory actions in the UCT-BR-1 assay, but was unable to achieve the major degree of inhibition demonstrated by the MDGIF preparation.

The material which eluted from the gel-filtration column (fraction B) was not yet pure. This judgement was based on the presence of numerous protein bands on SDS polyacrylamide gel electrophoresis (SDS-PAGE) of MDGIF preparation (Results

not shown). The possibility therefore existed, at least in principle, of obtaining inhibitory material of even greater specific activity. The decision to use reverse-phase HPLC to this end was based upon (i) the relative success ascribed to this approach in the identification of growth factors (ii) the apparent hydrophobicity of the growth inhibitor as assessed by preliminary qualitative experiments that utilized both hydrophobic-interaction chromatography and reverse-phase HPLC. However, reverse-phase HPLC chromatography of the growth inhibitor was unsuccessful in that it failed to enhance the inhibitory activity of the MDGIF preparation. There were several possible reasons for this failure. These included (i) partial inactivation of the growth inhibitor in the fairly harsh conditions used in reverse-phase HPLC (ii) separation of distinct molecular species, each with inhibitory activity, by the chromatographic procedure i.e. there might well have been multiple inhibitors, some of which either eluted prematurely under the isocratic loading conditions, or remained adherent to the column even in the presence of the non-polar solvent.

Even the major inhibitory fraction which eluted from the RP-HPLC contained several molecular species. An autoradiograph of an SDS-PAGE of "Fraction C" is displayed in Chart 19.

The presence of biologically active TGF-β in the inhibitory fractions which eluted from hydrophobic-interaction columns,

as well as the subsequent inability of purified TGF-β to fully reproduce the inhibitory spectrum of the MDGIF, provided circumstantial evidence that more than one inhibitor might have been present. Furthermore, multiple inhibitory peaks were detected in the gel-filtration chromatography of MDGIF preparation. Although these peaks may have represented aggregated or multimeric forms of a single molecular species, as was suggested earlier, they may have been a result of multiple distinct molecular forms.

In summary therefore, it was observed that the human cell line Bowes/sf, derived from a melanoma, (a tumour of neural crest origin) was able to proliferate in the complete absence of either serum, exogenous polypeptide growth factors, steroids or thyroid hormones. The Bowes/sf conditioned medium was growth inhibitory to a number of transformed human epithelial cell lines, especially a breast carcinoma UCT-BR-1. This inhibition followed a latent period of 1-2 days and was essentially irreversible after periods longer than 24 hours. Furthermore, inhibition occurred in the presence of 10% fetal calf serum which is rich in growth stimulatory factors. Additional experiments indicated that the inhibitory effect was due to a trypsin-sensitive, heat- and acid-stable polypeptide (or polypeptides) that was not a member of the interferon family.

The responsible polypeptide(s) appeared to have a molecular weight(s) in the 20 - 25000 dalton range. There was also evidence which indicated activation of previously latent growth inhibitory factor(s) during the characterization.

At least one of the factors was identified as TGF-β (140-142), an acid-stable polypeptide that is now known to be a major autocrine inhibitor of breast carcinoma cells in vitro and in vivo (139,143). Furthermore TGF-β is produced in an inactive high molecular weight form that is subsequently activated either by acid conditions or by limited proteolysis (140,142). Besides breast carcinoma cells, TGF-β is known to inhibit numerous other cells of epithelial origin. However, non-epithelial cells generally remain either unaffected by TGF-β or are stimulated by this growth factor (140,142). TGF-β thus is a prototype of a bifunctional growth regulatory polypeptide that can either stimulate or inhibit the target cells, depending on the nature of the cell being studied and the presence or absence of other factors. TGF-β belongs to a family of regulatory polypeptides which appear to be derived from a single ancestral gene. Many of these polypeptides have inhibitory actions, and include inhibin (a gonadal factor which inhibits pituitary FSH release) and the Müllerian inhibitory polypeptide (140,142) which is responsible for regression of the female urogenital system in male embryos.

When the MDGIF phenomenon is examined in the light of the TGF-β model, it is easy to conceive of a cell type that produces a factor that is either inactive or for which receptors are lacking on the producing cell, in this case the Bowes/sf line. Addition of this factor to the UCT-BR-1 cells produces growth inhibition because the UCT-BR-1 cells have functional receptors for the factor and in addition are able to activate the factor by, for example, producing proteases that cleave the factor at specific sites. In an alternative model, both the Bowes/sf and the UCT-BR-1 cells may have functional receptors but the nature of the response to receptor activation may be completely opposite in the two cell types.

CHAPTER 4

DIRECTIONS FOR THE FUTURE

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DIRECTIONS FOR THE FUTURE

In this thesis, an attempt was made to characterise the phenomenon in which conditioned medium obtained from a malignant cell line of neural crest origin irreversibly inhibited the growth of transformed human epithelial lines. Although many important biological and biochemical features of the putative growth inhibitor were delineated, the molecule (or molecules) responsible for the inhibitory action was not isolated in a pure form. Furthermore, the description of the Bowes/sf inhibitor was obtained only within the constraints of certain clearly defined parameters. These included:

(a) The response to the Bowes/sf medium and extracts thereof was defined as being inhibitory; this included either inhibition of cellular proliferation or inhibition of radiolabelled thymidine incorporation. Therefore, additional *potential* responses to the Bowes/sf factor (e.g. expression of novel cell surface antigens) were not examined.

(b) The inhibitory phenomenon was examined only in the presence of 10% fresh fetal calf serum. The latter is known to contain a multitude of growth factors.

(c) The affected target cells were all transformed, aneuploid human epithelial cell lines. The most

sensitive cell line appeared to be one derived from a metastatic breast cancer.

(d) The Bowes/sf inhibitor was detected only on the basis of its biological activity and not on any antigenic or catalytic properties.

It was essential to impose those constraints on the work performed for this thesis in order to allow the investigations to progress in a logical order.

What direction should this work take in the future? There are two critical issues which would have to be addressed before full insight could be gained into the mechanism of action and the biological significance of the growth inhibitor. Both issues are equally important and are not mutually exclusive. Firstly, the inhibitor must be purified. Secondly, once purified, the interaction of the inhibitor with a number of target cells under a variety of different conditions must be studied. The first issue is essentially a biochemical problem that requires a reductionist approach. The second issue, is one in which the cellular interactions of the inhibitor require to be investigated. Using a simple analogy, the distinction between the two approaches could be compared to the difference between, on the one hand, isolating an enzyme in a pure form and, on the other, completely defining the metabolic pathway (with all its control mechanisms) in which that enzyme participates. Indeed, it has recently become apparent that growth regulatory polypeptides form an

interacting network, a network of complex variables in which the effect produced by the factor depends upon the presence (or absence) of other polypeptides as well as the nature of the responding cell (144). This broader, more universal approach, is essential in order to make progress in the field of cell and cancer biology. However, it would be very difficult to investigate the full biological significance of a growth factor without having pure material at hand.

We therefore return to the first issue i.e. the problem of obtaining the growth inhibitory factor in pure form. Two questions must be answered. Why was it not possible to fully purify the active factor using the techniques described in this thesis? Furthermore, what are the new techniques that might be applied in the future in order to resolve this problem?

One of the major problems encountered during the purification of growth factors has been the quantity of starting material that is needed. These are typically, either liters of conditioned medium (55) or several kilograms of cultured cells (75). The explanation lies in the ability of polypeptide growth factors to exert their biological effects at very low concentrations, often less than 10^{-12}M. For this reason, they often exist only in trace amounts within biological sources. The above problem is compounded by the intrinsic hydrophobicity of many growth factor molecules, for this leads to unacceptable losses during the purification. Furthermore,

the protean nature of purely biological assays, especially those which are based upon cell growth kinetics, can make it extremely difficult to keep a systematic account of important parameters such as yields and specific activities.

Some of the solutions to these problems are self-evident. There is therefore a need, as mentioned earlier, to 'scale-up' the purification protocol so that use is made of enormous quantities of starting material. In addition, losses can be minimized e.g. by using hydrophilic filtration membranes and siliconized glassware whenever possible.

Because many of the assays for growth factors are operationally-defined biological assays, it is often useful to obtain a panel of antibodies, specific to known growth factors. These would help confirm whether or not a novel factor had indeed been isolated.

There are a number of novel approaches which, in principle, could facilitate the isolation of a polypeptide growth factor which is normally present only in minute concentrations. Firstly, one could attempt to develop a specific monoclonal antibody by immunizing mice with crude starting material. Following the spleen cell fusion stage and the subsequent generation of the immortal hybrid line, the aim would be to detect positive clones by virtue of their ability to secrete antibodies which specifically block the action of the growth factor. In the special case of the Bowes/sf MDGIF, the

anti-MDGIF monoclonal antibodies would be defined by their ability to interfere with the growth inhibitory assay i.e. UCT-BR-1 cells would be seen to grow normally in the presence of MDGIF and specific antibody. Positive clones could then be expanded to generate large quantities of monoclonal antibodies which could be linked to an affinity support. It would then be feasible to purify the growth factor in a single step by means of affinity chromatography. The monoclonal antibody technique is therefore extremely useful because there is no requirement for purified antigen in order to generate the antibody.

An alternative approach to the problem of identifying the factor would be to generate a cDNA library from Bowes/sf cells. The Bowes/sf cloned cDNAs could be inserted into expression vectors and then transfected into eukaryotic cells. Clearly, this is an enormous task, but it would be anticipated that a cDNA coding for the growth inhibitor should confer, upon the transfected cell, the property of synthesising MDGIF. In this manner it is possible, in principle, to identify the MDGIF cDNA without first achieving purification of the active polypeptide factor.

Whatever the approach that is finally adopted, the ultimate and ideal aim of all the investigations would be to develop potential therapeutic applications for the inhibitory factor. It would be naive to assume that a single polypeptide factor might be of major clinical significance, but it is possible that such a polypeptide may well have a role when used in combination with a number of other cell growth factors.

Even after a decade, the field of cell growth regulatory polypeptides remains in its infancy. Nevertheless, encouragement should be taken from the important insights which have been gained over this period. This field is one of the most rapidly expanding areas within modern cell biology. There is little doubt that this body of knowledge will have widespread future applications in such diverse areas as developmental biology, the enhancement of wound healing as well as in the control of malignancies.

APPENDIX

APPENDIX

1. CULTURE MEDIA AND SOLUTIONS

RPMI 1640 medium (Gibco No. 074-1800) containing 2mM glutamine and 24mM bicarbonate, and Dulbecco's modified Eagle's medium (DMEM; Gibco No. 074-2100) containing 0.45% (w/v) glucose were purchased as powdered reagents from Gibco, reconstituted as recommended by the suppliers and adjusted to contain 500 units/ml of penicillin G, 0.2mg/ml of streptomycin and 0.06mg/ml of tylocine (Gibco).

Fetal calf serum (FCS) was obtained as a gamma-irradiated product from the State Vaccine Laboratories, Pinelands, Cape Town. This was heat-inactivated (56°C, 30 minutes), sterile filtered (0.45 micron filter, Millipore HAWP04700) and stored as sterile 50 ml volumes at -20°C

Tris-buffered saline (TBS) was made by dissolving NaCl, 8g; KCl 0.38g; Na_2HPO_4 .$2H_20$ 0.125g; and Tris (hydroxymethylaminomethane) 3.0g in approximately 900 ml water. The solution was adjusted to pH 7.4 (at room

temperature) with 3M HCl, made up to 1000 ml with water and sterilized by autoclaving. This gave a final concentration of NaCl :137mM; KCl : 5mM; Na_2HPO_4 : 0.7mM and Tris : 25.0mM. Double strength tris-buffered saline (2xTBS) contained the same constituents at twice the concentration.

Phosphate buffered saline (PBS) pH 7.4 was made by dissolving NaCl, 8g; KCl, 0.2g, $Na_2HPO_4.2H_20$, 1.44g and KH2PO4, 0.2g in approximately 900 ml of water. When dissolved, the pH was checked, and the volume was adjusted to 1000ml with distilled water. This gave final concentrations of Na^+ 153mM; K^+ 4.2mM; Cl^- 140mM; and inorganic phosphate 9.6mM. The solution can be sterilized by autoclaving.

Versene Buffer. This was prepared by dissolving NaCl, 8g; KCl, 0.2g; $Na_2HPO4.2H_20$, 1.14g; KH_2PO_4, 0.2g and Na_2 EDTA 0.26g in 900ml of distilled water and then, when all components were dissolved, adjusting the volume to 1000ml. The solution was sterilized by autoclaving. This gave a final concentration of Na^+ 151mM; K^+ 4.2mM; Cl^- 140mM; inorganic phosphate 7.9mM and EDTA 0.8mM.

Trypsin solution. Working solutions were prepared from a stock solution of 0.5% w/v trypsin (Difco 1:250) in 1mM HCl. Phenol Red (Flow Laboratories) was added to the stock

solution: 1 ml of 0.5% Phenol Red in 500 ml of trypsin stock. The stock solution was sterilized by passage through a 0.45 micron Millipore filter and was stored frozen, in 50ml volumes at -20°C.

Working solution 1: 0.25% trypsin with EDTA was prepared by mixing equal volumes of 0.5% trypsin and 2xTBS. After mixing, 1 ml of 2% Na_2 EDTA (made up in TBS) was added to each 100ml solution to give 0.25% trypsin, 0.6mM EDTA in TBS.

Working solution 2: 0.05% trypsin with EDTA was prepared by mixing equal volumes of 0.5% trypsin stock solution and 2xTBS. The mixture was then diluted 1:5 with Versene buffer to give 0.05% trypsin, 0.6mM EDTA in tris-phosphate-buffered saline.

Tris-saline ultrafiltration buffer was made by dissolving NaCl, 8.0g and tris (hydroxymethylaminomethane), 3.0g in 900ml distilled water and then adjusting the pH to 7.4 with HCl. The solution was then made up to 1000ml with distilled water and was sterilized by autoclaving. This gave final concentrations of NaCl : 137mM and tris : 25mM.

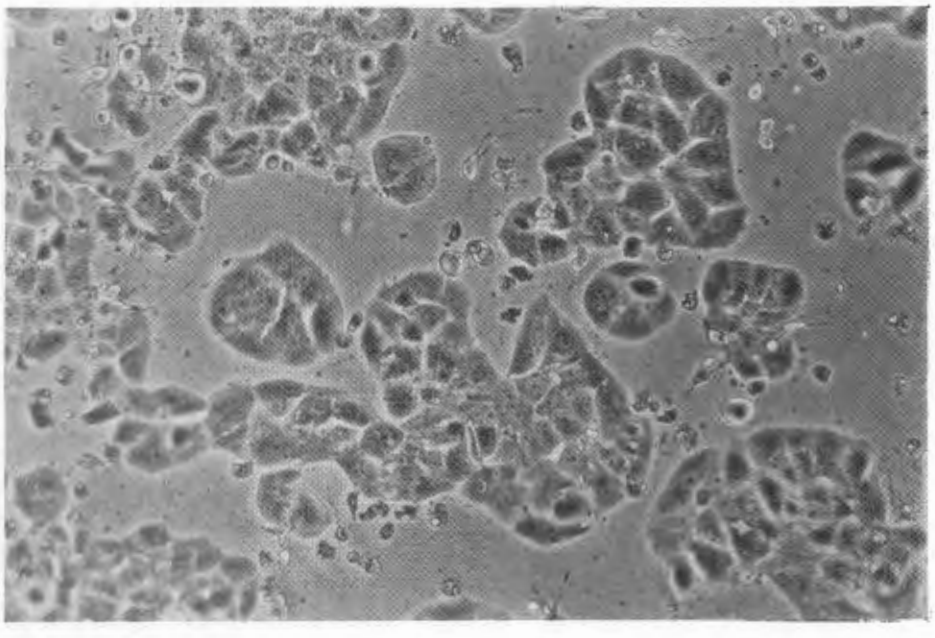

FIGURE 1

2. CELLS

The cells used in this study are described briefly in the paragraphs that follow. The prefix "UCT-" indicates that the cells are maintained as a continuous cell line that was established at the University of Cape Town, in this

laboratory, largely due to the efforts and expertise of Professor E.L. Wilson. "RPMI-10" or "RPMI-15" is the terminology used to indicate the percentage supplementation with fetal calf serum namely 10% and 15% FCS respectively in these two examples.

UCT-BR-1 (130)

This line was established in 1977 from a biopsy of an osseous metastasis of a primary carcinoma of the breast. The cells grow as an adherent monolayer with a characteristic polygonal, individual morphology and an equally characteristic social tendency to aggregate into acinar-like cell formations on the subtratum (Fig. 1). The cells are aneuploid and grow in RPMI supplemented with 10% fetal calf serum (RPMI-10) and antibiotics. The cells have a doubling time of approximately 60 hours. UCT-BR-1 responds to estrogens but not to progesterone. The cells

have a clear growth requirement for insulin and other growth factors that are present in serum and that are removed by treatment with activated charcoal (130). In routine use, the cells are released from the substratum using trypsin working solution 2 and are reseeded at a density of between 3 and $6x10^4$ cells/cm^2.

UCT-CA-2 was established from a metastatic carcinoma removed from a thoracic vertebra of a 40 year old male. A bronchogenic carcinoma was considered to be the most probable primary site, but this was not confirmed. The cells grow as an adherent monolayer with a doubling time of 24 hours in RPMI-10.

UCT-SQ-1 was derived from a lymph node metastasis; the primary was a carcinoma of the lip. The line is maintained in RPMI-10.

A431 cells were obtained from Dr. E. Reich of the Rockefeller University, New York, as a subculture of an epidermoid carcinoma line (131) that had been shown by Wrann and Fox (132) to be abundantly endowed with epidermal growth factor receptors.

The cells are maintained as adherent well-spread monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with FCS.

Trypsin, Working Solution 1, was used to release UCT-CA-2, UCT-SQ-1 and A431 cells from the substratum.

Bowes/sf cells

The Bowes melanoma cell line was originally established by Dr. George Moore, of Denver, Colorado, U.S.A. from a biopsy of a metastatic melanoma and is referred to, in the original reports, as RPMI-7272. A subculture of this line was provided by Dr. E. Reich of the Rockefeller University and was adapted by Professor E.L. Wilson to grow in serum-free medium. The line originally introduced into the laboratory and used in my initial studies was found to be heavily contaminated with mycoplasma. The method of mycoplasma detection, and the protocol used for the elimination of the mycoplasma is described in detail in a later section.

The cell line used in this thesis is called Bowes/sf (serum-free) to distinguish it from the serum-dependent Bowes line. Bowes/sf grows as a loosely adherent monolayer with a doubling time of 5 1/2 days. The cells are clearly less well-spread than the serum-dependent cells and have a

characteristic refractile, rounded morphology. Addition of serum to the cultures causes the cells to revert to the well-spread appearance of the original serum-dependent line.

Cultures were maintained in RPMI-1640 medium supplemented with antibiotics and were passaged by tapping the flask (to dislodge cells), collecting the detached cells by light centrifugation (400g; 5 minutes at room temperature) and reseeding at a density of $3x10^5/cm^2$.

The cells currently used to produce the growth inhibitor described in this thesis are free of mycoplasma, as established by two independent methods.

Maintenance of cell lines in culture

Generally speaking, cells were fed as frequently as the cultures seemed to require it. This depended upon the growth rate of the cells, the rate at which they liberated acid metabolites (sufficient to change the phenol red indicator in the medium from red to yellow) and the appearance of the cultures under phase-contrast microscopy.

All cell lines were kept as stocks frozen in liquid nitorgen. As far as possible all cells were used from expanded stocks that had been frozen at the same passage

number. In a typical case, cells from a single tube would be thawed, expanded and re-frozen to give a set of sub-cultures that provided a supply of experimental cells that had not drifted, genetically, too far from the reference culture.

Cells were frozen according to the following standard protocol: after detachment, the cells were washed once with complete medium containing 10% FCS (to neutralize any remaining trypsin) or with serum-free medium in the case of the Bowes/sf cells. The cells were then re-suspended at an approximate concentration of 10^6/ml in 1 ml of complete medium with serum containing 10% w/v of dimethyl sulfoxide (DMSO). The cells were then immediately transferred into freezing tubes (Nunc Cat. No. 363401) and placed on ice. The freezing tubes were placed in an automatic cell-freezer, programmed to freeze at the rate of 5°C/min to a temperature of -26°C. They were held at this temperature for twelve minutes and then frozen rapidly to -70°C. At this stage, the tubes could be transferred to liquid nitrogen storage tanks.

Cells were thawed rapidly by agitating the tube manually in a 37°C water bath until the last vestige of ice had melted, whereupon the contents of the tube were poured into a 10cm dish containing 10ml of complete medium. After six hours

(i.e. when the cells had adhered sufficiently to stand gentle washing) the monolayers were rinsed with warm (37°C) medium, re-fed and returned to the incubator.

Cells were counted with a haemocytometer or with an electronic cell counter (Coulter Electronics; "Model ZB $_I$") that had been calibrated against a haemocytometer and according to the manufacturers' instructions.

3. COLLECTION OF BOWES/SF CONDITIONED MEDIUM

Bowes/sf cells were maintained at 37°C and in a 95% air, 5% CO2 mixture as confluent monolayers (approximately $5x10^7$ cells/cm^2) in 150cm^2 flasks; (Costar; Cat. No. 3150) containing 100 ml of serum-free RPMI medium supplemented with 2mM glutamine, 24mM bicarbonate and antibiotics. Every 48h, the conditioned-medium (now appreciably acid) was decanted and replaced with fresh medium.

Provided the flasks did not become infected and the cells remained healthy, each flask yielded useful conditioned medium for several weeks to months. The natural tendency for the cells to detach when available plate substratum became limiting prevented loss of cultures from super-confluence.

The conditioned medium was centrifuged (400g; 4°C; 15 min) to sediment detached cells and a small amount of debris, after which the medium was filtered sequentially through an 8 micron filter (Millipore No. SCWP) and a hydrophilic 0.45 micron filter (Durapore HVLP Millipore). The clarified medium was made to 0.1% (w/v) with respect to sodium azide and was stored at 4°C.

Since 8 to 10 flasks of cells were maintained, approximately 3 litres of conditioned medium was prepared each week.

4. CONTROL OF MYCOPLASMA CONTAMINATION

Detection of mycoplasma contamination

Two methods were used to detect mycoplasma contamination of cell cultures.

(A) DNA fluorochrome stain (Hoechst Stain No. 33258) according to the method of Chen et al. (133). This stain was purchased in the form of a kit which contained both positive and negative control slides (Flow Laboratories, Cat. No. 30-100-00).

Briefly, Bowes/sf cells were seeded sparsely on four chambered glass slides (Lab-Tek (R) slides; Cat. No. 4804; Lab-Tek products; Division Miles Laboratories Inc., Naperville, Illinois 60540) in RPMI-10 and were allowed to settle and spread overnight. The next day the slides were fixed with Carnoy's fixative, (25% glacial acetic acid, 75% absolute methanol) according to the manufacturer's instructions and were then stained. This was followed by repeated distilled water washes. Coverslips were mounted after the slides had been covered with mounting medium comprising citric acid monohydrate 4662mg/L, disodium phosphate 8247 mg/L, glycerol 500ml/L. Slides were examined carefully

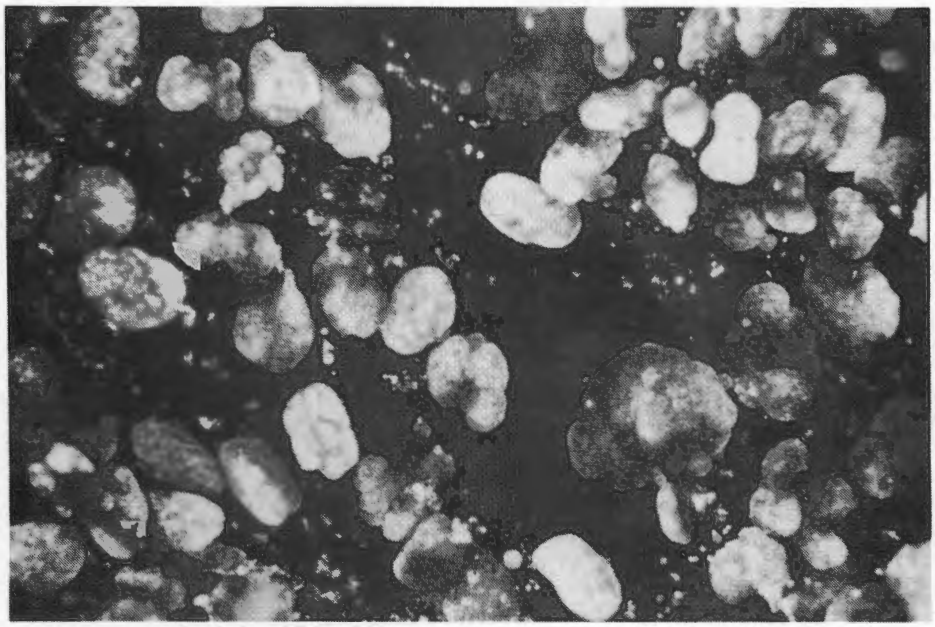

FIGURE 2

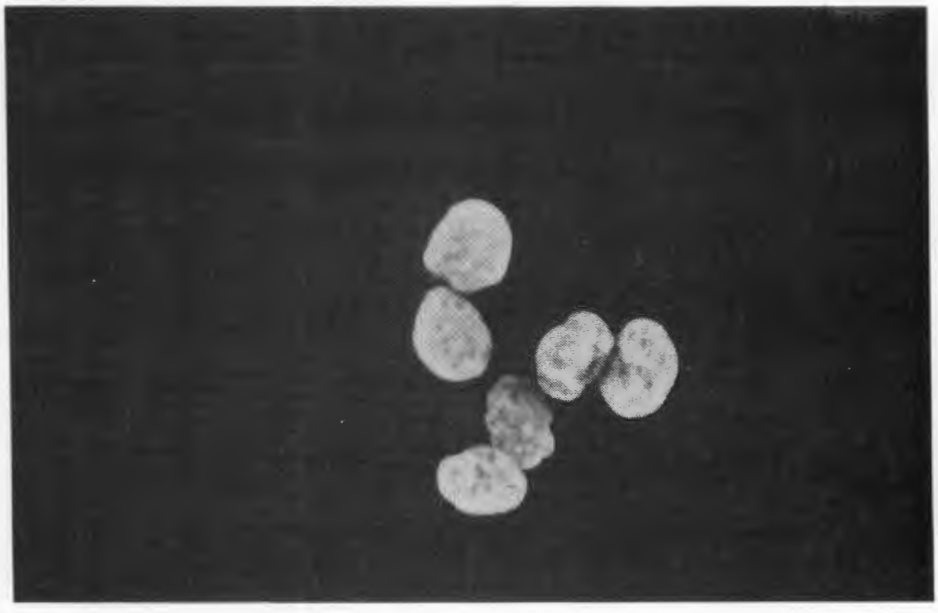

FIGURE 3

under 400x magnification using an Olympus BH-2 reflected light (Epi-illumination) fluorescence microscope with an ultraviolet light excitation filter. The dichroic mirror and barrier filter were chosen according to the emission peak of Hoechst Stain 33258 (490-500nm). The typical appearance of mycoplasma-contaminated cells is shown in Fig. 2.

(B) Mycotect ^{R} Assay (Bethesda Research Laboratories, Cat. No. 95765A). This assay derives from the knowledge that mycoplasmas contain the enzyme adenosine phosphorylase, and that this enzyme is lacking in mammalian cells. Adenosine phosphorylase converts 6-methylpurine deoxyriboside (6-MPDR), a non-toxic analogue of adenosine, into two products, 6-methylpurine and 6-methylpurine riboside, both of which are toxic to mammalian cells (134). Mammalian cells that are contaminated with mycoplasmas will therefore fail to grow in 6-MPDR (Mycotect ^{R}).

This assay was performed on Bowes/sf cells that had been seeded sparsely in 24-well plates (Costar No. 3524) in RPMI-10. Mycotect ^{R} (6-MPDR) was added to the medium in the wells to give the following final concentrations: 0, 15 uM/L and 30 uM/L. After 48-72

hours in culture, the cells were released by trypsinization and were counted using a haemocytometer.

Both the DNA fluorochrome stain and the Mycotect ^{R} were performed on Bowes/sf cells that had been grown in antibiotic-free medium for 72 hours prior to seeding for the assay.

Decontamination of mycoplasma-infected cells

Bowes/sf cells were decontaminated by being repeatedly passaged through nude mice. 2-5x10^{6} Bowes/sf cells were injected subcutaneously into the interscapular region of congenitally athymic nu/nu ("nude") mice that were maintained in a sterile pathogen-free environment. The resulting tumours were removed, and were cut into small pieces which were inserted subcutaneously via skin incisions into another group of nude mice.

After three such passages, through nude mice, the tumours were removed sterilely from the mice and were minced finely wth sterile scissors. This was followed by enzymatic digestion of the tumour, first in trypsin (working solution 1, 0.25%) for 15 min at 37°C, followed by collagenase (Sigma, type IV, 3mg/ml in DMEM, 5% FCS) for several hours at 37°C. The dispersed tumour cells were pelleted by

Contd./172

	MYCOTECT CONCENTRATION (uM/L)		
	0	15	30
Bowes/sf cells, prior to nude mouse injection	6.87	3.46	2.63
Bowes/sf cells passaged in nude mice	6.66	6.91	6.80

The above values, when multiplied by 10^5 give the cell counts of Bowes/sf cells in the Mycotect assay. Each value is the mean of duplicate counts. When grown in Mycotect, a reduced cell count (relative to the control) indicates the presence of mycoplasmas.

TABLE 1

centrifugation and were plated in 35 mm petri dishes (Falcon No. 3001) in RPMI-10. Stocks of Bowes cells thus obtained were expanded and were tested for the presence of mycoplasmas using the two methods described above. THEY

WERE FOUND TO BE FREE OF MYCOPLASMA CONTAMINATION (See Figure 3 and Table 1).

Mycoplasma-free Bowes cells were then adapted to grow in a serum-free environment. This was achieved simply by omitting serum from the culture medium. The Bowes cells adapted fairly rapidly and this adaptation was associated with a change in morphology of the cells. They became rounded up, but remained attached and thus they took on the typical appearance of Bowes/sf cells. These mycoplasma-free Bowes/sf cells were used for the definitive purification of the MDGIF.

5. ULTRAFILTRATION METHODS

The inhibitory material was present in Bowes/sf conditioned medium at a very low concentration and some degree of enrichment was required initially before purification could reasonably proceed. At several stages during the various purification procedures used, it was again essential to concentrate the MDGIF. This could conveniently be achieved

by ultrafiltration, a procedure that also provided a convenient estimate of the approximate molecular weight of the inhibitory factor.

The different forms of ultrafiltration apparatus used included:

(a) A hollow fibre concentrator (Amicon Model CH3) fitted wwith an H1P5 "Diafiber" membrane cartridge (nominal molecular weight cutoff 5000 daltons) and operated with a 400ml/min peristaltic pump. Pressure in the cartridge is controlled by means of a variable back-pressure valve.

(b) Stirred presure cells, of which 3 models are available.

Model	Volume	Diameter of Ultrafiltration membrane
Amicon Model 402	400ml	76mm
Amicon Model 202	200ml	62mm
Amicon 8MC micro-ultrafiltration system	100ml	25mm

The stirred cells are operated with Amicon "Diaflo" ultrafiltration membranes. These membranes are designated UM-2 (1000 dalton cutoff), PM-10 (10 000 dalton cutoff) and YM-10 (a more hydrophilic membrane with a 10 000 dalton cutoff).

(c) Millipore "Minitan" (Cat. No. XX42) concentrator that utilizes a tangential flow procedure. Five membranes (type PTGC) each with a nominal molecular weight limit of 10 000 daltons were used. During any "run", 6 to 7 liters of conditioned medium would be concentrated to about 700-800mls over about 10-15 hours.

6. THE MICROPLATE ASSAY FOR MDGIF

All incubations were performed at 37°C in an atmosphere of 5% CO2 and 95% air.

UCT-BR-1 cells wre seeded at 1.5 to $2x10^4$ cells per well of a 96-well flat-bottom tissue culture plate (Falcon Microtest II, No. 3040). After an overnight incubation, the seeding medium was aspirated and was replaced with 0.1ml of the medium to be tested. The plates were then returned to the incubator. After 54 hours, each well received 0.01ml of RPMI containing [3H]-thymidine (Amersham, TRK 61) at 20μCi/ml and 0.01mg/ml of unlabelled thymidine. This

provided a final concentration, in each well, of 2μCi/ml of [3H]-thymidine (3H-TdR) and 1μg/ml of non-radioactive thymidine. After a further 18 hours (i.e. after a total of 72 hr of exposure to the inhibitory factor) the cells were harvested and the incorporated radioactivity was counted.

This was performed as follows:

The microplates were removed from the incubator and the medium was gently aspirated from each well. The adherent cells were then washed by adding, in two successive cycles, approximately 0.2ml of pre-warmed (37°C) serum-free RPMI to each well and aspirating it. The cells were then released from the substratum by adding, to each well, 0.05ml of 0.25% trypsin (Working solution 1) containing 0.25M alpha-methyl-D-mannoside. The plate was then incubated at 37°C for 20 minutes and was shaken vigorously on a rotary plate shaker to release the cells. The radioactive cells were then harvested onto glass-fibre filter sheets (Titertek ^{R}, Cell Harvester filters, Flow Laboratories) using a multiple semi-automatic cell harvester that was constructed in this laboratory. The glass-fibre filter sheets were dried in a 45°C oven for several hours. Each circular area, containing the harvested cells from an individual corresponding well, was pressed out into a counting vial and was assayed for tritium content by liquid scintillation counting after the

addition of 3.0ml of Insta-gel ^{R} (United Technologies, Packard).

Inhibitory activity in test samples was measured as the percentage inhibition of 3H-TdR uptake relative to that of control cells (i.e. cells exposed to medium alone). This is calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Control cpm} - \text{test cpm}) \times 100}{\text{control cpm}}$$

Liquid scintillation counts were measured with either a Packard Tri-Carb Model 3380 or a Beckman LS3800 counter.

7. CHROMATOGRAPHY

Column chromatography was performed using established techniques. Details of the chromatographic condition for each run are described in the text or in the figure legends. The following chromatographic media were prepared and used according to the manufacturers instructions:

A. Bio-Gel P-60, fine, 100-200 mesh (wet). Fractionation range 3000 - 60000 daltons. (Bio-Rad Cat. No. 1501640).

B. Bio-Gel P-6, fine, 200-400 mesh (wet). Fractionation range 1000 - 6000 daltons. (Bio-Rad Cat. No. 1500750).

C. Bio-Gel P-6DG, Desalting Gel, 80-170 mesh (wet). Fractionation range 1000 - 6000 daltons. (Bio-Rad Cat. No. 150-0738).

D. CM52 carboxymethyl cellulose cation exchanger. Microgranular, pre-swollen form (Whatman, Cat. No. 6876).

E. SP - Trisacryl ^{R} M (LKB, Cat. No. 2205-400).

F. Phenyl-sepharose ^{R} CL-4B (Pharmacia Fine Chemicals, Cat. No. 17-0810-01).

Columns were run either under a gravity-induced flow or with a peristaltic pump. Column effluents were monitored spectrophotometrically at 280nm using a Uvicord ^{R} ultraviolet absorptiometer detector unit type 8303A linked to a type 8301A control unit (LKB). The column effluent was collected in a series of tubes that would change by means of an automatic fraction collector: Ultrorac ^{R} (LKB) or Frac-100 (Pharmacia).

High Performance Liquid Chromatography

The following columns were used:

(i) For Reverse-phase procedures

- uBondapak C18 (Waters) No. 27324, 3.9*mmx30cm. This is referred to in the text as an "analytical" column.
- uBondapak C18 (Waters) No. 84176, 7.8*mmx30cm. This is referred to in the text as a "semi-preparative" column.

(ii) For Ion-exchange procedures

TSK-IEX-545 DEAE, 6*mmx15cm (Toya Soda Manufacturing Company Limited, Japan).

* All cross-sectional diameters are internal diameters.

The HPLC system comprised the following components:

A. Pump 1 (Waters Model 6000A solvent delivery system).
B. Pump 2 (Waters Model M-45 solvent delivery system).
C. Automated Gradient Controller (Waters Model 680).
D. Spectrophotometer (Waters, Lamda-Max Model 480).

8. PROTEIN DETERMINATIONS

These were performed by either one of three methods.

A. Method of Lowry (135)

B. OD280/OD260 method; this is based on the absorption of ultraviolet light by aromatic amino acids. The following formula was applied:

Estimated protein concentration = (1.45xOD280nm) - (0.74xOD260nm)mg/ml.

C. Coomassie Blue G-250 Dye-binding Technique of Bradford (136). This is supplied as a commercial kit (Bio-Rad Cat. No. 500-0001 or 500-0002). The kit contains a dye reagent concentrate and a protein standard. The latter may be either bovine gamma globulin or bovine plasma albumin.

Assays were carried out according to the manufacturers' instructions.

The dye-binding technique was the most frequently used protein assay procedure in the thesis. It was always done

as a microassay procedure which typically detects protein in the 1 -25µg/ml range. The assay was read on a Unicam SP1800 ultraviolet spectrophotometer at 595nm. Standard curves were plotted by means of a Hewlett-Packard 85 computer using a linear regression program.

9. INTERFERON AND ANTI-INTERFERON

Human lymphoblastoid interferon (IFN) was obtained by courtesy of Professor B. Schoub (National Institute of Virology, Sandringham, Transvaal). The IFN was derived from Namalwa cells infected with Sendai virus and the activity of the preparation had been determined as 16 000 i.u./ml.

A sheep antiserum to human alpha IFN was obtained from Dr. K.C. Zoon, (NIH, Bethesda, Maryland, U.S.A.) while anti-IFN-beta was supplied by Dr. Jan Vilcek (NYU School of Medicine, New York). The anti-IFN-alpha contained 10^5 neutralizing units/ml while the anti IFN beta contained $2x10^5$ neutralizing units/ml.

Since preliminary experiments had shown that these antisera were directly toxic (for reasons unknown) to UCT-BR-1 cells in culture, it was decided to "desalt" the antibodies into phosphate buffered saline pH 7.4 (PBS) using gel-filtration chromatagraphy. For this purpose 2.4 mls of an anti-IFN

antibody preparation (consisting of a mixture of 2.1 mls of anti-IFN alpha and 0.3mls of anit-IFN beta) was chromatographed on a 20x1.2cm Bio-Gel P6 DG (Bio Rad) column previously equilibrated with PBS. The flow rate of the column was 11mls/hour/cm^2. 1ml fractions were collected. Only the void volume "peak" (10mls) was pooled and was concentrated to 3mls on an Amicon 8-Mc unit using a 25mm YM-10 'Diaflo' membrane. The 3 ml of retentate was sterile-filtered (for use in assays) using a Millex-GV 0.22 micron sterile filter unit.

10. TYPE BETA TRANSFORMING GROWTH FACTOR (TGF-BETA)

TGF-beta, purified from human platelets, was kindly supplied by Dr. Anita Roberts of the National Institutes of Health, Bethesda, Maryland. The TGF-beta sample was delivered in a reaction vial containing two micrograms of TGF-beta in 100 ul of 4mM HCl with 1mg/ml BSA(HB). This sample was diluted 1 in 10 with HB giving 200 ng/100ul TGF-beta. This was distributed in 50 microlitre aliquots into Cryotubes (Nunc Cat. No. 363401). Each aliquot therefore contained 100ng of pure TGF-beta. These were stored frozen at -20°C.

11. IODOGEN METHOD

1 mg Iodogen (Pierce Chemical Corporation) is dissolved in 1 ml dichloromethane. 20 ul of this solution is added to each of several 12 x 75 mm glass tubes which are then rotated in a 37°C water bath until the solvent has evaporated. These "coated" tubes can be stored in a dessicator at 4°C.

To a coated iodogen tube:

- Add 10 µl of a 0.25M sodium phosphate buffer, pH 7.5.
- Add 20 µl of protein to be iodinated.
- Add 5 µl of carrier-free $Na^{125}I$ at 100 µCi/µl.

Cover the tube and leave for 15 min at room temperature. Mix the contents of the tube at one minute intervals. At the end of the incubation, transfer the contents of the tube to the gel-filtration syringe. Wash out the iodogen tube with 70 µl of phosphate buffer.

Separation of iodinated proteins from free ^{125}I:

A Trisacryl GF05 gel (exclusion limit 3000 daltons) is used. This is packed into a 1 ml syringe and is washed with 0.25M phosphate buffer pH 7.5. Non-specific binding sites on the gel are blocked by addition of 100 ul of a 10 mg/ml bovine serum albumin (BSA) solution.

After addition of the BSA, the syringe is spun at 800 rpm for 2 min.

The iodinated solution is transferred from the coated tube to the Trisacryl GF05 syringe-column which is then centrifuged at 800 rpm for 2 min. to elute the iodinated protein.

REFERENCES

REFERENCES

1. Rous, P. and Jones, F.S. (1916).

J. Exp. Med. 23: 549-555

A method for obtaining suspensions of living cells from the fixed tissues, and for the plating out of individual cells.

2. Harrison, R.G. (1907).

Proc. Soc. Exp. Biol. Med. 4: 140-143.

Observations on the living developing nerve fiber.

3. Carrel, A. (1912).

J. Exp. Med. 15: 516-528.

On the permanent life of tissues outside the organism.

4. Eagle, H. (1955).

Science 122 (3168): 501-504.

Nutrition needs of mammalian cells in tissue culture.

5. Hayashi, I. and Sato, G.H. (1976).

Nature (London) 259 (5539): 132-134.

Replacement of serum by hormones permits growth of cells in a defined medium.

6. Baserga, R.(Editor)(1981).

Tissue Growth Factors. Handbook of Experimental Pharmacology Volume 57.

Springer-Verlag, Berlin, Heidelberg, New York.

7. Carpenter, G. and Cohen S. (1979).

Ann. Rev. Biochem. 48: 193-216.

Epidermal growth factor.

8. Deuel, T. and Huang, J.S. (1984).

J. Clin. Invest. 74: 669-676.

Platelet-derived growth factor. Structure, function and roles in normal and transformed cells.

9. Roberts, A.B., Frolik, C.A., Anzano, M.A. and Sporn, M.B. (1983).

Fed. Proc. 42(9): 2621-2626.

Transforming growth factors from neoplastic and non-neoplastic tissues.

10. Massagué, J. (1985).

Trends in Biochemical Sciences 10(6): 237-240.

The transforming growth factors.

11. Friedman, R.M. (1981).

Interferons. A Primer.

Academic Press. New York, London, Toronto, Sydney, San Fransisco.

12. Preble, O.T. and Friedman, R.M. (1983).

Laboratory Investigation 49(1): 4-18.

Interferon-induced alterations in cells: relevance to viral and non-viral disease.

13. Pestka, S. (1983)

Arch. Biochem. and Biophys. 221(1): 1-37.

The human interferons - from protein purification and sequence to cloning and expression in bacteria: before, between and beyond.

14. James, R. and Bradshaw, R.A. (1984).
Ann. Rev. Biochem. 53: 259-292.
Polypeptide growth factors.
15. Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979).
Nature (London) 279: 679-685.
Coated pits, coated vesicles, and receptor-mediated endocytosis.
16. Nilsson, J., Thyberg, J., Heldin, C-H, Westermark, B. and Wasteson, A. (1983).
Proc. Natl. Acad. Sci. (USA) 80: 5592-5596.
Surface binding and internalization of platelet-derived growth factor in human fibroblasts.
17. Hunter, T. (1984).
Nature (London) 311: 414-416.
The epidermal growth factor receptor gene and its product.
18. Hunter, T. (1985).
Nature (London) 313: 740-741.
At last the insulin receptor.
19. Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y-C, Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985).
Nature (London) 313: 756-761.
Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes.
20. Hunter, T. (1984).
Scientific American 251(2): 60-69.

The proteins of oncogenes.

21. Sporn, M.B. and Todaro, G.J. (1980).

N. Eng. J. Med. 303: 878-880.

Autocrine secretion and malignant transformation of cells.

22. Sporn, M.B. and Roberts, A.B. (1985).

Nature (London) 313: 745-747.

Autocrine growth factors and cancer.

23. Sporn, M.B., Roberts, A.B., Shull, J.H., Smith, J.M., Ward, J.M. and Sodek, J. (1983).

Science 219: 1329-1330.

Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo.

24. Seifert, R.A., Schwartz, S.M. and Bowen-Pope, D.F. (1984).

Nature (London) 311: 669-671.

Developmentally regulated production of platelet-derived growth factor-like molecules.

25. Okamoto, S. and Oka, T. (1984).

Proc. Natl. Acad. Sci. (USA) 81(9): 6059-6063.

Evidence for physiological function of epidermal growth factor: pregestational sialoadenectomy of mice decreases milk production and increases offspring mortality during the lactation period.

26. Savage, C.R. Jr. and Cohen, S. (1972).

J. Biol. Chem. 247(23): 7609-7611.

Epidermal growth factor and a new derivative.

27. Burgess, A.W., Knesel, J., Sparrow, L.G., Nicola, N.A. and Nice, E.C. (1982).
Proc. Natl. Acad. Sci. (USA) 79(19): 5753-5757.
Two forms of murine epidermal growth factor: rapid separation by using reverse-phase high performance liquid chromatography.
28. Gray, A., Dull, T.J. and Ullrich, A. (1983).
Nature (London) 303: 722-725.
Nucleotide sequence of epidermal growth factor C-DNA predicts a 128 000 molecular weight protein precursor.
29. Rall, L.B., Scott, J. and Bell, G.I. (1985).
Nature (London) 313: 228-231.
Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues.
30. Basu, M., Biswas, R. and Das, M. (1984).
Nature (London) 311: 477-480.
42 000-molecular weight EGF receptor has protein kinase activity.
31. Hunter, T., Ling, N. and Cooper, J.A. (1984).
Nature (London) 311: 480-483.
Protein kinase C phosphorylation of the EGF receptor at threonine residue close to the cytoplasmic face off the plasma membrane.
32. Downward, J., Parker, P. and Waterfield, M.d. (1984).
Nature (London) 311: 483-485.
Auophosporylation sites on the epidermal growth factor receptor.

33. Balk, S.D., Whitfield, J.F., Youdale, T. and Brown, A.C. (1973).
Proc. Natl. Acad. Sci (USA) 70(3): 675-679.
Roles of calcium, serum, plasma, and folic acid in the control of proliferation of normal and Rous sarcoma virus-infected chicken fibroblasts.
34. Ross, R., Glomset, J., Kariya, B. and Harker, L. (1974).
Proc. Natl. Acad. Sci. (USA) 71(4): 1207-1210.
A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro.
35. Kohler, N. and Lipton, A. (1974).
Exp. Cell Res. 87: 297-301.
Platelets as a source of fibroblast growth-promoting activity. 36. Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. (1977).
Proc. Natl. Acad. Sci. (USA) 74: 4481-4485.
Induction of DNA synthesis in Balb/c 3T3 cells by serum components: re-evaluation of the commitment process.
37. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., van Wyk, J.J. and Pledger, W.J. (1979).
Proc. Natl. Acad. Sci (USA) 76: 1279-1283.
Dual control of cell growth by somatomedins and platelet-derived growth factor.
38. Deuel, T.F., Huang, J.S., Profitt, R.T., Baenziger, J.U., Chang, D. and Kennedy, B.B. (1981).
J. Biol. Chem. 256(17): 8896-8899.

Human platelet-derived growth factor. Purification and resolution into two active protein fractions.

39. Heldin, C-H., Westermark, B. and Wasteson, A. (1981). Biochem. J. 193: 907-913.
Platelet-derived growth factor. Isolation by a large scale procedure and analysis of subunit composition.

40. Devare, S.G., Reddy, E.P., Law, J.D., Robbins, K.C. and Aaronson, S.A. (1983).
Proc. Natl. Acad. Sci (USA) 80: 731-735.
Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming gene product p28 sis.

41. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C-H, Huang, J.S. and Deuel, T.F. (1983).
Nature (London) 304: 35-39.
Platelet-derived growth factor is structurally related to the putative transforming protein p28 sis of the simian sarcoma virus.

42. Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983).
Science 221: 275-277.
Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet derived growth factor.

43. Deuel, T.F., Huang, J.S., Huang, S.S., Stoobant, P. and Waterfield, M.D. (1983).

Science 221: 1348-1350.
Expression of a platelet-derived growth factor-like protein in simian sarcoma virus transformed cells.
44. Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W. and Aaronson, S.A. (1983).
Nature (London) 305: 605-608.
Structural and immunological similarities between the simian sarcoma virus gene product(s) and human platelet-derived growth factor.
45. Huang, J.S., Huang, S.S. and Deuel, T.F. (1984).
Cell 39: 79-87.
Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors.
46. Garret, J.S. Coughlin, S.R., Nimon, H.L., Tremble, P.M., Giels, G.M. and Williams, L.T. (1984).
Proc. Natl. Acad. Sci. (USA) 81: 7466-7470.
Blockade of autocrine stimulation in simian sarcoma virus-transformed cells reverses down-regulation of PDGF receptors.
47. Clemmons, D.R. (1984).
J. Cell Physiol. 114: 61-67.
Age dependent production of a competence factor by human fibroblasts.
48. De Larco, J.E. and Todaro, G.J. (1978).
Proc. Natl. Acad. Sci. (USA) 75(8) 4001-4005.
Growth factors from murine sarcoma virus-transformed cells.

49. De Larco, J.E. and Todaro, G.J. (1978).
J. Cell. Physiol. 94: 335-342.
Epithelioid and fibroblastic rat kidney cell clones: epidermal growth factor (EGF) receptors and the effect of mouse sarcoma virus transformation.
50. Duc-Nguyen, H., Rosenblum, E.N. and Ziegel, R.F. (1966).
J. Bact. 92(4): 1133-1140.
Persistent infection of a rat kidney cell line with Rauscher murine leukaemia virus.
51. De Larco, J.E. and Todaro, G.J. (1980).
J. Cell. Physiol. 102: 267-277.
Sarcoma growth factor (SGF): specific binding to epidermal growth factor (EGF) membrane receptors.
52. De Larco, J.E., Reynolds, R., Carlberg, K., Engle, C. and Todaro, G.J. (1980).
J. Biol. Chem. 255(8): 3685-3690.
Sarcoma growth factor from mouse sarcoma virus-transformed cells. Purification by binding and elution from epidermal growth factor receptor-rich cells.
53. Todaro, G.J., Fryling, C. and De Larco, J.E. (1980).
Proc. Natl. Acad. Sci. (USA) 77(9): 5258-5262.
Transforming growth factors produced by certain human tumour cells: polypeptides that interact with epidermal growth factor receptors.
54. Tucker, R.F., Volkenant, M.E., Branum, E.L. and Moses H.L. (1983).
Cancer Research 43: 1581-1586.

Comparison of intra- and extracellular transforming growth factors from non transformed and chemically transformed mouse embryo cells.

55. Marquardt, H. and Todaro, G.J. (1982).
J. Biol. Chem. 257(9): 5220-5225.
Human transforming growth factor. Production by a melanoma cell line, purification and initial characterization.

56. Sherwin, S.A., Twardzik, D.R., Bohn, W.H., Cockley, K.D. and Todaro, G.J. (1983).
Cancer Research 43: 403-407.
High-molecular-weight transforming growth factor activity in the urine of patients with disseminated cancer.

57. Twardzik, D.R., Ranchalis, J.E. and Todaro, G.J. (1982).
Cancer Research 42: 590-593.
Mouse embryonic transforming growth factors related to those isolated from tumor cells.

58. Stromberg, K., Pigott, D.A., Ranchalis, J.E. and Twardzik, D.R. (1982).
Biochem. Biophys. Res. Commun. 106(2): 354-361.
Human term placenta contains transforming growth factors.

59. Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., De Larco, J.E. and Todaro, G.J. (1980).
Proc. Natl. Acad. Sci. (USA) 77(6): 3494-3498.
Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction.

60. Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981).
Proc. Natl. Acad. Sci. (USA) 78(9) 5339-5343.
New class of transforming factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues.
61. Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., Frolik, C.A., Marquardt, H., Todaro, G.J. and Sporn, M.B. (1982).
Nature (London) 295: 417-419.
Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF.
62. Roberts, A.B., Frolik, C.A., Anzano, M.A., Assoian, R.K. and Sporn, M.B. (1984).
In: Cell Culture Methods for Molecular and Cell Biology, Volume 1. Methods for Preparation of Media, Supplements, and Substrata for Serum-free Animal Cell Culture. Editors: Barnes, D.W., Sirbasku, D.A. and Sato, G.H. Alan R. Liss, Inc., New York.
Pages 181-194: Purification of type beta transforming growth factors from non-neoplastic tissues.
63. Frolik, C.A., Dart, L.L., Meyers, C.M., Smith, D.M. and Sporn, M.B. (1983).
Proc. Natl. Acad. Sci. (USA) 80: 3676-3680.
Purification and initial characterization of a type beta transforming growth factor from human placenta.
64. Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983).

J. Biol. Chem. 258(11): 7155-7160.
Transforming growth factor-beta in human platelets. Identification of a major storage site, purification and characterization.
65. Roberts, A.B., Anzano, M.A., Meyers, C.A., Wideman, J., Blacher, R., Pan, Y-C.E., Stein, S., Lehreman, S.R., Smith, J.M., Lamb, L.C. and Sporn, M.B. (1983).
Biochemistry 22(25): 5692-5698.
Purification and properties of a type beta transforming growth factor from bovine kidney.
66. Anzano, M.A., Roberts, A.B., Smith, J.M., Sporn, M.B. and De Larco, J. E. (1983).
Proc. Natl. Acad. Sci. (USA) 80: 6264-6268.
Sarcoma growth factor from conditioned medium of virally transformed cells is composed of type alpha and type beta tranforming growth factors.
67. Marquardt, H., Hunkapiller, M.W., Hood, L.E. and Todaro, G.J. (1984).
Science 223: 1079-1081.
Rat transforming growth factor type 1: structure and relation to epidermal growth factor.
68. Marquardt, H., Hunkapiller, M.W., Hood, L.E., Twardzik, D.R., De Larco, J.E., Stephenson, J.R. and Todaro, G.J. (1983).
Proc. Natl. Acad. Sci. (USA) 80: 4684-4688.
Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma

cells: amino acid sequence homology with epidermal growth factor.

69. Derynck,R., Roberts, A.B., Winkler, M.E., Chen, E.Y. and Goeddel, D.V. (1984).

Cell 38: 287-297.

Human transforming growth factor alpha: precursor structure and expression in E. coli.

70. Massagué, J. (1983).

J. Biol. Chem. 258(22): 13606-13613.

Epidermal growth factor-like transforming growth factors. Isolation, chemical characterization, and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells.

71. Derynck, R., Garrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. (1985).

Nature (London) 316: 701-705.

Human transforming growth factor-beta: complementary DNA sequence and expression in normal and transformed cells.

72. Tucker, R.F., Branum, E.L., Shipley, G.D., Ryan, R.J. and Moses, H.L. (1984).

Proc. Natl. Acad. Sci. (USA) 81: 6757-6761.

Specific binding to cultured cells of 125I-labelled type beta transforming growth factor from human platelets.

73. Frolik,C.A., Wakefield, L.M., Smith, D.M. and Sporn, M.B. (1984).

J. Biol. Chem. 259: 10995-11000.

Characterization of a membrane receptor for transforming growth factor-beta in normal rat kidney fibroblasts.

74. Massagué, J. and Like, B. (1985).

J. Biol. Chem. 260: 2636-2645.

Cellular receptors for type-beta transforming growth factor. Ligand binding and affinity labelling in human and rodent cell lines.

75. Anzano, M.A., Roberts, A.B., Smith J.M., Lamb, L.C. and Sporn, M.B. (1982)

Analytical Biochemistry 125: 217-224.

Purification by reverse-phase high-performance liquid chromatography of an epidermal growth factor-dependent transforming growth factor.

76. Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B. (1985).

Proc. Natl. Acad. Sci. (USA) 82: 119-123.

Type beta transforming growth factor: a bifunctional regulator of cellular growth.

77. Ibbotson, K.J., D'Souza, S.M., Ng, K.W., Osborne, C.K., Niall, M., Martin, T.J. and Mundy, G.R. (1983).

Science 221: 1292-1294.

Tumor-derived growth factor increases bone resorption in a tumor associated with humoral hypercalcemia of malignancy.

78. Ibbotson, K.J., D'Souza, S.M., Smith, D.D., Carpenter, G. and Mundy, G.R. (1985).

Endocrinology 116(1): 469-471.

EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with humoral hypercalcemia of malignancy.

79. Daughaday, W.H. (Editor) (1984).
Clinics in Endocrinology and Metabolism 13(1): Chapters 1-7.
W.B. Saunders Company, London, Philadelphia, Toronto.

80. Zapf, J., Watler, H. and Froesch, E.R. (1981).
J. Clin. Invest. 68: 1321-1330.
Radioimmunological determination of insulin-like growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumour hypoglycemia.

81. Schoenle, E., Zapf, J., Humbel, R.E. and Froesch, E.R. (1982).
Nature (London) 296: 252-253.
Insulin-like growth factor I stimulates growth in hypophysectomized rats.

82. Whitfield, H.J., Bruni,C.B., Frunzio, R., Terrell, J., Nissley, S.P. and Rechler, M.M. (1984).
Nature (London) 312: 277-280.
Isolation of a cDNA clone encoding rat insulin-like growth factor II precursor.

83. Moses, A.C., Nissley, S.P., Short, P.A., Rechler, M.M., White, R.M., Knight, A.B. and Higa, O.Z. (1980).
Proc. Natl. Acad. Sci. (USA) 77(6): 3649-3653.
Increased levels of multiplication-stimulating activity, an insulin-like growth factor, in fetal rat serum.

84. Adams, S.O., Nissley, S.P., Handwerger, S. and Rechler, M.M. (1983).
Nature (London) 302: 150-153.
Developmental patterns of insulin-like growth factor I and II synthesis in rat fibroblasts.
85. Rinderknecht, E. and Humbel, R.E. (1978).
J. Biol. Chem. 253(8): 2769-2776.
The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin.
86. Massagué, J., Kelly, B. and Mottola, C. (1985).
J. Biol. Chem. 260(8): 4551-4554.
Stimulation by insulin-like growth factors is required for cellular transformation by type beta transforming growth factor.
87. Houck, J.C. (Editor) (1976).
Chalones.
North-Holland Publishing Company, Amsterdam, Oxford, New York.
88. Pestka, S. and Baron, S. (1981).
Methods in Enzymology 78: 3-14.
Definition and classification of the interferons.
89. Baron S., Dianzani, F. and Stanton, G.J. (1981-1982).
Tex. Rep. Biol. Med. 41: 1-12.
General considerations of the interferon system.
90. Taylor-Papadimitriou, J. and Rozengurt, E. (1981-1982).
Tex. Rep. Biol. Med. 41: 509-516.

Modulation of interferons inhibitory effect on cell growth by the mitogenic stimulus.

91. Shibata, H. and Taylor-Papadimitriou, J. (1981).
Int. J. Cancer 28: 447-453.
Effects of human lymphoblastoid interferon on cultured breast cancer cells.

92. Balkwill, F., Taylor-Papadimitriou, J., Fantes, K.H. and Sebesteny, A. (1980).
Eur. J. Cancer 16: 569-573.
Human lymphoblastoid interferon can inhibit the growth of human breast cancer xenografts in athymic (nude) mice.

93. Taylor-Papadimitriou, J., Shearer, M., Balkwill, F.R. and Fantes, K.H. (1982).
J. Interferon Research 2(4): 479-491.
Effects of HuIFN-alpha2 ahd HuIFN-alpha (Namalwa) on breast cancer cells grown in culture and as xenografts in nude mice.

94. Trinchieri, G. and Perussia, B. (1985).
Immunology Today 6(4): 131-136.
Immune interferon: a pleiotropic lymphokine with multiple effects.

95. Bocci, V. (1983).
Trends in Biochemical Sciences 8: 432-434.
What is the role of carbohydrates in interferons?

96. Sagar, A.D., Sehgal, P.B., May, L.T., Inouye, M., Slate, D.L., Shulman, L. and Ruddle, F.H. (1984).
Science 223: 1312-1315.

Interferon-beta-related DNA is dispersed in the human genome. 97. Thompson, M.R., Zhang, Z., Fournier, A. and Tan, Y.H. (1985).
J. Biol. Chem. 260(1): 563-567.
Characterization of human beta-interferon binding sites on human cells.
98. Clemens, M. (1985).
Nature (London) 313: 531-532.
Interferons and oncogenes.
99. Jonak, G.J. and Knight, E. (1984).
Proc. Natl. Acad. Sci. (USA) 81: 1747-1750.
Selective reduction of c-myc mRNA in Daudi cells by human beta interferon.
100. Samid, D., Chang, E.H. and Friedman, R.M. (1984).
Biochem. Biophys. Res. Commun. 119: 21-28.
Biochemical correlates of phenotypic reversion in interferon-treated mouse cells transformed by a human oncogene.
101. Knight, E. (1975).
J. Biol. Chem. 250(11): 4139-4144.
Heterogeneity of purified mouse interferons.
102. Knight, E. (1976).
Proc. Natl. Acad. Sci. (USA) 78(2): 520-523.
Interferon: purification and initital characterization from human diploid cells.
103. Bridgen, P.J., Anfinsen, C.B., Corley, L.C., Bose, S., Zoon, K.C., Ruegg, U.T. and Buckler, C.E. (1977).

J. Biol. Chem. 252(19): 6585-6587.
Human lymphoblastoid interferon. Large scale production and partial purification.
104. Rubenstein, M., Rubinstein, S., Familletti, P.C., Miller, R.S., Waldman, A.A. and Pestka, S. (1979).
Proc. Natl. Acad. Sci. (USA) 76(2): 640-644.
Human leukocyte interferon: production, purification to homogeneity, and initial characterization.
105. Zoon, K.C., Smith, M.E., Bridgen, P.J., Zur Nedden, D. and Anfinsen, C.B. (1979).
Proc. Natl. Acad. Sci. (USA) 76(11): 5601-5605.
Purification and partial characterization of human lymphoblastoid interferon.
106. Mikulski, A.J., Heine, J.W., Le, H.V. and Sulkowski, E. (1980).
Preparative Biochemistry 10(2): 103-119.
Large scale purification of human fibroblast interferon.
107. Friesen, H-J., Stein, S., Evinger, M., Familletti, P.C., Moschera, J., Meienhafer, J., Shively, J. and Pestka, S. (1981).
Archives of Biochemistry and Biophysics 206(2): 432-450.
Purification and molecular characterization of human fibroblast interferon.
108. Knight, E. and Fahey, D. (1981).
J. Biol. Chem. 256(8): 3609-3611.
Human fibroblast interferon. An improved purification.
109. Davey, M.W., Sulkowski, E. and Carter, W.A. (1976).

J. Biol. Chem. 251(23): 7620-7625.
Hydrophobic interaction of human, mouse, and rabbit interferons with immobilized hydrocarbons.
110. Sulkowski,E., Davey, M.W. and Carter, W.A. (1976).
J. Biol. Chem. 251(17): 5381-5385.
Interaction of human interferons with immobilized hydrophobic amino acids and dipeptides.
111. Jankowski,W.J., von Muenchhausen, W., Sulkowski, E. and carter W.A. (1976).
Biochemistry 15(23): 5182-5187.
Binding of human interferons to immobilized Cibacron Blue F3GA. The nature of molecular interaction.
112. Sponsored by:
National Institute of Allergy and Infectious Diseases and World Health Organization - U.S. National Centre on Interferon. Held at National Institute of Health, Bethesda, Maryland on March 7th, 1980.
Nature (London)(1980) 286: 110.
Interferon nomenclature.
113. Holley, R.W., Bohlen, P., Fava, R., Baldwin, J.H., Kleeman, G. and Armour, R. (1980).
Proc. Natl. Acad. Sci. (USA) 77(10): 5989-5992.
Purification of kidney epithelial cell growth inhibitors.
114. Nilsen-Hamilton, M. and Holley, R.W. (1983).
Proc. Natl. Acad. Sci. (USA) 80: 5636-5640.

Rapid selective effects by a growth inhibitor and epidermal growth factor on the incorporation of (35S) methionine into proteins secreted by African Green Monkey (BSC-1) cells.

115. Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W. (1984).

Science 226: 705-707.

Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor.

116. Carswell, E.A., Old, L.J., Kassel, R.I., Green, S., Fiore, N. and Williamson, B. (1975).

Proc. Natl. Acad. Sci. (USA) 72(9): 3666-3670.

An endotoxin-induced serum factor that causes necrosis of tumors.

117. Sikora,K. (1984).

Nature (London) 312: 699-700.

Cancer toxin genes cloned.

118. Aggarwal, B.B., Moffat, B. and Harkins, R.N. (1984).

J. Biol.Chem. 259(1): 686-691.

Human lymphotoxin. Production by a lymphoblastoid cell line, purification and initial characterization.

119. Gray, P.W., Aggarwal, B.B., Benton, C.V., Bringman, T.S., Henzel, W.J., Jarrett, J.A., Leung, D.W., Moffat, B., Ng., P., Svedersky, L.P., Palladino, M.A. and Nedwin, G.E. (1984).

Nature (London) 312: 721-724.

Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity.

120. Pennica, D., Nedwin, G.E, Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B. and Goeddel, D.V. (1984).
Nature (London) 312: 724-729.
Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin.

121. Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V. and Harkins, R.N. (1985).
J. Biol. Chem. 260(4): 2345-2354.
Human tumor necrosis factor. Production, purification and characterization.

122. Wang, A.M., Creasy, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R. and Mark, D.F. (1985).
Science 228: 149-154.
Molecular cloning of the complementary DNA for human tumour necrosis factor.

123. Playfair, J.H.L., Taverne, J. and Matthews,N. (1984).
Immunology Today 5(6): 165-166.
What is tumour necrosis factor really for?

124. Collins, T., Ginsburg, D., Boss, J.M., Orkin, S.H. and Pober, J.S. (1985).
Nature (London) 316: 748-750.
Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis.

125. Bell, G.I., Merryweather, J.P., Sanchez-Pescador, R., Stempien, M.M., Priestley, L., Scott, J. and Rall, L.B. (1984).
Nature (London) 310: 775-777.
Sequence of a cDNA clone encoding human preproinsulin-like growth factor II.
126. Dull, T.J., Gray, A., Hayflick, J.S. and Ullrich, A. (1984).
Nature (London) 310: 777-781.
Insulin-like growth factor II precursor gene organization in relation to insulin gene family.
127. Brissenden, J.E., Ullrich, A. and Francke, U. (1984).
Nature (London) 310: 781-784.
Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor.
128. Tricoli, J.V., Rall, L.B., Scott, J., Bell, G.I. and Shows, T.B. (1984).
Nature (London) 310: 784-786.
Localization of insulin-like growth factor genes to chromosome 11 and 12.
129. Bell, G.I., Gerhard, D.S., Fong, N.M., Sanchez-Pescador, R. and Rall, L.B. (1985).
Proc. Natl. Acad. Sci. (USA) 82: 6450-6454.
Isolation of the human insulin-like growth factor genes: Insulin-like growth factor II and insulin genes are contiguous.

130. Wilson, E.L., Dutlow, C. and Dowdle E.B. (1982). Cold Spring Harbour Conferences on Cell Proliferation. Vol. 9. Growth of cells in hormonally defined media. Edited by Sato, G.H., Pardee, A. B. and Sirbasku, D.A. pp. 849-854. Effect of hormones on the secretion of plasminogen activator by a new line of human breast carcinoma cells, UCT-BR-1.

131. Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H. and Parks, W.P. (1973). J. Nat. Cancer Institute 51: 1417-1423. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors.

132. Wrann, M.M. and Fox, C.F. (1979). J. Biol. Chem. 254: 8083-8086. Identification of epidermal growth factor receptors in a hyperproducing human epidermoid carcinoma cell line.

133. Chen, T.R. (1977). Exp. Cell. Res. 104: 255-262. In situ detection of mycoplasma contamination in cell ucltures by fluorescent Hoechst 33258 stain.

134. BRL mycotect ™. Mycoplasma detection system. Cat. No. 9576SA. Bethesda Research Laboratories, Gaithersburg, Maryland 20877, U.S.A.

135. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R. (1951). J. Biol. Chem. 193: 265-275. Protein measurement with the folin phenol reagent.

136. Bradford, M.M. (1976).
Anal. Biochem. 72: 248-254.
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding.

137. Lane, H.C., Whalen, G. and Fauci, A.S. (1986)
In: Handbook of Experimental Immunology. Volume 2: Cellular Immunology. Editors: Weir, D.M., Herzenberg, L.A., Herzenberg, L.A. and Blackwell, C.
Blackwell Scientific Publications, Oxford, London, Edinburgh.
Chapter 66: In vitro evaluation of human lymphocyte function.

138. Davis, B.D., Dulbecco, R., Eisen, H.N, Ginsberg, and Wood, W.B. Jr. (1970).
In: Principles of Microbiology and Immunology (Harper International Edition). Harper and Row, New York, Evanston and London.
Chapter 16: Complement. Pages 509-524.

139. Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Kasid, A., Derynck, R. and Dickson, R.B. (1987).
Cell 48: 417-428
Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells.

140. Sporn, M.B., Roberts, A.B., Wakefield, L.M., and de Crombrugghe, B. (1987).
J. Cell Biol. 105: 1039-1045.
Some recent advances in the chemistry and biology of transforming growth factor - beta.
141. Moses, H.L., Coffey, R.J., Leof, E.B., Lyons, R.M., and Keoki-Oja, J. (1987)
J. Cell. Physiol., Supplement 5: 1-7.
Transforming growth factor β regulation of cell proliferation.
142. Massagué, J. (1987).
Cell 49: 437-438.
The TGF-β family of growth and differentiation factors.
143. Silberstein, G.B. and Daniel, C.W. (1987).
Science 237: 291-293.
Reversible inhibition of mammary gland growth by transforming growth factor-β.
144. Sporn, M.B. and Roberts, A.B. (1988)
Nature (London) 332: 217-219.
Peptide growth factors are multifunctional.