STUDIES OF CYTOKINES IN THE BIOLOGY AND TREATMENT OF CANCER.

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A thesis submitted in fulfilment of the requirements of the University of London for the degree of Doctor of Medicine.

April 1995

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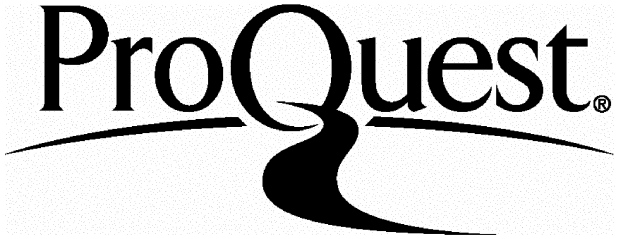

ABSTRACT.

While cytokines produced by infiltrating mononuclear cells in primary breast cancer might mediate a host immune response to tumour, they have other properties which could promote tumour development. The first part of the thesis describes the site and extent of tumour necrosis factor (TNF-α) expression in human breast tissue. Cells expressing TNF mRNA were rarely noted in normal breast tissue. Greater levels were noted in invasive breast cancer but expression was focal, scanty and found in the stroma adjacent to tumour islands. The presence of TNF-α protein was assessed and its production by cells of the monocyte lineage was confirmed. The number of infiltrating macrophages did not vary with tumour grade, but the proportion of them expressing TNF-α correlated with increasing tumour grade. This finding, coupled with the observation that TNF-receptors were not detected on tumour cells but rather on other infiltrating mononuclear cells and vascular endothelium, led to the hypothesis that the net effect of TNF-α expression in breast cancer could be to promote tumour progression. Further studies investigated expression of the TNF-α inducible enzyme, nitric oxide (NO) synthase which, by generating the vasoactive agent NO, may modulate tumour blood flow and promote angiogenesis. Biochemical assays demonstrated raised levels of activity of calcium dependent and independent isoforms of NO synthase in malignant compared with benign breast tissue. A correlation between NO synthase activity and tumour grade was noted and histochemical studies localised expression to macrophages, endothelium and myoepithelium. TNF-α may also regulate production and activation of the type IV collagenases. Zymography of breast tumour tissue demonstrated that the proportion of the 72kDa isoform of the enzyme in the activated state correlated with tumour grade and that expression of the 92kDa isoform was significantly raised in high grade tumours.

In the second part of the thesis the clinical and biological effects of prolonged infusions of interleukin-2, given in an attempt to augment the host immune response to tumour, were studied. Mechanisms of toxicity were investigated, with particular respect to induction of cytokines and nitric oxide, and comparison of lymphocyte trafficking to tumour and normal tissue in response to therapy were made. At the level of primary tumour and in metastatic disease in response to systemic therapy, any anti-tumour effects of cytokines may be offset by those which promote tumour progression or cause treatment toxicity.

ACKNOWLEDGEMENTS

Originally, the work for this thesis was based around the clinical trials of recombinant interleukin-2 which were carried out at the ICRF Clinical Oncology Unit, Guy's Hospital and are described in the second part of the thesis. The subject of induction of other cytokines such as TNF-α and IFN-γ as well as other biological effects of rIL-2 in the patients treated, were addressed when Dr. Frances Balkwill kindly allowed me time, space and guidance in the Biological Therapies Laboratory at the Imperial Cancer Research Fund. There I met Stuart Naylor who had developed *in situ* hybridisation in order to assess expression cytokines within human tumours. Under his patient tutoring, I examined TNF-α expression in primary breast cancer and observed that its presence was related to tumour grade. The possibility that TNF-α might actually promote the malignant process was further explored by investigating production of type IV collagenases with Bernard Davies, then a post-doctoral fellow in Dr Balkwill's Laboratory. Another aspect of TNF-α expression, both in primary tumours and as a consequence of therapy with interleukin-2, namely induction of the vasoactive compound nitric oxide, was investigated in collaboration with Salvador Moncada's group at the Wellcome Foundation and in particular, Lindy Thomsen. In all these studies immunolocalisation of TNF-α, collagenases and NO synthases has been a major feature and this was performed when I returned to the Clinical Oncology Unit at Guy's under the expert guidance of Lisa Happerfield with pathological advice from Lynda Bobrow. None of these studies would have been possible without the co-operation of patients, surgeons, pathologists and other staff of the ICRF Clinical Oncology Unit at Guy's or without the support and supervision of Prof. R.D.Rubens, director of the unit. As well as my sincere thanks to the above and those who supplied antibodies for the studies who are cited in the text, I would also like to thank the following for their support/assistance/advice: Dan Aderka, Frances Burke, Dorota Griffin, Peter Harper, Simon Joel, Steve Kelly, Richard Knowles, Saleem Malik, Robert Moore, Khaled Saraya, Ken Soo, Parames Thavasu, and David Wallach.

This thesis is dedicated to the patients of the Oncology Unit at Guy's whose courage knows no bounds and to Maria and Helena whose patience and tolerance know no bounds either.

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ABBREVIATIONS USED IN THIS THESIS.

ABC	avidin biotin complex
APAAP	alkaline phosphatase anti-alkaline phosphatase
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cNOS	constitutive nitric oxide synthase
CSF-1	colony stimulating factor-1
CTL	cytotoxic T-lymphocyte
CTP	cytidine triphosphate
DAB	3,3'-diaminobenzidine
DABCO	diazobicyclo-octane
DCIS	ductal carcinoma in situ
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DW	distilled water
EDRF	endothelial derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylenebis(oxy-ethylenitrilo)tetraacetic acid
ELAM-1	endothelial leucocyte adhesion molecule-1
ER	oestrogen receptor
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GTP	guanosine triphosphate
HLA	human leucocyte antigen
HPF	high power field
HPLC	high pressure liquid chromatography
ICAM-1	inter cellular adhesion molecule-1
IDO	indoleamine 2,3 dioxygenase
IFN	interferon
IL-	interleukin-
IL-2R	interleukin-2 receptor
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal

i.v.	intravenous
kDa	kilodalton
LAK	lymphokine activated killer cells
LFA-1	lymphocyte function associated antigen-1
L-NIO	L-imino ethyl arginine
L-NMMA	L-*N* monomethyl arginine
LPS	lipopolysaccharide
MCP	monocyte chemotactic protein
M-CSF	monocyte-colony stimulating factor
2-ME	2-mercaptoethanol
MEM	modified eagles medium
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MT-MMP	membrane type matrix metalloproteinase
NADPH	nicotinamide adenine dinucleotide phosphate
NK	natural killer cell
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitrite and nitrate
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF	platelet derive growth factor
PECAM	platelet endothelial cell adhesion molecule
PEM	polymorphic epithelial mucin
PFA	paraformaldehyde
PMN	polymorphonuclear leucocytes
PR	progesterone receptor
rIL-2	recombinant interleukin-2
RIA	radio-immunoassay
RNA	ribonucleic acid
RT	room temperature
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate buffer

sIL-2R	soluble interleukin-2 receptor
sTNF-R	soluble TNF receptor
TBS	tris buffered saline
TESPA	3-aminopropyltriethoxysilane
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TNF-R1	low molecular weight tumour necrosis factor receptor
TNF-R2	high molecular weight tumour necrosis factor receptor
TRITC	tetra methyl rhodamine isothiocyanate
UTP	uridine triphosphate
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late after activation antigen-4

Chapter 1.

MONONUCLEAR FUNCTION AND CYTOKINES IN CANCER.

1:1 The inflammatory infiltrate in primary breast cancer.

The presence of a lymphoplasmacytic infiltrate in primary breast cancer has been taken as evidence of a host immune response to tumour. Since spontaneous regression of tumours in general and infiltrating breast cancer in particular is rare, such an immune response is ultimately ineffective. Whether or not immune effector cells may at least impede tumour development is unclear and how the presence of a lymphoplasmacytic infiltrate relates to prognosis has been the subject of conflicting reports. Although studies agree that tumours with a large infiltrate are more likely to be oestrogen receptor (ER) negative, evidence of an independent association between infiltrate and tumour grade is conflicting (Champion et al 1972, An et al 1987). Hamblin (1968) noted that the presence of an inflammatory infiltrate had little effect in well-differentiated tumours, but was associated with a better prognosis in higher grade tumours. Similarly, Underwood (1974) suggested that an inflammatory infiltrate was associated with a good prognosis in poorly-differentiated, ER negative tumours. Although a more recent study also supports this view (Aaltoma et al 1992) other large studies have questioned this association (Rosen et al 1989, Kurtze et al 1990, Rilkhe et al 1991).

The development of monoclonal antibodies to leucocyte subsets has allowed the assessment of the relative proportions of effector cells such as T and B lymphocytes, natural killer (NK) cells and macrophages in primary breast cancer.

1:1:1 T-lymphocytes.

Initial studies suggested that T-cells were the predominant cell type (Schoorl et al 1976) being localised in stromal areas in contrast to normal breast in which they were found in epithelial areas (Lwin et al 1985). The predominance of cytotoxic/suppressor T-cells (CD8) has been reported by some groups (Bahn & DesMarais 1983, Rowe and Beverley 1984) whereas others have reported a predominance of CD4 (helper/inducer) T-cells (Whitewell et al 1984, An et al 1987). The reasons for these conflicting data are unclear, but the relatively small numbers of cases in each study and the focality of infiltrating T-cells (eg in perivascular areas) may contribute to the discrepancies. A further complicating factor is the observation that the CD4 antigen may be expressed on monocytes as well as helper/inducer T-cells (Moscicki et al 1983). In studies in which numbers of CD4 positive cells exceeded the number of T-cells defined by a pan-T-cell marker, expression of CD4 by infiltrating monocytes cells may have accounted for this discrepancy (Gottlinger et al 1985).

1:1:2 Monocytes/Macrophages.

Tumour-associated macrophages are a major component of the lymphoplasmacytic infiltrate in primary breast cancer. Early studies assessing the presence of macrophages used lysozyme as a marker (Nash et al 1981, Tanaka et al 1986) and may have underestimated the number of macrophages present. More recent studies using the marker UCHM-1 (CD14) demonstrated larger numbers of macrophages in benign and malignant breast disease (Lwin et al 1985, Steele et al 1985). Using a monoclonal antibody to CD68 (EBM/11), Kelly et al (1988) demonstrated that large numbers of macrophages were present in malignant breast tissue

occasionally out-numbering tumour cells, in this study there was no relationship between macrophage numbers and tumour grade. Other studies have suggested however that there is a correlation between the degree of macrophage infiltration and tumour grade (Zuk and Walker 1987, van Netten et al 1992). The relationship between the degree of macrophage infiltration in the primary tumour and other prognostic factors is also unclear. Lauder et al (1977), noted that high macrophage numbers were associated with absence of nodal metastases whereas the study by Zuk and Walker noted no correlation between regional lymph node status and macrophage numbers when the marker EBM/11 was used, but suggested that large numbers of macrophages in the primary tumour defined by the antibody Y1/82A (CD68) were associated with the presence of lymph node metastases.

1:1:3. Other cell types.

Most of the aforementioned studies agree that B-cells and antigen-independent natural killer (NK) cells are a minor component of the lymphoplasmacytic infiltrate in breast cancer.

1:2 Functional analysis of leucocytes in breast cancer.

Owing to the difficulty in isolating lymphocytes from sites of primary breast cancer, most studies have focused on the immunocompetence of immune effector cells in the peripheral circulation.

1:2:1. Natural killer cell activity.

Animal studies suggest that antigen independent, MHC unrestricted NK cells may be important in the host immune response to tumour (Hanna 1985) and most studies agree that NK numbers (Hajto and Lanzrein 1983) and function (Cunningham-Rundles et al 1981, Garner et al 1983) are reduced in untreated patients with breast cancer. Reduced NK activity in such patients is further depressed during adjuvant chemotherapy (Brenner et al 1986) though this could be overcome *in vitro* at least, by interleukin-2.

NK activity within primary breast tumours had been investigated in few studies but reduced cytotoxicity has been reported (Miescher et al 1987) which is generally lower than that observed in the peripheral circulation of the same patient (Gudmundsdottir et al 1992).

1:2:2. T-cell responses.

Specific immune responses to putative tumour associated antigens have been difficult to demonstrate in human breast cancer, so much of the assessment of cytotoxic T-cell activity has been through indirect means such as peripheral lymphocyte counts, proliferative responses of peripheral T-lymphocytes *in vitro* and response to recall antigens *in vivo* using delayed type hypersensitivity (DTH) testing. In 1971 Whittaker and Clark demonstrated reduced responsiveness of lymphocytes to mitogens *in vitro* and recall antigens *in vivo* in patients with advanced stage breast cancer. They also noted that addition of serum from these patients was capable of reducing the blastogenic response of lymphocytes isolated from normal individuals. Using a combination of *in vitro* and *in vivo* lymphocyte responses, Adler et al (1980) demonstrated reduced function with increasing

tumour stage and suggested that assessment of lymphocyte function may be of prognostic relevance within tumour stage. The observed lack of responsiveness of peripheral T-lymphocytes from patients with breast cancer has been attributed to reduced IL-2 receptor expression (Hakim 1988).

T-lymphocytes isolated from primary breast tumours show reduced proliferative activity when compared with autologous peripheral lymphocytes an effect which may be humoral (Whittaker and Clark 1971) or mediated by tumour cells directly (Miescher et al 1986).

Few studies have assessed specific anti-tumour activity of freshly isolated tumour associated lymphocytes but those which have report negligible cytotoxic activity against autologous tumour cells (Balch et al 1990, Schwartzentruber et al 1992).

1:2:3. Macrophage cytotoxicity.

Few studies have addressed the cytoxicity of cells of the monocyte/macrophage lineage specifically. Although lipopolysaccharide stimulated macrophages from patients with colon cancer were found to lyse tumour cell lines, no activity was noted in breast cancer patients and the presence of a plasma inhibitor of macrophage cytotoxicity was suggested (Cameron and O'Brien 1982).

1:2:4. Cytokine production.

A recognised parameter of the function of tumour associated leucocytes is the elaboration of cytokines which act as signalling molecules between

effector cells, may influence the proliferation of tumour cells and mediate lysis of tumour targets. Little data are currently available on the expression of cytokines within primary breast cancer. The expression of an archetypal cytokine, tumour necrosis factor-alpha (TNF-α), and some of the possible consequences of its expression are the subject of the first part of this thesis.

1:3. Cytokines: an introduction.

Cytokines are low molecular weight polypeptides (<80kDa) which bind to specific cell-surface receptors causing changes in DNA synthesis in target cells. Cytokines generally act at a local cellular level in an autocrine or paracrine manner. Most cytokines are pleiotropic having many biological activities. They include the interferons (α, β and γ); the tumour necrosis factors TNF-α and TNF-β (also known as lymphotoxin); the interleukins (IL1 to 15); the colony stimulating factors G-CSF, M-CSF and GM-CSF (granulocyte, monocyte and granulocyte-monocyte colony stimulating factor; the transforming growth factors, TGF-α and TGF-β; steel factor and leukaemia inhibitory factor (LIF). Growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) are also included in the general term 'cytokine' (reviewed Balkwill 1989).

Although a great deal is known about the cell regulatory effects of individual cytokines *in vitro* it is apparent that cytokines do not act in isolation *in vivo* but are part of a large network of cell regulators. Many cytokines have overlapping properties and have the ability to induce other cytokines as well as altering the expression of cell-surface receptors. Some cytokines antagonise each other while others may synergise to enhance the effects on target cells. In addition the presence of cytokine antagonists in the form of soluble receptors or proteins which bind to cell-surface receptors without

causing signal transduction, adds a further level of complexity to the cytokine network. The effect of a given cytokine on a target cell is probably dependent therefore upon the concentration of cytokine, the target cell type and the presence of other cytokines and regulatory molecules. Thus cytokines have been considered analogous to a complex signalling language in which the resultant cellular response reflects the net effect of the signals received at the cell-surface (Sporn and Roberts 1988).

Cytokines have varied and diverse roles in tumourigenesis *via* direct effects on tumour cells themselves or on the surrounding stromal cells including the tumour vasculature and cells which contribute to the host immune response to the tumour. Cytokines may inhibit tumour development in several ways by direct anti-proliferative or cytotoxic effects on malignant cells or by inducing differentiation. They may augment the immune response to tumour by causing the proliferation and activation of effector cells as well as augmenting antigen presentation and target cell recognition. Through their effects on vascular endothelium and haemostasis cytokines may affect on tumour blood flow.

Conversely, cytokines may promote the neoplastic process by direct effects on tumour cell growth and motility and indirect effects such as suppression of immune effector cell function. Some cytokines may promote the development of the tumour stroma and vasculature. Cytokines may also lead to up-regulation of cellular adhesion molecules and induce proteolytic enzymes capable of digesting the extra-cellular matrix, cartilage and bone all of which may contribute to local invasion and ultimately the metastatic spread of tumours. TNF-α is of particular relevance to this thesis and will now be described in some detail.

1:4. Tumour necrosis factor-α.

Tumour necrosis factor was so named following observations of the anti-tumour cell activity, primarily generated by macrophages *in vitro* (Carswell et al 1975). TNF is an archetypal cytokine and its given name engendered great promise for it as an anti-tumour agent. Following isolation of TNF-α (Aggarwal et al 1985a) and cloning of the gene (Wang et al 1985), details of its regulatory effects have been investigated extensively. It is clear that its activities are by no means confined to those of inducing tumour necrosis, rather it has a central role in many benign and malignant diseases in mediating effects which can be either beneficial or deleterious to the host.

1:4:1. TNF-α synthesis.

The gene for this cytokine is located on the short arm of chromosome 6 and lies close to the HLA-B locus in the major histocompatibility complex (Spies et al 1986). TNF is synthesised as a 26kDa protein and a 76 amino acid signal peptide sequence may be cleaved to yield the 17 kDa secreted form (Kriegler et al 1988). It is apparent that the signal peptide sequence, if uncleaved, may allow TNF-α to act as a transmembrane protein which could mediate cell killing by cell to cell contact. (Kriegler et al 1988). Although initial studies suggested that serine proteases may be involved in the release of soluble 17kDa TNF-α (Scuderi et al 1989) more recent data suggests the involvement of matrix metalloproteinases (Gearing et al 1994). The secreted form of TNF-α has an apparent size of 52kDa and a variety of techniques including gel filtration (Smith and Baglioni 1987), ultracentrifugation (Wingfield et al 1987) and gel electrophoresis (Eck et al 1988) have suggested that this form of TNF is trimeric. This trimeric form of TNF interacts with specific cell surface receptors and since each sub-unit of

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TNF-β (lymphotoxin).

In 1968 a factor, produced by activated lymphocytes, which was toxic to some tumour cell lines and embryonic fibroblasts was identified. This factor was initially named lymphotoxin after the cell of origin. It became apparent that it shared 34% sequence homology with TNF (α) and also shared some of its activities including cytotoxicity, induction of haemorrhagic necrosis in animal tumour models and activation of polymorphonuclear leucocytes (reviewed by Ruddle 1992†). As a consequence of these findings the nomenclature was changed such that TNF was termed TNF-α and lymphotoxin became known as TNF-β.

The genes for TNF-α and TNF-β are likely to have arisen by duplication since both reside within the MHC region and show similar organisation. The encoded proteins share many biological activities and compete for the same cell surface receptors. Differences do exist however at the level of gene regulation, protein sequence and function. In many cytoxicity assays TNF-α is more potent than TNF-β and may exist in a membrane bound form. TNF-α is produced primarily by activated macrophages whereas TNF-β is predominantly a T-cell product. It is intriguing that despite their similarities and their binding to the same receptors, TNF-α and TNF-β can cause different cellular responses. Further discussion in this chapter relates to the regulatory properties of TNF-α.

† Ruddle, N.H. (1992). Tumour necrosis factor (TNF-α) and lymphotoxin (TNF-β). Current Opinion in Immunology 4: 327-332.

TNF has a receptor binding site, it has been suggested that TNF may mediate biological activity by causing cross linking or aggregation of receptors (Eck and Sprang 1989). Indeed, Engelmann et al (1990) have demonstrated that aggregation of TNF receptors by multivalent anti-receptor antibodies leads to cytotoxicity in L929 cells. §

1:4:2. TNF receptors.

The existence of specific high affinity receptors was reported (Kull et al 1985) which were capable of binding both TNF-α and lymphotoxin (TNF-β) (Aggarwal et al 1985b) . When assessed by ligand binding, these receptors were found to be present on most cells types (Sugarman et al 1985, Beutler et al 1985). Although the number varies from 200 to 10,000 per cell, there is no correlation between receptor number and the magnitude of response. There are two distinct TNF receptors both of which bind TNF and transmit an intracellular signal (Brockhaus et al 1990). The p55 (TNF-R1) receptor is widely distributed on many cell types (Loetscher et al 1990). The p75 (TNF-R2) receptor has a more restricted distribution being found predominantly on haemopoetic cells (Ware et al 1991). The relative roles of the p55 and p75 receptors are unclear. Initial studies implied that p55 is essential for signal transduction while expression of p75 alone is insufficient (Thoma et al 1990) more recent data suggests that the p75 receptor alone may mediate signal transduction in some tumour cell lines (Heller et al 1992).

In common with other cytokine receptors, soluble forms of both types of TNF receptor are found in blood and urine (Engelmann et al 1990). Unlike other cytokine soluble receptors they are not a product of alternative splicing of TNF-receptor mRNA but are generated by proteolytic cleavage of the extracellular domain leading to rapid shedding of soluble receptors (Porteu

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In this study, the loss of gap junctions in tumour cell lines was promoted by treatment with 12-*o*-tetradecanoylphorbol-13-acetate and formation of gap junctions was induced by 8-bromo-cyclic adenosine monophosphate.

et al 1990). The function of such soluble receptors if any is unclear. Although they may inhibit the biological activity of TNF (Olsson et al 1989) they may also stabilise the molecule by preventing dissolution of trimeric TNF into its inactive monomers (Aderka et al 1992).

1:4:3. TNF-α: Biological effects *in vitro* and *in vivo*.

1:4:3:1. Mechanisms of TNF induced cytotoxicity in vitro.

Different cell types have varying sensitivities to TNF which are dose dependent (Ruggiero et al, 1987). Although TNF was originally described as a cytokine capable of cytotoxic and cytostatic effects on certain tumour cells while sparing normal cells (Carswell et al 1975), it may also result in cytotoxicity of actively proliferating endothelial cells in culture (Dealtry et al 1987). The relatively selective action of TNF does not appear to be due to the extent or type of cell surface receptors (Sugarman et al 1985, Kull et al 1985) since binding to less than 1% of receptors may still induce the cytolytic effect (Ruggiero and Baglioni 1987). There are conflicting views on which TNF receptor mediates the cytostatic/cytotoxic effects of TNF. Some studies suggest the involvement of the p55 receptor (Thoma et al 1990), or p55 and p75 together (Shalaby et al 1990), while other data suggest that the p75 receptor may mediate cytotoxicity in some tumour cell lines (Heller et al 1992).

Fletcher et al (1987) demonstrated an association between the ability to form tight junctions and target cell resistance to TNF. Manoeuvres to decrease gap junctions of TNF resistant cells rendered them sensitive to TNF. Conversely, induction of gap junctions in cells which were originally sensitive made them resistant to TNF.§

TNF increases intracellular superoxides, hydrogen peroxide and hydroxyl radicals in target cells (Yamauchi et al 1989). Agents which reduce or completely inhibit the formation of free radicals also reduce the cytotoxicity of TNF *in vitro* (Matthews 1983 and 1987). TNF cytotoxicity is associated with intracellular hydrogen peroxide production (Yamauchi et al 1989), alterations in arachidonic acid metabolism (Neale et al 1988), lipid peroxidation (Matthews et al 1987) and inhibition of electron transport (Lancaster et al 1989), features which are all related to excess free-radical synthesis. The ability of free radical scavengers such as manganese superoxide dismutase to protect against the cytotoxic effects of TNF (see below) also lends support to the view that induction of free radicals is an important mechanism in TNF induced cytotoxicity. This is discussed more fully in chapter 3.

TNF has also been shown to activate nuclear endonucleases leading to fragmentation of DNA into 200 base pair fragments, characteristic of programmed cell death or apoptosis (Dealtry et al 1987, Schmid et al 1987, Laster et al 1988). Although DNA fragmentation is observed in some cells lysed by TNF, a different form of lysis is also observed in which necrosis is characterised by formation of balloon like plasma membranes but in which there is no nuclear disintegration (Laster et al 1988).

1:4:3:2. Mechanisms of TNF induced resistance to TNF.

Although TNF is cytotoxic for some breast cancer cell lines, treatment with TNF induces resistance to subsequent exposure of the cytokine, a process which is dependent on protein synthesis (Hahn et al 1985). The induced resistance to TNF is sometimes associated with expression of TNF mRNA by tumour cells (Spriggs et al 1987) and transfection of anti-sense TNF cDNA

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More recent data have also suggested that inhibitors of MnSOD, such as sodium nitroprusside (SNP) and to a lesser extent diethyldithiocarbamic acid, also render cells more susceptible to TNF-α (Jia et al 1995†). It is noteworthy however, that different cell lines continued to show variability in their sensitivity to TNF-α in the presence of SNP, suggesting that resistance to TNF-α cytotoxicity is not wholly mediated by high levels of MnSOD.

† Jia, L,. Jiang, X-U., Wu, Y-L., Newland, A.C., Kelsy, S.M. (1995). TNF mediated killing of human leukaemia cells: Effects of endogenous anti-oxidant levels and TNF-α expression in leukaemia cell lines. Leukaemia Research 19: 187-194.

into cells in which resistance has been induced, renders them sensitive to killing by further exposure to TNF (Himeno et al 1990). The protection mediated by induced TNF may be due to inhibition of the binding of exogenous TNF-α to cell surface receptors (Rubin et al 1986).

Another mechanism by which TNF may induce resistance to its own cytotoxic effects is by induction of the free-radical scavenging enzyme manganese superoxide dismutase (MnSOD). Wong and Goeddel (1988) noted an association between target cell resistance to TNF and constitutively expressed levels of MnSOD. The role of MnSOD in TNF resistance was supported further by studies demonstrating that transfection of TNF sensitive cells with MnSOD cDNA rendered them significantly more resistant to TNF whereas transfection of anti-sense MnSOD mRNA into resistant cells made them sensitive to TNF (Wong et al 1989).§

Other conditions may also alter target cell susceptibility to TNF including several known inducers of heat shock proteins e.g. zinc (Dealtry et al 1987), anaerobic conditions (Matthews et al 1987), hydrogen peroxide (Baud et al 1990) and heat shock (Gromgowski et al 1989). Heat induced resistance to TNF requires protein synthesis but is not associated with an increase in the levels of MnSOD, indicating that this is a separate mechanism of induced resistance.

1:4:3:3. Effects of TNF-α on endothelium.

Increased vascular permeability is seen during infusion of TNF (Tracey et al 1986) and may be caused by several mechanisms observed *in vitro*. Although TNF was initially thought to be specifically cytotoxic for tumour cells it is also cytotoxic/cytostatic for proliferating endothelial cells *in vitro* (Dealtry

et al 1987). TNF also causes the reorganisation of actin filaments which leads to cell retraction and the appearance of intercellular gaps (Brett et al 1989) which may also contribute to the increase in permeability seen following treatment with TNF.

1:4:3:4. Effects of TNF-α on coagulation.

High dose TNF induces changes in coagulation (Tracey et al 1986) by several mechanisms. TNF inhibits endothelial production of thrombomodulin (Moore et al 1989) which results in a reduction in the thrombin-mediated activation of the normal endothelial associated anticoagulation mechanisms via the protein C/protein S inactivation of factor Va and VIIIa of the coagulation pathway. TNF also increases the synthesis of plasminogen activator inhibitor-1 (PAI-1) (Schleef et al 1988). Since activated protein C also inactivates PAI-1 (Sakata et al 1985), the decreased amounts of protein C induced by TNF may also lead to an enhancement of the effective levels of PAI-1.

TNF also induces endothelial cells to express procoagulant tissue factor which is a major initiator of coagulation (Bevilacqua et al 1986). Other factors produced by tumours themselves may also add to this effect as evidenced by the increased fibrin deposition in the tumour vascular bed only during systemic treatment of a sarcoma model (Nawroth et al 1988). Such tissue factors have been isolated from some tumour cell lines (Clauss et al 1990).

1:4:3:5. Effects of TNF-α on adhesion molecules.

TNF enhances adhesion of many cell types to endothelial cells. The increased adhesion of polymorphonuclear leucocytes (PMN) occurs rapidly following exposure to TNF (Gamble et al 1985). The effect is independent of RNA or protein synthesis which therefore implies a direct effect on PMN. The adhesion is blocked by antibodies to β2-integrin/CD18 and experiments using antibodies to specific α chains suggest that it is mediated by the LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) adhesion molecules (Lo et al 1989).

Exposure of endothelial cells to TNF also increases PMN adhesion but this takes place over a longer time course than the effect on PMN; it is dependent on RNA and protein synthesis (Gamble et al 1985) and is probably due to synthesis of ELAM-1 (Bevilaqua et al 1987). Antibodies to ELAM-1 do not entirely block this effect and other cellular adhesion molecules such as ICAM-1, also upregulated by TNF (Dustin et al 1986) and which are known to bind to LFA-1 (Marlin and Springer 1987), may also contribute to leucocyte/endothelial adhesion. The VLA-4 molecule on T-cells also mediates adhesion through interaction with the endothelium molecule VCAM-1 (Elices et al 1990) which is also upregulated by TNF (Carlos et al 1990).

TNF also leads to transendothelial migration of leucocytes (Moser et al 1989) *via* several mechanisms. Although antibodies to cellular adhesion molecules ICAM-1 and ELAM-1 inhibit this process (Smith et al 1988), the TNF induction of IL-8 (Strieter et al 1989) a known chemotactic factor for monocytes (Bagglioni et al 1989) may also contribute to TNF induced transendothelial migration. In addition TNF induces loss of the mel 14

antigen (Kishimoto et al 1989), described originally as the lymphocyte homing receptor (Gallatin et al 1986).The loss of this adhesion molecule following leucocyte activation may reduce adhesion to endothelial cells and therefore allow migration through vessel walls (Kishimoto et al 1989).

1:4:3:6. Effects of TNF-α on angiogenesis.

The effects of TNF on angiogenensis are unclear. Although some groups have demonstrated that TNF is a potent stimulator of angiogenensis (Frater-Schroder et al 1987, Leibovich et al 1987, Gerlach et al 1989) others have shown that tumour cell mediated angiogenesis may be inhibited by TNF (Sato et al 1986). In more recent studies, the effects of TNF on angiogenesis were dose dependent with low doses inducing angiogenesis and high doses inhibiting it (Fajardo et al 1992). Such apparently contradictory findings may be due to the proliferative state of the endothelium itself and the presence of other growth factors.

1:4:3:7. Effects of TNF-α on bone and cartilage.

In vitro studies have demonstrated that TNF promotes the differentiation of mononuclear cells into osteoclasts (Pfeilschifter et al 1989) and stimulates the osteoclastic resorption of bone possibly *via* a prostaglandin-mediated mechanism (Tashjian et al 1987). This latter effect has been shown to be enhanced by other agents including IL-1 and parathyroid hormone (Stashenko et al 1987, Dewhirst et al 1987). Local administration of TNF *in vivo* has been shown to induce osteoclastic bone resorption (Boyce et al 1989) and tumour cells transfected with the gene for TNF caused extensive osteoclastic bone resorption and led to hypercalcaemia (Johnson et al

1989). TNF further contributes to bone destruction by inhibiting the proliferation and differentiation of osteoblasts (Bertolini et al 1986).

1:4:3:8. Immunomodulatory effects of TNF.

As well as direct cytotoxic activity and anti-tumour effects mediated through effects on endothelium etc as described above, TNF activates many cells of the immune system increasing the cytotoxic capacity of macrophages (Talmadge et al 1987), cytotoxic T-cells (Ranges et al 1987, Nakano et al 1989), natural killer cells (Ostensen et al 1987) and neutrophils (Shalaby et al 1985).

1:4:3:9. In vivo effects of TNF-α: animal studies.

Animal models have been important in elucidating the effects of TNF in tumour development. Although administration of TNF to mice bearing the Meth A sarcoma led to rapid necrosis of the tumours in a dose dependent manner (Palladino et al 1987), the lack of effect of TNF on Meth A sarcoma cells *in vitro* suggested an indirect mechanism. Haemorrhagic necrosis was not seen in intra-peritoneal tumours or in small tumours suggesting a possible effect on tumour vasculature (Palladino et al 1987, Manda et al 1987). Such effects have been shown to include endothelial activation with increased HLA expression (Pober et al 1987), altered expression of adhesion molecules (Rice et al 1988) and induction of procoagulant activity (Bevilacqua et al 1986), leading to thrombin and fibrin deposition demonstrated in the Meth A sarcoma model (Nawroth et al 1988).

Effects on tumour vascular endothelium are not entirely responsible for tumour rejection in some animal models. In the SA1 sarcoma model,

complete tumour regression only occurred in immunocompetent mice (North and Havell 1988). In the meth A model, mice who had previously sustained regression of disease in response to TNF were resistant to subsequent challenge with the same tumour suggesting the development of a specific host-mediated immune response (Asher et al 1987).

Animal studies have shown that TNF inhibited the growth of several subcutaneous xenograft tumours including melanomas, colonic and gastric cancers, lymphomas and cervical cancers (Helson et al 1979, Haranaka et al 1984). In general a dose response relationship exists and repeated administration of the cytokine is required to prevent regrowth of tumour with greater efficacy noted where high doses may be achieved with local administration of TNF for example by intra-tumoural and intraperitoneal routes (Balkwill et al 1986).

Tumour cells transfected with the gene for TNF-α have shown reduced tumourigenicity (when injected subcutaneously) in animal models (Ollif et al 1987, Vanhaesebroeck et al 1991) an effect which is dependent on an intact host immune response. In contrast cells transfected with the gene for TNF injected by other routes may show increased invasive potential (see below).

1:4:4. Effects of TNF-α on tumour progression.

Although TNF has anti-tumour activity both *in vitro* and *in vivo,* several of its biological effects described above could promote the growth and invasive potential of tumours.

1:4:4:1 In vitro effects on tumour progression.

TNF-α is a growth factor for fibroblasts (Sugarman et al 1985) which may be important in the establishment and development of tumour stroma (Dvorak 1986). In this respect it is interesting to note that TNF may lead to upregulation of the epidermal growth factor receptor (EGF-R) on fibroblasts (Palombella et al 1987) and tumour cells (Adachi et al 1992). Thus in the presence of ligands for EGF-R, TNF may promote the growth of stromal elements as well as tumour cells through indirect mechanisms.

TNF may also promote angiogenesis (Frater-Schroder et al 1987, Leibovich et al 1987, Gerlach et al 1989) the establishment and maintenance of which is a prerequisite for continued tumour growth (Folkman and Klagsburn 1987).

TNF upregulates metalloproteinase genes (Brenner et al 1989, Ito et al 1990, Lefebvre et al 1991) which degrade basement membranes and other stromal elements which may promote local invasion and increase metastatic potential (reviewed Liotta and Stetler-Stevenson 1991). Invasion of tumour into vessels may be enhanced by changes in the expression of adhesion molecules which may promote interactions between tumour cells and the vascular endothelium (Gamble et al 1985, Rice et al 1988).

Of potential clinical importance to the cytotoxic chemotherapy of cancer are the observations that selection of tumour cell variants resistant to TNF results in increased expression of the gene for multi-drug resistance (Chapekar et al 1991) and is associated with development of drug resistance (Wright et al 1992).

1:4:4:2. In vivo effects on tumour progression.

The potential for TNF to promote local tumour invasion and enhance metastatic potential has been demonstrated in several animal model systems. In xenograft models of human ovarian cancer, intraperitoneal injection of TNF caused ascitic ovarian cancer cells to form clumps and form tumour nodules on the peritoneal surface (Malik et al 1989). In a later study, the cells were transfected with TNF and injected into the peritoneum. TNF transfected cells showed enhanced invasive capacity when compared with controls and this effect could be blocked using antibodies to TNF (Malik et al 1990). The use of TNF as therapy following intravenous injection of colon 26 cells was associated with an increase in incidence of liver metastases (Nishiyama et al 1989). Similarly, a single injection of TNF following intravenous injection of a fibrosarcoma cell line increased the frequency of pulmonary metastases, an effect which could be abrogated by the neutralisation of TNF (Orosz et al 1993).

1:4:5. TNF and cancer cachexia.

Cachexia is a complex syndrome frequently associated with chronic, debilitating illnesses including cancer. The weight loss and generalised asthenia are attributable to anorexia and changes in lipid and nitrogen metabolism. Beutler et al (1985) isolated a substance termed cachectin which led to rapid weight loss in a rabbit model. Subsequent sequencing of the protein showed cachectin to be identical to TNF. Tumours secreting TNF (Oliff et al 1987) and exogenously administered TNF (Darling et al 1990) have been shown to induce cachexia in animal models possibly by effects on lipoprotein lipase (Semb et al 1987) and reduced albumin gene expression (Brenner et al 1990). Some of these effects could also be

attributed to reduced intake of nutrients. The administration of neutralising antibodies to TNF has also been shown to abrogate the cachexia in a squamous cell tumour model (Yoneda 1991). Although the animal data support the view that TNF has a role in cancer-associated cachexia, the evidence for such a role in humans remains unclear.

1:4:6. TNF-α expression in human cancer.

Few studies have investigated expression of TNF-α in primary breast cancer. Spontaneous release of TNF-α from cultures of tumour associated lymphocytes was rarely found unless cultures were first stimulated with mitogens (Rubbert et al 1991). In a small series of cases expression of TNF-α mRNA was assessed *in situ* in cases of primary breast cancer and low levels of expression within stromal elements were found (Vitolo et al 1992).

The presence of TNF mRNA has been documented in colorectal carcinoma where *in situ* hybridisation localised expression to infiltrating macrophages. (Beissert et al 1989, Naylor et al 1990a). Similarly expression of TNF mRNA and protein by infiltrating macrophages has been documented in renal cell carcinoma (Waase et al 1992). In epithelial ovarian cancer, TNF mRNA expression was localised within epithelial areas (Naylor et al 1990b) though a more recent study suggests that expression of TNF protein may be confined to a subset of infiltrating macrophages (Naylor et al 1993).

1:5. Aims of studies described in this thesis.

In the first part of this thesis I have examined the expression of tumour necrosis factor and its receptors in primary breast cancer and, in view of the conflicting effects that TNF-α may have on tumour development, possible

correlations with established pathological features were studied. The observed distribution of TNF-α and its receptors within the tumour stroma suggested that the effects of TNF-α in primary breast cancer may well be indirect. This led to studies of other possible effects of TNF within the tumour stroma, including quantification and localisation of proteases, which could contribute to tumour progression, and assessment of another TNF-α induced enzyme, nitric oxide synthase, which, although implicated as an immune cell cytotoxic mechanism, may also modulate tumour blood flow.

The second part of the thesis describes studies of the potential use of another cytokine (interleukin-2) in the treatment of cancer in order to attempt to augment the host immune response to tumour. As noted above, individual cytokines may have multiple effects and the impact of recombinant IL-2 on aspects of the cytokine network, particularly with respect to TNF-α and interferon-gamma (IFN-γ), are described. The consequences of secondarily-induced cytokines as mediators of toxicity, through effects on induction of nitric oxide and leucocyte trafficking, have also been investigated.

Chapter 2.

EXPRESSION OF TNF-α IN PRIMARY BREAST CANCER.

2:1. Introduction

The background to the study of TNF in human breast cancer has been described in the preceding chapter. To determine the role that cytokines such as TNF-α play *in vivo* it is important not only to assess whether the genes encoding these molecules are expressed, but also determine the site and cell type expressing the mRNA for that cytokine. Immunohistochemistry techniques have developed to an extent that low levels of protein can be detected at the cellular level. However, cytokines are soluble factors that can be intracellular, membrane bound or bound to a specific receptor on another cell type. This means that antibody staining of cytokines within tissues may not necessarily identify the producer cell type or, indeed, detect cytokine protein at all. Also anti-cytokine antibodies are raised against recombinant cytokines that may differ from the naturally occurring form of that protein.

One way of determining the producer of a particular cytokine is to detect the expression of the gene encoding that cytokine at the level of messenger RNA (mRNA). This technique, termed *in situ* hybridisation, involves the hybridisation of a nucleic acid probe (tagged with an isotopic or non-isotopic label and complementary to the target nucleic acid sequence) to a tissue section that has been pre-treated to optimise the access of the probe to the target mRNA. A series of washes removes unbound probe leaving the tagged probe bound to its complementary target sequence. The probe is then detected by autoradiography, in the case of isotopic labels, or by chemical means in the case of non-isotopic labelling. The presence of mRNA does not

always imply the presence of encoded protein since post-transcriptional modification may occur. Thus the techniques of *in situ* hybridisation and immunohistochemistry are complementary and were used together in the studies described below.

In situ hybridisation is extremely specific for the tissue and the mRNA to be studied. One of the main problems of this procedure is the sensitivity of RNA to the ubiquitous ribonucleases. Throughout the technique ribonuclease inhibitors are employed and reagents are treated whenever possible to avoid ribonuclease contamination.

The optimal conditions for the *in situ* work were derived using a probe that would recognise an ubiquitous mRNA (β actin). The probe composition and method of labelling also had to be optimised. Other groups have used complementary DNA probes labelled with a ^{32}P-tagged nucleotide by the random priming method. Briefly, this involves the synthesis of a complementary strand of DNA using the target DNA sequence as a template. Labelled nucleotides are then incorporated at random along the length of the template. Although, after some development, the use of this type of probe proved effective for the detection of β actin mRNA, repeating the procedure with a TNF probe proved less successful. This was likely to be a question of sensitivity; the copy number of TNF mRNA is considerably less than that of β actin, and indicated that a more sensitive probe was required. *In vitro* transcription systems are available that generate complementary RNA to a high specific activity. Unlike the random priming method that can have variable levels of labelled nucleotide incorporated, the *in vitro* transcription systems use a virally derived RNA polymerase to synthesise a complementary strand that incorporates a labelled nucleotide at every complementary point on the template. This means that for every unit length of

probe synthesised, more labelled nucleotides are incorporated and therefore the specific activity is higher. The generation of complementary, 'antisense', RNA probes ('riboprobes') has an advantage over DNA probes in that the thermal stability of RNA:RNA hybrids is greater than that of DNA:RNA hybrids, thus hybridisation can be performed at higher temperatures reducing background signals. Unbound RNA probes can then be digested away following hybridisation using ribonuclease A, an enzyme specific for single stranded RNA. However, one problem with RNA probes is that they are more prone to digestion by nucleases, therefore synthesis and storage requires particular care.

The use of *in vitro* transcription to generate riboprobes required the DNA template to be in a specific *in vitro* transcription plasmid. These contain RNA polymerase binding sites to enable synthesis. Use of this probe form was successful for detection of TNF in control cell lines. Further developments were made to optimise the technique on cryostat tissue sections. These developments mainly focused on pretreatment of the tissues with optimal concentrations of protease to optimise access of the probe to the target mRNA.

Although conventional immunohistochemical techinques may be able to detect low levels of antigen, their use with respect to the detection of TNF-α may be problematic. The development of the colour reaction when conventional substrates such as diaminobenzidine (DAB) are used, relies on the presence of peroxidase conjugated to a secondary antibody raised against the species in which the primary antibody was produced. Activated monocytes/macrophages may also produce peroxidase and a colorimetric reaction may therefore occur within these cell types when no antigen is present, i.e. a false positive reaction. This is of particular concern when

potential macrophage products such as TNF-α are the antigen of interest. The use of an alkaline phosphatase system overcomes this problem of specificity and also allows amplification of the development steps in order to increase the sensitivity of the system.

Attempts were made to apply immunohistochemical techniques to sections which had undergone the process of *insitu* hybridisation in order to assess the co-localisation of TNF-α mRNA and TNF-α protein or cell specific determinants such as CD68. This was unsuccessful, probably as a result of the digestion of antigenic determinants during the protease steps which were necessary to aid probe entry into cells. The alternative approach would have been to carry out the immunohistochemistry first, followed by *in situ* hybridisation. In view of the fact that many of the reagents used in immunohistochemistry could not be rendered RNase free, this sequence was not attempted. Co-localisation of TNF-α message and protein depended upon analysis of adjacent sections of tissue using the techniques separately.

Adjacent sections of tissue were also used when localising expression of TNF-α protein although since such sections are necessarily not identical, two-colour immunofluorescence was also employed to improve accuracy. In this technique antibodies to the cytokine and cell-surface determinants were labelled with different fluorochromes and applied to the same tissue sections. Viewing the sections under specific wavelengths of light allows localisation and co-localisation of antigens.

2:2. Methods.

2:2:1 Tumour samples.

Tumour specimens were obtained from excision biopsies and mastectomy specimens and were snap frozen in liquid nitrogen. All patients had a total mastectomy and axillary clearance or a conservation technique comprising excision biopsy and axillary clearance followed by external beam radiotherapy supplemented by iridium implant at the time of surgery or external radiotherapy boost to the site of tumour. Steroid hormone receptor status was determined using a dextran-coated ligand binding assay (King et al 1979) with a value of $\geq$ 10 fmol mg^{-1} cytosol protein taken as positive. Histological examination was on paraffin-embedded formalin-fixed tissue and grading of ductal carcinomas was according to the modified Bloom and Richardson classification (Elston 1984).

Histology of the 77 cases examined by immunohistochemistry to be described in this chapter were normal breast/benign disease (18), infiltrating ductal carcinoma of varying grade (48), infiltrating lobular carcinomas (7) and carcinomas of the special type group (4), including mucoid (3) and medullary types (1). Sixty of these cases were also examined by *in situ* hybridisation for the presence of TNFα mRNA.

2:2:2. In situ hybridisation

2:2:2:1. Sample preparation

Solid tissue was snap frozen in liquid nitrogen then stored at -70^{o}C prior to sectioning. 5 μm cryostat sections were cut onto sterile, ribonuclease-free,

triethoxy-silylethanolamine (TESPA) coated slides. Cut sections were stored in slide boxes with silica gel for short periods (weeks) at -70°C.

Cells in suspension (TNF-α transfectants for positive controls) were prepared using a cytospin (Shandon). 100µl of cell suspension at a concentration of 1 x 10^6/ml was centrifuged at 1 x 10^6/ml at 500 rpm for 5 min. The procedure for *in situ* hybridisation used was as for cryostat sections but treatment with proteinase K was omitted.

The following protocols were developed within the course of study and are listed as a series of steps for brevity and comprehension.

2:2:2:2. Prehybridisation.

All incubations were carried out at room temperature on a rotary shaker
Slides were placed in racks and incubated in suitable slide troughs for the following steps.

-	**4% PFA**		**20**mins
1)	DEPC phosphate buffered saline (PBS)		5 mins
2)	DEPC distilled water (DW)		5 mins
3)	0.2M HCl		20 mins
4)	PBS		5 mins
5)	4% paraformaldehyde (PFA)		15 mins
6)	PBS	2 X	5 mins
7)	5mg/ml Proteinase K made up in appropriate buffer (50mM Tris, 5mM EDTA pH7.5)		7.5 mins
8)	PBS		5 mins
9)	4% PFA		5 mins
10)	DW dip		
11)	0.1M Triethanolamine + acetic anhydride (1ml/400ml)	2 X	10 mins
12)	PBS		5 mins

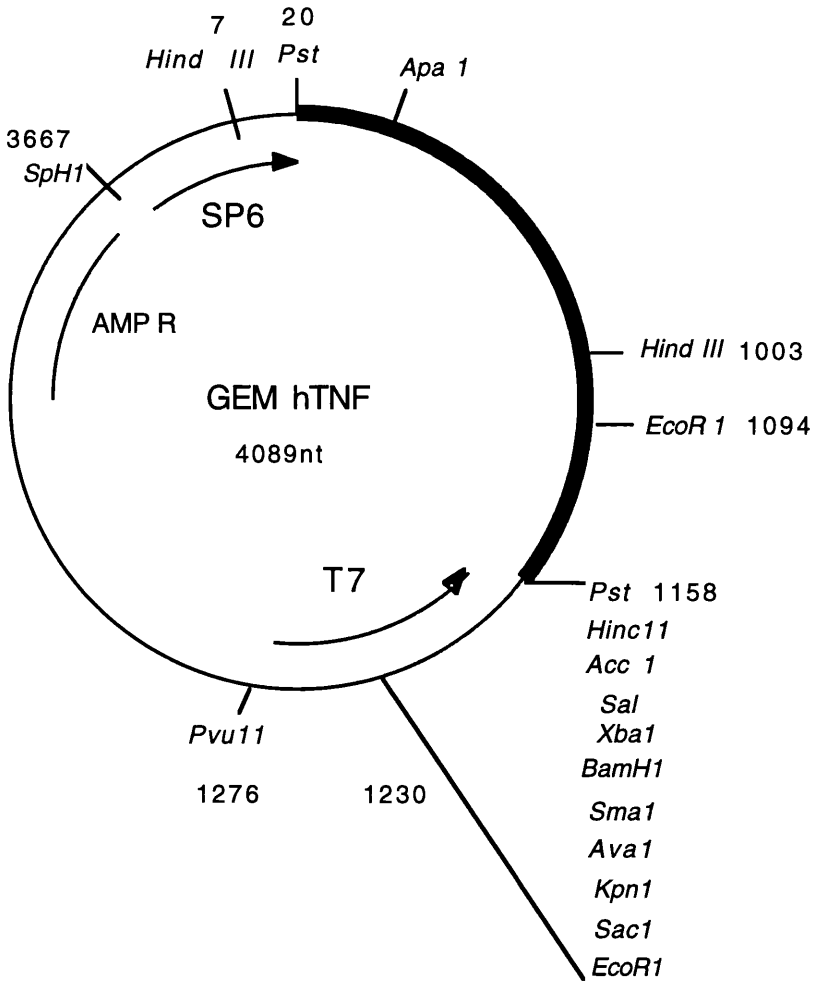
13) Dehydrate 30%, 50%, 70%, 95%, 100% 2 mins in each

14) Air dry in a dust free environment

2:2:2:3. Probe preparation.

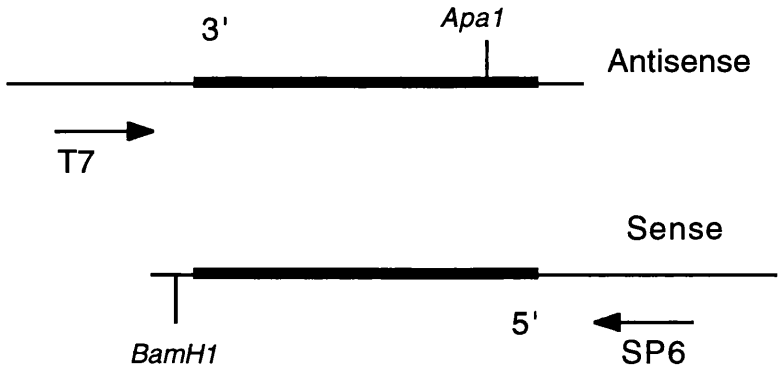
The antisense TNFα riboprobe was generated from the Apa-1 cleaved pGEM1-hTNF (figure 2:1) containing a 1kb sequence of the TNF-α cDNA (from Prof. W. Fiers, Universitiy of Ghent, Belgium) using T7 RNA polymerase (Promega Biotech, Madison, USA, figure 2:2).

Figure 2:1 Map of pGEM plasmid with TNF cDNA insert.



The negative control was sense TNF-α generated from Bam H1 cleaved pGEM1-hTNF using SP6 RNA polymerase (Promega Biotech, figure 2:2). *In vitro* transcriptions were performed using transcription kits (Promega Biotech UK) incorporating ^{35}S-UTP (Amersham International, UK) as described above. Restriction enzymes were obtained from Pharmacia.

Figure 2:2. Synthesis of riboprobes from linearised template.



2:2:2:4. Probe Labelling

1. To a sterile ribonuclease-free 1.5ml microfuge tube were added;

5 x Transcription buffer	4 µl
100mM DTT	2 µl
RNasin	0.8µl
ATP, CTP, GTP	4 µl
(mixed in a ratio of 1:1:1:1 with DW)	
Linearised transcription vector template	1 µl (=1µg)
^{35}S-UTP (>1000mCi/nmol)	10 µl
Relevant polymerase	1 µl

2. Pipetted up and down gently to mix. Incubate at 37°C for 1 hr.
3. 2 units of RQ1 DNase added, pipetted up and down gently to mix.
4. Incubated at 37°C for 30 min.

5. 80 µl of DW added, phenol extract, added 2µl of 10 mg/ml carrier RNA then precipitated with 20µl of 10M ammonium acetate and 300µl of absolute alcohol.
6. Dry ice for 10 min. Microfuged for 10 min at 4°C. Supernate.carefully removed.
7. Pellet dried in a vacuum desiccator (or inverted microfuge tube and dried in RNase free environment at room temperature) and taken up in 100µl of alkaline digestion buffer
8. Incubated in a dry block or water bath at 60°C for 75 mins.
9. Reaction stopped with 10µl 1M sodium acetate pH6
   10µl 5% acetic acid
   2µl 10mg/ml rRNA
10. Phenol extracted twice then precipitated as above, dried and resuspended in 50 µl of 10mM DTT.
11. Labelled probe counted; 1µl in approximately 10 ml of scintillant (picoflor).

2:2:2:5. Hybridisation

1. Hybridisation solution was prepared by adding the following (figures in number of µl) to 1.5 ml microfuge tube. Required volumes were selected according to number of sections.

reagent	µl	µl	final conc.
1M DTT	5	10	10mM
Deionised formamide	300	600	60%
100 X Denhardts	5	10	1 x
1M Tris pH 8	5	10	10mM
5M NaCl	30	60	0.3M
0.5M EDTA	5	10	1mM
10mg/ml Poly A	15	30	300µg/ml
10mg/ml carrier RNA	15	30	300µg/ml
20mM cold S-UTP	11	22	500mM
50% Dextran Sulphate	100	200	10%
total volume (µl)	491	982	

2. Probe added to a concentration of 5 X 10^4 cpm/ml
3. Heated in a dry block for 2 mins. at 80°C
4. 10-15 µl / section applied, covered with a siliconised coverslip
5. Incubated overnight in a 50°C slide incubator in a sealed slide box humidified with a tissue soaked in 50% formamide, 5 X SSC.

2:2:2:6. Post hybridisation washes

1. Slides were transferred to racks and incubated in the following buffers in 400 ml slide dishes equilibrated in 37°C, 50°C, and 65°C water baths as shown.

Buffer	Temperature	Time
a. 5 X SSC, 0.1% 2-mercaptoethanol (2-M.E.)	50°C	3 X 20 mins.
b. 50% formamide, 2 X SSC, 0.1% 2-M.E.	65°C	30 mins.
c. Ribonuclease Buffer (0.5M NaCl, 10mM Tris, 5mM EDTA pH 8)	37°C	2 X 10 mins.
d.Ribonuclease A, 20mg/ml in the above	37°C	30 mins.
e. wash in (c)	37°C	15 mins.
f. as (b)		
g. 2 X SSC	RT	15 mins.
h. 0.1 X SSC	RT	15 mins.

2. Dehydrate 30%, 50%, 70%, 95%, 100% — 2 mins. in each
3. Air dry in a dust free environment
4. Dip slides in filtered 0.1 % gelatine
5. Air dry in a dust free environment
6. Autoradiograph

2:2:2:7. Autoradiography

1. 10 ml of 0.1% gelatine was pre-warmed at 50^{o}C in a 50 ml measuring cylinder
2. Ilford K5 emulsion was added to 20 ml in a dark room
3. Emulsion was melted in a 50^{o}C water bath, rocking gently to mix.
4. Emulsion was poured into a slide mailing box
5. Slides were dipped with forceps, the backs wiped and slides allowed to dry for 2 hrs. in a light proof box in the dark room.
6. Slides were transferred to a slide box containing silica gel and which was then wrapped in foil and put at RT overnight, then put to 4^{o}C for 7-10 days.

2:2:2:8. Development

7. In a dark room, at RT, slides were incubated in the following;

a. D-19 developer 2.5 mins.

b. 1% acetic acid 0.5 min.

c. 30% sodium thiosulphate (freshly made) 5 mins.

8. Slides were then transferred into DW and removed from the dark room and washed in running DW tap for 60 mins.
9. Slides were counterstained with toluidine blue.

2:2:2:9. Interpretation

Sections were examined using a Leitz Diaplan microscope. A minimum of 10 randomly selected high power fields were counted using a x10 eyepiece and a x40 objective (field area 3.2mm^{2}). For *in situ* and immunohistochemistry, cells positive per high power field (hpf) were counted. Positive cells in the

case of *in situ* hybridisation were identified by deposition of silver grains over the perinuclear cytoplasm at levels higher than that of sense probed sections.

Probe localisation was detected under bright field microscopy as a perinuclear deposition of silver grains over the cells expressing the target message often giving a corona-like appearance. Dark field microscopy was used to accentuate this deposition but also increased the background considerably under high magnification.

Artefactual positives may occasionally occur for several reasons e.g. contamination of emulsion with talc from gloves, precipitate in the gelatine, precipitation of components in the hybridisation mix, bubbles in the emulsion. Negative controls were therefore screened thoroughly and employed widely.

2:2:2:10. Positive controls

Positive controls (cell lines transfected with the TNF gene as cytospins or established as tumours in nude mice, Malik et al 1990) were included and mRNA integrity in tissue was verified by probing for the ubiquitously expressed β-actin.

2:2:3. Histological techniques

Immunohistology was performed on acetone fixed frozen sections, acetone fixed cytospin preparations, and paraffin embedded sections according to the basic protocols listed below.

2:2:3:1. Processing of tissues

Frozen sections were prepared from tissue samples snap frozen in liquid nitrogen, mounted in OCT compound (Tissuetek, USA), and 5mm sections cut in a cryostat (Cryostat E, Reichert Jung, FGR). The sections were stored at -70^{o}C with silica gel until use.

Paraffin embedded sections were prepared from samples fixed in neutral buffered formalin for at least 24 hours. The tissues were dehydrated through graded alcohols, cleared with CNP 30 (Pentone Chemicals, UK) and embedded in paraffin wax. 5μm sections were then cut using a rotary microtome (American Optical, USA) and stored.

2:2:3:2. Antibodies used.

The antibodies used for immunohistological studies their source and dilution are shown below (table 2:1).

Table 2:1. Antibodies used for study of TNF and TNF receptor expression in primary breast cancer.

CD#/Ag	Antibody	Expression	Source	Dllution
monocyte/macrophage markers				
CD68	EBM/11	monocytes/mac	Dako	1 in 100
CD68	Y1/82A	monocytes/mac	DM	nil
tumour/epithelial cell markers				
PEM	HMFG-2	mucin	JT-P	nil
cytokeratin	CAM5.2	epithelial cells	WB	1 in 4
anti-human TNF monoclonal antibodies				
TNF	J1D9		JM	1 in 50
TNF	CB006		Celltech	1 in 40
anti-TNF receptor monoclonal antibodies				
CD120a (TNF-R p55)	htr	all cell types	MB	1 in 2000
CD120b (TNF-R p75)	utr	T-ly, monocytes	MB	1 in 2000

Key to table.
CD#/Ag: **PEM**-polymorphic epithelial mucin
Expression: **mac**=macrophages, **T-ly**=T-lymphocytes
Sources: **DM**; David Mason, Oxford.
JT-P; Joyce Taylor-Papdimitriou, ICRF, London.
WB; Walter Bodmer, ICRF, London.
JM Jez McLaughlin, Liverpool University.
MB Manfred Brockhaus, Hoffman La Roche.

2:2:3:3. Immunoperoxidase labelling technique

1. Sections dewaxed in xylene and taken down to 100% alcohol.
2. Endogenous peroxidase blocked by immersing slides in 240µl of 30% hydrogen peroxide (Sigma) in 400ml methanol for 15 mins. Wash in running water.
3. Sections trypsinised if required. Trypsinisation mixture:100 mg trypsin (Sigma), 100mg calcium chloride, in 100 ml distilled water (DW): adjusted pH to 7.8. Mixed and incubated at 37°C.
4. Washed in running tap water. Rinsed in tris buffered saline (TBS).
5. Sections incubate in either normal swine serum (1:25) for polyclonals or normal rabbit serum (1:25) for monoclonals for 15 mins.
6. Incubated for 30 mins in primary antibody at optimal dilution.
7. Washed in two changes of TBS, 5 mins. each.

8. Incubated for 30 mins. in biotinylated rabbit anti-mouse antibody 1:300 with 1:25 normal human serum added.
9. Washed in two changes of TBS, 5 mins. each.
10. Incubated for 30 mins. in ABC complex(1:100 of A, 1:100 of B) mix and allowed to stand for at least 30 mins before use.
11. Washed in two changes of TBS, 5 mins. each.
12. Peroxidase substrate prepared -5mg diaminobenzidine (DAB) (Sigma), 10 mls of TBS plus 10µl 30% hydrogen peroxide. Incubated in DAB for 3-5 mins.
13. Washed in water and counterstained in haematoxylin for 3 mins. Washed, and allowed to differentiate and blue.
14. Dehydrated, cleared and mounted in DPX mountant (BDH).

For frozen sections

1. Sections dried at room temperature for 30 mins, wash in TBS.
2. fixed in acetone for 5 mins. Rinse in TBS.
   Steps 5-14 as above.

2:2:3:4. Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining.

1. Sections dried at room temperature (RT) for 30 mins.
2. Fixed acetone for 30 mins. Rinsed in Tris-buffered saline (TBS). Preincubated with normal rabbit serum in the case monoclonal antibodies and normal swine serum in the case of polyclonal antibodies.
3. Incubated with primary murine monoclonal 30 minutes.
4. Washed in two changes of TBS, 5 mins. each.
5. Sections incubated with rabbit anti-mouse immunoglobulin diluted 1:25 in TBS and normal human serum 1:25 for 30 mins. at RT.
6. Washed in two changes of TBS, 5 mins. each.
7. Incubated with mouse anti-alkaline phosphatase complexed with alkaline phosphatase (APAAP) diluted 1:25, 30mins.
8. Washed in two changes of TBS, 5 mins. each.
9. Steps 5 to 8 repeated twice.
10. Slides incubated with substrate: 5mg fast red TR dissolved in 10mls of veronal acetate solution (sodium acetate 1.943gms plus sodium barbiturate 2.943 gms in 100mls distilled water: 5 mls of this solution

added to 0.25ml 0.1M HCL and 19.75 ml DW to pH 9.2). 5mg of naphthol-AS-BI (Sigma N2250) dissolved in 200µl dimethyl formamide. 10µl of stock 1M levamisole solution added and substrate filtered directly onto slides. Allowed to develop (approx. 5 mins.)

10. Sections washed in TBS.
11. Counterstained with haematoxylin and mounted in Apathy's medium (BDH).

2:2:3:5. Two colour immunofluorescence.

Cryostat sections were fixed in acetone and transferred directly to PBS, then placed in optimally diluted J1D9 (1 in 50) for 45 minutes. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG_1 (Sera-Lab) in 20% normal human serum, was incubated for a futher 30 minutes. Sections were absorbed for 20 minutes in 20% normal human serum prior to incubation with biotinylated EBM/11 (Dako). Tetra methyl rhodamine isothiocyanate (TRITC) conjugated streptavidin (Sera-Lab) was then added for 30 minutes. Sections were mounted in diazobicyclo-octane DABCO (Sigma) and viewed using a Nikon Optiphot microscope at 525 nm for FITC and 580 nm for TRITC.

All reagents, unless otherwise specified were obtained from Dako U.K. Ltd, High Wycombe, Bucks..

2:3. Results.

Using the techniques of *in situ* hybridisation and immunohistochemistry, expression of TNF-α mRNA and protein was assessed in a series of cases of benign and malignant breast tissue. The cases examined are shown in table 2:2.

Table 2:2. TNF-α expression in benign and malignant breast tissue: characteristics of cases studied.

Benign	18 (11)	Normal		10	(6)
		Fibroadenoma		6	(4)
		Gynaecomastia		2	(1)
Invasive carcinoma	59 (49)	Ductal		48	(39)
			grade I	8	(8)
			grade II	20	(16)
			grade III	20	(15)
		Lobular		7	(6)
		Mucoid		3	(3)
		Medullary		1	(1)

(cases also studied by in situ hybridisation in parentheses)

2:3:1. *In situ* hybridisation.

Positivity for TNFα mRNA was seen in one of seven normal cases examined and three of four fibroadenomas. In these cases, occasional positive cells were seen in the stroma. TNFα mRNA expression was found in a higher proportion of cases of invasive breast cancer compared with normal tissue (43/49 cases vs 1/7, p=0.01 Fishers exact test). Examples of some of the data

are shown in table 2:3 which reflects the focal nature of TNF expression in these tissues.

Table 2:3 Expression of TNF-α mRNA in benign and malignant breast tissue.

path no	tissue	range	median	mean
932/89	normal	0	0	0
512/89	lobular	0-4	1	0.7
776/89	lobular	0-5	1	1.8
354/90	lobular	0-2	1	0.8
357/90	lobular	0	0	0
788/89	lob/gde I	0	0	0
925/89	lob/gde I	0-1	1	0.6
775/89	grade I	0-7	2	3.5
806/89	grade I	1-3	1	1.1
954/89	grade I	0-5	1	1.7
387/88	grade II	0-2	1	0.8
521/88	grade II	0-5	1	1.6
571/88	grade II	0-1	0	0.5
175/89	grade II	0-1	0	0.1
819/89	grade II	0-2	0	0.6
841/89	grade II	0-10	0	1.6
884/89	grade II	0-5	0	0.6
906/89	grade II	0-1	0	0.2
942/89	grade II	0-24	4	6.1
961/89	grade II	0-4	1	1.5
429/88	grade III	0-4	1	1.7
579/88	grade III	0-24	5	7.0
747/88	grade III	0-1	0	0.4
155/89	grade III	0-2	0	0.7
821/89	grade III	0-1	0	0.2
939/89	grade III	3-8	5	5
941/89	grade III	0-7	0	1.4

Expression of TNF mRNA in 10 randomly selected high power fields (hpf). Results expressed as range, median and mean.

Although, in several cases the maximum number of TNF-α mRNA expressing cells/hpf. is high, the mean value is far lower e.g. in case 942/89 up to 24 cell/hpf expressed TNF-α mRNA the median and mean over the 10 fields examined were 4 and 6.1 cells positive respectively. In case 841/89 up to 10

positive cells were seen in an individual field but overall the median value was zero. This may be due to the nature of the field area selected i.e. stroma or tumour, as well as the 'clustered' nature of TNF expression.

Expression of TNFα mRNA was largely confined to stroma adjacent to tumour areas (figure 2:3), although positive cells were occasionally seen within tumour islands.

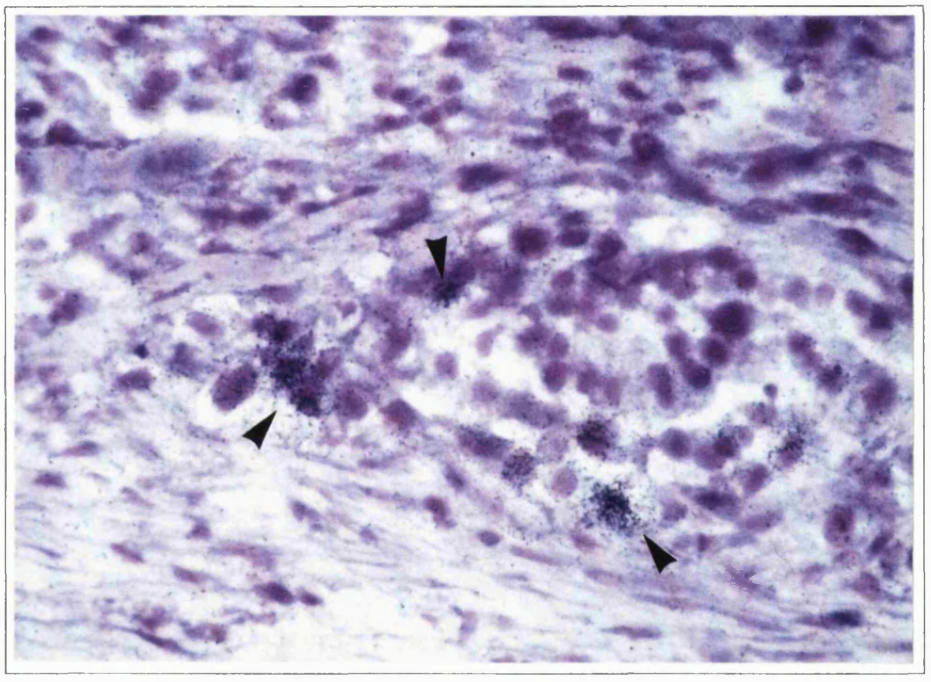


Figure 2:3. Cells expressing TNF-α mRNA in infiltrating breast carcinoma. Positive cells (arrowed) are in the stroma adjacent to an island of tumour (x400).

The median number of cells positive for TNFα mRNA/hpf was only 1.1 for the group of invasive carcinomas as a whole (table 2:4) which represents approximately 0.5% of cells present in a high power field. In 3 of the 39 cases of infiltrating ductal carcinoma studied however, over 20 cells/hpf (approximately 6% of cells present) were positive for TNFα mRNA. Overall, the number of cells per high power field expressing TNF-α mRNA was higher

in malignant compared with benign or normal breast tissue (p=0.002 Mann-Whitney U test).

Table 2:4. Median number of cells positive for TNF-α mRNA per high power field by tissue type.

Tissue		No of cases examined	Median no. cells positive/hpf
Normal & benign		11	0.0
Invasive carcinoma		49	1.1§
Ductal	grade I	8	1.4
	grade II	16	0.9
	grade III	15	1.7
Lobular & others		10	0.8

§ p=0.002 compared with benign cases (Mann-Whitney)

2:3:2. Immunohistochemistry.

The presence of TNFα protein was confirmed using two antibodies to TNFα. Considering all the cases studied, staining was more generalised and the number of cells positive/hpf using immunohistochemistry was significantly higher than the positivity seen with *in situ* hybridisation (median number of cells positive/hpf =10.5 [antibody J1D9] vs 0.8 [*in situ*], p<0.0001, and 6.35 [antibody CB006] vs 0.8 [*in situ*], p<0.0001, Mann-Whitney). Expression of mRNA and protein co-localised predominantly in the tumour stroma, particularly in those areas adjacent to infiltrating carcinoma (Fig 2:4, a&b). As with TNFα mRNA, occasional cells within tumour islands were found to express TNF protein.

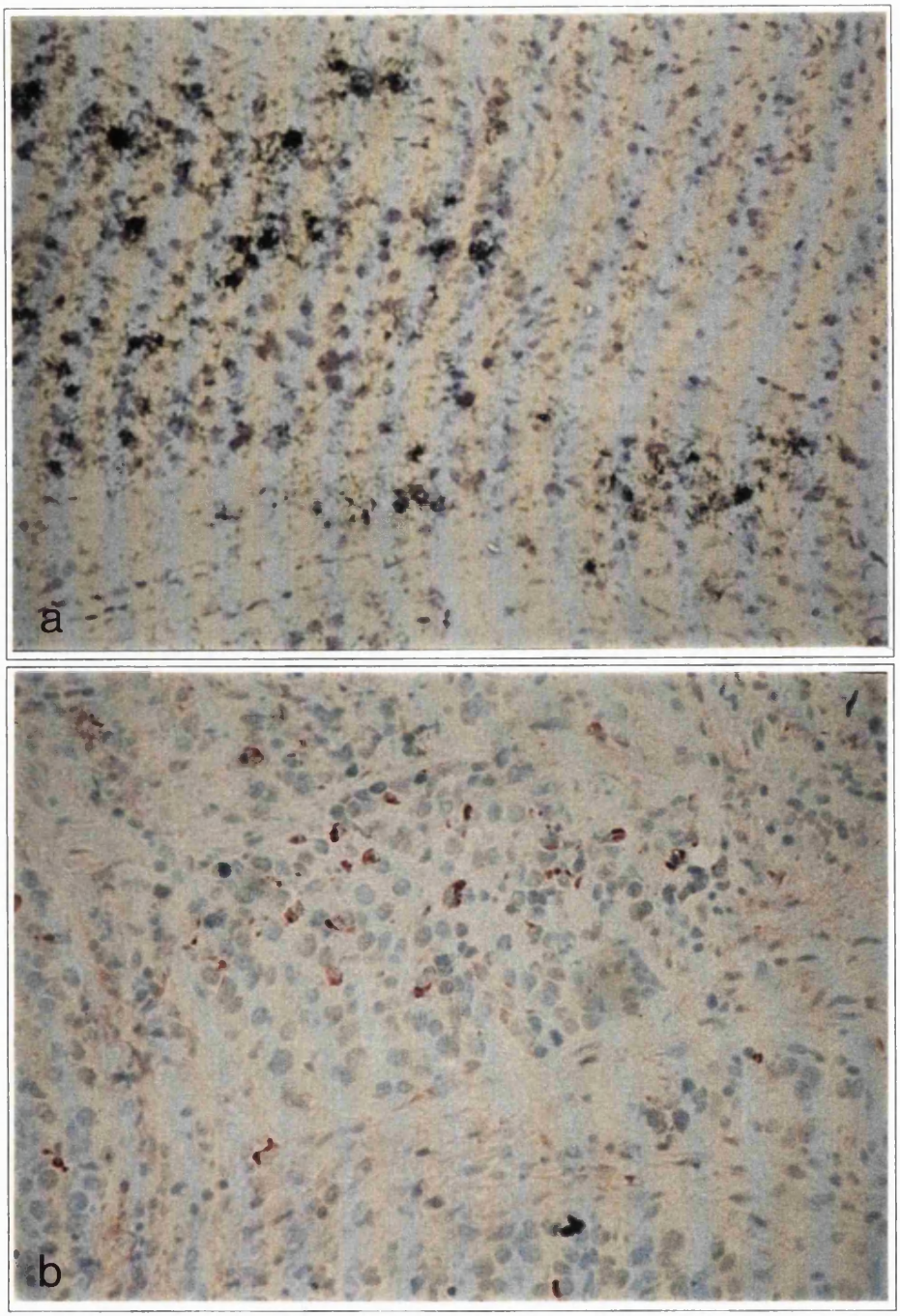


Figure 2:4 **Expression of TNFmRNA (a) and TNF protein (b) in adjacent sections from a case of infiltrating ductal carcinoma. TNF mRNA and protein colocalise predominantly in stroma, though occasional positive cells are seen within tumour islands (x200).**

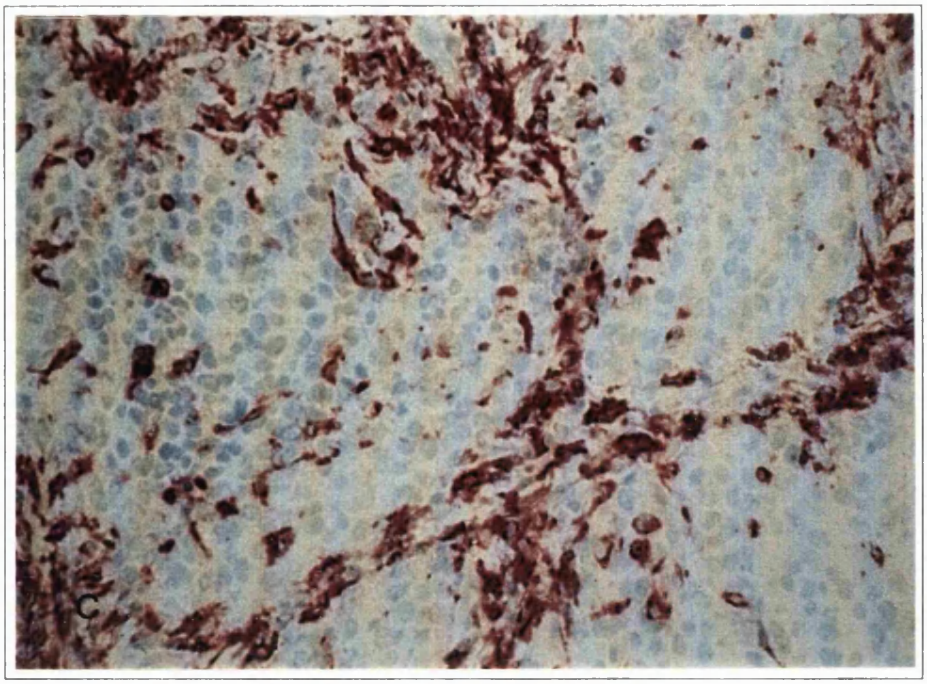


Figure 2:4. (c) CD68 positive cells (monocytes/macrophages) in an adjacent section (compare with figure 2:4, a&b on the previous page) x200.

When adjacent sections were stained with the CD68 monoclonal antibodies EBM/11 and Y1/82A it was apparent that TNFα expression was largely confined to cells of the monocyte/macrophage lineage (Fig 2:4c). Mononuclear cells were found to be present within tumour islands and were probably responsible for occasional TNFα message and protein positivity in such sites.

The presence of immunoreactive TNFα within EBM/11 positive cells was confirmed by two colour immunofluorescence (Fig 2:5 a&b).

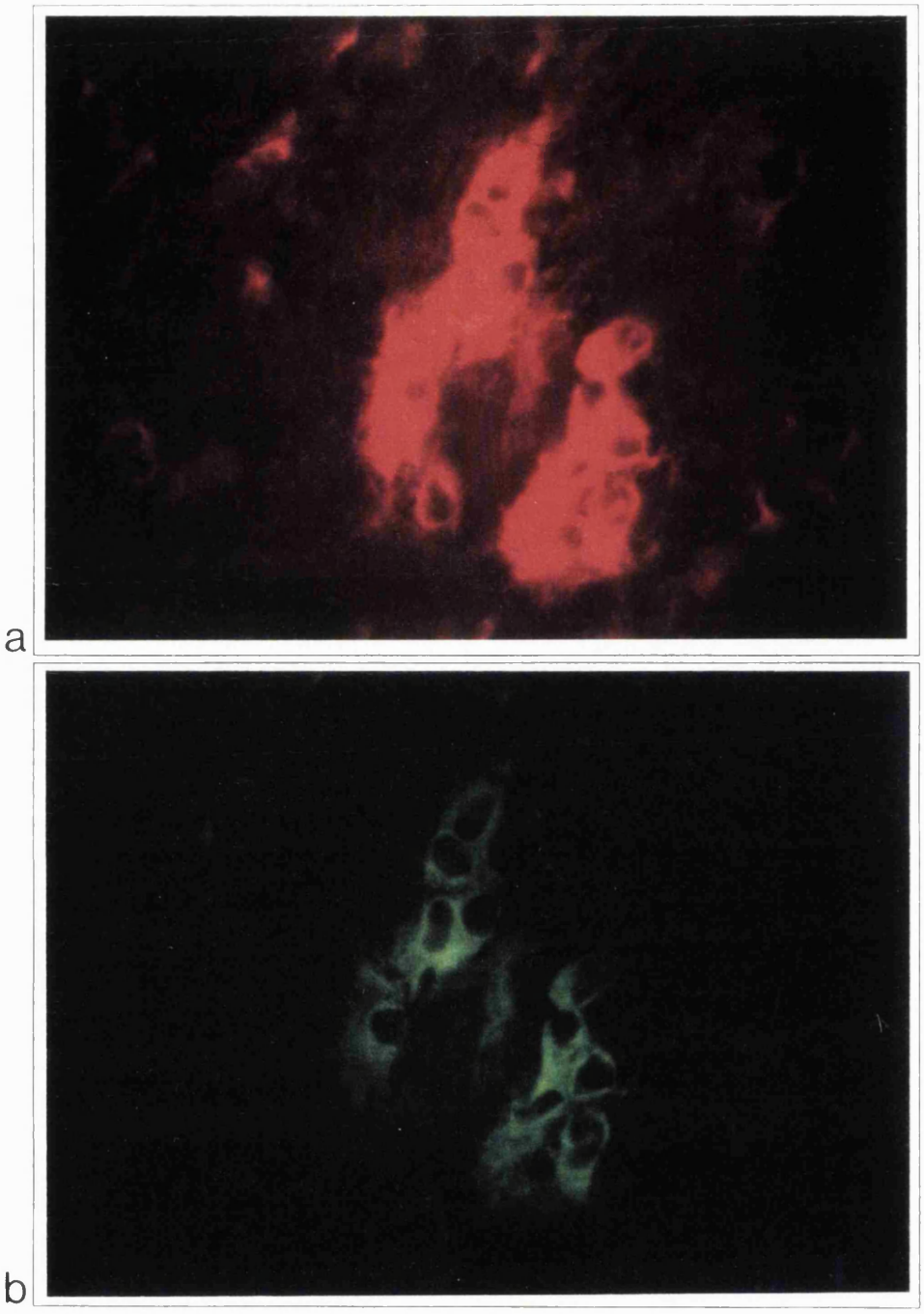


Figure 2:5 Expression of TNF protein by cells bearing the CD68 marker (a) and TNF protein (b) by two-colour immunofluoresence (x400).

The number of macrophages (defined by monoclonal antibody EBM/11) per hpf was significantly higher in malignant compared with normal/benign disease (median 67.6 vs 14.1 $p<0.001$, Mann-Whitney, table 2:5). There was

no difference between the numbers of macrophages present and tumour type or grade of ductal tumour.

Table 2:5. Median number of cells positive for TNF-α and CD68 marker (EBM/11) per high power field (hpf) by tissue type.

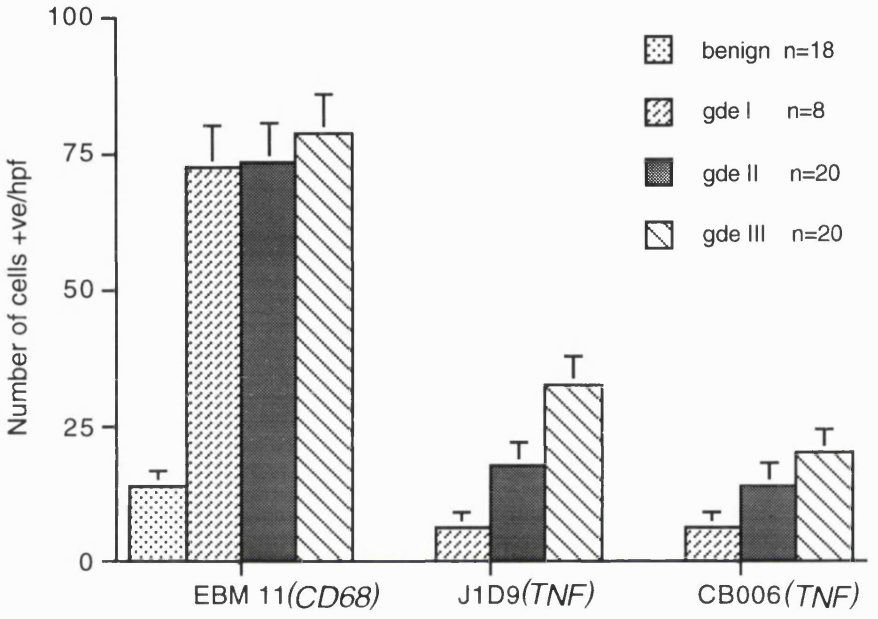
Tissue		(no)	Antibody (Median no. cells positive/hpf) J1D9	CB006	EBM/11
Normal & benign		18	1.4	0.2	14.
Invasive carcinoma		59	10.5	6.3	67.6*
Ductal	grade I	8	5.0	1.4	72
	grade II	16	15.3†	12‡	62.5
	grade III	15	35.5§	23#	77
Lobular & others		10	19.8	10.7	73.6

J1D9:- § p=0.002, c/w grade II, † p=0.02, c/w grade I. CB006:- # p=0.11, c/w grade II, ‡ p=0.04, c/w grade I. EBM/11:- * p<0.001, c/w normal/benign. EBM/11:- no significant differences within invasive carcinoma subgroups (Mann-Whitney).

Considering ductal carcinomas, there was however a correlation between the mean number of cells positive for TNFα protein per high power field and tumour grade (defined by antibodies J1D9 and CB006, test for trend R=0.63, p<0.001 and R=0.48, p<0.001 respectively, fig 2:6).

Expression of TNF-α protein was also seen in the stroma of lobular carcinomas and the frequency of expression was similar to that of grade II ductal carcinomas (table 2:5).

Figure 2:6. Macrophage and TNF-α expression in benign and malignant breast tissue: Cells positive per high power field vs grade.



J1D9: test for trend, R=0.63, p<0.001.
CB006: test for trend, R=0.48, p<0.001.

Nodal status was available on 34 of the cases of invasive carcinoma, in 16 of which nodes were uninvolved by carcinoma (negative) and 18 were involved (positive). There was no significant difference in either the number of macrophages/hpf or the number of cells/hpf expressing TNFα protein when comparing node positive and node negative cases.

2:3:4. Expression of TNF receptors.

Antibodies utr (p75) and htr (p55) to high and low molecular weight receptors demonstrated that as with TNFα protein, TNF receptor expression was predominantly stromal. In general, expression of the p75 receptor was more frequent than the p55 receptor. The p75 receptor was usually expressed by cells within the lymphoplasmacytic infiltrate which on adjacent sections

expressed T-cell markers (UCHT-1) and monocyte/macrophage markers (EBM/11, figure 2:7).

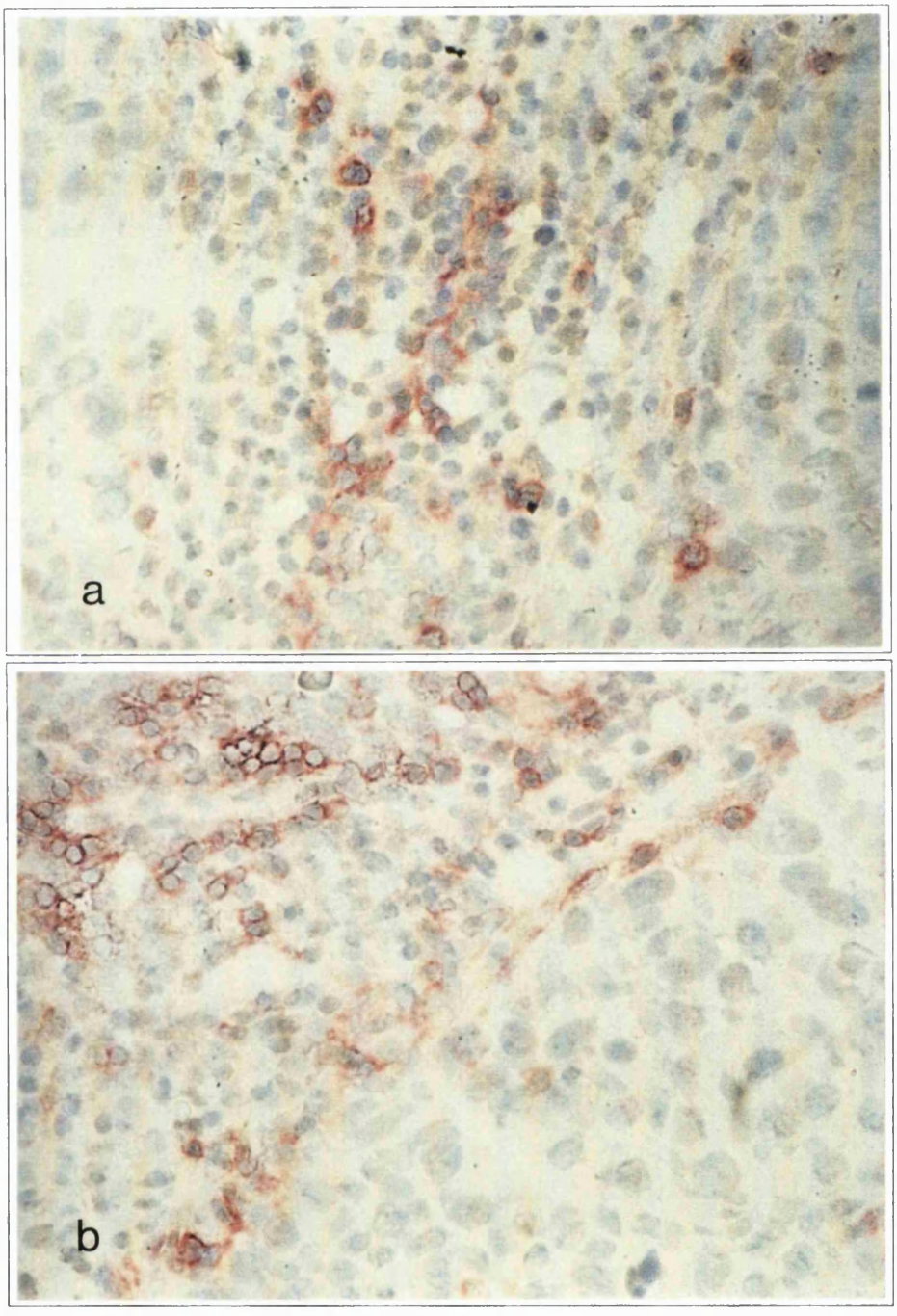


Figure 2:7. Expression of TNF receptor (TNF-R p75) in the stroma of infiltrating ductal carcinoma of the breast (a). Adjacent section demonstrating expression of UCH-T1 [T-lymphocytes] (b) in the same area. See overleaf for expression of CD68 (x400).

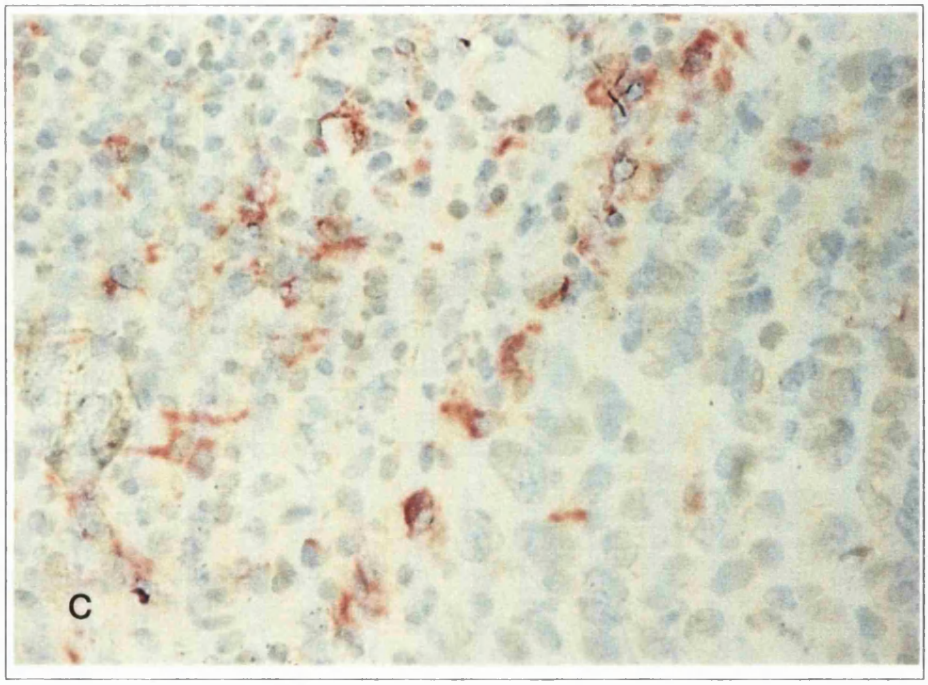


Figure 2:7. (c) CD68 positive cells (monocytes/macrophages) in an adjacent section (compare with figure 2:7, a&b on the previous page, x400)

There was a significant correlation between the number of cells positive per high power field for the TNF antibody J1D9 and the number of cells expressing the p75 TNF receptor ($R=0.8$, $p<0.001$). Similarly, there was a correlation between J1D9 expressing cells and expression of the lower molecular weight p55 TNF receptor ($R=0.81$, $p<0.001$).

In some cases, vascular endothelium was positive for both antibodies but adjacent tumour cells were negative (figure 2:8, a&b).

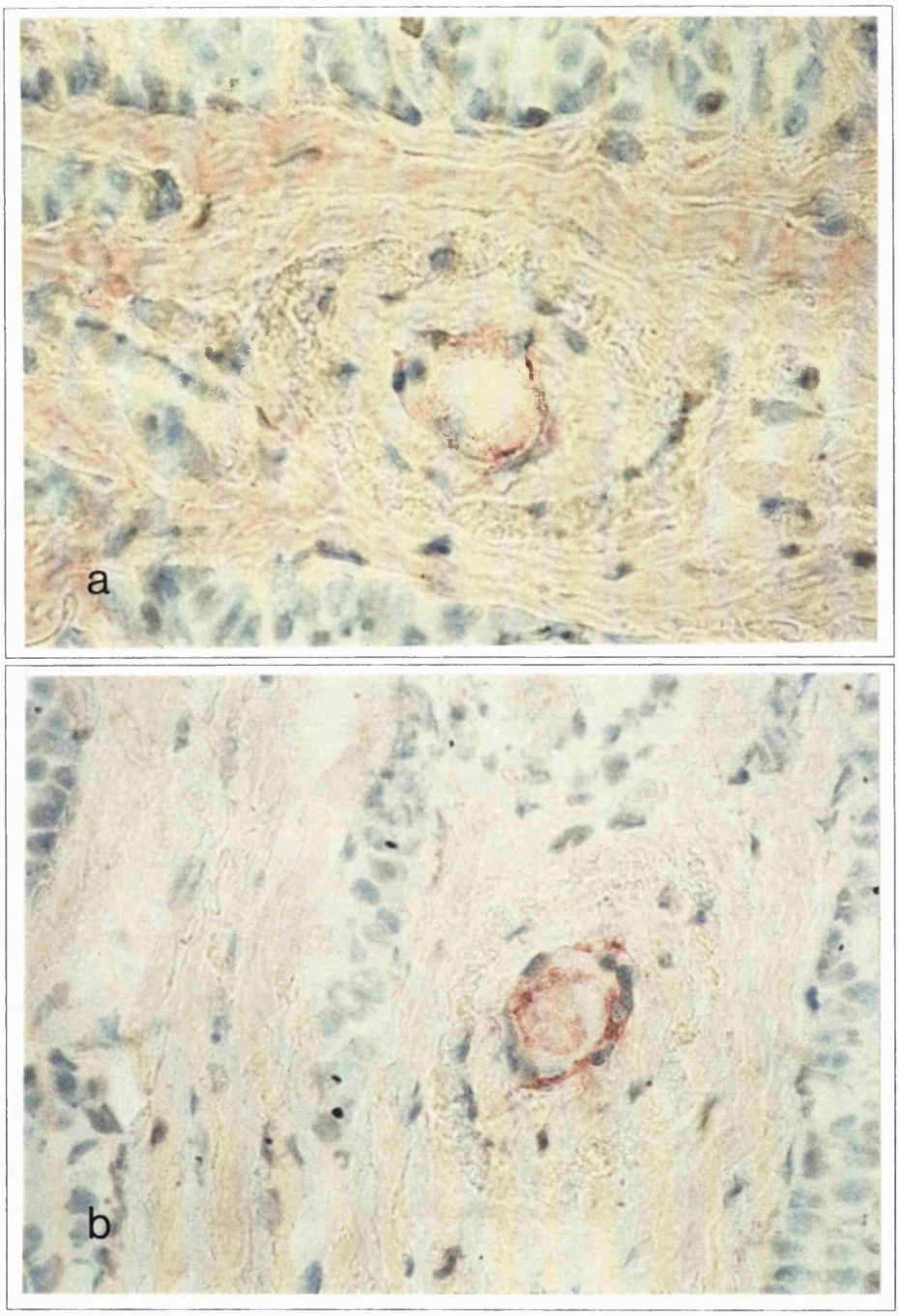


Figure 2:8. Expression of TNF-Rp55 [htr] (a) and TNF-Rp75 [utr] (b) on tumour vascular endothelium in a case of infiltrating ductal carcinoma. Note that adjacent tumour cells are negative (x400).

2:4. Discussion.

The presence of a lymphoplasmacytic infiltrate in primary breast cancer has been taken as evidence of a host immune response to tumour. This study has demonstrated, at the level of mRNA and protein, that one of the cytokines which may be responsible for mediating a host immune response to tumour, is present in the stroma of the majority of breast cancers studied. Expression of TNFα mRNA was scanty and focal, whereas TNFα protein expression was more frequent and widespread. This finding may reflect the relative instability of TNFα mRNA compared with protein (Ross et al 1991). TNFα mRNA and protein colocalised to the tumour stroma, particularly in areas of stroma adjacent to areas of invasive carcinoma. Staining of adjacent sections and two colour immunofluorescence indicated that immunoreactive TNFα was confined to a proportion of CD68 positive macrophages. Although no correlation between tumour grade and number of infiltrating monocytes-macrophages per high power field was found, the proportion of cells expressing tumour necrosis factor increased with increasing tumour grade. The number of specimens within a particular tumour grade examined was inadequate to determine whether or not TNFα expression is of any additional prognostic significance. No relationship between the frequency of macrophages or macrophages expressing TNFα and nodal status was noted.

The pattern of TNFα expression in this study is similar to that observed in colorectal cancer (Naylor et al 1990a), renal cell carcinoma (Waase et al 1992) and in cervical carcinoma (unpublished observations). In ovarian cancer TNFα mRNA expression was observed in epithelial tumour cells and infiltrating macrophages, whereas TNF protein localised primarily to a subpopulation of macrophages within and in close proximity to tumour areas

(Naylor et al 1993). In this series, a correlation was noted between grade of serous ovarian carcinoma and expression of TNFα.

In the current study, the p75 TNF receptor was expressed by cells in the mononuclear infiltrate, particularly T-cells. The p55 receptor was expressed to a lesser extent in the mononuclear infiltrate Both the p55 and p75 receptors were demonstrated on endothelial cells in cases of infiltrating carcinoma. Breast epithelial cells and tumour cells were negative for both types of receptor in all cases examined. Although TNF receptors have been demonstrated on a variety of cell lines using ligand binding, (Sugarman et al 1985) the above findings using immunohistochemistry are in accord with those of Ryffel et al (1991) who showed that neither the p55 or p75 receptors were detectable on non-lymphoid tissue. The lack of TNF receptors on epithelial tumour cells in carcinoma of the breast would suggest that TNFα may not be responsible for direct cytolysis of tumour cells in primary breast cancer. TNFα may be important however in mediating indirect anti-tumour mechanisms including cytotoxic T-cell responses (Ranges et al 1987, Nakano et al 1989) and the augmentation of non-MHC restricted cytotoxic mechanisms such as natural killer cell activity (Ostensen et al 1987) and lymphokine activated killer cell induction (Owen-Schaub et al 1988). TNFα may also be involved in the autocrine induction of macrophage cytotoxicity (Lake et al 1992). In addition, the anti-tumour activity of TNFα may also be mediated by its effects on tumour vascular endothelium on which both types of TNF receptor have been demonstrated.

As discussed in chapter 1, through a variety of mechanisms including endothelial activation and induction of proteases, TNFα may promote invasion and metastasis. The observed correlation between TNFα expression and tumour grade may explain therefore some of the differences in the biological

behaviour observed in higher grade tumours. Considering the pleiotropic effects of cytokines such as TNFα which may mediate the host immune response to tumour, it is perhaps not surprising that the effects of a lymphoplasmacytic infiltrate *per se* on prognosis in primary breast cancer are not clear.

2:5. Hypothesis.

It is becoming apparent that epithelial cells are capable of elaborating a range of chemotactic cytokines (chemokines) which result in trafficking of monocytes/macrophages to tumour sites. Considering the absence of TNF receptors on tumour cells and ultimately the lack of an effective immune response to tumour, the net effects of TNF expression in the stroma of primary breast cancer may be to promote tumour growth and invasive capacity. This hypothesis is supported by the observation that TNF expression is related to tumour grade. In order to study the potential consequences of TNF expression in relation to tumour grade further, assessment of the vasoactive molecule nitric oxide and proteases (72kDa and 92kDa type IV collagenase), both of which may be regulated by TNF, were studied in a series of primary breast cancers and are the subject of the following two chapters.

Chapter 3.

NITRIC OXIDE SYNTHASE ACTIVITY IN PRIMARY BREAST CANCER.

3:1. Introduction.

The sustained growth of solid tumours is dependent on the development of a vascular network (Folkman and Klagsburn 1987) and a variety of growth factors, including TNF-α, may be involved in this process (Leibovich et al 1987). Tumour associated vessels are morphologically distinct, having a more tortuous course, incomplete endothelium and a relative absence of smooth muscle (Jain 1988). In addition to, and possibly as a result of these morphological differences, tumour associated vessels have a decreased sensitivity to vasoactive agents such as endothelin, platelet activating factor and angiotensin II (Andrade et al 1991). More recent data have suggested that tumour vasculature is in a state of maximal dilation, possibly due to the presence of a potent vasodilator, nitric oxide (Peterson 1991).

3:1:1. Nitric oxide: a brief history.

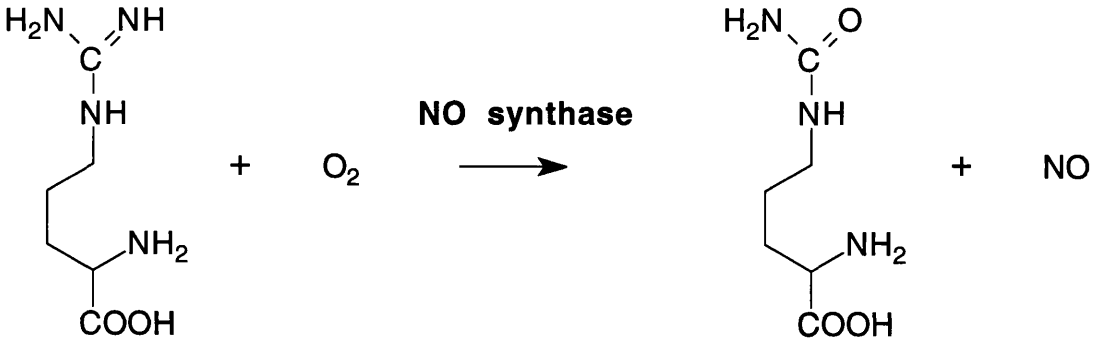
Following observations by Furchgott and Zawadski (1980) that a diffusible substance produced by endothelium (termed endothelium derived relaxing factor, EDRF) mediated the vasodilator action of acetyl-choline and the observation that the vasodilatory properties of nitrates were mediated by nitrosothiol and/or nitric oxide (Ignarro et al 1981), Moncada and colleagues, using simultaneous bioassay and chemiluminescence assay demonstrated subsequently that nitric oxide accounted for the biological activity of EDRF (Palmer et al 1987). It has since been shown that other vasoactive agents such as bradykinin, histamine adenine nucleotides, thrombin, substance P

and 5-hydroxytryptamine mediate their effects via nitric oxide and that other physical factors such as stretching, flow, stress or hypoxia also lead to the release of nitric oxide by endothelial cells (reviewed by Moncada and Higgs 1993). At about the same time that NO was shown to be equivalent to endothelium derived relaxing factor, its importance as a mediator of some forms of neurotransmission (reviewed by Snyder 1992) and macrophage mediated cytotoxicity (Stuehr and Nathan 1989) was recognised.

3:1:2. Synthesis and activities of nitric oxide.

Nitric oxide (NO) is synthesised during the conversion of L-arginine to L-citrulline (Palmer et al 1988) and is illustrated in figure 3:1. This reaction is catalysed by the enzyme NO synthase (NOS, Palmer and Moncada 1989) of which several isoforms have been identified (reviewed by Knowles and Moncada 1994).

Figure 3:1, Conversion of L-arginine to L-citrulline.



L-Arginine L-Citrulline

Broadly, the isoforms fall into two main categories constitutive and inducible. The constitutive form of the enzyme (cNOS) is present in neuronal tissue and endothelium, while an inducible form (iNOS) is present in cells of the

monocyte/macrophage lineage and vascular smooth muscle cells. The properties of these isoenzymes are compared in table 3:1.

Both constitutive and inducible isoforms of NO synthase require NADPH and tetrahydrobiopterin as cofactors, and are inhibited by competitive substrate analogues of L-arginine such as L-NMMA (N^G-monomethyl arginine) and L-NIO (L-iminoethyl ornithine, Moncada et al 1991).

Table 3:1. Comparison of constitutive and inducible isoforms of the enzyme NO synthase.

Constitutive	**Inducibie**
cytosolic	cytosolic
NADPH dependent	NADPH dependent
dioxygenase	dioxygenase
Ca^{2+}/calmodulin dependent	Ca^{2+}/calmodulin independent
picomoles released	nanomoles released
short acting release	long acting release
not affected by glucocorticoids	inhibited by glucocorticoids

Receptor activation by acetylcholine (endothelial cells) or glutamate (cerebellar cells) leads to an increase in cytosolic calcium which activates cNOS and thereby releases a short burst of NO. Being freely diffusible, the NO released may act on neighbouring target cells, e.g. adjacent smooth muscle cells or neurones by increasing intracellular cyclic GMP (Moncada et al 1991).

Inducible NOS is thought to be largely calcium independent and is expressed by a variety of cell types including macrophages (Marletta et al 1988),

neutrophils (McCall et al 1989), endothelial cells (Radomski et al 1990), vascular smooth muscle (Moncada 1991) and some tumour cell lines (Amber et al 1988). Inducible NOS in macrophages releases large amounts of NO (up to a thousand times the amount released by constitutive NOS). In this setting, NO is thought to act as a cytotoxic agent via effects on mitochondrial respiration and DNA replication of invading microrganisms and tumour cells (Drapier and Hibbs 1988, Stuehr and Nathan 1989).

Cytokine induction of nitric oxide has been demonstrated in endothelium (Kilbourn and Belloni 1990), and in smooth muscle (Busse and Mulsch 1990, Wood et al 1990, Beasley et al 1991) where IFNγ and TNF have been shown to have synergistic effects on the production of NO (Geng et al 1992). High levels of NO in response to cytokine stimulation has been demonstrated in macrophages in animal systems (Drapier et al 1988) though whether or not NO mediates the cytotoxic effects of cytokines such as TNF remains unclear. Although susceptibility of tumour targets to TNF were shown to correlate with the ability to produce NO (Fast et al 1992) abrogation of NO by the competitive substrate analogue L-NMMA did not affect response to TNF suggesting more complex mechanisms. Cyclic GMP (generated by NO induction of guanylate cyclase) has also been implicated in the mechanisms of TNF-mediated cytotoxicity (Higuchi et al 1991).

3:1:3. Nitric oxide and tumour vasculature.

Studies in animal models using tumour cell-lines carried in sponge implants have demonstrated that the pharmacological responses of tumour vasculature differ compared with responses of vasculature in normal tissue (Andrade et al 1991, Andrade et al 1992a). These studies demonstrated a loss of vasoconstrictor response to agents including endothelin-1, angiotensin II and

adrenalin. Other data have demonstrated that NO modulates blood flow in tumour vessels and maintains a dilator tone (Peterson 1991). This effect may be mediated by induction of NOS in tumour vasculature (Buttery et al 1993) and is supported by data which showed that competitive substrate analogues of NO synthase, L-NMMA and L-NAME (N^G-Nitro-L-arginine-methyl ester), leads to removal of the dilator tone (Andrade et al 1992b). It is possible therefore that the failure of tumour vasculature to respond to vasoactive mediators may be due to an increase of NO synthesis in cancer tissue.

Endothelial NO synthesis also leads to reduced platelet aggregation (Radomski et al 1987) which could therefore counteract the procoagulant activity of cytokines within sites of tumour, such as TNF and influence its anti-tumour activity.

Nitric oxide may also influence angiogenesis within tumour. It has been reported recently that the angiogenic activity of human monocytes requires an L-arginine-dependent NO synthase process (Leibovich et al 1994) and it is noteworthy that NO has also been shown to play an important role on the angiogenesis associated with tissue healing (Konturek et al 1993).

In other respects, NO may promote tumour growth, for example many of the physiological effects of NO are mediated by an increase in the cAMP/cGMP ratio, which is known to favour division in breast cancer cells lines (Cho-Chung et al 1981).

3:1:4. Aims of these studies.

Tumour necrosis factor is a potent inducer of iNOS. Having demonstrated the presence of TNF-α in primary breast cancer at the level of mRNA and protein, breast tumours were examined for evidence of NO synthase activity. Studies were designed to assess the total NO synthase and activity due to calcium dependent (constitutive) and calcium independent (inducible) isoforms of the enzymes. In addition, localisation of the enzyme was carried out using immunohistochemistry.

3:2. Methods.

Nitric oxide is a paramagnetic free radical gas with a half life in tissue of approximately 7 seconds. Assessment of nitric oxide activity was made by measurement of the conversion products nitrite (NO_2) and nitrate (NO_3) which are generated according to the equation:-

$$2NO^{\bullet} + O_2 \rightarrow 2NO_2 + H_2O \rightarrow \mathbf{NO_2 + NO_3} + H^+$$

The nitrate and nitrite thus formed (collectively termed NO_x) reflect the activity of NO and were measured in a colorimetric assay. The role of NO synthase in the generation of NOx was confirmed by coincubation with analogues of L-arginine which act as false substrates for the enzyme NO synthase and do not generate NO. The second method of assessing NO synthase activity measures the conversion of radiolabelled L-arginine to L-citrulline. Following collection of tissue, these assays were carried out by Lindy Thomsen at the Wellcome Foundation.

3:2:1. NOx estimation.

Fresh tissue was collected at the time of excision biopsy or mastectomy and placed in transport media. Breast tissue obtained from patients undergoing reduction mammoplasty provided a control group. Transport medium consisted of Dulbecco's modified Eagles medium (MEM) with Hepes buffer (25mM), sodium pyruvate (0.11g/L) and glucose (1g/ml) supplemented with amphotericin (3.6mg/l) and gentamicin (50mg/L)

Tissue was placed in culture medium (Dulbecco's MEM, Gibco) supplemented with glutamine (2mM) and divided into $1mm^3$ pieces. These were washed

twice in culture medium and placed into 96 well plates. Cultures were carried out in medium alone or with competitive substrate analogues N^G-monomethyl arginine (L-NMMA) and L-imino-ethyl ornithine (L-NIO) at concentrations of 0.5, 1.0 and 2.0 mM. Plates were cultured for 24 hours at 37°C in 95% air/5%CO_2. Supernatants were removed and stored at -70^0 C until assayed.

Nitrite and nitrate (NOx) was measured by chemiluminescence after reduction of nitrate using acid washed cadmium powder (Thomsen et al 1991). Nitrite was measured by chemiluminscence with a limit of detection of 0.1μM (Salter et al 1991). Total protein content of tissue pieces was determined with bicinchoninic acid reagent after solubilizing the tissue with 1M NaOH. NOx estimates are expressed as nmol/mg protein per 24 hour period.

3:2:2. Assay of NO synthase activity.

Frozen tissue was extracted at 0-4°C by homogenisation with an Ystral homogeniser in 2.5 volumes of a buffer containing 320mM sucrose, 20mM Hepes, 1 mM EDTA, 1 mM DL-dithiothreitol, 10μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor and 1 μg/ml pepstatin brought to pH 7.2 at 20° C with HCl. The homogenates were centrifuged at 10,000 g at 0-4°C for 30 minutes. Supernatants were passed through a 2 ml column of cation exchange resin (AG 50W-X8, Bio-Rad) to remove endogenous arginine and were stored on ice for up to 2 hours before use. Nitric oxide synthase in these supernatants was measured by the conversion of L-[U-^{14}C] arginine to [U-^{14}C] citrulline at 37°C for 10 minutes as described previously (Salter et al 1991). The activity of the calcium-dependent enzyme was determined from the difference between the [U-^{14}C] citrulline generated from control samples and samples containing 1mM [ethylenebis(oxy-ethylenitrilo)]tetraacetic acid (EGTA); the activity of calcium-independent enzyme was determined from the difference between

samples containing 1mM EGTA and 1mM L-NMMA. The limit of detection in this assay was 0.7pmol/min/mg protein.

3:2:3. Immunohistochemistry.

Sections (5μ thick) were cut from frozen tissue, air dried for 30 minutes then fixed in acetone for 30 minutes. Two antibodies were used for NO synthase detection: 1) rabbit antiserum raised against rat brain NO synthase which has proven reactivity with neuronal and endothelial constitutive NO synthase isoenzymes across several species including humans (Springall et al 1992) and 2) a monoclonal antibody raised in mice against a 21kDa protein fragment of murine macrophage inducible NO synthase (ANTI-macNOS; Transduction laboratories, Lexington Kentucky, USA). This antibody reacts with human inducible NO synthase (Ian Charles, Wellcome Research Laboratories, personal communication). Immunohistochemistry was carried out as described in chapter 2. Briefly, for the rabbit polyclonal antibody, sections were incubated in 4% normal swine serum for 15 minutes prior to incubation with primary antibody at a dilution of 1 in 1000 for 16 hours at 4°C. Following this sections were incubated with swine anti-rabbit (Fab_2) at a dilution of 1 in 300 with 1 in 25 normal human serum for 30 minutes. Following application of streptavidin at a dilution of 1 in 500 for 30 minutes, sections were developed in DAB. For the mouse monoclonal antibody, sections were incubated with normal rabbit serum for 15 minutes followed by incubation in primary antibody at a dilution of 1 in 100 for 16 hours at 4°C. Second layer consisted of rabbit anti-mouse (Fab_2) at a dilution of 1 in 250 with 1 in 25 normal human serum for 30 minutes. Antibody was visualised with streptavidin and DAB as described. For macrophage detection, adjacent sections were incubated for 30 minutes with a monoclonal antibody against the human

monocyte/macrophage marker CD 68 (EBM 11, DAKO UK). Sections without primary antibody were included as a negative control.

3:3. Results.

3:3:1. NOx accumulation.

NOx (nmol/mg protein) accumulated in culture medium supernatant during culture of tissue pieces over a 24 hour period. Results for benign and malignant breast tissue examined are shown in table 3:2. Levels of NOx produced by normal tissue were negligible (0.02±0.05, n=4). NOx production was significantly higher for the 14 invasive tumours ($p<0.018$, Mann-Whitney).

Table 3:2. Nitric oxide production in benign and malignant breast tissue.

Tissue		(no)	NOx production (ng/day/mg protein mean±s.e.)
Normal		5	0.067±0.03
Benign Cystic change		1	0.3
DCIS		1	1.46
Invasive carcinoma		14	1.92±0.45
Ductal	grade I	1	nd
	grade II	7	0.99±0.27
	grade III	4	3.75±0.85
Lobular		1	3.3
Phyllodes		1	1.77

Median values and non-parametric statistics (Mann-Whitney)
DCIS=ductal carcinoma in situ, nd=not detected

Normal vs Invasive carcinoma	0.06 vs 1.75, p=0.02
grade III vs grade II	3.65 vs 0.92, p=0.03
grade II vs normal	0.92 vs 0.06, p=0.04

A case of benign cystic change yielded a higher level than the normal controls but less than the mean value of the invasive tumours. NOx production in a case of *in situ* ductal carcinoma was higher than that observed in the normal/benign cases but again was lower than the mean value for invasive tumours. Within the group of ductal carcinomas, NOx production was greater in grade III tumours than in grade II tumours (median values, 3.65 vs 0.92 ng/day/mg protein, $p<0.03$, Mann-Whitney). NOx was not detectable in cultures of tissue from the grade I ductal carcinoma. An invasive lobular carcinoma and a phyllodes tumour also showed relatively high concentrations of NOx in culture medium after 24 hours.

The accumulation of NOx was inhibited by the competitive substrate analogues L-NMMA and to a lesser extent L-NIO (table 3:3) demonstrating that the presence of nitrite and nitrate was due to the activity of NO synthase.

Table 3:3. Inhibition of NOx production by competitive substrate analogues L-monomethyi arginine (L-NMMA) and L-iminoethyl ornithine (L-NIO).

	% inhibition of NOx production (± s.e.)	
	L-NMMA	L-NIO
Conc. inhibitor (μM)		
0.5	0±11	6±9
1.0	102±2	43±9
2.0	101±6	64±16

3:3:2. Nitric oxide synthase activity.

Nitric oxide synthase activity was detected only in tumour tissue (table3:4). For ductal carcinomas activity was detected in all grade III and one of two grade II tumours.

Table 3:4. NO synthase activity in benign and malignant breast tissue (n=23).

Tissue		(no)	NO synthase activity (pmol/min/mg protein, mean±SE)	
			Total	Ca^{2+} independent
Nomal		3	nd	nd
Benign lesions*		5	nd	nd
Invasive carcinoma		15	5.1±1.4	2.3±0.7
Ductal	grade I	1	nd	nd
	grade II	2	3.9±3.9	nd
	grade III	7	8.5±2.2	3.7±1.1
Lobular		4	3.3±1.8	2.0±1.2
poorly differentiated		1	4.9	0.98

nd=not detected. *1 case of benign cystic change, 1 case of stromal fibrosis and 3 fibroadenomas.

Nitric oxide synthase was not detectable in tissue from the grade I ductal carcinoma or benign conditions or in normal breast tissue. NO synthase activity was noted in 8 of 10 infiltrating ductal carcinomas, 2 of 4 lobular carcinomas and in a poorly differentiated carcinoma. In all but two cases of invasive ductal carcinoma (one grade II and one grade III) a proportion of the NO synthase activity was found to be calcium independent. Considering the

11 tumours which expressed NO synthase activity, the mean proportion (±SE) which was calcium independent was 42±10%.

3:3:3. Immunohistochemistry.

Rat brain NO synthase antibody.

This antibody labelled cells in the tumour stroma which had the morphology of macrophages (figure 3:2a). They were adjacent to islands of invasive tumour cell though occasionally, labelled cells were seen within solid islands of grade III tumours and within ducts involved by *in situ* carcinoma. It was usually possible to recognise that these cells morphologically as macrophages and serial sections stained with the CD68 marker EBM/11 confirmed their macrophage origin (figure 3:2b). In sections from normal and benign breast tissue occasional immunolabelling of stromal macrophages was seen.

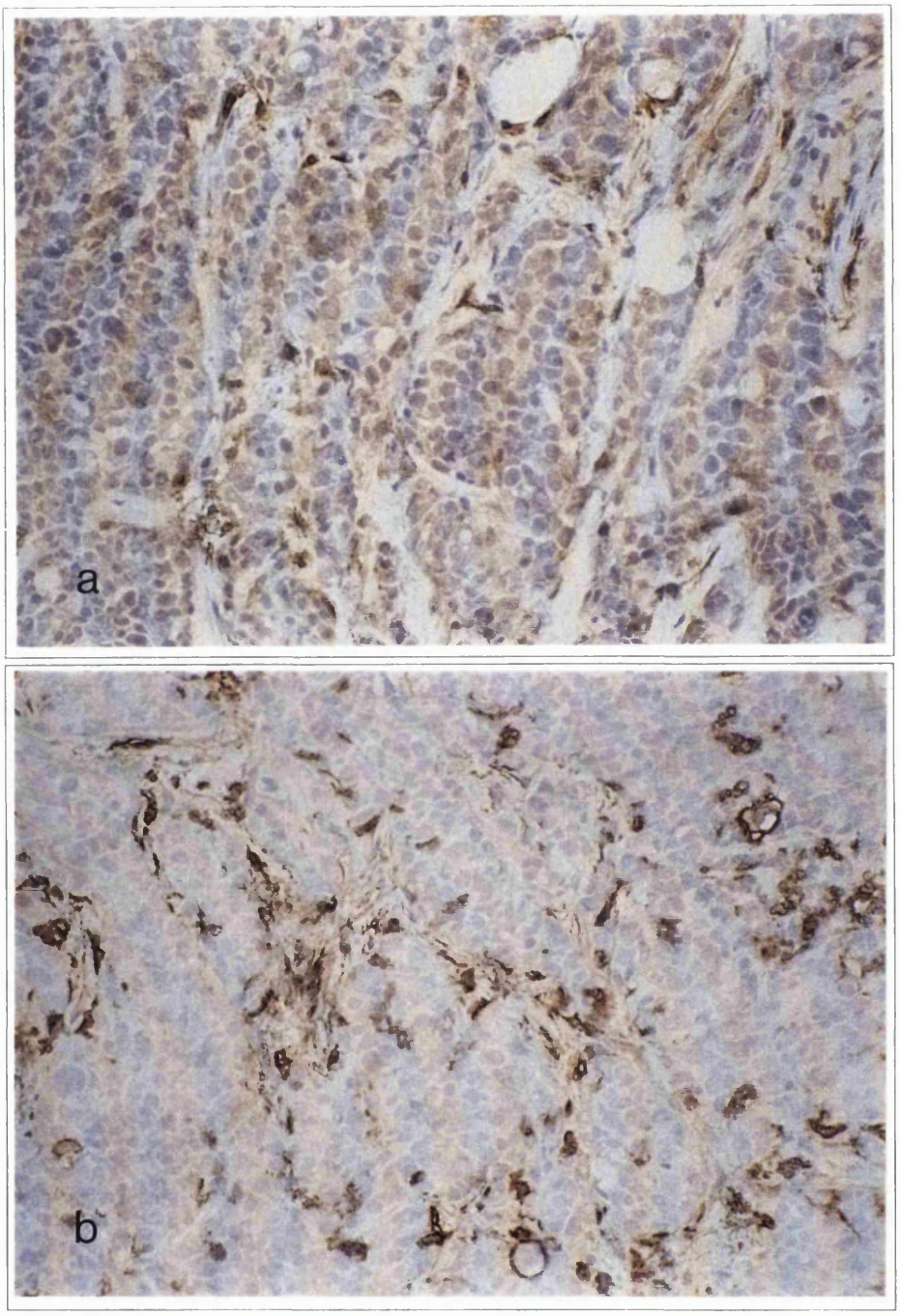


Figure 3:2. Expression of NO synthase (rat brain NO synthase antibody) in infiltrating ductal carcinoma (a). Cells have the morphology of macrophages and on an adjacent section express the monocyte-macrophage marker CD68 (b) x200.

In some cases of benign and malignant breast tissue, immunolabelling of myoepithelial cells (fig 3:3, fig 3:4, overleaf) and endothelial cells (fig 3:5, overleaf) was observed.

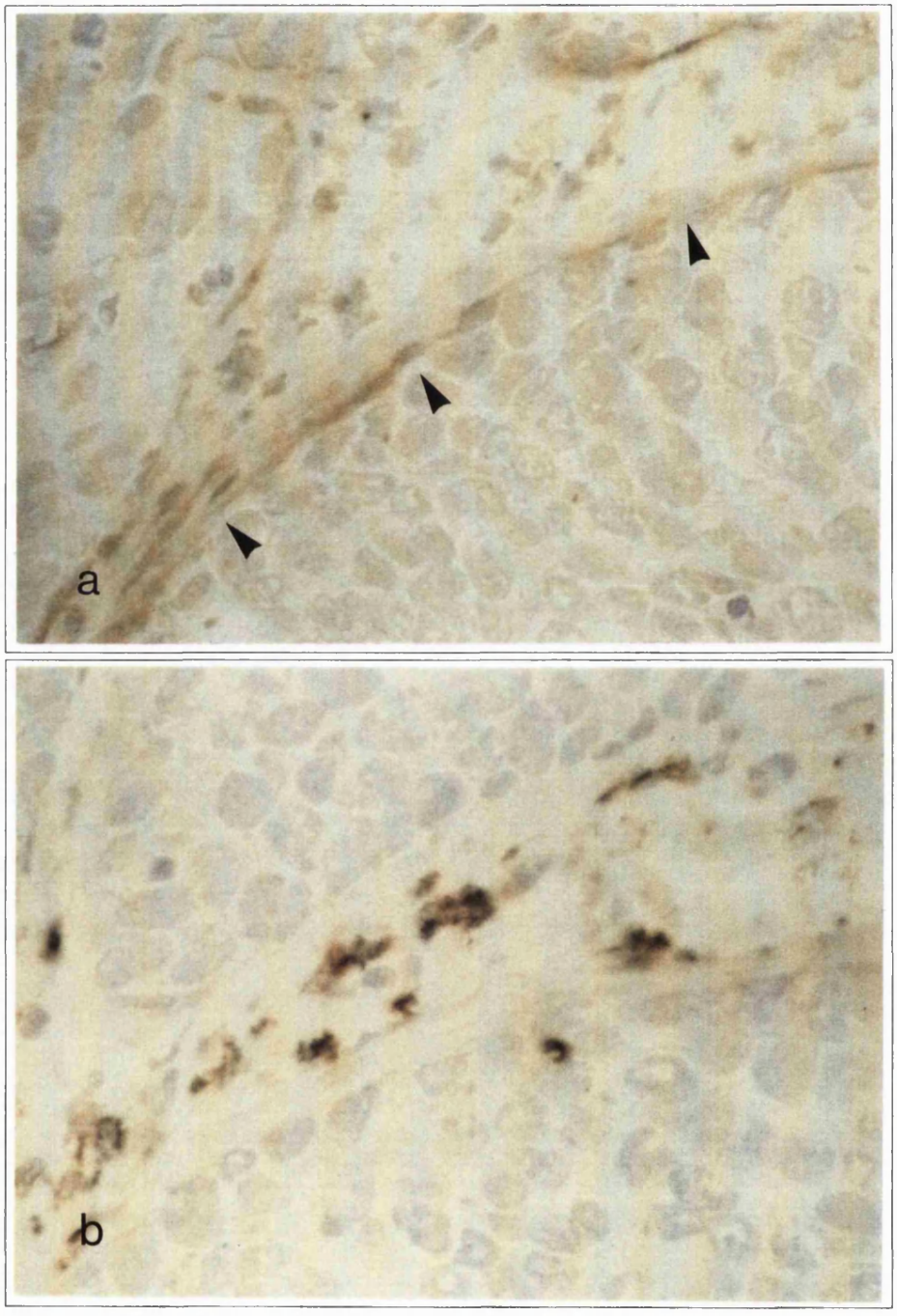


Figure 3:3. Expression of NO synthase (rat brain NO synthase antibody) in myoepithelium [arrow] surrounding an area of ductal carcinoma in situ (DCIS) [a)]. Although macrophages (CD68 positive) are present between areas of DCIS, they do not express NO synthase [b] x400.

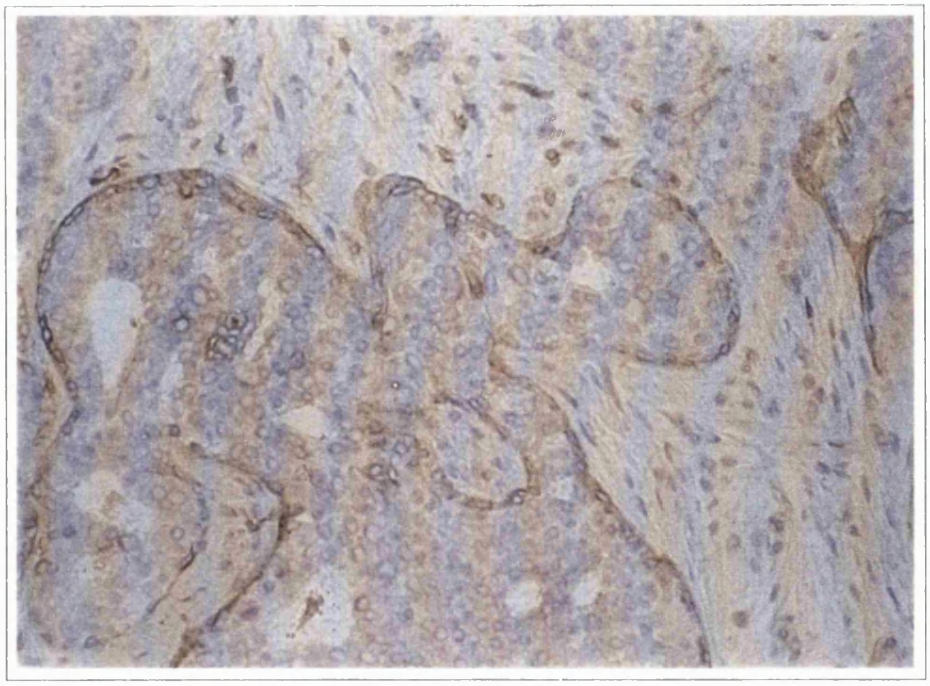


Figure 3:4. Expression of NO synthase (rat brain NO synthase antibody) in myoepithelium in an area of ductal carcinoma in situ x200.

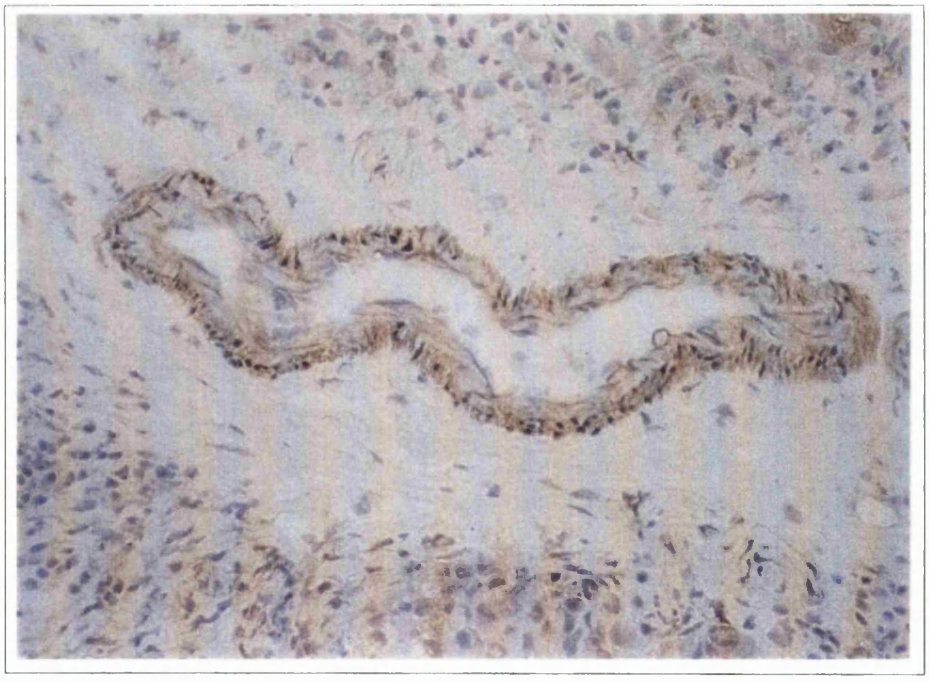


Figure 3:5. Expression of NO synthase (rat brain NO synthase antibody) in vascular endothelium in a case of infiltrating ductal carcinoma (x200).

Mouse macrophage NO synthase antibody.

Studies with this antibody showed a distribution of immunolabelling within tumour-associated macrophages (fig 3:6) similar to that observed with the rat brain NO synthase antibody (fig 3:2). Less labelling was observed in endothelium and myoepithelium.

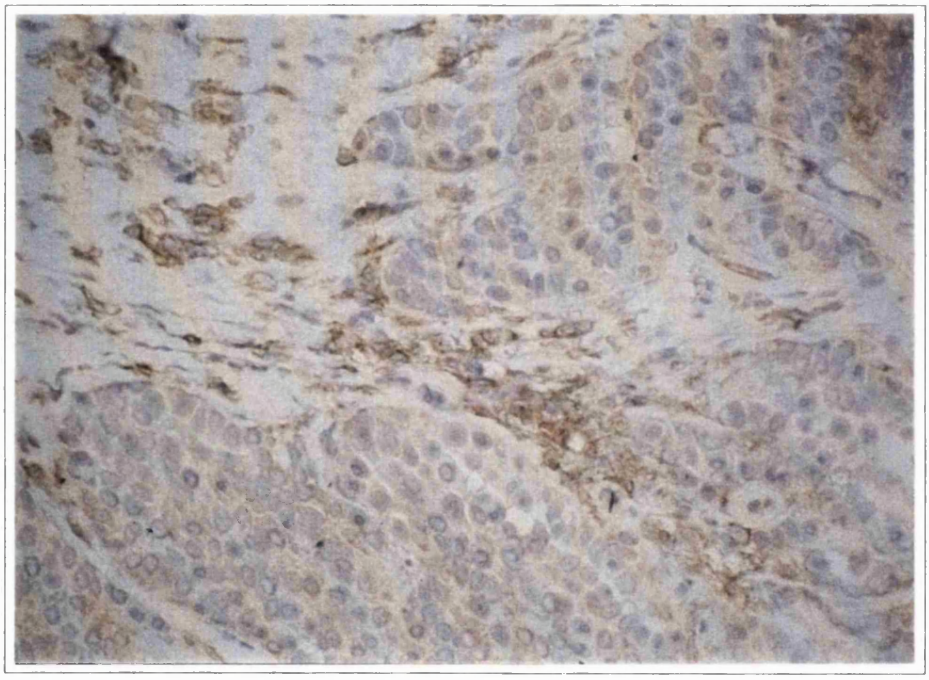


Figure 3:6. Expression of NO synthase (murine macrophage antibody) in a case of infiltrating ductal breast carcinoma (x200).

3:4. Discussion.

These studies have demonstrated that NO biosynthesis and NO synthase activity is higher in human primary breast cancers than either benign lesions or normal breast tissue in which activity was low or not detectable. Furthermore, NO biosynthesis was significantly greater in tissue from grade III compared with grade II invasive ductal carcinomas. The generation of NOx during culture of viable tumour tissue and demonstration of NO synthase activity in the presence of calcium chelators suggest the presence of the inducible form of NO synthase. Calcium dependent activity was also measurable, indicating the additional presence of a constitutive NO synthase isoform.

These quantitative biochemical assays are consistent with the localisation studies which revealed proteins immunoreactive with the inducible NO synthase antibody within macrophages. The polyclonal rat brain antibody, which cross-reacts with all three NO synthase isoforms (Springall et al 1992), labelled vascular endothelial and myoepithelial cells in addition to macrophages. Several tumour cell lines express NO synthases (Amber et al 1988, Forstmann et al 1990, Radomski et al 1991, Werner-Felmeyer et al 1993, Sherman et al 1993 and Jenkins et al 1994) and immunohistochemical studies in human ovarian cancer suggest NO synthase localisation in tumour cells (Thomsen et al 1994). NO synthase immunoreactivity was not observed within tumour cells in the breast cases studied here.

The generation of NO by cells of the immune system is an important aspect of non-specific immunity in animal models. (Hibbs et al 1990). Localisation of NO synthase within intratumoral macrophages indicates that NO may also be involved in the anti-tumour immune response in man. In the previous chapter

the presence of TNF-α was demonstrated in primary breast cancer at the level of mRNA and protein. Expression of TNF-α was noted in the tumour-associated macrophages and the frequency of expression in these cell types was also related to tumour grade. TNF-α is known to be a potent inducer of NO synthase in many cell types (see above) and it may regulate the expression of NO synthase in the tumour infiltrating macrophages in primary breast cancer. In this respect it is noteworthy that NO synthase activity was also related to tumour grade. A previous report examining NO synthase activity in gynaecological tumours also observed an association between activity and tumour grade (Thomsen et al 1994).

Like TNF, nitric oxide may play a paradoxical role in tumour biology. Although it is conceivable that NO is part of the host immune response to tumour, the activity of NO synthase reported in these studies of primary breast cancer are approximately 1 to 2 logs lower than levels associated with cytotoxicity (Moncada et al 1991) and apoptosis (Xie and Fidler 1993). Thus NO may have a dual role in cancer: produced at high concentrations, NO may have anti-tumour activity whilst at lower concentrations it may promote tumour growth through effects on angiogenesis (Leibovich et al 1994), and tumour blood flow (Wood et al 1993, Andrade et al 1992b).

A balance in favour of the vascular effects of NO may explain the positive correlation between NO synthesis and increasing tumour grade. This view is also supported by recent data demonstrating that although tumour cells transfected with the gene for iNOS grow more slowly *in vitro*, the tumours which develop *in vivo* behave more aggressively and have the histological appearance of higher grade tumours when compared with controls (Jenkins et al Proc. Natl. Acad. Sci. in press).

It is apparent from the data in the above studies that a major component of the NO synthase activity is calcium dependent and in part may be derived from the tumour vascular endothelium itself. The control of expression of calcium dependent isoforms of NO synthase is currently unclear but it is apparent that shear stress (Nishida et al 1992), exercise (Sessa et al 1994) and hormones such as oestradiol (Weiner et al 1994) may increase their expression. Although administration of competitive substrate analogues such as L-NMMA and L-NIO will necessarily inhibit production of NO by calcium dependent and calcium independent isoforms of NO synthase, their systemic administration in an attempt to modulate tumour blood flow would be complicated by inhibition of NO in the general circulation where continued production of NO is essential for maintenance of systemic vascular flow. Inhibitors of NO synthase, with little or no activity against constitutive NO synthase, might still prove useful for delaying the growth and progression of human breast cancer either alone or in combination with bioreductive agents or following conventional chemotherapy in order to increase retention of cytotoxic agents within the tumour vascular bed.

Chapter 4.

EXPRESSION OF TYPE IV COLLAGENASES IN PRIMARY BREAST CANCER.

4:1. Introduction

Malignant cells must attach to adhesion proteins of the extra-cellular matrix and degrade it in order to invade locally and ultimately metastasise (reviewed by Fidler 1991). In normal tissues a specialised form of extracellular matrix, the basement membrane, separates parenchymal cells from the underlying stroma. Basement membrane is preserved in benign breast disease and carcinoma *in situ* but variable loss of the basement membrane is a feature of invasive carcinoma of the breast (Siegel et al 1981). Basement membrane contains laminin, heparan sulphate proteoglycan but the major structural component is type IV collagen (Martinez-Hernandez and Amenta 1983). Degradation of type IV collagen could be part of the metastatic process.

4:1:1. Matrix metalloproteinases.

Matrix metalloproteinases are a family of highly homologous proteolytic enzymes, although each member has a different substrate specificty (table 4:1 adapted from Matrisian 1992). Matrix metalloproteinase-1 (MMP-1) degrades interstitial collagen, stromelysin (MMP-3) degrades proteoglycans whilst the two type IV collagenases, MMP-2 (molecular weight 72kDa) and MMP-9 (molecular weight 92kDa) degrade type IV collagen.

Table 4:1. Matrix-degrading metalloproteinases

	Name		kDa	Substrate
1.	**Interstitial collagenase** (type I collagenase)	**(MMP-1)**	52/57	I,II,III collagen
	PMN collagenase	**(MMP-5)**	75	I,II,III collagen
2.	**72kDa type IV collagenase** (gelatinase A)	**(MMP-2)**	72	IV,V,VII collagen fibronectin gelatins
	92kDa type IV collagenase (gelatinase B)	**(MMP-9)**	92	IV,V collagen gelatins
3.	**Stromelysin** (transin, proteoglycanase procollagen activating factor)	**(MMP-3)**	57/60	proteoglycans laminin, fibronectin III,IV,V collagen gelatins
	Stromelysin-2 (transin)	**(MMP-10)**	53	III,IV,V collagen fibronectin gelatins
	Stromelysin-3	**(MMP-11)**	55	serine proteases
	PUMP-1 (matrilysin)	**(MMP-7)**	28	gelatins fibronectin

Type IV collagenases are secreted in an inactive proenzyme form and activation results from removal of an 80-amino acid sequence from the N-terminus to yield a 62kDa form in the case of MMP-2 and an 81kDa activated form in the case of MMP-9 (Stetler-Stevenson et al 1989). This can be achieved *in vitro* by treatment with trypsin or organomercurials, but the precise sequence of events required for activation *in vivo* is unknown. Cathepsins and plasminogen activators themselves are incapable of degrading type IV collagen but may contribute to basement membrane damage by activating type IV collagenases (Yagel et al 1989; Goldberg et al 1990).

4:1:2. Regulation of type IV collagenase expression.

Type IV collagenase expression by tumour cell lines can be increased by serial passage (Bresalier et al 1987) and transfection with oncogenes, which in animal models lead to an increase capacity of tumour cells to metastasise and invade reconstituted basement membrane *in vitro* (Garbisa et al 1988). Reduction of expression has been observed by transfection with the adenovirus E1A gene (Frisch et al 1990), retinoic acid treatment (Nakajima et al 1989) or treatment with arachidonic acid analogues (Reich et al 1988). In each case, the level of type IV collagenase treatment was correlated with metastatic behaviour *in vivo* or *in vitro*. Inhibition of type IV collagenase expression by tissue inhibitors of metalloproteinases (TIMPs) also affects the behaviour of metastatic cell lines in animal models. Recombinant TIMP administered intravenously prevented formation of lung colonies in nude mice inoculated with a highly metastatic cell line (Alvarez et al 1990) and mRNA levels for TIMP in variants of a murine mammary adenocarcinoma showed an inverse relationship to their ability to metastasise (Ponton et al 1991).

4:1:3 Cytokine regulation of type IV collagenase expression.

Modulation of type IV collagenase production by cytokines and growth factors is poorly defined. Transforming growth factor-β (TGF-β) increases the secretion of both the 72kDa and 92kDa enzymes in tumour cells and fibroblasts (Overall et al 1989, Weinberg et al 1990, Welch et al 1990). TNF-α has been reported to increase production of the 92kDa enzyme but not the 72kDa type in tumour cells (Brenner et al 1989, Okada et al 1990). TNF-α regulation of the 72kDa form of the enzyme may however occur in non-neoplastic tissue including fibroblasts (Ito et al 1990) and endothelium (Skolnik et al 1992). In addition TNF-α is known to modulate production of

plasminogen activator in cells of monocyte macrophage lineage (Gyetko et al 1992) and this may be the first step in a protease cascade in which plasmin activates other metalloproteinases, which in turn leads to activation of type IV collagenases (Goldberg et al 1990). Thus as well as inducing type IV collagenases, TNF-α may also influence their activation state.

4:1:4. Type IV collagenase expression in breast cancer.

Studies using mouse polyclonal antibodies to type IV collagenase demonstrated staining in invading tumour cells but not in normal breast, benign lesions or *in situ* carcinomas (Barsky et al 1983). A more recent study using antibodies specific for the active and latent forms of the 72KDa enzyme did demonstrate however that both forms were present in normal and malignant breast tissue (Monteagudo et al 1900). In normal tissue type IV collagenase immunoreactivity was demonstrated in myoepithelial cells and occasionally in the epithelial cells of terminal ducts. In invasive carcinoma, a high proportion of tumour cells were positive for type IV collagenase. These findings suggested that myoepithelial cells, expressing type IV collagenase, are involved in normal basement membrane turnover but expression by tumour cells may be a marker of malignant transformation which may facilitate invasion. Studies have also demonstrated that the expression of type IV collagenase in breast cancer may be correlated with more conventional prognostic criteria such as the degree of lymph node involvement (D'Errico et al 1991). In node negative disease, expression of type IV collagenase by the primary tumour correlated with locoregional recurrence but not with time to relapse or overall survival (Daidone et al 1991).

4:1:5. Aims of these studies.

Tumour necrosis factor induces the production of type IV collagenases *in vitro* and in some animal model systems. Having demonstrated the presence of TNF-α in the lymphoplasmacytic infiltrate in primary breast cancer the expression of these enzymes was investigated.

Immunohistochemical studies of type IV collagenase reactivity are necessarily semi-quantitative and many antibodies do not distinguish between the latent and active forms, enzymes bound to co-secreted inhibitors or the different molecular weight isoforms. Quantitative estimation of enzyme activity by release of soluble material from radiolabelled substrate may also suffer from these drawbacks and requires considerable amounts of tissue.

Gel substrate analysis (zymography) using sodium dodecyl sulphate (SDS) polyacrylamide gels containing gelatin has several advantages over other methods. SDS dissociates enzyme/inhibitor complexes and activates proforms of the enzyme without causing a change in molecular weight (Birkedal-Hansen and Taylor 1982). Thus the assay can distinguish between latent and active forms of the collagenases and between the 72KDa and 92 kDa forms of the enzyme and is not affected by TIMPS's. Using computer-assisted image analysis, an accurate and reproducible method of quantifying type IV collagenases from zymograms was achieved allowing comparison between cases by expressing collagenase IV activity per unit of protein. Moreover, the method is sufficiently sensitive to enable assays to be performed on a single cryostat section.

Studies on the localisation of these enzymes at the levels of mRNA and protein were also performed on a subset of cases in which activity of the different forms of type IV collagenase activity was confirmed by zymography.

4:2. Methods

4:2:1. Tissue samples

Breast tissue removed at excision biopsy or mastectomy was cryopreserved in liquid nitrogen. Three cases of normal breast tissue, 8 of benign breast disease and 32 invasive carcinomas of varying type and histological grade were examined. From each tumour a 5 μm thick section was cut from a face area of tumour of approximately 5 mm x 5 mm. Samples were homogenised in 50 μl of SDS-PAGE sample buffer containing glycerol (10%v/v), SDS (1%w/v) and bromophenol blue using Treff microhomogenisers (Scott Lab). Adjacent sections were cut and either mounted for microscopic examination or used for protein estimations. Sections were also cut at varying depths in the block to assess reproducibility of zymography.

4:2:2. Zymography.

Gelatinolytic zymography was performed essentially as described by Brown et al 1990. Homogenised samples (50 μl) were applied directly without heating or reduction to a 5% stacking polyacrylamide gel laid over an 11% (w/v) polyacrylamide gel containing 1mg/ml gelatin and 0.1 % (w/v) SDS. Gels were run at room temp at 180 V for 3.5 hours. After incubation of gels in 2.5 % Triton X-100 for 30 min to remove SDS, the gels were incubated for 16 h in 50mM Tris-HCl, pH 7.6 containing 0.2M NaCl, 5mM $CaCl_2$ and 0.02 w/v Brij-35. Gels were stained for 3h in 30 % methanol/10 % glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G 250 and destained in the same solution in the absence of dye.

Conditioned media from RPMI-7951 cells (72 kDa) and HT-1080 stimulated with 10 ng/ml TPA for 36 hours were used as type IV collagenase standards (Brown et al 1990 Weinberg et al 1990). The same batches of conditioned media were used throughout all experiments.

4:2:3. Computer Assisted Image Analysis

Images of wet gels were captured using a Sony DXC-151P video camera and connected to capture hardware/software (Screen Machine/SM-camera). The integrated density of each band present on zymograms were determined using NIH Image 1.43 software equipped with gel plotting macros. 10 units of collagenase activity were defined as that activity present in 10 µl of the RPMI-7951 and HT-1080 conditioned media used as standards. Units of activity are not comparable between collagenase species of different molecular weight (92kDa, 72kDa and 62kDa).

4:2:4. Protein Estimation.

Single 5 µm cryostat sections from each tumour were homogenised in 1% (w/v) SDS and diluted 10 fold in water before measuring protein content against bovine serum albumin using a Bio-Rad protein assay reagent (Bradford, 1976)

4:2:5. *In situ* hybridisation.

Antisense probes to 72kDa and 92kDa type IV collagenases were generated from pGEM4-72K and pGEM4-92K (provided by British Biotechnology Ltd, Oxford, U.K.) using EcoR-1 cleaved vector using T7 RNA polymerase (Promega Biotech). Negative controls were sense RNA generated from the

same vector linearised in the opposite direction. *In vitro* transcriptions were carried out using Promega Biotech transcription kits to incorporate ^{35}S-UTP (Amersham Int.). *In situ* hybridisation was carried out on cryostat sections as described in chapter 2.

4:2:6. Immunohistochemistry.

A rabbit polyclonal antibody to human 72kDa collagenase was a gift of Professor Hideaki Nagase, University of Kansas Medical Centre. Following blocking with normal swine serum, primary antibody was applied at a dilution of 1 in 500. Following this sections were incubated with swine anti-rabbit (Fab_2) at a dilution of 1 in 300 with 1 in 25 normal human serum for 30 minutes. Following application of peroxidase conjugated streptavidin at a dilution of 1 in 500 for 30 minutes, sections were developed in DAB (as described in chapter 2).

A sheep polyclonal antibody to 92kDa collagenase was a gift of Dr Gillian Murphy, Strangeways Laboratory, Cambridge. Following blocking in normal rabbit serum, the primary antibody was applied in a 1 in 200 dilution. The second layer was biotinylated rabbit anti-goat 1 in 500 (Dako) for 30 minutes which had been previously absorbed with normal human serum. Sections were developed as described previously.

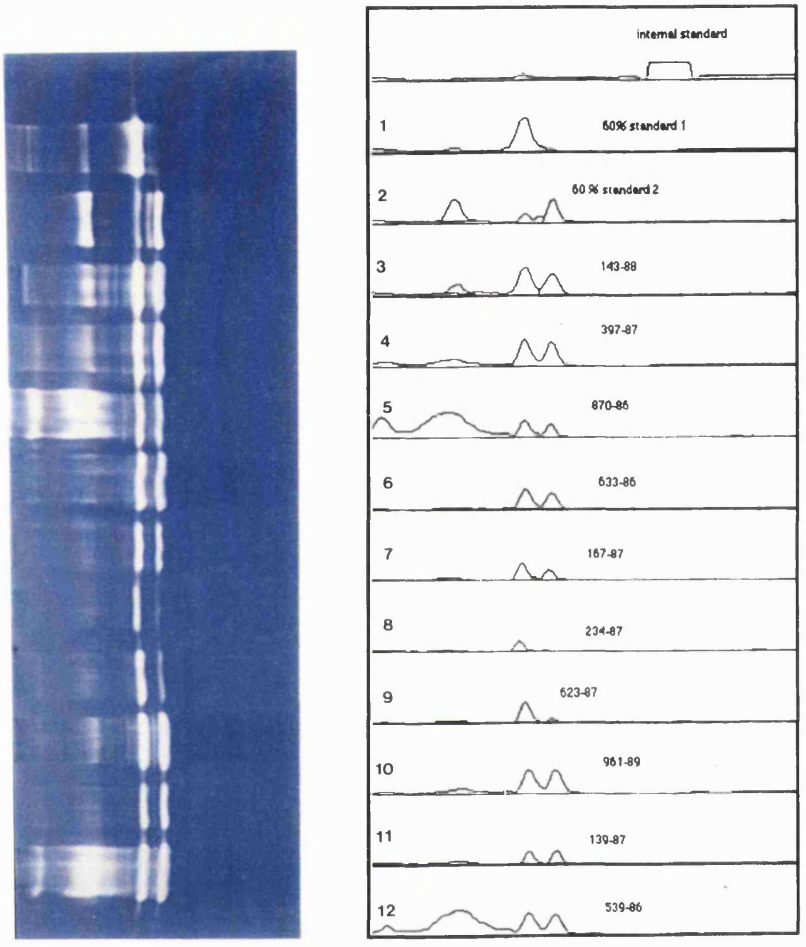
4:3. Results

4:3:1. Collagenolytic activity.

Type IV collagenase activity was detected in 11 cases of normal/benign breast disease and 32 cases of invasive ductal carcinoma of varying grade, by their gelatinolytic activity in gel substrate analysis. Conditioned media from human tumour cell lines which are known to contain type IV collagenases were employed as standards. The human melanoma cell line RPMI 7951, in common with other human melanoma cell lines constitutively secretes 72kDa type IV collagenase (Weinberg et al 1990)(lane 1, fig 4:1). The fibrosarcoma cell line HT1080 secretes 92 kDa type IV procollagenase and smaller quantities of the 72kDa species (lane 2, fig 4:1). The two smaller bands immediately below the 72kDa type with apparent molecular weights of 62 and 59 kDa are the activated forms of the 72kDa proenzyme. (Brown et al 1990). Due to the very close proximity of these bands, they were considered as a single entity when quantifying collagenase activity from the gels. The activity in 20µl of conditioned media from RPMI 7951 was defined as 100 units of 72kDa type IV procollagenase and the activities in 20µl of conditioned media from TPA-stimulated HT1080 cells were defined as 100 units of 92 kDa type IV procollagenase and 62/59 kDa type IV collagenase.

Computer assisted image analysis was used to quantify the results. In order to assess the reproducibility of the quantification technique, sections were cut from different portions of the same tumour and collagenase activity assessed. Variation in collagenolytic activity was a mean of 11.3% in 18 determinations.

Figure 4:1. Example zymogram of breast cancer tissue (a) with plot of integrated densitometric analysis of bands (b).



a b

Internal standard is a computer generated control to allow comparison between gels. Lane 1 = 20µl RPMI 7951 supernatant showing 72kDa band. Lane 2 = 20µl TPA stimulated HT-1080 supernatant showing 92/81kDa, 72kDa and the activated forms (59kDa/62kDa doublet which were considered together for the purposes of quantification). Lanes 3 to 12 are samples of breast cancer tissue.

In the example zymogram shown in figure 4:1 all 10 breast tumours express the 72 kDa type IV procollagenase. Using computer assisted image analysis, it was calculated that these activities ranged from 7.74 (sample no. 234-87) to 38.37 (sample no 143-88). The variation between levels of the 62kDa active enzyme was somewhat greater (0-sample 234-87 to 67.89 sample 397-87). Using this percentage of acrylamide gel we were unable to accurately distinguish between 92kDa procollagenase and its activation product of 81 kDa (Brown et al 1990). A considerable variation was observed in the levels of the 92kDa enzyme (240.36 sample 870-86 to 0 samples 234-87 and 633-86).

All cases examined expressed levels of the 72kDa type IV collagenase (table 4:2). The 62kDa activated form was found in 10/11 normal/benign cases, 7 of 9 grade I tumours and in all grade II, III and lobular tumours.

Table 4:2 **Proportion of cases expressing 72 and 62kDa collagenolytic activity**

	Collagenolytic Activity (no+ve/no)	
	72kDa	62kDa
Benign	11/11	10/11
Invasive carcinoma	32/32	30/32
Ductal		
grade I	9/9	7/9
grade II	9/9	9/9
grade III	11/11	11/11
Lobular	3/3	3/3

For each tumour sample the activities of 92/81kDa, 72kDa and 62kDa type IV collagenases were calculated in arbitrary units per 10 μg of protein loaded. For the 43 samples examined, the amount of protein loaded per zymogram varied between 4.4μg and 79μg (mean 17.38μg, S.D. 12.22). Table 4:3 shows the expression of different species of type IV collagenases in benign and malignant breast tissue and also by tumour grade.

Table 4:3. Activity of 72/62 kDa type IV collagenase in benign and malignant breast tissue.

		Mean collagenolytic activity (units/10μg protein± s.e.)			Ratio (mean±s.e.)
		62kDa	72kDa	62+72kDa	62/62+72
Tissue	(no)				
Benign	(11)	7.2±2.2	22.9±3.7	30.1±4.8	0.2±0.05
Invasive Ca	(32)	13.6±1.9	14.0±1.7	27.6±3.5	0.45±0.04
Ductal					
grade I	(9)	14.7±4.9	17.6±3.5	32.3±8	0.33±0.07
grade II	(9)	13.4±3.4	15.2±4.9	28.7±8	0.46±0.06
grade III	(11)	14.9±2.9	11.4±2.5	26.3±4.4	0.55±0.06
Lobular	(3)	5.7±2.4	9.8±1.6	15.5±2	0.36±0.10

Levels of 62kDa benign vs malignant, p=0.06. Levels of 72kDa benign vs malignant, p=0.03. Mean ratio of 62kDa to total (62+72kDa) benign vs malignant, 0.2±0.05 vs 0.45±0.04, p=0.003. Mean ratio of 62kDa to total (62+72kDa) within infiltrating ductal tumours vs grade, test for trend, p<0.001.

When the 72kDa and 62kDa types are considered together there was no difference between benign and malignant cases (30.1±4.8 vs 27.6±3.5, p=0.55). When the inactive (72kDa) form is considered alone, there are significantly higher levels in benign compared with malignant disease (22.9±3.7 vs 14.0±1.9, p=0.025. t-test). Conversely, the mean level of the

active (62kDa) form was higher in malignant compared with benign samples (13.6±1.9 vs 7.2±2.2), though this failed to reach standard levels of significance (p=0.06).

When the amount of the active (62kDa) enzyme is considered as a proportion of the total amount of the 72+62 species present, the proportion in malignant disease is significantly higher than that found in benign/normal breast tissue (0.45 vs 0.2, p=0.003). In addition, when considering infiltrating ductal carcinomas, the proportion of the active form of the enzyme increased with increasing tumour grade (test for trend: rank correlation, p<0.0001 table 4:3).

The 92 kDa form of type IV collagenase and its activation product was present in 3 of 11 benign cases, 3 of 9 grade I tumours, but in all eleven grade III tumours (table 4:4). Mean levels of this form were significantly higher in grade III tumours when compared with the other cases (32.25±10.67 vs 5.19±1.98, p<0.0001, t-test).

Table 4:4. Activity of 92/81 kDa type IV collagenase in benign and malignant breast tissue.

Tissue	no+ve/no	mean activity (units/10μg protein±s.e)
Benign	3/11	6.6±3.4
Invasive Carcinoma	18/32	14.0±4.57
Ductal		
grade I	3/9	1.3±0.8
grade II	4/9	6.8±4.3
grade III	11/11	32.3±10.7
Lobular	0/3	0

4:3:2. Localisation of type IV collagenases.

Localisation of the type IV collagenases was assessed at the level of mRNA and protein. *In situ* hybridisation was carried out on 6 cases of invasive breast cancer using riboprobes to 72kDa and 92kDa type IV collagenases. Immunohistochemical studies using polyclonal antibodies to 72kDa and 92kDa type IV collagenase were also carried out to confirm the presence of protein.

4:3:2:1. 72kDa type IV collagenase.

In the six cases of breast cancer examined for expression of 72kDa type IV collagenase, all cases showed stromal expression of signal (figure 4:2). In no case was expression of mRNA observed in tumour cells.

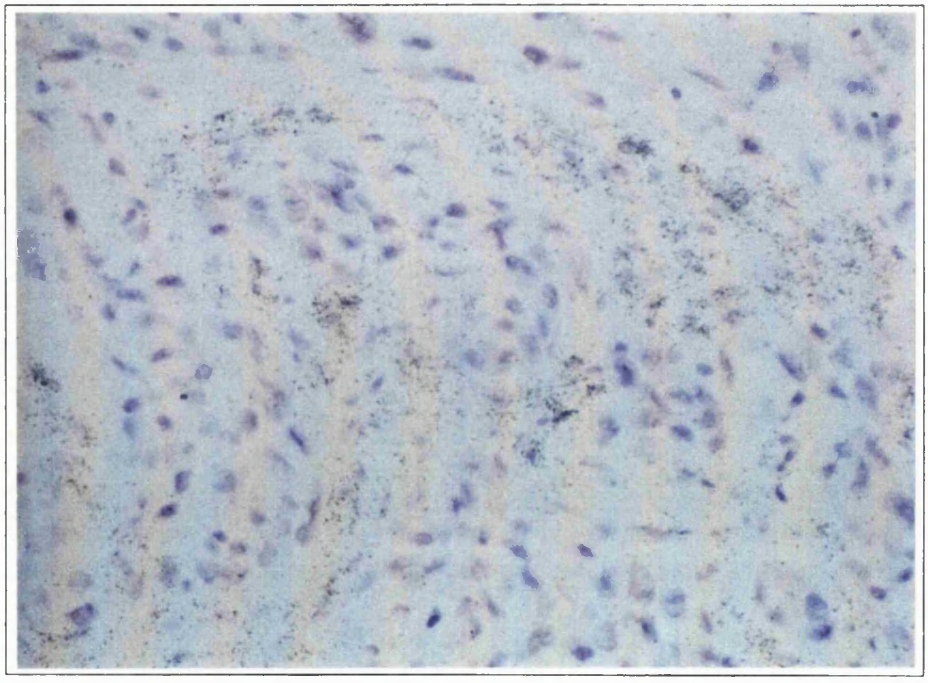


Figure 4:2. Expression of 72kDa type IV collagenase mRNA in a case of infiltrating ductal carcinoma. Signal overlies spindle shaped cells in the stroma. Tumour cells are negative. (x 200)

Stromal expression of 72kDa type IV collagenase protein was also noted in all cases of invasive carcinoma studied (example figure 4:3) where positivity in spindle shaped cells may represent fibroblast/monocyte expression.

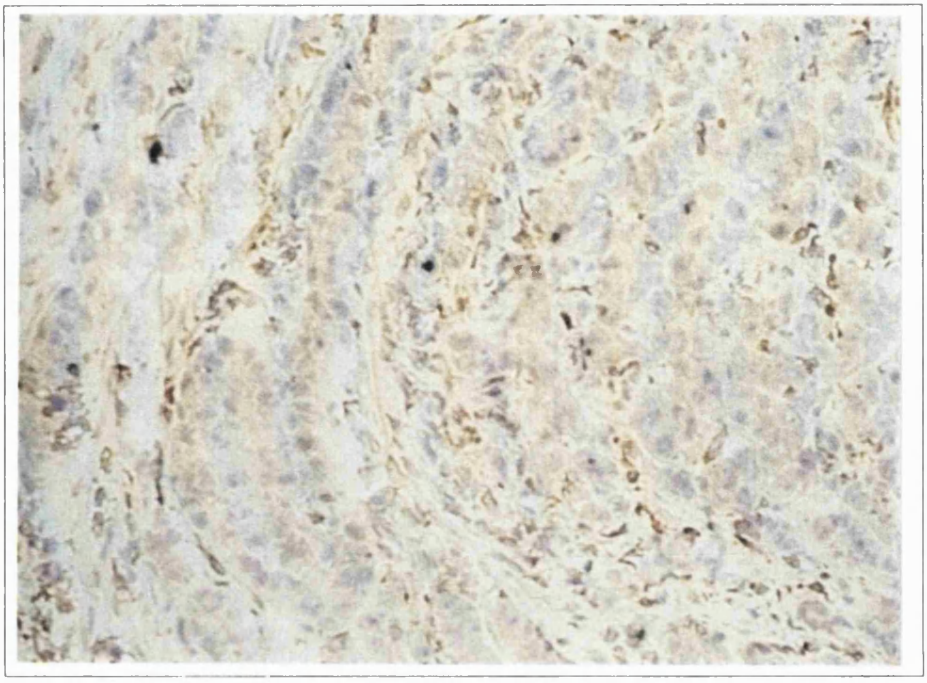


Figure 4:3. **Infiltrating ductal carcinoma labelled with antibody to 72kDa type IV collagenase. Expression is noted in cells of the tumour stroma and in this case tumour cells are negative (x 200)**

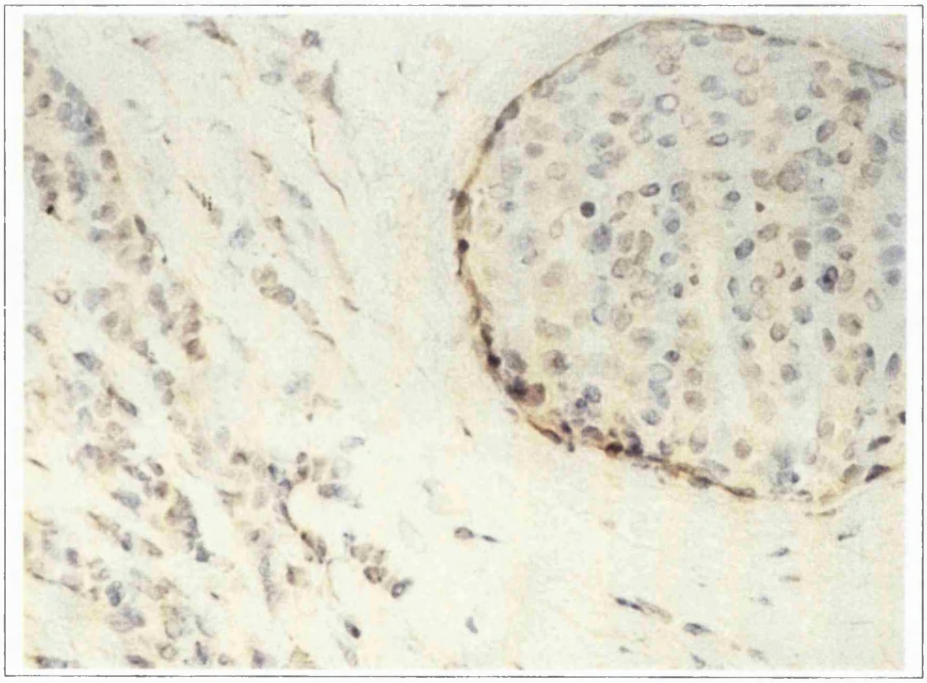


Figure 4:4. **Labelling of myoepithelial cells surrounding ductal carcinoma in situ with antibody to 72kDa type IV collagenase (x 200).**

In cases containing ductal carcinoma in situ expression was also noted in myoepithelium surrounding the ducts (figure 4:4).

In a proportion of infiltrating ductal carcinomas (including cases in which stromal expression of mRNA was noted) weak immunoreactivity with the antibody to 72kDa type IV collagenase protein was also observed in tumour cells (figure 4:5).

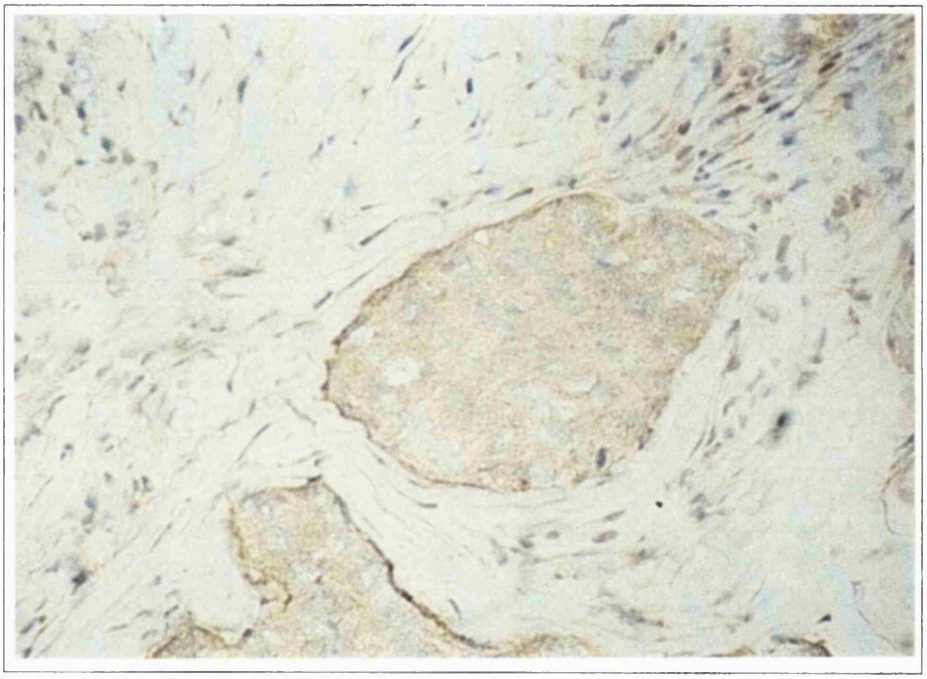


Figure 4:5. **Labelling of myoepithelial cells and weak staining of tumour cells with antibody to 72kDa type IV collagenase (x 200).**

4:3:2:2. 92kDa type IV collagenase.

Figure 4:6 illustrates expression of 92kDa type IV collagenase mRNA (a) and protein (b) in adjacent sections of invasive ductal carcinoma grade III.

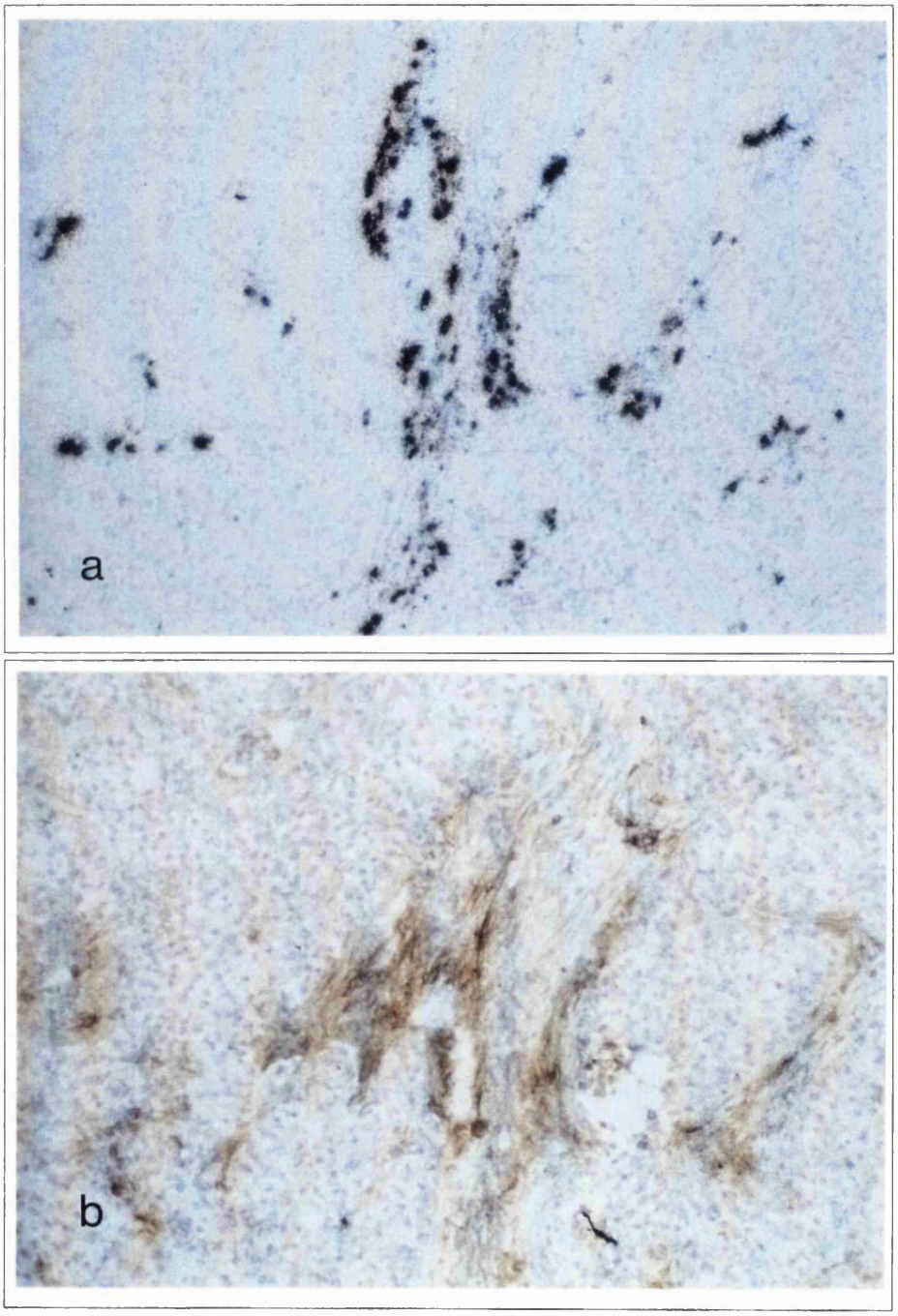


Figure 4:6. **Expression of 92kDa type IV collagenase mRNA (a) and protein (b) in a case of infiltrating ductal carcinoma grade III. Expression of mRNA and protein colocalises in tumour stroma (x 100).**

RNA expression was seen in elongated cells with fibroblast or macrophage morphology within tumour stroma. Protein expression was confirmed in adjacent sections using the polyclonal antibody to the 92kDa form of the enzyme and expression was found to be similar to that of the mRNA (figure 4:6). 92kDa type IV collagenase protein expression was also noted in myoepithelium and dendritic cells in the stroma as illustrated in figure 4:7.

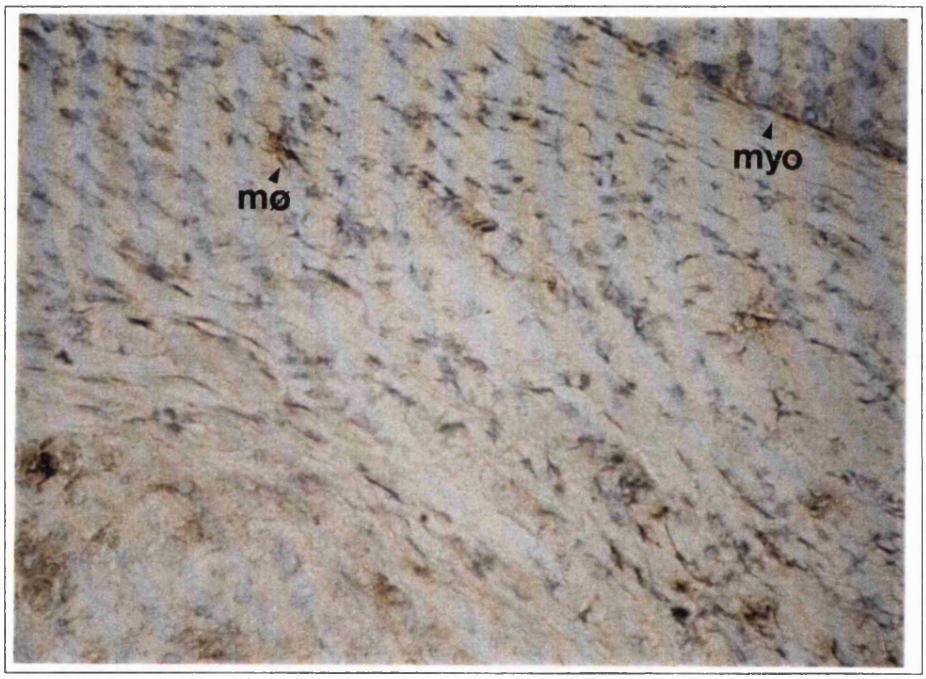


Figure 4:7. Expression of 92kDa type IV collagenase protein in myoepithelium (myo) and dendritic cells (mo) in the tumour stroma of a grade III infiltrating ductal carcinoma. (x200)

In none of the cases studied by *in situ* hybridisation or immunohistochemistry was expression of the 92kDa form of the enzyme noted in tumour cells.

4:4. Discussion

Evidence from experimental animal models suggests that type IV collagenases are required to breach the integrity of basement membranes thereby allowing metastasis to occur. Loss of basement membrane is a characteristic of primary breast cancer but a causative role for type IV collagenases in the spread and dissemination of breast neoplasms has not been established. Immunohistochemical studies correlating expression of 72kDa type IV collagenase with established prognostic criteria have yielded conflicting results (Daidone et al, 1991 D'Errico et al 1991). Although immunohistochemistry may give useful information regarding localisation of metalloproteinases, levels cannot be quantified accurately and antibodies may not distinguish between different molecular weight forms of the enzyme or whether they are in the pro-form or activated state.

In the studies described here the activity of the two different forms of the type IV collagenases has been quantified and expressed as arbitrary units per unit of protein present. In the case of the 72kDa type IV collagenase, the proportion of the enzyme in the activated form was also assessed.

Although the total amount (proform and activated form) of the 72kDa enzyme was similar in benign and malignant breast tissue, levels of the proform were significantly higher in benign compared with malignant disease. Conversely, levels of the activated form were higher in malignant tissue and in the case of infiltrating ductal carcinomas, the proportion of the 72kDa enzyme in the activated state correlated with tumour grade. Immunohistochemical methods alone would not have been able to detect this relationship.

In common with other matrix metalloproteinases, 72kDa type IV collagenase is secreted in an inactive pro-form and activation occurs by proteolytic cleavage of an N-terminal sequence (Stetler-Stevenson et al 1989). How activation occurs *in vivo* is unclear but other proteases may be involved (Yagel et al 1989, Goldberg et al 1990). Specifically, activation is also blocked by inhibitors of metalloproteinases (Ward et al 1991) and a membrane type matrix metalloproteinase (MT-MMP) capable of activating the 72kDa type IV collagenase has been identified recently (Sato et al 1994).

In the cases examined, expression of the 72kDa form of the enzyme at the level of mRNA was confined to the tumour stroma. This has been confirmed in subsequent studies in breast cancer (Soini et al 1994) and is consistent with the observations *in vitro* and *in vivo* of expression of this form of the enzyme by fibroblasts. (Goldberg et al 1986) and macrophages (Garbisa et al 1986). Immunohistochemistry confirmed the presence of protein in tumour stroma but in the majority of cases studied, expression was also noted on tumour cells. Expression of 72kDa type IV collagenase protein has been noted previously in tumour cells and in myoepithelial cells in normal breast and tumour (Monteagudo et al 1990). This apparent discrepancy between expression of message and protein reported by others in separate studies was confirmed in the series of cases described here. The differential expression of mRNA and protein observed is consistent with the finding that 72kDa type IV collagenase can bind to the plasma membrane of breast cancer cell lines (Emonard et al 1992) and localises to the tumour cells at the sites of cell invasion into the extracellular matrix in *in vitro* systems (Monsky et al 1993).

The 92kDa type IV collagenase was assessed using zymography although it was not possible to resolve it into pro-enzyme and activated forms. It was present in one third of normal /benign cases and a similar proportion of grade

I and II infiltrating ductal carcinomas. All grade III tumours examined had levels of the enzyme detectable by zymography. Basset et al (1991) demonstrated that a proportion of breast tumours expressed elevated levels of mRNA for 92kDa collagenase but no correlation with prognostic features was made.

Although TNF-α has been reported to increase expression of the 92kDa form of the enzyme in tumour cell lines (Brenner et al 1989, Okada et al 1990) the studies described here demonstrated that expression at the level of mRNA and protein was confined to the tumour stroma. Expression by stromal cells described here is consistent with reports that cells of the monocyte/macrophage lineage may secrete the 92kDa form of the enzyme particularly upon activation with lipopolysaccharide or concanavalin A (Hibbs et al 1987, Welgus et al 1990). Similarly, fibroblasts may produce the 92kDa form under certain conditions (Wilhelm et al 1989) and it is possible that fibroblasts may be induced to express this form of the enzyme by tumour derived factors. The stromal localisation of 92kDa mRNA in breast cancer has also been confirmed more recently (Soini et al 1994).

The relative roles of the 72kDa and 92kDa type IV collagenases in the metastatic process remain unclear. The 72kDa form of the enzyme has been demonstrated immunohistochemically in normal breast tissue where its presence could influence the normal process of tissue remodelling (Monteguedo et al 1991). The data presented above confirm similar levels of the total 72kDa enzyme activity in benign and malignant breast tissue but also demonstrate that the 92kDa form is not exclusively associated with the malignant phenotype. *In vitro* studies suggest the invasive phenotype is only associated with expression of the 92kDa form of the enzyme not the 72kDa type (Kubota et al 1991). These findings assume tumour cell expression of

enzyme *in vivo*, an assumption which the above and other data do not support, and fail to take into account the activation state of the enzymes or the ability of tumour cells to bind collagenases.

Using quantitative zymography a correlation between histological grade of infiltrating ductal carcinomas of the breast and expression of type IV collagenases has been established. No attempt was made in these studies to quantify the naturally occurring inhibitors of the type IV collagenases (TIMPs) and interpretation of the data is necessarily limited as a result. In addition, although TNF-α may regulate type IV collagenase expression *in vitro* and could influence activation *via* induction of plasminogen activator, the studies described here do not establish a causal relationship between TNF-α expression and collagenase activity. Nevertheless, the relationship described between type IV collagenase activity and tumour grade may partly explain the relationship between tumour grade and biological behaviour since degradation of the basement membrane is a prerequisite for invasion and metastasis.

Inhibitors of collagenase activity have been tested in animal tumour models (Davies et al 1993) and it is possible that inhibitors of collagenolytic activity could influence the clinical course of human breast cancer.

Chapter 5.

SUMMARY

5:1. Introduction.

The presence of a lymphoplasmacytic infiltrate in primary breast cancer has long been recognised but its biological and clinical significance remains unclear. Given the involvement of leucocytes in the eradication of invading micro-organisms it has been assumed that the presence of infiltrating leucocytes within tumours represents a host immune response to tumour. Spontaneous regression of human cancers generally and breast cancer in particular is a very rare event implying that the immune response is inadequate and/or that tumour cell variants which may subvert the immune response are selected for during tumour growth and development. As well as representing an inadequate response to tumour, evidence is also accumulating which suggests that some biological properties of the inflammatory infiltrate may actually promote tumour development. The observations in more recent analyses of the prognostic effect of lymphoplasmacytic infiltrate which suggest that a marked reaction is associated with a poor prognosis (Rosen et al 1989, Kurtz et al 1990, Rilkhe et al 1991) would support this view.

Past studies have probably underestimated the proportion of tumour associated leucocytes which are of the monocyte/macrophages lineage (Nash et al 1982, Tanaka et al 1986). More recent studies using antibodies to monocyte/macrophage determinants suggest that tumour associated macrophages represent a major component of the inflammatory infiltrate in

primary breast cancer representing up to 80% of the leucocytes present (Göttlinger et al 1985).

5:2. Recruitment of monocytes.

The mechanisms by which monocytes and macrophages are recruited to tumour are becoming clearer. A family of chemotactic cytokines (chemokines) is now recognised which are capable of recruiting and stimulating cells of the monocyte/macrophage lineage. This group includes monocyte chemotactic proteins 1, 2 and 3 (MCP-1,2 and 3), RANTES, and macrophage inflammatory proteins-1 α and -β (MIP-1α and MIP-1β) (Reviewed Schall 1991). In animal models, transfection of MCP-1 into the normally tumourigenic CHO cell line resulted in its failure to grow in nude mice associated with an influx of monocytes and macrophages at the injection site (Rollins and Sunday 1991). MCP-1 is known to be expressed by a variety of tumour cell lines (Graves et al 1989) and its presence in human melanoma has been documented (Graves et al 1992). As well as recruiting monocytes/macrophages, MCP-1 is also involved in monocyte activation in that it is known to elicit degranulation and respiratory bursts (Zachariae et al 1990), regulate adhesion molecule expression (Jiang et al 1992) and lead to stimulation of macrophage cytotoxicity and induction of cytokines such as IL-1, TNF-α and IL-6 (Fahey et al 1992).

Cytokines such as colony stimulating factor-1 (CSF-1) are capable of stimulating the survival, proliferation and differentiation of monocytes (Stanley et al 1983) and the presence of CSF-1 in the tumour cells of primary breast cancer and the expression of its receptor on cells of the associated mononuclear cell infiltrate has been demonstrated recently (Scholl et al 1994).

5:3. TNF-α and receptor expression in primary breast cancer.

Tumour necrosis factor is a cytokine known to be elaborated by activated macrophages (Carswell et al 1975). Although its name implies anti-tumour activities, many of its effects could contribute to tumour progression. The presence of TNF-α in primary breast cancer has been demonstrated at the level of mRNA and protein. Two-colour immunofluorescence confirmed the macrophage origin of TNF-α protein which is in agreement with studies in other malignancies such as renal cell carcinoma (Waase et al 1992) and colorectal cancer (Naylor et al 1990a) whereas in ovarian carcinoma TNF-α mRNA expression was localised to tumour islands although TNF-α protein expression was found predominantly in areas of mononuclear cells infiltrate (Naylor et al 1993). A correlation between the proportion of macrophages expressing TNF-α and increasing tumour grade was noted, a relationship that was also observed in the ovarian cancer study. This observation would support the view that the balance of the effects of TNF-α in human breast cancer might be a contribution to tumour growth and development. This hypothesis is supported by two further observations. Although macrophages producing TNF-α were observed in tumour cell islands their numbers relative to the tumour cells present were very small with most macrophages residing in the tumour stroma as noted by others (Vitolo et al 1992). Thus the ability of macrophages to lyse tumour cells is necessarily limited. Secondly, neither TNF receptor was noted, immunohistochemically at least, on tumour cells. Considering that by ligand binding all cell types studied expressed the p55 TNF receptor (Sugarman et al 1985) this observation was unexpected. Both types of receptor were demonstrated on tumour vascular endothelium and in the inflammatory infiltrate, where the predominant receptor was the p75 TNF-R. This finding provided a reassuring "positive" control though it is possible that much lower levels of receptor expression on tumour cells may not have

been detected using the described immunohistochemical techniques. In any event the relative paucity of TNF receptors on tumour cells could contribute to their relative resistance to the cytoxic effects of TNF. Other aspects of the known *in vitro* properties of TNF-α were investigated further with respect to its expression in primary breast cancer.

5:4. Nitric oxide activity in primary breast cancer.

Nitric oxide, which may modulate tumour blood flow and mediate angiogenesis, is known to be induced by TNF-α in animal systems through induction of the enzyme NO synthase. Evidence for activity of nitric oxide in human tissue is limited and means to identify it are necessarily indirect. Accumulation of nitrite and nitrate in the culture supernatants of cultured breast cancer tissue and its inhibition by competitive substrate analogues demonstrated NO synthase activity in breast tissue. Measurement of the conversion of radiolabelled arginine to citrulline provided supportive evidence for the presence of NO and the use of calcium chelators in this assay demonstrated that a proportion of the NO synthase activity was calcium independent and therefore presumably of macrophage origin. Correlation with known prognostic factors in primary breast cancer suggested a relationship with tumour grade which supports the hypothesis that TNF-α may be involved in NO induction in human breast cancer and that the presence of NO may influence tumour biology, promoting growth and development. This would be in keeping with known activities of NO on tumour vasculature (Andrade et al 1992b) and cell division *via* effects on reducing cAMP/cGMP ratios (Cho-Chung et al 1981). In addition although NO may mediate cell killing, levels of NO measured in malignant breast tissue were much lower than those required for cytotoxicity or induction of apoptosis, suggesting that the net effect of NO may be to promote tumour development. In the studies described above,

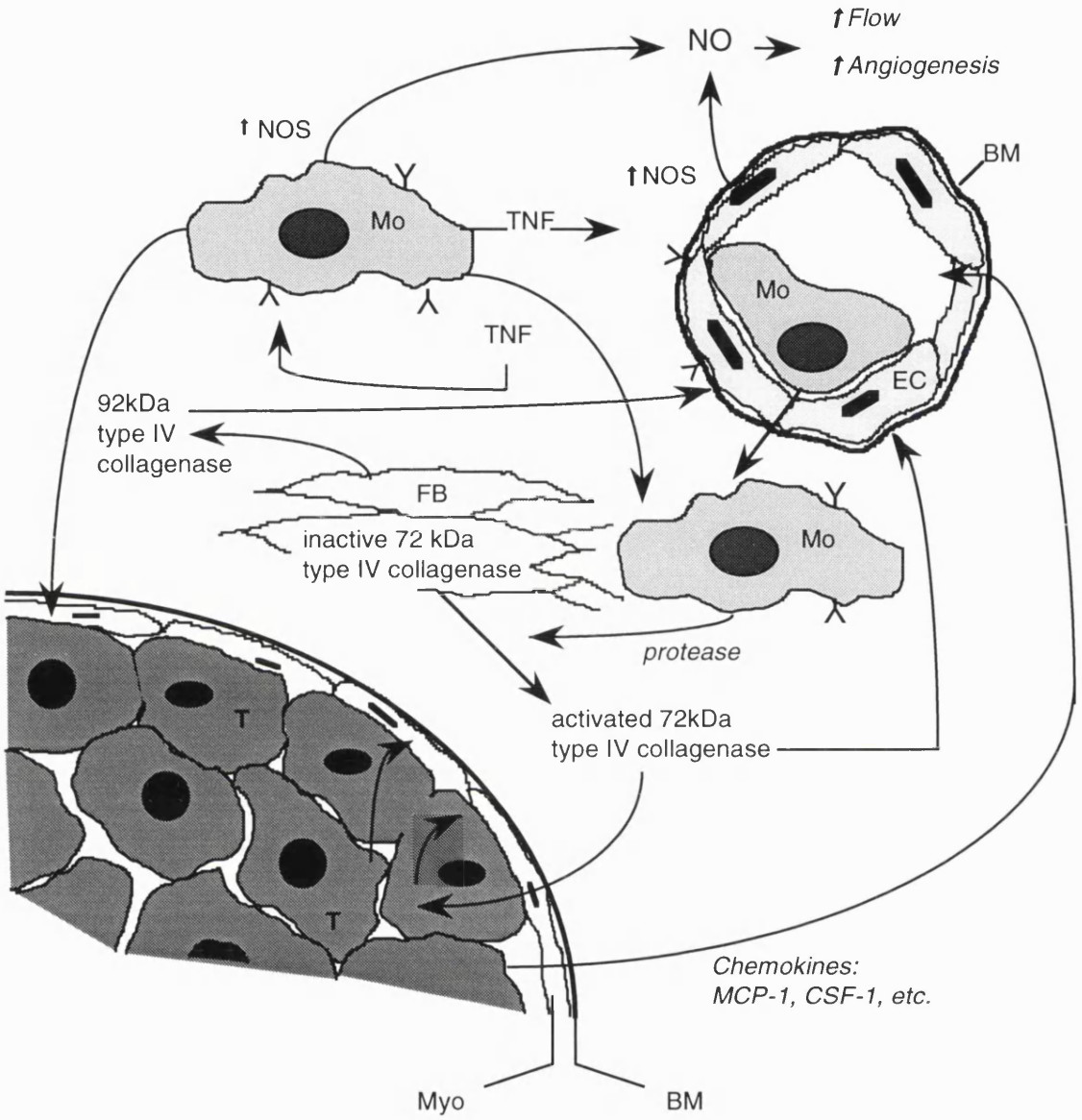
immunoreactive NO synthase was not found within tumour cells but was noted in tumour associated macrophages, myoepithelium and vascular endothelium, i.e. a stromal response to the presence of tumour.

5:5. Type IV collagenase expression in primary breast cancer.

Type IV collagen is a major component of the basement membrane and enzymes capable of degrading it may contribute to local invasion and ultimately the metastatic spread of tumour. There exists a more widespread literature on induction of the 92kDa form of type IV collagenases by TNF-α but induction of the 72kDa isoform has been documented and other proteases which could be involved in the activation of the pro-form of the enzyme, may be induced by TNF-α. The presence of the 72kDa form of the enzyme in the majority of the benign cases studied, supports the view that it may be involved in the normal process of basement membrane remodelling. In malignant breast tissue, the proportion of 72kDa type IV collagenase in the activated state correlated with increasing tumour grade suggesting that the presence of a converting enzyme is also grade related. Production of 72kDa type IV mRNA was by stromal cells though protein localised within stroma and tumour in most of the cases studied, consistent with the presence of a receptor for this enzyme on tumour cells. The 92kDa was found in approximately one third of benign cases and lower grade tumours studied, but in all the high grade tumours examined. RNA and protein for the 92kDa isoform localised to the stroma, specifically fibroblasts and macrophages, with no tumour cell expression.

The possible effects of macrophage recruitment and TNF-α expression on the biology of breast cancer with respect to the studies described in this thesis is illustrated in figure 5:1.

Figure 5:1. Schema for possible roles of macrophages in tumourigenesis in primary breast cancer.



Cells of the monocyte-macrophage lineage (**Mo**) may be attracted into the interstitium along a gradient of chemokines such as macrophage chemotactic protein (**MCP-1**) or colony stimulating factor-1 (**CSF-1**) produced by tumour cells (**T**). TNF, produced by activated macrophages, may act in an autocrine or paracrine manner on receptors (**Y**) on macrophages and endothelial cells (**EC**) to upregulate nitric oxide synthases (**NOS**), leading to raised levels of nitric oxide (**NO**) which may increase tumour blood flow and promote angiogenesis. Production of 92kDa type IV collagenase by fibroblasts (**FB**) and macrophages may lead to degradation of extra-cellular matrix (ECM) and basement membrane (**BM**) surrounding carcinoma *in situ*, allowing local infiltration of malignant cells. Similarly, 72kDa type IV collagenase, possibly activated by proteases produced by infiltrating macrophages or present on the surface of tumour cells, may degrade ECM/BM directly or by binding to tumour cells or myoepithelium (**Myo**) allowing degradation of BM from within the duct. The type IV collagenases could also degrade BM surrounding tumour vasculature allowing further influx of immune effector cells into the interstitium or efflux of tumour cells into the vasculature leading to distant metastatic spread. *Italicised text* refers to putative mechanisms or data not derived from this thesis.

5:6. Tumours: wounds that do not heal.†

It has been suggested that factors, such as the chemokines, are released by malignant cells and may recruit macrophages to sites of tumour where they misinterpret tumour-derived signals for those required in normal wound healing (Dvorak 1986). The critical difference between wounds and tumours may be that whereas the stimuli which induce invasive and proliferative behaviour in normal cells within a wound are self-limiting, signals which emanate from transformed cells are not (Whalen 1990). Angiogenesis, fibroblast proliferation and tissue remodelling through production of matrix-degrading proteases are central to the process of wound healing. Such features within tumour stroma could promote the tumourigenic process and several may be mediated by TNF-α.

Following the observation that activated macrophages induce vascular proliferation (Polverini et al 1977), TNF-α, a product of activated macrophages, was shown to stimulate angiogenesis (Leibovich et al 1987). More recent data suggest that nitric oxide may mediate this effect in tumours (Leibovich et al 1994) and in healing wounds (Konturek et al 1993). Although the association of type IV collagenase expression with the metastatic phenotype has been determined by upregulation in tumour cells, increases in collagenolytic activity derived from the tumour stroma may promote tumour spread by direct dissolution of the basement membrane or by the association of collagenases with the cell membrane of tumour cells.

The maintenance of a fibrovascular stroma is a prerequisite for tumour development and a variety of growth factors are implicated in this process

† Dvorak 1986. N. Eng. J. M.; 315: 1650-1659

(reviewed Whalen 1990). It is noteworthy that TNF-α is also known to upregulate epidermal growth factor (EGF-R) expression on fibroblasts (Palombella et al 1987) as well as tumour cells (Adachi et al 1992). Macrophages may therefore be involved directly in maintaining tumour stroma as well as tumour cell growth. Macrophages may also be the source of an appropriate ligand, O'Sullivan et al (1993) having demonstrated the production of epidermal growth factor by infiltrating macrophages in a third of primary breast cancers.

In an attempt to augment the host immune response to tumour in patients with breast cancer and other tumour types, the feasibility of administering another cytokine, interleukin-2 was examined and is the subject of the second part of this thesis.

PART 2: CLINICAL STUDIES.

Chapter 6.

INTRODUCTION.

6:1. Immunotherapy of human cancer.

The phenomenon of spontaneous tumour regression in patients with cancer suffering from concomitant acute bacterial infections was noted in the 18th century (Coley-Nauts 1989). In 1844, French physicians built on this observation and applied gauze dressings soaked in gangrenous discharges to superficial tumours (Coley-Nauts 1989). In the 1890s an American surgeon, William B. Coley noted occasional spontaneous regression of sarcomas in patients who were also suffering from erysipelas. In a attempt to reproduce this effect in patients with inoperable cancers, Coley initially induced erysipelas by sub-cutaneous injection of streptococci. Later he used crude filtrates of mixed bacterial cultures (*Streptococci and Bacillus prodigiosus*) which he termed 'Coley's mixed toxins' (Coley 1896). Although the preparations varied in their 'strength' Coley recognised the importance of a biological end-point as a guide to therapy and titrated the administered dose against the level of fever induced. Occasional dramatic responses were recorded. The establishment of other treatments for cancer, especially radiotherapy and chemotherapy led to a decline in the use of Coley's toxins, a position from which immunotherapy has been slow to recover.

Shear and colleagues, in attempting to isolate the active component in Coley's mixed toxins, purified a constituent of the bacterial cell wall of *Serratia marscescens*, termed lipopolysaccharide (LPS). LPS was shown to be

effective in inducing haemorrhagic necrosis in animal tumour models (Shear et al 1943). It was reported subsequently that LPS induced a factor in serum which could be transferred to, and lead to haemorrhagic necrosis of tumours in non-LPS treated animals (O'Malley et al 1962). Carswell et al (1975) using *Bacillus Calmette Guerin* (BCG) and LPS also demonstrated the induction of a transferable factor which would induce tumour necrosis. This agent, termed tumour necrosis factor (TNF), has since been well characterised and its properties are described in chapter 1. Human TNF was isolated by Aggarwal et al (1985a). Subsequent cloning (Wang et al 1985) and expression of the gene has allowed study of the *in vitro* effects of TNF as well as its potential use as an anti-tumour agent in human cancer.

As described in chapter 1, TNF activates many cells of the immune system, increasing the cytotoxic capacity of macrophages (Talmadge et al 1987), cytotoxic T-cells (Ranges et al 1987, Nakano et al 1989), natural killer cells (Ostensen et al 1987) and neutrophils (Shalaby et al 1985). In the animal meth A sarcoma model, administration of TNF led to a dose dependent induction of necrosis (Creasey et al 1986). Since these cells are not inhibited by TNF *in vitro*, an indirect mechanism is implicated *in vivo*, possibly through an effect on vascular endothelium (Nawroth et al 1988, Pober et al 1987). The subsequent development of a specific host-mediated immune response seems to be important for the sustained regression of tumours in animal models (North and Havell, 1988, Asher et al 1987).

6:2. TNF therapy in human cancer.

Phase I clinical trials of TNF in patients with advanced cancer began in 1985, shortly after the gene was cloned. The dose limiting side-effects observed have depended on the route of administration. When given intravenously by

either bolus or infusion, the major dose limiting toxicity was hypotension seen at doses of 150-225μg/m^2/day. Respiratory distress has also been noted and is probably due in part to a vascular leak syndrome as a consequence of an effect on endothelium (Spriggs et al 1988). With intramuscular or sub-cutaneous administration, local inflammation was dose limiting at doses greater than 75-150μg/m^2/day. Other constitutional symptoms included rigors, fevers, chills, fatigue and headache. Routine laboratory investigations also revealed leucopenia, thrombocytopenia, anaemia, renal impairment and transient alterations in liver transaminases (Sherman et al 1988). Although some of the side-effects, such as hypotension, myalgia and fatigue, could be abrogated by the concomitant administration of non-steroidal anti-inflammatory drugs and steroids, these agents could influence the potential efficacy of TNF.

Phase II trials of systemic rhTNF in human cancer have yielded disappointing results (reviewed by Balkwill 1989). Preclinical studies with TNF have suggested that a dose response relationship exists and that the optimal dose is close to the lethal dose of 3 to 4 mg/m^2 (Spriggs and Yates 1992) which is some 20 fold higher than the maximum tolerated dose in humans. Thus the low therapeutic index of TNF has precluded its more widespread use when administered systemically. As in animal models (chapter 1) local therapy with TNF allows high levels to be achieved but avoids systemic exposure and associated toxicities. This approach has shown more promise with short lived responses being seen with intra hepatic arterial infusion in patients with colorectal cancer (Mavligit et al 1990), intra-arterial infusion in brain tumours (Wakabayshi et al 1990) and intra-lesional therapy in Kaposi's sarcoma (Kahn et al 1989). Intra-peritoneal TNF has also been shown to reduce the extent of malignant ascites in patients with a variety of primary tumours (Raeth et al 1991) emphasising the importance of achieving high local levels. High levels

of TNF in combination with other agents has been has also been achieved using the technique of isolated limb perfusion with high response rates being recorded in melanoma and sarcoma (Lienard et al 1992).

6:3. Interleukin-2.

A growth factor present in the conditioned media of phytohaemagglutinin-stimulated peripheral lymphocytes which was capable of supporting the growth of bone marrow-derived T-cells was described by Morgan and colleagues in 1976 (Morgan et al 1976). This growth factor was termed interleukin-2 in 1979 and was later characterised as a glycoprotein with a molecular weight of 15-18kDa (Gillis et al 1980). The human gene was cloned and analysed by Taniguchi et al (1983). The gene encodes a 153 amino acid peptide which is processed into its mature form by the removal of a 20 amino acid peptide sequence. Secondary structure consists of four α-helices and two short β-helices. Tertiary structure, essential for receptor binding, is maintained by a disulphide bridge between cysteine residues (Robb 1984). Native IL-2 is variably glycosylated and sialated (Robb and Smith 1981) and although recombinant IL-2 produced from cDNA is not glycosylated (Taniguchi et al 1983) this does not have a significant effect on bioactivity (Robb 1984).

6:3:1. The IL-2 receptor (IL-2R).

IL-2 acts by binding to a specific cell-surface receptor which is expressed on the surfaces of T and B lymphocytes and monocytes after activation with antigens or mitogens. The IL-2 receptor consists of three components: an alpha chain (55kDa/p55, Tac, CD25) a beta chain (75kDa/p70-75) and a gamma subunit (95-110 kDa, p64). The p55 chain is a low affinity receptor

(Fuji et al 1986) and probably as a result of its short cytoplasmic domain (13 amino acids), it does not mediate signal transduction or internalise IL-2 (Weissman et al 1986). The p75 chain binds IL-2 with an intermediate affinity (Tsudo et al 1986) and *via* a 286 residue cytoplasmic tail, mediates the proliferative signal of T-cells and NK cells. (Siegel et al 1987). The interaction of IL-2 with the p75 subunit also results in the expression of the p55 subunit which forms a high-affinity heterodimeric receptor. More recently, the gamma sub-unit, expressed on intermediate and high-affinity receptor bearing cells has been shown to influence the affinity with which the p75 receptor binds IL-2. (Colamonici et al 1990).

6:3:2. Biological effects of IL-2.

6:3:2:1 T-lymphocyte responses.

The major sources of IL-2 are activated T-helper cells ($CD4^+$) following stimulation by foreign antigen in association with HLA class II determinants. Following antigen binding or occasionally non-specific triggering of CD3 or CD2 determinants, activation of T-cells occurs which results in responsiveness to IL-1. Induction of IL-2 and IL-2 receptor subunits also allows IL-2 to act in an autocrine and paracrine manner. The expression of the high affinity IL-2R and autocrine action of IL-2, allows further cell cycle progression probably *via* induction of the proto-oncogenes *c-myb* and *c-myc* which have been implicated in cell cycle control including proliferation and ultimately expansion of an antigen specific clone of cytotoxic T-cells (CTL) capable of killing antigen bearing cells (reviewed by Robb 1984). Production of secondary cytokines further modulates the cellular response which leads to T-cells having the ability to respond to the same antigen upon further encounter. The effector arm of the T-lymphocyte response to antigen is

mediated by cytotoxic T-cells which recognise antigen in association with MHC class I determinants. Animal studies suggest that low levels of IL-2 activate CTL killing (Weber et al 1988) and circumstantial evidence is provided the demonstration of T-cell infiltration in biopsies from regressing human melanomas (Cohen et al 1987).

The "recognition" and "effector" arms of the CTL response are therefore MHC dependent and the selection of tumour cell variants which do not express MHC determinants may be one way in which tumour cells could evade the immune response.

6:3:2:2. Non-MHC restricted killing.

Interleukin-2 augments the function and increases the target cell specificity of natural killer cells. The resultant activity has been attributed to lymphokine activated killer (LAK) cells. LAK cells were originally thought to be a novel class of effector cells distinct from NK cells or T-cells (Grimm et al 1982). As well as being morphologically similar to NK cells, having the appearance of large granular lymphocytes, the majority of LAK cells express the CD16 (Leu 11) and NKH1 antigens (Ortaldo et al 1986) suggesting that most LAK activity is attributable to IL-2 stimulation of NK growth and cytotoxicity. LAK cells are able to kill a broader range of tumour targets than NK cells. NK lysis is dependent on conjugate formation between effector and target cells. This is mediated by the cellular adhesion molecules upregulated four to six fold following rIL-2 such as LFA-1 (CD11a/CD18) and CD2 which bind target cell ligands ICAM-1 (CD54) and LFA-3 (CD58) respectively (Robertson et al 1990). Although LAK cells are preferentially cytotoxic for tumour cell targets, autologous lymphocytes (Sondel et al 1986) and endothelial cells (Damle et al 1987) may also be lysed by LAK cells

6:3:2:3. Effects of IL-2 on B-cells.

Activated normal B-cells and those from tumour bearing patients express high-affinity IL-2 receptors (Waldmann et al 1984). IL-2 increases this receptor expression and promotes the proliferation and immunoglobulin production of B cells (Nakagawa et al 1988).

6:3:2:4. Effects of IL-2 on monocytes.

Interferon-γ induces the expression of IL-2 receptors on monocytes (Herrmann et al 1985) and IL-2 itself increases the cytotoxic potential of monocytes against a range of tumour cell lines (Malkovsky et al 1987).

6:3:2:5. Secondary cytokine induction by IL-2.

As well as the simple proliferative effects of IL-2 on immune effector cells, the augmentation of their activity is also mediated through secondary induction of other cytokines, which, as well as increasing the cellular immune response, may additionally have anti-proliferative or cytotoxic activity.

IL-2 is one of a cascade of cytokines involved in the inflammatory/anti-tumour response. IL-2 may induce peripheral blood mononuclear cells to produce a variety of other cytokines *in vitro* such as TNF, IFN-γ, IL-1, IL-3, IL-5, IL-6, GM-CSF, and M-CSF (Kovacs et al 1989, Saraya 1989). Many of these cytokines can be demonstrated in the plasma of patients during treatment with rIL-2. The relative contribution of these induced cytokines to the overall effects of IL-2 is complex and dependent on the presence and distribution of the appropriate cytokine receptors which itself leads to the many changes in DNA transcription within the target cells. Secondary cytokine induction and

the possible contribution to the biological effects of rIL-2 are discussed below with respect to TNF-α and IFN-γ.

i) Tumour necrosis factor-α.

In vitro studies have shown that mRNA for TNF is present within 2 hours of stimulation of peripheral blood mononuclear cells with IL-2 (Saraya 1989). Several studies of IL-2 with and without LAK cells have demonstrated a rise in TNF during treatment (Lotze et al 1985, Gemlo et al 1988, Boccoli et al 1990) although others have shown no induction (Michie et al 1988).

The pleiotropic anti-tumour activities of TNF-α have been discussed previously (chapter 1). As well as inducing TNF-α, IL-2 also leads to upregulation of TNF receptors either directly (Owen-Schaub et al 1989) or *via* induction of IFN-γ (Ruggiero et al 1986). Such increases in TNF-α and its receptors are central to its known involvement in the generation of cytotoxic T-lymphocytes (Ranges et al 1987, Nakano et al 1989) and LAK cells (Owen-Schaub et al 1988, Ostensen et al 1987).

The clinical importance of TNF as a potential mediator of IL-2 anti-tumour activity is highlighted by the observation that induced levels of TNF may be a predictor of response to therapy with rIL-2 (Blay et al 1990).

Measurement of TNF-α in the peripheral circulation by radio-immunoassay and its use as a predictor of response or toxicity may be complicated by the presence of binding proteins which are capable of inhibiting its biological activity (Olsson et al 1990, Loetscher et al 1991). Conversely by acting as carriers and delaying renal excretion, binding proteins may increase the plasma half-life of TNF-α thereby prolonging its effects at tissue level (Aderka

1992). Two immunologically distinct TNF binding proteins (Mwt=30Kd) have been isolated, which inhibit the biological activity of TNF by preventing the binding of TNF to its cellular receptor (Seckinger et al 1988). Evidence suggests that these binding proteins (TNF-R1 and TNF-R2) are formed by proteolytic cleavage of the extracellular domain of the transmembrane portions of the p55 and p75 TNF receptors respectively (Porteau et al 1991). Induction of TNF binding proteins has been documented during therapy with TNF-α and IFN-γ, although injection of IFN-γ alone did not result in an increase in TNF binding protein (Lantz et al 1990). Although rIL-2 is known to induce TNF-α, induction of TNF binding proteins has not been demonstrated during therapy with IL-2.

ii) Interferon-γ.

Raised interferon-γ levels have been demonstrated during bolus IL-2 administration (Lotze et al 1985), but not during infusional therapy (Brown et al 1989). In the latter study however, markers of IFN-γ activity such as neopterin were found to be raised and serum tryptophan levels fell.

Interferon-γ may augment IL-2 mediated tumour rejection in several ways. IFN-γ is the most potent of the interferons in inducing class I MHC molecules (Rosa and Fellous 1984) and may therefore enhance the ability of CTL to recognise foreign antigens on tumour cells which may have low or absent class I expression. Expression of class II MHC is mostly restricted to cells of the monocyte lineage, activated T-cells and B-cells. IFN-γ increases class II expression on these cells and may also induce expression in cells such as fibroblasts and endothelial cells which are normally negative (Collins et al 1984). Thus IFN-γ may increase presentation of tumour associated antigens

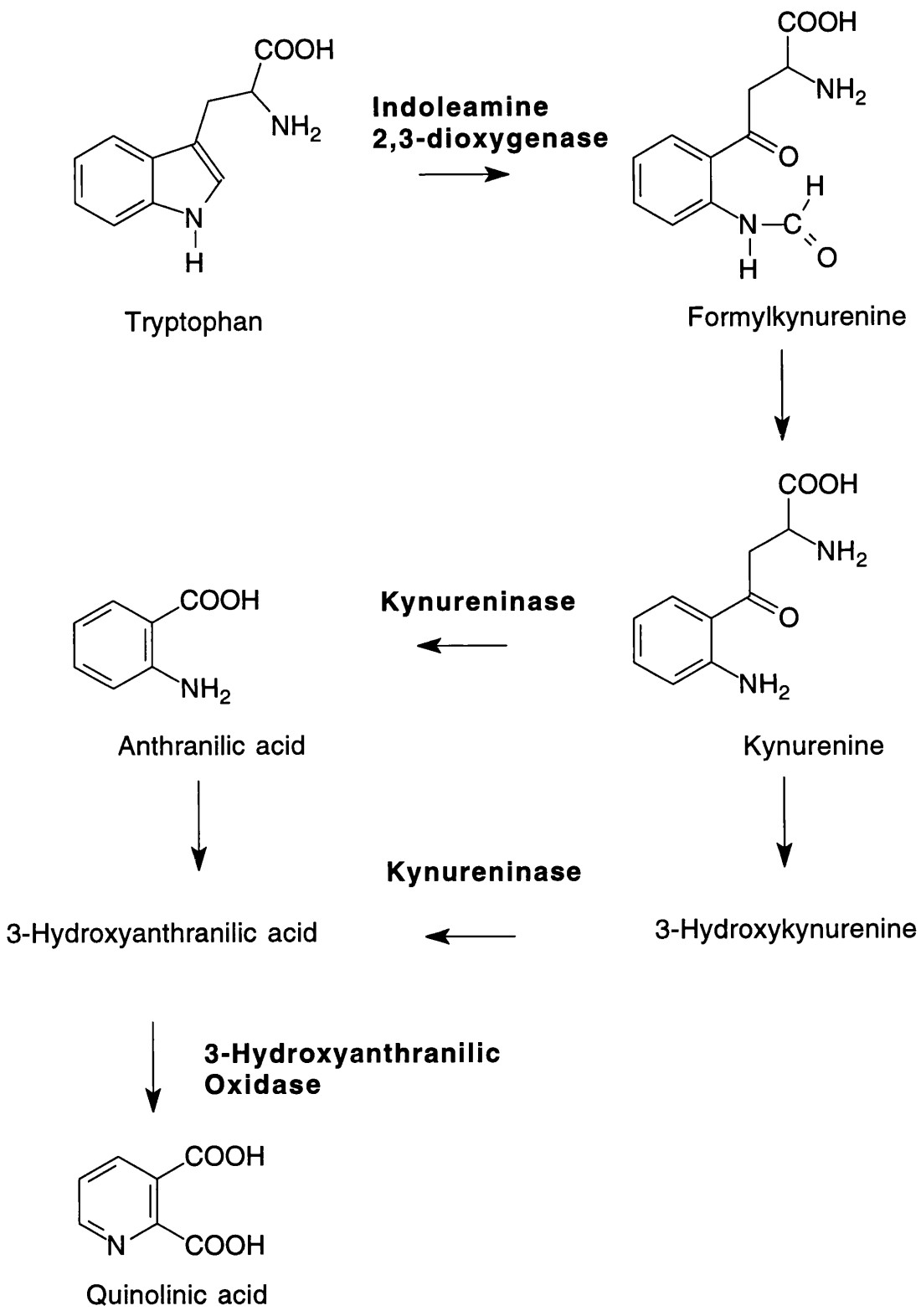
to T-helper cells. IFN-γ is further involved in MHC restricted cytotoxicity *via* induction of IL-2 receptor (Johnson and Farrer 1983).

IFN-γ also induces immunoglobulin Fc receptors on monocytes which may lead to an increase in antibody dependent cell mediated cytotoxicity. Induction of the TNF receptor by IFN-γ (Ruggiero et al 1986) may also promote non-MHC restricted killing.

Interferon-γ also has a direct anti-proliferative effect on tumour cell lines which may be mediated by depletion of tryptophan (Takikawa et al 1988). The breakdown of tryptophan to formylkynurenine is produced by an interferon inducible enzyme indoleamine 2,3-dioxygenase (IDO, figure 6:1). Further breakdown is mediated by kynureninases and 3-hydoxyanthranilic oxidase to produce quinolinic acid. The activity of IDO is dependent on the presence of superoxide anions possibly provided by another IFN-γ inducible enzyme xanthine oxidase (Datta et al 1987).

Decreased levels of serotonin result from depletion of tryptophan and metabolism of serotonin itself by IDO and have been implicated in mediating some of the side effects of IFN-γ and IL-2 therapy (Brown et al 1989).

Figure 6:1. Tryptophan metabolism.



Raised levels of neopterin, a metabolite of guanosine triphosphate (GTP) produced by macrophages as a result of induction of the enzyme GTP

cyclohydrolase (Werner et al 1990), have been reported in some cancer patients and in patients experiencing allogeneic immune stimulation. Indeed in renal transplant recipients, neopterin levels may be a useful early marker of transplant rejection (Reibnegger et al 1986). Interferon-γ is known to induce neopterin production in macrophages *in vitro* (Huber et al 1984) and raised levels observed in patients undergoing treatment with IFN-γ have been used to define a 'biologically active dose' of this cytokine when tested as a potential anti-cancer therapy (Aulitzky et al 1989). Raised levels of neopterin have been noted during treatment with IL-2 (Brown et al 1989, Lissoni et al 1991) and *in vitro* evidence suggests that this is mediated by the induction of IFN-γ. The effects on IDO and GTP cyclohydrolase are both therefore mediated by IFN-γ and *in vitro* evidence suggests that tryptophan metabolism and neopterin production are correlated (Ozaki et al 1986).

6:3:2:6. Anti-proliferative activity of IL-2.

In vitro evidence in cell lines and *in vivo* data from animal models also suggests that IL-2 may influence the cell proliferation (Paciotti and Tomartin 1988). In the hormone dependent MCF-7 cell line, picomolar levels of IL-2 inhibited growth while nanomolar levels promoted growth. No effect on cell proliferation was observed in the hormone independent cell line MDA231. Similarly in the *in vivo* studies, a dose dependent effect of IL-2 on tumour growth in the MCF-7 cell line was seen when injected into nude mice but not with the MDA231 cell line. Since the effects of IL-2 on tumour cell growth *in vivo* did not correlate with immunological changes (in terms of NK activity or T-cell proliferation) the authors inferred that the inhibitory effects of IL-2 on tumour cell growth *in vivo* were not modulated by immune stimulation by IL-2 but by a direct effect.

6:3:3 *In vivo* studies with Interleukin-2.

Pre-clinical studies demonstrated that *in vivo* administration of rIL-2 alone was capable of mediating regression of established metastases in animal models (Rosenberg et al 1985a) in a dose dependent fashion. The anti-tumour effect could also be augmented by the administration of exogenously stimulated autologous lymphocytes i.e. LAK cells (Mule et al 1986). These and other studies also established a dose response relationship for rIL-2 and suggested that efficacy was related to the number and activity of transferred LAK cells.

Phase I clinical trials of IL-2 began in 1985 (Lotze et al 1985) and the maximum tolerated dose when given by bolus was 10^6U/kg, equivalent to about $4x10^7$I.U./m^2. When given by continuous infusion, doses equivalent to 10^7U/m^2/day were only tolerated for 2 to 4 days. Dosage levels of $3x10^6$ U/m^2/day were tolerated for 6 to 7 days and levels of 10^6U/m^2 /day were tolerated for up to 21 days.

The schedules of bolus and infusional therapy developed by Rosenberg et (1985b) and West et al (1987) formed the basis of many phase II studies of rIL-2, with and without adoptive transfer of LAK cells in a variety of tumours. These included multi-centre trials in Europe of rIL-2 alone into which patients from our institution were enrolled and whose clinical course forms the second part of this thesis.

6:3:4. Immunomodulatory effects of rIL2.

It is unclear which of the many potential direct or indirect cytotoxic effects of rIL-2 which have been noted *in vitro* operate *in vivo*. Possible relevant anti-tumour Immunomodulatory effects have been inferred from assessment of

cell phenotypes and their changes during therapy with rIL-2, measurement of proliferative responses and induced cytotoxicity of peripheral blood mononuclear cells as well as assessment of cytokine induction.

Within hours of high-dose bolus injection (Lotze et al 1985) or 24 to 48 hour of continuous infusion rIL-2 (Sosman et al 1988), a transient and dose dependent fall in the peripheral total lymphocyte count occurs predominantly due to a fall in the NK and T-cell populations. If rIL-2 doses are repeated or infusion is continued, lymphocytes isolated from the periphery have a low capacity to proliferate or lyse tumour targets (Hank et al 1988). Following repeated bolus administration or at the end of an infusion period, the lymphocyte count increases, frequently to above pretreatment levels. At this point the proportion of T-cells may be increased and they may express activation markers such as expression of CD25 or class II HLA. An increase in the proportion of NK cells is also observed at this point. At the time of the rebound lymphocytosis, the peripheral blood mononuclear cells exhibit increased cytotoxicity (West et al 1987). During more prolonged lower dose treatment with rIL-2 a sustained lymphocytosis is observed largely due to expansion of NK cell numbers which show increased cytotoxic activity *in vitro* (Sleijer et al 1990). Although there is little change in the number of circulating T-cells, increases in activation markers such as CD25 and HLA-DR are comparable to those seen in higher dose studies. Other markers of peripheral mononuclear cell activation include the soluble form of the low affinity (p55) IL-2 receptor (sIL-2R) which can be detected in the serum during treatment with rIL-2 (Lissoni et al 1991) but although sIL-2R has been detected in the supernatants of activated T-lymphocytes, B-lymphocytes and monocytes *in vitro*, the source of sIL-2R *in vivo* is unclear (Rubin and Nelson 1990).

Data on the immunological changes at tumour sites during treatment with rIL-2 is necessarily limited. Studies have shown an increased mononuclear cell infiltrate consisting of CD4 and CD8 T-lymphocytes and macrophages but little change in the numbers of cells bearing NK markers (Cohen et al 1987, Rubin et al 1989). In the latter study, increased expression of class II HLA on tumour cells as well as infiltrating T-lymphocytes was observed and correlated with response to treatment.

6:3:5. Toxicity of rIL-2.

Treatment with rIL-2 is associated with considerable toxicity (Rosenberg et al 1989). Constitutional symptoms are common and include fever, rigors, malaise and anorexia. The serious adverse events which are dose-limiting are i) a vascular leak syndrome manifest by peripheral or pulmonary oedema, ascites and pleural effusions and ii) hypotension due to decreased peripheral vascular resistance, impaired left ventricular function and hypovolaemia as a consequence of the vascular leak syndrome. These changes may lead to pre-renal and respiratory failure.

As stated previously, the toxicity of IL-2 was found to be dose and schedule dependent, the dose level and the duration of treatment being limited by weight gain, hypotension and dyspnoea. In an animal model which studied the pulmonary toxicity of IL-2, the increase in high protein lymph flow from the lungs and the mononuclear/lymphoblastoid infiltrate were dependent on the dose of rIL-2 administered (Glauser et al 1988a). In a subsequent study, it was apparent that toxicity was greater when the IL-2 was given by infusion (Glauser et al 1988b). In clinical trials comparing schedules of administration, continuous infusion is associated with greater toxicity than with bolus administration (Sosman et al 1988, Thompson et al 1988).

Immunomodulatory parameters such as induced LAK activity and lymphocyte rebound are also dose-related (West et al 1987, Creekmore et al 1989). Indeed West suggested that, when given by infusion, a threshold level of $18x10^6$ I.U./m^2/day of IL-2 is required for significant lymphocyte activation. It is also evident that when considering the above indices of immunomodulation infused IL-2 may be up to ten times more potent than bolus administration (Thompson et al 1988). The reduction in dose which may be achieved as a result of using continuous infusion of IL-2 then probably outweighs the greater toxicity otherwise associated with this mode of administration. Claims that IL-2 is less toxic when given by infusion (West et al 1987) have been made after retrospective comparisons with patients who received a much higher overall dose given by bolus.

6:3:5:1. Hypotension: mechanisms of toxicity.

The systemic hypotension observed with IL-2 therapy is not only a consequence of a decreased intra-vascular volume due to the capillary leak syndrome, but is also dependent on reduced peripheral vascular resistance (Duke et al 1989). The physiological changes seen with IL-2 therapy are similar to those seen in septic shock in which TNF and IL-1 have been implicated (Tracey et al 1987). It is now recognised that cytokine/lipopolysaccharide induced hypotension is most likely mediated by nitric oxide (Kilbourn and Belloni 1990).

The properties of nitric oxide have been discussed in chapter 3. In summary, NO is generated during the conversion of L-arginine to L-citrulline by isoforms of the enzyme NO synthase. Several of the normal physiological actions of NO are attributed to the stimulation of guanylate cyclase (Moncada et al 1991) which converts GTP to GMP which itself leads to induction of second

messengers (protein kinases) within target cells. Constitutive forms of NO synthase in vascular endothelium produce low levels of NO which modulate vascular tone. Production in neural tissue mediates neurotransmission by acetylcholine and other muscarinic agents. The inducible form of the enzyme leads to a more prolonged production of higher levels of NO. Production of high levels of NO have been implicated in mediating the cardiovascular consequences of endotoxic shock. Cytokines such as IL-1, TNF-α and IFN-γ are known to induce this calcium independent form of the enzyme in a variety of cell types (see below) and since rIL-2 is also capable of inducing these cytokines, the induction of NO could mediate the cardiovascular consequences of rIL-2 therapy.

Cytokine induction of nitric oxide has been demonstrated in endothelium (Kilbourn and Belloni 1990), and in smooth muscle (Busse and Mulsch 1990, Beasley et al 1991). In the latter cell type IFNγ and TNF have been shown to have synergistic effects on the production of NO (Geng et al 1992). Induction of NO in hepatocytes (Nussler et al 1992) and fibroblasts (Werner-Felmayer 1990) has also been demonstrated. High levels of NO in response to cytokine stimulation has been demonstrated in macrophages in animal systems (Drapier et al 1988, Hibbs et al 1990).

Products of pterin metabolism, most notably 5,6,7,8 tetrahydrobiopterin and 7,8, dihydrobiopterin, are essential cofactors in the generation of NO by inducible NO synthase in a variety of cell types including macrophages (Kwon et al 1989, Werner et al 1990, Stuehr et al 1991), endothelial cells (Pollock et al 1991), cerebellum (Mayer et al 1990) and dermal fibroblasts (Werner-Felmayer et al 1990). Interferon-γ leads to induction of GTP cyclohydrolase I which catalyses formation of biopterin derivatives (Werner et al 1990), and

their presence during therapy with IFN-γ and IL-2 has been documented previously (see above).

It is possible therefore that the secondary induction of cytokines during rIL-2 which have been implicated in mediating its toxicity (Heberman et al 1989) may lead to induction of iNOS as well as generation of the essential co-factors.

As discussed in chapter 3, nitric oxide generated in macrophages from this L-arginine dependent pathway has been implicated in the cell mediated immune response to tumour (Hibbs et al 1987, Stuehr and Nathan 1989) by release of iron and inactivation of iron-sulphur containing enzymes in the electron transport system (Hibbs et al 1984, Drapier et al 1988) and the citric acid cycle (Hibbs et al 1987, Drapier et al 1988). The possible importance of the cytotoxic effect of nitric oxide is supported by a study which suggested that induced levels predicted response in patients treated with rIL-2 and flavone acetic acid (Thomsen et al 1992).

6:3:5:2. Capillary leak syndrome: mechanisms of toxicity.

Studies in animal models suggested that the capillary leak syndrome was not a direct effect of rIL-2 but was due to other cellular and humoral mediators (Rosenstein et al 1986). That IL-2 induced toxicity can be reduced by prior irradiation (Matory et al 1987) or administration of anti-asialo GM 1 (Anderson et al 1988) reinforces the importance of the cellular infiltrate in mediating toxicity. Although some studies have demonstrated retraction of endothelial cells in response to TNF-α (Brett et al 1989) neither cytokines alone (including IL-2) nor supernatants of LAK cell cultures had any effect on endothelial permeability in other studies (Damle and Doyle 1989).

In animal models the endothelial activation and increase in vascular permeability observed with rIL-2 is associated with migration of T-lymphocytes into the interstitium as well as ultrastructural changes in endothelium and the separation of endothelial cells from the basement membrane in the pulmonary vasculature (Goldblum et al 1990). The mechanisms by which the cellular infiltrate causes the changes observed in endothelium remain unclear. *In vitro* studies have demonstrated that lymphocytes cultured with IL-2 are capable of lysing pulmonary endothelial cells (Duke et al 1989). Other possibilities include neutrophil production of thromboxane (Klausner et al 1989) higher levels being associated with infusional IL-2 and not bolus therapy (Welbourn et al 1990). Free radicals (Klausner et al 1991) including nitric oxide (Palmer et al 1992) and leukotrienes (Klausner et al 1990) have also been implicated in endothelial damage. The adherence to and passage through endothelium of activated leucocytes also dependent upon activation of endothelial cells and cytokines induced during therapy with rIL-2 have been implicated in inducing such endothelial activation by increases in procoagulant activity (Bevilaqua et al 1986), prostacyclin production (Rossi et al 1985) HLA expression (Pober et al 1987).

Induction of leucocyte adhesion molecules is important in mediating leucocyte-endothelial cell interactions (Cavender et al 1987) and changes in ICAM-1 have been demonstrated in serial skin biopsies in patients undergoing therapy with IL-2 (Cotran et al 1987) which may contribute to the observed influx of mononuclear cells into normal skin following rIL-2 therapy (Gaspari et al 1987).

It is now clear that several adhesion molecules which may be upregulated by cytokines *in vitro*, are responsible for mediating leucocyte-endothelial cell

interactions *in vivo*. Their expression and upregulation has been assessed in a sub-group of patients during therapy with rIL-2 and consequently a brief summary of their distribution and ligands follows.

6:4. Endothelial/leucocyte adhesion molecules.

6:4:1. Immunoglobulin supergene family and their ligands.

6:4:1:1 Intercellular adhesion molecule-1 (ICAM-1).

ICAM-1, a member of the immunoglobulin (Ig) superfamily, is a single chain glycoprotein of 80-115kDa, which is expressed on endothelial cells, fibroblasts, epithelial cells and mucosal cells as well as on lymphocytes and monocytes (Hogg et al 1991). Expression of ICAM-1 on endothelial cells *in vitro* and *in vivo*, is upregulated by cytokines such as IL-1, TNFα and IFNγ (Pober et al 1986) and as noted above upregulation of ICAM-1 has been noted in uninvolved skin during therapy with rIL-2 (Cotran et al 1987). Known ligands of ICAM-1 are members of the β_2-integrin family, lymphocyte function associated antigen-1 [LFA-1, CD11a/CD18] (Marlin and Springer 1987) and CR3 [CD11b/CD18] (Diamond et al 1991) although the latter is bound less avidly than LFA-1. LFA-1 (CD11a/CD18) is expressed on all leucocytes (Krensky et al 1983) and mediates interactions between leucocytes and endothelium and other cell types expressing ICAM-1 (Marlin and Springer 1987). LFA-1 also mediates the adhesion dependent functions of immune effector cells such as T-helper cells, cytotoxic T-cells and NK cells (reviewed by Springer 1990). Although in general, the expression of integrin molecules is not influenced by the presence of cytokines, expression of LFA-1 on T-lymphocytes may be upregulated by IL-2 and IFN-γ (Shimuzu et al 1990).

6:4:1:2. Vascular cell adhesion molecule (VCAM-1).

VCAM-1, also a member of the Ig-like superfamily was thought to be expressed only on endothelial cells (Carlos et al 1990) but is now known to be expressed on renal tubular epithelium, lymphoid dendritic cells and tissue macrophages (Rice et al 1991). VCAM-1 is expressed at low levels on non-activated endothelial cells but is induced upon stimulation with IL-1 and TNF-α (Osborn et al 1989). VLA-4 (CD49d/CD29) a member of the β-1 integrin family which is expressed on monocytes, lymphocytes and eosinophils (Hemler et al 1990), has been shown to mediate the adhesion of these leucocytes to VCAM-1 on activated endothelium (Elices et al 1990).

6:4:1:3. Platelet/endothelial cell adhesion molecule (PECAM, CD31).

The site of initial contact between monocyte and endothelial cells appears to be random but monocytes then tend to accumulate at the endothelial cell boarders (Beekhuizen et al 1990). The cause for this localisation is unclear but may be related to the peripheral distribution of PECAM-1 (CD31) which may mediate the first monocyte-endothelial cell interaction. More recent studies have suggested that engagement of leucocyte CD31 results in signal transduction which increases adhesion mediated by β1 and β2 integrins (Tanaka et al 1992) thus the initial homophilic or heterophilic interactions of leucocyte CD31 may promote other adhesion mechanisms.

6:4:2. The selectins.

6:4:2:1. E-selectin: Endothelial-leucocyte adhesion molecule (ELAM-1).

ELAM-1 (CD62E) is expressed transiently on vascular endothelial cells following stimulation with IL-1α or TNF-α (Bevilacqua et al 1987). ELAM-1 recognises the carbohydrate moieties sialyl-Lewis X and sialyl-Lewis A and also binds carbohydrates on other members of the selectin family such as L-selectin which is expressed on virtually all types of leucocytes (Tedder et al 1990).

6:4:2:2. P-selectin (GMP 140).

P-selectin (CD62P) is a single chain glycoprotein of 140kDa found within secretory granules within platelets and vascular endothelial cells (Celi et al 1991). Following stimulation with histamine, thrombin (Zimmerman et al 1985) or leukotrienes (Hoover et al 1984) the cytoplasmic granules fuse with the plasma membrane and P-selectin is rapidly expressed. Like ELAM-1, P-selectin also recognises glycoproteins bearing sialyl-Lewis A and sialyl-Lewis X epitopes the latter being expressed on neutrophils (Geng et al 1990).

6:4:2:3. L-selectin (Mel14-Ag).

L-selectin (CD62L) is a 90 to 100kDa glycoprotein which is expressed on T and B lymphocytes where its function is related to the binding of lymphocytes to the high endothelial venules in lymph nodes and is also involved in lymphocyte recirculation (Yednock et al 1987). Monocytes and granulocytes also express L-selectin and has been shown to facilitate margination of these cell types during the inflammatory response prior to subsequent adhesion

through integrin molecules (Lawrence and Springer 1991). The effects of L-selectin are probably mediated through interaction with epitopes on E-selectin and P-selectin present on the surface of endothelial cells (Picker et al 1991). Like E-selectin, L-selectin also recognises sialyl-Lewis X and sialyl-Lewis A carbohydrate moieties (Berg et al 1992).

6:4:3. Other leucocyte adhesion molecules.

6:4:3:1. CD14.

The myeloid differentiation antigen CD14 is a 55kDa single chain glycoprotein found predominantly on monocytes (Hogg and Horton 1990). CD14 has been implicated in the binding of monocytes to cytokine activated endothelium though the endothelial ligand is as yet undefined (Beekhuizen et al 1991). CD14 may also be involved in signal transduction and monocyte activation (Schneider et al 1990).

6:4:3:2. p150,95 (CR4).

p150,95 (CD11c/CD18) is another member of the β_2-integrin family and is expressed on monocytes, tissue macrophages, granulocytes and activated T-lymphocytes. p150,95 has been implicated in monocyte and granulocyte adherence to endothelial cells (Stacker & Springer 1991) though as with CD14 the endothelial ligands for this integrin are not known.

6:4:4. Role of adhesion molecules in leucocyte/endothelial interactions.

The relative contribution of the above adhesion molecules to the interaction of different leucocyte subsets with cytokine stimulated endothelium remains unclear but the mechanism leading to the increase of adhesion of monocytes appears to be different from that reported for the adhesion of neutrophils or lymphocytes. Adhesion of neutrophils to cytokine stimulated endothelial cells is mediated predominantly by all three β_2-integrin molecules and by E-selectin and ICAM-1 on endothelial cells (reviewed by Beekhuizen and van Furth 1993). Lymphocytes can bind via LFA-1/ICAM-1 (Dustin and Springer 1988) and VLA-4/VCAM-1 (Elices et al 1990) on IL-1α and TNF-α stimulated endothelium. E-selectin has also been implicated in the binding of memory T-cells to activated endothelium (Shimuzu et al 1991). The binding of monocytes to cytokine activated endothelium does not appear to be dependent on β_2-integrin molecules or expression of ICAM-1 (Beekhuizen et al 1991). E-selectin may play a minor role in adhesion possibly via sialyl-Lewis A and X but antibodies to E-selectin inhibit adhesion by only 20 to 30% (Hakkert et al 1991). The potential role of VCAM-1 is supported by studies demonstrating that VCAM-1-transfected CHO cells bind VLA-4 expressed on monocytes and lymphocytes (Carlos et al 1991). Studies using monoclonal antibodies to the individual ligands VCAM-1 and VLA-4 did not demonstrate inhibition of adhesion (Verdegaal et al 1993). It is possible however that the VLA-4/VCAM-1 interaction is important but dependent upon other CD18 mediated adhesion mechanisms (Carlos et al 1991).

6:4. Clinical studies described in the second part of this thesis.

As part of multicentre trials to assess the potential use of rIL-2 in the immunotherapy of cancer, we treated several groups of patients with infusional rIL-2.

In addition to testing the feasibility of this approach, the possible mechanisms associated with the toxicity of rIL-2 were also explored. In particular, changes in TNF-α and IFN-γ were documented and the further consequences of their induction such as induction of TNF binding proteins and nitric oxide together with the production of pterin co-factors necessary for nitric oxide production were measured. Other consequences of secondary cytokine induction were also assessed at tissue level, namely the trafficking of leucocytes and induction of endothelial and leucocyte adhesion molecules which mediate their margination and migration into normal tissue and sites of metastatic tumour were assessed in a limited number of patients

Chapter 7.

METHODS.

Participation of the clinical oncology unit at Guy's hospital in phase I/II trials of rIL-2 provided the opportunity for additional studies assessing the biological effects of rIL-2 *in vivo* for the purpose of this thesis. These studies had the prior approval of the Guy's Hospital Committee on Ethical Practice.

7:1 Clinical.

Patients were treated with infusions rIL-2 (EuroCetus) on one of the following four studies:- metastatic breast cancer-EuroCetus protocol no. EC-L2-041 and Guy's Hospital protocol C74; renal cell carcinoma-EuroCetus protocol no. EC-MP-001; metastatic melanoma-EuroCetus protocol no. EC-MP-003. The details of the treatment regimens are described below. Because of the similar eligibility criteria, management details and response criteria, the clinical and immunological results for the whole group are considered together.

7:1:1. Study objectives.

The aims of the studies were:-

- to study the toxicity of infusions of rIL-2 alone in patients with metastatic breast cancer, melanoma and renal cell carcinoma.
- to evaluate the anti-tumour efficacy of the treatment regimens in these patient groups as part of European multi-centre trials.

- to investigate potential mechanisms of toxicity with respect to cytokine induction and its consequences (specific studies in the ICRF Clinical Oncology Unit at Guy's Hospital specifically for this thesis).

7:1:2. Study design.

The original aims of each of the studies was to accrue 14 patients in order to ensure that a response rate of ≥20% was not missed with a probability of 95%.

7:1:3. Patient eligibility.

Patients were eligible for participation in the studies providing that the following criteria were met:-

- histologically documented evidence of either carcinoma of the breast, melanoma or renal cell carcinoma.
- measurable progressive disease that could be assessed serially by physical examination or radiological procedures. Patients in whom one or more of the following were the only manifestations of disease were excluded: lymphoedema, hilar enlargement, pleural effusion, ascites, bone marrow suppression, osteoblastic skeletal lesions.
- no chemotherapy during the previous 3 weeks (6 weeks for nitrosoureas and mitomycin-C) and all toxic manifestations of prior treatment must have resolved.
- no prior endocrine treatment with oestrogens, androgens or progestogens during the previous 4 weeks.
- no prior radiation therapy to all areas of measurable or evaluable disease

- ambulatory performance status (ECOG 0-2, WHO 1979) and life expectancy of greater than 2 months.
- peripheral white cells count $\geq 4x10^9/L$, platelets $\geq 100x10^9L$ and haemoglobin ≥10.5 g/dl.
- serum bilirubin and creatinine within the normal range.
- age 70 years or less.

Patients were excluded from the study if:-

- there was a significant history or current evidence of cardiovascular disease (e.g. congestive heart failure, uncontrolled hypertension, coronary artery disease, serious arrhythmias or evidence of previous myocardial infarction on ECG.
- evidence of serious active infections requiring antibiotic therapy.
- contra-indications to the use of pressor agents.
- patients with meningeal or cerebral metastases or those considered to be at risk of spinal cord compression.
- patients with intercurrent disease treated by or likely to require corticosteroids.
- pregnant or lactating women.
- patients with prior malignancies (except adequately treated basal cell carcinoma of the skin or *in situ* carcinoma of the cervix).
- patients with concurrent other primary malignancies.

7:1:4. Treatment plans.

Prior to initiation of therapy a central venous catheter (Hickman Line) was inserted. Therapy with rIL-2 was administered by intravenous infusion according to the following protocols.

Metastatic breast cancer: EuroCetus protocol no. EC-L2-041.

Patients were treated with an escalating schedule, commencing at a dose of $9x10^6$ I.U./m^2/day for 4 days per week escalating by increments of $3x10^6$ I.U./m^2 per infusion each week to reach a final dose of $18x10^6$ I.U./m^2/day at week four

Metastatic breast cancer: Guy's protocol (C74).

Patients with metastatic breast cancer who had not received prior chemotherapy for metastatic disease were given a five day infusion of rIL-2 at a dose of $18x10^6$ I.U./m^2/day. Following a three day rest period, patients received epirubicin 75mg/m^2 i.v. bolus every three weeks to a maximum of six courses.

Renal cell carcinoma: EuroCetus protocol no. EC-MP-001.

Patients were treated with two 5 day infusions of IL-2 at a dose of $18x10^6$ I.U./m^2/day with an intervening two day rest period.

Metastatic melanoma: EuroCetus protocol no. EC-MP-003

Patients were treated with dacarbazine (DTIC) 250mg/m^2 on five consecutive days, followed two weeks later by two 5 day infusions of IL-2 at a dose of $18x10^6$ I.U./m^2/day with an intervening two day rest period.

7:1:5. Indications for continuation of therapy with rIL-2.

Having completed one cycle of therapy, patients who had stable disease or better were eligible to receive a second cycle according to the above protocols. Patients who had progressive disease at the end of one cycle did not continue the study. Following two cycles of therapy, responding patients with renal cell carcinoma were eligible to receive four further 5 day cycles of rIL-2 at 4 weekly intervals and responding patients with melanoma were eligible to receive 4 further courses of DTIC/rIL-2 as described above.

Patients off study due to progressive disease during therapy, unacceptable toxicity or progressive disease after study completion were offered alternative cytotoxic/supportive treatment as appropriate.

7:1:6. Schedule modification of rIL-2.

The dose of rIL-2 was interrupted for the following toxicities until they resolved (toxicity was graded according to WHO criteria, WHO 1979):

- hypotension grade 3 or 4 (change of systolic blood pressure greater than 40mm Hg or requiring fluids or pressor agents).
- significant cardiac arrhythmia.
- suspicion of myocardial ischaemia, anginal pain and or ST changes on electrocardiogram.
- agitation or persistent confusion.
- elevation of bilirubin to a level greater than 40μmol/L.
- elevation of serum creatinine to a level greater than 300μmol/L.
- bacterial sepsis.
- dyspnoea at rest.

- prolongation of prothrombin time (PT) ≥ 3 seconds over control or of the partial thromboplastin time (PTT) ≥10 seconds over control.
- if constitutional side effects were regarded as unacceptable despite supportive care.

7:1:7. Indications for dose reduction of rIL-2.

If the patients experienced the following toxicities subsequent rIL-2 was administered at 50% dose:

- hypotension ≥grade 3.
- rise in serum creatinine during the prior cycle to a level >300μmol/L or bilirubin to a level greater than 60μmol/L during the previous cycle.
- neurological toxicity greater than grade 3.

7:1:8. Contra-indications to subsequent rIL-2.

- documented myocardial ischaemia.
- grade 4 neurotoxicity.
- serum creatinine or bilirubin which fail to return to grade 1 toxicity or less.
- any toxicity deemed unacceptable by patient or physician.

7:1:9. Management of hypotension.

For patients experiencing grade 3 hypotension or greater:

- rIL-2 was discontinued until blood pressure returned to within 20mm Hg of baseline (grade 1 or less).
- albumin was infused at a rate of 12.5 grams/hour repeated as necessary.

- if hypotension did not respond to these measures, dopamine (at a dose of 5 to 10μg/kg/min) was given in order to maintain blood systolic pressure greater than 90mmHg.
- urine output was monitored with catheterisation if appropriate.

7:1:10. Management of fluid retention.

- patients were weighed every 12 hours.
- no supplemental i.v. fluids were given unless there was documented weight loss resulting from vomiting, diarrhoea or insensible loss.
- frusemide was given during rest periods if peripheral oedema was symptomatic.

7:1:11. Concomitant medications and management.

Other anti-tumour therapy, i.e. hormone-, chemo-, immuno- or radiotherapy was not given to patients while on study. The use of steroids was reserved for patients who developed life threatening signs or symptoms.

7:1:12. Study parameters.

Pre-study screening.

All inclusion and exclusion criteria were assessed together with all baseline parameters within 14 days before initiation of therapy.
Clinical assessment included:- history, physical examination, performance status, electrocardiograph, vital signs (pulse, blood pressure, temperature and respiratory rate) and weight.

Laboratory investigations included:- full blood count (FBC) including differential white cell count, PT and PTT, urea and electrolytes, bilirubin, creatinine, aspartate transaminase, alanine transaminase and alkaline phosphatase.

During rIL-2 infusions, the following were performed daily:- vital signs (four hourly), weight (twice daily) FBC, routine biochemistry. ECGs, chest radiographs and infection screens (if clinically indicated).

After completion of rIL-2 infusions the following were performed at approximately 4 week intervals:- Physical examination, performance status ECG, FBC and biochemistry.

Patients were followed up monthly and were observed for long term toxicity, response duration (where applicable) and survival.

7:1:13. Tumour response assessment.

Tumour response was assessed by clinical examination and analysis of appropriate radiological investigations (radiographs, CT scans).

All tumour measurements were recorded and consisted of the longest diameter and the perpendicular diameter in the widest portion of the tumour. Pleural effusions, ascites, osteoblastic skeletal lesions, lymphoedema and hilar enlargement were not considered evaluable.

The WHO criteria for response assessment were used (WHO 1979) i.e.:- complete response (CR): the disappearance of all known disease determined by two observations not less than 4 weeks apart;

partial response (PR): 50% or greater reduction in the sum of the products of the maximum perpendicular diameters of measurable tumour by two observations at least 4 weeks apart;
stable disease (SD) less than 50% reduction in tumour size (as determined above) but less than a 25% increase in the size of measurable lesions.
progressive disease (PD): a 25% or more increase in the size of measurable lesions or the appearance of new lesions.

The duration of response was defined as the interval between the time when response was documented and the time when progressive disease was first noted.

7:2. Biological studies.

Cytokine levels, markers of cytokine activity and soluble cytokine receptors were assessed in the peripheral circulation of patients during therapy with rIL-2. When possible, plasma samples were collected on a daily basis during the rIL-2 treatment period. Samples were collected into EDTA, centrifuged at 4^0C and stored at -20^0C prior to assay.

In addition, a small group of patients with metastatic breast cancer who were treated on the single 5 day infusion protocol at a dose of $18x10^6$ I.U./m^2/day underwent biopsy of accessible cutaneous lesions and adjacent normal skin, before and at the end of the rIL-2 infusion.

7:2:1. Cytokine and soluble receptor assays.

7:2:1:1. Tumour necrosis factor-α (TNF-α).

TNF-α levels were measured using immunoradiometric assay (IRMA) kits (Medgenix, Brussels, Belgium).The TNF-α IRMA range was from 15 to 5000pg/ml. The assay was calibrated with international reference preparation (87/650) from the National Institute of Biological Standards and Control (NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire U.K.) and was used at detection limits of 20 pg/ml plasma. The assay is specific for TNF-α and does not cross react with IL-1α, IL-1β, TNF-β, GM-CSF, IFN-α, IFN-β or IL-6. The calibration, standardisation and the assay format are specifically developed for measuring TNF-α in plasma/serum samples. Mean levels of TNF were below reliable detection limits in a random sampling of 25 normal individuals (7.4±0.6 pg/ml).

7:2:1:2. Soluble TNF receptors (sTNF-R).

ELISA plates (Maxisorp Nunc, Denmark) were coated with monoclonal antibodies to the soluble forms of either type 1 or type 2 TNF-R by incubation of the plates for 2 hours at 37^0 C with a solution of 25μg/ml of the antibodies in PBS containing 1% bovine serum albumin, 0.02% NaN_3. After an additional two hours of incubation at 37^0 C with phosphate buffered saline containing 1% bovine serum albumin, 0.02% NaN_3 and 0.05% Tween 20 (blocking solution) to block non-specific binding of protein, the plates were rinsed with PBS containing 0.05% Tween 20 (washing solution). Samples for testing were serially diluted in a solution containing 0.65 M NaCl, 10mM sodium phosphate buffer, pH 7.0, 0.05% Tween 20, 0.1% Nonidet P-40 and 0.02% NaN_3 before

being added to the plates in aliquots of 80μl/well. The plates were incubated for two hours at room temperature and rinsed three times with washing solution. Rabbit polyclonal antiserum against the relevant soluble receptor, diluted 1:500 in blocking solution was then added to the wells. After being incubated for a further 12 hours at room temperature, the plates were rinsed again with washing solution and incubated for 2 hours at 37^0 C with horseradish-peroxidase-conjugated affinity purified goat anti-rabbit IgG (Biomakor, Israel) The assay was developed for 30 minutes by using 2'2-azino-bis(3-ethylbenzthiazoline-6 sulphonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 405nm. Purified urine derived soluble forms of the two receptors served as standards. The detection limit of the assay was 30 pg/ml and no cross reactivity was found for the two species of receptors in the two assays. Addition of recombinant TNF at a concentration of 25 ng/ml, to tested samples of sTNF-Rs did not affect the estimates of binding protein. These assays were kindly performed by Dan Aderka of the Weizzman Institute, Rehovot, Israel.

7:2:1:3. Interferon-γ (IFN-γ).

Interferon-γ levels were measured using an IRMA kit (Medgenix, Brussels, Belgium). The IFN-γ IRMA range was from 0-90U/ml. The assay was calibrated with an interim reference preparation (88/606) (NIBSC) and was used at a detection limit of 2.5 U/ml plasma. No IFN-γ was detected in a sample of 25 normal individuals.

7:2:2. Markers of interferon-γ activity.

7:2:2:1. Neopterin.

Neopterin is a marker of the functional activation of the monocyte/macrophage series (Werner et al 1985). Neopterin levels were measured using a commercially available kit from Henning, Berlin GMBH, Berlin, Germany. The assay utilises a polyclonal anti-neopterin antisera raised in sheep precipitated by anti-sheep Ig antibody, a double antibody technique. The assay range was from 0.8-184 ng/ml. Mean levels of neopterin in a random sample of 75 normal individuals was 2.0±0.4 ng/ml.

7:2:2:2. Tryptophan/kynurenine.

Levels of tryptophan and kynurenine were assessed by reverse phase HPLC as described by Malik et al (1991) on a waters μBondpak C_{18} column (3.9x300 mm) with 4x12.5 C_{18} cartridge guard column a mobile phase consisting of 90% of (0.1 M ammonium acetate brought to ph4.5 with acetic acid), 10% methanol at 1 ml/min. Kynurinine (4.6 min) detected by absorbance at 350nm. Tryptophan (6.6min) detected by fluorescence, excitation 285 nm, emission 340 nm. These assays were kindly performed by Richard Knowles at the Wellcome Foundation.

7:2:3. Nitrate analysis.

Plasma nitrate levels were determined as described previously (Thomsen et al 1991). After precipitation of plasma proteins with 30% $ZnSO_4$ and reduction of nitrate in the supernatant to nitrite using acid-washed calcium powder, nitrite was measured using a microplate assay based on the Greiss reaction

(Green et al 1982). These assays were kindly performed by Lindy Thomsen at the Wellcome Foundation.

7:2:4. Skin and tumour biopsies.

The trafficking of leucocyte subsets and the expression of adhesion molecules which may play a role in their adherence to endothelium and subsequent extravasation, was assessed in normal skin and cutaneous metastases in three patients with metastatic breast cancer treated on the Guy's Hospital C74 protocol. Biopsies were carried out under local anaesthetic and tissue was immediately placed in liquid nitrogen. Frozen sections were prepared from tissue samples mounted in OCT compound (Tissuetek, USA), and 5μm sections cut in a cryostat (Cryostat E, Reichert Jung, FGR).

The primary antibodies used, antigens recognised and their source are noted overleaf in table 7:1. Antibodies were visualised using the APAAP method previously described (page 52). Cells positive per high power field were counted in dermal and tumour areas. A minimum of 5 high power fields were assessed and the mean number for each area calculated. As well as a comparison of absolute numbers of antigen expressing cells before and after rIL-2, data were also expressed as a ratio (post rIL-2/pre rIL-2).

Table 7:1. Antibodies used to assess changes in leucocyte subsets and adhesion molecule expression in normal skin and tumour before and after infusion of rIL-2.

CD#/Ag	Antibody	Expression	Alt. name	Source	Dilution
CD3	UCHT1	T-lymphocytes		PCLB	nil
CD45RA	SN130	Virgin T, B, Mo		GJ	nil
CD45RO	UCHL1	Memory T, B, Mo		PCLB	nil
CD4	UCHT4	helper/inducer T		PCLB	nil
CD8	UCHT8	cytotoxic/sup T		PCLB	nil
CD25	IL-2R	Activated T & B	Tac	BD7640	1 in 25
CD20	L26	B-Lymphocytes		Dako M755	1 in 100
CD68	EBM11	Mo, macrophages		Dako M718	1 in 100
CD14	UCHM1	Mo,	LeuM3	PCLB	nil
CD56	NKH1	NK cells	NCAM‡	Dako M852	1 in 50
CD57	HNK1	NK, some T&B		P.C.L.B.	nil
N/A	NP57	Neu	Elastase	Dako M852	1 in 200
CD66b		Neu	(CD67)	IT-0166	1 in 25
TNF	J1D9			JM	1 in 50
CD120a	htr	all cell types	TNF-Rp55	MB	1 in 2000
CD120b	utr	T, Mo,	TNF-Rp75	MB	1 in 2000
CD54	BBA3	EC,FB,Epi,Ly,Mo	ICAM-1	R&D	1 in 1000
CD62E*		EC	ELAM-1	DH	1 in 10
CD62P†	BTC71	EC,plt	GMP140	R&D	1 in 400
CD62L¶	leu 8	Mo,Neu,T&B	Mel14Ag	BD7440	nil
CD106		EC, Mo	VCAM	IT1224	1 in 100
CD31	JC10A	EC,plt	PECAM	Dako M823	1 in 20
CD49d/29	HP221	Mo,Ly,Eo	VLA-4	IT0764	1 in 50
CD11a/18		Mo,Ly,NK	LFA-1	Dako M782	1 in 100
CD11c/18		Mo,Neu,T	CR4, p150	Dako M732	1 in 100

Key to table.

CD#/Ag: **N/A**, not applicable. * E-selectin, † P-selectin, ¶ L-selectin.

Expression: **T**= T-lymphocytes, **B**=B-lymphocytes, **Ly**=lymphocytes, **Mo**=monocytes, **NK**=natural killer cells, **Neu**=neutrophils, **EC**=endothelial cells, **FB**=fibroblasts, **Epi**=epithelial cells, **plt**=platelets, **Eo**=eosinophils.

Alternative names: **NCAM‡**=isoform of neuronal cell adhesion molecule, **ICAM-1**=intercellular adhesion molecule 1, **ELAM-1**=endothelial-leucocyte adhesion molecule 1, **VCAM**=vascular cell adhesion molecule, **PECAM**=platelet/endothelial cell adhesion molecule, **VLA-4**=very late after activation antigen 4, **LFA-1**=lymphocyte function associated antigen-1.

Sources: **PCLB**; Peter Beverley, Human Tumour Immunology Group, ICRF.
GJ George Janossy, Royal Free Hospital, London.
JM Jez McLaughlin, Liverpool University.
MB Manfred Brockhaus, Hoffman La Roche.
DH Doreen Haskard, ICRF.
BD Beckton Dickenson, **IT** Immunotech, **R&D** R&D systems.

7:3. The problem of rIL-2 adherence

Our first experience with rIL-2 was in patients with metastatic breast cancer treated on EuroCetus protocol no. EC-L2-041. A previously reported phase II study of rIL-2 in breast cancer had used four consecutive infusions at doses of $18x10^6$ I.U./m^2/day for 5 days followed by a two day rest period (Israel et al 1989). Considerable toxicity had been documented in this study and in no case could the projected dose or duration of rIL-2 be tolerated. As well as the frequently observed dose-limiting toxicities of hypotension, pulmonary oedema and renal impairment, thrombocytopenia, in one instance fatal, was also reported. As a result of these findings we used the dose escalating schedule described above.

The toxicity of this treatment regimen is described in more detail in the results section below. Early in the study, we formed a strong clinical impression that although constitutional side effects were noted, the toxicity was not as severe as previously reported, even at the highest dose level, equivalent to that used in the previous reported study in patients with breast cancer.

The rIL-2 supplied by EuroCetus consisted of a lyophilised cake containing mannitol and sodium dodecyl sulphate but no protein carrier. Cytokines for laboratory use are routinely reconstituted with bovine serum albumin as a protein carrier in order to minimise their adsorption onto plastic surfaces. The absence of a protein carrier in the EuroCetus preparation of rIL-2 raised the possibility that patients in the dose escalating study were not receiving the anticipated dose because of adsorption of rIL-2 onto the giving sets used to administer rIL-2. A series of simple experiments was performed to explore this possibility.

7:3:1. Methods.

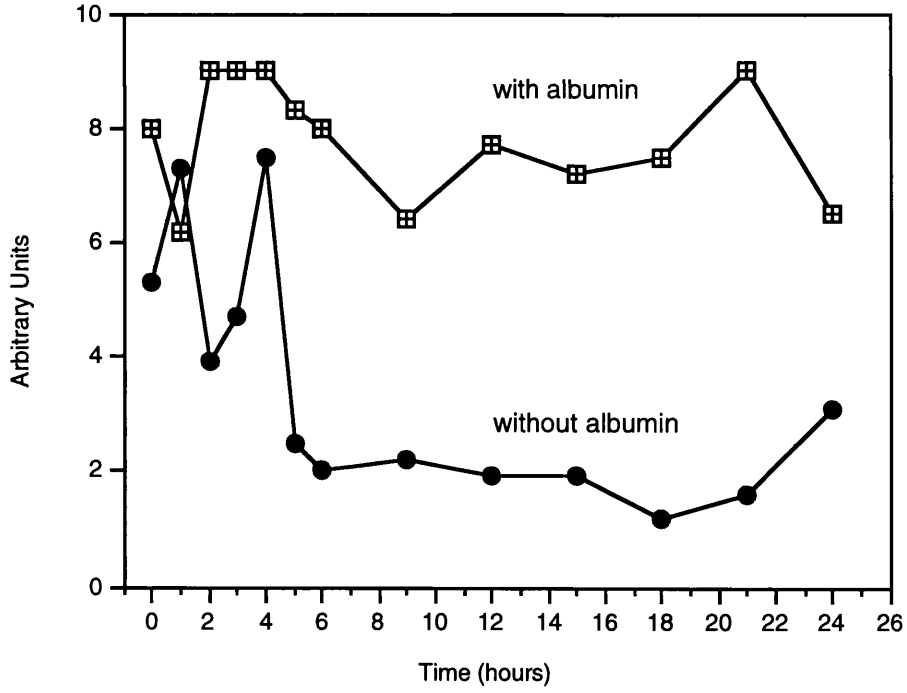
Proleukin (rIL-2 EuroCetus B.V., Amsterdam, The Netherlands, lot no LSP 911 expiry date 11/90) was initially reconstituted with 1.2 ml of sterile water for injection per vial. One millilitre of this solution was then made up to 50 mls with either 5% glucose alone (Baxter Healthcare Ltd, Lot 89120BD, expiry date 09/91) or 5% glucose with 2% human albumin (Travenol Laboratories Ltd, lot no 88L28A116B expiry date 12/91). Solutions were made up in Becton-Dickinson 50 ml syringes. Giving sets (Lectro-Cath 200cm, Vygon Ltd) were primed with the solution and the first millilitre was ejected manually (time = zero). The syringes were then placed in syringe drivers (Vickers Treonic IP4, Vickers Medical) and driven at 2 mls per hour. One millilitre aliquots were taken at various time points during the subsequent 24 hours. All samples were collected in Nunc vials containing 0.5 mls 5% human albumin. The volume in the vial was then measured and adjusted with 1% albumin in the case of samples originally reconstituted with glucose plus albumin and 5% albumin in the case of samples made up with glucose alone. This resulted in a two-fold dilution of the rIL-2 from the giving set and ensured that the concentration of albumin was equal in all samples. Solutions were then assayed for IL-2 activity by radioimmunoassay (Medgenix) and by bioassay using the CTLL-2 cell line (Gillis and Smith 1977). Cytokine induced proliferation was assessed by tritiated thymidine incorporation into DNA and scintillation counting (Gillis et al 1978). Titration of the WHO first International standard for IL-2 (100 I.U./ampoule) was included in each assay and assays were carried out in triplicate using several dilutions spaced on the dose response curve. Dose response relationships at each time interval (with and without albumin) were examined both graphically and statistically. The responses were analysed using weighted regression (Gaines-Das & Tydeman

1982) and the percentage potency for each sample was calculated relative to the concentration of rIL-2 at time = zero (with albumin)

7:3:2. Results.

When assessed by RIA, there was considerable hour to hour variation in the estimates of immunoreactive rIL-2 in the first four hours of infusion, particularly in the rIL-2 reconstituted without albumin. After 6 hours there was a considerable reduction in the estimate of immunoreactive rIL-2 in the infusion not reconstituted with albumin (figure 7:1).

Figure 7:1. Relative potency of rIL-2 when reconstituted with and without albumin (radioimmunoassay).



The dose response relationships for the CTLL bioassay for various time points during the continuous infusion period are illustrated in figure 7:2 (overleaf).

Figure 7:2.. CTLL bioassay of rIL-2 activity when reconstituted with and without albumin.

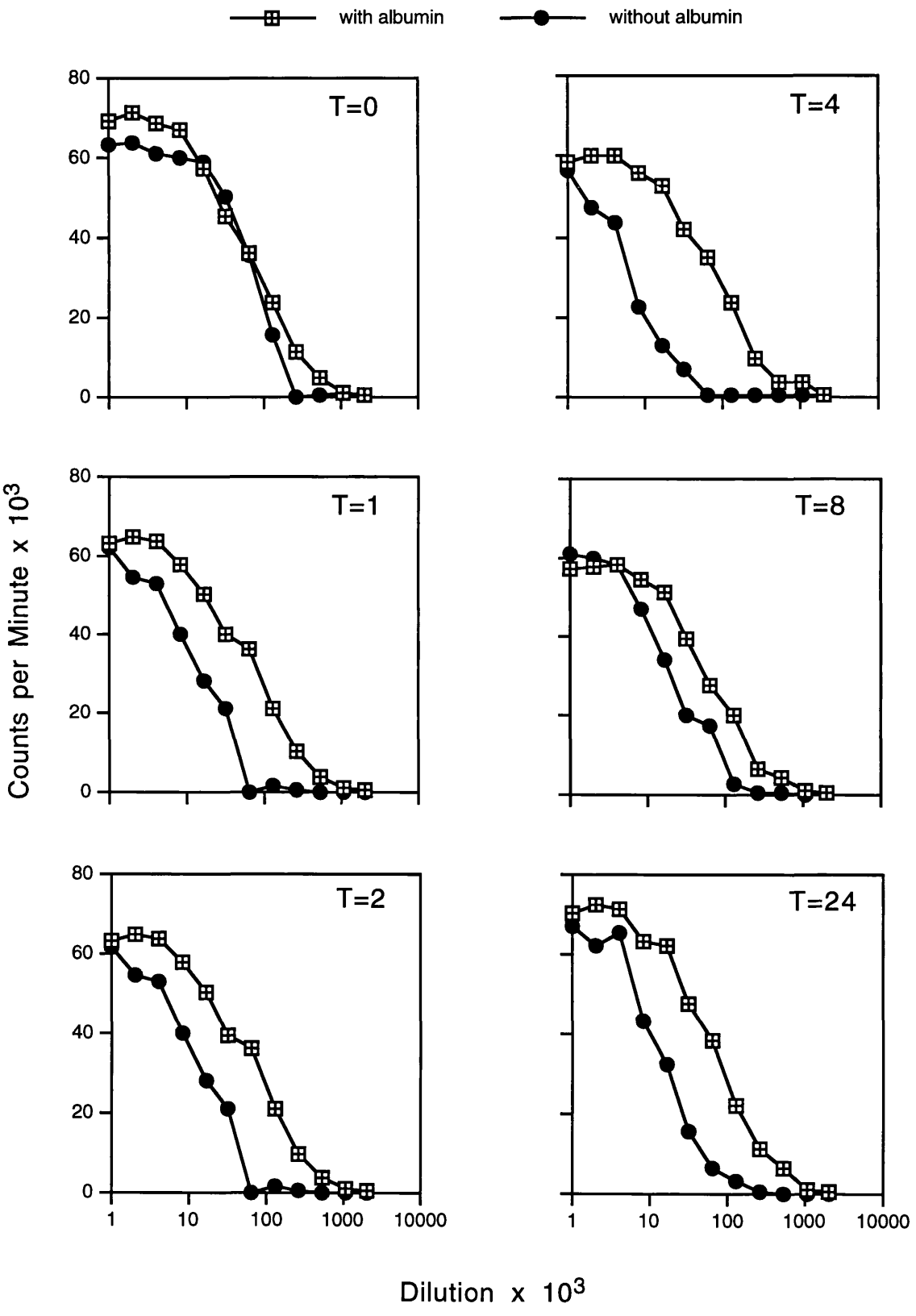
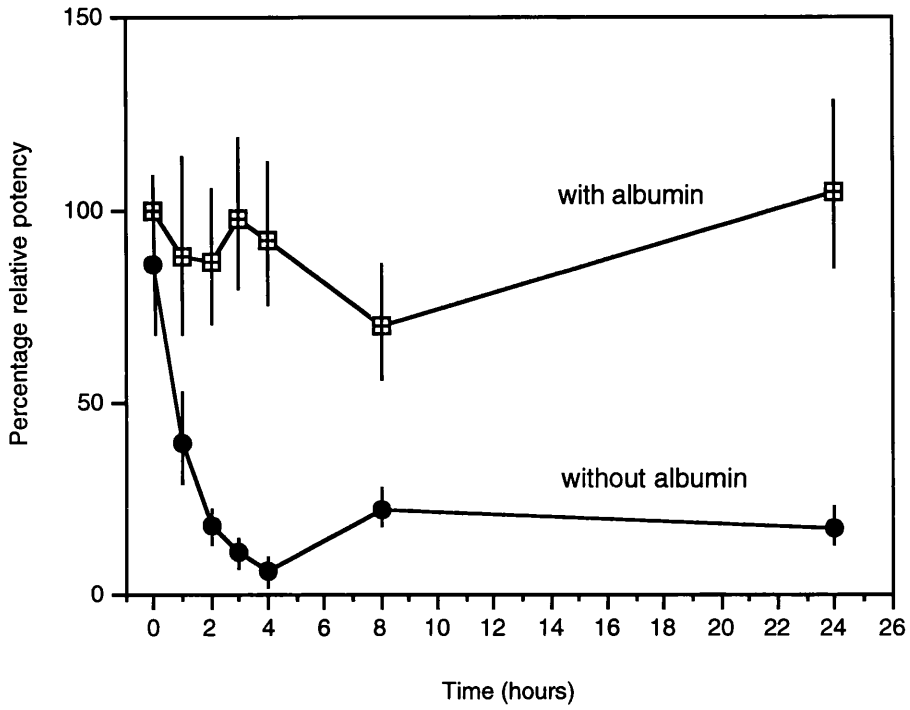


Figure 7:3. Relative potency of rIL-2 when reconstituted with and without albumin (bioassay).



The relative potency of rIL-2 was assessed by weighted iterative analysis and is illustrated in figure 7:3, where the error bars represent the biducial limits of the plotted values. The rIL-2 preparations were shown to be highly biologically active using the CTLL bioassay. Formulation with albumin did not affect biological potency (figures 7:2 and 7:3, T=0). During the infusion period the potency of rIL-2 reconstituted with albumin did not change significantly but the potency of rIL-2 diluted in 5% glucose alone declined markedly. The relative potency of samples diluted with albumin was unchanged over the 24 hour infusion period but by two hours the potency of rIL-2 diluted with 5% glucose alone had fallen to less than 25 % and it remained low in subsequent samples.

Evidence from these studies suggested that the bio-availability of rIL-2 was not consistent over a 24 hour period when using the particular giving set

described and as a consequence, the received dose of rIL-2 would be significantly less than projected. Further confirmatory evidence for this was provided by the following case.

7:3:3. Case report.

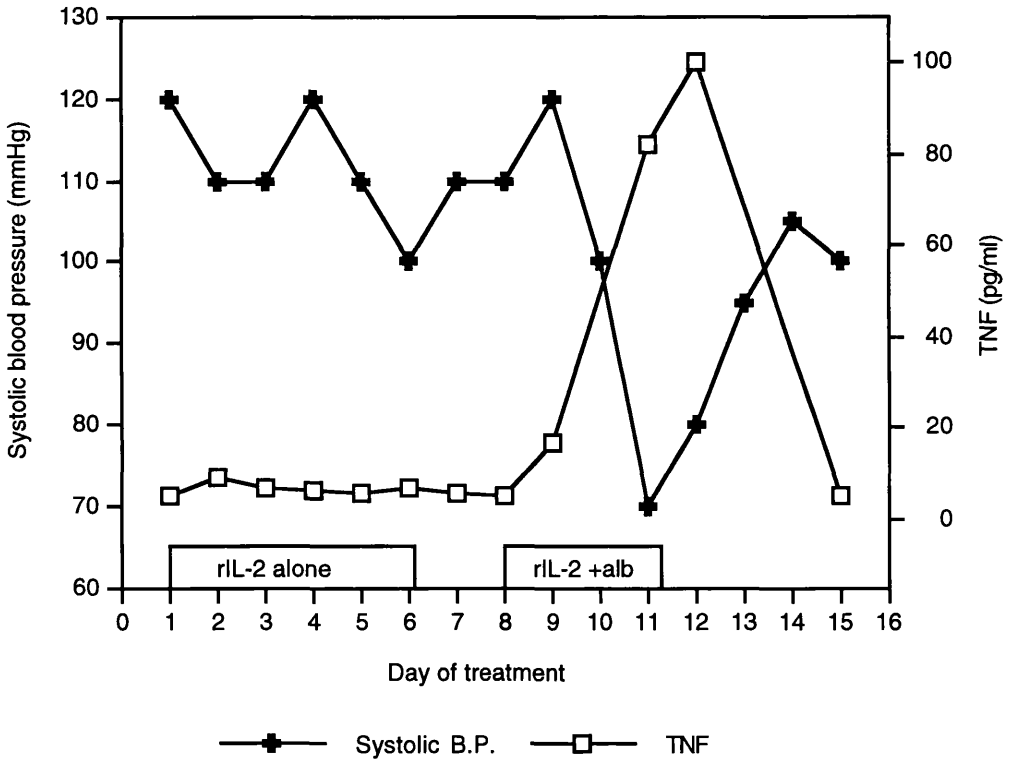
Patient JC was entered into a multi-centre phase II study of dacarbazine and rIL-2 (EuroCetus, protocol no. EC-MP-003). Following insertion of an intravenous Hickman line, the patient was given dacarbazine 250mg/m^2 i.v. on five consecutive days by infusion. She was treated subsequently with rIL-2 $18x10^6$ I.U./m^2/day. During the first five day infusion, rIL-2 was reconstituted with 5% glucose alone according to the manufacturers instructions and administered using a Beckton-Dickenson 50ml syringe and a Vygon lectro-cath giving set.

The patient tolerated the treatment well despite pyrexias of up to 38.5^{0}C. The maximum recorded fall in systolic blood pressure was 20mmHg (figure 7:4) and weight gain of 1 kg was noted.

During the second 5 day infusion, rIL-2 was reconstituted with 5% glucose with 1% human albumin. Within 48 hours of the start of the infusion, systolic blood pressure fell by 50mmHg. Interleukin-2 was discontinued and plasma expanders administered. The blood pressure recovered rapidly and rIL-2 was recommenced at a dose of $9x10^6$I.U./m^2/day. Despite dose reduction, significant hypotension was noted and treatment was stopped. The second 5 day infusion was associated with rigors and weight gain of 6 kg. Levels of TNF, IFN-γ and neopterin were also assessed in this patient. TNF levels remained low during the first infusion period (maximum induced level (9.4 pg/ml, upper limit of normal 6.1pg/ml, figure) and although plasma neopterin

increased from a baseline of 14.8 nmol/l to 32nmol/l (normal range 6±2 nmol/l), levels of IFN-γ remained below the reliable level of detection. After reconstitution with 5% glucose and 1% albumin, plasma TNF levels rose to 100pg/ml (figure 7:4), coinciding with the fall in blood pressure. Neopterin levels rose to higher levels than during the first infusion period (121.4 nmol/l) and IFN-γ levels of 1.04 U/ml were recorded.

Figure 7:4. Changes in systolic biood pressure and TNF levels during infusions of rIL-2 reconstituted with and without aibumin.



7:3:4. Discussion and conclusions.

Having demonstrated a reduction in bioavailability of rIL-2 of approximately two thirds if not reconstituted with albumin *in vitro*, the possible effects *in vivo.* were tested. Following reconstitution of rIL-2 with albumin, toxicity worsened, associated with raised levels of TNF and neopterin. Although the toxicity of

rIL-2 is related to the duration of treatment (Lotze et al 1985), the temporal relationship between the addition of albumin, the induction of TNF and the occurrence of hypotension would support the view that increased bioavailability of rIL-2 was the cause of the increase in toxicity. In addition we subsequently reconstituted rIL-2 with 5% glucose/1% albumin for all patients studied and noted significant rises in TNF and other markers during both infusion periods (see below). The albumin used for reconstitution could contain a small amount of endotoxin and administration of the albumin alone could conceivably lead to induction of TNF and its associated side effects. However, only a 1% solution of albumin was administered in a final volume of 50mls over 24 hours thus the dose of endotoxin would be extremely low and unlikely to cause such profound effects.

The data shown above with regard to the reduced bioavailability of rIL-2 if not reconstituted with albumin was subsequently validated by the National Institute for Biological Standards and Control. The recommendations for reconstitution of rIL-2 provided by EuroCetus were changed accordingly. The actual received dose of rIL-2 in the initial phase I study in patients with metastatic breast cancer is necessarily uncertain in view of these findings and the clinical and immunological results of these patients are discussed with that in mind.

Chapter 8.

RESULTS.

8:1. Patient characteristics.

The characteristics of patients studied are summarised in table 8:1 (overleaf).

8:1:1. Protocol EC-L2041/Guy's protocol C74: Metastatic breast cancer.

Patients on protocol EC-L2041 had received at least one line of endocrine treatment and at least one line of chemotherapy for metastatic breast cancer and most had had two. The five patients on Guy's Hospital protocol C74 were required not to have had chemotherapy for metastatic disease but included one patient (JS) who had received postoperative adjuvant chemotherapy 13 years previously with L-phenyl-alanine mustard. These patients had received more lines of endocrine therapy (median=3, range 1 to 4) compared with the group treated in the EC-L2041 study (median=1, range 0 to 2).

8:1:2. Protocol EC-MP001: metastatic renal cell carcinoma

Of the four patients entered onto the phase II study of rIL-2 in metastatic renal cell carcinoma, one (JT) had undergone nephrectomy 2 years prior to relapse in pulmonary hilar lymph nodes. This patient was then treated with medroxyprogesterone acetate for seven months with no response, prior to entry into this study. The other patients in this group had pulmonary metastases at the time of diagnosis of the primary tumour, which was made by incision biopsy of the associated renal mass.

Table 8:1 Characteristics of patients treated with rIL-2.

Patient	Δ	age	sex	DFi	Prior treatment					Sites of disease
					Surg.	R/T	E/T	C/T	I/T	
Protocol EC-L2041										
VW	BC	66	f	4	Mx	2	2	2	no	breast, nodes, pleura
MC	BC	45	f	5	Mx	0	no	2	no	pulmonary
MB	BC	53	f	4	Mx	0	1	2	no	breast, nodes, skin
ME	BC	47	f	3	e/b	1	1	1	no	breast, nodes
MT	BC	47	f	2	Mx	1	1	2	no	breast, nodes
MP	BC	40	f	3	e/b	2	2	2	no	breast, pleura, skin
SB	BC	45	f	1	e/b	1	1	3	no	skin, liver, pleura
Protocol ECMP001										
SM	RCC	47	m	0	i/b	no	no	no	no	renal, pulmonary
AG	RCC	54	f	0	i/b	no	no	no	no	renal, pulmonary
JT	RCC	52	m	2	neph	no	1	no	no	nodes
LS	RCC	66	f	0	i/b	1	no	no	no	renal, pulmonary
Protocol ECMP003										
JC	MM	49	f	7	e/b+	1	no	no	IFN	nodes
AH	MM	68	f	2	e/b	1	no	no	no	skin, marrow
EL	MM	64	f	2	e/b+	3	no	no	no	nodes, pulmonary
IC	MM	52	f	1	e/b+	no	no	no	no	nodes
PG	MM	48	m	0.5	e/b+	no	no	no	no	skin, nodes
MW	MM	38	m	1	e/b+	no	no	no	no	skin
JS	MM	56	m	0.5	e/b+	no	no	no	no	skin
Guy's protocol C74.										
BA	BC	72	f	7	Mx	no	2	no	no	nodes, skin, pleura
BC	BC	61	f	10	Mx	6	3	no	no	bone
SM	BC	49	f	0	no	1	1	no	no	skin, pulmonary
IP	BC	63	f	9	no	4	4	no	no	skin, bone
JS	BC	69	f	3	Mx	1	3	adj	no	skin, pleura

Δ=diagnosis, **BC**=breast carcinoma, **RCC**=renal cell carcinoma, **MM** malignant melanoma, **DFI** = disease free interval from time of diagnosis of primary tumour to first relapse (years): where DFI = 0, patients presented with metastatic disease. Sex, **m**=male, **f**=female. **Surg**=surgery: **Mx**=mastectomy with regional lymph node dissection, **e/b**=excison biopsy, **e/b+**=excision biopsy with regional lymph node block dissection, **i/b**=incision biopsy, **neph**=nephrectomy. **R/T**=radiotherapy (no. of courses of radiotherapy to different sites shown). **E/T**=endocrine therapy (no. of different types shown). **C/T**=chemotherapy for metastatic disease (no. of types, except **adj**=adjuvant chemotherapy). **I/T**=immunotherapy (**IFN**=interferon-alpha).

8:1:3. Protocol EC-MP003. Metastatic malignant melanoma.

Six of the patients with malignant melanoma had undergone excision biopsy of the primary disease with lymph node dissection of regional nodes prophylactically or for recurrence at intervals (median 6, range 0 to 72 months) following extirpation of primary disease. One patient received prophylactic radiotherapy to regional nodes and two other patients had received radiotherapy to sites of symptomatic bulk disease.

One patient (JC) had received systemic therapy with interferon-α for metastatic disease to which she had not responded. Interferon-α had been discontinued four months prior to entry into this study. Four patients had clinically assessable cutaneous metastases, one patient with additional nodal involvement. Patient JC had multiple mediastinal and para-aortic lymph nodes assessable on CT scan.

8:2. Toxicity.

8:2:1. Escalating dose schedule in metastatic breast cancer (ECL2041).

Patients on this protocol were given rIL-2 on an escalating dose schedule beginning at a dose of $9x10^6$I.U./m^2/day for four days increasing by $3x10^6$I.U./m^2/day increments for a total of four 96 hour infusion periods as described in methods (chapter 7). As noted previously, the received dose of rIL-2 may have been underestimated in this the first rIL-2 study, owing to adherence of cytokine to the giving set. Toxicity in this escalating dose schedule is considered for the first six patients only. The clinical course of patient number 7 (SB) is described separately. The worst toxicities experienced by the first six patients treated on protocol ECL2041 are shown in table 8:2:1.

Table 8:2:1. rIL-2 induced toxicity (maximum grade/patient): escalating schedule in metastatic breast cancer (n=6).

	WHO grade				
Type	0	1	2	3	4
Min. perf. status (ECOG)	2	1	1	2	0
Hypotension	4	2	0	0	0
Fever	0	1	4	1	0
Weight gain	3	3	0	0	0
Gastro (nausea/vomiting)	0	4	2	0	0
-intestinal (diarrhoea)	1	2	3	0	0
Cutaneous	1	4	1	0	0
Hepatic (AST)	4	1	1	0	0
(alk phos)	0	5	1	0	0
Renal (creatinine)	6	0	0	0	0
Haemoglobin	0	5	1	0	0
White cell count	6	0	0	0	0
Platelets	5	1	0	0	0
Cardiac	6	0	0	0	0
Respiratory	6	0	0	0	0

Systolic blood pressure fell in two patients by greater than 20mmHg but less than 30mmHg during the third infusion period. Therapy was not discontinued and no intervention was required. No arrhythmias or respiratory symptoms were noted during treatment. All patients experienced fever during treatment with maximum temperatures being recorded during the first infusion period in two patients. One patient (MC) experiencing rigors which required discontinuation of drug on two occasions during the third infusion period. Weight gain of >5% but <10% was noted in three patients during treatment. All patients experienced some gastro-intestinal toxicity with two patients requiring anti-emetic therapy for transient vomiting. Five patients experienced mild or moderate diarrhoea most commonly during the third infusion period. Five patients complained of an itchy erythematous rash which in one case led to mild desquamation.

Rises in serum transaminases were noted in two patients during the third infusion period (projected dose of $15x10^6$I.U./m^2/day). Increases in alkaline phosphatase were noted in all patients but no significant rises in serum creatinine (above 1.25 times the upper limit of normal) were observed. Haematological toxicity was minimal, with mild anaemia (grade 1, Hb<10.9 but>9.5) being recorded in 5 cases and moderate anaemia (grade 2, Hb<9.4 but>8.0) requiring transfusion in one case. In one patient, platelet count fell below $100x10^9$ on two occasions.

Three of four patients whose assessable disease included breast masses complained of pain in the affected breast during treatment and this was managed with analgesics.

One patient (MC) experienced a two point fall in ECOG performance status, the others experiencing a one point fall. Four patients remained ambulant for at least 50% of waking hours.

Following the observation that patients might be receiving less than the projected dose as a result of adherence of rIL-2 to the giving set, the rIL-2 administered to patient number 7 (SB) on this protocol was reconstituted with albumin. The first two four day cycles were tolerated reasonably well with moderate pyrexias controlled with paracetomol, weight gain of 3kg and rise in creatinine to 133μmol/L by the end of the second infusion. During the third infusion period the patient developed somnolence with a fall in performance status to ECOG 4 and significant peripheral oedema with weight gain of 8kg. Serum creatinine rose to 183μmol/L and the infusion of rIL-2 was stopped. Although serum creatinine fell following discontinuation of rIL-2, the patient developed hypostatic pneumonia from which she died 11 days following discontinuation of rIL-2 despite full supportive care. Following this, protocol ECL2041 was discontinued.

8:2:2. Phase II studies of rIL-2 in metastatic renal cell carcinoma (ECMP001) and DTIC/rIL-2 in metastatic malignant melanoma (ECMP003).

Patients on these studies received rIL-2 at a dose of $18x10^6$I.U./m^2/day for two five day periods separated by a two day rest period. Patients with metastatic malignant melanoma were also given DTIC 250mg/m^2 for five consecutive days, two weeks prior to the start of rIL-2. Antiemetics were given prophylactically with chemotherapy though most patients experienced transient nausea and vomiting. These gastro-intestinal symptoms had resolved by the time rIL-2 was commenced and the toxicities associated with the two five day infusions of rIL-2 in patients with malignant melanoma and

renal cell carcinoma are considered together. The maximum WHO grade of toxicity experienced by patients for the duration of rIL-2 therapy is documented in table 8:2:2.

Table 8:2:2. rIL-2 induced toxicity (maximum grade/patient): phase II studies in metastatic melanoma and renal cell carcinoma (n=11).

	WHO grade				
Type	0	1	2	3	4
Min. perf. status (ECOG)	0	1	2	7	1
Hypotension	5	3	2	1	0
Fever	0	0	11	0	0
Weight gain	9	1	1	0	0
Gastro- (nausea/vomiting)	0	7	4	0	0
intestinal (diarrhoea)	1	6	5	0	0
Cutaneous	1	6	4	0	0
Hepatic (AST)	6	4	1	0	0
(alk phos)	0	5	4	2	0
Renal (creatinine)	5	4	2	0	0
Haemoglobin	4	4	2	1	0
White cell count	10	1	0	0	0
Platelets	9	0	2	0	0
Cardiac	7	3	1	0	0
Respiratory	8	3	0	0	0

Hypotension, with a fall in systolic blood pressure of more than 20mmHg was noted in 6 patients during treatment and in two cases necessitated interruption of therapy. In one case blood pressure fell on the fourth day of the second cycle of rIL-2 but recovered rapidly after discontinuation of therapy which could not be recommenced because of a concomitant increase in plasma creatinine. In the other case blood pressure fell on the third day of the second cycle of treatment but restarting rIL-2 at a reduced dose (50%) led to further hypotension and therapy was discontinued.

Grade 2 pyrexia (temperature >38°C but <40°C) often associated with rigors was noted in all patients. Weight gain (less than 10%) was noted in one patient, and in another weight gain of greater than 10% associated with peripheral oedema was noted. All patients experienced nausea and in four transient vomiting requiring anti-emetic therapy was noted. Diarrhoea was recorded in most cases.

All but one patient experienced an erythematous skin rash and in four cases this was associated with pruritus and desquamation. Sinus tachycardia of greater than 110 was noted in three patients during therapy and patient IC developed atrial flutter with two to one block on day four of the first cycle of rIL-2. Following discontinuation of rIL-2 and digitalisation, the heart beat reverted to sinus rhythm. rIL-2 therapy was not reinstituted in this patient. Patient AG became increasingly somnolent during the second cycle of therapy and became unresponsive on day three of the second cycle. No obvious neurological or metabolic cause was noted and therapy with rIL-2 was stopped and not recommenced. Three patients complained of dyspnoea on minimal exertion during treatment.

Rises in transaminases were noted in 5 patients and in all patients a rise in alkaline phosphatase was recorded, which in two cases reached more than 5 times the upper limit of normal. Serum creatinine rose above 1.25 times the upper limit of normal in 6 cases and in one patient led to the discontinuation of therapy in association with an episode of hypotension on day 4 of the second 5 day cycle. Haematological toxicity was minimal with 3 patients experiencing grade 2 or 3 anaemia requiring transfusion. Platelet counts of <75 but >50 were noted in two patients but did not require intervention or discontinuation of therapy.

Overall, of 10 patients who commenced the second 5 day cycle of treatment with rIL-2, seven were capable of limited self care only and were confined to bed for more than 50% of waking hours. One patient was completely disabled (ECOG=4) being totally confined to bed.

8:2:3. Single 5 day infusion of rIL-2 in patients with metastatic breast cancer before chemotherapy (Protocol no. C74).

Patients treated on this protocol received rIL-2 at a dose of $18x10^6$I.U./m^2/day for five days prior to the start of chemotherapy. The worst toxicities experienced by this group of patients are summarised in table 8:2:3.

Table 8:2:3. rIL-2 induced toxicity (maximum grade/patient): single 5 day infusion in metastatic breast cancer (n=5)

	WHO grade				
Type	0	1	2	3	4
Min. perf. status (ECOG)	0	0	0	5	0
Hypotension	1	4	0	0	0
Fever	0	0	5	0	0
Weight gain	2	2	1	0	0
Gastro- (nausea/vomiting)	0	2	2	0	0
intestinal (diarrhoea)	1	2	2	0	0
Cutaneous	0	2	3	0	0
Hepatic (AST)	4	1	0	0	0
(alk phos)	0	3	2	0	0
Renal (creatinine)	5	0	0	0	0
Haemoglobin	2	2	1	0	0
White cell count	3	0	1	1	0
Platelets	4	0	1	0	0
Cardiac	3	2	0	0	0
Respiratory	4	1	0	0	0

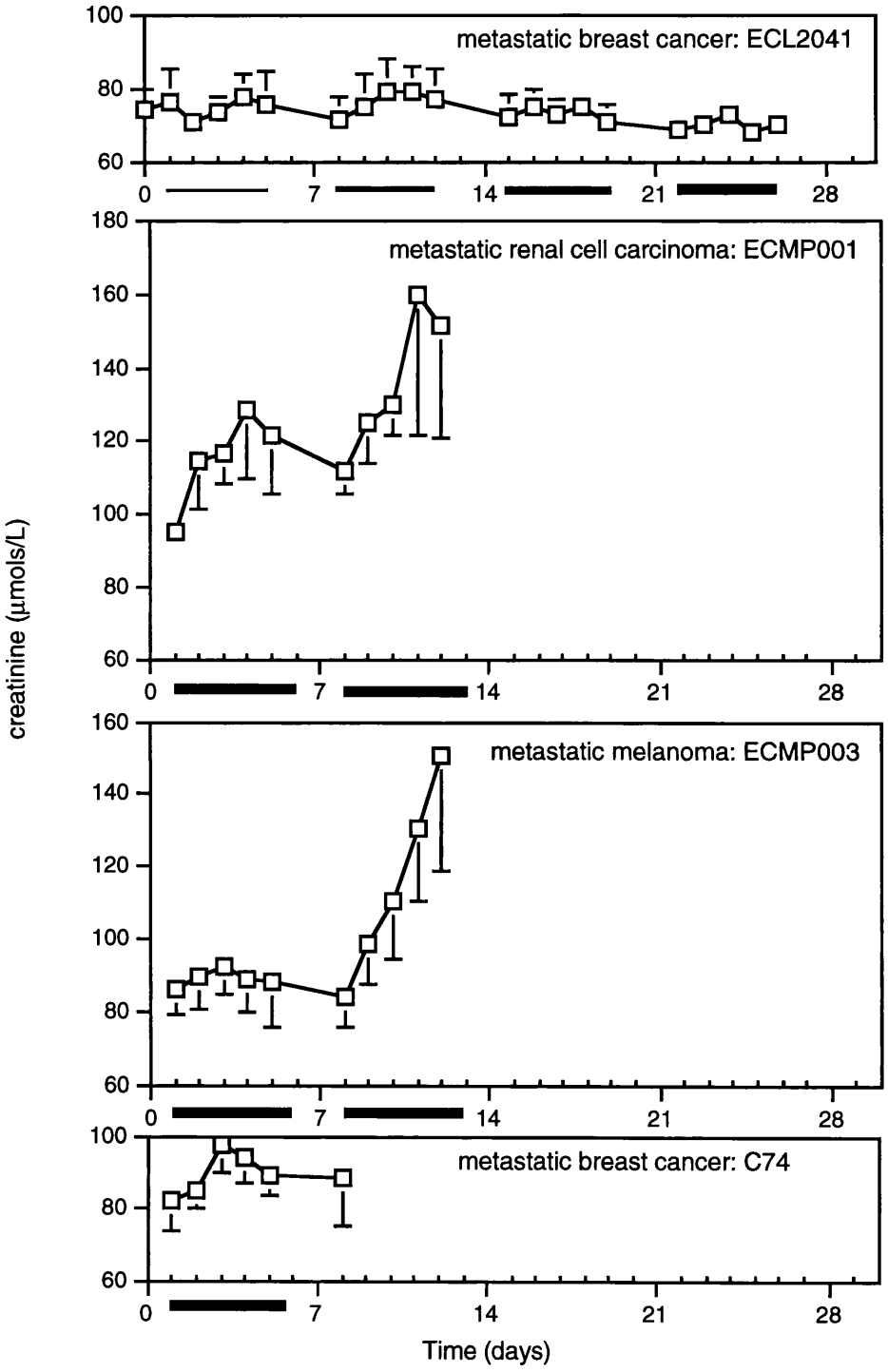
Transient falls in blood pressure of less than 30mmHg were recorded in two patients but rIL-2 was not discontinued. Fever of between 38°C and 40°C was recorded in all patients and in one patient (BC) rIL-2 was discontinued on day

four because of rigors uncontrolled by paracetamol. rIL-2 was not resumed in this patient. Weight gain of between 5 and 15% was recorded in three patients. All patients experienced nausea, three with transient vomiting. Four of five patients reported diarrhoea which in three patients lasted more than two days.

All patients reported an erythematous skin rash which in two patients was associated with pruritus. Rises in alkaline phosphatase were noted more frequently (5/5) than increases in aspartate transaminase (1/5). No significant increases in creatinine were noted. Haematological toxicity was generally mild though in one patient (IP), platelet count fell to $68x10^9/l$ on the final day of treatment. Overall, rIL-2 was associated with a fall in performance status in all patients who were confined to bed during treatment.

The comparative renal toxicity for all patients studied is illustrated in figure 8:2:1. In patients treated on protocol ECL2041, little change was noted in serum creatinine, even at the higher projected dose levels. In the other patient groups. Creatinine rose during the first infusion period but remained within the normal range. During the second infusion period, creatinine levels rose even further and exceeded the upper limit of normal in 6 of 11 cases and in one case necessitated discontinuation of therapy.

Figure 8:2:1. **Changes in serum creatinine levels during infusions of rIL-2.**



8:3. Patient outcome.

All patients on study ECL2041 (rIL-2 in metastatic breast cancer) completed the prescribed course of treatment and although the received dose of rIL-2 was probably less than projected owing to adherence of rIL-2 to the giving sets, patients who completed the prescribed course of treatment were considered evaluable for response. Patient outcome is summarised in table 8:3:1. Two of four patients on protocol ECMP001 and three of seven patients on protocol ECMP003 failed to complete one course of rIL-2 due treatment related toxicity described in section 8:2 and summarised in table 8:3:1.

Of six patients evaluable for response in study ECL2041, all had progressive disease when assessed four weeks following completion of rIL-2. No patients were eligible therefore to continue treatment with rIL-2.

Two patients who completed therapy with rIL-2 on study ECMP001 (metastatic renal cell carcinoma) had stable disease at the time of assessment and went on to have a further course of rIL-2.

Of the four patients evaluable for response on study ECMP003 (metastatic melanoma), three patients developed progressive disease. Patient AH experienced a less than 50% reduction in the sum of the areas of cutaneous lesions and underwent two further courses of DTIC/rIL-2, though maximal response remained stable disease. Although patient EL was not technically evaluable for response having discontinued therapy with rIL-2 on the penultimate day of the second cycle a partial response was noted on serial chest radiographs two weeks following discontinuation of therapy. Patient EL declined further treatment with rIL-2.

Table 8:3:1 rIL-2 studies: patient outcome.

Patient	Δ	no courses rIL-2	comments	response
Protocol EC-L2041				
VW	BC	one	-	PD
MC	BC	one	interrupted x 2 [1]	PD
MB	BC	one	-	PD
ME	BC	one	-	PD
MT	BC	one	-	PD
MP	BC	one	-	PD
SB	BC	-	discontinued [2]	NA
Protocol ECMP001				
SM	RCC	-	discontinued [3]	NA
AG	RCC	-	discontinued [4]	NA
JT	RCC	two	-	SD
LS	RCC	two	-	SD
Protocol ECMP003				
JC	MM	-	discontinued [3]	NA
AH	MM	three	-	SD
EL	MM	-	interrupted/discontinued [5]	NA
IC	MM	-	discontinued [6]	NA
PG	MM	one	-	PD
MW	MM	one	-	PD
JS	MM	one	-	PD
Guy's protocol C74.				
BA	BC	one	-	NA
BC	BC	one	discontinued [1]	NA
SM	BC	one	-	NA
IP	BC	one	-	NA
JS	BC	one	-	NA

1. because of fevers and rigors during third cycle of rIL-2. **2.** due to multiple toxicities.
3. hypotension day 4 of 2nd cycle. **4.** because of CNS toxicity. **5.** due to rise in creatinine.
6 . due to atrial flutter. **PD**= progressive disease, **NA**=not assessable, **SD**=stable disease.

Following the second course, responses were not observed and therapy with rIL-2 was discontinued. Patients entered into the single five day course of rIL-2 were not considered eligible for response.

8:4. Biological Evaluation: Leucocyte counts.

8:4:1. Pre-treatment lymphocyte counts.

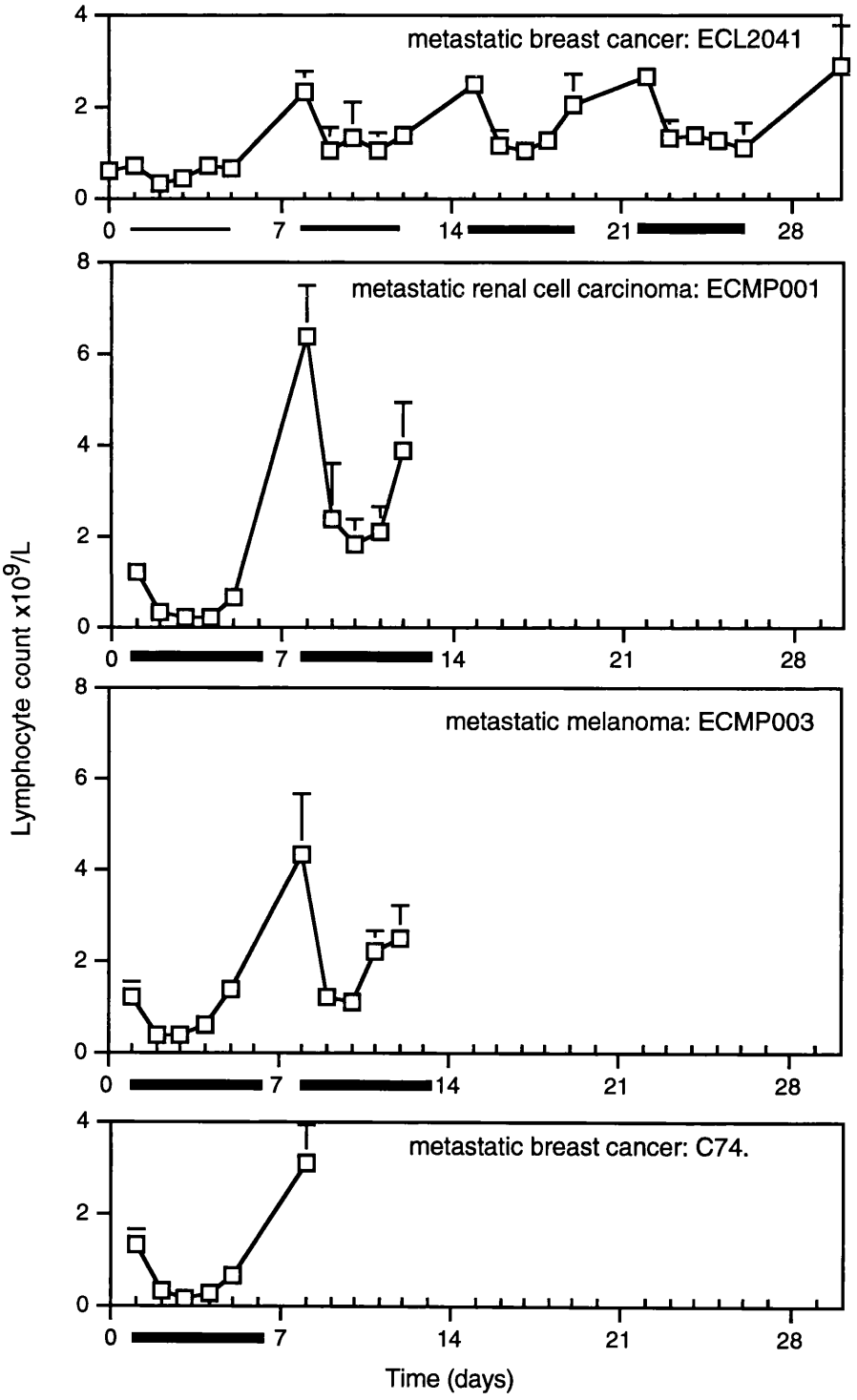
Differential blood counts were performed before and during treatment with rIL-2. Patients with metastatic breast cancer treated on protocol EC-L2041 had a low pre-treatment lymphocyte count compared with patients on the malignant melanoma/renal cell carcinoma protocols (medians= 0.5 and 0.9 respectively $p<0.03$ Mann-Whitney). The median pre-treatment lymphocyte count in patients with breast cancer who had not received chemotherapy for metastatic disease was $1.5x10^9$/L. Patient numbers in the two groups of patients with breast cancer were too small for a valid comparison to be made.

8:4:2. Lymphocyte counts during rIL-2.

Lymphocyte counts during infusion of rIL-2 in the different patient groups are illustrated in figure 8:4:1 (overleaf). Twenty four hours following the start of continuous infusion rIL-2, the peripheral lymphocyte count fell in all the patient groups studied and remained below pre-treatment levels for the duration of the infusion. Before the start of subsequent infusions (48 to 72 hours following cessation of rIL-2) lymphocyte levels were higher compared with values taken on the final day of the rIL-2 infusion in all groups studied. In patients on the escalating dose schedule this lymphocyte rebound did not vary greatly even at the highest projected dose, being a median of 1.7, 1.25, 1.6, and $1.1x10^9$/L following the first, second, third and fourth infusions of rIL-2 respectively. The rebound lymphocytosis was higher in the other groups being median of 2.68, 5.34 and $2.15x10^9$/L in patients treated on the melanoma, renal cell carcinoma and breast cancer protocols respectively. Statistical comparisons between groups are inappropriate because of small patient numbers. It was

not possible to assess the rebound lymphocytosis in patients receiving two five-day infusions owing to discharge home of all but two patients.

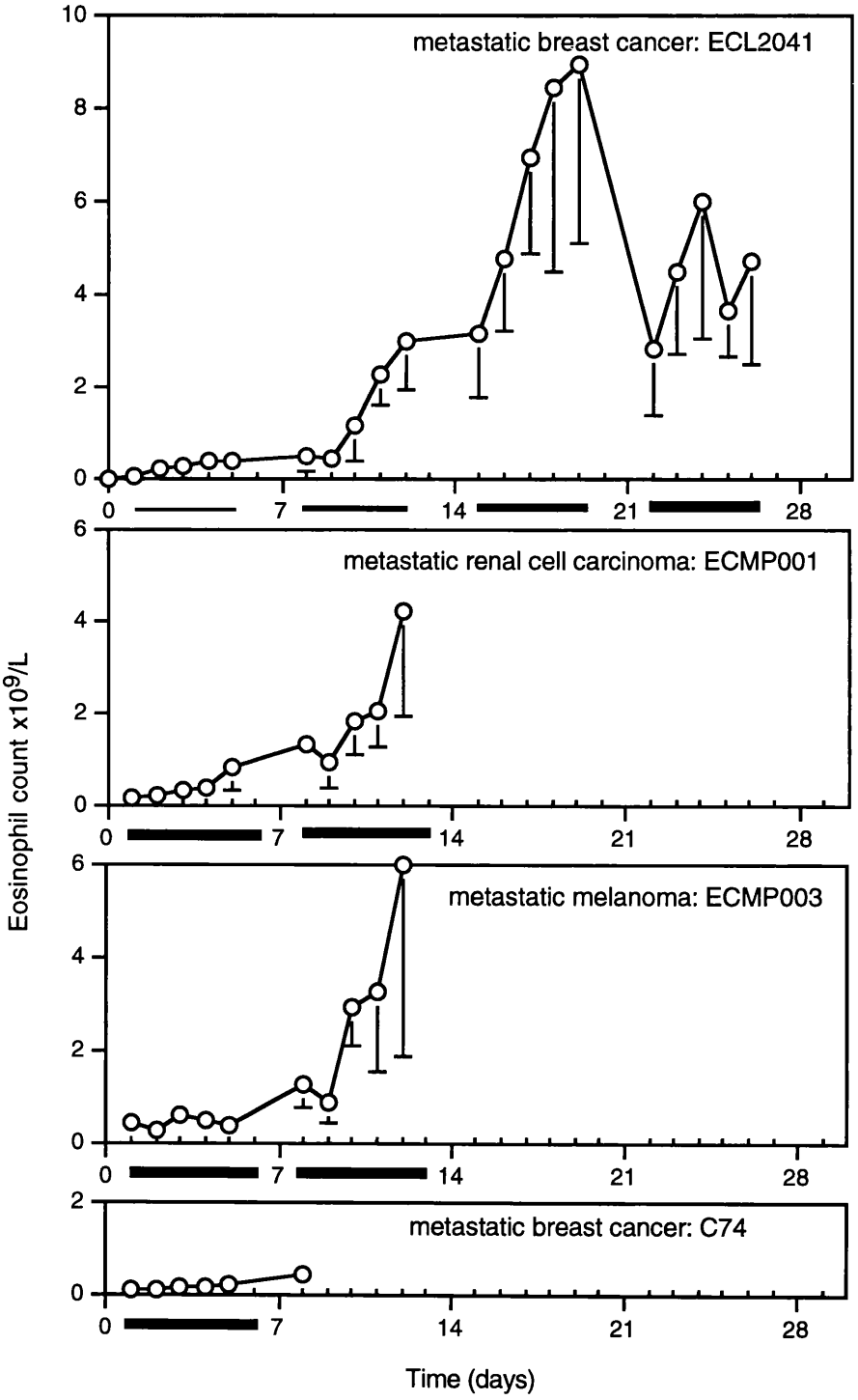
Figure 8:4:1. Changes in lymphocyte counts during infusions of rIL-2.



8:4:3. Eosinophil counts during rIL-2.

Changes in eosinophil counts during rIL-2 are illustrated in figure 8:4:2

Figure 8:4:2. Changes in eosinophil counts during infusions of rIL-2.



Pre-treatment eosinophil counts were low and changed little during the first infusion period in all protocols. During the second infusion period eosinophil counts increased considerably and were similar in all groups. In patients on the escalating dose schedule, eosinophil levels during the second infusion period were similar to those observed in the other patient groups even though the projected dose of rIL-2 was lower. The highest levels of eosinophils were noted during the third infusion period and not at the final dose level.

8:5. Biological Evaluation: Cytokine induction.

8:5:1. TNF-α and TNF soluble receptors.

TNF-α may be important in modulating the anti-tumour effect of rIL-2 but may also mediate some of the toxicities of rIL-2. Proteins formed by proteolytic cleavage of the extracellular domain of the low (p55) and high (p75) molecular weight TNF receptors are capable of binding and inhibiting the biological effects of TNF-α. Since increases in these binding proteins have been demonstrated during treatment with TNF-α, and therapy with rIL-2 is known to induce TNF-α, levels of TNF-α and its soluble receptors were measured during treatment with rIL-2.

TNF-α levels were measured by a commercially available IRMA kit in 22 patients receiving rIL-2 by infusion as part of the protocols described previously. Soluble receptors sTNF-Rp55 and sTNF-Rp75 were also measured by ELISA in a sub-group of 13 patients during therapy.

The mean pre-treatment level of TNF-α in this group of patients was not significantly different from level found in 25 normal controls (8.3±1.5 pg/ml *vs* 7.4±0.6 pg/ml), table 8:5:1. The mean pre-treatment level of sTNF-Rp55 was however significantly higher compared with 53 normal controls (1.6±0.1 ng/ml *vs* 0.7 ng/ml, $p<0.001$). Similarly, the mean pre-treatment level of sTNF-Rp75 in patients was 3.1±1.4 ng/ml compared with 2.1± pg/ml in normal controls ($p<0.001$).

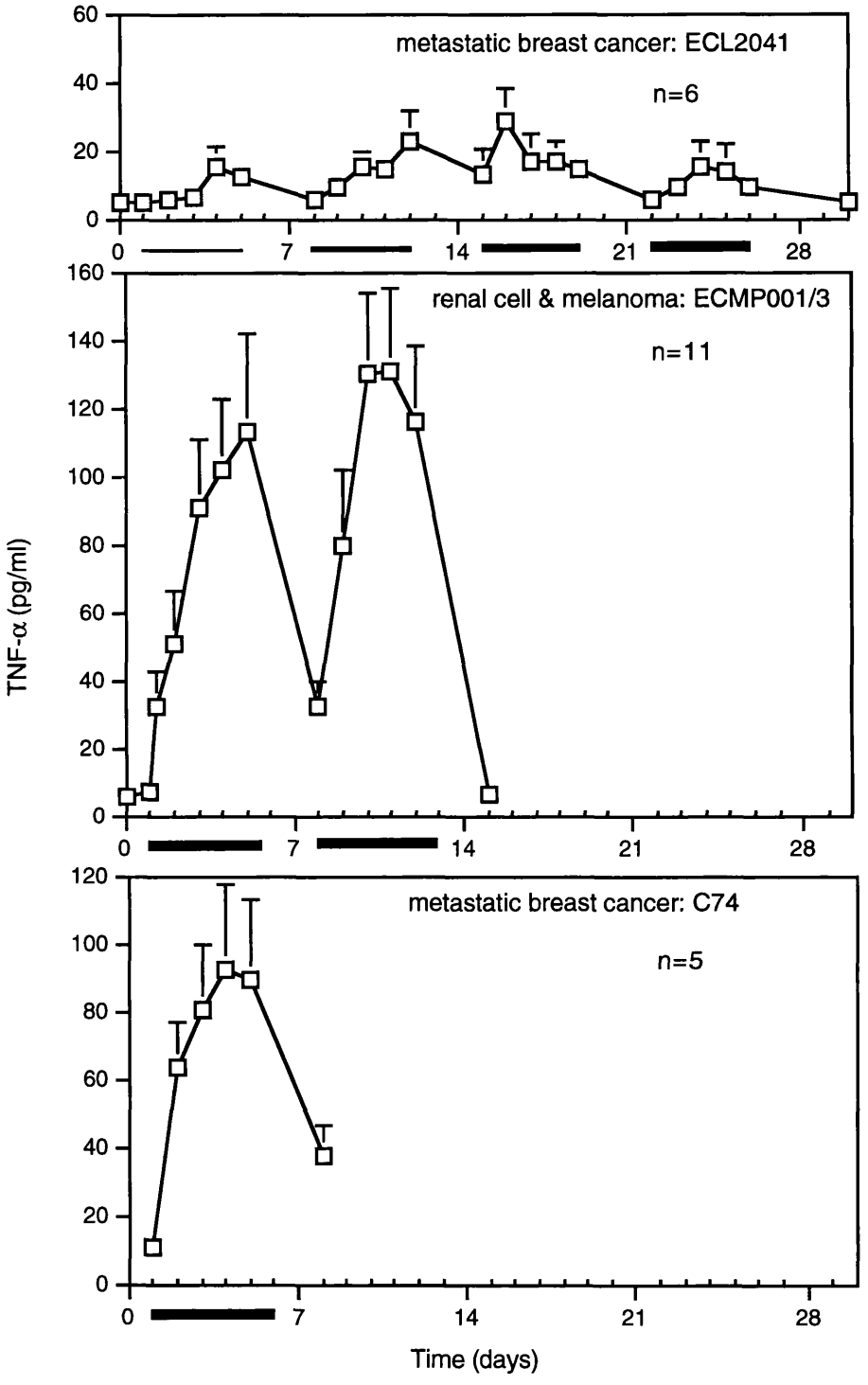
Table 8:5:1. Circulating TNF-α and TNF binding proteins in normals and patients with advanced cancer before rIL-2.

Group	TNF (pg/ml±s.e.)	sTNF-Rp55 (ng/ml±s.e.)	sTNF-Rp75 (ng/ml±s.e.)
Normals	7.4±0.6 *(n=25)*	0.7±0.1 *(n=53)*	2.1±0.1 *(n=53)*
Pre-treatment *(n=22)*	8.3±1.5	1.6±0.2*	3.1±0.3*

* statistically significantly different from normal samples ($p<0.001$, t-test).

The induction of TNF-α was assessed in all patients receiving rIL-2 and TNF levels by patient group are illustrated in figure 8:5:1. In all cases, treatment with rIL-2 led to induction of TNF. Induced levels of TNF rose throughout the period of rIL-2 infusion but levels fell rapidly (assessed 48 to 72 hours) following discontinuation of rIL-2. TNF levels in patients treated on the escalating dose schedule were low compared with the other groups even at the highest projected level of $18x10^6$ I.U./m^2. Indeed the highest levels of TNF-α in this group were recorded during the intermediate dose level $15x10^6$ I.U./m^2 and in two patients peak TNF levels fell with increasing doses of rIL-2. Levels of induced TNF-α during the single five day infusion period in patients with breast cancer were similar to those induced during the first five day infusion period of patients with melanoma and renal cell carcinoma treated with two five day infusion periods. Considering all patients studied, peak TNF-α levels were significantly higher in those patients whose therapy was discontinued due to toxicity compared with those who completed the prescribed course (median 120 pg/ml *vs* 85 pg/ml, $p=0.04$, Mann-Whitney)

Figure 8:5:1. Induction of TNF-α during rIL-2.



In nine patients, TNF and sTNF-Rs were measured six hours following the start of the rIL-2 infusion. In all nine patients, TNF levels were raised at six

hours compared with pre-treatment levels (25.1 pg/ml *vs* 6.8 pg/ml, p=0.005, paired T-test, table 8:5:2).

Table 8:5:2. Circulating TNF-α and TNF binding proteins pretreatment and 6 hours after the start of rIL-2 infusion (n=9).

Group		TNF (pg/ml±s.e.)	sTNF-Rp55 (ng/ml±s.e.)	sTNF-Rp75 (ng/ml±s.e.)
rIL-2	[t=0hrs]	6.8±1.1	0.7±0.1	2.1±0.1
	[t=6hrs]	25.1±5.6*	1.9±0.4	3.7±0.6

* statistically significantly different from pre-treatment samples (p<0.005, paired t-test).

In five of the nine patients, levels of sTNF-Rp55 and sTNF-Rp75 were raised at six hours compared with pre-treatment levels. However, the mean levels for the group as a whole were not significantly higher at this time point.

Figures 8:5:2 to 8:5:4 demonstrate the induction of TNF, sTNF-Rp55 and sTNF-Rp75 in the different patient groups during treatment with rIL-2. In all cases, treatment with rIL-2 led to an increase in levels of both type of soluble TNF receptor. Induced levels of sTNF-Rp75 were higher than sTNF-Rp55. Peak levels of sTNF-R were noted 24 to 48 hours following the peak of induced TNF and although levels of sTNF-R declined after the end of infusion periods the rate of decline tended to be less than that of TNF itself.

As in the case of TNF induction, levels of induced sTNF-R were considerably lower in patients treated on the escalating dose schedule but again, increases in sTNF-R tended to follow the rise in TNF (figure 8:5:2).

Figure 8:5:2. Induction of TNF-α and soluble TNF receptors during infusion of rIL-2 (escalating dose schedule).

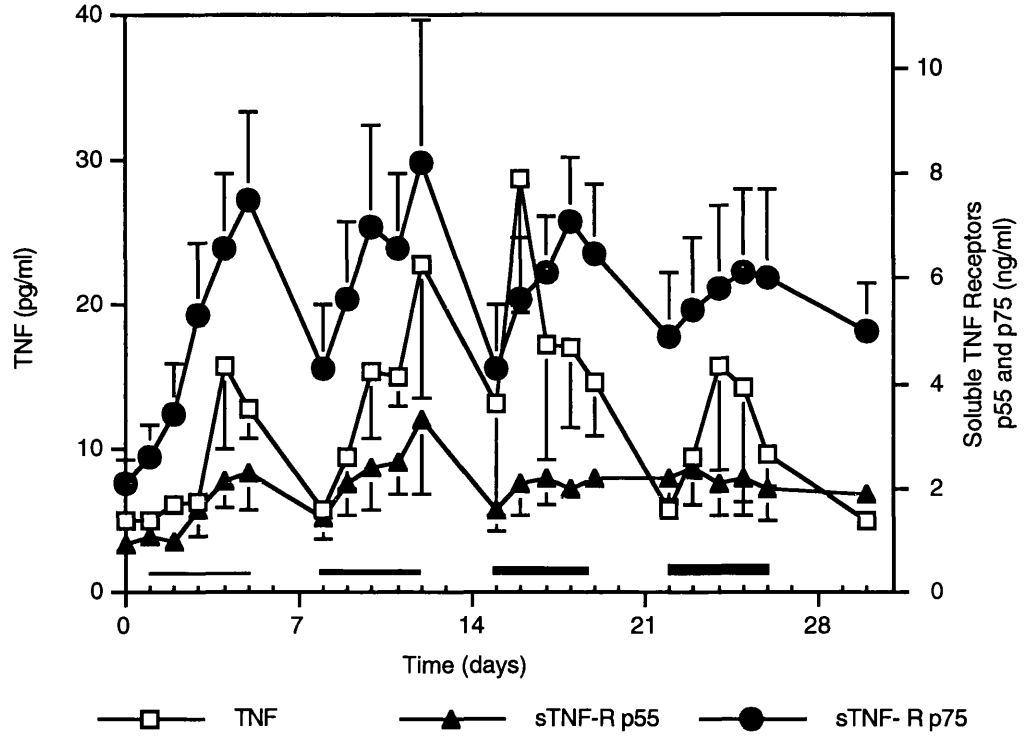


Figure 8:5:3. Induction of TNF-α and soluble TNF receptors during infusions of rIL-2 (two x 5 day infusions).

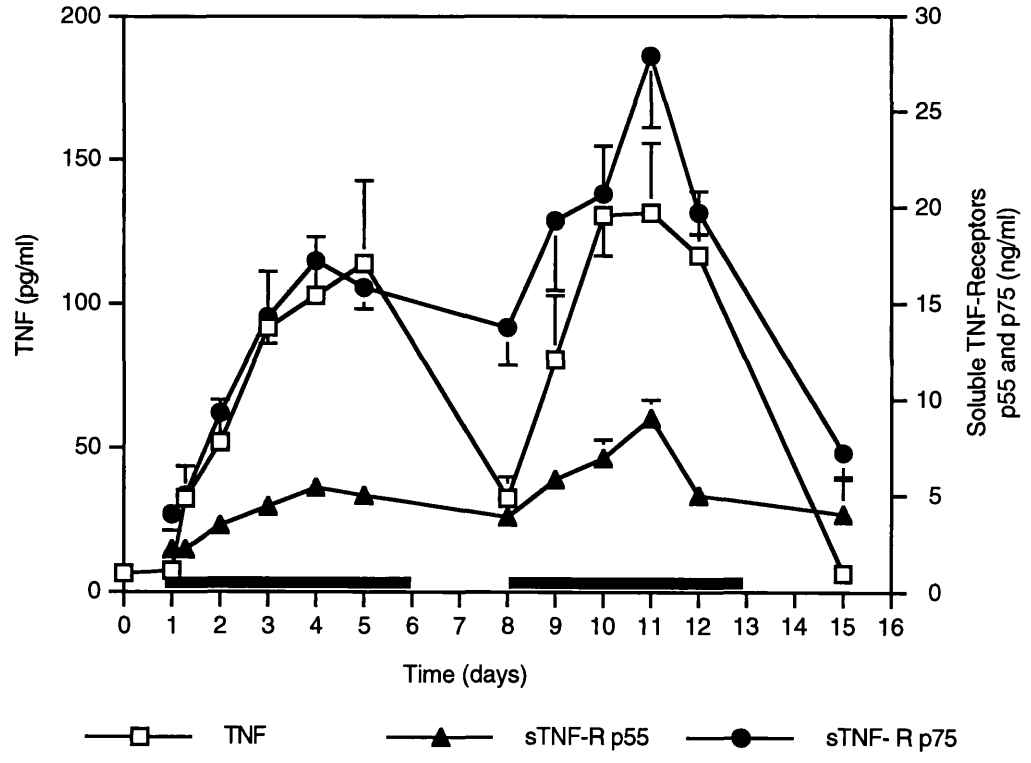
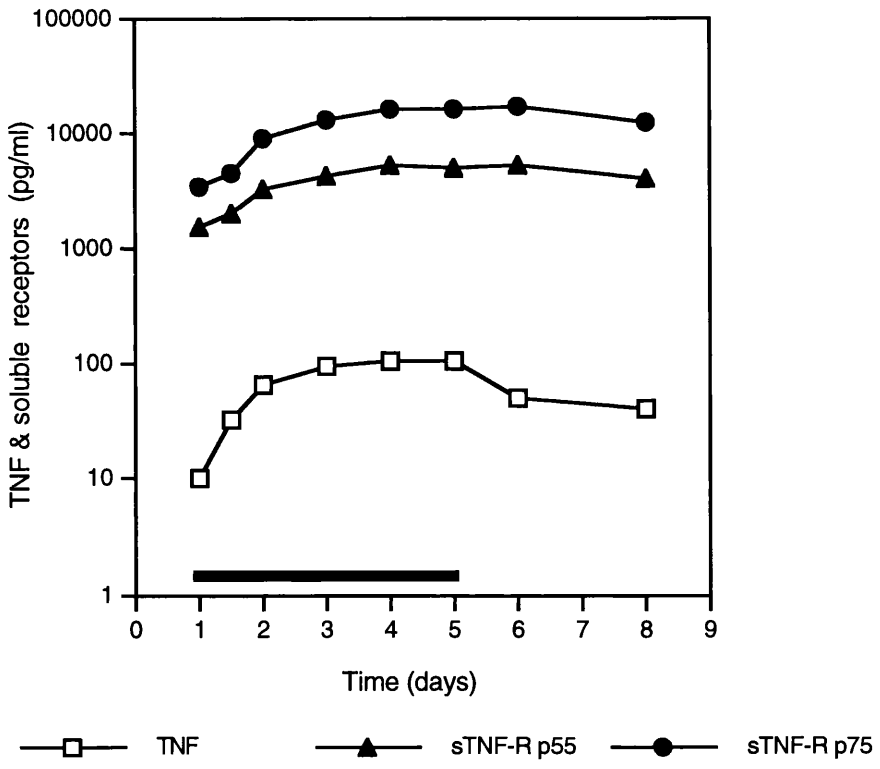


Figure 8:5:4. Induction of TNF-α and soluble TNF receptors during infusions of rIL-2 (single 5 day infusion, log scale).



Levels of induced TNF and sTNF-R for patients treated with the single five day infusion of rIL-2 are illustrated in figure 8:5:4. The data are plotted on a logarithmic scale. This highlights the relative concentrations of cytokine and binding proteins before and during treatment and the fact that levels of TNF, sTNF-Rp55 and sTNF-Rp75 rise concordantly.

Considering the group as a whole, there was a strong correlation between the maximum levels of induced TNF and peak levels of sTNF-Rp55 and sTNF-Rp75 ($R=0.835$, $p<0.001$ and $R=0.785$, $p<0.001$, respectively).

8:5:2. Interferon-γ and markers of its activity.

Interferon-γ may be important in mediating anti-tumour responses to rIL-2 by increasing MHC expression and therefore T-lymphocyte recognition, through effects on monocyte/macrophage cytotoxicity and *via* depletion of tryptophan. Raised levels of interferon-γ have been demonstrated during treatment with bolus rIL-2 but not during infusional therapy. In the following studies IFN-γ levels were measured using a commercially available IRMA kit at a detection limit of 2.5 U/ml plasma. Neopterin is a marker of monocyte/macrophage activation and has been used clinically to define interferon-γ activity. Raised levels of neopterin have been noted previously during therapy with rIL-2 but its measurement in these studies (using a commercially available kit) is in relation to the role of pterins in general as co-factors in the induction of the enzyme NO synthase which may contribute to the toxicity of rIL-2. Tryptophan depletion, induced by the enzyme indoleamine 2,3 dioxygenase has also been implicated in the anti-tumour activity of Interferon-γ but degradation of related compounds such as serotonin may contribute to IFN-γ toxicity. In these studies, tryptophan depletion was assessed and levels of the immediate metabolite kynurenine were measured in plasma. Tryptophan and kynurenine levels were measured using HPLC.

Pre-treatment values of IFN-γ, neopterin and tryptophan are shown in table 8:5:3. Levels of IFN-γ in normal and patients were below the reliable limits of detection. Levels of neopterin were significantly higher in this group of cancer patients compared with normal controls (14.3±3.7 ng/ml *vs* 2.0±0.4 ng/ml, $p<0.001$, Mann-Whitney). There was no significant difference in tryptophan levels between normals and pre-treatment levels in the patient group studied.

Table 8:5:3. Circulating IFN-γ, neopterin and tryptophan ieveis in normals and patients with advanced cancer before rIL-2.

Group	IFN-γ (U/ml±s.e.)	Neopterin (ng/ml±s.e.)	Tryptophan (μM±s.e.)
Normals (n=25)	0.2±0.01	2.0±0.4	38.7±1.04
Pre-treatment (n=22)	0.2±0.01*	14.3±3.7†	34.5±2.8§

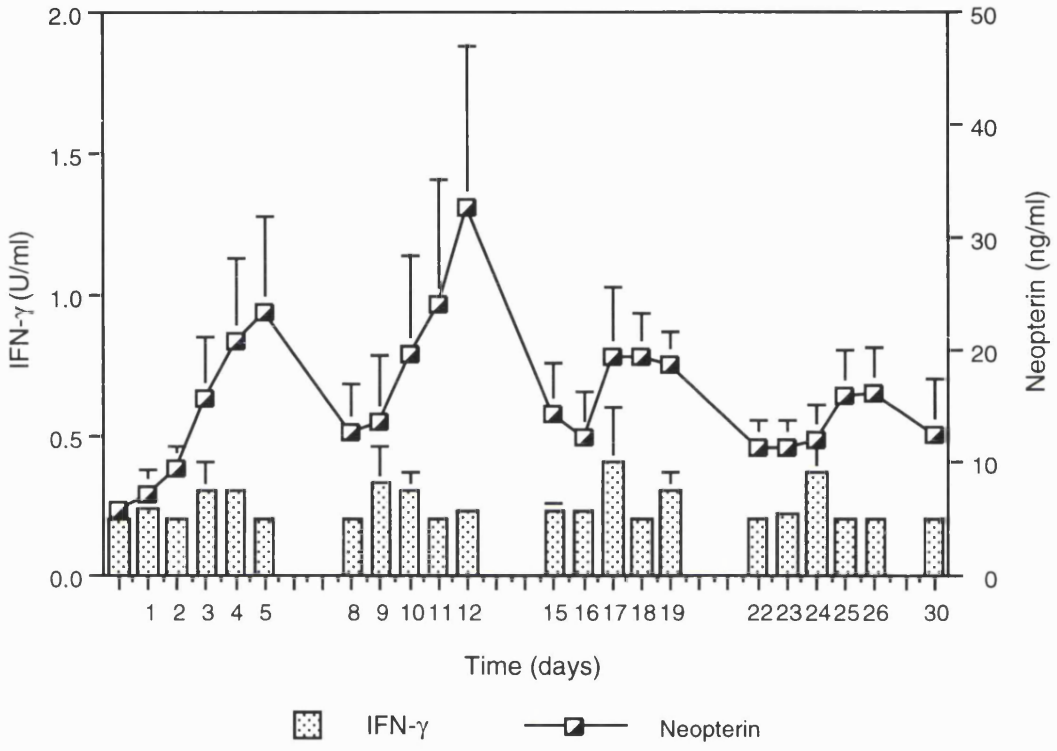
* limit of detection =2.5 U/ml
† significantly different from normal samples (p<0.001 Mann-Whitney).
§ p=not significant

In only two patients did the measured level of IFN-γ exceed the lower limit of detection during treatment with rIL-2. Levels of 3.4 and 5.1 U/ml were recorded both on day three of the first cycle of rIL-2 (18x10 I.U./m^2/day). Although interferon-γ levels are illustrated in the following figures, mean levels remained below the reliable limit of detection. Consistent patterns of change were noted in different patient groups.

Increased neopterin levels were noted in all cases following infusion of rIL-2. In nine patients receiving 18x10^6 I.U./m^2 levels of neopterin did not change significantly following 6 hours of continuous infusion of rIL-2 (23.3±8.2 *vs* 23±7.3 ng/ml, p=0.8) but had done so after 24 hours of rIL-2 (33.2± 8.3 *vs* 23±7.3 , p<0.03). Thereafter neopterin levels continued to rise throughout the course of the rIL-2 infusion reaching maximal values on the final day of infusion. Changes in IFN-γ and neopterin levels for the different patient groups treated with rIL-2 are illustrated in figures 8:5:5,8:5:6 and 8:5:7. Considering all patients maximum levels of induced neopterin were higher in patients whose therapy was discontinued because of toxicity compared with those who received the projected course of therapy though this difference failed to reach

standard levels of significance (median 121 *vs* 70 ng/ml, p=0.06, Mann-Whitney).

Figure 8:5:5. Changes in Interferon-γ and neopterin during infusions of rIL-2 (escalating dose schedule).



Considering patients in the escalating dose schedule (figure 8:5:5), measured levels of IFN-γ varied little throughout treatment and in no case did they exceed the reliable limit of detection. Increased levels of neopterin were noted in all cases but levels were consistently lower in patients treated on this schedule even at the highest projected dose levels compared with other patient groups. There was no consistent relationship between neopterin levels and projected dose administered, indeed the highest levels of neopterin were recorded at the intermediate dose of 12×10^6 I.U./m^2/day. Changes in IFN-γ and neopterin in patients receiving rIL-2 at a dose of 18×10^6I.U./m^2/day for two and one five day infusions are illustrated in figures 8:5:6 and 8:5:7 overleaf.

Figure 8:5:6. Changes in Interferon-γ and neopterin during infusions of rIL-2 (two x 5 day infusions).

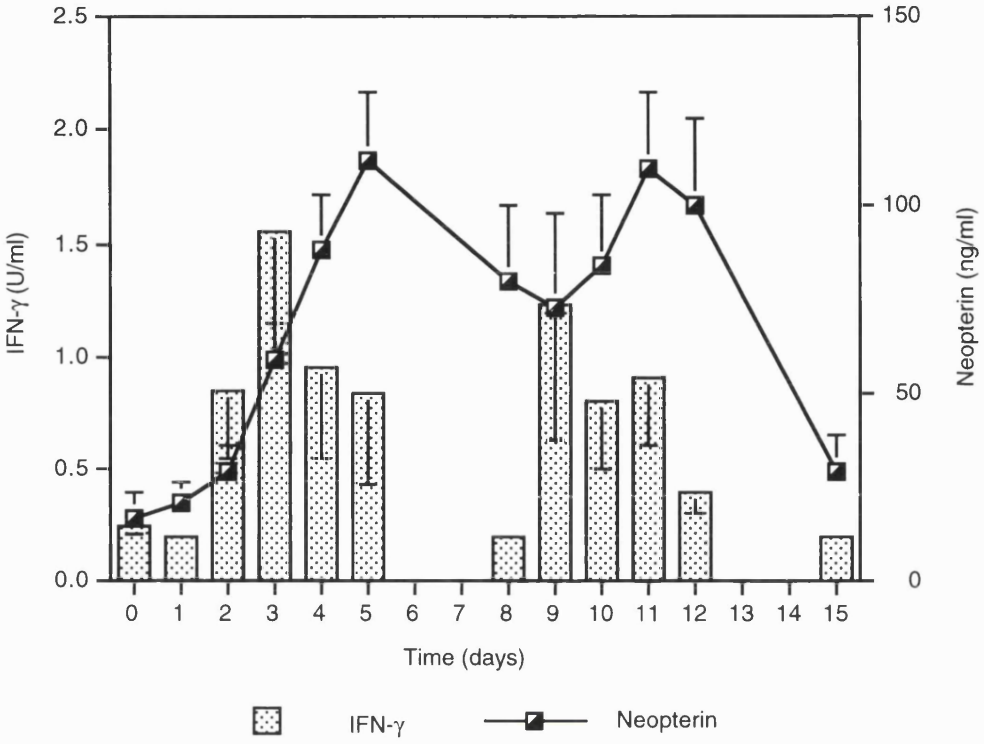
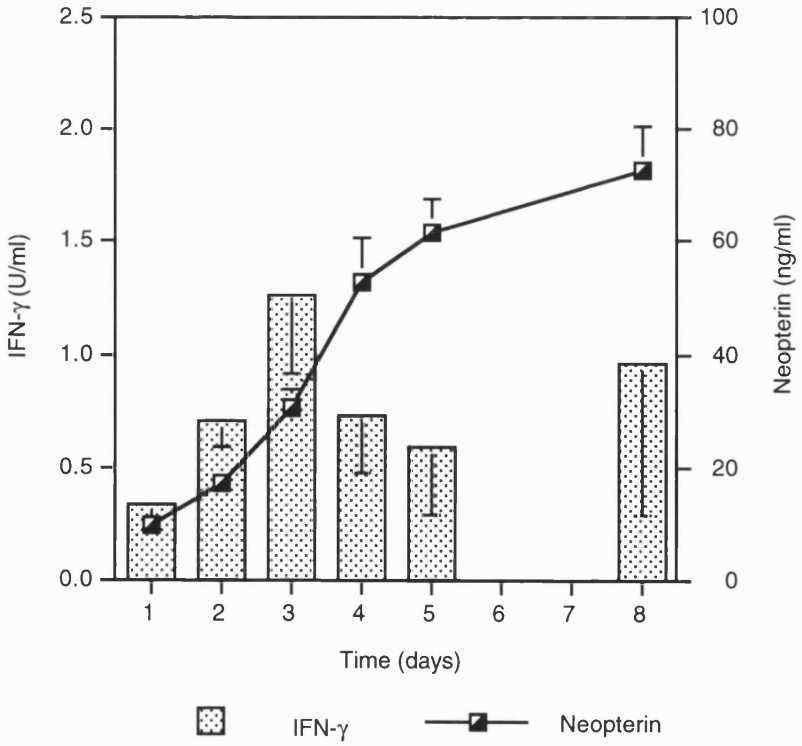


Figure 8:5:7. Changes in Interferon-γ and neopterin during infusion of rIL-2 (single 5 day infusion).



The levels of induced IFN-γ exceeded 2.5U/ml in two patients on day three of the first five day infusion of rIL-2. The levels in the other patients treated with two five day schedules as well as all the breast cancer patients treated with the single 5 day infusion remained below 2.5U/ml but in both these groups the pattern of change of IFN-γ was similar with maximum levels being noted on day three of the first infusion.

When considering patients who received rIL-2 at a dose of $18x10^6$ I.U./m^2/day, plasma neopterin levels were significantly higher 24 hours following the start of rIL-2 and continued to rise during the infusion reaching maximum values towards the end of the infusion periods. Levels of neopterin remained quite high following cessation of therapy. Levels again rose during the second infusion period again reaching a maximum towards the end of the infusion period.

Data for tryptophan and kynurenine six hours following the start of rIL-2 were not available but a significant decrease in plasma tryptophan was noted 24 hours following the start of infusion at the $18x10^6$ I.U./m^2/day dose level (24.6±4.3 *vs* 35±4μM p=0.03) with a reciprocal increase in plasma kynurenine (2.65±0.3 *vs* 2.2±0.33μM, p=0.002). Subsequent changes in tryptophan and kynurenine levels during infusions of rIL-2 for the different patient groups are illustrated in figures 8:5:8. 8:5:9, and 8:5:10. As with neopterin the most consistent reductions in tryptophan in those patients treated on the escalating dose schedule were seen at the intermediate projected dose levels of rIL-2 (fig.8:5:8). Considering patients receiving $18x10^6$I.U./m^2/day, a significant fall in tryptophan was noted with minimum levels reached on day four of the first five day infusion period (fig. 8:5:9 and 8:5:10). This was associated with a reciprocal rise in plasma kynurenine.

Figure 8:5:8. Changes in Interferon-γ, tryptophan and kynurenine during infusions of rIL-2 (escalating dose schedule).

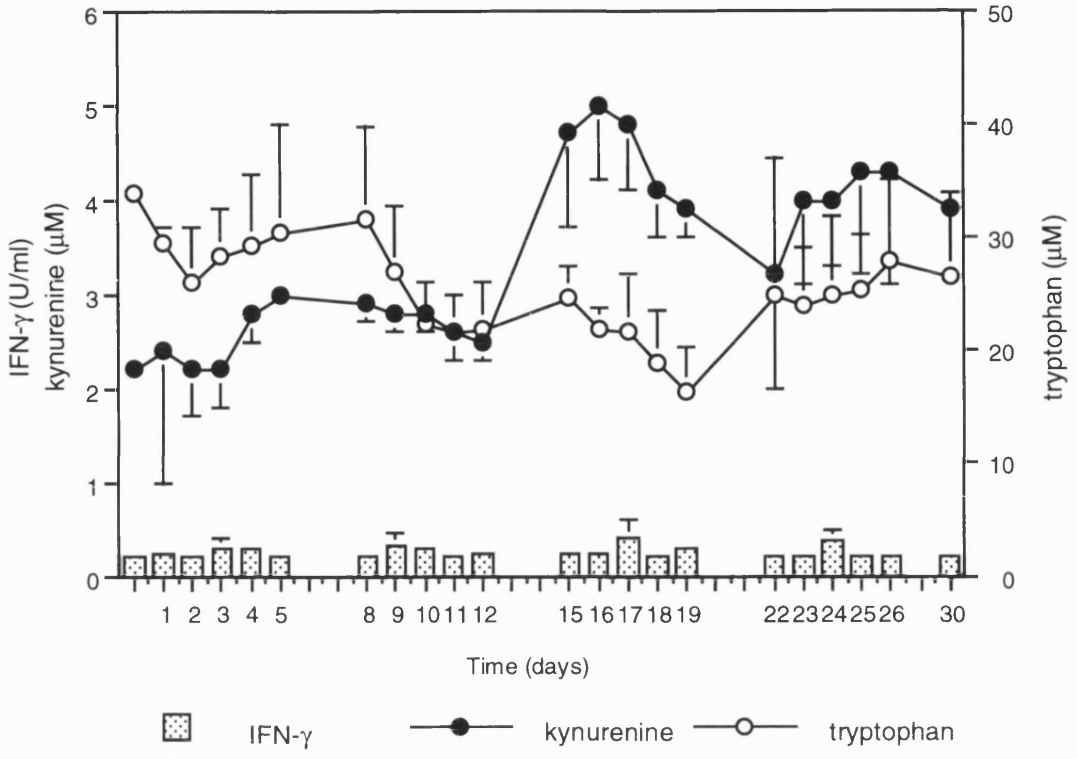


Figure 8:5:9. Changes in Interferon-γ, tryptophan and kynurenine during infusions of rIL-2 (two x 5 day infusions).

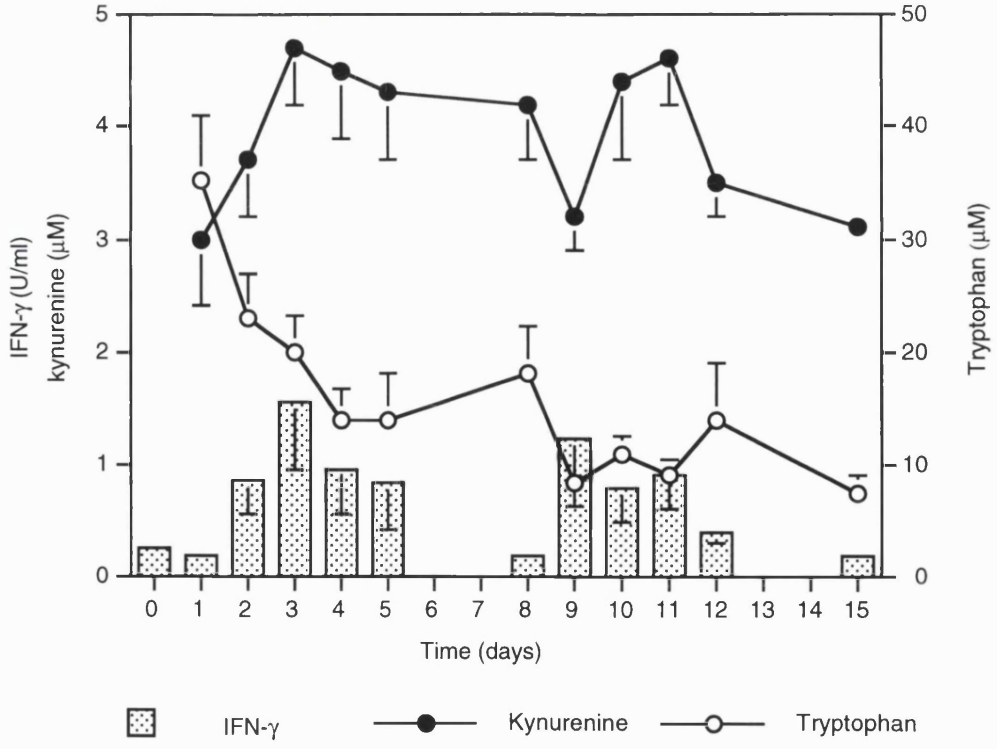
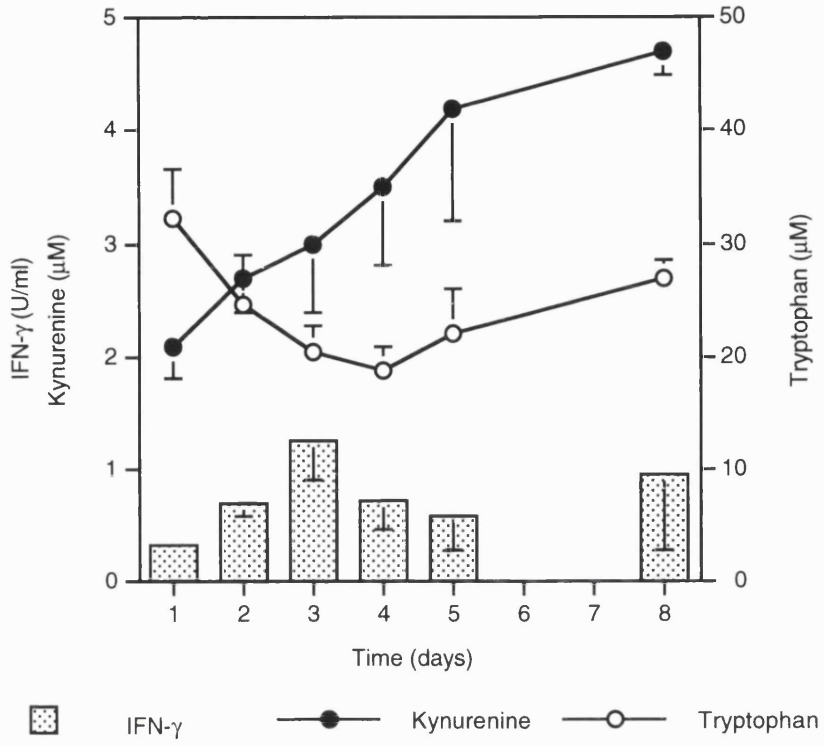


Figure 8:5:10. Changes in Interferon-γ, tryptophan and kynurenine during infusions of rIL-2 (single 5 day infusion).



Following the first five day infusion period, tryptophan levels had increased only slightly with little change in plasma kynurenine. During the second five day infusion period (fig.8:5:9) tryptophan levels fell significantly after 24 hours of rIL-2 (17.6±4.3 *vs* 8.4±1.2μM, p=0.05) but changed little after 24 hours of rIL-2. Kynurenine levels fell initially following 24 hours of rIL-2 during the second infusion period (4.2±0.5 *vs* 3.2±0.2μM, p<0.03) but increased significantly over the subsequent 24 hour period (day 9 to 10, 3.16±0.2 *vs* 4.42±0.6μM, p<0.03).

Considering all groups treated there was a significant correlation between the fall in plasma tryptophan and the rise in plasma kynurenine (r=0.6, p<0.04).

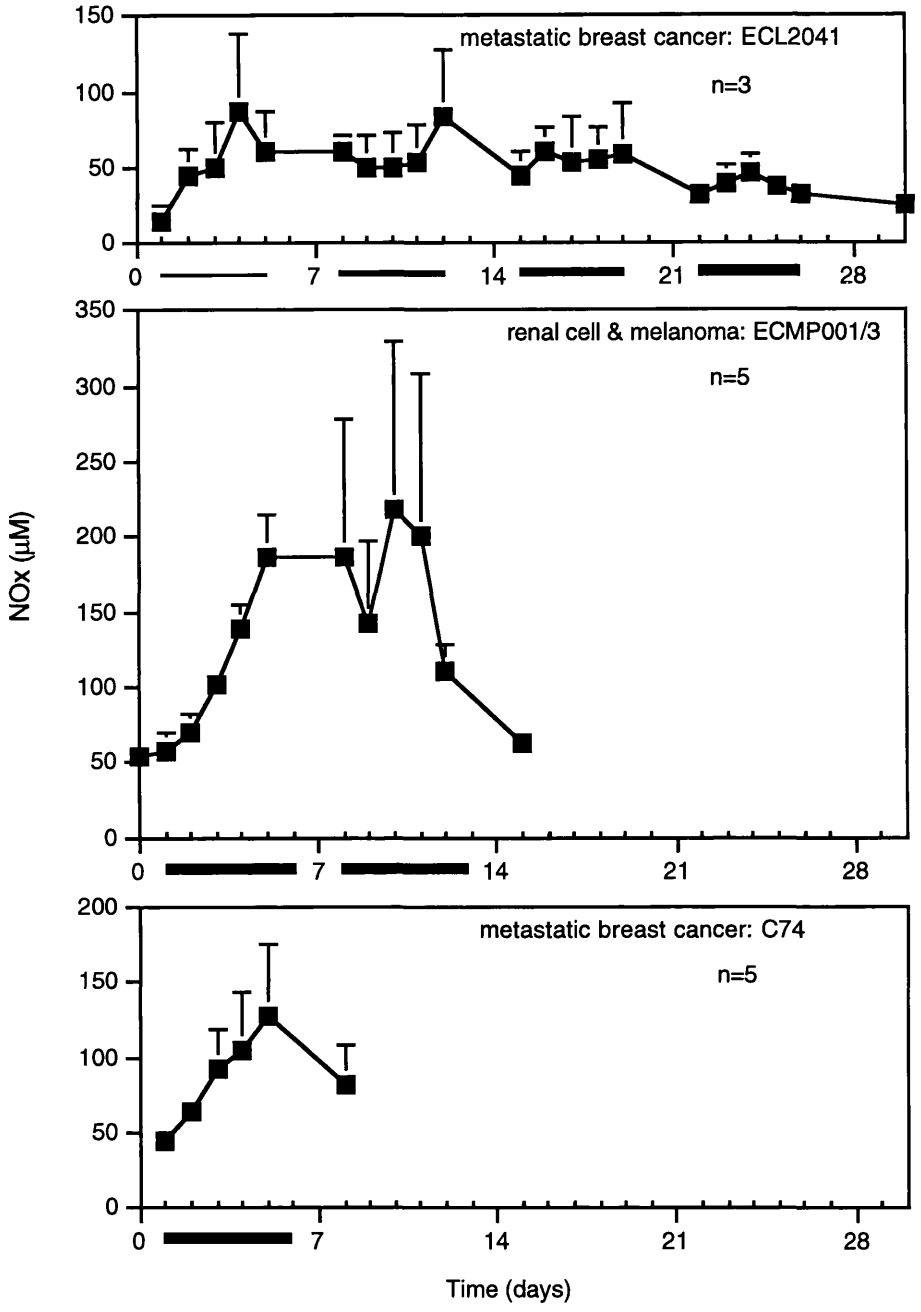
8:6. Biological evaluation: Induction of nitric oxide.

Nitric oxide (NO) activity was assessed in a sub-group of 13 patients receiving rIL-2 by measurement of plasma nitrate and nitrite (NOx) which reflect the presence of NO.

Pre-treatment plasma NOx levels were higher in patients with advanced cancer compared with normal controls (45±6 *vs* 28±2 μM, $p<0.005$ Mann-Whitney). Changes in plasma NOx by treatment protocol are illustrated in figure 8:6:1. NOx increased during the first infusion period but as with other parameters measured, induced levels during subsequent courses were not related to apparent dose, the maximum levels in the three patients being lower at the $18x10^6$ I.U./m^2 dose than during earlier infusions.

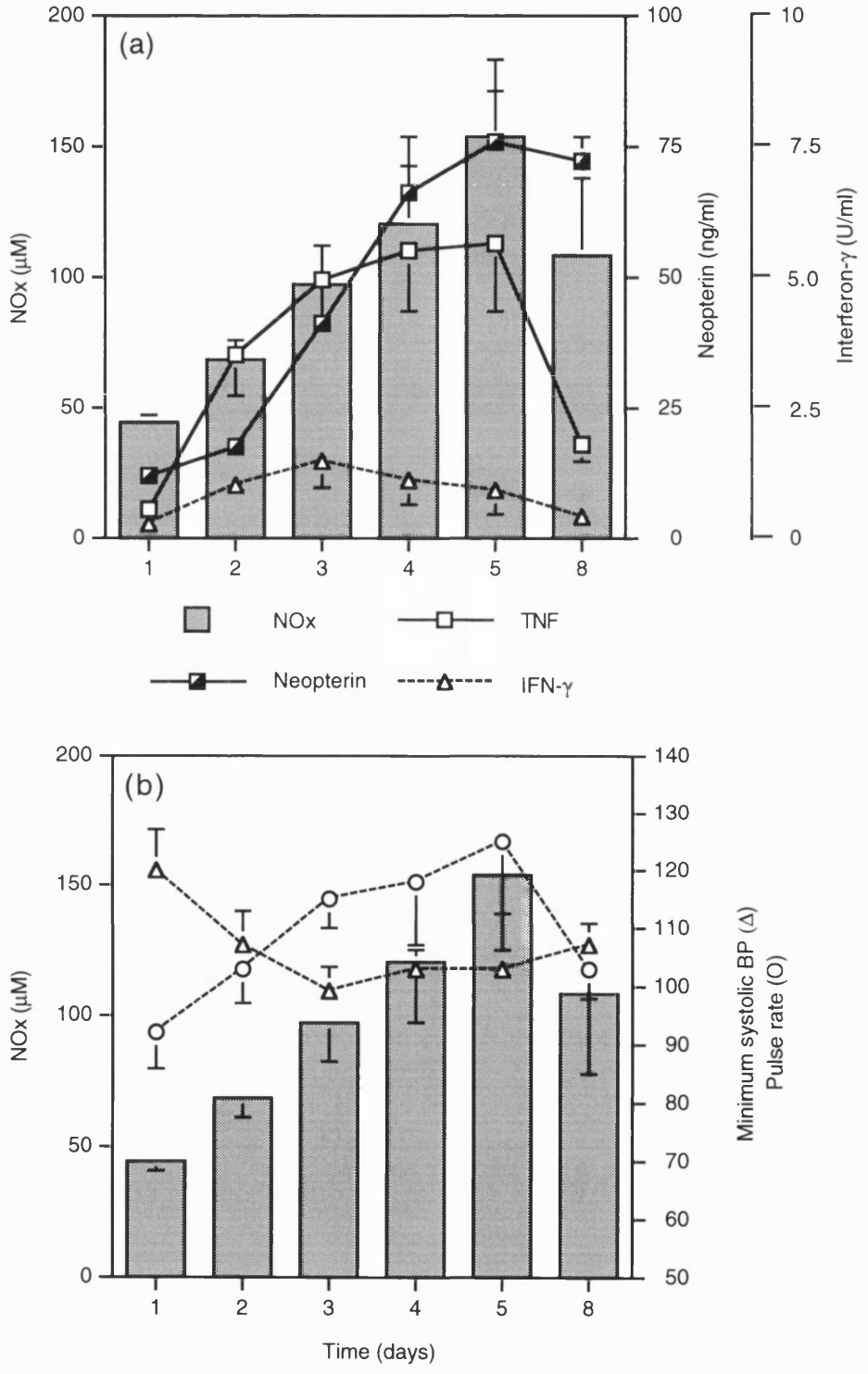
Patients treated on the single and two five day schedules of rIL-2 developed much higher levels of NOx (figure 8:6:1). Levels of NOx continued to rise throughout the infusion of rIL-2 and fell following discontinuation of therapy.

Figure 8:6:1. Changes in plasma NOx during infusions of rIL-2



Data for the 5 patients treated with a single 5 day infusion of rIL-2 were pooled with data from the first 5 day infusion of patients with malignant melanoma and renal cell carcinoma. Changes in induced cytokines, NOx and cardiovascular changes are illustrated in figure 8:6:2 (overleaf).

Figure 8:6:2. (a) Changes in plasma NOx, TNF-α, IFN-γ and neopterin during rIL-2 (b) changes in plasma NOx systolic blood pressure and pulse during rIL-2.



Over the five day infusion period, TNF-α, IFN-γ, neopterin and NOx increased significantly above pre-treatment values. Though levels of induced IFN-γ remained relatively low, the pattern of change suggested that maximum levels were reached at day 3. TNF-α levels peaked at day 4 and neopterin and NOx levels continued to rise reaching maximal values at the end of the infusion. Overall the maximum induced concentrations of NOx correlated with the maximum induced TNF-α ($r=0.6$, $p<0.04$), IFN-γ ($r=0.63$, $p<0.02$) and neopterin ($r=0.66$, $p<0.01$).

The mean minimum systolic blood pressure pulse rate and plasma NOx for these 10 patients are shown in figure 8:6:2(b). As plasma NOx concentrations increased, systolic blood pressure fell reaching a minimum at day 3, despite a continued rise in NOx concentrations. These changes were accompanied by a continuous increase in pulse rate throughout the infusion period. Although the highest level of NOx was noted in the patient in whom the largest fall in systolic blood pressure was recorded there was no overall correlation between maximum induced levels of NOx and change in systolic blood pressure ($r=0.53$, $p=0.14$).

8:7. Changes in leucocyte subsets and adhesion molecules in normal tissue and tumour following rIL-2.

Although cytokines induced during therapy with rIL-2 may have intrinsic anti-tumour activity the cellular response induced by rIL-2 is dependent upon the generation of effector cells and their trafficking to sites of tumour. The adherence to, and efflux through tumour vascular endothelium of effector cells, whether cytotoxic T-cells, activated NK cells or macrophages, depends upon the induction of adhesion molecules. TNF-α and IFN-γ are two of the cytokines responsible for induction of the relevant adhesion molecules on leucocytes and vascular endothelium and their presence in plasma of patients receiving rIL-2 has been described in the previous section. The toxicities associated with rIL-2 therapy, in particular the capillary leak syndrome and the frequent incidence of skin rash, suggest that these induced circulating cytokines may lead to widespread endothelial activation. Such changes in vascular endothelium may then promote the margination of leucocyte subsets and lead to the observed fall in peripheral lymphocyte counts observed following the start of therapy with rIL-2.

Changes in leucocyte subsets and the adhesion molecules associated with their adherence to endothelium was studied in biopsies of uninvolved skin and subcutaneous metastatic tumour deposits in patients with metastatic breast cancer before and at the end of a 5 day infusion of rIL-2 at a dose of $18x10^6$I.U./m^2/day (protocol C74). Following application of antibodies to frozen tissue, sections were stained and developed using the APAAP technique.

8:7:1. Changes in leucocyte subsets following rIL-2.

Changes in the number of T-lymphocytes (CD3 positive) in skin following rIL-2 are illustrated in figure 8:7:1.

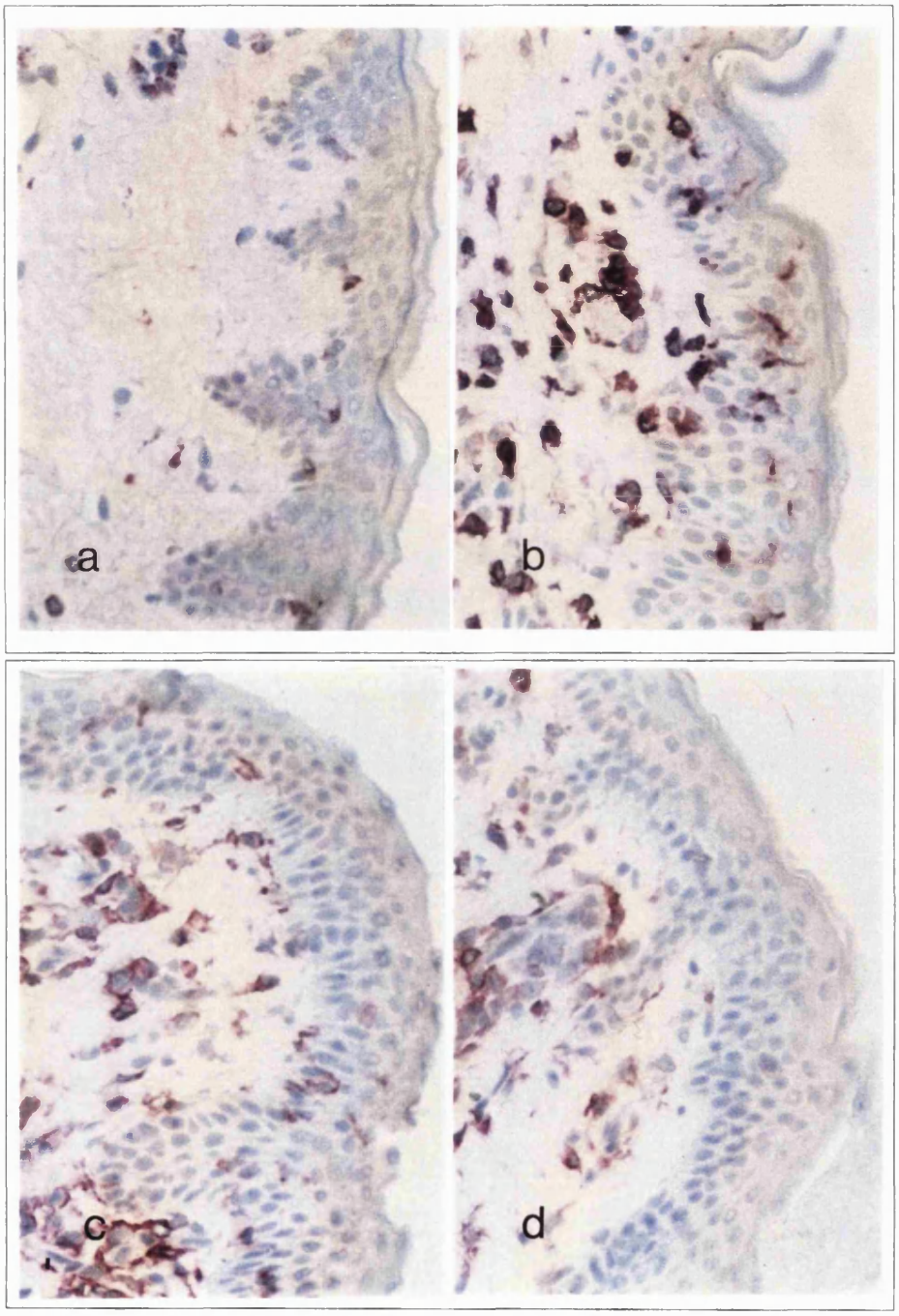


Figure 8:7:1. T-lymphocytes (CD3+ve, defined by antibody UCHT1) in normal skin before (a) and at the end (b) of rIL-2 infusion Following rIL-2, many cells also expressed the activation antigen CD25 [IL-2R] (c) and were CD45RO positive [memory T-cells] (d) x200.

A similar increase in the number of T-lymphocytes was noted in skin in the three cases studied with averages of 49, 57, and 61 CD3 positive cells per high power field following rIL-2. Most of the T-lymphocytes present were of the memory type (CD45RO positive) and many expressed the IL-2 receptor (CD25/Tac).

Similar increases in cells of the monocyte/macrophage lineage, defined by the antibody EBM/11 were observed in skin following rIL-2 (figure 8:7:2) with an average of 56, 44 and 68 CD68 positive cells per high power field following rIL-2 infusion in the cases studied.

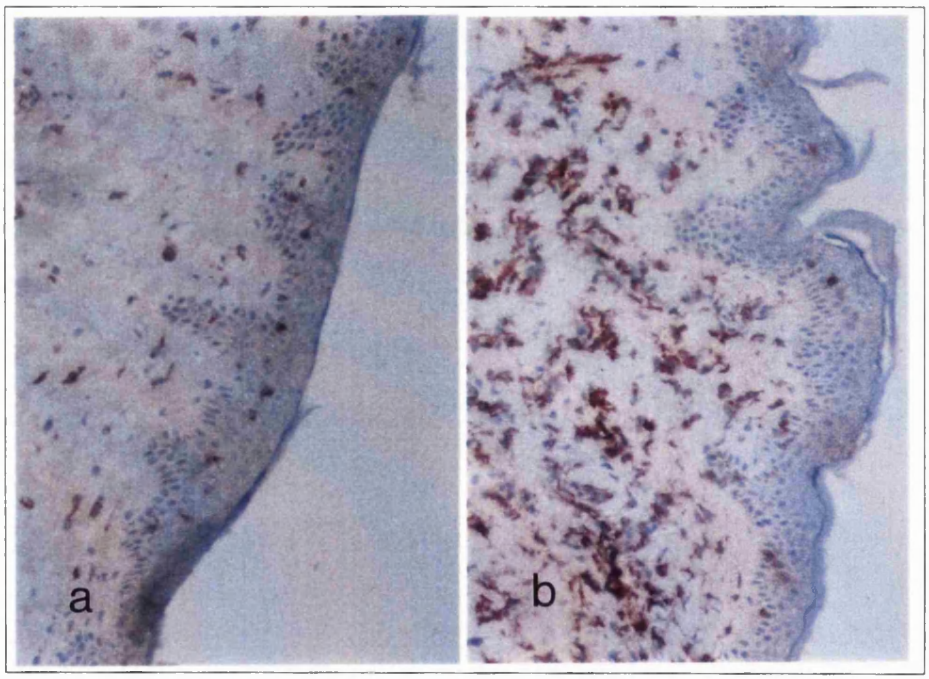


Figure 8:7:2. Cells of the monocyte/macrophage lineage (CD68, defined by antibody EBM/11) in normal skin before (a) and at the end (b) of rIL-2 infusion x100.

Increases in the numbers of neutrophils (defined by antibodies to CD67 and NP57) in the dermis were also noted following rIL-2 (figure 8:7:3) though the numbers observed were less than the T-lymphocyte or macrophage populations with an average of 18, 5 and 14 NP 57 positive cells per high

power field following rIL-2. Similarly, although an increase in the number of cells bearing NK markers was observed following rIL-2 the average number of cells per high power field bearing the NK marker CD56 (NKH-1) was only 4, 5 and 4 in the cases studied following rIL-2 (figure 8:7:3).

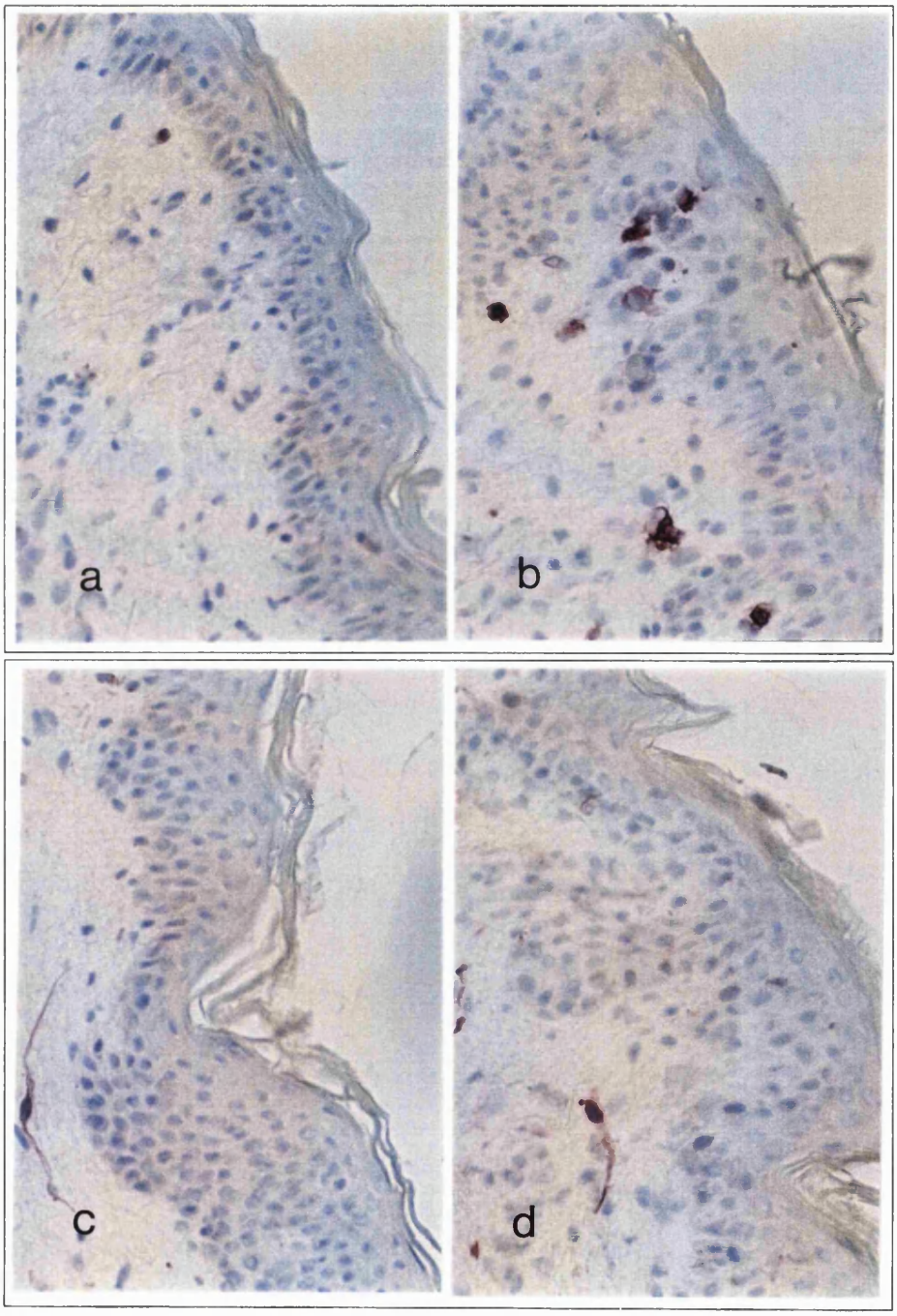


Figure 8:7:3. Neutrophils (NP57+ve) in normal skin before (a) and at the end (b) of rIL-2 infusion. NK cells (CD56) before (c) and at the end (d) of rIL-2 infusion x250.

The number of T-cells, monocyte/macrophages and neutrophils was higher in tumour tissue compared with skin before treatment with rIL-2 (figure 8:7:4).

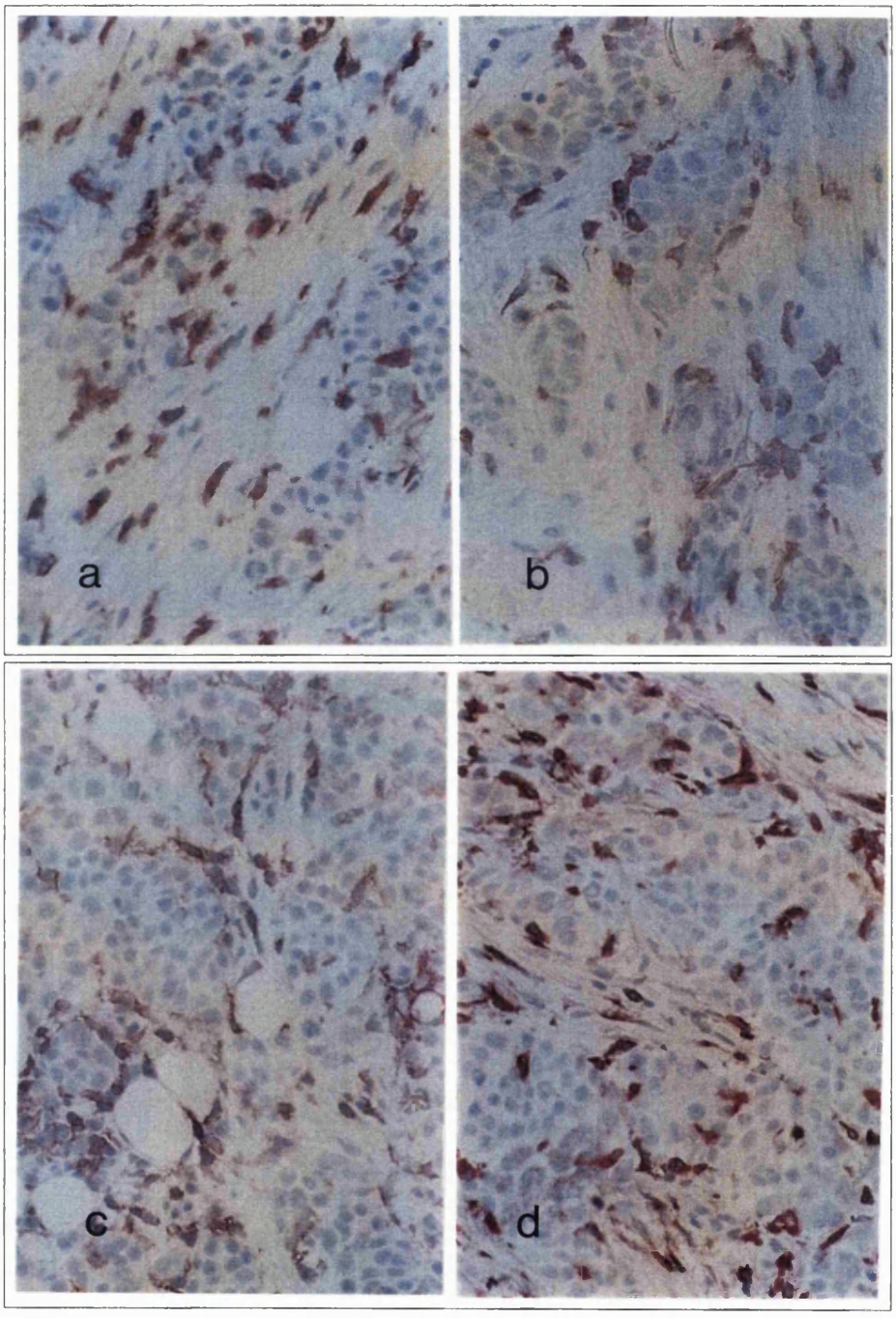


Figure 8:7:4. The majority of T-cells in tumour were CD4 positive [helper-inducer] (a), expressed CD25 [IL-2R] (b) and were CD45RO positive [memory T-cells] (c). Many infiltrating cells present were of monocyte/macrophage origin (CD68 +ve d) x200.

The majority of the T-cells present in the metastatic tumour infiltrate were CD4 positive (helper/inducer) type, expressed the IL-2 receptor (CD25) and were memory T-cells (CD45RO). Many cells of the monocyte/macrophage lineage (CD68) were also present in areas of metastatic tumour. The number of neutrophils and NK cells in the leucocyte infiltrate within areas of metastatic tumour was low.

The numbers of different leucocyte subsets varied little in tumour following the five day infusion of rIL-2. Average numbers of T-cells macrophages and neutrophils per high power field in skin and tumour before and after rIL-2 are illustrated in figure 8:7:5.

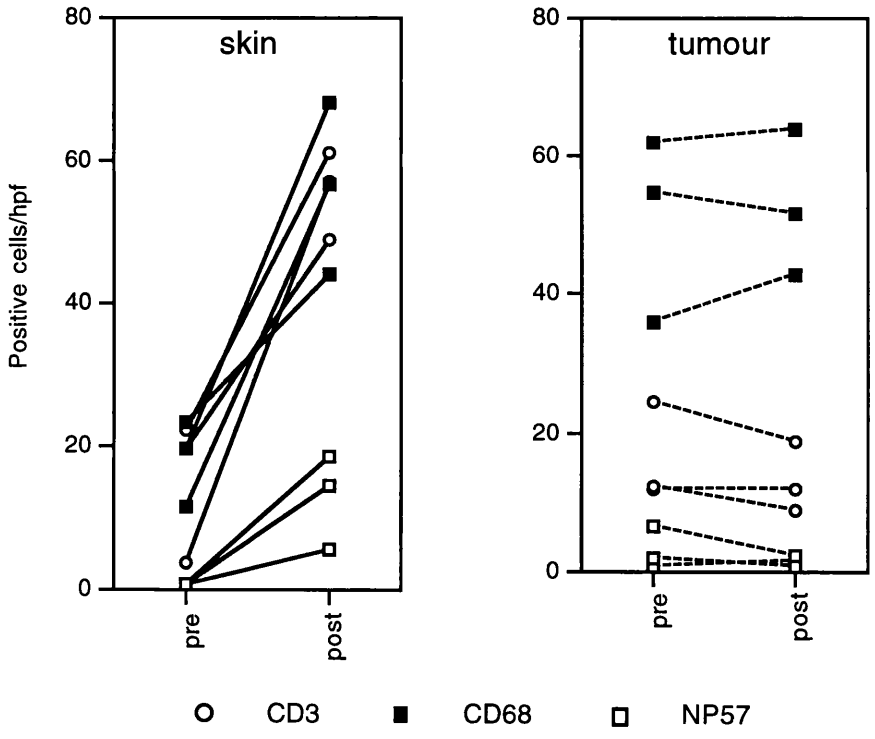


Figure 8:7:5. Numbers of cells per high power field (average of at least 5 high power fields) bearing T-lymphocyte (CD3), monocyte-macrophage (CD68) and neutrophil (NP57) markers in skin and tumour before and at the end of rIL-2 infusion (n=3).

The changes in all leucocyte markers assessed in skin and tumour before and at the end of rIL-2 are summarised in table 8:7:1. The data are expressed as ratio:- cells positive post/cells positive pre rIL-2.

Table 8:7:1. Ratio of cells positive for leucocyte antigens in normal skin and tumour following rIL-2 infusion.

		Ratio post/pre rIL-2 (±sem)	
	Cell type	**Skin**	**Tumour**
CD3	T-lymphocytes	6.8±4.1	0.8±0.1
CD4	helper/inducer	3.3±0.5	1.1±0.1
CD8	cytotoxic/supressor	4.4±1.2	1.0±0.2
CD45RA	naive T-cells	1.8±0.9	1.7±0.6
CD45RO	memory T-cells	3.4±0.2	0.9±0.1
CD25	IL-2 receptor	11.9±1.5	1.5±0.4
CD20	B-lymphocytes	1.4±1	1.4±0.4
CD68	monocytes	3.4±0.9	1.1±0.1
CD14	monocytes	3.4±0.7	0.8±0.1
NP57	neutrophils	17.8±5.9	1.5±1.1
CD67	neutrophils	11.8±4.2	3.3±2
CD56	NK/LAK cells	4.2±1.5	1.2±0.4
CD57	NK/LAK cells	7.1±2.1	1.0±0.1

Although the post/pre rIL-2 ratios for neutrophils and NK cells in skin are high, the absolute numbers of these cells before and after rIL-2 was low (figure 8:7:3 and 8:7:5). Numbers of naive (CD45RA) T-cells and B-cells remained low following rIL-2. Overall there was little change in the number of different leucocyte subsets following rIL-2 in tumour tissue.

8:7:2. Changes in TNF-α and TNF receptors in normal skin following rIL-2.

Cytokines induced during rIL-2 therapy such as TNF-α and IFN-γ are known to upregulate several endothelial and leucocyte adhesion molecules *in vitro*. Induction of TNF-α and soluble TNF-receptors are described in section 8:5:1. Changes in expression of TNF-α and its receptors in skin were assessed before and after rIL-2.

Prior to rIL-2 few infiltrating cells were noted in the dermis and little expression of TNF-α or the high molecular weight TNF-receptor was observed (figure 8:7:6).

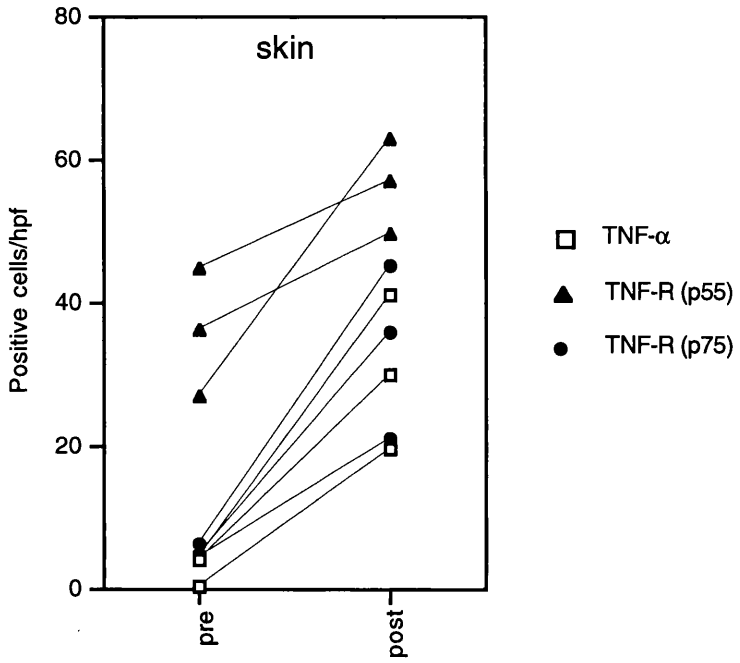


Figure 8:7:6. Numbers of cells per high power field (average of at least 5 high power fields) expressing TNF and TNF receptors TNF-R(p55) and TNF-R(p75) in skin biopsies before and at the end of rIL-2 infusion (n=3).

Expression of the low molecular weight TNF receptor was noted prior to treatment with rIL-2 and an example of expression on endothelial cells is shown in figure 8:7:7.

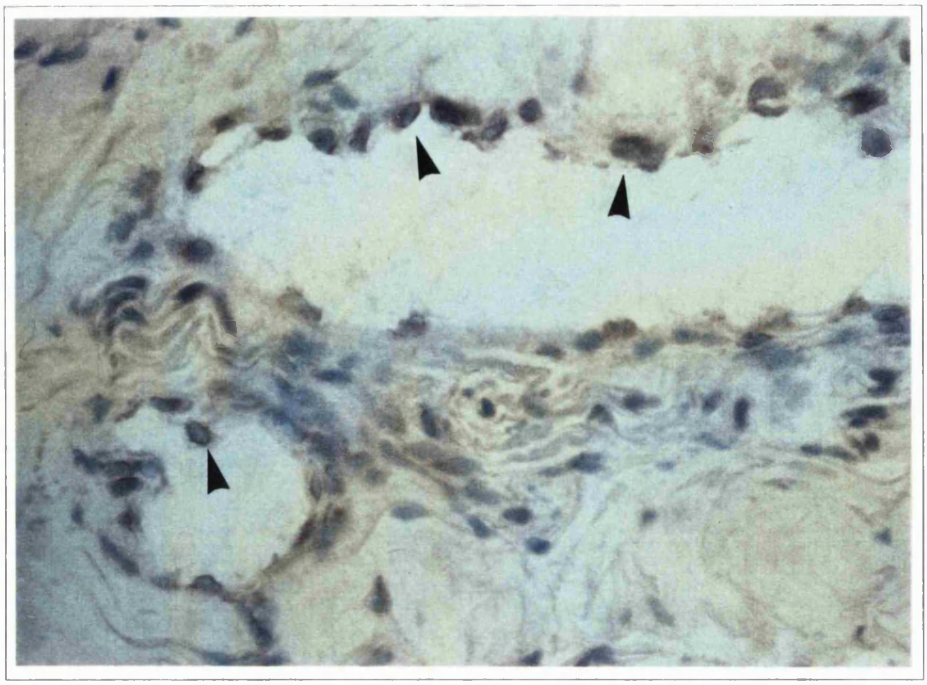


Figure 8:7:7. Expression of the low molecular weight TNF- receptor TNF-R(p55) in vascular endothelium before treatment with rIL-2 (x400).

Following infusion of rIL-2 many of the infiltrating mononuclear cells expressed TNF-α (figure 8:7:8).

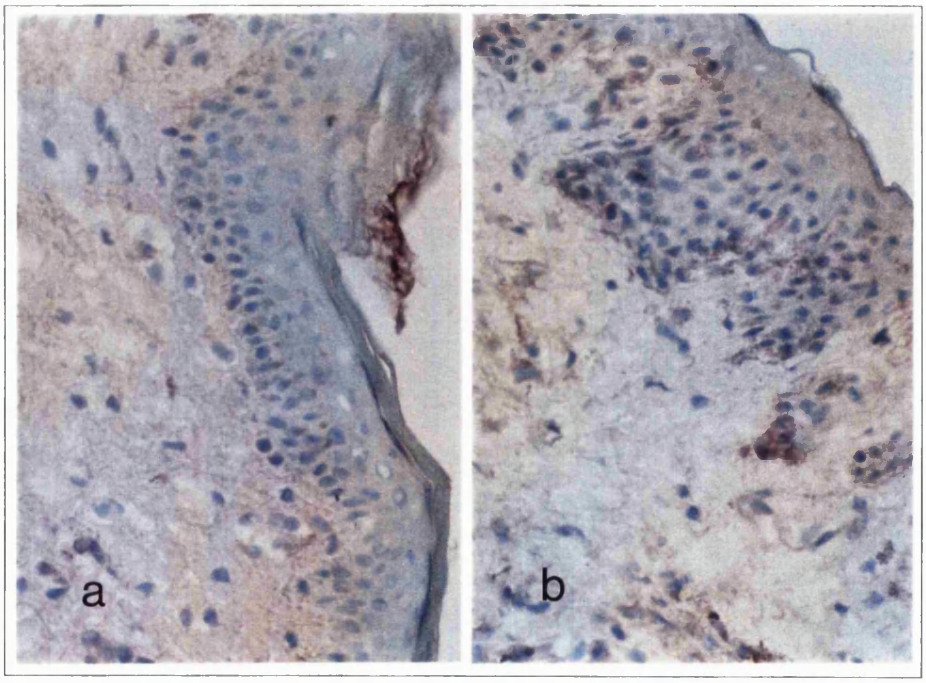


Figure 8:7:8. TNF-α expression by mononuclear cells before (a) and at the end (b) of rIL-2 infusion (x200).

Increases in the number of cells of TNF-R(p55) and TNF-R(p75) were noted following rIL-2 (figure 8:7:6) and were due to expression by infiltrating mononuclear cells within the dermis (not illustrated).

8:7:3. Changes in adhesion molecule expression in normal skin and tumour following rIL-2.

Changes in these adhesion molecules following rIL-2 some of which are known to be upregulated *in vitro* by cytokines induced by rIL-2 were assessed *in vivo* immunohistochemically in normal skin and metastatic tumour.

The increase in numbers of cells expressing the endothelial-monocyte adhesion molecule CD54 (ICAM-1) and its leucocyte associated ligand CD11a/CD18 (LFA-1) in skin and tumour, before and at the end of the rIL-2 infusion are illustrated in figure 8:7:9.

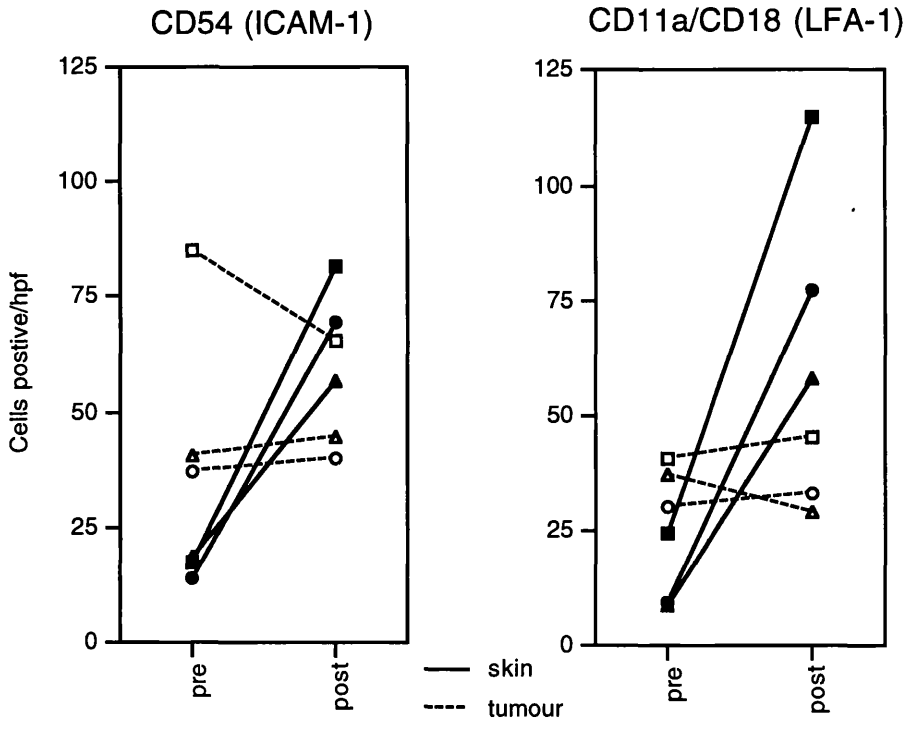


Figure 8:7:9. Numbers of cells per high power field (average of at least 5 high power fields) expressing CD54 (ICAM-1) and CD11a/CD18 (LFA-1) in skin (solid lines, closed symbols) and tumour (dotted lines, open symbols) before and at the end of rIL-2 infusion (n=3).

An example of the increase in ICAM-1 and LFA-1 expression in skin before and after rIL-2 is illustrated in figure 8:7:10.

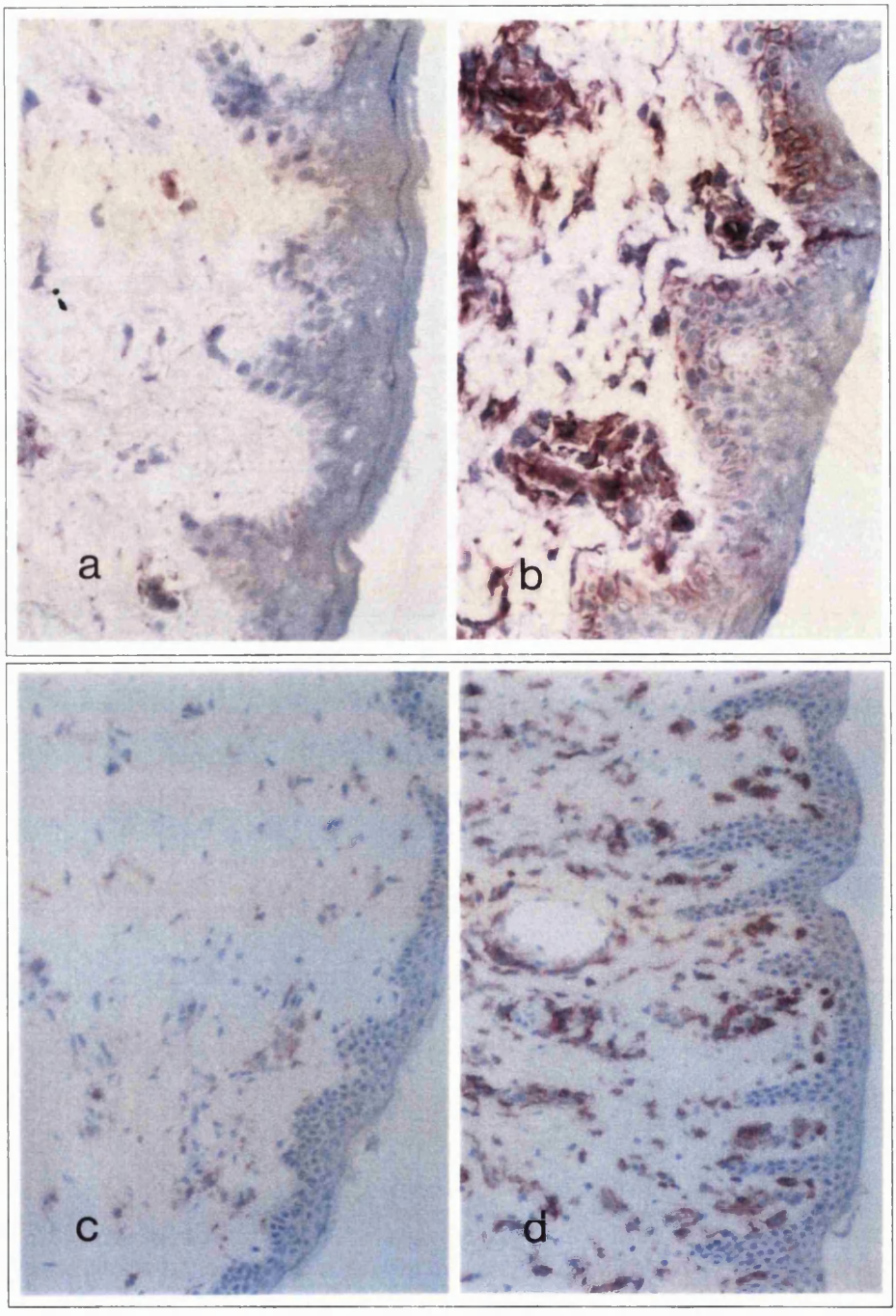


Figure 8:7:10. CD54 (ICAM-1) expression in normal skin before (a) and at the end (b) of rIL-2 infusion. CD11a/CD18 (LFA-1) expression before (c) and at the end (d) of rIL-2 infusion., x100.

Following rIL-2, increased expression of both adhesion molecules was noted in the dermal cellular infiltrate, with ICAM-1 expression also noted in vascular endothelium and epidermis (figure 8:7:10). Levels of ICAM-1 and LFA-1 were higher in tumour compared with skin prior to rIL-2 and expression was noted in the cellular infiltrate rather than tumour cells or vascular endothelium (figure 8:7:11).

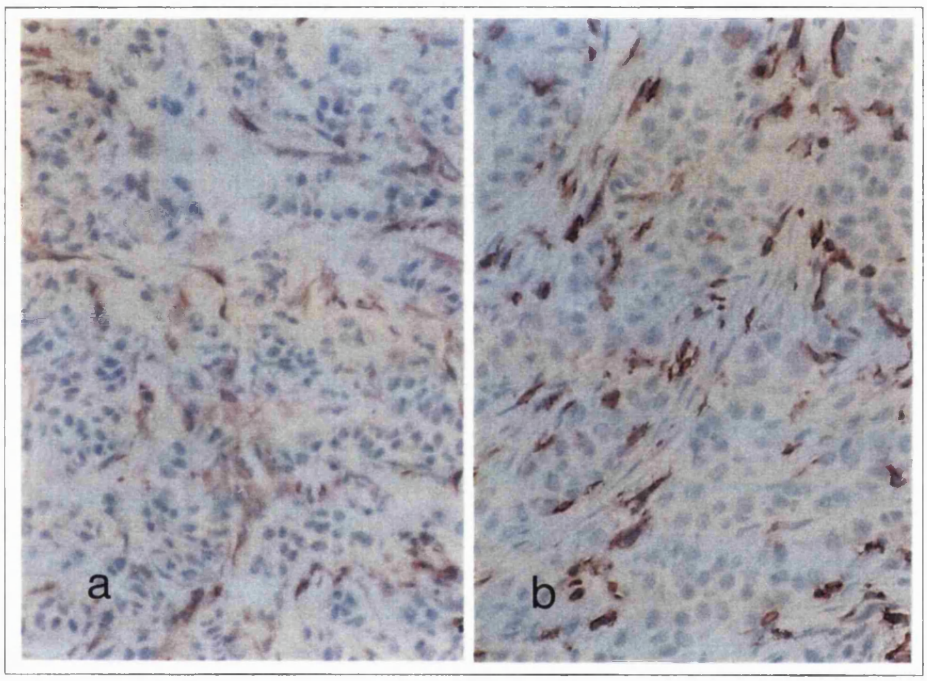


Figure 8:7:11. CD54 (ICAM-1) expression (a) and CD11a/CD18 (LFA-1) expression (b) in metastatic tumour prior to rIL-2. No changes were observed following rIL-2 infusion, x200.

No increases in expression of ICAM-1 or LFA-1 were noted in tumour following rIL-2 infusion (figure 8:7:9).

Changes in numbers of cells expressing CD106 (VCAM) and its leucocyte associated ligand (VLA-4) are illustrated in figure 8:7:12.

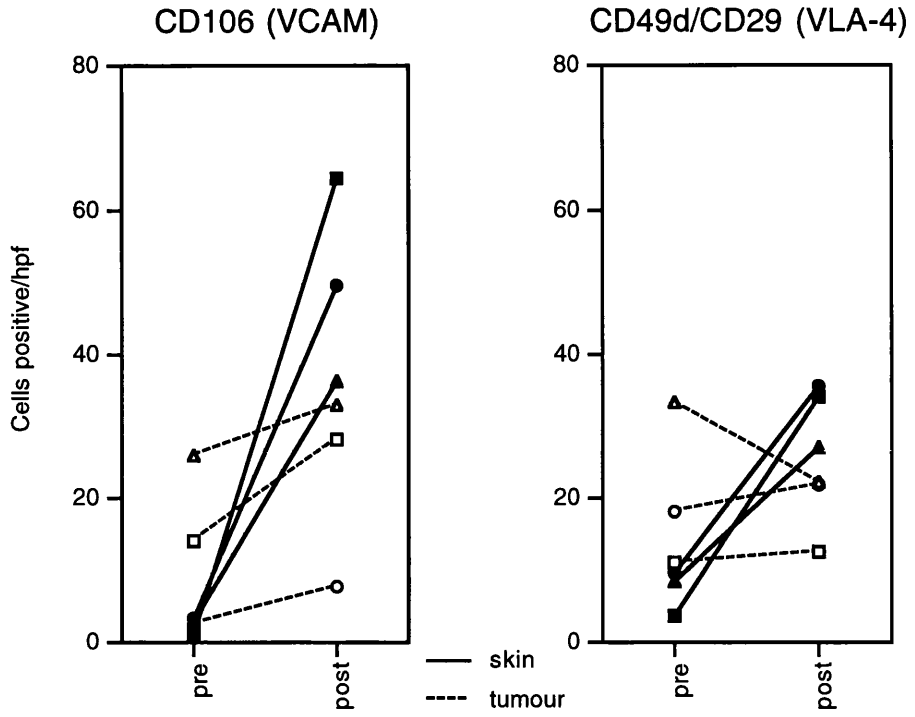


Figure 8:7:12. Numbers of cells per high power field (average of at least 5 high power fields) expressing CD106 (VCAM) and CD49d/CD29 (VLA-4) in skin (solid lines, closed symbols) and tumour (dotted lines, open symbols) before and at the end of rIL-2 infusion (n=3).

Following rIL-2, increased expression of both adhesion molecules was noted in the cellular infiltrate in the dermis (figure 8:7:13).

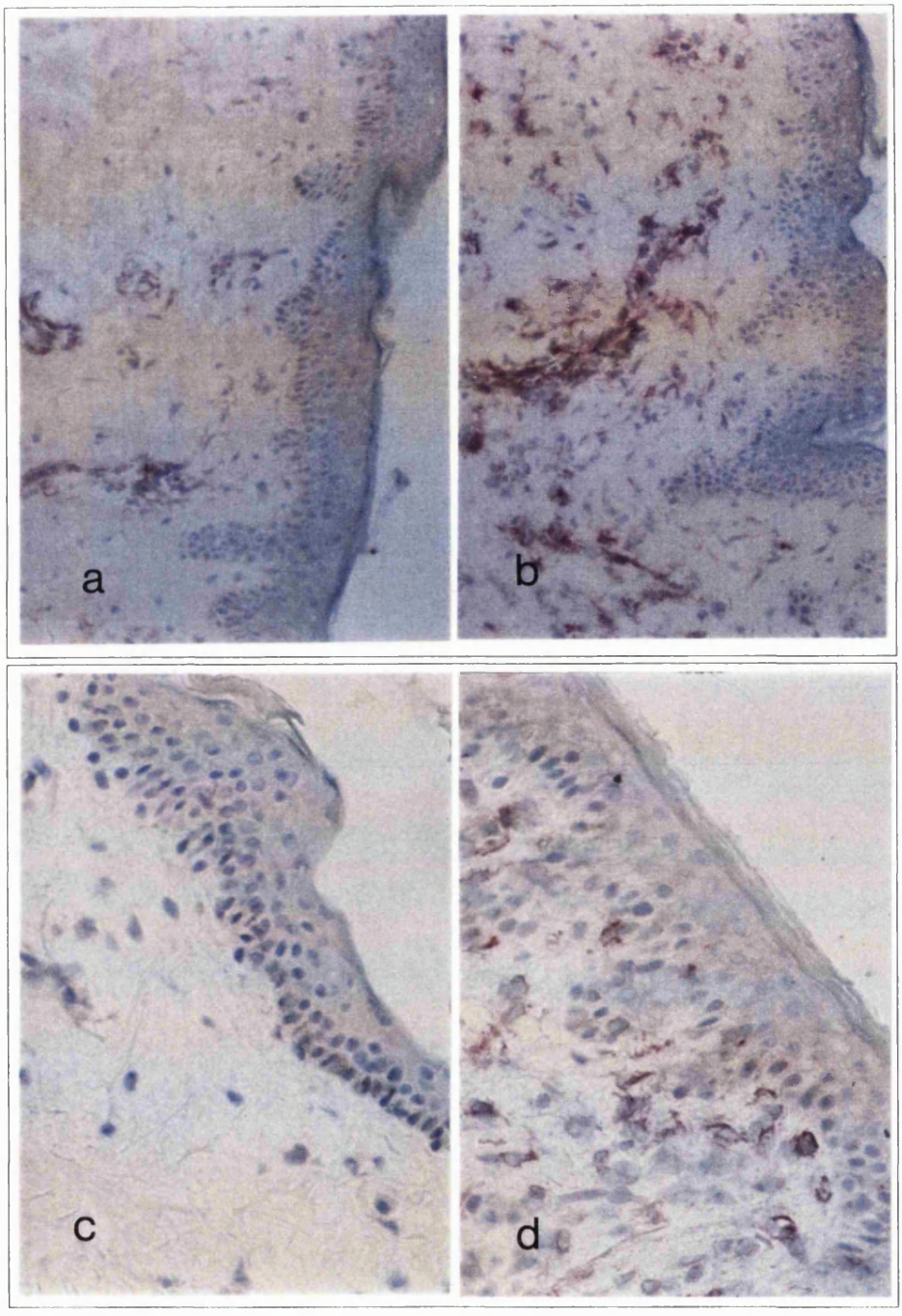


Figure 8:7:13. CD106 (VCAM) expression in normal skin before (a) and at the end (b) of rIL-2 infusion, x100. CD49a/CD29 (VLA-4) expression before (c) and at the end (d) of rIL-2 infusion, x200.

As with ICAM-1/LFA-1, the number of cells expressing VCAM and VLA-4 within areas of metastatic tumour were greater than in normal skin prior to rIL-2. (figure 8:7:14).

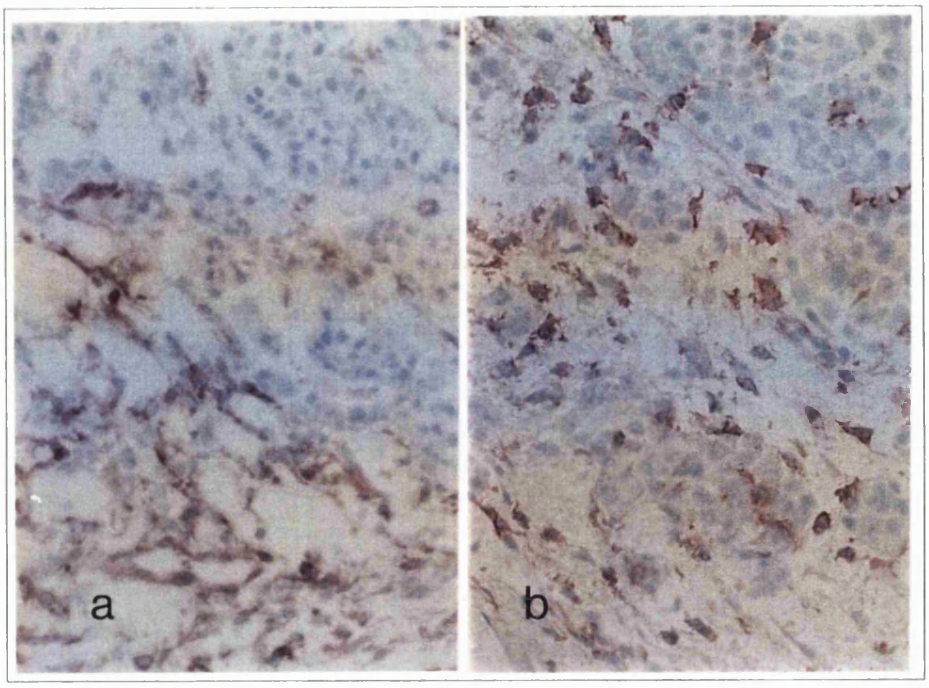


Figure 8:7:14. CD106 (VCAM) expression (a) and CD49d/CD129 (VLA-4) expression (b) in metastatic tumour before rIL-2 (x200).

In two cases of metastatic tumour, a rise in the number of cells expressing CD106 (VCAM) was noted but levels did not reach those observed in dermis. No increase in the number of cell expressing CD49d/CD29 (VLA-4) was observed in tumour following rIL-2.

Changes in the expression of the selectin group of adhesion molecules was also assessed before and after rIL-2 and data are shown in figure 8:7:15. Levels of the endothelial adhesion molecule ELAM-1 were relatively low in skin and tumour prior to rIL-2. Although an increase in the number of endothelial cells expressing ELAM-1 in the dermis was observed following rIL-2 numbers remained low compared with other adhesion molecules studied. A comparable increase in the number of endothelial cells expressing ELAM-1 was noted in one of the sites of metastatic tumour.

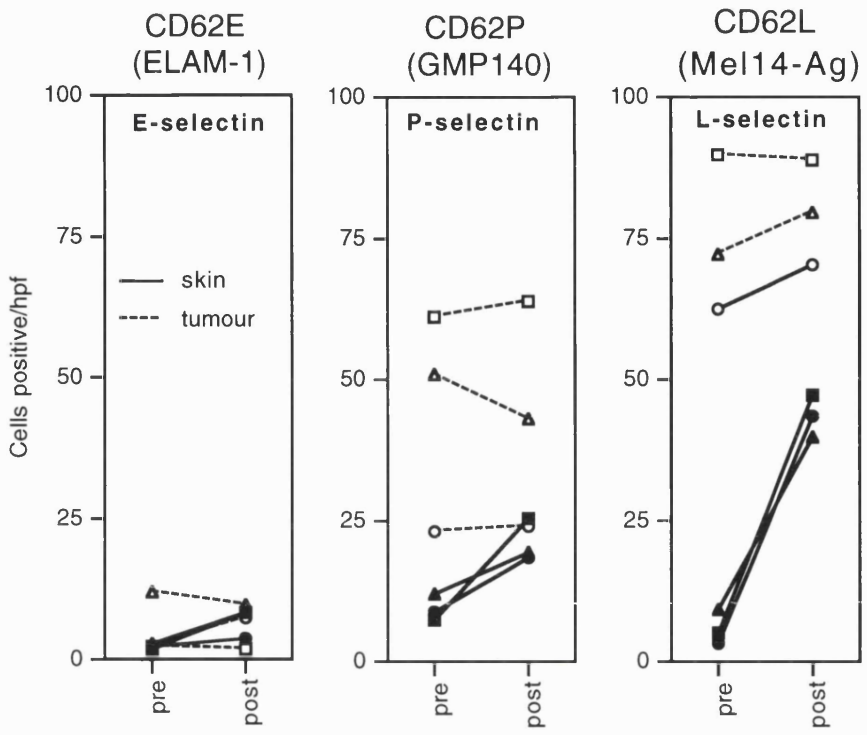


Figure 8:7:15. Numbers of cells per high power field (average of at least 5 high power fields) expressing CD62E (ELAM-1/E-selectin), CD62P (GMP140/P-selectin) and CD62L (Mel14-Ag/L-selectin) in skin (solid lines, closed symbols) and tumour (dotted lines, open symbols) before and at the end of rIL-2 infusion (n=3).

Numbers of cells expressing CD62P (P-selectin) were higher in skin and tumour before rIL-2 compared with E-selectin and increases in numbers of CD62P positive cells endothelial cells were noted in the dermis of the three patients (figure 8:7:15 and example figure 8:7:16) after rIL-2, whereas little change was noted in sites of metastatic tumour (not illustrated).

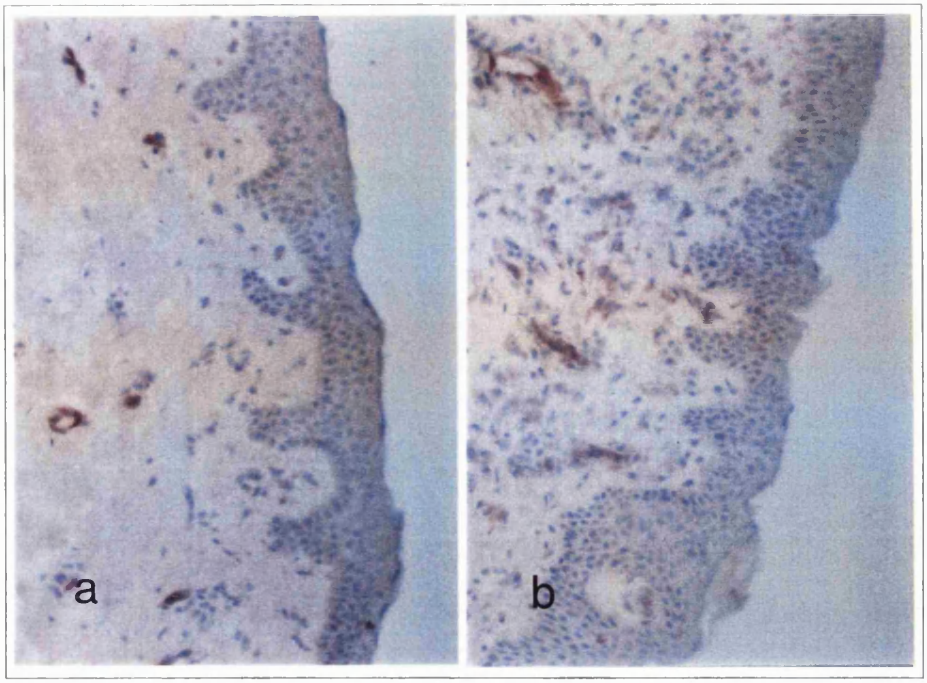


Figure 8:7:16. CD62P expression in normal skin before (a) and at the end (b) of infusion of rIL-2 . Staining of some endothelium is noted before rIL-2, and increased expression following rIL-2, predominantly in endothelium, is noted (x100).

Numbers of cells expressing CD62L(L-selectin) in dermis were low prior to rIL-2 but increases were noted with up to an average of 48 CD62L positive infiltrating leucocytes per high power field following rIL-2. The mean number of CD62L positive cells in tumour did not change significantly following rIL-2.

Another of the β_2-integrin family of adhesion molecules CD11c/CD18 known to be expressed on monocytes, neutrophils and T-lymphocytes was also assessed. Increase in expression following rIL-2 were noted in the infiltrating mononuclear cells with no changes observed in tumour. CD31 (PECAM, platelet/endothelial cell adhesion molecule) expression was also increased in skin but not tumour after rIL-2. Changes in expression of the adhesion molecules studied following continuous infusion rIL-2 is summarised in table 8:7:2, where data are expressed as data are expressed as the ratio:- cells positive post/cells positive pre rIL-2.

Table 8:7:2. Ratio of cells positive for adhesion molecules in normal skin and tumour following rIL-2 infusion.

		Ratio post/pre rIL-2 (±sem)	
		Skin	**Tumour**
CD54	(ICAM-1)	4.3±0.6	1.0±0.1
CD11a/CD18	(LFA-1)	6.5±1.1	1.0±0.1
CD106	(VCAM)	31.0±16	2.2±0.6
CD49d/CD29	(VLA-4)	5.4±1.9	1.0±0.2
CD62E	(E-selectin)	3.5±1	1.5±0.8
CD62P	(P-selectin)	2.4±0.6	1.0±0.1
CD62L	(L-selectin)	8.4±2.3	1.1±0.1
CD11c/CD18	(p150)	9.4±4.0	1.1±0.2
CD31	(PECAM)	4.5±2	1.0±0.1

With the exception of CD106 (VCAM), the ratio of cells positive for the adhesion molecules studied after rIL-2 compared with pre-treatment values was unity. Increases in all adhesion molecules studied was noted in the dermis following rIL-2.

Chapter 9.

CONCLUSIONS AND DISCUSSION.

9:1. Clinical aspects.

Following preliminary reports of tumour responses after treatment with bolus or continuous infusion rIL-2 in other malignancies, studies of rIL-2 given by continuous infusion in patients with metastatic breast cancer were undertaken in the ICRF Clinical Oncology Unit at Guy's Hospital. A previous study in metastatic breast cancer had noted considerable toxicity (Israel et al 1989) and so our initial experience with rIL-2 was in the context of the dose escalating study described above. The first six patients treated on this protocol (ECL2041) experienced some side effects, notably fever and rigors which in one case necessitated interruption of treatment. Skin rashes were noted in 5 of 6 patients treated on this regimen. Biological effects of infused rIL-2 were also evident including lymphopaenia during rIL-2 followed by a rebound lymphocytosis, eosinophilia and increases in TNF and markers of interferon-γ activity. Despite these changes, patients remained relatively well and all were ambulant for at least 50% of the time during treatment even at the higher dose levels. Deterioration in renal function had been anticipated but creatinine levels remained below the upper limit of normal throughout treatment. Our experience was at variance to that reported by other groups, which coupled with the apparent lack of relationship between dose level, toxicities and biological effects raised the question of bioavailability of rIL-2 being influenced by the giving sets used to administer rIL-2.

Human recombinant interleukin-2 for clinical use is usually supplied in a lyophilised from for ease of storage and distribution. Most preparations are supplied freeze dried with mannitol and sodium dodecyl sulphate. Some companies (Roche) include a carrier protein in their preparation whereas Cetus did not. Addition of a carrier protein when reconstituting cytokines is standard procedure in laboratory studies but Cetus' recommendations for reconstitution at this time did not include the use of a carrier protein. We therefore established an *in vitro* experiment to assess the effect of reconstitution of rIL-2 with and without albumin. Recombinant IL-2 was assayed using a commercially available radioimmunoassay and a loss of immunoreactive rIL-2 was noted after about 4 hours of the infusion. Activity of rIL-2 was also tested using a bioassay. This confirmed a loss of IL-2 activity after 2 hours in infusions not reconstituted with albumin. Both rIL-2 preparations were highly biologically active in the initial eluted sample demonstrating that formulation with albumin did not affect biological potency. The potency of samples diluted with albumin was unchanged over the 24 hour infusion but by 2 hours the potency of rIL-2 diluted with 5% glucose alone had fallen to less than 25% and it remained low in subsequent samples. It was deduced that the low potency after two hours resulted from binding of the cytokine to plastic in the syringe and giving set.

The initial work by Rosenberg and colleagues included the use of Cetus rIL-2 and treatment was associated with considerable toxicity (Rosenberg et al 1989). In these studies rIL-2 was given as a bolus injection in which case adherence to syringes and giving sets was of less relevance. It has been suggested that prolonged infusion of rIL-2 might yield valuable immunomodulatory effects with less toxicity than rapid intravenous injection (Thompson et al 1988, Sondel et al 1988). The principal reason for this apparent improvement in therapeutic index is that the total dose of rIL-2 given

by continuous infusion is lower compared with bolus injections. West et al (1987) used non-protein formulated rIL-2 by continuous infusion but did not state whether rIL-2 was reconstituted with albumin. Therefore the patients may not have received the intended dose. These findings may explain partly the lower frequency of toxicity when rIL-2 is given by continuous infusion. It is also possible that the potential efficacy of rIL-2 in these and other studies may also have been underestimated.

The observation that the bioavailability of rIL-2 could be compromised by failure to reconstitute it with a carrier protein led to a change in the recommendations for reconstitution by the manufacturers (Cetus) and co-incided with the start of our involvement in European multi-centre phase II clinical trials in melanoma and renal cell carcinoma. With the revised guidelines for reconstitution of rIL-2 the toxicity profile experienced by our patients on these studies was in keeping with previous reports.

In the case of patient J.C (malignant melanoma, protocol ECMP003) reconstitution of rIL-2 with albumin during the second 5 day infusion period was associated with hypotension which necessitated discontinuation of treatment. Treatment of 6 patients with metastatic breast cancer on the escalating dose schedule of rIL-2 (EC-L2041), in which cytokine was not reconstituted with albumin, was associated with only mild to moderate toxicity. In the case of the seventh patient (S.B.) reconstitution of rIL-2 with albumin led to development of the capillary leak syndrome and renal failure which necessitated discontinuation of therapy during the third infusion period. These two cases support the hypothesis that reduced bioavailablity of rIL-2 leads to underestimation of toxicity. This view has since been supported by others (Lamers et al 1992) who noted increased toxicity and inability to administer the projected dose of rIL-2 when reconstituted with albumin.

Of the 11 patients with renal cell carcinoma and malignant melanoma on protocols ECMP001 and ECMP003 receiving two five day infusion periods of rIL-2, 5 failed to receive the full dose rIL-2 because of toxicity. Only one partial response in a patient with malignant melanoma was noted in this group, although the patient was technically not assessable for response having discontinued treatment prematurely and in this case the response was maintained for only four weeks. Stable disease was noted in three further patients who went on to receive further courses of rIL-2.

The data from the phase II trial in renal cell carcinoma (ECMP001) as a whole have been reported recently (Gore et al 1994) and a response rate of 14% was noted in 109 evaluable patients. This response rate is consistent with other phase II trials (Parkinson et al 1990a, Bukowski et al 1990). Although response rates reported in these trials are greater than those seen with hormonal therapy (DeKernion 1983), they are equivalent to those noted with interferon-α (Horoszewicz and Murphy 1989) both of which are easily administered on an out-patient basis.

The application of conventional response criteria to assess efficacy of immunotherapeutic approaches may be inappropriate as it is widely believed that such approaches are most likely to be of benefit for minimal disease bulk. Moreover, response rates are essentially a surrogate for the desired end-point of increased survival. In this respect it is noteworthy that Jones et al (1993) in reviewing the data from rIL-2 studies in renal cell carcinoma concluded that a subset of patients with good prognostic features may have an improved survival. Prospective randomised studies to test this hypothesis have not yet been instituted.

The phase II study of DTIC/rIL-2 in malignant melanoma (ECMP003) has not yet been published. Other studies in which rIL-2 has been used in combination with DTIC in the treatment of metastatic melanoma have recorded response rates of 22% whether rIL-2 is given by bolus (Flaherty et al 1990) or continuous infusion (Shiloni et al 1989). Although melanoma is a relatively chemoresistant disease, DTIC is probably the most active single agent with response rates of up to 20% being recorded in selected patients (reviewed Miles and Souhami 1987). In the absence of randomised trials the contribution of rIL-2 in terms of response and survival benefit remain uncertain.

Considering the need for prolonged admission to hospital, the toxicity of rIL-2 and the lack of clear benefit of therapy, it is difficult to concur with the view that the side effects of rIL-2 given by infusion are "acceptable" (Hamblin 1990). Despite a product license for rIL-2 in renal cell carcinoma being granted, the use of infusional rIL-2 has declined. In an attempt to improve the therapeutic index, some groups have aimed to define those patients most likely to respond or to develop less toxic, more feasible schedules. These approaches are discussed at the end of this chapter.

9:2. Biological assessments.

In an attempt to elucidate the mechanisms of rIL-2 toxicity, aspects of its biological effects were studied in patients during treatment. As well as induction of the cytokines TNF and IFN-γ, nitric oxide activity, a potential mediator of the cardiovascular consequences of rIL-2 therapy, was measured. In a small group of patients trafficking of potential immune effector cells was assessed at sites of metastatic tumour and in uninvolved skin.

9:2:1. Induction of TNF-α and TNF soluble receptors.

Induction of TNF during treatment with IL-2 could have an important role in the response to treatment through its involvement in the CTL response to antigen and/or through its contribution to the generation of LAK activity. The lack of responders in the patient groups studied here does not allow confirmation of the findings of Blay et al (1990) who noted that induced levels of TNF-α correlated with response to rIL-2. The data described here do however support the view that induced TNF-α may mediated some of the toxic effects of rIL-2 in that maximum induced levels of TNF-α were significantly higher in those patients whose treatment was interrupted and/or discontinued because of toxicity. In addition, the induction of soluble TNF receptors which are capable of binding TNF-α has also been demonstrated.

Pretreatment levels of TNF-α in this group of patients with advanced cancer were similar to those found in normal controls, but levels of sTNF-Rp55 and p75 were significantly higher. Aderka et al (1991) previously noted elevated levels of sTNF-R in patients with advanced cancer and suggested a possible role as a tumour marker. The administration of rIL-2 leads to induction of both forms of the soluble TNF receptor as well as induction of TNF itself with induced levels of TNF-Rp75 being significantly higher than peak levels of TNF-Rp55. In patients from whom blood was taken at early time points, levels of TNF-α were raised in all patients after 6 hours of rIL-2, although levels of sTNF-R were raised in only 5 of the 9 patients at this time. After 24 hours of IL-2, levels of sTNF-R were raised in all patients treated. Early induction of sTNF-R may be due to a combination of upregulation of TNF receptors by induction of mRNA and protein and shedding of the extracellular domain of both TNF receptors. Activation of T-cells is associated with a rapid induction of TNF-Rp75 mRNA and protein (Ware et al 1991). Similarly, rapid induction

of TNF-Rp75 and subsequently TNF-Rp55 has been documented in activated B-lymphocytes (Heilig et al 1991). Ware et al also demonstrated that further stimulation of activated T-cells results in receptor down regulation possibly due to shedding of receptor. Preferential shedding of sTNF-Rp75 from neutrophils may occur within minutes of exposure to chemotactic factors by the action of neutrophil elastase (Porteu et al 1991).

Levels of sTNF-R peaked 24 to 48 hours after the peak level of induced TNF-α in 9 of 13 patients studied. In patients according to the escalating dose schedule levels of induced TNF and sTNF-R were much lower than in the other groups, possibly due to decreased bioavailability of the drug. Nevertheless, in the patients treated on this regimen there was no direct correlation between the projected dose of IL-2 administered and the levels of sTNF-R induced, indeed the highest levels of sTNF-Rp75 were seen at the intermediate doses.

Previous studies *in vitro* have suggested that the induction of TNF binding activity is induced directly by IL-2 (Owen-Schaub et al 1989). A clinical study of rTNF in patients with advanced cancer did however demonstrate that TNF itself could induce TNF binding proteins (Lantze et al 1990). The above data demonstrate that the induction of sTNF-receptors follows induction of TNF-α closely and the levels of soluble receptor induced correlate closely with levels of induced TNF-α, supporting the view that induced TNF-α may mediate this effect of rIL-2..

Stoichiometric studies of the binding of a recombinant sTNF-R1 have suggested that three molecules of sTNF-R1 bind to one TNFα trimer (Loetscher et al 1991). The same group also determined that a 10 to 100-fold excess of recombinant sTNF-R1 was required to neutralise TNFα activity.

Similarly, Olsson et al (1989) have previously shown that a 10-fold molar excess of TNF binding protein was required to reduce the cytotoxic effects of TNF in a WEHI assay by 50%. In our study the maximum mean induced level of sTNF-R1 for the patient group was 5.93ng/ml compared with a maximum induced TNF of 110 pg/ml. This represents a 30-fold molar excess of sTNF-R1. Similarly the maximum mean induced level of sTNF-R2 of 17.57ng/ml, represents a 90 fold molar excess of this binding protein. Thus, although immunoreactive TNF is induced during treatment with IL-2, binding proteins are also induced at levels which could theoretically neutralise its bioactivity, at least in the peripheral circulation. At the concentrations observed in this study, such binding proteins may also act as carriers for TNF and prolong its half life in the circulation (Aderka et al 1992). Thus although the bioactivity of TNF in the peripheral circulation may be reduced as a consequence of the presence of soluble receptors, end organ toxicity may be increased as a result of the prolongation of the half-life.

9:2:2. Interferon-γ and markers of its activity.

Through effects on non-MHC and MHC restricted cytotoxicity as well as effects on depletion of tryptophan, IFN-γ may also mediate any anti-tumour effects of rIL-2. Raised levels of IFN-γ have been noted during bolus therapy with rIL-2 (Lotze et al 1985) but not during infusional therapy (Brown et al 1989). In the latter study however, markers of IFN-γ activity, namely neopterin induction and tryptophan depletion were noted during rIL-2. In our studies, levels of IFN-γ were below the reliable limit of detection in normalise and patients with advanced cancer being 0.2U/ml in all cases studied. During therapy with rIL-2, IFN-γ levels above the reliable limit of detection (2.5U/ml) were noted in two patients, both on day three of the first of two five day infusions. Although mean levels of induced IFN-γ remained below the reliable

limits of detection throughout rIL-2 infusions, consistent patterns of changes were noted within patient groups such that the highest mean levels of induced IFN-γ were recorded on day 3 of infused rIL-2.

Neopterin.

Neopterin is recognised as an indicator of the activation state of the cellular immune system. It is synthesised from GTP by the enzyme GTP cyclohydrolase I, the most potent inducer of which is IFN-γ (Huber et al 1984) an effect which is known to be potentiated by TNF-α (Werner-Felmeyer et al 1989). Although pretreatment levels of TNF-α and IFN-γ were both below reliable detectable limits in the patients studied, the mean level of plasma neopterin was higher in this group of patients compared with normal controls. This suggests a pre-existing level of immune activation in this patients which is not reflected in cytokine levels in the peripheral circulation. The finding of raised neopterin levels in patients with advanced cancer is in keeping with previous reports in which neopterin levels are usually correlated with tumour stage and consequently with a poor prognosis (reviewed Reibnegger et al 1991). More recent data in carcinoma of the colon have suggested that levels of plasma neopterin may be independent of established prognostic criteria (Weiss et al 1993).

During treatment with infused rIL-2, plasma neopterin increased in all patients even at the lowest dose levels, although unlike IFN-γ, levels peaked towards the end of treatment, usually at day 5. Unlike previous studies a correlation has been noted between peak levels of induced neopterin and peak levels of induced IFN-γ. No comments can be made from these data regarding induction of IFN-γ or neopterin and response to treatment. These data do however implicate IFN-γ in mediating the toxic effects of rIL-2 since, as with

TNF-α, the levels of induced neopterin were higher in patients whose treatment had to be interrupted and/or discontinued because of toxicity although this difference failed to reach standard levels of significance (p=0.06).

Tryptophan and kynurenine.

Plasma tryptophan levels in our group of patients with advanced cancer were not significantly different from normal controls. IFN-γ is known to induce the enzyme indoleamine 2,3 dioxygenase (IDO) which converts tryptophan to kynurenine (Takikawa et al 1988). Although IL-2 has also been shown to lead to induction of IDO in peripheral blood mononuclear cells *in vitro*, this effect was inhibited by anti-sera to IFN-γ (Carlin et al 1987). Decreased plasma tryptophan was noted in all cases during infusion of rIL-2 even at the lowest dose levels and a reciprocal rise in plasma kynurenine was noted. The decrease in tryptophan is most likely to be the result of enhanced tryptophan catabolism rather than poor tryptophan intake in which decreases in kynurenine are usually observed (Vivian et al 1966). Although the levels to which tryptophan fell did not differ between groups whose treatment was interrupted or discontinued because of toxicity, depletion of tryptophan has been implicated in mediating some of the neurological consequences of rIL-2 therapy (Brown et al 1989), although how supplementation may influence toxicity or efficacy of rIL-2 has not yet been resolved.

9:2:3. Induction of nitric oxide.

In vitro studies have demonstrated that TNF-α and IFN-γ induce high levels of nitric oxide (endothelium derived relaxing factor) in macrophages (Drapier et al 1988), vascular endothelium (Kilbourn and Belloni 1990) and smooth

muscle (Busse and Mulsch 1990). Products of pterin metabolism also induced by cytokines such as IFN-γ via induction of GTP cyclohydrolase 1 (Werner et al 1990) demonstrated above, are essential co-factors in the generation of NO in smooth muscle (Gross and Levi 1992), macrophages (Tayeh and Marletta 1989) and fibroblasts (Werner-Felmeyer et al 1990). The toxic effects of these induced cytokines may be mediated therefore through nitric oxide leading to smooth muscle relaxation and therefore hypotension. NO has also been shown to induce lysis of pulmonary endothelial cells (Palmer et al 1992) which may therefore contribute to the observed capillary leak syndrome.

In these studies we have demonstrated that pretreatment levels of TNF and IFN-γ in patients with advanced cancer are not significantly higher than those found in normal controls. NO activity, assessed by nitrite production was shown to be raised in patients with disseminated carcinoma. Although nitrite levels do not appear to be influenced significantly by dietary factors (Lee et al 1986) they may be influenced by other factors such as renal impairment (Ochoa et al 1991). Normal renal function assessed by creatinine clearance was a prerequisite of entry into these trials. The aetiology of the raised nitrite levels may be a degree of immune activation at tissue level not reflected by raised levels of cytokines such as TNF-α and IFN-γ in the periphery. The production of NO by primary breast cancer reported in this thesis (chapter 3) and in other tumour types (Thomsen et al 1994) may explain the elevated NOx levels prior to treatment in this patient group.

Treatment with IL-2 increased the plasma concentration of NOx further and although deteriorating renal function could lead to increase in NOx, it is noteworthy that plasma creatinine did not increase above normal levels during the first five day infusion period of rIL-2. An observation not noted previously was the correlation between induced levels of nitrite and levels of TNF, IFN-γ

and neopterin production. Cessation of treatment led to a decrease in all values towards pretreatment levels. As NOx concentrations increased there was a decrease in systolic blood pressure. This is in agreement with a previous studies (Ochoa et al 1992, Hibbs et al 1992) and supports the suggestion that induction of NO synthase in the vasculature may be responsible for the hypotension observed. This effect on blood pressure was not sustained and despite increasing NOx levels with continued IL-2 treatment, blood pressure tended to return to pre-treatment values. The concomitant increase in pulse rate observed suggests that compensatory changes in vascular parameters elicited by the onset of hypotension may obscure the relationship between blood pressure and plasma NOx concentrations. Furthermore, NOx concentration in plasma is a pool of NO generated in several tissue types which most likely reflects a balance between generation and excretion. It is possible that post-induction accumulation of NOx occurs in plasma, alternatively induction of NO synthase may be longer lasting in other organs compared with the vasculature. Thus NOx concentration may not strictly correlate in time with the generation of NO in the vasculature. This dissociation which has been found in animals (Schulz et al 1992) predicts that a simple relationship between plasma NOx and blood pressure may not be observed.

Although the mechanism of action of IL-2 remains unclear, nitric oxide induced during treatment with IL-2 may be the cause of at least one of its dose limiting toxicities namely hypotension. Competitive substrate analogues such as *N*-monomethyl arginine inhibit the release of nitric oxide *in vitro*. Nitric oxide has been implicated in the aetiology of hypotension associated with septicaemia and analogues of NO synthase such as L-NMMA may be useful in its management (Petros et al 1991). Studies in animals have demonstrated that the administration of L-NMMA has no effect on the lymphocyte counts

induced by IL-2 *in vivo* and no effects on lymphokine activated killer cell activity *in vitro* but its use did abrogate IL-2 induced hypotension (Kilbourn et al 1992). It has also been suggested that inhibitors of tetrahydrobiopterin synthesis (an essential co-factor in the induction of iNOS) may also prevent cytokine induced hypotension (Kilbourn and Griffiths 1992). It may be possible therefore to inhibit the activity of NO synthase and reduce the side effects of IL-2 therapy without impairing the anti-cancer effects. It may even be feasible, using inhibitors of NO synthase to allow higher and possibly more effective doses of IL-2 to be given.

Conversely, *in vitro* data suggest that NO may mediate some of the tumourocidal effects of rIL-2. It is noteworthy that in an animal model, co-administration of L-arginine increased LAK activity and increased the efficacy of rIL-2 (Lieberman et al 1991). In addition, induced levels of nitrate and nitrite have been implicated as predictors of response to therapy with IL-2 and flavone acetic acid (Thomsen et al 1992).

9:2:4. Leucocyte trafficking and regulation of adhesion molecules.

As discussed previously the anti-tumour activity of rIL-2 may be mediated in part by the effect of other cytokines such as TNF-α and IFN-γ but may also depend on cell-mediated effects by macrophages, NK/LAK cells or cytotoxic T-cells. Similarly, the influx of leucocytes into normal tissue may contribute to the toxic effects of rIL-2. Previous studies examining the effects of rIL-2 in uninvolved tissue of patients receiving rIL-2 with or without LAK cells have demonstrated efflux of mononuclear cells (Gaspari et al 1987) and upregulation of one adhesion molecule, ICAM-1, which may mediate endothelial cell-leucocyte interactions, has been demonstrated following rIL-2 (Cotran et al 1987). In the studies described above a wide panel of markers for leucocyte subsets was used to define further the phenotype of extravasated cells into normal skin and cutaneous metastases following a five day infusion of rIL-2. In addition expression of more recently identified adhesion molecules and their ligands, many of which are known to be regulated *in vitro* by cytokines secondarily induced by rIL-2, was investigated.

Before treatment with rIL-2, few leucocytes were noted in the dermis, the predominant cell type being CD68 positive histiocytes. Following rIL-2 increases in T-cells (CD3), monocyte/macrophages (CD68/CD14) and neutrophils (CD67/NP57) were noted. Most T-cells were of the CD4 (helper/inducer), CD45RO (memory) phenotype. Increases in cells expression activation markers such as the IL-2 receptor (CD25) and TNF-α were observed following rIL-2. Very few B-cells (CD20) or virgin T-cells (CD45RA) were noted before or after rIL-2. Although an increase in the number of cells bearing NK markers (CD56 and CD57) was observed in the dermis following rIL-2, the numbers were low compared with other cell types.

T-lymphocytes and macrophages were the predominant cell type in the secondary cutaneous deposits studied. The IL-2 receptor was expressed by many of the T-cells present the majority of which also bore the CD4/CD45RO markers. Few B-cells, neutrophils or cells bearing NK markers were noted in these metastatic deposits. Following a five day course of rIL-2, there were no changes in the numbers of leucocyte subsets present. In particular no increases in T-cells or NK cells were noted.

It is now recognised that the margination and extravasation of leucocytes at sites of inflammation are mediated by adhesion molecules, many of which are regulated by cytokines induced by rIL-2. The upregulation of ICAM-1 expression in skin following rIL-2 observed by others was confirmed and increased expression of its ligand LFA-1 on mononuclear cells was also noted. Increases in expression of VCAM on endothelial cells and monocytes was noted as well as upregulation of its ligand VLA-4 by the infiltrating mononuclear cells. Similarly, changes in the selectin group of adhesion molecules (E, P and L-selectin) were noted following rIL-2. Upregulation of PECAM (CD31) which may be involved in homophilic interactions was also noted on the infiltrating mononuclear cells and endothelium.

Although all the adhesion molecules studied were expressed at higher levels in sites of metastatic tumour when compared with normal skin, little change was observed in any of the adhesion molecules following rIL-2.

Based on the experience of others, a single 5 day infusion is unlikely to yield a measurable tumour response. The demonstration here of widespread endothelial activation and leucocyte extravasation in uninvolved tissue after this period does, to some extent, explain the low therapeutic index of rIL-2 when administered in this way.

9:3. Increasing the therapeutic Index of rIL-2.

9:3:1. Predictors of response.

As discussed previously the effects of IL-2 *in vivo* are manifold. Knowing which mechanisms are involved in IL-2 mediated tumour regression and toxicity is important in helping to determine which immunological parameters should be used to define the optimum dose and schedule of administration. Such information might also contribute to predicting which patients would respond to IL-2 therapy. Reports of a correlation between the anti-tumour activity of rIL-2 and biological effects have been made including degree of lymphocyte rebound (West et al 1987), numbers of CD25+ve lymphocytes (Hermann et al 1991), prolonged TNF-α induction (Blay et al 1990), presence of infiltrates and HLA-DR expression in tumour (Cohen 1987, Rubin 1989), treatment induced hypothyroidism (Atkins et al 1988), levels of NK and LAK cell induction (Walewski et al 1989) and induced levels of C-reactive protein (Broom et al 1992). So far, no single parameter of biological effect or immunomodulation has consistently been shown to be predictive of response.

In the absence of immunological clues, clinical trials of IL-2 have primarily focused on the conventional end points of response rate and toxicity. Although the initial response rates with IL-2 in melanoma and renal cell carcinoma were encouraging, the costs in both quality of life and financial terms were high, leading to something of a backlash in the medical (Moertel 1986) and lay press. As a result, investigators have begun to study regimens of IL-2 with the aim of maintaining a worthwhile response rate, while minimising the inconvenience and toxicity experienced by patients. There are several ways in which this might be achieved.

Studies using animal models suggests that the received dose of rIL-2 and the schedule of administration are important determinants of response and toxicity. The small amount of comparative data on the treatment of human tumours also suggests that these parameters are important. Addition of *ex vivo* stimulated effector cells may allow a reduction in the dose of IL-2 received by patients and therefore lead to reduced toxicity.

9:3:2. Adoptive immunotherapy.

Although LAK cells do not accumulate specifically in tumours (Lotze et al 1980), their ability to lyse NK resistant targets and autologous tumour cells has been taken as evidence that LAK cells are indeed the effector cells associated with tumour regression. The addition of *ex vivo* stimulated LAK cells augmented the anti-tumour activity of IL-2 in a murine system, allowing a lower total daily dose of IL-2 to be given. This reinforced the view that LAK cells mediated tumour regression (Mule et al 1985).

The synergistic effect of IL-2 and LAK cells in this moderately immunogenic murine sarcoma model has not been reproduced in clinical trials in human cancer. The initial response rates noted in IL-2 and LAK cell therapy were encouraging (Rosenberg et al 1985b) particularly in view of the toxicity reported subsequently with high dose IL-2 alone (Lotze et al 1986). Initial reports from the NCI extra-mural units also suggested response rates approaching 50% with a less toxic regimen, using lower doses of IL-2 given over a longer period of time following LAK cell infusion (Eberlein et al 1988). The most recent review by Rosenberg however, suggests that addition of LAK cells may only improve complete response rates (Rosenberg et al 1989). Although not prospectively randomised trials, results from two large series in metastatic malignant melanoma reported response rates of 22% with IL-2

alone (Parkinson et al 1990b) and 14% using IL-2/LAK (Bar et al 1990). The more recent results of IL-2/LAK therapy in renal cell carcinoma have also been disappointing (Parkinson et al 1990a). European trials comparing IL-2 with and without LAK cell therapy in renal cell carcinoma also reported no significant difference in response rates (Negrier et al 1989). Although fractionating the daily dose of IL-2 given concurrently with LAK cells improved efficacy in animal models (Ottow et al 1987), the evidence from a randomised trial comparing bolus and continuous infusion of IL-2 during LAK cell therapy suggested no difference in efficacy (Weiss et al 1989). Rosenberg (1993) reporting the results of a randomised study of rIL-2 with or without LAK cell infusion demonstrated a trend towards increased survival in patients with melanoma receiving rIL-2/LAK but no difference in patients with renal cell carcinoma. Rosenberg's group reported no difference in toxicity between patients treated with rIL-2 alone compared with rIL-2/LAK.

The exact role of the adoptively transferred cells remains unclear and studies have suggested no correlation between response or toxicity and the number or cytolytic activity of LAK cells reinfused (Boldt et al 1988, Paciucci et al 1989). It is possible that LAK cells exert their effect by mechanisms other than direct cytolysis, for example induction of other cytokines.

Although Rosenberg's experience suggests that the administration of LAK cells does not enhance the toxicity of IL-2, data from Sondel's group have demonstrated greater toxicity when LAK cells were added to IL-2 given by infusion (Albertini et al 1990). Given the confidence intervals involved in the assessment of response rates in the phase II trials performed and the borderline results of Rosenberg's randomised study, infused IL-2 alone is probably as effective as moderate dose IL-2 /LAK cell therapy and considering the logistic and financial implications of *ex vivo* LAK cell

generation, it is perhaps not surprising that this mode of therapy has not been widely adopted.

Tumour infiltrating lymphocytes (TIL) can be extracted from solid tumours, expanded *in vitro* and used for adoptive immunotherapy. They are 50-100 times more effective than LAK cells in eradicating metastases in animal models (Rosenberg et al 1986), often exhibit absolute or relatively specific cytotoxicity for autologous tumour targets (Topalian et al 1987) and localise to tumour sites (Fisher et al 1989). Preliminary reports of this mode of adoptive immunotherapy in the treatment of metastatic malignant melanoma were impressive with response rates of 55% (Rosenberg et al 1988). The toxicity of the regimen as a whole was lower than during adoptive immunotherapy with LAK cells since the duration of IL-2 treatment was shorter (5 days compared with 15 days). The use of TIL may hold great promise for improved efficacy with reduced toxicity but again the logistic implications are likely to impede widespread use.

9:3:3. Inhibitors of toxicity.

In parallel with the increasing clinical experience of IL-2, the mechanisms which mediate its toxicity are becoming clearer. Some of these mechanisms have been shown to be schedule dependent and the use of specific inhibitors of such pathways may also reduce toxicity, hopefully without compromising the anti-tumour effect.

9:3:3:1. Phosphodiesterase Inhibitors.

Pentoxyifylline increases intracellular cAMP levels by inhibiting phosphodiesterases. Cyclic AMP may reduce vascular permeability directly

by preventing membrane gap formation and constricting gap junctions. In addition, raised levels of cAMP inhibit the secretory function of neutrophils (Nourshargh and Hoult 1986) and the release of TNF (Strieter et al 1988). In a guinea pig model, pretreatment with pentoxifylline reduced both the IL-2 induced increase in pulmonary vascular permeability and reduced the degree of inflammatory infiltrate (Ishizaka et al 1989). The effect of agents such as pentoxifylline on the anti-tumour activity of IL-2 are unclear but warrant further investigation in view of their potential effects in preventing the vascular leak syndrome.

9:3:3:2. Corticosteroids.

Glucocorticoids are known to inhibit the IL-2 stimulated expansion of T-cells (Arya et al 1984), reduce the expression of IL-2 receptors on lymphocytes (Reem & Yeh 1985) and inhibit the biosynthesis of TNF (Beutler et al 1986). A reduction in the vascular leak syndrome was noted with the concurrent use of cortisone acetate in the mouse model of IL-2/LAK therapy (Rosenstein et al 1986). The same group demonstrated subsequently that cortisone reduced *in vitro* and *in vivo* LAK cell generation in a dose dependent fashion. The anti-tumour effect of IL-2 alone was abrogated and the efficacy of IL-2/LAK was reduced (Papa et al 1986). In therapeutic trials of IL-2/LAK therapy, concurrent use of dexamethasone in a small group of patients was compared with retrospective controls (Vetto et al 1987). Dexamethasone significantly reduced the incidence and severity of dyspnoea, confusion and fever. Effects on creatinine and bilirubin were also less. Although patients were able to receive more of the scheduled IL-2 doses, no responses were seen in the six patients studied. In a similar study, the concurrent use of dexamethasone prevented the induction of TNF and C-reactive protein in most cases (Mier et al 1990). IL-2 associated toxicity was reduced and the maximum tolerated

dose was increased up to threefold. The antineoplastic activity was not completely abrogated with one partial and two minor responses being noted in 19 patients studied. The possible role of steroids as an adjunct to IL-2 therapy requires further assessment

9:3:3:3. Cyclooxygenase Inhibitors.

One of the effects of the cytokines induced as a result of IL-2 therapy is the activation of cyclooxygenase, leading to accumulation of prostaglandins and thromboxanes. The results of clinical trials of cyclooxygenase inhibitors as an adjunct to IL-2 based therapy are, at first sight, contradictory. Michie et al (1988) reported that the use of ibuprofen in association with bolus IL-2/LAK therapy was associated with reduced fever, tachycardia and attenuated the induction of stress hormones such as ACTH and cortisol. A simple symptom score was also estimated and found to be much lower in patients who received ibuprofen. The same group subsequently showed that ibuprofen did not compromise *in vitro* assessments of immunomodulation, or therapeutic efficacy (Eberlein et al 1989). Cyclooxygenase inhibitors may precipitate renal dysfunction and lead to fluid retention. Reports from other groups have demonstrated that creatinine levels and weight gain were significantly higher in patients who received indomethicin in addition to IL-2 given by infusion (Sosman et al 1988). The absence of comparative studies makes comment difficult, but cyclooxygenase inhibitors may relieve troublesome side effects of IL-2 therapy at the expense of renal toxicity

9:3:3:4. Other strategies.

Klausner's group have demonstrated that specific inhibitors of thromboxane synthesis and lipoxygenase inhibitors, as well as drugs to scavenge free

radicals, may reduce pulmonary and vascular toxicity in animal models (Klausner et al 1990 & 1991). Such agents have not yet been used in man in an attempt to abrogate the toxicity of IL-2.

More recent approaches to reducing toxicity include the use of cytokine antibodies and binding proteins. In an animal model, administration of polyvalent anti-sera to murine TNF led to a reduction in toxicity without compromising the anti-neoplastic effect (Fraker et al 1989). Anti-TNF antibodies allowed up to 60% more rIL-2 to be given and led to a further reduction in tumour burden.

9:3:4. Low dose schedules: subcutaneous administration.

The use of low dose IL-2 has been investigated using the subcutaneous route (s.c.) (Atzpodien et al 1990a). In this study, IL-2 was self administered in escalating doses, up to 14.4 x 10^6 I.U./m^2/day. Maintenance therapy was continued in patients with stable or responding disease at a level of 10.8 x 10^6 I.U./m^2/day. Toxicity was described as mild, although weight loss was noted. Local erythema and induration were seen at injection sites in the majority of patients. Peak serum levels of 25 to 30 I.U/ml of IL-2 occurred at 10-12 hours with detectable levels still present at 14 hours. One response was noted in a patient with renal cell carcinoma. Another pilot study of s.c. IL-2 in renal cell carcinoma documented 2 responses in 5 patients (Sleijfer et al 1990). Several parameters of immunological activation, namely increases in HLA-DR and CD 25 expression were similar to those seen in infusional therapy.

Although patient numbers are small, these studies demonstrate that IL-2 may be self-administered by out-patients. The s.c. route is associated with a high incidence of antibodies to IL-2, which in some studies are non-neutralising (Kolitz et al 1988), but in others the development of IL-2 specific IgG was

associated with neutralising activity in one third of patients treated (Whitehead et al 1990). Despite the variability in pharmacokinetics s.c. IL-2 seems to provide low, but prolonged, serum levels and measurable immune activation with documented responses. Perhaps most importantly, such regimens may provide the basis for combination therapy with other cytokines such as interferon-α. (Atzpodien et al 1990b).

9:3:5. Regional therapy.

The local administration of IL-2 may ensure high tumour levels which may lead to *in situ* activation of effector cells while keeping systemic levels and associated toxicities to a minimum. In animal models, doses of IL-2 as low as 100 I.U. have been shown to mediate regression of an immunogenic tumour when it is administered subcutaneously around the tumour (Vaage et al 1987). In human cancer, many metastatic sites are simply not accessible however certain clinical situations such as ovarian carcinoma and tumours of the head and neck may lend themselves to locoregional therapy.

9:3:5:1. Intraperitoneal (i.p.) therapy.

Although I.P. IL-2 may result in local levels 100 times those found in the peripheral circulation, this route is associated with a similar level of toxicity to i.v. bolus therapy (Lotze et al 1986). This may be a result of the low but sustained plasma levels measurable after i.p. IL-2. Indeed responses were recorded in melanoma both inside and outside the peritoneal cavity. More recent studies have investigated the use of IL-2 with i.p. LAK cells in a variety of tumour types (Steis et al 1990). This approach was associated with considerable toxicity, though responses were seen in 2 of 10 ovarian and 5 of 12 colorectal cancer patients. Trials studying the use of i.p IL-2 in patients

with ovarian cancer confined to the peritoneal cavity (stage III) who had failed chemotherapy are in progress, with some responses being recorded at low dose levels (Lemberski et al 1990). Patients with minimal residual disease after surgery and chemotherapy, may be more likely to respond to IL-2 in view of their low tumour burden. A small study of i.v. IL-2 has been reported in this patient group (Benedetti-Panici et al 1989) but no data is available on i.p. IL-2 in this situation. The case for i.p IL-2 is not yet proven and specific problems associated with it, such as difficulties with access catheters and the development of ascites and adhesions, may preclude the prolonged use of this route.

9:3:5:2. Peri-lymphatic therapy.

Squamous cell carcinoma of the head and neck may be amenable to local therapy. Cortesina et al (1988) noted responses in 6 of 10 patients with recurrent head and neck tumours, when IL-2 was injected perilymphatically. No major systemic toxicities were detected, an important consideration in view of the necessarily palliative nature of this treatment.

9:3:5:3. Other approaches.

Attempts have been made to activate potential sources of effector cells *in situ* by splenic or hepatic artery infusion of IL-2 (Thatcher et al 1989, Mavligit et al 1990) Although this approach is feasible and an increase in LAK activity was documented in the peripheral circulation, the toxicity was found to be equivalent to the i.v. route. In addition, the high incidence of hypersensitivity to iodine based contrast media following therapy with IL-2 (Zukiwski et al 1990) may add to the logistical problems of repeat arteriography

For localised, accessible disease, local therapy with IL-2 may produce useful responses with minimal toxicity. The intraperitoneal, intrasplenic and intrahepatic routes are probably associated with similar levels of toxicity to i.v. schedules. These types of loco-regional therapy have not been the subject of randomised, controlled trials, consequently the advantage of this approach is not clear.

9:3:6. Gene therapy.

The aim of gene therapy is to allow local expression of cytokine genes or other elements of the immune response either at sites of metastatic tumour or in an attempt to induce an effective host immune response. Rosenberg's group have continued to exploit the observation that tumour infiltrating lymphocytes traffic to sites of tumour and transfection of TIL with cytokine genes during their *ex vivo* expansion, may increase their cytolytic capacity following reinfusion (reviewed by Hwu and Rosenberg 1994). Another way of increasing the specificity of cytokine gene expression, is the use of constructs which contain tissue specific promoters which ensure that the gene of interest is expressed only in tissues which contain the appropriate transcription factors (reviewed by Vile 1995)

An alternative approach to the immunotherapy of human cancer may be the induction of a specific host immune response by "vaccination" with autologous tumour cells into which cytokine or other genes have been transfected. Transfection of genes for cytokines, costimulatory molecules such as B7 and MHC molecules are effective in animal models (reviewed by Pardoll 1993) although as with all approaches using gene therapy, considerable ethical and technical difficulties exist. Nevertheless these techniques may ensure specific

delivery of cytokines thereby increasing the therapeutic index of cytokine therapy in human cancer.

9:4. Summary.

The feasibility of rIL-2 administration by continuous infusion and an examination of some of the biological consequences of its use have been investigated in the second part of this thesis. Therapy with rIL-2 using these schedules was associated with prolonged hospitalisation and considerable toxicity. The disappointing response rates reported here and by others do not warrant its continued use at these doses and by this route.

The possible anti-tumour mechanisms of rIL-2 are manifold but the mechanisms which may operate *in vivo* are not defined. In these studies, systemic administration of rIL-2 led to induction of cytokines such as TNF-α and IFN-γ in the peripheral circulation which could mediate anti-tumour effects but which probably also mediate the toxicity associated with treatment through induction of nitric oxide and increased expression of adhesion molecules leading to extravasation of leucocytes into normal tissues. Whether reducing the secondary production of cytokines or nitric oxide will influence toxicity or efficacy requires further investigation. Cytokines or simpler molecules such as nitric oxide may represent a final common pathway as physiological and immunological mediators and it may not be possible to separate their desirable and undesirable properties.

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Publications.

Aspects of the work in this thesis have been published in the following articles. A paper describing the changes in leucocyte subsets and adhesion molecules in skin and tumour following rIL-2 is in preparation.

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