A Study of the Production of the Selected Cytokines Interleukin 1, Interleukin 6, and Tumour Necrosis Factor By Certain Tumours and Tumour Cell Lines.

A Thesis in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow, Scotland.

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Declaration:

I declare that, unless specified otherwise in the text, the work presented in this thesis is my own.

George Chambers, BSc.

Summary.

An investigation was carried out to examine the production of the inflammatory cytokines IL1α, IL1ß, IL6, TNFα, and TNFß in two tumour cell lines, the MCF-7 breast cell line and the T-24 bladder cell line, and in samples of **breast, bladder, lung and ovarian tumours.**

Two methods were used to investigate cytokine production. These were the polymerase chain reaction method (PCR) to examine cytokine mRNA production and immuno-staining of frozen or paraffin-embedded tissue sections to demonstrate the presence of the cytokine polypeptide directly. In the PCR experiments, the most frequently found cytokine was IL6, followed by IL1α. Only a few tumours of any type displayed TNFα, and even fewer produced TNFß. In the immunostaining experiments performed on frozen sections, IL1α and IL6 proteins were detected in sections of tumours which gave positive results with PCR. Cell phenotyping indicated that the IL1α and IL6 were probably being synthesised by the tumour cells themselves although there was lymphocyte infiltration in every section examined. In the immuno-histology study performed on the paraffin-embedded sections, a new collection of tumours was used. These tumours were not subjected to parallel PCR due to size of tumour samples being too small. The results obtained from these experiments conflicted with the results observed in the PCR study. IL1α was detected in all of the breast tumours used for immuno-histology but in none of the breast tumours in the PCR experiments.
This conflict was probably due to a lack of sensitivity in the method used to detect PCR products.

While the conflict could not definately be resolved, it was thought that the results of the immuno-histology experiments were more accurate as they detected expression of cytokine protein on a cellular scale. The immuno-histology experiments demonstrated that some tumour cells produced IL1α in breast and bladder carcinomas, and some produced IL6 in breast and lung carcinomas.

Overall, the results of the experiments descibed in this thesis demonstrated that all of the tumour types examined could produce inflammatory cytokines, and that two of these, IL1α and IL6 could be detected in with some frequency in tumour samples of all types. From the results presented in the thesis, and examination of the literature, IL6 would be a good candidate for development as a new biochemical tumour marker but limitations on the accurate measurement of IL1α in serum means it is not suitable as a biocheical marker. Expression of the inflammatory cytokines in breast tumours would be particularily worthy of future investigation, given the nature of the reported experimental resultsa and the absence of much evidence for cytokine production by this tumour type.

Chapter 1: Introduction.

1.1: General.

Cancer is one of the world's most serious medical problems. In the west, it is second only to heart disease in terms of adult mortality rates, and in childhood second only to accidental death. Indeed it has been forecast that it will become the major cause of death as medical advances in the treatment and management of other conditions continue. (Souhami and Tobias, 1986)

Unlike many other diseases, cancer is a broad term for a number of different disease states which have a variety of symptoms and causes. However, it seems possible that these disease states are caused by a similar basic mechanism. An infectious disease such as influenza has one specific cause - the influenza virus - and one set of symptoms arising from infection of a specific part of the body by the virus. By contrast, cancer displays no uniform set of symptoms and can arise in almost any type of tissue. At a gross level, it is a descriptive term for a group of conditions which if left untreated may result in the death of the afflicted individual due to overgrowth and spread of the cancer cells. At the cellular level it is a disease of abnormal cell proliferation and growth. The normal controls for regulation of cell growth and mortality are absent and the cancer cells keep dividing indefinitely (Devita et al, 1989).

Normal checks which prevent the overgrowth of one tissue by another are defective, as is the mechanism whereby cells of a particular type are fixed to a particular location. Cancer cells can migrate from their parent tissues to other parts of the body and grow there (this is called the formation of metastases). It is the burden induced on the body by tumour growth and the impairment of organ function by growth of invasive metastases which leads to death (Rubin, 1978).

Tumours can be either benign (simple is another term), or malignant. Benign tumours are localised, often surrounded by a capsule and rarely give rise to serious effects. When they do, this is usually due to their mass causing pressure on vital organs or by their secretion of abnormal amounts of biochemical products.

Malignant tumours are not localised although they do invade locally into surrounding tissues. They also form metastases in distant parts of the body, in which they then become invasive. They frequently cause death by extensive damage to the normal tissue they invade and by the metabolic effects of general tumour burden.

While tumours may be generally divided into the two types above, this classification is by no means absolute; indeed it is an operational one convenient for the pathologist or clinician.

Benign tumours may progress to become malignant. The common wart for example, which is a papilloma of the skin, never becomes malignant but papillomas of the bladder or colon do so with high frequency. (Calman and Paul, 1978)

One of the most important features of a tumour is that as it progresses to malignancy its cells transform. Tissue specific antigens are progressively lost and tissue specific functions disappear. This is associated with changes in the chromosomes of the tumour cells. Malignant cells are found to have very different morphology and biochemical properties from normal cells of their tissue of origin. This is very important in their pathological classification. Pathologists categorise tumours by their state (benign or malignant), by their embryonic tissue of origin and by their cell morphology (Moore, 1975). The table overleaf shows the most common types and how they are defined:

Table 1.1: Categorisation of Tumours (from Calman and Paul, 1978)

Tissue Type:	Normal Cell Type:	Name for Benign Tumour:	Name for Malignant Tumour:
Connective Tissue:	Fibrocyte Fat cell Osteocyte Muscle	Fibroma Lipoma Osteoma Myoma	Fibrosarcoma Liposarcoma Osteosarcoma Myosarcoma
Vascular Endothelium:	Endothelial cell	Haemangioma	Haemangio-sarcoma
Epithelium:	Squamous and transitional Glandular	Papilloma (Squamous and basal cell) Adenoma	Carcinoma Glandular Carcinoma
Neural:	Glial Nerve Melanoblast	Glioma Ganglionic Neuroma Melanoma	Neuroblastoma " " Malignant Melenoma
Haemopoetic:	Reticulum cell Plasma cell Leucocyte	Lymphoma	Lymphosarcoma Reticulosarcoma Myeloma or Leukaemia
Embryonal:	Embryonal	Teratoma	Terato-carcinoma

1.2: Importance of Biochemical Markers to the Diagnosis and Monitoring of Cancer.

Many tumours are difficult to diagnose in their early stages. Often the patient experiences no noticeable symptoms and there is nothing which can be detected externally. In many cases, by the time detectable symptoms or signs do manifest themselves, the tumour is quite advanced and has already metastasised, making a successful prognosis for the patient more unlikely. This is particularly true of cancers such as ovarian carcinoma, in which metastatic spread is very rapid and mortality rates are high. It is a consequence of the difficulty of making early diagnoses of cancer that clinicians wish to establish new diagnostic tools which may enable early diagnosis even before symptoms or signs become evident.

Following diagnosis it becomes important that clinicians can monitor the condition of the tumour as treatment proceeds.

To aid in the diagnosis and treatment of cancer much work has been reported, the aims of which are to find and study biochemical markers that may contribute either to diagnosis or therapy (see for example Beastall et al, 1991).

These so-called tumour markers are macromolecules, the appearance of which is related to the genesis and growth of malignant tumours and the concentrations of which may correlate with the tumour burden present in individuals. Tumour markers are usually classified as cellular or humoral in type.

1.3: Definitions of the Two Tumour Marker Types.

1.3.1: Cellular tumour markers. These are antigens located on the plasma membrane of cells and are often glycoproteins or complex carbohydrates. They may also be hormone or growth factor receptors. Cellular tumour markers enable the detection of tumour cells by a variety of means, such as immuno-staining, or in-situ hybridisation.

1.3.2: Humoral tumour markers. These are substances which can be detected in serum, urine and other body fluids at concentrations exceeding those found normally. They may be products synthesised and excreted by tumour tissue, shed cell surface receptors, substances released on tumour disintegration, or substances formed as a reaction of the body to the tumour.

1.4: Criteria for an Ideal Tumour Marker (from Beastall et al, 1991).

The criteria which an ideal tumour marker should display are:

1) High specificity, i.e. not detectable in benign disease or in healthy individuals.

2) High sensitivity, i.e. detectable very early when only a few cancer cells are present.

3) Organ specificity.

4) Correlation of concentration of marker with tumour stage and mass (the tumour burden).

5) Correlation with prognosis.

6) Reliable predictive value (i.e. the concentration of the marker giving a reliable prediction of the state or size of the tumour).

Unfortunately there are no markers which fulfil all of these criteria. However, some markers are in routine clinical use, having fulfilled several of the criteria and examples of these are:

1.5: Examples of Tumour Cell Markers.

1.5.1: Alpha fetoprotein (AFP).

This is a 70 kilodalton (kd) relative molecular mass protein with α_1 electrophoretic mobility that is synthesised by parenchymal cells of the liver, by the yolk sac, and by the gastrointestinal tract of the fetus. It was first characterised by Bergstrand et al (1956). It is the major protein in serum of the early fetus, and is present in the serum of adults at very low concentrations. In 1963, Ablev et al demonstrated that AFP was present in transplantable hepatomas of mice, and in 1964 Tatarinov detected AFP in the serum of human patients displaying hepatocellular carcinoma. Subsequent development of sensitive radio-immuno assays for AFP, including one by Waldman et al (1974), showed a strong association between AFP production and hepatocellular carcinoma. Raised concentrations of AFP in serum (greater than 40ng/ml) were observed by Waldman et al in over 72 % of patients with this carcinoma.

Since in normal individuals, AFP levels in serum occur only during pregnancy or in the first year of life, choice of an appropriate cut off level for AFP in the serum of individuals of normal physiology makes it quite a specific marker.

AFP exhibits a sensitivity in the range of 60- 80 % for hepatomas, and 50- 70% for non-seminomatous germ cell testicular carcinomas (according to Beastall et al, 1991). AFP is of greatest usefulness in diagnosis of hepatoma and in monitoring its response to therapy.

1.5.2: CA 125.

This is a glycoprotein with a relative molecular mass of approximately 200kd and is defined by its reactivity with the monoclonal antibody OC125. It was discovered in 1981 (Bast et al, 1981). In a further study, (Bast et al, 1983) it was demonstrated that raised concentrations of CA125 occurred in serum in 82% of patients with ovarian carcinomas and so CA 125 displays good sensitivity for this condition. The value of using CA125 to screen for ovarian cancer was further examined in a later study (Jacobs et al, 1988). Over 1000 apparently healthy postmenopausal women were examined for the presence of significant concentrations of CA125 and this was correlated with the successful diagnosis of ovarian cancer. The specificity of using CA125 to diagnose ovarian cancer was 97%, compared with 97.3% for diagnosis by vaginal examination. The main clinical application of CA125 today is in the monitoring of therapy and detection of clinically occult recurrences in patients with ovarian cancer. According to Jacobs et al, alterations of CA125 levels in serum correlated with disease response to therapy in 94% of the cases studied. Serial levels of CA125 can detect clinically occult recurrences with lead times of up to 17 months, with a median lead time of 3-4 months.

While these are two examples of currently used markers, there are a great many more, and it seems probable that the number of markers in use will grow as more is discovered about tumour biology.

1.6: The Relationship Between Tumours and Cytokine Production and the Possibility of their Selection as New Tumour Markers.

1.6.1: Introduction.

An essential part of the immune response (the way in which the body responds to invasion by foreign organisms) is the secretion of certain chemical messengers by a range of different cells in the body, including lymphocytes. These messengers help to determine, in turn, the responses of other cells and effects like inflammation which commonly accompany the immune response. These chemical messengers are called cytokines. Many of the cytokines also have other roles out with the immune response (see later) and there is a considerable body of evidence that some cytokines are associated with, or produced by, certain types of tumour: in particular the cytokines **Interleukin 1α (IL1α)**, **Interleukin 1ß (IL1ß)**, **Interleukin 6 (IL6)**, **Tumour Necrosis Factor α (TNFα)**, and **Tumour Necrosis Factor ß (TNFß)**. If production of any of these cytokines could be confirmed in a particular type of tumour, and found to exhibit suitable sensitivity and specificity, then they might make useful new markers for certain types of tumour

The following sections of this dissertation (1.6.2 - 1.6.5) review briefly in turn the characteristics and effects of the four cytokines including their interaction with one another. This is followed (1.6.6) by a review of the evidence for an association between each cytokine and tumours of various types.

1.6.2: Characteristics and Effects of Interleukin 1 (IL1).

Interleukins 1α, and 1ß are separate gene products, but are recognised by the same receptors and are very closely related both in terms of their three dimensional structure and biological activities. They are often considered together as Interleukin 1.

1.6.2.1: Discovery of IL1.

In 1972 Gery et al discovered a factor in the supernatants from stimulated human and murine leucocyte cultures which could induce mitogenic activity in murine thymocytes and in 1979, Togawa et al characterised a factor with similar biological properties and showed that it had two distinct forms with different molecular masses. The factor was named interleukin 1 (or IL1), by Aarden et al in 1979 who derived the name to denote a chemical messenger allowing signals to pass **between leucocytes**. The two forms are distinguished as IL1α and ß.

1.6.2.2: Size and structure of IL1.

cDNAs for IL1α and ß were cloned by Auron et al in 1984 (IL1ß), and Lomedico et al, also in 1984 (murine IL1α). Both forms are synthesised as 31 kd precursors which are then post-transcriptionally modified. The bio-active forms of IL1 have a relative molecular mass of 17kd. Mature human IL1ß has been crystallised and its three dimensional tertiary structure determined (Priestle et al 1989). It is folded into 12 ß strands held together by hydrogen bonds.

1.6.2.3: Constitutive Expression of IL1 in Healthy Tissue.

There is little evidence for constitutive expression of IL1 in healthy tissue. Hauser et al (1986) demonstrated that IL1 was present in normal human epidermis. Care had to be taken to ensure that the cells used in the experiment remained unstimulated. Unstimulated peripheral blood mononuclear cells (PBMCs) were used as a negative control and PBMCs stimulated with phytohaemagglutinin (PHA) were used as a positive control. The detection methods used were the Lymphocyte Stimulatory Factor (LAF) assay (the standard bio-assay for IL1 at the time) and an indirect assay which measured IL1-induced prostaglandin E 2 production.

Detectable IL1 was demonstrated in the unstimulated epidermis with both assays. No IL1 was found in the unstimulated PBMCs; IL1 activity was found in the stimulated PBMCs.

However, the results were inconclusive, as it could not be said for certain if the IL1 found in the epidermal tissue came from epidermal cells themselves or from lymphocytes also present in the tissue. The current view, as expressed by Dinarello (1992) is that there is no significant expression of IL1 in normal healthy unstimulated cells.

(Even in lymphocytes, production is always due to stimulation.)

1.6.2.4: Production of IL1 following Stimulation.

Following stimulation a variety of cell types can produce IL1. These include monocytes, tissue macrophages, vascular endothelial cells, astrocytes, microglia, glioma cells, and keratinocytes (Emery and Salmon 1991).

In the immune system, the production of IL1 is mainly from stimulated macrophages. T-cell factors, principally IL2 and interferon gamma (IFNΓ), activate macrophages and cause them to produce IL1. IL1 production is inhibited by IL4 (in humans only) and prostaglandin E2 and TNFα can stimulate IL1 production (Dinarello, 1992).

1.6.2.5: Method of Signal Transduction Caused by IL1.

The overall method of signal transduction caused by IL1 in its target cells is unclear. Both a cyclic AMP and protein kinase pathway and a serine kinase and G protein pathway have been proposed (Emery and Salmon 1991).

1.6.2.6: Effects of IL1.

IL1 can affect many kinds of cell. In the immune system Simic et al (1985) showed that IL1 could cause the production of IL-2 in lectin-stimulated T-cells, and also up-regulate their IL-2 receptors. IL1 can also affect B-cells by increasing B-cell proliferation and antibody formation. The effect is synergistic with IL6 (Dinarello 1992).

Bevilaqua et al (1985) showed that IL1 affected the vascular endothelium by causing increased adhesion of PBMCs to it. They proposed that this might be how leucocyte-vessel wall interactions are localised at sites of inflammation. Dejana et al (1987) demonstrated that IL1 could affect the vascular endothelium in other ways, by causing (a) prostaglandin I2 (PGI2) production, (b) platelet activating factor production and (c) plasminogen activator inhibitor production. It thus appears to play a role in thrombosis, probably by augmentation at the site of damage.

1.6.2.7: Systemic Role of IL1.

If IL1 is administered systemically it has a important role in the mediation of inflammation. Tewari et al (1990) demonstrated that following intravenous administration, recombinant IL1ß caused symptoms of acute inflammation, including increased heart rate, low grade fever and rigors, in human volunteers. Platelet counts rose by 50%, and neutrophil-dominated leucocytes rose by 100% .

Similar effects had previously been observed in rabbits in work by Okusawa et al (1988), who found that administration of recombinant IL1ß to rabbits caused increased heart rate, a fall in vascular resistance, a fall in arterial pressure, and a fall in central venous pressure. The effect was enhanced by co-administration with TNFα (see later), an example of cytokine synergy (for more information on the proinflammatory properties of IL1 see Dinarello 1991).

The table overleaf summarises the properties of IL1 (after Dinarello, 1992).

Table 1.2: A Summary of the Biological Properties of IL1.

(After Dinarello, 1992)

Immunological Properties:

a) T-cell activation: synergy with IL6 for IL2 synthesis.

b) Increased IL2 receptor expression.

c) B-cell activation via induction of IL6; synergy with IL4.

d) Stimulation of Natural Killer (NK) Cell activity; synergy with IL2 and interferon.

e) Lymphokine gene expression.

Pro-inflammatory properties:

a) Fever, anorexia, neuropeptide release.

b) Gene expression for complement and other acute phase proteins, suppression of cytochrome P450 synthesis.

c) Endothelial cell activation.

d) Neutrophilia.

e) Increased adhesion molecule expression.

f) Neutrophil tissue infiltration (via IL8).

g) Beta islet cell cytotoxicity.

h) Raised amino acid turnover; hyperlipidaemia.

i) Cyclooxygenase and lipoxygenase gene expression.

j) Increased synthesis of collagenases and collagens; osteoblast activation.

1.6.3: Characteristics and Effects of Interleukin-6 (IL6).

1.6.3.1: Discovery of IL6.

It was shown in the early 1980's that activated B-cells in culture required at least 2 different factors to stimulate antibody production (Yoshikazi et al, 1982). One was responsible for growth of the activated B-cells (B-cell growth factor) and the other for the subsequent induction of antibody production (B-cell differentiation factor). This second factor was first identified by Howard et al (1983) and named IL6 in 1988. Since 1983 it has been well characterised:

1.6.3.2: Size and Structure of IL6.

IL6 is a phosphoglycoprotein of (in the mature form) 184 amino acids, and has a relative molecular mass of 21-28kd. It shows homology with Granulocyte/Monocyte colony stimulating factor (GM-CSF). Other synonyms include:

B-cell Stimulating Factor-2 (BCSF-2), Interferon $ß_2$(IFN $ß_2$), 26 kd Protein, Myeloma/Plasmacytoma Growth Factor, Hepatocyte Stimulatory Factor, Macrophage/Granulocyte Inducing Factor 2, and Cytotoxic T-cell Differentiation Factor (see reviews by Kishimoto, 1989; and Hirano, 1992).

In the immune system, IL6 is produced principally by activated macrophages as is IL1. IL1, IL2, TNFα and IFNΓ (Interfearon Gamma) are inducers of IL6 production (Hirano, 1992) A summary of the large number of different cells producing IL6 is shown in the table overleaf (after Hirano, 1992).

Table 1.3: Cells Which Produce IL6 (after Hirano, 1992).

Normal Cells:	Cell Lines:
T-cells	T-cell lines (HTLV-1 transformed)
B-cells	Monocyte lines (U937, P388D1)
Monocytes	MG63 osteosarcoma line
Fibroblasts	T24 bladder line
Keratinocytes	A549 lung carcinoma line
Endothelial Cells	SK-MG-4 glioblastoma line
Astrocytes	U373 astrocyte line
Bone marrow	
Mesanglial cells	Tumour Cells:
	Cardiac Myxoma Cells
	Myeloma cells
	Hypernephroma cells

1.6.3.4: Effects of IL6 Within the Immune System.

In the immune system IL6 has a key role in the differentiation of B-cells. Hirano et al (1984) showed that cultured B-cells, which had been activated with the Staphylococcus aureus Cowan I mitogen, were induced to produce immunoglobulins IgG, IgM, and IgA when IL6 was administered to them at non-pharmacological concentrations. IL6 administered to unstimulated resting T-cells had no effect.

Garman et al (1987) showed that IL6 also had an effect on T-cells, at least in the mouse. They showed that IL6 could induce murine T-cells to produce IL2.

Khazarami et al, in 1989, showed that IL6 affected neutrophils and monocytes by priming the oxidative burst response. This is a key reaction in these cells, being the mechanism whereby they generate hydrogen peroxide and oxygen radicals, used in target cell elimination.

1.6.3.5: Systemic Effects Of IL6.

IL6 also has important systemic actions, the most important of which is the mediation of the acute phase response to injury. This is a response to tissue injury, neoplastic growth, infection or inflammation. It is characterised close to the area of cell damage by platelet aggregation, clot formation, dilation and leakage of local blood vessels and accumulation of granulocytes and monocytes, which in turn release the acute phase cytokines. These include IL1, IL6, and TNFα which cause fever, leucocytosis, increased erythrocyte sedimentation rate, complement activation and dramatic changes in the concentrations of some plasma proteins. These proteins, known as the acute phase proteins, include C-reactive protein (CRP) and serum amyloid-A, which are synthesised principally in the liver (Heinrich et al 1990). While both IL1 and TNFα are involved in the acute phase response, the principal mediator is IL6.

Gauldie et al (1987) showed that the synthesis of the acute phase proteins by hepatocytes was caused by IL6. They found that anti-IL6 antibodies could block the stimulatory effects on hepatocytes and that in a liver cell line, recombinant IL6 could induce synthesis of all the major positive acute phase proteins.

Nijstein et al (1987) demonstrated a close correlation between the concentrations in serum of IL6 and CRP synthesis in patients with severe burns, and concluded that IL6 was the primary agent of the acute phase response in humans. Further evidence was provided by Castell et al (1988) who showed that recombinant IL6 could induce the synthesis of serum amyloid A (SAA), CRP, haptoglobin α, antichymotrypsin, and fibrinogen.

Shenkin et al (1989) showed that there was a connection between IL6 production and the injury caused during surgery. A rise in the concentration of IL6 in serum was detected within 90 min of the initial surgical incision. The period of maximum IL6 concentrations correlated with the length of the operation. Shenkin et al concluded that IL6 might be of value in the study of the metabolic response to injury. The study was subsequently followed up by the same team (Cruikshank et al, 1990), who confirmed the increases in the concentration of IL6 in serum during surgery. There was also a correlation between the concentration of IL6 in serum and the synthesis of the acute phase protein C-reactive protein (CRP): the team speculated that the CRP synthesis was IL6 induced.

1.6.3.6: IL6 as a Stimulator or Inhibitor of Cell Growth:

There is also some evidence for IL6 being a growth factor/inhibitor. Chen et al (1988) showed that IL6 at non-pharmacological concentrations could inhibit the growth of a leukaemia cell line and two breast cell lines. These findings are discussed further in this dissertation (p 197).

Shimizu et al in 1988 showed that IL6 could also have a non-pharmacological growth promoting effect on a lymphoma-derived T-cell line which displayed IL6 receptors.

The table overleaf summarises the properties of IL6 (after Hirano, 1992).

Table 1.4: A Summary of the Biological Properties of IL6 (from Hirano, 1992).

1) Induction of Differentiation or Gene Expression:

IL6 Causes:

a) B-cell differentiation.

b) Induction of acute phase protein synthesis.

c) Cytotoxic T-cell differentiation.

d) Induction of IL2 production and IL2 receptor expression in T-cells.

e) Macrophage differentiation.

f) Neural cell differentiation.

g) Maturation of megakaryocytes.

h) Induction of ACTH synthesis.

2) Stimulation of cell growth:

IL6 Causes:

a) Hybridoma/plasmacytoma/myeloma growth.

b) T-cell growth.

c) Lennert's T-cell lymphoma growth.

d) EBV-transformed B-cell growth.

e) Haematopoietic stem cell growth.

f) Mesangial cell growth.

g) Renal carcinoma cell growth.

h) Keratinocyte growth.

Table 1.4 Cont.:

3) Inhibition of cell growth:

IL6 Causes inhibition of:

a) Myeloid leukemic cell line growth.

b) Breast carcinoma cell line growth.

1.6.4: Characteristics and Effects of TNFα:

1.6.4.1: Discovery of TNFα.

The name TNFα was coined by Carswell et al (1975) who demonstrated that it was cytotoxic against tumour cells. Another group (Caput et al, 1985) investigated a substance believed to be an important cause of cachexia and which they called cachectin. Cloning and sequencing of cachectin and TNFα cDNAs showed that they were identical.

1.6.4.2: Size and Structure of TNFα.

TNFα is a protein of relative molecular mass 17kd. It shares 28% homology with the cytokine TNFß, with which it also shares biological activity. Both appear to act on the same receptors.

1.6.4.3: Production of TNFα Following Stimulation.

Principal sources of TNFα are from macrophages, T-cells and natural killer (NK) cells (Akira et al, 1990). TNFα can be induced by a variety of stimuli including IL1, bacterial endotoxin, lipopolysaccharide (LPS), enterotoxin, toxic shock syndrome toxin-1 and other bacterial, viral, or parasitic antigens. TNFα also appears to be able to induce its own synthesis (Tracey et al, 1989).

1.6.4.4: Effects of TNFα.

TNFα can stimulate IL1 and IL6 production. It has a wide range of biological activities, many of which overlap with those of IL1 and IL6 (see Table 1.5).

1.6.4.5: Role of TNFα in the Mediation of Acute Inflammation.

TNF is closely implicated with IL1 in the mediation of acute inflammation. Tracy et al (1986) showed that recombinant TNFα administered to rats caused hypotension, metabolic acidosis, haemoconcentration and death. The following year Tracy et al (1987) went on to show that a similar response was produced when recombinant TNF was administered to dogs and that this response was similar to that observed with endotoxin. Hesse et al (1988) showed that administration of endotoxin to human volunteers caused a peak in TNF concentrations in serum corresponding to the onset of fever, rigors, myalgia and headache. The increased TNF concentrations were found before any IL1 activity could be detected, so Hesse proposed that it was the initial inducer of acute inflammatory effects. However, the actual effects may be mediated by a synergistic action of IL1 and TNF.

A summary of the properties of TNFα is given in the table overleaf (After Tracey et al, 1989; and Emery and Salmon, 1991)

Table 1.5: A Summary of the Biological Properties of TNFα.

(After Tracey et al, 1989; and Emery and Salmon, 1991)

1) Induction of Production or Release by TNFα:

TNFα stimulates production or release of:

a) IL1.

b) IL6.

c) GM-CSF.

d) TNFα.

e) TNFß.

f) prostaglandins.

g) leukotrienes.

h) PDGF (platelet-derived growth factor).

i) TGFß (transforming growth factor ß).

j) platelet activating factor.

k) cortisol.

l) adrenaline.

m) noradrenaline.

n) glucagon.

Table 1.5: A Summary of the Biological Properties of TNFα Cont.

Systemic effects of TNFα:

a) Mediation of endotoxic shock, with production of hypertriglyceridaemia.

b) Suppression of lipoprotein lipase in adipose tissue and other cells.

c) Activation of neutrophils with adhesion.

d) Endothelial cell toxicity.

e) Fever.

f) Induction of the acute phase response.

g) Leucocyte chemotaxis.

1.6.5: Cytokine Interactions and Synergy.

When considering the properties of the inflammatory cytokines, one of the key observations is their apparently common properties and general interaction with one another. Under experimental conditions, the effects seen may not be due to the cytokine under investigation alone but subsequent action of a different cytokine, or concurrent action of another cytokine with the one of interest. Some examples of cytokine interactions follow:

1.6.5.1: Interaction between IL1 and TNFα.

Dinarello et al (1986) demonstrated that recombinant TNFα injected intravenously into rabbits produced effects of acute inflammation which were similar to those seen with IL1. Dinarello and his colleagues also showed that in the case of PBMCs TNF was causing IL1 release. They suggested a possible synergy between IL1 and TNF.

Work by Holtman et al (1987), showed that TNFα receptors were down-regulated by IL1 in fibroblasts. TNFα can affect fibroblasts by causing them to make IL1, which lowers the number of TNFα receptors on the fibroblast cell surface and this in turn lessens the effects of TNFα on the cells.

1.6.5.2: Interaction Between TNFα and IL6.

Kohase et al (1986) showed that TNFα could also induce IL6 production. This was observed in cultured fibroblasts.

Recombinant TNF was shown to inhibit the replication of encephalomyocarditis virus in fibroblast cells. However this activity could be blocked by administration of anti-IL6 antibodies, indicating that the effect was due to the result of IL6, probably induced by the TNFα. They also showed that anti-IL6 antibodies could enhance the mitogenic effect of administered TNFα on confluent fibroblasts cultured in a serum-starved medium, thus demonstrating a possible negative feed-back mechanism for fibroblast proliferation involving IL6. Kohase et al then demonstrated that there was a complex cytokine network in fibroblasts (1987).

1:6.5.3: Interaction between IL1 and IL6.

Van Damme et al (1987 i) demonstrated that IL1 stimulates IL6 production in fibroblasts, even at very low doses. In a later paper (1987 ii), they confirmed that production of IL6 was also stimulated by TNFα.

1.6.5.4: Demonstration of Synergy Between IL1 and IL6.

Houssiau et al (1988) demonstrated that, while IL6 could stimulate PHA-activated T-cells, this activity was dependent on the presence of attendant accessory cells. T-cell proliferation was minimal when the activated cells were stimulated with IL6 alone. Very similar effects were seen with IL1. Use of IL1 and IL6 with the activated T-cells, in the absence of accessory cells, produced strong proliferation, suggesting synergy.

1.6.6: Evidence for the Production of Cytokines by Tumours.

1.6.6.1: Evidence for the production of IL1 by Tumours.

Prior to 1986, several authors (Folkman et al, 1971; Folkman and Haudenschild, 1980; Lobb et al, 1985) speculated that the spread of a tumour and its ability to invade tissue was dependent on both stimulation of blood vessel growth to the tumour, and generation of proteolytic activity as a means of invasion. Both of these processes involve the breakdown of the patient's extracellular matrix, and this is one of the characteristics associated with rheumatoid arthritis. Considerable work had already been done to demonstrate the involvement of IL1 in rheumatic disease, and so some researchers reasoned that IL1 might be involved in tumour spread. Therefore, these researchers began to investigate the possibility of IL1 production by tumours or tumour cell lines.

Using a liver tumour cell line (Sk hepatoma cell line), Moore et al, in 1986, successfully demonstrated the production of a factor very like IL1. It was pyrogenic, stimulated collagenase production in skin and synovial fibroblasts, induced bone resorption, caused thymocyte proliferation and had a relative molecular mass of 18kd.

1.6.6.1 Cont.:

These findings were characteristic of IL1 and the substance was therefore strongly suspected to be IL1. The IL1-like factor was also found (when administered to bone resorption assays in-vitro) to be a powerful stimulant of bone resorption, and so it was hypothesised by Moore and his colleagues that in-vivo production of IL1 by tumours in cancer patients could explain the hypercalcaemia often observed in these patients. However, this hypothesis was an extrapolation of the results from their in-vitro study and they did not examine IL1 production by actual tumours or investigate its presence in the serum of tumour patients.

Sato et al (1987) examined the apparent production of a factor which could cause bone resorption by a patient with hypercalcaemia consequent to a squamous cell carcinoma of the thyroid. They demonstrated that the factor had the known biochemical and biological properties of IL1. An anti-serum to IL1α blocked these effects, so the active factor was assumed to be IL1α. The results confirmed the hypothesis of Moore et al by showing that there was in-vivo production of IL1α in a patient with a tumour, that it could cause bone resorption in in-vitro assays, and that the patient displayed hypercalcaemia. In 1988 Sato et al produced further confirmation of the association of in-vivo IL1 production with bone resorption and hypercalcaemia in a patient with a squamous cell carcinoma of the oesophagus.

1.6.6.1 Cont.:

Using very similar methods to those of Sato et al, Freid et al (1989) demonstrated the production of IL1 in patients with carcinomas (they studied squamous cell carcinoma cell lines derived from patients with tumours of the tongue or facial epidermis) but without hypercalcaemia.

Work was also carried out to investigate whether IL1 might benefit the tumour. Orr et al (1988), working with cultured human endothelial cells in vitro, showed that medium from these cell cultures contained a substance which was an attractant for tumour cells. They found that secretion of this agent could be correlated with mRNA expression for IL1 in the cells, and that it had the biochemical properties of IL1. Indeed, their results could be mimicked by the administration of human recombinant IL1 ß, and the effect was found to be dose dependent. This suggested that IL1 might be involved in promoting tumour cell migration with formation of metastases.

Dejana et al (1987) showed that IL1 could affect the vascular endothelium, as well as being produced by it. IL1 seemed to make the endothelium more adhesive to tumour cells, allowing them to stick to it. The effect was both time and dose dependent. They postulated that it could cause the localisation of metastases.

1.6.6.1 Cont:

Giavazzi et al (1990) showed a definite augmentation in the formation of metastases in nude mice with human melanomas (the mice were infected with the human melanoma cell line A375M). The effect was concentration dependent and short lived. They postulated that IL1 production might contribute to the malignant behaviour of tumours by favouring the implantation of tumour cells at selected sites. The effect of IL1 was apparent even though it was injected, therefore the effect was not due to any local production at a specific site. Circulating IL1 from the tumour, therefore might have the same effect of contributing to the formation of metastases in general.

Further evidence for the production of IL1 by tumour cells came from a major study by Miyauchi et al (1988), who examined medium, taken from 64 malignant cell lines, for IL1 activity. Analysis was by the murine thymocyte proliferation assay, backed up by biochemical characterisation to check that the active factor causing the results in the assay was IL1. 16 cell lines showed high IL1 activity including glioblastoma, oral squamous cell, lung squamous cells, hepatoma, pancreatic cancer, cervical cancer and malignant melanoma.

1.6.6.1 Cont:

Some other cell lines, including the breast cell line MCF-7 and the bladder cell line T-24, produced small amounts of IL1

Malignant melanoma proved useful in the study of IL1 production. Following the work of Miyauchi et al, Kock et al (1988) examined the production of IL1 by malignant melanoma cell lines. Most of the work was carried out using a combination of bioassay, and biochemical techniques. In addition molecular biological techniques were used, one of the earliest examples of this approach. Northern blotting confirmed the existence of messenger RNA for IL1α in the cells.

Further molecular biological work (Shabon et al, 1989) was the first to confirm that the genes in malignant melanoma cells expressing IL1α were the same as those expressed in the "natural" source of macrophages. The genes for IL1 α and IL1 ß were cloned and sequenced in the malignant melanoma cell line studied (WM1158), the first time this had been done in tumour cells and provided strong evidence that tumour cell lines could produce IL1.

1.6.6.1 Cont.:

Work has continued to investigate the extent of IL1 production by various types of cells. For example, in 1992, Li et al observed that human ovarian cell lines cultured, in vitro, expressed genes for both IL1α and IL1ß. They found that 5 different ovarian cancer cell lines expressed the genes for IL1 α and ß constitutively. This expression was also found in cells harvested from ascitic fluid of patients with ovarian cancer, thus indicating the possibility of IL1 production in vivo as well as in vitro.

1.6.6.2: Evidence for the production of IL6 by Tumours.

As with IL1, the effects of IL6, or those which could be caused by its secretion, are seen in patients with certain tumours. This, and evidence for IL6 production by some cell lines, created interest in its possible use as a tumour marker. Subsequently, studies have been undertaken to determine if it is produced by tumours.

Tabibzadeh et al (1989), in a wide-ranging study, used antibodies to IL6 to examine any evidence of its production by certain solid tumours. This was achieved using immuno-staining on frozen sections of freshly removed tumours with the anti-IL6 antibody; a second biotin-labelled antibody was used to detect the first.

1.6.6.2 Cont.:

A wide variety of tumours was examined. IL6 was detected in neoplastic (cancerous) elements in primary squamal cell carcinomas of colonic, ovarian, and endometrial origin, metastatic adenocarcinomas, and lymph nodes and cells of soft tissue tumours such as leiomyosarcoma. Poorer staining was present in the other tissues examined. However, it was sufficient to show that most tumours of epithelial or mesenchymal origin displayed the presence of IL6.

The presence of a pool of IL6 seemed to be a general feature of neoplastic tissues, backing up and extending the results of previous work which showed the presence of IL6 in cardiac myxoma (as seen by Hirano et al in 1987). Tabibzadeh et al postulated that such a generalised production of IL6 by tumours might suggest that IL6 plays a major role in the modulation of host-tumour interactions both locally and systemically, by altering the physiological, biochemical and immunological status of the host.

Miki et al (1989) examined renal carcinomas and found that when these carcinomas were grown in vitro, like many cell lines, they required foetal calf serum (FCS) for growth. However they found that if recombinant human IL6 was supplied to the cultures, (in the absence of FCS) the cells could grow.

1.6.6.2 Cont.:

Miki et al suspected that IL6 was needed as a growth factor for the cells and that it was being produced by the cells in response to another factor in the FCS suggesting that IL6 is an autocrine growth factor for the cells. Using immuno-staining for IL6, it was found that renal carcinoma cells were producing IL6. Analysis of extracted mRNA from the cells by northern blotting showed the presence of mRNA for IL6. Use of another anti-IL6 antibody administered to the cells in culture inhibited their growth.

The evidence seemed to support the theory that, in vitro, IL6 was an autocrine growth factor for renal carcinomas. However, at that time, there was no evidence of production of IL6 in vivo in patients with renal carcinomas, although patients with these tumours often showed clinical symptoms, such as elevated concentrations in serum of acute phase proteins, which could be caused by the effects of IL6.

Takenawa et al (1991) used northern blotting to examine whether IL6 was present in fresh tissue taken, immediately following surgery, from patients with renal carcinoma. A semi-quantitative technique was used to compare results with those taken from non-neoplastic tissue from other patients.

1.6.6.2 Cont.

10 out of 43 tumour samples displayed as much as a 40-fold increase in IL6 mRNA expression as compared to the control tissue (normal renal glomeruli, mesangial cells, and vascular endothelium express low concentrations of IL6). Immuno-staining of the tumour tissue indicated that it was not normal cells producing the IL6, but the tumour cells themselves. Following on the ideas of Miki et al, they also looked for mRNA expression for the IL6 receptor. If IL6 was an autocrine growth factor then the renal carcinoma cells would have to express the IL6 receptor as well as expressing IL6.

Northern blotting gave a negative result for presence of the receptor, but use of the very sensitive polymerase chain reaction to amplify cDNA prepared from the extracted mRNA did show that the renal carcinoma cells expressed the IL6 receptor.

Takenawa et al also examined the relationship between the production of IL6 in patients with renal carcinomas and the clinical conditions which the patients displayed. They showed that patients with tumours showing enhanced IL6 production had apparently higher concentrations of C-reactive protein in serum and a higher erythrocyte sedimentation rate, though these were not statistically significant.

1.6.6.2 Cont.:

The incidence of lymph node metastases was significantly increased in patients with enhanced concentrations of IL6. Thus, the findings and hypotheses of Miki et al were verified and it appeared likely that IL6 could make a good marker for renal carcinoma.

Interest has also been shown in the possibility of IL6 being a marker in other cancers, and in particular those which are difficult to monitor or diagnose by other means. In ovarian carcinoma, as has been previously mentioned, the marker CA125 has recently been characterised. However, IL6 has been examined as a possible marker also. Watson et al, in 1990, demonstrated that primary cultures from ovarian tumours, and 3 ovarian cell lines, could secrete IL6. In 1991, members of the same team, in a paper by Berek et al, looked at IL6 concentrations in serum from patients with ovarian carcinomas to determine if they would correlate with the disease state of the patients. A bio-assay was used to detect the IL6 (using the MH60.BSF-2 cell line, an IL6-dependent murine hybridoma cell line).

1.6.6.2 Cont.:

IL6 concentrations in 90 separate samples of serum were measured. In those patients with macroscopic disease, 16 out of 21 patients had significantly raised concentrations of IL6 compared to the concentrations measured in the serum of normal women used as controls.

Out of 9 patients with bulky tumours, 8 had significantly raised concentrations, and 8 out of 12 patients with residual disease also had high concentrations. There was also a definite correlation between raised CA125 concentrations and high IL6 concentrations in 12 out of 14 patients. Studies of IL6 concentrations in serum over a period of 3 to 4 months showed that in 9 out of 16 patients, concentrations of IL6 were seen to rise. This increase was also seen to correlate with tumour progression. There also appeared to be a link between IL6 concentrations in serum and patient survival rate, the patients with higher IL6 concentrations being less likely to survive. These data suggest that, as with renal carcinoma, IL6 might be a useful marker for the monitoring of ovarian carcinoma.

1.6.6.2 Cont.:

Meyers et al (1991) investigated bladder carcinoma to see if IL6 expression could correlate with prognostic factors. The treatment of bladder carcinoma is dependent on the accurate assessment of the malignant potential of the tumour. Usually this is determined by staging (assessing the extent of invasion of the surrounding tissue by the tumour) and histological categorisation (low grade versus high grade. Meyers et al used polymerase chain reaction (PCR) amplification of prepared cDNA transcripts of extracted mRNA (this technique is discussed in section 2), which has the particular advantage of allowing probing for several gene products in the same sample simultaneously. All of the ex-vivo transitional cell carcinomas, one squamous carcinoma and 2 transitional cell carcinoma cell lines (including the cell line T-24) expressed the majority of HLA genes. All of the samples and cell lines expressed the IL6 receptor. One of the cell lines and all of the high grade tumours expressed mRNA for IL6.

No useful correlation of IL6 expression with prognostic or diagnostic factors was demonstrated in the study and no investigation was undertaken to examine if the detected IL6 expression was due to the carcinoma cells or due to infiltrating cells in the tumour (e.g. macrophages). The expression of the IL6 receptor and IL6 in high grade transitional cell carcinomas suggested that in these too, IL6 might be an autocrine growth factor, as is the case with renal cell carcinomas.

As with IL1, efforts have been made to link IL6 to clinical conditions often observed in cancer patients, such as cachexia. This involves depletion of muscle and fat, anorexia, asthenia, hyperglycaemia and anaemia and is often an important contributor to the death of patients.

1.6.6.2 Cont.

As will be examined later, efforts have been made to link cachexia with tumour necrosis factor (TNF) (see **1.6.6.3**.) This has included studies with animal models, usually in rats or mice. Strassman et al (1992) set up an animal model of cachexia in mice. They used a cell line derived from murine colon 26 adenocarcinoma, an undifferentiated tumour induced by a carcinogen, which caused cachexia. However, rather than examining any evidence for TNF production, they measured IL6 concentrations in serum in the mice, using the B-9 bioassay. They found a definite correlation between concentrations in serum of IL6 and the state of the cachexia in the mice.

When the tumours were resected, the concentrations of IL6 in serum fell (thus indicating that the tumours were the source of the IL6) and the mice also began to gain weight. That the IL6 was involved in the cachexia was confirmed when it was observed that administration of a monoclonal antibody to IL6 to the mice caused significant suppression of the development of cachexia. Whether this is also true in humans remains to be demonstrated.

1.6.6.3: Evidence for the production of TNFα by Tumours.

The first significant investigation into TNFα production in human cancer patients was undertaken by Balkwill et al (1987). In an attempt to demonstrate that TNFα was responsible for cachexia, they assessed 226 samples of serum taken from cancer patients, using a sensitive enzyme linked immuno-sorbant assay (ELISA). Samples of serum were also taken from normal individuals to provide a control group. 50% of the samples from cancer patients yielded detectable TNF, but TNFα was found in only 3% of the control group. Of 39 samples from patients with tumours, who displayed no clinical symptoms of cancer, 18% had detectable concentrations of TNFα. A greater proportion of samples were TNF-positive in patients with ovarian or oat-cell carcinomas (69 and 67% respectively), while in lymphomas 26% were positive.

In the same study, efforts were also made to identify the source of the TNFα. Northern blotting of mRNA extracted from peripheral blood mononuclear cells and from tumour samples taken from patients with colorectal carcinomas known to have TNFα in serum was carried out, using a probe for TNFα. Peripheral blood mononuclear cells taken from normal individuals were also probed. TNFα mRNA was found in 8 out of 11 samples of peripheral blood mononuclear cells from the patients with cancer, but in only 1 of 8 samples from the normal group.

1.6.6.3 Cont.:

TNFα mRNA was detected in 2 out of 6 colorectal tumours examined. Thus it appeared that while the majority of the TNFα was from peripheral blood mononuclear cells, probably in response to the tumour, some tumours may produce TNFα. Balkwill et al proposed that circulating TNF was responsible for the cachexia seen in some patients.

Further evidence for the production of TNFα by tumours in vitro was provided in a later study (Takayama et al, 1990). Takayama and his colleagues used immuno-staining to examine evidence of TNFα in ovarian carcinomas and found that of 20 samples examined 16 were positive for TNFα. In-situ hybridisation was also carried out and confirmed the immuno-staining findings with the presence of TNFα mRNA being detected in the tumour tissue. Subsequently, Roby et al (1991), demonstrated that TNFα could be expressed in normal ovarian tissue in vivo.

1.6.6.3 Cont.:

In vivo studies were still unclear however. The research by Balkwill et al showed that TNF could be produced by peripheral blood mononuclear cells in cancer patients. Ishi et al (1990), detected TNFα from a pleural effusion of a patient with lung cancer. The fluid could inhibit growth (in vitro) of lung tumour cells taken from the same patient. The origin of the TNF was suspected to be monocytes or macrophages. In another study, by Ikemoto et al (1989), monocytes were shown to produce TNF in patients with bladder carcinomas.

In vitro studies were also carried out to examine if cell lines could produce TNFα. Spriggs et al (1988) demonstrated that some tumour cell lines could produce TNFα. 2 out of 4 ovarian cell lines, and 1 out of 3 lung tumour-derived cell lines expressed mRNA for TNFα, as detected by Northern blotting.

These results were supported by Kronke et al (1988), who examined 17 tumour cell lines of various origins and found (again by northern blotting to show expression of TNFα mRNA) that 8 cell lines could express TNFα without any external stimulus (i.e constitutively). Probing for TNFß, Kronke and his colleagues found that 5 of the 8 also displayed TNFß production. Of 9 cell lines not showing constitutive expression, 5 were induced to express TNFα if stimulated by phorbol ester and/or various cytokines.

1.6.6.3 Cont:

The cell lines displaying constitutive TNFα mRNA expression included those derived from solid tumours, and myelomas. Significantly, TNFß was expressed only by cell lines of myeloid origin.

Kronke et al also proposed that TNFα could have a beneficial effect on some tumours (presumably those resistant to its cytotoxic effects), especially with regard to the formation of metastases. They proposed that the mechanism for this might be promotion of growth of blood vessels to the tumour and possibly also promotion of tumour invasion. Kronke et al further proposed that TNF production could be involved in the hypercalcaemia observed in some patients with myeloma.

Rosen et al (1991) found that TNFα could stimulate the migration of epithelial tumour cells in vitro and promote the invasion of several tumour cell lines through reconstituted basal membrane, giving support to the ideas expressed by Kronke et al.

1.6.6.3 Cont.

Work also continued on the link between TNFα and cachexia. Much of this was carried out on rats. Stavroff et al (1989), using a bio-assay to determine the concentration of TNF in the serum of sarcoma-bearing rats, established a correlation between TNFα concentrations in the serum and tumour burden. There was also an inverse relationship between the concentration of TNF and rate of food intake and body weight change. After resection of the tumours, TNF concentrations in serum fell to undetectable levels, indicating that the TNF production was due to the presence of the tumour, even if that was not necessarily its source.

However, animal models cannot always be relied on to give results predictive of human disease. In a similar study carried out in humans, Knapp et al (1991) examined the relationship between weight loss and TNF production in patients with advanced breast cancer. They found that TNFα was present in significant amounts in the serum of all the patients, the concentrations being significantly higher in patients who had lost weight (one of the symptoms of cachexia). Concentrations of TNFα were also seen to increase significantly as the patients' disease progressed.

1.7: Aims of the Research Presented in this Dissertation

The original aim of the research presented in this dissertation was to study the synthesis of IL1α, IL1β, IL6, TNFα, and TNFβ by tumour cells, and to develop methods for the detection of cytokines and their mRNA in primary samples of tumours. None of these cytokines could be considered as tumour markers if they were not produced by a suitable variety of tumours, or indeed by any types of tumour. It was not the aim of the research to develop any of the cytokines as tumour markers, but it was hoped that the results of the work would indicate which cytokines (if any) could be developed as markers in future studies.

The types of tumour used in the study would be determined by a number of criteria, including: availability, frequency, and ease of collection of tumour samples, and as wide a variety of different tumours as possible would be collected. It was also decided to avoid collecting certain types of tumour which were already known to produce the cytokines of interest, in favour of those whose cytokine production profiles (in 1989) were largely unknown.

1.8: Plan of Investigation of this Present Study.

At the time of commencement of this investigation in October 1989, very little was known about the production of IL1α, IL1ß, IL6, TNFα, and TNFß, by tumour cells themselves. Those investigations that had been undertaken had used for the most part methods which were non-specific. Only a few used more specific methods based on molecular biology techniques (examination of cytokine mRNA production), or immunological techniques (immuno-histology or immuno-assay of the cytokine polypeptide). In addition, much of the work had been carried out on cell lines or primary explants of tumours grown in culture. It was therefore decided that the investigations that are now reported here would examine the production of these cytokines in samples of solid tumours taken from patients as soon as possible following surgery.

The studies were performed in three phases, as follows:-

a) Methods would be verified in studies on tumour cell lines whose cytokine production profile was known. The cell lines would also enable some other experiments which would be difficult to perform with the tumour samples themselves, e.g. to examine whether any of the cytokines were growth factors for the cell lines (and so possibly also for the tumours).

b) Experiments would then be performed to explore cytokine production in the solid tumour samples themselves.

i) By examining cytokine mRNA production in the tumours.

ii) By using immuno-histology to confirm that tumours were producing cytokine polypeptides.

c) The medical records of patients donating tumour samples would be checked to assess if any patients displayed clinical symptoms or conditions which could be attributed to production of relevant cytokines by the tumour.

Chapter 2: Methods.

2.1: List of Reagents and Materials.

From BDH Ltd., Poole, Dorset, U.K.:

Chloroform, reagent grade

Ethanol, reagent grade

Formalin

Acetone

Xylene

Paraffin wax

Sodium Acetate, 3M (pH 5.2)

From Biogenesis Ltd., 12 Yeoman's Park, Bournemouth, U.K.:

RNA Sol solution

From Boehringer Mannheim Gmbh.

10mM deoxynucleotide mix (equal mixture of deoxyadenosine trisphosphate, deoxythymidine trisphosphate, deoxycytosine trisphosphate, and deoxyguanosine trisphosphate)

Restriction Enzymes: Eco R I E.C. no. 3.1.27.1

Hinf I E.C. no. 3.1.30.1

Msp I E.C. no. 3.1.30.2

Taq polymerase, E.C. no.2.7.7.7

Taq Polymerase buffer (Tris 100mM, $MgCl_2$ 15mM, KCl 500mM, gelatin 1mg/ml, ph 8.3)

From FMC Ltd., Sittingbourne ,U.K.:

Seakem agarose

From Gibco Ltd., Renfrew Road, Paisley, U.K.:

10mM deoxynucleotide mix (equal mixture of deoxyadenosine trisphosphate, deoxythymidine trisphosphate, deoxycytosine trisphosphate, and deoxyguanosine trisphosphate)

0.1M dithiothreitol (DTT)

Foetal calf serum

Glutamine

2-Mercaptoethanol

Oligo dT primer

Penicillin/ streptomycin solution

10x reverse transcriptase synthesis buffer

RNase H, E.C. no. 3.1.27.1

RPMI 1640 cell culture medium

RPMI 1640 cell culture medium, phenol red free

Superscript ™ reverse transcriptase, E.C. no. 2.7.7.7

Trypsin

From Glasgow University Biochemistry Department, Glasgow, U.K.:

Oligonucleotide primers 10mM

From Janssen Biochemica:

Hybridoma Growth factor/ crude IL6

From Nat. Institute of Biological Standards and Control, Potter's Bar, U.K.:

Anti- IL1 antibody (Sheep)

Anti- IL6 antibody (Goat)

From Scottish Antibody Production Unit, Law Hospital, U.K.:

Anti- CD8 Antibody (Mouse)

Anti- CD14 Antibody (Mouse)

Anti- CD21 Antibody (Mouse)

FITC-Conjugated Anti-Mouse Antibody

From Sigma Ltd., Fancy Road, Poole, Dorset, U.K.:

Diazo-bicyclo 2,2,2, octane (DABCO)

Diethylpyrocarbonate (DEPC)

Ethidium Bromide

FITC-Conjugated anti-sheep antibody

FITC-Conjugated anti-goat antibody

Alkaline Phosphatese-Conjugated anti-sheep antibody

Alkaline Phosphatase-Conjugated anti-goat antibody

Fast Red™ Alkaline Phosphatase detection system

Gelatin

Isopropanol

MTT dye

Phenol, molecular biology grade

Triton X-100 detergent

From Dept. of Pathology, Western Infirmary, Glasgow, U.K.:

Haematoxylin

Eosin

Tris-borate buffer (x5 stock Solution):

0.089 M Tris-borate: 54 g tris base

0.089 M boric acid: 27.5 g boric acid

0.002 M EDTA: 20 ml 0.5 M EDTA (pH 8.0)

made up to 1 l with distilled water.

Sample Loading Buffer (x6 stock solution):

0.25% bromophenol blue, 40% (w/v) sucrose in distilled water

(Keep at 4°C)

2.2: RNA Extraction.

2.2.1: Introduction.

In initial experiments to extract mRNA from cells or tissues the guanidinium isothiocyanate/ phenol/ chloroform method developed by Chomczynski and Sacchi (1987), and utilising locally prepared reagents, was employed. Testing of the extracted material prepared by this method resulted in only degraded mRNA being found. It was thought that this was due to either one of the reagents being contaminated, or contamination of glassware with RNAse, but the cause was not definitely established. Subsequent experiments, using commercial RNAsol (Biogenesis Ltd.), which is a ready-mixed guanidinium isothiocyanate/phenol solution, and use of a slightly adapted protocol using isopropanol to precipitate the RNA instead of ethanol, did provide intact mRNA. All glassware used with this protocol was treated in a similar manner to that in experiments with the locally prepared reagents.

2.2.2. Method employed for mRNA Extraction:

(1) The tissue sample was frozen (-70°C). A portion of approximately 1cm^3 was removed with a scalpel, and placed in a small petri dish. The remainder of the sample was then plunged into liquid nitrogen to ensure it remained fully frozen before being returned to storage in the -70°C freezer.

2.2.2 Cont.

(2) 1ml of RNAsol was added to the sample on the dish, thus ensuring that the tissue would be in RNAsol as it thawed. The tissue was then chopped into as small pieces as possible and pipetted into a sterile 2ml polypropylene microcentrifuge tube (Sarstedt Ltd.) together with all the liquid. A further 0.5ml of RNAsol was then used to wash out the petri dish and this wash added to the tube. 150µl of chloroform was added and the tube left to stand upright in ice for 5 min.

(3) The tube was then centrifuged in a pre-cooled refrigerated microfuge at 10000g for 15 min. The contents of the tube were now identifiable as three layers, a clear aqueous upper layer, an interface layer of solid material, and a lower blue organic layer. The top (aqueous) layer was carefully removed, without disturbing the layers below, and placed in another 2ml microfuge along with an equal volume of cold isopropanol (taken from -30°C freezer and used immediately). The tube was left to stand on ice for 15 min and then centrifuged using the same conditions as before.

(4) The resulting precipitate was drained carefully, washed twice in a mixture of 75% ethanol, 25% DEPC treated water, and centrifuged as before, but for 10 min between each wash. The sample was then air-dried in the tube, in the flow hood at room temperature, and the resultant pellet of material dissolved in 104µl of DEPC treated water.

2.2.2 Cont.

(5) 4µl of this solution was removed and placed into a fresh microfuge tube and 396µl of DEPC water added to give a total volume of 400µl. The absorbance of this solution was then measured on a spectrophotometer with a deuterium light source, previously blanked with plain DEPC water, at 260 and 280 nm, using suitable quartz cuvettes.

The ratio of Absorbance at 260 nm / Absorbance at 280 nm is indicative of the purity of the RNA. A ratio of >1.8 indicates good purity of RNA, though in practice samples with ratios of as low as 1.3 were still found to yield RNA pure enough to be reverse transcribed. The absorbance at 260 nm was used to calculate the yield using the principle that an absorbance value of 1 optical density (O.D.) at that wavelength equals 0.04 mg of RNA, i.e.:

RNA YIELD (mg/ml) = Absorbance at 260 nm x 0.04 x 100

(x100 because the sample is a 1/100 dilution ie. 4µl/ 400µl)

(6) 300µl of absolute alcohol was added to the remaining 100 µl of stock sample prepared in (4), and the sample stored at -70°C until needed.

2.2.3: Explanation of Method.

The guanidinium isothiocyanate in the RNAsol is a potent inhibitor of RNAse and causes immediate cell lysis and protein denaturation. The phenol in the solution deproteinises the nucleic acids. The added chloroform acts as an organic solvent.

The extraction process, after addition of the RNAsol and chloroform to the sample and centrifugation (see method for details), results in the creation of an upper clear aqueous layer containing RNA, an interphase layer of proteins, and a lower blue layer with DNA. The aqueous layer is transferred into a separate tube and the RNA precipitated using isopropanol. RNA is more insoluble in isopropanol than ethanol, resulting in a faster precipitation. The RNA must be washed in alcohol prior to being used for any purpose to remove contaminating traces of phenol or chloroform.

2.2.4: Precautions Necessary for Working with RNA.

Stringent precautions have to be observed when working with RNA due to the danger of contamination by the enzyme RNase which can rapidly break down any RNA that is present. This enzyme is very stable and it is only broken down by extreme conditions e.g. a temperature in excess of 180°C. It is also very prevalent. All work involving RNA has to be carried out in a laminar flow hood (to prevent airborne contamination) and gloves have to be worn at all times as RNase can be secreted in sweat. Glassware has to be baked at 250°C for at least an hour but where possible, it is better to use sterile disposable plastics. All aqueous solutions must be made up with distilled water treated with diethyl-pyrocarbonate (DEPC water) which breaks down RNase. Autoclaving is necessary to remove traces of DEPC and to sterilise the solution. Microfuge tubes must be soaked in DEPC water for at least an hour and then autoclaved.

2.3: Reverse Transcription of RNA into cDNA.

2.3.1: Introduction.

Reverse transcription was used to convert the extracted mRNA into its corresponding complementary DNA, or cDNA, prior to any further analysis. RNA is unstable and prone to digestion by RNAse, a very common enzyme. It is also single stranded, rather than double stranded and often forms loops due to complementary base pairing. It is therefore best to convert it into a corresponding, and more stable, cDNA. The method for doing this uses the enzyme, Reverse Transcriptase. Oligo dT primer was used to prime the reaction as it ensures amplification of mRNA, which contains a poly-adenylated tail.

Enzyme and Oligo dT primer were supplied in kit form with all the necessary reagents. The manufacturer's protocol was used without modification and the kit used was from Gibco BRL.

2.3.2: Method for Reverse Transcription.

2.3.2.1: Extraction of mRNA from Storage in Alcohol.

The following method was performed to retrieve mRNA from storage in alcohol prior to reverse transcription (It was not performed where reverse transcription was carried out immediately following RNA extraction).

(1) A volume of the stock solution was removed so that it contained approximately 5µg of RNA, and placed in an RNAse-free 0.5 ml microfuge tube (see 2.1.2. p61) To this was added 1/10th of the volume of 4M ice-cold sodium acetate (taken from the -30°C freezer and used immediately) and the tube was left to stand at -70°C for 15 min.

(2) The tube was then centrifuged at 10000g for 15 min at 4°C in a refrigerated microfuge.

(3) The resulting pellet (often invisible or only a smear) was washed twice in a 75% ethanol, 25% DEPC water mixture (centrifuged as in (2), but for 10 min). It was then air dried in the tube, in the laminar flow hood at room temperature, and dissolved in 13µl of DEPC water. The sample was now ready for reverse transcription.

2.3.2.2: Reverse transcription.

(1) 1µl of oligo dT primer (provided in the reverse transcription kit) was added to the tube, and the tube heated to 70°C in a Hybaid thermal reactor for 10 min.

(2) The contents were collected by centrifugation for 15s at 10000g and quick-chilled on ice. The following were then added in order: 2µl of 10x synthesis buffer, 1µl of 10mM dNTP mix, 2µl 0.1M DTT, 1µl of SUPERSCRIPT RT reverse transcriptase (EC no.2.7.7.7)

(3) The mixture was mixed gently and collected by brief centrifugation (as in 2) and left at room temperature for 10 min. It was then heated to 42°C in the thermal reactor, and left to incubate for 50 min. The reaction was terminated by incubating the tube at 95°C for a further 10 min.

(4) The tube was cooled on ice for 10 min, and the contents collected by brief centrifugation (as in 2). 1µl of RNase H (E.C. no. 3.1.27.5) was added to the tube and incubated at 37°C for 20 min. The contents of the tube were collected by brief centrifugation (as in 2) and 79µl of DEPC water added to give a final volume of 100µl. The sample was stored in the fridge at 4°C.

Table 2.1: Thermocycler Programme for Reverse Transcription *.

Step 1) 70°C for 10 min

Step 2) 42°C for 50 min

Step 3) 90°c for 5 min

if adding RNase H:

Step 4) 37°C for 20 min

(* using a Hybaid T2 thermocycler)

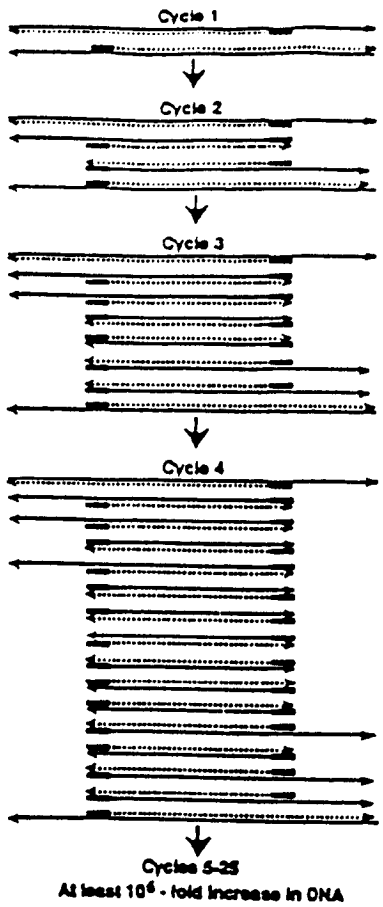


Figure 2.1: Illustration of the Principle of PCR.

The figure shows repeated cycles of DNA denaturation, oligonucleotide primer annealing, and elongation with DNA polymerase, which results in an exponential increase in the DNA flanked by the primers.

2.4: Polymerase Chain Reaction Analysis of cDNA for Cytokine Expression.

2.4.1: Theory and Background to the Polymerase Chain Reaction.

The polymerase chain reaction (PCR) is one of the most powerful new molecular biology techniques. Originally developed by Saiki et al (1985), and Mullis et al (1986), PCR is used to amplify, many times, a segment of DNA (or cDNA, complementary DNA made by reverse transcription of mRNA) that lies between two regions of known sequence. Two oligonucleotide primers, usually of different sequences, which are complementary to the known flanking regions of the sequence to be transcribed, are used together with the key enzyme, a DNA polymerase.

Figure 2.1 opposite illustrates the process:

2.4.1 Cont.:

Firstly, the template DNA is thermally denatured by heating at approximately 90°C for 2 to 3 min, and then it is hybridised with the primers. The DNA polymerase extends each primer to give a copy of each of the original strands. The primers are designed so that the products of each extension have overlapping ends. This means they can act as new templates to be primed and extended by the opposing oligonucleotide in a new round of synthesis and this is repeated many times. The number of copies made of the region of interest defined by the primers increases exponentially as a function of the number of cycles. Each cycle of the reaction can be completed in a few minutes, and therefore in three to five hours a sequence can be amplified from $3x10^5$ to $2x10^7$ times. Importantly, the majority of strands synthesised during the PCR are the same length, as defined by the distance between the binding sites of the two primers, so that each product can be visualised as a discretely migrating band by agarose gel electrophoresis.

2.4.1 Cont.:

The sequence chosen for amplification often has one or more restriction enzyme sites built into it. This means that the identity of the amplified product can be checked by restriction enzyme digestion following the PCR process.

The first protocols for PCR used, as the polymerase, the Klenow fragment of E. coli DNA polymerase, which was not stable at the high denaturing temperature of the reaction (95°C) and so had to be added after the denaturing step in each round of the reaction (Saiki et al ,1985; Mullis et al, 1986) This has been replaced by a polymerase from Thermus aquaticus (Taq DNA polymerase), which is stable at 95°C, and can survive at least 40 cycles (Chien et al, 1976). This enzyme can be added entirely at the start of the reaction and needs no replacement, allowing the reaction to be mechanised using a programmable thermocycling machine. It also increases the specificity of the PCR, while decreasing the amount of starting material that is required (Saiki et al 1988). PCR can be used to amplify DNA from almost any source, including cDNA prepared from extracted mRNA.

2.4.2: Artifacts Which Can Occur in PCR.

PCR, no matter how well performed, can produce artefacts. The most common of these are primer dimers. While primers are chosen to minimise the possibility of cross-hybridisation, dimers can be formed. Since dimers are of small size, they can easily be distinguished from products, which have a much larger size. In an agarose gel, which separates DNA or RNA depending on the mobility of each fragment when a current is passed through the gel, the primer dimers travel very far along the gel (beyond the bromophenol blue dye front) but the products travel less far as they are much larger and are retarded by the gel.

Other artefacts can occur e.g. nucleotide or degraded cDNA fragments. These are also of very small size and can be distinguished from products in the same way as primer dimers.

While care in choosing primer sequences which are intron-spanning should eliminate the chances of genomic DNA being amplified, this can occur. Genomic DNA products amplified are of large size and appear towards the bottom of agarose gels (near the sample wells) The presence of artefacts means that the identity of each PCR product has to be checked by comparing its observed size to the expected size and by restriction enzyme digestion.

2.4.3. Method used for PCR:

2.4.3.1: Precautions Necessary to Prevent Contamination by Extraneous DNA.

The PCR method is very vulnerable to false results caused by accidental contamination by extraneous DNA. Stringent precautions have to be taken as follows:

1) Gloves to be worn at all times and changed frequently.

2) The reaction should be carried out in a good laminar flow hood, preferably a class II, with an ultraviolet light fitted, which is washed with a detergent (Decon) and then a 70% alcohol, 30% DEPC water mixture before use.

3) The following are then placed in the hood:

(i) Two pipettes, thoroughly cleaned with Decon/ alcohol (200µl and 20 µl sizes).

(ii) A vial of sterile water used only for PCR.

(iii) A new box of standard yellow pipette tips.

(iv) A new container of 0.5 ml microcentrifuge tubes, previously autoclaved.

(v) A marker pen

(vi) Reaction components (buffer, etc.)

(vii) Oligonucleotide primers.

2.4.3.1 Cont.:

The Taq Polymerase enzyme, which performs the reaction, is kept in the freezer (-20°C) until required for use. The U.V. light is switched on and the contents of the hood exposed for at least 20 min. U.V. light cross-links any DNA which may be present, preventing it from being a possible contaminant.

2.4.3.2: Method employed for PCR.

The reaction volume used was 50µl. The following reagents were used:

1) dNTP mixture: An equal mixture of the nucleotides deoxyadenosine trisphosphate (dATP), deoxycytosine trisphosphate (dCTP), deoxyguanosine trisphosphate (dGTP), deoxythymidine trisphosphate (dTTP) each at a concentration of 10mM. (Nucleotides supplied by Boehringer Mannheim Gmbh)

2) 10x Reaction buffer (supplied with the Taq polymerase): Tris 100mM, $MgCl_2$ 15 mM, KCl 500mM, gelatin 1mg/ml (pH 8.3)

3) Oligonucleotide primers, ready mixed at a concentration of 10µM (obtained from Glasgow University Biochemistry Department)

4) Taq DNA polymerase (E.C. no.2.7.7.7) (Boehringer-Mannheim Gmbh)

2.4.3.2 Cont.:

The reaction was performed as follows:

1) 0.5µl microfuge tubes were labelled appropriately and the following added to each tube:

4µl of dNTP mix

5µl 10x reaction buffer

30.75 µl PCR water

0.25 µl Taq polymerase

2) 5µl of the appropriate oligonucleotide primer was added to each tube.

3) All components were removed from the hood, except for the reaction tubes and 5 µl of sample cDNA added to each tube. The contents of each tube were then gently mixed and overlaid with three drops of mineral oil (Sigma, molecular biology grade) to prevent evaporation of the contents during incubation. All tubes were then incubated in a Hybaid thermal reactor using an appropriate programme.

4) On completion the samples were stored at -4°C until they could be analyzed.

Table 2.2: Thermocycler Programme for PCR *.

1 Cycle of: 95°C for 3 min

65°C for 1 min

72°C for 1 min

followed by

33 Cycles of: 95°C for 50 sec

65°C for 1 min

72°C for 1 min

followed by

1 Cycle of : 95°C for 50 sec

65°C for 1 min

72 °C for 5 min

(* using a Hybaid T2 thermocycler)

Table 2.3: Primer Sequences Used in the PCR

IL1α Upstream:

No. of bases: 23

Molecular mass (Daltons): 7032

Sequence: 5' > GGC CAT CGC CAA TGA CTC AGA GG < 3'

IL1α Downstream:

No. of bases: 23

Molecular mass (Daltons): 7026

Sequence: 5' > GCA CTG GTT GGT CTT CAT CTT GG < 3'

IL1ß Upstream:

No. of bases: 24

Molecular mass (Daltons): 7359

Sequence: 5' > GAA GTA CCT GAG CTC GCC AGT GAA < 3'

IL1ß Downstream:

No. of bases: 24

Molecular mass (Daltons): 7381

Sequence: 5' > CGT GCA GTT CAG TGA TCG TAC AGG < 3'

Table 2.3 Cont.

IL6 Upstream:

No. of bases: 23

Molecular mass (Daltons): 6841

Sequence: 5' > CCA CAC AGA CAG CCA CTC ACC TC < 3'

IL6 Downstream:

No. of bases: 23

Molecular mass (Daltons): 6866

Sequence: 5' > CTG GCT TGT TCC TCA CTA CTC TC < 3'

TNFα Upstream:

No. of bases: 23

Molecular mass (Daltons): 6965

Sequence: 5' > AGC TGC CAG GCA GGT TCT CTT CC < 3'

TNFα Downstream:

No. of bases: 23

Molecular mass (Daltons): 6909

Sequence: 5' > GGT TAT CTC TCA GCT CCA CGC CA < 3'

Table 2.3 Cont.

TNFß Upstream:

No. of bases: 24

Molecular mass (Daltons): 7117

Sequence: 5' > CAC CAC CTG AAC GTC TCT TCC TCC < 3'

TNFß Downstream:

No. of bases: 23

Molecular mass (Daltons): 7120

Sequence: 5' > GGA GTA GAC GAA GTA GAT GCC AC < 3'

ß2M Upstream:

No. of bases: 24

Molecular mass (Daltons): 7237

Sequence: 5' > CCT TGA GGC TAT CCA GCG TAC TCC < 3'

ß2M Downstream:

No. of bases: 23

Molecular mass (Daltons): 6939

Sequence: 5' > CCA TGA TGC TGC TTA CAT GCT TC < 3'

2.5: Analysis of cDNA Products.

2.5.1: Introduction.

The cDNA products amplified by the PCR process were analyzed by agarose gel electrophoresis. A Hybaid mini-gel apparatus was used. This is a horizontal slab gel system designed specifically for molecular biology applications.

2.5.2: Method Employed for Electrophoresis.

1) A 1.8% agarose gel was prepared in TBE buffer, using Seakem agarose (FMC Ltd.) in a clean 250 ml glass conical flask. The agarose was melted by placing the flask in a domestic microwave oven on a high setting for 2 min. It was then allowed to cool slightly and 15µl of ethidium bromide solution added (Sigma Ltd.). When hand hot, the solution was poured into a mould, with a comb (to make the wells in the gel) and allowed to set.

2) As the gel was setting, the samples to be analyzed were prepared. Using 2 ml microfuge tubes, 3 µl of loading buffer (see appendices for details) was mixed with 12 µl of sample cDNA for each tube. The samples were then briefly centrifuged in a microfuge at 10000g for 10s to collect the liquid in the bottom of the tubes.

2.5.2 Cont.

3) The cast gel was placed in the gel tank, TBE buffer added to a depth to cover sufficiently the wells of the gel, and the comb removed. Each sample was then added to a well and the cover placed on the gel. The power supply was switched on and electrophoresis performed at room temperature for approximately 1 hour, at 100 volts.

4) Once the electrophoresis was complete, the products were visualised by using UV light. The gel was placed on a UV-emitting light box (on long wave setting). Ethidium bromide cross links DNA and causes it to fluoresce under UV light. Therefore, each product showed up as a fluorescent band. As each product has a different molecular size, each appears at a different place on the gel, due to the fact that short lengths of DNA can, under electrophoresis, travel faster through the gel than larger pieces. A molecular size marker, consisting of a mixture of DNA fragments of different, but known sizes, was used alongside the samples, allowing their sizes to be worked out. The identity of the samples was confirmed by comparing their sizes to the known sizes of the primer products.

5) A permanent record of each set of results was taken by photographing the developed gel under UV light, using a Polaroid instant black and white camera, with Polaroid 665 film and an exposure time of about 25s. The film also gave negatives from which multiple prints were made.

2.6: Restriction Digestion of PCR Products.

2.6.1: Introduction.

To verify the identity of the PCR products it was necessary to perform restriction digestion. The use of each oligonucelotide primer resulted in the formation of a unique product containing an appropriate restriction enzyme site. This site was present in the original sequence chosen for amplification.

Upon reaction with the appropriate restriction enzyme, particular fragments of known size are produced; analysis of these confirms the identity of the PCR product. The table overleaf shows the expected size of each product, the restriction enzyme which cuts it and the expected size of each digested fragment.

Table 2.4: Expected Results of Restriction Enzyme Digestion of PCR Products.

PCR Product	Size (bp)*	Res. Enzyme#	Fragment Size* (bp)
IL1α	324	EcoR I	112, 212
IL1ß	376	Hinf I	142, 234
IL6	313	Hinf I	197, 116
TNFα	450	Msp I	54, 96, 300
$ß_2$M	322	EcoR I	108, 214

* bp = base pairs.

Res. Enzyme = Restriction Enzyme.

(E.C. nos for enzymes are: EcoR I: 3.1.27.1; Hinf I: 3.1.30.1; Msp I: 3.1.30.2)

N.B. : Information on the sequences of the PCR primers, the sizes of the products they give, the location of the restriction enzyme sites in the products and the sizes of the fragments produced by successful restriction enzyme digestion can be obtained from the GCG gene data bank. The following are the GCG codes for each primer:

IL1α: Pr:HUMIL1AA.GB IL1β : Pr:HUMIL1BA.GB IL6: Pr:HUMIFNB2.GB

TNFα: Pr: HUMTNFAA.GB TNFβ : Pr:HUMLT.GB β_2M: Pr:HUMB2M.GB

2.6.2: Method Used for Restriction Digestion.

The entire procedure was performed in a laminar flow hood

1) Two PCR products for each sample (ones which gave strong fluorescent bands on electrophoresis) were selected to give a volume of 100 µl per sample and combined in a 2 ml microfuge tube.

2) An equal volume of 50% phenol (molecular biology grade - Sigma) and 50% chloroform (BDH Ltd.) was added (i.e 100 µl) to each sample and the whole lightly vortex mixed.

3) The samples were centrifuged at 10000g for 3 min at 4°C.

4) The supernatants were removed and placed in fresh 2 ml microfuge tubes, 2.5 volumes of 100% ethanol added to each, along with 1/10 volume of 3M sodium acetate (pH 5.2), and the tubes placed at -20 °C for at least 15 min.

5) The tubes were centrifuged at 10000g for 15 min at 4°C and the supernatants removed carefully to leave the pellets in the bottom of the tubes. These pellets were then washed twice in a 75% ethanol, 25% DEPC water mixture and centrifuged at 10000g for 10s at 4°C between each wash.

2.6.2 Cont.:

6) The pellets were air-dried for 5 min and resuspended in 24 µl of sterile water.

7) The following were added to each tube:

8 µl of the freshly extracted sample cDNA (prepared in points 1-6)

2 µl of restriction enzyme buffer (this is supplied with the enzyme and is specific for it)

1 µl of the appropriate restriction enzyme

11 µl of sterile distilled water (so the final volume is 20 µl)

The tubes were incubated at 37°C (in a water bath) for at least 1h. Following this the products of the digestion were visualised by electrophoresis on a 2% agarose (Seakem) slab gel at 100 volts for 1 h, followed by ethidium bromide staining and the result photographed.

2.7: Methods Used for Immuno-Staining.

2.7.1: Introduction.

Unlike the other techniques described, immuno-staining deals with detection of the protein of interest, not its mRNA. Investigation of mRNA alone does not prove that the protein is actually translated or secreted, so immuno-staining is important as a sensitive technique which directly detects the protein. The technique employs antibodies to the protein of interest to detect its presence in fixed or frozen tissue sections. Once the antibodies have reacted with the tissue, the unbound antibodies are washed off and the bound detected by using another suitably labelled secondary antibody which will bind to the primary antibody. The secondary antibody was fluorescent (e.g. FITC-conjugated). or Alkaline Phosphatese labelled (depending on the experiment) Fluorescent-conjugated antibody gives very good sensitivity, but alkaline phosphatase-conjugated antibody allows identification of the cells in which the protein has been detected and shows the morphology of the tissue. This technique does not give as accurate localisation of product as in-situ hybridisation since it will detect secreted protein in the matrix of the tissue as well as intra-cellular material. However, it is effective in showing that a particular tissue is producing and secreting the protein.

2.7.1 Cont.:

For immunofluorescent investigation original samples of tissue, from which portions had previously been removed for mRNA analysis were used. Frozen sections were prepared from them on a cryostat and mounted on standard glass slides. The slides were stored at -20°C until they could be stained. The staining protocol was based on methods employed by the University Department of Orthopaedics, Western Infirmary, Glasgow. Primary antibody dilutions were determined in a series of experiments, but secondary (fluorescent) antibody dilutions were those recommended by the manufacturers.

2.7.2: Method Employed for Immuno-Staining Frozen Sections with FITC-Conjugated Antibody.

1) The slides with the frozen sections were fixed in 0.5% formalin for approximately 3 min and washed in phosphate buffered saline for 5 min.

2) The slides were then reacted with the primary antibody (see Table 2.2 for details) at 4°C overnight and rinsed 3 times in PBS. The antibody dilutant used to make up the dilutions of primary antibody (and secondary antibody) was PBS antibody dilutant (87ml of 0.01 M PBS and 3ml of 30 % bovine serum albumin)

3) The secondary antibody was then applied (this was FITC-conjugated) for 30 min in the dark, followed by washing 3 times in PBS (5 min per wash) and mounted in diazobicyclo (2,2,2) octane mountant (D.A.B.C.O.). They were viewed immediately using a microscope with UV illumination.

The table overleaf details the antibodies used, and their dilutions:

Table 2.5: Antibodies Used for Immunofluorescent Staining of Frozen Sections and their Dilutions.

Primary Antibody	Antibody Dilution	Secondary Antibody	Antibody Dilution
IL1α (Sheep)	1:4000	FITC-Conjugated Anti-Sheep (Sigma)	1:128
IL6 (Goat)	1:4000	FITC-Conjugated Anti-Goat (Sigma)	1:64
CD8 (Mouse)	1:4000	FITC-Conjugated Anti-Mouse*	1:80
CD14 (Mouse)	1:4000	FITC-Conjugated Anti-Mouse	1:80
CD21 (Mouse)	1:4000	FITC-Conjugated Anti-Mouse	1:80

*CD marker antibodies supplied by Scottish Antibody Production Unit.

2.7.3: Protocol Used for Immuno-Histological Staining Using Alkaline Phosphatase Conjugated Secondary Antibodies Detected by the Fast Red™ Method on Paraffin-Embedded Sections.

1)Tissue was collected as soon as possible after surgery and placed in 0.5% formalin for 24h.

2) Following this it was placed in 70% ethanol for 24h, then 90% ethanol for 3h, then 100% ethanol for 24h to ensure that the tissue was fully dehydrated.

3) The tissue was placed in Xylene ("Histoclear") for a minimum of 4h or until it was microscopically clear.

4) The tissue was placed in a bath of molten paraffin for 24h, and the embedded in paraffin.

5) The embedded tissue was sectioned on a microtome, and the sections mounted on glass slides coated with 3-amino-propyl-tri-ethoxy silane (APES)

2.7.3 Cont.

6) The mounted sections were washed in tris-buffered saline (TBS), and subjected to digestion by a 0.5% trypsin solution (0.1g trypsin, activity 8980 U/mg, E.C. no. 3.4.21.4; 0.1g calcium chloride; dissolved in 100ml of TBS at 37°C) at 37°C for 15 min.

7) The slides were then washed in TBS and the primary antibody added at the appropriate dilution (see Table 2.6). One slide for each sample was used as a negative control and received only antibody dilutant. The antibody dilutant was TBS antibody dilutant (made as for PBS antibody dilutant on p105 but substituting 87ml of 0.85% TBS for the PBS). The slides were then left at 4°C for 24h and rinsed 3 times in TBS.

8) The appropriate secondary antibody was then applied to each slide and left to react for 1h in the dark. The slides were then washed 3 times in TBS.

9) Detection of the secondary antibody was carried out using a Sigma Fast Red ™ detection kit in accordance with the manufacturer's instructions, summarised as follows:

a) Tablets of 0.1ml tris buffer (supplied) were dissolved in 1ml of distilled water each (one per slide).

2.7.3 Cont.

b) To these were added 1 tablet each of Fast Red TR/Napthol AS-MX solution, and the tablets dissolved. The Fast Red / buffer solution was then applied to the slides immediately, and the slides monitored under a microscope at x10 magnification until a pink or red colour was observed. The staining reaction was then terminated by placing the slides in tap water.

10) The developed slides were then counter-stained with haematoxylin for 12s, mounted in gelatin and stored. Where required, they were photographed using Kodak EPT-160T colour slide film.

TABLE 2.6: Antibodies Used for Immuno-Histological Staining Using Alkaline Phosphatase - Conjugated Secondary Antibodies and Their Dilutions.

Primary Antibody	Antibody Dilution	Secondary Antibody	Antibody Dilution
IL1a (Sheep)	1:60	Alkaline Phosphatase - Conjugated Anti - Sheep	1:125
IL6 (Goat)	1:60	Alkaline Phosphatase - Conjugated Anti - Goat	1:64
CD68 (Clone KP-1) Macrophage Marker (Mouse)*	1:75	Alkaline Phosphatase - Conjugated Anti - Mouse	1:50

*Tissue being stained with this marker had to be visually examined for mast cells (distiguishable from macrophges by their cell morphology) as these also display the CD68 marker.

2.8: Method Employed for Haematoxylin and Eosin Staining.

1) The slides were stained with 1 drop of haematoxylin (obtained from the hospital Pathology Department) for 5 min and rinsed carefully with tap water.

2) The slides were then stained with 1 drop of eosin (obtained from the hospital Pathology Department) for 5 min and rinsed carefully in tap water.

3) The finished slides were then mounted in gelatin and kept safe.

2.9: Tissue Culture Methods Employed.

2.9.1: Maintenance of Adherent Cell Lines.

The adherent cell lines (MCF-7 and T-24) were routinely grown in 75 cm^2 polystyrene culture flasks (Bibby) in RPMI 1640 culture medium (GIBCO Ltd.) with the following supplementations: 10% foetal calf serum, 1% penicillin/ streptomycin, 1% glutamine

(all GIBCO Ltd.), until confluent. The medium used for MCF-7 cells was phenol red free.

When confluent, the cells were removed by trypsinisation. 5ml of trypsin (0.25% crude) was added to each flask, and the flasks incubated in an ordinary incubator at 37°C for approximately 10 min until the cells were observed floating free. The Trypsin was then removed by washing the cells in 10ml of culture medium, followed by centrifugation at 5000g for 10 min at room temperature. The resulting pellet of cells was drained and resuspended in 1ml of culture medium. The cells were counted in a haemocytometer using trypan blue dye to measure their viability (cells taking up the dye considered as dead) and reseeded at an appropriate concentration in fresh flasks with fresh medium. Incubation conditions were 37°C, 5%CO_2, 95% air.

2.9.2: Maintenance of Non-adherent Cell Lines.

Culture conditions were similar for the B9 cell line although it was non-adherent. The medium was as described in 2.8.1, except for the addition of ß-mercaptoethanol to a final concentration of $5x10^{-5}$ M. This cell line also required supplementation with crude IL 6 (hybridoma growth factor, Janssen) each time the cells were subcultured, at a final concentration of approximately 0.1%.

The B9 cells could not be grown above a concentration of $5x10^5$ cells per ml otherwise they were found to lose their sensitivity to IL 6. They were always subcultured before reaching this concentration, usually being diluted by a factor of 1/10. Cell transfer or subculture did not require the use of trypsin, as the cells float free in the medium.

2.10: Methods Employed in Growth Studies on Cells.

2.10.1: Introduction.

The effects of IL6 on growth of cells was studied in two separate experiments. These were similar in design, but differed from one another in the type of IL6 used (crude natural, or recombinant), and in the way that the cell proliferation was measured.

2.10.2: Method Employed for Cell Growth Studies Using Crude Hybridoma Growth Factor.

The first method was designed to study the effects of IL6 on the MCF-7 cell line, and used cells cultured in 24 well flat-bottomed microtitre plates.

1) The cells were distributed into the wells on the plate. In all, 8 wells were used, one row of 4 as an untreated control, and one row of 4 as the treated populations. Approximately 3000 cells were added to each well in 1 ml of culture medium. The wells in the control set also received 100 µl of PBS, while those in the treated set received 100 µl of hybridoma growth factor of initial activity 400 U/ml, so that the final concentration in each well was approximately 36 U/ml.

2.10.2 Cont.:

1 Cont.) The medium used was RPMI 1640 with the usual supplements (see 2.9.1 p 113) and no phenol red as this is weakly oestrogenic and might interfere with the experiment. Following this the plate was placed in an incubator at standard conditions (2.9.1. p113).

2) The cells in one well for the treated population, and one well for the control population were harvested by trypsinisation each day, giving an experiment lasting 4 days. The cell concentration in each well was determined using a haemocytometer.

3) At the end of 4 days, graphs of cell number versus time were plotted for the control and treated populations and these graphs compared to determine whether IL6 caused any effect on growth

2.10.3: Method Employed for Cell Growth Studies Using Human Recombinant IL6.

M.T.T. dye absorbance was employed in the second study to determine the cell population. It used only 2 incubation periods, 48 and 72 h,and recombinant IL6 at different concentrations.

2.10.3 Cont.:

1) Two 96 well flat-bottomed microtitre plates were prepared as shown in table 2.6 overleaf. Serial IL6 dilutions were prepared down the plate and cells of each type were added to the wells (see table 2.7 for details). All cell types were added to the wells to give the same initial concentration, $5x10^4$ cells/ml. This is a higher initial concentration than was used in 2.10.2 and was used to enable the detection method to give measurable results.

The detection method is based on dye absorbance, the greater the number of cells, the greater the final dye absorbance. However, the initial cell concentration has to be large enough to ensure that the final cell concentrations will be sufficient to give easily measurable absorbance values.

2) At the conclusion of the incubation periods (either 48 or 72 h) cell concentrations were determined using the M.T.T. dye method. 10µl of M.T.T. dye (5mg/ml, Sigma) was added to each well on the plate. The plate was then incubated for 4 h at standard conditions (2.9.1 p113). 50µl of Triton-x detergent (10%, made up in 0.05M HCl) was added to each well to solubilise the dye precipitate. This process required the plate to be returned to the incubator for a further 12 h.

2.10.3 Cont.:

3) The absorbances of each well were then measured in a plate reader at 540nm.

4) Graphs were drawn of concentration of IL6 versus absorbance (growth) for each cell line for each incubation period and the effects of IL6 observed from the graphs.

Table 2.7: Cell Growth Study Experiment Using Recombinant IL6 over 48-72 h.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Reagent Blank	Reagent Blank	T-24 No IL6	T-24 No IL6	T-24 No IL6	T-24 IL6 100pg/ml	T-24 IL6 100pg/ml	T-24 IL6 100pg/ml	MCF-7 No IL6	MCF-7 IL6 100pg/ml	B-9 No IL6	B-9 IL6 100pg/ml
B	" "	" "	" "	" "	" "	T-24 IL6 50pg/ml	T-24 IL6 50pg/ml	T-24 IL6 50pg/ml	" "	MCF-7 IL6 50pg/ml	" "	B-9 IL6 50pg/ml
C	" "	" "	" "	" "	" "	T-24 IL6 25pg/ml	T-24 IL6 25pg/ml	T-24 IL6 25pg/ml	" "	MCF-7 IL6 25pg/ml	" "	B-9 IL6 25pg/ml
D	" "	" "	" "	" "	" "	T-24 IL6 12.5pg/ml	T-24 IL6 12.5pg/ml	T-24 IL6 12.5pg/ml	" "	MCF-7 IL6 12.5pg/ml	" "	B-9 IL6 12.5pg/ml
E	" "	" "	" "	" "	" "	T-24 IL6 6.3pg/ml	T-24 IL6 6.3pg/ml	T-24 IL6 6.3pg/ml	" "	MCF-7 IL6 6.3pg/ml	" "	B-9 IL6 6.3pg/ml
F	" "	" "	" "	" "	" "	T-24 IL6 3.1pg/ml	T-24 IL6 3.1pg/ml	T-24 IL6 3.1pg/ml	" "	MCF-7 IL6 3.1pg/ml	" "	B-9 IL6 3.1pg/ml
G	" "	" "	" "	" "	" "	T-24 IL6 1.6pg/ml	T-24 IL6 1.6pg/ml	T-24 IL6 1.6pg/ml	" "	MCF-7 IL6 1.6pg/ml	" "	B-9 IL6 1.6pg/ml
H	" "	" "	" "	" "	" "	T-24 IL6 0.8pg/ml	T-24 IL6 0.8pg/ml	T-24 IL6 0.8pg/ml	" "	MCF-7 IL6 0.8pg/ml	" "	B-9 IL6 0.8pg/ml

Chapter 3: Results

3.1: Validation of the Method and Primers used in the PCR Experiments.

As has been explained on p89, the PCR technique can produce artefacts and care has to be taken to avoid contamination with extraneous DNA which will lead to false positive results. However, care also has to be taken to avoid the generation of false negative results. These can occur if the primers for the PCR reaction are incorrect, or if the conditions of the PCR reaction are not at their optimum. To ensure this, a set of experiments was carried out to ensure that the primers were performing correctly and that the method was using optimum conditions. It should be pointed out that all the PCR experiments were repeated at least twice to ensure reproducibility of results. All the results reported in this section and those in sections 3.2 and 3.3 were reproducible.

Stimulated macrophages produce IL1α, IL1ß, IL6, TNFα, and TNFß. Almost all nucleated cells produce ß2 microglobulin (ß2M) including macrophages so it should be detected as well. Macrophages were extracted from blood and stimulated with Lipopolysaccharide (LPS). After culture in RPMI medium for 24h at standard conditions (p113), mRNA was harvested as described (p77), reverse transcribed (p83), and PCR performed (p90). Results were obtained by agarose gel electrophoresis followed by ethidium bromide staining (p97).

Figure 3.1 shows the results obtained. Lanes 1 and 8 show the molecular size markers. In lane 2 there is a broad faint band of molecular size approximately 320. This corresponds to the expected mass of the IL1α product (see table 2.4 p101). In the same lane is another faint band which is above the last of the molecular size markers and is likely to be primer dimer (due to it being so small). Lane 2 used the primers for IL1α and the band observed was near to the expected size of the IL1α product (324 bp) so a positive result can be concluded for IL1α expression. Lane 3 (using material prepared with primers for IL1ß) shows a similar result to lane 2, 2 bands, one of molecular size approximately 350 bp and one of very small size. The small product is probably primer dimer and the other product is around the expected size for IL1ß (376 bp). This would indicate a positive result for IL1ß. Lane 4 (using material prepared with primers for IL6) shows 1 strong band of around 310 bp, which is close to the expected size of the IL6 product (313 bp), indicating a positive result for IL6. Lane 5 (using material prepared with primers for TNFα) shows a band of molecular size approximately 420bp, which is close to the expected size of the TNFα product, indicating a positive result for TNFα. Lane 6 shows 2 bands, the smaller of which is probably primer dimer, and the other is about 320 bp. This is close to the expected size of the TNFß product (344 bp), indicating a positive result for TNFß.

Lane 7 shows one very strong band of molecular size approximately 320 bp, which is near the expected mass of the ß2M product (322 bp), indicating a positive result for ß2M. However, there are other bands visible in this lane, all of smaller sizes than the β2M product, giving a laddering effect. They are probably products of partial PCR caused by the annealing temperature for the primer being too high.

A positive result for every primer set was obtained indicating that the primers were suitable and the method satisfactory. However, it was necessary to check that the products observed were appropriate to each primer set and not false positive results. Restriction digestion of each macrophage primer product was carried out as described on p100. Following this, electrophoresis (as described on p97) was performed on both restriction digested and undigested primer products. The results obtained are shown in figure 3.2. Table 2.4 (p101) gives the mol sizes of each intact product (obtained from GCG gene bank) and the number of fragments and fragment mass for each digested product.

Lane 1 shows the molecular size markers, as 8 distinct bands, of sizes (bottom to top) 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2 (using material prepared with the IL1α primers, undigested) shows a band near 324bp, the expected size of the intact IL1α product.

Lane 3 (using the same material as that in lane 1, but digested with EcoR I) shows a bright band of around 212 bp, and a slightly fainter band at around 112 bp, the expected sizes of the fragments for digested IL1α product. Lane 4 (using material prepared with the IL1ß primers, undigested) shows 1 bright band around 376 bp, the expected size of the intact IL1ß product. Lane 5 (using the same material as lane 4, but digested with Hinf I) shows 1 band around 234 bp and another around 142 bp, the expected sizes of the fragments for digested IL1ß product. There is also a band at the top of the lane which is probably primer dimer. Lane 6 (using material prepared with the TNFα primers, undigested) shows 1 band of around 450 bp, the expected size for intact TNFα product. Lane 7 (using the same material as lane 6, but digested with MSP I) shows a bright band around 300 bp, and another fainter band of around 94 bp, corresponding to the expected masses of 2 of the fragments of digested TNFα product. The expected third fragment of 54 bp is not present. Lane 8 (using material prepared with the TNFß primers), shows a faint band of 400 bp, a bright band of 344 bp, and a faint band of 95 bp. The band of 344 bp is approximately the size of the expected product, the other 2 bands are artefacts. Lane 9 (using the same material as 8, but digested with MSP I) shows a bright band of 236 bp, another band of 95 bp, and a faint band at the top of the lane. These correspond to the expected fragment sizes of the digested TNFß product.

Lane 10 (using material prepared with the IL6 primers shows 1 bright band of around 313 bp, the expected size of the IL6 product. Lane 11 (using the same material as 10, but digested with Hinf I) shows 2 bright bands of around 197 and 116 bp, the expected fragment sizes of the digested IL6 product. Lane 12 (using material prepared with the β_2M primers) shows 1 band around 322 bp, the expected size of the β_2M product. Lane 13 (using the same material as 12, but digested with EcoR I) shows 1 band of around 214 bp, the expected size of one of the fragments of the digested β_2M product. There should also be a fragment of 108 bp, but this cannot be observed.

The results confirmed that the primers and method used could successfully detect cDNA for all of the cytokines of interest in experiments in which macrophages were the source of mRNA.

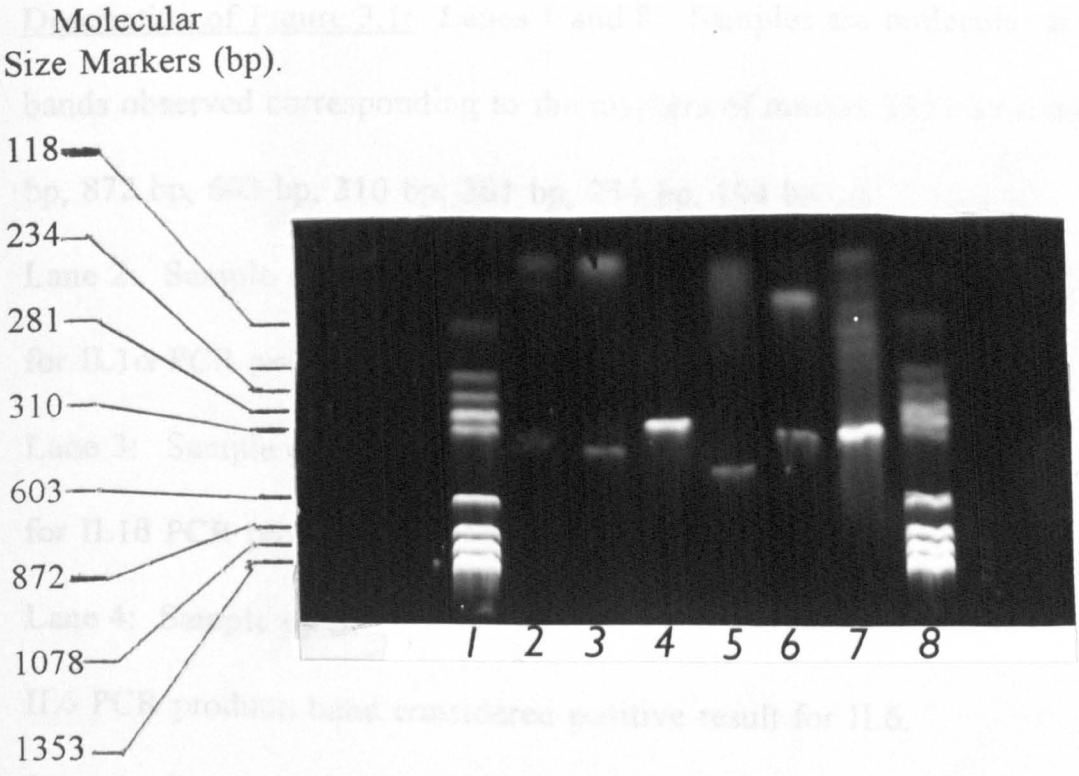


Figure 3.1: Photograph of PCR result using intact PCR products prepared (p90) from cDNA reverse transcribed from mRNA extracted from peripheral blood macrophages cultured for 24h and stimulated with lipopolysaccaride (culture conditions p113). Photograph shows PCR products on an agarose slab gel, on which electrophoresis was performed as on p97, and staining carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s. A detailed description is given overleaf.

Description of Figure 3.1: Lanes 1 and 8: Samples are molecular size markers. 8 bands observed corresponding to the markers of masses 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2: Sample probed for IL1α. 1 band observed approx. 324 bp, expected size for IL1α PCR product, band considered positive result for IL1α.

Lane 3: Sample probed for IL1ß. 1 band observed approx. 376 bp, expected size for IL1ß PCR product, band considered positive result for IL1ß.

Lane 4: Sample probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Lane 5: Sample probed for TNFα. 1 band observed approx. 450 bp, expected size for TNFα PCR product, band considered positive result for TNFα.

Lane 6: Sample probed for TNFß. 1 band observed approx. 344 bp, expected size for TNFß PCR product, band considered positive result for TNFß.

Lane 7: Sample probed for ß2M. 1 band observed approx. 322 bp, expected mass for ß2M PCR product, band considered positive result for ß2M.

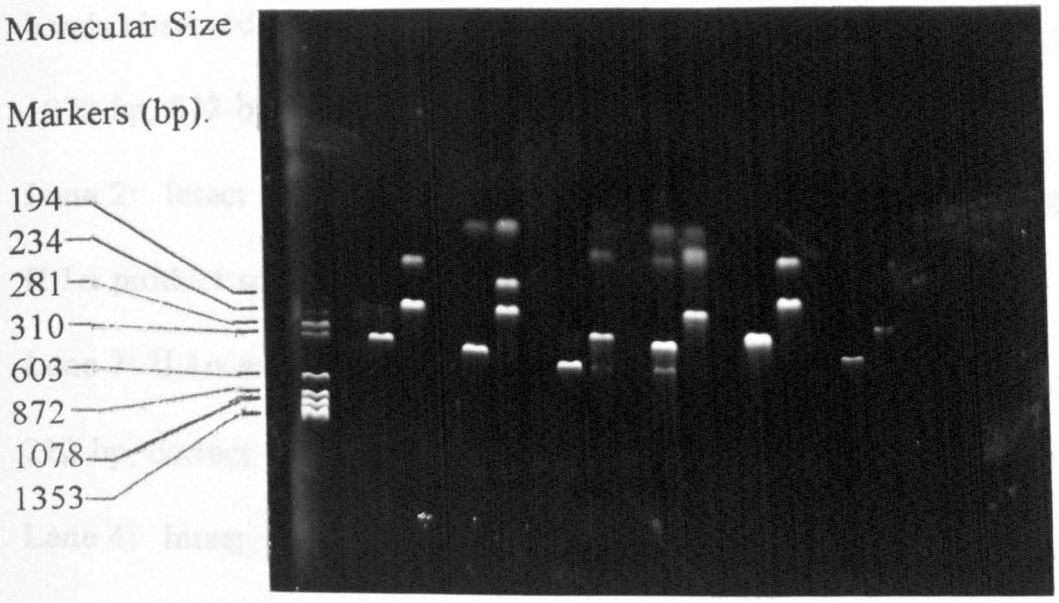


Figure 3.2: Photograph of PCR result using intact PCR products and restriction enzyme digested PCR products (see p100 for restriction digestion method) prepared from cDNA reverse transcribed from mRNA extracted from peripheral blood macrophages cultured for 24 h and stimulated with lipopolysaccaride (culture conditions p113). Photograph shows PCR products on an agarose slab gel, on which electrophoresis was performed as on p97, and staining carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s. A detailed description is given overleaf.

Description of Figure 3.2: Lanes 1: Samples are molecular size markers. 8 bands observed corresponding to the markers.of masses 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2: Intact sample probed for IL1α. 1 band of correct sizes (324 bp) for IL1α product so result positive for IL1α.

Lane 3: IL1α sample digested with EcoR I giving 2 fragments of approx 112 and 212 bp, correct sizes of fragments, therefore product confirmed as IL1α product.

Lane 4: Intact sample probed for IL1ß. 1 band of correct size (376 bp) for IL1ß product so result positive for IL1ß.

Lane 5: IL1ß sample digested with Hinf I giving 2 fragments of approx 142 and 234 bp, correct sizes of fragments, therefore product confirmed as IL1ß product.

Lane 6: Intact sample probed for TNFα. 1 band of correct size (450 bp) for TNFα product so result positive for TNFα.

Lane 7: TNFα sample digested with Msp I giving 3 fragments of approx 54 bp, 96 bp, and 300 bp, correct sizes of fragments, therefore product confirmed as TNFα product.

Lane 8: Intact sample probed for TNFß. 1 band of correct size (344 bp) for TNFß product so result positive for TNFß.

Lane 9: TNFß sample digested with Msp I giving 3 fragments of approx 13 bp, 95 bp, and 236 bp, correct sizes of fragments, therefore product confirmed as TNFß product.

Description of Figure 3.2 Cont.:

Lane 10: Intact sample probed for IL6. 1 band of correct size (313 bp) for IL6 product so result positive for IL6.

Lane 11: IL6 sample digested with Hinf I giving 2 fragments of approx 116 bp, and 197 bp, correct size of fragments, therefore product confirmed as IL6 product.

Lane 12: Intact sample probed for ß2M. 1 band of correct size (322 bp) for ß2M product so result positive for ß2M.

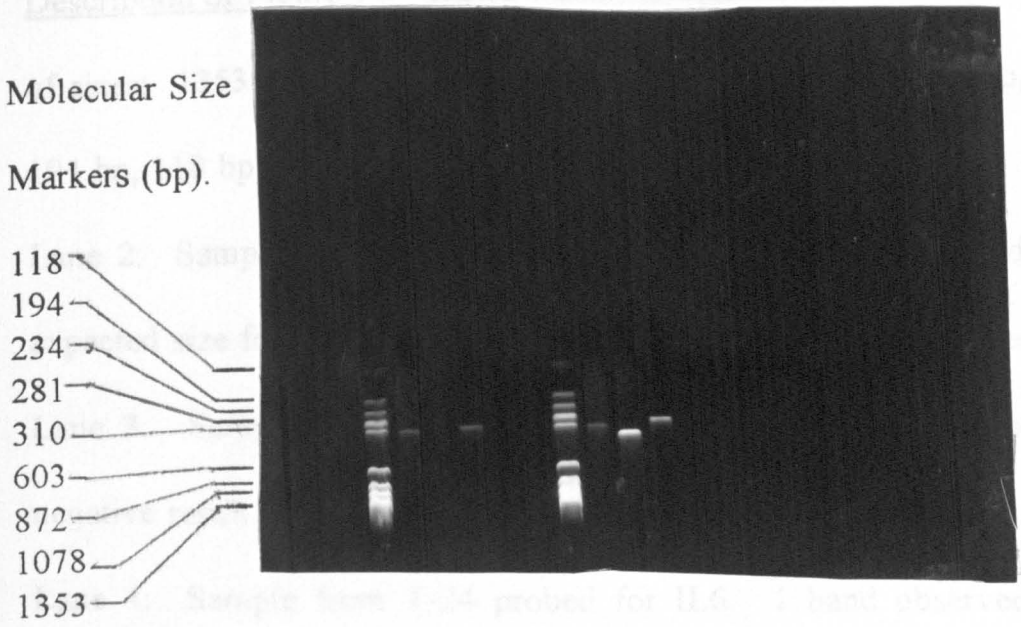
Lane 13: ß2M sample digested with EcoR I giving 2 fragments of approx 108 bp, and 214 bp, correct sizes of fragments, therefore product confirmed as ß2M product.

3.2: Investigation of the Patterns of Cytokine Production in Tumour Cell Lines.

A study by Tabibzadeh et al (1989) indicated that the bladder cell line T-24, and the breast cell line MCF-7 produced IL6. A series of experiments were carried out to confirm that the primers and PCR method could detect this. Production of IL1α, IL1ß, TNFα, TNFß, and ß2M (as a positive control) were also to be examined. For these experiments, cells of the appropriate lines were cultured for approximately 72 h, until confluent. mRNA was extracted (p77), reverse transcribed, (p83), and PCR performed (p90). The results were analysed by agarose gel electrophoresis (p97) and are shown in figure 3.3. Lanes 1 and 7 show 10 bands corresponding to the molecular size markers of sizes (bottom to top) 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp. Lane 2 (material from T-24 cell line prepared with the IL1α primers) shows a band of around 324 bp, the expected size of the IL1α product. Lane 3 (material from T-24 cell line prepared with the IL1ß primers) shows no bands, indicating a negative result. Lane 4 (material from T-24 cell line prepared with the IL6 primers) shows a band around 313 bp, the expected size of the IL6 product. Lane 5 (material from T-24 cell line prepared with the TNFα primers) shows no bands indicating a negative result. Lane 6 (material from T-24 cell line prepared with the TNFß primers) shows no bands, indicating a negative result.

Lane 8 (material from MCF-7 cell line prepared with the IL1α primers) shows a band of around 324 bp, the expected size of the IL1α product. Lane 9 (material from MCF-7 cell line prepared with the IL1ß primers) shows a band of around 376 bp, the expected size of the IL1ß product. Lane 10 (material from MCF-7 cell line prepared with the IL6 primers) shows a band around 313 bp, the expected size of the IL6 product (sizing is approximate and in a strong band like the one here, there is a resolution limit of about 20bp and this is why the product looks larger than it is). Lane 11 (material from MCF-7 cell line prepared with the TNFα primers) shows no bands, indicating a negative result. Lane 12 (material from MCF-7 cell line prepared with the TNFß primers) shows no bands, indicating a negative result.

The results of the experiments showed that the T-24 cell line constitutively produced IL1α and IL6, and the MCF-7 cell line constitutively produced IL1α, IL1ß, and IL6.



Lanes

Figure 3.3: Photograph of the PCR result obtained using PCR products prepared (p90) from cDNA reverse transcribed from mRNA extracted from the cell line T-24 (bladder) and the cell line MCF-7 (breast), cultured to confluence as described on p112. Photograph shows PCR products on an agarose slab gel on which electrophoresis was performed as on p97, staining was carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s. A detailed description is given overleaf.

Description of Figure 3.3: Lanes 1 and 7 molecular size markers. 9 bands observed of sizes: 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp.

Lane 2: Sample from T-24 probed for IL1α. 1 band observed approx. 324 bp, expected size for IL1α PCR product, band considered positive result for IL1α.

Lane 3: Sample from T-24 probed for IL1ß. No bands observed, considered negative result for IL1ß

Lane 4: Sample from T-24 probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Lane 5: Sample from T-24 probed for TNFα. No band observed, considered negative result for TNFα.

Lane 6: Sample from T-24 probed for TNFß. No band observed, considered negative result for TNFß.

Lane 8: Sample from MCF-7 probed for IL1α. 1 band observed approx. 324 bp, expected size for IL1α PCR product, band considered positive result for IL1α.

Lane 9: Sample from MCF-7 probed for IL1ß. 1 band observed approx. 376 bp, expected size for IL1ß PCR product, band considered positive result for IL1ß.

Lane 10: Sample from MCF-7 probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Description of Figure 3.3 Cont.:

Lane 11: Sample from MCF-7 probed for TNFα. No band observed, considered negative result for TNFα.

Lane 12: Sample from MCF-7 probed for TNFß. No band observed, considered negative result for TNFß.

3.3: Investigation of Cytokine Production in Tumours.

Tumour samples were collected as soon as possible after surgery, snap frozen in liquid nitrogen and the mRNA extracted as detailed, reverse transcribed (p77-83 respectively), PCR was performed to analyse the resulting cDNA (p90), and the result analysed by agarose gel electrophoresis (p97). In this way a cytokine production profile could be built up for each tumour sample. The results of the profiling were collated for each tumour type and represented graphically.

Figure 3.4 shows the collated results for the breast tumour samples. No samples had detectable cDNA for IL1α or IL1ß, 5 out of 9 samples had detectable cDNA for IL6, 2 out of 9 samples had detectable cDNA for TNFα, 1 out of 9 samples had detectable cDNA for TNFß, and all 9 samples had detectable cDNA for ß2M.

Figure 3.5 shows a sample result for one of the breast tumours. Lanes 1 and 9 show 9 bands corresponding to the molecular size markers of sizes (top to bottom) 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp. Lanes 2, 3, and 6 show no bands indicating a negative result for IL1α, IL1ß, and TNFß. Lane 4 (using material prepared with the IL6 primers) shows a bright band at 313 bp, the expected size for the IL6 product.

Lane 5 (using material prepared with the TNFα primers) shows a band around 450 bp, the expected size of the TNFα product. Lane 7 (using material prepared with the ß2M primers) shows 1 bright band around 322 bp, the expected size of the ß2M product. Lane 8 (using material prepared with no cDNA, only ß2M primers as a negative control) shows no bands, as expected.

Figure 3.6 shows the collated results for the bladder tumour samples. 4 out of 13 samples gave detectable cDNA for IL1α, none gave cDNA for IL1ß, 7 out of 13 gave detectable cDNA for IL6, 1 out of 13 gave detectable cDNA for TNFα, 1 out of 13 gave detectable cDNA for TNFß, and all 13 gave detectable cDNA for ß2M.

Figure 3.7 shows a sample result for one of the bladder tumours. Lanes 1 and 8 show the molecular size markers, observed as 9 bands of sizes (bottom to top) 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp. Lane 2 (using material prepared with the ß2M primers) shows a band of approx. 322 bp, the expected size of the ß2M product. Lanes 3, 4, 6, and 7 show no bands, indicating a negative result for TNFα, TNFß, IL1α and IL1ß respectively. Lane 5 (using material prepared with the IL6 primers) shows 1 band of approx. 313 bp, the expected size for the IL6 product.

Figure 3.8 shows the collated results for the lung tumour samples. All the tumours were malignant but were not all of the same type (see 3.6). No tumours gave detectable IL1α cDNA, no tumours gave detectable IL1β cDNA, and 6 tumours gave detectable TNFβ cDNA.

Figure 3.9 shows the collated results for the non-malignant lung samples. All of these came from the same patients as the tumours, but from the regions as far from the area of malignancy as possible. No samples gave detectable IL1α or IL1β cDNA, 3 out of 4 samples gave detectable IL6 cDNA, no samples gave detectable TNFα or TNFβ cDNA, and 4 samples gave detectable β_2M cDNA.

Figure 3.10 shows a sample result for the lung tumours with the result for the corresponding sampe of non-malignant lung. Lanes 1, 5, and 11 are the molecular size markers seen as 8 bands of sizes 1353 _ 194 bp. Lane 4 (using tumour material prepared with the IL6 primers) shows 1 band around 313 bp, the expected size of the IL6 product.

Lane 8 (using non-malignant lung material prepared with the IL6 primers) shows 1 band around 313 bp, the expected size of the IL6 product. Lane 9 (using non-malignant lung material prepared with the TNFα primers) shows 1 band of about 300bp. This is too small to be TNFα as its expected size is 450 bp. The band is some sort of contaminant and illustrates the danger of misinterpreting false positive results in PCR.

Lanes 2 and 3 showed no bands, indicating that no cDNA was detected for IL1α and IL1ß in the lung tumour. No bands were also observed in lanes 6, 7, and 10, indicating that no cDNA was detected for IL1α, IL1ß, and TNFβ in the non-malignant lung.

Only 2 of the 4 ovarian tumours sampled had intact mRNA. One gave a positive result for IL1α, IL1ß, and ß2M, and the other a positive result for TNFα, and ß2M only. 3 normal ovaries were also profiled. All 3 were positive for ß2M, and 2 out of the 3 were positive for IL1α. The results were too few to put on a graph. Figure 3.11 shows the result obtained for one of the tumours, and one of the normal ovaries (not from the same patient). Lanes 1 and 14 show the molecular size markers seen as 8 bands of sizes (bottom to top) 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2 (using ovarian tumour material prepared with the IL1α primers) shows 1 faint band around 324 bp, the expected size of the IL1α product. Lane 3 (using ovarian tumour material prepared with the IL1ß primers) shows 1 strong band around 376 bp, the expected size of the IL1ß product. Lane 7 (using ovarian tumour material prepared with the ß2M primers) shows 1 faint band around 322 bp, the expected size of the ß2M product. Lanes 4, 5, and 6, show no bands indicating negative results for IL6, TNFα, and TNFß in the ovarian tumour.

In lanes 8-13, the results from a normal ovary (not from the same patient), lanes 8-12 show no bands indicating negative results for IL1α, IL1ß, IL6, TNFα, and TNFß, but lane 13 (using normal ovarian material prepared with the ß2M primers) shows 1 faint band around 322 bp, the expected size of the ß2M product.

Figure 3.4: Breast Tumour Results from PCR Experiments

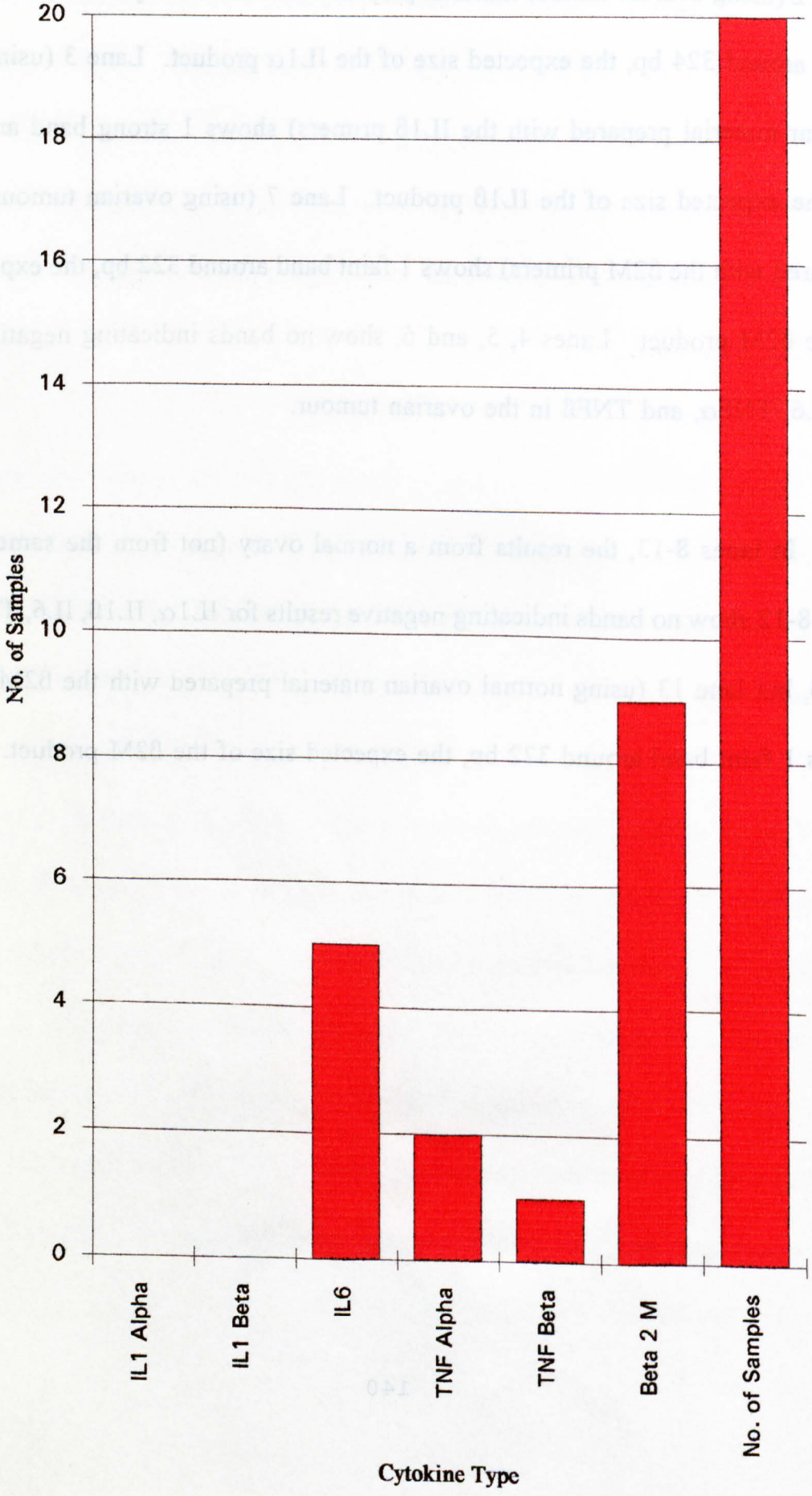


Figure 3.4: Collated results of cytokine profiles for the breast tumour samples prepared by PCR analysis of cDNA prepared from extracted tumour mRNA (see p77-90 for methods). 20 samples were collected and profiled out of which 9 gave a positive result for ß2M. All samples which have undergone PCR successfully give a positive result for ß2M. Therefore only the 9 samples positive for ß2M gave valid results. The graph shows the number of tumour samples giving a positive result for each cytokine probed for.

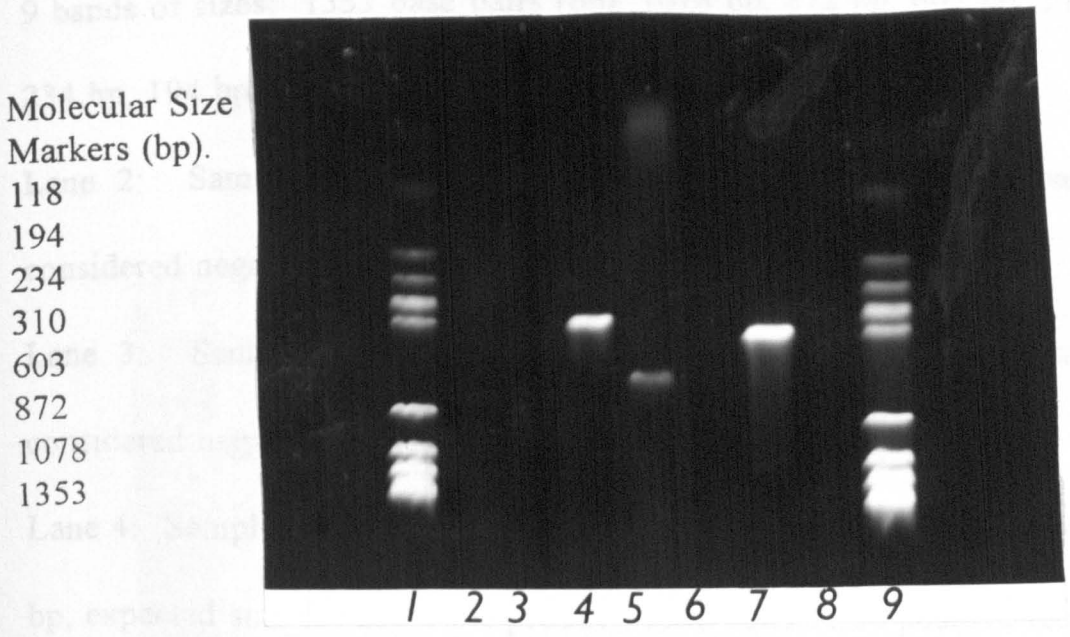


Figure 3.5: Photograph of the PCR result obtained from a ductal cell breast carcinoma. Tissue was collected and snap frozen immediately after surgery, mRNA extracted (p77), reverse transcribed (p83), and PCR performed (p90). Photograph shows PCR products on an agarose slab gel on which electrophoresis was performed (p97), staining following electrophoresis was carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s.

A detailed description is given overleaf.

Description of Figure 3.5: Lanes 1 and 9 are molecular size markers, observed as 9 bands of sizes: 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp.

Lane 2: Sample from breast tumour probed for IL1α. No bands observed, considered negative result for IL1α.

Lane 3: Sample from breast tumour probed for IL1ß. No bands observed, considered negative result for IL1ß.

Lane 4: Sample from breast tumour probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Lane 5: Sample from breast tumour probed for TNFα. 1 band observed approx. 450 bp, expected size for TNFα PCR product, band considered positive result for TNFα.

Lane 6: Sample from breast tumour probed for TNFß. No bands observed, considered negative result for TNFß.

Lane 7: Sample from Breast tumour probed for ß2M. 1 band observed approx. 322 bp, expected size for ß2M PCR product, band considered positive result for ß2M.

Lane 8: No sample, only PCR reaction mix and primers, as negative control. Lane blank, therefore no contamination by extraneous DNA.

Figure 3.6: Bladder Tumour Results From PCR Experiments

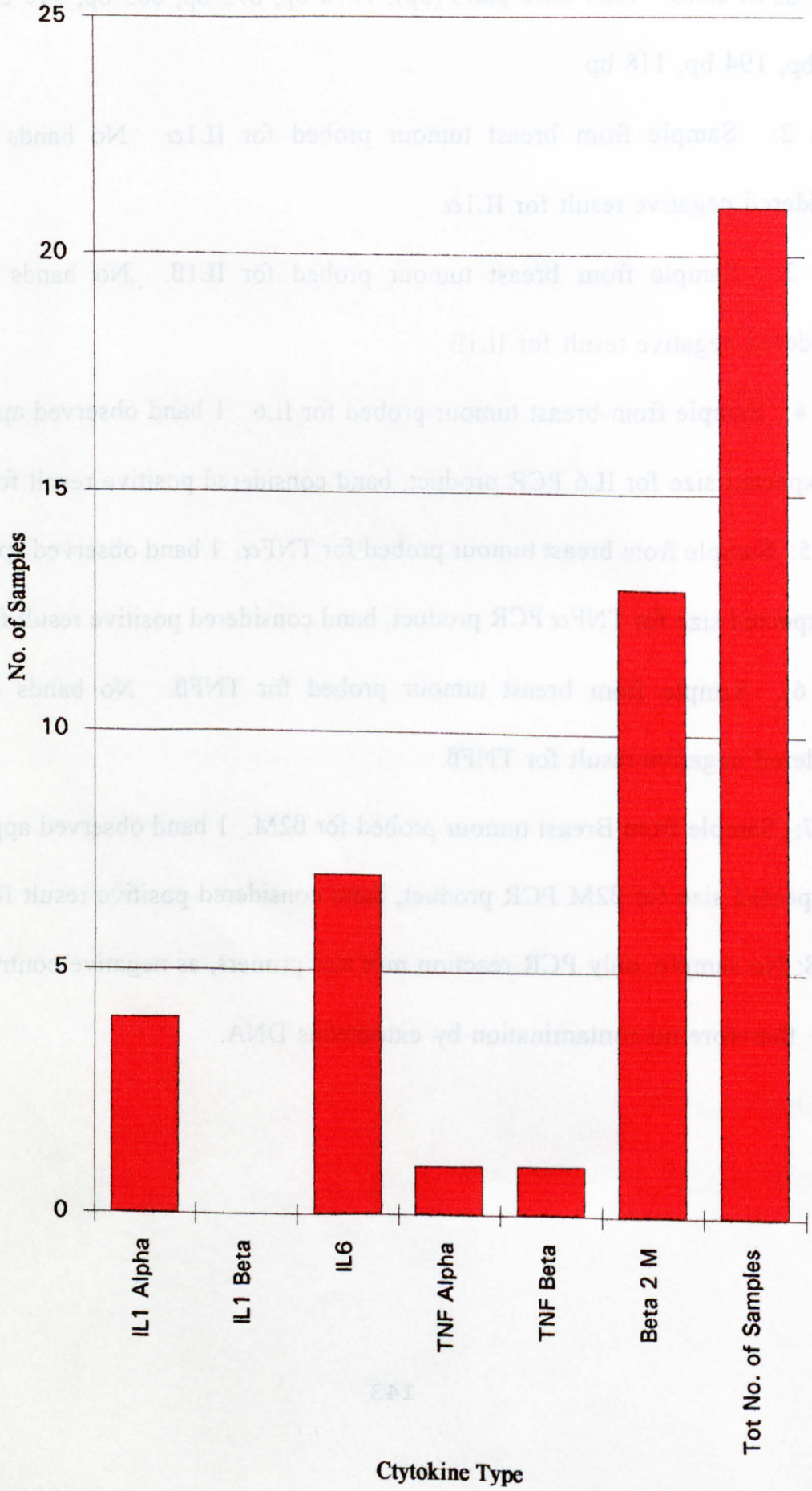


Figure 3.6: Collated results of cytokine profiles for the bladder tumour samples prepared by PCR analysis of cDNA prepared from extracted tumour mRNA (see p77-90 for methods). 21 samples were collected and profiled out of which 13 gave a positive result for ß2M. All samples which have undergone PCR successfully give a positive result for ß2M. Therefore only the 13 samples positive for ß2M gave valid results. The graph shows the number of tumour samples giving a positive result for each cytokine probed for.

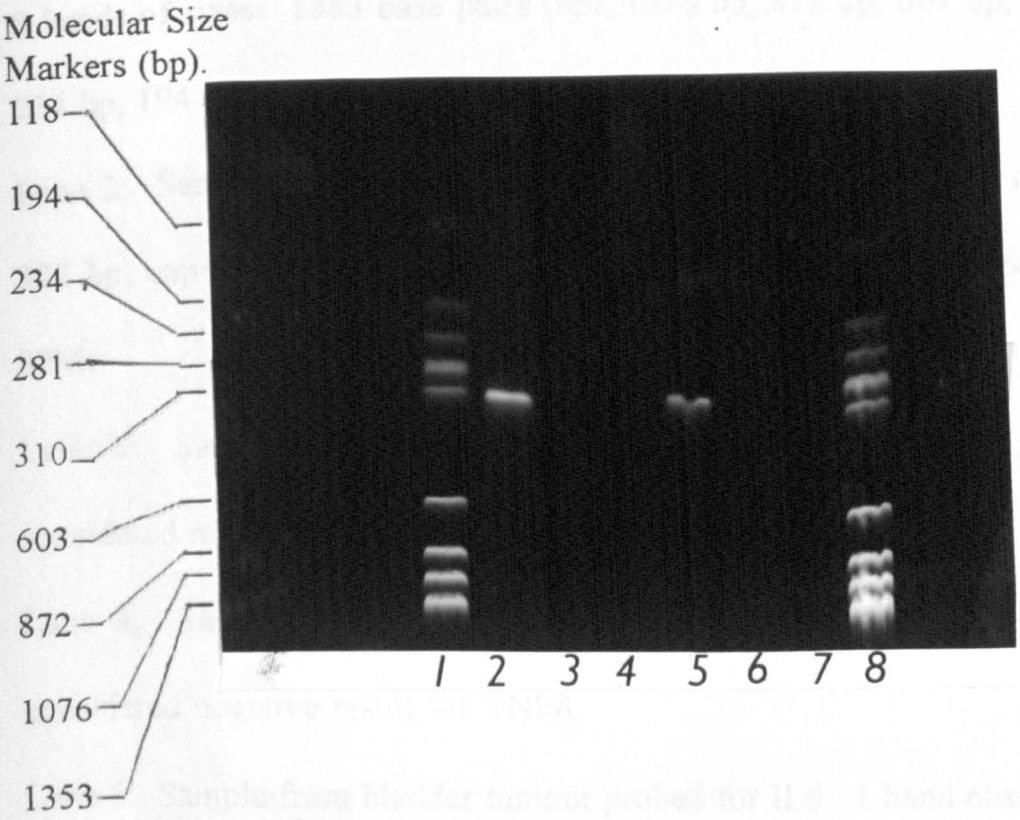


Figure 3.7: Photograph of the PCR result obtained from a transitional cell bladder carcinoma. Tissue was collected and snap frozen immediately after surgery, mRNA extracted (p77), reverse transcribed (p83), and PCR performed (p90). Photograph shows PCR products on an agarose slab gel on which electrophoresis was performed (p97), staining following electrophoresis was carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s.

Description of Figure 3.7: Lanes 1 and 8 are molecular size markers, observed as 9 bands of sizes: 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp, 118 bp.

Lane 2: Sample from bladder tumour probed for ß2M. 1 band observed approx. 322 bp, expected size for ß2M PCR product, band considered positive result for ß2M.

Lane 3: Sample from bladder tumour probed for TNFα. No bands observed, considered negative result for TNFα.

Lane 4: Sample from bladder tumour probed for TNFß. No bands observed, considered negative result for TNFß.

Lane 5: Sample from bladder tumour probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Lane 6: Sample from bladder tumour probed for IL1α. No bands observed, considered negative result for IL1α.

Lane 7: Sample from bladder tumour probed for IL1ß. No bands observed, considered negative result for IL1ß.

Figure 3.8: Lung Tumour Results from PCR Experiments.

Figure 3.8: Collated results of cytokine profiles for the lung tumour samples prepared by PCR analysis of cDNA prepared from extracted tumour mRNA (see p77-90 for methods). 11 samples were collected and profiled out of which 6 gave a positive result for ß2M. All samples which have undergone PCR successfully give a positive result for ß2M. Therefore only the 6 samples positive for ß2M gave valid results. The graph shows the number of tumour samples giving a positive result for each cytokine probed for.

Figure 3.9: Non-malignant Lung Results from PCR Experiments

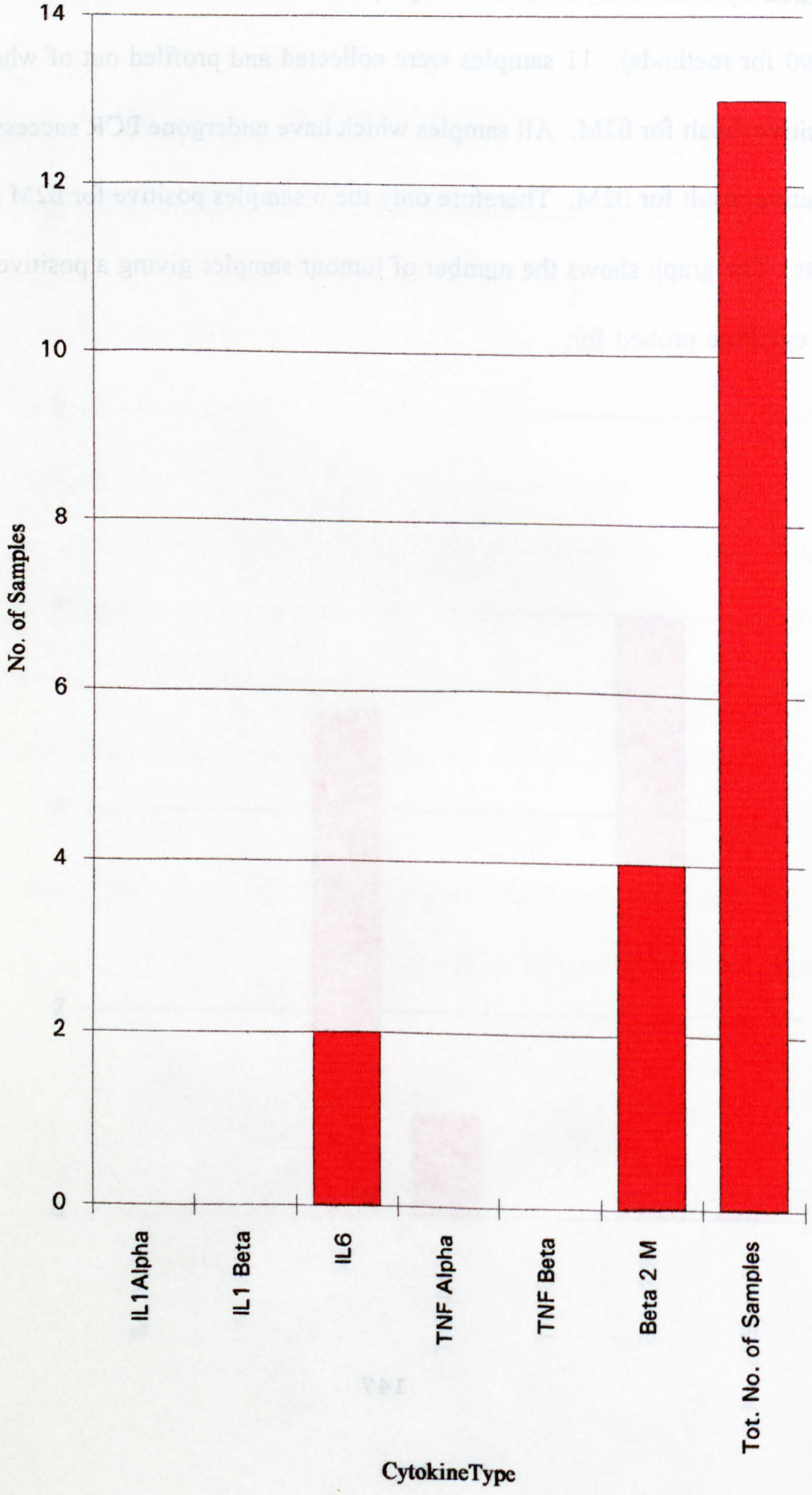


Figure 3.9: Collated results of cytokine profiles for the non-malignant lung samples prepared by PCR analysis of cDNA prepared from extracted tumour mRNA (see p77-90 for methods). 13 samples were collected and profiled out of which 4 gave a positive result for ß2M. All samples which have undergone PCR successfully give a positive result for ß2M. Therefore only the 4 samples positive for ß2M gave valid results. The graph shows the number of tumour samples giving a positive result for each cytokine probed for.

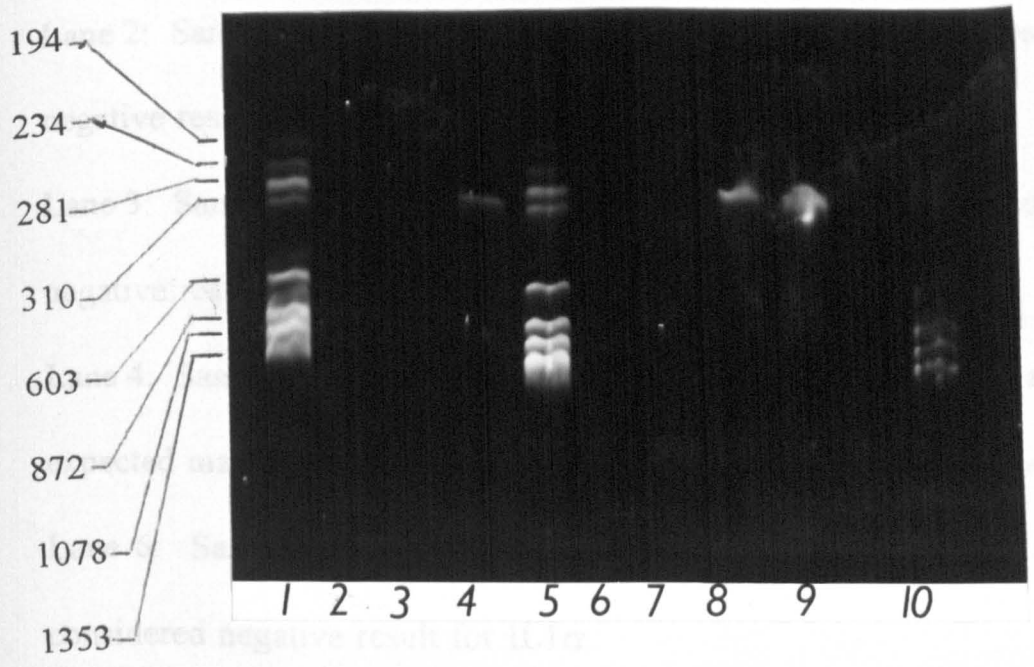


Figure 3.10: Photograph of the PCR result obtained from a lung tumour and from a non-malignant lung (not from the same patient). Tissue was collected and snap frozen immediately after surgery, mRNA extracted (p77), reverse transcribed (p83), and PCR performed (p90). Photograph shows PCR products on an agarose slab gel on which electrophoresis was performed (p97), staining following electrophoresis was carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s. A detailed description is given overleaf.

Description of Figure 3.10: Lanes 1, 5, and 10 are molecular size markers, observed as 8 bands of sizes: 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2: Sample from lung tumour probed for IL1α. No bands observed, considered negative result for IL1α.

Lane 3: Sample from lung tumour probed for IL1ß. No bands observed, considered negative result for IL1ß.

Lane 4: Sample from lung tumour probed for IL6. 1 band observed approx. 313 bp, expected mass for IL6 PCR product, band considered positive result for IL6.

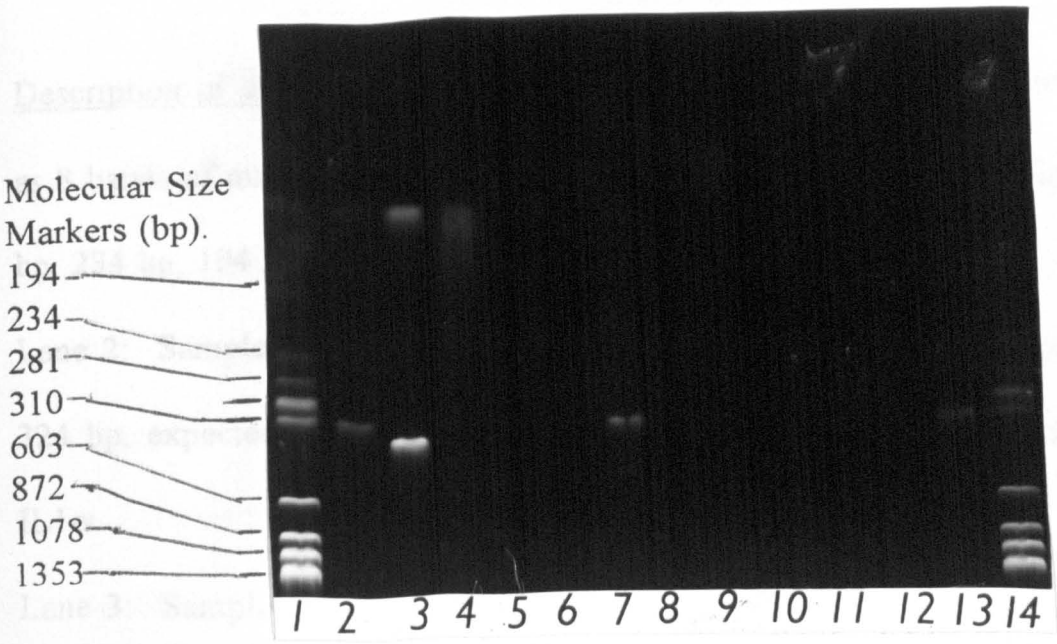
Lane 6: Sample from non-malignant lung probed for IL1α. No bands observed, considered negative result for IL1α.

Lane 7: Sample from non-malignant lung probed for IL1ß. No bands observed, considered negative result for IL1ß.

Lane 8: Sample from non-malignant lung probed for IL6. 1 band observed approx. 313 bp, expected size for IL6 PCR product, band considered positive result for IL6.

Lane 9: Sample from non-malignant lung probed for TNFα. 1 band observed approx. 300 bp, **not** expected size for TNFα PCR product (approx. 450bp), band considered to be artefact and so counted as negative result for TNFα.

Lane 10: Sample from lung probed for TNFß. No bands observed, considered negative result for TNFß.



Lanes

Figure 3.11: Photograph of the PCR result obtained from a papillary adenocarcinoma of the ovary. The PCR result obtained from a normal ovary is also shown (not from the same patient). Tissue was collected and snap frozen immediately after surgery, mRNA extracted (p77), reverse transcribed (p83), and PCR performed (p90). Photograph shows PCR products on an agarose slab gel on which electrophoresis was performed (p97), staining following electrophoresis was carried out with ethidium bromide. Film used for photography was polaroid 665 and the exposure time for the photograph was 25s. A detailed description is given overleaf.

Description of Figure 3.11: Lanes 1 and 14 are molecular size markers, observed as 8 bands of masses: 1353 base pairs (bp), 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 234 bp, 194 bp.

Lane 2: Sample from ovarian tumour probed for IL1α. 1 band observed approx. 324 bp, expected size for IL1α PCR product, band considered positive result for IL1α.

Lane 3: Sample from ovarian tumour probed for IL1ß. 1 band observed approx. 376 bp, expected size for IL1ß PCR product, band considered positive result for IL1ß.

Lane 4: Sample from ovarian tumour probed for IL6. No bands observed, considered negative result for IL6.

Lane 5: Sample from ovarian tumour probed for TNFα. No bands observed, considered negative result for TNFα.

Lane 6: Sample from ovarian tumour probed for TNFß. No bands observed, considered negative result for TNFß.

Lane 7: Sample from ovarian tumour probed for ß2M. 1 band observed approx. 322 bp, expected size for ß2M PCR product, band taken as positive result for ß2M.

Lane 8: Sample from normal ovary probed for IL1α. No bands observed, negative result for IL1α.

Lane 9: Sample as for (8),but probed for IL1ß. No bands observed, negative result for IL1ß.

Description of Figure 3.11 Cont.:

Lane 10: Sample from normal ovary probed for IL6. No bands observed, considered negative result for IL6.

Lane 11: Sample from normal ovary probed for TNFα. No bands observed, considered negative result for TNFα.

Lane 12: Sample from normal ovary probed for TNFß. No bands observed, considered negative result for TNFß.

Lane 13: Sample from normal ovary probed for ß2M. 1 band observed approx. 322 bp, expected size for ß2M PCR product, band considered positive result for ß2M.

3.4: Immuno-Histology Experiments.

The results of the PCR experiments gave an indication of the patterns of cytokine expression in each tumour type. However the information was derived entirely from cDNA transcript analysis and so satisfactory conclusions could not be drawn from this material alone. There is always the possibility that any detected cDNA has been reverse transcribed from spurious mRNA and not translated into the protein. Production of the correct mRNA might not mean production of the protein. Also, assuming the tumour cells were producing the protein, the PCR results do not show the source of the detected cytokine, or the types of cell that are present in the tumour. There is always the possibility that the detected cytokines are being produced by infiltrating lymphocytes in the tumour and are not tumour cell derived.

3.4.1 Immuno-histological Experiments Using Indirect Immuno-Fluorescence.

A series of immuno-fluorescent staining experiments were undertaken. It was decided to examine only those cytokines which had occurred most frequently in the PCR results, IL1α and IL6, and only samples which were positive for these in the PCR experiments were chosen.

No normal breast tissue was used as it was found to be fatty and difficult to section adequately, no ovarian tissue was used due to the lack of material. A number of frozen tissue sections were prepared from each sample.

Each sample was to be stained for IL1α and IL6 and also with phenotyping antibodies. These were antibodies for the cell surface markers CD8 (Cytotoxic/ Suppressor T-lymphocytes), CD14 (macrophages) and CD 21 (Activated B-lymphocytes). A series of experiments, using sections prepared from each sample chosen, was carried out to optimise the dilutions for each primary antibody. Dilutions of 1:2000 to 1:8000 were used for each antibody. A 1:4000 dilution was optimal for the anti-IL1α, anti-IL6 and anti-CD antibodies. The secondary antibody was employed at the manufacturer's recommended dilution (see methodologies for details). Negative controls were used for each set of sections, each being a section stained with PBS instead of primary antibody, but stained with secondary antibody. Each negative control demonstrated minimal fluorescence. A positive result was one in which there was noticeable fluorescence compared to that seen with the negative controls. Cells grown on cover-slips at standard conditions (see p) were stained for IL1α and IL6. Both the MCF-7 and T-24 cell lines gave positive responses for these cytokines, but only the results for the MCF-7 cell line were bright enough to photograph. These are shown in figures 3.12-14. For the frozen sections, those samples which stained positive did not give fluorescence bright enough or long-lasting enough to photograph, although for each result the fluorescence was clearly brighter than that seen on the negative controls and was cell-associated. The results independently verified by a skilled histologist who confirmed their unsuitability for photography. They are shown in table 3.1 overleaf.

Table 3.1 Results of the Immunohistology Experiments Using Indirect Immuno-Fluorescence.

Cell Line or Tumour Type[1]	IL1α Antibody	IL6 Antibody	CD8 Marker (Cytotoxic/ Suppressor T-Cells)	CD14 Marker (Activated B-Cells)	CD21 Marker (Activated B-Cells)
MCF-7 Cell Line	Strong +	Stong +	N.A.	N.A.	N.A.
T-24 Cell Line	+	+	N.A.	N.A.	N.A.
Breast Tumour 4	-	+	+	+	+
Bladdder Tumour 2	+	+	-	-	+
Lung Tumour 4	-	+	+	+	+
Non-malignant Lung 4	-	+	+	+	+

N.A.= Not Applicable

[1] Only 1 sample of each type probed, number is identifier used on tables 3.3-3.13

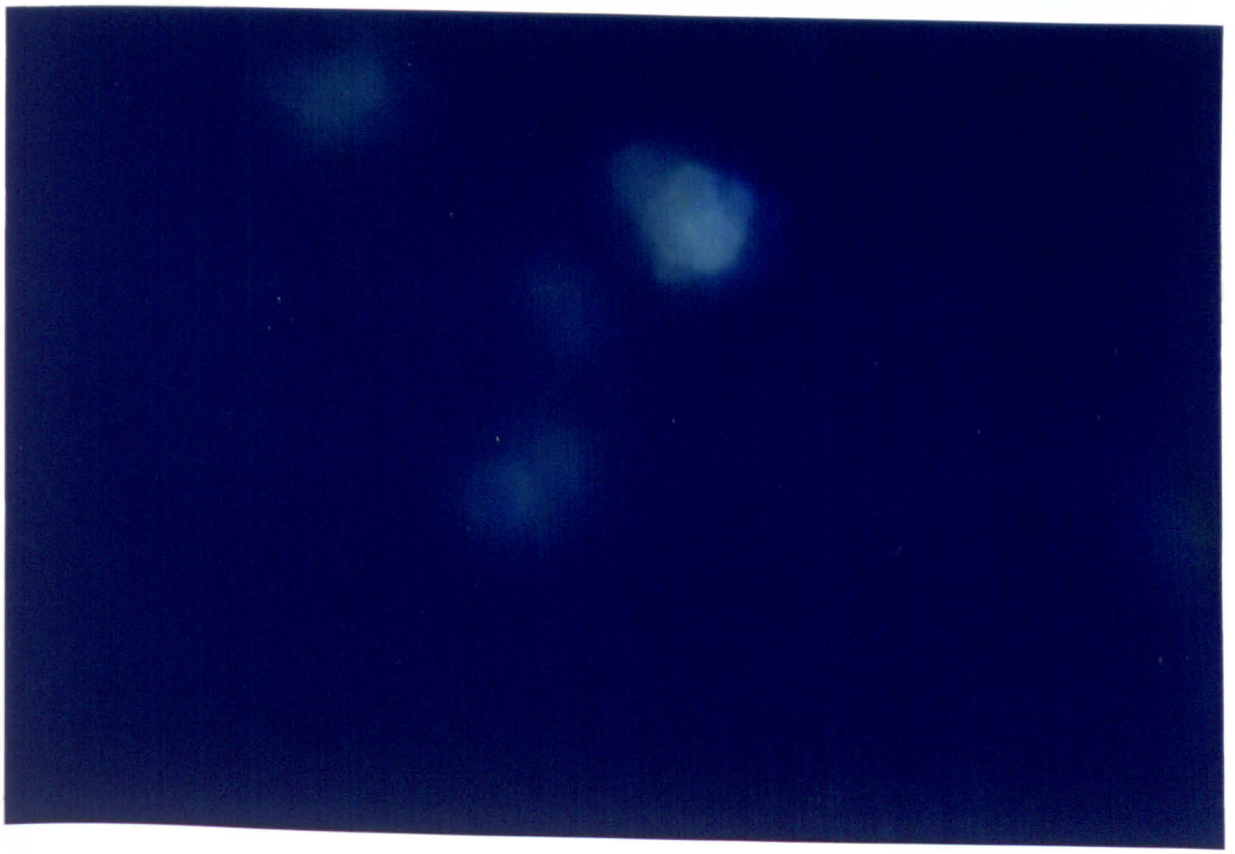


Figure 3.12: MCF-7 cells stained for IL1α using indirect immuno-fluorescence. Objective magnification x 40, film speed ASA 400.

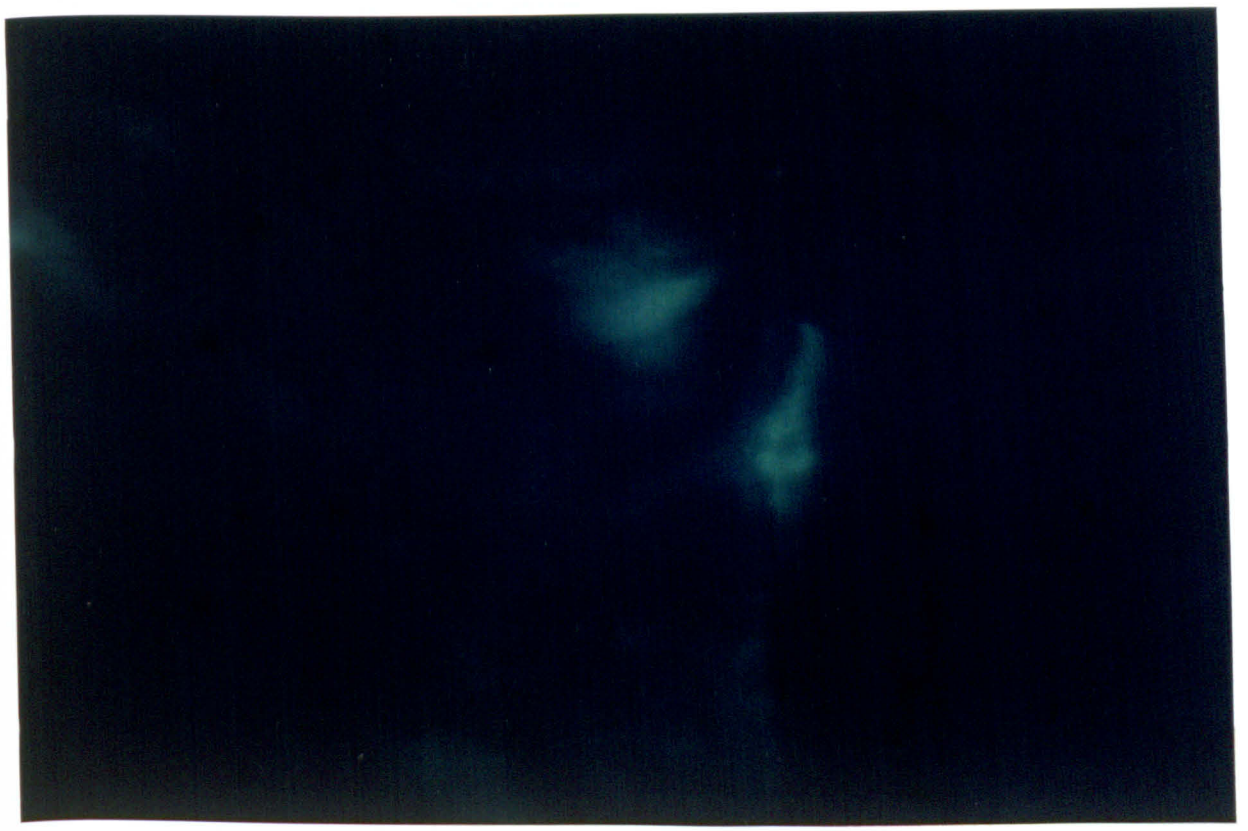


Figure 3.13: MCF-7 cells stained for IL6 using indirect immuno-fluorescence.

Objective magnification x 40, film speed ASA 400.

Figure 3.14: MCF-7 cells stained with haematoxylin and eosin. Objective magnification x 10, film speed ASA 400.

Table 3.2: Results of Immuno-histology Experiments Performed on Paraffin -Embedded Sections Using an Alkaline Phosphatase conjugated Secondary Antibody.

Tumour Type	IL1 Alpha	IL6	CD68 (macrophage marker)
Breast Tumour 10	+	+	+
Breast Tumour 11	+	+	N.D.
Breast Tumour 12	+	+	N.D.
Breast Tumour 13	+	+	N.D.
Breast Tumour 14	+	+	N.D.
Bladder Tumour 14	weak +	-	-
Lung Tumour 7	-	+	-
#Non-malignant Lung 7	-	+	+
*Non-malignant Lung 8	-	-	N.D.

Notes: #Non-malignant lung 7 from same patient as lung tumour 7
*No lung tumour 8 (lung tuberculated)

3.4.2: Immuno-Histological Experiments Using An Alkaline Phosphatase-Conjugated Secondary Antibody Carried Out on Paraffin-Embedded Sections.

The previous set of experiments had demonstrated that IL1α and IL6 proteins could be detected in tumour samples which gave positive results when probed for these cytokines by PCR. However, although the results of the experiments could be independently verified, they could not be presented in this thesis in photographic form (apart from the results for the MCF-7 cell line). Nor could the results determine in which type of cells the cytokines originated. A new series of experiments was undertaken. These used a different immuno-histological technique to investigate the presence of IL1α and IL6. This was one which could be performed on paraffin-embedded sections and it used an alkaline phosphatase-conjugated secondary antibody. This antibody, when developed gives positive results which are permanent and which, if the slide is counter-stained with haematoxylin, will show what cell type the stained protein is associated with. The stained material can be seen in the cellular cytoplasm in the golgi body. In this way, any positive results for IL1α and IL6 would also give the cell type of origin. The previous set of experiments used only material in which IL1α and IL6 were expected. To compensate for this, in these experiments all of the tissue chosen was not previously subjected to PCR, and so there was no expectation of what cytokines would be detected in what tissue. The results of the experiments are shown in table 3.2 opposite, and photographs of the best results given in figures 3.15-3.25.

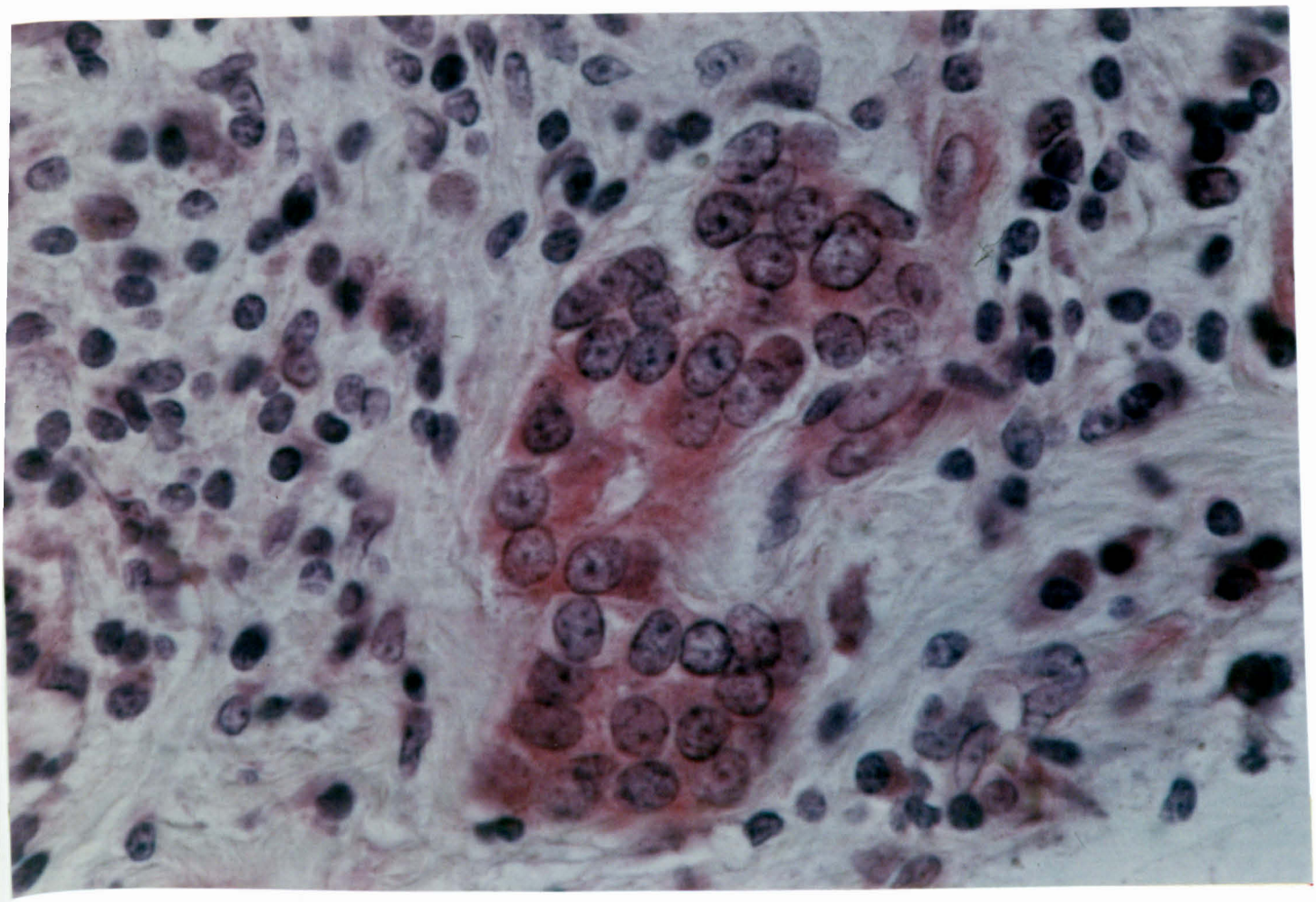


Figure 3.15: Breast Tumour 10 stained for IL1α using an alkaline phosphatase conjugated secondary antibody. A strong positive result (red staining) can be seen in the large ductal cells in the centre of the picture. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

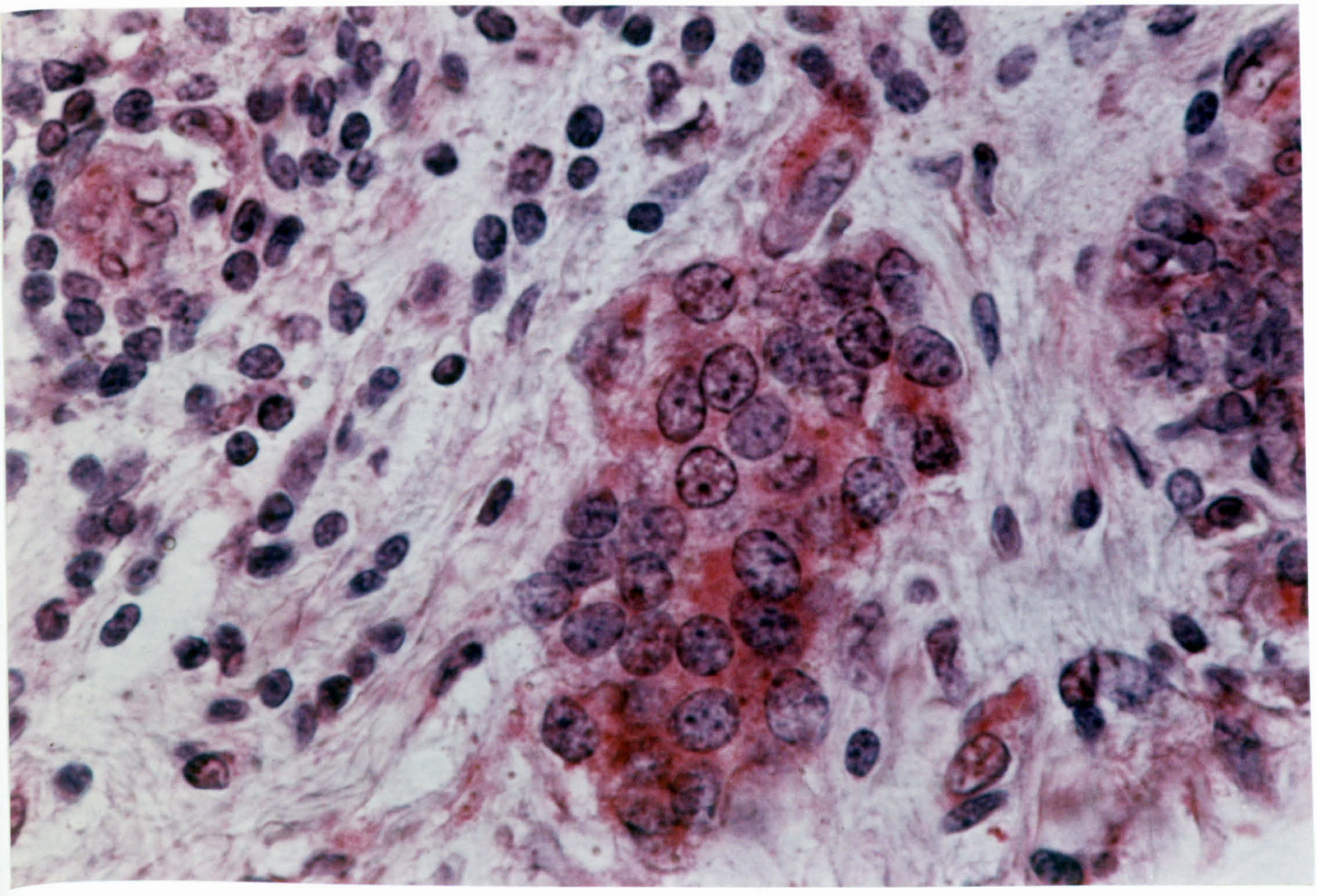


Figure 3.16: Breast Tumour 10 stained for IL6 using an alkaline phosphatase conjugated secondary antibody. A strong positive result (red staining) can be seen in the large ductal cells in the centre of the picture. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

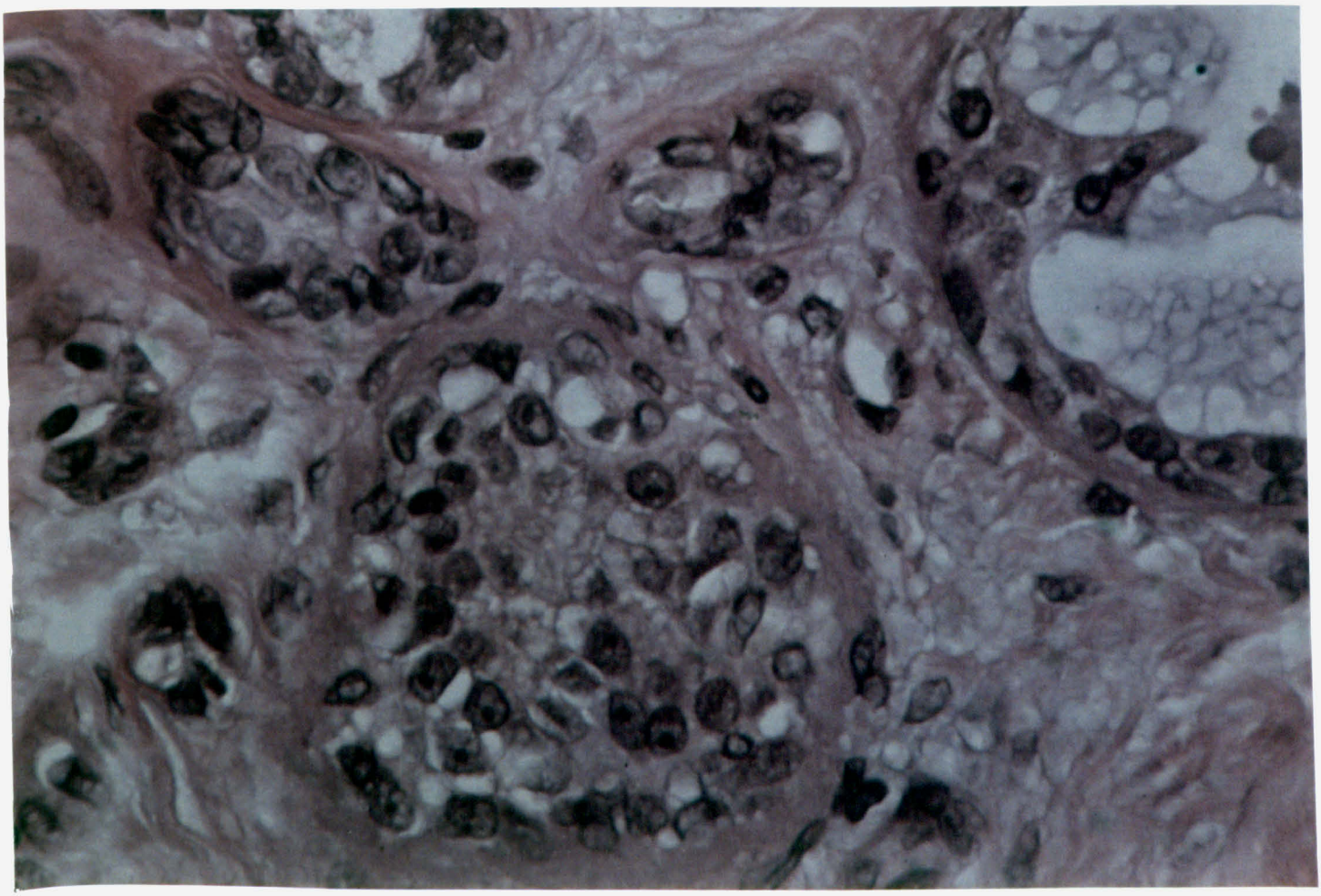


Figure 3.17: Breast Tumour 10 stained with only the secondary antibody and then haematoxylin and eosin as a negative control, area shows large ductal cells. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

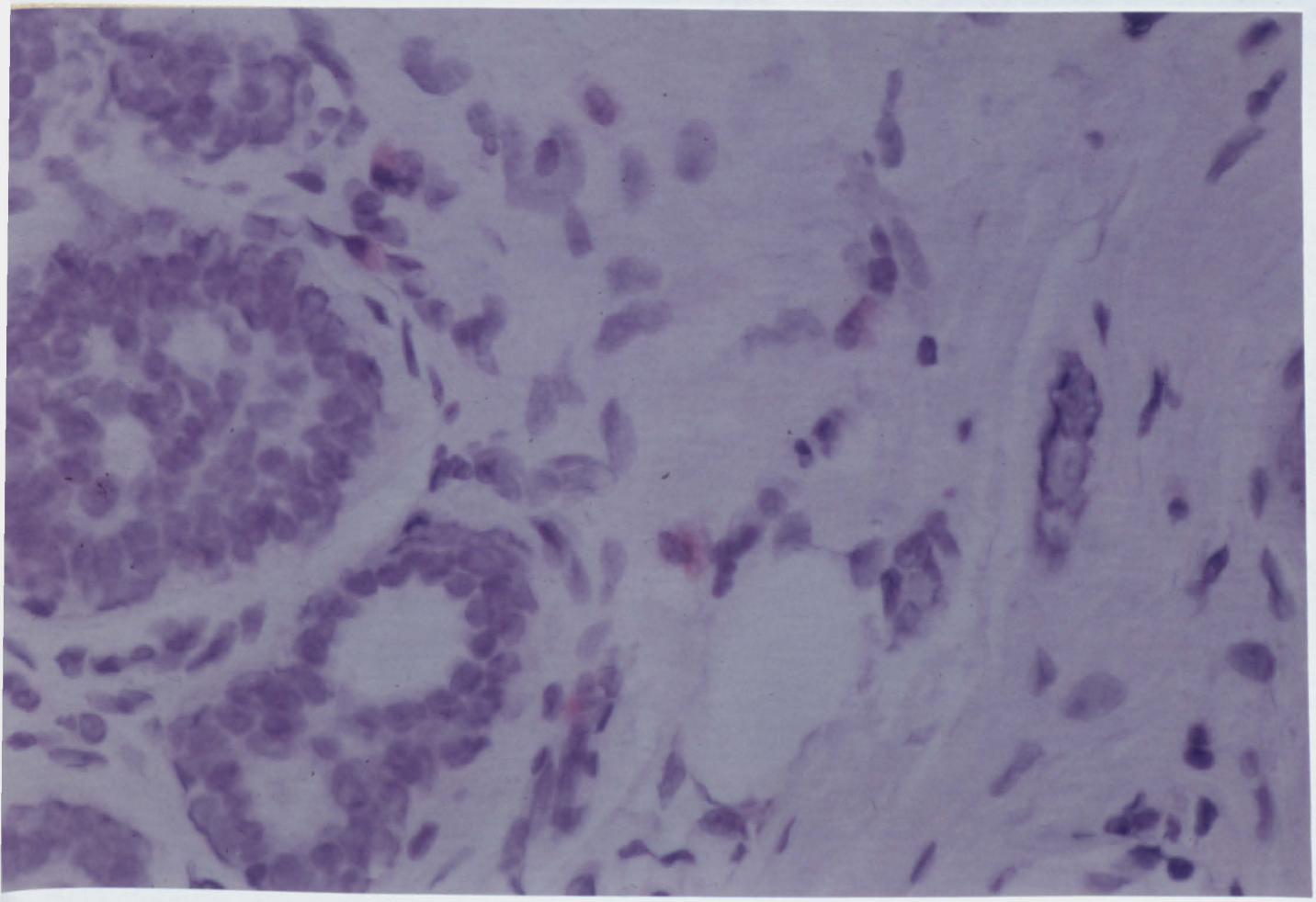


Figure 3.18: Breast Tumour 10 stained for CD68 (macrophage marker). Area shows positive-staining macropages around large ductal cells, but these macrophages are not in the same region of the tissue as the IL1α or IL6-positive stained areas shown in figures 3.15 and 3.16. Objective magnification is x25 and film speed is ASA160, with a tungsten light source.

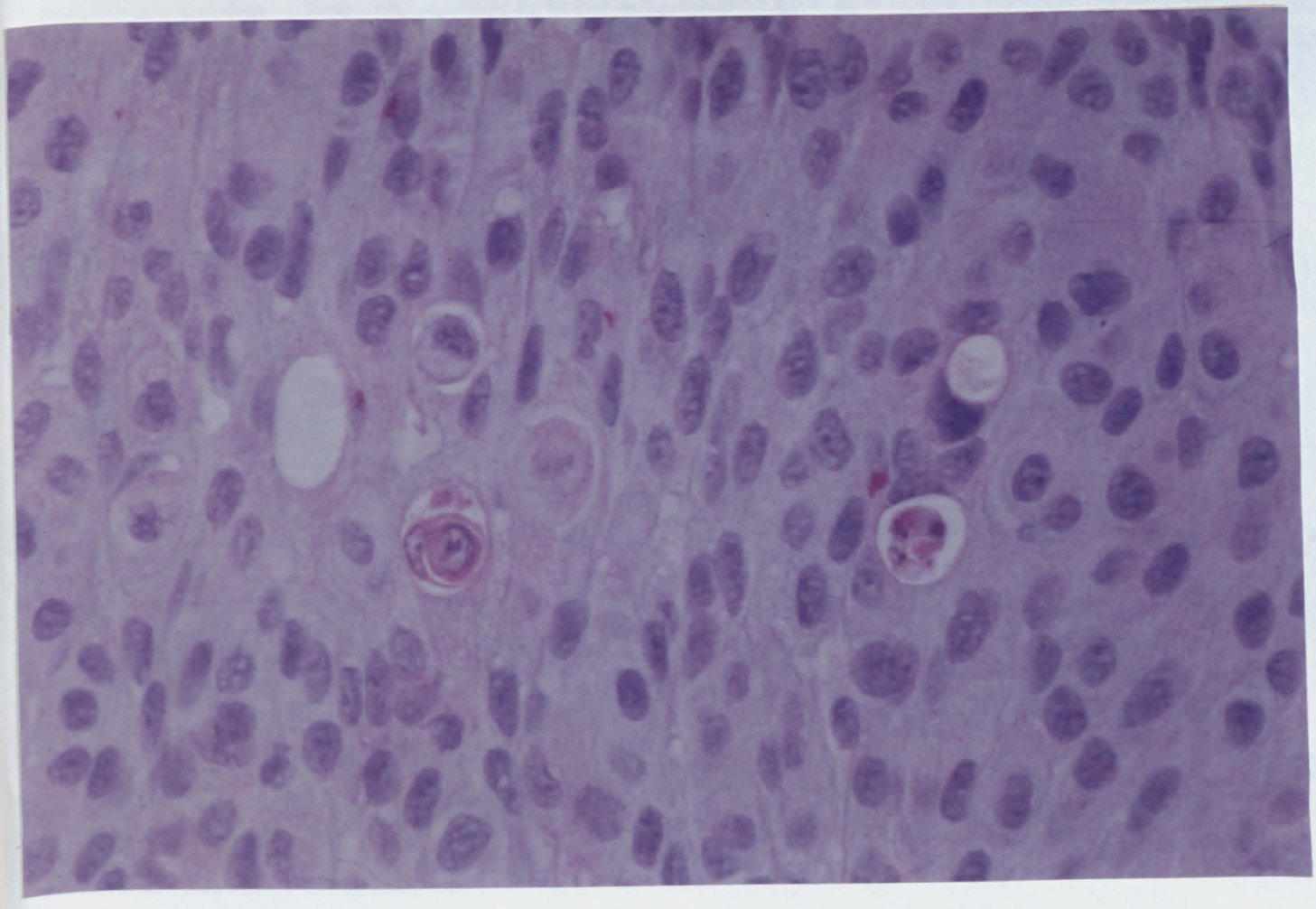


Figure 3.19: Bladder Tumour 14 stained for IL1α using an alkaline phosphatase conjugated secondary antibody. A positive result (red staining) can be seen in two cells in the centre of the picture. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

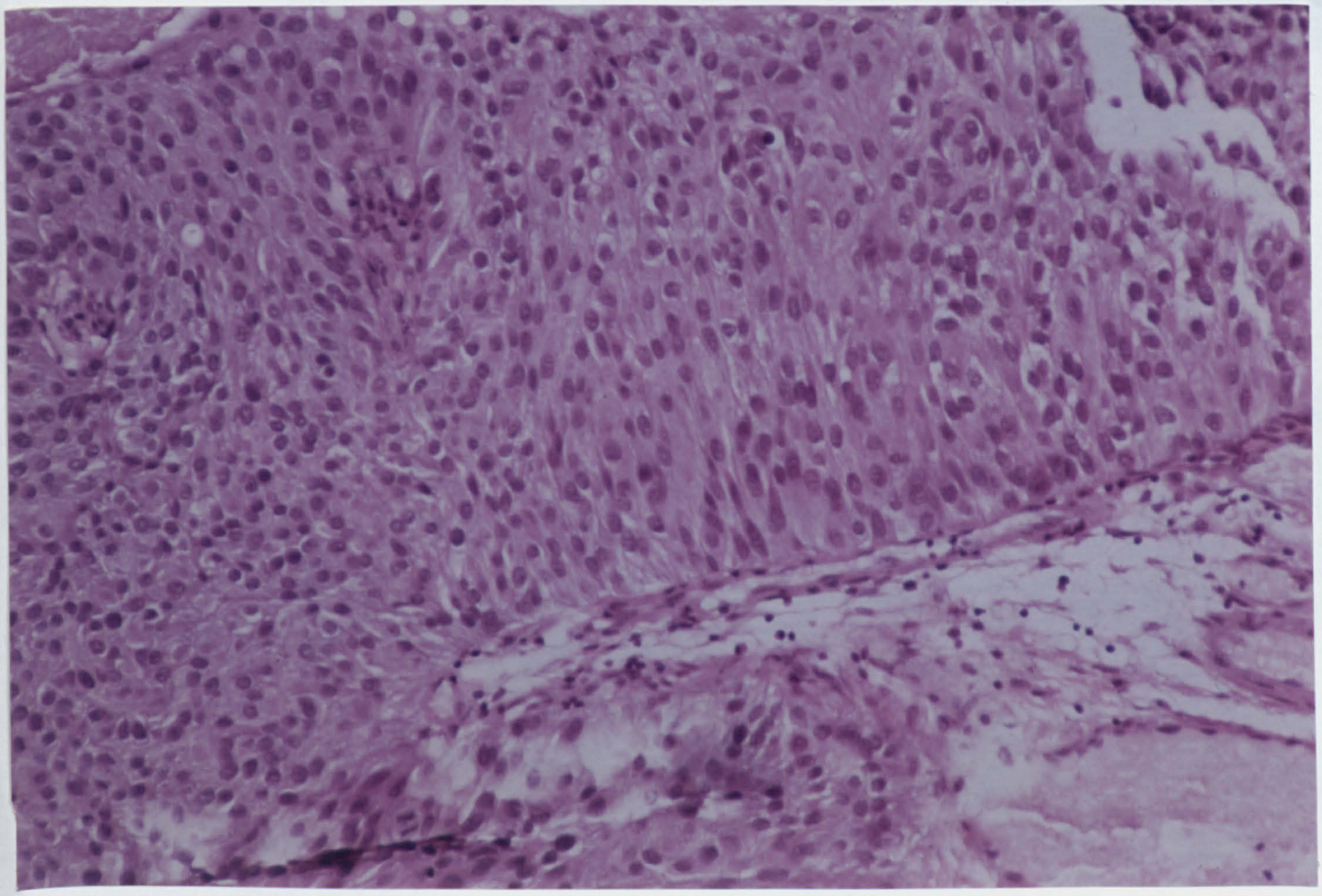


Figure 3.20: Bladder Tumour 14 stained with only the secondary antibody and then haematoxylin and eosin as a negative control. Objective magnification is x10 and film speed ASA 160, with a tungsten light source.

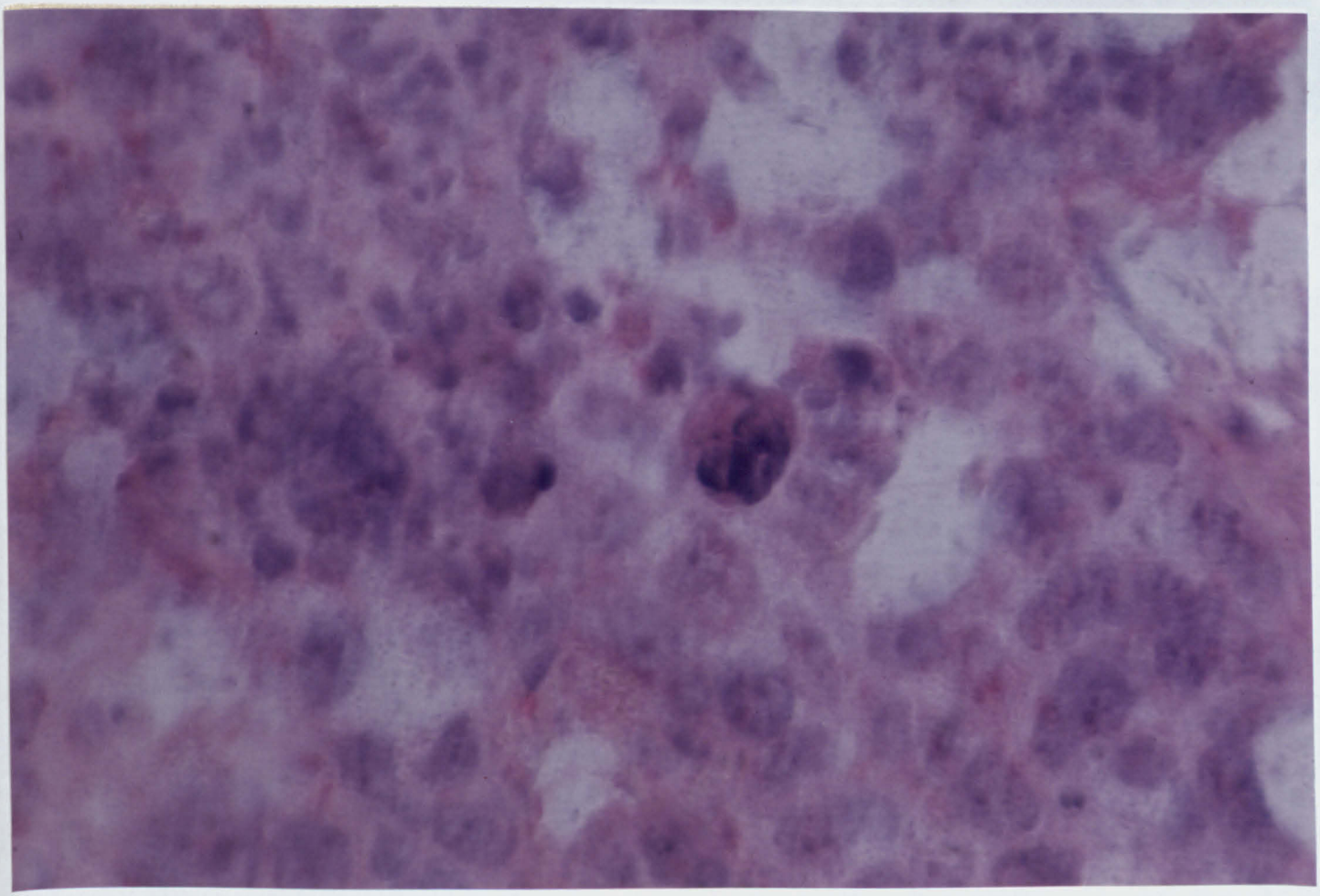


Figure 3.21: Lung Tumour 7 stained for IL6 using an alkaline phosphatase conjugated secondary antibody. A positive result (red staining) can be seen in the large cell in the centre of the picture. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

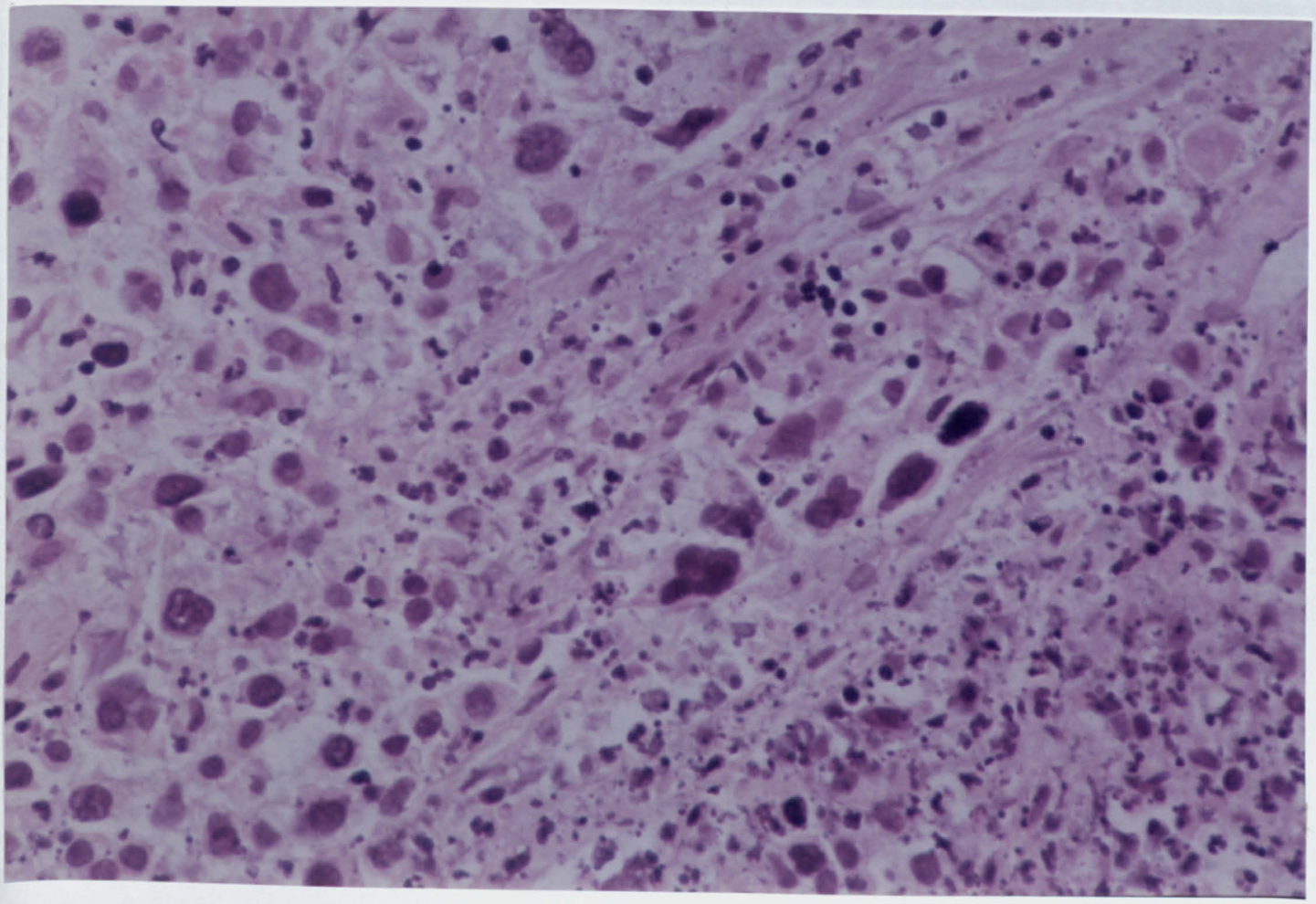


Figure 3.22: Lung Tumour 7 stained with only the secondary antibody, and then haematoxylin and eosin, as a negative control. Area shows similar large cells to those which stained positive for IL6 in figure 3.21 Objective magnification is x25 and film speed ASA 160, with a tungsten light source.

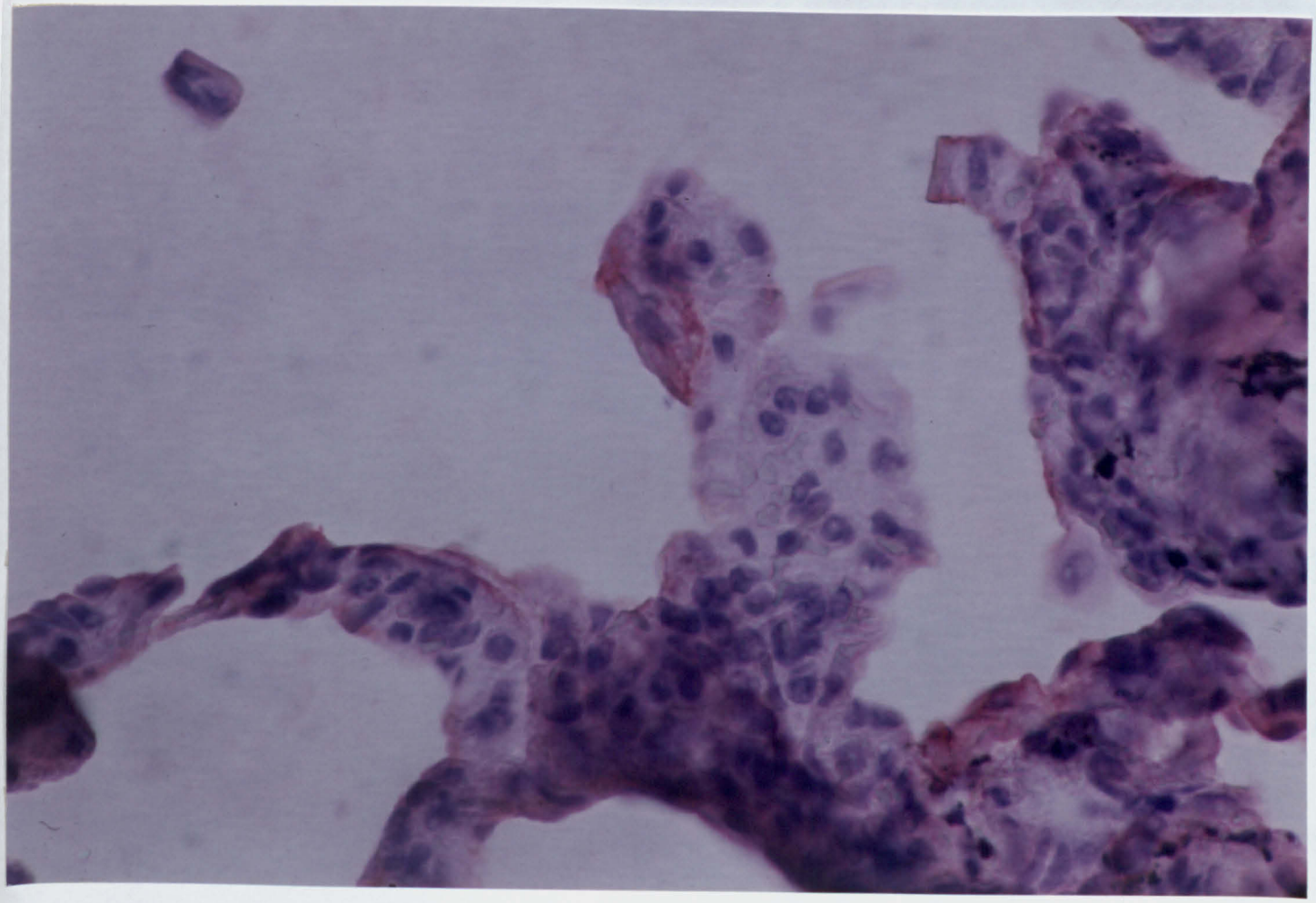


Figure 3.23: Non-malignant Lung 7 stained for IL6 using an alkaline phosphatase conjugated secondary antibody. A positive result (red staining) can be seen in some of the large surface cells, especially in the centre of the picture. Objective magnification is x40 and film speed ASA 160, with a tungsten light source.

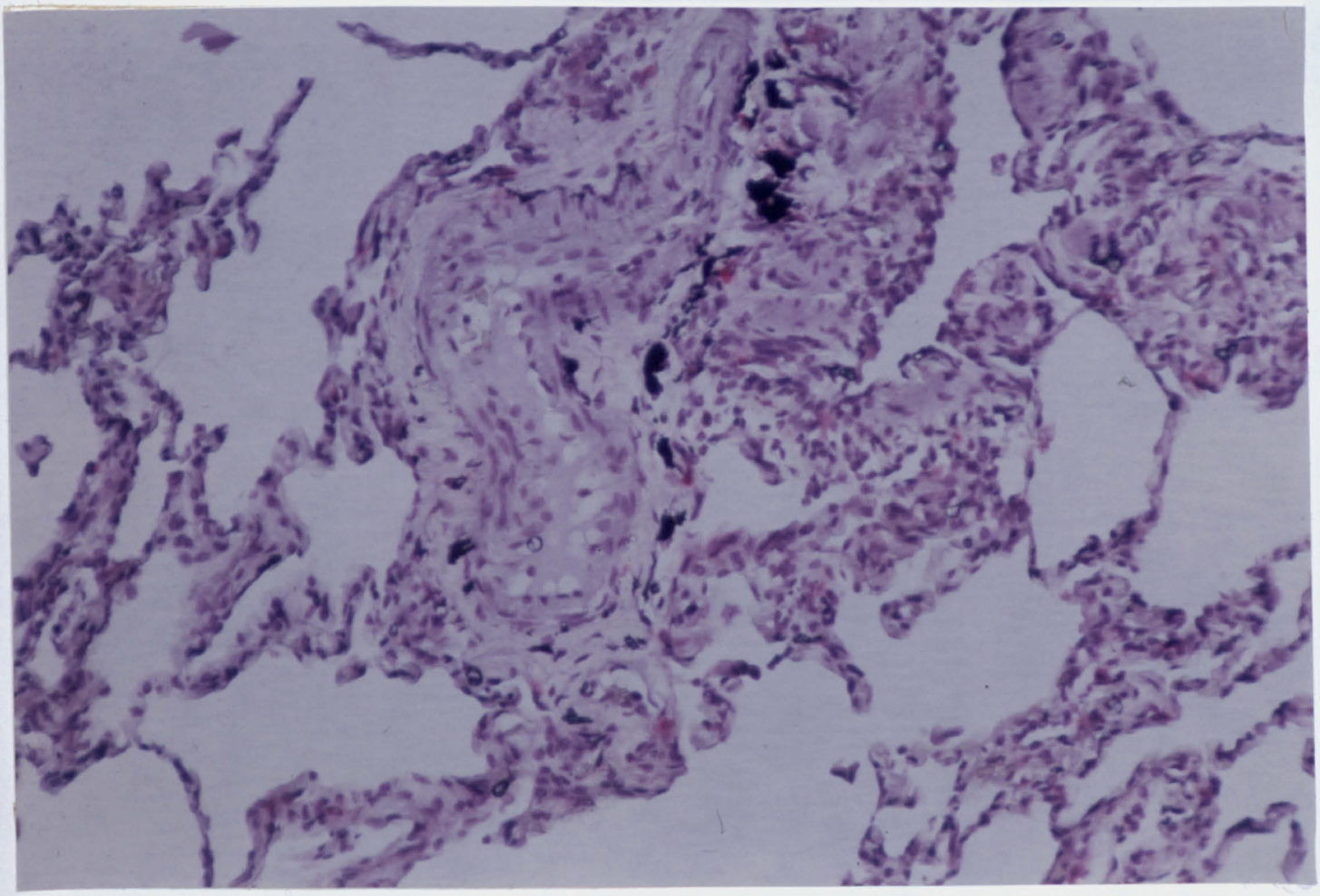


Figure 3.24: Non-malignant lung 7 stained for CD68 (macrophage marker). Macrophages can be observed as red staining cells scattered over the field. They are of a different morphology to the IL6- positive cells shown in figure 3.23. Objective magnification is x25, film sped ASA 160, with a tungsten light source.

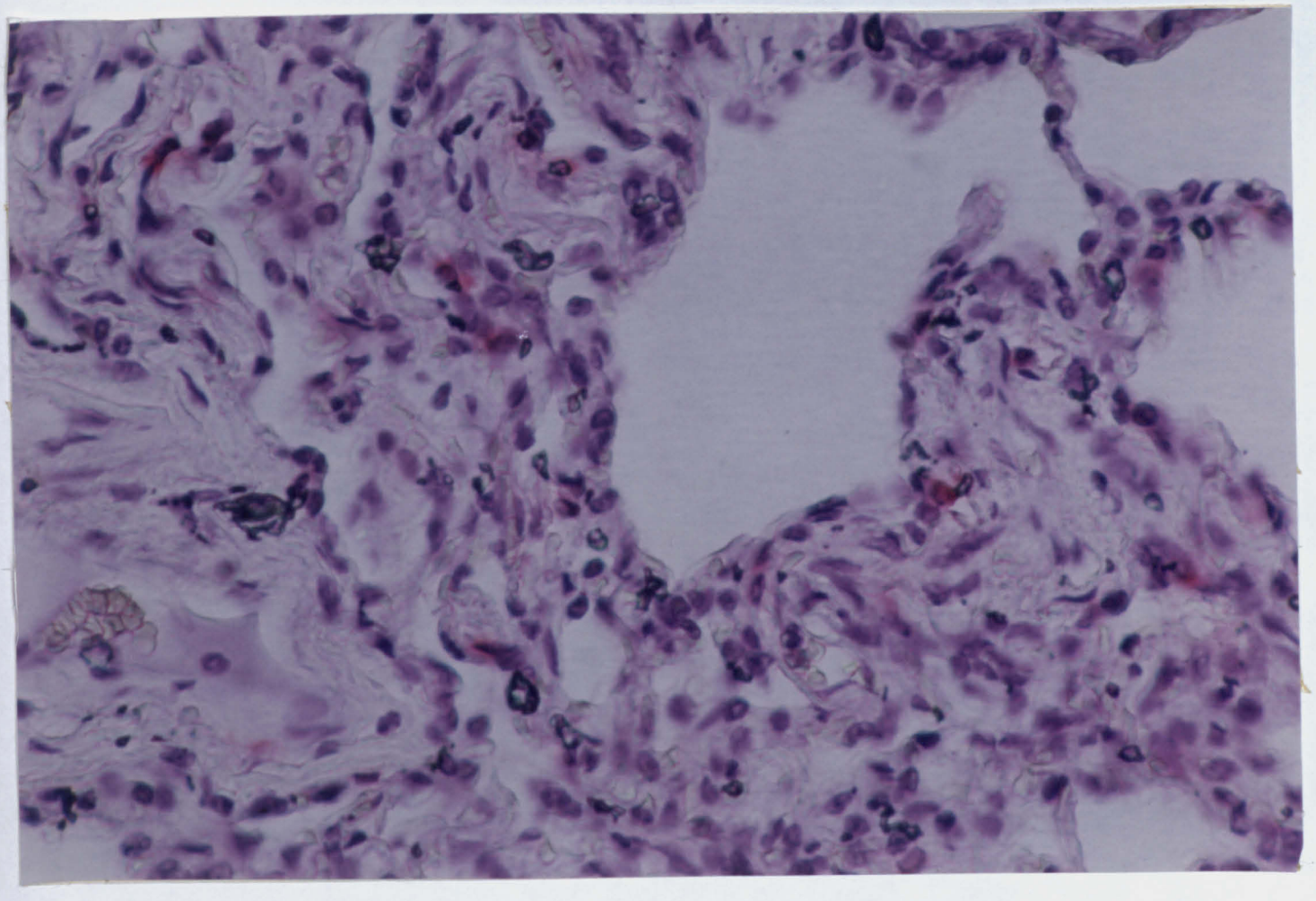


Figure 3.25: Close-up of non-malignant lung 7 stained for CD68 (macrophage marker). Macrophages can be observed as red staining cells scattered over the field. They are of a different morphology to the IL6- positive cells shown in figure 3.23. Objective magnification is x40, film sped ASA 160, with a tungsten light source.

3.5: Investigation of Patients' Medical Records.

An investigation of the medical records of the patients supplying tumours for both the PCR portion of the research, and the immuno-histological part of the research was carried out. Records of all the patients giving positive PCR results were checked where possible. This was also carried out for the patients supplying tumours for the experiments using immuno-histology on paraffin-embedded sections. For various reasons not all of the records were available and so could not be consulted. The information from the records was collated into tables. The first table gives general clinical information, the second biochemistry and haematology information, and the last table gives individual cytokine profiles for each sample examined. In the tables showing the biochemistry and haematology results, the levels of c-reactive protein and the erythrocyte sedimentation rates could not be reported as these were not measured routinely.

Table 3.3: Clinical Results From Records of Breast Tumour Patients

Tumour Type	Pathology of Tumour	Weight Loss: Yes/No	Appetite: Poor/Good	Temp. on Day of Adm. (degrees C)
Breast Tumour 1	Invasive Ductal Cell Carcinoma (Bloom + Richardson II)	Yes	Poor	36
Breast Tumour 2	Mucinoid Carcinoma of Breast with Surrounding Interductal Carcinoma	N.A.	Poor	36.7
Breast Tumour 3	Intraductal and Invasive Breast carcinoma (Bloom + Richardson II)	N.A.	Good	36.2
Breast Tumour 4	In-situ and Infiltrating Ductal Cell Carcinoma (Bloom + Richardson III)	No	Good	36.4
Breast Tumour 5	In-Situ Ductal Cell Carcinoma, and seperate Invasive Ductal Darcinoma (Bloom +Richardson III)	Yes	Poor	36.6
Breast Tumour 6	Paget's Disease of Left Nipple and Intraductal Carcinoma	N.A.	Good	36
Breast Tumour 7	Intraductal and Infiltrating carcinoma (Bloom +Richardson II)	N.A.	N.A.	36.2
Breast Tumour 8	In-situ and infiltrating moderately well differentiated ductal carcinoma of the breast (Bloom + Richardson II)	No	Good	36
Breast Tumour 10	Intra-ductal and infiltrating ductal carcinoma (grade I) .No involvement of lymph nodes	N.A.	Good	36
Breast Tumour 11	Infiltrating grade III ductal carcinoma with a prominent lymphocytic reaction to the tumour	N.A.	Good	37
Breast Tumour 12	lobular carcinoma in situ associated with infiltrating breast carcinoma (grade II)	N.A.	Good	36.5
Breast tumour 13	Infiltrating lobular carcinoma (grade II) with foci of lobular carcinoma in-situ	No (had wt. gain)	Good	36.5

Notes: 1) Bloom + Richardson is grading system. Higher no. = higher grade
2) Records for breast tumours 9 and 14 were unavailible.
3) N.A. = Not Availible

Table 3.4: Clinical Results from Records of Bladder Tumour Patients

Tumour Type	Pathology of Tumour	Weight Loss: Yes/No	Appetite: Poor/Good	Temp. on Day of Adm. (degrees C)
Bladder Tumour 1 (male)	Well-differentiated papillary transitional cell carcinoma	N.A.	Good, but Small	35.5
Bladder Tumour 2 (male)	Invasive poorly-differentiated transitional cell carcinoma (Ash IV), with focal squamous differentiation	Yes	Poor	36.8
Bladder Tumour 3 (male)	Papillary transitional cell carcinoma(Ash III)with areas of infarction, and invasion of the superficial lamia propria	No	Good	36.2
Bladder Tumour 4 (female)	Mainly papillary superficial transitional cell carcinoma (Ash II) with no evidence of sub-mucosal invasion	No	Good	36.2
Bladder Tumour 5 (male)	Moderately-well differentiated transitional cell carcinoma (Ash II) with one focus of early stromal invasion	No	Good	36.2
Bladder Tumour 6 (male)	Well-differentiated papillary transitional cell carcinoma, with no invasion of underlying tissue	N.A.	Good	36.3
Bladder Tumour 7 (male)	Superficial transitional cell carcinoma (Ash II) which is mainly papillary. No evidence of Invasion of sub-mucosa or muscle. Mild chronic inflammation present.	No	Good	35.7
Bladder Tumour 8 (male)	Well-differentiated papillary transitional cell carcinoma with focal superficial invasion of the underlying stroma (Ash II).	N.A.	Good	36.5
Bladder Tumour 9 (male)	Papillary transitional cell carcinoma (Ash II-III), with focal invasion of the submucosa)	N.A.	Good	36.2

Notes: 1) Ash is grading system. Higher no. = higher grade
2) Only results for Bladder tumours 1- 9, are shown. Records for rest were unavailible.
N.A. = Not Availible

Table 3.5: Clinical Results from Records of Lung Tumour Patients

Tumour Type	Pathology of Tumour	Weight Loss: Yes/No	Appetite: Poor/Good	Temp. on Day of Adm. (degrees C)
Lung Tumour 1 (male)	Poorly-differentiated mucus-secretory adenocarcinoma	No	Good	36
Lung Tumour 2 (male)	Moderately -differentiated squamous carcinoma arising in a bronchus	Yes	Good	36.1
Lung Tumour 3 (male)	Invasive moderately-differentiated squamous carcinoma arising from disrupted and ulcerated mucosa of the upper second order bronchus	No	Good	35.8
Lung Tumour 4 (male)	Moderately-differentiated squamous carcinoma	No	Good	36.5
Lung Tumour 5 (male)	Invasive large cell anaplastic carcinoma of the bronchus	N.A.	Good	36
Lung Tumour 6 (female)	Bronchial carcinoid tumour composed of packets and trabeculae of eosinophilic cells with open nucleai	Yes	Poor	36.5

Notes: 1) Records for lung tumour 7 were unavailible.
2) N.A. = Not Availible

Table 3.6: Clinical Results from Records of Ovarian Tumour Patients

Tumour Type	Pathology of Tumour	Weight Loss: Yes/No	Appetite: Poor/Good	Temp. on Day of Adm. (degrees C)
Malignant Ovarian Tumour 1	Papillary Adenocarcinoma with numerous calcospherocytes consistent with metatastic spread from an ovarian primary	No	Poor	36.5
Malignant Ovarian Tumour 2	Papillary Adenocarcinoma with numerous calcospherocytes consistent with metatastic spread from an ovarian primary	No	Good	36
Normal Ovary 1*	Right Ovary containing a theca lutein csyst	No	Good	36

Notes: * Not from same patient as Ovarian Tumour 1

Table 3.7 (overleaf): This table shows the results taken from the biochemical and haematological analyses preformed on the breast tumour patients on the day prior to surgery. The records for patients 9 and 14 were unavailible. Only brest tumour 5 had elevated serum levels of calcium (2.72 mmol/l) This tumour expressed IL6 only. No patients displayed abnormal globulin levels. Breast tumours 2 and 6 had elevated white blood cell counts (17.5 and 12 $x10^9$ cells/ l respectively)However, neither of these patients had expression for any cytokine. All patients had normal lymphocyte, monocyte and platelet counts, except for breast tumour 6, which had an elevated platelet count of 471 $x10^9$ cells / l. In the table 1 $x10^x$ is given as 1 x 10 exp X, and N.A. = not availible.

Table 3.7 Biochemistry and Haematology Results from Records of Breast Tumour Patients

Tumour Type	Serum Calcium mmol/l (Norm.2.2-2.65 mmol/l)	Serum Total Protein g/l (norm. 60-77 g/l)	Serum Albumin g/l (Norm. 36-50 g/l)	Serum Globulin (Abnormal >40 g/l)	White Blood Cell Count in Cells x10 exp 9/l (Norm. 4 - 11 x10 exp 9/l)	Neutrophil Count in Cells x10 exp 9/l (Norm. 2.3-7.5 x 10 exp 9/l)	Lymphocyte Count in Cells x10 exp 9/l (norm. 1.5-3.5 x10 exp 9/l)	Monocyte Count in Cells x10 exp 9/l (Norm. 0.2-0.8 x10 exp 9 /l)	Platelet Count in Cells x10 exp 9/l (Norm. 150-400 exp 9 /l)
Breast Tumour 1	2.4	66	43	23	9.5	7.3	1.44	0.21	294
Breast Tumour 2	2.36	73	45	28	17.5	15.1	1.86	0.44	344
Breast Tumour 3	2.59	75	47	28	5.8	4.05	1.47	0.28	350
Breast Tumour 4	2.24	64	41	23	5.7	3.41	2.02	0.27	263
Breast Tumour 5	2.72	84	N.A.	N.S.	7.83	5.83	1.81	0.16	322
Breast Tumour 6	2.47	71	44	27	12	9.47	2.28	0.25	471
Breast Tumour 7	2.54	77	50	27	9.5	6.76	2.32	0.42	250
Breast Tumour 8	2.39	71	43	28	7.9	5.47	2.1	0.33	270

N.B.: Exp means "To the Power of". Eg. 1x10 exp 3 would be 1000.
N.A. means "Not Availible".
Records for Breast tumour 9 were unavailible.

Table 3.7 Biochemistry and Haematology Results from Records of Breast Tumour Patients

Tumour Type	Serum Calcium mmol/l (Norm.2.2-2.65 mmol/l)	Serum Total Protein g/l (norm. 60-77 g/l)	Serum Albumin g/l (Norm. 36-50 g/l)	Serum Globulin (Abnormal >40 g/l)	White Blood Cell Count in Cells x10 exp 9/l (Norm. 4 - 11 x10 exp 9/l)	Neutrophil Count in Cells x10 exp 9/l (Norm. 2.3-7.5 x 10 exp 9/l)	Lymphocyte Count in Cells x10 exp 9/l (norm. 1.5-3.5 x10 exp 9/l)	Monocyte Count in Cells x10 exp 9/l (Norm. 0.2-0.8 x10 exp 9 /l)	Platelet Count in Cells x10 exp 9/l (Norm. 150-400 exp 9 /l)
Breast Tumour 10	2.36	65	43	22	9.47	6.45	2.58	0.39	192
Breast Tumour 11	2.5	80	50	30	7.32	4.49	2.15	0.39	240
Breast Tumour 12	2.29	64	43	19	7.33	5.28	1.71	0.24	261
Breast Tumour 13	2.43	69	45	24	6.52	4.93	1.14	0.32	218

N.B.: Exp means "To the Power of". Eg. 1x10 exp 3 would be 1000.
N.A. means "Not Availible".
Records for Breast tumour 9 were unavailible.

Table 3.8 (overleaf): This table shows the results cells taken from the biochemical and haematological analyses performed on the bladder tumour patients on the day before surgery. The records for bladder tumours 10 - 14 were unavailible. No bladder tumour patients had elevated levels of calcium in serum, or elevated levels of globulins. In the haematological analysis, bladder tumour 2 had an elevated white blood cell count (13.3 $x10^9$ cells / l) and bladder tumour 3 had an elevated platelet count (648 x 10^9 cells / l) all other bladder tumour patients had normal white blood cell, neutrophil, lymphocyte, monocyte, and platelet counts. Bladder tumour 2 expressed IL1α and IL6, but bladder tumour 3 expressed no cytokines.

In the table, 1 $x10^x$ is shown as 1 x 10 exp X.

Table 3.8: Biochemistry and Haematology Results from Records of Bladder Tumour Patients

Tumour Type	Serum Calcium mmol/l (Norm.2.2-2.65 mmol/l)	Serum Total Protein g/l (Norm. 60-77 g/l)	Serum Albumin g/l (Norm. 36-50 g/l)	Serum Globulin (Abnormal is >40 g/ l)	White Blood Cell Count in Cells x10 exp 9/l (Norm. 4 - 11 x10 exp 9/l	Neutrophil Count in Cells x10 exp 9/l (Norm. 2.3-7.5 x 10 exp 9/l)	Lympho-cyte Count in Cells x10 exp 9/l (Norm. 1.5-3.5 x10 exp 9/l)	Monocyte Count in Cells x10 exp 9/l (Norm. 0.2-0.8 x10 exp 9 /l)	Platelet Count in Cells x 10 exp 9/l (Norm 150 - 400 x10 exp 9 /l)
Bladder Tumour 1	2.41	71	41	30	8	4.03	2.08	N.A.	245
Bladder Tumour 2	2.18	58	32	26	13.3	N.A.	2.39	N.A.	309
Bladder Tumour 3	2.39	64	36	28	8.3	5.47	2.03	0.81	648
Bladder Tumour 4	2.4	70	43	27	9.2	5.41	3.21	0.58	191
Bladder Tumour 5	2.49	67	43	24	10.8	7.67	2.72	0.41	247
Bladder Tumour 6	N.A.	N.A.	N.A.	N.A.	7.1	5.21	1.43	0.43	262
Bladder Tumour 7	2.47	74	44	30	10.2	6.82	2.59	0.79	283
Bladder Tumour 8	2.26	65	N.A.	N.A.	10	7.28	2.2	0.52	252
Bladder Tumour 9	2.44	69	43	26	8.7	5.8	2.37	0.39	244

Table 3.9 (overleaf): This table shows the results cells taken from the biochemical and haematological analyses performed on the lung tumour patients on the day before surgery. All of the lung tumour patients had normal levels of calcium in serum, and all had normal levels of globulins except lung tumour 6, which had a globulin level of 46 g / l. This tumour expressed IL6. Lung tumours 3, 4, and 6, expressed high white blood cell counts (17.7 x 10^9 cells / l, 14.8 x10^9 cells / l, and 14.9 x10^9 cells / l respectively), and high neutrophil counts (15.7 x10^9 cells / l, 7.65 x10^9 cells / l and 13 x10^9 cells / l respectively) Lung tumours 3, 4, and 6 expressed IL6. IL6 was expressed in the non-malignant lungs of lung tumour patients 4 and 6. No platelet counts were available for the lung tumour patients.

In the table, 1 x10^x is shown as 1 x 10 esp X.

N.A. = not available.

Table 3.9: Biochemistry And Haematology Results from Records of Lung Tumour Patients.

Tumour Type	Serum Calcium mmol/l (Norm.2.2-2.65 mmol/l)	Serum Total Protein g/l (Norm. 60-77 g/l)	Serum Albumin g/l (Norm. 36-50 g/l)	Serum Globulin (Abnormal > 40 g/l)	White Blood Cell Count in Cells x10 exp 9/l (Norm. 4 - 11 x10 exp 9/l	Neutrophil Count in Cells x10 exp 9/l (Norm. 2.3-7.5 x 10 exp 9/l)	Lymphocyte Count in Cells x10 exp 9/l (Norm. 1.5-3.5 x10 exp 9/l)	Monocyte Count in Cells x10 exp 9/l (Norm. 0.2-0.8 x10 exp 9/l)
Lung Tumour 1	2.26	62	42	20	11.2	9.32	1.21	0.67
Lung Tumour 2	2.24	62	36	26	10.3	8.33	1.67	0.3
Lung Tumour 3	2.38	66	40	26	17.7	15.7	1.4	0.7
Lung Tumour 4	2.43	N.A.	40	N.A.	14.8	7.65	0.71	0.23
Lung Tumour 5	2.1	66	42	24	8.7	6.91	1.57	0.23
Lung Tumour 6	2.49	79	33	46	14.9	13	1.3	0.58

Exp. means "to the power of", e.g. 1x10 3 = 1000.
N.A. = Not Availible

Table 3.10 (overleaf): This table shows the results cells taken from the biochemical and haematological analyses performed on the ovarian tumour patients on the day before surgery. No patients have elevated calcium concentrations or globulin concentrations in their serum. Malignant tumour patient 1 has an elevated white blood cell count and an elevated platelet count. In the table, 1 $x10^x$ is shown as 1 x 10 exp X.

N.A. = not availible.

Table 3.10: Biochemistry and Haematology Results From Records of Ovarian Tumour Patients

Tumour Type	Serum Calcium mmol/l (Norm.2.2-2.65 mmol/l)	Serum Total Protein g/l (norm. 60-77 g/l)	Serum Albumin g/l (Norm. 36-50 g/l)	Serum Globulin (Abnormal >40 g/l)	White Blood Cell Count in Cells x10 exp 9/l (Norm. 4 - 11 x10 exp 9/l)	Neutrophil Count in Cells x10 exp 9/l (Norm. 2.3-7.5 x 10 exp 9/l)	Lymphocyte Count in Cells x10 exp 9/l (norm. 1.5-3.5 x10 exp 9/l)	Monocyte Count in Cells x10 exp 9/l (Norm. 0.2-0.8 x10 exp 9 /l)	Platelet Count in Cells x10 exp 9/l (Norm. 150-400 exp 9 /l)
Malignant Ovarian Tumour 1	2.52	58	26	32	16.6	N.A.	1.64	N.A.	484
Malignant Ovarian Tumour 2	2.5	71	49	22	7.6	4.48	2.56	0.55	248
Normal Ovary 1 *	2.4	73	45	28	7.9	N.A.	3.17	N.A.	278

Table 3.11: Cytokine Profiles for Breast Tumours.

Tumour Type	IL-1 Alpha	IL-1 Beta	IL6	TNF Alpha	TNF Beta	Beta -2 M
Breast Tumour 1	-	-	-	-	-	+
Breast Tumour 2	-	-	-	-	-	+
Breast Tumour 3	-	-	-	-	-	+
Breast Tumour 4	-	-	+	-	-	+
Breast Tumour 5	-	-	+	-	-	+
Breast Tumour 6	-	-	-	-	-	+
Breast Tumour 7	-	-	+	+	-	+
Breast Tumour 8	-	-	+	-	-	+
Breast Tumour 9	-	-	+	+	+	+
Breast Tumour 10*	+	N.D.	+	N.D.	N.D.	N.D.
Breast Tumour 11*	+	N.D.	+	N.D.	N.D.	N.D.
Breast Tumour 12*	+	N.D.	+	N.D.	N.D.	N.D.
Breast Tumour 13*	+	N.D.	+	N.D.	N.D.	N.D.
Breast Tumour 14*	+	N.D.	+	N.D.	N.D.	N.D.

Notes: * These profiles were obtained from the second set of immuno-histology experiments, not from PCR

3.12: Cytokine Profiles For Bladder Tumour Samples.

Tumour Type	IL-1 Alpha	IL-1 Beta	IL6	TNF Alpha	TNF Beta	Beta -2 M
Bladder Tumour 1	-	-	+	-	-	+
Bladder Tumour 2	+	-	+	-	-	+
Bladder Tumour 3	-	-	-	-	-	+
Bladder Tumour 4	-	-	+	-	-	+
Bladder Tumour 5	-	-	-	-	-	+
Bladder Tumour 6	-	-	-	-	-	+
Bladder Tumour 7	+	-	+	-	-	+
Bladder Tumour 8	-	-	+	-	-	+
Bladder Tumour 9	+	-	-	+	+	+
Bladder Tumour 10	+	-	+	-	-	+
Bladder Tumour 11	-	-	+	-	-	+
Bladder Tumour 12	-	-	-	-	-	+
Bladder Tumour 13	-	-	-	-	-	+
Bladder Tumour 14*	+	N.D.	N.D.	N.D.	N.D.	N.D.

Notes: *Profile for Bladder tumour 14 from second set of Immuno-histology experiments, not from PCR

Table 3.13: Cytokine Profiles for Lung Tumour Samples and Samples of Non- Malignant Lung From the Same Patients

Tumour Type	IL-1 Alpha	IL-1 Beta	IL6	TNF Alpha	TNF Beta	Beta -2 M
Lung Tumour 1	-	-	+	+	-	+
Lung Tumour 2	-	-	-	-	-	+
Lung Tumour 3	-	-	+	-	-	+
Lung Tumour 4	-	-	+	-	-	+
Lung Tumour 5	-	-	+	-	-	+
Lung Tumour 6	-	-	+	-	-	+
Non-Malignant Lung Sample 1	-	-	-	-	-	-
Non-Malignant Lung Sample 2	-	-	-	-	-	+
Non-Malignant Lung Sample 3	-	-	-	-	-	-
Non-Malignant Lung Sample 4	-	-	+	-	-	+
Non-Malignant Lung Sample 5	-	-	-	-	-	+
Non-Malignant Lung Sample 6	-	-	+	-	-	+

3.6: Effects of IL6 on the Growth of the Cell Lines.

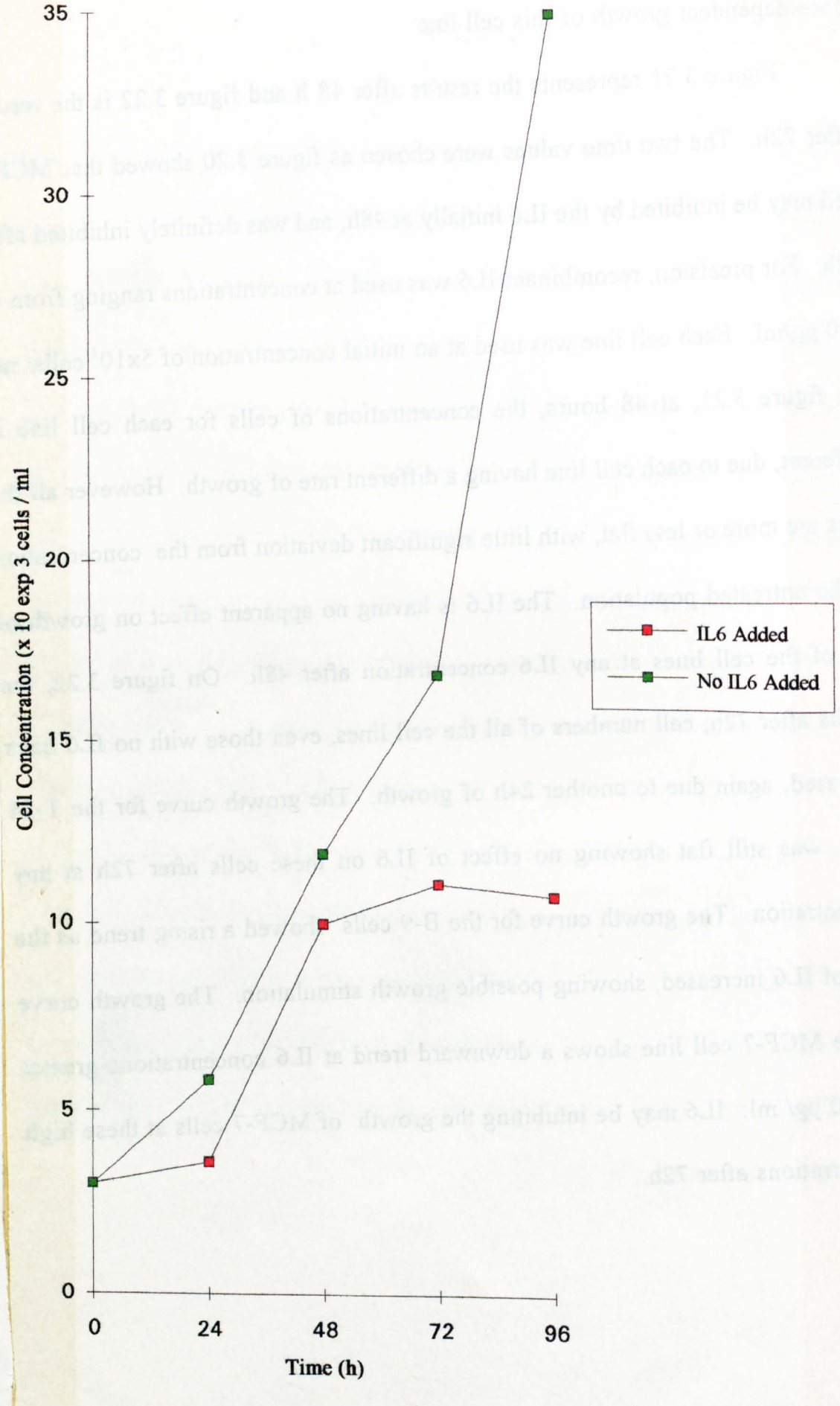
A series of experiments were carried out to investigate the effect of IL6 on the cell lines. The first experiment consisted of a study of the effects of IL6 (overall the most commonly detected cytokine) on the breast cell line, MCF-7. The method used is described on p115. The experiment studied the effect of a set dose of crude IL6 on a population of MCF-7 cells over 96h. The results are shown in figure 3.26 Two separate populations were used, a control group, and a population treated at the start of the experiment with 36 U/ml of crude IL6. Both cell concentrations were initially 3000 cells/ml. Over the course of the experiment the line derived from the control population demonstrated an exponential growth curve typical of uninhibited cell growth. In contrast the growth curve for the treated cells, flattened out after 72h; this is typical of growth inhibition.

A follow-up experiment was carried out to determine if this apparent inhibition was due to IL6 and if it was dose responsive. The result is shown in figures 3.27 and 3.28. The method used to measure growth was different, as detailed on p115. Cell numbers were estimated by dye absorbance. The greater the number of cells, the greater is the absorbance.

The B-9 cell line was used as a positive control as it was known that IL6 causes dose-dependent growth of this cell line.

Figure 3.27 represents the results after 48 h and figure 3.28 is the results after 72h. The two time values were chosen as figure 3.26 showed that MCF-7 cell may be inhibited by the IL6 initially at 48h, and was definitely inhibited after 72h. For precision, recombinant IL6 was used at concentrations ranging from 0-100 pg/ml. Each cell line was used at an initial concentration of $5x10^4$ cells/ ml. On figure 3.27, at 48 hours, the concentrations of cells for each cell line is different, due to each cell line having a different rate of growth. However all the lines are more or less flat, with little significant deviation from the concentration of the untreated population. The IL6 is having no apparent effect on growth of any of the cell lines at any IL6 concentration after 48h. On figure 3.28, the results after 72h, cell numbers of all the cell lines, even those with no IL6 have increased, again due to another 24h of growth. The growth curve for the T-24 cells was still flat showing no effect of IL6 on these cells after 72h at any concentration. The growth curve for the B-9 cells showed a rising trend as the dose of IL6 increased, showing possible growth stimulation. The growth curve for the MCF-7 cell line shows a downward trend at IL6 concentrations greater than 50 pg/ ml: IL6 may be inhibiting the growth of MCF-7 cells at these high concentrations after 72h.

Figure 3.26: Effects of IL6 on the Growth of MCF-7 Cells Over 96 h.



Note: Exp = "to the Power of", e.g. 1 x 10 exp 3 = 1000)

Figure 3.26 (Opp): Effects of Crude IL6 on the Growth of MCF-7 cells Over 96h.

The procedure for this experiment, the medium used and the culture conditions are given on page 115. Both initial cell populations were 3000 cells / ml.

Figure 3.27: Effects of Increasing Concentrations of Human Recombinant IL6 on the T-24, MCF-7, and B-9 Cell Lines After 48h.

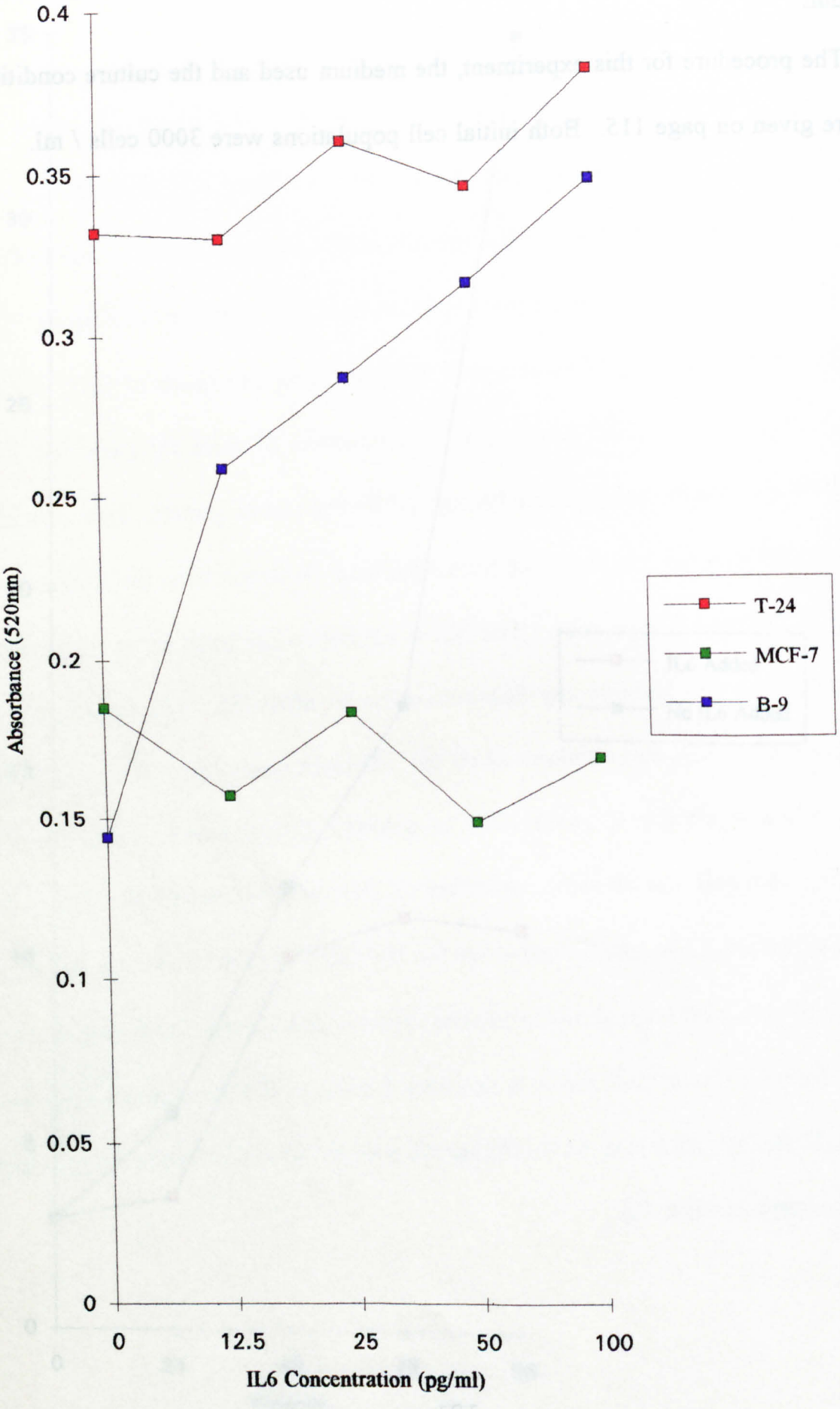


Figure 3.27 (Opp.): Effect of Increasing Concentrations (pg/ml) of Human Recombinant IL6 on the T-24, MCF-7, and B-9 Cell Lines After 48h.

The procedure for this experiment, the medium used and the culture conditions are given on page 115. Cell concentrations were evaluated as a function of absorbance as measured on a standard plate reader at 520 nm. Each point on the graph is individual, and not a mean. For this reason no error bars are plotted.

Figure 3.28: Effects of Increasing Concentrations of Human Recombinant IL6 on the T-24, MCF-7 and B-9 Cell Lines After 72h.

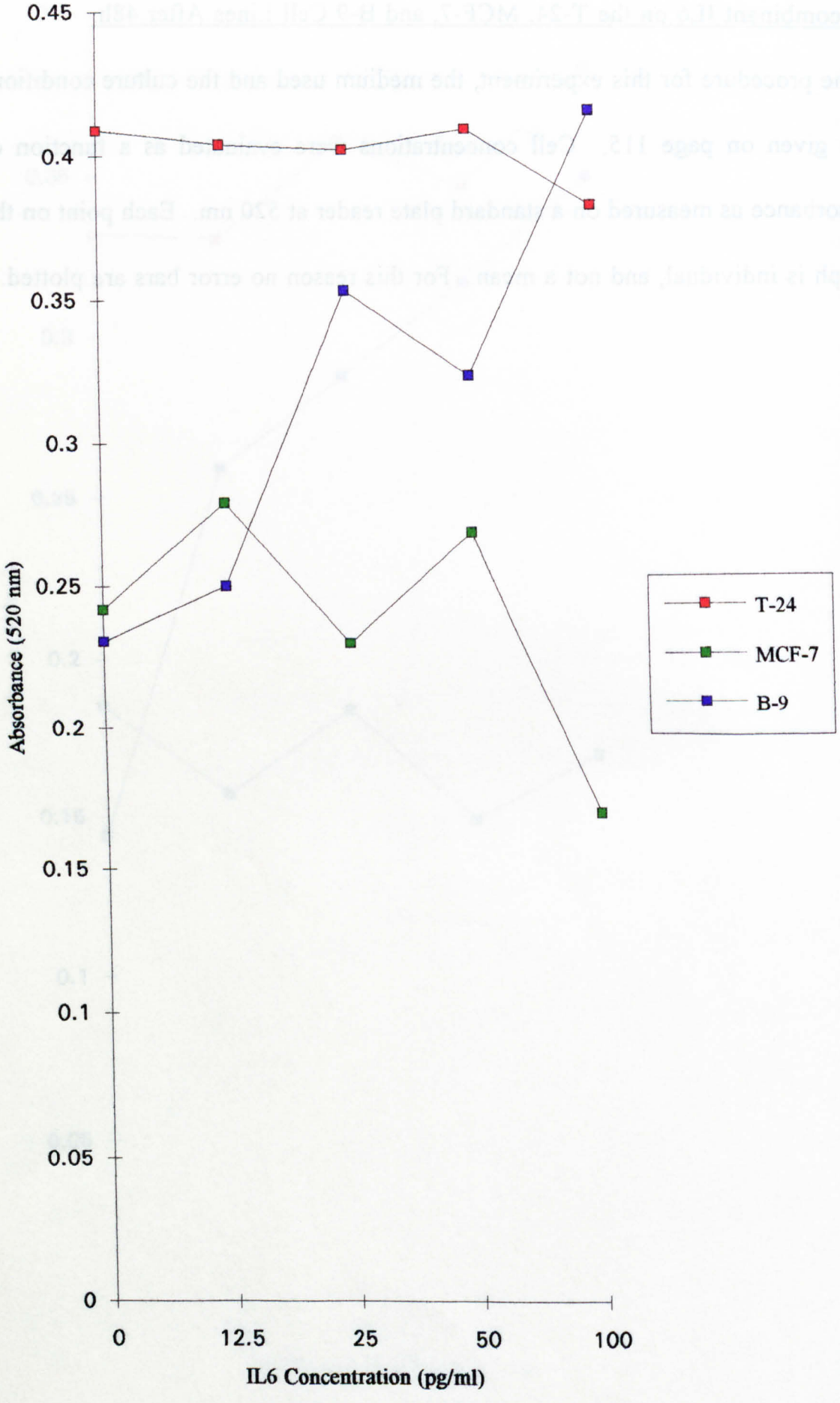


Figure 3.28 (Opp.): Effect of Increasing Concentrations (pg/ml) of Human Recombinant IL6 on the T-24, MCF-7, and B-9 Cell Lines After 72h.

The procedure for this experiment, the medium used and the culture conditions are given on page 115. Cell concentrations were evaluated as a function of absorbance as measured on a standard plate reader at 520 nm. Each point on the graph is individual, and not a mean. For this reason no error bars are plotted.

Chapter 4: Discussion.

4.1 General:

The investigations presented in this thesis were prompted by reports that some tumours could produce inflammatory cytokines. Miyauchi et al (1988) had reported the in vitro production of IL1 by 16 cell lines of various types, and Kock et al (1988) demonstrated IL1 production by malignant melanoma cell lines. In an in-vivo study, Sato et al (1987) found a factor with IL1-like bio-activity in serum from a patient with thyroid carcinoma. The T-24 bladder epithelial cell line was found to produce IL6 by Yasukawa et al (1987) and in-vivo production of IL6 was detected by Meyers et al (1991) in high grade bladder tumours. Expression of IL6 was also detected in renal carcinoma cell lines and solid renal tumours (Miki et al, 1989; and Takenawa et al, 1991). Tabibzadeh et al (1989) detected IL6 by immuno-histology in primary squamous cell carcinomas of colonic, ovarian, and endometrial origin. TNFα expression was reported in various cell lines including ovarian and lung (Spriggs et al, 1988; Kronke et al 1988) and it was detected in-vivo in high concentrations in the serum of approximately 70% of patients with ovarian and oat-cell carcinomas by Balkwill et al (1987).

The aim of the research presented here was to expand this work by examining whether the inflammatory cytokines could be produced in-vivo by some common tumour types. To this end methods were developed to detect the inflammatory cytokines in primary samples of tumours.

It was hoped that if this work were to be successful, it might lead to the development of one or more of the inflammatory cytokines as a tumour marker. The types of tumour chosen had not, at the time of commencement of the research, been previously examined for cytokine production. They were also readily and frequently available.

The methods chosen for cytokine detection were PCR followed by two types of immuno-histology. PCR was chosen, despite the possible difficulties of analysing mRNA discussed later, because of its specificity and rapidity. One PCR experiment could simultaneously profile the entire inflammatory cytokine expression of several tumours in a few days. Immuno-histology was performed on a limited number of tumours to check the results obtained in the PCR experiments and ensure that detected cytokine mRNA was being translated into protein. Examination of tumour sections by indirect immuno-fluorescence using an FITC-conjugated secondary antibody was chosen initially because of its sensitivity. At a later stage a method employing an alkaline-phosphatase conjugated secondary antibody was used because the immuno-fluorescence method did not give photographically recordable results for some tumours and could not show cell morphology clearly.

4.2: Cytokine Production by Breast Tumours.

4.2.1 Cytokine production by a Breast Cell Line:

When the breast cell line MCF-7 was examined in the PCR study, mRNA for IL1α, IL1ß, and IL6 was detected. In the indirect immuno-fluorescence experiments, there was a strong positive result for IL1α and IL6. The result for IL6 is partly corroborated by a report that a cell line similar to the MCF-7, the T-47D cell line, produces IL6 detectable by immuno-histology (Tabibzadeh et al 1989).

4.2.2 Cytokine Production in the Breast Tumours as Detected by PCR:

Twenty breast tumour samples were collected and snap frozen in liquid nitrogen as soon as possible after removal. Of these, 9 tumours had intact mRNA as determined by positive $ß_2$ microglobulin. Seven of these tumours were invasive ductal cell carcinomas of Bloom and Richardson grade II or III, one was an intraductal carcinoma ,one a mucinoid carcinoma and the last was of unknown type (its' record was unavailable). Non-malignant breast tissue was also collected from each patient for use as positive controls. None of this material had intact mRNA despite numerous attempts to extract it and re-evaluation of the methodology using macrophage-derived mRNA. PCR profiling of the 9 intact tumour samples showed no IL1α or IL1ß, but detection of IL6 in 5 tumours, TNFα in 2 tumours, and TNFß in 1 tumour.

4.2.3 Cytokine Production in Breast Tumours as Detected by Indirect Immuno-fluorescence.

Frozen sections of breast tumour 4 were selected for the immuno-fluorescence experiments. Staining was carried out for IL1α and IL6 only, because from the PCR profiling of all tumour types these were the most frequently observed cytokines. From the cytokine profile obtained from PCR analysis, breast tumour 4 was expected to give a negative result for IL1α but a positive result for IL6. As expected breast tumour 4 was observed to fluoresce only when stained for IL6. However, the fluorescence was not of sufficient duration for photographic recording (the result was independently witnessed). Subsequently, indirect immuno-fluorescence was also performed on other sections of breast tumour 4 to examine the degree of T-cell, B-cell, and macrophage infiltration in the tumour (using antibodies to the cell surface markers CD8, CD21, and CD14 respectively). All three types of lymphocyte were detected in the tumour (again fluorescence was too unstable to photograph) and all three lymphocyte types could potentially be the source of the detected IL6. However, since the indirect immuno-fluorescence technique does not show cell morphology clearly (see fig 3.12 p157) it could not be determined whether the IL6 expression was tumour-cell associated or lymphocyte associated. Preparation of frozen sections of non-malignant breast from breast tumour patient 4 was attempted, but was unsuccessful due to the tissue containing a high proportion of fat, which dissolved during the mounting process, removing the tissue as well.

4.2.4: Cytokine Production in Breast Tumours as Detected by Immuno-histology using an Alkaline Phosphatase-Conjugated Secondary Antibody.

The short duration of fluorescence observed with positive results for breast tumour 4 was repeated with sections of other tumour types subjected to the same technique. Therefore a new immuno-histology technique was adopted. This used the same primary anti-cytokine antibodies but a different secondary antibody. This was an alkaline-phosphatase conjugate capable of giving positive results of a permanent nature observable by an ordinary light microscope. Positive staining tissue would show as a bright red colour once reacted with the "fast red" enzyme substrate. Sections could also be counter-stained to highlight cell morphology.

Unfortunately, before any of these experiments could be performed, a power failure resulted in spoilage of the original frozen tumour samples. The tissue archives kept by the hospital pathology department did have paraffin-embedded samples of the tumours. However after expert consultation, the amount of material left in each sample was judged inadequate for use so a new set of tumour samples had to be collected.

Improvements in the immuno-histological technique meant that it could be used on paraffin-embedded sections. Using tissue in this way made sample collection simpler, as material could be placed in formalin by the surgeon or pathologist as soon as possible after removal and uplifted for further processing later. Paraffin-embedded tissue also offered more secure long-term storage.

However the immuno-histology technique required modification by using the primary antibodies at higher concentration.

Five breast tumours were collected and subjected to the new immuno-histology method. While it was intended to collect non-malignant breast tissue from the same patients, this was not possible as in each case the tumours were so large compared to surrounding tissue that the pathologist could not supply tissue which was guaranteed tumour-free. Three of the tumours were infiltrating ductal cell carcinomas (of grades Bloom and Richardson I, III and II respectively) and the other two infiltrating lobular carcinomas (both grade II). All five tumours gave clearly positive results when stained for IL1α and IL6. In both cases the positive staining was observed in some large ductal cell structures and surrounding stroma. Staining of the five samples using an anti-macrophage marker (the CD68 marker, chosen because it was more sensitive than the CD14 marker used previously) showed that these were present in at least one of the tumour samples but were not associated with the regions of IL1α or IL6 expression.

While expression of IL6 by the breast tumours correlated with the results obtained from the first group of tumours using PCR, expression of IL1α by the new collection of tumours did not correlate with these results as its expression was not seen in any of the tumours in the PCR group. PCR was not performed on any of the new tumours as they had all been fixed in formalin, which renders it very difficult to extract intact mRNA..

Because of this, the conflict between the lack of IL1α expression detected in the first group of tumours and the expression detected in the second group of tumours could not be adequately resolved.

It is probable that some of the tumours in the PCR group had a low level of IL1α expression. The PCR technique was based on bulk analysis of mRNA in the whole sample. RNA is easily degraded and often present at low concentrations so the PCR technique may not have been sufficiently sensitive to detect small amounts of cytokine mRNA compared to high background levels of non cytokine material. It must be remembered that of the 20 samples collected for PCR analysis, just under half expressed $ß_2$-microglobulin, and approximately half of these expressed IL6. $ß_2$-microglobulin should be expressed by all of the nucleated cells in all of the samples, but was not, indicating possible lack of sensitivity or ability to extract intact mRNA. If IL6 was being expressed (and this is suspected from the immuno-histology results) then it might be detected in some of these tumours but low level expression of IL1α might be beyond the detection limit of the PCR technique.

By contrast, the immuno-histology technique not only detected cytokine protein, which is much more stable than mRNA, but was also able to do this with great sensitivity, on a cellular scale. Cytokine expression was detected in individual cells scattered in patches throughout the tumour sections, with some sections showing good detection of IL1α.

In balance therefore, it seems probable that the results of the immuno-histology experiments were more valid than those of the PCR experiments, and therefore the final conclusion for the breast material is that some large ductal cells in these tumours can express IL1α and IL6.

If a new study were to be performed (as outlined later) then the conflict between the results could be resolved by collecting both fixed and snap-frozen tissue and examining cytokine production in each group by both immuno-histology and PCR. This study would also include, wherever possible, non-malignant breast tissue from the same patients and non-malignant material from breast reduction mammoplasties (which would require the co-operation of a plastic surgery unit at another hospital).

4.2.5 Examination of the Medical Records of the Breast Tumour Patients.

The records of all of the patients donating tumour samples (where available) were examined for features which could be due to the systemic action of the inflammatory cytokines. As previously mentioned in the introduction (pp62-63, p67) there is some evidence for an association between cachexia (characterized by weight loss) and TNFα and IL6 expression in cancer patients and animal models. There is also an association between hypercalcaemia and IL1 expression in some cancer patients (p 50).

General indicators of inflammation such as increased platelet counts, increased white blood cell counts and increased concentrations in serum of globulins were also examined, although much of the best information of this type e.g. concentrations in serum of acute phase proteins such as C-reactive protein (CRP) or erythrocyte sedimentation rates could not be used as they were not recorded in the patients' notes.

Scrutiny of the available clinical records revealed that breast tumour patients 1 and 5 had both weight loss and poor appetite. While breast tumour 5 expressed IL6, breast tumour 1 displayed no cytokine production. In the majority of the patients, weight loss was not recorded. Breast tumour patient 2 also had a poor appetite, but this patient's tumour displayed no cytokine expression. Breast tumour patients 4, 8, and 13 all had tumours expressing IL6, but showed no weight loss (breast tumour 13 showed weight gain). It appears that in all of the breast tumour patients, there was no correlation between tumour IL6 expression and weight loss. No correlations could be made between the TNFα expression in breast tumours 7 and 9 and weight loss as the records for the patients donating these tumours were either unavailable or made no record of it. Only breast tumour patient 5 had a raised serum concentration of calcium. The corresponding tumour was observed to express only IL6. No breast tumour patients expressed raised concentrations in serum of globulins. Breast tumour patients 2 and 6 displayed elevated white blood cell counts and neutrophil counts, with breast tumour patient 6 also having an elevated platelet count. Neither patient had a tumour expressing any of the inflammatory cytokines.

In conclusion, while there was good evidence for the production of IL1α and IL6 in some of the breast tumours examined (probably by the tumour cells) and weaker evidence for the production of TNFα or TNFß in a few tumours, no correlation was demonstrable between the clinical features observed in the patients and systemic action of the inflammatory cytokines.

4.3: Production of Inflammatory cytokines by a Bladder Cell Line and by Bladder Tumours.

4.3.1 Cytokine production by a Bladder Cell Line:

The bladder epithelial cell line T-24 was found to express IL1α mRNA as determined by PCR. The first set of immuno-histology experiments (using indirect immuno-fluorescence) showed that it also expressed IL1α protein, although the observed result was not long-lasting enough to photograph. Production of IL1α by the T-24 cell line has also been demonstrated by Hayashi et al (1994) confirming the result. The bladder cell line also produced IL6, which was detected both by PCR and indirect immunofluorescence. This was a good positive control for both detection methods as this cell line is a known constitutive producer of IL6 (Yasukawa et al, 1987).

4.3.2 Cytokine Production in the Bladder Tumours as Detected by PCR:

Twenty-one bladder tumours, all transitional cell carcinomas, were collected from patients undergoing cystoscopy. Of these, 13 gave detectable expression of $ß_2$-microglobulin and were judged to have intact mRNA.. In these, 7 tumours expressed IL6, 4 expressed IL1α, and 1 each expressed TNFα and TNFß. There appeared to be no correlation between cytokine expression and tumour grade or invasiveness, although the number of tumours examined was too small for meaningful correlations to be made.

4.3.3 Cytokine Production in Bladder Tumours as Detected by Indirect Immuno-fluorescence.

Frozen sections of bladder tumour 2 were selected for the immuno-fluorescence experiments. Staining was carried out for IL1α and IL6 only because from the PCR profiling of all tumour types they were the most frequently observed cytokines. From the cytokine profile obtained from PCR analysis, bladder tumour 2 was expected to give a positive result for both IL1α and IL6 and this was confirmed by the presence of fluorescence. However, the fluorescence was not of sufficient duration for photographic recording although the result was independently witnessed. Subsequently, indirect immuno-fluorescence was also performed on further sections of bladder tumour 2 to examine the degree of T-cell, B-cell, and macrophage infiltration in the tumour (using antibodies to the cell surface markers CD8, CD21, and CD14 respectively). Only B-cells were detected (again fluorescence was too unstable to photograph) and these could potentially be the source of the detected IL6.

However, since the indirect immuno-fluorescence technique does not show cell morphology clearly (see fig 3.12 p157) it could not be determined whether the IL6 expression was tumour-cell associated or B-cell associated.

4.3.4: Cytokine Production in a Bladder Tumour as Detected by Immuno-histology using an Alkaline Phosphatase-Conjugated Secondary Antibody.

Three bladder tumours were collected, all of which were transitional cell carcinomas. Of these only one was suitable for immuno-histology, as the others were necrotic. This tumour, bladder tumour 14, gave a weak positive result for IL1α. The cytokine was observed in the cytoplasm of two large cells of unusual morphology thought to be malignant cells. Use of an antibody to the macrophage marker CD 68 failed to detect macrophages in the tumour tissue. Therefore it is probable that IL1α production in bladder tumours is tumour-cell associated.

4.3.5 Examination of the Medical Records of the Bladder Tumour Patients.

Examination of the clinical records of all of the patients donating bladder tumours yielded the following: Only one patient displayed weight loss concurrent with the detection of IL6 in the donated tumour (bladder tumour 2). However bladder tumour patients 4 and 7, both having tumours expressing IL6, had no weight loss. None of the bladder tumour patients had increased concentrations of calcium or globulins in their serum.

Bladder tumour patient 2 had an elevated white blood cell count, and bladder tumour patient 3 had an elevated platelet count. Bladder tumour 2 had detectable IL1α and IL6, but bladder tumour 3 expressed no cytokines. These revealed no apparent correlation between cytokine expression in the bladder tumours and corresponding clinical conditions in the patients.

4.4: Production of Inflammatory Cytokines by Lung Tumours and Non-Malignant Lung Tissue.

4.4.1: Cytokine Production in Lung Tumours and Non-Malignant Lung as Determined by PCR.

Eleven tumour samples were collected, 6 of which gave a positive result for $ß_2$-microglobulin, and hence contained intact mRNA.. Three of the tumours were moderately differentiated squamous carcinomas, one was a poorly-differentiated adenocarcinoma, one was an invasive large cell carcinoma, and one was a bronchial carcinoid tumour. Intact mRNA was obtained from non-malignant tissue obtained from tumour-bearing patients 2, 4, 5, and 6. None of the tumours or non-malignant lungs expressed detectable IL1α or IL1ß, but IL6 was detected in 5 tumour samples, and 2 non-malignant lung samples. TNFα was detected in 1 tumour sample and in none of the non-malignant samples.

4.4.2 Cytokine Production in Lung Tumours and Non-Malignant Lung as Detected by Indirect Immuno-fluorescence.

Frozen sections of lung tumour 4 and non-malignant lung 4 were selected for the immuno-fluorescence experiments. Staining was carried out for IL1α and IL6 only because from the PCR profiling of all tumour types they were the most frequently observed cytokines. From the cytokine profile obtained from PCR analysis, lung tumour 4 was expected to give a positive result for IL6 only and as expected lung tumour 4 was observed to fluoresce only when stained for this cytokine. However, the fluorescence was not of sufficient duration for photographic recording although the result was independently witnessed. Subsequently, indirect immuno-fluorescence was also performed on other sections of lung tumour 4 to examine the degree of T-cell, B-cell, and macrophage infiltration in the tumour (using antibodies to the cell surface markers CD8, CD21, and CD14 respectively). All three types of lymphocyte were detected in the tumour (again fluorescence was not sufficient to photograph) and any of these lymphocyte types could potentially be the source of the detected IL6. The non-malignant lung tissue from the same patient expressed IL6 only, in accordance with the result expected from PCR profiling. It also contained all three types of lymphocytes, any of which could be the source of IL6 expression. However, since the indirect immuno-fluorescence technique does not show cell morphology clearly (see fig 3.12 p157) the type of cells expressing it could not be determined.

4.4.3: Cytokine Production in a Lung Tumour and Non-malignant Lung Tissue as Detected by Immuno-histology using an Alkaline Phosphatase-Conjugated Secondary Antibody.

One lung tumour was used, of unknown histological type (the record for this tumour patient was unavailable). Non-malignant lung from the same patient was also used along with a sample of non-malignant lung from a non-cancer patient. The lung tumour gave positive staining for IL6 only, which was observed in a large cell suspected to be malignant. Staining of the tumour with macrophages failed to detect their presence. The non-malignant lung from this patient also stained positively for IL6 only, this time the result was observed in large surface cells. Staining for macrophages showed these to be present in the non-malignant tissue, but they were not of the same cell morphology as the large surface cells producing IL6. The non-malignant lung from a non-cancer patient showed no expression of IL1α or IL6 and no macrophages present. Partial corroboration of the expression of IL6 by the lung tumour cells is provided by Mizuno et al (1994) who reported the production of IL6 in-vitro by some lung tumour cell lines. Absence of IL1α in the lung tumours in all the studies is confirmed by Arias-Dias (1994).

4.4.4 Examination of the Medical Records of the Lung Tumour Patients.

Examination of the available clinical records of all the patients donating lung tumours yielded the following: None of the lung tumour patients showed any correlation between type or grade of tumour and IL6 expression. Weight loss was observed in lung tumour patient 2. However, this did not correlate with cytokine expression as lung tumour 6 expressed IL6 but lung tumour 2 did not. Lung tumour patient 6 displayed an elevated globulin count, white blood cell count and neutrophil count. Lung tumour patients 3 and 4 had elevated white blood cell counts and neutrophil counts. All 3 of the corresponding tumours displayed detectable IL6. This might indicate a tenuous link between IL6 expression in these patients and systemic inflammation. However, since lung tumour patient 5 had a tumour displaying IL6 but no signs of inflammation, and the correlations between elevated globulin concentrations, white blood cell counts, and neutrophil counts and IL6 expression are not as good as those between elevated acute phase protein concentrations and IL6 expression, no clear conclusions can be made.

4.5: Production of Inflammatory Cytokines by Ovarian Tumours.

Only two ovarian tumours were collected, as few tumours of this type were available. Two non-malignant cystic ovaries from a non-cancer patient were also collected.

4.5.1: Cytokine Production in Ovarian Tumours and Non-Malignant Ovarian tissue as Determined by PCR.

Malignant ovarian tumour 1 expressed IL1 α and IL1 ß only, and malignant ovarian tumour 2 expressed TNFα only. The 2 non-malignant ovaries from different patients both displayed IL1α only. The results might imply an association between IL1ß production and ovarian tumours, but the number of tumours examined was too small to say this with any degree of certainty. The fact that no IL6 was detected in either tumour is contradicted by Plante et al (1994) who found that there was IL6 in the serum of many ovarian cancer patients. These concentrations correlated with tumour size and ascites volume, but not with survival time or grade. further studies are required with a larger number of malignant ovarian tumours in order to establish the possible association between IL1βand these tumours.

There was insufficient ovarian tumour or non-malignant ovarian material for use in the immuno-fluorescence experiments, and no tumours or non-malignant material were available during the course of the immuno-histology experiments carried out using the alkaline phosphatase method.

4.5.2Examination of the Medical Records of the Ovarian Tumour Patients.

Examination of the available clinical records of all the patients donating material yielded the following: Neither of the two ovarian tumour patients displayed weight loss and neither had an elevated concentration in serum of globulin, but malignant ovary patient 1 had elevated white blood cell and platelet counts. This may possibly have been due to systemic action of IL1, but this cannot be concluded for certain from the available data.

4.6: Possible Effects of IL6 on Tumour Cells.

Since IL6 was the most commonly observed cytokine in most of the tumour types studied, experiments were carried out on the T-24 bladder and MCF-7 cell lines to determine if IL6 had any effect on cell growth A previous study by Miki et al (1989) showed that IL6 was an autocrine growth factor for a renal carcinoma cell line. It was therefore possible that IL6 might be an autocrine growth factor for either cell line as both were observed to produce it. Two experiments were performed

. The first examined the effects of crude IL6 on the growth of the MCF-7 cell line over a 96h period. Inhibition of growth was observed after 48h at a dose of 36 U/ml.

The second experiment examined the effect of recombinant IL6 on growth over a 72h period. The effects of IL6 were also measured on the T-24 cell line and the B-9 hybridoma cell line (which is dependant on IL6 for growth) which was used as a positive control. This experiment showed that growth of the MCF-7 cell line was inhibited by concentrations of recombinant IL6 greater than 30 pg/ml after 48h, but that the growth of the T-24 cell line was unaffected by any dose of IL6 used. As expected, IL6 promoted the growth of the B-9 hybridoma in a dose-dependant manner.

The results of the experiments may point to IL6 being an autocrine growth inhibitor for MCF-7 cells. The findings agree with those of Chen et al (1988), who have reported that IL6 causes growth inhibition in this cell line at doses of 10 - 250 U/ml. Danforth et al (1993) also report growth inhibition of MCF-7 cells by IL6, but at a higher concentration of 1000 U/ml. Significantly, they also observed growth inhibition by IL1α at the same concentration. This growth inhibition was even greater than that seen with IL6. However, most noticeable of all was a synergistic effect of IL1α with IL6. This may be very significant, as the MCF-7 cell line was seen to produce both cytokines constitutively in the experiments described in this thesis.

The mechanism whereby IL6 might be an autocrine growth inhibitor for MCF-7 cells is unclear. The fact that it was only detected in cell cultures at full confluence may have some significance. These cultures were incapable of further growth due to no more free surface area being available for expansion. It might be logical to assume that this set of circumstances could result in the production of a growth inhibitor by the cells. However, it is also worth noting that due to the work of Danforth et al, the growth inhibition is probably a result of added IL6 working in synergy with constitutively produced IL1α. In the experiments described here, this effect of IL1α was unknown as the work predated that of Danforth et al. Therefore, no effort was made to block the synthesis of IL1α by the cells, or add it with IL6 to see if this augmented the growth inhibition. It is also possible that neither IL1α or IL6 is the primary mediator of the growth inhibition.

It is possible that they stimulate the production of a secondary factor, and this may explain growth inhibition occurring after 48h. A likely candidate for this substance is Transforming Growth Factor Beta (TGFß). Knabbe et al (1987), and Zugmier and Lippman (1990), have both demonstrated that TGFß is a potent growth inhibitor for the MCF-7 cell line. Danforth et al demonstrated that IL1α (which had more of a growth inhibiting effect than IL6) increased the production of TGFß, lending some credence to the idea of the involvement of TGFß in the growth inhibition.

The true nature of growth inhibition in the MCF-7 cell line would seem therefore to be complex, but does seem to involve IL6. It would be worthwhile to perform future experiments, perhaps using antibodies to IL1α, IL6, and TGFß to see how this might affect the growth inhibition. This study could also be expanded to examine the precise mechanism of IL1α and IL6 expression in the MCF-7 cells.

4.7: Final Conclusions and Viability of the Inflammatory Cytokines as Tumour Markers.

The research presented in this thesis showed that all of the inflammatory cytokines could be observed in primary samples of tumours by the detection methods employed. However, because of the small number of tumours examined, further studies need to be undertaken to confirm the findings and investigate the usefulness of the inflammatory cytokines as tumour markers. These would incorporate larger numbers of tumours, and where possible, the use of non-malignant control tissue from both tumour-bearing patients and normal individuals. An examination of concentrations of cytokines in serum from tumour patients and normal individuals would also be necessary.

4.7.1 Recommendations for Methodologies to Detect Cytokines in Samples of Primary Tumours.

The studies presented in this thesis have demonstrated that reverse-transcription-PCR (RT-PCR) can detect cytokines in some bulk tumour samples. However, there are problems with the technique as it stands. In nearly all cases only 50% of tumours collected had intact mRNA as assessed by the presence of $ß_2$ microglobulin. This is probably due to time delays in obtaining tumour samples These may be unavoidable due to the need for samples to be examined by a pathologist prior to being snap frozen. In the case of bladder tumours the nature of tumour removal leads to destruction of the integrity of the tumour. It was also thought that the RT-PCR method was not sufficiently sensitive enough to detect low levels of cytokines in some of the material.

IL1α could not be detected in breast tumours by this method and could be detected by immuno-histology. Clearly the sensitivity of the technique would need to be increased. One approach to this would be to employ a PCR technique using nested primers.
In this technique, PCR is performed as normal, but the products of the amplification are then subjected to a further round of PCR using internal primers to sequences in the product DNA. This results in much higher amplification and therefore is a good method for detecting very small amounts of mRNA in bulk tissue samples. One example of a successful application of this technique has been the use of a nested-primer PCR protocol to detect very small amounts of endothelin-2 and endothelin-3 (both low molecular mass vasoactive polypeptides) in human endometrium (O'Reilly et al 1992).

Both the indirect immuno-fluorescence and alkaline-phosphatase based immuno-histology techniques successfully detected cytokine polypeptide in all of the tumour samples in which they were used. Comparison of the results of the two methods would suggest that the second technique is superior. Not only is it sensitive but it also gives a clear picture of cell morphology. This is not the case with indirect immuno-fluorescence. However immuno-histology techniques have limitations. While they can detect some cytokine polypeptide in cells, most is observed in the surrounding stroma (this was particularity the case with IL6 in the breast tumours) so direct cell-association with particular cytokines often cannot be clearly demonstrated. While better verification of cell-associated cytokine protein can be obtained using confocal microscopy it is only in-situ hybridisation that achieves this beyond doubt.

First developed by Gall and Pardue in 1969, this method can detect particular sequences of mRNA in cells. If it were used to detect cytokine mRNA in tumour sections then any cell association would be clearly demonstrated.

4.7.2: Assessment of the Usefulness of Inflammatory Cytokines as Tumour Markers.

The findings presented in this thesis, when considered in association with other published work would suggest that all of the inflammatory cytokines could potentially be used as tumour markers, with IL1α and IL6 being the best candidates. This is based on their frequency of occurrence in most tumour types. However, their usefulness as tumour markers would depend on how well they fulfil the criteria of Beastall et al, outlined on p23. Assessment of specificity or sensitivity as outlined by Beastall et al cannot be determined. Lack of suitable control material for most of the tumour types examined meant that numbers of false positive or false negative results could not be determined and these are necessary to work out true specificity or sensitivity of a tumour marker. False positive or negative results can only be determined by using healthy individuals as controls, and this cannot be done unless the assessment of the tumour marker is based on its measurement in serum or bodily fluids. Where the terms are mentioned in the following sections, they are used in a different sense ie: specificity of detection, meaning that the cytokine was detected in the tumour tissue but not in non-malignant material of the same type (often from the same patient), and sensitivity of detection, meaning how easily the cytokine was detected in the tumour sample.

4.7.2.1.: Usefulness of IL1α as a Tumour Marker.

In the experiments reported in the thesis IL1α was observed in breast tumours, bladder tumours and ovarian tumours. The sensitivity of detection of it was good in all three tumour types, but the degree of specificity of detection could not be adequately assessed due to control tissue only being available for the ovarian tumours. This demonstrated that both malignant and non-malignant ovarian tissue could produce IL1α. There was no correlation between IL1α expression in the tumours and tumour grade, stage, or mass. However, these features, which are necessary to define a prognostic marker, would be best observed from measurements of IL1α concentration in bodily fluids (especially serum). This was beyond the scope of this thesis but could be the subject of further study if adequate methods for measurement of IL1 in bodily fluids became available. Eastgate et al (1990) demonstrated that IL1 is bound to a binding protein in plasma. While Capper et al (1990) showed that IL1ß can be dissociated from its binding protein by acid treatment it is not possible to do this with IL1α. The bound form of IL1α in serum cannot accurately be measured by sandwich ELISA (enzyme-linked immuno-sorbant assay). Bio-assays for IL1α will also not give an accurate result due to the presence of an IL1 antagonist in serum and other bodily fluids (Liao et al 1984; Mazzei et al ,1990). It therefore seems unlikely that IL1α would make a useful biochemical marker in cancer. Indeed the difficulty in its accurate measurement may explain why, for example, Moradai et al (1993) failed to detect IL1α in the serum of ovarian cancer patients.

4.7.2.2.: Usefulness of IL6 as a Tumour Marker.

IL6 occurred with reasonable frequency in breast, bladder, and lung tumours (10 /13, 7/13, and 6/7 samples respectively). Sensitivity of detection (judging from the immuno-histology results) was good. Specificity of detection was not good, as IL6 could be detected in non-malignant lung (3/5 samples) and IL6 expression has also been reported in non-malignant breast tissue (Basolo et al, 1993). There were no apparent correlations between tumour IL6 expression and prognostic factors. However to ascertain this properly it would be necessary to measure IL6 concentrations in bodily fluids of large numbers of cancer patients and compare them with similar measurements in healthy individuals. Unlike IL1α, IL6 can be measured in serum. It does bind to a carrier protein but this does not affect its measurement by ELISA and its bound form is bio-active. It has been successfully measured in the urine of bladder cancer patients (Seguchi et al, 1992) and found to be specific (39 cancer patients had raised concentrations of IL6 in their urine but there was no significant expression in 15 healthy individuals) and to correlate with progression of tumour stage. Ferdighini et al (1994) successfully showed a correlation between raised concentrations of IL6 in the serum of cervical cancer patients and tumour stage, and Plante et al (1994) demonstrated that concentrations of IL6 in serum from ovarian cancer patients correlates with tumour size and ascitic volume. Gogusev et al (1993) report good specificity for IL6 in renal carcinomas but no clinical correlations.

It therefore seems possible that IL6 is a good candidate for a new tumour marker and it would be rewarding to measure concentrations in serum of IL6 in breast, bladder and lung tumour-bearing patients to establish whether there is concentration-dependent specificity and correlation between IL6 concentrations in serum and prognostic features.

4.7.2.3.: Usefulness of TNFα as a Tumour Marker.

In the studies performed in the thesis, TNFα was seen to occur with fairly low frequency but was seen in a few samples from all the tumour groups (2/9 breast tumours, 1/13 bladder tumours, 1/6 lung tumours). The number of tumours of any type was too small to establish whether correlations could be made between TNFα expression and prognostic factors such as tumour stage or grade.

However, the method used to detect TNFα was RT-PCR, which showed poor sensitivity. In the breast tumours, TNFα was detected in only 2 out of 9 tumours. Miles et al (1994), using immuno-histology, found TNFα expression in stromal cells in 43 out of 49 breast tumours examined, and this expression correlated with tumour grade. Bebok et al (1994) also detected TNFα by immuno-histology in malignant breast tumours and found that its expression correlated with clinical staging, tumour grade, lymph node metastasis, tumour recurrence and survival time of the patient. Immuno-histological methods have also successfully detected TNFα in ovarian tumours.

Takeyama et al (1991) found TNFα expression in 16 out of 20 ovarian tumours and in 4 out of 5 ascitic fluid specimens. In the RT-PCR experiments, 1 out of 2 malignant ovarian tumours expressed TNFα so this tenuously agrees with their results. It seems likely therefore that further work on TNFα as a potential histological marker for some types of tumour is warranted. Furthermore Knapp et al (1991) have demostrated increased concentrations of TNFα in the serum of cachectic breast tumour patients with stage IV tumours using an ELISA.. This work should be followed up.

4.8: Outlines for Future Work.

Of all the results presented in the thesis, those obtained from the breast tumours offer the best area for future investigation. At present published confirmatory evidence that these tumours produce IL6 and IL1α is not available. It would therefore be desirable to initiate a new study to examine IL1α and IL6 expression in breast tumours in more detail. The study could be set up as follows:

1) A new collection of breast tumours would be gathered, the number of samples being large enough (preferably over 30) to give enough material for proper statistical correlations. Non-malignant breast tissue from the same patients would be collected, if possible, to determine the specificity of any cytokine expression and additional controls would be introduced through the input of normal breast material, for example from breast reduction mammoplasties.

2) PCR and immuno-histology using the alkaline phosphatase technique would be employed in tandem where tumour samples were large enough (tissue would have to be collected both snap-frozen and fixed in formalin) and thus reduce the possibility of conflicting results such as those already observed in the thesis.

3) Blood samples from all patients in the study would be collected, allowing measurement of serum cytokine concentrations. Cytokines expressed by tumours may not necessarily enter the bloodstream of the patients and this could only be determined by comparing cytokine concentrations in the serum samples from the tumour group with those in the control group. Limitations on the ability of methods to measure IL1α accurately in serum would have to be taken into account.

4) Both erythrocyte sedimentation rates, and concentrations in serum of acute phase proteins such as CRP correlate well with increased concentrations in serum of IL6 (Bedell et al, 1985) Consequently, the intention would be to introduce such assays into the work.

5) The samples used in the second immuno-histology experiments could be re-examined (they are held in storage) using in-situ hybridisation to probe for IL1α mRNA as successful detection of this would provide a means of resolving the conflicting results already reported between the original PCR and immuno-histology study. If the technique proved successful on these samples it could also be used on the material collected in the new study, allowing the cellular source of detected cytokines to be verified.

The information gained from this extended study when added to the data already reported in this thesis would establish whether IL6 is a useful biochemical breast tumour marker and perhaps establish IL1α as a histological marker. This plan of investigation could also be adopted to examine the production of cytokines by other tumour types not covered by the thesis. The scope for further research is considerable and many questions remain to be answered.

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