A feasibility study of oncogene transgenic mice as therapeutic models in cytokine research

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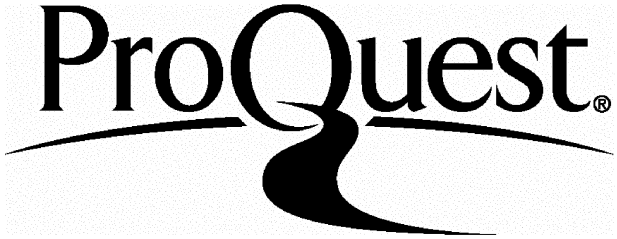

Abstract

Transgenic mice carrying the activated rat c-*neu* oncogene under transcriptional control of the MMTV promoter were backcrossed to BALB/c mice, with the aim of developing a model for cancer therapy. A total of 86 of 268 transgene positive mice in the first five generations developed 93 histologically diverse tumours (median age of onset 18 months). The cumulative incidence of breast tumours at 24 months was 18%, and overall tumour incidence 31%. As well as expected c-*neu* expressing breast cancers, lymphomas and Harderian gland carcinomas developed. All of the mammary carcinomas, Harderian gland carcinomas and lymphomas expressed c-*erb* B2. Virgin mice had fewer mammary tumours than those with two litters ($p= 0.006$), further litters did not affect tumour incidence. Breast carcinomas metastasised to the lungs, and lymphomas were widely disseminated. The tumours showed a range of architectural patterns which resembled human breast cancers or lymphomas. This diversity was reflected in S-phase fraction and aneuploidy.

Mammary tumours transplanted to nude mice showed variable responses to IFN-α and -γ. A tumour transplanted to BALB/c mice responded to IL-12. Groups of mice treated prophylactically with IFN-α and -γ, and IL-2 and IL-7 from the third and fourth generations respectively were studied for tumour development. No statistically significant differences were noted between the groups except an increased incidence of B-cell lymphomas in the IL-7 treated group.

There was a significant decline in transgene positivity with successive generations (X^2 test for trend $p<0.001$) but transmission of transgene, litter number and offspring viablity was not changed in homozygotes. The diversity, histologic and biologic resemblance to human cancer suggests the model has potential for evaluating novel therapies. Genetic and environmental manipulations are underway to increase tumour incidence and decrease age of onset.

Acknowledgements

I now understand that the development of an animal model is a lengthy and unpredictable process. Compounded by my return to a busy clinical post in November 1994 this has resulted in the elephantine gestation of this thesis. As a result many colleagues, particularly those in the Biological Therapy Lab, have had to bear with me in a protracted process. The brunt of this has been borne by my supervisor, Fran Balkwill, who has suffered the vagaries of this study throughout and has diligently honed each chapter. Thank you for your patience and tenacity. Peter Beverley, my second supervisor, has similarly had to tolerate the endless phone messages, and all with good grace. I am grateful to them both for the careful and helpful way in which they have shaped this project.

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This thesis is dedicated to

Nick, Isobel and Phoebe

Abbreviations

bp	base pair
CD	Control diluent
CTL	cytotoxic T Lymphocytes
CO	Corn Oil
CSF	Cerebrospinal fluid
DCIS	Ductal carcinoma in situ
DEPC	Diethylpyrocarbonate
DMBA	7,12-dimethylbenzanthracene
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal gorwth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen receptor
FLC	Friend Leukaemia cell
GIT	Guanidine isothiocyanate
h	hours
HCl	Hydrochloric acid
HCL	Hairy cell leukaemia
IC	Intermediate cell
IFN	Interferon
Igh	Immunoglobulin heavy chain
IL-2	Interleukin-2
IL-7	Interleukin-7
IL-12	Interleukin-12
ip	intraperitoneal
IRF	insulin regulatory factor
iv	intravenous
k	kilobase
kDa	kilodalton
LAK	Lymphokine-activated killer
LC	Large cell
LIF	Leukaemia inhibitory factor
LT	Lymphotoxin
LTR	Long Terminal Repeat
MCA	3-methylcholanthrene

µg	microgrammes
mg	milligrammes
MMP	Matrix metalloproteinase
MMTV	Murine mammary tumour virus
MTD	maximum tolerated dose
mRNA	messenger ribonucleic acid
MU	Megaunits
NC	Negative control
NIH	National Institutes of Health
NK	Natural killer
OD	Optical Density
PC	Positive control
PCR	Polymerase chain reaction
PEM	Polymorphic epithelial mucin
PK	protein Kinase
PR	Progesterone Receptor
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
sc	subcutaneous
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulphate
SPF	Specified Pathogen Free
TBE	Tris Borate EDTA buffer
TBS	Tris buffered saline
TE	Tris EDTA buffer
TGF	Transforming growth factor
TNF	Tumour necrosis factor
WAP	Whey Acidic Protein
UICC	International union against cancer
UV	ultraviolet

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Chapter One

Introduction

Cancer as a multistep process

A wealth of evidence illustrates that cancer is a multistep process associated with changes at a genetic level. Cytogenetic and histological changes can be recognised in the development of an increasing number of human tumours. Histologically these tissues range from apparently normal tissue with a relatively low proliferative rate through a spectrum to rapidly proliferative solid tumours which behave aggressively and are likely to metastasise. It is the ability of cancer cells to invade other tissues and spread to other parts of the body where they can generate metastases which is the major property leading to the death of the host.

Over a period of years independent cellular events result in cumulative changes in cell behaviour most of which are explicable by accumulated changes in genes (Fearon & Vogelstein, 1990). Many of these mutations will occur in non-coding DNA and be of no consequence. Some will alter genes with profound effects on cell behaviour. Greater understanding of these changes and how they influence the development of cancer is essential to progress in the treatment and prevention of the disease.

The Problem of Breast Cancer

Epidemiology

On a worldwide basis breast cancer is the most common malignancy in women, with 570,000 new cases in the world each year (18% of all female cancers). The lifetime risk for a woman in the UK has now reached 1 in 12, and the incidence continues to rise in the West (Program, 1990). The age standardised incidence in the United Kingdom is the highest in the world with nearly 30,000 new cases and 15,000 deaths each year, it being the commonest single cause of death in those aged 35-54 years (Austoker, 1994; McPherson K, 1994).

Aetiology and Risk factors

The reason for the recent rise in incidence is not clear, and is not explained by improved life expectancy. It may be related to longer duration of exposure to oestrogens as a consequence of earlier menarche and later menopause (King *et al.*, 1993; Ottman *et al.*,

1986; Program, 1990), Nevertheless only 30% of women who develop the disease have an identifiable risk factor making it difficult to define high risk groups for interventional studies (Atiba & Meysken, 1992). Hence long term success in breast cancer must be linked to decreasing the incidence (Robert, 1994). The likelihood is that the development of breast cancer is a multistage process, influenced by different factors extrinsic and intrinsic to the host. Although the cause is unknown epidemiologic evidence points to three areas: endocrine factors, environment and genetics.

Endocrine Factors

The age of menarche, menopause and first pregnancy have been linked to the incidence of breast cancer in numerous studies. The most important interval appears to be the time between menarche and first pregnancy. In one study almost a two-fold increase was seen in the incidence of breast cancer in women with menarche before 12 compared with those where it occurred after 13 (Pike *et al.*, 1981). Starvation and strenuous physical activity, which delay menarche, may underlie some of the international differences in breast cancer incidence. Nulliparous women have a higher incidence than parous women, although age at first pregnancy is an even more powerful determinant and a first pregnancy after the age of 35 may actually increase the risk (Trichopoulos *et al.*, 1983).

Evidence concerning the influence of hormone replacement therapy and the potential effect of oral contraceptives on breast cancer incidence is equivocal (McPherson & Drife, 1986)

Environmental Factors and Diet

The importance of endocrine factors is suggested by the incidence of breast cancer in Japanese women who migrate from Japan, where the incidence of breast cancer is low, to North America, where it is high (Buell, 1973). Cohort studies in countries with a gradual increase in incidence demonstrate that risk is related to year of birth (Lilienfeld *et al.*, 1975).

A relationship between fat or cholesterol intake and steroid hormone metabolism has led to emphasis on dietary fat as a possible aetiologic agent. International studies relating age-adjusted cancer mortality rates and national per capita fat intake demonstrate a direct correlation (Wynder *et al.*, 1986). In spite of this compelling indirect data, epidemiologic studies correlating dietary fat and breast cancer incidence have been inconclusive. Animal model work suggests fat is a tumour promoter, although in rats some studies show that total calorie intake and not dietary fat, is the significant factor (Boissonneault *et al.*, 1986; Klurfeld *et al.*, 1989).

There also appears to be an increased risk of breast cancer, after a latency of 10-15 years, in women exposed to radiation before the age of 40 years. (Boice *et al.*, 1979). This relates to large radiation exposures such as experienced by survivors of atomic bombs or repeated fluoroscopies during therapy for tuberculosis.

Genetics

There is now unequivocal evidence that a small proportion of breast cancer cases, 4-5%, are due to highly penetrant dominant genes (Claus *et al.*, 1991). Familial breast cancer is more likely with early age at presentation, several affected relatives, bilaterality and a history of related cancers (Anderson & Badzioch, 1985; Evans *et al.*, 1994).. The genes are relevant to both sporadic and inherited forms of the disease (Marcus *et al.*, 1996). There may be more than five genes causing familial breast cancer; the most important of which (*BRCA*1) was first mapped to chromosome 17q21 in 1990 (Hall *et al.*, 1990). The breast and ovarian cancer susceptibility gene, *BRCA*1, is mutated in the germline and the normal allele is lost in tumour tissue from hereditary breast and ovarian cancer (Hall *et al.*, 1990; Miki *et al.*, 1994). Somatic point mutations in *BRCA*1 in sporadic tumours are very rare but complete somatic deletion of one allele of *BRCA*1 occurs in approximately 50% of sporadic breast cancers (Takahashi *et al.*, 1995). The candidate gene, a tumour suppressor gene, encodes a 190kD protein with sequence homology and biochemical analogy to the granin protein family (Jensen *et al.*, 1996). It is a regulated secretory protein whose expression increases during pregnancy (Lane *et al.*, 1995). It appears to function through a novel mechanism not previously described for tumour suppressor gene products (Jensen *et al.*, 1996) Recently a second important gene associated with a predisposition to breast cancer has been cloned and given the nomenclature BRCA-2 (Schutte *et al.*, 1995)

Natural History

Most patients present with a lump in the breast or increasingly, through breast cancer screening programmes, with a mammographic abnormality and a small or indetectable lump. Up to 5% of patients present with metastatic disease where the primary may be occult. The natural history of breast cancer is characterised by a long duration and marked heterogeneity within and between patients. The preclinical and clinical phases can be measured in years and decades. Patients lie on a continuum from aggressive disease at one end to indolent disease at the other. Similar heterogeneity is found in a range of measurable biological indices.

Pathology

Breast cancers are derived from the epithelial cells lining the terminal duct lobular unit (TDLU). Cancer cells within the basement membrane of the TDLU are classified as *in situ* or non-invasive, whereas in invasive breast cancer cells have disseminated into the surrounding normal tissue. *In situ* carcinoma is divided into ductal and lobular types. Ductal carcinoma *in situ* (DCIS) is classified on the basis of cytological features and within the categorisation there are clear cytological differences between the major types.

Broadly DCIS can be divided into comedo and non-comedo, or high-grade and low-grade. Comedo DCIS is associated with a much higher incidence of invasive cancers in patients treated conservatively. Comedo DCIS usually expresses the c-*erb*B2 protein, has a high proliferation rate and is oestrogen receptor (ER) negative. Microscopically comedo lesions show a solid proliferation of malignant cells within the ducts, with characteristic central necrosis, which may be extensive. A new classification for DCIS was proposed in 1994, based on cytonuclear differentiation and architictural differentiation. Three categories have been defined:

Poorly differentiated DCIS: composed of cells with very pleomorphic, irregulalry spaced nuclei, with coarse, clumped chromatin, prominent nucleoli and frequent mitoses. Architectural differentiation is absent or minimal. The growth pattern is solid or pseudo-cribriform and micropapillary. Necrosis is usually present.

Well-differentiated DCIS: composed of cells with monomorphic, regularly spaced nuclei containing fine chromatin, inconspicuous nucleoli and few mitoses. Necrosis is uncommon. the third category lies between these two groups (Holland *et al.*, 1994).

The previous classification of invasive breast cancers divided them into ductal and lobular types - both arise from the TDLU and the terminology is no longer appropriate. *In situ* and invasive cancers are now identified on the basis of characteristic patterns of growth and cellular morphology (Sainsbury *et al.*, 1994). Some have specific features - classified as invasive cancers of special type whilst others are of 'no special type' (the commonest subgroup of infiltrating mammary carcinomas). This has prognostic significance as certain special type tumours are of much better prognosis than those of no special type.

The most important morphological prognostic features of breast tumours are histologic grade and the presence of lymphatic invasion. Grade describes the degree of tumour differentiation. Lymphatic invasion (presence of tumour emboli within breast lymphatics) occurs in 25% of tumours and is associated with a poorer outlook, as is

vascular invasion (tumour emboli in tumour blood vessels). These features are then related to stage of disease and the number of involved axillary nodes.

Receptor status

To obtain more quantitative information about the biology of a tumour, for prognostic and therapeutic reasons, other parameters have been introduced. These include oestrogen and progesterone receptor status (ER and PR), measurement of DNA, tumour cell kinetics and oncogene expression. In addition to hormone receptor status, epidermal growth factor receptor status is a useful prognostic predictor (Sainsbury *et al.*, 1994).

Staging

Staging is the grouping of patients according to their extent of disease. The most widely used staging system is that adopted by the UICC (International Union against Cancer), the TNM (acronym of Tumour, Node, Metastasis) system.

Management

Surgery

Most patients with early breast cancer are managed with surgery initially. Modern breast conserving surgery entails a wide local excision of the tumour along with dissection of the axillary lymph nodes. The latter is undertaken for prognostic information.

Radiotherapy

Radiotherapy has been used to treat inoperable cancers since the 1920s (Keynes, 1929). More recently it has been used after surgery to prevent local recurrence. With moderate doses of radiation, eradication of any subclinical burden of residual cancer cells is possible whilst enabling breast conservation. Wide local excision without subsequent irradiation results in a 15-40% risk of local recurrence compared with 5-10% after radiotherapy (Jacobson *et al.*, 1995; McCormick, 1994).

Adjuvant therapy

Local control in breast cancer has not translated into improved survival. Either the patient is cured by local treatment or she dies of distant metastases at around the same time she would have died without local intervention. This is because metastases were established prior to the time of diagnosis.

The rationale for adjuvant systemic treatment is based on the premise that systemic therapy is more likely to cure microscopic metastatic disease than treatment at the time of relapse, with macroscopic metastases. It is a complex issue influenced by intrinsic sensitivity to hormones, chemotherapy, risk of relapse and time to relapse. Oversimplifying a complex subject: the treatment of choice for postmenopausal women is hormonal therapy and, for pre- and perimenopausal women with involved lymph nodes, it is chemotherapy.

Hormonal Therapy-Tamoxifen

Tamoxifen, an oestrogen receptor partial agonist, is the most widely used adjuvant therapy in the treatment of breast cancer. When ER and PR status first became widely available they were used to predict hormone responsiveness, ER and PR positive tumours being more likely to respond to hormonal manipulation. Nevertheless the accuracy was not absolute - some ER positive tumours being hormone resistant and some ER negative ones hormone responsive. These findings, and the lack of toxicity of tamoxifen, have lead to tamoxifen being given to virtually all postmenopausal women, and increasingly to premenopausal women, with invasive breast cancer. In the UK there has been an improvement in the outlook of breast cancer in recent years. The reasons are unclear but the greater involvement of specialists in its management and hence wider use of tamoxifen is believed to explain much of this improvement.

Tamoxifen is also used to prevent breast cancer in genetically predisposed women. For prophylaxis only women at high risk of the disease could justify taking a drug regularly on a long term basis. In this respect the only drug sufficiently well tolerated to be acceptable is tamoxifen. The question of preventitive tamoxifen use is considered further in Chapter 3.

Chemotherapy

The use of adjuvant chemotherapy in women with high risk disease started in the 1970s. It translated into a survival benefit for women with four or more positive lymph nodes (Bonadonna *et al.*, 1985) and now the use of systemic chemotherapy is widespread in the management of breast cancer.

The need for new treatments

The concept of breast cancer as a systemic disease at presentation explains the relatively high morbidity and mortality. Existing therapeutic modalities have not altered the prognosis significantly in decades. The accepted management has not changed greatly in recent years - early detection, optimal surgery combined with

radiotherapy and adjuvant treatment are all standard but cure rates have plateaued, with one-third of patients with localised disease eventually dying from it and two-thirds of those with nodal involvement (Gamel *et al.*, 1996). Unless early detection can be improved the only hope for further improvement lies in new systemic therapies (McVie, 1995).

Although there are some exciting new agents appearing, drug resistance remains a problem. Chemotherapy is apparently able to reduce the volume of disease dramatically but a significant risk of subsequent relapse remains (Overmoyer, 1995). Dealing with this 'minimal residual disease' still represents a challenge in eradicating breast cancer in high risk patients. It seems logical at this point that an immunological modality has a role where cytotoxic therapy has failed.

The genes involved in breast cancer development

Genes involved in the development of breast cancer, which are transforming or activated by specific events, have been identified from the study of tumour virology, cytogenetics, cell biology and carcinogens (Barbacid, 1986; Bishop, 1983; Hunter & Cooper, 1985; Klein & Klein, 1985);. These transforming genes are collectively termed oncogenes. The term is used to describe any gene which contributes directly to malignant change in a cell. Oncogenes which have been activated by mutation are often called dominant oncogenes reflecting the fact that they exert their cellular effects in spite of the presence of a normal gene product from the homologous allele.

Many oncogenes are mutated normal cellular genes essential to normal biological processes such as cell division. These are called proto-oncogenes. Their cellular function is enhanced by mutations which activate or enhance the cellular functions of the encoded proteins. Examples include the over-expression of growth factor receptors and the expression of mutant *ras* signalling proteins.

Conversely, tumour suppressor genes are normal cellular genes whose functions are inactivated by mutation (Ponder, 1988). They inactivate the cellular functions of the encoded proteins. One example is inactivation of normal BRCA-1 gene.

The characteristics and terminology associated with proto-oncogenes and tumour suppressor genes are summarised in Table 1.1.

	Proto-oncogenes	Tumour suppressor genes
Number of alleles in normal somatic cells	Two	Two
Effect of mutations on cellular function of gene product	Enhanced	Reduced

Table 1.1 Characteristics of proto-oncogenes and tumour suppressor genes

Regulation of cell growth

Cellular homeostasis depends on a balance between cellular proliferation, cell growth and apoptosis (Evan *et al.*, 1992). Proliferation is controlled by a series of signalling mechanisms between and within cells. the genes involved in this process include cytokines, cytokine receptors, including tyrosine kinase transmembrane receptors, nuclear transcription regulators, cell adhesion molecules and tumour suppressor gene products.

Breast cancer progresses as a result of the acquisition of progressive genetic changes. These are likely to involve oncogenes or tumour suppressor genes. The growth of normal and malignant glands is controlled by endocrine hormones and local growth factors. Here I will concentrate on cytokines and their receptors as relevant to the control mechanisms involved in the development of breast cancer.

c-erbB2 and Breast Cancer

Phosphorylation is a frequent biochemical process in cell metabolism. Transfer of a high energy phosphate group from ATP or GTP to another molecule by kinases is widespread on the amino acids serine and threonine but on tyrosine residues it is restricted and highly significant. The phosphorylation of tyrosine residues is performed by tyrosine kinases specific for particular substrates, for example specific growth factor receptors. Tyrosine phosphorylation then alters the activity of a molecule, activating or inactivating an enzymic function or modifying the binding of proteins.

The transmembrane protein tyrosine kinases are the principle receptor type implicated in neoplastic transformation and several 'types' are now recognised. The members of the type I subgroup are relevant to this thesis. The type I growth factor

receptor family consists of at least four genes (c-*erb* B1-4). Each gene encodes a distinct protein which functions as a transmembrane protein tyrosine kinase (Ullrich & Sclessinger, 1990). *erb*B2 was identified independently by different groups (King *et al.*, 1985; Schechter *et al.*, 1985; Semba *et al.*, 1985) and this gave rise to synonymous designations as c-*erb*B2 and HER-2. The initial identification was facilitated through its amplification in a primary human adenocarcinoma (King *et al.*, 1985). There is considerable homology between specific functional regions in each of the receptor molecules, in particular the kinase domain, suggesting that they are all derived from a common ancestral precursor (Hanks *et al.*, 1988).

Relevance of *erb*B2 to human cancer

A role for EGFR and *erb* B2 in human cancer is based on a number of observations:

- Overexpression of EGFR and *erb*B2 mRNA/ protein is documented in a range of human epithelial tumours as a result of gene amplification, transcriptional upregulation or a combination of the two (Gullick, 1991). By contrast protein overexpression to the same degree is not seen in normal tissue.

- Transfection of EGFR into immortalised NIH3T3 fibroblasts in the presence of an activating ligand results in a transformed phenotype (Di Fiore *et al.*, 1987a). Transfection of *erb* B2 into NIH3T3 fibroblasts is also transforming.

- Transgenic mice with the mutated *neu* gene (rodent homologue of *erb*B2), under the control of the MMTV promoter, develop bilateral mammary tumours with a high frequency (Muller *et al.*, 1988).

- Antibodies directed against the *erb*B2 protein limit the growth of malignant cells *in vitro* and *in vivo* (Wels *et al.*, 1995).

Relationship between rodent gene, *neu* and human counterpart, c-*erb*B2

The c-*neu* proto-oncogene (rat homologue of the human c-*erb*B2 oncogene) is a membrane bound 185kDa receptor molecule with tyrosine kinase activity. In a chemically transformed neuroblastoma cell line, rat c-*neu* is activated by a point mutation which results in a single amino-acid substitution (valine to glutamic acid) in the transmembrane domain of the protein (Barbacid, 1986; Bargmann *et al.*, 1986a). This results in constitutive activation of its intrinsic tyrosine kinase function (Press *et al.*, 1990). Hence, in the apparent absence of ligand, constitutive levels of tyrosine phosphorylation are seen and thus a constitutive mitogenic signal is applied. The mutant *neu* gene, but not the normal *neu* gene, can transform NIH3T3 cells (Bargmann *et*

al., 1986b). In contrast, spontaneous human neoplasia involving *erb*-B2 appears to involve gene amplification and/or overexpression of structurally intact transcripts and proteins. Substitution of the corresponding amino-acid in human c-*erb*B2 protein would require two mutations in the gene although the human c-*erb*B2 gene can transform the fibroblasts by overexpression (Di Fiore *et al.*, 1987b). The overexpression of c-*erb*B2 mRNA and protein has been demonstrated in human breast cancer using northern analysis and immunohistochemistry of tumour biopsies and cell lines, respectively (Slamon *et al.*, 1987). In human adenocarcinomas, particularly breast and stomach cancers, it is overexpression of c-*erb*B2 (determined by northern blot or immunocytochemistry), and not activation, which is found (van de Vijver *et al.*, 1987; Yokota *et al.*, 1986). Overexpression usually occurs as a result of amplification but increased transcript levels have also been found in the absence of amplification.

Clinical significance of c-*erb*B2

The most significant oncogene study was carried out by Slamon and colleagues. They correlated the amplification of the c-*erb*B2 (HER-2/*neu*) oncogene with prognosis in 189 patients and found, on multivariate analysis, that it was an independent prognostic marker (Slamon *et al.*, 1987). The interpretation of c-erbB2 as a prognostic factor in breast cancer is still not clear (Harada *et al.*, 1994; Ravdin & Chamness, 1995).

It is now known that *in vitro* the adjuvant hormonal agent tamoxifen influences the regulation of both erbB2 and erbB3. Whether such *in vitro* findings will translate into clinical relevance is uncertain. However this must be borne in mind given increasing detection of DCIS (the high grade, comedo-type expresses c-erbB2 highly) and the more widespread use of tamoxifen in breast cancer patients (Brinster & Palmiter, 1984).

Cytokines and Breast Cancer

In spite of encouraging advances in the management of breast cancer the prognosis for patients has not changed significantly(Robert, 1994). The biology of the disease is such that it is likely to be systemic at presentation and therefore its appropriate management should also be systemic. There is controversy surrounding optimal treatment, ranging from high dose chemotherapy with autologous bone marrow support, to less intensive chemotherapy or hormonal therapy. The disease encompasses a broad spectrum and it is not yet possible to predict ideal therapy for each individual tumour.

Most of the growth and differentiation of the mammary gland occurs during sexual

maturation, and then cyclically during pregnancy and lactation. The process is controlled by endocrine hormones and local cytokines *in vivo*. The changes in normal and malignant breast ductal epithelium are influenced by a number of biological agents (Lippman *et al.*, 1986). Conditioned media from oestradiol-treated breast cancer cell lines stimulate the growth of other cell lines. Monoclonal antibodies specific for tumour-derived cytokines can inhibit this growth (Ennis *et al.*, 1991). This suggests that cytokines act in an autocrine or paracrine fashion to induce their effects on breast cancer cells (Bates *et al.*, 1989; Dickson & Lippman, 1995; Lippman *et al.*, 1986). The growth and regulation of breast cancer cells is complex. It involves the interaction of tumour cells, stroma, circulating hormones and the microenvironment, possibly mediated by tumour or stromal derived cytokines. The role of exogenous cytokines with their pleiotropic effects within this 'network' is uncertain, though some may modulate the release of specific endogenous cytokines.

The outcome of clinical trials using cytokines in the treatment of breast cancer has been disappointing for a number of reasons:

- Inclusion of patients with advanced refractory disease
- Use of cytokines alone where combination therapy may be more effective
- Assessment of their efficacy against criteria used for cytotoxic agents

Clinical experience with the IFNs is outline in Table 5.1.

Interferon	Dose range	No. of patients	Response	References
Human Leukocyte IFN-α	3-9 mu	55	20 (36%)	(Bordon *et al.*, 1982; Gutterman *et al.*, 1980; Medenica & Slack, 1985);
Lymphoblastoid IFN-α	0.5-30 mu	138	1 (1%)	(Usui *et al.*, 1983) (Goodwin & W, 1985; Usui *et al.*, 1983); (Laszlo *et al.*, 1986 Silver, *et al*, 1983; Sarna *et al*, 1985;
Recombinant IFN-α	3-86 mu	101	3 (3%)	(Quesada & Gutterman, 1983; Quesada *et al.*, 1984; Sherwin *et al.*, 1983 Padmanabhan, *et al*, 1985; Muss, *et al*, 1984; Nethersell, *et al*, 1984 Lenzhofer *et al*, 1984;
Recombinant or Fibroblast IFN-β	3-30 mu	19^	1(5%)	(Barreras *et al.*, 1988; Bruntsch *et al.*, 1984; Quesada *et al.*, 1982; Wakasugi *et al.*, 1982)
Recombinant IFN-γ	2mg/m^2	15	0 (0%)	(Muss *et al.*, 1986)

Table 1.1 Outcome of clinical trials of IFNs in the treatment of breast cancer

^Excludes one trial in which responses were observed in the skin in 10 of 11 patients receiving IFN-β but criteria not fulfilled for complete or partial response.

In addition to the trials outlined in evaluable disease (Table 1.1), human leukocyte IFN-α has been used in an adjuvant setting. Thirty-two patients with loco-regional recurrence of breast carcinoma were entered into a controlled trial of adjuvant IFN-α. All had histological confirmation of recurrence, local treatment with radiotherapy, and negative staging investigations. The patients were then randomised to either observation alone, or treatment with human IFN-α 3 x 10^6 U sc daily for one year. There were no differences in the rate of local or distant relapse between the groups and it was therefore concluded that IFN-α was of no value in this setting (Fentiman *et al.*, 1987). Intralesional IFN-α alone and in combination with IFN-γ has been succesfully used to treat cutanous metastases. In combination with tamoxifen evidence also

suggests that IFN-α has a role in upregulating oestrogen receptor expression (Seymour & Bezwoda, 1993; van den Berg *et al.*, 1987).

Clinical experience with IFN-γ in breast cancer is more limited and as a single agent it appears to have a different spectrum of activity from that of IFN-α. IFN-γ has been found to have potent *in vitro* activity and activity in a number of murine tumour models. There are few direct clinical comparisons of the two agents. IFN-γ is less effective than IFN-α in both AIDS-associated KS (Krown *et al.*, 1987) and melanoma (Ishihara *et al.*, 1989). In metastatic renal cell carcinoma responses were comparable with those seen with IFN-α (Aulitzky *et al.*, 1994). In ovarian cancer IFN-γ has been used intraperitoneally (ip) as an adjunct to conventional chemotherapy (Pujade Lauraine *et al.*, 1996; Pujade-Lauraine *et al.*, 1993; Welander *et al.*, 1988).

Other cytokines have not been as widely used in the treatment of breast cancer although small numbers of patients treated with IL-2 were included in two of the early trials of IL-2 with disappointing outcomes (Rosenberg *et al.*, 1987; West *et al.*, 1987)

In the long term the role of cytokines is likely to be in the setting of minimal residual disease or as an adjunct to conventional treatment (Dutcher, 1996). In spite of the largely disappointing long-term results from the clinical use of interferons to date they remain an appropriate model for cytokine therapy as they are potent inhibitors of epithelial cell growth.

Cytokines and Tumourigenesis

Cytokines are regulatory proteins which influence many aspects of tumour cell biology, including proliferation, survival and death, motility, surface antigen expression, and cell:cell or cell:matrix interactions. They may also control neovascularization, extracellular matrix synthesis, leucocyte infiltration, stromal cell proliferation, and local immune response through intercellular communication with a wide range of cell types (reviewed in (Balkwill, 1994; Gutterman, 1994). Despite the diversity of molecular structures and properties they possess a number of common features. They are all low molecular weight polypeptides (usually less than 80kD), which bind to high affinity cell surface receptors and induce changes in macromolecular synthesis in target cells. Cytokines may act in a paracrine, autocrine and juxtacrine manner at picomolar concentrations. They occupy an intermediate position between endocrine secretions, which act systemically, and neurotransmitters, which act locally at synaptic gaps. The natural range of cytokine action is within a zone of tissue, although they may also spill over into the circulation. Their *in vivo* production is usually transient and

tightly controlled. Most cytokines are produced by more than one cell type, and show pleiotropy in their actions. They play a wide role in development, immunity, inflammation, tissue repair, regulation of cell growth and differentiated function.

The cytokine family includes the interferons, IFNs, α,β, γ and ϖ; the tumour necrosis factors, TNFs, TNF-α and lymphotoxin-α and -β (LT-α and -β); interleukins, ILs, 1-16, the colony stimulating factors, CSFs, G-CSF, M-CSF, GM-CSF; the transforming growth factors, TGF-α,and β, steel factors and LIF (leukaemia inhibitory factor) as well as growth factors such as epidermal growth factor, EGF, and platelet derived growth factor, PDGF (reviewed in Burke et al, 1993)(Burke *et al.*, 1993). One of the most important aspects of this group of cell regulators is their ability to act in a network, whereby cytokines can induce other cytokines and alter the expression of othercytokine receptors. The response of a target cell or tissue to a given cytokine is dependent on the local concentration of the cytokine, the target cell type and the other cytokines and regulatory molecules interacting with the cell or tissue at that moment in time. Sporn and Roberts drew an analogy between cytokines and a code or alphabet, a complex signalling language in which the ultimate cellular response is determined by the sum of the signals received at the cell surface (Sporn & Roberts, 1988). Cytokines may antagonise or synergise to enhance effects on target cells. The existence of natural cytokine antagonists - in the form of soluble receptors, viral homologues to receptors, or proteins that bind to target receptors without causing signal transduction- further complicates the network. Cytokine receptors transmit regulatory 'information' to the next signalling component in the intracellular environment. These then connect with cytoplasmic pathways which transmit proliferative signals to the nucleus.

Cytokines have a broad role in tumourigenesis and tumour therapy. This includes direct effects on tumour cells or actions on stromal components such as vasculature, extracellular matrix and cells of the immune system. Their effects are summarised below:

Antitumour effects of exogenous cytokine therapy

* Inhibit tumour development and progression.
* Augment immune response to a tumour through effector cells
* Enhanced target cell recognition.
* Deleterious effects on tumour vasculature.
* Influence nutritional status of tumour.

Tumour-enhancing effects of endogenous tumour cytokines

* Act as autocrine or paracrine growth factors

* Indirect effects - suppression of immune cell effector function.

* Enhanced establishment of tumour stroma and vasculature

* Local effects on adhesion molecules.

* Enhanced spread by mediation of bone, cartilage and metalloproteinase ECM destruction.

When investigating the cytokine network attention is now shifting from individual cells to tissues in order to understand their true physiological role. The complexity of the cytokine network - the ability to cross regulate each others production, dysregulated cytokine/ cytokine receptor production and overall response to cytokines - demands a more complex experimental system. Consequently animal models are highly relevant for looking at cytokines in the tissue microenvironment.

As well as the cellular components of a particular tissue, the intercellular matrix is now recognised as an important participant in the cytokine network (Liotta & Stetler Stevenson, 1991). There is increasing evidence to suggest that adherence to matrix induces cells to make cytokines, that cytokines induce cells to alter matrix and that matrix can present cytokines to cells. The process of cell-to-cell adhesion influences cytokine production and response to cytokines (Nathan & Sporn, 1991). In normal glandular tissue the basement membrane, a form of extracellular matrix, separates epithelial cells from the underlying stroma. This basement membrane is partially lost in invasive carcinoma of the breast (Siegal *et al.*, 1981). Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in degradation of the basement membrane and degradation of extracellular proteins such as type IV collagen. Until now the role of endogenous cytokines in the tumour microenvironment has received litle attention and greater understanding of this aspect of tumour biology may be fundamental to overcoming the failure of systemic cytokine therapy evident hitherto in the treatment of solid tumours (Balkwill, 1994). The metalloproteinases appear to be elevated in tumours of high grade and metastatic potential. The two type IV collagenases (gelatinases) of molecular weights 72kDa (MMP-2) and 92kDa (MMP-9) are capable of degrading type IV collagen, gelatin and fibronectin (Liotta & Stetler Stevenson, 1991).

Cytokines in Clinical Use

The interferons

The interferons, IFNs, are a family of secreted multifunctional proteins first identified in 1957 as being produced by vertebrate cells in response to viral infection (Isaacs and Lindemann, 1957). Of the four types: alpha(α), beta(β), gamma(γ) and omega(ω), the first two are termed type I interferons and are made by virtually all cells, whereas IFN-γ (type II) is made only by T lymphocytes and large granular lymphocytes. To date there have been nearly 30 genes identified which encode for IFN-α species, two for β, and at least 5 for ω (Charlier *et al.*, 1993; Mege *et al.*, 1991) and one for IFN-γ. A large number of stimuli can induce the synthesis of type I and type II interferons (IFNs). In relation to breast cancer the interferons (IFNs) α and β are known to modulate TGF-β expression, increase oestrogen receptor (ER) content and inhibit cellular proliferation. They augment the cytostatic effect of tamoxifen and the cytotoxic effect of a number of chemotherapeutic agents (Kerr *et al.*, 1989; Kerr, 1989; van den Berg *et al*, 1987; Laszlo *et al*, 1986; Wadler *et al*, 1990; Pouillart *et al*, 1982).The interferons are components of the vertebrate defence system against viral, bacterial and parasitic infections as well as certain tumours. They exert their activities by inducing a large variety of proteins, several of which may have tumour-suppressor activities (Lengyel, 1993). Some proteins encoded by the tumour virus oncogenes (eg. EBNA-2 from Epstein Barr virus) impair the induction or action of particular proteins by IFNs and thereby may overcome their tumour suppressor function. The IFNs are known to have activity against a number of tumour types.

Historically, the aim of cytokine therapies was to increase the immune response to tumour cell antigens, but anti-tumour effects can also be achieved by direct cytokine action on tumour cells, destruction of the tumour microvasculature, and/or alteration of the local nutritional balance (Balkwill, 1994; Gutterman, 1994; Urabe, 1994) . Optimising the dose and schedule of cytokines in clinical use is complicated and does not follow the rationale of administration of conventional chemotherapy. The anti-proliferative effects of the cytokines are dose-related. Hence the dose regimen will vary whether maximal antiproliferative effect is required or optimal immunomodulatory dose. It is not known which of the effects of the interferons is most important for obtaining a tumour response and hence what should influence scheduling and dose. Clinical trials of conventional chemotherapy seek to determine the maximum tolerated dose (MTD) (Phase I) and then the antitumour activity of this dose (Phase II). *In vivo* and clinical work with IFNs to date suggest a disparity between MTD and optimal antitumour dose (Gutterman *et al.*, 1982; Maluish *et al.*, 1988); . Doses below the MTD have been administered in melanoma and other solid

tumours (Kopp *et al.*, 1993; Satake *et al.*, 1993). Clinical and animal studies have attempted to define the optimum mode of administration and regimen. To compare the toxicity of anticancer agents in different species a formula was devised in which surface area to volume ratios were taken into account (Freireich *et al.*, 1966).

Interferon -α

Clinical trials of systemically administered cytokines have met with some success: most notably interferon (IFN)-α in hematologic malignancies (Talpaz, 1994; Urabe, 1994). In hairy cell leukaemia chronic treatment has been shown to improve survival (Frassoldati *et al.*, 1994). The overall response rate was 90% with 70% of patients showing complete normalisation of their blood. Nevertheless many patients appeared to develop neutralising antibodies, This may interfere with the efficacy of recombinant interferons (Steis *et al.*, 1988). The toxicity of IFN-α is dose-related and the main symptom is of a chronic flu-like illness. Prolonged administration is better tolerated at lower doses.

At doses near the MTD clinically meaningful responses to IFN-α have been seen in low-grade lymphoma, AIDS-associated Kaposi's sarcoma, melanoma, myeloma, renal cell carcinoma, ovarian carcinoma and glioma reviewed in (Mier & Atkins, 1993) A number of studies have evaluated the role of IFN-α, alone and in combination with chemotherapy, in patients with follicular lymphoma (Rohatiner & Lister, 1991). In a comparison of chlorambucil plus IFN-α2b and chlorambucil alone there was no difference in actuarial survival at 3 years (75% in both arms), but a significant prolongation of remission duration in favour of maintenance IFN α–2b (Andres *et al.*, 1988). In a small phase II study in refractory myeloma responses were disappointing but subsidence of pain and improvement of performance status were seen (Ganjoo *et al.*, 1993).

The most encouraging use of IFN-α in solid tumours to date is a recent study in high-risk melanoma. In patients with deep tumours or those with lymph node disease at high risk of relapse, IFN-α2b has been used as an adjuvant (Kirkwood *et al.*, 1996). The Eastern Cooperative Oncology Group (ECOG) conducted a randomised controlled trial of high dose intravenous IFN-α treatment for one month followed by intermediate dose sc for 48 weeks, versus observation, in 287 patients. The treated group had significant prolongation of relapse-free and overall survival compared with the control group. With a median follow-up time of 6.9 years, there was an increase in median disease free survival from 1 to 1.7 years and overall survival from 2.8 to 3.8 years. The greatest benefit was seen in patients with involved lymph nodes. No adjuvant therapy has previously shown an impact on survival in this disease.

There has been little experience of IFN therapy alone in the management of breast cancer. In the limited number of studies where it has been used this is generally in combination with other therapy where a synergistic effect was required and a direct antitumour effect was not the primary aim (Kennedy *et al.*, 1994). IFN–α was used as an adjuvant agent in 32 breast cancer patients after loco-regional recurrence, but results were disappointing (Fentiman *et al.*, 1987).

Interferon-γ

As a single agent IFN-γ appears to have a different spectrum of activity from IFN-α, although experience is more limited. Whilst sharing many antiproliferative and antiviral effects with IFN-α and β, IFN–γ has distinct immunomodulatory effects including macrophage activation, induction of MHC class II genes and more pronounced synergistic interactions with cytokines such as TNF-α and IL-2. For these reasons, the early clinical studies with IFN-γ concentrated on treatment doses and schedules that were capable of enhancing the immune function in patients with both malignant and infectious diseases(Jaffe & Sherwin, 1986) . IFN-γ has now been tested in many advanced cancers, however in solid tumours the results have been largely disappointing. Its use is in part limited by its greater toxicity than IFN-α. IFN-γ has been used in head and neck cancer (Richtsmeier *et al.*, 1990) , colorectal cancer (Brown *et al.*, 1991) , renal cell carcinoma(Aulitzky *et al.*, 1989; Aulitzky *et al.*, 1994) and ovarian cancer (Pujade Lauraine *et al.*, 1996; Pujade-Lauraine *et al.*, 1993). In 100 patients in complete remission from SCLC, IFN-γ had no activity as adjuvant therapy (Jett *et al.*, 1994), but was effective in a very small trial (8 patients) of head and neck cancer patients, receiving IFN-γ in 24 hour infusions once per week for 4 weeks (Richtsmeier *et al.*, 1990). Renal cell carcinoma has been the focus of a number of trials. Early indications were that a biologically defined low dose of IFN-γ once per week for a median period of 10 months resulted in 30% of patients responding with either partial or complete response (Aulitzky *et al.*, 1989). These results were not confirmed in a larger study of unselected patients (Aulitzky *et al.*, 1994) , as those with poor prognosis features and large tumour burden did not respond to this non-toxic dose. In another study with 35 patients with renal cell carcinoma a 15% response rate was demonstrated (Ellerhorst *et al.*, 1994). In this latter study toxicity was minimal and treatment compared favourably with IL-2 or IFN-α.

A study involving interferon-γ at a dose of 2mg/m2 in 15 patients with advanced breast cancer did not show any clinical responses (Muss *et al.*, 1986). However, in the context of advanced disease this is not surprising. The potential role of the interferons in the treatment of breast cancer might be missed as their use to date has been restricted to heavily pretreated patients where they are known to be ineffective in

overcoming mechanisms of acquired drug resistance (Wadler & Schwartz, 1990; Wadler *et al.*, 1989).

IFN-γ has also been used in a number of studies in patients with ovarian cancer (D'Acquisto *et al.*, 1988; Pujade Lauraine *et al.*, 1996) . Some responses were observed in patients with minimal residual disease. A large European multicentre phase II trial of i.p. IFN-γ in patients after chemotherapy with persistent residual disease at second look laparotomy has recently been conducted. A dose of 20 MU/m^2 was administered twice per week for 3-4 months. 31% of patients responded to therapy (23%, complete response, 8% partial response), with fever being the most common clinical adverse reaction. Both young age and tumour burden were predicitive factors of response, with figures of 52%, 35% and 16% response in patients less than 50 years, between 50-59 years and more than 60 years respectively (Pujade Lauraine *et al.*, 1996).

There is evidence that IFN-γ acts synergistically with other cytokines and more conventional therapeutic agents. IFN-β and -γ combined with cytotoxic drugs produced a remission rate of 47% in patients with metastatic colorectal cancer (Klein *et al.*, 1991). In contrast IFN-γ alone had no effect on a group of 50 patients with advanced colorectal cancer (Brown *et al.*, 1991) .

There is some indication that low dose IFN-γ adjuvant therapy may have adverse effects. A randomised Phase III trial in melanoma was halted prematurely as the number of relapses and deaths suggested those receiving IFN-γ fared worse than untreated patients (Meyskens *et al.*, 1990) . Likewise, time to progression and survival were inferior in patients with SCLC treated in complete remission with IFN-γ compared to control patients, although the differences were not statistically significant (Jett *et al.*, 1994) .

IL-2

Interleukin-2 (IL-2) is a 15kDa protein which was the first of a series of lymphocytotrophic hormones to be recognised and characterised as pivotal for the generation and regulation of the immune response. Originally recognised as T cell growth factor (Morgan *et al.*, 1976), it is responsible for signalling T lymphocyte proliferation (Smith, 1988). Its administration, either alone or with LAK cells, can mediate the regression of metastatic cancer in mice and humans (Puri & Rosenberg, 1989). The availability of recombinant cytokines has made its use feasible within the setting of clinical trials. IL-2 has been asssessed alone and in combination with other cytokines or adoptive cellular therapy in patients with metastatic cancer.

The early clinical studies with IL-2 (Quaife *et al.*, 1987; Rosenberg, 1985); West, 1987

#33 showed that it could bring about the regression of metastatic disease in some patients with advanced malignancy. With greater experience it has become apparent that response rates vary with different modes of administration. Toxicity was marked and mainly attributable to high dose therapy. Most side effects are explained by a capillary leak syndrome which leads to fluid retention and organ dysfunction, a high proportion of patients also experience hypotension during high dose therapy. Approximately 15-20% of patients with advanced renal cell cancer and malignant melanoma respond to 'high dose' therapy. The initial enthusiasm for IL-2, based on the results of Rosenberg and colleagues, has waned. Lower dose therapy, which is less toxic, and can hence be given in an outpatient setting, appears to have a lower response rate but the long-term survival figures from such an approach are not yet known (Facendola *et al.*, 1995; Hjelm *et al.*, 1995).

In vivo study of the immunologic consequences of continuous infusion of low dose rIL-2 has been undertaken in 90 patients with advanced cancer (Caligiuri *et al.*, 1993). At doses of 10 and 30 μg/m2 per day a gradual expansion of NK cells was seen in the blood with no evidence of a plateau over a three month period. At this dose level only high affinity IL-2 receptors were saturated such that NK cells were selectively expanded with only minimal toxicity. Unlike the conventional paradigms of cytotoxic chemotherapy where a direct dose response curve appears to exist this novel approach may result in responses to more physiologic levels without the associated severe systemic toxicity (Caligiuri *et al.*, 1993).

IL-7

Interleukin-7 (IL-7) is a 25kDa glycoprotein, first purified in 1988 by Namen and co-workers (Namen *et al.*, 1988). In spite of promising *in vitro* and *in vivo* properties, which influence B and T cells (see Chapter 6), the toxicity in humans has limited its clinical use and there are no published studies of its use in this setting.

IL-12

Interleukin-12 (IL-12) is a heterodimeric cytokine which plays an important part in induction of cell-mediated immunity. Human IL-12 is inactive on mouse cells but murine IL-12 is active on human cells (Schoenhaut *et al.*, 1992) . Through its effects on T cells recombinant IL-12 (rIL-12) has therapeutic activity in a variety of syngeneic murine tumour models and is being evaluated in clinical trials in cancer. In murine tumour models it appears to have an antitumour effect which is greater than IL-2. As yet the clinical use of IL-12 has been hampered by toxicity (Cohen, 1995)..Occasional responses have been seen in renal cell cancer during Phase I studies (Dutcher, 1996).

Nevertheless dose, schedule and route of administration are yet to be determined(Cohen, 1995; Stern *et al.*, 1996).

The best established indications for cytokine therapy are the treatment of hairy cell leukaemia, HCL, with IFN-α and the amelioration of myelosuppression by G-CSF and GM-CSF. Elsewhere the success of cytokine therapy has been sporadic and often unpredictable (Oettgen, 1991). In addition to responding cells other tissue components are important determinants of cytokine action. Consequently the effects of the interactions of stromal cells, matrix and adhesion molecules and their receptors can only be fully evaluated in intact organisms.

Cytokines have a different modality of action, as an adjunct to the immune system, from that of conventional chemotherapy. In the long term the role of cytokines is likely to be in the setting of minimal residual disease or as an adjunct to conventional treatment.

Cytokines and animal models

The need for new animal models

Much of the clinical use of cytokines has been based on empirical principles. Better *in vivo* information is required to predict the optimal dosage, combinations, routes of administration more accurately. At the present time the major animal models - in syngeneic mice or human tumour xenografts in nude mice have significant limitations.

Problems with existing animal models and cytokine therapy

i) *Species specificity of some cytokines*

There are limitations when extrapolating from animal model work using human cytokines to clinical use, related to the species specificity of the different cytokines. Some cytokines, such as IFNs, are strongly species specific whereas others TNF, IL-1 and IL-2 cross species barriers. The availability of purified murine cytokines has facilitated their study and demonstrated their marked potency. Human IFNs induce 2,5A synthetase activity in human tumour xenografts but do not enhance murine NK activity or alter the level of the enzyme in murine tissue. Similarly murine IFNs do not induce 2,5A synthetase in human tissues (Balkwill *et al.*, 1982). Human TNF binds to the murine p55 receptor but not to murine p75 receptor. Murine TNF binds to both human receptors. Theoretically species specific cytokines could induce other non species specific cytokines in the human tumour xenograft model.

Of the cytokines used in this study IL-2 and IL-7 are cross species reactive whilst IFN-

α and IFN-γ are species specific. Murine IL-12 works on human cells but the converse is not true.

ii) *Comparison with human clinical studies*

Many studies have demonstrated that cytokine therapy prolongs survival of tumour bearing mice, but partial and complete remission as defined in clinical studies (greater than 50% regression and complete disappearance of all assessable tumour, respectively) may not be seen, or can only be assessed post mortem. In animal models the end points of tumour stasis, reduction in growth rate, or a reduction in number of metastases, may be useful in terms of understanding mechanisms, but would not necessarily translate into a useful clinical outcome.

iii) *Wide variation in scheduling and differing pharmacokinetics between humans and animals*

Due to the incompletely defined mode of action of most cytokines and the apparent lack of a dose-response relationship in many studies, clinical and animal studies have attempted to define the optimal mode of administration and regimen. A comparison of the toxicity of anticancer agents in several species and man was devised based on a formula in which surface area to volume ratios between species were taken into account (Freireich *et al.*, 1966). The value of this formula has been confirmed by its use to calculate appropriate doses when using recombinant human IFN-γ to treat a human ovarian tumour xenograft in nude mice (Malik *et al.*, 1991). Using this method of calculation comparable peak plasma and intraperitoneal levels of rhIFN-γ were obtained in mouse and human (Malik *et al.*, 1991).

iv) *Few tumour lines have been studied*

Only a small number of tumour lines have been studied and many behave as highly aggressive, malignant tumours. In humans renal cell carcinoma and malignant melanoma are the two main solid tumours in which cytokine therapy has been used (Rosenberg *et al.*, 1987; West *et al.*, 1987). Those tumours which respond are likely to be of low grade and slow growing. This makes the highly proliferative cell lines described poor models for tumours responsive to cytokine therapy in clinical practice.

v) *Immunogenicity of some syngeneic tumours*

Some syngeneic tumours are highly immunogenic. When treated with cytokines a response to the allograft, and not the tumour itself, may be seen.

Cytokine therapy of experimental animal models

Interferon - α

IFN-α and nude mouse xenografts

Interferon-α therapy prolongs survival of tumour bearing mice in a dose and schedule dependent manner. Xenografts from breast, colon, melanoma, osteosarcoma and ovarian tumours have been widely used to look at the direct antitumour activities of the various types of IFN-α (Balkwill, 1985; Balkwill *et al.*, 1982; Balkwill *et al.*, 1980; Brosjo *et al.*, 1985; Crane *et al.*, 1978). More recently it has been combined with other agents such as hormones and cytotoxic chemotherapy (French *et al.*, 1995; Josui *et al.*, 1992; Laurent *et al.*, 1994; Tanaka *et al.*, 1994);.

IFN-α in syngeneic systems

The indirect antitumour effects of IFNs have been well documented in transplantable murine tumour models. They are most effective where tumour burden is low and tumour cytostasis is the usual finding (Gresser, 1989). Regression of established tumour was rarely seen, the effects being to increase survival rather than effect a cure (Balkwill *et al.*, 1989; Gresser *et al.*, 1985). IFN-α has also been reported to act on tumour vasculature in these models (Dvorak & Gresser, 1989).

IFN-α and Metastases

The majority of models of metastasis are based on the iv injection of transplantable tumour cells which are then entrapped in organs such as the lungs and spleen. IFNs-α/β generally have activity in these models and a host component is necessary for response (Gresser *et al.*, 1990; Kaido *et al.*, 1993; Yasui *et al.*, 1990). A competent immune system was required for optimal effects, and mice were resistant to rechallenge with tumor cells (Gresser *et al.*, 1990). However, in the Colon 26 murine colon carcinoma model, treatment with IFN–α commencing 2 hours after the injection of tumor cells had a significant antimetastatic effect that was also apparent in NK deficient mice (Ramani & Balkwill, 1987; Ramani *et al.*, 1986). A significant antimetastatic effect was also seen in the Colon 26 murine colon carcinoma model treating with IFN–α.

Interferon-γ

IFN-γ and nude mouse xenografts

IFN-γ, like IFN-α, shows strict species specificity. As a result its direct effects on growth of tumour cells can be examined in the nude mouse xenograft system. Many

studies have compared the effects of IFN-α and IFN-γ on sc xenografts. Generally, IFN-γ was not as effective as IFN-α whatever the injection route, despite being able to alter surface antigen expression (Balkwill *et al.*, 1987). However, IFN-γ was active against ovarian cancer xenografts growing ip in nude mice (Balkwill *et al.*, 1986b; Balkwill & Proietti, 1986; Malik **et al, 1991).** IFN-γ, administered ip at doses equivalent to those given in clinical trials, increased the survival of mice bearing human ovarian cancer xenografts growing as ascites or solid tumour (Malik *et al.*, 1991). Mice bearing ascites were cured of peritoneal disease. In solid ovarian tumours, there was a gradual decline in the number of proliferating cells following IFN-γ treatment. Endlabelling studies showed an increase in DNA fragmentation which reached a maximum at 14 days. These changes were associated with an upregulation of p53 and hypophosphorylation of the product of the retinoblastoma protein, Rb. Continual exposure to the cytokine was more effective than higher doses three times per week, and the animals only needed 14 days treatment to achieve a significant increase in survival (Burke *et al,* submitted).

IFN-γ in syngeneic systems and metastatic models.

IFN-γ inhibited tumours growing in syngeneic mice including murine osteogenic sarcoma and a chemically induced fibrosarcoma (Crane *et al.*, 1978; Giovarelli *et al.*, 1986), although in many cases tumour stasis was the most common finding (Balkwill, 1985). Response to treatment depends on tumour burden and schedule. IFN-γ had antitumour effects in experimental lung metastases models of colorectal cancer and melanoma (Kondo *et al.*, 1987). In contrast to antitumour effects *in vivo,* pretreatment of colon 26 cells with mIFN-γ *in vitro* significantly increased the number of lung tumour nodules when cells were injected i.v. into immunocompetent mice and nude mice. NK cells appeared to be involved since no effect was observed in NK depleted or deficient mice (Ramani & Balkwill, 1987).

IFN-α and -γ in combination

IFN-α, in combination with IFN-γ, was effective in the Renca model of murine renal cell carcinoma. A synergistic effect was observed when murine IFN-α was combined with low dose IFN-γ (1-10 units/ml). This combination was less effective in nude mice and 75% of the euthymic were shown to be immune to rechallenge. Although IFNs may directly inhibit the growth of Renca tumour this study demonstrates that specific immune response plays a dominant role although a direct effect is evident.

IL-2

IL-2 and nude mice xenografts

Early animal experiments were limited by problems with toxicity. This included development of ascites, splenomegaly, lymphadenopathy, gastrointestinal effects, general weakness and malaise (Ettinghausen & Rosenberg, 1986; Marincola *et al.*, 1992; Moore *et al.*, 1991; Papa *et al.*, 1986).

IL-2 has been used in combination with allogeneic human LAK cells in tumor xenograft models. Antitumour effects are seen in human squamous cell carcinoma tumours of of the head and neck (Sacchi *et al.*, 1990; Sacchi *et al.*, 1991)., xenograft models of haematological malignancies (Greenberg, 1986; Mule *et al.*, 1987; Talmadge *et al.*, 1987 Greenberg *et al*, 1986; Mule *et al*, 1987; Talmadge *et al*, 1987; Foa *et al*, 1990).l the MCF-7 breast carcinoma cell line both *in vitro* and in nude mice (Paciotti & Tamarkin, 1988).

IL-2 and syngeneic and metastatic models

Much of the early work with IL-2 investigated the combination of IL-2 and LAK cells (Rosenberg *et al.*, 1986; Schwarz *et al.*, 1989). Therapeutic efficacy was dependent on tumour burden and the dose of both IL-2 and LAK (Mule *et al.*, 1985). Most animal experiments have entailed metastatic models of tumour lines syngeneic to C57/BL6 mice. These demonstrated tumour regression , primarily by enhancing the cytolytic activity of NK and LAK cells (Herberman *et al.*, 1975; Hirabayashi *et al.*, 1995; Lafreniere & Rosenberg, 1985; Mule *et al.*, 1985).

IL-2 in combination with other agents.

Combinations of IL-2 with other cytokines have been extensively studied (reviewed in Brunda, 1992)(Brunda, 1992). Synergistic interactions between IL-2 and IFN-α were first demonstrated in the M5076 reticulum cell sarcoma model (Brunda *et al.*, 1987). and have since been shown in other models (Hornung *et al.*, 1988; Rosenberg *et al.*, 1988; Sakura *et al.*, 1989; Truitt *et al.*, 1989) following perilesional (Silagi *et al.*, 1988) or systemic treatment (Iigo *et al.*, 1988).

IL-7

IL-7 has a role in the early development of B lymphocytes (Namen *et al.*, 1988) and stimulates the proliferation of murine (Grabstein *et al.*, 1993) and human mature T-lymphocytes (Armitage *et al.*, 1990).

IL-7 and nude mouse xenografts

IL-7 alone has no effect on the survival of human colon carcinoma-bearing nude mice. However the addition of human T-cells significantly promoted survival of the nude mice in comparison with mice receiving either treatment alone. The effect was abrogated by IFN-γ as it by the addition of antibodies to IFN-γ. Systemic administration of IFN-γ was also less effective than IL-7 and human T-cells, suggesting that the continuous local release of cytokines is important in the action of IL-7 (Murphy *et al.*, 1993).

IL-7 and syngeneic models

Antitumour cytotoxic T lymphocytes (CTLs) generated in IL-7 are of equivalent potency to those generated with IL-2 (Jicha, 1991). Presence of IL-7 in the medium in which CTL are cultured can support their growth *in vitro* for prolonged periods in the absence of repeated stimulation with tumour stimulating cells or tumour antigen. They also retain antigenic specificity and the ability to reject tumours *in vivo* when subsequently injected intravenously (Lynch & Miller, 1994). In the Renca renal cancer and the MCA-38 colon carcinoma models twice daily rhuIL-7 injections ip substantially decreased the number of pulmonary metastases formed (Komschlies *et al.*, 1994).

IL-12

Syngeneic systems and models of metastases

IL-12 has shown promising preclinical results in a range of animal cancer models, such as B16F10 melanoma, Renca renal adenocarcinoma and M5076 reticulum cell sarcoma (Brunda *et al.*, 1993). Injected peritumourally or systemically IL-12 caused complete regression of large tumours and antimetastatic effects in these and other models (Brunda *et al.*, 1993); Nastala, 1994 #112; Gately, 1994 #111 (reviewed by Gately *et al*, 1994). IL-12 mediates its antitumour effects through the immune system. It is effective in NK deficient mice injected with B16F10 melanoma (Brunda *et al.*, 1993) or bearing Renca or MCA-207 tumours, suggesting that these cells do not play a major role. The anti-tumour effect was reduced in nude mice in both of these models, suggesting a role for T cells. In contrast to these studies IL-12 was effective as an anti-tumour agent in T- and B-cell deficient SCID mice bearing a X5563 B cell lymphoma (O'Toole *et al.*, 1993). In some models the IL-12 effect was abrogated by treating the mice with neutralizing anti-IFN-γ antibodies (Nastala *et al.*, 1994). Whilst the induction of IFN-γ may be necessary for the anti-tumour effect of IL-12, it is not sufficient since IL-

12 therapy in tumour-bearing nude mice resulted in an 8 fold increase in serum IFN-γ as compared to euthymic controls (Brunda *et al.*, 1993). Moreover, murine IFN-γ was not as effective as IL-12 in producing an anti-tumour effect in the Renca tumour bearing mice.

IL-12 is more effective than IL-2 in a number of models. Synergistic effects were observed with the combination at low doses but the most effective therapy was still an optimal dose of IL-12 alone (Brunda *et al.*, 1993). Investigation of cytokine expression in tumours of mice receiving murine rIL-12 suggests that IL-12 initiates a cytokine network in which IL-12 responsive cells within the tumour express IFN-γ which acts in a paracrine fashion to induce chemokines which attract T cells which are then activated and act cytolytically (Tannenbaum *et al.*, 1996). Further work using cytokine gene-engineered tumour vaccines is investigating the use of systemic IL-12 with carcinoma cells engineered to release local IL-2 (Vagliani *et al.*, 1996).

Animal Models - The advantages and limitations of each

The advantages and disadvantages of existing animal models of human cancers are summarised in Table 1.3 below.

Model	Advantages	Disadvantages
Nude mouse and human tumour xenografts	Histology and ultrastructure of human counterpart maintained. Direct actions of species specific cytokines can be examined. Close histological resemblance to human disease. Response to chemotherapy comparable to human tumours. Metastatic behaviour assessable.	Take rate of primary tumours variable, often low. No competent immune system, hence effects secondary to cytokines not evaluable. Xenogeneic response induced against tumour with some cytokines, eg.IL-2. Only orthotopic transplants metastasise.
Transplantable tumours	Readily available. Easy to use, reproducible. Tumour/ host relationships can be studied.	Selected by successive passage - only serves as model for anaplastic tumour. Immunogenic. Lack diversity. Different mechanism of metastasis. Few mammary tumour models. Histology different from human tumours.
Spontaneous models - MMTV Types	Tumours develop at reproducible times and frequencies. Uniform genetic background.	Morphology does not resemble human morphology.
Carcinogen-induced tumours	Readily available. Tumours develop readily.	Immunogenic. Need for manipulation (ie. Rx with mutagen).
Metastatic models -Spontaneous -Experimental	Can observe metastatic process. Wide range of histological types available. Develop metastases sooner than spontaneous model.	Tumours may be anaplastic. Tumours often immunogenic. Metastases arise after entrapment (Different mechanism from that in humans).

Table 1.3 The advantages and disadvantages of exisiting animal models

Having outlined the pitfalls with existing animal models it is clear that a transgenic model which develops tumours stochastically, in an immunocompetent mouse, with histology which resembles the human tumour counterpart and a non-immunogenic tumour would have several advantages.

Transgenic Oncogene Mice

Background

Molecular analysis of tumorigenesis is often hampered by the accessibility of tissue specimens at different stages in the multistep pathway. This has made transgenic mice a valuable tool for the study of molecular events involved in this stepwise progression to the tumour phenotype. The transgenic mouse model system has become the experimental system of choice for the assessment of the transforming activity of oncogenes in the mammary epithelium (Cardiff, 1996).

As with the genes involved with the development of cancer, most mouse mutations created by transgenic technology fall into two groups - dominant gain-of function and recessive loss-of function. The gain-of-function mutations are modelled by introducing a gene sequence attached to a promoter which determines the developmental stage and tissue specificities of expression. The production of novel protein results in a corresponding phenotypic alteration in the mouse. Loss of function mutations requires the inactivation of both alleles in order to exert a cellular effect, hence the description as recessive. Loss of function has also been modelled using knockout mice where coding sequences of genes are interrupted.

History

The technology to introduce exogenous foreign genes stably into chromosomal DNA has been available for over a decade now and over this period transgenic mice have been used to study complex processes such as immunity, development, gene regulation and pathogenesis (Jaenisch, 1988).

Production

A number of methods have been used for the production of transgenic mice. These include microinjection into the one-cell embryo (Brinster *et al.*, 1985); infection of preimplantation embryos with retroviral vectors containing foreign DNA (Brinster & Palmiter, 1984); infection and transfection of embryonic stem (ES) cells and infection of postimplantation animals (Jaenisch, 1988). The most common method of producing transgenic mice is by microinjection of linear DNA into the pronucleus of the mouse

zygote (Figure 1.1). This leads to stable integration of the injected DNA in 10-40% of the injected embryos. In the majority of animals this integration takes place at the one cell stage, leading to the integration of foreign DNA into every cell of the transgenic animal including the primordial germ cells. Integration usually occurs at a single site but multiple copies of the transgene can be incorporated. The transgenic 'founders' thus produced will then transmit the gene in a Mendelian fashion to approximately 50% of their offspring.

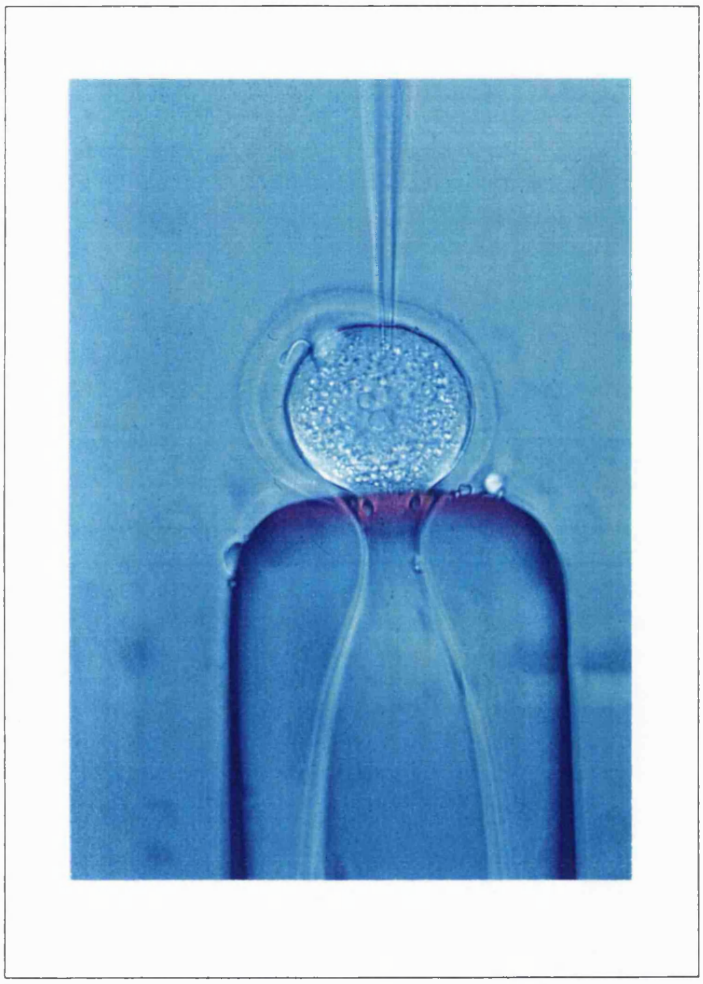


Figure 1.1 Microinjection of linear DNA into the pronucleus of the mouse zygote.

Using microinjection the site of transgene integration is essentially random, preventing selection of specific gene mutations and therefore limiting the utility of this approach. In the past year or so powerful technical advances have made it possible to generate and select site-specific homologous recombination events in pluripotent embryonic stem (ES) cells. ES cells can be maintained *in vitro,* genetically modified through homologous recombination, and returned to the blastocoel, where they may ultimately contribute to the germ line of the resulting mouse. This technique of gene targetting enables the production of transgenic mice bearing a selective modification or

inactivation of virtually any gene for which cloned DNA is available. By crossing mice heterozygous with respect to the inactivated target gene, homozygous null mutants can then be generated. As a result animal models can be generated with the same mutations as human syndromes. These animals have proved to be highly susceptible to tumour development indicating that they are a potent *in vivo* assay system for tumour suppressor genes (Matzuk & Bradley, 1994).

Promoter

Expression of a transgene in a mouse is directed by a tissue specific promoter. The mammary gland has been a popular organ for targeting of transgenes because of the availability of relatively mammary-specific promoters (Pattengale *et al.*, 1989). Although the promoters used in mammary transgenic mice are better than those in other transgenic models they still have significant limitations (Edwards *et al.*, 1996).. The mammary tumour virus long terminal repeat (MMTV LTR) has been used most widely and, to a lesser extent, milk-protein promoters such as beta-lactoglobulin (Watson *et al.*, 1991) and whey acidic protein (WAP) promoter/ enhancer elements (Andres *et al.*, 1988). All three promoters direct high level expression of linked genes to the mammary epithelium, but their behaviours differ in several important respects.

All the promoters are hormone sensitive and are only fully active during pregnancy and lactation, although MMTV LTR is transcriptionally active during all stages of mammary epithelial differentiation. WAP promoter/enhancers are expressed at high levels only in mid pregnant and lactating mammary glands (Andres *et al.*, 1987; Andres *et al.*, 1988). Consequently the epithelial cell types expressing the MMTV oncogene or WAP oncogene fusion genes may differ in their states of differentiation. Clearly it is the resting gland which is of greatest relevance to the development of cancer in humans (Webster & Muller, 1994).

Like many promoters the MMTV LTR has proven to be promiscuous, it is active in the Harderian gland, seminal vesicles, epididymis, salivary glands and prostate gland (Pattengale *et al.*, 1989). This lack of complete specificity of the promoters may result in the oncogene being expressed elsewhere. Such unwanted expression may be lethal (Stocklin *et al.*, 1993) or may produce tumours in other organs which are difficult to distinguish from metastases arising from the mammary tumours (Guy *et al.*, 1992a).

Transgenic Mice As Models of Malignancy

The constructs introduced into the germline usually have an oncogene (activated, cellular or viral) or tumour suppressor gene under transcriptional control of a promoter. Normal tumourigenesis involves two events, mutation of a single cell and expansion of

the resultant clone. In transgenic mice the presence of a heterologous promoter expedites the normal process of carcinogenesis by one step. The transgene is usually expressed in a tissue specific manner, the tissue being determined by the regulatory elements of the hybrid gene. Thus the promoter region of the mouse mammary tumour virus (MMTV) placed upstream of the coding sequences for c-*myc* and H-*ras*, targets their expression preferentially to tissues in which MMTV is expressed in greatest quantities, ie. breast, salivary epithelium, and lymphoid tissues. For this reason models of breast cancer often involve the oncogene of interest under the control of the MMTV promoter. However in most murine transgenic models of cancer, tumours are monoclonal and only become apparent after a variable latency period. It seems highly likely that synergistic mutations are required for tumour formation.

Specific examples of transgenic oncogene mice as models of different malignancies are detailed below. Many of these illustrate a process of multistep tumour progression and enable the molecular characterisation of tumour cell proliferation and tumour angiogenesis.

Non-mammary models

Haematological and lymphoid tumours

Representatives of most classes of genes implicated in neoplasia and proliferation have been tested in relation to the haemopoietic system. Oncogene transgenic mouse models of lymphomas, acute and chronic leukaemias and lympho- and myelo-proliferative syndromes have all been developed.

Transgenic mice have made it possible to test the hypothesis that deregulated *myc* expression promotes the development of B lymphoid tumours within a few months of birth (Adams *et al.*, 1985). In mice expressing a mutated H-*ras* human cDNA controlled by CD2 regulatory sequences, high thymic expression of H-*ras* is seen and some lines develop transplantable clonal T lymphoid tumours (Suda *et al.*, 1987)..

Gastrointestinal tumours.

Liver carcinogenesis has been investigated in a number of transgenic models. Two models involving the HBV large surface antigen and the HBx gene suggest that HBV may predispose individuals to cancer by altering hepatocyte differentiation and driving regeneration (Kim *et al.*, 1991). Sustained proliferation is also a feature of liver tumours seen in TGF-α transgenic mice (Halter *et al.*, 1992).

The SV40 T antigen is tumourigenic in liver and pancreatic tumours (Sandgren *et al.*,

1989). The antigen appears to promote karyotypic instability which may be central to many forms of tumorigenesis. In the pancreas a mutant H-*ras* allele or SV40 T antigen(Ornitz *et al.*, 1992) were associated with development of acinar tumours, whereas *myc* produced mixed acinar-ductal tumours (Sandgren *et al.*, 1989).

Skin cancer models

The epidermis is composed of one main cell type - the keratinocyte, which undergoes a progression of steps in differentiation to produce a hardy and renewal protective covering. In transgenic mice the four major epidermal keratin promoters have been used to target differentiation-specific and keratinocyte- or epidermal- expression of a growth factor or oncogene.

Models of breast cancer

The overexpression or amplification of a number of oncogenes has been demonstrated in breast cancer using Southern and northern analysis and immunohistochemistry of tumour biopsies and cell lines (Slamon *et al.*, 1987). Nevertheless transgenic models are better able to address questions about the causal association between expression of a gene and tumour progression.

As described above targeting oncogene expression to mammary epithelium has been possible by using a mammary specific transcriptional element such as the MMTV Long Terminal Repeat (LTR) or the Whey acidic protein gene (WAP) promoter (Muller, 1991). These promoters stimulate high levels of transgene expression in mammary epithelium. Ha-*ras* strains promoted by MMTV LTR have displayed a high incidence of mammary tumours (Sinn *et al.*, 1987; Tremblay *et al.*, 1989), whereas in the WAP/v-Ha-ras transgenic strains mammary tumours are relatively rare and follow pregnancy (Andres *et al.*, 1988; Nielsen *et al*, 1992; In transgenic animals bearing the *ras* gene under the zeta-globin promoter sporadic mammary tumours arise which are morphologically identical to those arising in the mice with MMTV-driven oncogenes and hence are likely to be caused by expression of the same transgene(Cardiff, 1996) .

Two models involving the activated c-*neu* oncogene have been developed. In the model of Muller *et al* tumours arose synchronously in all mice, involved the entire gland and were polyclonal in origin. Development of tumours in these mice appeared to involve a single genetic event (Muller *et al.*, 1988). This is the only transgenic model of mammary cancer where a single oncogene has been found sufficient for the malignant transformation of mammary cancer. Muller and coworkers noted a 100% incidence of polyclonal tumours with a median onset of 89 days in their mice(Muller *et al.*, 1988). In mice with the same transgene, developed by Bouchard *et al* , tumours were monoclonal

and appeared between 5 and 10 months in a stochastic pattern in 28 to 50% of mice, depending on the line (Bouchard *et al.*, 1989). A model developed more recently with the unactivated *neu* oncogene under control of an MMTV promoter was also associated with the development of mammary tumours in transgenic mice. These tumours appeared to metastasise to lung with higher frequency than described in the two models of Muller and Bouchard, but the latency to tumour development is 8 months or more (Guy *et al.*, 1992a). This suggests that there may be two or more genetic events required prior to mammary tumorigenesis in this model. Primary tumours arising in models with the activated gene also behave more aggressively. This unactivated *neu* model may be more clinically relevant as in human breast cancer overexpression of unactivated *neu* is associated with poor clinical outcome (Guy *et al.*, 1992b).

Mice bearing MMTV-*wnt*-1, MMTV- *int*-2 and MMTV-TGF-α transgenes develop mammary hyperplasia (Dickson & Lippman, 1995; Kwan *et al.*, 1992; Matsui *et al.*, 1990). Double transgenic mice have been used to investigate oncogene cooperativity (Muller, 1991) . The *Wnt*-1 and *int*-2 protooncogenes cooperate in mammary carcinogenesis - tumours (hyperplasias) arising earlier in the transgenic mice possessing both oncogenes (Kwan *et al.*, 1992).

Importance of genetic background

It is well documented that genetic background influences tumour susceptibility. The effect is illustrated well in haemopoietic tumours. Placing Eμ-*myc* transgenes on differing genetic backgrounds alters the kinetics and tumour type as well as tumour susceptibility. On a C57Bl/6, SJL or BALB/c background Eμ-*myc* transgenes resulted in B lymphoid tumours almost exclusively, but seven of eight founder C3H/HeJ transgenic mice developed T lymphomas. In the both C57Bl/6 and C3H/HeJ mice an increase in size and numbers of pre-B cells was seen along with similar levels of Eμ-*myc* transcripts in the spleen and thymus, consistent with B lymphoid expression of the transgene (Yukawa *et al.*, 1989). This suggests the difference in tumour type is unlikely to be an effect of the transgene alone - the stromal environment may be important as conventional C3H/HeJ mice repopulated with transgenic C57Bl/6 cells also developed T lymphomas (Yukawa *et al.*, 1989). The effect of genetic background is also seen with mice harbouring the SV40 large T antigen gene in a C57Bl/6J genetic background. In this model the level of transgene RNA expression is considerably higher than in transgenic mice harbouring the same transgene on an F1 genetic background. The F1 hybrids are C56Bl mice crossed with NZW mice which appears to have a dominant negative effect on SV40 large T antigen expression. Choroid plexus papillomas appear later and less frequently, resulting in longer survival of the animal (Cho *et al.*, 1989). Our own experience also suggests that background is likely to modulate incidence,

natural history and biology of the tumour.

Use of transgenic mice for therapeutic studies

Relatively little has been published about the use of trasgenic mice for the assessment of anticancer therapy. Attempts to develop transgenic mice commercially have proved difficult. The Harvard 'oncomouse' was patented in 1988, and licensed exclusively to DuPont for development. The mice which possess the v-Ha-ras transgene and develop mammary tumours were used to assess the efficacy of cancer chemotherapy agents in breast cancer (Dexter *et al.*, 1993). A similar, earlier study used the WAP *ras* transgenic mouse with an activated, human c-Ha-*ras* gene on the Y chromosome. Adult males develop salivary and/or mammary adenocarcinomas (Nielsen *et al.*, 1992)..

Tumour regression of mammary and salivary gland carcinomas in ras transgenic mice has been shown using inhibitors of the enzyme farnesyl-protein transferase (FPTase) which interferes with the function of ras oncoproteins. The tumours reappear when treatment is stopped but most regress again when it is reintroduced (Kohl *et al.*, 1995). Another study involved transgenic mice that express the rat *neu* oncogene in mammary epithelial cells. Intraperitoneal injection of a monoclonal anti-receptor antibody specific for the oncogene product prevented tumour development in these transgenic mice in a dose-dependent manner. Half the mice, when injected with anti-receptor antibodies, did not develop tumours even after 90 weeks of age (Katsumata *et al.*, 1995).

Rationale

Although mice transgenic for oncogenes have been available for a decade, their use as a model for experimental therapeutics has been limited. If feasible such mice could be useful for preclinical investigation of prophylaxis, primary therapy and adjuvant treatment.

The rationale for this study was to investigate the potential of oncogene transgenic mice as models for new cancer therapies which more closely resemble human cancer. There was a need for a model with the following salient characteristics:

* Inbred

* Immunocompetent

* Develops spontaneous tumours

* Tumours resemble those seen in humans - biologically and morphologically

Transgenic mice carrying the activated rat c-*neu* oncogene under the transcriptional control of the MMTV promoter were bred to BALB/c mice. This PhD thesis entailed a detailed examination and documentation of the diverse tumours which arose in five successive generations of such mice in respect of biology, natural history, proliferation and other indices. The cytokine sensitivity of the tumours has been investigated after transplantation into nude mice and , in one case, syngeneic mice. Longterm prophylactic treatment with cytokines has been carried out to investigate the effect on tumour development. This baseline information provided an assessment of the feasibility of this model as a tool for cancer, and in particular cytokine, therapy.

Chapter 2

Materials and Methods

All reagents used were obtained from Sigma (Gillingham, U.K.) unless otherwise stated.

Mice

Nude mice

Female nude mice of mixed genetic background (bred by the ICRF animal unit, Clare Hall) aged 6-12 weeks were used for transplant experiments. The mice were allowed food and water ad libitum and housed in sterile isolators at 20°C (La Calhene Ltd, Cambridge). All isolator supplies were sterilised, and all items entering the isolators were sprayed with 2% Tegedor (TH Goldschmidt, Middlesex).

BALB/c mice

Inbred male and female BALB/c mice (ICRF breeding unit, Clare Hall) aged 6-12 weeks were used for backcrossing, test-crossing and tumour transplantation. The mice were allowed food and water ad libitum and housed in sterile isolators at 20°C (La Calhene Ltd, Cambridge) or the specified pathogen-free unit at Clare Hall from birth until tumour development or death from other causes.

Founder mice

Transgene-positive male founder mice of the F1 generation (C57BL/6 x C3H) were obtained from Professor Paul Jolicoeur, Instituts de Recherches de cliniques de Montreal, Montreal, Canada. These had been generated by microinjecting a 8.2kb SacII-EcoRI chimeric DNA fragment containing the activated rat c-*neu* cDNA under transcriptional control of the MMTV LTR, as described in Bouchard *et al.* (1989)(Bouchard *et al.*, 1989). One-cell (C57BL/6 x C3H) F2 embryos were collected, microinjected and transferred into pseudopregnant CD-1 females as described in (Hogan *et al.*, 1986),.

The founder mice were initially maintained in sterile isolators at 20°C (La Calhene Ltd, Cambridge) in the quarantine unit until the next generation was bred after successful uterine transfer. These mice were then backcrossed onto BALB/c mice.

All animal experiments were performed under Home Office licence.

Uterine transfer

Embryos from pregnant female mice in the quarantine colony were collected, introduced into a pseudopregnant female (mated with a vasectomised male) and at 21 days the offspring were tailsnipped to screen for the transgene.

***In vivo* passage**

Tumours transplanted into nude mice were minced finely with scissors and 0.1 or 0.05ml tumour suspension with RPMI and injected subcutaneously into a lateral site on each mouse. The tumours were assessed histologically for any changes in morphology at successive early passages and at regular intervals thereafter. The virulence of the lymphoma (see Chapter 4) led to its being frozen down once recoverability had been established. The tumour passaged into BALB/c mice (see Chapter 5) was initially injected into offspring from subsequent generations and when no suitable transgene-negative offspring remained it was injected into mice from earlier generations and female BALB/c mice.

Animal techniques

Protocol for nude mouse transplants

In experiments with tumour transplants the same principles were applied throughout: IFN-α was given sc and IFN-γ ip and later sc. The diameters of tumours were measured weekly with calipers and tumour volumes calculated from the formula: Vol = (longest diameter) x (shortest diameter)^2 (Gately *et al.*, 1994). The mice were killed when the maximum tumour diameter approached 2cm. In each experiment the intention was to treat eight mice in each group, and in every case at least seven mice were assessable. Cytokine sensitivity was assessed by logrank survival of the eight mice in each group and by changes in tumour volume over time.

Home Office regulations do not state explicitly the exact maximum size of a tumour prior to an animal being killed. In an animal who is not distressed and in reasonable

condition, where the tumour is not ulcerated the maximum diameter of any tumour is taken as up to 20 mm. Hence the majority of mice are killed when their tumours reach this size. Because of the way in which tumour volume is calculated a 20mm long thin tumour would have a comparatively smaller volume than a spherical tumour. In each graph of tumour volume the last points in the line represent the final time point at which the volume of tumour could be measured before it was necessary to sacrifice the mouse.

Peripheral blood

Mice were bled by cardiac puncture and blood collected in EDTA tubes for determination of peripheral blood counts. A full blood count was carried out using a Coulter S plus 6 cell counter (Coulter Electronics Ltd., Bedfordshire ,U.K.) and blood films were made to determine the differential count.

Diet

Transgenic mice in the experiment involving dietary fat manipulation were divided into three groups. One group was fed the ICRF Rodent Diet, modified from a formula devised by Glaxo with 4.9% groundnut meal. The high fat group received a 15% fat diet, based on greoundnut meal. Both diets were supplied by Special Diet Services, Ware, UK. The 10% fat diet was supplied by Harlan -Olac. All diets were packed in vacuum packaging for use in SPF Units

Molecular Biology Techniques - DNA

Restriction enzyme digests

Restriction enzyme digests were performed as previously described(Sambrook *et al.*, 1989) .

Preparation of L.agar ampicillin plates

These were prepared as previously described (Sambrook *et al.*, 1989).

Transformation of Epicurian Coli Sure™ competent cells

Epicurian Coli Sure™ competent cells (Stratagene,) were used as hosts for ampicillin resistant plasmids. The competent cells were stored on ice and gently mixed by hand. 100µl was aliquoted into a prechilled tube and 1.7µl of 1.44M β-mercaptoethanol was added. Following gentle mixing the cells were incubated on ice for 10 mins. Ten ng of DNA was added to the cells and incubated for 30 mins on ice. The cells were subsequently heat shocked at 42°C for 45s and incubated for a further 2 mins on ice. 0.9ml of L. broth was added to the cells and incubated at 37°C for 1h with shaking. Ten-100µl of transformation mix was spread onto L.agar plates with ampicillin added at a final concentration of 50µg/ml. Control transformations were included in all cases.

Plasmid purification

Plasmid mini-preparation

Small scale plasmid purifications were performed using the alkaline lysis method as described previously (Sambrook *et al.*, 1989). The protocol is based on a modified alkaline lysis method.

Large scale plasmid purification

Large scale plasmid purification was performed using the alkaline lysis maxi-preparation method as prescribed previously (Sambrook *et al.*, 1989). Superhelical plasmid was isolated by double banding on caesium chloride-ethidium bromide gradients by standard methods (Sambrook *et al.*, 1989).

Determination of DNA/RNA concentration

DNA/RNA samples were diluted in D.W. and the OD_{260nm}/OD_{280nm} was determined. 1 $OD_{260\ nm}$ unit corresponds to 50mg/ml for double stranded DNA and 40mg/ml for single stranded DNA or RNA. The ratio of OD_{260nm}/OD_{280nm} provides an estimate of the sample purity, pure preparations of DNA and RNA have an OD_{260nm}/OD_{280nm} of 1.8 and 2.0 respectively.

Organic extraction and precipitation of nucleic acid

Unless otherwise stated protein contaminants were removed from nucleic acid solutions by phenol/chloroform extraction. Nucleic acid solutions were extracted with an equal volume of phenol, once with phenol/chloroform (1:1) and once with chloroform. Phenol (redistilled nucleic acid grade, BRL) was buffered with TE pH 8.0 and 0.1% (w/v) hydroxyquinoline added as an antioxidant. Chloroform was a 24:1 (v/v) mixture of chloroform and isoamyl-alcohol unless otherwise stated. Nucleic acids were precipitated from aqueous solution by the addition of either 0.1 vol 3M sodium acetate pH 5.2 and two volumes of absolute ethanol (2.5 vols was used for precipitation of RNA), or by addition of an equal volume of isopropanol. Precipitated nucleic acid was washed once with 70% (v/v) ethanol, air-dried and dissolved in the appropriate buffer.

Extraction of genomic DNA

A method suitable for processing large numbers of samples was used to prepare DNA which digests with most restriction enzymes and is of sufficiently high molecular weight for Southern blotting of restriction fragments of up to 10kb. Tailsnip DNA was digested in Tris-based buffer and proteinase K solution at 55^{o}C overnight. Nucleic acid solutions were extracted with an equal volume of phenol once and twice with phenol/chloroform (1:1). Phenol (redistilled nucleic acid grade, BRL) was buffered with TE pH 8.0 and 0.1% (w/v) hydroxyquinoline added as an antioxidant. Chloroform was a 24:1 (v/v) mixture of chloroform and isoamyl-alcohol. Nucleic acids were precipitated from aqueous solution by the addition of 0.6 volumes of isopropanol. Precipitated nucleic acid was washed once with 70% (v/v) ethanol, air-dried and dissolved in TE buffer.

Probe for screening

Screening for the transgene was established initially using Southern hybridisation analysis of tail DNA using a *neu*-specific probe from the DNA fragment microinjected.

The 4.6kB fragment was obtained by cleavage of MMTV/neuT DNA with Hind III and Sal I. The plasmid was mapped with restriction enzyme digestion (see Figure 3.2(b)) and the 4.6kB fragment excised. This did not hybridise to tailsnip DNA from transgene

negative C57 Bl/6 and BALB/c mice at a detectable level confirming that it did not detect endogenous murine c-*neu*.

Radiolabelling of cDNA probes

DNA probe fragments were excised from LMP-agarose gels (BRL) and 3mls D.W. was added per g of agarose. The agarose was melted by boiling for 5 mins and stored at -20^{o} C. DNA probes were labelled by the random priming technique (Feinberg & Vogelstein, 1984) using [^{32}P]-dCTP (~3,000Ci/mmol, Amersham International). Unincorporated precursors were removed by passing through a pre-prepared Chroma spin^{TM} column 100 (Clontech, Palo Alto).

Agarose gel electrophoresis

An appropriate quantity of agarose (BRL) was added to 1 x TBE (Tris-Borate EDTA, pH 8.0) buffer and heated in a microwave. The agarose solution was allowed to cool, ethidium bromide was added to a final concentration of 0.5μg/ml and the gel was cast (Sambrook *et al.*, 1989) DNA samples in agarose gel loading buffer were loaded and electrophoresed at ~5V/cm with 1 x TBE pH 8.0 as running buffer as previously described (Sambrook *et al.*, 1989).

Alkaline transfer

The gel was depurinated in 0.25M hydrochloric acid for not more than 15 minutes. It was then neutralised by rinsing twice in 0.4M NaOH for 5 minutes and alkaline blotted. Southern transfer was established with prewetted Hybond N+(Amersham) in 0.4M NaOH overnight. The following day the membrane was rinsed in 2 x SSC and allowed to air dry.

Membranes were prehybridised using Church conditions for 30 minutes before hybridising at 50^{o}C to the labelled probes using a standard method (Church and Gilbert 1984). After high stringency washing, the membranes were exposed to film (Kodar XAR5) for up to 7 days at -70^{o}C.

Slot blotting

Once backchecking of Southern hybridisation had been performed slot blotting was established using a modified alkaline blotting method for Southern analysis. After blocking the slot-blot apparatus with nonhomologous DNA (100-200μg/ml Herring Sperm DNA) the tailsnip DNA was dissolved to a final concentration of 0.4M NaOH and then loaded with 5μl of DNA per slot. The wells were then rinsed with 0.4MNaOH. The membrane was crosslinked in a UV crosslinker then neutralised with 5x SSC for 5 minutes twice. The membrane was placed DNA side facing upwards in 0.4M NaOH for 20 mins. Finally the mebrane was rinsed in 2xSSC for 5 minutes twice. Thereafter prehybridisation and hybridisation were performed using "Church conditions". and the same probe as describedpreviously.

Molecular Biology Techniques - RNA

Preparation of RNA from tumour samples and organs

Solid tumour specimens and organs were removed from the transgenic mice and snap frozen in liquid nitrogen, LN_2,. For RNA preparation, solid tumours were homogenised using a Ultraturrax T25 (Janke and Kunkel, Staufen, Germany) and transferred into a guanidinium thiocyanate (GIT) based lysis buffer. Cell lines were lysed by the addition of 5M GIT buffer.

RNA extraction

Total cellular RNA was isolated using one of two methods as described below. The tumours and organswere thawed and homogenised. For large quantities of tissue, total RNA was isolated after centrifugation through caesium chloride followed by precipitation with 3M sodium acetate and ethanol as described by Chirgwin *et al* (Chirgwin *et al.*, 1979). Alternatively, the method of Chomczynski and Sacchi 1987 was followed (Chomczynski & Sacchi, 1987). Tumour tissue was homogenised in denaturing solution D (4M guanidium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl 0.1M β-mercaptoethanol). One ml of solution D was used for each 100mg of tissue 0.1ml of 2M sodium acetate, pH 4, 1ml of phenol (water saturated) and 0.2ml of chloroform/isoamyl alcohol (49:1) was added to 1ml of homogenate. Following mixing and cooling for 15 mins on ice, the samples were centrifuged at 14,000 rpm for 20 mins. The aqueous phase was removed and mixed with an equal volume of isopropanol for 1 h at 4°C to precipitate

the RNA. Following a further centrifugation the pellet was dissolved in 4M LiCl and centrifuged to pellet the insoluble RNA. The pellet was subsequently dissolved in 0.2ml of 10mM Tris pH 7.5, 1mM EDTA, 0.5% SDS, chloroform was added (0.2 ml) and the upper phase collected after centrifugation and precipitated with an equal volume of isopropanol in 0.2M sodium acetate pH5.0. The pellet was air dried and resuspended in diethylpyrocarbonate (DEPC) treated water buffer. The RNA quantity and purity was obtained by spectrophotometry.

Northern blotting

Northern blotting analysis was carried out following standard protocols. 15μg total cellular RNA was electrophoresed through a 1.4% agarose-formaldehyde denaturing gel and capillary blotted onto 'Biodyne A' membranes (Pall Ultrafine Filtration Corp, Glen Cove, New York, USA). cDNA was labelled with ^{32}P-dCTP by a random priming method(Feinberg & Vogelstein, 1984) . Membranes were hybridised to the ^{32}P-labelled inserts of human cDNA probes under standard conditions as outlined by Church & Gilbert, 1984 (Church & Gilbert, 1984). Membranes were subsequently washed to high-stringency and exposed to Kodak XAR5 film at -70°C with 2 intensifying screens (Dupont, Stevenage, U.K.).

cDNA Probes

β-actin

This was provided by Dr L. Kedes (Stanford). The 0.7kb EcoR1-Hind III fragment of pβact Bluescript was labelled by random priming.

c-neu

This was provided by Professor Paul Jolicouer (Montreal). The HindIII-SalI 4.6kB fragment was labelled by random priming.

Cytokines

All cytokines except IFN-γ and IL-12 cytokines were diluted in phosphate buffered saline plus 3 mg/ml bovine serum albumin (Sigma, Dorset, United Kingdom) and stored in single dose aliquots at -70°C until required. IFN-γ and IL-12 were reconstituted in

sterile water. Mice were given 0.1 ml injections of the cytokine or the same volume of diluent. All cytokines were stored in single dose aliquots at -70°C until required.

Interferon-α

Recombinant human interferon-αA/D (IFN-α) is a hybrid molecule produced by joining the amino terminal segment (amino acids 1-62) of rHuIFN-αA to the carboxyterminal segment (amino acids 64-166) of rHuIFN-αD at the Bgl 1 site (Rehberg *et al.*, 1982). It was used in these experiments because rmIFN-α was not available in large quantities. IFN-α was obtained from Dr. M. Brunda, Hoffman La Roche, Nutley, NJ. It was more than 99% pure with a specific activity of $2x10^8$units/mg protein.

Interferon-γ

Recombinant rat interferon gamma (IFN-γ) with an extra methionine residue at the N-terminus of the natural sequence molecule was used. This was obtained from Dr Daniel Lando, Roussel-Uclaf, Romainville, France. It was at least 95% pure with a specific activity of 1×10^7 units/mg protein.

Interleukin-2

Human recombinant IL-2 was obtained from Dr Daniel Lando, Roussel-Uclaf, Romainville, France. It was at least 95% pure with a specific activity of 1×10^7 units/mg protein.

Interleukin-7

Recombinant human IL-7 was a kind gift of Dr Connie Faltynek, Sterling Drug Inc., Malvern, Philadelphia, having been manufactured and purified by Immunex Corporation. The purity was not confirmed but specific activity was 4.0×10^7 units/mg protein.

Interleukin-12

Recombinant murine IL-12 was the kind gift of Dr Brunda and was used at a dose of 1μg per animal per day. It had a bioactivity of 2.3×10^8 Roche units/mg.

Control diluent

Control diluent comprised 3mg/ml of bovine serum albumen (BSA).

Histological techniques

Morphological analysis

The animals were inspected for general condition and tumours at least twice a week. If the animals became unwell, or tumours ulcerated or approached 2cm in diameter, they were sacrificed and a post-mortem examination performed. In most cases only one tumour was evident at this point. Tumor tissue, lungs, liver and spleen were fixed in neutral buffered formalin (NBF) and embedded in paraffin wax. Parallel samples were also snap frozen. In a subset, the skeleton was also routinely examined, to document the distribution of occult metastases.

Sections were cut and stained with haematoxylin and eosin (H & E) and parallel sections cut for immunohistochemistry and flow cytometric analysis (see below).

Immunohistochemistry

Processing of tissues

Frozen sections were prepared from tissue samples snap frozen in liquid nitrogen, mounted in OCT compound (Tissuetak, USA), and 6mm sections cut in a cryostat (Cryostat E, Reichert Jung, FGR). The sections were stored at -20^{o}C until used.

Classification of lymphomas in mice is largely based on immunohistochemistry or FACS analysis as well as morphological characteristics. Immunohistochemistry was performed using two B cell markers, surface IgG and B220; and two T cell markers α/β TCR and Thy 1.2 (figure 4.8). The manufacturers instructions for the antibodies α/β TCR, surface IgG and B220 immunostaining suggested the use of frozen tissue sections. This was adapted for paraffin sections by prior microwaving of the sections followed by rapid cooling to avoid deleterious drying. This worked effectively and enabled the tissue architecture to be better preserved.

Parrafin embedded sections were prepared from samples fixed in neutral buffered formalin for at least 24 hours. The tissues were dehydrated through graded alcohols, cleared with CNP 30 (Pentone Chemicals, UK) and embedded in paraffin wax. 5mm

sections were then cut using a rotary microtome (American Optical, USA) and stored at room temperature. Sections were immunostained with an antibody to human c-*erb* B2 (1/50 dilution) (Dakopatts, Denmark) and in the case of lymphomas, the murine T/B lineage antibodies to α/βTCR(1/1000 diluton) (Pharmingen, USA), B220(1/300 dilution) (Pharmingen, USA), Surface Ig(1/25 dilution) (Sigma Chemical Co., USA) and Thy 1.2(1/100 dilution) (Becton-Dickinson, USA). These antibodies were employed in conjunction with a standard streptavidin-biotin technique. A brown reaction product was obtained using a peroxidase substrate (diaminobenzidine, PBS, 0.3% hydrogen peroxide). All antibodies except Thy 1.2 worked well and appropriately on formalin fixed material after prior microwaving of the sections. Unstained sections were immersed in 0.01M sodium citrate buffer solution at pH 6 in which they were microwaved at 700 watts for 10 minutes with rapid cooling by running water thereafter to avoid deleterious drying. The antibody to Thy 1.2 worked without microwaving sections. All histopathology assessment was performed by a consultant pathologist with an interest in breast cancer (A.M.Hanby).

Immunohistochemistry controls

A human mammary carcinoma known to be positive for *c-erb*B-2 was used as a positive control for the c-*erb*B2 antibody. Mouse lymph node and tonsillar tissue, in which there are distinct patterns of T and B lymphocyte localisation, acted as both positive and negative controls for the T and B lineage markers.

Tumor growth and flow cytometric analysis

Flow cytometry was performed on nuclear suspensions prepared from formalin fixed paraffin embedded sections as described elsewhere (Camplejohn *et al.*, 1989). Three 50μm paraffin sections were dewaxed and rehydrated through a series of alcohols into double distilled water. Nuclei were extracted by the addition of pepsin (5mg/ml) at 37°C for 30 min at pH 1.5. Following filtration through a 35mm pore size nylon filter and incubation with with propidium iodide 250mg/ml, the samples were analysed using a Becton-Dickinson FACS Analyser powered by a mercury arc lamp. 10^5 particles were scanned to to construct a DNA histogram. The DNA index was calculatedby relating DNA content of the aneuploid G0/G1 peak to that for the diploid G0/G1 peak. The S-phase fraction (SPF) for the diploid tumours was measured using the method of Baisch et al (1975) (Baisch *et al.*, 1975). The number of cells in S-phase was calculated from a rectangle fitted between the peak channels of the G0/G1 and G2/M peaks. For the DNA

aneuploid histogram, the percentage of aneuploid S-phase cells as a percentage of total aneuploid cells was estimated in a similar way (Camplejohn *et al.*, 1989).

Zymographic analysis

Using the technique of zymography small amounts of homogenised tissue are run on polyacrylamide gels impregnanted with gelatin. Collagenase in the tissue digests the gelatin, leaving a clear band after the gel is stained for protein. The technique can distinguish between the 92 and 72kDa enzymes and between the inactive and active forms of the 72 kDa enzyme, because SDS causes enzyme activation. Using a modification of the method of Heussen and Dowdle (Heussen & Dowdle, 1980), similar to that used by Unemori and Werb (Unemori & Werb, 1988) the technique was adapted for densitometric analysis using computer assisted image analysis (Davies *et al.*, 1993). This study in breast tissue showed a clear relationship between production of type IV collagenases and increasing tumour grade in malignant breast disease.

Tissue Samples

Tumours removed from mice were cryopreserved in liquid nitrogen. A 5 µm thick section was cut from a face area of each tumour. Samples were homogenised in 50 µl of SDS-PAGE sample buffer containing glycerol (10%v/v) SDS (1%w/v) and bromophenol blue using treff microhomogenisers (Scott Lab). Adjacent sections were cut and used for protein estimation.

Gelatin Zymography

Gelatinolytic zymography was performed as described by Brown et al., 1990 (Brown *et al.*, 1990). This technique can distinguish between the 72 and 92KDa type IV collagenases. Additionally, the method can detect the inactive proforms of collagenases because SDS causes activation without changing the molecular weight of the enzymes (Birkedal Hansen & Taylor, 1982). Homogenised samples (50 µL) were applied directly without heating or reduction to a 5% w/v stacking polyacrylamide gel laid over an 11% (w/v) polyacrylamide gel containing 1mg/ml gelatin and 0.1 % (w/v) SDS. Gels were run at room temp at 180 V. After incubation of gels in 2.5 % Triton X-100 for 30 min to remove SDS, the gels were incubated for 16 h in 50mM Tris-HCl, pH 7.6 containing 0.2M NaCl, 5mM CaCl2 and 0.02 w/v Brij-35. Gels were stained for 3h in 30 % methanol/10 %

glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G 250 and destained in the same solution in the absence of dye.

Controls for zymography

Conditioned media from human melanoma RPMI 7951 cells and from TPA stimulated HT1080 fibrosarcoma cells were used as type IV collagenase standards (Brown *et al.*, 1990; Weinberg *et al.*, 1990).

Statistical analysis

The Fisher's exact test was used to compare proportions of tumours between different groups in Chapter 3 where the groups were small. The Wilcoxon matched pairs signed rank test was used to compare passage times between transplants in Chapter 5. The tests used were on the advice of the ICRF Medical Statistics Unit, Oxford. The logrank test was used for survival analysis and was carried out by Joanna Hadley and Sharon Love at the ICRF Medical Statistics Unit, Oxford.

Chapter Three

The Natural History of the Model

Background and Rationale

Over the past forty years a variety of animal models have been used in the development of anticancer therapies (Geran *et al.*, 1974)., Initially syngeneic transplantable murine leukaemias and solid tumours were used and more recently, human tumour xenografts growing in nude mice (Curt, 1994; Mattern *et al.*, 1988). These models have not proved completely reliable in predicting the efficacy of novel therapies in human disease, particularly solid tumours (Fiebig *et al.*, 1984; Grindey, 1990). There is a need for models which can predict the efficacy of new therapies more accurately.

The aim of this study was to develop an animal model of breast cancer for use in preclinical assessment of cancer therapy, in particular cytokine therapy. In the past decade the availability of an increasing number of cytokines for therapeutic use has resulted in a new mode of cancer therapy. Nevertheless clinical trial results have been varied and the number of patients for whom cytokine therapy is appropriate remains proportionately very small. With our greater understanding of the molecular biology of cancer, the need for data from animal models to predict efficacy, toxicity and mechanisms of these and other novel treatments, is greater than ever (Thomas & Balkwill, 1994) .

Existing murine models of mammary cancer

Spontaneous models

The majority of spontaneous tumours arising in mice are related to endogenous or vertically transmitted murine mammary tumour virus (MMTV). These spontaneous mammary tumours arise in inbred mice. They tend to be adenocarcinomas and can be modulated by a number of factors including chemical carcinogens, irradiation, hormones, genetic background, diet and the immune status of the host.

Spontaneous tumours metastasise with a frequency which depends upon the strain (Consolandi *et al.*, 1958); Anderson, 1974 #235, type of MMTV (Squartini & Bistocchi, 1977) amongst other factors (Liebelt *et al.*, 1968). Cross-fostering BALB/c mice offspring onto C3H mice infected with MMTV at birth, leads to a high incidence of

spontaneous mammary tumours. The incidence of lung metastases in these cross-fostered mice is 9.5% (Frith *et al.*, 1981). One study involving several different strains showed that metastasis was increased by number of pregnancies, administration of oestrogen, pituitary isografts and tumourectomy. Incidence did not relate to tumour size, location, histological type or latent period of tumour growth (Liebelt *et al.*, 1968). One disadvanatage extrapolating from MMTV induced mammary tumours to human studies is that most tumours arising in the MMTV model do not resemble human tumours either morphologically (see Chapter 4) or biologically.

A further limitation of the MMTV model is that several cytokines possess antiviral activity (Dianzani, 1992; Dorr, 1993) thus any effect may be difficult to interpret. Thirty years ago mice were treated with the antiviral protein interferon (IFN) which not only produced a reduction in the incidence of virus-induced tumours but also slowed the development of transplantable tumours (Burke, 1979; reviewed in Gresser, 1991) (Burke, 1979; Gresser, 1991).

Transplantable models

Transplantable models of breast cancer are readily available, their growth is reproducible and their use is well documented. A wide range of histological types can be assessed and tumour/ host relationships can be studied. In addition they are useful as models of metastases. Transplantable models also have a number of disadvantages. Those tumours which arise may be immunogenic and selected by successive passages. Multiple passages may result in changing histological characteristics unrepresentative of the original tumour. Such tumours are often rapidly growing and anaplastic. They may not develop a host-tumour relationship, which is being found to be increasingly relevant in the biology of human cancer. Transplantable models also lack the diversity of transgenic models and the histological resemblance to human tumours. Finally, only a relatively small number of transplantable mammary tumour models are available.

Nude mouse and human tumour xenografts

In relation to the *in vivo* testing of cytokine therapy the human tumour xenograft system has both advantages and disadvantages. One advantage is that the histology and ultrastructure of the human counterpart is preserved in the mouse, as well as chromosome number, DNA content, tumour markers and hormone secretion (Fiebig *et al.*, 1984; Mattern *et al.*, 1988). Using the nude mouse model the direct actions of species specific cytokines can be determined as the cytokines will either act directly on human tumour cells or on murine host derived stroma.

The drawbacks of this model include the fact that the 'take rate' of the original tumour to be xenografted is variable and generally low (Fidler, 1986). The disadvantages for assessing cytokine therapy *in vivo* lie in the fact that the nude mouse does not have a competent immune system, and with some cytokine therapies, such as IL-2, a xenogeneic response against the tumour may be induced. In addition there are only three mammary xenograft models involving tumours of epithelial origin which are known to metastasise. These are the MDA MB human breast cancer sublines 231, 435 and 453 (Chetrite, 1993; Leone *et al.*, 1993). The SCID mouse is an alternative model for xenotransplantation and comparative studies show a relatively higher take rate in scid mice (Bosma *et al.*, 1983).

Carcinogen-induced tumours

Some murine and rat models have been developed by treating strains with a low tumour incidence with a variety of mutagens to induce tumours. The tumours which arise may be highly immunogenic and hence not suitable for assessing cytokine therapy.

Models of metastases

i) Spontaneous

Here transplantable tumour cells are injected locally and the animal is observed for progression of metastases. The primary tumour may be excised to prolong survival of the mouse or excision may enhance metastasis (O'Reilly *et al.*, 1995). The advantages of this model are that a wide range of histological types are available and they resemble metastatic tumours in the clinical situation. The disadvantages are that the tumours are derived from small numbers of cells, are highly anaplastic and often immunogenic in nature.

ii) Experimental

Here a single cell suspension of tumour cells is injected into the mouse (usually via the tail vein). After an interval the number of metastatic deposits is counted. The deposits themselves usually develop in a specific site and are useful in the study of the metastatic process without the delay necessitated in the spontaneous model. However the metastases arise after intravenous injection and then entrapment - they are consequently not an optimum model for the mechanism of metastases in humans where cells are shed into the lymphatics or bloodstream from the primary site.

Oncogene Transgenic Mice

To address questions about the causal association between expression of a gene in a particular tissue and tumour progression, oncogene transgenic mice have been developed. There is a wide range of transgenic mouse models of malignancy. These mice express an oncogene under the control of a specific promoter and develop spontaneous tumours of diverse histological types (Adams & Cory, 1991; Jaenisch, 1988; Jenkins & Copeland, 1989). They have provided some important insights to the molecular mechanisms of tumourigenesis, particularly oncogene cooperativity, but there is little published information about their use for the assessment of anticancer therapy (Dexter *et al.*, 1993; Katsumata *et al.*, 1995; Kohl *et al.*, 1995; Nielsen *et al.*, 1992); Oncogene transgenic mice have some advantages over existing cancer models, particularly with regard to possession of an intact immune system, the existence of tumour stroma relationships analogous to those in humans and the slower evolution of the tumours than in other models.

The majority of founder transgenic oncogene mice are bred as F1 hybrids, but for models involving cytokine therapy an inbred strain is required. In some cases this has been overcome by microinjecting the transgene into an inbred mouse strain, FVB, where the cellular characteristics make microinjection of the pronucleii more straightforward than in other inbred strains (See Discussion section) (Taketo *et al.*, 1991). For this study the BALB/c background was selected for its susceptibility to the effects of the mouse mammary tumour virus. One hundred percent of BALB/c mice cross-fostered onto MMTV-infected C3H mice will develop mammary tumours.

Mammary tumour transgenics

The promoter

Particular promoters are used in transgenic models to direct and modulate expression of the gene of interest. Only a few are known to function preferentially in mammary tissue and promoter control elements help to restrict the expression of transgenes to selected cell populations.

Targeted expression of oncogenes to the mammary epithelium has been achieved by linking the oncogene of interest to a mammary specific transcriptional element such as the MMTV Long Terminal Repeat (LTR), Whey Acidic Protein gene (WAP) promoter and β-casein genes. These promoters direct transgene expression in mammary epithelium (Muller, 1991). Nevertheless they differ greatly in their level of expression during pregnancy and lactation with respect to the timing and response to lactogenic stimuli. WAP transcription is very low in the developing mammary gland

but is induced several thousandfold at lactation. Consequently many of the tumours or hyperplasias developed in mice involving the WAP promoter are lactation-dependent.

The MMTV-LTR contains one of the most widely studied mammary 'restricted' promoters. Essential for proviral expression, the LTR is also responsible for the activation of the *int* genes, whose name derives from their proximity to the integration site of the provirus. The MMTV-LTR is also transcriptionally active throughout all stages of mammary gland development and its activity is further induced during pregnancy (Pattengale *et al.*, 1989). Although proviral MMTV is expressed predominantly in the lactating mammary gland, LTR-based transgene constructs have been found to be expressed in mammary gland, salivary gland, lung, thymus, spleen and testes of transgenic mice (Cho *et al.*, 1989; Leder *et al.*, 1986; Ross & Solter, 1985; Stewart *et al.*, 1984);. A problem in generating transgenic mice with such constructs is that expression in the testis often results in sterile males. Restriction to one tissue type or even cell type is rare. The problem is overcome in a few transgenic models by having a promoter with greater tissue specificity, for example the tyrosinase promoter in melanoma, which is relatively specific for melanocytes.

The transgene

The *c-neu* proto-oncogene (rat homologue of the human c-*erb*B2 oncogene) is a membrane bound 185kDa receptor molecule with tyrosine kinase activity. It shares partial homology with the epidermal growth factor receptor and its role in mammary cancer has been extensively investigated (Slamon *et al.*, 1987). In a chemically transformed neuroblastoma cell line, rat c-*neu* is activated by a point mutation which results in a single amino-acid substitution (valine to glutamic acid) in the transmembrane domain of the protein (Bargmann *et al.*, 1986a; Bargmann *et al.*, 1986b). This results in constitutive activation of its intrinsic tyrosine kinase function (Press *et al.*, 1990). Hence, in the apparent absence of ligand constitutive levels of tyrosine phosphorylation are seen and a constitutive mitogenic signal is applied. The mutant *neu* gene, but not the normal *neu* gene, can transform NIH3T3 cells (Bargmann *et al.*, 1986b). Unlike rodent neoplasia spontaneous human neoplasia involving *erb*-B2 appears to involve gene amplification and/ or overexpression of structurally intact transcripts and proteins (determined by northern blot or immunocytochemistry) and not activation. Substitution of the corresponding amino-acid in human c-*erb*B2 protein would require two mutations in the gene. The human c-*erb*B2 gene can transform fibroblasts by overexpression (Di Fiore *et al.*, 1987a). Overexpression of c-*erb*B2 mRNA and protein has been demonstrated in human breast cancer using northern analysis and immunohistochemistry of tumour biopsies and cell lines, respectively (Slamon *et al.*,

1987; van de Vijver *et al.*, 1987; Yokota *et al.*, 1986);. Overexpression usually occurs as a result of gene amplification but increased transcript levels have also been found in the absence of amplification.

At the time of embarking on this study two transgenic mouse models of breast cancer involving the activated c-*neu* oncogene were described(Bouchard *et al.*, 1989; Muller *et al.*, 1988) (see Introductory Chapter). As described above the oncogene was constitutively activated in both of these (Press *et al.*, 1990)).

Choice of founder mice

The aim of this study was to establish an inbred model of mammary cancer in which tumours developed thet bore histological resemblance to human mammary cancers, arose stochastically and metastasised in a pattern comparable to the human disease. The most expedient way of establishing such a model of known characteristics was to obtain founder mice. After a careful search of the available literature at the time (1990-91) it was felt that the model of Bouchard *et al* was the most suitable for this study. This was based on the relatively late onset of the tumours, allowing time for treatment, their monoclonal origin and, importantly, their close histological resemblance to human tumours. Other models in which mice develop pregnancy dependent hyperplasias and tumours, such as *int*-2 (*wnt*-2) mice, or where all the mammary glands develop tumours at an early stage, would have been less suitable for cytokine studies(Ornitz *et al.*, 1992; Stamp *et al.*, 1992). Professor Paul Jolicoeur kindly provided three founder male mice from the line with highest incidence of tumours as the basis for the study. The BALB/c background was selected for its susceptibility to the effects of the mouse mammary tumour virus.

Experience of Jolicouer's group with founder mice

The founder mice of the colony described in this thesis were reported to develop comedo-type metastatic breast tumours, in a stochastic fashion, at 7-14 months of age (Bouchard *et al.*, 1989). The overall mammary tumour incidence, in four separate lines, was 53/127 female mice at 10 months of age. Affected mice generally had multiple tumours of different size arising independently and asynchronously. Although established tumours continued to grow in the absence of further pregnancy, no tumours were seen in 20 virgin mice kept for over one year (Jolicouer, personal communication). The tumours that developed in the mated mice were poorly differentiated adenocarcinomas of the mammary gland with intratumour necrosis and calcification. In some areas papillary formations were occasionally seen. The tumours also gave rise to lung metastases and all eleven tested could be transplanted in nude mice, underlining

their malignant potential. The histology paralleled the pattern seen in comedo-type ductal carcinoma in humans, a histological type of tumour associated with amplification of unactivated c-*erb B2* (Bartkova *et al.*, 1990).

In addition to the mammary tumours described, salivary and Harderian gland tumours (the Harderian gland is a modified sebaceous gland found deep in the orbit), enlarged or abnormal seminal vesicles, enlarged epididymis and splenomegaly were also observed. In one of the four lines of mice, MN-10, five of 81 mice developed unilateral proptosis (protruberance of eye). On dissection this was found to be a Harderian gland tumour with severe bilateral hyperplasia, dysplasia of the epithelium and possible malignant transformation. Unilateral proptosis was noted in five of 81 male and female mice of this line (Bouchard *et al.*, 1989). In this same line two female mice developed salivary gland tumours. Males became infertile by 3-4 months of age.

Bouchard *et al* investigated transgene expression in undiseased mice from their best characterised line, MN-9, and found it to be high in lactating mammary glands of females and the epididymis of males. Low but detectable levels were found in seminal vesicles of males and muscles of females. In two other lines, MN-12 and MN-10 high transgene expression was observed in salivary and Harderian glands, respectively (Bouchard *et al.*, 1989).

This chapter details our experience in establishing a colony of mice backcrossed onto BALB/c mice and the characteristics and phenotype of that colony.

Results

Screening for the transgene

Screening for the transgene was initially performed by Southern blotting of tail DNA. The MMTV/*neu* fusion gene was obtained in the plasmid vector pJRD184 from Professor Jolicoeur. A map of the transgene is shown in Figure 3.1.

The 4.6kB Hind III-Sal I fragment of this construct was used as a probe in Southern analysis (Figure 3.1) (See Chapter 2). It did not hybridise to tailsnip DNA from transgene negative C57 Bl/6 and BALB/c mice (see Chapter 2). Initially DNA was digested with the restriction enzyme Eco RI for Southern analysis, but the probe detected a band of high molecular weight (>10kB) near the top of the gel such that the possibility of non-specific binding could not be excluded (Figure 3.2(a)). Hind III digestion of the DNA yielded a lower molecular weight band (Figure 3.2 (b)). Thereafter screening was performed using this enzyme.

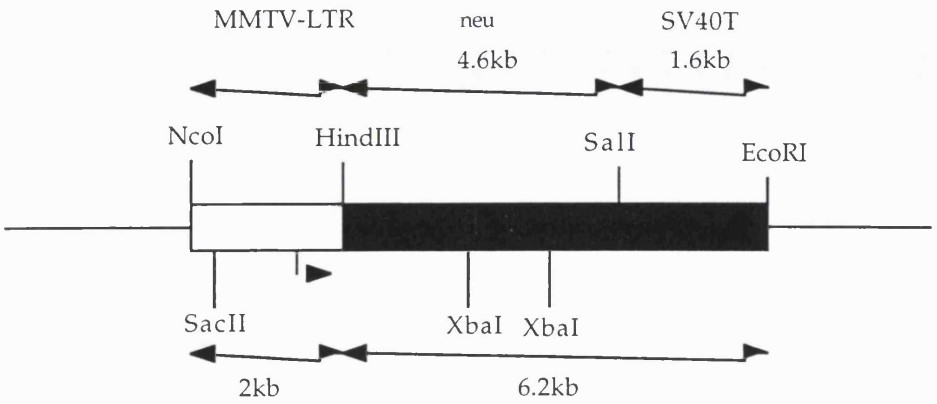


Figure 3.1. Restriction map of MMTV neuT fusion gene, adapted from Bouchard *et al*, Cell 57, 931 - 936,1989.

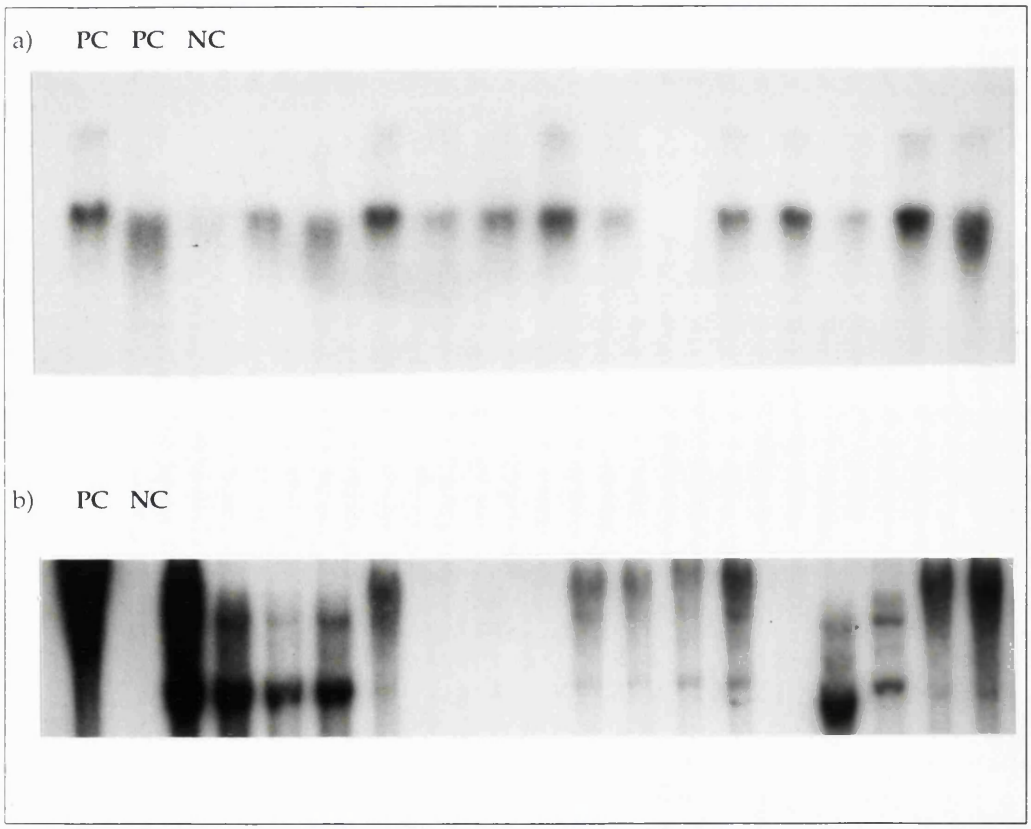


Figure 3.2 A Southern blot of DNA a) digested with ECoR1 b) digested with HindIII **PC** - Positive control **NC** - Negative control

Once screening by Southern analysis was working efficiently, a slot-blotting technique was developed (see Chapter 2). Tailsnip DNA from six mice from the colony were run alongside unscreened DNA on each occasion. Three of each were previously established as transgene positive and negative. An early slot blot shows positive and negative controls at serial dilutions in which the amount loaded was based on

spectrophotometric readings and other known positives and negatives (Figure 3.3). Once the sensitivity of slot-blotting was established 15µl of tailsnip DNA was loaded routinely for each sample. Measurements are given in µl and not µg of DNA as densitometric analysis on genomic DNA is not reliable. However as is illustrated in Figure 3.3 DNA yields from mouse tail preparations are remarkably consistent.

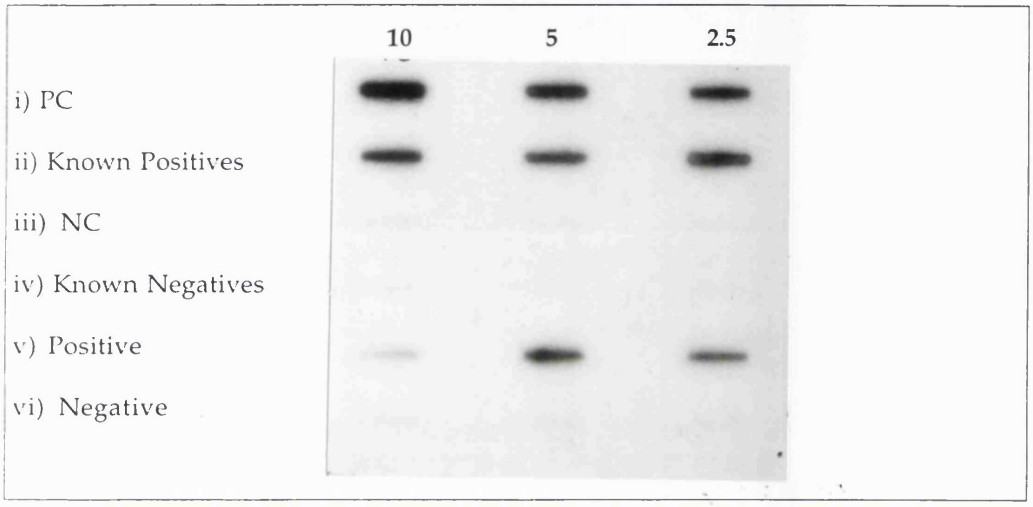


Figure 3.3 Slot blot illustrating positive and negative controls and samples. The numbers 10, 5, 2.5 above blot relate to the volumes (in µl), of positive and negative control genomic DNA used in rows i) and iii) and the known negative in row vi).; In row v) 2.5, 7.5 and 4.0 µl of DNA from a known positive mouse were used. 5µl of DNA was used in all wells in rows ii) and iv) - three known positive and negative mice in each .

mRNA expression of the transgene

Transgene mRNA expression was investigated in three livers, and in spleen, lung, thymus, brain, ovary, non-lactating mammary gland and salivary gland from two different animals as well as one lymph node, an angioma and an angiosarcoma from female mice. Transgene mRNA expression could not be detected in any of the normal tissues by northern analysis. High levels of expression were found in both mammary tumour and Harderian gland tumours. No detectable transgene expression was seen in an angiosarcoma (Figure 3.4).

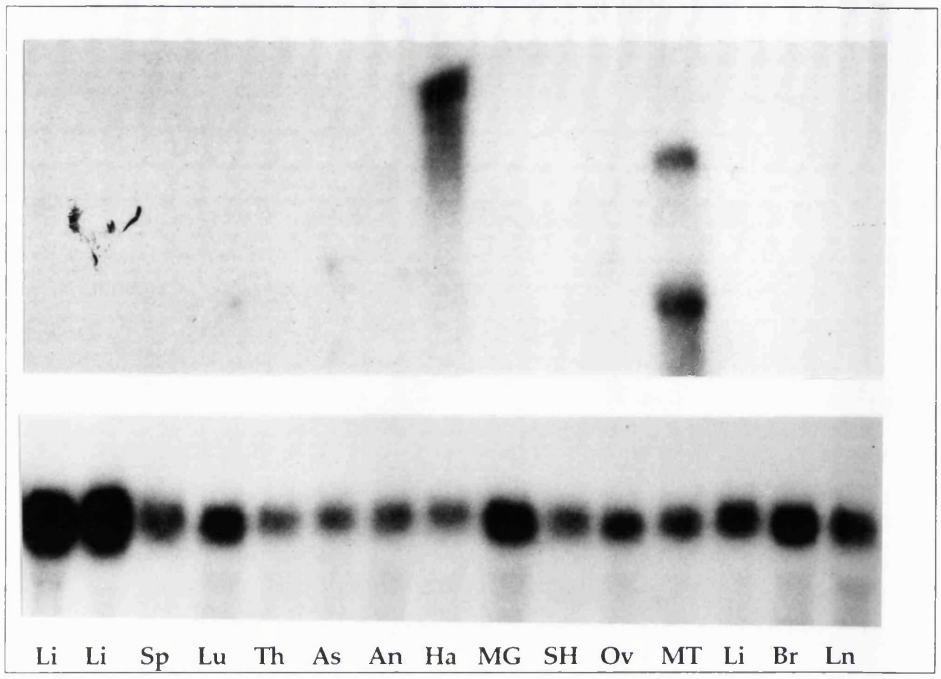


Figure 3.4 Northern blot showing transgene expression and β-actin as control.

Breeding history

To assess cytokine therapy experiments a homogeneous H2 background was required which necessitated an inbred colony. The mice were backcrossed onto a BALB/c background for eight successive generations and in the ninth generation a colony homozygous for the transgene was established. Seven hundred and thirty-eight female mice were bred in the first seven generations, of which 391 were transgene positive (53%). Two hundred and eighteen of the transgene positive females littered at least twice and were thus at risk of tumour development.

Ten mice littered only once and a further 31 did not litter in spite of pairing. Given the latency of tumour development it was only possible to assess this in the first five generations at 25 months.

Tumour development

Eighty-six of 269 transgene positive female mice in the first five generations developed 93 histologically distinct tumors, 83 arising in tissues shown, by Jolicoeur (Bouchard et al, 1990), to overexpress the transgene in some lines. Age of onset of the different tumours is shown graphically in Figure 3.5. Fifty-three breast carcinomas (57%), 24 Harderian gland tumours (26%), 6 lymphomas (6%) and 5 angiosarcomas developed as well as 6 of less common histologic types. This is summarised for the first

five generations in Table 3.1 and Figure 3.6. The column c-*erb*B2 positivity refers to the results of immunohistochemical analysis using an antibody to human c-*erb*B2 which showed cross-reactivity with the rat oncogene. Immunohistochemical analysis showed that the transgene was expressed in the three most common tumour types - mammary carcinomas, lymphomas and Harderian gland carcinomas.

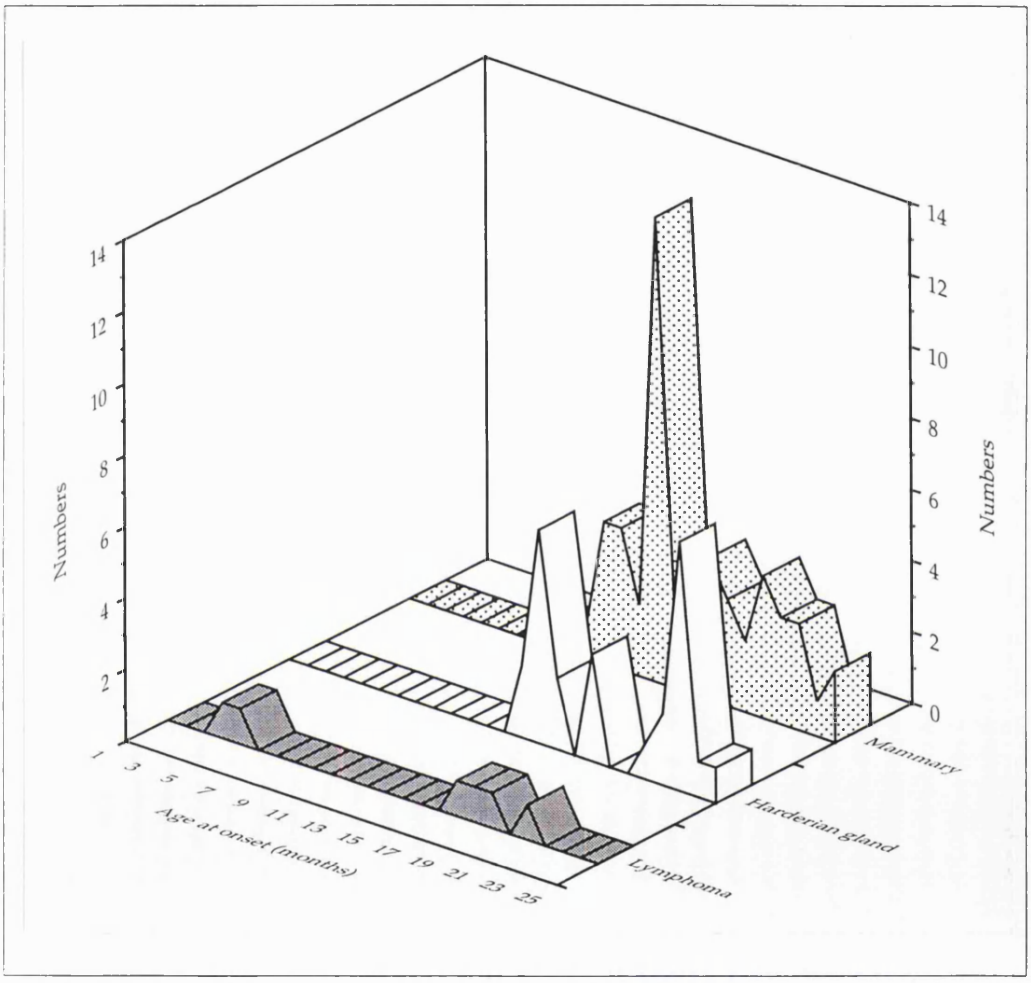


Figure 3.5 Age at onset of different tumours

For the whole colony the tumour incidence was 13.7% at 15 months, with a median age of onset of 18 months. At 24 months the cumulative incidence of breast tumours was 18% with an incidence of 34% for all tumour types (Figure 3.7 and Figure 3.8). Tumour incidence is based on the number of mice succumbing as a result of tumour and not the total tumour numbers.

In the process of investigating the effect of cytokines on tumour development, two large long-term prophylaxis experiments were established in the third and fourth generations, involving treatment with IFN-α and IFN-γ, or IL-2 and IL-7 respectively. Some groups of these mice were administered regular cytokine injections and observed

for tumour development. These mice are not included in this analysis but are considered separately in Chapter 5. Other mice received injections with control diluent. Of the tumours documented here a total of 16 arose in mice who had received injections with control diluent (CD). Comparing the CD-treated and untreated groups of mice from both prophylaxis experiments there was no statistically significant difference between the overall incidence of tumours (Fisher's Exact Test for Prophylaxis experiment 1 $p = 0.5$, for Prophylaxis 2 $p = 0.4$) or the proportion of mammary tumours developed (Fisher's Exact Test for two groups $p = 0.2$). The CD-treated mice have therefore been included in this analysis.

Metastases of tumours

The breast carcinomas and Harderian gland carcinomas metastasised predominantly to the lungs and the lymphomas were widely disseminated at post mortem. Eleven mice were found to have lung metastases from an occult primary at post mortem examination, six of these had Harderian gland carcinomas and five had mammary tumours. As there is a risk of leukaemia development in older mice it is arguable that the six tumours described in untreated mice were unrelated to the presence of the transgene. Against this is the fact that all of the lymphomas stained positively with an antibody to human c-*erb*B2, which is not a characteristic of spontaneous lymphomas in mice, and suggests a role for the transgene.

Tumour type	Number of tumours (%)	Median age of onset (range)	c-*erb*B2 positivity
Mammary carcinoma	53 (57%)	15.0 (10 - 25)	+
Harderian gland carcinoma	24 (26%)	18.0 (14 - 25)	+
Lymphoma	6 (6%)	17.5 (4 - 21)	+
Angiosarcoma	4 (4%)	22.5 (21 - 24)	–
Others	6 (6%)	19.0 (14 - 25)	–

Table 3.1. Different tumor types for the first five generations

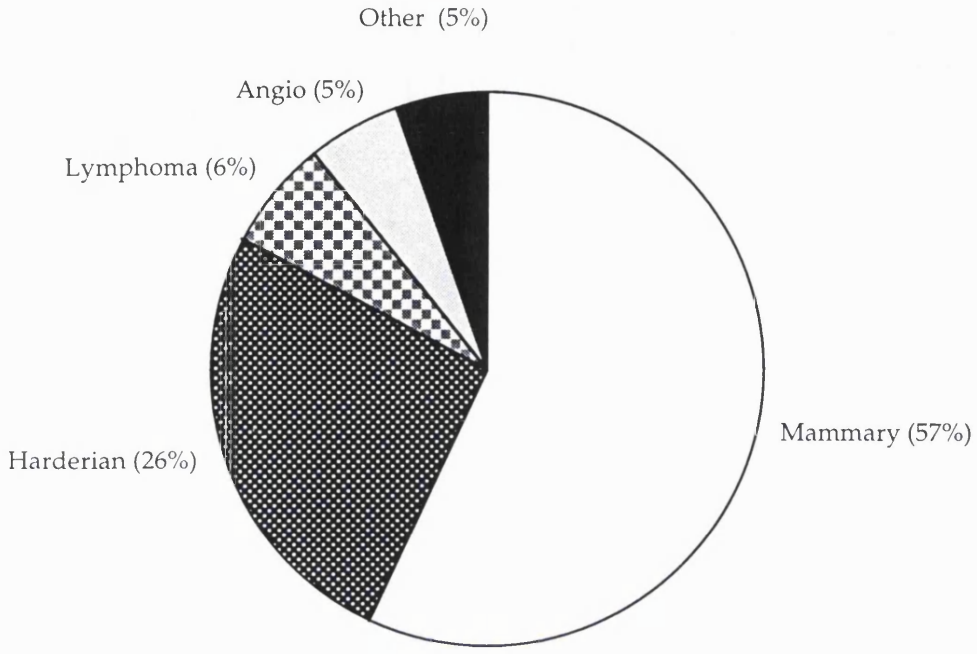


Figure 3.6 Pie chart of all tumours

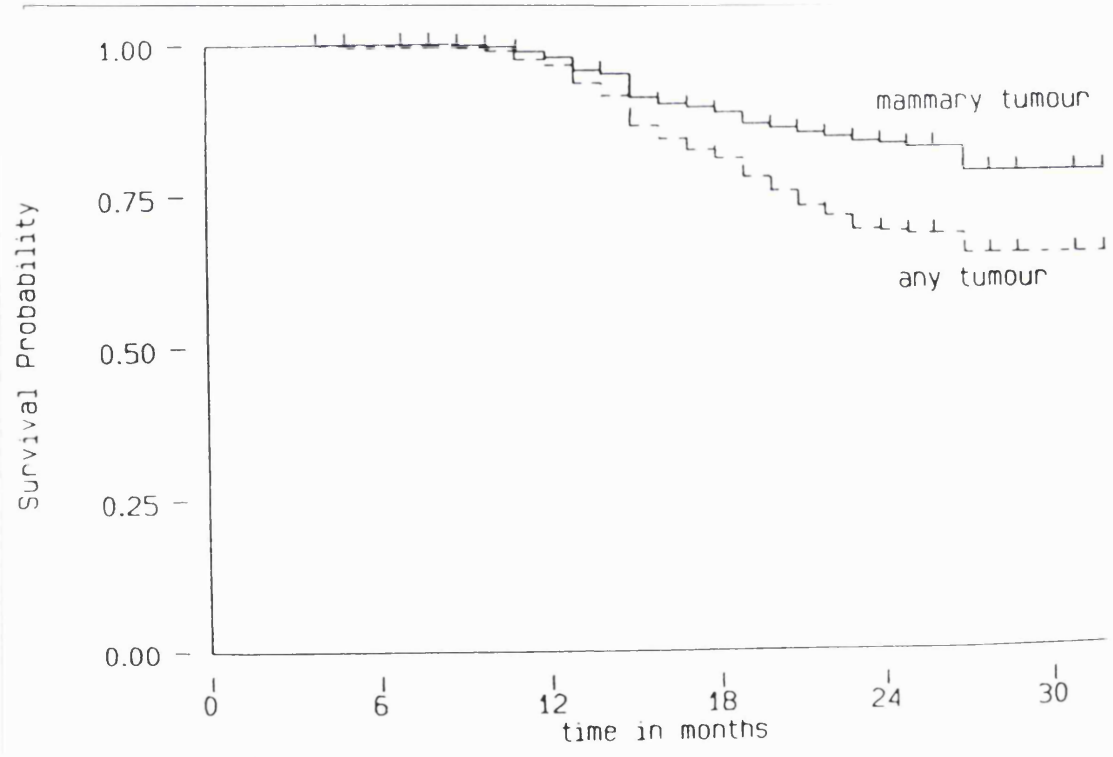


Figure 3.7 Graph of tumour incidence overall and mammary tumour incidence in all mice.

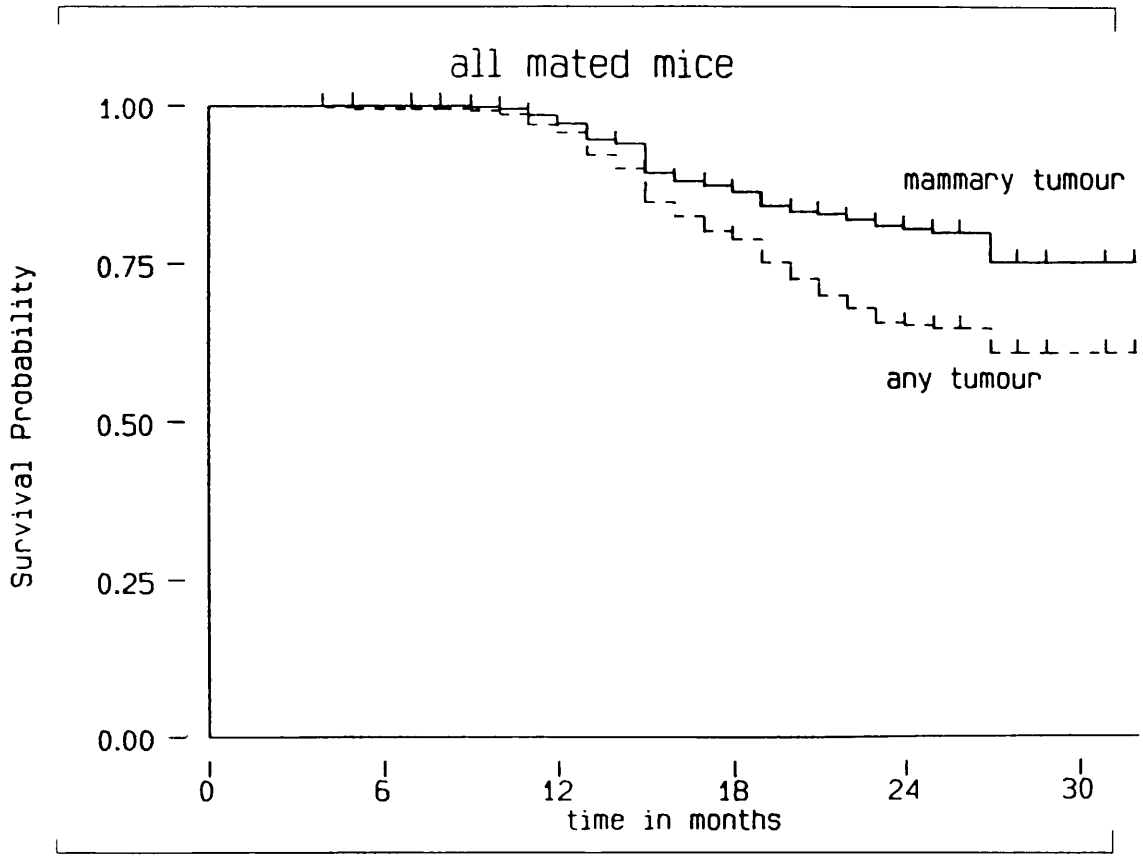


Figure 3.8 Graph of tumour incidence overall and mammary tumour incidence in all mated mice.

In addition to the development of tumours, other effects of the transgene, as previously described in the F1 generation (Bouchard *et al.*, 1989), were observed. Splenomegaly was a common finding. This appeared to manifest as red pulp, with some evidence of extramedullary haemopoiesis. In male mice fertility was not affected so early. Mice continued to breed up until 7 months, as compared with 3-4 months in the F1 generation mice (Bouchard *et al.*, 1989). At post mortem examination male mice were found to have enlarged or abnormal seminal vesicles and enlarged epididymis. Harderian gland hyperplasia was often evident and in two mice lung metastases were found consistent with Harderian gland carcinoma in the presence of an apparently benign tumour of the Harderian gland.

Comparison cannot be made with the natural history of the transgene negative littermates as the numbers of mice entailed in the study necessitated large amounts of shelfspace in a specified pathogen free unit. As a result only a small number of transgene negative mice were kept for particular comparisons such as haematological values.

Inbreeding the colony

Backcrossing onto a BALB/c background was performed for eight successive generations. With successive generations the incidence of mammary tumours declined (Table 3.2). A number of factors may have influenced this observation, in particular genetic background. The founder mice, as in many transgenic models, were hybrid F1 mice of C57Bl/6/C3H background. These are used for their litter size and breeding vigour. The BALB/c background was selected for its susceptibility to the effects of the mouse mammary tumour virus. One hundred percent of BALB/c mice cross-fostered onto MMTV-infected C3H mice will develop mammary tumours. However the BALB/c background may affect susceptibility to the genetic events required for development of spontanoues transgene-related tumours.

FVB mice have been crossed with homozygous mice from the ninth generation of the colony but after two consecutive matings all offspring were transgene negative.

The tumour incidence and characteristics of each generation are outlined in Table 3.2.

Generation	1	2	3	4	5	6	7
Proportion of transgene positive females	3/4(75%)	23/36 (64%)	101/159 (64%)	207/392 (53%)	45/110 (41%)	5/6 (31%)	7/21 (33%)
At risk females*	3	17	101	160	41	5	-
Unmated females	0	6	0	47	0	0	0
Number of tumours	2	13	22	46	3	0	0
Age of generation (m)	23	25	25	24	19	16	13
Median age of tumour development (m) (range)	Too few for anaysis	20 (11-24)	18.5 (4-25)	15.5 (5-23)	Too few for analysis	No tumours to date	No tumours to date
Number of mammary tumours	1	6	15	23	3	0	0
Median age of mammary tumour development (m) (range)	Too few for anaysis	17 (11-20)	15 (10-25)	15 (12-23)	Too few for anaysis	No tumours to date	No tumours to date
Duration of observation (m)	23	up to 26	up to 26	up to 26	up to 23	up to 20	up to 18

Table 3. 2 Characteristics of first seven generations of the colony

Changes in colony with inbreeding

With the increasing extent of inbreeding onto the BALB/c background there appeared to be a gradual change in the proportion of transgene positive offspring (from 64% to 31%), the proportion of mammary tumours developed in transgene positive females and age at their development. (Table 3.2 documents the change in the proportion of transgene positive females with each generation, this is shown graphically in Figure 3.8.). Analysis of the second, third and fourth generation showed a slight decline in the median age of tumour development (17, 15 and 15 months respectively). The median age of onset of tumours in the whole colony was 18 months. The cumulative incidence of breast tumours at 24 months was 18%, the proportion of tumours that were of mammary origin remained the same in successive generations. This compares with a background incidence of 3-5% in retired breeding BALB/c mice(Medina, 1982) .

Although the numbers of mice in early and later generations were small the changes between the generations are highly statistically significant. For all the mice there was a difference in transgene positivity between the generations giving a χ^2 value of 9.097, with 2 degrees of freedom (df), p=0.01. Given that the proportion appears to be declining, on looking for a trend, the Chi squared test for trend value is $\chi^2 = 20.6$, df =1, $p<0.001$. This suggests that there was a difference between the different generations and this difference was progressive, occurring in a particular direction.

In the eighth generation five different mating pairs were established between male and female mice heterozygous for the transgene. Of the 73 offspring from these pairs, 54 were transgene positive (74%) and 19 (26%) transgene negative, close to the expected proportions of 3/4 and 1/4, expected with heterozygote-heterozygote matings. In contrast in matings between heterozygotes and wild-type BALB/c mice there were fewer transgene positive offspring than the expected 50% and some whole litters were transgene negative. This is illustrated graphically in Figure 3.9.

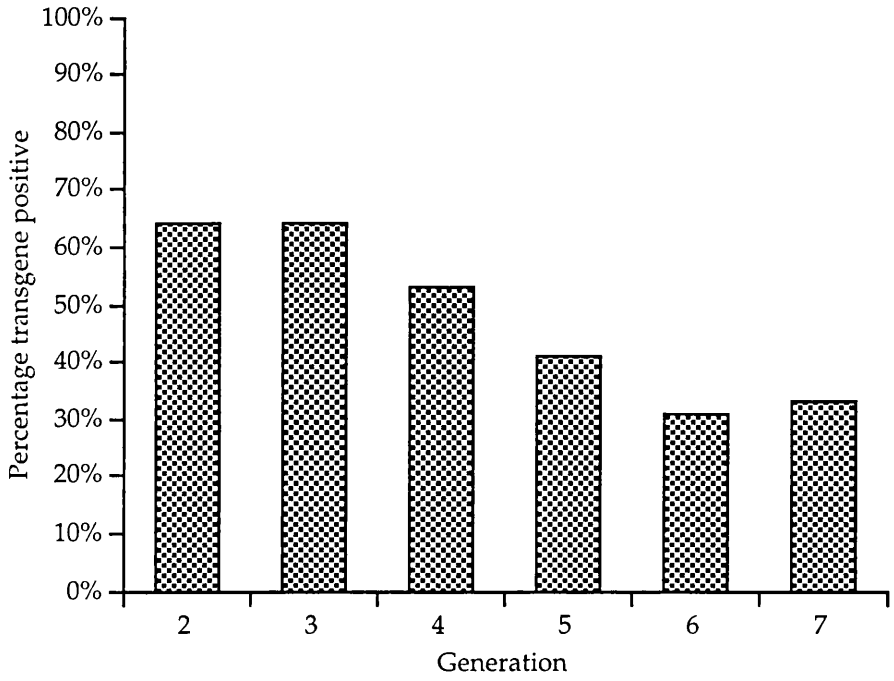


Figure 3.9 Percentage of transgene positivity by generation.

Attempts to increase tumour incidence

Once this colony had been established for about fifteen months, it became apparent that a higher incidence of tumours was required to make the model useful for therapy experiments. A number of manipulations were initiated to increase the incidence of tumours. Some of these are still ongoing.

Homozygotes

A homozygous colony has now been established in the hope of a higher incidence of tumour development. As heterozygosity may be sufficient for the oncogenic effect of the transgene or at least maximal enough to perturb signalling through *neu* a higher incidence of tumours is not inevitable in homozygous mice. Longterm experience of homozygous mice in this colony is based on the offspring of a limited number of heterozygote-heterozygote matings, performed in the second generation. Two homozygous mice were identified when testcrossed with BALB/c males, on the basis of 18 and 16 transgene positive offspring, respectively. Both have developed tumours - one a papillary mammary tumour at thirteen months and the other a lymphoma at 21 months. Age of onset and type of tumours do not suggest a dramatic effect of homozygosity. The main practical advantage of a homozygous colony is that it abrogates the need for screening.

Identification of homozygous mice from Southern blots is only possible using a single copy control and densitometry and involves a large number of variables. Consequently there is a wide potential margin of error. The only failsafe method is test-crossing with transgene negative mice. If at least eight offspring are transgene positive the possibility of heterozygosity is 1 in 256 or less.

Using slot-blotting to determine zygosity

To establish whether slot-blotting could be used to judge zygosity we compared subjective assessment of the density of slot blot wells with test-crossing. This assumes similar DNA yields from tailsnip preparations, similar loading in wells and then homogeneous probe hybridisation. After probing with the gene of interest the blots were probed with a housekeeping gene (β-actin) to confirm this homogeneity. Groups of mice with apparent 'light' and 'dark' wells (greater than two fold difference) on slot blot hybridisation have been set up and test-crossed against transgene negative BALB/c mice. Based on a minimum of 8 offspring all four mice with 'dark' wells who mated are homozygous. The four mice with 'light', but screen-positive wells, are heterozygous on testcrossing (Fisher's exact Test p=0.03).

Homozygous mice were established in the ninth generation and initially did not litter as frequently as when heteozygous mice were crossed with BALB/c mice. However in generations eleven and twelve the litter size and frequency is comparable with that in the earlier heterozygous matings.

The influence of litter number on tumour development

In the first two heterozygous generations, mice were mated freely in order to expand the colony. Thereafter they were routinely mated twice in order to place them at risk of tumour development (Bouchard *et al.*, 1989). In the fourth generation, the effect of litter number was assessed systematically. Two groups - one of virgin mice and one of mice mated four times, were established. The overall difference in tumour development and the difference in mammary tumour incidence between the groups is outlined in Table 3.3 and Figure 3.10. The virgin mice developed fewer mammary tumours (at 5.7% overall), than those having at least two litters (22% overall). Those mice mated only once (only 10 mice) developed no tumours. It appears that the risk of a mammary tumour is not increased further by four litters than two (Table 3.3).

Litter number	Total no. of mice	No. of mice with tumours (%)	Incidence of mammary tumours
Virgin	53	11 (20.8%)	3/53 (5.7%)
One litter	10	1 (10.0%)	0/10 (0.0%)
Two litters	141	62 (44.8%)	34/141 (24.1%)
Three litters	7	3 (42.8%)	2/7 (28.6%)
Four litters	29	6 (27.6%)	3/29 (10.3%)

Table 3.3. Influence of litter number on mammary tumour development. There is an overall difference between the groups in relation to litter number, p = 0.003, Fisher's Exact Test. Comparing the incidence of mammary tumours between the groups is also statistically significant, p = 0.006, Fisher's Exact Test.

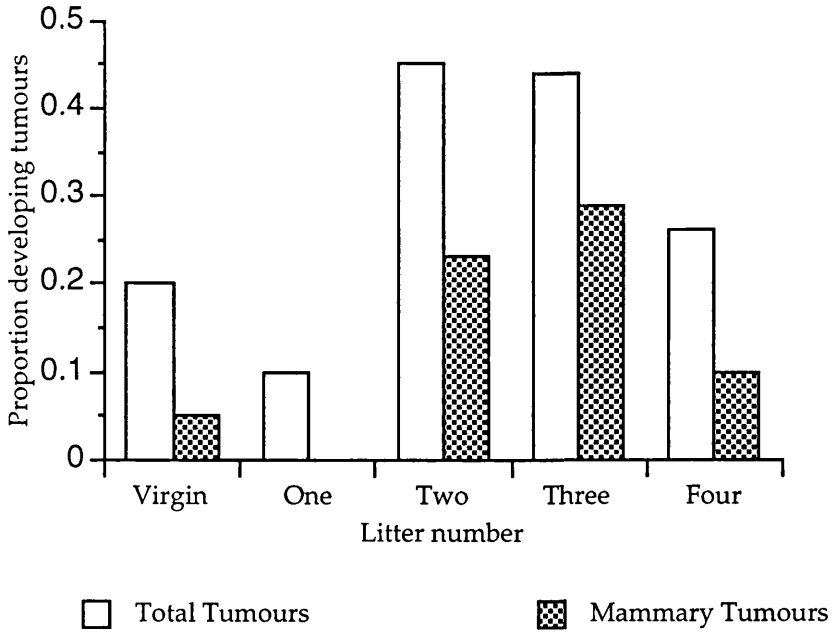


Figure 3.10 Overall incidence, up to 25 months, of all tumours and mammary tumours in relation to litter number in transgene positive animals.

Tumour Transplantation in Syngeneic Mice

Seven tumours from seven mice have been transplanted directly into BALB/c mice. The details and outcome of each transplant are documented in Table 3.4. Experiments involving these transplants are described in Chapter 5.

Transplant	Origin	Histology	Destination	Outcome
1	Generation 2	Comedo-type mammary	2 TG-ve Generation 4 Offspring	No take
2	Generation 2	Comedo-type mammary	2 TG +ve Generation 3 Offspring	No take
3	Generation 3	Solid-type mammary	2 TG-ve Generation 4 Offspring	No take
4	Generation 3	Comedo-type mammary	i) 2 TG -ve Gen.^{n} 4 offspring ii) 2 TG +ve Gen.^{n} 4 offspring iii) 1 TG+ve Gen.^{n} 5 mouse, 12 BALB/c mice	Passage 1 i)Established after 4/12 Passage 2 ii)Established after 3/52
5	Generation 4	Mammary	3 BALB/c mice	Observed for over 40 weeks - no tumour
6	Generation 4	Mammary	3 BALB/c mice	Observed for over 40 weeks - no tumour
7	Generation 3	Mammary	3 BALB/c mice	Observed for over 40 weeks - no tumour

Table 3.4 Details of tumours arising in transgene positive females, transplanted into immunocompetent mice.

Tamoxifen

Currently the only drug which is acceptable as prophylactic therapy in women at high risk for breast cancer is tamoxifen. The laboratory and clinical data available on tamoxifen makes it the most important chemopreventive agent available in breast cancer. Its great advantage is the relative lack of side effects such that well women would be prepared to accept its administration if they perceived the risk of breast cancer as significant. A North American study conducted by the National Surgical Adjuvant Breast and Bowel Project began to address the use of tamoxifen as a chemopreventive agent in 1992. In the UK a longterm trial of tamoxifen in women with a strong family history of breast cancer is currently recruiting (Powles, 1992).

A long term experiment has been established to investigate the influence of tamoxifen on the development of mammary tumours in the mice. Two groups of 36 heterozygous females aged between 2 and 5.5 months are being compared. One group is being given water ad libitum and the other receives 400µg tamoxifen citrate in the drinking water twice weekly, assuming an average water consumption of 5ml per mouse per day. The treatment will be maintained for 9 months and the mice will be observed for early onset of tumours and mortality. This experiment is ongoing and to date one tumours has arisen at 16 months of age in each group.

Chemical carcinogenesis

Mammary tumours can be induced in a number of strains of mice by a variety of chemical carcinogens. By weight 7,12-dimethylbenzanthracene (DMBA) is the most potent chemical carcinogen of the three studied most extensively, the others being 3-methylcholanthrene (MC) and urethane. In most studies a dose of 4-9mg of DMBA induces a 40-70% mammary tumour incidence within 10-14 months in virgin mice (Lenzhofer *et al.*, 1984; Wakasugi *et al.*, 1982). However non-mammary tumours arise in 25% of mice and consequently experiments have to be terminated between 10-12 months.

In BALB/c mice administration of exogenous hormone, or pseudopregnancy (Goodwin & W, 1985) concomitantly with DMBA, facilitates induction of mammary tumours. Recently 0.5-1.0 mg DMBA given to TGF-α transgenic mice at 56 days of age was found to increase the incidence of mammary tumours (Coffey *et al.*, 1994). Fifty-six days appears to be a critical time in the development and proliferative state of the mammary gland and has been shown to result in a shorter latency period for tumour formation (Coffey *et al.*, 1994).

Two groups of 25 at risk female mice have been given 1.0mg DMBA in corn oil or ordinary corn oil, by gastric gavage, at around 56-days, as described by Coffey *et al* (Coffey *et al.*, 1994) . The mice have been left to litter twice and are being kept on the shelf and monitored for tumour development. To date one tumour has been observed in the DMBA-treated group at 13 months.

Diet

Evidence from a number of experimental models supports the hypothesis that dietary fat has a tumour promoting effect, although the mechanism remains unknown. Some of these studies have directly investigated the influence of dietary fat on tumourigenesis. Comparable chemopreventive studies to those outlined have investigated the influence of dietary manipulation on mammary tumorigenesis. One such study involved feeding female MMTV/v-Ha-*ras* transgenics diets providing 0, 5 and 25% of calories from corn oil (CO) (DeWille *et al.*, 1993). The mammary tumour incidence was significantly higher in mice fed the 5 and 25% CO diets and in the 25% group *ras* mRNA levels were found to be increased in the mammary tumours. Another study involved manipulating dietary folate intake. Several retinoids have also been evaluated for prevention of mammary carcinogenesis in rats and mice (Moon *et al.*, 1992). They have been found to be effective in carcinogen-induced mammary tumour models and to have a synergistic effect with tamoxifen.

We have established three groups of mice from the ninth generation of the colony on diets of differing total fat content. They were fed diets providing 5 (normal), 10 and 15% of calories from corn oil to assess the influence on tumour incidence. The experiment has now been established for over eighteen months and, although too early to analyse formally, a difference between the groups is apparent (Table 3.5).

Diet	Total no.of mice	No. of mice died(%)	No. died with tumour (%)
Normal (4.9% fat)	34	13 (38.2%)	7 (20.6%)
10% Fat	49	20 (40.8%)	15 (30.6%)
15% Fat	37	19 (51.3%)	13 (35.1%)

Table 3.5 Incidence of definite tumours in mice fed varying total fat content diet. If all deaths are assumed due to tumour (as appears possible, in retrospect) the incidence at 20 months is high.

Mating with MUC1 mice

The human MUC1 gene encodes the core protein of a mucin, polymorphic epithelial mucin (PEM), expressed by glandular epithelia and the tumours arising from these tissues (Peat *et al.*, 1992). The core protein is aberrantly glycosylated in many cancers, including mammary tumours, and some antibodies react specifically with the cancer-associated mucin, which also has epitopes recognised by T cells from breast and pancreatic cancer patients. A group of mice which develop tumours expressing the MUC1 gene product as a self antigen has potential for use as a preclinical model for the evaluation of PEM-based antibodies directed at PEM in cancer therapy.

Using a 10.6 kB SacII fragment as the transgene, from a larger fragment containing 1.6kB of 5' sequence and 1.9kB of 3' flanking sequence, a novel strain of transgenic mice (TG18) was established (Peat *et al.*, 1992). The strain showed tissue specificity of expression of the MUC1 gene very similar to the profile of expression seen in human tissues. The antibody SM-3 is directed to a core protein epitope, which is selectively exposed in breast cancers and shows a more restricted distribution on normal human tissues. The distribution of the SM-3 epitope of PEM in the tissues of these mice shows good agreement with that seen in human tissues (Peat *et al.*, 1992). TG18 mice homozygous for MUC1 have been mated with heterozygous MMTV-*neu* male mice from the third generation and the offspring then screened for MMTV-*neu*. To assess the influence of litter number on tumour incidence these mice have been divided into three groups and mated to produce 0, 2 and 4 litters, respectively. These mice should develop tumours in PEM-expressing tissues. To date one mammary tumour and three Harderian gland tumours have arisen. The mammary tumour has been stained for MUC1 expression and successfully transplanted into nude and BALB/c mice. Unfortunately only very low and sporadic expression was observed using the antibody SM3 suggesting that MUC1 expression is not a salient feature of this tumour.

Discussion

There have been a number of drawbacks to any attempt to treat tumours arsising spontaneously in this colony. Most noteable are:

- The low incidence of tumours
- Tumours arise late in the lifespan
- Some tumours are large at the time of diagnosis, making them less likely to respond to cytokine therapy.
- The diverse types of tumour in this colony .

The most important feature limiting the significance and value of this colony as a model of breast cancer is the relatively low incidence of mammary tumours. There are likely to be a number of factors which have influenced this finding.

The colony described here and the founder mice from which it was established have not been exposed to comparable conditions. There are differences in animal husbandry, diet, endemic infection and relative crowding of the animals in the two colonies. The Montreal colony was fed a diet of higher fat content and the mice were kept in more stressful, overcrowded conditions (Jolicouer, personal communication). There was murine hepatitis in the colony in Montreal whereas our mice have been kept in cleaner SPF conditions. As tumours arise after a number of genetic events in this particular model, all the above factors are likely to have contributed to the lower incidence of tumours in our colony.

Some of the findings with this colony have not been described by others working with mice transgenic for activated c-*neu*. The spectrum of tumours more closely resembles that described by Suda *et al* in mice with the unactivated c-*erb*B2 oncogene (Suda *et al.*, 1990) and that seen with MMTV-Ha-*ras* transgenic mice (Cardiff *et al.*, 1993). The incidence of Harderian gland tumours in Bouchard's experience was lower than in ours but at 5% it is higher than would be found in normal control mice suggesting that the transgene is responsible for the initiation, maintenance or both. Harderian gland tumours have also been documented arising in transgenic mice with the MMTV promoter and oncogenes other than *neu* suggesting that the promoter may also influence site of tumour development. The development of B cell lymphomas was a further unexpected finding which is closer to the experience of Suda et al(1990) than Bouchard et al(1989) (Bouchard *et al.*, 1989; Suda *et al.*, 1990).

Further strategies are required to increase the mammary tumour incidence to a level more appropriate for the conduct of therapeutic experiments. One possibility is the manipulation of dietary fat content, outlined above. The effect on mammary tumourigenesis has been investigated in MMTV/v-Ha-*ras* female transgenics fed diets providing 0, 5 and 25% of calories from corn oil (DeWille *et al.*, 1993). The mammary tumour incidence was 7, 36 and 52% respectively. Another study showed delayed tumour onset in transgenic mice, carrying the T-lymphotropic viruse type 1 tax_1 gene,, fed a low folate diet (Bills *et al.*, 1992) Whilst diet is undoubtedly an important aspect of the risk factors involved in the development of mammary cancer, the link between diet and fat in murine models does not translate readily to the findings in epidemiological studies in humans (Goodwin & Boyd, 1988; Tannenbaum, 1942). Comparative international studies have shown an association between fat intake and breast cancer in a number of countries. There is conflicting evidence epidemiologically and from case control studies as to whether it is fat intake or total calorie intake which is important (Goodwin & Boyd, 1988; Prentice *et al.*, 1988);. One combined analysis of 12 case control studies shows a highly significant correlation between saturated fat intake and breast cancer risk in postmenopausal women but no evidence for an association between fat intake and increased risk of premenopausal breast cancer, or between total calorie intake and risk of breast cancer (Howe *et al.*, 1991). There is no doubt that measurement error is inherent in the technique of dietary assessment and this may contribute to the conflicting evidence from these two types of study (Greenwald, 1989).

Radiation carcinogenesis in experimental animals is well documented. Irradiation-induced mammary cancer in rats is common but there are few reports in mice, two of which involved neutron irradiation, which is not widely available (Medina *et al.*, 1973; Ullrich *et al.*, 1977). Inbred BALB/c mice are known to be sensitive to radiation in comparison with other inbred strains of mice (Roderick, 1963). In a large study in BALB/c female mice 2Gy of gamma irradiation increased mammary tumour incidence from 7 to 20% (Ullrich *et al.*, 1977). Mammary carcinomas induced by irradiation of BALB/c mice exhibited a 55% incidence of metastases (Ullrich *et al.*, 1977).

Based on these and other studies in mice (Medina *et al.*, 1973) we plan to expose female mice to a single dose of 2Gy whole body irradiation, at 21 days after the birth of their first litter. Thereafter the mice will be compared with a similar control group of mice exposed to the same stresses who have not been irradiated. The relevance of such a manoeuvre must be considered. It appears that irradiation does influence biology but its effect is not readily predictable and will again necessitate careful documentation.

The tumour incidence may also be increased by exposing the mice to carcinogens known to be effective in rodents such as 7,12-dimethylbenzanthracene (DMBA) or 3-methylcholanthracene (MC) (Biancifiori *et al.*, 1959; Medina & Warner, 1976). The direct or indirect administration of hormones, either using pituitary isografts or oestrogen and diethylstilboestrol, also influences tumour incidence. The effects of hormones and carcinogens are very strain dependent and would require careful research and pilot studies before embarking on a long-term undertaking, particularly as there is evidence in BALB/c mice that dysplastic lesions of limited growth and tumorigenic potential may arise (Medina & Warner, 1976).

Experience with other transgenic models of malignancy would suggest that the incidence and age of onset of tumours may be increased in homozygous mice. For this reason a homozygous colony has been established from the ninth generation onwards.

The BALB/c background was chosen for this study for two reasons. Firstly, based on the experience of Jolicouer and colleagues, who routinely mated the F1 generation with BALB/c mice, and secondly the knowledge that this strain is predisposed to the development of MMTV-induced mammary tumours. For comparison, the incidence of spontaneous tumours arising in BALB/c mice is outlined in Table 3.6.

Tumour type	Incidence	Reference
Mammary - normal	5%	(Bordon *et al.*, 1982)
Mammary - C3H fostered	100% at 7/12	(Heston & Vlahakis, 1971)
Mammary - virgin	1%	(Bordon *et al.*, 1982)
Lung tumours	21%	(Smith & Pilgrim, 1971)
Angiomas	6%	(Smith & Pilgrim, 1971)
Lymphomas	11-20%	(Sass *et al.*, 1976)
Leukaemias (Myeloid)	5%	(Myers *et al.*, 1970)

Table 3.6 Incidence of spontaneous tumours in BALB/c mice

Since we originally embarked on this study another inbred strain, FVB/N, with specific advantages for transgenic analyses, has been used increasingly to develop oncogene transgenic mice. One of the advantages most relevant to this study is an

apparent increased susceptibility to tumours (Hennings *et al.*, 1993; Taketo *et al.*, 1991). Transgenic mice have usually been generated by pronuclear injection of F1 zygotes rather than inbred strains as the latter have small pronuclei which are difficult to inject and have a relatively poor reproductive performance. FVB/N is an inbred strain characterised by vigorous reproductive performance and consistently large litters and the fertilised eggs have large prominent pronucleii, which facilitate microinjection of DNA. The strain was originally developed from an outbred colony of Swiss mice established at the National Institutes of Health in 1935. These have been selected out firstly for histamine resistance in a study of pertussis vaccine and then they were found to carry the *Fv-1*^{b} allele for sensitivity to the B strain of Friend leukaemia virus. They were then inbred, selected for *Fv-1*^{b} homozygosity and designated as FVB for Friend virus B-type susceptibility (Taketo *et al.*, 1991). It appears that they may have a higher incidence of spontaneous tumours than BALB/c mice and might therefore prove to be a useful genetic background for this model.

Another important observation has been the statistically significant decline in the proportion of transgene negative offspring with successive generations of mice. This finding has significant implications in a heterozygous breeding programme. It also appears that this decline is related to the fact that in some cases whole litters are transgene negative, suggesting that the gene may have been eliminated in the germline. Similar experience appears to be emerging from other groups (Perbal, personal communication) and any explanation is likely to be complex. It may be that the transgenes are not inherited as a result of the abnormal 'state' of the oncogene in these mice. This could be explained by the influence of another gene or genes in the BALB/c haplotype which disadvantages embryos heterozygous for the transgene relative to transgene negative BALB/c mice. Such a disadvantage would not be evident where animals heterozygous for the transgene are mated. This is consistent with our finding in the ninth generation where two homozygous colonies have been established (with and without MUC-1).

Further investigation of the mechanisms of activated c-*neu* in oncogenesis could be studied in this model. By extracting protein from tissues and tumours known to express the transgene the biochemical effects of *neu* could be investigated using phosphotryosine blots. This may highlight differences between the types of tumours or within the group of mammary tumours which relate to metastatic behaviour or morphology.

Conclusion

This model has yielded unexpected findings which have not been previously described. Some of these limit its usefulness as a model for cancer, and in particular, cytokine, therapy. The baseline outlined here, in terms of the natural history of the model and the biology of the individual tumours, will enable it to be manipulated appropriately to provide a model of greater applicability.

Chapter Four

The Biology of Individual Tumours

Introduction

The morphological characteristics of spontaneous murine mammary tumours were first systematically described by Dunn (1959)(Dunn, 1959), extended by van Nie (1967)(Nie, 1967) and then revised by Sass and Dunn (1979)(Sass & Dunn, 1979). The majority of tumours classified by Dunn (1959) were MMTV-induced and did not resemble human mammary tumours morphologically. They were divided into three main categories - carcinomas, carcinomas with squamous differentiation and carcinosarcomas. The carcinomas were further divided into six types of adenocarcinoma, the majority being type A and B.

- Type A - small acini lined by a single layer of cuboidal cells - adenoma, tubular carcinoma or alveolar carcinoma.

- Type B - variable histology with poorly and well-differentiated regions. This included irregular cords and sheets of cells or papillomatous areas.

As outlined in Chapter One, a number of oncogene transgenic mice strains in which mammary tumours arise have been developed. Particular transgenes are associated with characteristic histological patterns. Indeed the association appears to be strong enough to predict the genotype of the mouse (Cardiff *et al.*, 1991; Halter *et al.*, 1992; Suda *et al.*, 1990). Mammary tumours arising in mice bearing *neu*, *ras* or *myc* oncogenes have been found to exhibit characteristic phenotypes which differed from each other. Only 9% of the tumours arising in these three groups of mice could be classified using the standard categories described by Dunn's nomenclature, compared with 95% of those seen in tumours in MMTV-infected mice (Dunn, 1959). The histological patterns of tumours arising in transgenic mice with the different oncogenes, were:

- A small eosinophilic cell papillary tumour associated with the *ras* transgene.

- A large basophilic cell lesion which differentiated into an adenocarcinoma and was associated with the *myc* transgene.

- An intermediate cell tumour with eosinophilic cytoplasm associated with the *neu* transgene. This usually resulted in the development of nodular tumours.

This also held true for groups of *ras* and *myc* bigenic animals where the *myc* phenotype appeared to be dominant, and in trigenic animals with all three oncogenes where again *myc* appeared dominant (Cardiff *et al.*, 1991).

Two models involving the activated c-*neu* oncogene are mentioned in Chapter Three. In the model developed by Bouchard and colleagues the tumours bore a close histological resemblance to human mammary tumours (Bouchard *et al.*, 1989). They were poorly differentiated adenocarcinomas arising adjacent to morphologically normal epithelium which sometimes showed microcalcifications without significant dysplasia or hyperplasia. In some tumours papillary formations were occasionally seen. Intra-tumour necrosis and calcifications were frequent findings. These tumours were similar to human ductal breast carcinomas (Bouchard *et al.*, 1989). They metastasised to lung and could be transplanted into nude mice, confirming their malignant behaviour.

The Harderian gland is a modified sebaceous gland found deep in the orbit of animals with a nictitating membrane, (the inner eyelid present in many animals which serves to protect the eye from dust and keep it moist) (Davies, 1929). Named after Harder (1963)(Harder, 1963), it is bilobed and horseshoe-shaped. Spontaneous tumours occur naturally in the Harderian gland in a number of strains of mice (Murphy, 1966). The vast majority arising spontaneously in BALB/c mice are categorised as adenomas. These naturally occurring tumours arise later in life and grow slowly (Tucker & Baker, 1967). They vary in size, shape and colour but the larger ones are white in colour. Growth in the restricted space often causes protrusion of the eye. Microscopically, most of the tumours are papillary cystadenomas.

Lymphomas in the mouse are similar to those seen in other species. Dunn described neoplasms of the mouse reticular system as arising from stem cells, granulocytes, lymphocytes, reticular cells and plasma cells (Dunn, 1954). With immunocytochemistry and the more recent classification of murine malignant lymphomas (Morse *et al.*, 1987; Pattengale & Frith, 1983; Pattengale & Frith, 1986), lymphoblastic lymphoma has been reported as one of the more common neoplasms in mice and resembles Dunn's lymphocytic leukaemia. Although the incidence increases slightly with age in BALB/c mice, overall the incidence of spontaneous lymphomas in BALB/c mice kept in germ-free conditions is less than 3% (Smith & Pilgrim, 1971). Immunoblastic lymphoma of B cell origin is rare in the mouse, which differs from our findings in this colony (Pattengale & Frith, 1983). It is characterised by large

lymphoid cells with round to oval vesicular nuclei with prominent and distinct nucleoli. The nuclei also sometimes have plasmacytoid features.

In the mouse, angiosarcomas usually arise in the spleen, liver and subcutaneous tissues, although they may also arise in the ovaries, mammary tissue, uterus and urinary bladder (Frith *et al.*, 1993). They tend to be locally invasive and for this reason it is often difficult to distinguish multicentric origin from secondary metastases (Frith *et al.*, 1993). They may metastasise to the lungs. The incidence of angiomas in BALB/c mice is about 6%.

This aims of the work outlined here were:

- To investigate the range of tumours arising in the model
- To assess the proliferative status of one subgroup
- To assess the suitability of such a model for therapeutic studies

Results

Mice in the colony developed a range of tumours, the most common being mammary, lymphomas and Harderian gland tumours. Within each tumour subgroup there was diversity in the biological behaviour of individual tumours. This diversity is also illustrated by the age at onset of the different tumour types, as outlined previously in Table 3.1. and Figure 3.5. The breast carcinomas and Harderian gland carcinomas metastasised predominantly to the lungs and the lymphomas were widely disseminated at post mortem. Some mice developed more than one histological tumour type and others, at post mortem, were found to have lung metastases from a primary tumour which was not evident. The range of tumour types are discussed below.

One group of mice in each of the third and fourth generations were treated with control diluent as part of a prophylaxis study (see Chapter Six). A comparison of the type of tumours developed, their biological behaviour and the survival of the mice showed no difference between the untreated transgene positive mice and control diluent-treated transgene positive littermates. They were therefore included in this analysis.

Mammary tumours

Of the 93 tumours documented in control diluent or untreated mice in the first five generations, 53 were mammary (57%). They were characterised phenotypically by a subcutaneous tumour in an otherwise well animal. Most arose in the mammary line, making them apparent over the scapula or thigh. Twenty-three of these 53 tumours metastasised to the lung (43%). Histologically, all the mammary tumours shared high grade cytomorphology, with a significant degree of mitotic activity and pleomorphism consistent with the classification of poorly differentiated DCIS described in Chapter One. This describes cells with pleomorphic, irregularly spaced nuclei, with coarse, clumped chromatin, prominent nucleoli and frequent mitoses. Necrosis is usually present (Holland, 1994). However in the tumours found in this colony no definite *in situ* carcinoma was seen.

The architectural pattern showed a range of appearances with the following types of growth pattern merging with one another, and sometimes co-existing in the same tumour. The first three histological types account for the majority of tumours:

- Tumours showing islands of interlocking large cells with areas of necrosis accounted for 21 of the mammary tumours. These were characteristic of the classical comedo-type tumours described by Bouchard *et al* in the founder mice and associated with c-*erb* B2 positivity(Bouchard *et al.*, 1989). Unlike human comedo carcinoma (large cell ductal carcinoma *in situ* with central necrosis) the tumours were not confined to ducts (figure 4.1.i).

- Thirteen of the mammary tumours were predominantly solid, consisting of sheets and well defined islands of tumour (figure 4.1.ii).

- Sixteen tumours were micropapillary in nature. In 12 this was the predominant growth pattern (figure 4.1.iii). Tumours consisted of numerous duct like structures in which the malignant epithelium was thrown into small papillae. These structures were more numerous than the usual number of ducts and were believed to represent invasive disease. In humans micropapillary histology is also associated with c-*erb*B2 positivity.

- Two tumours resembled human intracystic papillary carcinoma architecturally (but not cytologically), with the papillary growth pattern contained within a cystic space (figure 4.1.iv).

- Two tumours showed apocrine features, a finding which is not uncommon in human carcinomas, where the infiltrating component of the tumour may show apocrine differentiation. Here the lumenal epithelial cells resemble those of apocrine glands. The cells are larger than normal and have abundant granular eosinophilic cytoplasm which shows apical lumenal blebbing (figure 4.1.v).

- In one tumour, a spindle cell epithelial element was seen evolving from more typical solid type carcinoma in keeping with a so-called 'metaplastic' carcinoma.

- A further tumour was entirely of metaplastic type (figure 4.1.vi).

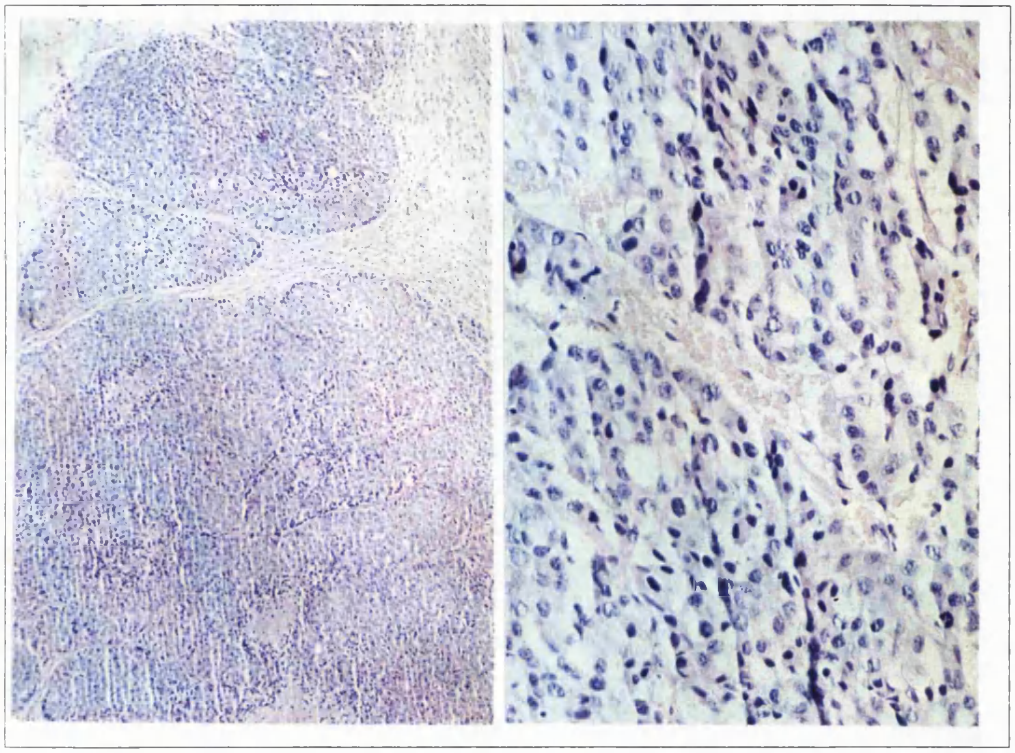


Figure 4.1(i) Comedo-type mammary tumour Figure 4.1(ii) Solid mammary tumour

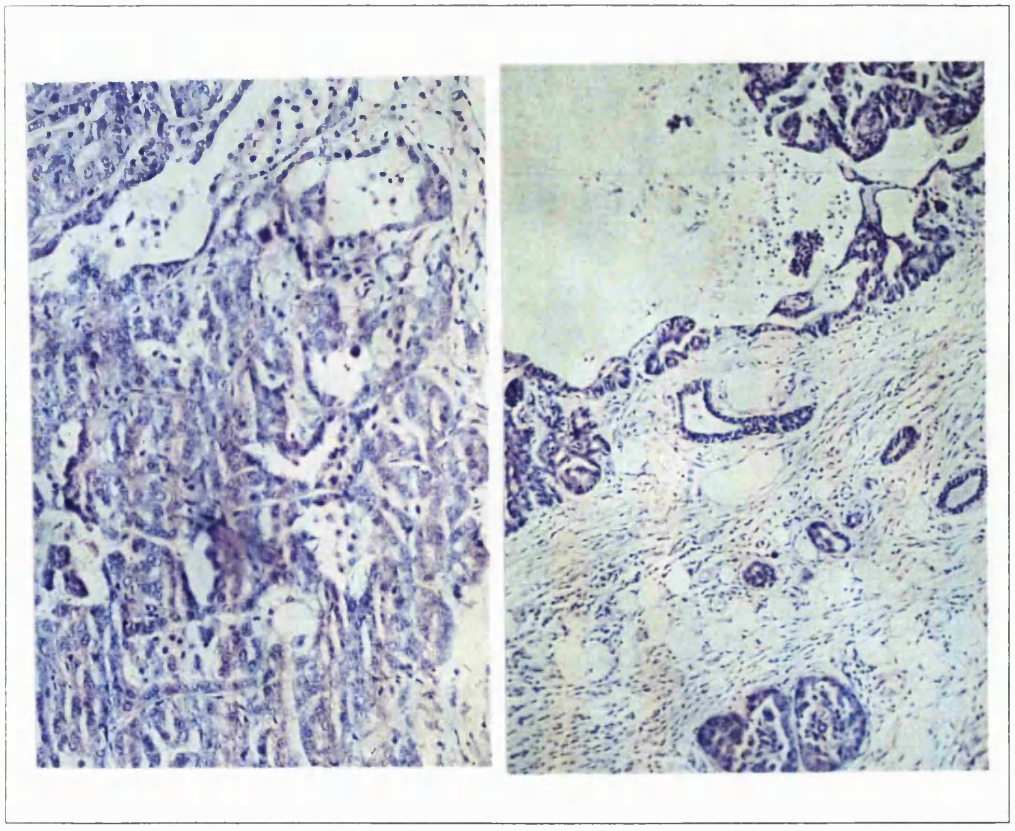


Figure 4.1 (iii) Papillary tumour Figure 4.1 (iv) Intracystic papillary tumour

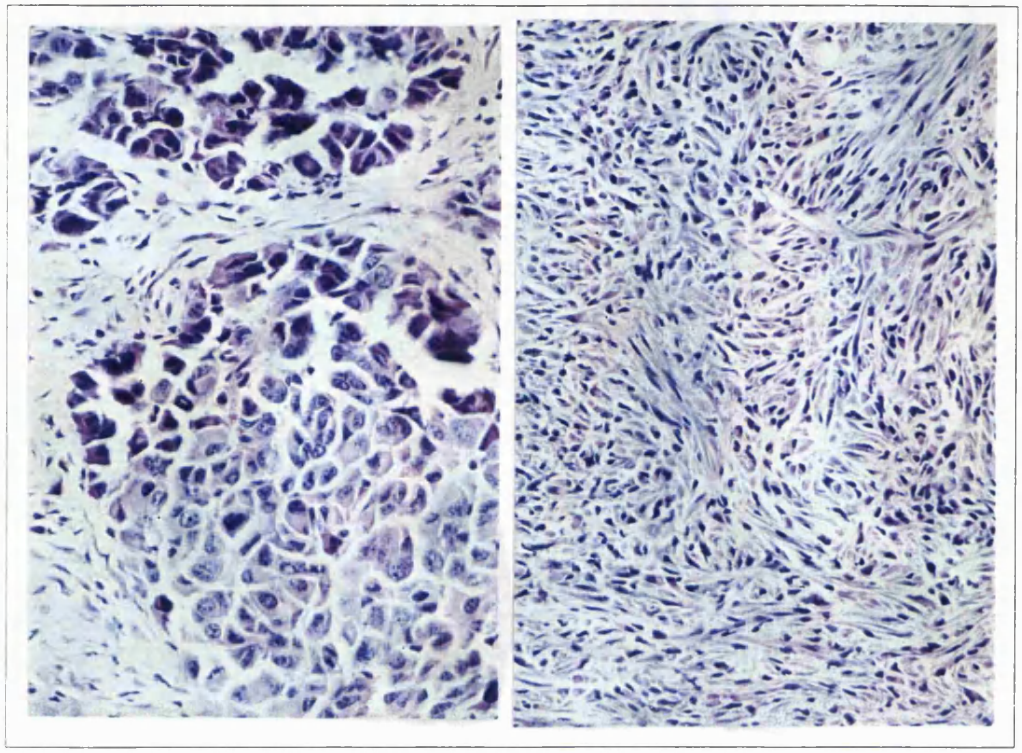


Figure 4.1 (v)Apocrine tumour Figure 4.1 (vi)Metaplastic tumour

Figure 4.1. Different histological features of mammary tumours in colony

In summary the characteristics of the tumours were as follows:

- All the lesions graded as III using the criteria laid down by Bloom and Richardson (Bloom & Richardson, 1957).
- All of these tumours stained positively with an antibody to human c-*erb*B-2 (figure 4.2.i and 4.2.ii). Membrane staining was demonstrated in all 53 tumours
- The tumours did not readily fit into the Dunn classification of MMTV-induced murine mammary tumours (Dunn, 1959; Sass & Dunn, 1979) though some of the solid type lesions bore resemblance to the type B group.
- None of the tumours showed a significant host inflammatory response.

Thus we have found close similarities between the grade and cytopathology of murine mammary cancer associated with c-*neu* and the human disease associated with c-*erb*B2.

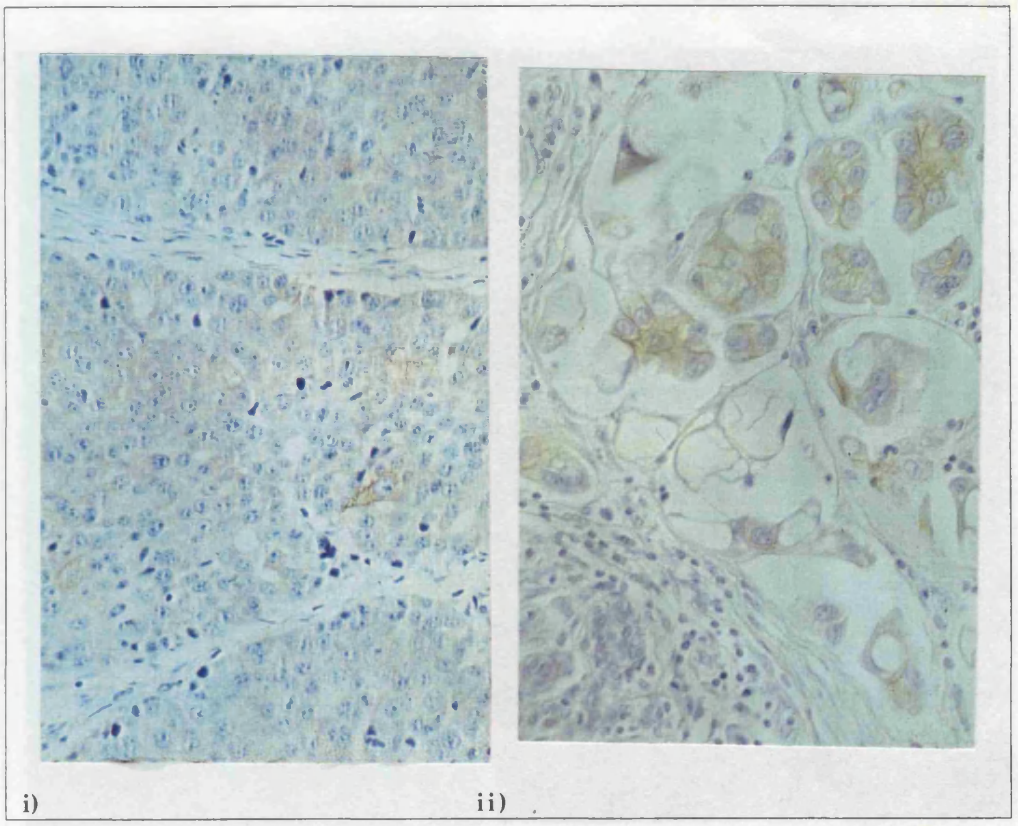


Figure 4.2(i)Immunohistochemistry with antibody to c-erbB2
(ii) Positive control (human)

Metastases from mammary tumours

Twenty-three of the 53 (43%) mammary tumours metastasised to lung. Deposits were found in the spleen in two mice. No metastases were recorded in bone - these were sought by systematically sectioning spinal cord in 20 mice.

Of the different pathological categories, the micropapillary histological pattern appeared most likely to metastasise to lung, with an incidence of 11/16 (69%) as compared with 12/37 (32%) of the non-papillary tumours. (Fisher's exact test, p=0.003). In two cases where a small element of micropapillary histology was seen in a tumour of mixed histology, the lung metastases were entirely papillary in morphology. Five animals were unwell with breathing difficulties and at post mortem examination extensive lung metastases from an occult mammary tumour were evident. In four of these five cases the tumour was papillary in pattern (Fig 4.3.i and ii).

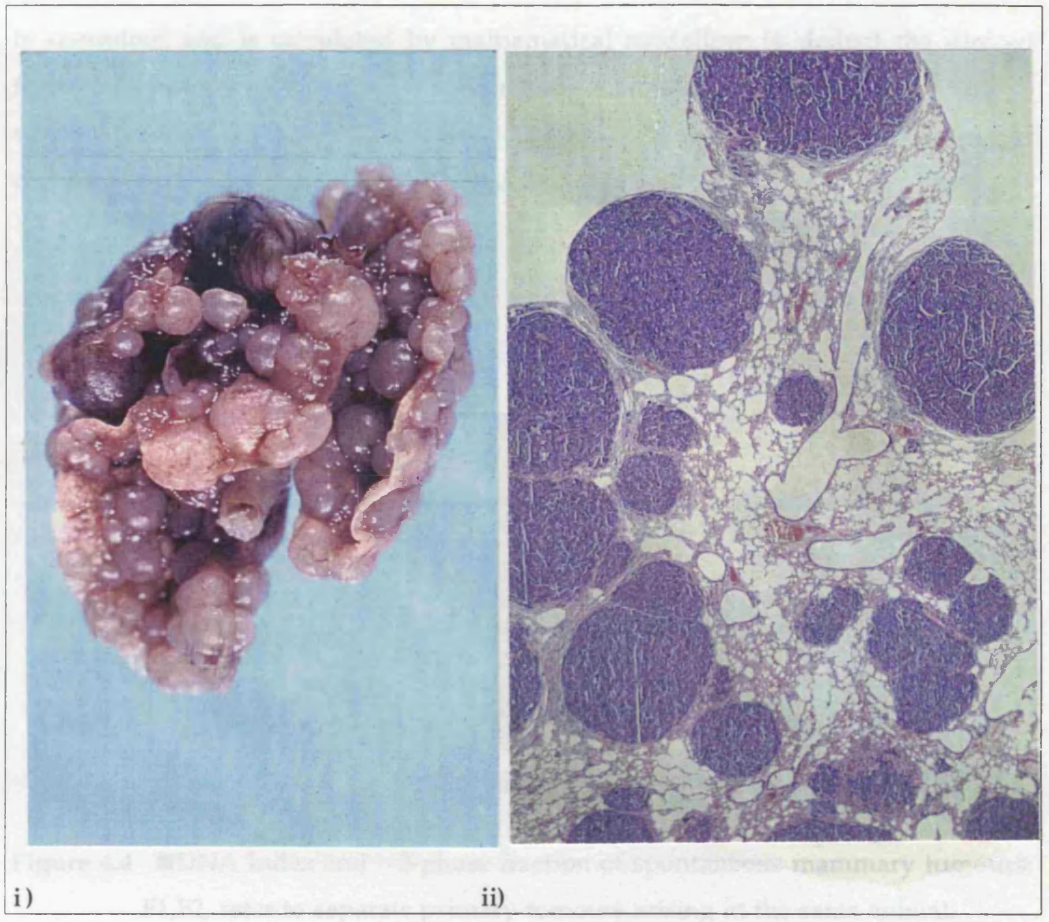


Figure 4.3.i) Macroscopic picture of lungs with metastases ii) Microscopy of same.

S-phase fraction analysis in mammary tumours

In order to confirm the subjective impression of diversity in this model, both within and between tumour subcategories, we examined the proliferative rate of mammary tumours using S-phase fraction. DNA index and S-phase fraction (SPF) were measured by flow cytometry of paraffin-embedded tissue. Thirteen primary mammary tumours were examined and 11 found to exhibit a wide range of SPF (range 5.6 - 11.9, median 9.0). In two other mammary tumours there were two clones of tumour of differing ploidy preventing analysis of the SPF of the two different peaks. Thus analysis of both aneuploidy and SPF was possible in 9 of the 13 tumours and these data are shown in Figure 4.4 and Table 4.1.

Of the 13 mammary tumours, one was diploid and 12 (92%) were aneuploid. A DNA Index around 2.0 suggested that eight of the 10 primary tumours examined were tetraploid, the other two having DNA indices of 1.0 and 2.7 respectively. As two of the tumours had two clones of tumour of differing ploidy the SPF could not be analysed. The percentage of aneuploidy indicates the proportion of a tumour which

is aneuploid and is calculated by mathematical modelling to deduct the diploid fraction (see Chapter Two). The higher the percentage the greater the degree to which a tumour has become aneuploid. Of the nine aneuploid tumours where this could be analysed, six had over 60% aneuploid cells.

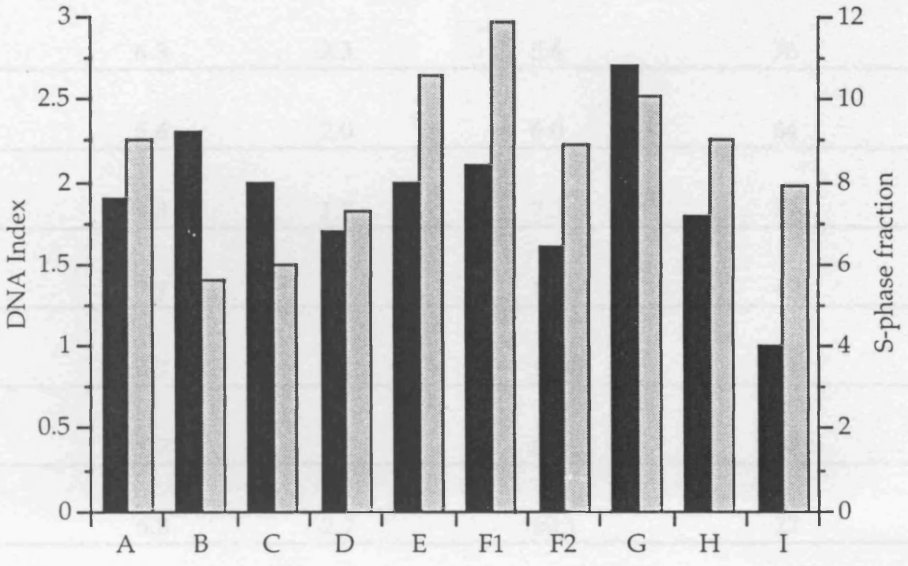


Figure 4.4 ■DNA Index and ▨ S-phase fraction of spontaneous mammary tumours. F1,F2, refer to separate primary tumours arising in the same animal.

Tumour	CV	DNA Index	S-phase fraction	% Aneuploidy
A	3.6	1.9	9.0	75
B	6.3	2.3	5.6	76
C	5.6	2.0	6.0	64
D	4.3	1.7	7.3	79
E	7.7	2.0	10.6	47
F1	5.7	2.1	11.9	41
F2	4.7	1.6	8.9	70
G	6.8	2.7	10.1	27
H	2.4	1.8	9.0	85
I	4.4	1.0	7.9	-

Table 4.1 Flow cytometric analysis of primary mammary tumours. CV = Coefficient of variance; % Aneuploidy = Width of aneuploid peak; S-phase fraction analyses on aneuploid tumours where proportion of aneuploid cells is over 10%.

Harderian gland carcinomas

Twenty-four Harderian gland carcinomas were seen in the colony. Age of onset ranged from 11 to 25 months with median being 18 months. Two were documented in the second, five in the third, 16 in the fourth, and one in the fifth generation. Harderian gland carcinomas were diagnosed on the basis of a protruberant eye and the presence of fluid and solid tumour behind the eye at post mortem. In six cases lung metastases were found on pathological assessment although no primary was noted at post mortem. Histologically these tumours were papillary in pattern and resembled the more poorly differentiated end of the spectrum of Harderian gland carcinomas found to occur naturally (Figure 4.5.i). All 24 of the Harderian gland carcinomas stained positively for c-*erb* B2 (Figure 4.5.ii). There was a higher proportion of these tumours in virgin mice than in mated mice.

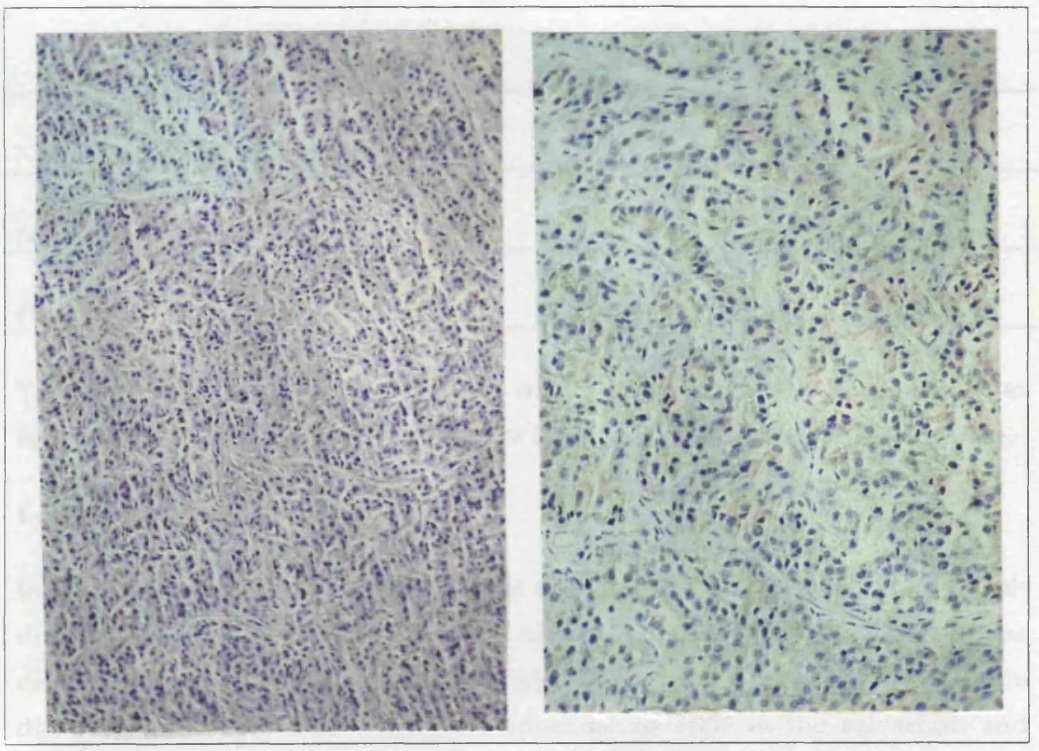


Figure 4.5 i) Haematoxylin and eosin stain of Harderian gland tumour
ii) Immunohistochemistry with c-erbB2 antibody of Harderian gland carcinoma

Seventeen of 24 (71%) of Harderian gland carcinomas metastasised to lung. Metastases did not correlate with the grade of the primary tumour. Indeed, in one case the primary tumour had the appearance of an adenoma but metastases in the lungs were consistent with a malignant Harderian gland carcinoma. Two of three tumours arising in mice in the second generation, all five in the third generation and 13 of 17 cases in the fourth generation had metastasised to lung. Three of the latter which had not metastasised being virgin mice. There was a statistically significant difference between the proportion of Harderian gland tumours metastasising to lung in mated mice as compared with proportion arising in virgin mice (Table 4.2). This is consistent with the findings of Liebelt (1968) and Fry (1976) who found, independently, that hormones influence the biological behaviour of spontaneous and radiation-induced Harderian gland tumours in inbred strains of mice (Fry *et al.*, 1976; Liebelt *et al.*, 1968).

	Virgin	Mated	Total
Number of Harderian gland tumours	6/53	18/187	24/240
Number with lung metastases	1	16	17
Percentage with lung metastases	16.7%	88.8%	70.8%

Table 4.2 Proportion of lung metastases from Harderian gland tumours related to reproductive history. Fisher's Exact Test p= 0.003, comparing mated and virgin mice.

Lymphomas

In total there were 6 malignant lymphoid tumours arising in untreated or control-diluent treated mice in this colony. All the lymphomas were found to be disseminated at post mortem. Microscopically the spleen, liver and lungs were diffusely infiltrated. One lymphoma appeared to arise in the calvarium and subsequently disseminated into the brain and systemically. In other cases lymphoma was found to be infiltrating the spine, lung, large intestine and skin. The age at onset ranged from four to 21 months, with a median of 17.5 months. In one case the tumour had the morphology of an immunoblastic lymphoma with lymphoplasmacytoid features (figure 4.6.i), while the rest manifested as a lymphoblastic lymphoma/acute lymphoblastic leukaemia (figure 4.6.ii).

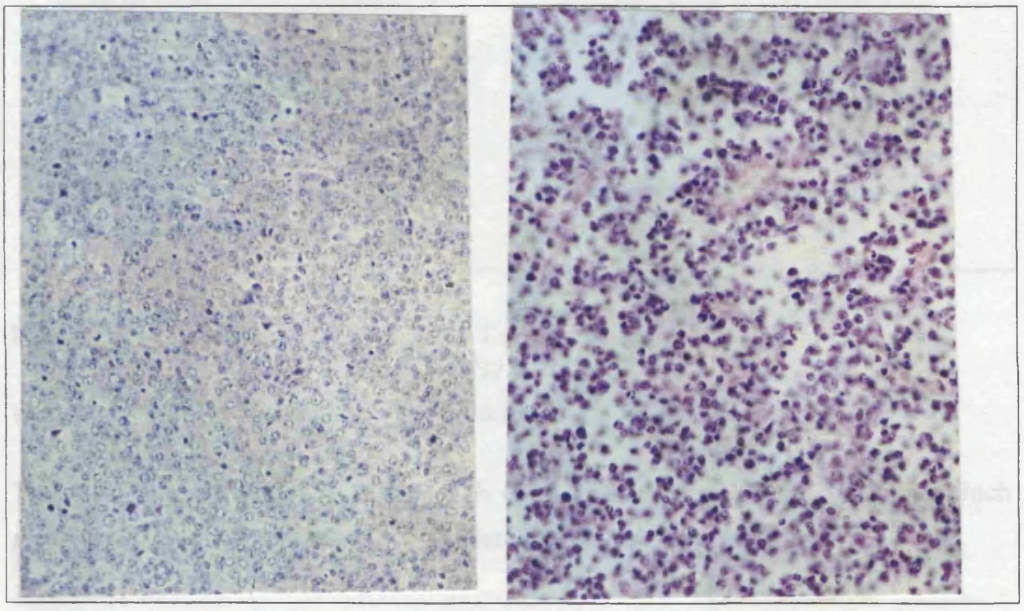


Figure 4.6 i) An immunoblastic lymphoma with lymphoplasmacytoid features.
ii) Lymphoblastic lymphoma

Classification of lymphomas in mice is largely based on immunohistochemistry or FACS analysis as well as morphological characteristics. All of the 6 lymphomas thus far characterised in the c-neu colony showed a B-cell phenotype using the four murine antibodies against α/β TCR, Thy 1.2, surface IgG and B220 (figure 4.7). Described in Chapter Two.

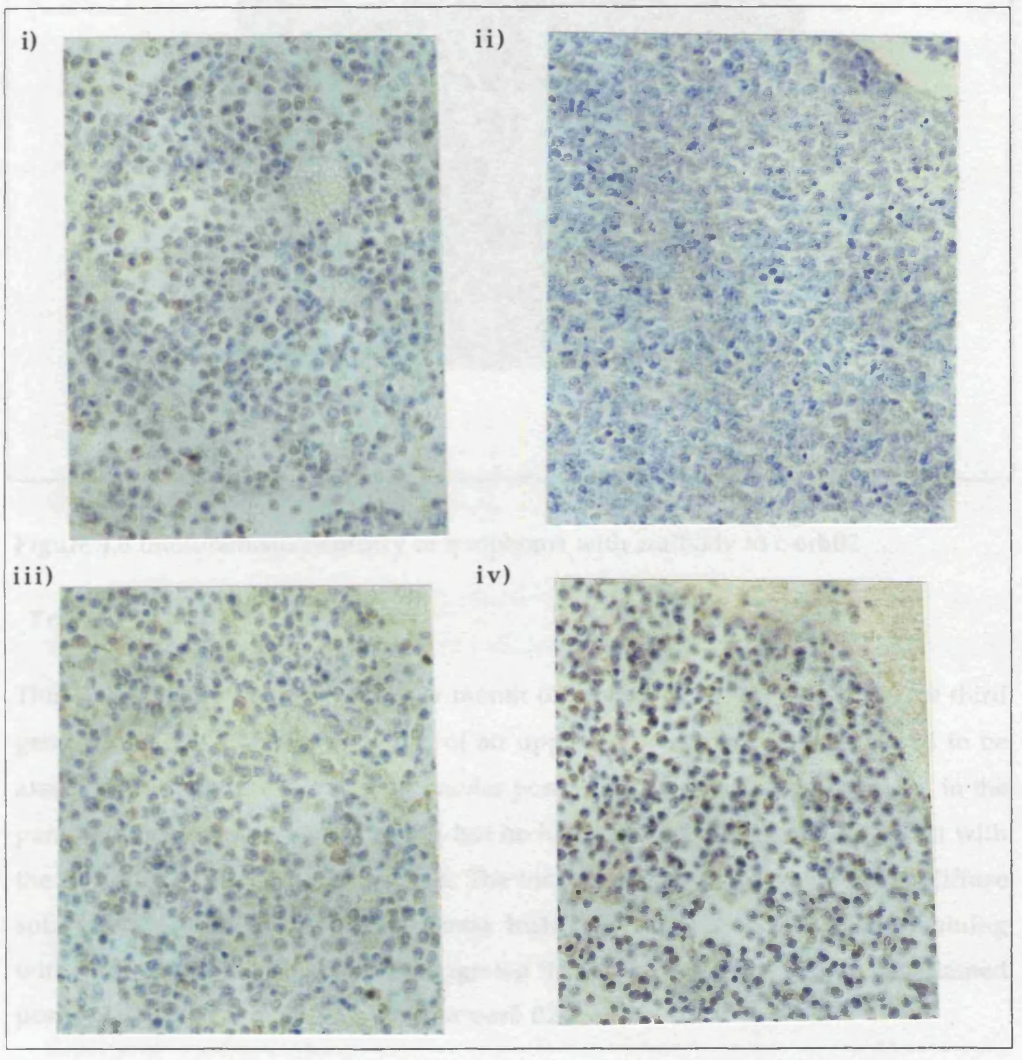


Figure 4.7 Immunohistochemistry with T and B cell markers

i)α/β TCR ii) Thy 1.2 iii) Surface IgG iv) B220

The lymphomas stained positively with the antibody to c-*erb*B2 (Figure 4.8) which suggests that their development was related to the transgene.

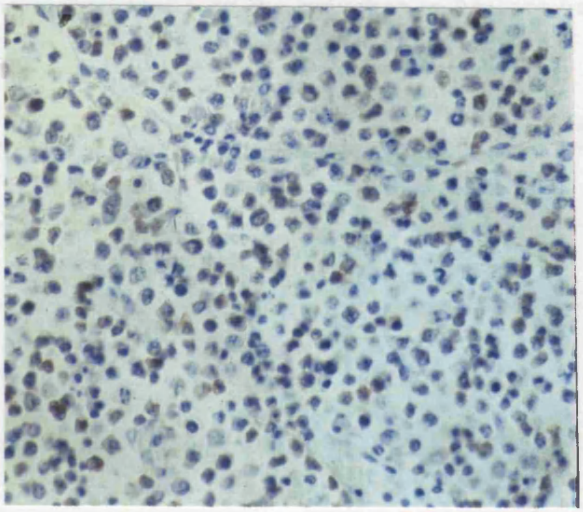


Figure 4.8 Immunohistochemistry of lymphoma with antibody to c-erbB2

Transplant 3, Lymphoma line

This lymphoma developed in a four-month old mated female mouse from the third generation. The tumour, at the site of an upper mammary gland, was found to be associated with contralateral lymph nodes post mortem. There were also nodes in the para-aortic chain and into the pelvis, but no lung metastases. This is in contrast with the behaviour of mammary tumours. The tumour was of high grade with a diffuse solid pattern, resembling a lymphoma histologically and cytologically. Staining with murine T and B cell markers suggested that it was a B cell lymphoma. It stained positively with an antibody to human c-*erb* B2.

At this point the colony had been backcrossed onto BALB/c mice for only three generations. By Mendelian genetics this results in BALB/c genome by the third generation. Concern that this was not sufficiently inbred for transplantation into siblings, offspring or BALB/c mice, led to the tumour being passaged into nude mice initially for further investigation of its characteristics (see Chapter Five). Here it grew very rapidly, becoming established within days, whether injected subcutaneously or intraperitoneally. When re-injected into BALB/c mice it grew very rapidly and was locally invasive. In both strains the mice had to be killed with ascitic disease within 4-5 days of injection. The highly malignant nature of this tumour made it unsuitable for therapy experiments.

The proliferative rate of different passages of this tumour in nude mice was examined using S-phase fraction analysis. The progression in S-phase fraction between passages 1 and 6 was consistent with the reduction in passage times seen *in vivo* (Table 4.3).

Passage No.	C.V.	DNA Index	S-phase fraction	Passage times (days)
1	4.1	1.0	13.9	21d
6	3.6	2.0	23.5	11d
18	3.6	2.0	24.7	4d

Table 4.3. Proliferative rate of different passages of a lymphoma passaged in nude mice.

Vascular tumours

In this colony five angiosarcomas and one angioma were documented. These tumours were present in a variety of sites (Table 4.4) and were evident macroscopically as very vascular tumours, with obvious blood-filled spaces. Microscopically they were composed of elongated, flattened spindle-shaped or polyhedral endothelial cells that line vascular clefts and spaces and sometimes form solid sheets (Figure 4.9). None of these tumours stained positively with an antibody to c-*erb* B2 (Fig 4.10).

Generation	Number of tumours	Site of origin
One	1	Spleen
Two	2	Spleen
		Subcutaneous tissue overlying neck
Three	3	Bladder base - attached by a pedicle
		Uterus - benign angioma
		Spleen

Table 4.4 Characteristics of vascular tumours arising in the colony

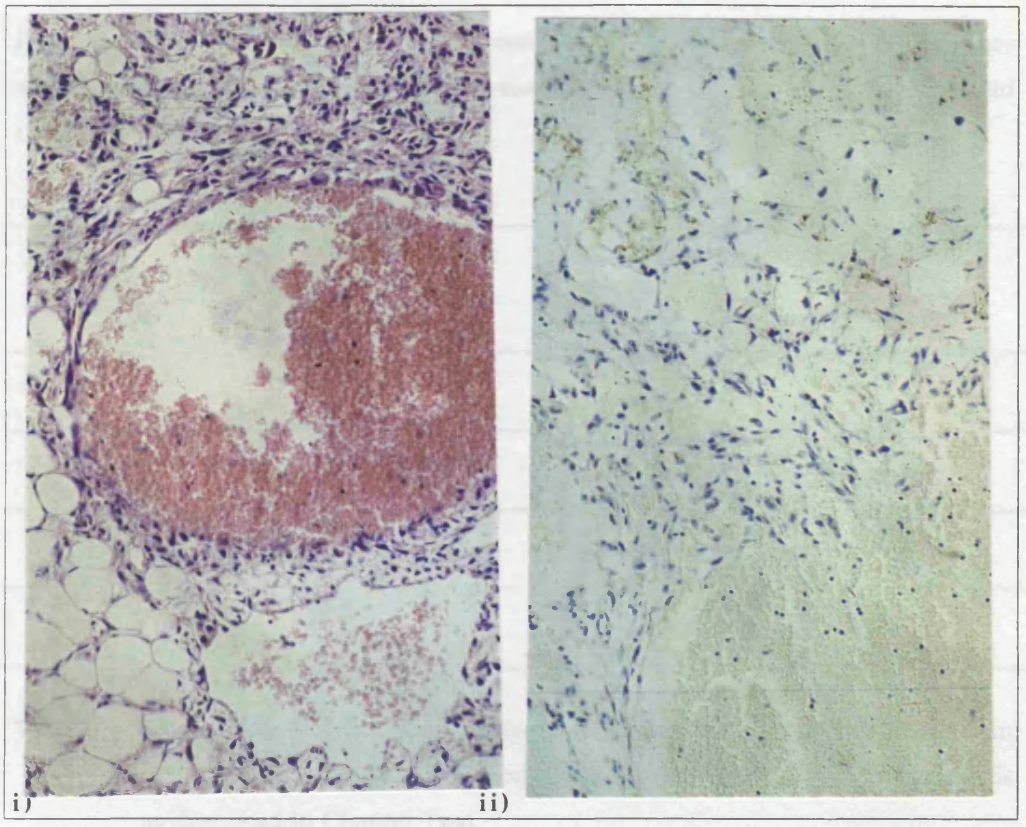


Figure 4.9 i) Angiosarcoma ii) Immunohistochemistry with antibody to c-erbB2

Other tumours

One of the 93 tumours arose in the parotid gland. Salivary gland tissue is known to overexpress the transgene and this tumour expressed the transgene weakly. Histologically this tumour bore a close resemblance to the Harderian gland carcinomas. Spontaneous tumours of the mouse salivary glands, other than myoepitheliomas, are extremely rare (Dunn & Andervont, 1963).

Zymographic Analysis

To date we have assessed 31 tumours, (25 mammary, four Harderian gland carcinomas, one angiosarcoma and one lymphoma). 72kD collagenase was expressed in all of these, probably a function of the presence of stromal tissue. As the levels are too low to quantify it is not possible to say what proportion of this is in active, cleaved form, but a further band, presumed to be the 62kD collagenase, was visible macroscopically in all but two, suggesting some activation (see Figure 4.10). A band consistent with the 92kD form was visible in all the mammary tumours. In 15 of the 25 mammary tumours two distinct bands could be distinguished at a level consistent with the 92kD form. Of the 25 mammary tumours, six had metastases in the lungs.

However there was no correlation between expression of the different collagenases and metastatic behaviour. Of the six non-mammary tumours examined none showed two 92kD bands (Table 4.5).

		Type IV Collagenase			
Tumour	Number of tumours	72	72A	92	92A
Mammary	25	25	23	25	15
Harderian	4	4	4	2	0
Lymphoma	1	1	1	0	1
Angiosarcoma	1	1	0	0	1

Table 4. 5 Expression of different collagenase enzymes by zymographic analysis. 5µm sections of each tumour were homogenized and run on polyacrylamide gels as described in Chapter Two.

Figure 4.10 A zymogram illustrating differential expression of collagenases in a number of mouse tumours. Lane A, Conditioned media from PMA-stimulated HT-1080 cells. Lane B, Conditioned medium from RPMI 7951 cells.

Discussion

An important feature of this model has been the development of diverse tumours, not only in the range of tumours developed but also the diverse biological behaviour within different tumour categories. This is illustrated by their wide-ranging histological appearances and proliferative indices, growth characteristics in nude mice and differential cytokine sensitivity (see Chapter Five).

The advantages and disadvantages of the transgenic mouse model in general have been outlined in the Introductory Chapter. In this colony diversity presents both benefits and drawbacks. An important feature of this colony, and a salient reason for selecting the founder mice is that the mammary tumours resemble human breast cancer both histologically and biologically, in contrast with the phenotype seen in other mammary tumour transgenic mice described. Of the broad range of mammary tumours described in the model most have a similar counterpart within human breast tumours, both architecturally and in some cases cytologically and with respect to c-*erb*B2 immunohistochemistry. In humans the term poorly differentiated ductal carcinoma in situ (DCIS) describes cells with pleomorphic, irregularly spaced nuclei, with coarse, clumped chromatin, prominent nucleoli and frequent mitoses. Necrosis is usually present (Holland, 1994). Although no definite *in situ* carcinoma was seen in the tumours found in this colony the cellular morphology fits this description. It is this resemblance to human tumours which is one of the most relevant aspects of the model as a means of assessing cytokine therapy in humans.

Heterogeneity is itself a characteristic of both human and murine mammary cancer. From a single murine mammary tumour Dexter and colleagues isolated four cell lines with markedly different morphology, *in vitro* growth properties, expression of MMTV antigen and karyotype. Yet these yielded histologically similar tumours upon *in vivo* transplantation (Dexter *et al.*, 1978).

Studies in human breast cancer have shown that abnormal DNA ploidy indicates a worse prognosis than diploid tumours (Merkel & McGuire, 1990). This is consistent with our findings looking at proliferative fraction and suggests that these tumours are predominantly at the more aggressive end of the spectrum, which again is consistent with the comedo-type histological subgroup.

The lymphomas which arose in the colony stained positively with the antibody to c-*erb*B2 - this phenotype is consistent with that seen in association with the human homologue of c-*neu*, c-*erb*B2.

A further aspect of this model is the unpredictability of metastasis. As in human breast cancer, there is no straightforward correlation between tumour size and metastasis, nor age of onset and rate of progression. This lack of homogeneity is a challenge for a new therapeutic agent but its assessment is likely to be closer to a clinical trial than *in vivo* testing on existing murine models of mammary cancer. As outlined in Chapter Three, overexpression of the unactivated *neu* oncogene has been reported to be associated with a higher incidence of lung metastases than that seen in the model of Muller (Guy *et al.*, 1992b). The authors postulate that this overexpression may confer enhanced metastatic potential upon the mammary tumour cell. In the colony described here the overall incidence of metastases was 23 of 53 mice (43%). This is comparable to the findings of Guy *et al* (1992) (Guy *et al.*, 1992b) with the unactivated *neu* oncogene model and make this colony a useful model for the study of metastasis.

In those mice who developed Harderian gland carcinomas metastases did not correlate with the grade of the primary tumour. Indeed, in one case the primary tumour had the appearance of an adenoma but metastases in the lungs were consistent with a malignant Harderian gland carcinoma. This suggests that either a small subclone of cells within the primary tumour transformed prior to metastasis or, less likely, that the metastases themselves changed phenotypically in a different environment.

The specific disadvantages of the colony include the tumour latency, the need to breed an inbred strain and the time and resource costs of that exercise. The most important disadvantage is the unexpectedly high proportion of non-mammary tumours. Not only does this limit the potential pool of mice for therapeutic experiments it carries the risk of mice being treated inappropriately as it is not until after treatment commences that the precise histology will be known. With this particular colony there is also an apparent relationship between the number of litters, the type of tumour developed, and its subsequent behaviour. It is therefore important that all mice have the same reproductive history to place them at a comparable level of risk.

A further problem is the change in phenotype which appears to be occurring with each successive backcross. For this reason any experimentation should be performed on animals from the same generation and successive generations should not be pooled.

In summary, this is a diverse model which has certain parallels with human breast cancer. This makes it an adjunct to existing animal models in the assessment of novel therapies for cancer. In an attempt to predict suitable therapies for human cancer we

have successfully transplanted a number of tumours into nude mice and used these as the basis for modelling cytokine therapy. These are discussed in detail in Chapter Six. We have also treated groups of mice from generations three and four of the colony with longterm cytokine therapy as prophylaxis for tumour development. These mice, the spectrum of tumours which they developed, and the influence of cytokines on this tumour development are discussed in detail in the following chapter.

Chapter Five

Therapy of tumour transplants

Nude mice - Historical Background

Human malignant tissues were first successfully transplanted into athymic (nu/nu) mice in 1969. The nude mouse has since become established as the model for the transplantation of human tumour xenografts and is a major tool for tumour biology studies, preclinical drug testing and assessment of the tumourigenic potential of cancer cell populations (Winograd *et al.*, 1987).

Rationale

One reason for establishing a colony of oncogene transgenic mice was to obtain a source of spontaneous tumours which could be used to test new cancer therapies. Because of the time-scale involved in establishing an inbred colony under specified-pathogen free conditions, we sought a means of assessing tumour cytokine sensitivity, appropriate routes and schedules, while the colony was being developed. Mammary tumours arising in the transgenic mice were therefore transplanted into nude mice and used for cytokine therapy experiments. As early generations of the colony were not genetically homogeneous they could not be transplanted into immunocompetent mice. In this way a range of tumours could be assessed using different cytokines, alone and in combination, and different schedules. Because of the limitations of assessing cytokines in nude mice, which have been discussed in Chapter 3, attempts continued to be made to transplant tumours into siblings or offspring once the colony had reached generation three.

Interferons and Breast Cancer

We have examined the role of two IFNs with cross-species specificity in the nude mouse tumour transplants of our model: IFN-α A/D hybrid, a recombinant human hybrid molecule with strong activity on murine cells; (Balkwill *et al.*, 1982; Rehberg *et al.*, 1982) (Table 5.1) and recombinant rat IFN-γ, which has cross species specificity and is more readily available than the murine homologue(Balkwill *et al.*, 1986b; Ramani *et al.*, 1986).

The experiments outlined in this chapter were used to predict the interferon responsiveness of the tumours arising spontaneously in the colony.

Transplanted tumours

Tumours used for transplantation into nude and BALB/c mice

Seven mammary tumours arising in the colony were transplanted into nude mice successfully. One lymphoma (Tumour 8 - Table 5.1), was transplanted into nude mice successfully and a ninth tumour (mammary) from a later generation was successfully transplanted into BALB/c mice. Three other tumours transplanted into BALB/c mice did not become established. The characterisitics of the seven mammary tumours transplanted into nude mice are outlined below in Table 5.2.

In each of the nine transplants (eight into nude mice - seven mammary, one lymphoma; and one into BALB/c - mammary) the interval between passages became shorter with each successive passage. This is demonstrated for all nine transplants (Table 5.2). For each transplant a comparison has been made of the interval between passage numbers 1-7 and that between passage numbers 37-44. These were chosen in order to compare different tumour transplants at a comparable point in their life cycle.

Mean passage time Tumour	1	2	3	4	5	7	8*	9^
First 7 passages (days)	32	42	49	39	37	34	44	21
Passages 37-44 (days)	16	24	13	23	16	12	12	10

Table 5.1 Mean passage time of first 7 passages and passages 37-44 of Transplants 1-9

*Lymphoma ^Transplanted into BALB/c mice. Statistical Significance p = 0.006 (Wilcoxon matched pairs signed rank sum test)

Tumour	Age of mouse	Histological features	Human c-*erb*B2	Other immuno	Metastases at first transplant	Changes with passage
1	8/12	Poorly differentiated comedo-type breast carcinoma	+	Collagen 4 - BM invasion seen	None	Became more pleomorphic
2	10/12	Epithelial and sarcomatous characteristics	After passage 1	S-100 & smooth muscle actin neg	None	More carcinoma-like features
3	12/12	Mixed solid and papillary type	+	-	None	None
4	12/12	Solid-type with small papillary area	+	-	None	Comedo necrosis more pronounced
5	12/12	Solid-type tumour	+	-	None	None
6	13/12	Large cell, high grade tumour with areas of comedo-necrosis	+	-	In lungs	None
7	14/12	Mixed papillary and comedo elements	+	-	Numerous in lungs	Comedo necrosis more pronounced

Table 5.2 Characteristics of seven mammary tumours arising in colony transplanted into nude mice

Experiments with nude mouse transplants of mammary tumours

Using Freireich's formula, described previously, a dose of $5x10^4$ U/animal/day of both rhIFN-α A/D hybrid and rat IFN-γ was used, equivalent to a dose of $5x10^6$ U/m^2 in a human (Freireich *et al.*, 1966). Initially the IFN-α was given subcutaneously (sc) and the IFN-γ intraperitoneally (ip) in order to achieve optimal circulating levels, based on previous pharmacokinetic studies (Balkwill, 1986). Regular ip injections were poorly tolerated, and occasionally associated with haemorrhage which led to investigating the use of sc rat IFN-γ further. An experiment was set up in one of the mammary tumours growing in nude mice (Tumour 1) to compare sc and ip administration. The median survival of mice in the control diluent-treated group was 28 days compared with 39 days in the sc group and 31 days in the ip group ($p = 0.23$). As there was no statistically significant difference in survival or tumour volume between the two treatment arms it was decided to switch to sc administration thereafter.

Initial experiments involved Tumour 1. They formed the basis for later experiments to assess the value of the cytokines individually and in combination and establish the optimum schedule and duration of therapy (see Table 5.3).

Experiment (passage number)	Type	Cytokine treatment Duration	End-points	Statistical Significance	Conclusions
A (3)	Fixed time-point	IFN-α or -γ 3 weeks	a) tumour volume b) tumour wt on sacrifice	a) NS b) NS	Start therapy sooner (D7 not D12). Smaller tumour volume (0.05 not 0.1ml)
B (5)	Survival	IFN-α or -γ 3 weeks	a) tumour volume b) tumour wt on sacrifice c) median survival	a) CD vs IFN-α, p=0.001 CD vs IFN-γ, p = 0.005 b) NS c) Overall logrank, p = 0.02	Use combination therapy
C (8)	Survival	IFN-α , γ and α + γ 3 weeks	a) tumour volume b) tumour wt on sacrifice c) median survival	a) CD vs IFN-α, CD vs IFN-γ, NS. CD vs IFN-α+γ, p = 0.006 b) NS c) Logrank on CD vs IFN-α+γ, p = 0.037	Six weeks duration of therapy. No further evaluation of tumour weight on sacrifice.
D (13)	Survival	IFN-α, -γ and α + γ 6 weeks	a) tumour volume - 4/52 b) median survival	a) NS b) NS	Cytokine sensitivity of Tumour 1 has changed between passage 8 and passage 13.

Table 5.3 Summary of experiments carried out with passages of Tumour 1

Experimental design

The endpoints used in clinical studies - complete and partial regression (complete disappearance of all assessable tumour and greater than 50% regression, respectively) are usually defined on the basis of investigations such as tumour markers and radiology (X-rays, computerised tomography etc). In animal models the end points of tumour stasis, reduction in growth rate, or a reduction in number of metastases, may be useful in terms of understanding mechanisms, but would not necessarily translate into a useful clinical outcome. Two main endpoints were used in the initial experiments - both with advantages and disadvantages.

- *Fixed time-point experiments* have the advantage that tumour weights and volumes can be compared at the same time point from the intervention being assessed. However they do not enable a difference in survival to be seen.

- *Survival experiments* in mice are influenced by more subjective factors relating to the general condition of the animal which may be unrelated to the presence of the tumour.

As a consequence of animal husbandry regulations fewer mice survive to the later time points in either type of experiment and hence less data is available on these points.

Tumour1

The details of the four experiments carried out on different passages of tumour 1 (A,B,C and D) are summarised in Table 5.3.

In the first experiment 0.1ml of tumour was injected on Day 0 and cytokine therapy with $5x10^4$ U/animal/day of either rhIFN-α A/D hybrid or rat IFN-γ was started on Day 12. The tumour grew rapidly in all groups and the experiment had to be terminated at 22 days. Tumours in the cytokine-treated groups were smaller but this was not statistically significant.

Subsequently only 0.05ml tumour was injected and cytokine therapy was started on Day 7. Cytokine sensitivity was demonstrated by logrank survival of the control diluent versus IFN-α alone and the overall logrank survival for all three groups was statistically significantly different. Mean tumour volumes at 21 days were statistically significantly smaller in both cytokine-treated groups than control (Figure 5.1). On this basis we went on to use the two cytokines in combination which resulted in a statistically significantly prolonged survival compared with either agent alone (logrank survival, data not shown).

Conclusion: Longer period of therapy warranted, subsequent experiments entailed six weeks treatment.

In the final experiment with this transplant the cytokine sensitivity appeared to have changed and this phenomenon is discussed further at the end of this chapter (Figure 5.2).

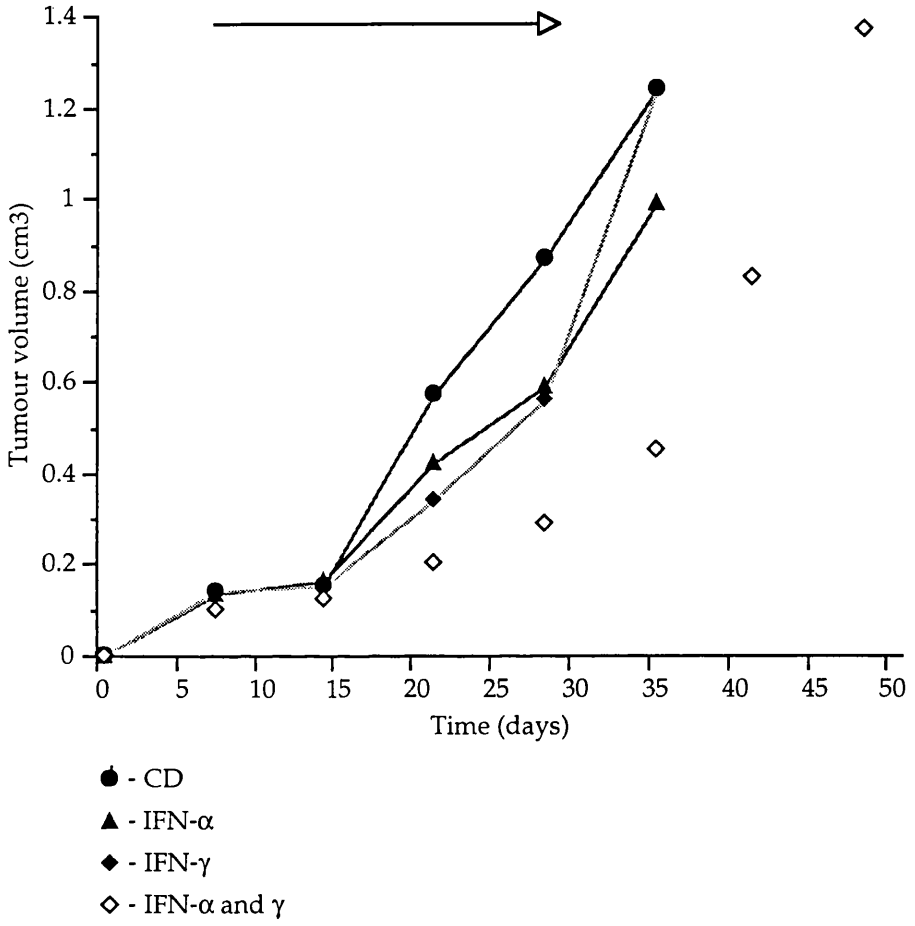


Figure 5.1 Experiment using passage 8 of tumour 1. Graph of mean tumour volumes (cm^3) with time. Cytokine therapy between Day 7 and Day 28. Bold arrow denotes period of cytokine therapy.

Survival

Overall logrank survival for all four groups p = 0.230

Logrank on CD vs IFN-α p = 0.444

Logrank on CD vs IFN-γ p = 0.297

Logrank on CD vs IFN-α and γ = 0.037

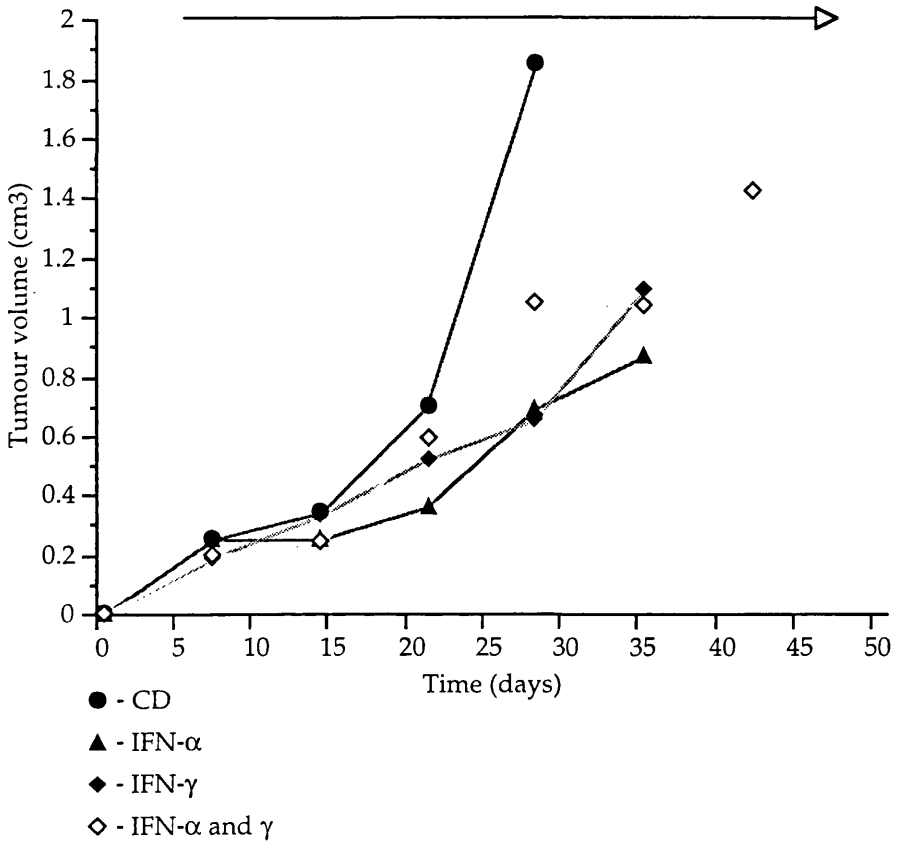


Figure 5.2 Final experiment using passage 13 of tumour 1. Graph of mean tumour volumes (cm^3) with time. Cytokine therapy between Day 7 and Day 49. Bold arrow denotes period of cytokine therapy.

Survival

Overall logrank survival for all four groups p = 0.570

Logrank on CD vs IFN-α p = 0.555

Logrank on CD vs IFN-γ p = 0.226

Logrank on CD vs IFN-α and γ = 0.557

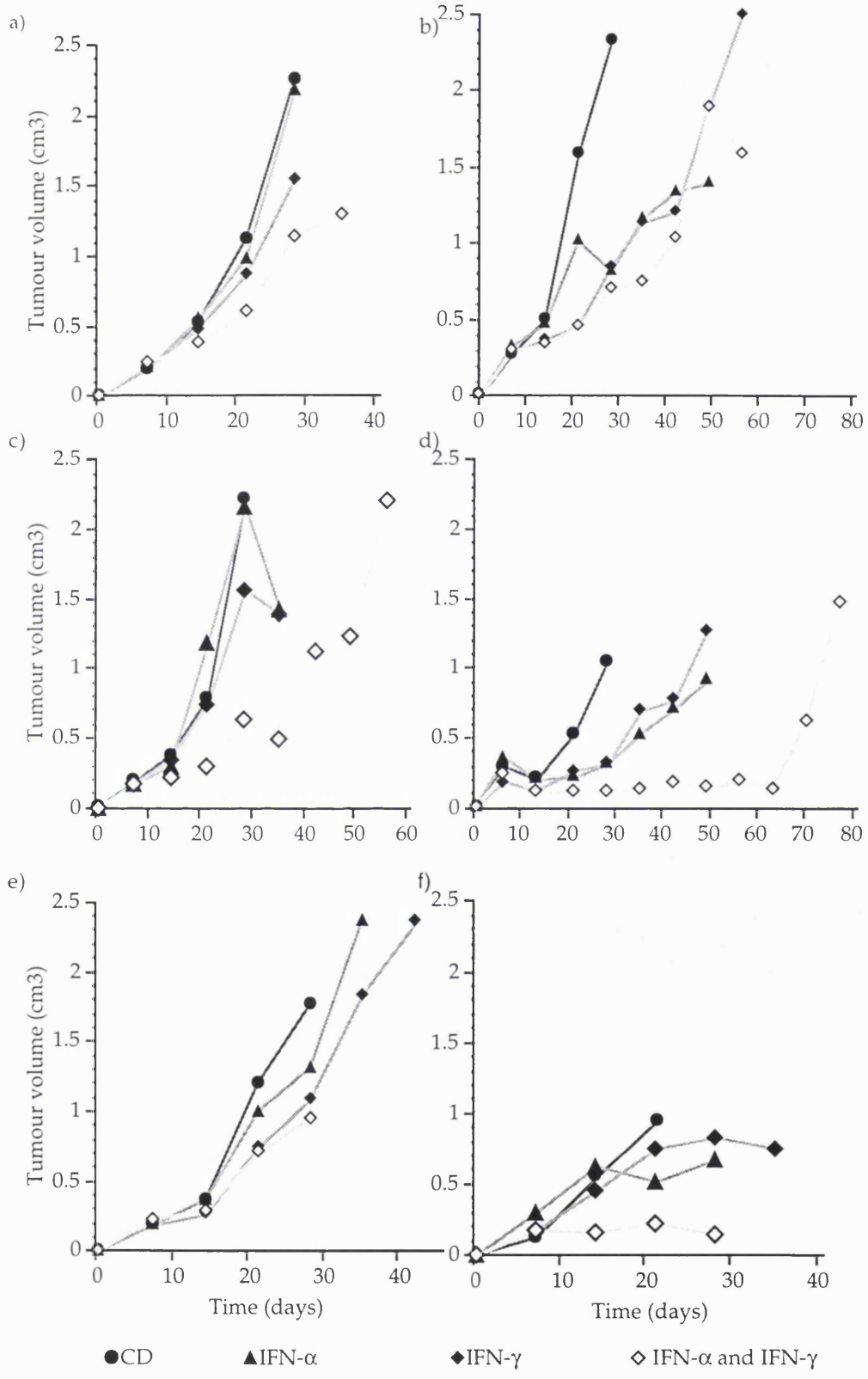
In each experiment with Tumour 1 there was evidence of tumour growth retardation during the period of therapy, in comparison with the control diluent treated groups. This was not always statistically significant. Looking at changes in tumour volume throughout the experiments tumour growth appears to accelerate at the cessation of therapy. This is best illustrated graphically in Figure 5.1.

Translating results with tumour 1 to other tumours

Having established the principles of injecting mice with 0.05ml tumour on Day 1, commencing therapy on Day 7 and continuing for six weeks duration, these were then applied to the other transplanted tumours. All experiments were survival experiments investigating the effect of each interferon alone and the two in combination. The outcomes measured were tumour volumes and survival. The tumour volume data for each of the transplants is collated in Table 5.4 and represented graphically in Figure 5.3. The logrank and median survival data is colated in Table 5.5 and represented graphically in Figure 5.4 .

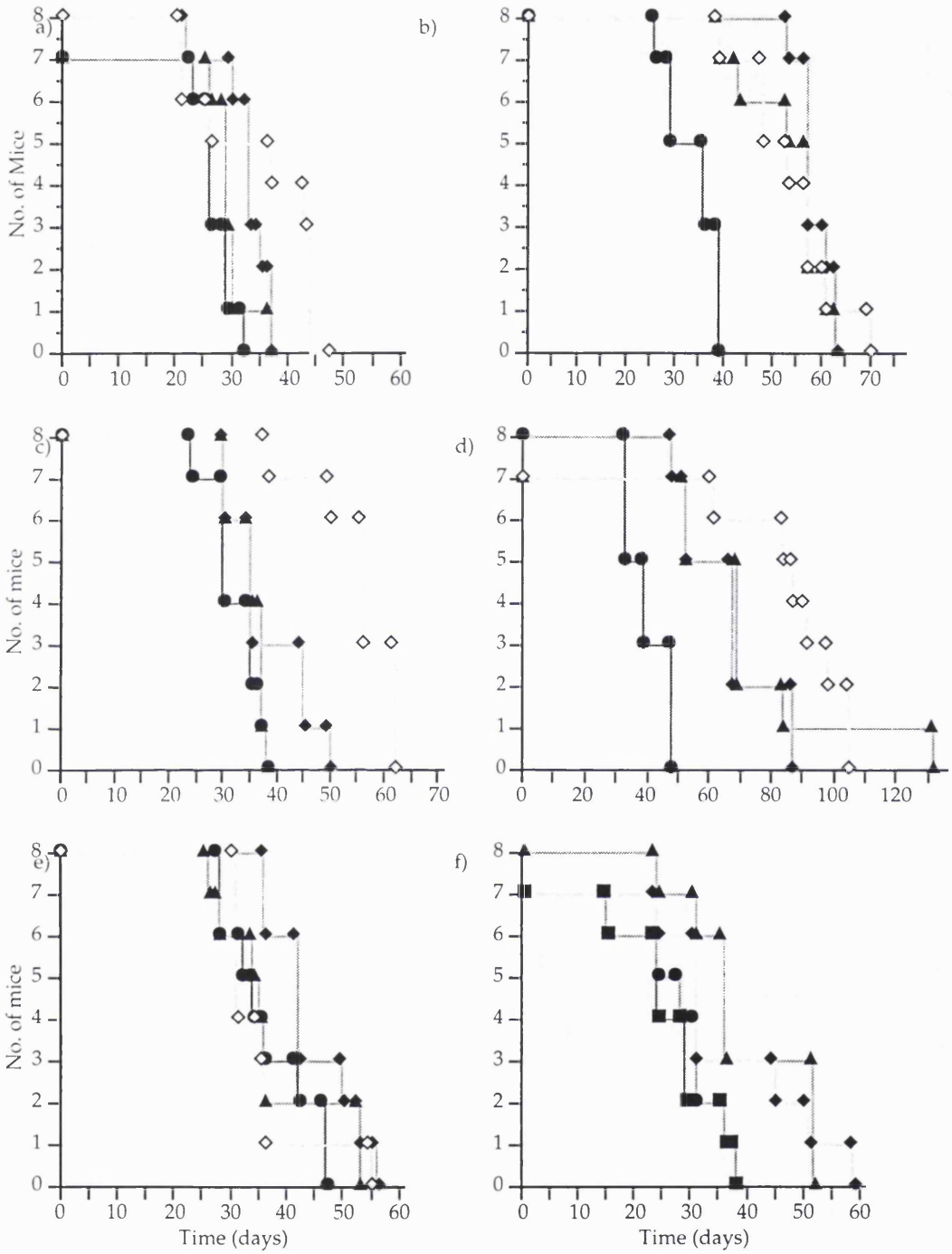
Transplant	Mean tumour volume at 4 weeks (cm^3)				Student's t-test		
	CD	IFN-α	IFN-γ	IFN-α+γ	CD vs IFN-α	CD vs IFN-γ	CD vs IFN-α+γ
1	1.61	0.69	1.34	1.04	0.201	0.720	0.423
2	1.74	1.80	1.37	1.14	0.872	0.274	0.068
3	2.32	0.82	0.84	0.70	0.014	0.015	0.013
4	2.21	1.81	1.55	1.23	0.353	0.192	0.025
5	1.05	0.34	0.31	0.11	0.004	0.003	0.000
6	1.77	1.30	1.08	0.95	0.257	0.093	0.044
7	1.43	0.77	0.88	0.39	0.022	0.069	0.001

Table 5.4 Summary of mean tumour volume at 4 weeks for each Transplant, with statistical significance. The values for Tumour 1 were obtained from the fourth experiment in which therapy lasted 6 weeks.



a) Tumour 2 b) Tumour 3 c) Tumour 4 d) Tumour 5 e) Tumour 6 f) Tumour 7

Figure 5. 3 Graphs of mean tumour volume (cm^3) with time for each transplanted tumour. Cytokine therapy administered from Day 7 to Day 49



a) Tumour 2 b) Tumour 3 c) Tumour 4 d) Tumour 5 e) Tumour 6 f) Tumour 7

Figure 5.4 Logrank survival curves of all six transplanted tumours 2 - 6. Cytokine therapy administered from Day 7 to Day 49.

Transplant	Logrank survival				Median survival (days)			
	CD vs IFN-α	CD vs IFN-γ	CD vs IFN-a +γ	Overall (all 4 groups)	CD	IFN-α	IFN-γ	IFN-a +γ
1	0.56	0.23	0.56	0.57	34	42	56	47
2	0.27	0.02	0.14	0.06	26	29	33	40
3	0.003	0.001	p<0.001	0.003	36	57	57	55
4	0.53	0.18	0.001	0.006	32	36	35	56
5	0.003	0.004	0.003	p<0.001	39	69	67	91
6	0.64	0.10	0.99	0.45	35	36	42	34
7	0.84	0.07	0.08	0.13	29	31	36	31

Table 5.5 Summary of logrank survival and median survival data for each transplant. The values for Tumour 1 were again obtained from the fourth experiment in which therapy lasted 6 weeks.

Tumour 2

Passage 19 of Tumour 2 was used for this experiment. Taking tumour volume at the 4 week time-point, or log-rank survival, there was no statistically significant evidence of sensitivity to IFN-α alone or the combination of IFN-α and IFN-γ. The graph of tumour volume (Figure 5.3(a)) with time suggests the tumour was sensitive to IFN-γ, although the p-value was not statistically significant (p= 0.068). Survival was statistically significantly prolonged for the group receiving IFN-γ although the combination of the two approached significance (Figure 5.4 (a)). Only the log-rank survival statistic for IFN-γ alone was significant

Conclusion: With Tumour 2 IFN-γ alone was more effective than the cytokine combination.

Tumour 3

Passage 9 of Tumour 3 was used for this experiment. In terms of tumour volume, fixed time-point data at 4 weeks showed the combination to be more effective than either

cytokine alone. Figure 5.3(b), a graph of mean tumour volume with time, shows the benefit of the combination to be particularly evident after completion of treatment, when the IFN-γ alone and combination groups diverge. Taking log-rank survival into account IFN-γ alone was more effective than the combination, although median survivals were similar in all three cytokine-treated groups (Figure 5.4(b).

Conclusion: There was sensitivity to the individual cytokines and the combination, with the latter being the most effective.

Tumour 4

Passage 12 of Tumour 4 was used for this experiment. Once again an increase in tumour volume was evident after therapy had been discontinued (Figure 5. 3 (c)). The combination of IFN-α and -γ had a significant effect on survival (Figure 5.4(c)).

Conclusion: This tumour had cytokine sensitivity and the combination of IFN-α and IFN-γ was more effective than either cytokine alone.

Tumour 5

Passage 6 of Tumour 5 was used for this experiment. The best anti-tumour effect was seen in the combination group . Tumours in this group grew more slowly for some time after treatment had finished (Figure 5.3(d)). Again cytokine sensitivity was demonstrated, with individual cytokines and their combination being effective in respect of both tumour volume and survival (Figure 5.4 (d).

Conclusion: These results suggest this tumour had cytokine sensitivity and that the combination of IFN-α and IFN-γ was more effective than either cytokine alone.

Tumour 6

Passage 6 of Tumour 6 was used for this experiment. There was no statistically significant difference between any of the groups in relation to logrank survival (Figure 5.4(e)) or tumour volume with time (Figure 5.3 (e). The only distinction (not apparent from the graphs) is that the tumours in the combination therapy group were smaller at sacrifice than those in the other groups. This is illustrated by the different mean tumour weights at sacrifice (CD vs IFN-α+IFN-γ, $p = 0.002$). The reason for killing the mice in this group was their general condition, as opposed to tumour size. Many of them were unwell and losing weight, and some had ulcerating tumours.

Conclusion: It was felt there may be an interaction between tumour and cytokine in this group which caused the apparent toxicity of the combination of the two

cytokines. There was no statistically significant difference between the treatment groups in relation to logrank survival or tumour volume with time (Figure 5.3 (e)).

Tumour 7

Passage number 5 of tumour 7 was used for this experiment. Tumours in the groups receiving combination therapy were significantly smaller at the time of the mice being killed than those in other groups. Of note at post mortem three of the mice were noted to have a jelly-like substance around the tumour and the overlying skin was thickened and yellow. There was no statistically significant difference between the groups in relation to logrank survival (graph not shown). As in experiment 9 these mice were sacrificed as a result of their general condition and not tumour size - all mice had been killed prior to completion of 42 days therapy (Figure 5.3 (f)).

Conclusion: There was no statistically significant difference between the groups in relation to logrank survival

Summary of IFN sensitivity of tumours transplanted into nude mice.

In Figure 5.5 the percentage increase or decrease in survival of the cytokine treated mice is calculated in relation to survival of the control diluent treated mice. This figure demonstrates the marked diversity between different tumours in their sensitivity to interferons. With one exception, all the cytokine treated animals survived longer (but not always statistically significantly so) than control diluent treated mice. IFN-α treatment caused a significant increase in survival in two of seven different tumour lines ($p=0.003$), IFN-γ in 3 of 7 ($p=0.02$, $p=0.003$, $p=0.001$) and IFN-α/γ combinations in three of seven ($p=0.001$ or $p=0.003$). Three of the tumours failed to respond significantly to either IFN or their combination. Two of the tumours responded significantly to both IFN-α and γ, and in only one case, did the combination of these work in the absence of a response to the individual cytokine.

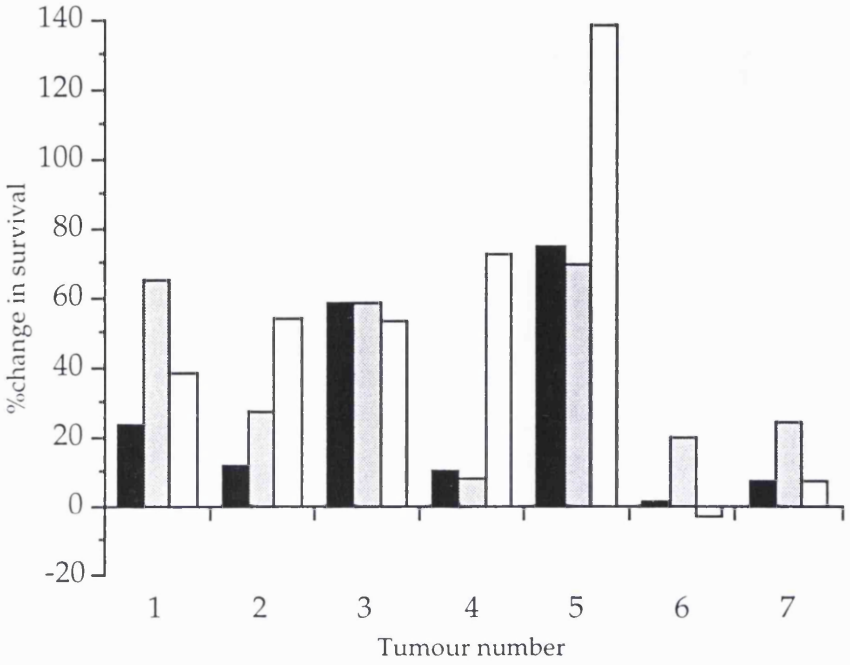


Figure 5.5. Percentage increase in survival of nude mice bearing transplanted mammary tumours and receiving interferon therapy ■IFN-α ▒ IFN-γ□ IFN-α +-γ

The diversity and cytokine sensitivity of the seven different tumour transplants is also reflected in the percentage decrease in mean tumour volume at 4 weeks illustrated graphically in Figure 5.6 and in Table 5.4.

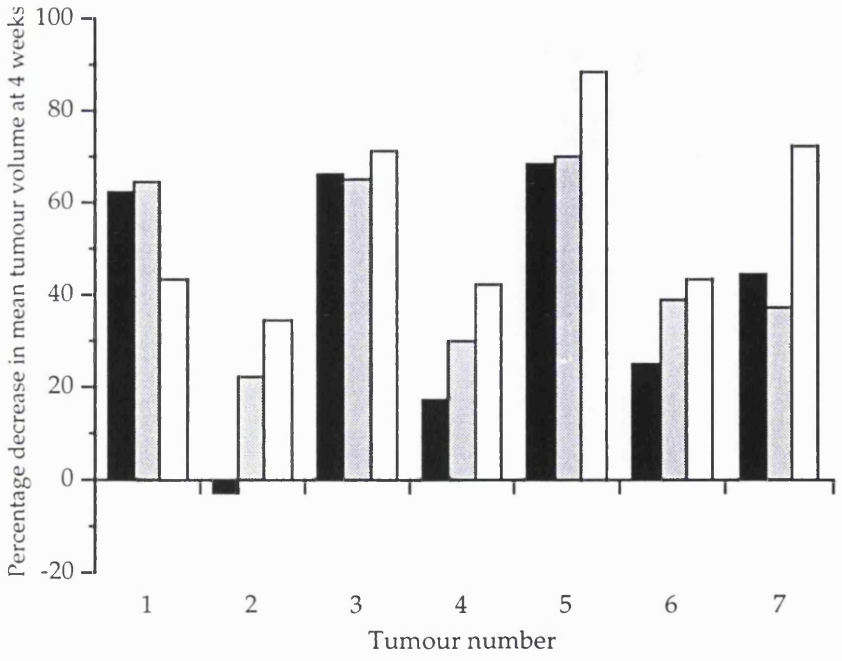


Figure 5.6. Change in mean tumour volume of mammary tumours at 4 weeks comparing IFN therapy with CD treated nude mice. ■IFN-α ▒ IFN-γ□ IFN-α +-γ

Importance of passage number

Some of the transplanted tumours changed phenotypically with successive passages. This phenomenon was best demonstrated with Tumour 1, where a change in cytokine sensitivity was evident between Experiments C and D (Table 5.4). Experiment D was performed with passage 13 whereas Experiment C utilised passage 8. There was no convincing evidence of significant cytokine sensitivity, either in terms of mean tumour volume (Figure 5.2) or logrank survival. However mean tumour volumes with time were all smaller in the cytokine-treated groups, and median survivals were all longer.

These changes in cytokine sensitivity parallel other objective changes. Early passages grow more slowly than later ones, as illustrated in Table 5.3, which compares the mean passage time of the first 7 passages and passages 37-44 of Transplants 1-9 (See Table 5.3). Secondly data from S-phase fraction analysis shows a rise in this value between early and later passages (see Table 5.6). Thirdly morphological changes are seen histologically (increased numbers of mitoses, greater pleomorphism, poorer differentiation) - all of these features are consistent with a change in cytokine sensitivity. This is likely to have influenced the comparability of experiments perfomed on different passages and underlines the importance of carrying out cytokine therapy experiments in as early a passage as is feasible. The phenomenon of murine tumours changing when passaged in mice was described first in the 1950s by Foulds(Foulds, 1954).

S-phase fraction analysis was performed on three different transplants of Tumour 1. By passage 14 there was a marked increase in the S-phase fraction in comparison with the first two passages.

Tumour 1 (passage no.)	DNA Index	S-phase fraction (%)	% Aneuploidy
1	1.9	9.0	75
2	1.9, 2.2	7.7	84
14	1.9	14.6	76

Table 5.6 S-phase fraction data on passages of Tumour 1. S-phase fraction calculated from aneuploid fraction , DNA index in passage 2 - two peaks.

An interesting observation made at post mortem in tumours 2 and 7 in the IFN-α and combination-treated groups is that they were surrounded by thickened skin, which

was jelly-like under the outer layer, with a well-developed network of blood vessels, making the tumours bloody in appearance.

Syngeneic Transplanted Mammary Tumour

One tumour from the fourth generation arose in a 13-month old female transgene-positive mouse which had littered twice. The tumour arose over the left shoulder in the mammary line and there were no other abnormalities at post mortem examination. Injected into the flank of two offspring it became established after about sixteen weeks and was then passaged into other mice from the same litters. By passage three it was found to grow readily in ordinary BALB/c mice. This tumour, named HTH-K, has been further passaged successfully and used in cytokine therapy experiments. Histologically it is a mammary tumour with a comedo-type pattern, which has an extensive network of blood vessels and areas of necrosis. No metastases have been seen in the transplanted mice at post mortem examination to date. The tumour has become progressively more aggressive on passaging and it is currently necessary to passage it every 12 days. S-phase analysis is detailed in Chapter 4.

Survival Experiment with Transgenic Mammary Tumour

Interleukin-12 (IL-12) is a heterodimeric cytokine which plays an important part in induction of cell-mediated immunity. Its exerts its effects through T cells and hence was of relevant here to examine its effects in an immunocompetent model.

Two groups of eight mice were treated with daily injections of 0.1ml control diluent or 1μg IL-12 (in 0.1ml). The CD group had all been killed by Day 32, whereas of the eight mice in the IL-12 treated group, one survived until Day 233 and two had complete tumour regression and lived for more than 250 days (Figure 5.7). The mean survivals were 30.1 days (SE 0.92) in the CD-treated group and 122.8 days (SE 35.86) in the IL-12 treated group. The experiment was repeated with a later passage of the tumour, and the findings were similar. The tumour regressed completely in one IL-12 treated mouse (Figure 5.8).

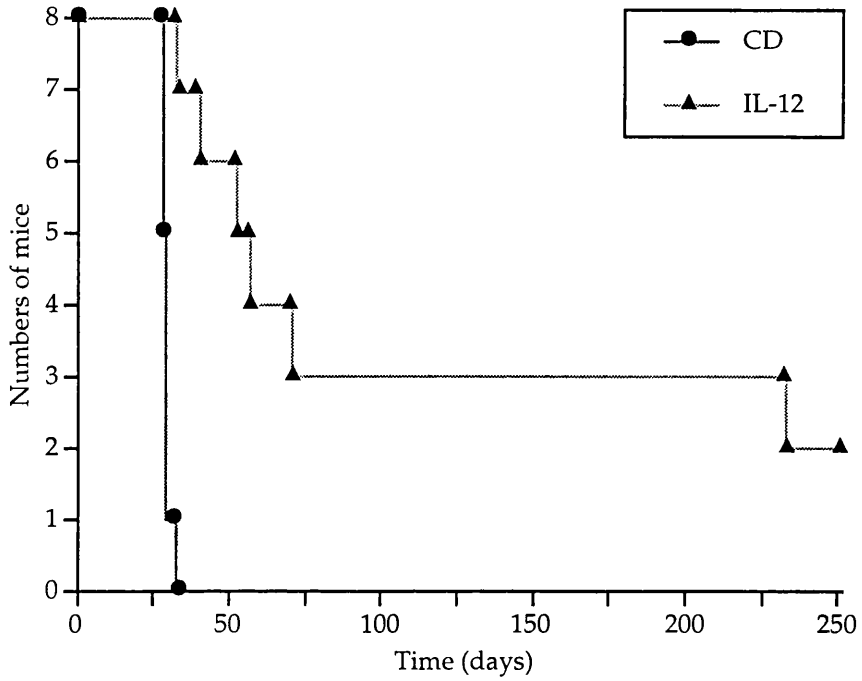


Figure 5.7 Survival of BALB/c mice bearing transgenic-mouse murine mammary tumour treated with control diluent or rmIL-12 ($X^2 = 6.3$, $p = 0.01$).

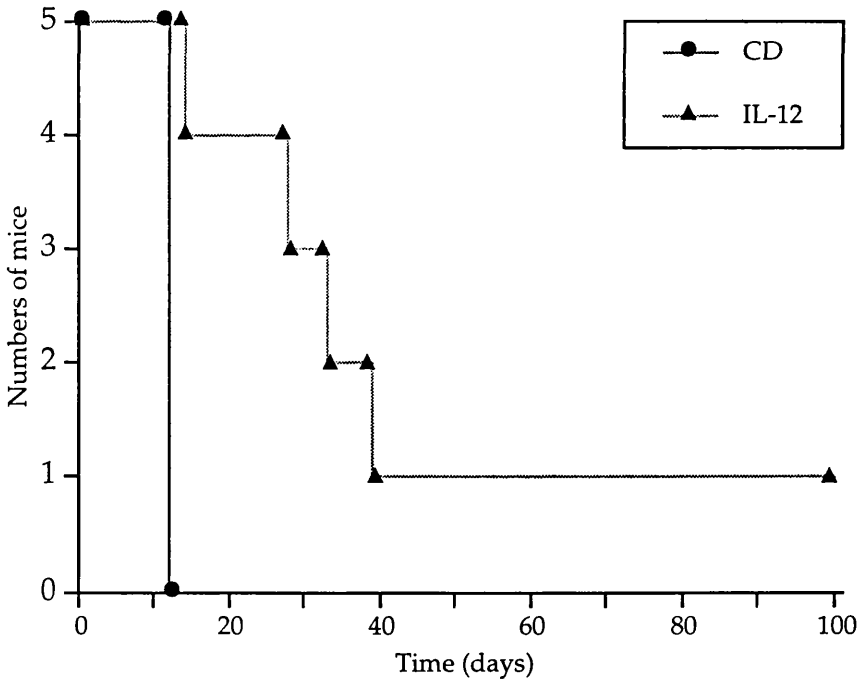


Figure 5.8 A later experiment of the same type as that performed in Figure 5.7 using a later passage of the same tumour.

Discussion

There is extensive experience of the interferons in the therapy of human tumour xenografts, some of which relates to breast cancer xenografts. Xenografts have been used to look at the direct antitumour activity of a wide range of IFN types and subtypes and to determine optimal doses and regimens (Crane *et al.*, 1978). The antitumour effect is most noticeable if therapy is commenced shortly after tumour injection (Hofmann *et al.*, 1985) which supports the contention that IFNs are best used in the presence if minimal residual disease or in an adjuvant setting. Balkwill *et al*, (1986), looked at the mechanisms of antitumour action of interferons on human tumour xenografts (Balkwill *et al.*, 1986a). Using human lymphoblastoid interferon (IFN-α(Ly)) the growth of 3 early passage human tumour xenografts was significantly inhibited. As with our experience, cessation of therapy resulted in regrowth of tumour after a lag period of 1-3 weeks. This growth inhibition has been shown to be the result of a direct effect on the tumour and not a modulatory effect on the nude mouse host (Balkwill *et al.*, 1982). Using rHuIFN-γ at the same dose the growth of only one of these three breast xenografts was inhibited (Balkwill *et al.*, 1986a). Direct administration of rHu IFN-γ into the tumour did not improve its therapeutic efficacy (Balkwill *et al.*, 1989). Much of our understanding of IFN-γ is based on *in vitro* evidence (Kumar & Mendelsohn, 1994; Marth *et al.*, 1993) . Some of the *in vivo* evidence is also conflicting - Kelly *et al* showed in a metastasis model that preincubation with IFN-γ produces a significant increase in experimental metastases in syngeneic BALB/c and BALB/c nude mice (Kelly *et al.*, 1991). Matthys et al have shown that early treatment with anti-interferon-γ antibody inhibited tumour growth (Matthys *et al.*, 1991). Both of these findings suggest a role for endogenous interferon gamma in the metastatic process and tumour cell proliferation, respectively.

The work outlined here demonstrates that the mammary tumours arising in the transgenic mouse colony grew readily in nude mice and such transplants were used in preliminary cytokine therapy experiments. The aim of these experiments was to develop treatment schedules which could be translated to spontaneously arising tumours; assess the inherent cytokine sensitivity of these tumours, and to assess the inter-tumour variation in response. In general IFN therapy had a modest beneficial effect on survival, only two complete regressions being recorded in over 150 treated tumours. Taking both tumour volume and survival into account the combination of both IFNs delayed tumour growth most effectively in the majority of transplants. Three of the tumour lines failed to respond significantly to either IFN or their combination in terms of survival. This lack of response is similar to the human experience with these cytokines in solid tumours (Gutterman, 1994). The diversity of response of individual

tumours is again analogous to results obtained in clinical trials with several cytokines (Sparano & O'Boyle, 1992).

There are very few published *in vivo* experiments entailing the combination of IFNs α and γ. The combination was effective in the Renca syngeneic model of murine renal carcinoma. A synergistic effect was observed in this model when murine IFN-α was given in combination with low dose IFN-γ (1-10 Units/ml). This combination was less effective in nude mice and 75% of the euthymic mice were shown to be immune to rechallenge. Although IFNs may directly inhibit the growth of the Renca tumour, this study demonstrates that specific immune response plays a dominant role (Sayers *et al.*, 1990). In a murine bladder cancer model combinations of a number of cytokines, incuding IFN-α and -γ were more effective than therapy with either agent alone (Riggs *et al.*, 1992). In breast cancer xenografts the combination has been used for intralesional treatment and this was further potentiated by recombinant human TNF-α(Riggs *et al.*, 1992). In humans combination therapy with cytokines is generally more toxic than the effect of individual cytokines.

Another interesting phenomenon is the increase in growth of transplanted tumours after the cytokine therapy has been discontinued. This is best demonstrated in Tumours 4 and 5. It clearly underlines the importance of long-term administration of cytokines and the appropriateness of cytokine therapy in the setting of minimal residual disease. This is illustrated graphically for a number of the tumours and is most consistently evident in the combination group (See Figure 5.1 - Tumour 1(best seen in combination group); Figure 5.3(b) - Tumour 3 (IFN-γ group); Figure 5.3 (c) - Tumour 4 (combination group); Figure 5.3(d) - Tumour 5 (combination group)). This suggests a suppression of tumour growth by the cytokines. It is difficult to predict whether this would be clinically useful.

An important feature of this model, which differentiates it from other available models, is diversity. This is reflected in several ways in the tumours which arise - their histological diversity, their biological diversity and the diversity of response to cytokine therapy.

The management of breast cancer is at the beginning of a new phase. With the advent of improved support, in the form of colony-stimulating factors, it is now feasible to undertake intensive chemotherapy in the hope that micrometastatic disease can be treated. Treatment on the assumption of microscopic deposits placing patients at risk of relapse will become more widespread. Alongside the model of follicular lymphoma, myeloma and melanoma it is conceiveable that a role for the interferons,

in an adjuvant setting, after high dose treatment or peripheral stem cell rescue, will emerge.

Chapter Six

Prevention of mammary tumours with cytokine therapy

Tumour Prophylaxis - Introduction and Rationale

Chemoprevention is the use of a pharmacologic or dietary compound to block or inhibit development of a malignancy from normal or preneoplastic tissue. The chemoprevention of breast cancer is not a new concept and in mammary tumour models candidate preventive agents are being identified. However these necessitate a working model of breast carcinogenesis, which relates to the epidemiologic, clinical and laboratory data.

The growth inhibitory effects of many cytokines make them interesting candidates for novel preventive or therapeutic modalities. The closest clinical application to a prophylactic role is the use of cytokines, particularly IFN-α, as maintenance therapy after bone marrow transplantation for myeloma or lymphoma (Mandelli *et al.*, 1992; Solal-Celigny *et al.*, 1993); The use of cytokines at low dose, on a long term basis, may stimulate the above processes on early undetectable tumours (Kirkwood *et al.*, 1996); Smith, 1992 #153 .

Experience in melanoma

Interferon α-2b has been used in the treatment of melanoma in patients with deep tumours or involved lymph nodes at high risk of relapse. The Eastern Cooperative Oncology Group (ECOG) conducted a randomised controlled trial in 287 patients (20MU/m2/day iv for 1 month followed by 10MU sc three times weekly for 48 weeks,) versus observation (Kirkwood *et al.*, 1996). The treated group had significant prolongation of relapse-free and overall survival compared with the control group. The median follow-up time is now 6.9 years and there was an increase in median disease free survival from 1 to 1.7 years and overall survival from 2.8 to 3.8 years. The greatest benefit was seen in patients with positive lymph nodes. No adjuvant therapy has previously shown an impact on survival in this disease. Previous studies in melanoma entailed low doses for prolonged periods or very high doses for short periods (Cascinelli *et al.*, 1994; Creagan *et al.*, 1995). Further studies are now underway in an attempt to define those groups of patients most likely to benefit from this therapy. The ECOG study suggests intermediate doses for a period of about one year are the most effective regime used to date (Balch & Buzaid, 1996).

This study

The aim of these experiments was to examine the chemopreventive potential of different cytokines, as a preclinical study for human breast cancer. By determining whether they had a role in the prevention of spontaneously arising *neu*-associated mammary tumours in transgenic mice, it was hoped this could be translated into an application in c-*erb* B2 associated human breast cancer.

Before the tumour incidence in the colony was apparent two prophylaxis experiments were established with mice from the third and fourth generations. Once transgene positivity had been established, all female mice in the colony were mated for two litters and then recruited. The time and duration of cytokine therapy was different in the two experiments, but having completed the prescribed intervention the mice were maintained on the shelf and observed for tumour development.

Prophylaxis Experiment 1

These experiments required cytokines with cross-species specificity or murine cytokines which were readily available. Extrapolating from experiments in the nude mouse tumour transplants, outlined in Chapter Five, the two cytokines IFN-α A/D hybrid and rat IFN-γ were chosen as prophylaxis for mammary tumours.

The alpha interferons are a family of inducible secreted proteins which confer resistance to viruses on target cells, inhibit cell proliferation and regulate expression of MHC class I antigens. IFN-α A/D hybrid is a recombinant human hybrid molecule with strong activity on murine cells (Balkwill *et al.*, 1982; Rehberg *et al.*, 1982). IFN-γ is a pleiotropic cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including activation, growth and differentiation of T cells, B cells, macrophages, NK cells and others such as endothelial cells and fibroblasts.

The intention was to treat mice with the cytokines, alone and in combination, three times weekly between 6 and 12 months of age (Figure 6.1). Administering two injections three times per week became progressively more distressing for a proportion of the mice and hence the duration of prophylactic treatment was reduced to a three month period (6-9 months) in the combination group.

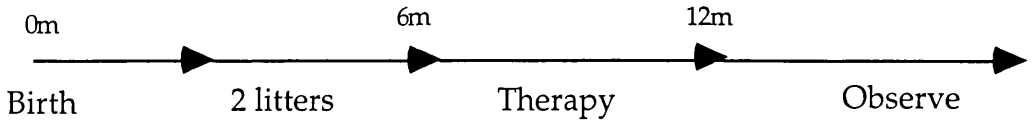


Figure 6.1 Time-course of prophylaxis experiment 1

Littermates were divided in different treatment arms to decrease the likelihood of genetic factors confounding the outcome. The cytokines and the rationale for using them are described in detail in Chapter 5. Those used: IFN-α, IFN-γ, IL-2 and IL-7 were all given in a volume of 0.1ml per injection. Control diluent and 5 x 10^4 U of IFNs α and γ were injected subcutaneously (sc). The dose used was based on Freireich's formula and clinical trials of maintenance IFN therapy (See Chapter 1) (Freireich *et al.*, 1966). A dose of 5 x 10^4 U of IFNs-α and -γ is comparable to 5 x 10^6/U m^2 in humans, an intermediate dose.

The numbers of mice in each group represents the number assessable, although the intention was to recruit 25 mice to each group. The main reason for variation in group size was that some mice had not weaned two litters in time to be recruited at six months. Many of the animals in the combination therapy group were unable to tolerate two injections, some became unwell with anorexia and lassitude and had to be withdrawn from the experiment. Two mice bled as a result of the intraperitoneal injection of rat IFN-γ. Consequently the numbers of mice in the combination group were smaller.

On the basis of previous pharmacokinetic studies it was felt that optimal circulating levels of rat IFN-γ would be achieved with ip injection, (Balkwill *et al*, 1986). However problems with administering regular ip injections led us to investigate sc use of rat IFN-γ instead. An experiment comparing sc and ip administration of IFN-γ was performed in one of the mammary tumours transplanted into nude mice (Tumour 1). There was no statistically significant difference in survival or tumour volume between the two treatment arms and it was decided to switch to sc administration thereafter.

Group 1 (Control)

Twenty-two female mice observed for tumour incidence and kept on the shelf indefinitely.

Group 2 (Control 2)

Nineteen female mice injected sc three times weekly with 0.1ml control diluent (CD) from 6 until 12 months. Then observed for tumour incidence and kept on the shelf indefinitely.

Group 3 (IFN-α A/D Hybrid)

Twenty female mice injected sc three times weekly with 0.1ml IFN-α A/D Hybrid (IFN-α) from 6 until 12 months. Then observed for tumour incidence and kept on shelf indefinitely.

Group 4 (rat IFN-γ)

Twenty-one female mice injected sc three times weekly with 0.1ml rat interferon-γ (IFN-γ) from 6 until 12 months. Then observed for tumour incidence and kept on shelf indefinitely.

Group 5 (IFN-α A/D Hybrid and rat IFN-γ)

Eighteen female mice injected sc with 0.1ml IFN-α A/D Hybrid (IFN-α) and sc with 0.1ml rat IFN-γ three times weekly from 6 until 9 months. Then observed for tumour incidence and kept on the shelf indefinitely. As described above the experimental period was shortened for some of the mice in this group.

Results

Outlined below (Table 6.1) are the numbers and percentages of assessable mice in each group developing tumours, the categories of tumour developed and the number surviving at 25 months.

Group	Cytokine	Total no. of mice	Total no. of tumours (%)	Mammary tumours (%)	Other tumours	Non-malignant death	Surviving at 25 months (%)
1	Nil	22	13 (59)	10 (45)	2H, 1L	2	7 (32)
2	Control	19	8 (42)	5 (26)	3H	5	6 (32)
3	IFN-α	20	9 (45)	5 (25)	1H,1A, 1L, 1O	4	7 (35)
4	IFN-γ	21	6 (29)	5 (24)	1L	2	13 (62)
5	IFNs α+γ	18	7 (39)	4 (22)	2H, 1L	3	8 (44)

Table 6.1. Outcome of first prophylaxis experiment - tumours types developed.

H - Harderian gland carcinoma; L - Lymphoma; A - Angiosarcoma; O - Other malignant tumour.

Taking into consideration survival of the five groups of mice with respect to death from mammary tumour, lymphoma or any tumour there was no significant difference between the survival of the five groups. Analysis entailed the logrank test to compare survival between the groups (Table 6.2).

Overall logrank survival	mammary	any tumour	lymphoma
p value	0.7027	0.7612	0.8916

Table 6.2 Overall logrank survival comparing the groups with respect to tumour death.

This suggests that the use of interferons in this model does not influence tumour development sufficiently for an effect to be seen in groups of this size with this prophylactic therapy regimen. It also shows that the trauma of an injection three times weekly has no effect on tumour development.

The proportion of all tumour-bearing mice surviving from birth in each group is illustrated in Figure 6.2 and for those developing mammary tumours in Figure 6.3. No tumours were documented before eight months and mice were excluded from the analysis after 25 months.

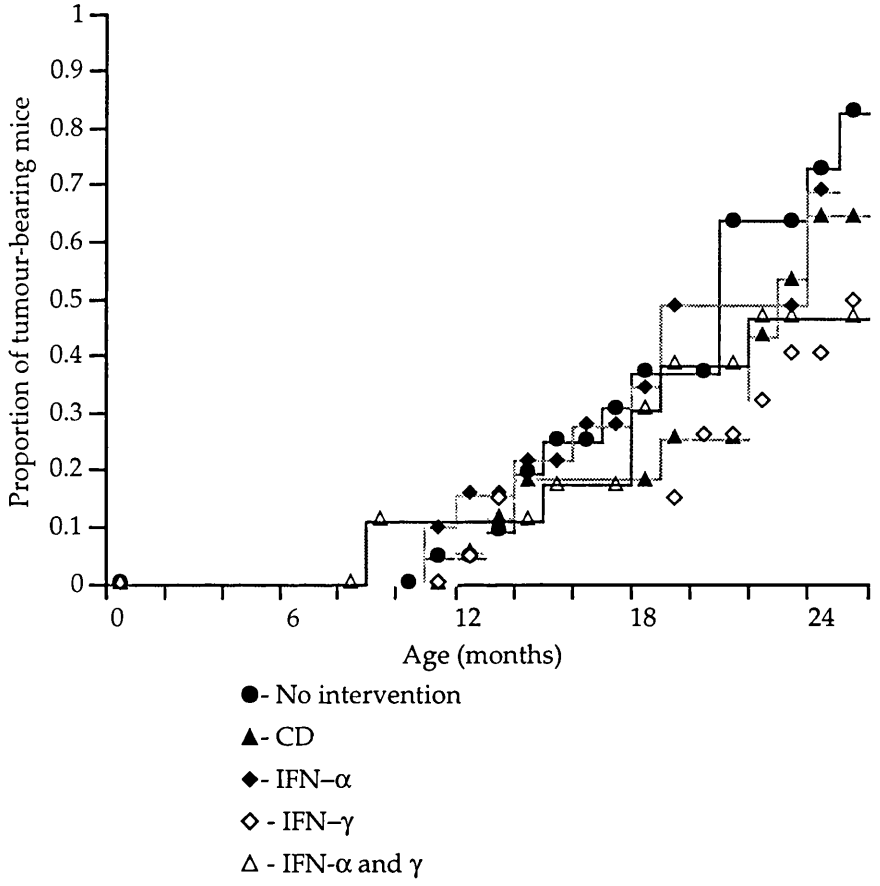


Figure 6.2 Proportion of mice bearing tumours of all types with increasing age.

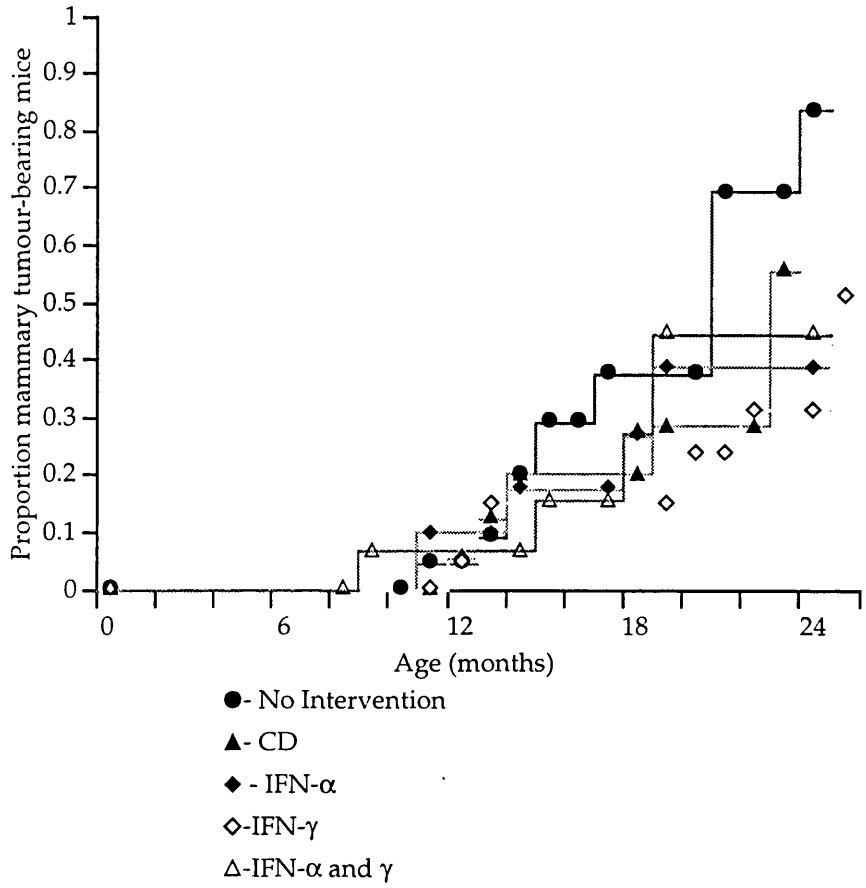


Figure 6.3 Proportion of mice bearing mammary tumours with increasing age.

Haematological profiles in mice

Baseline haematological values were established on transgene negative littermates in the colony, using the mean of values obtained from six mice (Figure 6.3). These compare with those in animals who have been treated with IFN-α, IFN-γ and the combination for 2, 4 and 6 months respectively.

	Mean	Range	SD
Haemoglobin (Hb)	10.59	10.11 - 11.19	0.347
WBC	7.98	5.5 - 10.4	1.967
Neutrophils	12.5	3.0 - 23.0	7.662
Lymphocytes	85.5	70 - 95	9.05
Monocytes	0.83	0 - 2	0.983
Eosinophils	0.33	0 - 1	0.516
Platelets	1060	694 - 1912	576

Table 6.3 Haematological profiles in untreated mice in the colony.

The values in the three tables (Table 6.4-6.6) for treated mice are based on values in 3 mice treated with the relevant cytokine or control for the allotted period.

The haemoglobin values and white blood cell counts of all the 'treated' groups were higher than those found in the untreated group, although the platelet counts were comparable. This is unlikely to relate to therapy as it was true of the CD-treated mice as well as the cytokine-treated groups. The higher valuse in the CD-treated mice than the untreated controls may be explained by the presence of endotoxin. The neutrophil and lymphocyte counts were very consistent throughout all three groups and at each of the time points. The eosinophil counts were slightly higher in the IFN-α treated mice in comparison with those of the IFN-γ treated group at all three time points. With that minor exception no consistent trend or pattern was seen with either cytokine or duration of therapy.

	CD	IFN-α	IFN-γ
Hb	16.5	15.0	15.4
WBC	12.0	14.3	9.0
Neuts (%)	23	22	24
Lymphos (%)	70	70	70
Monos (%)	5	3	3
Eosinos (%)	2	5	2
Platelets (%)	568	719	818

Table 6.4 Haematological profiles in mice treated for two months.

	CD	IFN-α	IFN-γ
Hb	15.6	13.8	15.8
WBC	12.8	17.5	11.2
Neuts (%)	21	31	22
Lymphos (%)	72	63	74
Monos (%)	1	1	3
Eosinos (%)	6	5	2
Platelets (%)	1030	1360	876

Table 6.5 Haematological profiles in mice treated for four months.

	CD	IFN-α	IFN-γ
Hb	15.7	15.8	15.8
WBC	12.7	8.2	14.8
Neuts (%)	20	23	21
Lymphos (%)	70	70	74
Monos (%)	4	2	3
Eosinos (%)	6	5	2
Platelets (%)	984	1007	876

Table 6.6 Haematological profiles in mice treated for six months

Prophylaxis Experiment 2

The aim of this long term study was to determine the effect of rhIL-2 or rhIL-7 on the incidence of mammary tumours in generation four of the colony. The treatment groups were recruited at seven months of age and treated with three times weekly injections of cytokine for three months.

All positive female MMTV-*neu* transgenic mice were recruited after mating them twice. Injections were given daily for a shorter period in keeping with the more low-dose, regular administration of these cytokines used in previous animal and human work (Smith, 1992);. Mice were divided into four groups - individuals from the same litter being divided into different treatment arms to decrease the likelihood of genetic factors confounding the outcome.

IL-2

Interleukin-2 (IL-2) is a 15kDa protein which was the first of a series of lymphocytotrophic hormones to be recognised as pivotal for the generation and regulation of the immune response. The human form of interleukin-2 is known to work in mice. The results of clinical trials are mixed and a better understanding of the

observed clinical responses is needed in order to design better treatment schedules (see Introductory Chapter).

On the basis of clinical studies in metastatic cancers in humans utilising rhIL-2 a dose of 0.3μg was selected. This was comparable with the immunomodulatory experiments of KA Smith (Smith, 1993). This is equivalent to a dose of 50μg in humans, the upper limit of the immunomodulatory dose used by Smith and co-workers (Caligiuri *et al.*, 1993) .

IL-7

Interleukin-7 (IL-7) is a 25kDa glycoprotein, first purified in 1988 by Namen and co-workers (Namen *et al.*, 1988). It has growth promoting effects on lymphocytes of B and T cell lineage and increases the cytotoxic activity of effector cells, making it a potential antitumour agent. *In vitro* , it has been found to beneficially augment the generation, and enhance the long-term growth, of antitumour cytotoxic T lymphocytes from lymph nodes draining a tumour site (Lynch & Miller, 1994). The human recombinant form acts on mice. *In vivo,* in experimental metastases models, it has antitumour activity against some tumours and toxicity appears to be lower than that of rhIL-2 (see Introductory Chapter).

In *in vivo* experiments performed to date a range of rhIL-7 doses have been used. The higher doses (25μg per injection twice daily) were usually only administered for short periods of time (Komschlies *et al.*, 1994). This protocol attempted to define whether the effect and toxicity were comparable. Disappointingly the clinical use of IL-7 has been limited to date, with no published studies. The dose of IL-7 (5μg daily) which we chose to use was based on the *in vivo* experience of Wiltrout and coworkers (1992) where treatment continued for a longer duration (Wiltrout *et al.*, 1995) .

There were twenty-five mice in each group which were mated twice and after the relevant intervention were kept on the shelf indefinitely and observed for tumour incidence.

Group 1 (Control Group 1)

No intervention

Group 2 (Control Group 2)

Injected sc daily with 0.1ml control diluent (CD) from 7 until 10 months (twelve weeks).

Group 3 (rhIL-2)

Injected sc daily with 0.1ml recombinant human interleukin-2 (rhIL-2) from 7 until 10 months (twelve weeks).

Group 4 (rhIL-7)

Injected sc daily with 0.1ml recombinant human interleukin-7 (rhIL-7) from 7 until 10 months (twelve weeks).

Results

The ages at development of the mammary tumours and lymphomas are outlined in Table 6.6. Outlined below are the outcomes of treatment for the four different groups of mice in the second prophylaxis experiment (Table 6.7).

Group	Cytokine	Number of lymphomas	Number of mammary tumours	Median age at lymphoma development	Median age at mammary tumour development
1	nil	1	9	21	15
2	Control	2	3	20.5	19
3	IL-2	3	7	18	15
4	IL-7	9	3	15	17

Table 6.7 Ages at development of lymphomas and mammary tumours

Taking account of survival from any tumour, survival from a mammary tumour and survival from lymphoma into account, a comparison has been made between the two treated groups (3 and 4) and each of the control groups (1 and 2). Analysis entailed the logrank test to compare survival between the groups. All of the p values relating to survival from any tumour or mammary tumour were not significant at the 5% level.. However in group 4, where an unexpectedly high incidence of lymphomas was observed, with a significant difference between the number of lymphomas arising in comparison with both groups 1 and 2 (p= 0.003 and p= 0.007, respectively).

The proportion of all tumour-bearing mice surviving in each group is illustrated in Figure 6.4 and for those developing mammary tumours in Figure 6.5. No tumours were documented before eight months and mice were excluded from the analysis after 25 months.

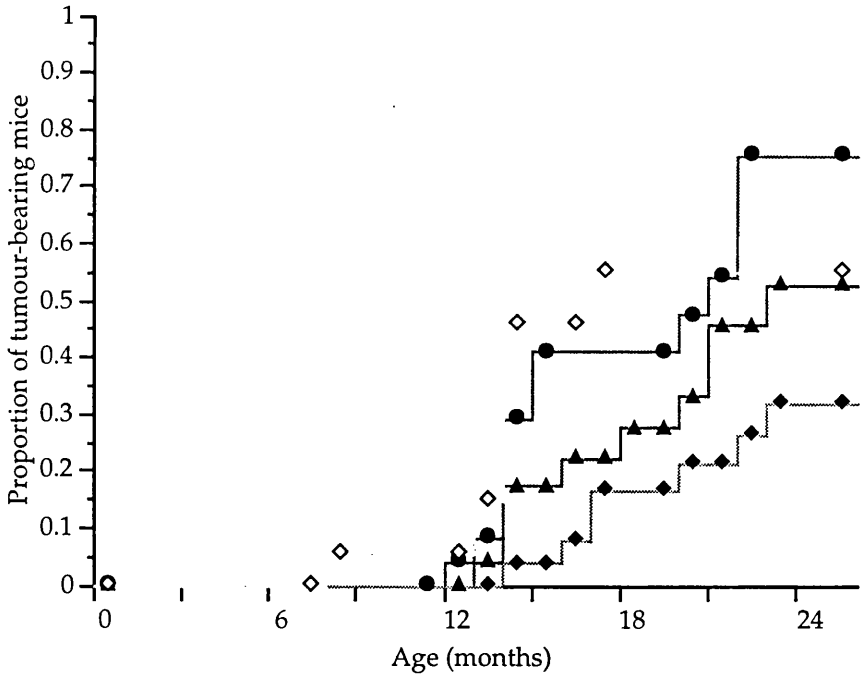


Figure 6.4 Proportion of tumour-bearing mice with increasing age

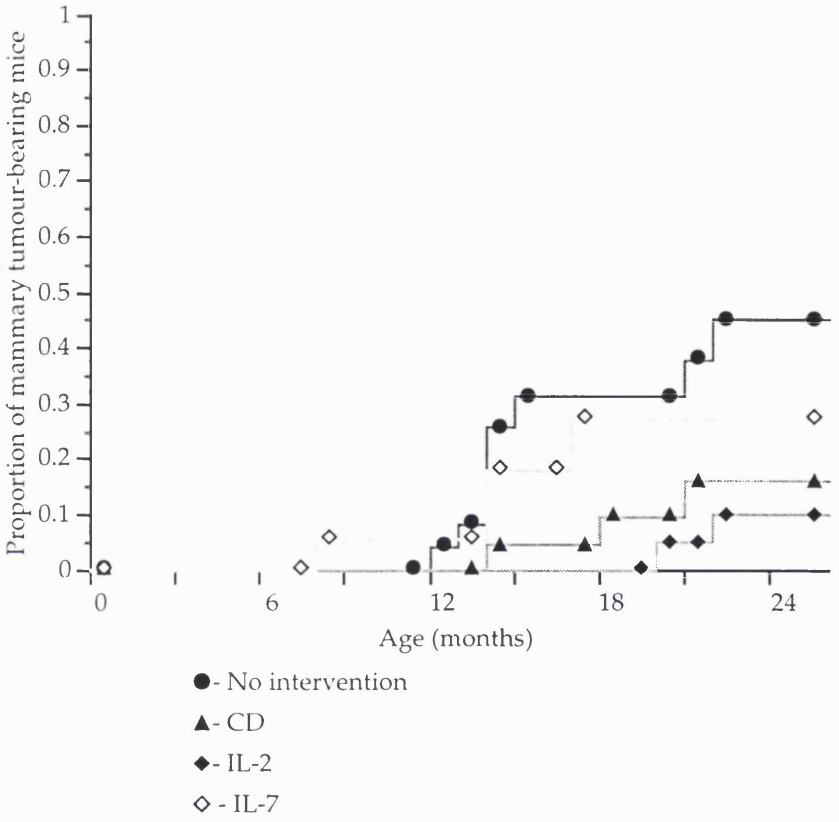


Figure 6.5 Proportion of mammary tumour-bearing mice with increasing age

Individual histology

In respect of the histological appearance of tumours there were no noteable differences between the groups of mice whether treated or untreated, with the exception of mice treated with interleukin-2. In the IL-2 group both mammary tumours and lymphomas arose which differed microscopically from others seen in the general colony and other prophylaxis experiments.

One mouse was killed with abdominal swelling and found to have an enlarged spleen. Histologically there was widespread low-grade lymphoma infiltrating the liver, lungs, spleen, spinal cord and and brain. Another mouse was similarly bloated and the spleen was noted to be enlarged as well as the liver being mottled in appearance. A third mouse had a perforated Peyer's patch post mortem and the mesenteric lymph nodes were grossly enlarged. Microscopically the lymphomas arising in IL-2 treated animals were of a lower grade, and better differentiated than those in other groups, with smaller more even nuclei, less pleomorphy and lower mitotic rates. In summary the lymphomas which arose in the IL-2 treated group are best regarded as low grade

lymphomas and those in the IL-7 treated group as high grade. Both are B cell lymphomas.

Because of difficulties measuring haematological profiles of transgene negative littermates in parallel with Prophylaxis Experiment 1 the possibility of performing a similar study in parallel with Prophylaxis Experiment 2 was reconsidered. The need for large numbers of mice (at least 5 per group and time-point), in addition to the restricted availability of rhIL-7, made this labour and resource-dependent and it was decided not to repeat the work in the second prophylaxis experiment.

Discussion

Attempts at chemoprophylaxis of tumours in humans using agents such as retinoids are becoming increasingly widespread (**ref**). Nevertheless, in spite of its epidemiological importance very few trials are being conducted on the chemoprevention of breast cancer (Veronesi & Costa, 1992). The inevitable length of such trials is an obstacle to both progress and recruitment and consequently intermediate end-points or surrogate markers are needed. An animal model which could accurately predict some of the clinical findings would be invaluable.

The animal studies presented here illustrate some of the known disadvantages with this particular model. As detailed in Chapter 1 the MMTV-LTR promoter targets expression of c-*neu* to a number of epithelial tissues. This results in transgene overexpression in tissues such as Harderian gland and salivary gland and consequently predisposes to tumours at these sites. This effect of the promoter is unlikely to be perturbed by chemoprophylaxis alone and is evident from the spectrum of tumours which arise.

The outcome of these long-term experiments has been influenced by factors such as the relatively late onset of the tumours, the range of tumours arising, their stochastic nature and that tumours only occur in a proportion of mice. Consequently, even using groups of up to 25 mice, there are too few mice to eliminate the many confounding variables and provide statistically significant conclusions about the incidence of mammary tumours themselves. The experiments might be redesigned with much larger groups and further manipulation of the model to increase the incidence and age of onset of tumours might be considered (see Future Studies).

As increased cell proliferation plays a key role in the development of all human cancers (Preston Martin *et al.*, 1990) it follows that growth inhibitors should delay or prevent the development of a malignant metastatic tumour. TGF-β is the only cytokine which has been used in the chemoprevention of experimental mammary cancer. Using exogenous TGF-β to inhibit the growth of established tumours in nude mice has not been very successful (Zugmaier *et al.*, 1991), although it has been used in the chemoprevention of mammary cancer by Sporn and colleagues (Wakefield *et al.*, 1992). Similarly other cytokines, such as IFNs-γ and -α are more likely to work in the setting of small volume or minimal residual disease due to their local, rather than systemic effects and their tendency to induce cytostasis rather than direct cytotoxicity.

At the time of establishing this experiment the time-course of tumour development was not known and for this reason cytokine prophylaxis was administered to the mice

aged 6-12 months. In the light of the median tumour development being 18 months treatment at 12 - 18 months might have been more appropriate.

Six lymphomas arose in the first five generations of untreated and control diluent treated mice, accounting for 6% of all the tumours. The overall incidence of spontaneous lymphomas in this colony was 6 in the 218 untreated and control diluent treated mice (2.8%), comparable with the incidence of spontaneous lymphomas in BALB/c mice kept in germ-free conditions which is less than 3% (Smith & Pilgrim, 1971). There is an increase in lymphoma incidence with age in mice (Dunn, 1969) but this did not explain the incidence documented in the IL-7 treated group.

Generally the lymphomas found in IL-2 treated-mice were of lower grade than the heterogeneous, aggressive appearance of the lymphomas arising in IL-7 mice, or those arising in control-treated mice. The mechanism for this is unclear but IL-2 is known to have a role in growth and differentiation of T cells, B cells, NK cells, LAK cells, monocytes, macrophages and oligodendrocytes (Kuziel & Greene, 1990; Smith, 1988).. The median age at development of the lymphomas in both treated groups was earlier than in the untreated groups, although that in the IL-2 group was later than the IL-7 group. The high incidence of lymphomas seen in association with transplantation and the necessary associated immunosuppression has been well documented since the late 1960s (Kreis & Legendre, 1989). It is now believed that much of this is explained by Epstrein Barr Virus (EBV) infection. In humans the major risk factors appear to be duration of immunosuppression, dosage of immunosuppressive agents and the number used together or sequentially (Kreis & Legendre, 1989). There have been many studies of lymphoma incidence in populations known to have disease or take medication which alters immunity. Nevertheless we have little insight into the mechanisms of lymphomagenesis (Hoover, 1992). The lymphomas which characteristically arise are non-Hodgkin's lymphomas, most of which are of B-cell origin and extranodal in distribution (Penn, 1993). Cytokines are also known to be immunosuppressive. IL-7 has been shown in vitro to regulate the proliferation of B cell progenitors (Namen *et al.*, 1988). It has also been shown to stimulate the proliferation of murine and human mature T lymphocytes (Grabstein *et al.*, 1993). IL-7 undoubtedly has a promoting effect on the development of lymphoma. BALB/c mice are known to be susceptible to lymphoma induction by radiation and this susceptibility is believed to be related to this promoting effect (Haran Ghera, 1985) . It seems likely that altered immunoregulation or impaired immune surveillance on the background of genetic susceptibility to lymphomagenesis played a part in the high incidence of lymphomas in the IL-7 treated mice.

This interesting finding should be developed further. The role of IL-7 in lymphomagenesis needs to be investigated with this model. A more detailed comparison between the lymphomas arising in the IL-7 treated mice and those arising in other mice in the colony - both treated and untreated - needs to be undertaken. Other possibilities include looking at changes in the expression and regulation of rodent c-*neu* in the presence of IL-7 and alterations in B cell subpopulations within the mice .

Chapter Seven

Future Studies

The diversity and unpredictability of this model has posed as many questions as it has answered.

Increasing the incidence of tumours in the colony and facilitating their development at an earlier age is a major aim. This might be achieved through a number of approaches. Preliminary data suggests that dietary manipulation and treatment with carcinogens may be effective, although the tumours are still arising around 18 months, the incidence may be rising. Radiation at the time of mammary gland development is also known to predispose to mammary tumour development in mice of certain strains, including BALB/c mice. Crossing with transgenic mice possessing other oncogenes, or lacking tumour suppressor genes, may predispose to earlier onset of tumours.

The genetic background is clearly important for the development of tumours and placing this particular transgene on the FVB background may result in tumours of greater frequency and earlier onset than seen on the BALB/c background. Alternatively direct microinjection of eggs from recent generations of mice in this colony with other mammary directed transgenes, to produce a bigenic model, may give further information.

It is apparent that homozygote mice, bred from the ninth generation onwards continue to develop tumours in a similar proportion of animals. There is a suggestion that tumour onset may be earlier and more frequent. Clearly the model provides a sound basis for further development and this should now include careful monitoring and observation for tumour development in a non-invasive way. This should enable earlier detection such that genetic, epigenetic and environmental influences to be defined more carefully. Sequential sampling of mammary glands in a cohort of mice to look for preinvasive lesions and tumours at an earlier stage would facilitate this investigation.

The increased incidence of lymphomas in the IL-7 treated prophylaxis group is perhaps the most unexpected finding. Further investigation of the effect of IL-7 administration in other transgenic models, particularly those which develop spontaneous lymphomas is indicated. IL-2 and IL-7 appear to be acting in a paracrine

and autocrine fashion on the development of lymphoma and more information about their effects on the immune system is required.

Transgenic models have an important, and now fully established, role in the investigation of tumorigenesis. Although this study was beset with some difficulties - predominantly a low incidence and late onset of tumours we have now reached a point - at twelve generations - where the model can be manipulated. The original aim of this thesis - to determine the feasibility of developing such a model - has been fulfilled. With further manipulation, either genetic or epigenetic, an ethical, reliable and reproducible model which develops tumours within a reasonable period may be achieved.

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An inbred colony of oncogene transgenic mice: diversity of tumours and potential as a therapeutic model

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Summary Transgenic mice carrying the activated rat c-*neu* oncogene under transcriptional control of the MMTV promoter were backcrossed to BALB/c mice, with the aim of developing a model for cancer therapy. A total of 86 of 268 transgene-positive mice in the first five generations developed 93 histologically diverse tumours (median age of onset 18 months). The cumulative incidence of breast tumours at 24 months was 18%, and overall tumour incidence 31%. As well as expected c-*neu* expressing breast cancers, lymphomas and Harderian gland carcinomas developed. Virgin mice had fewer mammary tumours than those with two litters. Breast carcinomas metastasised to the lungs, and lymphomas were widely disseminated. The tumours showed a range of architectural patterns, which resembled human breast cancers or lymphomas. This diversity was reflected in S-phase fraction and aneuploidy. Breast tumours transplanted to nude mice showed variable responses to interferon (IFN)-α and γ. A tumour transplanted to BALB/c mice responded to interleukin (IL)-12. There was significant decline in transgene positivity with successive generations. The diversity, histological and biological resemblance to human cancer suggests that the model has potential for evaluating novel therapies. However, further genetic and environmental manipulations are required to increase tumour incidence and decrease age of onset.

Keywords: oncogene; transgenic mice; cytokines; murine cancer models

Existing murine tumour models have a number of disadvantages that limit their usefulness in the investigation of cancer therapy, particularly cytokine therapy. Some syngeneic tumours are immunogenic and when treated with cytokines an allograft response may predominate. Transplantable tumours are often derived from cell lines and produce rapidly growing tumours that are a model for poorly differentiated or anaplastic tumours. Such tumours are not analogous to those human malignancies that respond to cytokines and also may not develop the complex host-tumour relationship of slow growing tumours. Similar disadvantages apply to models of metastases. Human tumour xenografts growing in nude mice are obviously inappropriate for studying any cytokine that may act via the host immune system. Consequently, there is a need for a murine tumour system that more closely resembles human cancer, is metastatic and arises in an immunocompetent animal. A model that also reflects the diversity of growth patterns encountered in human carcinomas, and uses an oncogene implicated in a particular cancer, would have further advantages.

Human c-*erb*B-2, the human equivalent of the rodent *neu* oncogene, was found to be amplified in 30% of 189 primary human breast cancers (Slamon *et al.*, 1987). This amplification had greater predictive value in lymph node-positive disease than existing prognostic factors. In both invasive, and certain types of *in situ* carcinoma, a high cytological grade was associated with up-regulation of this gene. In particular, comedo-type ductal carcinoma was a histological type of tumour more frequently associated with c-*erb*B-2 amplification (Van de Vijver *et al.*, 1988). This gene is therefore an appropriate candidate in a model tumour system. There are two well-characterised transgenic mouse models of mammary cancer that possess the activated *neu* oncogene under control of the MMTV-promoter (Muller *et al.*, 1988; Bouchard *et al.*, 1989). In the model of Muller *et al.*, tumours arise synchronously in all mice, involve the entire gland and are polyclonal in origin. In the *neu* transgenic mice developed by Bouchard *et al.*, tumours are monoclonal and appear later, in a stochastic pattern, in approximately 30-50% of mated female mice (Bouchard *et al.*, 1989). Because of its closer resemblance to the biology of human disease, we have used the latter model to establish a colony of inbred mice. In this paper we describe the range of tumours, their morphology, biological diversity, metastatic pattern and growth characteristics. We compare these features with the similar data available on c-*erb*B-2-positive human mammary carcinoma. To enable a preliminary assessment of the potential of these mice as a model for cancer therapy, we have established eight tumours from the colony in nude mice or transgene-negative mice and treated these with a range of cytokines.

Materials and methods

Mice

Three male founder mice on a C57B1/6 x C3H background were obtained from Professor Paul Jolicoeur. These had been generated by microinjecting a 8.2 kb *Sac*II-*Eco*RI chimeric DNA fragment containing the activated rat c-*neu* cDNA under transcriptional control of the MMTV long terminal repeat (LTR) (Bouchard *et al.*, 1989). One-cell embryos were collected, microinjected and transferred into pseudopregnant CD-1 females (Hogan *et al.*, 1986). Transgene-positive female mice have now been backcrossed onto inbred BALB/c males for eight generations. Inbred BALB/c mice were obtained from ICRF breeding unit, Clare Hall, South Mimms, Hertfordshire. All mice studied were housed in the specified pathogen-free unit at Clare Hall from birth until tumour development or death from other causes. Female nu/nu mice of mixed genetic background were obtained from the ICRF breeding unit and maintained in negative pressure isolators. Tumours were implanted in mice aged 6-8 weeks.

Screening and colony

Screening for the transgene was established initially using Southern hybridisation analysis of tail DNA using a *neu*-

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specific 4.6 kb probe which has the *Hind*III–*Sal*I digest from the microinjected DNA fragment. This did not hybridise to tailsnip DNA from transgene negative C57 Bl/6 and BALB/c mice. Once back-checking had been performed, slot-blotting was established using 10 μg of DNA per slot and the 4.6 kb probe, labelled with ^{32}P. In the first six generations positive females were placed 'at risk' of tumour development by mating them against BALB/c mice for two litters.

Histopathology

Morphological analysis The animals were inspected for general condition and tumours at least twice a week. If the animals became unwell, or tumours ulcerated or approached 2 cm in diameter, they were sacrificed and a post mortem examination performed. In most cases only one tumour was evident at this point. Tumour tissue, lungs, liver and spleen were fixed in neutral buffered formalin (NBF) and embedded in paraffin wax. Parallel samples were also snap frozen.

Immunohistochemistry Sections were immunostained with an antibody to human c-*erb*B-2 (1:50 dilution) (Dakopatts, Denmark) and in the case of lymphomas, the murine T/B lineage antibodies to α/βTCR(1:1000 dilution) (Pharmingen, USA), B220(1:300 dilution) (Pharmingen, USA), Surface Ig(1:25 dilution) (Sigma, USA) and Thy 1.2(1:100 dilution) (Becton-Dickinson, USA). These antibodies were employed in conjunction with a standard streptavidin–biotin technique. A brown reaction product was obtained using a peroxidase substrate [diaminobenzidine, phosphate-buffered saline (PBS) 0.3% hydrogen peroxide]. All antibodies except Thy 1.2 worked well and appropriately on formalin-fixed material after prior microwaving of the sections. For microwaving the unstained sections were immersed in 0.01 M sodium citrate buffer solution at pH 6 in which they were microwaved at 700 W for 10 min with rapid cooling by running water thereafter to avoid deleterious drying. The antibody to Thy 1.2 worked without microwaving sections. All histopathology assessment was performed by a consultant pathologist with an interest in breast cancer (AMH). A human mammary carcinoma known to be positive for human c-*erb*B-2 was used as a positive control for the c-*erb*B-2 antibody. Mouse lymph node and tonsillar tissue, in which there are distinct patterns of T and B lymphocyte localisation, acted as both positive and negative controls for the murine T and B lineage markers.

Tumour growth and flow cytometric analysis Flow cytometry was performed on nuclear suspensions prepared from formalin-fixed paraffin-embedded sections as described elsewhere (Camplejohn *et al.*, 1989). Three 50 μm paraffin sections were dewaxed and rehydrated through a series of alcohols into double distilled water. Nuclei were extracted by the addition of pepsin (5 mg ml^{-1}) at 37°C for 30 min at pH 1.5. Following filtration through a 35 mm pore size nylon filter and incubation with 250 mg ml^{-1} of propidium iodide, the samples were analysed using a Becton-Dickinson FACS Analyser powered by a mercury arc lamp. Approximately 10^5 particles were scanned to construct a DNA histogram. The DNA index was calculated by relating DNA content of the aneuploid G_0/G_1 peak to that for the diploid G_0/G_1 peak. The S-phase fraction (SPF) for the diploid tumours was measured using the method of Baisch *et al.* (1975). The number of cells in S-phase was calculated from a rectangle fitted between the peak channels of the G_0/G_1 and G_2/M peaks. For the DNA aneuploid histogram the percentage of aneuploid S-phase cells as a percentage of total aneuploid cells was estimated in a similar way (Camplejohn *et al.*, 1989).

Cytokine therapy As a result of the incompletely defined mode of action of most cytokines and the apparent lack of a dose–response relationship in many studies, both clinical and animal studies have attempted to define the optimal mode of administration and regimen. A comparison of the toxicity of anti-cancer agents in mouse, rat, hamster, dog, monkey and man was devised based on a formula in which surface area to volume ratios between species were taken into account (Freireich *et al.*, 1966). Using Freireich's formula we have used a dose of 5×10^4 U per animal per day of both rh interferon (IFN)-α A/D hybrid and rat IFN-γ, equivalent to a dose of 11×10^6 U per day in a human. In each case the mice were injected with 0.05 ml of tumour on day 0 and treatment with control diluent or cytokines commenced on day 7, and continued for 42 days, or less if the animal was unwell. FN-α A/D hybrid was the kind gift of Dr Michael Brunda, Hoffmann La Roche, New Jersey USA. Recombinant rat IFN-γ was the kind gift of Roussel UCLAF, Romainville, France. Recombinant murine interleukin (IL)-12 was the kind gift of Dr Brunda and was used at a dose of 1 μg per animal per day.

Results

Overall tumour incidence in transgene-positive animals

A total of 86 of 268 female mice in these first five generations developed 93 histologically diverse tumours over a period of 25 months. Of these 83 arose in tissues known to express the transgene. Fifty-three breast carcinomas, 24 Harderian gland tumours, six lymphomas and five vascular tumours developed as well as five of less common histological types. The median age of tumour development was 18 months. At 24 months the cumulative incidence of breast tumours was 18%, with an incidence of 34% for all tumour types. The development of the three major tumour types in this colony is shown in Figure 1 and Table I. Four mice developed two different histological tumour types simultaneously. Consequently tumour incidence is based on number of mice succumbing as a result of tumours and not on numbers of tumours.

Tumour incidence in successive generations

Analysis of the second, third and fourth generation showed a slight decline in the median age of tumour development (17,15 and 15 months respectively). The proportion of tumours that were of mammary origin remained the same.

Change in transgene positivity with successive generation

A total of 738 female mice were bred onto a BALB/c background in the first seven generations, of which 391 were transgene positive. There was a gradual and significant decline in the proportion of transgene-positive females born with each successive generation (Figure 2). This observation was originally based on slot-blot results but was confirmed by Southern blotting. The difference between the generations

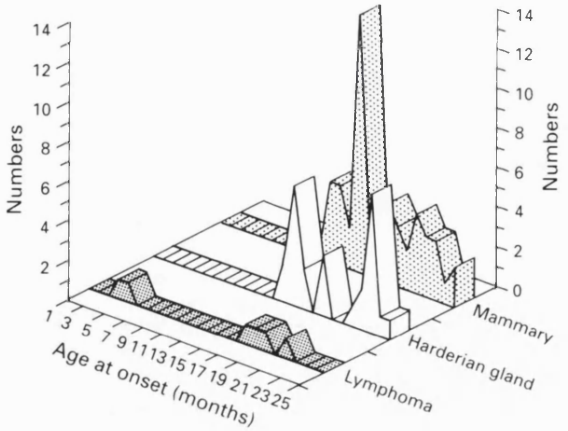

Figure 1 Age at onset of three major tumour types in colony. ▨, mammary; □, Harderian gland; ■, lymphoma.

gave a χ^2 value of 9.097, with 2 degrees of freedom (d.f.), $P = 0.01$. Looking for a trend, given that the proportion appeared to be declining, the test for trend value was $\chi^2 = 20.6$, d.f. = 1, $P < 0.001$. This suggests that there is not only a difference between the generations but this difference is occurring in a particular direction. The transgene was transmitted normally when homozygous matings were established and litter number and offspring viability of the homozygous mice was the same as in heterozygotes. However, in the heterozygous matings there were fewer transgene-positive offspring than expected with successive generations and sometimes whole litters were transgene negative.

Influence of litter number on tumour development

The influence of litter number on tumour development was studied in the fourth generation. Virgin mice developed fewer mammary tumours, those mice mated only once developed no mammary tumours whereas those mated two or more times had a higher incidence of tumours overall (Figure 3). There was an overall difference between the groups in relation to litter number, ($P = 0.003$ by Fisher's exact test). Comparing the incidence of mammary tumours between the groups is also statistically significant ($P = 0.006$, Fisher's exact test). It appears that the risk of a mammary tumour development is not increased by further litters.

Pathological description of tumours

Mammary carcinomas Of the 93 tumours, 53 were mammary (57%). Phenotypically they were characterised by a subcutaneous tumour in an otherwise well animal. The age at onset ranged from 10 to 25 months, with a median of 15 months. These tumours all shared high-grade cytomorphology with a high degree of mitotic activity and pleomorphism. No definite *in situ* carcinoma was seen. The architectural pattern showed a range of appearances with the following types of growth pattern merging one with another and sometimes co-existing in the same tumour. These patterns were generally as follows:

(1) Tumours showing islands of interlocking large cells with areas of necrosis, characteristic of the classical comedo-type tumours described by Bouchard *et al.* (1989) in the founder mice and associated with c-*erb*B-2 positivity. Unlike human comedo carcinoma, characterised by a large cell ductal carcinoma *in situ* with central necrosis, the tumours were not confined to ducts (Figure 4a).
(2) Solid tumours in which sheets and well-defined islands of tumour were present but no large areas of necrosis.
(3) Tumours which were completely or partly (micro) papillary in nature. Though a minor component of four of the tumours, in a further eight tumours this was the predominant growth pattern (Figure 4b). Pure tumours of this type consisted of numerous duct-like structures, in which the malignant epithelium contained therein was thrown into small papillae. The number of these structures considerably exceeded the number of ducts normally expected and it was deduced that the appearances represented invasive disease. In two tumours some of the papillary growth pattern was contained within a cystic space thus architecturally (but not cytologically) mimicking human intracystic papillary carcinoma.
(4) In one tumour a spindle cell epithelial element was seen evolving from more typical solid-type carcinoma in keeping with a so-called 'metaplastic' carcinoma. A further tumour was entirely of metaplastic type.

None of the tumours showed a significant host inflammatory response and all of the tumours stained positively with an antibody to human c-*erb*B-2. Though this was occasionally patchy and included much diffuse cytoplasmic staining, convincing appropriate membrane staining was demonstrated in all 53 tumours.

Twenty-three of the 53 (43%) mammary tumours metastasised to lung (an example is shown in Figure 4c). Lymph node deposits were sometimes seen near the primary site and carcinoma cells were also seen in the liver sinusoids and the spleen. No metastases were recorded in bone. However these were sought by sectioning spinal cord in 20 of the mice. In humans, the more sensitive technique of bone scintigraphy would normally be used. Of the spectrum of pathology, the micropapillary histological pattern appeared most likely to metastasise to lung, with an incidence of 11/16 (69%) as compared with 12/37(32%) of the non-papillary tumours ($P = 0.003$, Fisher's exact test).

Harderian gland carcinomas The Harderian gland is a modified sebaceous gland found deep in the orbit of animals with a nictitating membrane. Twenty-four Harderian gland carcinomas were seen, being diagnosed mainly on the basis of a protruberant eye and the presence of fluid and solid tumour behind the eye at post-mortem. Age of onset ranged from 11 to 25 months with median being 18 months. In four cases lung metastases were found on pathological assessment, although no primary was noted post-mortem. Histologically these tumours were papillary in pattern and resembled the more poorly differentiated end of the spectrum found to occur naturally (see Figure 4d). There was a higher proportion of these tumours in virgin mice than in mated mice. Sixty per cent of Harderian gland carcinomas metastasised to lung. Metastases did not appear to correlate with the grade of the primary tumour. Indeed, in one case, the primary

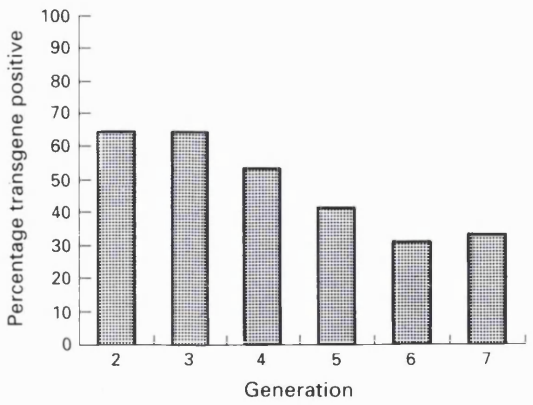

Figure 2 Percentage of females born transgene positive in the first seven generations. The difference between the generations: χ^2value of 9.097, with 2 degrees of freedom (d.f.), $P = 0.01$. Chi-squared test for trend value was $\chi^2 = 20.6$, d.f. = 1, $P < 0.001$.

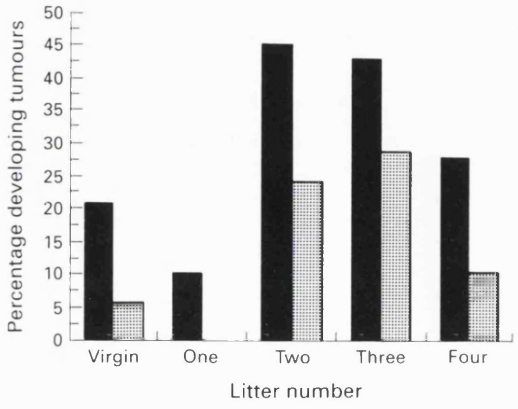

Figure 3 Influence of litter number on mammary tumour development. Overall difference between the groups in relation to litter number, $P = 0.003$, Fisher's exact test. Comparing the incidence of mammary tumours between the groups was also statistically significant, $P = 0.006$, Fisher's exact test. ■, Total tumours; ▨, mammary tumours.

tumour had the appearance of an adenoma but metastases in the lungs were consistent with a malignant Harderian gland carcinoma. There was a statistically significant difference ($P = 0.003$) between the proportion of Harderian gland tumours metastasising to lung in mated mice as compared with the proportion arising in virgin mice (Table II). All the 24 Harderian gland tumours stained positively for c-*erb*B-2.

Lymphomas Six malignant lymphoid tumours arose in the transgenic mice under observation in this colony. All were disseminated at post mortem examination. Microscopically the spleen, liver and lungs were diffusely infiltrated. One lymphoma appeared to arise in the calvarium and subsequently disseminated into the brain and systemically. In other cases lymphoma was found to be infiltrating the spine, lung, large intestine and skin. The age at onset ranged from 4 to 21 months, with a median of 17.5 months. In one case the tumour had the morphology of an immunoblastic lymphoma with lymphoplasmacytoid features, while the rest manifested as a lymphoblastic lymphoma/acute lymphoblastic leukaemia

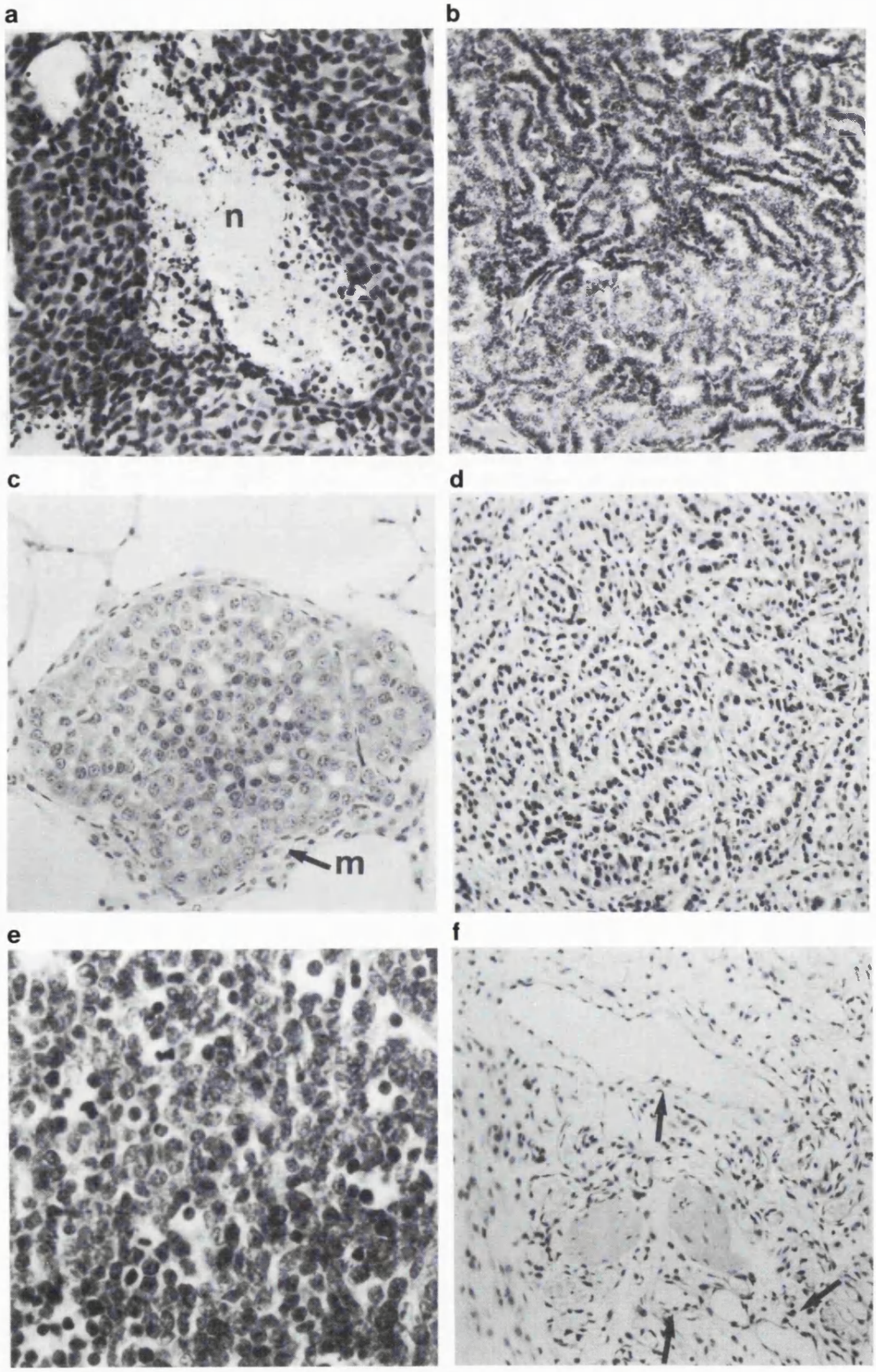

Figure 4 Histology of tumours in transgene-positive females (**a**) Comedo-type mammary tumour. n, area of necrosis. (**b**) Papillary-type mammary tumour. (**c**) Lung metastasis (m) from mammary carcinoma. (**d**) Harderian gland carcinoma. (**e**) Lymphoblastoid lymphoma. (**f**) Angiosarcoma, arrows mark blood vessels.

(Figure 4e). All six lymphomas demonstrated a B-cell phenotype using the four murine antibodies against α/β TCR, Thy 1.2, surface IgG and B220. They also stained positively with the antibody to c-*erb*B-2.

Vascular tumours In five mice vascular tumours were seen, of which three were undoubted angiosarcomas (Figure 4f) and the other two suggestive of angiosarcoma. These tumours were present in a variety of sites and were evident macroscopically as very vascular, with obvious blood-filled spaces. One involved the spleen, another was attached to a pedicle arising from the bladder base and another appeared to derive from subcutaneous tissue overlying the neck. In two mice tumour was found in the spleen as well as another site. Two arose in conjunction with Harderian gland carcinomas. None of these stained positively with an antibody to c-*erb*B-2.

Others In total there were five other tumours. One metaplastic carcinoma of uncertain site of origin, one spindle cell sarcoma, not otherwise specified, one tumour resembling a papillary mesothelioma morphologically and two adenocarcinomas in which the lung appeared to be the primary site. None of these tumours stained positively with an antibody to c-*erb*B-2.

S-phase fraction analysis

In order to confirm the subjective impression of diversity in this model, both within and between tumour subcategories, we have examined their proliferative rate using S-phase fraction. Thirteen primary mammary tumours were examined and 11 found to exhibit a wide range of S-phase fraction (range 5.6–11.9, median 9.0). In two other mammary tumours there were two clones of tumour preventing analysis of the S-phase fraction of the different aneuploid peaks. One of the 13 mammary tumours was diploid and 12 were aneuploid. Analysis of both aneuploidy and S-phase fraction was possible in 9 of the 13 tumours and these data are shown in Figure 5. Two primary lymphomas were also examined and the S-phase fraction values were 6.1 and 13.9.

Transplantation of tumours into BALB/c mice

Three attempts at tumour transplantation from second and third generation mice into other mice of the colony failed. However, one tumour from the fourth generation, which arose in a 13-month-old female mouse, has been successfully passaged. The tumour arose over the left shoulder in the mammary line and there were no other abnormalities at post-mortem examination. Injected into the flank of two offspring, it became established after about 16 weeks and was then passaged into other mice from the same litters. By passage 3 it was found to grow readily in ordinary BALB/c mice. Histologically this was a mammary tumour with a comedo-type pattern and extensive areas of necrosis. No metastases have been seen at post mortem examination to date. This tumour has been further passaged successfully and has been used in preliminary cytokine therapy experiments as described below.

IL-12 therapy of transplanted tumour

Aliquots of 0.05 ml of this murine mammary carcinoma were injected into two groups of eight female BALB/c mice on day 0. Injection with control diluent or rmIL-12 daily was commenced on day 7, for a total period of 42 days. The cytokine appeared to be well tolerated. Tumours grew in all control-injected mice, but only six of eight IL-12 injected mice. Median survival of the control and treated groups was 32 and 70 days respectively. (Log-rank survival, χ^2 $P = 0.01$) Two IL-12-injected mice were still alive with no evidence of tumour some 233 days later (Figure 6).

Interferon sensitivity of tumours transplanted into nude mice further demonstrates biological diversity

Seven of seven mammary tumours were successfully transplanted and passaged in nude mice. We have examined the anti-tumour activity of two interferons in these transplants. Interferon-α A/D hybrid, a recombinant human hybrid molecule with strong activity on murine cells (Rehberg *et al*., 1982) was used because it is more readily available than purified murine IFN-α. The other cytokine used, recombinant rat IFN-γ, also has cross-species specificity. Figure 7 shows the percentage change in survival of IFN-treated mice compared with a group of control diluent-treated mice. The animals were killed when the tumour size reached 2 cm, or on the basis of factors such as poor health of the animal. In

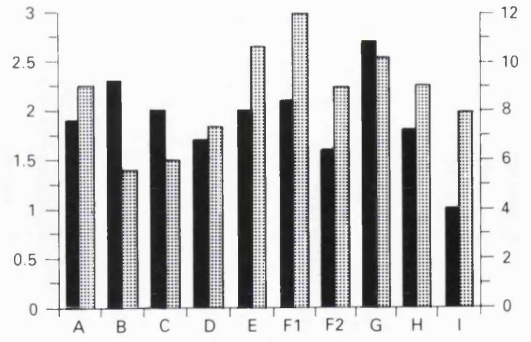

Figure 5 S-phase fraction and DNA index in nine spontaneously arising tumours in the colony. ■, DNA index; ▨, SPF (S-phase fraction).

Table I Tumour development in transgenic mice

Tumour Type	*Number of tumours (%)*	*Median age of onset (range)*	*C-erbB-2 positivity*
Mamary carcinoma	53 (57)	15.0 (10–25)	+
Harderian gland	24 (26)	18.0 (14–25)	+
carcinoma	6 (6)	17.5 (4–21)	+
Lymphoma			
Angiosarcoma	4 (4)	22.5 (21–24)	–
Others	6 (6)	19.0 (14–25)	–

Table II Metastasis of Harderian gland tumours

	Virgin	*Mated*	*Total*
Number of Harderian gland tumours	6/53	18/187	24/240
Number with lung metastases	1	16	17
Percentage with lung metastases	16.7%	88.8%	70.8%

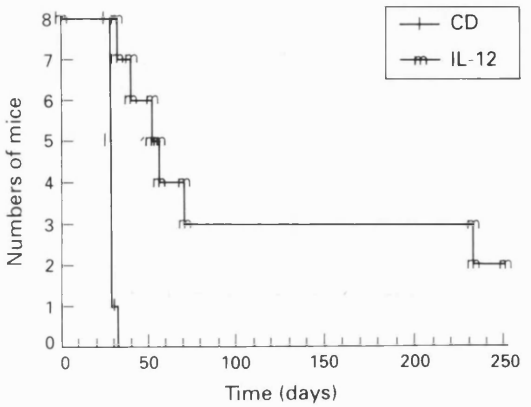

Figure 6 Percentage increase in survival of nude mice bearing transplanted breast tumours and receiving interferon therapy.

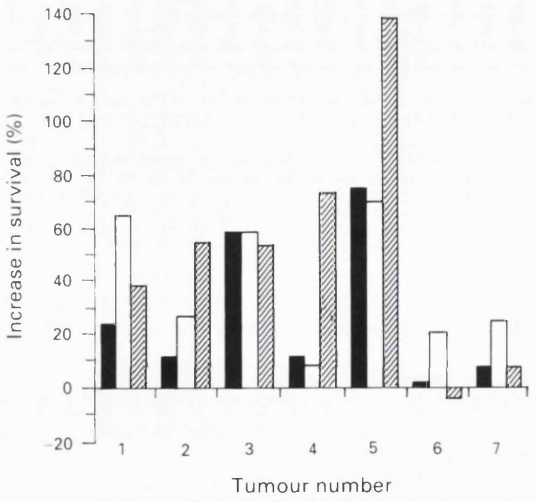

Figure 7 Percentage increase in survival of nude mice bearing transplanted breast tumours and receiving interferon therapy. ■, IFN-α; □, IFN-γ; ▨, IFN-α + IFN-γ.

each group seven or eight animals were assessable and median survival for the group calculated.

There was a marked diversity between different tumours in their sensitivity to the individual interferons and their combination. With one exception, all the cytokine-treated animals survived longer (but not always statistically so) than control diluent treated. IFN-α treatment caused a significant increase in survival in two of seven different tumour lines ($P = 0.003$), IFN-γ in three of seven ($P = 0.02$, $P = 0.003$, $P = 0.001$) and IFN-α/γ combinations in three of seven ($P = 0.001$ or $P = 0.003$). Three of the tumours failed to respond significantly to either IFN or their combination. Two of the tumours responded significantly to both IFN-α and γ, and in only one case did the combination of these work in the absence of a response to the individual cytokine.

Discussion

The rationale for this study was to develop a model for use in preclinical assessment of cancer therapy, in particular cytokine therapy. An unexpectedly diverse range of tumour types and biological behaviours has been observed. The founder mice were reported to develop poorly differentiated metastatic adenocarcinomas of the breast at 7–14 months of age in a stochastic and asynchronous fashion (Bouchard *et al*., 1989). As these mice have been backcrossed onto a BALB/c background for eight successive generations, the tumour incidence has been lower and the tumour types have been more varied and have arisen later than in the founder mice (Thomas and Balkwill, 1994). This may be the result of the BALB/c background, but may also have been affected by differences in animal husbandry, diet, endemic infection and relative crowding of the animals in the two colonies. In this particular model tumours arise after a number of genetic events and all the above factors may contribute.

One notable observation was the decline in transgene positivity with successive generations. Any explanation for this is likely to be complex. It may be that the transgene is not inherited or expressed as a result of the abnormal 'state' of the oncogene in these mice. As a result it may not be feasible to maintain a reproducible and stable model using a colony of transgenic mice. This potential drawback for the assessment of therapy could be overcome by homozygous matings. This is our current strategy now that the colony has reached the eleventh generation. Another option may be to use a different inbred mouse, such as FVB, which is more suitable for microinjection of DNA, and is more amenable to tumour development. There is no apparent change in the expression or structure of the transgene being transmitted on the basis of Southern analysis with three different enzymes. Similarly protein expression has not altered on the basis of immunohistochemical analysis of transgene-positive tumours from different generations. The change in transgene transmission may be explained by a disadvantage to the heterozygous mice that results in death *in utero*.

Of the two mammary tumour transgenic models involving MMTV-activated *neu*, the tumours described by Bouchard *et al*. (1989) bore a greater histological resemblance to human mammary tumours than those described by Muller *et al*. (1988). We have seen this characteristic morphology in 35 of the 47 mammary tumours. This bears some resemblance to the histological pattern seen in large-cell or comedo-type DCIS in humans, a histological type of tumour associated with c-*erb*B-2 amplification (Bartkova *et al*., 1990). Features of papillary carcinoma, present to varying degrees in twelve of the tumours, are also consistent with findings in humans and associated with c-*erb*B-2 positivity. The close similarities between the grade and cytopathology of murine mammary cancer associated with c-*neu* and the human disease associated with c-*erb*B-2, is in contrast with those seen in other mammary tumours in oncogene transgenic mice (Halter *et al*., 1992; Cardiff *et al*., 1993).

The resemblance to human tumours also extends to many of the non-mammary tumours arising in the colony. Lymphomas have previously been described in mice transgenic for the normal human c-*erb*B-2 oncogene (Suda *et al*., 1990). These lymphomas were predominantly B cell in origin. All the lymphomas in our colony stained positively with the antibody to c-*erb*B-2 and were B cell in origin, suggesting that they were related to expression of the transgene. There were five angiosarcomas arising at a number of different sites that did not express the transgene. In the mouse angiosarcomas usually arise in the spleen, liver and subcutaneous tissues, although they account for fewer than 3% of spontaneously arising tumours (Smith and Pilgrim, 1971). Angiosarcomas tend to be locally invasive and may metastasise to the lungs. This suggests these tumours may not be related to transgene expression, although the incidence is rather high, angiosarcomas being rare in BALB/c mice.

Neoplasms of the Harderian gland form a spectrum and the vast majority arising spontaneously in BALB/c mice tumours are categorised as adenomas. A few progress to adenocarcinomas and metastasise to lung, although the incidence of metastases may be increased by exposure to a number of mutagens and chemicals (Della Porta *et al*., 1963; Fry *et al*., 1975; Vesselinovitch *et al*., 1975). In our experience Harderian gland carcinomas frequently metastasised to lung in mice that had two litters, but not in virgin mice. This did not appear to correlate with the grade of the primary tumour, which is comparable with the behaviour of spontaneously arising carcinomas. The Harderian gland carcinomas stained positive for c-*erb*B-2.

The incidence of mammary tumours arising spontaneously in BALB/c mice kept in germ-free conditions varies widely in different studies. They appear to have a relatively low incidence of spontaneous mammary tumours (up to 5% in retired breeding females) (Foster *et al*., 1982). Other sources suggest up to 3% in breeding females and 1% in virgin mice (Smith and Pilgrim, 1971; Kalra *et al*., 1993) during the normal lifespan of the mouse. The incidence of spontaneous lymphomas in BALB/c mice kept in germ-free conditions is less than 3% but again the overexpression of *neu* suggests that the transgene is involved. The median S-phase fraction value for murine mammary carcinomas arising in this colony is similar to that of human mammary carcinomas (9.0% in these tumours; 9.6% in humans) (Camplejohn *et al*., 1995). A higher proportion of the murine tumours were aneuploid than in many human series (11/12 in this study as compared with 18/29 human tumours in Kalra *et al*. (1993)) but this is entirely consistent with the poor differentiation of these tumours, and characteristic of their *neu* positivity.

The unactivated *neu* oncogene has been reported to be associated with the development of mammary tumours that

metastasise to lung in older transgenic mice (Guy *et al.*, 1992) but the activated gene has been linked with aggressive primary tumours with a relatively low incidence of metastasis (Muller *et al.*, 1988; Bouchard *et al.*, 1989). In our model, in tumours approaching the 2 cm diameter limit, the incidence of metastases approached 70%, being similar to that seen with the unactivated *neu* oncogene and making this a useful model for the study of metastasis. This is most likely a consequence of the BALB/c genetic background and the fact that tumours arise later in this model in comparison with the founder mice of Bouchard *et al.* (1989).

Our findings with this colony have not been described by others working with mice transgenic for activated c-*neu*. Indeed the spectrum of tumours more closely resembles that described by Suda *et al.* (1990) in mice with the unactivated c-*erb*B-2 oncogene and that seen with MMTV-Ha-*ras* (Cardiff *et al.*, 1993). The c-*neu* proto-oncogene (rat homologue of the human c-*erb*B-2 oncogene) is a membrane-bound 185 kDa receptor molecule with tyrosine kinase activity. It shares partial homology with the epidermal growth factor receptor and its role in mammary cancer has been extensively investigated (Slamon *et al.*, 1987). In a chemically transformed neuroblastoma cell line, rat c-*neu* is activated by a point mutation, which results in a single amino-acid substitution (valine to glutamic acid) in the transmembrane domain of the protein (Bargmann *et al.*, 1986*a*). The mutant *neu* gene, but not the normal *neu* gene, can transform NIH3T3 cells (Bargmann *et al.*, 1986*b*). Substitution of the corresponding amino acid in human c-*erb*B-2 protein would require two mutations in the gene. The human c-*erb*B-2 gene can transform the fibroblasts by overexpression (Di Fiore *et al.*, 1987). Overexpression of c-*erb*B-2 and not activation is found in human adenocarcinomas, particularly breast and stomach cancers (Yokota *et al.*, 1986; Van der Vijver *et al.*, 1987).

The breast tumours arising in the colony grew readily in nude mice and such transplants were used in preliminary cytokine therapy experiments. The aim of these experiments was to develop treatment schedules that could be translated to spontaneously arising tumours at a later date; to assess the inherent cytokine sensitivity of these tumours and to assess the inter-tumour variation in response. In general IFN therapy had a modest beneficial effect on survival but the response was not dramatic and only two complete regressions were recorded in over 150 treated tumours. Three of the tumour lines failed to respond significantly to either IFN or their combination. This lack of response is similar to the human experience with these cytokines in solid tumours (Sparano and O'Boyle, 1992). The diversity of response of individual tumours is again analogous to results obtained in clinical trials with several cytokines (Gutterman, 1994).

Recombinant murine IL-12 has been tested against a number of murine tumour models (Brunda *et al.*, 1993) and shown to have potent *in vivo* anti-tumour and anti-metastatic effects. The preliminary results were encouraging and certainly warrant further investigation. IL-12 would seem to be the most suitable candidate for treatment of spontaneous tumours in this model.

To date there has been limited use of transgenic mice for preclinical assessment of cancer therapy. One of the few studies involved the use of chemotherapy in hybrid transgenic mice (Dexter *et al.*, 1993). However the histopathology of the tumours was not comparable with that seen in humans.

In summary, this model, which demonstrates a histological and biological convergence of human and murine mammary cancer, has potential for evaluating the spectrum of cancer therapies and as such is highly relevant to the assessment of novel therapies for c-*erb*B-2-positive breast cancer. However further manipulations, such as dietary change, hormonal therapy or administration of mild carcinogens, are required to increase the incidence, and decrease the age of onset, of tumours in the colony.

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Assessing new anti-tumour agents and strategies in oncogene transgenic mice

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Key words: oncogene, transgenic, experimental therapeutics, cytokine, screening

Introduction

In the past fifteen years transgenic technology has been used to study immunity, development, gene regulation and pathogenesis. Although the use of transgenic mice as models for therapy and preclinical screening of new agents has been theoretically possible, this area has been slow to expand. Oncogene transgenic mice develop heritable tumours in a diverse range of tissue types, which exhibit reproducible patterns of growth and development. They are therefore likely to provide insight not only into pathogenesis, but also treatment, of human malignancy. In this article we examine the use of oncogene transgenic mice for experimental cancer treatment. We discuss their advantages and pitfalls and give examples of their use and future applications.

Murine tumour models have long been a significant component in the development of cancer therapies. However the use of animal models remains a relatively expensive part of that process and as such has been restricted [1]. Existing mouse tumour models have various complementary features but none is an ideal model of human cancer. Human tumour xenografts involve a human tumour within murine stroma in a mouse which does not have T cells [2]. Thus the influence of both T-cells and syngeneic tumour-stroma interactions cannot be studied. Murine syngeneic models involve rapidly growing tumours which are sometimes highly immunogenic. These tumours are often anaplastic and therefore not a good model for the slow-growing well-differentiated human tumours. In addition, rapidly growing models may not develop the complex tumour/stroma relationships which are targeted by many novel therapies. Transplantable tumours have often been derived from cell lines selected for high viability *in vitro,* and thus may be more resistant to apoptosis than their *in vivo* counterparts. Metastatic models also involve tumour cell lines which localise with reliable frequency to particular sites, and may have been selected for properties which are not necessarily representative of metastatic human tumours. Consequently there is a need for a spontaneous and metastatic tumour model in immunocompetent mice. Oncogene transgenic mice may offer a suitable model. Table 1 summarises the ideal characteristics of an oncogene transgenic mouse model of spontaneous cancer.

Experience with transgenic models to date

A wide range of transgenic mouse models of malignancy express an oncogene and develop spontaneous tumours of various histological types [3, 4]. As they have been used in dissecting the role of cellular and viral oncogenes in the development of cancer they also have potential in cancer drug discovery in treatment [5]. MMTV-Ha-*ras* transgenic mice have been used to investigate the influence of dietary fat on mammary tumourigenesis [6]. Female transgen-

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ic mice were fed diets providing 0,5 or 25% of calories from corn oil (CO). The mammary tumour incidence was 7% (0% CO), 36% (5% CO) and 52% (25% CO), and in mammary tumours in the latter group mRNA levels for *ras* were increased. One component of CO, Apolipoprotein E, may be a link between dietary fat and tumour promotion in these mice. Its synthesis is influenced by dietary fat and it can alter cellular growth and differentiation by altering the interaction between growth factors and cell matrix glycoproteins [7–9].

Another potential use of transgenic mice is to assess different immunotherapy strategies. In the development of T-cell therapy, Hu *et al.* [10] generated transgenic mice which expressed low levels of Friend murine leukaemia virus envelope protein in lymphoid cells under the control of an immunoglobulin promoter. This protein has been shown to serve as an efficient tumor-specific rejection antigen in B6 mice. The transgenic mice were tolerant to the virus envelope protein, whereas envelope specific T-cells transferred from immunised non-transgenic B6 mice mediated complete eradication of the tumour cells [10]. Their results suggest that these and analogous strains of mice may be useful in elucidating principles for the generation and therapeutic use of tumour-reactive T cells specific for tumour-associated antigens.

Another transgenic mouse system which has been used to test anticancer drugs is the wap-*ras* transgenic mouse [11]. Line 69 wap-*ras* transgenic mice have an activated human c-Ha-*ras* gene on their Y chromosome. Adult males develop salivary and/or mammary adenocarcinomas and both tumour types express high levels of human ras oncoprotein. One subline has been created by selective inbreeding which develops multiple mammary tumours at puberty. This has been used for testing chemotherapeutic regimes designed to interfere with processes occurring early in tumourigenesis, before palpable tumour presentation. Tumour sensitivity has also been tested in adult males with palpable mammary and salivary tumours [11].

Female transgenic mice carrying the V-Ha-Ras transgene under the control of the MMTV promoter, develop mammary carcinomas, and have been treated with selected cancer chemotherapy agents. Agents were given intraperitoneally on a daily schedule for 9 days once the mice had developed tumours 50–100 mg in size. Doxorubicin produced significant responses, with several tumours undergoing shrinkage. Two novel anthrapyrazoles were as effective as doxorubicin and showed more activity than mitoxantrone, a synthetic agent with similar properties. As would be predicted, Cisplatinum only caused modest antitumour responses [12]. A quantitative analysis of the tumour growth curve showed a dose response relationship for each drug tested. Thus this model may have a role in identifying drugs with efficacy for breast cancer in women.

Importance of genetic background

It is well documented that genetic background modulates tumour susceptibility. Studies with Eμ-*myc* transgenes illustrate that the kinetics and tumour type can be influenced as well as tumour susceptibility. On a C57 B1/6, SJL or BALB/c background Eμ-*myc* transgenes provoked B lymphoid tumours almost exclusively, but seven of eight founder C3H/HeJ transgenic mice developed T lymphomas [13]. Thus tumour histology is unlikely to be an effect of the transgene alone. They also showed that the stromal environment may influence tumour phenotype [13]. The effect of genetic background is also seen with transgenic mice harbouring the SV40 large T antigen gene in a C57B1/6J genetic background. In this model the level of transgene RNA expression is considerably higher than in mice harbouring the same transgene on an F1 genetic background. In the F1 hybrids there is a dominant negative effect on SV40 large T antigen expression. The

Table 1. Characteristics of the ideal oncogene transgenic model for cancer therapy

- Easily diagnosed/monitored tumour – ideally superficial
- Histological resemblance to equivalent human tumour
- Tumours arise at suitable point in life-span for therapy to be given
- Inbred model ideally – possibility of immunotherapy and transplantation
- High incidence of tumours in transgene positive mice
- Tumours should metastasise

choroid plexus papillomas appear later with less frequency and animals survive longer [14].

Advantages of transgenic mice as a model of cancer therapy

The major advantage of this model is a closer resemblance to the biological mechanisms of the human disease (see Table 2). Non- or weakly-immunogenic tumours arise spontaneously and stochastically in immunocompetent mice. This provides a better parallel for the development of human malignancy and makes it more likely that metastases will arise via the same mechanisms as in human cancer. This is of potential value in preclinical studies of therapy, adjuvant or maintenance, and chemoprophylaxis. These mice are also predisposed to viral and chemical carcinogenesis and should be of value in tests of putative carcinogens.

A broad and increasing range of tumours can be studied. Our experience (see below) would suggest that these tumours are biologically diverse, like human tumours, both between and within individual mice and transgenic strains.

Table 2. Advantages and disadvantages of oncogene transgenic mouse models

Advantages

- Non-immunogenic tumours arise in immunocompetent mice
- Spontaneous tumours arise in a stochastic fashion
- Many tumours resemble human tumours histologically
- Metastases more likely to resemble the human process
- Broad range of tumours can be studied
- Tumours are biologically diverse
- Mice can be used for therapy, chemoprophylaxis and carcinogen screening
- In inbred model, tumours can also be transplanted for experimental therapeutics

Disadvantages

- Wastage of mice.
- Screening for transgene may be labour-intensive.
- Experimental therapeutics may necessitate a macroscopically visible tumour, ideally with a long natural history.
- Tumours may develop too early or too late for experimental therapeutics.
- An inbred strain is needed for cytokine/immunotherapy experiments.
- The cytokine may induce the promoter, altering transgene expression artificially.
- Integration site of the transgene has an uncertain effect on the genome of the animal.
- The genetic background of the mouse will affect tumour type and, maybe, response.

Disadvantages of transgenic mice as a model of cancer therapy

The main disadvantage of this model is the wastage of mice (see Table 1). Many tumours arise only in one sex. Furthermore heterozygous breeding is usually necessary, meaning that only one quarter of the mice born can be used. In most cases attempts to breed homozygous mice have been largely unsuccessful, the animals often develop tumours which are rapidly progressive at an early age. The mice may also be prone to development of other disorders limiting their usefulness in therapeutic experiments.

In most models, only a proportion of transgene positive mice will develop tumours. With most transgenes some founder mice show no biological effect from the presence of the transgene and up to a third may be mosaic for the transgene. On entry into the experiment animals from the same litter must be allocated randomly between groups to reduce bias. The incidence and natural history of tumour development must be carefully documented in each group. This entails greater space than many current animal experiments require.

In many transgenic models, tumours or hyperplasias (especially mammary) may be pregnancy-dependent, regressing when the animal is no longer pregnant or lactating. In addition some tumours are unlikely to develop in virgin females. Breeding either involves geometric expansion of the colony or further wastage of mice. Screening for the transgene makes the model labour-intensive and expensive. The time taken to develop a tumour is dependent on the promoter, transgene and strain and needs to be considered when embarking on therapy experiments.

Where transgenic mice are to be used to test ther-

apies which function by modulating the immune system, such as cytokines, a homogeneous genetic background of inbred strains is important. The contribution of different H2 haplotypes may influence results. A genetically inbred colony is necessary and this entails either micro injection of eggs from inbred animals or backcrossing offspring from an F1 hybrid founder for several generations. In practice it is often difficult to generate isogenic transgenic mice. The eggs from inbred strains are technically more difficult to microinject than the hybrid strains. Inbred mice also tend to have smaller litters, making the establishment of such a colony more problematic. Backcrossing takes a number of generations (ideally twenty) to obtain an inbred background.

The degree and distribution of transgene overexpression in transgenic animals may stack the odds against a therapeutic modality being effective, as constitutive expression of the transgene, which may be an important early event in the development of a tumour, must be overcome. Another potential problem is the integration site of the transgene and its uncertain effect on the genome of the animal. Different lines of the same transgenic animal can behave differently, not only in respect of tumour natural history but also in terms of lifespan and viability.

Many tumours in oncogene transgenic mice, for example solid gastrointestinal tract or haematological neoplasms, can only be diagnosed by the general condition of the animal. By this point the animal is usually too unwell to be recruited to a therapeutic experiment. Hence experiments are likely to be limited to superficial, slowly developing tumours unless some non-invasive method of cancer detection can be developed.

Many of these difficulties have been encountered by companies developing transgenic mice for use in molecular biology and cancer research, and the market has proved disappointing. The Harvard 'oncomouse' was patented in 1988, and licensed exclusively to Du Pont for commercial development. It has now been conceded by Massachusetts Institute of Technology that, if the primary use of the mouse is basic research, a patent application is not justified [15]. The NIH has decided to establish a repository to accept and distribute transgenic mice at cost for the sake of furthering basic biomedical research, thereby buttressing existing commerical facilities. Currently the most promising commerical use is by environmental protection agencies who are using these mice to assess possible carcinogens. The ideal oncogene transgenic model would thus encompass the characteristics outlined in Table 1.

Experience with c-*neu* transgenic mice

We have developed a colony of transgenic mice carrying the activated rat c-*neu* oncogene under the transcriptional control of the MMTV promoter. The founder mice were obtained from Prof Paul Jolicoeur and were reported to develop comedotype metastatic breast tumours in a stochastic and asynchronous fashion at 7–14 months of age [16]. These mice have been bred onto a BALB/c background for nine successive generations.

Although initial studies reported a tumour incidence of over 40% at 7 months, once the mice were crossed to BALB/c the tumour incidence was only 31.4% in mated female mice at 25 months with a median age of onset of 18 months (Thomas *et al.*, manuscript submitted). The tumours were morphologically and biologically diverse with a variety of metastatic patterns and histological features. In later generations a higher proportion of non-mammary tumours arose including lymphomas. Lymphomas have previously been noted in other mice with the *neu* oncogene [17] but not in this founder colony. Eight of the tumours were transplanted and successfully passaged in nude mice or BALB/c mice. These showed a spectrum of cytokine sensitivity in therapeutic experiments. The diversity, histologic and biologic resemblance to human cancer, and cytokine responsiveness suggests that the model has potential for evaluating novel therapies. However, further genetic or environmental manipulations are required to increase the incidence of mammary tumours and decrease the age of tumour onset.

Conclusion

Oncogene transgenic models have potential in assessing conventional and novel cancer therapies. However, these spontaneous models have a number of scientific and economic limitations. We believe they are unlikely to replace existing models for the foreseeable future, but that they may be complementary.

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