"The Tumour Necrosis Factor (TNF) locus in Human Breast Cancer."

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ABSTRACT.

The Tumour Necrosis Factor (TNF) locus lies within the MHC locus on human chromosome 6. It encompasses the genes for TNF, Lymphotoxin alpha (LTα) and Lymphotoxin Beta (LTβ). The location of the locus has intimated a role for TNF in the progression of MHC-linked and also malignant diseases. Ten polymorphic sites have been detected within the locus (comprising of RFLPs and microsatellites) and consequently, disease-associated alleles are now being looked for. Certain alleles have been linked to increased levels of TNF expression and these higher levels are thought to contribute to the aetiology of disease states. In this study I have examined six of the polymorphic sites spanning the TNF locus in 84 unrelated breast cancer patients, presenting at a follow-up clinic in Glasgow Royal Infirmary. I obtained allelotype and genotype data on each individual and compared the results with those previously obtained within our group for two further adenocarcinoma populations (gastric, n=45 and colorectal, n=106) and a control population, n=115. The results show that there is not a defined malignant genotype at the TNF locus, but the breast population does differ significantly to the control population in allelotype expression at two of the polymorphic sites, the TNFc and TNFa microsatellites (p=0.0282 and 0.0023 respectively). The colorectal cancer population is significantly different to the controls with regard to allelotype expression at the TNFc locus (p=0.0111) and also shows a significantly different genotype expression pattern at this site (p=0.0248) (data supplied by group member, Hui-Hui Oh). I also found that, in the breast cancer population, there was an overexpression of two alleles and a genotype previously demonstrated to be associated with high TNF expression; TNFa2, TNFc2 and the TNFB*1, B*2 genotype at a Nco-1 endonuclease RFLP site. This leads us to suggest that the predicted higher levels of TNF in these patients may play a role in the progression to the malignant state.

CONTENTS.

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ABBREVIATIONS.

a.a.	Amino acid
B.C-G	Bacillus Calmette-Guerin
BM	Basement Membrane
bp	Base Pair
CAT	Chloramphenicol Acetyltransferase
CLA	Calyculin
DAG	Diacylglycerol
ECM	Extra Cellular Membrane
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
H_2O_2	Hydrogen Peroxide
HLA	Human Leukocyte Antigen
IDDM	Insulin Dependant Diabetes Melitus
IL	Interleukin
IFN	Interferon
LPS	Lipopolysaccharide
LTα	Lymphotoxin Alpha
LTβ	Lymphotoxin Beta
MCT	Macrophage Cytotoxin
Meth-A	Methylcholanthrene
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MnSOD	Manganous Superoxide Dismutase
MS	Multiple Sclerosis
NGF-R	Nerve Growth Factor Receptor
NO	Nitrous Oxide
nt	Nucleotide
OKA	Okadaic Acid
PAF	Platelet Activating Factor
PBLs	Peripheral Blood Leukocytes
PBMCs	Peripheral Blood Mononucleocytes
PBMNCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PGE_2	Prostaglandin E_2
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
RFLP	Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SLE	Systemic Lupus Erythematosis
SSCP	Single-Strand Conformational Polymorphism
Tam	Tamoxifen
TILs	Tumour Infiltrating Lymphocytes
TNF	Tumour Necrosis Factor
UT	Untranslated

FIGURES and TABLES.

INTRODUCTION

In 1893, Dr. W.B. Coley first described the phenomenon of tumour necrosis, which he observed in human cancer patients injected with bacterial toxins derived from *Streptococcus* and *Serratia*. This lead to the initial supposition that the albeit short-term regression resulted directly from the "mixed toxin" administration. Years later, O'Malley *et al* (1962) demonstrated that a serum factor which was induced in mice by the injection of lipopolysaccharide (LPS) caused haemorrhagic necrosis. It was not until 1975 however, that Carswell confirmed the presence of this serum factor which could mimic the necrotic actions of endotoxin. He was studying bacillus Calmette-Guerin (BCG)- infected mice after treatment with LPS from E. coli and observed secondary "haemorrhagic necrosis" of transplanted tumours. His early tests indicated that the factor was probably released from macrophages in response to endotoxin. Due to the biological actions observed, he named this factor Tumour Necrosis Factor (TNF). This was followed by the isolation of TNF from rabbit and human sera (Green *et al*, 1976 ; Matthews *et al*, 1978).

Twenty years on, a vast spectrum of biological activities has now been determined for TNF and its two closely related cytokines; Lymphotoxin Alpha (LTα) (previously TNFβ or LT) and Lymphotoxin Beta (LTβ). These three molecules are produced by a variety of cell types in response to various insults. Through interaction with the "cytokine network" and intracellular signalling via cell-surface receptors, their range of activities includes roles in the host defence against infection, immunomodulation, anti-tumour actions and an active role in septic shock and cachexia.

Discovery.

It was not until 1984, some 10 years after its initial detection, that the gene sequence of TNF was established, cloned and the protein product expressed in two independant studies in California; Pennica at Genentech and Shirai at the Beckman Research Institute. Both groups showed purified TNF to have a relative molecular mass of 17,000 by SDS-PAGE, but nearer 40,000 after non-denaturing gel filtration, indicating that TNF may occur naturally in a multimeric form. Both also demonstrated its ability to induce haemorrhagic necrosis of murine tumours *in vivo*.

Concurrent studies by Beutler *et al* (1985), were focusing on the metabolic changes which occur during chronic infection, including anorexia and suppression of lipoprotein lipase. This wasting, termed "Cachexia" was one of the biological effects induced by the macrophage-secreted protein cachectin. Beutler isolated the hormone from macrophages and on sequencing, reported a homology to TNF so strong, that he concluded they were one and the same molecule. This was evidence that TNF was capable not only of acting on tumour cells, but could also modulate the metabolic activity of normal cells.

In the same year, Gray *et al* (1985) successfully cloned and expressed the cDNA of Lymphotoxin (now LTα) in *E.coli*..They showed it to be a glycoprotein with a relative molecular mass of 60,000-70,000 using molecular sieve chromatography, whereas the monomer was only 25,000. Its anti-tumour activity was tested in an *in vivo* tumour necrosis assay involving growing methylcholanthrene-induced (Meth-A) sarcomas in mice then injecting the tumours directly with Lymphotoxin. The extent of tumour necrosis was determined 24 hours postmortem and shown to be significantly high, with up to 75% necrosis being observed.

In 1985, human TNF and LT were localised to chromosome 6 between 6p23 and 6q12 by Nedwin *et al*, using Southern blot analysis of human/murine somatic cell hybrids. Sequencing revealed that the two genes were at least 30% homologous. This fact, along with the apparent close linkage, suggested they were ancestrally related.

The precise localisation of the genes was determined in the following year by Spies *et al* (1986). Using Southern blot analysis of various MHC deletion mutants and *in situ* hybridisation, he assigned TNF and LT to between HLA-DR and HLA-A.

It was not until 1993 that the third gene in the TNF locus was identified (Browning *et al*, 1993). They showed that the cell-surface form of the Lymphotoxin molecule formed a complex with a 33kD glycoprotein. Cloning and sequencing of this molecule revealed it to have significant homology to both TNF and LT, although more with LT. Furthermore, its genomic location was found to be next to the previously mapped TNF-LT locus. To this end it was named LTβ to distinguish it from LTα.

Cells of origin and production regulation.

Until 1985, TNF was also known as Macrophage Cytotoxin (MCT) because the macrophage was thought to be the only source. Since then, various other cell types have been shown to produce TNF and indeed LT. Under normal physiological conditions neither TNF nor LT are detectable, but become so in response to various stimuli including lipopolysaccharide (LPS), concanavalin A, bacterial proteins, viral agents, phorbol diesters, Ca^{2+} ionophores, phytohaemagglutinin and fungi. LPS stimulates TNF and LT production from monocytes/macrophages (Aggarwal *et al*, 1985), mast cells (Young *et al*, 1987) and brain astrocytes (Chung *et al*, 1990). Haematopoietic cells can be induced to express TNF and LT, but not in response to LPS. Functional T cells secrete TNF on stimulation with concanavalin A (English *et al*, 1991) and LT in response to antigenic or mitogenic stimulation (Early *et al*, 1986). Lymphocytes and natural killer cells can produce TNF in response to the crosslinking of the cell-surface antigen CD16 (Anegon *et al*, 1988).

Viruses such as Epstein-Barr, herpes simplex and Sendai alter TNF expression in PBLs, with the latter inducing TNF mRNA synthesis. (Berent *et al*, 1986 ; Gosselin *et al*, 1992). The same induced expression is seen with mycobacterial proteins (Wallis *et al*, 1990), malarial parasite antigens (Picot *et al*, 1990), tumour cell membranes and some tumour cell proteins (Hasday *et al*, 1990 ; DeMarco *et al*, 1992). Many cytokines are involved in the control of TNF and LT expression. Upregulation is induced by INF-γ (Beutler *et al*, 1986), IL-1β (Bethea *et al*, 1992), IL-1 (Nedwin *et al*, 1985), GM-CSF (in concert with INF-γ in human monocytes) (Hart *et al*, 1988), platelet activating factor (PAF) (Maier *et al*, 1992) and TNF itself. Cytokines inducing downregulation include IL-4 (Essner *et al*, 1989) and IL-10 (Ralph *et al*, 1992). Thus TNF plays an integral role in the inflammatory response.

Biological Effects.

The ability of TNF and LT to modulate cellular function has been studied closely *in vivo* and *in vitro*. Their respective individual activities as well as in combination with other polypeptides such as INF-γ and the interleukins has been examined. In general, the main effects are on growth and immunomodulation, but as described below, these multifunctional cytokines have diverse effects on different cells and tissues.

(a) In vitro studies.

Cell lines can be categorised on the basis of their response to LT and TNF (Goeddel *et al*, 1986); (1) Cytostatic or cytolytic, (2) Unaffected, (3) Growth stimulation. TNF was originally described by Carswell (1975) as an anti-tumour agent. It has subsequently been shown that TNF and LT are cytotoxic to normal cells as well as tumour cells (Kawakami *et al*, 1989). Approximately 40% of described tumour cell lines are sensitive to the antiproliferative (cytostatic) or necrotic (cytolytic) actions. In some cases, as with the transformed murine fibroblasts, L929, viability can be reduced by as much as 50%. Cell lines which are resistant to TNF or LT cytotoxicity appear not to differ in the amount of cell surface receptors, or in the ability to internalise the cytokines (Sugarman *et al*, 1985). This suggests the resistance to cytokines is due to a defect in the signal transduction pathways following receptor-binding. In addition, some TNF-resistant tumour cell lines have been shown to secrete small amounts of TNF (Spriggs *et al*, 1987). This protective capacity of low dose TNF results in the induction of protective proteins such as manganous superoxide dismutase (MnSOD), the mitochondrial antioxidant enzyme that protects from oxygen free radicals (Wong *et al*, 1992).

The cytotoxic effects of TNF and LT can be enhanced by other cytokines, anti-tumour drugs and heat. Both TNF and LT synergise with IFN-γ to kill tumour cells (Lewis *et al*, 1987 ; Matsuo *et al*, 1992). IL-1 also increases the cytotoxicity towards some tumour cell lines (Ruggerio *et al*, 1987), while cytotoxicity can also be further enhanced when TNF is administered along with IFN-γ and heat treatment (Dubois *et al*, 1989). The cytotoxic effect of the drug Tamoxifen is increased by TNF when given to MCF-7 cells (Matsuo *et al*, 1992).

As well as inhibiting proliferation, LT and TNF can also have the opposite effect ie. to induce growth. This is not limited to normal cells, as growth stimulation has been demonstrated on several tumour cell lines including B cell leukaemias (Digel *et al*, 1989) and cervical carcinomas (ME-180neo; Lewis *et al*, 1987). Suggested mechanisms for this have been reviewed (Vilcek and Palombella, 1992) and include induction of responsiveness to other growth factors and induction of genes mediating progression through the cell cycle. In 1985, Sugarman *et al* demonstrated the ability of LT to act as a growth stimulator and a cytotoxic agent towards the same cell lines. For example, at low doses of LT, WI38 cells were stimulated, whereas at high LT concentrations, given in concert with INF-γ, these cells sucummed to the cytotoxic effects.

The immunomodulatory roles of TNF and LT have been demonstrated by various *in vitro* studies of their effects on haematopoeitic cells. Both are secreted by activated T (Gray *et al*, 1984 ; Green *et al*, 1986) and B (Nedwin *et al*, 1985 ; Sung *et al*, 1988) cells and act as autocrine growth factors by upregulating cell-surface receptor expression and further TNF production. MHC class-I molecule expression is also upregulated on T cells (Schewrich *et al*, 1987), whereas MHC class-II expression is unaffected. Both TNF and LT mediate neutrophil activation and increase oxygen free radical production (Ozaki *et al*, 1988). As well as being the major source of TNF, macrophages are also activated by it. The cells secrete cytotoxic molecules such as nitrous oxide (NO) and H_2O_2. The tumoricidal activity of TNF is enhanced by its ability to induce monocyte chemotaxis and lymphocyte cell adhesion molecules on endothelial cells (Bevilacqua *et al*, 1987). These activities may attract phagocytes from the blood to the sites of tumours.

The catabolic actions of TNF and LT stem from their actions on adipose tissue and bone. TNF (by its other name cachectin), was identified as the hormone able to modulate metabolic activity in adipocytes (Beutler *et al*, 1985 ; Torti *et al*, 1985). Lipoprotein lipase and fatty acid synthase are suppressed and glycerol release is stimulated. These actions serve to mobilise energy stores in times of acute illness. TNF is regarded as a mediator of protein catabolism in muscle cells (Tracey *et al*, 1986) and also of the decreased transmembrane potential observed in shock (Tracey *et al*, 1986).

Both TNF and LT alter the properties of endothelial cells. Upregulation of cell surface adhesion molecules promotes procoagulant properties and the inflammatory state (Wertheimer *et al,* 1992). This is further induced by the downregulation of thrombomodulin and protein C (Nawroth *et al,* 1986) and endothelin 1, the vasoconstrictor is induced in endothelial cells, contributing to organ dysfunction in septic shock (Marsden & Brenner, 1992).

TNF and LT have anti-parasitic and anti-bacterial properties, each mediating through different cell types. TNF enhances the cytotoxicity of eosinophils for schistosomula (Silberstein *et al,* 1986), whereas LT, in combination with IL-2, activates macrophages allowing the killing of *Mycobacterium avium* organisms.

(b) In vivo activities.

The *in vivo* effects of TNF and LT are largely dependant on the site of production or administration and the amount produced. At low concentrations, they can be beneficial to the host by controlling infections by viruses, bacteria and parasites, promoting an inflammatory response and also contributing to tumour cytotoxicity. However at high doses, tissue injury, organ failure and septic shock are all induced. The anti-tumour effect of TNF varies with the site of administration; intratumoral, intraperitoneal or intravenous (Spriggs & Yates, 1992).

Chronic TNF exposure has been implicated as a mediator of the wasting state cachexia (Beutler & Cerami, 1986). Further to this, Tracey *et al,* (1990) showed that grafting a TNF-producing tumour to the leg of mice caused cachexia, whereas grafting 1 million TNF-secreting cells to the brain caused severe anorexia, with a 25% reduction in body weight observed after 10 days.. TNF and LT induce septic shock which can be fatal (For review see Strieter *et al,* 1993).

A role for both cytokines is also suggested in the pathogenesis of non-infectious disorders and autoimmune diseases. Large amounts of LT are produced in rats undergoing experimental allergic encephalomyelitis, the animal model for human multiple sclerosis (M.S.) (Ellison *et al,* 1971). Sharief *et al* (1991) showed an association between increased TNF levels and disease progression in patients with M.S. Insulin dependant diabetes mellitus (IDDM) may be made worse by LT; LT synergises with INF-γ to upregulate MHC class II expression on β-cells in the islets of Langerhans, which could contribute to cellular destruction (Pujol-Borrell *et al,* 1987). There is increasing evidence for the role of TNF in the pathogenesis of other autoimmune diseases, namedly rheumatoid arthritis (Saxne *et al,* 1988), primary billiary cirrhosis (Miller *et al,* 1992) and systemic lupus erythematosis (SLE) (Maury *et al,* 1989).

The anti-tumour effects of TNF and LT on animal and human tumours have both been demonstrated in animal models. Studies in mice have shown that TNF augments T cell cytotoxicity (Tomazic *et al*, 1988), as well as directly causing activation of procoagulant activity in tumour endothelium (Gerlach *et al*, 1989). Recombinant human (rh)TNF displays anti-tumour activity against several different human tumour types *in vivo*, including mammary carcinomas, ovarian tumours, lung cancer and gastric tumours (Creasy *et al*, 1986; Sohmura *et al*, 1986 ; Manetta *et al*, 1989). Several general observations have arisen from rhTNF studies; (a) Intratumoral, high dose administration is most effective, (b) repeated administration is necessary for sustained anti-tumour activity, (c) actual hemorrhagic necrosis is rare and (d) a relatively high local dose is necessary. *In vivo* clinical work has been carried out, without a large amount of success (see later).

As with the *in vitro* studies, the anti-tumour response can be enhanced by other cytokines. TNF plus INF-γ show synergistic effects against rat and human ovarian tumour xenografts in nude mice (Manetta *et al*, 1989). Intravenous administration of TNF with IL-2 was shown by Winkelhake *et al* (1987) to inhibit growth of subcutaneous murine tumours. Synergy has also been demonstrated with IL-6 against pulmonary metastatic fibrosarcoma tumours (Mule *et al*, 1990) and IL-1 against pulmonary metastases of Lewis lung carcinoma (Ohkwra *et al*, 1990). TNF also augments the anti-tumour actions of irradiation (Leonard *et al*, 1992) and cytotoxic drugs such as adriamycin (Maruo *et al*, 1992).

The genes and their gene products.

In 1985, Nedwin *et al* localised the TNF and LTα (TNFβ) genes to chromosome 6. Southern blot analysis of DNA from human-murine somatic cell hybrids was carried out with cDNA probes. Further analysis of hybrids containing only fragments of human chromosome 6 localised it more precisely to between 6p23 and 6q12. A year later, Spies *et al* mapped them within the chromosomal segment between HLA-DR and HLA-A, within the MHC locus. A panel of MHC deletion mutants was investigated by genomic blot hybridisation and the results corroborated by *in situ* examination of metaphase chromosomes to a TNF cDNA probe. This result was confirmed and refined by Dunham *et al* in 1987. Using pulsed-field gel electrophoresis and 'cosmid walking', they established a map of the entire MHC locus, mapping TNF to approximately 250kb centromeric to the MHC class I genes.

Mature LT is made up of three 171 residue monomer units (Gray *et al*, 1984). His sequencing of the cDNA revealed a 14S transcript, 1,329bp in length with 79bp of 5' untranslated (UT) sequence and 626bp of 3' UT sequence. He also showed the presence of an additional polyadenylation sequence (AATAAA) upstream of the conventional poly(A) tail. Recombinant LT, purified on a mAb (LT-B) column generated a product with a relative molecular mass of 18K. A further species was also detected at 25K, made heavier due to N-linked glycosylation.

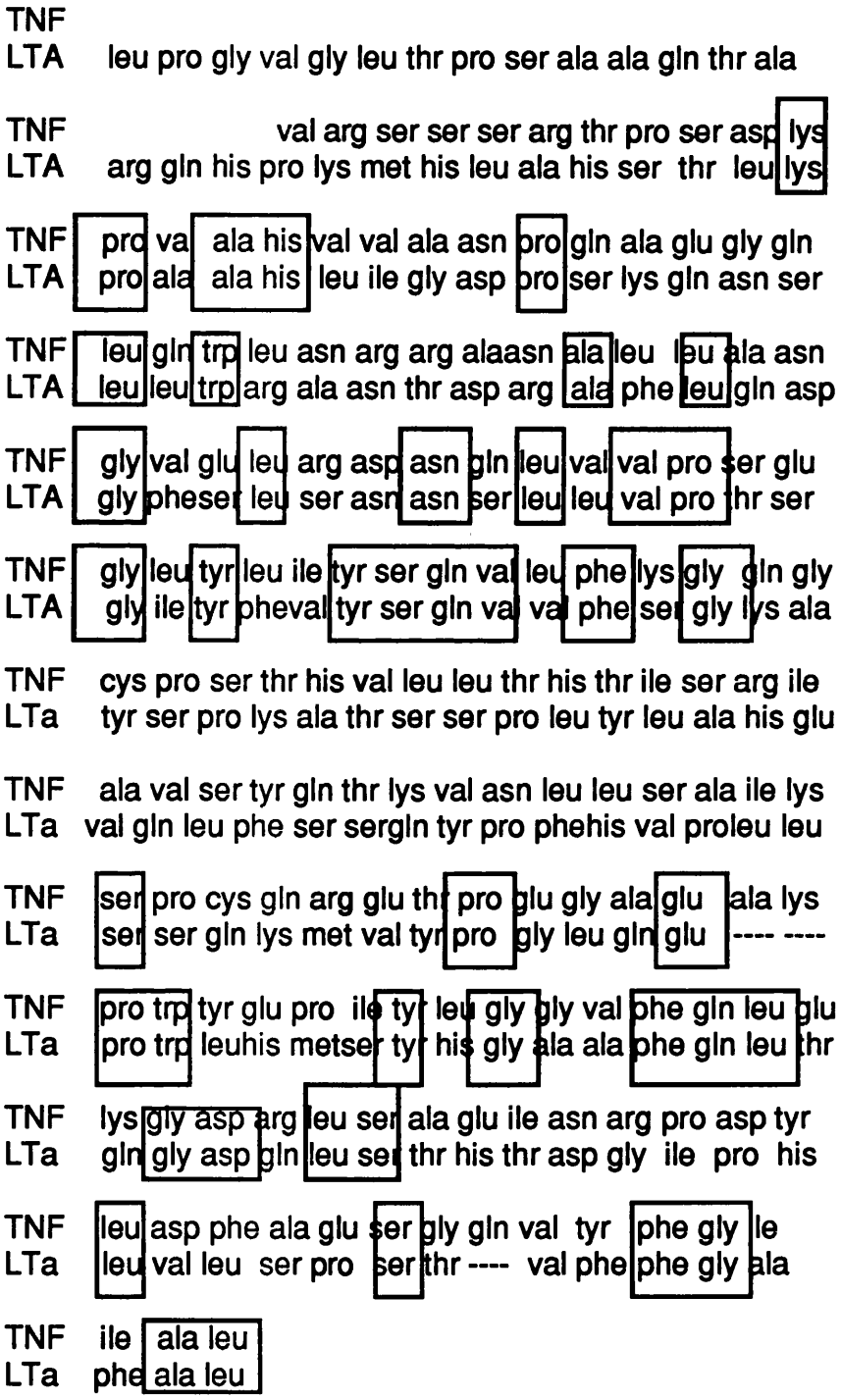
Pennica *et al* (1984) were the first to obtain the cDNA sequence of TNF and predict from this, the amino acid sequence. TNF was purified from PMA-stimulated HL60 cells. SDS-PAGE revealed a 17kDa protein. The cDNA was an 18S transcript of approximately 1,300bp;125 nucleotides (nt) 5'UT, followed by a Methionine residue and an open reading frame of 233 amino acids. The context of this Met. AUG codon conformed to a consensus sequence for eukaryote initiator sites [CCA/GCCAUGG]. Following the termination codon, were 792nt of 3'UT sequence. A long hydrophobic pre-sequence of 76 residues was also predicted.

Pennica compared the amino acid sequence of TNF with that of LT and showed the two molecules displayed 28% residue homology. Furthermore, many of the differences resulted from conservative amino acid changes [FIGURE 1].

At this time, another group (Shirai *et al* , 1984) successfully cloned the TNF gene and expressed it in *E.coli*. Their results were consistent with those of Pennica *et al*, in that SDS-PAGE yielded a 17kDa protein and the deduced amino acid sequence was also in agreement. Gel filtration under non-denaturing conditions revealed a 45kDa protein, suggesting that the active TNF molecule exists as a trimer.

A year later in 1985, Nedwin *et al* gained a more detailed insight to the structure of both the TNF and LTα genes. They were isolated from a human genomic DNA-l library then sequenced by the di-deoxy chain termination method. The LTα primary transcript contained three introns (Intron 1, at 287bp in length was inserted 9bp before the coding sequence. Intron 2, at 86bp in length was inserted one residue before the mature sequence and intron 3, at 247bp interrupts the 35th codon of the mature protein). The 5' UT region was shown to comprise 170 bases. Similarly to LTα, TNF was revealed to comprise of three introns, but only the third was in a homologous position to LTα. The 5'UT region was 180nt. The last exon, encoding 80% of the secreted LTα, and 89% of TNF showed the greatest homology (35%).The LTβ gene was not characterised until 1993, by Browning *et al*. Southern blot analysis confirmed its presence in the genome as a single copy gene. It was shown to be contained within 4 exons, in an arrangement similar to LTα and TNF, but being oriented in the opposite direction [FIGURE 2].

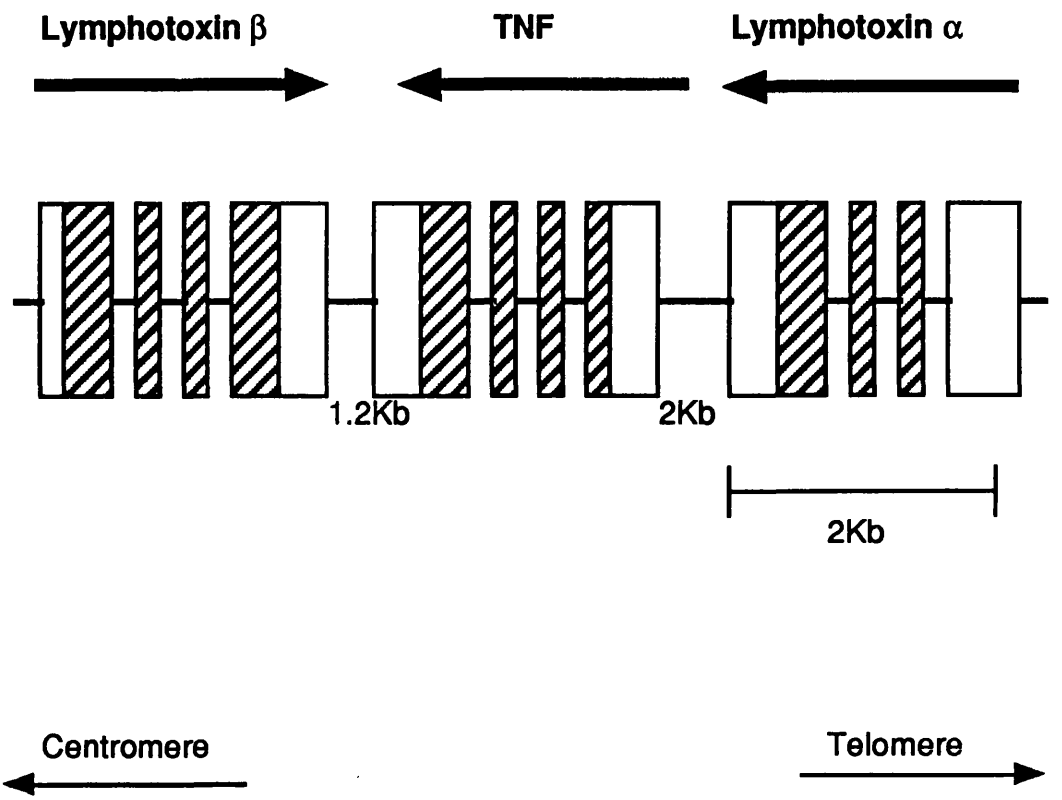
FIGURE 1. Comparison of the amino acid sequence of human TNF with human LTα.



The sequences have been aligned so as to show maximum homology, by adding two gaps in the LTa sequence (----). Identical amino acids have been boxed.

Adapted from Pennica et al, 1984, Nature 312, p728

FIGURE 2. Schematic representation of the region of human chromosome 6 encompassing TNF, LTα & LTβ.



Thick arrows indicate transcriptional orientation.
Hatched boxes represent translated portions of the genes and open boxes indicate untranslated portions.

The biologically active TNF molecule exists as a homotrimer composed of three 157 amino acid polypeptide subunits (Eck and Sprang, 1989). X-ray crystallography has revealed the structure to be a β-sandwich comprised of 2 anti-parallel β-sheets [FIGURE 3a]. The N-terminus is flexible, whereas the C-terminus is rigid. The N-terminus is not necessary for activity. On the other hand, deletions at the C-terminus can render the molecule inactive (Creasey *et al,* 1987).

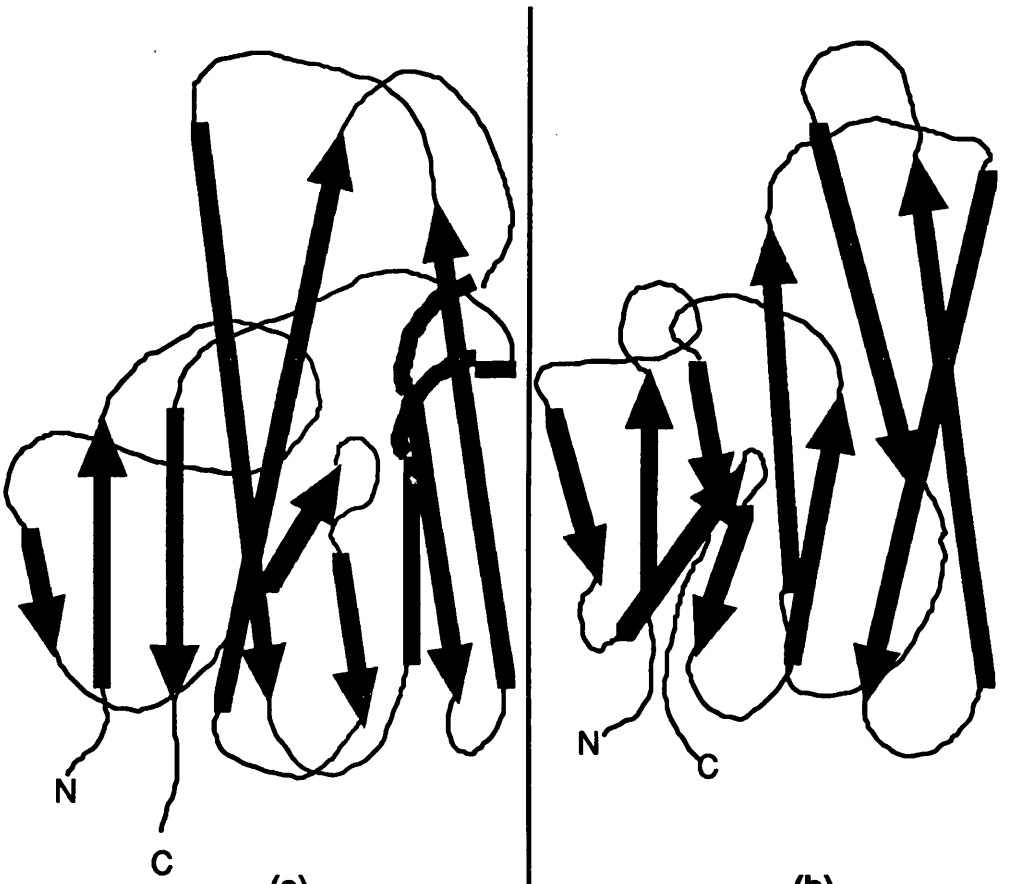
As with TNF, LT is folded into a β-sheet sandwich (Eck *et al,* 1992) [FIGURE 3b]. Hydrophobic interactions act to stabilise the trimeric structure. The residues conserved between LT and TNF are found mainly at the base of the trimer in the polypeptide loops joining the β–sheets. Results of site-specific mutagenesis of TNF (Smith *et al,* 1987) and LTα (Goh *et al,* 1991) have localised the domains involved in receptor interaction to the cleft between subunits near the base of the trimer. Indeed, the active sites of TNF were localised to 3 regions clustered at the base (Van Ostade *et al,* 1991). A mutation at one of these sites (Ala 84 to Val) abolishes cytotoxicity, while amino acid alterations in close proximity also reduced the cytotoxic activity. A mutation at position 91 (Val to Ala) decreased bioactivity, suggesting it is important in receptor binding.

The Receptors.

TNF and LT mediate their actions through binding to surface receptors which triggers a range of signalling pathways. Two forms of receptor have been identified and the cDNAs cloned [FIGURE 4]. The first has a relative molecular mass of 55kDa and is termed p55 or TNF-R1 (Loetscher *et al,* 1990). The second has a MW of 75kDa and is termed p75 or TNF-R2 (Schall *et al,* 1990). The p55 gene has been mapped to human chromosome 12, whereas p75 maps to human chromosome 1 (Milatowich *et al,* 1991). The structure of the receptors is homologous to the family of proteins displaying 4 extracellular cysteine-rich domains. This includes the nerve growth factor receptor (NGF-R).

The cDNA for the p55 encodes a 455a.a. sequence, comprising a 29a.a. hydrophobic leader sequence, a 182a.a. N-terminal extracellular domain, a 21a.a. transmembrane domain and a 233a.a. C-terminal intracellular domain. The cDNA of p75 encodes a 461a.a. protein containing a 22a.a. signal sequence, a 234a.a. extracellular domain, a 28a.a. transmembrane domain and a 176a.a. cytoplasmic domain. Most mammalian cells carry both types of receptor, although the ratio of p55 to p75 varies, as does the cells' response to the signals induced by the binding of either TNF or LT.

FIGURE 3. Schematic representations of (a) TNF monomer and (b) Lymphotoxin monomer.



(a)

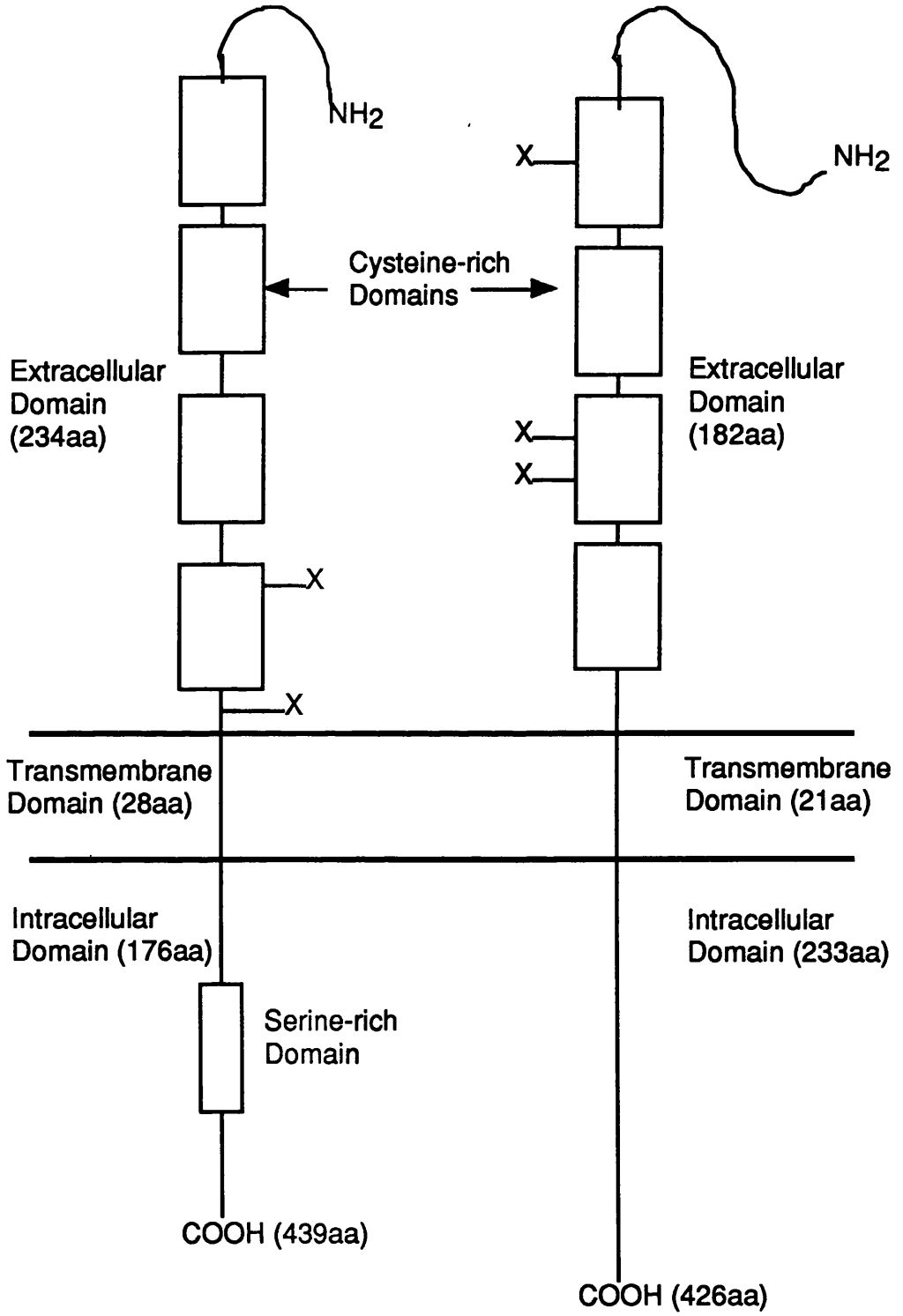
Adapted from Jones et al, Nature 338, p227.

(b)

Adapted from Eck et al, 1992, J. Biol. Chem. 267, p2121.

The beta sheets are represented by the thick arrows and the alpha chains by the thin lines.

FIGURE 4. Domain structure of receptors for human tumour necrosis factors.



RECEPTOR R2
[p75]

RECEPTOR R1
[p55]

X represents the glycosylation sites.
Figures in ( ) represent the number of amino acids in each domain.

Because the intracellular regions of both receptors show no homology, this suggested that they cause the activation of different cell signalling pathways (Lewis *et al,* 1991). There is still conflicting evidence regarding which receptor mediates cell proliferation and which is responsible for the induction of cell death or apoptosis. Heller *et al* (1992) showed that human HeLa cells which express mainly p55 are not killed by TNF, but if p75 expression is induced by transfection with the p75 gene, TNF causes cell death. Contrary to this result, Tartaglia *et al* (1991), using a murine model showed that p55 initiates cytotoxicity and also the induction of protective activity (manganese superoxide dismutase) in murine NIH3T3 cells, whereas p75 initiates the proliferation of murine thymocytes. These conflicting results suggest that the cellular response is dependant upon cell type.

Naturally-occurring soluble fragments of both receptors have been identified in human urine and serum (Engelmann *et al* ,1990 ; Schutze *et al,* 1990). They are truncated portions of the extracellular domains and could probably act to regulate the amount of active TNF and LT in circulation.

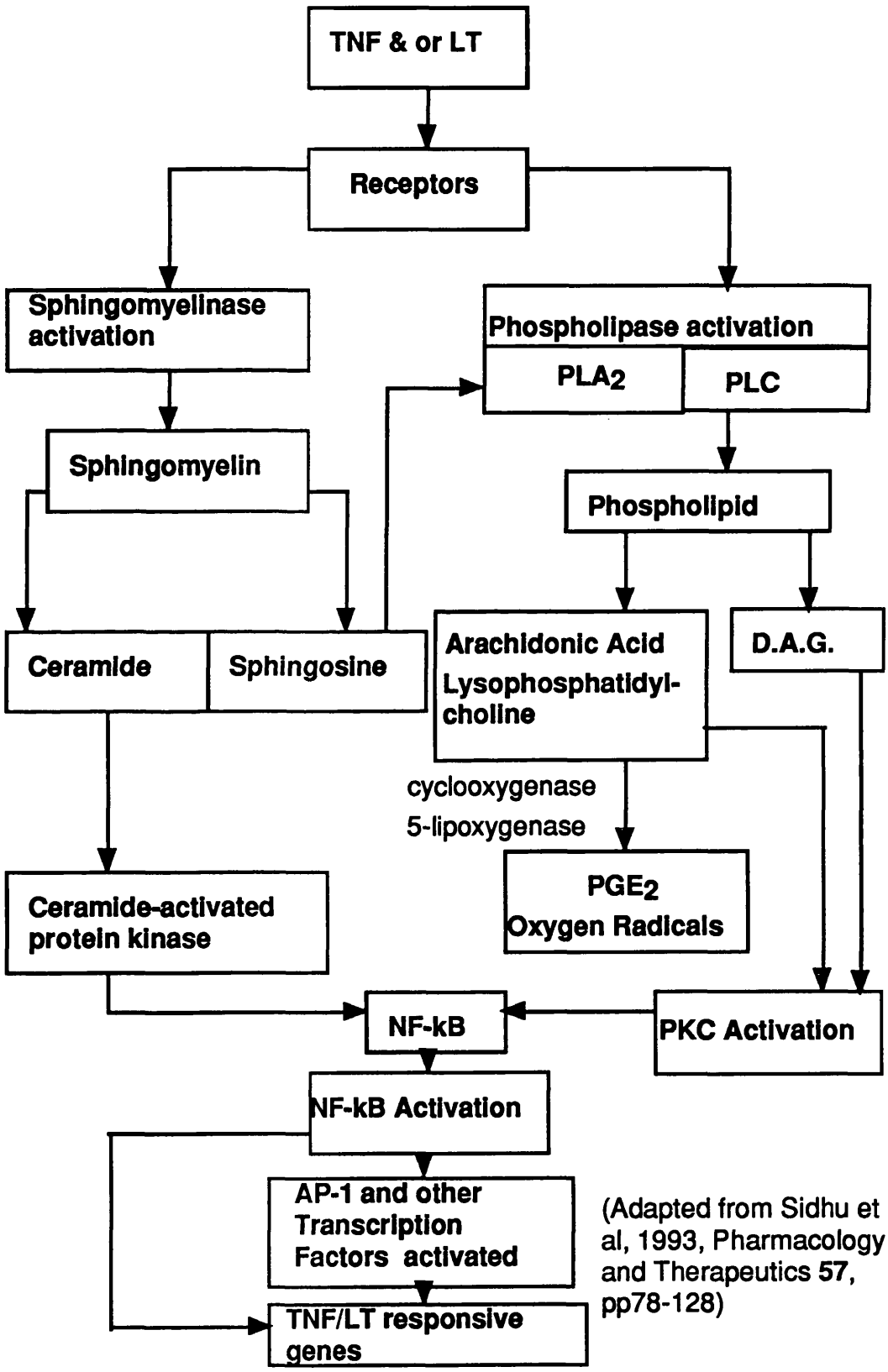
Cell Signalling.

On binding to their cell surface receptors, TNF and LT elicit a number of signal transduction pathways, mainly involving the activation of protein kinases, with the end result of the induction of TNF responsive genes [FIGURE 5]. The receptor sequences show that they do not possess any intrinsic protein kinase activity, but evidence is growing which suggests that TNF causes the activation of phospholipases as its main signalling mechanism.

Phosphatidlycholine-specific phospholipase C (PC-PLC) is activated by TNF binding to p55. This causes the production of diacylglycerol (DAG), which in turn, is responsible for protein kinase C (PKC) activation (Schutze *et al,* 1991).

Another phospholipase, PLA_2 is activated by TNF in a variety of cells (Wiegman *et al,* 1992; Palombella *et al,* 1989 ; Neale *et al,* 1988). As a consequence of this, unsaturated fatty acids such as arachidonic acid and lysophosphatidlycholine are generated. Both are capable of PKC activation. The actions of cyclooxygenase and 5-lipoygenase on the two afore mentioned products of PLA_2 activation can cause prostaglandin (PGE_2) and oxygen radicals to be produced, which in turn, lead to cellular damage. There is some evidence that pertussis toxin-sensitive GTP-binding proteins may couple the TNF receptors to PLA_2 (Yanaga *et al,* 1992 ; Imamura *et al,* 1988).

FIGURE 5.
Cell Signalling Pathways



(Adapted from Sidhu et al, 1993, Pharmacology and Therapeutics **57**, pp78-128)

Dressler *et al* (1992) showed that the activation of sphingomyelinase may be involved in TNF-induced signalling. Sphingomyelin is hydrolysed by this enzyme to ceramide and sphingosine (Kim *et al*, 1991). Ceramide stimulates the activation of magnesium-dependent protein kinases, including ceramide-activated protein kinase, whilst sphingosine as well as causing PKC activation, stimulates both PLA_2 and cyclooxygenase activity (Candela *et al*, 1992).

Studies involving protein phosphatases (protein kinase inhibitors) have confirmed the role of protein kinases in TNF-mediated signalling. Schutze *et al* (1990) showed that PKC activation occurred on TNF administration. Wright *et al* (1993) demonstrated that serine-threonine phosphatase inhibitors such as okadaic acid (OKA) and calyculin (CLA) synergistically augmented TNF-induced apoptosis in several TNF-sensitive tumour cell lines, including BT-20 mammary carcinoma and LNCap prostatic tumour cell lines.

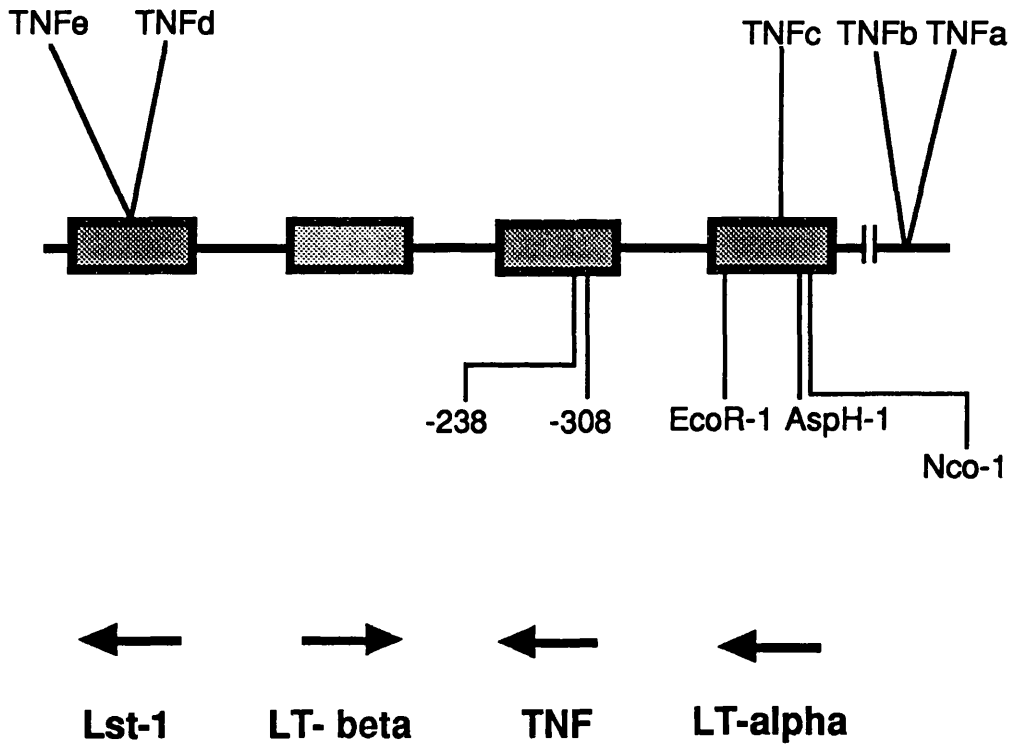
The subsequently activated protein kinases are involved in the activation of the nuclear transcription factor NF-kB. This can occur without protein synthesis, so that transcription of TNF responsive genes happens rapidly (Hohmann *et al*, 1990), or at the level of induction of the transcription of the NF-kB gene. Other transcription factors such as AP-1 (comprising c-fos and c-jun) are also activated by TNF (Brenner *et al*,1989), but only at the level of gene transcription. Thus, NF-kB is the only factor directly activated by TNF. The transcription factors go on to induce 'TNF-responsive genes' (Kronke, 1992).

TNF-responsive genes include transcription factors, growth factors, cytokines and cell surface antigens. Some of the primary response genes include a zinc finger protein, A20 (Opipari *et al*, 1992), which has been implicated in protecting cells from TNF cytotoxicity. The presence of the zinc finger structure suggests that A20 is a transcription factor which would control cell-survival through the modulation of genes induced in a protein synthesis-dependent response to TNF.

Genetic Polymorphisms.

Genetic polymorphism is essential for the function of certain proteins such as the MHC class I and class II cell-surface antigens. Studies recently have concentrated on the hypothesis that such polymorphic genes may play a role in the pathogenesis of HLA- and non-HLA-associated diseases. The TNF locus is the site of multiple polymorphic loci; to date, ten have been identified [FIGURE 6]. They are divided into three classes; naturally occurring restriction fragment length polymorphisms (RFLPs), single strand conformational polymorphisms (SSCPs) and

FIGURE 6. The polymorphic regions of the TNF Locus.



TNFe and TNFd lie within intron 2 of Lst-1. -238 & -308 lie within the promoter region of TNF. EcoR-1 lies within intron 4 of LTα. TNFc, AspH-1 & Nco-1 all lie within intron 1 of LTα and TNFb and TNFa lie 3.5Kb upstream of LTα.

microsatellites. Two of the polymorphisms originally identified as SSCPs lie within the promoter region of TNF at positions -308 and -238 (Wilson *et al,* 1993 ; D'Alfonso *et al,* 1994) and the three RFLPs are located within the LTα gene (Partanen et al, 1988; Webb et al, 1990 ; Ferencik *et al,* 1992). The five microsatellites (TNFa, b, c, d and e) are scattered along the entire length of the locus (Nedospasov *et al,* 1991; Jongeneel *et al,* 1991 ; Udalova *et al* , 1993). No polymorphic sites have yet been assigned to the LTβ gene.

Partanen *et al* (1988) were the first group to identify a polymorphic region in the TNF locus. Using various restriction endonucleases, they located a polymorphic EcoRI site in the 3'UT region of LTα exon 4. Two alleles were detected by Southern blotting, measuring 2.4kb and 2.5kb. The latter allele was present in only 4 of the 67 individuals tested. Because the RFLP is so uninformative (the rare allele only presenting in 6% of the population), little work has since been carried out on it. Partanen also showed a strong association between the rare allele and HLA-B40. In 1991, Verjans *et al* showed an association between the rare 2.5kb allele and HLA-Bw60, while demonstrating no association between the EcoR1 RFLP and the autoimmune disease, anklyosing spondylitis.

Nedospasov *et al* (1986) identified a polymorphic Nco-1 restriction site by comparisons of cloned genomic LTα sequences prior to the exact localisation of the TNF genes. Originally this site was wrongly assigned to the TNF gene (Badenhoop *et al,* 1989, Dawkins *et al,* 1989 and Fugger *et al,* 1989a & 1989b). It was not until two years later that its location within intron 1 of LTα was established by genomic Southern blotting using TNF and LTα probes, restriction site mapping of cloned genes and direct genomic sequencing (Webb *et al,* 1990; Abraham *et al,* 1991; Messer *et al;* 1991; Verjans *et al,* 1991). Messer *et al* (1991) showed that the polymorphic Nco-1 site is linked to an amino acid substitution in the native protein. The amino-terminal asparagine at position 26 is conserved in the rare TNFB*1 allele and threonine in the common TNFB*2 allele. This site is far more informative than the EcoR1 RFLP, with frequencies of 67% to 71% and 29% to 33% for B*2 and B*1 respectively (Fugger *et al,* 1989 and Messer *et al,* 1991). Because of this, several studies were undertaken to determine whether either of the Nco-1 alleles were associated with disease states or differential cytokine production.

Fugger *et al* (1989a) showed a decreased frequency of the more common B*2 allele in patients with primary biliary cirrhosis and in a subsequent paper, reported this decrease in four auto-immune diseases, along with a significant increase in the frequency of the rare B*1 allele in the patients (Fugger *et al,* 1989b).

Regarding differential cytokine production, Sachs *et al* (1990) reported decreased LTα secretion in IDDM patients. In the same year, Jacob *et al* (1990) stimulated monocytes from SLE patients with LPS and showed no association between TNF production and either allele, but demonstrated that B*1 homozygotes displayed increased LTα production compared to B*2 homozygotes. This was also shown by Messer *et al* (1991) using phytohaemagglutinin (PHA)-stimulated PBMCs. These two studies are in direct contrast to that undertaken by Pociot *et al* (1993) on a normal population. There appeared to be no difference in LTα secretion by PHA-stimulated PBMCs, regardless of Nco-1 allelotype, but LPS-stimulated monocytes from B*2 homozygotes displayed increased TNF secretion compared to cells from B*1 homozygotes, with heterozygotes displaying intermediate levels. This result is surprising considering the Nco-1 site is within the LTα gene. It may be explained by the demonstration that a strong positive association exists between B*1, HLA-DR3 and HLA-B8, alleles linked with an MHC-extended haplotype associated with immuno-inflammatory diseases. Earlier this year, Derkx *et al*, (1995) carried out a series of experiments which gave results in contrast to the study by Pociot *et al* (1993). They demonstrated that LPS-induced TNF release from PBMNCs was greatest in B*1/B*2 heterozygotes, followed by B*1 homozygotes, then B*2 homozygotes and suggested that the contradictory results were due to differing cell populations and stimuli.

The next polymorphic region to be defined (by SSCP) was the -308 site (Wilson *et al*, 1992). This lies within the promoter region of the TNF gene and results from a G to A transition polymorphism at position -308. Two alleles were detected following Nco-1 endonuclease digestion. Allele 1 (T1) gave two fragments of 87bp and 20bp whereas allele 2 (T2) gave a single 107bp fragment. The allele frequencies in 40 unrelated individuals were 0.84 and 0.16 respectively. The following year, the same group using PCR and again SSCP in HLA-typed individuals, showed the existance of a strong positive association between the rare T2 allele and the extended haplotype HLA-A1, B8, DR3. As this haplotype is associated with autoimmune diseases, the possibility arose of an additional link with the TNF locus. This was disputed however by Verjans *et al* (1994) in relation to ankylosing spondylitis. They found no significant differences in allele frequencies between AS patients and controls, thus concluding that predisposition to AS is not due to an association between TNF alleles and HLA-B27.

In 1994, Wilson *et al* suggested a possible functional role for the polymorphism. Using constructs with the TNF promoter linked to the CAT reporter gene, they showed the T2 allele to be associated with higher constitutive and inducible levels of TNF transcription than the T1 allele. Following this work, McGuire *et al* (1994) demonstrated a role for the polymorphism in the pathogenesis of fatal cerebral malaria. The disease severity is increased with higher circulating levels of TNF.

They showed a higher than expected gene frequency of the T2 allele in the Gambian population studied (0.16). This could be because the disadvantage is counter-balanced by another biological advantage. Heterozygotes may possess optimal TNF levels for response to other infections or alternatively the T2 homozygotes are protected from fatal conditions other than cerebral malaria.

Also in 1992, a biallelic RFLP was detected within intron 1 of the LTα gene by Ferencik *et al.* The group carried out a systematic analysis of 15 different endonucleases using a LTα-specific cDNA probe amongst 74 reference cell lines. Only AspH-1 showed a polymorphism resulting from a G to C base change. The two alleles detected gave fragments of 10kb and 8+2kb fragments with allele frequencies of 0.66 and 0.34 respectively. With regard to allelic associations with HLA alleles, the 8+2kb fragments associated with B7 and DR2, whereas the 10kb fragment associated with DR1, 3, 8 and 9. Using data previously obtained by Badenhoop *et al* (1990), Ferencik *et al* showed there is no correlation between Nco-1 and AspH-1 alleles despite their close proximity. To date, no further work has been undertaken on this polymorphism.

The most recently determined polymorphic site was identified in 1994 by D'Alfonso and Richiardi, using SSCP. Concentrating on the region containing most of the sequences reported critical for TNF gene regulation, a diallelic polymorphism was detected at position -238. This site is located within a sequence similar to the regulative motif called the Y-box. Detection was based on the influence of sequence variation on DNA conformation. A transition of G to A at position -238 altered the curvature of the molecule and therefore caused altered electrophoretic retardation. 127 random individuals were tested; 110 were homozygous for the G allele and 17 were heterozygous ie. had both the A and G alleles, giving gene frequencies of 0.93 and 0.07 respectively. The group also considered associations between the two -238 alleles and HLA markers and observed that individuals positive for all the markers constituting 2 of the well-known Caucasoid extended haplotypes (DQw2) and (DQw9) were also positive for the rare A allele. To date, this is the only study regarding this polymorphism.

The use of RFLPs as allelic markers is limited due to the small degree of polymorphism. For this reason, the technique of microsatellite mapping was developed. The repeat number of a dinucleotide repeat (usually CA or CT) is measured after PCR amplification and can be used as an allelic marker. Since 1991, 5 such microsatellite regions have been identified spanning the TNF locus (Nedospasov *et al*, 1991; Jongeneel *et al*, 1991 ; Udalova *et al*, 1993).

The TNFa and TNFb microsatellites were identified in 1991 (Nedospasov *et al* ; Jongeneel *et al*). They consist of AC/GT and TC/GA repeat units respectively. They are located 3.5kb upstream (telomeric) of the LTα gene and have 13 and 7 alleles respectively. The TNFc microsatellite, also identified by both groups consists of a TC/GA repeat unit and is located within intron 1 of the LTα gene; two alleles have been identified. The remaining two microsatellites TNFd and TNFe were identified in 1993 by Udalova *et al*.. These linked repeats; TC/GA and TC/GA-like respectively, lie 8-10kb downstream of the TNF gene, within intron 2 of the Lst-1 gene. (No function has yet been ascribed to this gene, which is homologous to the murine B144 gene). They consist of 7 and 4 alleles respectively. Family studies verified that all the microsatellites are stably inherited and segregate as Mendelian alleles.

Three studies have attempted to correlate levels of TNF secretion with microsatellite alleles; Turner *et al* (1993) showed a possible association between TNFd3 and TNF level following endotoxin stimulation of monocytes from heart transplant recipients. Patients homozygous for d3 had elevated TNF levels compared to d3 heterozygotes. The group suggest this could be due to an association between the TNFd alleles and a transcription regulatory sequence.

Also in 1993, Pociot *et al* showed that high TNF secretion by monocytes following LPS stimulation associated with a2, whereas low secretion correlated with a6. They also showed that monocytes from c2 positive individuals secrete higher levels of TNF than monocytes from c1 positive individuals and that there are no differences between TNFa, TNFb or TNFc in terms of PHA-stimulated LTα secretion from PBMCs. Furthermore, differences in TNF secretion were greater when differences in DR type were accounted for.

In 1995, Derkx *et al* showed that endotoxin-induced TNF release from whole blood was significantly lower in individuals with a2, a6 or a10 than those individuals with a4 or a11. They found no associations in stimulated PBMNCs, only the whole blood samples. This may explain the difference to the Pociot study. In agreement with this study was the observation that there was no TNFb genotype relationship to TNF release upon LPS-stimulation.

Several of the microsatellite alleles form part of extended haplotypes; a2 and b3 associate with the markers making up the 8.1 ancestral haplotype (A1, B8, C4AQ0, C4B1, DR3) (Abraham *et al*, 1993). Pociot *et al* (1993) identified 4 extended haplotypes which correlate with TNF secretion levels. They incorporate some of the TNFa alleles and are listed below in ascending order of TNF secretion;

DRw6, B*1, *a4*, B40, A2.
DQw8, DR4, B*1, *a6*, B44 , A1.
DQw2, DR3, B*1, *a2*, B8, A1.
DQw8, DR4, B*2, *a2*, B15, A2.

These results fit well with all the work regarding allelic links with TNF secretion, in that both the B*1 and a6 alleles had been linked to reduced TNF secretion and B*2 and a2 with higher secretion levels. This is clearly demonstrated in the context of DR4 individuals.

The authors also suggested a direct role for the TNF locus in the pathogenesis of IDDM in that patients display a higher frequency of a2 and lower frequency of a6 than controls and also the DR3/4 IDDM haplotype (DQw8, DR4, C4A3, B*2, *a2*, B15) is identical to a higher TNF response.

A large study was undertaken in 1993 by Crouau-Roy *et al* comparing the TNFa, b and c microsatellites in 4 European populations (French, Danes, Basques and Greeks) (see discussion for data). Of the 208 possible haplotypes, eight accounted for almost 60% of those individuals studied. Each population differed significantly in allele frequency and the haplotype data demonstrated the heterogeneity of the 4 populations, showing it may be useful for discriminating between different ethnic groups.

TNF/LT and Breast Cancer.

Breast cancer is the second most common cancer to kill women in the Western world. As many as 1 in 12 women will develop it at some point during their lives. Because of the ambiguous relationship with cancer, TNF expression has been extensively studied in populations with this often fatal adenocarcinoma.

The vast majority of studies have shown that TNF expression is increased in the cancer populations. Mallmann *et al* (1991) showed that non-metastatic breast cancer patients had serum TNF levels greater than the controls and also that patients with progressive disease had the highest levels.

In the following year, Abbate *et al* (1992) showed that serum TNF and also soluble IL-2 receptor levels are increased in breast (and moreso ovarian) cancer patients, when compared to controls. However, contrary to the previous study, they also demonstrated that the levels were not indicative of disease status.

In 1994, Miles *et al* demonstrated that TNF mRNA is expressed more in invasive than benign mammary tumours and also that this expression, along with the expression of the protein itself is confined mainly to the stroma. They also showed that along with increasing tumour grade,was an increase in the number of cells expressing TNF.

Several roles for TNF within the tumour microenvironment have been postulated, ranging from a direct role in metastasis to the converse role of tumour vascular destruction. The process of metastasis is a series of interactions between the cancer cell and the environment (see below) leading to tumour cell migration and escape from the site of the primary tumour via the lymphatics and/or vasculature and the colonisation of lymph nodes or secondary organs respectively.

Metastatic Processes.

1. Transformation of the normal cells.
2. Neovascularisation.
3. Invasion of the extracellular matrix (ECM).
4. Escape into the lymph or vasculature systems.
5. Specific or non-specific arrest of tumour cells in capillary beds of organs.
6. Extravasation into secondary organs.
7. Proliferation to form metastases.

The ECM is the main barrier against invasion. It comprises a stromal layer and a basement membrane. Proteases can be induced by cytokines or indirectly by tumour infiltrating lymphocytes (TILs) to degrade this barrier. There are several sources of cytokines in and around the site of the tumour, including the tumour cells themselves, normal infiltrating host cells and cells in the ECM. They will provide the tumour environment with molecules capable of regulating tumour cell behaviour. TNF is known to induce the expression of the matrix metalloproteinases MMP-2 and MMP-9, which are among the enzymes primarily involved in the processes altering the ECM to allow cell migration. In addition, in concert with INF-γ, TNF alters the proteinase: inhibitor balance leading to the degradation of BM proteins underlying the endothelial cells (Stolpen *et al*, 1986).

In 1990, Basset *et al* identified a novel MMP gene, expressed specifically in the stromal cells surrounding the invasive breast carcinomas. It was named Stromelysin-3 on the basis of its gene sequence. Also in 1990, Monteagudo *et al* investigated the distribution of the MMP type IV collagenase in normal, benign and malignant breast tissue. They found an increase of activity in invasive carcinomas of the enzyme. Furthermore, in normal tissue activity was confined mainly to myoepithelial cells, whereas in malignant tissue there was a redistribution mainly to the epithelial cells. They concluded that the enzyme may play a role in tumour invasion and metastasis and also that the tumour cells themselves were a souce of the enzyme.

From these, and studies like them, it is clear that MMPs are present in the vicinity of the tumour, so can be acted upon by TNF to potentiate metastasis. A more direct role for TNF in metastasis comes from work by Malik *et al* (1989). In studies on ovarian cancer, they showed that TNF therapy promoted invasion and solid tumour formation. Further studies by the same group (Malik *et al*, 1990) showed that Chinese hamster ovary cells transfected with TNF exhibited enhanced invasion and metastasis in nude mice, further suggesting the promotion of metastasis by TNF.

As well as enhancing invasion and metastasis, TNF has the potential to play the opposing role, that of tumour suppression. As described below, several mechanisms have been suggested for this role.

TNF is known for its cytostatic and cytotoxic activities both *in vitro* and *in vivo* against malignant and non-malignant cell types. Indeed, Sgagias *et al* (1991) showed this effect on the growth of the human breast cancer cell line MCF-7. Growth inhibition by TNF is also seen in ZR-75-1 and BT-20 cell lines (Bellomo *et al*, 1992). Pusztai *et al* (1993) demonstrated the dose-dependent, reversible inhibition of [^{3}H] thymidine incorporation and cell growth of the human breast cell line T47D by TNF. The cytostatic effect occurs at the G1/S transition and is seemingly mediated by a pathway not involving PKA or C or phosphatases PP1 or PP2B.

Van haesenbroeck *et al* (1991) demonstrated that a constitutive production of low levels of TNF by neoplastic cells correlated with decreased tumorigenicity and reduced invasiveness. Several mechanisms for this result were suggested.

1. Autocrine production of TNF induces other cytokines such as IL-1 and IL-6, which are involved in immune reactivity modulation.
2. TNF causes the development of T-cell-mediated immunity and in doing so, enhances antigen presentation by T-lymphocytes.
3. TNF activates macrophage-mediated tumour cell cytotoxicity.

The group further suggested that continuous low-level exposure to local tumour-derived TNF doses could have an important role in host-mediated tumour suppression *in vivo*.

Obviously there is a delicate balance to strike regarding the level of TNF wanted in the tumour. The positive effects of a low dose could be outweighed by too much TNF leading to increased invasiveness. Treatment regimes involving the administration of TNF have been attempted, although none too successfully.

In 1992, Budd *et al* carried out a Phase II trial with recombinant TNF. It was administered intravenously to patients who had been given one round of chemotherapy for metastatic disease. The results of the trial were poor, with little or no tumour regression seen, suggesting that as a single agent, rTNF is inactive in these patients.

Matsou *et al* (1992) demonstrated an antiproliferative role for recombinant human TNF in combination with INF or Tamoxifen (Tam) to the human breast cancer cell lines MCF-7 and ZR-75-1. MCF-7 cells show increased sensitivity to TNF than INF, but when both agents are given in combination, the cytotoxic effect was synergised. ZR-75-1 cells are resistant to TNF but sensitive to INF and when given in combination, display an additive cytotoxic effect. They also showed that pre-treatment of both cell lines with INF increased the cytotoxicity of TNF. Finally the group gave Tam plus TNF or INF to ZR-75-1 cells. This increased the cytotoxicity over giving Tam alone, the suggestion being that INF causes an increase in the expression of oestrogen receptors, allowing further action by Tamoxifen.

AIMS.

The genetic control over TNF and LT expression must be an important factor in cases of cancer. The sheer number of polymorphic sites within the locus suggests their importance in this control. The fact that two of these lie within the TNF promoter (at positions -238 and -308) further fuels the hypothesis. Taking into account the already known information linking genotype expression with levels of TNF/LT and disease status, several aims were drawn up for this project;

(1) To carry out a thorough allelotype and genotype analysis of the polymorphic sites within the TNF locus on human chromosome 6 in a population of 88 breast cancer patients.

(1b) In doing so, to optimise the reaction conditions for the detection of the alleles where necessary at each polymorphic site.

(2) To compare the data obtained with that previously collected within the group for a normal population and two further adenocarcinoma groups (gastric and colorectal). This would allow the detection of any differences and/or similarities between the populations to be noted, with a possible clue as to whether genotype expression patterns were a feature of these malignancies.

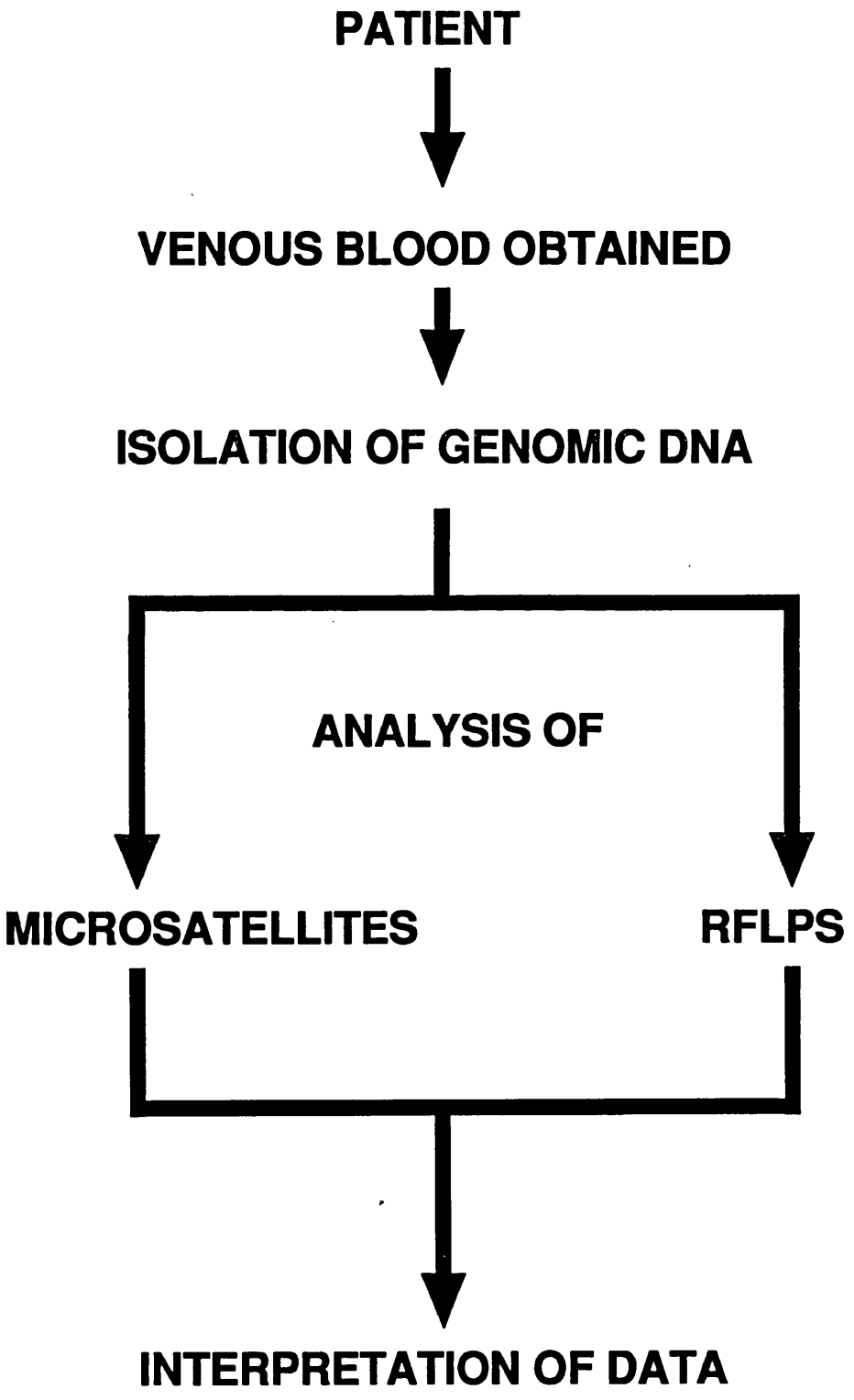
(3) To look for correlations between clinical data and allelotype or genotype.

(4) To determine whether the breast cancer population shows similarities to other malignant populations where certain alleles linked to high TNF production are overexpressed.

(5) To draw conclusions as to the role of high TNF in the malignant state.

MATERIALS AND METHODS.

OVERVIEW OF THE METHODS SECTION.



1. Patients.

This study involved 88 unrelated breast cancer patients attending the follow-up clinics of Professor Colin McArdle and Mr. Peter Stanton at Glasgow Royal Infirmary.

2. Collection of blood.

10mls of peripheral venous blood was collected from each patient into a vacutainer containing tri-potassium ethylene diamine tetraacetic acid (K_3 EDTA). The tubes were stored on ice or at 4°C for a maximum of two hours prior to the isolation of the peripheral blood leukocytes (PBLs).

3. Isolation of peripheral blood leucocytes (PBLs).

The peripheral venous blood, collected as above, was divided equally into two 25ml universal tubes with three volumes of red blood cell (RBC) lysis solution (0.83% w/v ammonium chloride, 0.037g/l EDTA, 1g/l potassium hydrogen carbonate). The contents were gently mixed then incubated at room temperature for 15 minutes. The remaining whole cells were collected by centrifugation at 1,400 rpm for 10 minutes. The supernatant was discarded and the remaining cell pellet resuspended in fresh RBC lysis solution. The incubation and centrifugation steps were repeated a further twice. If at this stage, the cell pellet was free of red blood cell contamination, the cells were resuspended in 500µl of sterile phosphate buffered saline (PBS) (0.8% w/v sodium chloride, 0.144% w/v disodium hydrogen phosphate, 0.02% w/v potassium chloride, 0.02% potassium dihydrogen phosphate, pH 7.4). If however the cell pellet showed contamination with red blood cells, it was subjected to a further incubation and centrifugation step prior to resuspension in PBS. The PBLs were stored at -20°C until required.

4. Isolation of genomic DNA from human PBLs.

The PBLs were isolated as detailed above and allowed to thaw prior to DNA isolation. 500µl of a 0.4M solution of sodiun acetate was added to each 500µl aliquot and mixed gently. 150µl of a 10% w/v aqueous solution of sodium dodecyl sulphate (SDS), 150µl of a 1 mg/ml solution of proteinase-K (Stratagene) and 200µl sterile water were then added. The digestion was incubated at 37°C for 48 hours. The resultant product was divided equally between two 1.5ml microcentrifuge tubes and subjected to phenol:chloroform extraction as follows;

an equal volume of Tris/EDTA- saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added (10mM Tris-Cl, 1.0mM EDTA, pH8.0). The tubes were vortexed then centrifuged at 13,500 rpm for 10 minutes. The upper aqueous layer (containing the DNA) was removed into another sterile tube. A further equal volume of the Tris/EDTA saturated phenol:chloroform was added and the tubes centrifuged for 10 minutes. The upper aqueous layer was again removed to a sterile tube and an equal volume of chloroform added. Following a further 10 minute centrifugation, the upper layer was transferred to a fresh tube. Genomic DNA was precipitated by adding a 10th volume of 5M ammonium acetate and 1ml of 100% ethanol. The DNA was removed by spooling onto a sealed glass pasteur pipette. It was then washed in 70% ethanol and left overnight to resuspend in 500µl sterile water.

5. Standardisation of the DNA concentration.

10µl of the resultant DNA solution was then diluted in 990µl of sterile water and the absorbances at 260nm and 280nm measures using a spectrophotometer. If the 260:280nm ratio was >1.5, the DNA was determined pure enough to have the concentration calculated. One $O.D._{260}$ unit was taken to represent 50µg/ml of genomic DNA. Each sample was diluted accordingly in sterile water to a final concentration of 50µg/ml. Samples with a 260:280 ratio <1.5 were deemed too impure to accurately calculate the DNA concentration, so were subjected to further phenol:chloroform extraction. 5µl of the DNA was then run out on a 1% agarose gel (LE SeaKem) to check its integrity.

6. Determination of genotype at the LTαNco-1 RFLP locus.

(a) PCR conditions.

Oligonucleotide PCR primers were synthesised according to the published sequences (Messer *et al*, 1991).

5' Primer: 5'-CCG TGC TTC GTG CTT TGG ACT G -3'
3' Primer: 3'-AGA GCT GGT GGG GAC ATG TCT G -5'

PCR reactions were carried out in 50µl volumes containing 250ng genomic DNA, 1µM each primer, 1 unit Taq DNA polymerase, 1.5mM $MgCl_2$, 1x PCR buffer IV (all Advanced Biotechnologies) and 2mM dATP, dGTP, dCTP & dTTP (GIBCO BRL). Each tube was overlaid with 50µl paraffin to prevent evaporation during the reaction. Amplification was carried out on a Biometra Thermocycler for 40 cycles (1min. at 95°C, 1 min. at 64°C, 1min. at 72°C), preceeded by an initial

denaturation of 95°C for 6 minutes and concluding with a 5 minute extension time at 72°C. Prior to further analysis, the PCR products were stored at 4°C.

(b) Nco-1 restriction endonuclease digestion of PCR products.

The above PCR amplification generates a 750bp fragment which spans the polymorphic Nco-1 site. The presence of a threonine residue at position 26 results in the sequence recognised by the endonuclease. The 750bp fragment is cleaved to give two smaller fragments of 500bp and 250bp. The presence of an asparagine residue at the polymorphic site abolishes the cleavage site, thus generating only the 750bp fragment on endonuclease digestion.

10µl aliquots of the PCR products were subjected to Nco-1 restriction endonuclease digestion in a total volume of 15µl with 5 units of Nco-1 and 1x Nco-1 buffer 4 (both N.E.B.). The reagents were centrifuged briefly then incubated at 37°C for 2 hours. The resultant products were analysed on a 2% LE SeaKem agarose gel (see below).

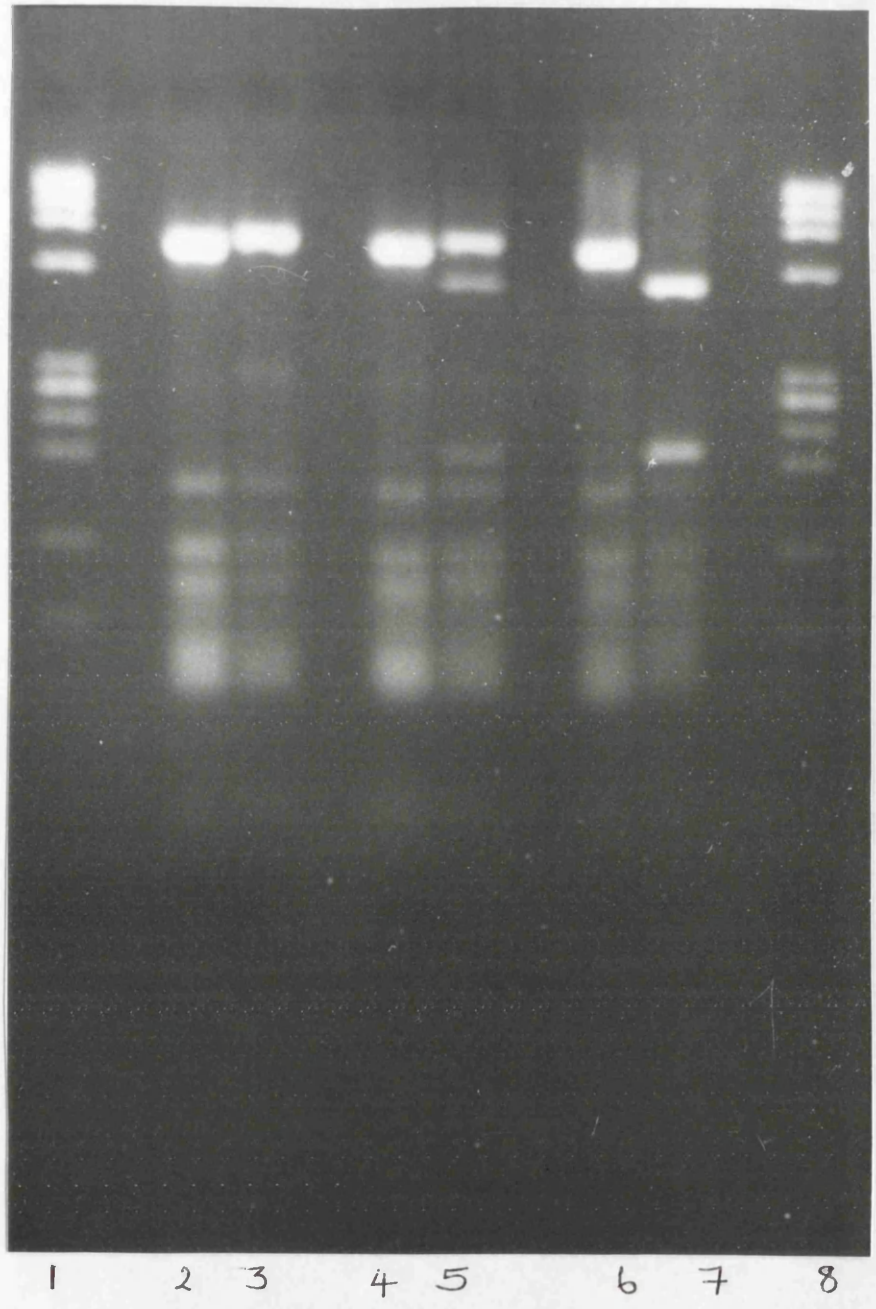
(c) Agarose gel electrophoresis of digestion products.

10µl of digested and 10µl of sham-digested product were electrophoresed in parallel on a 2% LE SeaKem agarose gel containing 0.25mg/ml ethidium bromide (EtBr). 5µl of Orange-G loading buffer was added to each sample. The gel was run in 1x TAE buffer at 50mA until the bands were clearly resolved. Hae-III digested ΦX174 was used as a molecular weight marker. (It gives bands of 1353bp, 1087bp, 872bp, 603bp, 310bp, 271bp, 234bp, 194bp, 118bp and 72bp). The banding pattern was visualised under transillumination with an ultraviolet (UV) light source.

(d) Genotype determination.

(i) TNFB*1 homozygosity was represented by the presence of the 500bp and 250bp fragments.
(ii) TNFB*2 homozygosity was represented by the presence of the 750bp fragment only.
(iii) Heterozygosity ie. (B*1,B*2) was represented by the presence of all three bands [FIGURE 20].

FIGURE 20. Gel showing possible genotypes at the Nco-1 locus.



This figure shows the three possible genotypes at the Nco-1 locus. Lanes 1 and 8 contain the size marker Hae-III digested ΦX174. Lanes 2, 4 and 6 contain the undigested PCR product of 750bp. Lane 3 contains a sample homozygous for the B*2 allele. Lane 5 contains a heterozygous sample (B*1, B*2) and lane 7 contains a sample homozygous for the B*1 allele.

7. Determination of genotype at the AspH1 RFLP locus.

(a) PCR conditions.

The primers previously obtained for the above Nco-1 RFLP analysis were used for this reaction. PCR reactions were also identical to those described above.

(b) BsiHKA-1 digestion of the PCR products.

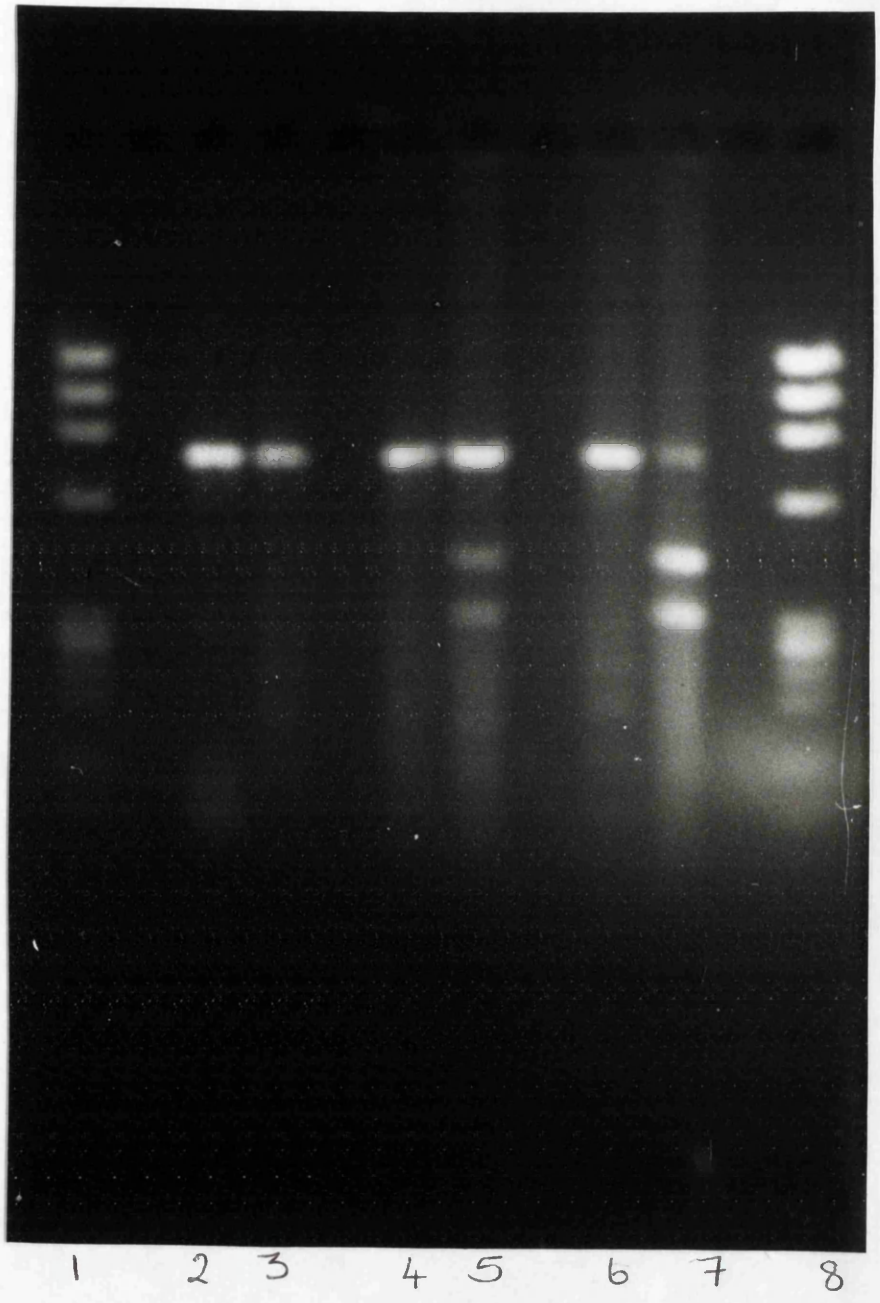
The amplification generates a 750bp fragment which straddles a polymorphic AspH1 restriction endonuclease site. The presence of the sequence G(A/T)GC(A/T)'C generates two fragments of 425bp and 325bp on digestion with the endonuclease. A G to C transversion abrogates the site, thus generating a single 750bp fragment. The endonuclease BsiHKA-1 (an isoschizomer of AspH-1) was used for the digestions.

10µl aliquots of the PCR products were digested with BsiHKA-1 in a total reaction volume of 15µl in the presence of 5 units of BsiHKA-1, 1x BsiHKA-1 buffer 3 and 100g/ml B.S.A. (all Advanced Biotechnologies). The reagents were centrifuged and incubated overnight at 60°C, after being overlaid with paraffin. 10µl of the digested and 10µl of sham-digested products were analysed in parallel on a 2% LE SeaKem agarose gel as detailed above. The products were again visualised under UV light.

(d) Genotype determination.

(i) Allele 1 homozygosity was represented by the presence of the 425bp and 325bp fragments.
(ii) Allele 2 homozygosity was determined by the presence of the 750bp fragment.
(iii) Heterozygosity ie. (1,2) was represented by the presence of all three fragments [FIGURE 21].

FIGURE 21. Gel showing the various genotypes at the AspH-1 locus.



Lanes 1 and 8 contain Hae-III digested ΦX174, run as a molecular weight marker. Lanes 2 and 3 show the (2,2) homozygote (with undigested sample in lane 2). Lanes 4 and 5 show a (1,2) heterozygote, with lane 4 containing undigested sample. Lanes 6 and 7 show a (1,1) homozygote, with undigested sample in lane 6.

8. Determination of genotype at the TNF -308 locus.

(a) PCR conditions.

Oligonucleotide PCR primers were synthesised according to published sequences (Wilson et al, 1992). The 5' primer was designed to incorporate the -308 polymorphic site into a Nco-1 restriction site. This was created by a single base change (underlined).

5' Primer: 5'-AGG CAA TAG GTT TTG AGG G<u>C</u>C AT-3'
3' Primer: 5'-TCC TCC CTG CTC CGA TTC CG-3'

PCR reactions were carried out in 50µl volumes containing 250ng genomic DNA, 2mM dATP, dTTP, dCTP & dGTP (GIBCO BRL), 1µM each primer, 1.5mM $MgCl_2$, 1x PCR buffer-IV and 1 unit Taq DNA Polymerase (all Advanced Biotechnologies). Each tube was overlaid with 50µl paraffin to prevent evaporation. Amplification was carried out on a Biometra Thermocycler for 35 cycles (1 min. at 94°C, 1 min. at 60°C, 1 min. at 72°C), preceeded with an initial cycle (3 mins. at 94°C, 1 min. at 60°C, 1 min. at 72°C) and concluding with a 5 minute extension time at 72°C. Prior to further analysis, the PCR products were stored at 4°C.

(b) Nco-1 restriction endonuclease digestion of -308 PCR products.

The PCR generates a 107bp fragment which contains a polymorphic Nco-1 restriction endonuclease site. The presence of the site results, upon digestion in the genetration of two fragments of 97bp and 20bp.
10µl aliquots of the PCR products were subjected to Nco-1 restriction endonuclease digestion in a final volume of 15µl, containing 5 units of Nco-1 and 1x Nco-1 buffer-4 (both N.E.B). After brief centrifugation, the reagents were incubated overnight at 37°C. The resultant products were analysed on an 8% polyacrylamide gel (see below).

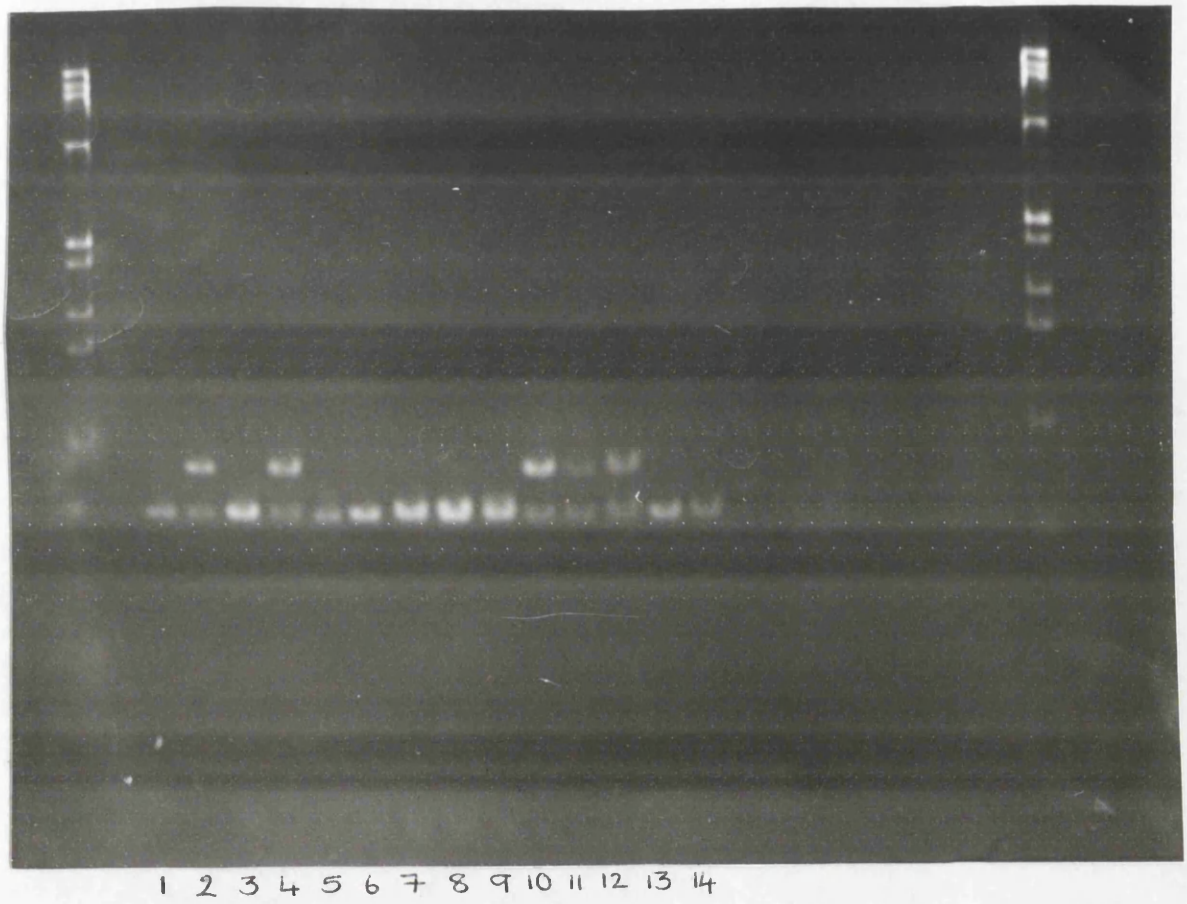
(c) Polyacrlyamide gel electrophoresis (PAGE) of -308 RFLP digestion products.

10µl of digested and 10µl sham-digested products were electrophoresed in parallel on a 20cm x 20cm, 8% polyacrylmide gel rig (Bio-Rad). 1x TAE was used as running buffer and Hae-III- digested ΦX174 as the molecular weight marker. 5µl of bromophenol blue/xylene cyanol buffer was added to each sample and electrophoresis carried out until the bromophenol blue ran to within 2cm of the end of the gel. The gel was then stained by submerging in 1xTAE containing 0.4mg/ml EtBr for 30 minutes. The banding pattern was visualised under transillumination with UV light.

(d) Genotype determination.

(i) Homozygosity for allele T1 was represented by two fragments of 97bp and 20bp.
(ii) Homozygosity for allele T2 was represented by a single fragment of 107bp.
(iii) Heterozygosity (T1,T2) was represented by the presence of all three fragments [Figure 22].

FIGURE 22. Gel showing various genotypes at the -308 locus.



In this figure, Hae-III digestedΦX174 was used as a molecular weight marker. Lanes 1, 3, 5 to 9, 13 and 14 show samples homozygous for the T1 allele. Lanes 2, 4, 10, 11 and 12 show heterozygous samples (T1, T2).

9. Determination of genotype at the five microsatellite loci.

Only three of the previously described microsatellites were examined in the TNF locus, those of TNFa, TNFc and TNFe. There were several reasons for this. Firstly the tandem positioning of TNFb and TNFd with TNFa and TNFe respectively, suggesting a redundancy effect of the former two loci. Secondly, we were unable to fully optimise the reaction conditions for the TNFb and TNFd microsatellites. Thirdly, data already accrued suggested that the main area of interest in the locus was at the telomeric end, so it was deemed unnecessary to continue working on the TNFd locus.

Initially, each microsatellite region was amplified according to the protocol below and the products run out on a 15% non-denaturing polyacrylamide gel. In the latter stages of the project, attempts were made to optimise the conditions for each individual microsatellite locus (see later).

(a) PCR conditions.

Oligonucleotide PCR primers were synthesised according to published sequences (Udalova et al, 1993).

TNFa 5': 5'-GCC TCT AGA TTT CAT CCA GCC ACA G-3'
TNFa 3': 5'-CCT CTC TCC CCT GCA ACA CAC A-3'

TNFc 5': 5'-GGG AGG TCT GTC TTC CGC CG-3'
TNFc 3': 5'-CGT TCA GGT GGT GTC ATG GG-3'

TNFe 5': 5'-GTG CCT GGT TCT GGA GCC TCT C-3'
TNFe 3': 5'-TGA GAC AGA GGA TAG GAG AGA GAC AG-3'

PCR reactions were carried out in 20µl volumes containing 100ng genomic DNA, 0.2mM dATP, dGTP, dTTP (Pharmacia), 0.02mM α^{32}P-labelled (Amersham) dCTP, 1µM each primer, 1.5mM $MgCl_2$, 1x PCR buffer-IV and 0.4 units Taq DNA polymerase (all Advanced Biotechnologies). Each tube was overlaid with 20µl paraffin to prevent evaporation. Amplification was carried out on a 96 well Biometra Thermocycler for 40 cycles (25 secs. at 94°C, 60 secs. at 60°C, 60 secs. at 74°C), preceeded with an initial melting time of 10 mins. at 94°C and concluding with a final extension time of 10 mins. at 74°C.

(b) Non-denaturing PAGE of microsatellite PCR products.

3µl aliquots of the radiolabelled products were electrophoresed on a 15% non-denaturing polyacrylamide gel [29:1, acrylamide (GIBCO BRL) : bisacrylamide (Sigma)]. The gel was run on a Base Ace sequencing gel rig (Stratagene) in 1xTBE buffer. The samples were loaded with a bromophenol blue/ xylene cyanol loading buffer and run overnight at 22mA until the bromophenol blue had run off the bottom and the xylene cyanol was within 5cm of the end of the gel. (The xylene cyanol runs with an apparent molecular weight of approximately 45). The gel was then dried on a vacuum dryer (Biorad) for 1 hour and exposed to photographic film (Kodak) to produce an autoradiographic image. The alleles at each polymorphic locus were determined by measuring their mobility in relation to a labelled ladder (see below) and in comparison to the cosmid M31A, which has the known genotype a8, b4, c1, d3, e3.

10. Production of γ^{32}P-labelled 10bp ladder.

1µl of ladder (GIBCO BRL) was end-labelled with γ^{32}P ATP (Amersham) by incubating with 4µl 5x Exchange reaction buffer (GIBCO BRL), 5µl γ^{32}P ATP, 1µl T4 Kinase (GIBCO BRL) and 9µl water for 30 mins. at 37°C followed by 5 mins. at 55°C. An equal volume of the bromophenol blue/xylele cyanol loading buffer was added to the mix.

11. Production of γ^{32}P-labelled 100bp ladder.

1µl of ladder (GIBCO BRL) was end-labelled by incubation with 4µl 5x Exchange Reaction Buffer (GIBCO BRL), 5µl γ^{32}P ATP, 1µl T4 Kinase (GIBCO BRL) and 9µl water for 30 mins. at 37°C followed by 5 mins. at 55°C. An equal volume of the bromophenol blue/xylene cyanol loading buffer was added to the mix.

12. Production of γ^{32}P-labelled HaeIII-digested ΦX174 ladder.

2µl ladder (Sigma) was end-labelled by incubation with 4µl 5x Exchange Reaction Buffer (GIBCO BRL), 5µl γ^{32}P ATP, 1µl T4 Kinase (GIBCO BRL) and 8µl water for 30 mins. at 37°C followed by 5 mins. at 55°C. An equal volume of the bromophenol blue/ xylene cyanol loading buffer was added to the mix.

13. Optimisation of the protocol for determining genotype at the TNFa microsatellite locus.

As mentioned above, attempts were made to optimise the PCR and gel conditions of the individual microsatellites. Various components of each reaction mix were titrated to determine the optimum concentrations.

(a) $MgCl_2$ concentration.

This was varied from 0.5mM to 2.0mM in increments of 0.5mM.

(b) Primer concentration.

This was varied from 0.2μM to 1.0μM in increments of 0.2μM.

(c) Reaction volume.

Initially each PCR amplification was carried out in a total volume of 20μl. Each was subsequently duplicated in 50μl volumes.

(d) PCR programme.

A touchdown programme of cycling was tried as an alternative to the original programme.

(e) Denaturing PAGE versus non-denaturing PAGE.

(f) Source of Taq DNA Polymerase.

Taq was tested from both Advanced Biotechnologies and Primezyme. In the latter case, the enzyme was supplied with pre-optimised PCR buffer (1.5mM $MgCl_2$) and Mg^{2+}-free buffer. Both were tried during the optimisation process.

Genotype determination was tested on a 6%, 8% and a 10% denaturing polyacrylamide gel.

The complete optimised protocol is detailed below.

14. Optimised protocol for haplotype determination at the TNFa microsatellite.

(a) PCR conditions.

Oligonucleotide PCR primers were synthesised according to published sequences (Nedospasov et al, 1991).

PCR reactions were carried out in 20μl reaction volumes containing 100ng genomic DNA, 0.2mM dATP, dGTP, dTTP and 0.02mM α^{32}P-labelled dCTP, 1μM each primer, optimised PCR buffer (1.5mM $MgCl_2$) & 0.25 units Taq DNA Polymerase (both Primezyme). Each tube was overlaid with 20μl paraffin to prevent evaporation during the reaction. Amplification was carried out in a 96-well Biometra Thermocycler for 40 cycles (25 secs. at 94°C, 60 secs. at 60°C, 60 secs.

at 74°C), preceeded by an initial melting time of 10 mins. at 94°C and concluding with an extension time of 10 mins. at 74°C.

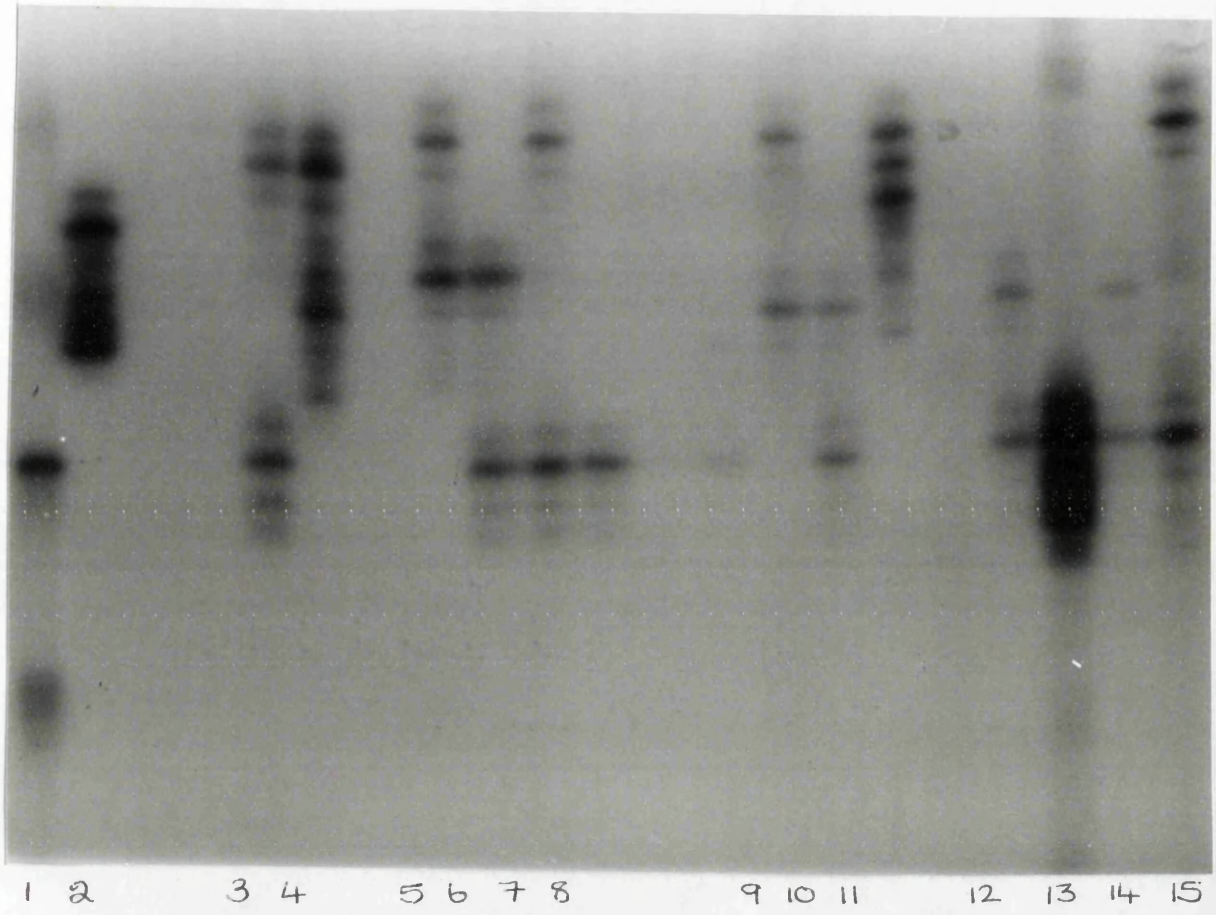
(b) Denaturing PAGE of TNFa microsatellite PCR products.

3µl aliquots of the radiolabelled products were electrophoresed on a 6% denaturing polyacrylamide gel [19:1 acrylamide (GIBCO BRL): bisacrylamide (Sigma)], containing 7.65M urea. The gel was run on a Base Ace sequencing rig (Stratagene) in 1x TBE. The gel was preheated for 1 hour prior to sample loading by running it at 75W. The samples were heated to 80°C for 10 mins. in 6µl of a formamide-based buffer (1mg/ml bromophenol blue, 1mg/ml xylene cyanol, 80% formamide, 10mM EDTA pH 8.0) before being loaded directly from ice. Labelled ladders were run, along with the cosmid M31A (a8) and cell-line markers T7527 (a6), OLL (a6), IBW9 (a4) and LZL (a2) (ECACC), chosen with reference to Udalova *et al*, 1993. The gel was run for 2.5 hours at 69W, until the bromophenol blue had run off the bottom and the xylene cyanol was within 8cm of the end. On this 6% gel, the latter dye front runs at 110bp. The gel was then dried for 50 minutes on a vacuum drier (Bio-Rad) and exposed to photographic film to produce an autoradiographic image.

(c) Genotype determination.

The alleles were called with the aid of the cell line markers and the cosmid M31A. A γ^{32}ATP- labelled 10bp or 100bp ladder was run on each gel as a size marker. This was made according to the original protocol, with the exception that a formamide-based loading buffer was added to each [FIGURE 23].

FIGURE 23. Gel showing various genotypes at the TNFa locus.



This figure shows a typical TNFa gel. Lane 1 contains the labelled 100bp ladder, with the 100bp band represented by the dark band. Lane 2 contains M31A (8,8). The remaining lanes contain a selection of the breast cancer patient samples, displaying a range of alleles; lane 3 (2,10), lane 4 (6,10), lane 5 (7,11), lane 6 (2,7), lane 7 (2,11), lane 8 (2,2), lane 9 (2,5), lane 10 (6,11), lane 11 (2,6), lane 12 (9,11), lane 13 (2,6), lane 14 (2,6) and lane 15 (2,11).

15. Optimised protocol for genotype determination at the TNFc microsatellite locus.

These optimised conditions were determined by a further member of our research group, Mrs. Joyce Eskdale.

(a) PCR Conditions.

Oligonucleotide PCR primers were synthesised according to published sequences (Nedospasov et al, 1991).

PCR reactions were carried out in 20µl volumes containing 100ng genomic DNA, 0.2mM dATP, dGTP, dTTP and 0.02mM α^{32}P-labelled dCTP, 1µM each primer, 1.5mM $MgCl_2$, 1xPCR buffer & 0.4units Taq DNA polymerase (all Advanced Biotechnologies). Each tube was overlaid with 20µl paraffin to prevent evaporation during the reaction. Amplification was carried out according to the programme used for the TNFa microsatellite.

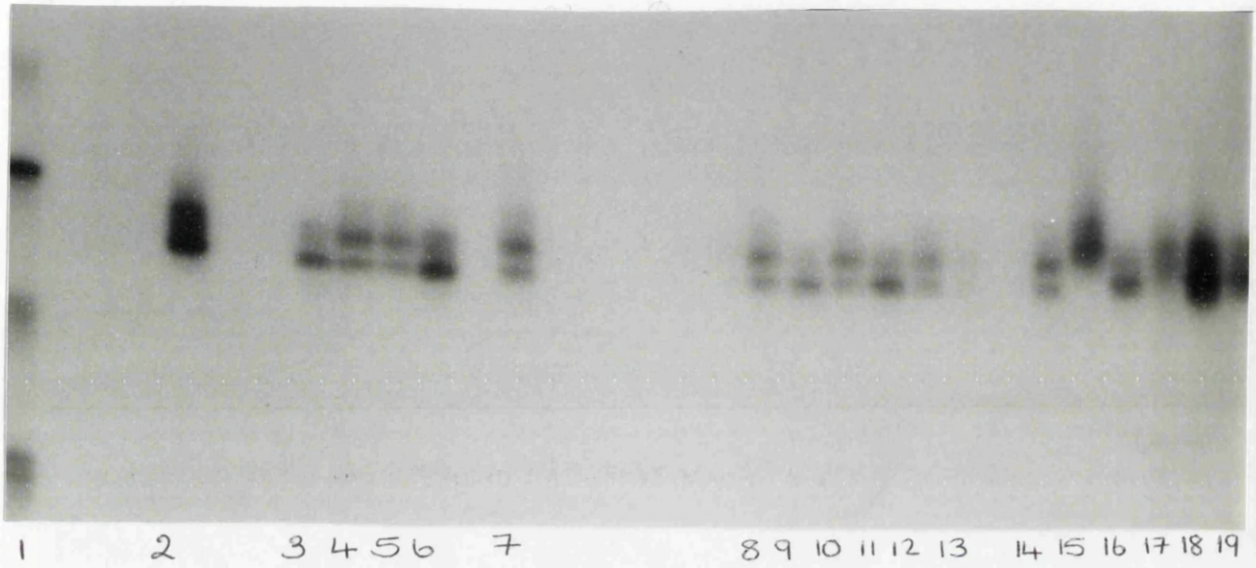
(b) Denaturing PAGE of TNFc microsatellite products.

3µl aliquots of the radiolabelled products were electrophoresed on a 10% denaturing polyacrylamide gel (19:1) acrylamide : bisacrylamide, containing 7.65M urea. The gel was run on a Base Ace sequencing rig (Stratagene) in 1x TBE. The gel was preheated for 1 hour prior to loading the samples by running it at 69W. The samples were heated to 80°C for 10 mins. in 6µl of a formamide-based loading buffer prior to loading directly from ice. The gel was run for 3 hours at 69W until the bromophenol blue dye front had run off the bottom of the gel and the xylene cyanol was within 5cm of the end. The gel was then dried for 90 minutes on a vacuum drier (Bio-Rad) and exposed to photographic film to produce an autoradiographic image.

(c) Genotype Determination.

A 10bp ladder was run on the gel as a size marker, as the two alleles at this locus are 97 and 99 base pairs. The cosmid M31A was also run alongside the samples as it is known to be homozygous for allele 1 [FIGURE 24].

FIGURE 24. Gel showing various genotypes at the TNFc locus.

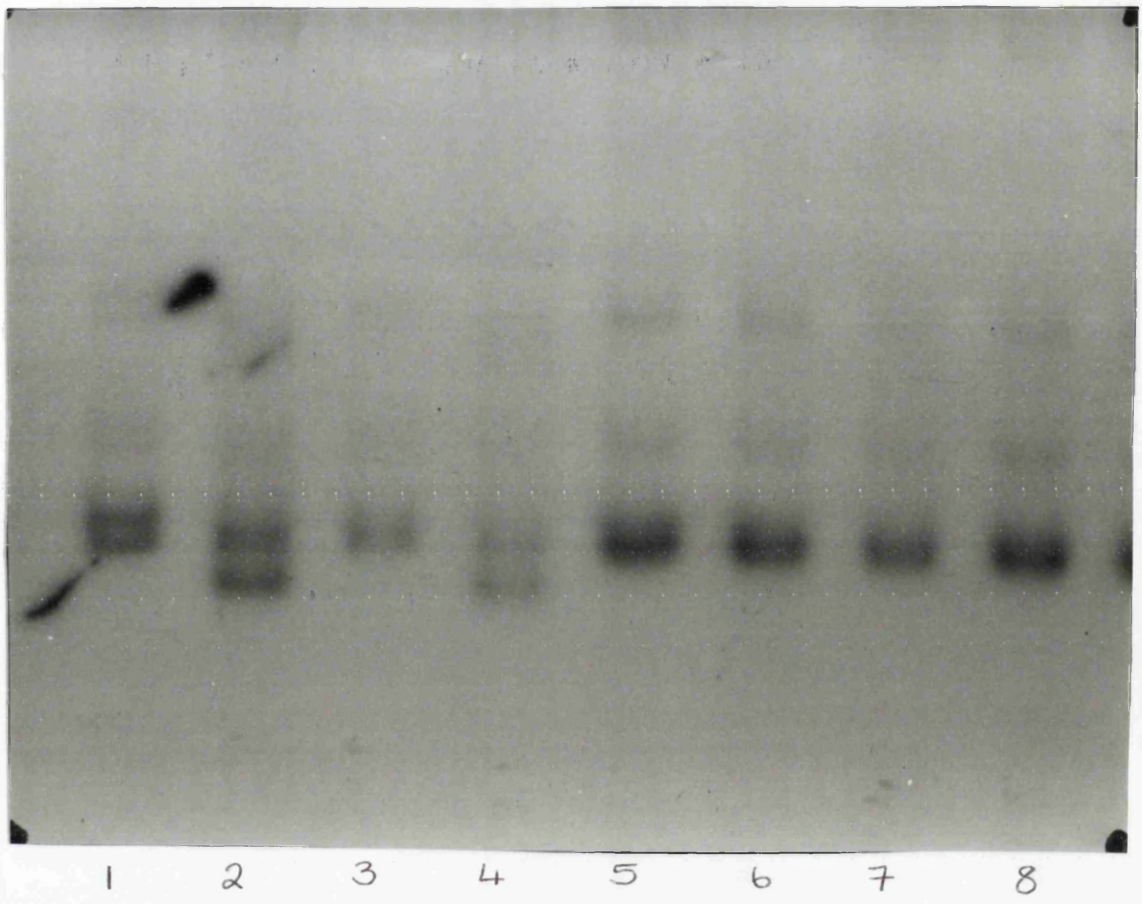


This figure shows a typical TNFc gel. Lane 1 contains the labelled 10bp ladder, with the dark band representing 100bp. Lane 2 contains the cosmid M31A (1,1). Lanes 3, 6, 9, 11, 16, 18 & 19 contain samples which are also (1,1). Lanes 4, 5, 7, 8, 10, 12, 13 & 14 contain samples which are (1,2) and lanes 15 & 17 contain samples which are (2,2).

16. Protocol for genotype determination at the TNFe microsatellite locus.

The reaction conditions of this locus were unchanged from the original non-denaturing protocol stipulated earlier due to an initial lack of success of various optimisation steps carried out by another member of the group, Miss Hui-Hui Oh. Genotype determination was decided upon by visualisation only [FIGURE 25].

FIGURE 25. Gel showing various genotypes at the TNFe locus.



This figure demonstrates a typical TNFe gel. The sample in lane 1 shows the alleles (2,3), samples in lanes 2 and 4 are (1,2) and those in lanes 3, 5, 6, 7 and 8 are (2,2).

RESULTS.

(a) Methodology

1. Optimisation of the reaction conditions for the microsatellite reactions.

In the original protocol employed, there was a large quantity of non-specific banding on the gels. This could not be explained soley by slippage error on the part of the Taq DNA polymerase, so steps were taken to optimise the conditions of each reaction.

The necessity for optimising the PCR and allele assignment of the microsatellite reactions was further demonstrated by the striking results demonstrated when the 88 breast cancer samples underwent amplification to determine their haplotype at the biallelic TNFc microsatellite locus under both the original and optimised protocol conditions (see materials and methods for details).

Seventy seven of the 88 samples successfully amplified under both sets of conditions and the results are summarised in Table 1.

From these results it was obvious that many of the samples had their genotype reassigned from the optimised protocol conditions. Indeed, 35 changes were made out of the original 77 allele calls [Table 2].

Confirmation of the allele calls was provided by running DNA from the cosmid M31A on the gels along with the samples. It is homozygous for allele 1 at the TNFc locus.

2. TNFa Optimisation.

The initial protocol used a non-denaturing PAGE system, but it was determined that clearer definition of alleles and also far less non-specific banding were seen if a denaturing system was employed (the denaturing agent being 7.65M urea). The various components of the PCR reaction mix were individually titrated to find optimim concentrations. Varying the $MgCl_2$ concentration from 0.5mM to 2.0mM was shown to effect the clarity of the gel, with 1.5mM being chosen as optimal. The concentration of primers in each reaction was also titrated from 0.2μM to 1.0μM and 1.0μM deemed optimal. The effect of overall reaction volume on the results was tested by duplicating samples on 20μl and 50μl final volumes. The 20μl volume was chosen as optimal.To reduce the non-specific binding made by the primers, a touchdown PCR amplification programme was tried, but the results failed to show much improvement, so the original programme was retained. The concentration of acrylamide in the gel was optimised by duplicating samples on 6%, 8% and 10% gels (containing acrylamide:bisacrylamide at a ratio of 19:1)

Table 1

Non-denaturing conditions (original protocol).

Genotype	No. of samples
(1,1)	22
(1,2)	27
(2,2)	28

Denaturing conditions (optimised protocol).

Genotype	No. of samples
(1,1)	37
(1,2)	30
(2,2)	10

Table 2

Original Conditions	Optimised Conditions	No.of samples changed
(1,1)	(1,2)	1
(1,1)	(2,2)	1
(1,2)	(1,1)	6
(1,2)	(2,2)	4
(2,2)	(1,1)	11
(2,2)	(1,2)	12

resulting in the 6% gels being used as the allele separation was far clearer. The final component of the reaction mix to be optimised was the Taq DNA polymerase. The initial protocol used agents from Advanced Biotechnologies. However, it was found that using Primezyme Taq and optimised PCR buffer (1.5mM $MgCl_2$) from Biometra gave the clearest results. This result is clearly demonstrated on two 6% gels in which the only difference is the source of Taq and PCR buffer. They demonstrate the increased clarity of the Primezyme reagents over those from Advanced Biotechnologies on which all the alleles are represented by 'doublets', which makes allele determination very unreliable [FIGURE 26 a & b].

To aid the allele determination and give a greater range of standards, cell line DNA was obtained from 4 lines as well as DNA from cosmid M31A. All five are homozygous at the TNFa locus as previously mentioned;

Marker	TNFa Alleles
M31A	**(8,8)**
T7527	**(6,6)**
OLL	**(6,6)**
IBW9	**(4,4)**
LZL	**(2,2)**

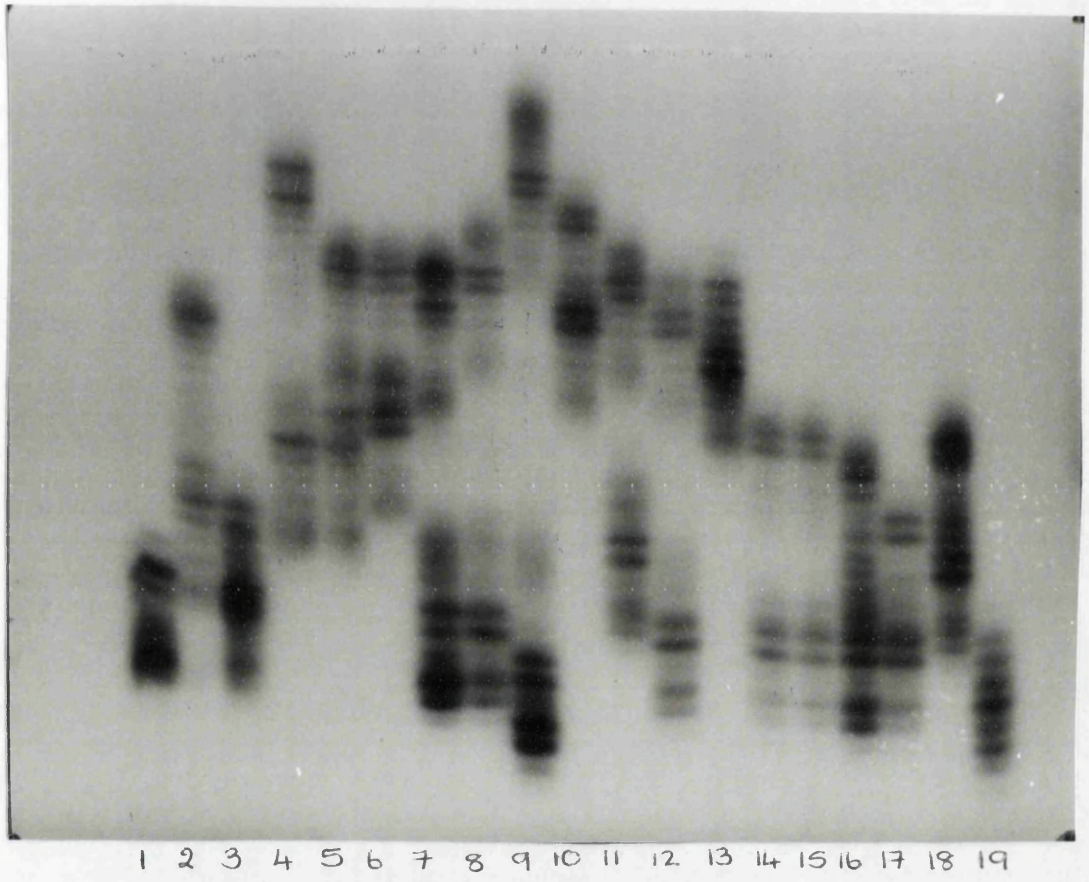
The cell lines were acquired because cosmids and plasmids are subject to allele instability (Marshall *et al*, 1993). A γ^{32}P-labelled 10bp ladder was also run on the gels as a size indicator [FIGURE 27].

Allelotype and Genotype data of 88 Breast Cancer patients at various polymorphic loci spanning the TNF locus.

Blood samples from 88 patients attending the follow up breast cancer clinic were obtained. Each was subjected to genotype analysis at the 6 polymorphic sites under study, following the lengthy optimisation process (see earlier). Occasionally there was a failure in the amplification process, but on the whole the PCR reactions were successfully carried out.

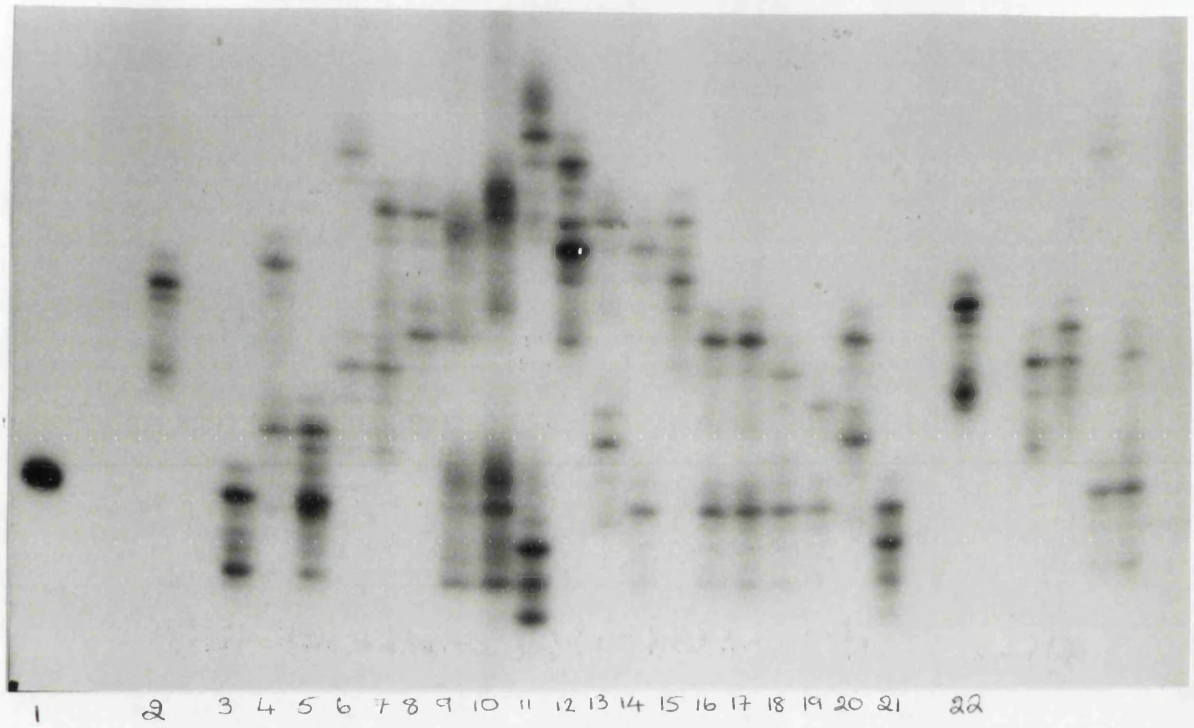
Table 3 shows the complete data obtained for each sample at the various polymorphic sites across the TNF locus and figures 7 to 15 show the individual breakdown of the data at each site, with allelotype and genotype frequencies. (In Table 3, grade refers to the degree of differentiation of the tumour, with 1 being most differentiated and 3 being least differentiated. With regard to size, T1 represents a tumour of <20mm, T2 represents a tumour of 21-50mm and T3 represents a tumour of >50mm in diameter). In figures 7 to 15, genotype 1 = (1,1), genotpye 2 = (1,2) and genotype 3 = (2,2).

FIGURE 26(a). Gels showing the difference between (a) Advanced Biotechnologies and (b) Primezyme Taq and PCR buffers when amplifying the TNFa locus.



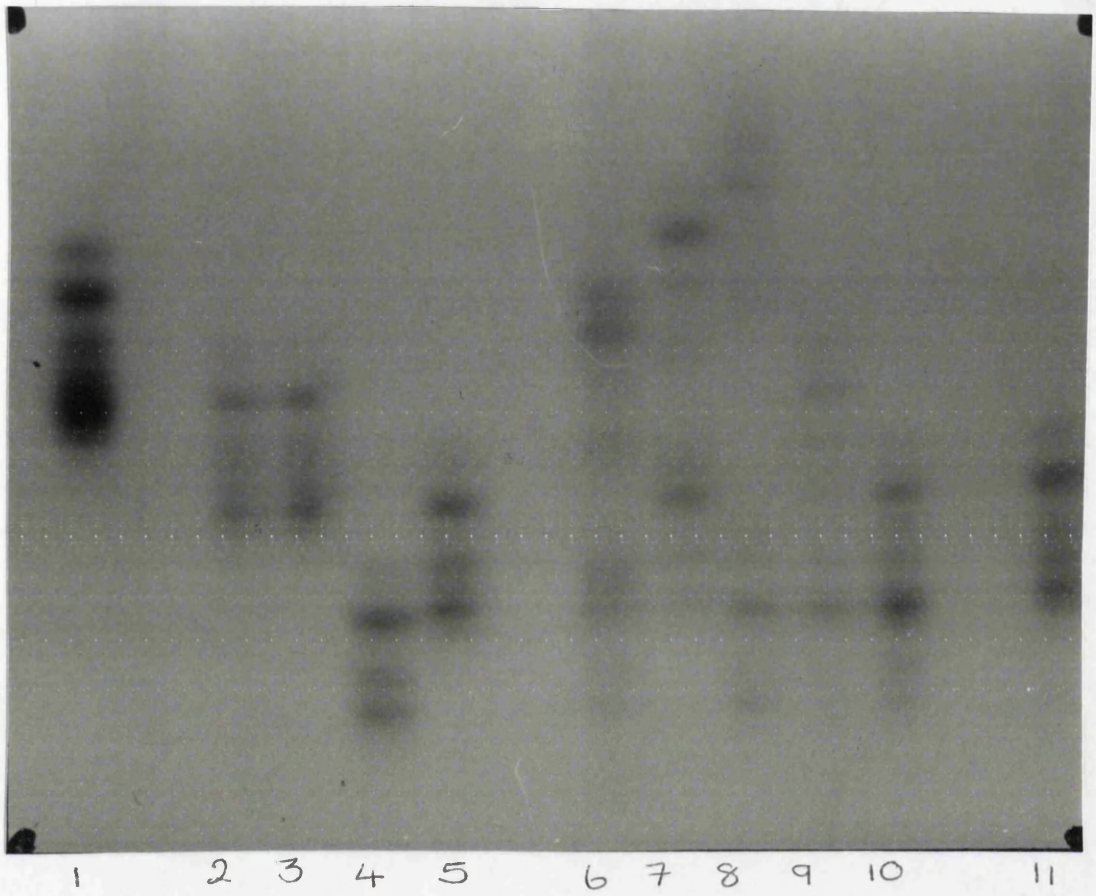
This gel was run using Taq DNA polymerase and PCR buffer from Advanced Biotechnologies. Compared to the results obtained when using Primezyme reagents (Fig. 26b), the latter system is far easier to use for allele determination. The gel above displays the 'doublet' banding pattern. The samples in each lane were selected to cover the entire range of alleles at this locus. Lane 1 (1,2), lane 2 (4,9), lane 3 (2,4), lane 4 (6,13), lane 5 (6,11), lane 6 (7,11), lane 7 (2,10), lane 8 (2,11), lane 9 (1,14), lane 10 (10,13), lane 11 (4,11), lane 12 (2,10) lane 13 (9,11) lane 14 (2,7), lane 15 (2,7), lane 16 (2,6), lane 17 (2,5), lane 18 (4,7) and lane 19 (1,2).

FIGURE 26(b). Gels showing the difference between (a) Advanced Biotechnologies and (b) Primezyme Taq and PCR buffers when amplifying the TNFa locus.



This TNFa gel was run with Primezyme Taq DNA polymerase and optimised PCR buffer. When compared to the previous gel showing the same samples, this is easier to read as the alleles appear as single, dark bands, rather than 'doublets'. The samples in each lane were again selected to cover the entire range of alleles at this locus. Lane 1 contains the 100bp ladder, with the band representing 100bp present only. Lanes 2 & 22 contain M31A (8,8). Lane 3 (1,2), lane 4 (4,9), lane 5 (2,4), lane 6 (6,13), lane 7 (6,11), lane 8 (7,11), lane 9 (2,10), lane 10 (2,11), lane 911 (1,14), lane 12 (10,13), lane 13 (4,11), lane 14 (2,10) lane 15 (9,11) lane 16 (2,7), lane 19 (2,7), lane 18 (2,6), lane 19 (2,5), lane 20 (4,7) and lane 21 (1,2).

FIGURE 27. Gel showing the four cell-lines and the cosmid M31A used to aid allele determination.



Lane 1 contains M31A (8,8). Lane 2 contains the cell-line T7526 (6,6). Lane 3 contains the cell-line OLL (6,6). Lane 4 contains the cell-line LZL (4,4) and lane 5 contains the cell-line IBW9 (2,2). The remaining lanes contain samples which cover a range of alleles; Lane 6 (2,7), lane 7 (4,9), lane 8 (2,10), lane 9 (2,6), lanes 10 & 11 (2,4).

TABLE 3. Genotype and clinical data of the 84 Breast Cancer patients.

Sample	TNFe	-308	TNFc	AspH-1Nco-1		TNFa	Node	Grade	size
90001	2/2	T1/T1	1/1	-	B*2/B*2	11/12	+ve	?	T1
90002	1/2	T1/T2	1/2	2/2	B*1/B*2	1/2	?	?	?
90003	2/2	T1/T1	1/1	1/1	B*2/B*2	7/10	+ve	?	?
90004	2/2	T1/T1	1/2	2/2	B*1/B*2	4/6	-ve	3	T1
90005	2/2	T1/T1	1/2	2/2	B*1/B*2	2/6	-ve	?	T2
90006	-	T1/T1	1/1	1/2	B*1/B*2	6/11	+ve	2	T1
90007	2/2	T1/T2	1/2	1/2	B*1/B*2	-	-ve	?	?
90008	2/2	T1/T1	1/2	1/2	B*2/B*2	2/7	-ve	?	?
90009	2/2	T1/T2	1/1	1/2	B*1/B*2	2/11	-ve	?	T2
90010	2/2	T1/T2	2/2	2/2	B*2/B*2	2/4	-ve	?	T2
90011	2/2	T1/T2	1/1	1/2	B*1/B*2	2/13	+ve	2	T1
90012	2/2	T1/T1	1/1	1/2	B*1/B*2	6/7	+ve	?	?

Sample	TNFe	-308	TNFc	AspH-1	Nco-1	TNFa	Node	Grade	size
90013	1/2	T1/T2	1/2	2/2	B*1/B*2	2/2	?	?	T1
90014	2/2	T1/T2	1/1	-	B*2/B*2	11/13	-ve	1	T1
90015	2/2	T1/T2	1/1	1/2	B*1/B*2	2/7	+ve	?	?
90016	2/2	T1/T2	1/1	1/2	B*2/B*2	6/11	?	?	?
90017	2/3	T1/T1	1/1	1/2	B*1/B*2	4/7	+ve	3	T2
90018	2/3	T1/T1	1/1	2/2	B*1/B*2	6/7	+ve	1	T2
90019	1/2	T1/T2	1/2	2/2	B*1/B*2	1/2	+ve	?	?
90020	2/2	T1/T1	1/2	1/2	B*2/B*2	2/11	+ve	?	T1
90021	1/2	T1/T1	2/2	2/2	B*2/B*2	2/4	-ve	?	T2
90022	2/2	-	1/1	1/2	B*1/B*2	2/11	-ve	2	T2
90023	2/2	T1/T2	2/2	1/2	B*1/B*2	7/7	-ve	3	T3
90024	2/2	T2/T2	1/1	1/2	B*1/B*2	3/7	+ve	?	?

Sample	TNFe	-308	TNFc	AspH-1	Nco-1	TNFa	Node	Grade	size
90025	2/2	T1/T1	1/1	1/2	B*1/B*2	5/12	-ve	?	T1
90026	2/2	T1/T1	1/1	2/2	B*1/B*2	6/6	-ve	3	T2
90027	2/2	T1/T2	1/1	2/2	B*1/B*2	2/5	-ve	?	T1
90028	1/2	T1/T1	1/2	1/2	B*2/B*2	2/10	+ve	?	T1
90029	1/2	T1/T2	1/2	2/2	B*1/B*2	2/2	-ve	?	?
90030	2/2	T1/T1	1/1	1/2	B*1/B*2	6/11	-ve	?	?
90032	2/2	T1/T1	1/1	1/1	B*2/B*2	7/11	+ve	2	T2
90033	1/2	T1/T1	1/2	1/2	B*2/B*2	2/10	+ve	1	T1
90034	2/3	T1/T1	1/2	1/2	B*2/B*2	2/11	?	?	?
90035	2/3	T1/T1	1/2	1/2	B*2/B*2	1/14	+ve	?	?
90036	2/3	T1/T2	1/1	1/2	B*1/B*2	5/10	+ve	?	T2
90037	1/3	T1/T1	1/2	1/2	B*2/B*2	2/11	-ve	3	T1
90038	2/2	T1/T1	1/1	1/1	B*2/B*2	6/11	+ve	?	?

Sample	TNFe	-308	TNFc	AspH-1	Nco-1	TNFa	Node	Grade	size
90039	2/3	T1/T2	1/1	1/2	B*1/B*2	2/7	+ve	?	?
90040	-	T1/T2	1/1	1/2	B*1/B*2	-	+ve	?	T2
90041	-	T1/T1	1/1	2/2	B*1/B*1	2/6	-ve	3	T1
90043	2/2	T1/T2	1/1	1/2	B*1/B*2	4/10	-ve	?	?
90044	1/2	T1/T2	1/2	2/2	B*1/B*2	1/2	+ve	2	T1
90045	1/2	T1/T1	1/2	1/2	B*2/B*2	2/11	-ve	?	T2
90046	2/2	T1/T2	1/1	1/2	B*1/B*2	2/11	-ve	?	T1
90047	-	T1/T1	1/1	1/1	B*2/B*2	7/7	-ve	?	T1
90048	2/2	T2/T2	1/1	2/2	B*1/B*1	2/2	-ve	?	?
90049	1/1	T1/T2	1/2	2/2	B*1/B*2	2/2	+ve	?	?
90050	2/2	T1/T1	1/1	1/2	B*1/B*2	6/11	+ve	2	T2
90051	2/2	T1/T1	1/1	1/2	B*1/B*2	6/11	+ve	2	T2

Sample	TNFe	-308	TNFc	AspH-1	Nco-1	TNFa	Node	Grade	size
90052	2/2	T1/T2	1/1	1/2	B*1/B*2	3/3	-ve	?	T1
90053	2/2	T1/T1	1/1	1/1	B*2/B*2	7/7	+ve	?	?
90054	-	T1/T2	1/1	-	-	3/10	+ve	?	T1
90055	-	T1/T1	1/2	2/2	B*1/B*2	2/6	+ve	3	T3
90056	-	T1/T2	1/2	2/2	B*1/B*2	2/4	+ve	?	T3
90057	-	T1/T2	1/1	1/2	B*1/B*2	2/11	-ve	?	T1
90058	3/3	T1/T1	1/2	1/2	B*2/B*2	7/9	+ve	?	T2
90059	3/3	T1/T2	1/2	2/2	B*1/B*2	2/4	-ve	?	T1
90060	3/3	T1/T1	1/1	1/2	B*1/B*2	6/13	+ve	1	T2
90061	3/3	T1/T1	1/1	1/2	B*1/B*2	6/11	+ve	3	T3
90062	3/3	T1/T1	1/2	1/2	B*2/B*2	4/7	+ve	?	T2
90063	3/3	T1/T1	1/2	2/2	B*1/B*2	-	-ve	1	T1
90064	2/3	T1/T1	1/2	1/2	B*2/B*2	2/11	-ve	3	T1
90065	2/3	T1/T1	2/2	2/2	B*2/B*2	2/4	-ve	?	T1

Sample	TNFe	-308	TNFc	AspH-1Nco-1		TNFa	Node	Grade	size
90066	3/3	T1/T1	1/1	1/2	B*1/B*2	-	+ve	?	T2
90068	3/3	T1/T1	-	1/2	B*1/B*2	6/10	-ve	2	T1
90069	3/3	T1/T1	2/2	2/2	B*2/B*2	2/5	-ve	?	?
90070	3/3	T1/T1	1/1	1/1	B*2/B*2	7/11	-ve	?	?
90071	-	T1/T1	2/2	1/2	B*2/B*2	2/7	-ve	2	T2
90072	2/2	T1/T1	2/2	1/2	B*2/B*2	2/11	?	?	T2
90073	1/2	T2/T2	2/2	2/2	B*2/B*2	2/2	-ve	?	T2
90074	1/2	-	2/2	2/2	B*2/B*2	-	-ve	1	T1
90075	2/2	-	1/1	2/2	B*1/B*1	2/5	-ve	1	T1
90076	2/2	T2/T2	1/2	1/2	B*1/B*2	6/11	-ve	?	?
90077	-	T1/T2	1/2	2/2	B*1/B*1	2/6	-ve	?	T2
90078	-	T2/T2	1/2	1/1	B*2/B*2	9/11	-ve	2	T1

Sample	TNFe	-308	TNFc	AspH-1	Nco-1	TNFa	Node	Grade	size
90079	-	-	-	1/2	B*1/B*2	-	-ve	3	T2
90080	-	T2/T2	2/2	2/2	B*1/B*2	2/6	+ve	?	?
90082	-	T1/T2	1/1	2/2	B*1/B*1	2/6	?	?	?
90083	-	T2/T2	1/2	1/2	B*2/B*2	2/7	+ve	3	T3
90084	-	T2/T2	1/2	1/2	B*2/B*2	2/11	?	?	?
90085	-	T2/T2	1/1	1/1	B*2/B*2	10/13	+ve	2	T1
90086	-	T1/T2	-	1/2	B*1/B*2	-	-ve	?	T1
90087	-	T2/T2	1/2	1/2	B*2/B*2	4/11	?	?	?
90088	-	T1/T2	1/2	2/2	B*1/B*2	1/2	-ve	1	T1

The TNFe Locus.

In the breast cancer population at this locus, allele e2 is the most commonly expressed, with a frequency of 0.66, followed by allele e3 at 0.23 and allele e1 at 0.11. These data are significantly different to those obtained by Dr. D. Campbell in our group who looked at this locus in 65 control samples, obtaining frequencies of 0.03, 0.75 and 0.22 for alleles 2, 3 and 1 respectively (Figure 7).

The protocol employed to obtain allelic data at this locus employed the use of the non-denaturing system which as previously shown is very unreliable. Furthermore the data are not relevant to the study as there has yet to be evidence produced suggesting a link between this locus and the expression of TNF. The locus was only looked at briefly as part of an attempt to obtain data spanning the whole TNF locus.

The -308 Locus.

The results at this locus show that allele T1 is by far the most commonly expressed in both the breast cancer and control populations (frequencies of 0.69 and 0.80 respectively) (Figure 8). This expression pattern is also, as expected, reflected in the genotype expression pattern observed, with the T1 homozygote frequency of 0.51 comparing with the heterozygote frequency of 0.37 and T2 homozygotes making up the remaining 12% of samples in the breast cancer population. In the control population, 64% were (T1,T1), 32% were (T1, T2) and 4% were (T2, T2) (Figure 9).

The Nco-1 Locus.

The allelotype expression at this locus shows B*1 at a frequency of 0.33, with B*2 at 0.67 in the breast cancer population. In the control population the allele frequencies are 0.35 and 0.65 respectively (Figure 10). With regard to genotype expression, in the breast cancer population only 6% of patients were homozygous for B*1, 40% were homozygous for B*2 and the remaining 54% were heterozygous. In the control population, 13% were homozygous for B*1, 45% were homozygous for allele B*2 and 13% were heterozygous (Figure 11).

Figure7.

A comparison of allelotype data between the control population (n=115) and the breast cancer population (n=84) at the TNFe microsatellite locus. The two populations are significantly different.

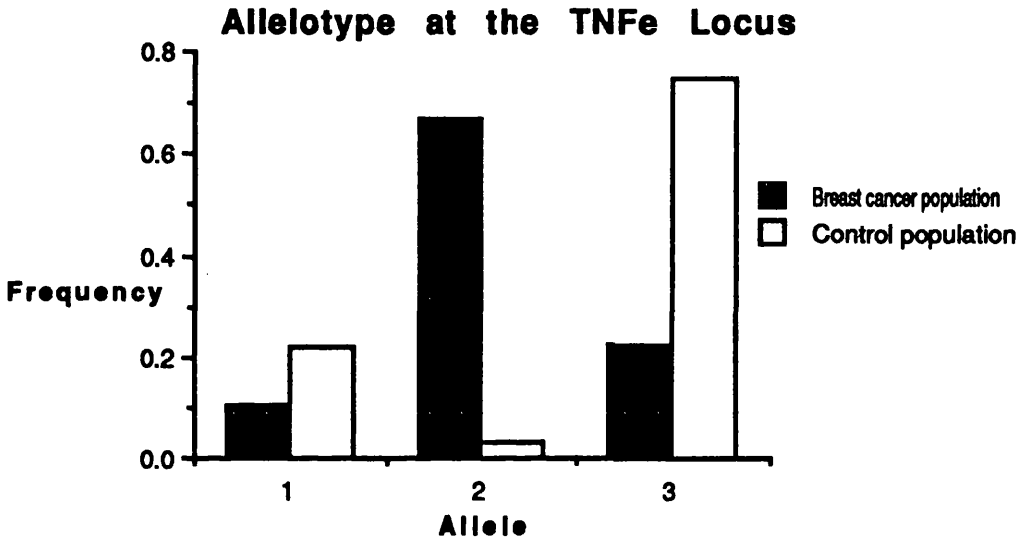


Figure 8

A comparison of allelotype data between the control population (n=115) and the breast cancer population (n=84) at the -308 polymorphic site in the promoter region of the TNF gene. The two populations do not differ significantly.

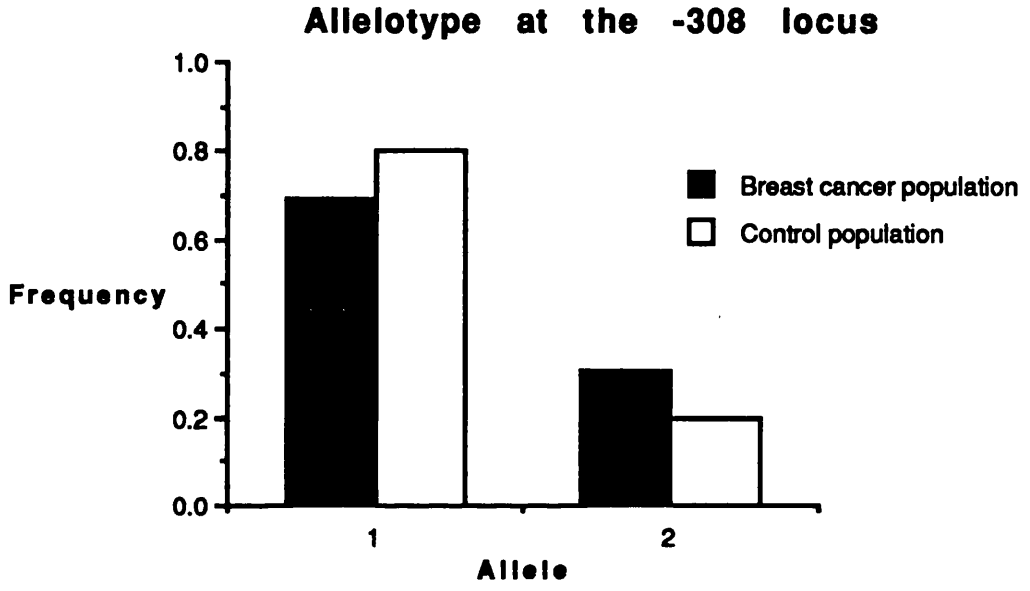


Figure 9

A comparison of the genotype data at the -308 locus between the control (n=115) and breast cancer population (n=84). The two populations do not differ significantly in genotype expression.

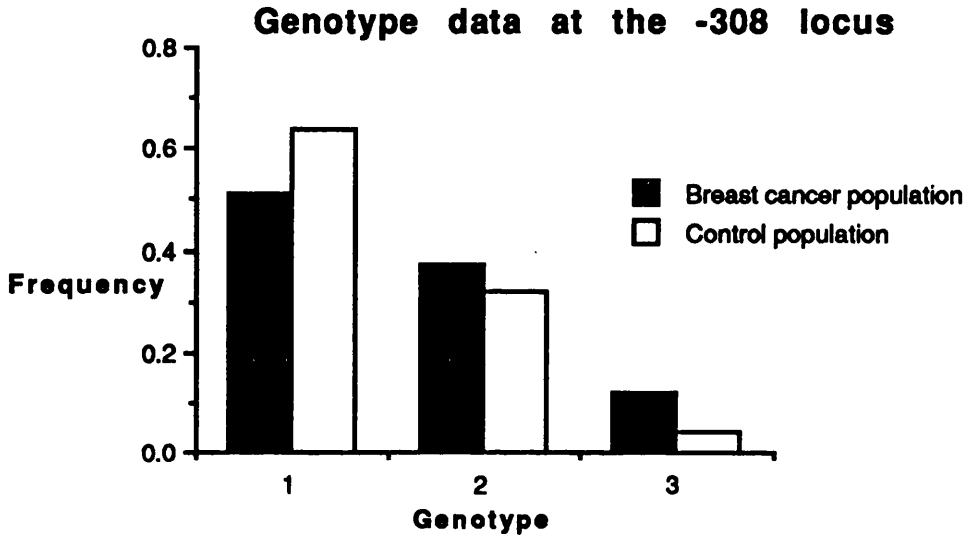


Figure 10

A comparison of allelotype data between the control population (n=115) and the breast cancer population (n=84) at the Nco-1 RFLP locus. The two populations are not significantly different.

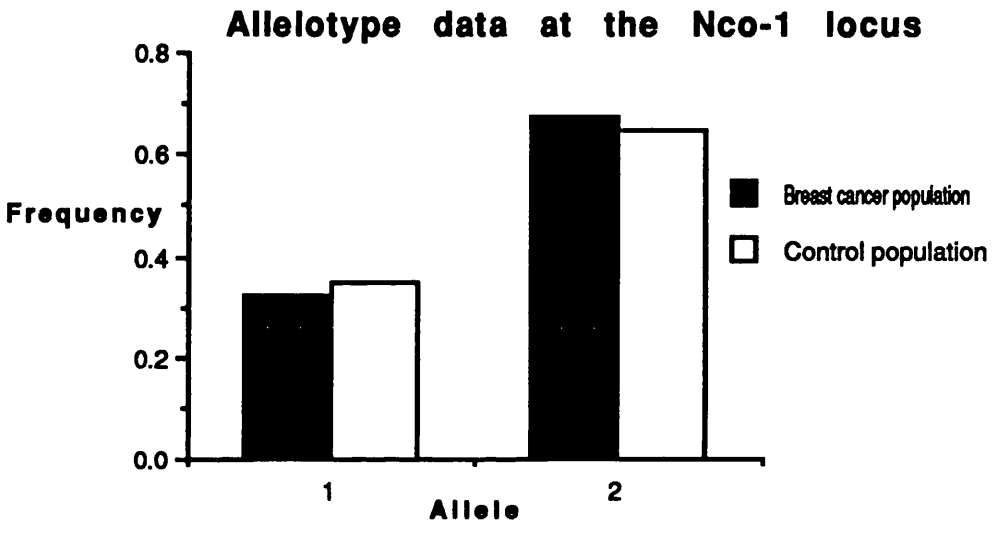


Figure 11

A comparison of the genotype data at the Nco-1 RFLP locus between the control (n=115) and breast cancer population (n=84). The two populations do not differ significantly in genotype expression.

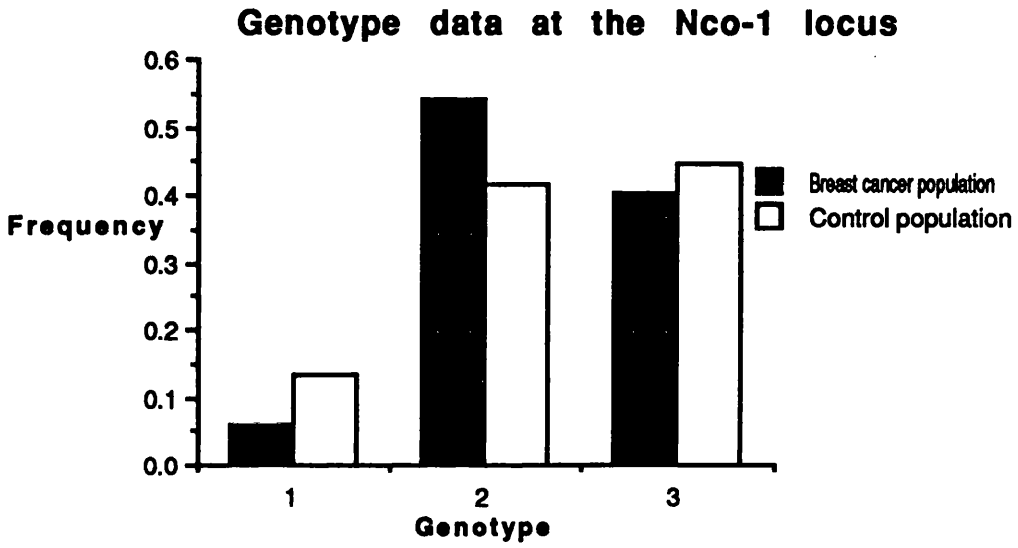


Figure 12

A comparison of allelotype data between the control population (n=115) and the breast cancer population (n=84) at the AspH-1 RFLP locus. The two populations are not significantly different.

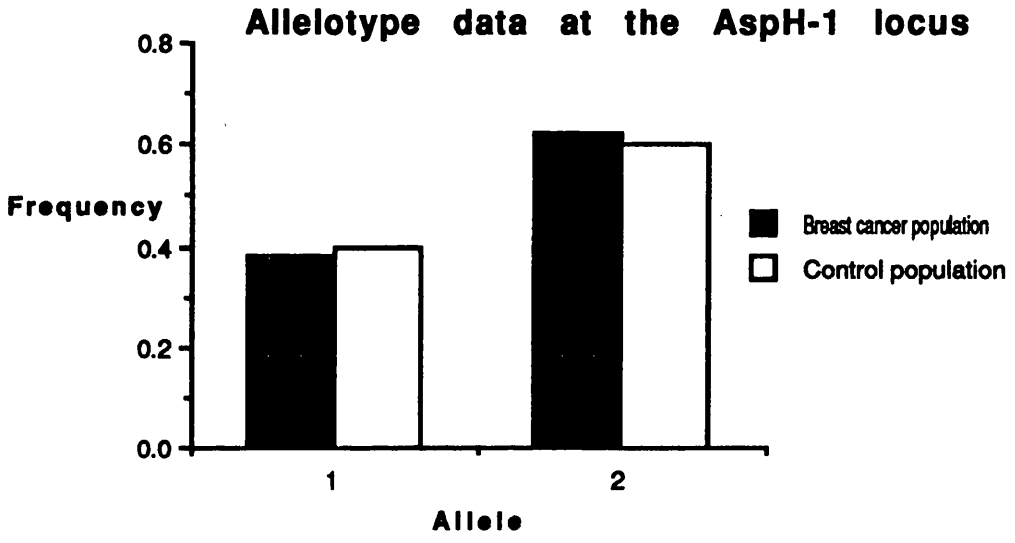
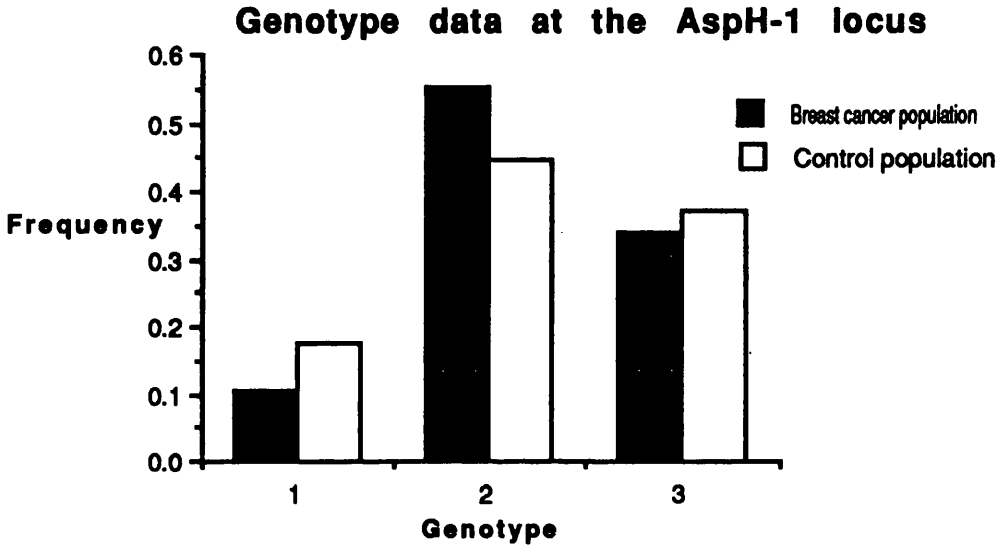


Figure 13

A comparison of genotype data between the control population (n=115) and the breast cancer population (n=84) at the AspH-1 RFLP locus. The two populations are not significantly different.



The AspH-1 Locus.

The most commonly expressed allele, in both populations at this biallelic locus is allele 2 (frequencies of 0.62 and 0.60). This is not reflected directly in the genotype expression pattern, where in both populations the majority of samples are heterozygous 55% and 45% in the breast cancer and control populations respectively (Figures 12 and 13). The allelotype data are similar to that obtained by Ferencik *et al* (1992) who made the original discovery of the polymorphic site, although his group used the technique of Southern Blotting.

The TNFc Locus.

Allele c1 is most frequently expressed at this biallelic microsatellite locus, with a frequency of 0.67 in the breast cancer and 0.79 in the control populations. With regard to genotype expression, the breast cancer population showed 48% of the samples to be homozygous for c1, 11% for c2 and the remaining 41% are heterozygous. In the control samples, 64% were homozygous for c1, 5% for c2 and 31% are heterozygous (Figures 14 and 15).

Figure 14

A comparison of allelotype data between the control population (n=115) and the breast cancer population (n=84) at the TNFc microsatellite locus. The two populations are significantly different (chisquare=4.904, p=0.0268).

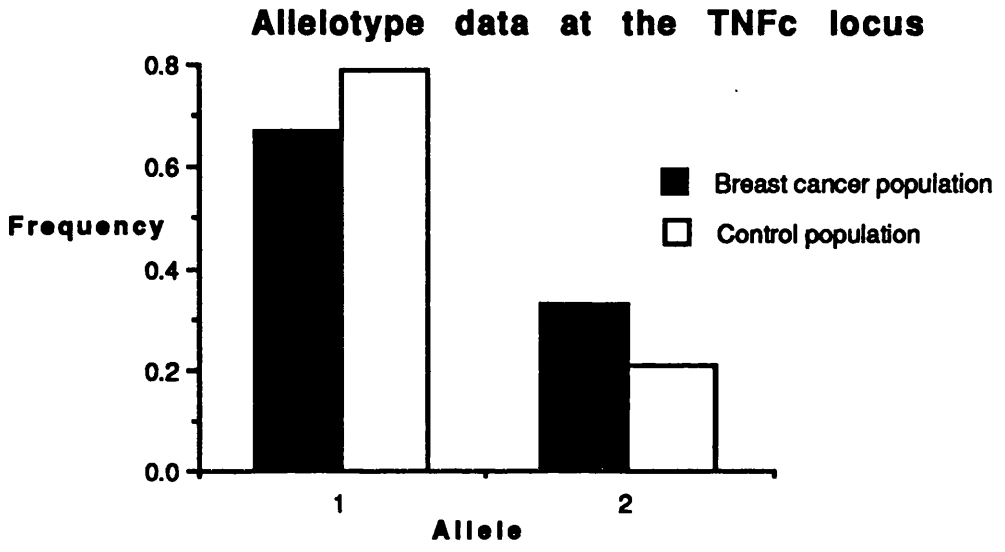
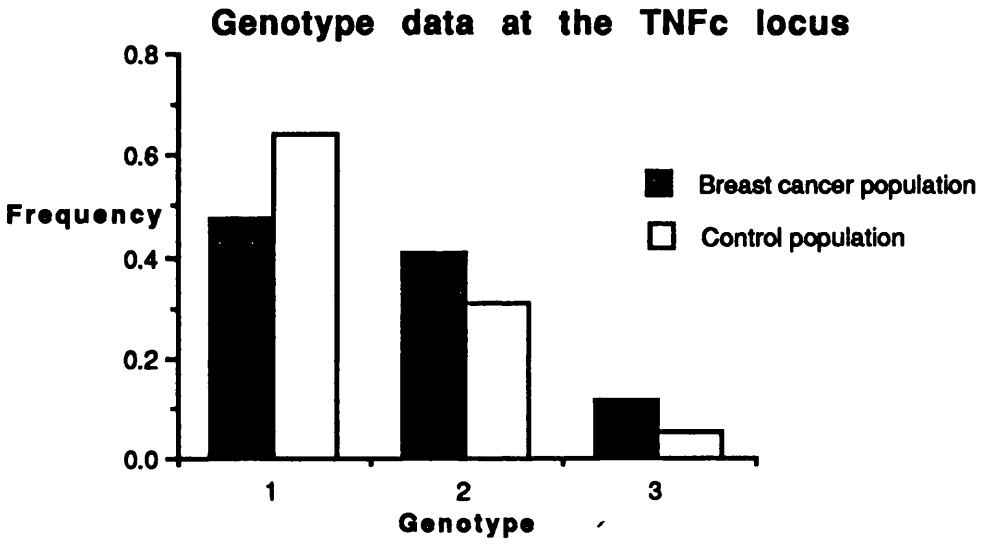


Figure 15

A comparison of genotype data between the control population (n=115) and the breast cancer population (n=84) at the TNFc microsatellite locus. The two populations are not significantly different despite the overexpression of genotype (2,2) in the breast cancer population (chisquare=5.252, p=0.0724).



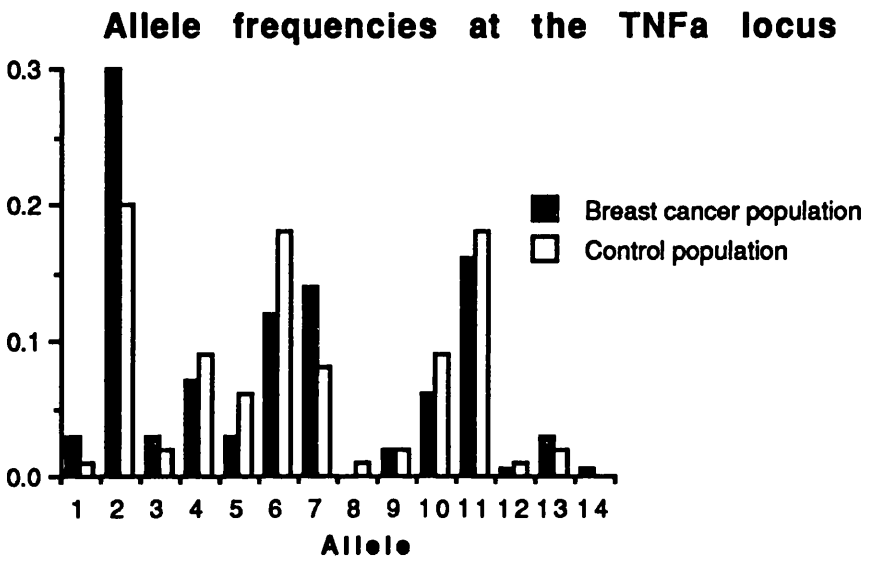
Comparison of allelotype data at the TNFa locus between the breast cancer population and the control population.

The allele frequencies at this locus are shown below, and in figure 16. Allele a2 is by far expressed most frequently (0.31), with a11 the next most common allele, at 0.16. Allele a8 is non-existent in this population of breast cancer patients. Of note is the presence of an allele 2bp larger than the original 13 alleles determined at this locus. Due to its size, it is referred to here as a14. This was found in only one sample where the patient was (1,14). Chi-square analysis revealed that individual alleles 2 and 7 differed significantly between populations (p=0.029 and 0.0445 respectively). The Monte Carlo significance test showed that taken over all the alleles, the two populations are significantly different (p=0.023).

Allele frequencies at the TNFa locus in 84 breast cancer and 115 control samples.

Allele	Breast	Control
1	0.03	0.01
2	0.31	0.20
3	0.03	0.02
4	0.06	0.09
5	0.03	0.06
6	0.14	0.18
7	0.13	0.08
8	0.00	0.01
9	0.01	0.02
10	0.05	0.09
11	0.16	0.18
12	0.01	0.01
13	0.03	0.02
14	0.006	0.00

Figure 16


Allele frequencies at the TNFa locus
0.3
0.2
0.1
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Allele
Breast cancer population
Control population

Comparison of allelotype and genotype data obtained at the three polymorphic sites within intron 1 of LTα between the breast cancer population, 2 further adenocarcinomas (colorectal and gastric) and a control population.

I thought it would be interesting to examine the relationships between various alleles within the TNF locus, looking for linkage between them in the control group and various tumour populations. (I would like to thank Hui-Hui Oh, Joyce Eskdale and Dr. David Campbell for the provision of the additional data on which I did the analysis).

Since three of the polymorphic sites all lie within 157bp of each other within the first intron of the LTα gene (the Nco-1 and AspH-1 RFLPs and the TNFc microsatellite), it was deemed reasonable to examine whether, in any of the 4 populations, patterns pertaining to disease-specificity could be observed and to look for linkage between alleles at the three sites.

Initially, allelotype expression was determined at the three sites in the malignant populations; breast (n=84), colorectal (n=106) and gastric (n=45) to see whether any of these populations showed a tendency to over- or under-express either allele at the biallelic sites when compared to the control population (n=115). This data was obtained with the aid of other members of the research group (Dr. D Campbell, Miss HH Oh & Mrs. J Eskdale). The results, shown in Tables 4 to 6, indicate that none of the adenocarcenoma populations differ significantly to the control population in terms of allelotype expression at the Nco-1 and AspH-1 loci, but the breast and colorectal cancer populations differ at the TNFc locus.

Following this, genotype expression data was obtained for each adenocarcenoma populations and compared against the control population. At each of the three loci there were three possible genotypes; (1,1), (1,2) and (2,2).

The results indicated that none of the malignant populations differed significantly to the controls at the Nco-1 locus (Table 7). At this locus, allele 2 is known to be the most commonly expressed. The data obtained here confirms this with the majority (44.8%) of the control population expressing genotype (2,2). This pattern was also seen in the colorectal population, but not in the gastric or breast cancer populations, where genotype (1,2) is more frequently expressed than (2,2). However, taken over all three possible genotypes, neither of the latter two populations differ significantly to the controls.

Table 4. Allelotype expression at the Nco-1 locus, expressed as frequency, with the nos. of alleles observed in ( ).

Population	Allele 1	Allele 2
Control	0.35 (67)	0.65 (128)
Breast	0.33 (55)	0.67 (111)
Gastric	0.35 (29)	0.65 (53)
Colorectal	0.29 (39)	0.71 (97)

Table 5. Allelotype expression at the AspH-1 locus, expressed as frequency, with the nos. of alleles observed in ( ).

Population	Allele 1	Allele 2
Control	0.40 (41)	0.60 (61)
Breast	0.37 (61)	0.63 (101)
Gastric	0.29 (27)	0.71 (53)
Colorectal	0.34 (26)	0.66 (66)

Table 6. Allelotype expression at the TNFc locus, expressed as frequency, with the nos. of alleles observed in ( ).

Population	Allele 1	Allele 2
Control	0.79 (156)	0.21 (42)
Breast	0.69 (111)	0.31 (51)
Gastric	0.69 (62)	0.31 (28)
Colorectal	0.67 (128)	0.33 (62)

Table 7. Genotypes at the Nco-1 locus, expressed as a percentage, with actual sample nos. are in ( ).

Population	(1,1)	(1,2)	(2,2)
Control	13.5 (13)	41.7 (40)	44.8 (43)
Breast	5.8 (5)	54.0 (47)	40.2 (35)
Gastric	7.3 (3)	56.1 (23)	36.6 (15)
Colorectal	5.9 (4)	45.6 (47)	48.5 (33)

Table 8. Genotypes at the AspH-1 locus, expressed as a percentage, with actual sample nos. in ( ).

Population	(1,1)	(1,2)	(2,2)
Control	17.6 (9)	45.1 (23)	37.3 (19)
Breast	6.0 (5)	54.0 (45)	33.8 (27)
Gastric	12.5 (5)	42.5 (17)	45.0 (18)
Colorectal	8.7 (4)	39.1 (18)	52.2 (24)

Table 9. Genotypes at the TNFc locus, expressed as a percentage, with actual sample nos. in ( ).

Population	(1,1)	(1,2)	(2,2)
Control	64.3 (63)	30.6 (30)	5.10 (5)
Breast	49.3 (40)	38.3 (31)	12.4 (10)
Gastric	50.0 (21)	45.2 (19)	4.80 (2)
Colorectal	45.3 (43)	44.2 (42)	10.5 (10)

At the AspH-1 locus, the control and breast cancer populations most frequently expressed genotype (1,2), whereas the gastric and colorectal populations showed genotype (2,2) to be most commonly expressed. However, taken over the three possible genotypes, none of the cancer populations differed significantly to the controls (Table 8).

At the TNFc locus, genotype (1,1) was the most commonly expressed in each of the four populations, which was to be expected due to the vast overexpression of allele 1 compared to allele 2. Despite the seemingly similar expression pattern observed, the colorectal cancer population does in fact differ significantly to the controls (chisquare=7.395, p=0.0248). The frequency of genotype (2,2) is greatly increased in the colorectal and breast cancer populations, but taken across all three possible genotypes, the breast population is not significantly different (chisquare=5.252, p=0.0724) (Table 9).

Finally, it was of interest to determine whether, within any of the four populations, any of the genotypes at the three loci were significantly linked to those at either of the other two loci ie. whether there was any evidence of extended genotypes in any of the adenocarcinoma groups. A significant linkage was found which was exclusive to the colorectal cancer population, between AspH-1 (2,2) and TNFc (1,1); p corrected=0.0256. Other significant linkages were found, either exclusive to the control population or common to more than one population (Table 10).

Table 10. Significant linkages between Genotypes at the three polymorphic loci (corrected by Bonferoni's factor).

Population	Nco-1	AspH-1	TNFc	p value
Colorectal		(2,2)	(1,1)	0.0256
Control	(2,2)	(2,2)		0.0064
Control	(1,1)	(2,2)		0.0024
Control	(2,2)	(1,1)		0.0008
Gastric	(2,2)	(1,1)		0.0040
Breast	(2,2)	(1,1)		0.0040

Comparison of allelotype and genotype between node positive and node negative breast cancer patients reveals significant differences at both the TNFa and TNFc microsatellite loci.

Nodal status is a reflection of the metastatic behaviour of the tumour at the time of surgery. It is used to predict the prognostic outcome of the patient, with a positive status equating to poor prognosis and a negative status to a good prognosis.

I separated the breast cancer population on the basis of being either node positive (N+ve) or node negative (N-ve) and determined allelotype and genotype data for each group at the TNFa, TNFc and Nco-1 loci (those linked with TNF levels and prognosis) [Tables 12 and 13].

At the Nco-1 locus, neither the allelotype nor genotype differed significantly between the two groups (chisquare=0.539, p=0.537 and chisquare=3.531, p=0.1711 respectively).

At the TNFc locus, the two populations differed significantly on the basis of allelotype (chisquare=5.540, p=0.0186) and genotype (chisquare=6.433, p=0.0401). Interestingly, the c1 allele was overexpressed in the N+ve group and the (2,2) genotype underexpressed in these patients. This was surprising considering that it is allele c2 which is linked with increased TNF levels and hence poor prognosis.

At the TNFa locus, the two groups differed significantly when the number of a2 alleles is compared with the number of non-a2 alleles (chisquare=4.814, p=0.0282). Again as with the TNFc result, it is somewhat surprising in that a2 is underexpressed in the node +ve population and it is this allele which has been linked with high TNF expression and poor prognosis. With regard to genotype at this locus, a greater percentage of node positive patients do not carry an a2 allele compared to the node negative patients (61% versus 36%), making the two populations significantly different (chisquare=4.140, p=0.0419).

Table 12. Allelotype data of node positive and node negative breast cancer patients at the TNFa, TNFc and Nco-1 loci.

TNFa locus.

Node status	no. of a2 alleles	no. of non-a2 alleles
Positive	12	52
Negative	25	45

TNFc locus.

Node status	allele c1	allele c2
Positive	56	14
Negative	46	28

Nco-1 locus.

Node status	allele B*1	allele B*2
Positive	21	47
Negative	30	52

Table 13. Genotype data of node positive and node negative breast cancer patients at the TNFa, TNFc and Nco-1 loci.

TNFa locus.

Node status	no. of genotypes with allele a2.	no. of genotypes without allele a2.
Positive	13	20
Negative	23	13

TNFc locus.

Node status	(1,1)	(1,2)	(2,2)
Positive	22	12	1
Negative	16	14	8

Nco-1 locus.

Node status	(1,1)	(1,2)	(2,2)
Positive	0	21	13
Negative	4	22	16

DISCUSSION

Usefulness of RFLP and microsatellite markers in studies involving immunorelevant genes.

Restriction fragment length polymorphisms (RFLPs) were the initial markers used in any such studies. The presence or absence of a restriction endonuclease cleavage site within a region of DNA gives rise, on digestion with that enzyme, to a possible biallelic pattern. The haplotype tends to be inherited in a Mendelian fashion allowing the inheritance of genes to be monitored. The main drawback however is the low degree of informativity available from a biallelic site. This is where the use of microsatellite polymorphisms has revolutionised the field of DNA markers. They involve the presence of di- (or less frequently tri-) nucleotide sequences, tandemly arranged and each allele defined by the varying number of repetitions of the sequence. Their high abundance throughout the genome and high degree of informativity makes them ideal candidates for studying allelic variation in genes or loci of interest (in this study, the region of interest being the TNF locus).

PCR-based determination of DNA markers.

The polymerase chain reaction is a quick, simple method of amplifying a region of DNA within which RFLP and/or microsatellite sites lie. The effectiveness of the technique is very much dependant upon the optimisation of the reaction conditions, as clearly shown in this study where significant differences were demonstrated during the optimisation of the conditions for the TNFa and also the TNFc microsatellite reaction conditions.

Genotype determination at the TNFa microsatellite locus in a normal population as a means of establishing a technique suitable for use on further populations.

In order to establish that the technique set up to determine allelotype at the TNFa microsatellite locus was indeed suitable, 115 control samples, ie. samples from people with no known disease, were obtained from the tissue typing department at Glasgow Royal Infirmary and the allele frequencies obtained compared with those from several previous studies carried out worldwide over the past 4 years (Table 11).

From this comparison we were happy with the overall similarities between this study and the others in terms of allele frequency patterns, so were felt justified to
(a) Transfer the technique to further populations (the breast cancer population in the case of this study) and (b) Use the date obtained to draw further conclusions.

Table 11. Comparison of allele frequency in control populations at the TNFa microsatellite locus between this study and a further 7 populations.

TNFa allele	This study	[1]	[2]	[3]	[4]	[5]	[6]	[7]
1	0.01	ND	0.020	0.007	0.116	-	0.031	0.007
2	0.20	0.11	0.215	0.307	0.250	0.219	0.208	0.320
3	0.02	ND	0.034	0.033	0.035	0.012	0.031	0.033
4	0.09	0.09	0.040	0.113	0.045	0.037	0.042	0.100
5	0.06	0.04	0.040	0.067	0.036	0.110	0.031	0.067
6	0.18	0.22	0.101	0.140	0.071	0.134	0.094	0.140
7	0.08	0.15	0.134	0.033	0.170	0.073	0.125	0.040
8	0.01	ND	0.040	-	-	-	0.052	-
9	0.02	0.07	-	0.033	0.027	0.085	-	0.033
10	0.09	0.15	0.154	0.087	0.107	0.134	0.104	0.093
11	0.18	0.13	0.128	0.160	0.089	0.098	0.187	0.147
12	0.01	0.02	0.074	0.013	-	0.012	0.073	0.0133
13	0.02	0.02	0.013	0.007	0.054	0.085	0.021	0.007

[1] Nedospasov *et al*, 1991.
[2] Jongeneel *et al*, 1991: French population.
[3] Pociot *et al*,1993: Danish population.
[4] Crouau-Roy *et al*, 1993: Basque population.
[5] Crouau-Roy *et al*, 1993: Greek population.
[6] Crouau-Roy *et al*, 1993: French population.
[7] Crouau-Roy *et al*, 1993 : Danish population.

As further 'quality control' checks, three samples which had been previously genotyped were run on each TNFa microsatellite gel, along with the cosmid M31A and the four cell lines. This confirmed that all the samples ran at the same pace relative to several standards. An additional 'internal control' was also employed, involving the use of related individuals. The microsatellite alleles are inherited in a Mendelian manner, so by following transmission from parents to siblings or observing groups of siblings, the reliability of allele calling could be determined. Six family groups were available for study (Figure 17).We were unable to obtain three samples (within families 3, 5 & 6), but despite this, were clearly able to demonstrate that a Mendelian pattern of inheritance of the alleles was feasible in each family group.

Where data were unavailable for the TNFa locus, the alleles determined at the other loci under examination were considered in the same manner (data shown below TNFa data), and it was found that within each family, the alleles determined could all have been transmitted in a Mendelian fashion further supporting our allele calling.

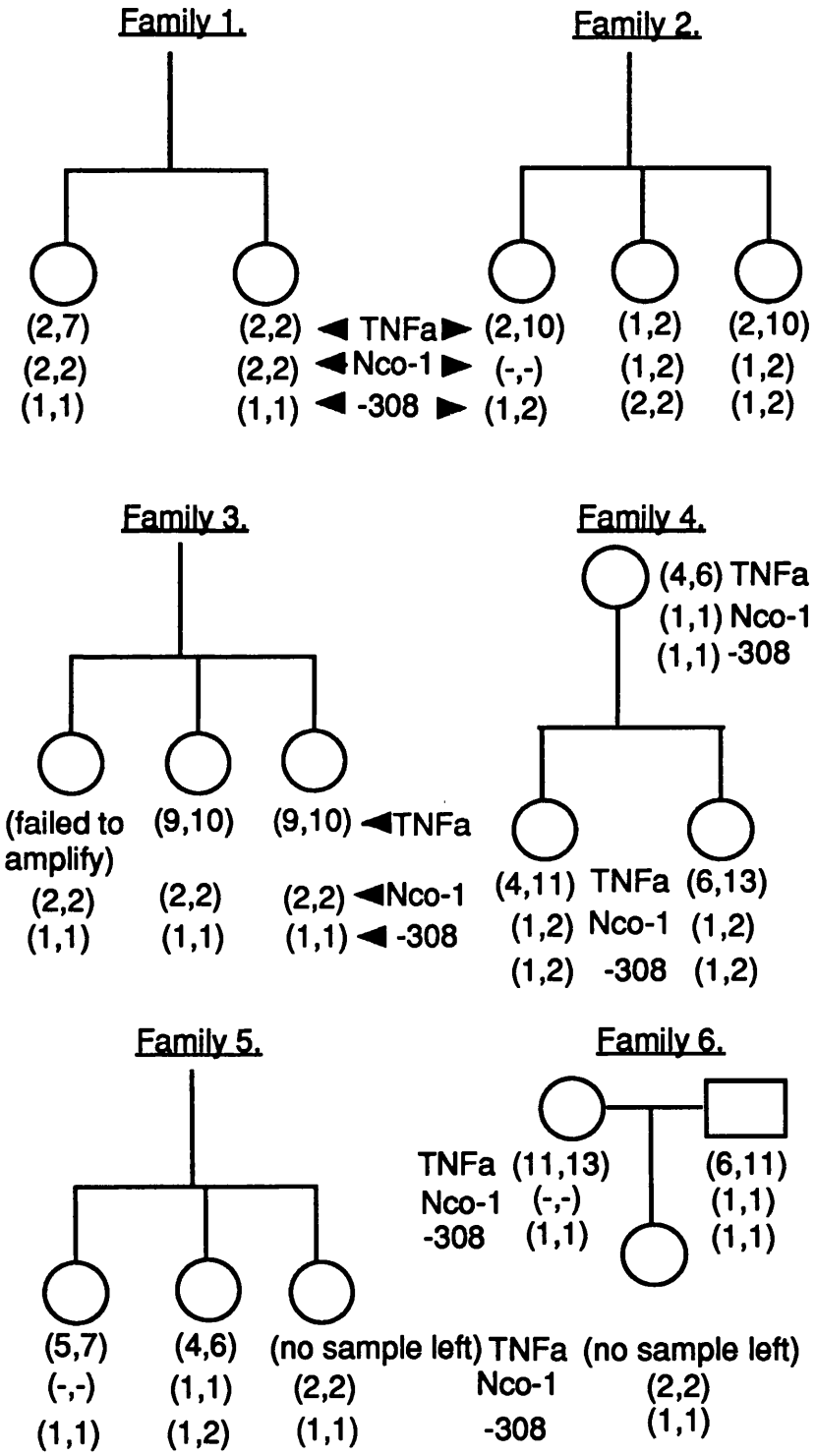
Allelotype data at the TNFe microsatellite locus in the breast cancer population does differ significantly to that obtained for a control population.

The TNFe locus, originally identified in 1993 by Udalova et al is a TC/GA-like repeat lying 8-10kb downstrean of the TNF gene, within intron 2 of Lst-1 (Holzinger *et al,* 1993). Originally thought to be a triallelic locus, the smallest allele being 101bp, data obtained from detailed cell-line typing has concluded the presence of a larger e4 allele (present in the cell lines TAB089 and T7527).

Because of the distance away from the TNF locus, no groups have attempted to relate allelotype or genotype at the site to TNF/LT synthesis or expression. The reason for TNFe typing the breast cancer population in this study was primarily to produce a more comprehensive 'whole-locus' study. The fact that the reaction conditions were not fully optimised was not essential because of the above reasons. Furthermore, with previous studies showing that the loci appearing to have linkage with TNF levels lie at the telomeric, as opposed to the centromeric, end of the locus, it was felt unnecessary to give too much time to this site.

Figure 17.

Family groups within the control samples used as internal controls for allele determination.



Comparing the data obtained for the breast cancer population with that previously obtained by Dr. D. Campbell in the group for a control population suggests that the allele expression pattern shown by the cancer population at the TNFe locus could be unique. However the absence of optimal reaction conditions meant that there were several non-specific bands present on the gel, which caused some ambiguity when the alleles were being called. This result therefore was not looked on as important.

One of the polymorphic loci not examined in this study, that of the TNFd microsatellite, lies linked to the TNFe locus. It too displays a TC/GA repeat sequence, but consists of 7 alleles, the smallest being 129bp (Holzinger *et al*, 1993). Data was not obtained for this locus for a variety of reasons. Only one previous study (Turner *et al*, 1993) has attempted to correlate allelic variation at this site with TNF levels. The results showed only a tenuous link between d3 and increased TNF levels, which was not deamed sufficient evidence to look at the site ourselves. Furthermore, as with the TNFa and B loci, the TNFd microsatellite lies in close linkage with TNFe, the site at which we had already gathered data. Finally the TNFd locus is situated at a distance from the TNF gene cluster, certainly far from sites previously linked to TNF expression, so we felt justified in omitting it from this study.

The breast cancer population does not show a unique allele expression pattern at the -308 RFLP, located in the TNF gene promoter region.

The interest in this locus stemmed from its positioning in the TNF promoter. Had the breast cancer population shown a unique expression pattern, it could have been postulated that this polymorphism was therefore involved in controlling the level of TNF expression and even LT production. Whether this increased or indeed decreased the cytokine levels would have been of particular interest as other polymorphic loci have shown linkage with increased TNF levels.

The results of the allelotyping however show the common allele (T1) to be expressed with a frequency of 0.69 and allele 2 (T2) with a frequency of 0.31. This is similar to the original study by Wilson *et al* in 1992 where values of 0.84 and 0.16 respectively were obtained for his 40 control samples. Furthermore, it is also similar to the results obtained by workers in our own research group on a control population of 91 samples where frequencies of 0.8 and 0.2 respectively were obtained.

The second polymorphic site not to be considered in this study lies 70bp downstream from the -308 locus, at position -238. This also lies within the promoter region of the TNF gene. It was felt that the biallelic site displayed too low an informativity (less than 7% of the population carry the rarer allele) to be considered for detailed study. Nedospasov *et al* have shown (personal communication) that deletion mutants lacking the -238 site do not have altered LPS-induced TNF production, suggesting a lack of involvement for this site in the regulation of TNF expression. They have also shown that the -238 and -308 loci are in linkage equilibrium with each other, which is somewhat unexpected due to their close proximity. This could suggest that they are both ancient polymorphic sites.

The third of the polymorphic sites within the TNF locus not to be studied for this project is the next polymorphic site upstream of the -308 polymorphism. It is the biallelic EcoR-1 RFLP, located in the 3'UT region of LTα exon 4. This site displays low informativity (Partanen *et al,* 1988) and no linkage between either of its alleles and TNF levels has been detected, so it was deemed unnecessary to investigate.

Intron 1 of the Lymphotoxin alpha gene.

With data available on a further 2 adenocarcinoma populations (gastric and colorectal) as well as the control population, a more detailed comparison was possible for the three polymorphic loci located within intron 1 of the LTα gene, namely the TNFc microsatellite and the AspH-1 and Nco-1 RFLPs. As well as determining allele expression at these loci, we wanted to ascertain whether within each population there was a unique pattern of allele expression which may suggest the importance of a particular site in the control of expression of TNF in the progression to malignancy. We also wanted to determine whether there were significant linkages between alleles at the three loci which would suggest if the sites were under a single control factor or are independant of each other with regard to the control of TNF expression.

(a) Allelotype expression within intron 1 of Lymphotoxin α.

With regard to the allelotyping results at each of the three loci, none of the malignant populations differed significantly to the control population at both the Nco-1 and AspH-1 RFLPs, but both the breast and colorectal cancer populations differed significantly to the control population at the TNFc locus (chisquare=4.904, p=0.0268) and (chisquare=6.445, p=0.0111) respectively. Both these populations express allele 2, the rarer allele, at a slightly higher frequency than the gastric cancer population. The TNFc2 allele has been linked to increased TNF expression, so the results could suggest a role for higher TNF levels in the progression to malignancy in breast and colorectal cancers.

(b) Genotype expression within intron 1.

Two previous studies (Pociot *et al*, 1991 & Derkx *et al*, 1995) showed contradicitng results as to genotypic associations at the Nco-1 locus. Using activated monocyte cultures, Pociot demonstrated that the B*2 homozygote genotype was linked to high TNF production. Derkx on the other hand, using stimulated PBMNCs showed that samples with the heterozygous genotype (B*1, B*2) released significantly higher levels of TNF than the B*1 homozygotes and moreso than the B*2 homozygotes. With regard to the genotype expression patterns determined in our study at the Nco-1 locus, none of the malignant populations differed significantly to the control population. The high TNF expression genotype as determined by Pociot (B*2,B*2) was only most common in one of our malignant populations (colorectal) and also in the controls. As Pociot's data was obtained only from activated monocytes, this is less likely to reflect the *in vivo* situation than the data obtained by Derkx from activated PBMNCs. In accordance with his results, we have shown that both the breast cancer and gastric cancer populations display the heterozygous genotype (B*1, B*2) as the most common genotype, suggesting that high TNF levels may be associated with the progression to malignancy. However, as neither population differed significantly to the control population, it does suggest that this locus does not play an important role in the control of TNF or LT production in relation to malignancy.

At the AspH-1 locus the genotype expression patterns of the malignant populations is more site-specific. (The choice of adenocarcinoma groups allows site-specificity to be tested because the other two populations develop tumours in the gastro-intestinal tract whereas the breast cancer group tend to develop them in the bones). Both the gastric and colorectal groups most frequently express (2,2) whereas in the breast cancer group, (1,2) is most common. However, taken across the three genotypes, no malignant population differs significantly to the controls. The results again suggest that the locus does not play any role in the progression to malignancy through the control of TNF or LT due to the overall similarities to the control population.

At the TNFc locus,there is an overexpression of allele 2 in the breast cancer population although the population does not differ significantly to the controls (chisquare=5.252, p=0.0724). The colorectal cancer population however, differs significantly to the controls (chisquare=7.395, p=0.0248) due to the same overexpression of allele 2 (data from Miss. H.H. Oh).The c2 homozygous genotype is associated with high TNF expression (Pociot et al, 1993). Furthermore, studies by Mallmann *et al* and Miles *et al* both showed that increased TNF levels were found in breast cancer patients with poorer prognoses. Taking these two facts together, the TNFc locus may be one of the controlling factors in TNF expression and this increased level may play a role in the progression to malignancy.

Some clinical data was obtained on most of the breast cancer patients. The subjects were divided according to whether they were node positive or node negative, with positivity being taken as an indicator of poor prognosis. It was found that none of the node positive patients were homozygous for allele 2 at the TNFc locus whereas 7 out of 31 node negative patients were c2 homozygotes. With reference to Pociot's data, these results are in contradiction of what would have been expected again suggesting that looking only at activated monocytes does not reflect the *in vivo* situation. Our data for the TNFc locus suggests that this locus alone is not the controlling factor in malignancy progression. (The differences in allelotype and genotype expression at the TNFc locus between the node positive and node negative patients were both statistically significant (p=0.0186 and 0.0401 respectively). As TNF levels were not measured in this study, there was no confirmation that high levels necessarily correlate with the c2 homozygous genotype.

(c) Genotype linkages within Intron 1.

As the three loci in intron 1 lie within 157bp of each other it was expected that they would be in linkage disequilibrium ie.that certain alleles would be inherited as a group, as in an extended genotype. This was not the case due to the number and variety of significant genotype linkages present in the four populations, especially between alleles at the Nco-1 and AspH-1 loci. From this it could be concluded that the three sites are in fact independent of each other. Furthermore it could be postulated that there are putative recombination hot-spots flanking the AspH-1 locus, but within the boundaries of the TNFc and Nco-1 loci.

The TNFa microsatellite locus.

The two remaining polymorphic sites within the TNF locus are the TNFa and b microsatellites, which lie 3.5kb telomeric of the LTα gene.The TNFb locus was omitted from this study. Initial optimisation attempts proved unsuccessful and the fact that the alleles only differ by one base instead of the usual two, made the gels harder to read with confidence. Since the locus is linked to the TNFa site, it was decided that data from only one of these sites would be necessary. Furthermore, little work has previously been done on this locus, whereas several studies have already looked at TNFa linkage with TNF levels. It was therefore thought more pertinamt to concentrate on TNFa and omit the TNFb locus.

At the TNFa locus several interesting observations arose. Firstly an extra allele was detected in one of the breast cancer samples. As it appeared 2bp larger than the a13 allele it was named a14. This naming system has to be questioned at this point. Had the allele been 2bp smaller than allele 1, it would have been named a0. Any more than 2bps less, would have given it a minus number. A more sensible system would be to base the allele name on the number of repeats present. For example, a1 has 6 repeats of the TC unit so would be referred to as TNFa $(TC)_6$ (Epplen *et al*, 1995). Secondly, no sample contained the allele a8. This has previously been shown by Crouau-Roy *et al*, 1993 and Nedospasov *et al*, 1991 although both studies used healthy populations. This is perhaps further confirmation of our allele calling procedure. As with the previously reported studies, the a2 allele is the most frequently expressed, although it is overexpressed in the breast cancer population. Ten of the 84 samples expressed the genotype (2,11) which may suggest linkage disequilibrium between these two alleles.

In our control population, (2,11) was also the most frequently expressed genotype with 7 samples expressing it. In the study of four different populations by Crouau-Roy, they found linkage disequilibrium at the TNFa locus between three sets of alleles; (6,11), (6,12) and (7,11). The second most frequent genotype in the breast population was also (6,11), with 8 samples expressing it.

Previous studies by Pociot *et al*, 1993 showed that both the a2 and a6 alleles correlate with TNF secretion levels in monocytes stimulated with LPS. High secretion correlated with the presence of a2 whereas low secretion correlated with allele a6. Contrading these results are the observations of Derkx *et al* (1995). They showed that the endotoxin-induced TNF release in whole blood (but not PBMNCs) was significantly lower in individuals with alleles a2, a6 and a10 than individuals with alleles a4 and a11. Returning however to the results of Pociot *et al*, where a2 was associated with high TNF levels. The a2 allele was significantly overexpressed in the breast cancer population compared to the control samples which further fits with the hypothesis that there is a role for high TNF in the early progression of breast cancer, a result also shown by both Mallmann and Miles. Both groups found high TNF correlated with poor prognosis.Furthermore, as the breast cancer population differs significantly to the control population when all the TNFa alleles are taken into account, this suggests a role for the TNFa locus in the development of breast cancer.

Extended genotypes involving the Nco-1, TNFa and TNFc loci.

As already mentioned, certain alleles at the Nco-1, TNFa and TNFc loci have all shown linkage with TNF levels (Nco-1 B*2, TNFa2 and TNFc2 all correlate with high levels of TNF whereas TNFa6 is linked with low TNF secretion). A study earlier this year by Derkx *et al*, linked a2, a6 and a10 to low TNF secretion and a4 and a11 along with the Nco-1 genotype (B*1,B*2) to high TNF secretion. In the breast cancer population, the frequency of the (2,2) genotype at the TNFc locus is increased compared to the control population (0.123 versus 0.051). This is also seen in the colorectal cancer population. At the Nco-1 locus, the (1,2) frequency is higher in the breast cancer and gastric cancer populations compared to the controls and there is a large decrease in the (1,1) genotype frequency (0.54 versus 0.130), which is linked to decreased TNF expression. Finally, allele 2 is overexpressed at the TNFa locus in the breast cancer population.

Both the Pociot and Derkx studies show that the TNF response correlates with several genetic markers within the TNF gene so on this basis, an extended genotype involving all three loci was looked for in the breast cancer population which would further substantiate the hypothesis that high TNF expression is involved in the aetiology of the malignancy.

When all those samples homozygous for TNFc2 were analysed across the Nco-1 and TNFa loci, the following data was obtained;

Sample	TNFc	Nco-1	TNFa
90010	(2,2)	(2,2)	(2,4)
90021	(2,2)	(2,2)	(2,4)
90023	(2,2)	(1,2)	(7,7)
90065	(2,2)	(2,2)	(2,4)
90069	(2,2)	(2,2)	(2,5)
90071	(2,2)	(2,2)	(2,7)
90072	(2,2)	(2,2)	(2,11)
90073	(2,2)	(2,2)	(2,2)
90074	(2,2)	(2,2)	(-,-)
90080	(2,2)	(1,2)	(2,6)

These results show that the alleles linked with high TNF expression do seem to form part of an extended haplotype in that the majority are not only homozygous for TNFc2, but also homozygous for Nco-1 B*2 and carry a TNFa2 allele. (In the control population, all the c2 homozygotes (only 6 out of 115) were also homozygous for the B*2 allele).

When looking at all those samples homozygous for TNFa2, the following data was obtained;

Sample	TNFa	Nco-1	TNFc
90013	(2,2)	(1,2)	(1,2)
90029	(2,2)	(1,2)	(1,2)
90048	(2,2)	(1,1)	(1,1)
90049	(2,2)	(1,2)	(1,2)
90073	(2,2)	(2,2)	(2,2)

Here the most common haplotype is (2,2), (1,2), (1,2). This evidence is interesting when the results from Derkx study are noted, where the heterozygous Nco-1 genotype associated with high TNF.

Looking at the samples homozygous for the Nco-1 B*2 allele, 16 out of the 34 were heterozygous for TNFa2, 1 was homozygous for TNFa2 and the remainder did not express a2. As for the TNFc locus of these samples, 16 were heterozygous, 10 homozygous for allele c1 and the remaining 8 homozygous for allele c2. These results are less suggestive of the extended haplotype between the three sites although fewer of the samples are homozygous for the TNF c1 allele.

Taking those samples heterozygous at the Nco-1 locus, at the TNFc locus, 15 out of 42 were heterozygous, 25 were homozygous for allele c1 and 2 were homozygous for allele c2. As for the TNFa locus in these samples, 20 expressed allele a2, a further 19 did not and the remainder failed to amplify.

Examining the samples homozygous for the Nco-1 allele B*1, 3 of the 5 expressed an a6 allele. The combination of B*1, a6 is linked to low TNF expression levels. More convincing of this, is the fact that 29 out of the 31 (B*2,B*2) samples did not express the a6 allele.

From the above data it can be concluded that several of the samples do display an extended genotype including the three alleles previously correlated with high TNF expression which further substantiates its role in the aetiology of the disease. Also, a second subset of samples also exists displaying a6, B*1 which are both linked with low TNF expression. In the node negative patients, 8 out of 26 who carried a B*1 allele also carried an a6 allele. In the node positive group, 9 out of 21 with B*1 also carried a6.

HLA expression and associations with TNF.

Because of the localisation of the TNF genes to within the MHC class I and class II genes and the biological roles of the gene products, it is worth considering the expression patterns of the HLA molecules in tumours and associations between these antigens and TNF alleles in non-immune as well as autoimmune diseases.

In most cell types, HLA class II expression is not constitutive, but can be induced by physiological inducers such as TNF and interferon gamma. The pattern of expression of these cell surface antigens changes in relation to the cells' functional capacity.

Alterations such as *de novo* expression, a decrease or even complete loss of expression are often associated with oncogenesis and also autoimmune disease. A study in 1994 by Cabrera *et al* investigated the biological implications of HLA-DR expression in tumours.They found that 36% of tumours derived from HLA-DR positive normal breast tissue retained their ability to express the HLA molecules and moreover that there was a clear relationship between DR expression and tumour differentiation. Tumours which still expressed the HLA-DR molecules tended to be less well differentiated and the patients were given a better prognosis. This could perhaps be explained by the fact that antigen presentation at the site of the tumour will be enhanced, as consequently will be the immune response, leading to heightened tumour destruction. As TNF is known to be one of the molecules able to induce HLA-DR expression, it could be postulated that this is one of the roles of the higher levels of TNF seen in some cancers in patients given better prognoses (Mallmann *et al*, 1991).

An earlier study by Bendtzen *et al* in 1988 concentrated on the association between HLA-DR2 and the LPS-induced production of TNF in the mononuclear cells from healthy individuals. They found that cells from HLA-DR2$^+$ individuals secreted significantly less TNF than those from HLA-DR2$^-$ individuals. This is evidence that TNF is influenced by genes in the HLA-DR region. This is more interesting when it was shown that there were no other correlations between TNF and other HLA molecules, including the HLA-B antigens. The HLA-B locus is nearer the TNF locus than the HLA-DR locus, so stronger linkage disequilibrium would therefore be expected between HLA-B and allelic variants within or in close proximity to the TNF gene. This is an argument against the possibility that the TNF/HLA-DR association involves a variant of the TNF promoter. It could however be that the HLA-DR molecules are involved in the release or uptake of TNF. Alternatively, regulatory genes located close to the HLA-DR locus are involved in the expression of the TNF gene.

As already mentioned TNF-HLA associations are involved in autoimmune diseases such as rheumatoid arthritis (RA) and insulin dependent diabetes mellitus (IDDM). HLA-DR2 is associated with resistance in these two cases and may be explained if TNF is involved with the destruction of cartilage and bone in RA and islet-β cells in IDDM. As well as associations with single HLA loci and indeed single HLA antigens, there is now substantial evidence for the association of TNF alleles with ancestral haplotypes.

Such 'ancestral haplotypes' refer to haplotypes carrying particular combinations of MHC alleles, which have been serologically and RFLP typed, spanning particular physical genomic distances. They account for between 30 and 90% of all MHC extended haplotypes or supratypes and consequently account for most linkage disequilibrium between MHC alleles. The 'extended haplotype' describes a series of allele combinations occurring more frequently than expected from their individual allele frequencies.

In 1991, Abraham *et al* showed that the two most common ancestral haplotypes, 57.1 and 8.1, are allelic in that they differ in their LTα sequences. They demonstrated that 57.1 carries the Nco-1 B*2 allele whereas 8.1 carries the B*1 allele. Verjans *et al* (1992) carried out further work on associations between the TNF locus and HLA antigens and found the Nco-1 B*1 allele to be associated with DR4 and B15, whereas the B*2 allele associated with the haplotype A1, B8, DR3. This latter association was also seen by Peruccio *et al* (1993). Also in 1993, Wilson *et al* showed that the -308 allele T2 associated with the A1, B8, DR3 haplotype, whereas the T1 allele associated with DR4 and DR6. Due to the proximity of the two genes they postulated that the association was a result of linkage disequilibrium.

HLA expression in Breast cancer.

Since the late 1970s, evidence has been coming to light that certain HLA antigens are associated with susceptibility or resistance to cancer. Rogentine *et al* (1977) showed an association with the presence of HLA-Aw19 and/or B5 and resistance to the progression of lung carcinomas in a study of 70 cancer patients. This positive association was also found in 1979 by Weiss *et al* who showed that 50% of lung cancer patients who survived for at least one year carried these antigens. In 1977, Oliver *et al* demonstrated that acute myeloid leukaemia patients expressing A1and B8 and/or A2 and B12 survived longer than patients without either of these pairs of antigens in association. In direct contrast to this, Tongio *et al* (1981) showed that B12 is overexpressed in lung cancer patients and concluded that this increase is associated with susceptibility to the disease rather than resistance to it. Further studies noting an association with HLA antigens and disease susceptibility include Osoba *et al* (1980) who observed significantly poorer survival rates in Hodgkin's lymphoma patients who expressed Aw19 and Markman *et al* (1984) who found that small cell lung carcinoma patients possessing A1 had a significantly poorer 1-year survival rate.

We were unable to carry out HLA-typing of the 88 breast cancer samples obtained for this study. Furthermore, there seems to be a general lack of up-to-date data available on this front. However, a study was recently carried out in India by Biswal *et al* (1994), where 100 female patients with invasive breast carcinomas were analysed for HLA class I expression, as an approach to show a genetic correlation in this familial disease.

At the HLA-A locus two antigens, A2 and A11 displayed an increased frequency of expression in the breast cancer population when compared to aged matched controls (p=0.02 and 0.005 respectively). The group were also able to demonstrate an increased expression of A2 and Aw31 in patients whose lymphocyte cytotoxicity test results were high.

The results at the HLA-B locus obtained in this Indian study do not appear similar to work previously done. An early study by Lynch *et al* (1977) reported an increased frequency of B15, B35 and B40 in breast cancer. Hammond *et al* (1979) found B52 to be increased, while Garam *et al* (1980) showed a significant decrease in B14 expression. Biswal *et al* however found HLA-B8 and B14 to be increased to statistically significant levels (p= 0.0046 and 0.005) respectively. The variations in findings could be due to the vast heterozygosity observed at the HLA-B locus, which would be even more striking when different ethnic populations and geographic locations are studied. Biswal's results at this locus link with work done by Jongeneel *et al* (1991), who investigated extended haplotypes. They found HLA-B8 to be associated with the TNFa2 allele. This may suggest that other markers within the MHC locus could also be linked to the control of TNF secretion levels.

Only one antigen at the HLA-C locus showed an association with breast cancer. Cw6 appeared to be negatively associated (p=0.05). Song *et al* (1989) reported a positive association with Cw4.

A study by Concha *et al,* (1991) investigated whether the HLA class I phenotype could be used as a prognostic indicator in patients with breast carcinomas. They observed that 39% of patient tumours showed selective or complete loss of HLA class I molecules. This negative phenotype was associated with the increased presence of metastases and short survival time. It is unclear however whether the loss of HLA expression is due to immune selection or a secondary event occuring during tumour progression.

The overall variations in the association of HLA-A, B and C antigens and breast cancer seem to suggest the presence of susceptibility genes located within or in close proximity to the MHC locus. Interesting is Biswal's finding that B8 is increased in the breast cancer population, since B8 is part of the extended haplotype (A1, B8, DR3) which also associates with the Nco-1 B*2 allele, linked to increased TNF expression. This could be further evidence for the role of high TNF levels in the malignancy.

As far as class II expression is concerned, in 1994, Sheen-Chen investigated the prognostic significance of HLA-DR expression in node-negative breast cancer patients. Planning post-operative treatment for this group of patients is difficult. It would be unfortunate to subject patients to unnecessary, toxic and expensive adjuvant treatments if their prognosis following surgery alone was good enough that it merited no further treatment. Sheen-Chen *et al,* in an attempt to identify such patients, showed that the 5-year disease-free survival rate of DR+ patients was significantly greater than DR- patients. The overall survival rate in the DR+ group was also greater, but not significantly so. When the combination of HLA-DR expression and oestrogen receptor status was used, both the 5-year and the overall survival rates of patients staining positive for both were significantly better than patients staining negative for both. From this study, it could be concluded that HLA-DR expression may be a predictive factor to node-negative breast cancer, especially when taken together with oestrogen receptor expression. Remembering that TNF can induce HLA-DR expression, this may be further evidence that it is TNF levels in these patients which is involved in the better prognosis.

The role of high TNF levels in cancer.

There is conflicting information regarding the role of high TNF levels in cancer patients. In this study, there seems to be an overall trend towards an increased expression of TNF alleles associated with elevated TNF levels, although it was not possible to measure actual TNF levels in the samples to confirm this.

The question then arises of the role that these suggested higher levels of TNF play in the cancer populations. It would fall into two opposing categories;

1) Potentiating the disease.
2) Protecting from metastases once the disease has initially developed.

Evidence for the former suggestion comes from such studies as those by Bertolini *et al* (1986) who showed that TNF and LT stimulate bone resorption and inhibition of bone formation. Coupling this with the fact that TNF and LT have been detected in breast stromal cells (Miles *et al*, 1992) and also that IL-1 synergises with TNF and LT (Stashenko *et al*, 1987), TGF-α (Lorenzo *et al*, 1988) and Parathyroid hormone-related peptide (PTHrP) (Sato *et al*, 1989) in bone resorption assays, is evidence for a role for TNF and LT in the formation of bone metastases (Figure 18). This is especially pertanent to breast cancer, as the first sites of metastases tend to be the bones.

As already mentioned TNF has been implicated in the upregulation of tumour angiogenesis (Frater-Schroder *et al*, 1987) and the induction of metalloproteinases, the latter property leading to increased tumour cell motility and invasive potential.

On the other hand, TNF also has the ability to induce other cytokines and attract lymphocytes to the site of the tumour, thus potentiating an immune response against the tumour. HLA class II expression has been increased in several tumour cells (Cabrera *et al*, 1995). This expression allows the cells to present antigens, thus reducing the degree of tumour aggressiveness. Obviously the more TNF present, the greater the class II induction leading potentially to a better prognosis (Figure 19).

It would seem however that the negative roles of TNF at the site of a tumour outweigh the positive effects, thus supporting the hypothesis that high levels of TNF, suggested by the presence of alleles linked to higher levels, in breast cancer patients associates with poor prognosis.

Figure 18

Mechanism of Osteolysis causing bone metastases.

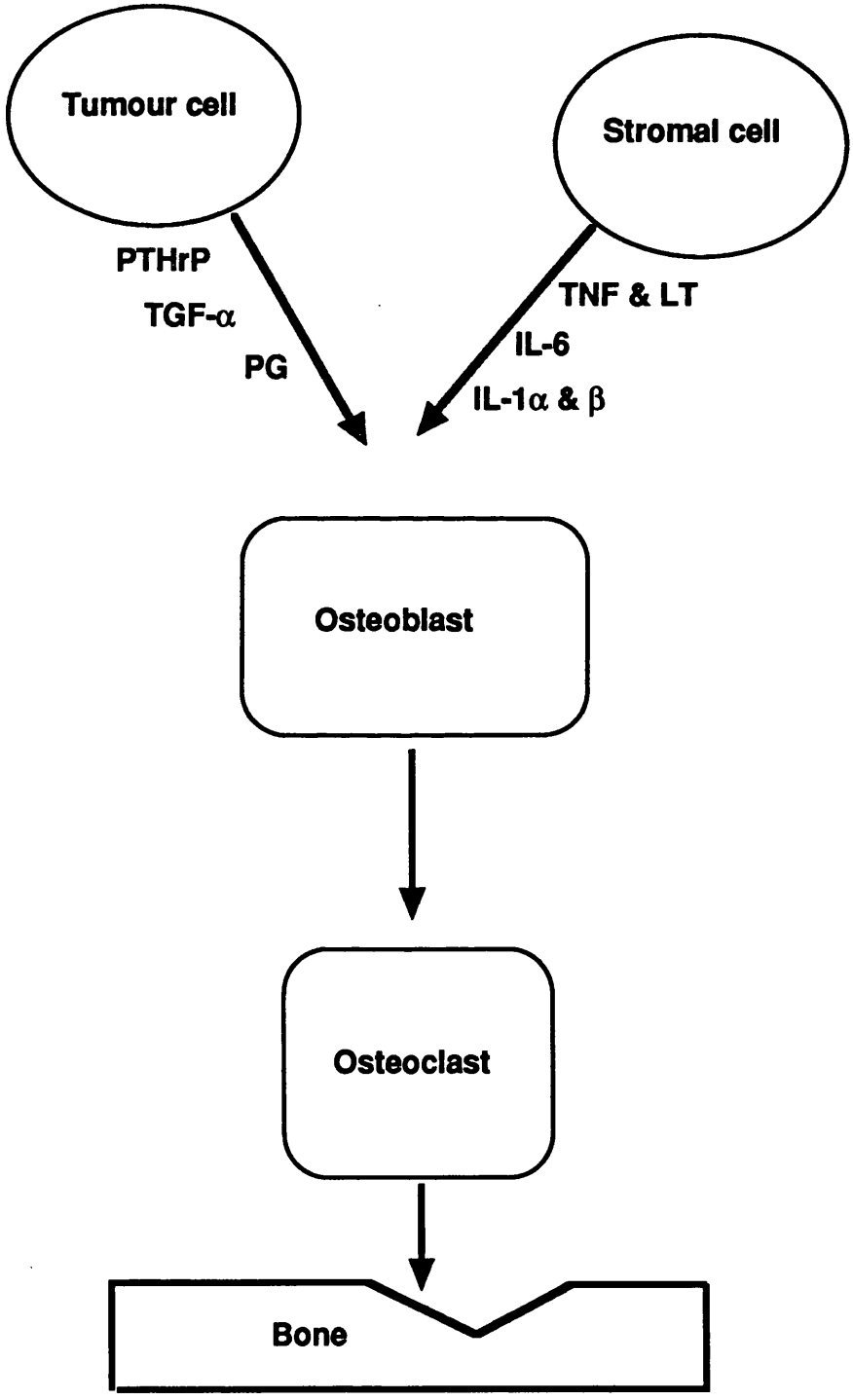
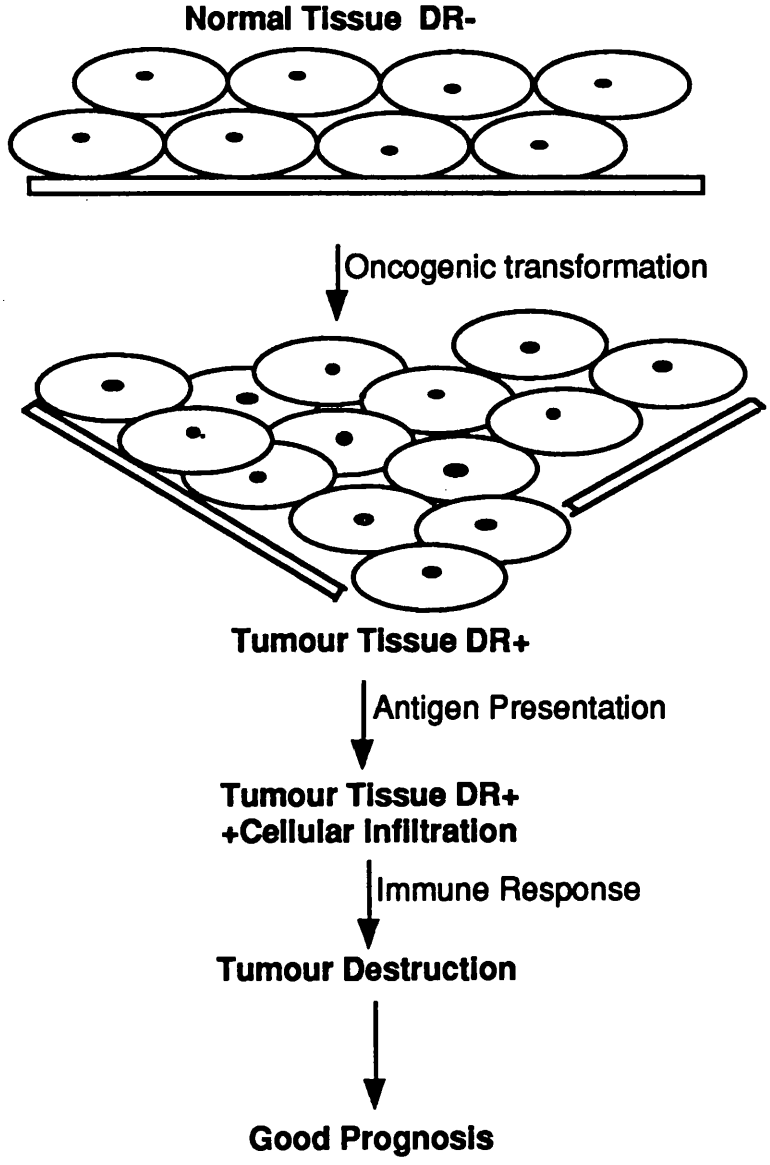


Figure 19.

Development of tumour destruction



Adapted from
Cabrera et al, 1995,
Scan. J. Immunol.
41, 4, p401.

TNF as a therapeutic strategy.

Despite having such a variety of effects on a wide range of cells, TNF has been investigated as a cancer therapy. Ever since the very early work by Coley and his 'mixed toxins' showed antitumour activity in animals, attempts have been made to dulpicate this property in humans. Unfortunately clinical trials using TNF as a single agent have not met with much success. Several reasons for this failure have been postulated. Firstly, the relative amount of TNF required to cause toxicity in humans is less than in mice (8-10mg/kg compared to 400mg/kg). This could prevent therapeutic dose levels being reached before treatment with the cytokine is stopped. Secondly TNF has a short half-life *in vivo* of 20 minutes, which could reduce the response generated. Thirdly, the original 'mixed toxins' probably produced many cytokines, not just TNF and it was the combination effect that caused the tumour regression. (Studies to date have been more successful when TNF is given in combination with other cytokines or therapeutic agents) (see later). Finally, the involvement of the immune system is an important factor in the production of an anti-tumour response and this may differ between the human and animal models. Certainly a better response is seen against immunogenic tumours (murine) compared with non-immunogenic tumours (human).

Several phase I and a few phase II trials have been carried out using TNF as a single agent in the treatment of cancer. The former aim to determine the dose which can be tolerated before toxicity becomes too great and also the pharmacokinetics of TNF. Phase II trials are concerned with determining the effectiveness of a drug, or in this case, TNF. The results of the phase I trials demonstrated that the maximum tolerated dose is 200$\mu g/m^2$ (Spriggs and Yates, 1992), the serum half-life is 20 minutes and that the dose-limiting side effect is hypotension. The phase II trials where TNF has been given alone have been disappointing. In summarising the results of several trials, no more than 1% of patients showed complete tumour regression.

Phase I trials where TNF is given in combination with other cytokines or antitumour drugs have had greater success. If administered with INF-γ, the toxicity of TNF can be increased by as much as 10-fold and the maximum tolerated dose decreased by 2 to 5 fold. A phase II study by Lienard *et al* (1992) has shown the triple combination of TNF, INF-γ and the alkylating agent Melphalan to be successful in the treatment of metastatic melanoma and sarcoma patients, with 19 out of 23 patients showing complete regression of their tumours.

From these limited studies, the potential is there to develop TNF and LT as a possible anticancer therapy. Several new approaches are now being attempted in order to overcome the various problems which, up until now, have limited progress in this field. To increase the TNF concentration at the site of the tumour, local treatment rather than systemic is being attempted; Rosenberg *et al* (1992) developed a method of gene therapy where TIL cells were transduced with the TNF gene so that on infiltration, large doses of TNF were delivered to the tumour. Making TNF fusion proteins would be another strategy for targetting TNF to the site of the tumour. TNF analogues are yet another possibility for a therapeutic strategy, where the toxicity of the cytokine is decreased but the antitumour effects are not compromised. A further angle of approach is to increase the short half-life shown by TNF *in vivo*. The attachment of polyethylene glycol (PEG) to proteins has been shown to increase half-life (Davis *et al*, 1981) so provided that this modification would not decrease the antitumour effects, then it is another possible strategy.

It must be borne in mind however that increasing the TNF dose at the site of the tumour may in fact potentiate the metastatic process, if the treatment fails, so a balance needs to be achieved where enough is given to be of therapeutic use, but not too much is administered to counterbalance this. With insights constantly being gained into the mechanisms of action of TNF and also LT, pharmaceutical drug design to prevent cell injury and conversly, how best to use TNF therapeutically should not be too far away.

Concluding remarks.

Several points can be concluded from the work undertaken for this project.

Firstly is the fact that allelotyping at the polymorphic sites within the TNF locus is possible by employing the optimised PCR-based techniques determined here. Secondly is the large degree of heterogeneity observed within the study populations at the TNF locus. However despite this heterogeneity, allelotype expression at the polymorphic sites seldom differs significantly between the control and malignant populations.The breast and colorectal cancer populations differed at the TNFc locus. The breast cancer population alone differed in allelotype expression at the TNFa locus ($p=0.023$). Genotype expression failed to show a malignant-specific pattern, with only the colorectal population differing significantly to the control population at the TNFc locus. (The breast cancer population only narrowly failed to differ significantly to the control population ($p=0.0724$)) at this locus. This would seem to suggest a lack of involvement of the TNF locus in the progression of the malignancies investigated. Previous studies linked the expression of certain alleles and genotypes within the locus to increased TNF-expression levels. In this study we have observed a significantly increased expression of two of these alleles (c2 and a2) and one of the genotypes (Nco-1 heterozygosity) in the breast cancer population. Although we were unable to measure the actual TNF levels in our samples due to time constraints, we can suggest that the predicted higher levels of TNF in the breast cancer population could be a contributing factor in the aetiology of the disease, bearing in mind the previously observed roles of TNF in metastatic progression. TNF reportedly also shows anti-tumour activity, so provided the dosage was optimised, the potential is there to use this multifunctional cytokine in the treatment of malignant diseases, which conversly enough it may play a role in advancing.

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