An investigation of the biological role of the neural cell adhesion molecule in lung cancer and its clinical potential as a target for antibody-directed therapy.

A Thesis submitted to the University of London for the Degree of Doctor of Medicine

by

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To my parents
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Abstract

The neural cell adhesion molecule (NCAM) is strongly expressed by small cell lung cancers (SCLCs). NCAM functions as a homophilic and heterophilic adhesion molecule in many biological systems and its binding function can be modified by a unique post translational modification with polysialic acid. Previous studies have suggested an association between NCAM and morphology of SCLC in culture and it has been proposed that polysialylated NCAM contributes to the highly metastatic nature of SCLC. Using an aggregation assay, the biological function of NCAM in SCLC was investigated by attempting to inhibit its function with antibodies, peptides and antisense molecules. No differences in the rate of aggregation were observed except in clonal cell lines with high expression of PSA which aggregated at a slower rate than lines with low PSA expression.

Approximately 20% of non-SCLCs express NCAM and they appear to have a worse prognosis, higher metastatic rate and greater chemosensitivity. To investigate the role of NCAM, the NSCLC cell line L23 was transfected with NCAM and its biological characteristics were compared with the parent cell line. No differences were seen in growth rate, invasiveness, chemo- or radiosensitivity in vitro, and growth rate in vivo after transfection with NCAM. However the tranfectants expressed PSA which suggested that polysialylation was activated by transfection with NCAM.

Anti-NCAM monoclonal antibodies (MAbs) localise well in SCLC xenografts in mice and produce regression of tumours when conjugated to a radioactive label. To investigate whether NCAM was a suitable target for antibody directed therapy in man, an anti-NCAM MAb NY3D11 and its F(ab')_2 fragment were radiolabelled and injected into patients with SCLC. No evidence of localisation in tumours was seen but antibody localised preferentially to bone marrow and liver. The most likely explanation for this distribution was binding to circulating natural killer cells.
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Abbreviations

CAM  Cell adhesion molecule
CEA  Carcinoembryonic antigen
Chr A Chromogranin A
Endo N Endoneuraminidase N
EM  Electron microscopy
FACS  Fluorescence activated cell sorting
GPI Glycosylphosphatidylinositol
HITES Hydrocortisone, insulin, transferrin, estrogen, selenium
Ig  Immunoglobulin
Ln  Natural log
MAb Monoclonal antibody
NaCl Sodium chloride
NaOH Sodium hydroxide
NCAM Neural cell adhesion molecule
NE Neuroendocrine
NE-NSCLC Neuroendocrine non-small cell lung cancer
NSCLC Non-small cell lung cancer
NSE Neurone specific enolase
PBS Phosphate buffered saline
PSA Polysialic acid
PSA-NCAM Polysialylated NCAM
SCLC Small cell lung cancer
TBS Tris buffered saline
CHAPTER 1.
Introduction

1.1 Introduction

This thesis addresses two questions about the neural cell adhesion molecule (NCAM) in lung cancer: Firstly, whether expression of NCAM in lung cancer has a role in its biological behaviour and secondly whether NCAM is a suitable target for antibody directed therapy of small cell lung cancer (SCLC). The introduction begins with a review of the structure and function of NCAM and the evidence that has suggested a role for NCAM in the biology of lung cancer and other malignancies. The second part of the introduction presents a general overview of antibody therapy in cancer and is followed by a more specific discussion of antibody studies in lung cancer. The introduction concludes with an outline of the studies performed in this thesis.

1.2 History of NCAM

In the course of studying mechanisms of cell adhesion among cells from neural tissues of chick embryos, a cell surface protein was identified on retinal and brain cells that appeared to be involved in the formation of cell-cell bonds (Rutishauser et al., 1976). Antibodies to this protein, subsequently termed the neural cell adhesion molecule or NCAM, inhibited aggregation of cells from nerve tissue, perturbed normal patterns of differentiation in retinal tissue and prevented binding of nerve cells to muscle cells in vitro (Brackenbury et al., 1977; Buskirk et al., 1980; Grumet et al., 1982).

Chemical characterisation of purified NCAM showed that it was an integral membrane glycoprotein with a high and variable sialic acid content (Hoffman et al., 1982). The sialic acid of NCAM was present in a
unique form as alpha 2-8 linked polysialosyl units which had not previously been found in mammalian tissues (Finne et al., 1983). The NCAM molecule was a single polypeptide chain divided into three domains, an amino terminal region that formed a binding site, a central region that contained the majority of carbohydrate and a carboxyterminal region associated with the cell membrane (Cunningham et al., 1983).

Several observations suggested that NCAM was a ligand involved in the formation of cell-cell bonds and that the binding process involved a direct or 'homophilic' interaction between NCAM molecules (Rutishauser et al., 1982). Large changes in the amount of cell-surface NCAM were observed in early embryonic and neural crest cells which correlated with their migration and suggested a role in development (Thiery et al., 1982). In vitro experiments demonstrated that even small changes in the amount of NCAM could substantially alter binding. For example, when NCAM was conjugated to the surface of synthetic vesicles a two-fold increase in the NCAM-to-lipid ratio of reconstituted vesicles resulted in a greater than 30 fold increase in their rate of aggregation. It has subsequently been shown that the function of NCAM as an adhesion molecule is important in many biological systems (see below).

In man, NCAM is expressed both by normal tissues including nerve, muscle, thyroid and testis and by tumours including lung cancer, neuroblastoma and Wilms tumour. Although the structure and role in the biology of the nerve and muscle have been extensively studied, it was only recently recognised that NCAM is a major immunodominant antigen expressed by SCLC and by 20% of non-SCLC (NSCLC). It has been suggested that expression of this adhesion molecule, which has an important role in cell-cell interactions in other tissues, might influence the highly metastatic behaviour of SCLC (Michalides et al., 1994a). While it was soon appreciated that NCAM might be a suitable target for
antibody-targeted therapy of SCLC, it is not known whether NCAM has a role in the biology of SCLC or whether it represents part of the oncophenotype and has no important function. One of the aims of this thesis was to investigate the biological function of NCAM in lung cancer.

1.3 Structure of NCAM

1.3.1 Gene structure and protein isoforms

The NCAM gene is present as a single copy in the human genome located on chromosome 11 at band q23 proximal to DRD2 (Nguyen et al., 1986; Telatar et al., 1995). Sequence analysis has shown that NCAM is a member of the immunoglobulin gene superfamily of recognition molecules (Hemperly et al., 1985). The genes for two other cell surface molecules involved in cell-cell interactions, Thy-1 and the T cell receptor delta chain are also localised to the same region in man (Van den Elsen et al., 1985; Van Rijs et al., 1985). This region of chromosome 11 appears to be syntenic to a region of mouse chromosome 9 that contains the staggerer locus (D'Eustachio et al., 1985). Staggerer mice have abnormal neurological features which may be related to abnormalities in the conversion of embryonic NCAM to the adult form (Edelman et al., 1982).

The intron-exon structure of the NCAM gene has now been extensively studied (Owens et al., 1987; Thompson et al., 1989). Twenty six exons have been identified in addition to the 5' regulatory region which has also been characterised (Barton et al., 1990; Hirsch et al., 1990). The size and position of the exons appears to remain remarkably constant across species although the size of the introns may vary.

Although more than 20 different forms of NCAM have been identified, the protein products of the NCAM gene can be grouped into 3 main size classes derived from alternative splicing: The transmembrane 180 kDa and 140 kDa forms, the 120-125 kDa glycosylphosphatidylinositol...
(GPI) membrane-linked form and the soluble 110 kDa forms (fig. 1). All three forms have five extracellular immunoglobulin (Ig)-like domains, each domain being encoded by two exons rather than the more usual single exon in Ig molecules (Cunningham et al., 1987; Owens et al., 1987). Each Ig domain has a pair of cysteine residues about 60 amino acids apart that form a disulphide bond which is a feature shared with many other Ig superfamily molecules, particularly those involved in brain development. It has been suggested that this structure may have evolved to resist proteolytic degradation (Walsh et al., 1991).

There is little variation in the structure of the immunoglobulin-like domains with the exception of a region encoded by an alternatively spliced exon located between exons 7 and 8. This exon, termed VASE (variable alternatively spliced exon), encodes a 30 base pair sequence for a 10 amino acid insertion within the fourth domain (Small et al., 1988; Small et al., 1990). The VASE sequence is found in a variety of cell types and appears to become more abundant at later stages of brain development. Its function is unknown but as it is located close to putative sites of homophilic binding and a carbohydrate attachment site it may modulate NCAM-NCAM interactions. Furthermore, the position of VASE insertion is similar to the hypervariable region of an immunoglobulin molecule.

The Ig domains are the major site of carbohydrate attachment. There are five potential sites of N-linked carbohydrate attachment on domains 3-5 but the precise location is unknown. The site(s) of homophilic binding are also uncertain but blocking antibody studies have suggested that Ig domain 3 may be important (Frelinger et al., 1986 Watanabe et al., 1986).
Figure 1. Diagram showing exon structure of NCAM gene and structure and membrane attachment of the major NCAM isoforms.
Heparin can disrupt NCAM-NCAM interactions in tissue culture models (Cole et al., 1989a). A heparin-binding domain has been located to a region of Ig domain 2 suggesting that heparin might block homophilic binding through steric effects (Cole and Akeson, 1989a).

Alternative splicing occurs extensively in the NCAM gene between exons 11 and 14. There are 9 exons in this region generating at least 8 alternatively spliced variants of NCAM. The region encoded by exon 11 appears to be an immunodominant region of the NCAM molecule. Several anti-NCAM MAbs react with this sequence including Leu 19, NKH1, UJ13A, Eric 1, Lu 243 and Lu 246. The region encoded by exons 12 and 13 is similar to the type III repeats of the fibronectin molecule. Located between exon 12 and 13 is a region of 6 exons which is associated with at least 8 individual splicing pathways (Santoni et al., 1989). In skeletal myotubes this block comprises 4 exons termed the MSD 1 region (Dickson et al., 1987). This region is unique to skeletal muscle but various other combinations are found in brain. It has similarities with the hinge region of the Ig molecule and the contact site A protein of the slime mould Dictyostelium.

The MSD 1a exon encodes a string of proline residues that may induce a hinge in the NCAM molecule and thus confer a degree of flexibility in the structure. However, as not all NCAM isoforms utilise the MSD 1a exon, it is unlikely to be essential for NCAM-NCAM binding. The MSD 1b and 1c exons encode sequences rich in serine and threonine residues which act as a site of O-linked carbohydrate attachment in muscle (Walsh et al., 1989). This O-linked carbohydrate appears to be exclusive to skeletal muscle and does not appear on brain NCAM (Nybroe et al., 1988). Myoblasts that synthesise NCAM without the MSD 1 region do not bear O-linked carbohydrate whereas myotubes are rich in this form. Several functions for O-linked carbohydrate have been suggested:
O-linked carbohydrate can induce proteins to have a stiffer extended structure, thereby increasing their distance away from the plasma membrane. In the case of NCAM it has been suggested that this modification might extend the Ig recognition domain outside the glycocalyx and increase the probability of cell-cell interactions mediated by NCAM (Walsh and Doherty, 1991). Other possible functions of O-linked carbohydrate include side-to-side interactions that might sequester NCAM to certain membrane regions or as a regulator of NCAM turnover time in the plasma membrane.

The SEC exon of 239 base pairs is located downstream of MSD 1c and upstream of exon 13. It closes the reading frame of the protein and generates a secreted NCAM isoform that contains only one fibronectin repeat (Gower et al., 1988). The final exon, AAG, located immediately before exon 13 is the smallest and encodes just a single amino acid. Its function is not known although its usage appears to be highly regulated.

1.3.2 Attachment to the cell membrane

The mechanism by which NCAM attaches to the cell membrane is determined by differential use of specific exons. The 120-125kDa GPI-linked form uses exon 15 which encodes a 27 amino acid carboxy-terminal region (Cunningham et al., 1987; Barbas et al., 1988). Exon 15 contains two polyadenylation sites which result in the generation of 2.9 kb and 5.2 kb mRNAs. The transmembrane form of NCAM uses exon 16 to traverse the membrane which encodes a 70 amino acid sequence with an alpha helical conformation over about 20 amino acids. The 180 kDa form uses exons 16-19 but the 140 kDa form omits exon 18. Both forms use the single polyadenylation site in exon 19.

It is thought that the mechanism by which a protein is attached to the cell membrane may influence its signalling functions. When
signalling across a membrane is required the transmembrane form is used whereas if the cell needs to provide a recognition signal without responding to it, the GPI-linked form is used. Neurons mainly synthesise 140 kDa and 180 kDa transmembrane forms of NCAM while most of the NCAM synthesised by skeletal muscle myotubes is a GPI-linked 125 kDa isoform.

1.3.3 Polysialic acid

A unique feature of the carbohydrate associated with NCAM is the presence of long chains (up to 200 residues) of unbranched homopolymers of alpha-2,8-linked sialic acid residues (Finne et al., 1983). This polysialic acid (PSA) appears to be virtually unique to NCAM, having only been described elsewhere in bacterial capsular polysaccharides (Troy, 1979) and the perivitelline zone of trout eggs (Inoue et al., 1986). The key enzyme of eukaryotic PSA synthesis is polysialyltransferase-1 which has now been characterised (Eckhardt et al., 1995). A bacterial end neuraminidase (Endo-N) can specifically cleave the alpha-2,8-linked PSA units from NCAM without affecting other sialoglycoproteins (Rutishauser et al., 1985).

Recent evidence suggests that translocation of PSA-NCAM to the cell surface occurs via regulated exocytosis and is linked to cell activity. Following enzymatic removal of PSA, rapid redistribution of PSA-NCAM to the surface of cultured cortical neurons occurs during depolarisation and to the surface of insulin-secreting β cells under conditions that stimulate insulin secretion (Kiss et al., 1994). Furthermore, subcellular fractionation indicates that the secretory vesicle fraction of insulin-secreting cells is highly enriched in PSA-NCAM.

The embryonic (E) form of NCAM contains large amounts of sialic acid which decrease during conversion to the adult (A) form (Hoffman et
1982). Expression of PSA is spatio-temporally regulated and a common feature of NCAM-PSA expressing cells is developmental plasticity (Edelman, 1986). It has been proposed that by modulating the binding ability of NCAM, PSA optimises the conditions for normal development of the nervous system (Rutishauser et al., 1988). In some tissues the embryonic, highly sialylated form of NCAM persists in adult structures capable of morphological plasticity such as the hypothalamo-neurohypophyseal system and the olfactory bulb (Miragall et al., 1988; Kiss et al., 1993).

1.3.4 Soluble NCAM

Soluble NCAM has been found in cerebrospinal fluid, in cell culture media from retinal cells, astrocytes and neurons and in amniotic fluid and serum (Jorgensen et al., 1975; Rutishauser et al., 1976; Ibsen et al., 1983; Nybroe et al., 1989). It has also been demonstrated in the extracellular matrix of striated and smooth muscle and peripheral nerve (Martini et al., 1986; Rieger et al., 1988a; Gulbenkian et al., 1989). In brain, soluble NCAM constitutes approximately 1-2% of total NCAM (Dalseg et al., 1989). Levels of soluble NCAM in the CSF fall in an age dependent manner during the first year of life (Weisgerber et al., 1990).

Three soluble NCAM polypeptide classes of Mr values 190000 (NCAM-s1), 135000 (NCAM-s2) and 115000-110000 (NCAM-s3) have been demonstrated in rat brain and CSF (Krog et al., 1992). NCAM-s3, which is quantitatively the major soluble NCAM class, appears to be a heterogeneous population which may arise from release of GPI-linked NCAM C (Mr 115000-110000) and from extracellularly cleaved transmembrane isoforms NCAM-A (Mr 190000) and NCAM-B (Mr 135000) (He et al., 1987; Nybroe et al., 1989). Spontaneously-released NCAM-C may still be attached to the GPI-anchor as NCAM in the
extracellular matrix is still susceptible to enzymatic cleavage (Theveniau et al., 1991). The generation of NCAM-s3 is likely to be regulated in vivo (Theveniau et al., 1992). It has now been demonstrated that the larger soluble NCAM forms s1 and s2 probably represent intact released transmembrane NCAM-A and NCAM-B (Olsen et al., 1993). These soluble transmembrane forms are likely to exist in vivo as they are readily demonstrated in CSF.

NCAM-expressing rat glioma cells can bind to soluble NCAM and soluble NCAM can inhibit binding of glioma cells to immobilised NCAM suggesting that soluble NCAM may be capable of modulating NCAM-mediated cell-behaviour (Krog et al., 1992; Olsen et al., 1993). The ability of NCAM to modulate behaviour would depend on local concentrations of soluble NCAM and cell surface expression and on the relative contribution of other cell adhesion molecules (Olsen et al., 1993).

Binding of soluble NCAM to cell-surface NCAM may trigger signal transduction to the cell interior. Binding of NCAM antibodies or extracellular NCAM fragments to neuronal cells leads to changes in the level of myo-inositol phosphates, intracellular calcium and pH (Schuch et al., 1989; Frei et al., 1992). Release of NCAM from the cell surface may allow a change from localised action to regional action by triggering transmembrane signalling in a larger area of cells and soluble NCAM in the extracellular matrix may act as a chemotactic factor to guide migrating cells or growing axons (Dalseg et al., 1989).

NCAM is present in the basal lamina of frog muscle (Rieger et al., 1988b). Addition of NCAM antibodies after denervation resulted in abnormal patterns of re-innervation with both old synaptic sites and extra-synaptic regions becoming reinnervated. It was suggested that selective adhesivity due to NCAM in the basal lamina at synaptic sites was decreased by anti-NCAM antibodies. Finally there is evidence that
soluble NCAM is able to influence the configuration of extracellular matrix constituents. Formation of collagen type-I fibrils is retarded and the diameter of fibrils increased in the presence of soluble NCAM (Probstmeier et al., 1992).

1.3.5 Regulation of NCAM expression

The 5' regulatory region of the NCAM gene has been isolated and contains a single promoter region spanning 840 bp (Barton et al., 1990; Hirsch et al., 1990). Exon 0 contains most of the signal peptide and the initiator methionine codon. The transcription initiation site has been mapped to nucleotides -193 and -194 and upstream of this site are binding sites for several regulatory factors including Sp1 and neurofilament-1. Levels of NCAM expression are controlled by regulatory elements binding to the NCAM promoter in a defined pattern.

1.4 Function of NCAM

1.4.1 Homophilic binding mechanism

Studies demonstrating inhibition of adhesion in cell-monolayer or cell-substratum binding assays and inhibition of aggregation of membrane vesicles by anti-NCAM antibody fragments suggested that the mechanism of NCAM adhesion involved homophilic interactions between two NCAM molecules (Rutishauser et al., 1982; Hoffman et al., 1983; Cole et al., 1985b). Transfection of mouse L cells, which do not express any known primary cell adhesion molecules, with either of the transmembrane forms of NCAM resulted in aggregation with each other or with bound membrane vesicles from chick brain (Edelman et al., 1987). Binding was specifically inhibited by Fab' fragments of anti-NCAM antibodies. The transfected cells also exhibited phenotypic changes acquiring more rounded morphologies and spreading inefficiently in
culture. Cells that were transfected with the soluble form of NCAM did not show these morphological changes. Similarly, transfection of mouse 3T3 fibroblasts with cDNAs encoding either transmembrane or GPI-linked NCAM isoforms increased intercellular adhesiveness while the secreted isoform was without effect (Pizzey et al., 1989). Cells transfected with cDNAs either containing or lacking the MSD 1 sequence were equally adhesive.

1.4.2 Homophilic binding site

Early antibody mapping studies suggested that the homophilic binding site was localised to Ig-like domains 3 and 4 (Frelinger and Rutishauser, 1986; Watanabe et al., 1986). A more detailed analysis of domain deletion mutations, epitope mapping of monoclonal antibodies and the use of synthetic peptides to inhibit NCAM activity localised the homophilic binding site to a 10 amino acid sequence (KYSFNYDGSE) between Lys-243 and Glu-252 in the third immunoglobulin domain of chick NCAM (Rao et al., 1992). This sequence appears to be unique to NCAM and has not been found in other cell adhesion molecules. Deletion of this decapeptide sequence from chick NCAM abolishes homophilic binding activity (Rao et al., 1993). Substitution of aromatic residues Tyr-244 and Phe-246 with Ala abolished NCAM binding activity suggesting that hydrophobic and/or aromatic interactions may be important in homophilic binding. In addition, substitution of amino acids in the predicted β-strand portion of the decapeptide binding site led to loss of binding activity suggesting that this backbone structure is also important (Rao et al., 1993). It has subsequently been shown in covaspheres and transfected L cells that this decapeptide sequence interacts isologously with the same sequence on opposing molecules.
providing further support for a homophilic mechanism of NCAM interaction (Rao et al., 1994).

1.4.3 **Localisation of NCAM at cell-cell contacts**

NCAM clusters preferentially at sites of cell-cell contact and not at sites of cell-substrate contact in several NCAM-expressing cell lines (Bloch, 1992). Photobleaching experiments on mouse N2A neuroblastoma cells suggested that the 140 kDa sd (small cytoplasmic domain) isoform was freely mobile in the plane of the membrane but that the 180 kDa ld (large cytoplasmic domain) isoform was relatively immobile following *in vitro* differentiation induced by culture in low serum concentration with dimethyl sulfoxide (Pollerberg et al., 1986). It was subsequently demonstrated that the ld isoform accumulates at areas of cell-cell contact, synapses and at points of cellular contact with latex beads coated with extracellular matrix molecules (Pollerberg et al., 1987; Persohn et al., 1989). These changes were accompanied by a change in expression to the ld isoform alone upon differentiation in these cell lines *in vitro* (Pollerberg et al., 1985). However, when mouse N2A neuroblastoma cells were transfected with full-length cDNA clones of the 140 and 180 kDa isoforms, both isoforms accumulated at areas of cell-cell contact after several days of culture or *in vitro* differentiation (Woo et al., 1993a). These findings indicated that the large cytoplasmic domain of NCAM 180 was not necessary for localisation to points of cell-cell contact.

GPI-membrane anchoring may act as a targeting signal for expression on the apical surface of polarised epithelial cells. When polarised epithelial cells (MDCK cells) were transfected with the cDNAs for GPI-anchored and transmembrane forms of NCAM, the GPI-anchored form was expressed on the apical surface whereas the transmembrane forms were expressed on the basolateral surface (Powell et al., 1991).
1.4.4 *Modulation of function by PSA*

The observation that neuraminidase treatment of NCAM appeared to increase binding of soluble NCAM to retinal cells led to the hypothesis that there was an inverse relationship between the binding strength of NCAM and its sialic acid content (Cunningham *et al.*, 1983; Edelman, 1983). This was supported by studies which measured the rate of aggregation of vesicles reconstituted from lipid and purified NCAM or of native membrane vesicles (Hoffman and Edelman, 1983). Aggregation was inversely related to the sialic acid content of the NCAM molecules with full de-sialylation resulting in a four-fold increase over the sialylated E-form of NCAM. However, in other studies of purified NCAM, homophilic interactions were only slightly increased or remained unchanged after removal of PSA (Hall *et al.*, 1987; Hall *et al.*, 1990).

Subsequent studies suggested that PSA on NCAM could have a regulatory effect on adhesion between living cells and was critical for normal morphogenesis of neural tissue (Rutishauser *et al.*, 1985). Injection of Endo-N into the eyes of chick embryos produced an array of abnormalities in the neural retina including a thickening of the neural epithelium in the posterior eye, a failure of cells to elongate radially, formation of an ectopic fiber layer and incomplete association of the presumptive pigmented epithelium with the neural retina.

1.4.5 *Mechanism of Modulation of NCAM function by PSA*

Removal of PSA from embryonic chick brain cells results in a 5-fold increase in the area of closely opposed regions (Rutishauser *et al.*, 1988). PSA can comprise up to one third of the mass of NCAM and it has been suggested that the steric properties of the negatively charged PSA moiety can affect not only NCAM-NCAM binding, but in view of the abundance of NCAM in the membrane could produce a thin screen of
carbohydrate around the cell and consequently exert broad steric effects. In F11 neuron/neuroblastoma hybrid cells, the measured density and size of PSA suggests that a substantial fraction of the space between two opposed cell surface membranes is sterically influenced by the presence of PSA (Yang et al., 1992). Removal of PSA causes a 25% decrease in the distance between two opposed cells. Studies of membrane vesicle aggregation under conditions of varying ionic strength have provided further evidence that the charge and hydration properties of PSA can impede both molecular interactions between opposing membranes and direct contact of NCAM with other proteins at the cell surface (Yang et al., 1994).

For NCAM with relatively low PSA content as found in early development and adult tissues, the formation of NCAM-NCAM bonds would increase the extent or duration of membrane contact and promote other interactions. For NCAM with a high PSA content, the large volume occupied by carbohydrate impedes membrane contact so that not only NCAM-NCAM interactions are hindered but also the function of other ligands (fig. 2). This hypothesis was directly supported by experiments with the F11 neuroblastoma/sensory neuron cell hybrid where removal of PSA augmented cell-cell aggregation mediated by the L1 cell adhesion molecule as well as attachment to a variety of tissue culture substrates (Acheson et al., 1991).

Other mechanisms by which PSA could exert broad effects on cell-cell interaction are mediated via the NCAM polypeptide, either by generation of some form of transmembrane signal that alters cell biochemistry or by affecting the association of other receptors such as L1 with NCAM (Schuch et al., 1989; Doherty et al., 1990a; Kadmon et al., 1990a).
Figure 2. Models of NCAM-mediated adhesion. (A) shows NCAM-NCAM homophilic binding, NCAM-heterophilic binding, and interaction between other adhesion molecules. In (B), polysialylation of NCAM inhibits all 3 types of interaction.

1.4.6 Heterophilic binding mechanism

Aggregation amongst retinal and mouse neuroblastoma cells occurs vigorously by NCAM-dependent mechanisms but aggregation of NCAM transfectants in other experiments may be relatively poor (Brackenbury et al., 1977; Rathjen et al., 1984; Edelman et al., 1987; Pizzey et al., 1989). In a direct binding assay no homophilic interaction between soluble NCAM and immobilised NCAM was detected (Probstmeier et al., 1989). Thus it was suggested that homophilic NCAM-mediated binding
may be relatively weak and that heterophilic binding interactions may also be important (Kadmon et al., 1990b).

A heterophilic mechanism of NCAM-mediated cell-substratum binding was supported by studies involving a quantitative centrifugal adhesion assay (Murray et al., 1992). Embryonic chick retinal cells and mouse N2A neuroblastoma cells both adhered to purified NCAM adsorbed to a solid substrate and the adhesion could be inhibited by monovalent anti-NCAM fragments. However, CHO cells transfected with NCAM cDNAs did not adhere specifically to substratum-bound NCAM and pretreatment of both the chick and mouse cells with anti-NCAM fragments did not inhibit adhesion to substratum-bound NCAM. These results supported the hypothesis that a heterophilic interaction was occurring between substratum-adsorbed NCAM and a non-NCAM ligand on the surface of the probe cells.

In the same study the effects of temperature, cation availability and cytoskeletal inhibitors on cell adhesion to substrate-immobilised NCAM were investigated. NCAM binding is cation-independent in contrast to other cell adhesion systems involving integrins or cadherins. Although adhesion was reduced by low temperatures it was not influenced by cytoskeletal inhibitors. This suggested that lateral mobility of NCAM and its receptors was important for time-dependent strengthening of adhesion but that strengthening of adhesion was independent of the cytoskeleton. Cell adhesion to NCAM substrates is also independent of inhibitors of cellular metabolism suggesting that NCAM function does not depend on metabolic activity (Storms et al., 1994).

In a subsequent study, no difference was detected in the force required to dislodge either untransfected mouse L cells or L cells transfected with NCAM 140 from a monolayer of L cell NCAM 140 transfectants (Woo et al., 1993b). In contrast to previous experiments
(Edelman et al., 1987), adhesion among transfected L cells in suspension aggregation experiments was no greater than that of parental L cells and the force required to dislodge embryonic chick neural retina cells from a retinal cell monolayer was not reduced by treatment with anti-NCAM antibody fragments. These results suggested that NCAM-mediated homophilic binding was weak and that the major interaction was more likely to be heterophilic.

1.4.7 NCAM has the ability to bind different ligands

Several studies have demonstrated that NCAM can bind to different ligands. NCAM binds to heparin and heparan sulphate with the physiological ligand likely to be a heparan sulphate proteoglycan (Cole et al., 1985a; Cole et al., 1986a; Cole et al., 1989b) Exogenous heparin or heparan sulphate competitively inhibits both NCAM-mediated cell-substratum and cell-cell adhesion suggesting that binding of this proteoglycan may be involved in NCAM function (Cole and Glaser, 1986a; Cole et al., 1986b). The heparin binding site has been mapped to a 17AA segment within the second Ig-like domain (Cole and Akeson, 1989a; Reyes et al., 1990).

The cell adhesion molecule L1 is invariably cooexpressed with NCAM on neural cells (Kadmon et al., 1990b). A functional cooperation was suggested by the finding that the molecules copurified by immunoaffinity chromatography and accumulated at sites of cell contact between cultured cells (Pollerberg et al., 1987). Studies have suggested that NCAM can modulate L1 on one cell (cis-binding) by forming a putative molecular complex which results in an increased interaction with L1 on a neighbouring cell (trans-binding) (Kadmon et al., 1990b). The functional cooperation between L1 and NCAM has been shown to be carbohydrate dependent (Kadmon et al., 1990a).
NCAM can also bind to chondroitin sulphate proteoglycans, galectin-3, a β-galactosidase binding animal lectin and protein H, a bacterial surface protein with affinity for both the immunoglobulin and fibronectin domains (Milev et al., 1994; Frick et al., 1995; Probstmeier et al., 1995).

1.4.8 Extracellular domains of NCAM are involved in different functions

In addition to the putative homophilic binding site in Ig domain III, individual domains of NCAM may also have specific functions. When domains of mouse NCAM were synthesised as protein fragments in a bacterial expression system, it was found that Ig-like domains I and II and the combined type III fibronectin repeats I-II were the most efficient for adhesion of neuronal cell bodies when coated as substrates (Frei et al., 1992). Neurite outgrowth was best on substrate-coated combined type III fibronectin repeats I-II as was spreading of neuronal cell bodies. However, metabolism of inositol phosphate, and intracellular pH and calcium were all increased by Ig domains I and II whereas the fibronectin repeats had no effect. These experiments suggested that the domains of NCAM may have different roles in cell adhesion and signal transduction and that they were functionally competent without carbohydrate.

The alternatively spliced 30-bp VASE exon in the fourth immunoglobulin-like domain is the structural variation nearest those portions of the polypeptide proposed to mediate cell-cell adhesion. When L cells were transfected with expression vectors encoding rat 140 kD NCAM +/- the VASE exon, cells expressing NCAM-VASE formed aggregates which tended to exclude cells expressing NCAM+VASE and vice versa (Chen et al., 1994). Thus a small difference in NCAM structure appears to be sufficient to allow segregation of cells.
1.4.9 NCAM and transmembrane signalling

The morphoregulatory activities of NCAM can involve activation of intracellular second messenger pathways. When PC12 cells are cultured on monolayers of 3T3 cells expressing transfected NCAM, the morphology of PC12 cells is changed from an adrenal to a neuronal phenotype in a transcription-independent manner. These changes appear to be directly induced by NCAM through G protein-dependent activation of L- and N-type neuronal calcium channels (Doherty et al., 1991). Protein phosphatases and tyrosine kinases have also been implicated as components of N-CAM intracellular signalling pathways (Klinz et al., 1995).

1.4.10 NCAM can modulate formation of junctions involving other cell adhesion molecules

Adherens-type junctions (AJ) are specialised intercellular contacts, mediated by cadherins and characterised by the association with actin filaments through a vinculin- and catenin-rich submembrane plaque. The transfection of NIH-3T3 cells, which have poor cadherin- and vinculin-containing intercellular junctions, with cDNA encoding the 140kD NCAM resulted in the extensive formation of cadherin- and vinculin-rich AJ, demonstrating a cooperativity between the two junctional systems (Michalides et al., 1994).

1.5 The role of NCAM in biological systems

NCAM has a role in axonal growth during development and regeneration and both the transmembrane and the GPI-linked isoforms are potent substrates for neurite outgrowth (Landmesser et al., 1990; Doherty et al., 1989). Antibodies to NCAM can inhibit neurite regeneration over cells that express either native or transfected NCAM.
(Bixby et al., 1987; Doherty et al., 1990a). There is a critical threshold of NCAM expression required for increased neurite outgrowth above which small increases in NCAM induce substantial increases in neurite outgrowth (Doherty et al., 1990b). Neurons can show a considerable variability in their responsiveness to NCAM in a cellular substrate. For example, retinal ganglion cells lose their ability to respond to a given level of NCAM expression over a short developmental period (Doherty et al., 1990a). The loss of responsiveness cannot be correlated with reduced expression of NCAM and is likely to be a consequence of differential splicing and post-translational processing.

NCAM expression is tightly regulated during skeletal muscle development. In proliferating myoblasts the predominant NCAM isoform is the 140-KD transmembrane species whereas in myotubes the 125-kD GPI linked form is expressed (Moore et al., 1987). During myogenesis, NCAM expression is upregulated by 4-5 fold due to increased transcriptional rate (Roubin et al., 1992). If the 125-Kd GPI-linked NCAM is enzymatically removed, myoblast fusion is inhibited while overexpression of 125-kD NCAM after transfection enhances fusion (Dickson et al., 1990; Knudsen et al., 1990).

NCAM and N-cadherin play an important role during in vitro chondrogenesis of limb bud mesenchymal structures (Widelitz et al., 1993). Cell-cell contact is thought to be a key event in mesenchymal cell condensation in the chick limb, the starting event of chondrogenesis. Expression of NCAM is developmentally regulated in differentiating chondrocytes (Tavella et al., 1994). NCAM is detectable in prechondrogenic cells, increases during cell aggregation, becomes undetectable in hypertrophic chondrocytes and is subsequently reexpressed during maturation to osteoblast-like cells.
Glial proliferation is a key process during neural development and in the regenerative response of neural tissue to injury. Astrocytes express NCAM both in vivo and in culture although the amount decreases significantly during CNS maturation as it does elsewhere in the nervous system (Bartsch et al., 1989; Smith et al., 1993; Linnemann et al., 1993). However, following neurotoxin-induced brain damage, glial cells express high levels of sialylated embryonic form of NCAM (Le et al., 1992). Similarly peripheral nerve injury leads to the expression of high levels of sialylated NCAM in both neural and glial tissue (Danilloff et al., 1986). A functional role for NCAM in neural regeneration is supported by evidence that astrocyte proliferation in culture is inhibited by addition of purified NCAM, antibodies to NCAM and synthetic peptides with homologous sequences to the putative homophilic binding region (Sporns et al., 1995).

Several observations have suggested that NCAM and related molecules may be involved in the processes of learning and memory. In the marine mollusc, Aplysia, a form of learning (long-term sensitisation of the gill- and siphon-withdrawal reflex), is associated with neurotransmitter mediated down-regulation of apCAM, an adhesion molecule which closely resembles NCAM, on the cell surface of presynaptic neurons involved in the learning reflex (Mayford et al., 1992). However in humans, the expression of NCAM in Alzheimer's disease does not appear to be different from age-matched controls (Gillian et al., 1994).

NCAM-deficient mice generated by gene targeting remain healthy and fertile but adults have a 10% overall reduction in brain weight and a 36% decline in size of the olfactory bulb (Cremer et al., 1994). This region maintains a high degree of plasticity in the adult and is a major region of the brain in which NCAM proteins retain a highly polysialylated form.
Loss of NCAM was also associated with an almost total loss of alpha-2,8-linked PSA. Although the activity and motor abilities of the NCAM-deficient mice appeared normal, they showed deficits in spatial learning.

There is a significant reduction in the expression of the embryonic polysialylated form of NCAM in the hippocampal region of brains of schizophrenic patients (Barbeau et al., 1995). While the overall expression of NCAM does not differ from control brains the decrease in PSA-NCAM immunoreactivity in schizophrenic hippocampi suggests an altered plasticity of this structure in schizophrenic brains (Breese et al., 1995).

1.6 Expression of NCAM in malignancy

1.6.1 The role of adhesion molecules in malignancy

Tumour growth and metastasis are likely to involve a variety of cell interactions with cell adhesion molecules. Although most tumours are thought to originate from a single transformed cell, the cells within a growing tumour quickly become heterogeneous with respect to many properties including growth rate, drug resistance and metastatic capacity. Thus, some cells may detach from the tumour mass, invade through the basement membrane and underlying connective tissue and enter the vasculature in which they are carried to distant sites. They eventually arrest in capillaries where they penetrate and migrate through the basement membrane and connective tissue to a metastatic site (fig. 3).

Adhesion molecules may be involved in either cell-cell or cell-substrate interactions during the process of metastasis. A decrease in cell-cell adhesiveness may facilitate cell separation and subsequent detachment from the main tumour. The cells may subsequently interact with components of the extracellular matrix allowing them to adhere to and penetrate basement membranes and to invade connective tissue.
The regulation of the expression or activity of adhesion molecules is likely to be of great importance in malignancy.

Figure 3. The major stages of metastasis.

Studies of the epithelial adhesion molecule E-cadherin (also known as uvomorulin, L-CAM, cell-CAM 120/80) showed that Madin-Darby canine kidney (MDCK) epithelial cells acquired invasive properties when intercellular adhesion was inhibited by anti-E-cadherin antibodies (Behrens et al., 1989). Ras-transformed MDCK cells which did not express E-cadherin were constitutively invasive without antibody treatment. Similarly, transfection of E-cadherin into these highly invasive ras-transformed MDCK cells reduced invasiveness which could be reinduced with anti-E-cadherin antibodies or transfection with anti-E-cadherin antisense (Vleminckx et al., 1991). In vitro invasion of non-E-cadherin expressing human cell lines derived from bladder, breast, lung and pancreas could be prevented by transfection with E-cadherin cDNA and again reinduced by treatment with E-cadherin antibodies (Frixen et al., 1991).
This evidence supported the hypothesis that adhesion molecules can act as invasion suppressors. In colorectal cancer, deletions in the q21 region of chromosome 18 are found in over 70% of tumours leading to the identification of a gene termed DCC (deleted in colorectal carcinoma) (Fearon et al., 1990b). Sequencing of the exons revealed that DCC encoded a molecule of the Ig supergene family which showed greatest sequence similarity to NCAM. The loss of DCC appears to be a relatively early event in the stepwise progression of colorectal carcinomas through precursor lesions including hyperplasia, benign adenomas and carcinoma in situ and might contribute to disruption of normal cell-cell adhesion (Fearon et al., 1990a).

NCAM is a cell-cell adhesion molecule that mediates both homophilic and heterophilic interactions between cells and therefore might be expected to reduce rather than promote invasion and metastasis. However, NCAM in malignancy is often expressed in a 'cell-repulsion form' similar to the highly polysialylated embryonic form found during times of cell migration and developmental plasticity (Johnson, 1991). The expression of NCAM in malignancy and the possible functional significance of polysialylation are discussed below.

An alternative mechanism by which overexpression of a CAM might promote metastasis is suggested by the example of carcinoembryonic antigen (CEA), an adhesion molecule that is frequently overexpressed in colon carcinoma and secreted into the circulation (Wanebo et al., 1978). Although this CAM normally promotes adhesive interactions, the overexpression and altered distribution of CEA on malignant epithelial cells may disturb normal intercellular adhesion and lead to disruption of the tissue architecture (Benchimol et al., 1989). CEA may also play a direct role in development of metastasis. CEA is cleared from the circulation by the liver and can be detected on the surface of
Kupffer cells and hepatocytes where it may act as a receptor for circulating CEA-positive tumour cells. This is supported by experiments in nude mice where liver metastasis and implantation of colorectal carcinomas could be enhanced by prior intravenous injection of CEA (Hostetter et al., 1990). This selective trapping may increase the probability that these cells successfully establish hepatic metastases.

It is possible that NCAM may function in a similar way as a 'homing receptor' for NCAM-expressing tumour cells. NCAM is widely expressed throughout the normal nervous system and detectable as soluble NCAM in the circulation. There is high incidence of brain and liver metastases in SCLC which may be associated with NCAM expression in a similar manner to CEA in colon carcinoma.

A third group of interactions which may involve NCAM in malignancy are those with the cell matrix. Having escaped from the primary tumour site, a metastasising cell must invade through the extracellular matrix prior to invading the vascular basement membrane and entering the circulation. Interaction with components of the matrix including type I collagen, fibronectin, vitronectin and hyaluronic acid are mediated through molecules including integrins and lymphocyte attachment molecules such as CD44 (Zetter, 1993). NCAM can also function as a cell-substrate adhesion molecule through interactions with extracellular ligands such as heparan sulphate proteoglycans and various collagens. Recent evidence supporting a role for NCAM interaction with the extracellular matrix in malignancy is presented below.

In summary, NCAM may play a role in tumour metastasis through modulation of both cell-cell interactions and cell-matrix interactions and by acting as tumour receptor signal for circulating tumour cells. The expression of NCAM in lung cancer and other malignancies is described
below and is followed by a review of the evidence supporting a functional role for NCAM in tumours.

1.6.2 Expression of NCAM by lung cancer

NCAM was identified as an antigen of SCLC following the first International Workshop on SCLC antigens (Souhami et al., 1987). There was interest in the antigens of SCLC for detection of tumour in foreign sites, as targets for antibody-mediated attack, and to improve understanding of biological behaviour. By the late 1980s, at least 100 MAbs had been raised throughout the world against SCLC and there was need for a central analysis modelled on the previous international workshops on human leucocyte antigens (Knapp et al., 1989). Participants in the workshops were provided with a coded panel of MAbs with which they carried out immunohistochemical, immunocytological or flow cytometric analysis on a range of target tissues or cells. The results of these assays were analysed centrally and the MAbs grouped into clusters on the basis of patterns of similar reactivity. After three workshops a total of fifteen clusters have been identified and many of the antigens have been identified or characterised (Stahel et al., 1994) (table 1).

The major cluster identified at the first international SCLC workshop was termed cluster 1. At that time the identity of the antigen was not known but it was recognised that the tissue specificity of cluster-1 antibodies which bound to SCLC and other neuroectodermal tissues/tumours was similar to that of "cluster-1-related" antibodies from a parallel Neuroblastoma Workshop (Kemshead, 1987) which bound to neuro-ectodermal tumours/tissues, Wilms' tumours, foetal kidney/muscle and rhabdomyosarcomas. It also became apparent that the tissue specificity of cluster 1/cluster-1-related group of antibodies was similar to the known distribution of NCAM.
<table>
<thead>
<tr>
<th>Cluster</th>
<th>Antigen</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NCAM</td>
<td>SCLC, neuroblastoma, brain, nerve, testicular leydig cells, thyroid</td>
</tr>
<tr>
<td>2</td>
<td>Epithelial glycoprotein</td>
<td>Broad epithelial distribution</td>
</tr>
<tr>
<td>EGP-2/GA733-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CD24 leucocyte activation antigen</td>
<td>SCLC, ductal epithelium, granulocytes, neural tissue</td>
</tr>
<tr>
<td>5a</td>
<td>Sialoglycoproteins</td>
<td>50% SCLC, not epithelial tissues or granulocytes</td>
</tr>
<tr>
<td>6</td>
<td>Lewis y hapten</td>
<td>SCLC, NSCLC, broad epithelial but not granulocytes or neural</td>
</tr>
<tr>
<td>w7</td>
<td>High MW mucins</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Mucins Tag-72</td>
<td>NSCLC, broad epithelial</td>
</tr>
<tr>
<td>9</td>
<td>Mucins MUC-1</td>
<td>SCLC, NSCLC, epithelial, granulocytes</td>
</tr>
<tr>
<td>10</td>
<td>Neurone specific proteins</td>
<td>Intracellular expression in NE tissues</td>
</tr>
<tr>
<td>11</td>
<td>40kD protein (nag gene)</td>
<td>NSCLC, weak epithelial binding</td>
</tr>
<tr>
<td>12</td>
<td>Folic acid receptor</td>
<td>NSCLC, limited epithelial binding, monocytes</td>
</tr>
<tr>
<td>13</td>
<td>EGP-1/GA733-1 (transmembrane proteins)</td>
<td>NSCLC, some epithelia</td>
</tr>
<tr>
<td>14</td>
<td>EGF receptor, p185\text{\textsuperscript{neu}}</td>
<td>NSCLC, limited epithelia</td>
</tr>
<tr>
<td>15</td>
<td>mesothelial membrane antigen</td>
<td>Mesothelioma, squamous cell lung carcinoma, some epithelia</td>
</tr>
</tbody>
</table>

Table 1. Lung cancer antigens
To demonstrate that the cluster 1 antigen was NCAM, 3T3 cells were transfected with full length cDNA coding for the 125kDa isoform of human NCAM (Patel et al., 1989). Indirect immunofluorescence showed binding of cluster 1/cluster-1-related antibodies to 3T3 transfectants but not to control 3T3 cells.

1.6.3 Expression of NCAM isoforms in SCLC

NCAM is present on 100% of classic SCLC cell lines and 88% of variant SCLC cell lines (Carbone et al., 1991). In the human SCLC cell line H69, only the 180kd and 140kd NCAM isoforms are expressed (Moolenaar et al., 1990). When expression of NCAM isoforms in H69 was investigated by analysis of NCAM mRNAs, four major mRNA size classes of 7.2, 6.7, 4.3 and 4.0kb were identified (Moolenaar et al., 1992). Only the 4.0kb transcript is not found normal tissues. The 7.2-kb and 6.7kb mRNA code for NCAM-180 and NCAM-140 respectively. Additional alternative splicing events further increase the diversity of NCAM transcripts: The trinucleotide AAG occurs in most NCAM mRNAs as an insert between exon 12 and 13 (the hinge region of NCAM). It is usually associated with other alternatively spliced exons (SEC,MSD) but in H69 it occurs without these exons. A second alternatively spliced 30 nucleotide VASE exon is present in all NCAM transcript isoforms at the exon7/exon 8 junction. This results in the insertion of 10 amino acids into the 4th immunoglobulin loop of the NCAM protein.

In a study of expression of SCLC isoforms in SCLC tissues and lines (GLC-14, -16, -19, -28 and -1), the 140- and 120-kDa isoforms and their corresponding mRNAs were present in all lines/tissues, whereas the 7.4-kb transcript coding for 180-kDa isoform was only found occasionally (Van Duijnoven et al., 1992). The VASE mini-exon was detected in some of the mRNAs. A 5-kb mRNA transcript was identified in all SCLC
samples but the NCAM isoform encoded by this mRNA remains unknown. From these limited studies, it appears that SCLC has the potential to express the 180, 140 and 120-kDa isoforms of NCAM with further minor variations in protein size arising from VASE and AAG alternative splice variants. It is not known whether these minor alterations can affect the binding properties of NCAM.

1.6.4 Polysialylation in SCLC

Several studies have demonstrated that NCAM of surgically resected SCLCs, cell lines and xenografts is polysialylated. When paraffin sections of resected lung tumours were stained with MAb 735 (which binds specifically to (2,8) linked sialic acid polymers consisting of at least eight molecules), all SCLC (19) stained focally or diffusely whereas only 3/33 NSCLC were faintly and focally positive with MAb 735 (Roth et al., 1988; Kibbelar et al., 1991). Polysialylation of NCAM in tumour cell lines and xenografts has also been identified by the presence of smearing on Western blots, when it was detected in 14/18 NCAM expressing SCLC tumours (Rygaard et al., 1992). The possibility of a lower level of PSA in the apparently negative tumours could not be excluded.

FACS analysis of the SCLC cell line H69 with MAbs 735 and 123C3 (anti-NCAM) reveals that two forms of NCAM are present - a highly polysialylated form termed NCAM-H and a less sialylated form, NCAM-L but it is unclear whether downregulation of PSA expression on some cells is a result of tissue culture adaptation (Moolenaar et al., 1990). Cell heterogeneity in PSA expression by MAb735 immunoreactivity has also been studied in 50 primary lung tumours and the metastases from 15 of these. Two patterns of immunostaining for PSA were seen: 1) SCLC composed of all cells exhibiting immunostaining (54%) and 2) SCLC with a variable number of cells immunoreactive for PSA (46%). In samples
where sections from both primary tumour and metastases were available, the pattern of PSA staining remained consistent between the two.

A study of NCAM-associated PSA expression in 99 surgically removed neuroendocrine primary lung tumours showed increasing prevalence of positivity from typical carcinoid (4/12), to atypical carcinoid (36/47) and SCLC (39/41). It was suggested that there is an association between expression of PSA and an aggressive and immature subtype of tumour (Michalides et al., 1994).

In a human SCLC cell line, there is evidence that synthesis of PSA occurs in the golgi apparatus (Kalafonos et al., 1988). After enzymatic removal, PSA only reappears on the cell surface at control levels after 5 days. However, when transport from golgi apparatus to cell membrane is blocked, the presence of PSA can be demonstrated in the golgi apparatus.

1.6.5 Kidney tumours

The polysialylated embryonic form of NCAM is detectable in embryonic kidney but not in the adult kidney. Wilms' tumour (nephroblastoma) a highly malignant kidney tumour, is characterised by reexpression of polysialic acid characteristic of the embryonic form of NCAM (Roth et al., 1988).

1.6.6 Haematological malignancies

The expression of NCAM is a rare phenomenon in malignant lymphoma. A group of NCAM-positive lymphomas have been identified which appear to have a predilection for extranodal sites of involvement (including CNS, muscle, GI tract, skin and nasopharynx) and an aggressive clinical course (Kern et al., 1993; Wong et al., 1994; Nakamura et al., 1995). The disease affects mostly middle-aged adults who present with fever, skin rash and hepatosplenomegaly in the absence
of peripheral lymphadenopathy. Similarly, a case of NCAM-positive peripheral T cell lymphoma associated with HTLV-1 is described which also shows unusual involvement of the GI tract (Hayashi et al., 1994). It is suggested that the presence of NCAM on lymphoma may have biological and clinical significance in terms of tumour behaviour and spread.

The expression of certain cell-adhesion molecules on leukaemic blasts may determine a tendency to develop extramedullary relapse. For example, a patient with NCAM-positive acute myeloid leukaemia (AML) who had successful induction chemotherapy subsequently relapsed after 8 months with a paraspinal granulocytic sarcoma (Krishnan et al., 1994). In AML, CD56 positivity is associated with VCAM-1 expression and leukaemic cells of B cell lineage. Clinically, this subset was associated with dermal infiltrates (Reuss et al., 1995).

N-CAM expression is a common finding in multiple myeloma (Barker et al., 1992). In a study of 21 patients with multiple myeloma, N-CAM was found in 62% of patients (Drach et al., 1991).

1.6.7 CNS tumours

The 140-kDa isoform of NCAM appears to be expressed by all meningiomas (Figarella et al., 1994). The expression of polysialylated isoforms is associated with high-grade tumours and convexity meningiomas. In choroid plexus tumours and ependymomas, polysialylated NCAM immunoreactivity has been found to correlate with the emergence of anaplastic features (Figarella et al., 1995).

1.6.8 Skin tumours

NCAM immunoreactivity has been demonstrated in normal Merkel cells of human skin and in neuroendocrine carcinomas of the skin (Merkel cell carcinomas) (Gallego et al., 1995).
1.6.9 GI tumours

In the normal gastric mucosa, NCAM immunoreactivity has been detected in the deep gastric gland and the islets of Langerhans (Sakamoto et al., 1994). No NCAM positivity was detected in gastric carcinomas or cell lines regardless of their differentiation status. However all gastric carcinoid tumours are NCAM-positive suggesting that they might be derived from NCAM-positive cells in deep gastric gland.

In gall bladder cancer, perineural invasion is significantly more common in patients with NCAM-positive tumours (88% in patients with NCAM-positive tumours and 22% in NCAM-negative tumours) (Seki et al., 1995).

1.6.10 Muscle tumours

Embryonal rhabdomyosarcoma expresses NCAM. Tissue specific expression of the MSD1 region which was originally detected in the 120-kDa isoform of NCAM in muscle may allow this tumour to be differentiated from neuroblastoma. The MSD1c mini-exon was only detected in NCAM from rhabdomyosarcoma cells and not in any of the neuroblastoma cell lines investigated (Phimister et al., 1994). However, in a study of three rhabdomyosarcoma-derived human cell lines, only one expressed a highly sialylated NCAM whereas two of the lines had no detectable NCAM expression (Soler et al., 1993).

1.6.11 Soluble NCAM as a marker of disease

Soluble NCAM has been proposed as a potential serum marker of disease in SCLC (Jaques et al., 1993; Ledermann et al., 1994). Serum NCAM levels correlate with serum neurone specific enolase (NSE), an established marker of disease in SCLC. As with NSE, levels of serum
NCAM are significantly higher in extensive than limited disease and high levels correlate with shorter survival times.

In human serum at least two molecular species of NCAM with MW 110000-130000 and 150000-180000 are identified by Western blotting (Takamatsu et al., 1994). Both are characterised by absence of the intracellular domains of the transmembrane isoforms. The size difference between the two serum species is due to the presence of PSA. When PSA is removed from the 150-180 kDa species, it is indistinguishable from the 110-130 kDa species. Furthermore, while the smaller molecule is detectable in the serum of healthy subjects, the PSA-associated species is only detected in the serum of patients with tumours, suggesting that it may be a tumour-specific marker.

1.7 The role of NCAM in malignancy

1.7.1 NCAM and SCLC morphology in culture

NCAM expression in SCLC may have a role in the morphology of cells in culture. Several observations have suggested an association between NCAM immunoreactivity and the adhesive properties of lung cancer cells. SCLC cell lines in culture usually grow as floating aggregates whereas NSCLC cell lines grow as adherent monolayers (Carney et al., 1985). Classic SCLC lines grow as tight spheroids with central necrosis while variant SCLC (SCLC-v) lines grow as looser branching chains. A small number of SCLC-v lines (referred to as type 4 morphology) grow as adherent monolayers. Of these lines, NCI-H446 (derived from the pleural effusion of a SCLC patient) has been described as growing with coexistent adherent and floating subpopulations (Doyle et al., 1990). After segregating the adherent and floating sublines over many passages, expression of NCAM as detected by immunoreactivity with MAbs UJ13A and HNK-1 was found to be downregulated in the adherent subline. Two
other SCLC-v adherent lines, H196-B and H1607 were also found to be non-reactive with UJ13A and HNK-1 and it was suggested that NCAM immunoreactivity was associated with SCLC growth as floating aggregates.

The hypothesis is supported by observations on SCLC-v cells treated with retinoic acid (Doyle et al., 1991). These cells underwent a marked phenotypic change with loosely aggregated cells becoming tightly aggregated spheroids associated with increased NCAM expression in the retinoid-treated cells. In addition, some cell lines established from NSCLC tumours with features of neuroendocrine differentiation (including NCAM positivity) are unusual in growing as floating aggregates (Gazdar et al., 1987).

The variant SCLC line (SCLC-v) is characterised by gene amplification and overexpression of \textit{c-myc} protooncogene (Little et al., 1983). In addition to altered morphology in culture compared with classic SCLC, SCLC-v has increased doubling time and decreased SCLC biochemical markers. It has been shown that stable transfection of classic SCLC with multiple copies of \textit{c-myc} protooncogene leads to development of SCLC-v growth and morphological characteristics (Johnson et al., 1985). Furthermore, insertion of v-Ha-ras gene into SCLC-v can induce features of typical large cell undifferentiated lung carcinoma (NSCLC) including growth as an adherent monolayer (Mabry et al., 1988). When the classic SCLC cell line H209 was transfected with multiple copies of \textit{c-myc}, expression of NCAM was downregulated and the cells became more loosely associated than the parent line (Doyle et al., 1991). Cells that were subsequently cotransformed with \textit{c-myc} and v-Ha-ras showed a further decrease in NCAM expression and formed a predominantly adherent monolayer.
These observations strengthened the evidence that changes in NCAM expression were associated with changes in the adhesive properties of cells. However, changes in cell culture environment may also alter SCLC cell morphology without associated changes in NCAM expression. Cultivation of the classic SCLC cell line NCI-H69 on a substratum provided by pretreatment with medium conditioned by growth of the lingual squamous carcinoma cell line HN5 induces an adherent phenotype within 24 hr of plating (Walker et al., 1992). An adherent subline, H69A, was established from these cultures but when compared with the parent NCI-H69 cells, H69A retained expression of NCAM. Thus downregulation of NCAM is not essential for SCLC cell lines to adopt an adherent morphology in culture.

1.7.2 Functional studies of the role of PSA in SCLC

There is some evidence to support the hypothesis that polysialylated NCAM in SCLC is associated with decreased cell adherence and might potentiate metastasis. The heterogeneity of PSA expression in the SCLC line H69 has been exploited to establish clonal sublines, which express PSA on either none or 95 percent of the cells (Scheidegger et al., 1994). Expression of PSA in these sublines was found to be stable in serial subculture. In aggregation and disaggregation assays, high PSA expression was found to correlate with reduced cell-cell adherence. There were no measurable differences in attachment to various substrates but the PSA positive clonal subline formed significantly more colonies in soft agar and more intracutaneous metastases in the nude mice. Similarly, removal of PSA from H69 cells by treatment with Endo N can result in a small but significant increase in cell aggregation compared with untreated cells and adhesion of H69 onto the extracellular-matrix components laminin and fibronectin was two-fold higher for treated cells (Michalides
et al., 1994). These observations were consistent with a role for PSA in modulating NCAM binding function in SCLC.

1.7.3 NCAM expression and cell motility, growth and invasion

The coordination between NCAM expression and cell motility during neural crest cell migration provides indirect evidence that changes in NCAM expression in malignancy might relate to decreases in cell-cell adhesiveness or increased motility (Thiery et al., 1982). Neural crest cells express NCAM when they appear at the neural tube but down-regulate expression during migration. Upon arrival at their respective target tissues and during reaggregation the cells re-express NCAM on their surfaces. Thus the migratory potential of cells and NCAM expression seems to be inversely related.

Direct evidence for a role of NCAM in migration derives from experiments with chick neuroepithelial cells which after transformation with Rous sarcoma virus (RSV) express about one tenth of the normal level of NCAM compared with control cells (Brackenbury et al., 1984). The transformed cells exhibit markedly reduced cell-cell adhesiveness and become highly motile, migrating rapidly over the culture dish. Similarly, NCAM expression can lead to migration inhibition (Meyer et al., 1995). When mouse L cells are transfected with cDNA encoding the human transmembrane NCAM isoform of 140 kDa (NCAM-B), the migration of NCAM-expressing cells in collagen is inhibited compared to that of NCAM-negative control cells.

In the WC5 rat cerebellar cell line, loss of NCAM-mediated cell-cell adhesion is not necessary for invasion (Brady et al., 1991). WC5, which is infected with a Rous sarcoma virus, shows temperature-dependent expression of NCAM. At both permissive (NCAM expression 4-10x reduced) and non-permissive temperatures, cells move at similar rates
and are invasive in *in vitro* assays. Furthermore when transformed, low-NCAM-expressing cells are transfected with NCAM, high-NCAM-expressing clones show no reduction in invasiveness (Brady et al., 1993).

The rat glioma cell line, BT4Cn, derived from the parental BT4C cell line, is characterised by a more aggressive behaviour and enhanced growth rate in a subcutaneous location (Mella et al., 1990). BT4Cn cells lose NCAM expression and have increased cell motility (Andersson et al., 1991; Edvardsen et al., 1993a). They invade as single cells into surrounding brain tissue and in contrast to the NCAM-expressing BT4C parental line, they do not form multicellular spheroids in vitro. However following transfection with NCAM, BT4Cn cells aggregate into multicellular tumour spheroids suggesting that this process is mediated by NCAM and that the observed capacity to invade as single cells may be related to reduced cell-cell adhesiveness (Edvardsen et al., 1993b). Transfection of BT4Cn cells with NCAM-140 also modulates the *in vivo* growth of tumours derived from these cells (Edvardsen et al., 1994). NCAM-transfected cells show a slower rate of growth after sub-cutaneous injection than parental cells. When transplanted into rat brain, NCAM-transfectants were less invasive and caused less brain destruction than control cells. This data provided direct evidence that NCAM can modulate the malignant behaviour of rat glioma cells and that loss of NCAM expression is associated with a more invasive and rapidly growing tumour population.

1.7.4 *NCAM can regulate the metabolism of surrounding matrix*

There is some evidence that NCAM can regulate the metabolism of surrounding cell matrix. The NCAM-negative BT4Cn cell line secretes substantial amounts of metalloproteinases whereas its NCAM-positive mother cell line BT4C does not (Edvardsen et al., 1993b). When the
BTC4n cell line was transfected with transmembrane NCAM-140 kDa, there was down-regulation of 92-kDa gelatinase (matrix metalloproteinase 9) and interstitial collagenase (matrix metalloproteinase 1). Transfection with the GPI-linked NCAM-120 kDa isoform did not have this effect. This NCAM-associated modulation of extracellular matrix structure would be consistent with other changes in NCAM expression seen during migratory events in embryogenesis. Thus, a down-regulation of NCAM expression during neural crest cell migration might be associated with an increased secretion of matrix metalloproteinases to facilitate migration. It is possible that similar interactions with the matrix mediated by NCAM might occur in malignancy.

1.7.5 Involvement of NCAM in adhesion of tumour cell lines to endothelial cells

During invasion into blood vessels or lymphatics the initial stage of crossing the endothelium involves tumour cell-endothelial cell adhesion (Zetter, 1993). At least three adhesion systems have been reported to take part in these interactions: the E-selectin (ELAM-1) and its ligands sLe^X and Le^X (CD15), the VLA4 integrin in the spreading of malignant melanomas and binding the vascular cell adhesion molecule (VCAM1) and the CD44 homing receptor whose ligand is hyaluronic acid. It has now been demonstrated that NCAM can mediate both lymphocyte-endothelial cell adhesion and tumour cell-endothelial cell binding (Zocchi et al., 1993; Zocchi et al., 1995).

In tumour cell lines derived from human renal cell carcinomas, an increase in growth rate and loss of adhesiveness to inert substrate is accompanied by NCAM expression and the appearance of specific binding to endothelial heparan sulfate (Zocchi et al., 1994). Similarly when the
renal cell line COS7 was transfected with NCAM, there was a marked increase in ability to bind endothelium and heparan sulfate. *In vivo* studies demonstrate the presence of NCAM at tumour sites of active proliferation in addition to heparan sulfate in the walls of blood vessels close to the tumour. These studies provide further evidence that interactions of NCAM with components of the extracellular matrix and complexed growth factors may be important in the malignant proliferation of tumours.

1.8 NCAM and the neuroendocrine phenotype in lung cancer

1.8.1 The neuroendocrine phenotype in lung cancer

Neuroendocrine cells are found in fetal and infant lungs where they are thought to have a paracrine role in lung development (Aguayo et al., 1992b). In the adult lung, hyperplasia of NE cells to form carcinoid tumourlets is found in areas of fibrosis associated with bronchiectasis but also occurs in other chronic lung diseases (Aguayo et al., 1992a). In the WHO classification of neuroendocrine carcinomas of the lung, a range of NE tumours is recognised from carcinoid tumours at the benign end of the spectrum to SCLC at the highly malignant end (World Health Organisation, 1982). Between the two, there is a subgroup of carcinoid tumours termed atypical carcinoid with histological and clinical evidence of malignancy. Since the early 1980s it became apparent that a small proportion of NSCLC also contained dense core secretory granules when examined under EM which established them as neuroendocrine tumours (Mcdowell et al., 1981) and it was suggested that these NE-NSCLC behaved more aggressively like SCLC (Mooi et al., 1988).

The hallmark of the NE phenotype is the presence of dense core neurosecretory granules seen under EM. In practice however, workers have tended to use immunocytochemistry to detect various markers
associated with the NE phenotype. These include cytoplasmic enzymes and components of intracellular secretory systems such as NSE, PGP 9.5, synaptophysin, CK-BB and Chromogranin A and hormonal secretory products such as GRP, ACTH, calcitonin, calcitonin gene related peptide and leucine encephalin. The natural killer cell membrane antigen Leu-7 (CD57 or HNK1) is also expressed by NE cells. A major problem in the recognition of the NE phenotype is the absence of defining criteria for its detection and the apparent lack of specificity of some markers for NE differentiation. In a study of the reliability of commercial immunocytochemical markers used to identify the NE phenotype, PGP 9.5 and CK-BB were neither sensitive or specific and NSE labelled more NSCLC than SCLC (Gosney et al., 1994). Of five secretory products sought, only GRP was detectable in 1/83 tumours. Synaptophysin and Chromogranin A were the only markers sufficiently specific and sensitive for NE differentiation. In another study, varying dilutions of antibodies and criteria for detection of positivity when immunolabelling NSCLC gave positivity rates between <10% and >90% (Burnett et al., 1993). Because of these difficulties there is little agreement in the literature about the proportion of NSCLC tumours which should be described as NE-NSCLC but 10-20% would be generally accepted (Gazdar et al., 1988).

A strong correlation has been found between expression of NCAM and the NE phenotype in both lung cancer (100% SCLC and 15-20% NE-NSCLC), other neuroendocrine tumours and neuroendocrine tissues (Carbone et al., 1991). At present there is no "gold standard" for NE differentiation, but NCAM expression would seem to be as reliable as synaptophysin and and chromogranin which are currently considered to be the most specific markers of NE phenotype in lung cancers (Gosney et al., 1994).
1.8.2 NE differentiation, NCAM expression and chemosensitivity in NSCLC

There has been considerable interest in whether NE differentiation might identify tumours which behave more aggressively or exhibit greater sensitivity to chemotherapy and radiotherapy. If neuroendocrine tumours of the lung are considered, both ends of the spectrum are represented - SCLC is associated with highly malignant behaviour and response to chemotherapy while typical carcinoids have a more benign behaviour. The possibility that the NE phenotype might identify a small group of NSCLC with atypical behaviour and treatment sensitivity has been the subject of several studies.

_In vitro_ drug sensitivity testing has suggested that SCLC and NE-NSCLC cell lines are more responsive to cytotoxic drugs than NSCLC (Gazdar et al., 1991). Several clinical studies have suggested that patients with NE-NSCLC show increased response to chemotherapy compared to patients with non-NE-NSCLC tumours. Graziano undertook a retrospective analysis of 52 NSCLC patients who had been treated with chemotherapy, of whom 26 were responders and 26 non-responders (Graziano et al., 1989). Diagnostic slides were analysed for the presence of the NE markers NSE, Leu-7 and chromogranin A. Responders with two or more positive markers showed superior survival (median 79 weeks) compared with responders with fewer than two positive markers (median 51 weeks) and non-responders (median 27 weeks). It was suggested that the presence of NE markers in NSCLC was associated with an increased likelihood of response to chemotherapy.

Skov evaluated the expression of NSE and Chr A in the tumours of 114 patients with inoperable adenocarcinoma of the lung who were previously untreated and received chemotherapy in a prospective randomised trial (Skov et al., 1991). In 16% tumours, more than 10% of
cells stained positive for NSE. 44% of these patients responded to chemotherapy compared to 17% of the patients with less than 10% positive cells (p<0.025). The difference was not significant for chromogranin. Although patients with >10% positive NSE tumours were more likely to respond to chemotherapy, no statistical impact on survival was detected.

The Eastern Cooperative Oncology Group reported a large retrospective study looking at NE markers in patients with advanced lung cancer and found that NSE was correlated with response to chemotherapy and Leu-7 expression with longer survival (Ruckdeschel et al., 1991).

1.8.3 NE differentiation, NCAM expression and prognosis in NSCLC

It has been suggested that NCAM expression in NSCLC is associated with the emergence of a highly malignant clone, tumour progression and a poor prognosis (Pujol et al., 1989). When MAb123C3 was used to study the expression of NCAM in resected NSCLC, about 20% of tumours were NCAM positive (Kibbelar et al., 1991). Within a subgroup of 226 radically resected NSCLC, NCAM positive tumours showed a significantly shorter overall survival and disease-free survival. However the strongest prognostic factor was tumour stage.

In a prospective study of 88 surgically treated patients with NSCLC, NCAM expression was detected in 16 tumours (18%) and was significantly higher in poorly differentiated squamous carcinoma and in stage N2 tumours (Pujol et al., 1993). Although patients with NCAM-positive NSCLC were found to have a shorter survival than those with NCAM-negative NSCLC, the main determinants of prognosis were nodal status and histology.

Three other studies of the prognostic importance of NE phenotype in NSCLC, in which additional or alternative NE markers have been
studied, have not shown any association with survival. Sundaresan
examined tissue sections of NSCLC tumours obtained from surgically
treated cases of lung cancer (Sundaresan et al., 1991). Tissues were stained
for NSE, CK-BB, bombesin, neurotensin, Chr A, synaptophysin and UJ-
13A. The NE phenotype was defined as expression of two or more
markers which was observed in 30% of cases. A significant correlation
between nodal status and NE differentiation and disease stage and NE
differentiation was found. There was no correlation between NE
differentiation and survival but these findings suggested that NE-NSCLC
was more highly metastatic than non-NE-NSCLC.

Graziano retrospectively analysed tumours from 260 patients with
surgically resected stage I and II NSCLC for expression of NSE (70% +ve),
Chr A (14.2%), Leu 7 (7.7%), synaptophysin (11.2%) and CEA (68.5%)
(Graziano et al., 1994). Twenty-three percent were positive for ≥ 2 NE
markers. No significant differences in stage, site of relapse (distant vs
local), disease-free or overall survival were found either for an
individual marker or for tumours with ≥2 NE markers. Thus NE
markers were not found to be of prognostic significance in this group of
patients with resected stage I and II NSCLC. Similarly, Linnoila studied
expression of the NE markers NSE, Chr A, Leu 7 and GRP, epithelial
markers CEA and mucin, and three non-NE markers including vimentin
in 237 resected NSCLC (Linnoila et al., 1994). 12% of tumours were
positive for ≥2 NE markers. Expression of NE markers did not correlate
with recurrence but the presence of CEA was strongly associated with
improved survival whereas mucin was associated with a worse outcome.

In summary, several studies have suggested that the NE phenotype
or NCAM expression may be associated with increased response to
chemotherapy and a worse prognosis. But many of the studies were
small and retrospective and should be interpreted cautiously (Souhami,
The most recent studies have failed to show a correlation between NE phenotype and prognosis. Although NCAM is a marker of the NE phenotype, few studies have examined the prognostic impact of NCAM expression in favour of analysing conventional biochemical markers of the NE phenotype. A large prospective study of the prognostic value of expression of NCAM and other NE markers is needed to determine whether these variables carry information independent of tumour stage, performance status and other clinical factors predictive of outcome.

1.9 Antibody targeting in malignancy

1.9.1 Introduction

Hybridoma technology has produced monoclonal antibodies directed against antigens on the tumour cell surface. Many tumour antigens have been identified as potential targets for antibody-directed therapy but most are also expressed by normal tissues and are tumour-related molecules occurring at greater concentrations in tumours (Kohler et al., 1975).

The ability to grow human tumours as xenografts in nude mice has provided a useful model for the study of antibody localisation in tumours. The superiority of specific over non-specific antibody was demonstrated in early studies when radiolabelled antibodies to carcinoembryonic antigen (CEA) localised specifically in colon cancers growing in mice (Mach et al., 1974). With external gamma scintigraphic imaging it became possible to image tumours, first in mice and subsequently in man when deposits of colon cancer were imaged following intravenous injection of a radiolabelled anti-CEA monoclonal antibody (Goldenberg et al., 1974; Goldenberg et al., 1978). It was hoped that monoclonal antibodies would revolutionise the diagnosis and treatment of hitherto untreatable tumours and initial pilot studies were
encouraging (Miller et al., 1982; Sears et al., 1982). However, it has become clear that there are problems with antibody targeting in man that differ from the mouse xenograft model.

The success of antibody-directed tumour detection and therapy depends on the ability of the antibody to localise specifically in a tumour. The tumour to normal tissue uptake ratio should be high and an adequate dose of antibody needs to be delivered to the tumour. The fundamental problem with antibody targeting in man is that after intravenous administration of antibodies, only a very small proportion, about 0.005%, localises to tumours. This differs from the animal model where accumulation of antibody in xenografts is 100-1000 times greater (Schlom, 1986). It is difficult to deliver adequate amounts of antibody to achieve a tumouricidal effect without damaging other normal tissues such as bone marrow, liver and kidney. In addition to improving delivery of immunoconjugate to tumour, a further challenge has been to overcome the human anti-mouse antibody response (HAMA) (Schroff et al., 1985). Tumours are rarely cured by a single treatment and strategies to overcome HAMA have been sought to allow administration of repeated therapies.

1.9.2 Therapeutic strategies

Most tumours are poorly immunogenic in the host and naturally occurring antibody responses are not effective. Limited success has been reported following the administration of anti-idiotype antibodies to tumours such as lymphomas which express unique antigens (Miller et al., 1982) but results in solid tumours are generally disappointing. The mechanism of action is not understood but probably involves recruitment of natural effector mechanisms including cytotoxic T cells, complement and antibody-dependent cellular cytotoxicity.
For therapy and imaging antibodies are usually conjugated to a radioactive isotope most commonly iodine-131. This emits β radiation capable of penetrating about 1mm, about 40 cell diameters, which helps to overcome the disadvantages of unequal antibody distribution within tumours. In animal models, injection of radioimmunoconjugates has produced growth delay of human xenograft tumours including colon carcinoma (Buchegger et al., 1989), neuroblastoma (Cheung et al., 1986) and lung cancer (see below). In humans, radiolabelled antibodies have been successfully used in the treatment of hepatoma and lymphoma with response rates in the order of 40% (Lenhard et al., 1985; Order et al., 1985). However in trials with colorectal cancer, melanoma and neuroblastoma only limited responses have been seen which are not sustained.

Antibodies have also been conjugated to cytotoxic agents and high potency toxins such as ricin, a protein synthesis inhibitor derived from beans. In a clinical study where ricin A chain was conjugated to an antimelanoma antibody some responses were observed (Spitler et al., 1987). By linking antibodies to enzymes which can convert a prodrug to its active form, large amounts of active drug can be generated at tumour sites. ADEPT (antibody directed enzyme prodrug therapy) can eradicate xenografts of human choriocarcinoma in nude mice (Springer et al., 1991) but as yet no responses in patients have been reported.

1.9.3 Factors effecting localisation in tumours

When radiolabelled antibodies are injected intravenously into mice bearing human tumour xenografts, antibody accumulates rapidly in tumour within a few hours and persists for several days while levels in the circulation remain high. However, persistent circulating antibody results in low tumour to blood ratios which both delays the time at which
imaging can be performed and limits the dose of therapeutic antibody by causing damage to normal tissues.

Antibody fragments are cleared more rapidly from the circulation and have better tumour penetration making them an alternative to intact antibody for tumour localisation and therapy. In animal models, radiolabelled F(ab')\textsubscript{2} antibodies have superior tumour to blood ratios and reduced toxicity compared with intact antibody (Pedley et al., 1993). However twice the amount of F(ab')\textsubscript{2} must be administered as intact antibody to achieve similar therapeutic effects as intact antibody. This is because F(ab')\textsubscript{2} is cleared more rapidly during the first few hours after administration in mice so that a lower absolute amount of antibody is delivered to tumour. Conjugation of polyethylene glycol (PEG) to proteins can alter their pharmacological properties resulting in extended plasma half-life, reduced immunogenicity and antigenicity, increased solubility and resistance to proteolysis (Buchegger et al., 1989). PEG modification of antibody fragments has been shown to enhance tumour accumulation and reduce normal tissue to blood ratios and may additionally reduce immunogenicity (Cheung et al., 1986).

In man F(ab')\textsubscript{2} fragments penetrate more rapidly into tumours compared with intact antibody but clearance rates are similar and cumulative dose rates in tumour are comparable (Kohler et al., 1975). It has been suggested that F(ab')\textsubscript{2} might be a good compromise as a therapeutic molecule in man giving high absolute amounts of radiotherapy in tumour with rapid penetration. Smaller antibody fragments including Fab' and single chain Fv antibodies penetrate tumours well but their therapeutic efficacy is limited by rapid clearance and accumulation in kidney.

A potential approach to reduce the toxicity of circulating radiolabelled antibodies is to inject a second antibody directed against the
first resulting in the formation of immune complexes that are rapidly cleared by the reticuloendothelial system (Miller et al., 1982; Schlom, 1986). More recent work has examined the use of avidin or streptavidin which have high affinity for biotin to complex with circulating biotinylated antibodies and thereby increase clearance of the complexes (Schroff et al., 1985; Pedley et al., 1993). The presence of circulating tumour antigen may also affect clearance and tumour uptake of antibodies. In mice bearing human pancreatic xenografts, elevated serum CEA was associated with rapid clearance of antibody-antigen complexes and reduced tumour localisation to one quarter of that seen in mice with low serum CEA (Pedley et al., 1989).

Factors which may influence localisation of antibodies to tumours include the concentration gradient between blood and interstitial fluid surrounding the tumour and the ability of the antibody to permeate the interstitial spaces and into the tumour itself. There is much variability in uptake of antibody in individual tumours in patients for reasons that are poorly understood. Injected antibodies are not distributed uniformly in tumours and do not always penetrate efficiently into some areas even if they are antigen-rich (Lenhard et al., 1985; Order et al., 1985). The basal lamina may present a significant barrier to extravasated antibodies (Dvorak et al., 1991) and preferential expression of antigens on tumour cells may render them inaccessible to antibodies (Pervez et al., 1988; Boxer et al., 1994)

The importance of the affinity of the antibody for the antigen is unclear. In animals, specific antibody is retained in tumours longer than a non-specific antibody and uptake of antibody has been shown to be greater with antibodies of high affinity (Spitler et al., 1987). Alternatively it has been suggested that high affinity antibodies interact strongly with antigen at the tumour surface resulting in reduced penetration (Shawler
et al., 1985). Antibody uptake may also depend on the physiochemical properties of antibodies (Reichmann et al., 1988)

Tumour vasculature is likely to differ between xenografts in mice and tumours in man and may account for some of the differences in localisation that are seen. Approaches to improve therapy have included the use of vasodilators or interleukin-2 to increase perfusion or permeability of tumours before delivery of immunoconjugate (De Leij et al., 1987; Souhami et al., 1990) or the administration of synthetic flavonoids such as flavone-8-acetic acid (FAA) which reduce blood flow and induce necrosis after antibody has been delivered (Krishnamurthy et al., 1990).

1.9.4 HAMA

In addition to the problems of achieving good localisation and adequate therapeutic ratios with minimal systemic toxicity, a third limitation of antibody treatment arises when repeated therapy is considered. In humans this is limited not only by cumulative toxicity of the immunoconjugates but also by host immune responses to mouse antibodies (HAMA or human anti-mouse antibodies) (Schroff et al., 1985). Repeated administration of antibody results in hypersensitivity reactions, rapid clearance of immunoconjugate and failure of tumour localisation (Shawler et al., 1985). Strategies to overcome this have included the administration of immunosuppressant drugs such as cyclosporin A which can both delay and reduce the magnitude of the humoral immune response to mouse anti-CEA (Ledermann et al., 1988). More recently, the use of molecular biology has allowed the production of 'humanised' mouse antibodies with reduced immunogenicity (Reichmann et al., 1988).
1.10 Antibody targeting of Lung Cancer

1.10.1 Introduction

Small cell lung cancer may be particularly suitable for antibody directed therapy. It is highly chemosensitive and radiosensitive and over half of the patients who present with this disease will have a partial or complete remission following treatment (De Leij et al., 1987). However the disease invariably relapses so that 95% of patients will die from metastatic disease within two years of diagnosis (Souhami and Law, 1990). It is believed that antibody-directed therapies are likely to be most effective in eradicating small tumour bulk rather than large primaries (Pedley et al., 1987). In patients with SCLC, microdeposits of residual tumour remain following initial therapy and later regrow as recurrent disease. Antibody treatment may help to improve outcome in SCLC by scavenging the remaining tumour cells following conventional treatment.

Following the development of monoclonal antibodies against lung cancer associated antigens, several studies were undertaken to assess their potential use for diagnosis, staging and therapy.

1.10.2 Diagnosis of lung cancer (SCLC and NSCLC)

Radiolabelled antibodies are unlikely to be superior to conventional diagnostic investigations in the diagnosis of lung cancer and few studies have assessed their usefulness for the detection of primary lung cancer. However primary tumours can be detected by antibody imaging: In twenty patients suspected of having lung cancer, 12/16 primary cancers were detected by localisation with an indium-labelled anti-CEA antibody ZCE-025 (Krishnamurthy et al., 1990), and 57/63 patients with primary lung cancer (54 NSCLC, 6 SCLC) had positive
scans with another indium-labelled anti-CEA, FO23C5 F(ab)\(_2\), (Biggi et al., 1991).

NSCLC tumours can be reliably imaged with indium-labelled anti-human milk fat globulin (HMFG) F(ab')\(_2\) but non-specific antibodies also localise in 70% of tumours (Kalafonos et al., 1988; Behrens et al., 1989). To investigate the importance of using specific antibodies, 27 patients with squamous cell lung cancer were imaged with a specific \(^{131}\)I-labelled MAb, PO66, which recognises an intracellular antigen (Brichory et al., 1996) and a non-specific antibody. Tumour uptake was three times greater with the specific antibody (Bourget et al., 1990).

1.10.3 Staging of SCLC

Radiolabelled antibodies are potentially more useful for staging tumours and particularly for SCLC which metastasises early and may be present as microdeposits of cells which are undetectable by standard clinical imaging techniques.

The antibody NR-LU-10 recognises a 40-kD cell surface glycoprotein expressed by many epithelial carcinomas and virtually all SCLC. A staging study of SCLC was undertaken with \(^{99}\)mTc-labelled NR-LU-10 Fab' (Nelp et al., 1992). As Fab' fragments clear from blood more rapidly than F(ab')\(_2\) or intact antibodies, they provide optimal tumour to background ratios for imaging tumour at earlier times. Ninety-six patients from 21 centres were reviewed. The positive predictive value of antibody imaging in patients with extensive disease was 95-100% compared with 96-100% for a standard battery of tests (CXR, CT scan chest, brain, abdomen, bone scan and bone marrow aspiration). Although 10% patients were understaged, 15% patients who were thought to have limited disease were upstaged to extensive disease by antibody imaging, which was subsequently corroborated by imaging or bone marrow biopsy.
This study concluded that if antibody imaging showed extensive disease, the positive predictive value was so high that no further tests were necessary. However, if limited disease was shown, further tests were indicated to identify the 15% of patients who were understaged.

1.10.4 Staging of NSCLC

In a study of 36 patients with NSCLC, $^{99m}$Tc-labelled NR-LU-10 Fab was used to image both surgically resectable and non-resectable disease (Friedman et al., 1991). Primary lung lesions and diseased nodes imaged with a sensitivity of 94% and 95% respectively, whereas the sensitivity of CT in assessment of nodal metastasis was 81-88%.

In a study of 19 patients with inoperable NSCLC, all primary tumours were imaged with the EGF receptor monoclonal antibody 225 at doses of 20mg or greater and metastases > 1cm were seen at 40mg or greater (Divgi et al., 1991).

1.10.5 Biodistribution studies and immunotherapy with anti-NCAM monoclonal antibodies in nude mice bearing SCLC xenografts

NCAM is strongly expressed by SCLC tumours and its potential as a target for immunotherapy has been studied in SCLC xenografts grown in the nude mouse model. In nude mice bearing NCIH69 xenografts $^{111}$Indium-labelled LS2D617, an anti-NCAM MAb, localised well to the tumours with peak values of about 35% injected dose/g on day 3 compared with 8% injected dose/g for an irrelevant IgG1 antibody (Wilson et al., 1990). The normal tissue accumulation for both antibodies was 2-8% injected dose/g. The ability of LS2D617 to accumulate in vivo in tissues that normally expressed NCAM was evaluated by injecting rabbits with labelled antibody or control labelled antibody, sacrificing them at 48hr, and examining tissues by gamma well counting,
autoradiography and immunohistochemical staining. Specific uptake was seen in sites known to express NCAM including heart, liver bile duct, peripheral nerves, pituitary and adrenal. Uptake in brain and spinal cord was not seen presumably because of the blood-brain barrier.

The biodistribution of the cluster 1 MAb RNL-1, radiolabelled with either 111-Indium or 125-iodine, was studied in nude mice bearing NCI-H82 SCLC xenografts (Boerman et al., 1991). The maximum tumour dose was 11.8% ID/g for $^{111}$In-RNL 1 and 6.5% ID/g for $^{125}$I-RNL 1. The higher localisation with $^{111}$In was consistent with previous studies that had shown metabolism of radioiodinated antibodies in tumour with subsequent release of free $^{125}$I and rapid removal via the blood. Autoradiographs of whole-body sections from animals injected with $^{125}$I-RNL-1 showed that activity in SCLC xenografts was mainly peripheral. Histological examination of xenografts indicated that vascularisation was minimal and that distribution of activity within the tumour was similar to the distribution of blood vessels.

Michalides used the anti-NCAM monoclonal, MAb 123C3, to image SCLC-H69 xenograft tumours in nude mice (Michalides et al., 1994). After 7 days the tumour-to-blood ratio was approximately 4 and a significant fraction of the injected dose (14%) was retained in the tumour. Immunotherapy studies were carried out in the same xenograft model using MAb 123C3 labelled with 600μCi $^{131}$I. Regression was seen in all tumours with complete regression in 2 out of 6. Regrowth occurred after 4-9 weeks. When 2 doses of 500μCi were administered, growth delay up to 16 weeks was seen but no delay was seen in mice injected with a non-specific radiolabelled MAb. It was proposed that the response to radioimmunotherapy was due to cellular internalisation of the antibody. This was supported by a comparison of the efficacy of three cluster-1 antibodies, 123C3, 123A8, and MOC191 for immunscintigraphy of SCLC.
xenografts in nude mice after intraperitoneal injection. The tumour uptake was most favourable for MAb 123C which was the only antibody found to be internalised (Kwa et al., 1996).

The cluster 1 MAb, SEN 7, recognises a unique epitope on NCAM (Waibel et al., 1993). Although the antibody bound to NCAM-transfected mice lymphoma cells, it failed to bind to peripheral blood mononuclear cells which usually stain with NCAM antibodies. In biodistribution studies with radiolabelled SEN 7 in nude mice with SCLC xenografts, the antibody localised selectively in the tumours with more than 30% of total injected dose/g tissue at 4 days. It has been suggested that 67Cu might have an advantage over 131I as a therapeutic isotope in possessing similar levels of beta emission but reduced gamma emissions and thus reducing the radiation burden to the patient. To investigate the potential of 67Cu as a therapeutic isotope in SCLC, the distribution of whole SEN7 and its F(ab')2 fragment each labelled with 67Cu or 131I was compared in SCLC xenografts (Smith et al., 1994). Absolute levels of tumour localisation with intact SEN7 were similar with either label, but tumour:blood ratios were more favourable with 67Cu. However with 67Cu-labelled F(ab')2 high levels of accumulation were seen in kidney, liver and spleen compared with the 131I-labelled fragment.

After conjugation to blocked ricin (bR) or pseudomonas exotoxin A (PE), SEN7 immunotoxin has demonstrated potent and selective cytotoxic activities against SCLC cells in culture (Zangemeister-Wittke et al., 1994). Previously, ricin A chain immunotoxins directed against NCAM had not proved to be potent cytotoxic agents (Wawrzynczak et al., 1991) but in this case it was found that coupling of toxin to antibody by a thioether linkage promoted internalisation which did not occur spontaneously when the antibody was unconjugated.
H69 xenografts in nude mice treated with 11.1 MBq of 131I- anti-NCAM MAb, NE150, became invisible after 30 days and demonstrated a 60-day mean growth delay when compared with sham-treated tumours (Hosono et al., 1994a). In mice treated at 43°C, there was an increase in tumour uptake of labelled antibody suggesting that hyperthermia might be a potential method of increasing accumulation of radiolabelled antibody in tumours (Hosono et al., 1994b).

1.10.6 Biodistribution of antibodies to other antigens on SCLC xenografts in nude mice

The antibody SWA11 recognises a glycoprotein assigned to cluster w4 in SCLC. Radiolabelled SWA11 localises in SCLC xenografts in nude mice (Smith et al., 1989) and therapeutic doses of 131I SWA11 have irradiated tumours in mice (Smith et al., 1991). In conjugation with ricin A to form an immunotoxin, SWA11 has also demonstrated cytotoxic effects in SCLC cell lines (Derbyshire et al., 1992).

1.10.7 Imaging of SCLC patients with radiolabelled anti-NCAM monoclonal antibodies

Four patients with SCLC have undergone imaging studies with 1mg 131I-labelled MAb 123C3 (Michalides et al., 1994). The primary tumour was not detected in any patient but metastases were seen in the liver of one subject. Background binding of 123C3 to NK cells was not seen. The reasons suggested for the failure to localise included the presence of variable levels of circulating NK cells or soluble NCAM in serum or that the dose of antibody was too low.
1.10.8 Imaging of SCLC patients using antibodies directed against antigens other than NCAM

The biodistribution and tumour localisation of 131I-SWA11, a cluster w4 MAb, was investigated in five patients with SCLC (Ledermann et al., 1993). Although tumour was detected by gamma camera in two patients, most of the activity was located in the spine, pelvis and spleen. The probable explanation for this distribution was that the antibody bound to the CD24 molecule expressed on human granulocytes which was subsequently shown to be almost identical to the cluster w4 antigen (Jackson et al., 1992).

Carcinoembryonic antigen (CEA) is expressed by up to 70% of SCLC cell lines (Goslin et al., 1981). Using In-111-labelled F(ab')2 fragments or Tc-99m-labelled intact antibody to image SCLC in 21 patients, tumour was detected in 62% of patients (Macmillan et al., 1993). There was no significant difference in performance between the two antibody preparations. Of 38 known sites of disease, 18 were positively detected. Tumour localisation was seen both in patients whose tumour biopsies showed weak or absent CEA immunoreactivity and in patients with normal CEA levels (8/15).

Six patients with SCLC were studied with indium labelled monoclonal antibody MOC-31, a cluster-2 antibody which detects the epithelial glycoprotein EGP-2 (Kosterink et al., 1995). The primary tumour or metastases were imaged in five out of the six patients with restricted localisation in normal tissue.
1.11 Plan of studies

1.11.1 Plan of studies to investigate the biological role of NCAM in lung cancer

NCAM is an adhesion molecule strongly expressed by SCLC tumours and cell lines which has an important function in other biological systems. However its function in the biology of SCLC has not previously been investigated. An initial approach to this question was suggested by the observation that there may be an association between NCAM expression and morphology of SCLC in culture. Most SCLC cell lines grow as floating spheroids or tight aggregates in culture but variant adherent sublines of SCLC have been described which have downregulated expression of NCAM. Using antibodies, peptides and antisense oligonucleotides to inhibit NCAM, the contribution of NCAM to cell-cell adhesion in SCLC cell cultures is studied with an *in vitro* aggregation assay to measure the effect of blocking NCAM interactions on the aggregation of cells in culture.

Polysialylation of NCAM increases repulsive interactions between NCAM molecules and it has been suggested that the highly polysialylated form of NCAM found in SCLC may contribute to its aggressively metastatic nature. The effect of polysialic acid on SCLC cell-cell aggregation in culture is studied using clonal SCLC cell lines which express high and low levels of PSA and by enzymatic removal of PSA from NCAM. Attempts to downregulate NCAM expression in SCLC cell lines with antisense oligonucleotides are also explored.

The small subgroup of NSCLC tumours which are NCAM-positive may have a worse prognosis in patients, a higher metastatic rate and greater chemosensitivity than NCAM-negative NSCLC. To investigate whether NCAM expression changes the biological characteristics of NSCLC, a plasmid vector is constructed containing the full length cDNA
for the human NCAM 140 kDa isoform. An NCAM-negative NSCLC cell line, L23, is transfected with this vector and a population of cells with high NCAM expression are selected by sequential fluorescent activated cell sorting (FACS). The growth rate, invasiveness, chemosensitivity and radiosensitivity of the transfected cell line are compared with the parent cell line to observe whether NCAM expression alters its biological characteristics.

1.11.2 Plan of antibody targeting studies

NCAM is the major immunodominant antigen of SCLC. Anti-NCAM MAbs localise well to human SCLC xenografts in nude mice and radioimmunotherapy can result in complete regression or growth delay of these tumours. Only one study has previously investigated targeting with anti-NCAM monoclonals in patients with SCLC. The preparation, labelling and administration of the anti-NCAM MAb NY3D11 to patients with SCLC is described. A F(ab')\textsubscript{2} fragment of NY3D11 is produced for clinical use and localisation in SCLC xenografts growing in nude mice is compared to whole NY3D11. The results of administration of radiolabelled NY3D11 F(ab')\textsubscript{2} fragments to patients are presented.
CHAPTER 2.
The role of NCAM in the morphology and biology of SCLC in culture

2.1 Introduction

Several studies have suggested that there is an association between NCAM expression and the morphology of SCLC cells in culture (see section 1.7.1). NCAM functions as a homophilic or heterophilic binding molecule in other tissues but the role of NCAM as an adhesion molecule in the biology of SCLC has not been investigated previously. If NCAM-NCAM homophilic binding interactions are important in SCLC, function blocking experiments may result in altered cell morphology or aggregation in culture.

The traditional approach to block the function of an adhesion molecule has used monoclonal antibodies and this technique has successfully been used to inhibit NCAM-NCAM binding (Rutishauser et al., 1982; Hoffman et al., 1983; Cole et al., 1985b). Alternatively, peptides with sequences homologous to the binding regions of adhesion molecules can block binding function. Rao has described a peptide P10 that inhibits homophilic binding of NCAM (see section 1.4.2) (Rao et al., 1992). A third approach is to use specific antisense oligonucleotides to downregulate expression of a molecule. In this chapter, the results of blocking NCAM function in SCLC cells in culture using these approaches will be presented.

Highly polysialylated NCAM, which occurs on the surface of SCLC, shows reduced homophilic binding compared to NCAM with a low PSA content. It has been suggested that polysialylated NCAM may have a role in the highly metastatic nature of SCLC by reducing cell-cell binding interactions. To investigate the role of PSA in SCLC biology, aggregation experiments are performed using clonal lines with high or low PSA
expression derived from the parent SCLC line H69. The effect of PSA removal by treatment with the enzyme ENDO N is also studied.

2.2 Materials and methods

2.2.1 Cell lines and antibodies

SCLC cell lines were maintained at 37°C and 5% CO₂ in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Sigma, Poole, UK) and 2mM glutamine (Gibco). Cells were passaged weekly by splitting in a ratio of 1:20 and adding fresh medium. The SCLC cell lines NCI-H69 and POC-1 were kindly donated by Peter Twentyman (MRC, Cambridge, UK). POC-1 grows as spheroids in suspension while H69 grows as tightly packed aggregates. The cell lines H119-54.2 and H119-45 were kindly provided by Rita Gerardy-Schahn, Hannover, Germany. They were derived by clonal selection from the parent cell line NCI-H69 on the basis of PSA expression detected by reactivity with the monoclonal antibody MAb735 (which binds specifically to alpha 2-8 linked PSA). H119-54.2 is PSA positive and H119-45 is PSA negative. All lines were tested for mycoplasma and found to be negative.

NY3D11 antibody was previously produced by Shona Ward at UCL from NY3D11 cell culture supernatant by purification on protein A sepharose. SWA11, a kind gift from Rolf Stahel (Geneva, Switzerland), is a mouse IgG₂a antibody belonging to cluster w4 group defined by the SCLC international workshops. It binds to the leukocyte activation antigen CD24 and was chosen as a non-adhesion molecule control. Eric-1 antibody is an anti-NCAM monoclonal kindly provided by Frank Walsh, London, UK. MAb 735 was a gift from R. Gerardy-Schahn. Characteristics of the cell lines and antibodies are summarised in table 9, p226.
2.2.2 Inhibition of spheroid formation by NY3D11

POC cells were harvested 4-6 days after passage and resuspended to a concentration of $1 \times 10^5$ cells/ml. Cells were gently disaggregated by continuous pipetting until only single cells or small clumps were visible. One ml of cell suspension was added to the wells of a 48 well tissue culture plate (Costar, Bucks, UK) and NY3D11 antibody was added at a concentration of 20μg/ml. Control wells contained either no antibody or SWA11. A calibrated grid inserted into the eyepiece of the microscope was used to measure the size of spheroids. At 24 hour intervals, the size of 15 spheroids in each well was measured in a randomly chosen field. Fresh antibody was added every 48 hours.

2.2.3 Aggregation assay

The cell lines H69, H119-54.2 and H119-45 were used for aggregation experiments. The spheroids of the POC cell line contain a core of necrotic cells and overall viability after disaggregation through a 19 gauge needle was approximately 40% measured by toludine blue staining. Other non-mechanical methods of disaggregating spheroids such as addition of trypsin:versene were not found to be effective in breaking up the spheroids. The use of Ficoll-triosil mixture (Sigma) to separate viable from non-viable cells (De Vries et al., 1973) improved viability to 80-90% but preliminary experiments indicated that cells which had undergone this process had increased adherence to each other. Thus the POC cell line was not used for aggregation experiments.

Cells were harvested 4-6 days after passaging and washed twice in RPMI by centrifuging at 700rpm for 5 minutes. The cells were resuspended in RPMI containing 20μg/ml DNAase (Promega, Southampton, UK) and disaggregated by passing gently 7-8 times through a 19 gauge needle. The concentration of cells was checked on a haemocytometer (Weber Scientific
International Ltd, Teddington, UK) and adjusted to the desired concentration for the experiments. The cell suspension was left on ice until the start of the assay. For each experiment, 200μl of cell suspension was transferred to each of six tubes from which an aliquot of 12μl was taken immediately to check the starting concentration in each tube. The tubes were placed in a rack and transferred to an incubator at 37°C with 5% CO₂. If the assay was performed under rotation, a plate shaker was placed inside the incubator. Further aliquots of 12μl were taken from each tube at the appropriate time. Prior to sampling, tubes were gently swirled to aid homogenous sampling. The aliquots were transferred to a haemocytometer where the number of non-aggregated cells remaining was recorded. Non-aggregated cells were defined as single cells or doublets of cells.

2.2.4 Treatment with Endoneuraminidase N (Endo N).

Endo N was kindly provided by R. Gerardy-Schahn. This enzyme specifically cleaves the PSA moiety from NCAM. Cells were harvested, washed twice in RPMI and resuspended in 1ml RPMI to which 1μl of 1:10 Endo N was added. After 1 hr at 37°C, the cells were washed once in RPMI and brought to a single cell suspension in RPMI with 20μg/ml DNAase.

2.2.5 Peptides

The synthetic peptide P10 containing the amino acid sequence KYSFYNDGSE is the shortest active peptide that blocks NCAM homophilic interactions in the third Ig-like domain (Rao et al., 1992). This was synthesised by Peptide and Protein Research Consultants, University of Exeter, Devon. The peptide was dissolved in RPMI to give a concentration of 1mg/ml. Cells were washed twice in RPMI and resuspended as a single cell suspension at twice the experimental concentration. An equal volume of P10 (1mg/ml) was added to give a final concentration of 0.5mg/ml peptide.
2.2.6 Antisense oligonucleotides

Antisense oligonucleotides were obtained from Clare Hall Laboratories, Imperial Cancer Research Fund, Potters Bar, UK. The complementary DNA sequence encoding the major NCAM isoform in a human small cell lung cancer cell line has been described (Saito et al., 1994). The oligonucleotides were pentadecamers chosen to be complementary to mRNA sequences derived from NCAM cDNA immediately after the ATG start codon, spanning the ATG codon and downstream from the ATG. Thus the three sequences chosen were 5'-CTGCAAACTAAGGAT-3', 5'-ATTACAATGCTGCAA-3', and 5'-CCAAGTGCGAGGAGA-3'. Oligonucleotides with a sense orientation to these sequences were obtained as controls. Homology of these sequences with other human genome sequences was checked by submitting the sequences to the FASTA database on the world wide web at FASTA@ebi.ac.uk.

2.2.7 Antisense experiments on cells

Oligonucleotides are degraded by nucleases in serum and therefore experiments on cells were performed in HITES A serum-free media (see appendix A for composition) (Carney et al., 1981; Ballie-Johnson et al., 1985). H69 cells were grown in HITES A media for one week prior to experiments. Solutions of oligonucleotides in HITES A were prepared at a concentration of 100μM. H69 cells were harvested and brought to a concentration of approximately 13 x 10^5. Aliquots of 450μl cell suspension were plated on a 48 well tissue culture plate and oligonucleotides were added at a concentration of 1μM (5μl oligonucleotide solution with 45μl HITES A), 5μM (25μl oligonucleotide solution with 25μl HITES A), 10μM (50μl oligonucleotide solution) or 20μM (removed 50μl medium and added 100μl oligonucleotide solution). Cells were harvested at 24 hours and NCAM expression was studied by FACS analysis.
2.2.8 Labelling of oligonucleotides

To investigate whether oligonucleotides were being taken up into cells, oligonucleotides were radiolabelled with gamma P³² (Amersham Life Sciences, Amersham, UK) and incubated with cells. An aliquot of 0.5μl oligonucleotide solution (100μM) was added to 17.5μl H₂O to which were added 5ml 5X forward buffer, 1.5μl gamma P³² and 1μl T4 polynucleotide kinase (Promega). A Bio-Spin column (Bio-Rad, Hemel Hempstead, UK)) was prepared as follows: the column was inverted several times before snapping off tip and allowing buffer to drain by gravity. The column was placed in a collection tube and centrifuged in a swinging bucket rotor centrifuge for two minutes at 1100 x g. The radiolabelled oligonucleotide plus 25μl H₂O was added to the centre of the column and allowed to drain into the gel bed. The applied sample was eluted into a clean collection tube by centrifuging for 4 minutes at 1100 x g. Labelling of the oligonucleotides was checked on a gamma counter (Packard Instrumentation, Groningen, Netherlands).

2.2.9 Cellular uptake of labelled oligonucleotides

Aliquots of 400μl H69 cells in HITES A (at 5 x10⁵/ml) were placed into ependorf tubes. 100μl of unlabelled oligonucleotide and 10μl of labelled oligonucleotide was added to the cell suspension (final concentration 20 μM). The cell samples were analysed at 0, 4, 8 and 24 hours for uptake of oligonucleotides. Cells were spun at 1000rpm for 1 minute and the supernatant collected (supernatant 1). Cells were washed with 400 μl PBS, spun at 1000 rpm for 1 minute and the supernatant collected (supernatant 2). This process was repeated again and supernatant 3 collected. The cells were resuspended in 400 μl PBS and added to a scintillation vial with 3ml scintillation fluid. The activity of all samples was counted on a gamma counter.
2.2.10 Fluorescence activated cell sorting (FACS) analysis

A 96 well plate (Costar) was seeded with $2 \times 10^5$ cells/well. Five micrograms of antibody were added to each well and the plate was incubated at $4^\circ$C for 20 minutes. The plate was centrifuged for 3 minutes at 1000rpm, the media carefully discarded and the cells resuspended in 100 µl 2% FCS/RPMI. The plate was centrifuged again for 3 minutes at 1000rpm, the media discarded and the cells were resuspended in 100µl 1:200 dilution of IgG1 FITC. The plate was incubated at $4^\circ$C for 20 minutes and then cells were washed three times in 2% FCS/RPMI by centrifuging the plate and discarding media as described above. Finally the cells were resuspended in 100µl 2% FCS/RPMI by gentle mixing with a pipette and transferred to fax vials for analysis. FACS analysis was performed on a Becton Dickson FACS analyser.

2.2.11 Statistical analysis

Data were analysed using the Mann-Whitney U test. Statistical significance was taken as $p < 0.05$.

2.3 Results

2.3.1 Inhibition of spheroid formation by NY3D11 in the POC cell line

Formation and growth of spheroids by the POC cell line was not inhibited in the presence of NY3D11 antibody (fig. 4). Although there was some delay in the growth of spheroids incubated with NY3D11, this was also observed in the control wells containing the antibody SWA11.
Figure 4. Growth of spheroids formed by POC cells in the presence of NY3D11 and control antibody SWA11. Results are mean of 6 wells with standard deviations.

2.3.2 Effect of cell concentration on rate of aggregation

Cells were suspended at four different concentrations: 4, 7, 9 and 11 x 10^5 cells/ml. Samples were rotated at 100rpm and aliquots taken at 10 minute intervals up to 30 minutes (fig. 5). At the two higher concentrations, there was little or no further aggregation after 20 minutes. At the lower concentrations, aggregation was mostly completed by 30 minutes. Cell concentration clearly had an effect on the rate of aggregation and on the basis of these findings it was decided to study the effects of anti-NCAM antibodies on 20 minute cell aggregation at a starting concentration of 10 x 10^5 cells/ml. At this time point and concentration aggregation would be expected to be almost complete and the effect of blocking NCAM function on cell aggregation might be apparent.
Figure 5. The effect of cell concentration on aggregation of H69 cells rotated at 100rpm. Results shown are mean and standard deviation of 6 samples at each time point.

2.3.3 Effect of anti-NCAM monoclonal antibodies (NY3D11 and ERIC-1) on aggregation of H69

Cells were harvested, washed and formed into a single cell suspension as described above. Whole antibody in PBS was added to a final concentration of 0.5mg/ml with the cells at a starting concentration of 10 x 10^5 cells/ml. The samples were placed on ice for 15 minutes to allow time for antibody to bind to the cells. Preliminary studies had shown that there is little aggregation at 4°C after 15 minutes but immediately before starting the assay, cells were again passed twice through a 19 gauge needle to ensure a single cell suspension. The control cells without antibody were treated similarly. There was no significant difference in aggregation in the presence of either antibody compared with controls (fig 6).
Figure 6. The effect of addition of anti-NCAM antibodies on 20 minute aggregation of H69 cells under rotation at 100rpm. Mean and standard deviation of 3 experiments.

2.3.4 Aggregation of H69 in still medium: Effect of varying cell concentration

After observing no effect on aggregation in an assay under rotation, it was decided to study aggregation in a still medium. H69 cells were brought to concentrations of 4.8, 6.1, 14 and \(20 \times 10^5\) cells/ml. The cells were incubated in still medium and aggregation was assessed after 10 minutes. Aggregation of cells occurred more rapidly under still conditions compared with rotation, particularly at the higher cell concentrations (fig. 7). At a concentration of \(20 \times 10^5\) cells/ml there was little further aggregation after 10 minutes.
2.3.5 Aggregation of H69 in still medium with NY3D11

The effect of adding NY3D11 on the 10 minute aggregation of a single cell suspension of H69 in still medium was studied. H69 cells were brought to a concentration of $18 \times 10^5$ cells/ml and aggregation of cells in the presence of NY3D11 at a concentration of 0.5mg/ml was compared with controls. No significant difference was found between the two groups (fig. 8).

2.3.6 Aggregation of H69 cells with P10 peptide

P10 peptide was added at a final concentration of 0.5mg/ml to a single cell suspension of H69 cells ($16-19 \times 10^5$/ml) and aggregation after 10 minutes in still medium at 37°C was measured. Cells aggregated approximately twice as quickly in the presence of P10 peptide ($p=0.01$) (fig.9).
Figure 8. The effect of addition of NY3D11 on 10 minute aggregation of H69 cells in still medium. Mean and standard deviation of 3 experiments.

Figure 9. The effect of addition of P10 peptide on 10 minute aggregation of H69 cells in still medium. Mean and standard deviation of 3 experiments.
2.3.7 Effect of antisense oligonucleotides on expression of NCAM

Expression of NCAM by H69 cells after 24hr incubation with antisense oligonucleotides was examined by FACS analysis. NCAM was expressed strongly by all cells and there was no evidence of downregulation by antisense. After radiolabelling, the oligonucleotide activity was only detected in supernatant 1 at all time points which suggested that oligonucleotides were not taken up by the cells (table 2).

<table>
<thead>
<tr>
<th>Time/hrs</th>
<th>SN 1</th>
<th>SN 2</th>
<th>SN 3</th>
<th>Cells</th>
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<td>19058</td>
<td>2180</td>
<td>114</td>
</tr>
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<td>24</td>
<td>673440  (98%)</td>
<td>10789</td>
<td>859</td>
<td>104</td>
</tr>
</tbody>
</table>

Table 2. Distribution of activity in H69 cells following incubation with radiolabelled antisense oligonucleotides. Abbreviations: SN = supernatant

2.3.8 Expression of NCAM and PSA by H69, H119-54.2 and H119-45

The cell lines H69, H119-54.2 and H119-45 were studied by FACS after incubation with NY3D11 and MAb735 to determine expression of NCAM and PSA. H69 and H119-54.2 both expressed NCAM and PSA strongly. H119-45 expressed NCAM but not PSA (fig. 10). Following treatment with Endo N, H69 no longer expressed PSA but continued to express NCAM (fig. 11).
Figure 10. Expression of NCAM and PSA by H69, H119-54.2 and H119-45.

Figure 11. Expression of PSA by H69 cells (a) before treatment with Endo N (+ve) and (b) after treatment (-ve)
2.3.9. Aggregation of PSA positive and negative clonal cell lines before and after treatment with endoneuraminidase N.

Cells were brought to a concentration of $10 \times 10^5$ cells/ml. Aggregation of cell lines was compared after rotation at 100rpm for 20 minutes. The PSA +ve cell line aggregated less than the PSA -ve cell line (fig. 12). This difference was small but significant ($p=0.002$) and was consistent with the hypothesis that polysialylation reduced NCAM-NCAM interactions. After treatment with Endo N, aggregation of the PSA +ve line was similar to the PSA -ve line suggesting that removal of PSA could influence aggregation of cells in vitro. Aggregation of the PSA -ve line after treatment with ENDO N was not affected. Aggregation of the parent cell line H69 was unaffected by treatment with Endo N although these cells are also polysialylated.

Figure 12. Comparison of the 20 minute aggregation of H69 and PSA +ve and -ve clones at 100rpm rotation and the effect of treatment with Endo N.
2.4 Discussion

The aim of these experiments was to block NCAM-NCAM homophilic binding in SCLC cell lines and to observe whether spheroid formation or rates of aggregation were altered as a result. If an effect was seen it would suggest that the binding functions of NCAM are important in the biological behaviour of SCLC. To perform these experiments a method of blocking NCAM homophilic binding function was required. Historically, workers have used monoclonal antibodies to block function. Anti-NCAM antibody fragments could block homophilic interactions between NCAM molecules in studies of adhesion in cell-monolayer or cell-substratum binding assays and inhibition of aggregation of membrane vesicles (Rutishauser et al., 1982; Hoffman & Edelman, 1983; Cole et al., 1985a). The anti-NCAM monoclonal antibodies NY3D11 and Eric-1 were both available and Eric-1 had been previously shown to have NCAM function blocking activity in nerve outgrowth studies (Doherty et al., 1990).

Classic SCLC cell lines are relatively unusual in growing as spheroids in culture and it was hypothesised that NCAM-NCAM interactions might have a role in this morphology. This was supported by previous studies in which SCLC cell lines that grew as adherent monolayers were found to have downregulated NCAM expression but treatment with retinoids caused reversion to the spheroid morphology associated with increased NCAM expression (Doyle et al., 1990; Doyle et al., 1991). In the present studies the addition of NY3D11 to a suspension of POC cells did not prevent formation of spheroids. NCAM may not be essential for spheroid formation and other adhesion molecules might be involved or possibly electrostatic interactions between cells are more important than adhesion molecules in determining morphology in culture. A third possibility is that NCAM is important but NY3D11 is not a function blocking antibody.
In the next series of studies, the cell line H69 was used to investigate whether aggregation of cells in single cell suspension could be perturbed by addition of the anti-NCAM monoclonals NY3D11 or Eric-1. Initially, it was necessary to develop an aggregation assay and to determine the effect of cell concentration, rate of rotation and temperature on the behaviour of the cells. When single cells were rotated on a shaker aggregation occurred rapidly and was complete within 20-30 minutes. These experiments indicated that aggregation rates were sensitive to cell concentration and this would be an important parameter to control in future experiments. When NY3D11 or Eric-1 antibodies were added to the cell suspension undergoing rotation, no difference in the rate of aggregation was seen at twenty minutes. It was concluded that either NCAM was not important in aggregation or that the antibodies were not blocking function. A third possibility was that the conditions of the assay were not suitable to demonstrate a difference and it was decided to investigate aggregation of cells under still conditions and at earlier time points when differences in rates of aggregation might be seen more clearly.

When aggregation was studied under still conditions after 10 minutes, H69 cells aggregated rapidly and significant differences were seen between different cell concentrations again indicating the importance of uniform cell concentrations in assays of this type. However, as previously, addition of NY3D11 or Eric-1 did not influence the rate of aggregation in still medium after 10 minutes.

Having failed to demonstrate any difference in aggregation rate with NY3D11 or Eric-1, it was probable that these antibodies were not function-blocking and that further studies were unlikely to yield further information. An alternative approach using a peptide to block the binding function of NCAM was explored. The peptide PIO has been shown to inhibit homophilic binding of NCAM (Rao et al., 1992). However in the H69 cell
aggregation assay no difference was observed and in fact aggregation appeared to be even more rapid. If the P10 peptide was blocking NCAM-NCAM binding in this aggregation assay then the results imply that NCAM-NCAM interactions might be repulsive in H69 cells and therefore blocking NCAM with P10 led to an observed increase in aggregation. Alternatively P10 was not an effective NCAM binding blocker in this assay and the observed effects were due to other non-specific factors.

Antisense oligonucleotides were used in an attempt to downregulate expression of NCAM in H69 cells. Pentadecamer sequences were chosen as an optimum compromise between a size that is able to enter cells and one with sufficient specificity for the desired target sequence. Unfortunately no downregulation of NCAM expression was observed and subsequent radiolabelling experiments suggested that the oligonucleotides were not entering the cells. The uptake of oligonucleotides into cells is dependent on many variables including the size of the oligonucleotide, its chemical modifications and the cell type itself. At this stage it was decided not to pursue antisense experiments further due to the potential cost in time and resources.

Although it was not possible to inhibit the function of NCAM in these studies it was possible to show that changes in PSA expression on the cell surface could influence aggregation rates. Using cell lines clonally derived from H69 which either expressed high or low PSA on their surface, it was shown that aggregation in the PSA positive cell line was significantly slower over twenty minutes than the PSA negative cell line. When the PSA positive cell line was treated with Endo-N to remove PSA the rate of aggregation became similar to the PSA negative line which itself was not affected by the addition of Endo-N. As PSA expression is linked to NCAM and the expression of NCAM by these two lines was similar it may be concluded that PSA expression appears to reduce cell-cell interactions with a resulting lower
rate of aggregation. These findings were similar to those of Scheidegger who also demonstrated that PSA in clonal sublines of SCLC cell lines was able to modulate cell-cell adhesion (Scheidegger et al., 1994). In those studies the differences in rates of aggregation were also small but statistically significant (70 ± 9.5% in the PSA positive line versus 83% ± 7.5% in the PSA negative line, p ≤ 0.001). They suggested that PSA expression was also associated with greater clonogenic ability in semi-solid media and higher incidence of intracutaneous metastases in nude mice. However in the present study Endo N treatment of the parent H69 cell line which expresses PSA did not result in a reduced aggregation rate. The reason for this is not clear but a possible explanation is that PSA expression by H69 cells is heterogeneous and the effects of PSA removal were less marked than in the clonal cell lines.

As PSA is uniquely associated with NCAM and these experiments demonstrate that changes in surface PSA may influence aggregation rates in clonal derivatives of H69 cells, it can be inferred that NCAM might have a biological role in SCLC. The relationship between expression of adhesion molecules and metastatic potential is usually inverse so that a highly metastatic tumour is found to have downregulation of the adhesion molecule whereas a less metastatic line may have upregulation e.g. expression of E-cadherin by MDCK cells. However NCAM is strongly expressed by SCLC tumours which are nevertheless highly metastatic. Although polysialylation may reduce NCAM-NCAM interactions and modify other NCAM heterophilic or non-NCAM interactions, overall it is unlikely that polysialylation is an important factor in the metastatic potential of SCLC. However expression of NCAM by normal tissues such as brain may act as receptor or target for metastatic SCLC cells promoting their arrest which leads to subsequent growth of tumours and this might be a factor in the high incidence of cerebral metastases seen in patients with SCLC.
CHAPTER 3.
Transfection of an NSCLC cell line with NCAM cDNA.

3.1 Introduction

Approximately 20% of NSCLC tumours express NCAM and other neuroendocrine features. Several studies have suggested that NCAM-positive NSCLC tumours may have a worse prognosis in patients and greater chemosensitivity compared with NCAM-negative NSCLC tumours (see sections 1.8.2 and 1.8.3). To investigate whether NCAM expression might influence biological behaviour in NSCLC, a NSCLC cell line was transfected with NCAM and the morphology, *in vitro* growth rate, invasiveness, chemosensitivity/radiosensitivity and *in vivo* growth rate of the transfected cell line was compared with that of the parent NSCLC cell line.

This chapter will describe the preparation of a plasmid containing the full length cDNA for the 140kB NCAM isoform which was used to transfect a NSCLC cell line, L23, which did not express NCAM. A transfected cell line L23T, which expressed NCAM and a control cell line L23C transfected with vector lacking the NCAM insert were produced. The biological characteristics of these transfected lines are compared to those of the parent cell line in experiments which are described in chapter 4.

3.2 Materials and methods

3.2.1 Plasmids

The plasmid pcDM8 (Invitrogen) containing the full length cDNA for the 140kB NCAM isoform was a gift from Rita Gerardy-Schahn. The plasmid pcDNA3 (Invitrogen) is a mammalian expression vector which contains the CMV promoter to drive expression of inserted genes and the neomycin resistance marker for selection of stable transformants in the presence of
G418 (Geneticin, Gibco) (fig. 13). G418 is an aminoglycoside related to gentamicin which is toxic to mammalian cells. The pSV-β-Galactosidase Control vector (Promega) was used as a positive control vector to monitor transfection efficiency in cells. The bacterial lacZ gene, driven by the SV40 early promoter and enhancer, is translated into the reporter enzyme β-galactosidase which can be assayed in cell extracts using a spectrophotometric assay.

![Diagram of pcDNA3 plasmid](from Invitrogen product information)

**Figure 13.** Diagram of pcDNA3 plasmid (from Invitrogen product information)

### 3.2.2 Cell lines

Two NSCLC cell lines L23 and MOR were kindly donated by Dr Peter Twentyman for these studies. The cells were maintained at 37°C and 5% CO₂ in RPMI 1640 medium supplemented with 10% heat inactivated FCS
and 2mM glutamine. Cells were passaged weekly using trypsin/versene and split 1: 10 into fresh medium. NIH 3T3 cells, a fibroblast cell line, was provided by Professor Peter Beverley and used for test transfections. These cells were maintained as above but split 1:20 due to their more rapid growth rate.

3.2.3 Restriction enzymes

The restriction enzymes Hind III (Gibco) and XbaI (Gibco) were used to cut out DNA fragments from the plasmids. Reactions were performed in 0.5 ml Ependorf tubes, the reaction mixture having a final volume of 20μl. Thus for a typical double digestion, the reaction mixture consisted of 1μl DNA, 2μl restriction enzymes (1μl of each), 2μl of REact 2 Buffer (Gibco), and 15 μl H2O. For uncut controls, 1μl DNA was diluted in 19μl H2O. The reaction mixture was incubated at 37°C for 2 hours in a heat block.

3.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used analytically to resolve nucleic acids after digestion or ligation and to separate mixtures of nucleic acids prior to isolation and purification of a desired fragment. For a 1% gel, 1g of agarose slurry (electrophoresis grade, Gibco) in TAE buffer (40mM Tris-acetate (Sigma) and 1mM EDTA (Sigma), pH 8.3) was melted in a microwave and allowed to cool to approximately 60°C when 5μl of ethidium bromide (Sigma) were added to the solution. The agarose/ethidium bromide mixture was immediately poured into a gel mould and allowed to set with a well divider in position. When set, the gel was placed on the gel running apparatus which was filled with TAE running buffer. DNA samples were mixed with 0.1 volume gel loading buffer and carefully loaded into the wells. A 1 Kb DNA ladder (Gibco) was run concurrently as a size marker for DNA fragments. Electrophoresis was carried out at a constant 70V.
3.2.5 Agarose Gel Extraction

A QIAEX II agarose gel extraction kit (Qiagen, Dorking, UK) was used to extract DNA fragments from agarose gels. The DNA band for purification was carefully excised from the agarose gel with a clean, sharp scalpel ensuring that as much excess agarose as possible was removed. The band was transferred to a preweighed 1.5ml microfuge tube and the gel slice was weighed. The gel slice was dissolved in Buffer QX1 by adding 3 volumes of QX1 to 1 volume of gel (i.e. 300μl Buffer QX1 to each 100 mg of gel). The pH of the dissolved gel slice was checked and if greater than 7.5, 10ml of 3M sodium acetate pH 5.0 were added and the pH rechecked. The sample was then incubated at room temp for a further 10 minutes. QIAEX II was resuspended by vortexing for 30 seconds and 10 μl were added to the dissolved gel. The sample was mixed by vortexing and incubated in a heat block at 50°C for 10 minutes. The sample was vortexed every 2 minutes to keep QIAEX II in suspension. After incubation, the sample was centrifuged at high speed (13000 rpm) and the supernatant carefully removed. To remove any residual agarose, the pellet was resuspended by vortexing in 500μl Buffer QX1, centrifuged at high speed for 30 seconds and the supernatant removed. To remove residual salt contaminants the pellet was washed twice with 500μl of Buffer PE. The pellet was resuspended by vortexing, centrifuged at high speed for 30 seconds and the supernatant removed. The pellet was air-dried for 10-15 minutes or until white. The DNA was eluted by resuspending in 20 μl H₂O and incubating at 50°C for 5 minutes. Finally the suspension was centrifuged at high speed for 30 seconds, and the supernatant containing the purified DNA was carefully pipetted into a clean tube.
3.2.6 Chloroform/phenol extraction and DNA precipitation

This method was used to concentrate DNA prior to ligation reactions. An equal volume of Phenol: chloroform (BDH, Leicester, UK) was added to the DNA. The mixture was vortexed and centrifuged at high speed for 10 minutes. To an eppendorf tube containing the DNA the following reagents were added: 1/10 DNA volume 3M sodium acetate (Sigma) pH 5.4 and 2x DNA volume 96% ethanol (BDH). The solution was mixed by vortexing and left on dry ice for 20 minutes. After centrifuging at 12000 rpm for 10 minutes at 4°C the supernatant was removed and the remaining DNA pellet vortexed in cold 70% ethanol. The solution was centrifuged again at 12000 rpm for 5 minutes at 4°C. After removal of the supernatant, the DNA pellet was air-dried in a hood for approximately 10 minutes. The pellet was then resuspended in H₂O.

3.2.7 Ligation of DNA fragments

T4 DNA ligase (Gibco) was used for ligation of DNA fragments into cut vectors. Vector and insert were combined in a ratio of 1μl vector to 3μl insert to which were added 1μl of 5X T4 DNA ligase buffer (Gibco) and 0.5μl T4 DNA ligase. The reaction mixture was incubated at room temperature for 1.5 hours.

3.2.8 Preparation of LB agar plates

The required volume of LB agar was prepared by adding 1 tablet LB agar (Sigma) to 50 ml H₂O. The solution was autoclaved and retained for future use. To prepare agar plates the solution was melted at low temperature in a microwave. After allowing the agar to cool antibiotic was added to the desired concentration. Twenty ml were added to each petri dish in a sterile hood and allowed to dry. The plates were stored at 4°C but prior to use were dried at room temperature in a hood.
3.2.9 Preparation of competent bacteria

A small amount of XL1 blue bacteria cells (<10μl) were spread over an LB plate without antibiotic and grown overnight at 37°C. A colony was selected and grown in 100ml LB (without ampicillin) at 37°C on a shaker for 5 hours. The bacterial suspension was centrifuged (in 2 X 50 ml tubes) at 3000 rpm for 10 minutes. The supernatant was discarded and the bacterial pellet gently resuspended in 10ml ice-cold 0.1M calcium chloride by flicking the tube with a finger. The bacteria were spun again at 3000 rpm for 10 minutes and resuspended in 5 ml cold 0.1M calcium chloride on ice.

3.2.10 Transformation of competent bacteria

Competent bacteria were freshly prepared and 100μl were added to the DNA and kept on ice for half an hour. For the heat shock stage, the bacteria were transferred to a Falcon 15 ml tube and placed in a water bath at 42°C for 90 seconds. The tube was then immediately put on ice for a further 2 minutes after which the bacteria were transferred to an agar plate and spread with a sterile glass pipette. The plates were inverted and incubated overnight at 37°C (not longer than 18 hours).

3.2.11 Miniprep plasmid purification protocol

To analyse plasmid DNA from bacterial colonies prior to large scale purification, a Promega Wizard Minipreps DNA purification kit (Promega) was used. Each colony to be tested was inoculated into 2ml of LB broth containing ampicillin (Sigma) 50mg/ml and grown overnight at 37°C on a shaker. For each colony, one ml of cells were added to a 1.5ml ependorf tube and pelleted by centrifugation for 2 minutes in a microcentrifuge. The supernatant was discarded and the cell pellet resuspended in 200μl of Cell Resuspension Solution. 200 μl Cell Lysis Solution were added and mixed by gentle inversion of the tube several times resulting in clearing of the cell.
suspension. Next, 200μl Neutralisation Solution were added and mixed by inversion. The solution was spun at 12000 rpm in a microcentrifuge for 5 minutes and the cleared supernatant was decanted to a fresh ependorf tube. One ml of Wizard Minipreps DNA Purification Resin was added to the supernatant and mixed by inversion. For each miniprep a Wizard minicolumn was prepared by removing the plunger from a 2 ml syringe and attaching the syringe barrel to the leuer lock extension of the column. The resin/DNA mixture was carefully pipetted into the syringe barrel and slowly pushed into the minicolumn with the syringe plunger. The syringe was removed from the column, the plunger removed and the barrel reattached to the Minicolumn. Two ml of column wash solution were pipetted into the syringe and pushed through the minicolumn with the plunger. The syringe was removed and the minicolumn transferred to a 1.5 ml Ependorf tube prior to centrifugation at 12000 rpm for 2 minutes to dry the resin. The Minicolumn was transferred to a new Ependorf tube and 50 μl of water preheated to 70°C were added to the Minicolumn and left for 1 minute. The DNA was eluted by spinning the Minicolumn at 12000 rpm for 20 seconds. The minicolumn was discarded and the DNA analysed by gel electrophoresis as described above.

3.2.12 Maxiprep plasmid purification protocol

A single colony was inoculated into 2ml LB broth containing ampicillin 50mg/ml and grown to saturation. One ml of this saturated miniculture was diluted into 100ml LB broth with ampicillin and regrown overnight to approximately 1 x 10⁹ cells/ml (A₆₀₀ = 1.0 - 1.5). A Quiagen Plasmid Maxi Kit (Qiagen) was used to purify plasmid from this culture.

Bacterial cells were harvested by centrifugation at 4°C for 15 minutes at 6000g in a Sorval GSA rotor. The supernatant was carefully removed and the centrifuge tube was inverted to allow remaining medium to drain away.
The bacterial pellet was resuspended in 10 ml of buffer P1 (to which RNase A had been added) ensuring that no cell clumps remained. Ten ml of buffer P2 (lysis buffer) were added to the suspension and the solution was mixed gently by inverting the tube 4-6 times. The mixture was incubated at room temperature for exactly 5 minutes after which 10 ml of buffer P3 (neutralisation buffer) were added. The solution was mixed immediately by inverting the tube 5-6 times and incubated on ice for 15 minutes. The addition of P3 caused the solution to become cloudy and viscous. The mixture was mixed again and centrifuged in plastic tubes at 4°C for 30 minutes at 30000xg in a Sorvall SS-34. The clear supernatant was removed promptly and filtered over a folded mesh filter to remove any remaining particulate matter. While the suspension was being centrifuged a Quiagen-tip 500 was equilibrated by applying 10ml buffer QBT (equilibration buffer) and allowing the column to empty by gravity. The filtered supernatant was applied onto the Quiagen tip and allowed to enter the resin by gravity. The Quiagen tip was then washed through twice with 30 ml buffer QC (wash buffer). DNA was eluted with 15 ml buffer QF (elution buffer) and collected in a 30 ml tube. DNA was precipitated by adding 0.7 volumes of isopropanol equilibrated to room temperature and centrifuging at 15,000xg at 4°C for 30 minutes. The supernatant was carefully removed leaving the glassy isopropanol pellet undisturbed. The DNA pellet was washed with 15 ml cold 70% ethanol and centrifuged again at 15000xg for 15 minutes. The ethanol supernatant was completely removed and the pellet was air dried in a hood for approximately 10 minutes. Finally the DNA pellet was redissolved in a small volume of sterile H2O. The concentration of DNA was checked on a spectrophotometer and a sample was run on agarose gel for analysis.
3.2.13 Determination of G418 concentration for selection of transfectants

Cells were seeded in a 24 well plate at a concentration of $5 \times 10^4$ cells/well with normal growth medium. G418 was added to wells at increasing concentrations ranging from 100μg/ml to 1000μg/ml in 100μg/ml increments. Each concentration was added to duplicate wells with two wells kept free of G418 to act as controls. Medium and G418 were replaced every 2-3 days or when medium turned yellow. Cells were examined daily under a phase contrast microscope through which dead or dying cells could be identified.

3.2.14 Procedure for transfection

For the transfection of NSCLC cell lines, LipofectAMINE reagent (Gibco), a liposome formulation for high efficiency transfer of DNA into eukaryotic cells was used. Prior to transfection $3 \times 10^5$ cells were seeded in 35 mm tissue culture dishes with 2 ml of their normal growth medium. The cells were incubated at 37°C with 5% CO$_2$ and allowed to grow to approximately 70% confluence. This would usually take approximately 24 hours but could take longer depending on the cell line. In sterile 1.5 ml eppendorf tubes the following solutions were prepared: Solution A - for each transfection, 1μg DNA were diluted into 100μl OPTI-MEM Reduced Serum Medium (Gibco); Solution B - for each transfection 6-12 μl LipofectAMINE reagent were diluted into 100μl OPTI-MEM. The two solutions were combined, mixed gently and incubated at room temperature for 45 minutes to allow the formation of DNA-liposome complexes. While the complexes were forming cells were rinsed once with 2 ml of OPTI-MEM. For each transfection, 0.8 ml of OPTI-MEM were added to the tube containing the complexes, mixed gently and overlaid onto the rinsed cells. The cells were incubated with the complexes in an incubator at 37°C with 5% CO$_2$ for 6-24 hours, the optimal time being determined for each cell line as described
below. Following incubation periods of less than 24 hrs, one ml of growth medium containing twice the normal concentration of serum was added to the cells without removing the transfection mixture. After 24 hours the medium was completely replaced with fresh growth medium. At 72 hours the cells were split 1:10 into new 35 mm dishes and the selection agent Geneticin (Gibco) was added to the growth medium at the appropriate concentration.

3.2.15 β-Galactosidase enzyme assay

Cell extracts were prepared for analysis at 48 hours post-transfection. To determine endogenous levels of β-galactosidase a control extract was prepared from cells which had not been transfected with the β-galactosidase gene. The assay was performed according to the protocol of the β-galactosidase enzyme assay system from Promega. Growth medium was removed from the cells to be assayed. The cells were washed twice with PBS buffer (Mg$^{2+}$ and Ca$^{2+}$ free) and residual PBS removed with a pipette tip. The cells were covered with 200μL of Reporter Lysis Buffer and the dish rocked slowly to ensure complete coverage of the cells. The cells were incubated at room temperature for 15 minutes rocking the dish halfway through the incubation period. The plate surface was then scraped and all visible cell lysate tilted and scraped down to the lower edge of the plate. The cell lysate was transferred to a microcentrifuge tube and the samples placed on ice. The tubes were vortexed for 15 seconds and then centrifuged at 12000 rpm in a microcentrifuge for 2 minutes at 4°C. The supernatant was transferred to a fresh tube and assayed directly.

One hundred and fifty μl of cell extract were pipetted into labelled tubes to which 150μl of Assay 2X Buffer were added. The samples were mixed by vortexing and incubated at 37°C for 30 minutes or until a yellow
colour had developed. The reactions were stopped by adding 500µl of 1M Sodium Carbonate and vortexing. The absorbance was read at 410nm.

3.2.16 Live cell sorting by FACS

This was performed by Ray Hicks on a live cell FACS machine (Becton Dickinson, UK). Cells were trypsinised and prepared as for FACS analysis (see section 2.2.10).

3.2.17 Immunohistochemistry

3.2.17.1 Cells

H69, L23, L23C and L23T cells were trypsinised and then centrifuged at 3000 rpm (1559g) for 5 minutes to obtain a pellet. The supernatant was removed, the cells were resuspended in tris buffered saline (pH 7.6) and then centrifuged once more. Almost all the supernatant was removed and the solution gently agitated to leave a drop of fluid containing a dense population of cells. 50 µl aliquots of the cell preparations were dropped onto glass microscope slides coated with 3-amino™propyltriethoxysilane (Sigma). A smear was made by gently running an additional glass slide over the surface to produce a diffuse spread of cells. Cell smears were allowed to air-dry for 10 minutes at room temperature. Cell preparations were reacted immunohistochemically with monoclonal antibodies to NCAM (NY3D11), Pgp8.5, chromogranin and NSE (Dako Ltd, UK).

3.2.17.2 Human tissues

Fresh samples of non-neoplastic human tissue including lung, liver, kidney, spleen, cervix and endometrium (x3 samples of each), were snap-frozen in isopentane, cooled in liquid nitrogen and cryostat sections (5µm thick) were cut (Brights Ltd, Huntingdon, U.K.).
3.2.17.3 Immunohistochemistry methods

Cell preparations and cryostat sections were fixed in cold acetone for 10 minutes, washed in tap water and then immersed in tris buffered saline. A blocking agent (normal horse serum) was applied for 10 minutes. Slides were drained and the sections/cell preparations were incubated with primary antibody at a concentration of 20μg/ml for 40 minutes. After washing in tris buffered saline (pH 7.6), three changes over 15 minutes, biotinylated horse anti-mouse immunoglobulins (Vector Laboratories, U.K.) were applied at a dilution of 1:250, for 35 minutes. After a further wash in TBS slides were covered with avidin biotin-peroxidase complexes reagent (Vector Laboratories, U.K.) and left for 50 minutes. Slides were washed with three more changes of TBS over 15 minutes. 3'3' diamobenzidine tetrahydrochloride (Sigma) was freshly prepared in tris buffered saline (pH 7.6) to a concentration of 0.6mg/ml. After filtering, hydrogen peroxide (Sigma) was added to a concentration of 0.03% and the reagent then applied immediately to the slides and left for 6 minutes. After stopping the reaction in tap water, slides were counterstained in Harris' haematoxylin for 30 seconds and then washed in running tap water for 5 minutes. Slides were dehydrated by rinsing in 70%, 90% and two changes of 100% industrial methylated spirits (Flowindex Ltd, U.K.) for 1 minute each, transferred to inhibisol (Penetone Chemicals, U.K.) and mounted under glass coverslips with a drop of mountant (Ralmount, Merck Ltd, UK). Immunohistochemical reactivity of antibody with antigen (brown granular reaction product) was assessed using a light microscope (Axioskop © Zeiss Ltd, U.K.) under bright field illumination, by two observers (D.O. & G.B.) and photomicrographs taken using Fujichrome daylight film with a blue 80b filter (Cokin Ltd, France). Control slides included omission of the primary antibody and substitution of the primary antibody with one of irrelevant specificity (antibody to CEA, A5B7). In the case of liver and kidney sections, an avidin
biotin technique was avoided due to the presence of endogenous biotin in the samples. Instead of a biotinylated secondary antibody a peroxidase labelled sheep anti-mouse immunoglobulins was used.

3.3 Results

3.3.1 Construction of pcDNA3/NCAM 140Kb plasmid

To obtain stable transfectants that expressed NCAM 140 Kb, it was necessary to construct a plasmid vector which contained both the NCAM 140 cDNA and the neomycin resistance gene which would allow selection of stable transfectants in G418. As the pCDM8/NCAM construct did not contain the neomycin resistance gene, it was necessary to cut out the NCAM gene from pCDM8 and insert it into the pcDNA3 vector. The NCAM gene was cut out from pCDM8 using HIND III and Xba I and ligated into the cloning site of pcDNA3 which was cut with the same restriction enzymes.

3.3.1.1 Digestion of pCDM8/NCAM 140 and pcDNA3 with Hind III and Xba I

The pCDM8/NCAM construct and the plasmid pCDNA3 were both cut with Hind III and XbaI. The result of a 1% agarose gel electrophoresis is shown in figure 14. Digestion of pCDM8 yielded two main bands - one of approximately 4kb (the plasmid) and one of 3.2 kb (NCAM gene). After cutting, pCDNA3 was seen as a single band of approximately 5.4 kb.

3.3.1.2 Extraction of the NCAM 140kb band

The 3.2kb NCAM cDNA band was cut from the gel and purified as described above. The purified DNA was precipitated and concentrated into a final volume of 5µl H2O. A 1% agarose gel was run to confirm the presence of the insert (fig. 15).
3.3.1.3 Preparation of pCDNA3

It was not necessary to purify the cut pCDNA3 vector from the gel. The vector was digested with Hind III and Xba I, extracted by the phenol: chloroform:ISAr method and concentrated into a final volume of 10μl H2O.
3.3.1.4 Ligation of 3.2kb NCAM insert into pCDNA3 and transformation of bacteria

The 3.2kb NCAM insert was ligated into the pCDNA3 vector and the ligation mixture used to transform freshly-prepared competent XL1 blue bacterial cells. The cells were diluted in 5ml H2O and plated on an agar/ampicillin plate prior to overnight incubation at 37°C. A negative control of untransformed bacteria alone and a positive control of bacteria transformed with pCDNA3 vector only were also plated overnight. To confirm the presence of the ligated pCDNA3/NCAM 140 vector in transformed bacteria after overnight incubation, 12 colonies were picked for miniprep analysis. The purified plasmid DNA from each colony was cut with Hind III and Xba I and both intact and cut plasmid DNA were analysed on a 1% agarose gel (fig 16). Colonies 3, 7, 11 and 12 contained the successfully ligated pCDNA3/NCAM 140 plasmid which after digestion yielded two bands of appropriate size. The remaining colonies had mostly been transformed with pCDNA3 vector only which had not been successfully ligated.

![1% agarose gel analysis of minipreps from 12 transformed bacterial colonies. For each colony two DNA lanes were analysed: uncut (left) and cut with Hind III and XbaI (right). Colonies 3, 7, 11 and 12 contain the ligated vector pcDNA3/NCAM 140.](image-url)
3.3.1.5 Maxiprep purification of pCDNA3/NCAM 140 vector and confirmation of vector

LB broth from one of the pCDNA3/NCAM 140 positive colonies was used to grow up bacteria for maxiprep purification of the plasmid. To confirm the nature of the ligated vector a 1% agarose gel was run with undigested pCDNA3 and pCDNA3/NCAM 140 and each vector digested by Xba I only, Hind III only and Xba I and Hind III together (fig. 17). This gel clearly shows the presence of the pCDNA3 vector (5Kb+) and the 3.2kb NCAM 140 insert after double digestion.

Figure 17. Confirmation of ligated pcDNA3/NCAM140 vector in transformed colony. pcDNA3 vector is shown cut with a) XbaI b) HindIII and c) XbaI/Hind III. pcDNA3/NCAM140 is shown u) uncut and cut with a, b and c as above. The vector pcDNA3 and NCAM 140 insert are clearly seen in lane c.

3.3.2 Test transfection in NIH 3T3 cells

To test that cells transfected with the pCDNA3/NCAM 140 vector would express NCAM, NIH 3T3 cells were transfected with the vector and underwent FACS analysis at 48 hrs with MAb NY3D11. A positive 'tail' was seen indicating that approximately 20% of transfected cells were expressing NCAM at 48 hours (fig. 18).
Figure 18. FACS analysis showing results of transfection of NIH 3T3 cells with pcDNA3/NCAM140 vector. a) control b) parent 3T3 cells with NY3D11 c) Transfected 3T3 cells with NY3D11

3.3.3 Measurement of transfection efficiency in L23 and MOR cells with β-galactosidase assay

To determine transfection efficiency in L23 and MOR cells using Lipofectamine reagent, a β-galactosidase assay was performed in which β-galactosidase activity was compared in control cells (untransfected) with activity in cells transfected using 6µl, 8µl and 10µl of lipofectamine respectively. In L23 cells, increased β-galactosidase activity was detected in all transfectants, the peak activity occurring in cells transfected with 8µl of lipofectamine (fig. 19). In MOR cells peak β-galactosidase activity was also seen with 8µl Lipofectamine but levels were substantially lower compared to L23.

3.3.4 Determination of G418 concentration for selection of cell lines

In the L23 cell line, all cells were dead by day 8 at a G418 concentrations of 300 µg/ml or greater. Control cells and cells in 100µg/ml G418 remained alive while some cells remained viable at 200µg/ml. For the MOR cell line, all cells died by day 8 at a concentration of 700µg/ml or greater.
3.3.5 Transfection of L23

The cell line L23 was transfected with 1μg pCDNA3/NCAM 140 and 8μl Lipofectamine. A control line was transfected with 1μg of pCDNA3 vector only. Selection in G418 was commenced after 72 hours at a concentration of 500μg/ml. Medium and G418 were replaced (and dead cells removed) every 3-4 days or when the medium turned yellow. Resistant colonies of L23 were observed after one week and grew sufficiently after two weeks to split into 25cm³ flasks. Untransfected control L23 cells died after approximately one week. At three weeks after transfection, G418 resistant L23 had grown up sufficiently to allow FACS analysis. At this time approximately 20% of cells were NCAM positive although all cells were G418 resistant at this stage (fig. 20).
To improve the proportion of NCAM-positive cells, NCAM-expressing L23 transfectants were sorted from non-expressing cells by live FACS. Approximately 8.5 million cells were prepared for FACS. Two populations were sorted: from the first 4 million cells, all NCAM positive cells were sorted (approximately $2 \times 10^5$) while from the remaining cells only the top 5% NCAM positive cells were retained (only a few thousand cells). The sorted cells were transferred to 35 mm cell culture dishes. FACS undoubtedly caused considerable trauma to the cells, many of which died shortly after the procedure. For this reason, G418 was not added to the media until 48 hours after sorting to allow cells to recover to some degree. The sorted transfectants were grown for three weeks when they underwent FACS analysis again. The proportion of NCAM positive cells in populations derived from both sorts had now increased to approximately 50% (fig. 21).

There was no substantial difference in the proportion of NCAM positive cells derived from the "top 5% positive" sort compared with cells derived from the "all NCAM positive" sort. There was also a broad range of fluorescence intensity in both populations suggesting that cells were expressing NCAM at varying levels from high to relatively low.
Over the following two months NCAM positive transfectants were sorted and regrown two further times. FACS analysis of transfectants grown up after the second sort demonstrated that the proportion of NCAM positive cells was in excess of 60-70%. The proportion of NCAM positive cells did not increase substantially following a third sort. It seemed unlikely that further FACS would yield a population with a higher percentage of NCAM positive cells and the population of L23 transfectants obtained after the third sort was grown up and used in future studies. This population of NCAM-expressing transfectants was named L23T. The expression of NCAM by L23T was about 70% (fig. 22). The control population of L23 which was transfected with pCDNA3 vector only and selected in G418 was termed L23C. There was no NCAM expression by this population or the parent population L23.

3.3.6 Expression of PSA by L23T

FACS analysis of L23T with MAb735 demonstrated that NCAM expressed by transfectants was polysialylated (fig. 23). Parental L23 and L23C were not immunoreactive with MAb 735.
3.3.7 Expression of NCAM and other neuroendocrine markers detected by immunohistochemistry in L23 transfectants (L23T)

Immunohistochemistry of L23T, L23C and L23 with NY3D11 as primary antibody confirmed expression of NCAM by L23T (fig. 24) but not by L23C or L23. To determine whether transfection with NCAM might have altered the expression of other neuroendocrine markers, expression of NSE, PGP 8.5 and chromogranin A were also studied by immunohistochemistry (table 3). No difference in expression of these markers was seen in L23T compared with L23C or L23.
Figure 24. Immunohistochemistry of L23T with NY3D11 showing expression of NCAM
<table>
<thead>
<tr>
<th>cell line</th>
<th>NY3D11</th>
<th>NSE</th>
<th>Chromogranin</th>
<th>PGP 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>H69</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L23</td>
<td>-</td>
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<td>L23C</td>
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<tr>
<td>L23T</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. Expression of NCAM and neuroendocrine markers in H69, L23, L23C and L23T by immunohistochemistry

3.4 Discussion

This chapter has described the first successful transfection of a NSCLC cell line with full length cDNA for NCAM. A transfected cell line termed L23T was derived which expressed NCAM strongly when examined by FACS analysis with NY3D11 and immunohistochemistry. A control cell line L23C was transfected with the pCDNA3 vector only and did not express NCAM. This confirmed that the procedure of transfection itself did not produce expression of NCAM.

It would have been desirable to transfect more than one NSCLC cell line with NCAM cDNA but the MOR cell line appeared to have a low transfection efficiency as measured by the galactosidase assay. Several attempts to transfect MOR with NCAM were made but no NCAM expression was detected in this cell line.

The expression of NCAM by transfected L23 after three weeks of selection in G418 was only about 20%. It was apparent that not all G418-resistant cells were expressing NCAM. Possible explanations for this observation were that a proportion of L23 cells were not able to produce the NCAM molecule or that the vector was being degraded inside the cells due to the relatively large size of the insert. It was necessary to use a further selection process to obtain a higher proportion of cells which expressed...
NCAM. The two possibilities considered were further live sorting by FACS or clonal selection. Live FACS was chosen for two reasons: Firstly it would be quicker and allow sorting of the L23 transfectants which expressed NCAM most strongly. Secondly, L23 cells grow poorly or fail to grow at low concentrations in culture and clonal selection would be very slow or unsuccessful. Using live FACS a population of L23 cells which expressed NCAM strongly was obtained. Despite repeated sorts NCAM expression by transfectants was not greater than 60%. Ideally, for the function experiments described in the next chapter, a clonally-derived transfected cell line would have been desirable but the FACS and immunohistochemical studies on the L23T cell line described here demonstrated unequivocally that NCAM was expressed strongly. Expression of NCAM by L23T was stable for 4-6 weeks in culture before further sorting was necessary but this was adequate time to perform the function experiments described in the next chapter.

An unexpected but interesting finding was that the L23T cell line also expressed PSA. This implies that the recently characterised enzyme for eukaryotic PSA synthesis, polysialyltranferase-1 (Eckhardt et al., 1995) must have been present in L23 cells and may have been induced by the expression of NCAM. Alternatively the mRNA message was already present in the cells but there was no prior substrate for polysialylation as PSA is uniquely associated with NCAM.

The expression of several neuroendocrine markers by H69, L23 and the transfected cell lines was examined by immunohistochemistry. The results showed that L23 already expressed several common NE markers and this expression was not altered by transfection. However NCAM was not expressed by the parent cell line and as discussed in the introduction it is currently accepted that NCAM is one of the most reliable markers of the NE phenotype. Further studies are needed to determine whether transfection
with NCAM of a NSCLC cell line which did not previously express NE markers might subsequently alter their expression.

For additional discussion on the choice of transfection method see pages 227-28.
CHAPTER 4.  
Biological effects of NCAM transfection into L23.

4.1 Introduction

The purpose of the studies outlined in this chapter was to determine whether transfection of NCAM into a non NCAM-expressing NSCLC cell line L23 could alter its biological behaviour. Three cell lines were compared: the parent line L23, the transfected cell line L23T which expressed NCAM and the control cell line L23C transfected with the vector pcDNA3 only. The biological characteristics investigated were growth rate in vitro, invasive ability through Matrigel, a synthetic gel matrix, sensitivity to chemotherapeutic agents, sensitivity to radiation and growth rate of xenograft tumours implanted subcutaneously in nude mice.

4.2 Materials and methods

4.2.1 Materials

The derivation, culture and maintenance of the cell lines L23, L23T and L23C are described in chapter 3. Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) is a solubilised basement membrane preparation extracted from Englebreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin followed by collagen IV, heparan sulphate proteoglycans, entactin and nidogen. It also contains growth factors including TGF-beta, fibroblast growth factor and tissue plasminogen activator. Matrigel was supplied at a concentration of 15.5 mg/ml and diluted to 0.2 mg/ml with cold distilled H$_2$O. Matrigel is stored at -20°C and gels rapidly at 22-35°C. Prior to use it was thawed overnight at 4°C and kept on ice. Pre-cooled pipettes and tubes were used when handling matrigel. Following dilution, the material was dispensed into aliquots and refrozen immediately.
Transwell chambers (Costar) with 6.5 mm diameter filters and 8μm pores in polycarbonate membranes were used to study the invasion of cells through matrigel-coated membranes. A twenty four well culture plate (Costar) was used as the lower well.

Conditioned medium for use as a chemoattractant was obtained by incubating almost confluent mouse L cells in RPMI without FCS for 24 hours. The medium was collected and centrifuged at 1500 rpm for 10 minutes to remove cellular debris. This conditioned medium was aliquoted and stored at -20°C until required.

MTT (Sigma) was used as a colorimetric assay. Dimethyl sulfoxide (Sigma) was used to dissolve formation crystals of MTT. Doxorubicin (Faulding Pharmaceuticals Plc, Warwick, UK) and cisplatin (Faulding Pharmaceuticals Plc) were used as chemotherapeutic agents.

4.2.2 Growth rate assay

The MTT assay was used to compare growth rates between cell lines. Cells were seeded at the desired concentration in two columns of a 96 well tissue culture plate. The outermost wells of the plate were excluded as spectrophotometric readings from these wells are unreliable. For each time point to be analysed, a separate plate was prepared. Growth medium was added to each well to bring the total volume to 200μl. The plates were placed into a sterile plastic box containing a bijou filled with sterile water to prevent dehydration and incubated at 37°C with 5% CO₂. Yellow growth medium was replaced with fresh medium as necessary. For the MTT assay, 20μl of a solution of MTT 5mg/ml (Sigma, UK) were added to each well. Plates were incubated at 37°C for 4 hours to allow formation of the blue MTT formazan crystals by live cells. The plates were spun in a centrifuge at 2000 rpm for 10 minutes and medium was carefully but thoroughly removed from the wells with a pipette tip. To dissolve the crystals, 200μl DMSO (Sigma)
were added to each well mixed well with a pipette. After 10 minutes, the absorbance was read on a plate reader at 570nm.

4.2.3 Invasion assay

The invasive ability of cell lines was compared in an invasion assay described by Imamura et al., 1994. The inside walls of transwell chambers were carefully wiped with small blocks of paraffin wax and the residue removed with a cotton swab. The purpose of this procedure was to prevent meniscus formation when coating the filters with matrigel. In a conventional chamber untreated with paraffin, it has been suggested that the coating of matrigel after drying in the central area of the filter may be thin or absent due to meniscus formation. This allowed cells to pass easily through matrigel-coated filters because of uneven thickness or absence of matrigel. However, following treatment with paraffin the meniscus is abolished which produces a more even and uniform layer of matrigel on the filter after drying (fig. 25).

Figure 25. The effect of modifying transwell invasion chambers with paraffin wax
The transwell filters were coated with 70μl 0.2 mg/ml Matrigel and dried overnight under a laminar flow hood. Matrigel was reconstituted with 100 μl of RPMI. Cells were trypsinised and washed once in RPMI before resuspending at a concentration of 20 x 10⁵ cells/ml in RPMI (without FCS). Each upper well was seeded with 2 x 10⁵ cells in 100 μl RPMI. In the lower wells, 750 μl of L cell conditioned medium were placed as a chemoattractant. The chamber was incubated at 37°C with 5% CO₂ (fig. 26A). After 24 hours of incubation, cells with invasive ability invaded through the matrigel-coated filter appearing on the lower surface of the filter (fig. 26B). Following incubation, the medium from the upper wells was carefully removed with a pipette and the non-invasive cells and matrigel were wiped away with a cotton wool bud (fig. 26C). To detect the cells that had invaded into the filter pores or that remained on the lower surface of the filter, 20 μl of a 5 mg/ml MTT solution were added to the medium in the lower well and the filters were incubated for 4 hours at 37°C (fig. 26D). The live cells that remained formed dark blue crystals of MTT formazan. The transwell was then transferred into the well of another 24 well culture plate containing 200 μl of dimethyl sulfoxide (figs. 26E and F). After 10 minutes the crystals were fully dissolved and the formazan solution was transferred to a 96 well microplate (fig. 26G). The absorbance of the solution was measured on a microplate reader (MR 700, Dynatech Laboratories) at 570 nm. For each experiment, invasion of a cell line was measured in four wells and experiments were performed in triplicate.
Figure 26. MTT invasion assay (see text for explanation)
4.2.4 Irradiation of cells

Each of the three cell lines were seeded in two columns of a 96 well tissue culture plate at the experimental cell concentration. The central columns 4-9 were used and results were recorded from the rows B-G to minimise errors in absorbance measurements at the peripheries of the plate. Thus, for each dose of radiation, the three cell lines were present on the same plate and results from 12 wells per cell line were analysed. Cells were irradiated at room temperature 40cm from the source at a rate of 2.6 Gy/minute. Control cell lines were not irradiated. Survival of cells was analysed after 3 days or 1 week using the MTT assay and results for each cell line are expressed as percent survival compared with the control line.

4.2.5 Chemosensitivity assay

The chemosensitivity of cell lines to doxorubicin and cisplatin was investigated using the MTT assay. Each cell line was plated at $10^4$ cells/well along two rows of a 96 well plate. After 48 hours growth, the drug was added at increasing concentration across the plate commencing with a control well (no drug), then drug at 0.01μM, 0.03μM, 0.1μM, 0.3μM, 1.0μM continuing up to 100μM. Drug was added for 1 hour and then removed by washing twice with RPMI/FCS. Survival was analysed after 4 days by the MTT assay and results are presented as percent survival (compared to control) for each drug concentration.

4.2.6 In vivo growth of subcutaneous tumours in nude mice

To compare growth of tumour cells in vivo, 3 million cells were injected subcutaneously into the flanks of nude mice. Each tumour cell line was injected into three mice except for L23C which was only injected into 2 mice due to a shortage of animals. Tumour size was measured in three dimensions at 3 day intervals. Tumour volume was calculated from the
formula $\pi/6 \times L \times B \times D$. Mice were sacrificed when tumours grew too large. This work was performed in conjunction with Barbara Pedley and Joan Boden at the Animal Unit, Royal Free Hospital.

4.3 Results

4.3.1 Growth curve of L23 determined by MTT assay

To determine the optimum starting concentration for measuring growth of L23 with the MTT assay, cells were seeded at three initial concentrations of $10^4$, $3 \times 10^4$, and $10^5$. The results indicated that an initial concentration of $10^4$ cells/well was the most suitable for future comparison of growth rates (fig. 27).

![Growth curve of L23 determined by MTT assay](image)

**Figure 27.** Effect of cell concentration on growth of L23 measured by the MTT assay. Each point represents the mean value from 6 wells.

4.3.2 Comparison of growth rate of L23, L23T and L23T by the MTT assay

The growth rate of the parental cell line L23 was compared with that of L23T and the control transfectant L23C. Cells were seeded at an initial
concentration of $10^4$ cells/well and growth rate was measured up to 9 days (216 hours). No significant difference was apparent until 216 hours by which time the cells had completed their exponential growth phase and the wells were overcrowded with cells (fig. 28). These studies suggested that transfection with NCAM did not influence the growth rate of L23.

![Graph showing growth rates of L23, L23T, and L23C](image)

**Figure 28.** Comparison of growth rates of L23, L23T and L23C by the MTT assay. Each point represents the mean result from 12 wells.

### 4.3.3 Invasion through Matrigel

The ability of L23, L23T and L23C to invade through Matrigel was compared at 24 hours. There was no significant difference in invasion between the three cell lines and thus transfection with NCAM did not appear to alter invasive ability (fig. 29).

### 4.3.4 Chemosensitivity assay

The chemosensitivity of the three cell lines to cisplatin and doxorubicin at 4 days after a one hour exposure to the drug was investigated
by the MTT assay. No difference in chemosensitivity to either drug was seen between the three cell lines (figs. 30 and 31).

**Figure 29.** Invasion of L23, L23T and L23C through Matrigel. Results are the mean of three experiments and show standard deviation.

**Figure 30.** % Survival at 4 days following one hour exposure to Cisplatin.
4.3.5 Radiosensitivity

The radiosensitivity of L23, L23T and L23C was compared by the MTT assay. In the first experiment cells were irradiated with 2 or 4 Gy and MTT assay was performed at 3 days. Control cells were not irradiated. Two initial cell concentrations were investigated: $10^4$ cells/well or $5 \times 10^3$ cells/well. There was no difference between the three cell lines under these conditions (figs. 32 and 33). These studies also demonstrated that the cell lines were poorly radiosensitive and subsequently cells at an initial cell concentration of $10^4$ cells/well were irradiated with either 4 or 8 Gy and survival measured at 7 days in order to allow the cells to go through more life cycles. It was hoped that this would increase the chance of observing any differences in radiosensitivity between the cell lines. After irradiation with 8 Gy, survival of L23 was significantly less than L23T but did not differ significantly from L23C.
Figure 32. % survival of irradiated cells at 3 days post irradiation. Initial concentration of cells $10^4$/well.

Figure 33. % survival of irradiated cells at 3 days post irradiation. Initial cell concentration cells $3 \times 10^5$/well.
Figure 34. % survival of irradiated cells at 7 day post irradiation.

4.3.6 In vivo growth rate

When serial growth rates of subcutaneous xenografts were compared, the transfected cell lines L23T and L23C grew at a slower rate than the parent cell line. However due to the small numbers of mice it is not possible to make firm conclusions from these observations. L23C grew at the slowest rate and these observations did not suggest that NCAM expression was influencing in vivo growth in these xenografts.
Figure 34. In vivo growth of cell lines implanted subcutaneously in nude mice.

4.4 Discussion

Transfection of L23 with NCAM 140 did not alter the biological behaviour of this NSCLC cell line with respect to growth rate, invasive ability in vitro, chemo- or radiosensitivity and in vivo growth rate. The basis for these studies were observations in both cell culture and patients that NSCLC cell lines and tumours with the neuroendocrine phenotype exhibited greater chemosensitivity than non-NE-NSCLC tumours (see section 1.8.2). It was also suggested that NCAM expression in NSCLC was a feature of phenotypic diversification associated with the emergence of a highly malignant clone and subsequent tumour progression (see section 8.1.3). Patients with NCAM-positive tumours appeared to have a worse prognosis and the neuroendocrine phenotype was suggested to be associated with metastasis although its importance as a prognostic indicator of survival was less clear.

The contemporary view on the origin of lung cancer is that all types are derived from a single progenitor cell which is capable of differentiating
into SCLC, squamous, large cell and adeno- carcinomas. This is supported by observations of mixed appearances in tumours such as SCLC/squamous, SCLC/adenocarcinoma etc. SCLC is a highly metastatic tumour which is also chemosensitive and radiosensitive and is characterised by expression of NE markers. It is proposed that expression of the NE phenotype by NSCLC cell lines and tumours confers a more 'SCLC-like' behaviour. The studies in this chapter sought to determine whether expression of one marker of the NE phenotype, NCAM expression, could alter the biological behaviour of the NSCLC line L23.

The NE phenotype is characterised by the presence of many amines and peptides which are expressed to varying degrees by different NE tumours (see section 1.8.1). There is no clear definition of what constitutes the NE phenotype in terms of the quantity or type of NE markers expressed. Thus there is little or no conformity between the criteria used in the various studies which looked at chemosensitivity, metastasis and prognosis in NE-NSCLC cell lines and patient tumours. Recent studies have failed to show a correlation between NE phenotype and survival but there has not been a large scale prospective study looking at the prognostic importance of the NE phenotype. NCAM expression is the most consistent marker of the NE phenotype whereas expression of other markers may vary and in these experiments only the effect of changes in NCAM expression on cell behaviour were studied.

Transfection with an adhesion molecule such as NCAM might have been expected to alter the morphology or growth rate of L23 cells. The non-NCAM expressing cell line BT4Cn aggregates into spheroids and grows at a slower rate following transfection with NCAM (see section 1.7.3) and differences in the morphology of SCLC cell lines in culture have been related to their level of NCAM expression (see section 1.7.1). However the introduction of NCAM into the L23 cell line did not appear to alter its
morphology in culture. L23, in common with most other NSCLC cell lines grows as a monolayer in cell culture. It is possible that NCAM-NCAM interactions were insufficiently strong to overcome other factors influencing the morphology of L23. Alternatively the transfected NCAM expressed by L23T cells was not functioning properly as an adhesion molecule. When growth rates of L23, L23T and L23C in culture were compared by the MTT assay no differences were observed. The explanation for this could either be that NCAM-NCAM interactions were not a significant factor influencing growth rates or that the transfected NCAM was not functioning normally in its capacity as a transmembrane signalling molecule.

In previous studies, cells transfected with NCAM have exhibited reduced invasiveness (e.g BT4Cn cells - see section 1.7.3). In the present study all three cell lines were able to invade through matrigel but no differences were observed between them. Chemoinvasion assays through matrigel measure the ability of cells to attach to the matrix, degrade the matrix and migrate in the direction of a chemoattractant. Both invasive and non-invasive cell lines can usually attach to a matrix and migrate towards a chemoattractant. However, invasive cell lines also possess the ability to degrade basement membranes and matrix. Previous studies have demonstrated that the matrigel assay can distinguish between invasive and non-invasive cell lines and can also be used to compare invasiveness between cell lines (Imamura et al., 1994). In previous studies transfection with NCAM has reduced invasion possibly by increasing cell-cell adhesiveness. As no effect was seen in L23T compared with the parent line, it is possible that NCAM transfection did not increase cell-cell adhesiveness or that transfected NCAM was non-functional.

It would have been unexpected if NCAM transfection resulted in differences in chemosensitivity or radiosensitivity. The reason for performing these studies was that the NE phenotype may be associated with
differences in chemosensitivity and the aim was to observe the effect of NCAM expression as a single feature of the NE phenotype. Neuroendocrine differentiation of NSCLC cell lines has also been correlated with radiosensitivity in vitro (Duchesne et al., 1988). However no differences were seen in the present studies and it can be concluded that transfection with NCAM does not alter chemosensitivity or radiosensitivity in L23.

Limited in vivo growth studies were undertaken which also did not show any substantial differences in growth rates of tumours in nude mice. Xenografts do not metastasise in nude mice and thus it was not possible to study whether NCAM expression might alter metastatic potential.

In summary, transfection of L23 with NCAM 140 cDNA did not alter its biological properties in the assays and growth studies performed here. It is not possible to be sure that the NCAM expressed by the transfectants was a normally configured and functioning molecule or whether it was only expressing its immunodominant regions which were detectable by FACS and immunohistochemistry with NY3D11. Western blotting of the NCAM expressed by H69 cells and L23T was attempted to demonstrate that the whole protein was present but technical difficulties were experienced with both cell lines and although the results were suggestive of NCAM being normally expressed by L23T cells they are not presented here. An alternative control to show that transfection with the vector pCDNA3/NCAM 140 could produce normally functioning NCAM might have been to transfect fibroblast cells with NCAM and develop an aggregation assay similar to that used for H69 cells in chapter two to demonstrate increased cell-cell adhesiveness. These controls would have been helpful in drawing firmer conclusions about these results.

These studies could also be criticised on the basis that the L23T transfected cell population used for the biological studies was not clonally derived and was a heterogeneous population derived by successive cell
sorting. The reasons for choosing this method of selection were discussed in the previous chapter. Assuming that transfected NCAM was functioning normally, the high level of NCAM expression by the L23T cells would probably have been adequate to reveal any biological differences from the parent cell line and if positive differences had been observed it would have been appropriate to produce a clonal population for further study. As no differences were observed in the present experiments it is doubtful whether a clonally derived L23 NCAM transfectant would have yielded different results.

The identification of tumour cell antigens which are of prognostic significance will allow clinicians to make more informed decisions about treatment regimens and assess probability of response to therapy. Some studies have suggested that expression of NCAM and the neuroendocrine phenotype in NSCLC tumours is associated with a worse prognosis and perhaps paradoxically, an enhanced response to chemotherapy. However the size and methodology of these studies can be criticised and a large prospective study is required to firmly establish whether these observations reflect a true association. Expression of a tumour cell antigen such as NCAM may represent a phenotypic regression to a more primitive, embryonic cell type and perhaps in a malignant cell its expression may not have any functional role in the biology of the cell. There are multiple changes in lung cancer cells both at the genetic and phenotypic levels and it is unlikely that any single change can bring about the transformation of a non-malignant cell to a malignant one.
CHAPTER 5.
Biodistribution of radiolabelled NY3D11 after injection into patients with small cell lung cancer.

5.1 Introduction

This chapter will describe a study of the distribution of radiolabelled NY3D11, a monoclonal antibody against NCAM, following intravenous injection into patients with SCLC. The aim of the study was to determine whether the antibody could localise to tumours in patients and whether the blood clearance characteristics and localisation of antibody in the tumour relative to normal organs was favourable for therapy.

The mortality of patients with small cell lung cancer (SCLC) remains greater than 90% at two years after diagnosis and new therapeutic approaches are urgently needed to improve the outcome of the disease (Kardamakis et al., 1988; Souhami et al., 1990). The tumour is characterised by its initial responsiveness to chemotherapy producing complete remission in about 50% of patients and early relapse. Eradication of persistent micrometastases at the end of chemotherapy would prevent the development of chemoresistant tumour relapse. Antibody-directed therapy is one strategy used to target therapy specifically to the tumour site. Monoclonal antibodies recognising tumour associated surface antigens have been shown to localise specifically in many different tumour types. Therapy is most likely to be effective in small tumour foci when the total tumour burden is low as antibody uptake is most efficient (Pedley et al., 1987; Olabiran et al., 1994). Adjuvant antibody therapy in patients with colorectal cancer has produced encouraging results (Riethmuller et al., 1994) and antibody directed therapy of micrometastases of SCLC in the adjuvant setting could also be an effective new treatment.
NCAM might be a suitable target for antibody directed therapy in SCLC as it is strongly expressed by tumour biopsies and cell lines (see section 1.6.3). Several studies have shown that anti-NCAM monoclonal antibodies can localise successfully and produce regression of human SCLC xenografts in nude mice. Only one clinical study in patients with SCLC has previously been reported where the anti-NCAM MAb123C failed to localise to primary tumours in four patients but a single liver metastasis was identified (Michalides et al., 1994a). Although immunohistochemical studies with anti-NCAM monoclonal antibodies demonstrate crossreactivity with neural tissue, muscle, thyroid epithelium, testicular leydig cells and circulating natural killer (NK) cells (Souhami et al., 1991) no evidence of localisation or toxicity in these tissues was seen in this study.

5.2 Materials and methods

5.2.1 Production of antibody

The NY3D11 hybridoma was produced in 1992 by Yemi Olabrian at the Department of Oncology, UCLMS, London. RBS/DNJ mice (Robertsonian 8:12 translocation) were immunised with live UCH 10 cells (Kardamakis et al., 1988). After fusion of RBS/DNJ mice spleen cells with Fox NY (NS1 variant myeloma line), clonal colonies were derived by limiting dilution and their supernatants were screened on mouse fibroblasts transfected with the human muscle NCAM gene (a gift from Professor F. S Walsh, Guy’s Hospital, London, UK). The antibody NY3D11 was an IgG1 subtype which bound to NCAM transfectants and to the product of a cloned sequence from NCAM DNA. In common with most other anti-NCAM monoclonal antibodies, NY3D11 binds to immunodominant epitopes located in the "stem region" C-terminal of the 5th Ig-like domain of the NCAM molecule (Gerardy-Schahn et al., 1994).
NY3D11 antibody for patient administration was prepared by Shona Ward. The antibody was purified by protein A-sepharose chromatography from tissue culture supernatant grown by Celltech laboratories and tested prior to clinical use to ensure compliance with the guidelines in "Operation manual for the control of production, preclinical toxicity and phase I trials of anti-tumour antibodies and drug antibody conjugates" (CRC, 1986)

5.2.2 Radioiodination of antibody

5.2.2.1 Chloramine T method

Solutions of chloramine T (Pierce Ltd, UK) and L tyrosine (Sigma) in 0.05M phosphate buffer were made up at a concentration of 2mg/ml and autoclaved prior to use. In a sterile cabinet, the following reagents were drawn up in sterile syringes allowing 0.5ml air between the plunger bottom and the reagent: 0.5mg NY3D11 as previously aliquoted; 100µl 1M sodium phosphate buffer (pH 7.5); 100µl chloramine T; 100µl tyrosine; 2ml 0.05M sodium phosphate buffer pH7.5; 3.5ml 0.05M sodium phosphate buffer pH7.5; 4ml 0.05M sodium phosphate buffer pH7.5. A PD10 column (Pharmacia, St. Albans, UK) was blocked with 0.5ml 20% human albumin solution and equilibrated with 25ml 0.05M sodium phosphate pH7.5. Labelling was performed in a laboratory radiation cabinet. The vessel containing the antibody was placed in a lead container on crushed ice. 100µl 1M phosphate was added to the antibody. The rubber septum of the vial containing sodium $^{131}$Iodide was wiped with a steret. Using a sterile Hamilton syringe washed in 70% methanol/pyrogen-free water, 3mCi $^{131}$Iodide (Amersham, UK) was drawn up and added to the antibody. 100µl chloramine T was added and after 3 minutes 100µl tyrosine was added. After a further 1 minute the total volume was transferred to a PD10 column and allowed to wash in. The total volume added to the column was brought to 2.5ml with 0.05M sodium phosphate buffer. The first 2.5ml eluted from
column was collected into a 7.5ml Sterilin bijou labelled 'Void'. A further 3.5ml 0.05M sodium phosphate buffer pH7.5 was added to the column and the eluent collected in a bijou labelled "Protein". This fraction should contain the labelled antibody. Finally 4ml 0.05M sodium phosphate buffer pH7.5 was added to the column and 4ml was collected in a bijou labelled "Waste". This fraction may contain a small amount of free sodium $^{131}$Iodide. The radioactivity in each fraction was counted to confirm that most of the $^{131}$Iodide label was in the protein fraction. Thin layer chromatography was performed to determine the proportion of $^{131}$Iodide incorporated into antibody.

5.2.2.2 Iodogen labelling method

Ten glass tubes (internal diameter 0.7-1.0cm) were washed in 70% methanol followed by sterile pyrogen-free water. In a glass flask prewashed in 70% methanol and pyrogen-free water 10mg iodogen (Pierce Ltd) was added to 10ml dichloromethane (1mg/ml solution). In a prewashed glass tube 0.2 ml of 1mg/ml iodogen solution was added to 9.8ml dichloromethane to give a final iodogen concentration of 20ug/ml. Using a sterile glass pipette, 700μl of 20μg/ml iodogen solution was added to each glass tube which were left to dry in a sterile cabinet. Tubes must be completely dry before labelling. Iodogen-coated tubes were stored at 4°C with dessicant in a sterile container. In a sterile cabinet, the following reagents were drawn up in sterile syringes allowing 0.5ml air between the plunger bottom and the reagent: 0.5mg NY3D11 as previously aliquoted; 100μl 1M sodium phosphate buffer (pH 7.5); 2ml 0.05M sodium phosphate buffer pH7.5; 3.5ml 0.05M sodium phosphate buffer pH7.5; 4ml 0.05M sodium phosphate buffer pH7.5. A PD10 column was blocked with 0.5ml 20% human albumin solution and equilibrated with 25ml 0.05M sodium phosphate pH7.5. Labelling was performed in a laboratory radiation cabinet.
The following were added to an iodogen-coated tube: 0.5mg NY3D11; 100μl 1M sodium phosphate buffer pH7.5; 3mCi 131I drawn up as previously described. The tube was shaken gently every 5 minutes for 20 minutes at room temperature. The mixture was drawn up from the iodogen tube with a sterile syringe and applied to the PD10 column. Three fractions were collected as previously described.

5.2.3 Thin layer chromatography

A longitudinal line was drawn in pencil down the centre of a TLC plate (HPTLC-Merck, UK). A horizontal line was drawn near the bottom of the plate. A small drop of radiolabelled antibody was placed on the horizontal line on either side of the central dividing line. After drying, the plate was placed into a beaker containing 80% methanol ensuring that the level of the methanol meniscus was below the dots. The beaker was covered and the methanol was allowed to migrate up the plate by capillary action drawing any unreacted radioiodine with it. When the methanol meniscus was nearing the top of the plate (after approximately half an hour) it was removed from the beaker and the plate above the meniscus was cut off. The plate was dried and then divided longitudinally. Each half was cut into 10 strips of equal width which were placed into counting vials and counted on a gamma counter. The labelling ratio was expressed as the ratio of counts from labelled antibody (usually on the second lowest strip) to the sum of counts from free radioiodine (on the top strip) and labelled antibody.

5.2.4 Immunoreactivity assay

Immunoreactivity of the labelled antibody with glutathione-S-transferase (GST) NCAM fusion protein (kindly provided by Prof. Frank Walsh, UMDS, London) was compared with immunoreactivity of unlabelled antibody by enzyme immunoabsorbent assay (ELISA). The GST NCAM
fusion protein contained a peptide sequence from full length human NCAM previously shown to bind anti-NCAM antibodies. A 96 well plate was coated with 100µl NCAM fusion protein 5µg/ml in carbonate buffer for 2hrs at 37°C. The plate was washed three times with phosphate buffered saline (PBS)/Tween (Sigma). Non-specific binding was blocked by adding 150µl 3% bovine serum albumin (BSA)/PBS (Sigma) to each well for 2hrs at 37°C. The plate was washed again 3 times with PBS/Tween. 200µl PBS were added to the first well of the row and 100µl PBS to the remaining wells. 1µg antibody was added to the first well (to give concentration of 5µg/ml). A serial 1:2 dilution was made across the rows and the plate was incubated at 37°C for 1hr. After washing 3 times with PBS/Tween, 100µl alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was added to each well. The plate was incubated at 37°C for 1hr and washed 3 times with PBS/Tween. Finally, 100µl of fresh p-nitrophenyl phosphate substrate (Sigma) was added to each well and colour was allowed to develop for 1hr at 37°C. The absorbance of each well was read on a plate reader at 410nm. Experiments were performed in duplicate on the same plate.

5.2.5 Aggregate analysis by gel filtration on S300 column

Antibodies in solution may aggregate to form dimers or larger aggregates. The presence of aggregates can be detected by high resolution gel filtration which separates the monomer from aggregates. The S-300 gel is a sephacryl acrylamide-agarose combination gel which is capable of good resolution at comparatively high flow rates and is especially useful for fractionating large serum proteins including immune complexes.

One milligram (200µl) of unlabelled NY3D11 antibody was loaded onto the S300 column (Pharmacia) and washed in with 0.6ml PBS. Fractions of 1.7ml were collected by an automated fraction collector and the O.D of 200µl aliquots was measured on a spectrophotometer at 280nm. Results are
presented as a graph of OD 280nm against fraction number. For studies on radiolabelled antibody, NY3D11 was labelled with \(^{125}\)Iodine by the iodogen method and the labelling ratio was checked by TLC. The radiolabelled antibody was diluted 1:50 in PBS/azide. Approximately 100000cpm were loaded onto the S300 column and washed in with 0.6ml of PBS. After the antibody was on the column, 1.7ml fractions were collected by an automated fraction collector. A 200μl aliquot was taken from each fraction and activity was measured on a gamma counter. The results are presented as a graph of counts per minute (cpm) against fraction number. Non-aggregated antibody is seen as a single peak while aggregated antibody will appear as additional peak(s) in earlier fractions.

5.2.6 Fluorescence activated cell sorting analysis of antibody binding to cells

This work was carried out by technicians in the laboratory of Dr Peter Amlot at the Royal Free Hospital. Staining of NK and H69 cells with NY3D11 was investigated using double immunofluorescence on a BD FACScan and three different markers of NK cells, CD8, CD16, and CD57. A commercial monoclonal antibody for NCAM, NKH1:CD56 (Coulter, UK) was used as a positive control. A non-specific antibody was used to determine the gate between positive and negative cells and both a narrow gate ( 'Low gate' ) and a wider gate ( 'high gate' ) were used. The results of staining of cells from the blood of two different donors, PLA and IV, are presented.

5.2.7 Immunohistochemistry of bone marrow

Bone marrow aspirates from normal donors were obtained and smears prepared. Bone marrow smears were fixed in cold acetone for 10 minutes, washed in tap water and then immersed in tris buffered saline. Immunohistochemistry was carried out as described in section 3.2.17.
5.2.8 Study Design

5.2.8.1 Patient eligibility

Patients with SCLC, newly diagnosed or undergoing treatment, age 16-80 years, and WHO performance status 0-3 were eligible for the study. Patients with a history of allergy to iodine or immunoglobulins or a positive skin test to intradermal administration of antibody were excluded. Written informed consent was obtained from participants and the study was approved by the ethical committees of participating centres.

5.2.8.2 Preliminary investigations

Chest radiography, bone scanning and ultrasonography of the liver were used to assess tumour site(s). Full blood count, urea, electrolytes, creatinine, liver function tests and thyroid function tests were performed prior to antibody injection.

5.2.8.3 Administration of antibody

To prevent accumulation of radioactivity in the thyroid gland, patients received potassium iodate 85mg tds for seven days commencing 24hrs before administration of radiolabelled antibody. Prior to antibody injection, patients received an intradermal injection in the forearm of 0.1ml (1-2µg) radiolabelled antibody. At an adjacent site 0.1ml of 0.9% saline was injected intradermally and the skin reaction at both injection sites was compared after 15 minutes. If erythema at the test site was greater than at the control site, intravenous administration of antibody would not proceed.

The antibody was injected intravenously through a butterfly needle over 5 minutes after which the line was flushed with 0.9% saline. Intravenous injections of chlorpheniramine, hydrocortisone and adrenaline were available during this period in the event of an acute reaction.
intravenous line was left in situ for 20 minutes and then removed if no immediate adverse reaction occurred.

5.2.8.4 Gamma camera imaging

Patients were scanned at 24, 48 and 72 hrs after injection of antibody. Scanning was performed in the department of nuclear medicine at the Middlesex Hospital using a large field of view gamma camera with a high energy parallel hole collimator and an on-line computer.

5.2.8.5 Venous blood sampling

To determine the elimination of antibody from the circulation, 5ml of venous blood were withdrawn immediately after injection (from the contralateral arm) and at 24, 48 and 72 hrs. Blood was stored in pre-weighed EDTA bottles and activity was measured after several weeks when sufficient decay allowed accurate counting. β-half lives of antibody in blood at 24 hours were calculated using the formula: Half life = -slope/ln(2) where slope = ln (activity at 24 hr) - ln (initial activity)/24.

5.3 Results

5.3.1 Imaging studies

Subject 1: JL was a 64yr old male whose CXR showed a large opacity in the right mid-zone subsequently found to be SCLC. A bone scan and ultrasound scan showed no evidence of metastases. NY3D11 was labelled with approximately 1mCi 131Iodine by the chloramine T method. Thin layer chromatography indicated that 95% antibody was labelled. Whole body images at 24, 48 and 72 hours post injection showed no evidence of localisation to the tumour (fig. 36). However, uptake was noted in the bone marrow and liver at these time intervals.
Subject 2: AG was an 80 year old male who had presented 19 months previously with polypoid SCLC in the right main and upper lobe bronchi and signs of superior vena caval obstruction. He was treated with three courses of chemotherapy (etoposide/chloramambucil) and radiotherapy to the mediastinum and primary tumour. Sixteen months later he presented with a right pleural effusion and was found to have extensive solid tumour in the right lung on ultrasound. A bone scan demonstrated extensive metastases in the ribs with additional hot spots in the left trochanter and right upper femur. He received two cycles of oral etoposide which were discontinued after disease remained stable. At the time of his antibody study, his CXR showed a "white-out" of the right lung. An ultrasound scan demonstrated that this was mainly due to solid tumour. NY3D11 was labelled with 1.7mCi of $^{131}$Iodine by the chlorammine T method. Scans were performed at 24 and 48 hours post injection. Uptake was seen in the liver, spleen and bone marrow but there was no evidence of localisation in the right lung tumour (fig. 37).

![Image](image-url)

**Figure 36.** Gamma camera images of patient 脒 (posterior views) at 24, 48 and 72 hours after intravenous injection of $^{131}$I-NY3D11.
Subject 3: AP was a 68 year old male who presented with a cough and was found to have a left hilar mass. Histology was confirmed as SCLC and a CT scan indicated involvement of regional lymph nodes. Other staging investigations revealed no evidence of metastatic spread. The patient received no treatment prior to the antibody scan. NY3D11 was labelled with 0.8 mCi $^{131}$Iodine. Scans were performed at 24, 48 and 72 hours. The antibody did not localise to the tumour but uptake was again noted in the bone marrow and liver (fig. 38).

5.3.2 Clearance of antibody from blood

Labelled antibody was cleared rapidly from blood in all three subjects (fig. 39). By 24hrs only 15-28% of injected activity remained. The beta half-lives after 24 hours were 51.1, 46.2 and 46.8 hours respectively.
Figure 38. Gamma camera images of subject AP at 24 and 72 hours post injection.

Figure 39. Clearance of $^{131}$I-NY3D11 from blood following intravenous injection.
5.3.3 Experiments to determine whether NY3D11 became aggregated after storage, radiolabelling, or incubation with human serum

The scans in all three subjects showed that radiolabelled antibody was localising to bone marrow and liver and failing to localise to tumour. A possible explanation was that NY3D11 became aggregated before or after radiolabelling and was subsequently being taken up by the reticuloendothelial system. To investigate this possibility, cold and radiolabelled NY3D11 were run on an S300 column.

5.3.3.1 Unlabelled ("cold") antibody: One milligram of cold NY3D11 (200μl) was put onto an S300 column. Antibody was eluted as a single peak with no evidence of aggregation in this sample (fig. 40).

5.3.3.2 Radiolabelled antibody: 0.5mg NY3D11 were labelled with 1.7mCi by the chloramine T method and 230μl of a 1:50 dilution run an S300 column. A small amount of aggregation was noted in this sample but the majority of labelled antibody in this sample was non-aggregated (fig. 41).

![Figure 40. Plot of O.D. 280 nm vs fraction number after running 1mg 'cold' NY3D11 down an S300 column.](image)
5.3.3.3 Incubation with human serum

Serum from patients with SCLC may contain variable amounts of soluble NCAM. To investigate the possibility that NY3D11 aggregated in serum, $^{125}$I-labelled antibody was incubated with serum from the subject JL (unknown level of serum NCAM) and with serum previously stored from a patient with a high level of soluble NCAM. The antibody was mixed with serum in a ratio of 1:50 and incubated at 37°C for 24 hours. The samples were run on an S300 column to determine whether aggregation had occurred. Although there was evidence of minimal aggregation in the serum of patient JL and in the 'high-NCAM' serum, most of the antibody remained disaggregated (figs. 42 and 43).
Figure 42. Incubation of $^{125}$I-labelled NY3D11 with serum from patient JL. Data from S300 column.

Figure 43. Incubation of $^{125}$I-labelled NY3D11 with serum from a patient with high serum NCAM. Data from S300 column.
5.3.4 Effect of radiolabelling on immunoreactivity of NY3D11

Radiolabelling of antibodies can cause loss of binding affinity for antigen which may result in failure to localise to tumour. To investigate this possibility the immunoreactivity of NY3D11 labelled with $^{131}$Iodine by the chloramine T method was compared with 'cold' clinical grade NY3D11 and a sample of stock non-clinical grade NY3D11. Clinical grade and stock NY3D11 had similar immunoreactivities but there was at least 50\% loss of immunoreactivity after radiolabelling (fig. 44).

![Graph showing immunoreactivity of NY3D11](image)

**Figure 44.** Comparison of immunoreactivity of 'cold' clinical grade and laboratory stock NY3D11 with $^{131}$I-labelled clinical-grade NY3D11.

5.3.4.1 Comparison of the immunoreactivity of $^{131}$I labelled NY3D11 (administered to subject AG) and immunoreactivity of unlabelled NY3D11

As there appeared to be a large loss of immunoreactivity after labelling by the chloramine T method, a small amount of $^{131}$Iodine-labelled NY3D11 prepared for administration to patient AG was retained and its immunoreactivity was compared with "cold" stock (fig. 45). This experiment
again suggested that radioactive labelling by the chloramine T method was reducing the binding affinity of NY3D11.

![Graph showing comparison of immunoreactivity of 131I-NY3D11 administered to patient AG with cold NY3D11.](image)

**Figure 45.** Comparison of immunoreactivity of $^{131}$I-NY3D11 administered to patient AG with cold NY3D11.

5.3.4.2 *Comparison of immunoreactivity of 131I NY3D11 labelled by iodogen method and immunoreactivity of unlabelled NY3D11*

Using the iodogen method, 0.25mg of stock NY3D11 were labelled with 1.8mCi $^{131}$Iodine. The immunoreactivity compared with cold NY3D11 is shown in figure 46. This experiment suggested that the immunoreactivity of NY3D11 after labelling by the iodogen method was very similar to that of unlabelled NY3D11. It was thought that the failure of localisation in patients might be explained by reduced affinity of NY3D11 for NCAM due to chloramine T labelling conditions. It was therefore decided to change the method for radiolabelling antibody to the iodogen method which appeared to preserve immunoreactivity.
A small amount of $^{131}$I NY3D11 administered to the subject AP was retained to compare immunoreactivity with cold stock NY3D11. The immunoreactivity of labelled antibody was slightly reduced but to a lesser extent than observed previously after chloramine T labelling (fig. 47)
5.3.5 Immunohistochemistry of bone marrow with NY3D11

The unexpected localisation of NY3D11 to bone marrow in all three subjects might be explained by reaction of the antibody with a cellular element in the bone marrow. To investigate this possibility, fresh frozen bone marrow aspirates from 10 normal patients were stained with NY3D11. No evidence of staining was seen in any of the sections.

5.3.6 Immunoreactivity of NY3D11 with NK cells

FACS studies suggested that NY3D11 had a much lower affinity for NCAM than the control anti-NCAM MAb NKH1. On the H69 control cell line at least 6-7 times as much NKH1 bound as NY3D11 despite the use of NY3D11 over a concentration range of 1-100μg/ml (table 4). An alternative interpretation of these results is that NY3D11 and NKH1 recognise different epitopes and the epitope seen by NY3D11 is less abundant or accessible.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration μg/ml</th>
<th>MFI*</th>
<th>MFI Ratio NKH1/NY3D11</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY3D11</td>
<td>100</td>
<td>437</td>
<td>6.5</td>
</tr>
<tr>
<td>NY3D11</td>
<td>10</td>
<td>381</td>
<td>7.5</td>
</tr>
<tr>
<td>NY3D11</td>
<td>1</td>
<td>497</td>
<td>5.8</td>
</tr>
<tr>
<td>NKH1</td>
<td>1</td>
<td>2858</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 4. FACS analysis comparing binding of NY3D11 and NKH1 to H69 cells
*MFI = mean fluorescence intensity

Staining of cells from the blood of two different donors PLA and IV was also investigated. The results paralleled those with H69 cells in that the percentage of CD8, CD16 or CD57 cells that stained with NY3D11 or NKH1 was always much less for NY3D11 than NKH1.
% positive cells

<table>
<thead>
<tr>
<th></th>
<th>Donor PLA</th>
<th></th>
<th>Donor IV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8</td>
<td>CD16</td>
<td>CD57</td>
<td>CD8</td>
</tr>
<tr>
<td>NY3D11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.6</td>
<td>44.7</td>
<td>35.4</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>36.4</td>
<td>19.7</td>
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</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td>41.3</td>
<td>14.9</td>
<td>0.7</td>
</tr>
<tr>
<td>NKH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>42.9</td>
<td>87.9</td>
<td>84.0</td>
<td>17.2</td>
</tr>
<tr>
<td>CD4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>78.5</td>
<td>70.6</td>
<td>68.8</td>
<td>69.6</td>
</tr>
</tbody>
</table>

Table 5. Double immunofluorescence FACS staining of NK cells with 3 markers of NK cells (CD8, CD16 and CD57) and NY3D11, NKH1 and CD4 ('low gate')

Although NY3D11 bound much less well than NKH1, MFI values using the higher more discriminating gate suggested that NY3D11 could still bind to a considerable number of NK cells (table 6).

MFI of positive cells

<table>
<thead>
<tr>
<th></th>
<th>Donor PLA</th>
<th></th>
<th>Donor IV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8</td>
<td>CD16</td>
<td>CD57</td>
<td>CD8</td>
</tr>
<tr>
<td>NY3D11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>310</td>
<td>239</td>
<td>40</td>
<td>81</td>
</tr>
<tr>
<td>10</td>
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<td>74</td>
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<td>29</td>
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<tr>
<td>1</td>
<td>2</td>
<td>63</td>
<td>223</td>
<td>52</td>
</tr>
<tr>
<td>NKH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>132</td>
<td>106</td>
<td>114</td>
</tr>
<tr>
<td>CD4</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>597</td>
<td>607</td>
<td>648</td>
<td>723</td>
</tr>
</tbody>
</table>

Table 6. Double immunofluorescence FACS staining of NK cells with 3 markers of NK cells (CD8, CD16 and CD57) and NY3D11, NKH1 and CD4 ('high gate')
5.4 Discussion

The results of these patient studies demonstrated that $^{131}$Iodine-labelled NY3D11 antibody did not localise to tumour but accumulated in the bone marrow and liver. The blood clearance data showed that antibody was cleared rapidly from blood over the first 24 hours with a subsequent second phase half life of approximately 48 hours. NY3D11 antibody had localised well in preclinical studies with human SCLC xenografts in nude mice and it was therefore important to investigate why this discrepancy in imaging between human and mouse occurred. Several possible explanations for the observed findings were considered: i) Antibody was aggregating either before or after radiolabelling resulting in uptake by the reticuloendothelial system (RES); ii) radiolabelling of antibody caused loss of binding affinity for NCAM; iii) antibody was reacting with a cellular component of bone marrow; iv) antibody was reacting with NK cells in blood and subsequently being removed from circulation by the RE system or v) non-specific interactions with the Fc component of whole NY3D11 were occurring resulting in the observed distribution pattern.

Aggregation of antibody could explain the observed pattern of distribution if large particles were formed and recognised as particulate matter by the RES. Monocytes and macrophages in both mouse and humans possess specialised receptors for the Fc portion of IgG which are part of the mechanism by which microorganisms coated with IgG are recognised and ingested. The RES can also recognise and remove foreign particles from the circulation. Aggregation of antibody might have occurred during storage, after radiolabelling, or after introduction into patients. Gel filtration experiments on an S300 column were undertaken to determine whether aggregation was occurring. The results showed that unlabelled NY3D11 was detected as a single peak just after fraction 30, which is the expected fraction for unaggregated IgG. After radioactive labelling, there is a very small
amount of aggregate seen as a higher molecular weight fraction immediately prior to the main peak.

The presence of soluble NCAM in the serum of patients raised the possibility that NY3D11 was binding to circulating antigen. This could result in rapid clearance and promote aggregation. It is known, for example, that high levels of circulating CEA result in more rapid clearance of antibody and reduce accumulation in tumour (Pedley et al., 1989). To investigate this possibility, radiolabelled NY3D11 was incubated with serum stored from a patient with high levels of circulating NCAM and also with serum from subject JL taken prior to the study. Analysis on the S300 column suggested that a small amount of aggregation had occurred, to a greater extent after incubation with the 'high NCAM' serum. However most of the antibody remained non-aggregated. From these studies it was concluded that aggregation was not likely to be the major cause of the observed distribution patterns although it is probable that circulating serum NCAM does contribute to the rapid clearance of antibody from circulation. It was not feasible to measure the serum NCAM levels of patients in this study but in the presence of limited disease it is more likely that serum NCAM levels were low (Ledermann et al., 1994)

The observed distribution of NY3D11 was not entirely compatible with uptake by the RES when activity should also have been seen in the spleen (only 1/3 patients) and to some extent in the lung and gut where no increased uptake was seen. However, localisation to the spine, ribs and pelvis occurred in all patients suggesting that antibody was accumulating in the bone marrow. Although cells of the RES are present in bone marrow, the preferential localisation to this area suggested that NY3D11 might also be binding to an epitope present on a cellular component within bone marrow. The binding of NY3D11 to normal bone marrow aspirates was therefore investigated by immunohistochemistry but no immunoreactivity was seen in
any of the sections (N=10). However a negative result using immunohistochemistry does not completely exclude the possibility that NY3D11 was reacting with bone marrow. If an epitope is expressed at low levels or affinity for the epitope is weak, immunohistochemistry may not be sensitive enough to detect binding. FACS analysis is a more sensitive technique and it was therefore decided to examine binding of NY3D11 to blood and bone marrow cells using this technique.

The antigens expressed by leucocytes have been extensively characterised in the leucocyte workshops. The CD56 antigen expressed on the surface of natural killer (NK) cells is identical to NCAM (Hida et al., 1991). Prior to commencing this study the possibility that an NCAM monoclonal might bind to circulating NK cells was considered. Using FACS to compare binding of several NCAM monoclonals to a population of NK enriched cells, it was found that NY3D11 had weak reactivity with NK cells (Shona Ward, unpublished data). This was thought to be a favourable factor for clinical use of NY3D11. In the present study, binding of NY3D11 to circulating NK cells was another likely factor contributing to the observed distribution of the antibody. The results of FACS analysis of binding of NY3D11 and NKH1 to NK cells suggest two important conclusions: Firstly, that NY3D11 appeared to be substantially less immunoreactive with NCAM on control H69 cells than NKH1 and this was mirrored by a similar reduction in the observed binding on NK cells. An alternative explanation might have been that the antibodies were recognising different epitopes but they are believed to be identical (Doyle et al., 1991). Secondly, FACS analysis indicated that despite reduced immunoreactivity, NK cells were still able to bind large amounts of NY3D11. Thus it is highly probable that some of the labelled antibody bound to circulating NK cells after injection.

The aim of the above studies was principally to determine why NY3D11 was localising to bone marrow and liver. The reasons for failure of
the antibody to localise to the tumour were also considered. An omission from this study was that tumour biopsies from the patients were not tested for NCAM expression. Previous studies of NCAM expression in SCLC tumours from patients and SCLC cell lines have shown that NCAM is expressed by almost 100% of tumours (Schol et al., 1988; Rygaard et al., 1992). In clinical practice, biopsies taken at bronchoscopy are often small and unsuitable for immunohistochemistry. In addition, NCAM monoclonals react poorly or are non-reactive with specimens fixed in formalin (Broers et al., 1994). Unfixed specimens would therefore have had to be obtained from all suspected tumours at biopsy. For these reasons, it is not known for certain whether the tumours in this study expressed NCAM but it is likely that they did.

The importance of immunoreactivity of an antibody with its epitope for tumour localisation remains uncertain. Some authors have suggested that antibody uptake and penetration into tumours is enhanced by high affinity radioimmunoconjugates (Schlom et al., 1992) while others propose that interaction of high-affinity antibodies at the surface of a tumour prevents penetration (Fujimori et al., 1989). The possibility that radioactive labelling of NY3D11 reduced the affinity of the antibody for NCAM was considered in this study. Using an assay to compare immunoreactivities, it was apparent that iodination of antibody by the chloramine T method led to a substantial loss of immunoreactivity compared to unlabelled antibody. When this assay was performed on a sample of chloramine T-labelled NY3D11 administered to patient AG, the difference was less than first seen but still indicated a sizeable reduction in immunoreactivity. After the labelling technique was changed to the iodogen method, the reduction in immunoreactivity appeared to be less (fig. 46) and this method was retained for the third subject. However, the improvement in immunoreactivity failed to enhance tumour localisation of antibody in this patient.
The accessibility of epitopes on tumour cells is also likely to be important for successful antibody localisation (Pervez et al., 1988). The limited success of radioimmunotherapy and wide variations in antibody localisation amongst patients with the same cancer has been attributed in part to heterogeneity of antigen distribution (Edwards, 1985). Antigens in vivo may be expressed at preferential sites. For example, in a study of the distribution of two antigens expressed by colonic adenocarcinoma in vivo, one was expressed lumenally and the other basolaterally (Pervez et al., 1989). The basal lamina may represent a physical barrier to extravasated antibodies (Dvorak et al., 1991) as may tight junctions and desmosomal intercellular junctions. In colorectal adenocarcinomas there is evidence that CEA epitopes expressed on the lumenal surface of malignant acini or cytoplasmically may be inaccessible to antibodies in vivo whereas epitopes on the basal or basolateral aspects of glandular structures are more readily accessible (Schlom et al., 1992; Boxer et al., 1994). The distribution of NCAM expression in SCLC in vivo has not been investigated but there is evidence in vitro that NCAM accumulates preferentially at sites of cell-cell contact over sites of cell-substrate contact (Bloch, 1992). It is possible that NCAM epitopes were poorly accessible to antibody in these patients and consequently failed to localise.

In vitro studies using a human tumour spheroid model of SCLC have suggested that uptake and penetration of NY3D11 depends on the local concentration of antibody (Olabiran et al., 1994). Rapid clearance of NY3D11 antibody from blood would have produced a poor local concentration gradient which was unfavourable for antibody localisation. This may have been exacerbated by poor blood supply although the vascularisation of tumours in this study was unknown. The size of tumour in this study did not influence localisation but a small tumour size is considered more favourable (Pedley et al., 1987).
In summary, the reasons why NY3D11 failed to localise were thought to include rapid first phase clearance from blood due to non-specific Fc receptor interactions with cells of the RES and specific reactions with circulating NCAM and CD56 on NK cells. The effect of a relatively low affinity for the NCAM epitope is uncertain and may not be significant as the antibody localises well in human SCLC xenografts in mice (see chapter 6). It was therefore speculated that removal of the Fc portion of NY3D11 to produce a F(ab')2 fragment might alter its clearance behaviour in humans resulting in more favourable conditions for tumour localisation. The results of these studies are described in the following chapter.
CHAPTER 6.
Preparation and administration of clinical grade radiolabelled NY3D11 F(ab')\textsubscript{2} to patients with SCLC.

6.1 Introduction

This chapter will describe the preparation of clinical grade NY3D11 F(ab')\textsubscript{2} for localisation studies in patients with SCLC. F(ab')\textsubscript{2} fragments may be useful alternatives to intact antibodies for radioimmunotherapy (Buchegger et al., 1989; Pedley et al., 1993). In the mouse, removal of the Fc binding moiety results in more rapid circulatory clearance thus reducing exposure of normal tissue and enhancing the tumour: blood (therapeutic) ratio, while the smaller size allows greater tumour penetration. In addition, removal of Fc from mouse monoclonal antibodies results in a less immunogenic product for administration to patients (Seccamani et al., 1989). However, absolute tumour levels with antibody fragments are lower than with intact antibody and tumour removal is more rapid both of which may limit their effectiveness as therapeutic agents. Moreover, the affinity constant and hence the immunoreactivity of antibody fragments decreases with size so that \( K_{IgG} > K_{F(ab')2} > K_{Fab} \) (Ferrone et al., 1988).

Mouse IgGs, particularly IgG1, are relatively resistant to the classic method of F(ab')\textsubscript{2} production by pepsin digestion. This method works well with mouse IgG2a and IgG2b but with high pepsin to antibody ratios (Parham et al., 1982). The mouse gamma-1 chain sequence lacks the residue corresponding to the pepsin cleavage site on human gamma-1 chain (leu 234) that is highly conserved in other isotypes and species (Burton, 1985). Digestion of mouse IgG1 by alternative enzymes, including bromelain and ficin, is rapid and stable and will reliably produce high yields of F(ab')\textsubscript{2} (Mariani et al., 1991). Furthermore, immunoreactivity of the F(ab')\textsubscript{2} is highly
conserved, approaching that of intact IgG, whereas pepsin digestion substantially reduces the antigen binding capacity.

The results of localisation studies with intact NY3D11 in patients with SCLC suggested that interactions with the Fc moiety might be contributing to rapid first phase clearance and failure to localise in tumours. This chapter will describe the preparation of F(ab’)2 fragments of NY3D11 by bromelain digestion of intact antibody and testing to comply with the CRC guidelines for preparation and administration of antibodies in phase I trials (CRC, 1986). Prior to injection into patients, it was demonstrated that radioactive labelling of the F(ab’)2 did not result in loss of immunoreactivity and that immunoreactivity of radiolabelled F(ab’)2 was preserved after incubation with human serum. An in vivo localisation study in nude mice bearing human SCLC xenografts comparing F(ab’)2 with whole antibody confirmed that F(ab’)2 was likely to be favourable for tumour targeting. Studies of tumour localisation in patients with SCLC were therefore undertaken and the results are presented at the end of this chapter.

6.2 Materials and methods

6.2.1 Purification of NY3D11 antibody from supernatant

Prior to full scale preparation of clinical grade material a trial run was undertaken at Celltech Ltd, Slough, UK under the supervision of Dr Leslie Chaplin. The clinical material was subsequently prepared in an area designated for patient antibody production at the Royal Free Hospital.

A cell seed lot of NY3D11 hybridoma was established and maintained by Celltech Ltd. Starting material was 50 ml sterile NY3D11 supernatant concentrated and filtered at Celltech to a final concentration of 0.7mg/ml. NaCl (Sigma) was added to 3.5M and 1/40 volume 2M Tris pH 8.5 (Sigma). A Pharmacia column C16/20 was depyrogenised by running 1M NaOH (Sigma) for 90 minutes followed by 60% Ethanol/1M acetic acid for a further
90 minutes. The column was then washed with pyrogen free water and packed with 25ml fresh protein A. The column was equilibrated with 5 column volumes (CV) 4M NaCl/50mM Glycine pH8.0 at flow rate 50ml/cm²/hr (1.7ml/min). Before proceeding an Atlas Pyrogen kit was used to check that column was pyrogen-free. A Pharmacia GM1 gradient mixer (excluding motor but including stirrer blade) was autoclaved, depyrogenised in 1M NaOH followed by 60% ethanol/1M acetic acid and washed with pyrogen free water. The sample was loaded onto the column which was washed with 10 CV 4mM NaCl/50mM glycine pH8.0. The antibody was eluted with a 10CV gradient of 0.15M Na₂HPO₄ pH9/0.1M citric acid pH2. One hundred and twenty-five ml of each solution were put into the gradient mixer and samples were eluted in 5ml fractions at 1.7ml/min starting with the high pH. The chart trace indicated when the protein peak came off the column. The pH of protein-containing fractions was checked and neutralised with 2M Tris pH8.5 to pH5.0-7.0 if necessary. The O.D. of protein-containing fractions was measured and fractions with A₂₈₀ > 0.05 were pooled. The O.D of the pooled fractions was rechecked. An Amicon concentrating cell was washed in 0.2M sodium hydroxide followed by 60% Ethanol/1M Acetic acid and rinsed well with pyrogen free water prior to use. Prior to use, an Amicon filter (PM10) was soaked in pyrogen-free water for 10 minutes. The antibody was concentrated to approximately 10mg/ml, filtered through a 0.22um sterile filter and the O.D. was rechecked. A Pharmacia Biotech PD10 column was equilibrated with 25ml O.1M sodium acetate buffer pH 5.5 containing 3mM EDTA. The antibody sample was applied to the column and eluted with 0.5ml aliquots of 0.1M sodium acetate /3mM EDTA pH5.5. Fractions were collected as 0.5ml aliquots and the O.D. was checked. The most concentrated fractions (usually 4-6) were pooled and the O.D. measured. The less concentrated fractions were pooled separately and retained.
6.2.2 Activation of Bromelain

One ml of Bromelain (Boehringer) was incubated with 4.0 ml of 50 mM cysteine in 0.1 M sodium acetate buffer /3 mM EDTA pH5.5 for 30 minutes at 37°C. Cysteine-containing buffer must be made up fresh. A Pharmacia Biotech PD10 column was equilibrated with 25 ml 0.1 M sodium acetate /3 mM EDTA pH5.5. One ml of the activated Bromelain solution was put onto the column and 1 ml fractions were eluted. The protein concentration in the fractions was measured and the fraction with the highest concentration (usually fraction 4) was retained.

6.2.3 Digestion of Intact NY3D11 to F(ab')2 fragments

A C10/10 column (Pharmacia) was depyrogenised as described above and packed with 10 ml SP Sepharose fast flow (Pharmacia Biotech). The column was equilibrated with 5 column volumes (50 ml) of 0.1 M sodium acetate buffer pH6. Freshly activated and desalted bromelain was added to the antibody in 0.1 M sodium acetate/3 mM EDTA pH5.5 in a ratio of 1:50 (Bromelain: antibody). The mixture was incubated for 1 hour at 37°C. The mixture was then cooled to 4°C and a small amount of 0.1 M NaOH was added to bring the pH to 6.0 (100 ul of NaOH will bring the pH of 1 ml 0.1 M sodium acetate/3 mM EDTA pH5.5 to 6.0). The antibody/bromelain reaction mixture was immediately applied to the SP sepharose column. The column was washed with a 5CV gradient of 0.2 M NaCl/0.1 M NaAc pH6.0 at a rate of 1 ml/min and 1 ml fractions were collected. The first protein peak contained the Fc fragments. The second protein peak was the F(ab')2. The fractions containing this peak were pooled and the O.D. was measured. In the final stage of purification, F(ab')2 was passed down a protein A column to remove any residual whole IgG. A depyrogenised C10/10 column (Pharmacia) was packed with 2 ml Protein A sepharose fast flow (Pharmacia). F(ab')2 was run through the column and collected as a broad peak. A typical
Purification of NY3D11 from supernatant by elution on Protein A sepharose fast flow column.

Elution of Fc (1st peak) and F(ab')2 (2nd peak) on SP sepharose fast flow column following bromelain digestion.

Purification of F(ab')2 on Protein A sepharose fast flow column.

Figure 4. A spectrophotometer trace showing elution of proteins at each stage of purification.
spectrophotometer trace illustrating the elution of protein fractions during each stage of the procedure is shown in fig 48. Finally, the F(ab')$_2$ was dialysed in sterile pyrogen-free phosphate buffered saline, concentrated on an Amicon concentrating cell and the concentration measured. The purity of the F(ab')$_2$ was assessed on SDS-PAGE gel and HPLC.

6.2.4 SDS-PAGE gel

Assessment of protein purification was carried out by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Prior to loading onto a 4-20% polyacrylamide resolving gel (Biorad, UK), samples were diluted 1:2 with SDS sample buffer (2-mercaptoethanol was added 1:20 to buffer for reducing conditions) and boiled for 2 minutes. Using a Hamilton syringe (Hamilton, Nevada, USA) 3|Xg of each sample was pipetted into the wells at the top of the gel. A molecular weight marker (See Blue, Novex, San Diego, USA) was used in one of the ten wells. Samples were run at 30mA until the bromophenol blue had reached the bottom of the gradient gel. The gel was soaked in Coomassie blue (Sigma) to stain the protein bands for 2 hours and immersed in destain overnight.

6.2.5 Purity and toxicity testing of NY3D11 F(ab')$_2$ patient aliquots

In a sterile area of a hospital pharmacy the sample was divided into 0.5mg aliquots in sterile tubes and sealed. The aliquots were stored at 4°C in an area reserved for clinical grade material.

Culture of antibody for contamination by bacteria or fungi was undertaken at the Microbiology Department, University College London Hospitals. Virus culture was performed by the Royal Free Hospital Virology Department. Mycoplasma detection was carried out at Clare Hall Laboratories (ICRF) by microbiological cultivation on cell enriched agar and fluorescent staining with bisbenzimide using vero cells as indicators.
Pyrogen testing in rabbits was performed at SafePharm Laboratories, Derby. Three rabbits were injected with 35µg/kg of antibody and subsequent temperature rise monitored. Toxicity testing was performed by Dr B. Pedley at the Royal Free Hospital. Ten times the patient dose (0.5mg/kg) was given to 5 guinea pigs and 6 balb c mice. Each guinea pig received 0.025mg antibody intraperitoneally (ip) in 0.2ml saline. Each mouse received 0.002mg antibody ip in 0.2ml saline. The animals were observed for 14 days for signs of toxicity then sacrificed and gross pathology carried out. A haematological study was performed on the guinea pig bloods.

6.2.6 Animal studies
6.2.6.1 Establishment of H69 xenografts in nude mice

The human small cell lung cancer cell line H69 was used to establish a xenograft model subcutaneously in the flanks of nude (nu/nu) mice. The mice used were female, 2-3 months old and weighed between 20 and 25g. This work was performed by Dr Barbara Pedley and Joan Boden at the Animal Unit, Royal Free Hospital. Subsequent passaging of tumours was carried out by subcutaneous implantation of small tumour pieces (approximately 1mm³). The biodistribution study was commenced when the tumours reached a size of 0.5 and 1.0cm³.

6.2.6.2 Biodistribution study

Labelled antibody was injected into the tail veins of the mice. Animals were given food and water ad libitum. The water contained 0.1% potassium iodide to prevent thyroid uptake of iodide. At 24hr and 72hr, 4 mice from each group were bled and the following tissues removed for counting: liver, tumour, spleen, rectum, kidney, lung and muscle. Femur was taken from two mice in each group at both time points. Tissues were placed into preweighed tubes which were then reweighed and filled with 7M
KOH to dissolve the tissues. Three days after the 72hr time point (when all tissues had completely dissolved), the samples were counted on a gamma counter (Pharmacia, 1470 Wizard). The results are presented graphically as the percentage of injected dose per gram of tissue and as the ratio of tissue to blood activity.

6.27 Isolation of peripheral blood leukocytes

Peripheral venous blood (40ml) was collected in a 60 ml syringe containing 5ml acid citrate dextrose (ACD) as an anticoagulant. This was mixed with an equal volume of 6% hydroxyethyl starch (w/v) and 2% (v/v) ACD to sediment erythrocytes. The leukocyte rich serum was removed and centrifuged at 312g for 7 minutes at 4°C. The cell pellet was resuspended in 5ml serum, gently layered on top of 7.5ml 75% (v/v) Percoll and centrifuged at 1200g for 30 minutes at 18°C. This procedure separated the leukocytes which are held at the interface between the serum fraction and the Percoll. The leukocytes were recovered by gentle aspiration.

6.3 Results

6.3.1 Preparation of F(ab')2 from whole NY3D11 by bromelain digestion

Preliminary experiments were undertaken to determine the time length of bromelain digestion which gave an optimal yield of F(ab')2. Samples of whole NY3D11 digested by bromelain were analysed by HPLC after 25 and 50 minutes at 37°C, and after overnight incubation at 4°C.
<table>
<thead>
<tr>
<th>Bromelain digestion/time</th>
<th>Proportion of antibody remaining/%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>25 minutes</td>
<td>7</td>
</tr>
<tr>
<td>50 minutes</td>
<td>2</td>
</tr>
<tr>
<td>Overnight (4°C)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7. HPLC analysis of relative amount of whole antibody, F(ab')\_2 and Fc present after incubation of NY3D11 with Bromelain

Although overnight incubation produced the optimal yield of F(ab')\_2, the absolute amounts of antibody remaining were found to be substantially reduced in comparison with the shorter digestion times. It was thought that this occurred due to continued digestion of both products by bromelain. For the subsequent large scale production of F(ab')\_2 a bromelain digestion time of 1 hour was used which was thought to represent an optimal balance between digestion of IgG to F(ab')\_2 and loss of total protein amount.

6.3.2 SDS-PAGE analysis of products during preparation of NY3D11 F(ab')\_2

The products of bromelain digestion and protein fractions derived from purification on gel filtration and affinity columns were analysed by SDS-PAGE. Approximately 3\( \mu \)g of protein were loaded per well. The results of 4-20\% SDS-PAGE analysis on a non-reduced gel are shown in figure 49. Samples of bromelain digest after 25 minutes and overnight yielded two bands representing F(ab')\_2 and Fc respectively (lanes 3 and 4). After separation of products on the SP sepharose column, the first protein peak contained Fc (lane 5) and the second peak contained F(ab')\_2 (lane 6). After further purification on protein A, the final product contained a single band representing F(ab')\_2. These findings were also confirmed by HPLC analysis.
In the reduced gel (fig. 50), NY3D11 (lane 2) is reduced to heavy and light chain. After bromelain digestion (lanes 3 and 4), F(\(\text{ab}'\))\(_2\) is reduced to Fc and heavy + light chain of F(\(\text{ab}'\))\(_2\). After SP-sepharose purification, Fc appears in the first peak (lane 5) and heavy + light chain of F(\(\text{ab}'\))\(_2\) in the second peak (lane 6). After further purification on protein A, reduced F(\(\text{ab}'\))\(_2\) is seen in lane 7.

<table>
<thead>
<tr>
<th>Gel slot</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marker</td>
</tr>
<tr>
<td>2</td>
<td>NY3D11 purified</td>
</tr>
<tr>
<td>3</td>
<td>Bromelain digest (25 mins)</td>
</tr>
<tr>
<td>4</td>
<td>Bromelain digest (overnight)</td>
</tr>
<tr>
<td>5</td>
<td>Post SP-sepharose I</td>
</tr>
<tr>
<td>6</td>
<td>Post SP-sepharose II</td>
</tr>
<tr>
<td>7</td>
<td>Post SP/Protein A purification</td>
</tr>
</tbody>
</table>

![Image](image.png)

**Figure 49.** SDS-PAGE analysis of preparation of F(\(\text{ab}'\))\(_2\) from NY3D11 (non-reduced gel).

![Image](image.png)

**Figure 50.** SDS-PAGE analysis of preparation of F(\(\text{ab}'\))\(_2\) from NY3D11 (reduced gel).
6.3.3 Purity and toxicity testing of clinical grade NY3D11 F(ab')2

The purity and toxicity of the clinical grade F(ab')2 product was tested to comply with CRC guidelines for the control of production and preclinical toxicology of anti-tumour antibodies in phase I trials (CRC, 1986). The results are summarised in the table below.

<table>
<thead>
<tr>
<th>Method</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE</td>
<td>Single band (approx 100kD)</td>
</tr>
<tr>
<td>HPLC</td>
<td>100% purity</td>
</tr>
<tr>
<td>Microbiology</td>
<td>Cultures sterile after 10 days</td>
</tr>
<tr>
<td>Virology</td>
<td>No virus detected on culture</td>
</tr>
<tr>
<td>Mycoplasma</td>
<td>None detected</td>
</tr>
<tr>
<td>Pyrogen testing</td>
<td>Pass</td>
</tr>
<tr>
<td>Toxicity testing</td>
<td>No evidence of haematological or tissue toxicity</td>
</tr>
</tbody>
</table>

Table 8. Summary of purity and toxicity testing results on clinical grade NY3D11 F(ab')2

6.3.4 Immunoreactivity of cold NY3D11 F(ab')2 vs intact NY3D11

The immunoreactivity of 1μg NY3D11 F(ab')2 was compared with 1μg intact NY3D11 in the immunoassay previously described (fig. 51). In molar concentration the amount of intact antibody applied was approximately two thirds of the amount of F(ab')2. The O.D ratio of NY3D11: F(ab')2 between dilutions 1-32 is approximately 0.6. Therefore the immunoreactivity of NY3D11 F(ab')2 is similar to that of the intact antibody.

6.3.5 Immunoreactivity of NY3D11 F(ab')2 after labelling with 125iodine

NY3D11 F(ab')2 (0.1mg) was labelled with sodium 125Iodine (0.6mCi) by the iodogen method. The ratio of labelled-protein:free iodine measured by thin layer chromatography was 99.4%. The immunoreactivity of 125I-F(ab')2 was then compared with unlabelled F(ab')2 in an immunoreactivity
assay (fig. 52). There was no loss of immunoreactivity following radioactive labelling.

**Figure 51.** Comparison of immunoreactivity of 1μg whole NY3D11 with 1μg F(ab')2.

**Figure 52.** Comparison of immunoreactivity of cold and ^125I-labelled NY3D11 F(ab')2.
6.3.6 Stability of $^{125}$I-labelled NY3D11 F(ab)$_2$ in human serum

The stability of $^{125}$I-labelled NY3D11 F(ab)$_2$ in human serum was assessed by mixing the labelled antibody at a ratio of 1:50 with human serum from a healthy donor. The immunoreactivity was compared after incubating the antibody for 1, 3 and 24 hrs in serum (fig. 53). There was no difference in immunoreactivity of the antibody in serum after incubation up to 24 hr. There was a small reduction in immunoreactivity compared to the antibody without serum.

![Figure 53. Immunoreactivity of $^{125}$I-labelled NY3D11 F(ab')$_2$ after incubation in human serum up to 24 hours](image)

6.3.7 Biodistribution study of NY3D11 F(ab')$_2$ and intact NY3D11 in nude mice bearing subcutaneous H69 xenografts.

Immediately prior to this study, a xenograft was examined by immunohistochemistry to confirm immunoreactivity with NY3D11 and F(ab')$_2$ which was positive (fig. 54). NY3D11 F(ab')$_2$ (0.25 mg) was labelled with 0.7 mCi $^{125}$Iodine. Intact clinical grade NY3D11 (185 μg) was labelled with 0.4 mCi $^{125}$Iodine. Thin layer chromatography confirmed that 98% and
99% of radioactivity was protein-bound respectively. On day 0, eight mice received labelled F(ab)$_2$ (21μCi/7.1μg/0.1ml) and eight mice received labelled intact antibody (11.4μCi/5.3μg/0.1ml). The biodistributions at 24 and 72 hours are shown in figs. 55 and 56.

Figure 54. Immunohistochemistry of H69 xenografts with NY3D11 and NY3D11 F(ab')$_2$.
Figure 55 Biodistribution of whole $^{125}$I-NY3D11 and F(ab')$_2$ in nude mice bearing H69 SCLC xenografts (expressed as percentage of injected dose per gram of tissue)
Figure 56. Biodistribution of whole $^{125}$I-NY3D11 and F(ab')2 in nude mice bearing H69 SCLC xenografts (expressed as tissue to blood ratios).
At 24 hour, the percent injected dose g$^{-1}$ tumour of 3D11 vs F(ab')$_2$ was 19.7% vs 7.1%. At 72 hours, the values were 26.7% vs 3.81% respectively. There was no evidence of localisation to bone marrow or liver. The tumour: blood ratios of 3D11 vs F(ab')$_2$ were 1.42 vs 6.18 at 24 hours and 2.54 vs 23.1 at 72 hours.

6.3.8 Clinical studies with NY3D11 F(ab')$_2$ in patients with SCLC

Subject 4 (FH): The first subject was a 68 year old male with a large left-sided tumour. Investigations revealed no evidence of metastatic disease. The patient was injected with 0.5mg F(ab')$_2$ labelled with 114.3 MBq of $^{131}$I by the iodogen method. The labelling efficiency was 99%. Scans were performed at 24, 48, 72 and 168 hours (fig. 57). No evidence of localisation to the tumour was detected. As seen previously with the intact antibody there was some evidence of accumulation in bone marrow (spine and pelvis) although to a lesser extent.

Subject 5: RB was a 48 year old male with a SCLC in the left lower lobe and a metastasis in the liver. There was no evidence of bony metastases.

Figure 57. Gamma camera scans of subject FH at 24 and 72 hours post injection of NY3D11 F(ab')$_2$. 
After labelling by the iodogen method, 1mg of NY3D11 was conjugated to 26 mBq of $^{131}$Iodide. Thin layer chromatography showed a labelling efficiency of 98%. Scans were performed at 24 and 48 hours (not shown). The antibody accumulated in the spine, pelvis and liver with no evidence of localisation to the primary lung tumour or liver metastasis.

Subject 6: GB was a 59 yr old female with extensive SCLC. At entry to the study there was a small left hilar mass, upper mediastinal lymphadenopathy, a large mass in the left supraclavicular fossa and a 2cm lymph node in the right anterior cervical chain. The patient received 1mg NY3D11 F(ab')$_2$ labelled with 2.08mCi $^{131}$Iodide. The labelling efficiency was 98%. None of the tumour sites were imaged but spine, pelvis and liver were seen as previously (fig. 58).

![Gamma camera images of patient GB at 24 and 48 hours post injection of 1mg NY3D11 F(ab')$_2$.](image)

**Figure 58.** Gamma camera images of patient GB at 24 and 48 hours post injection of 1mg NY3D11 F(ab')$_2$.

6.3.9 Clearance of antibody from blood

Antibody was cleared rapidly from subjects 5 and 6 but appeared to clear less rapidly in the first 24 hours from the blood of subject 4 (fig. 59).
6.3.10 Analysis of radioactivity associated with white cell fraction of blood

The proportion of radioactivity associated with white cells in the blood of subject 4 was estimated by separating the white cells from whole blood by Percoll spin and comparing the activity in this fraction with blood. Blood samples were taken at 4 and 24 hours when 5.3% and 4.3% of whole blood radioactivity was associated with the white cells.

6.3.11 Immunoreactivity of the radiolabelled antibody administered to patients

The immunoreactivities of radiolabelled F(ab')₂ fragments administered to patients 5 and 6 were well preserved compared with unlabelled F(ab)₂. (figs. 60 and 61).
Figure 60. Immunoreactivity of $\text{P(ab')}_2$ administered to subject 5

Figure 61. Immunoreactivity of $\text{F(ab')}_2$ administered to subject 6.
6.4 Discussion

This study demonstrated that bromelain digestion of NY3D11 was a rapid and efficient method of producing F(\(ab')_2\) for clinical use. The immunoreactivity of the F(\(ab')_2\) was well preserved compared with whole IgG and the fragment localised well to human SCLC xenografts in nude mice with a good therapeutic ratio. However in patients with SCLC no evidence of tumour localisation was seen and the F(\(ab')_2\) appeared to accumulate in the bone marrow and liver in a similar manner to the whole antibody.

The clearance of the F(\(ab')_2\) from patient serum was similar to that of the whole with the exception of one subject (FH). The clearances of all six subjects are shown below (fig. 62).

![Figure 62. Clearance of antibody from serum of all patients.](image)

Although murine F(\(ab')_2\) fragments are cleared more rapidly from blood than intact IgG in mice, clearance in man has previously been shown to be similar (Lane et al., 1994). In this study, the clearance of intact antibody
and F(ab')₂ fragments in SCLC patients was similar implying that Fc interactions were not an important factor in clearance.

The distribution of intact antibody and F(ab')₂ fragments in tissue was also similar. Thus the hypothesis that the Fc portion of NY3D11 antibody was recognised by the RES resulting in uptake appears to be incorrect. As with intact antibody, the F(ab')₂ fragments failed to localise in the tumours (primary or metastatic) and localised preferentially in the bone marrow and liver. This was a disappointing result as the mouse xenograft studies had suggested that F(ab')₂ localised well to the SCLC tumours. The tumour to blood ratios of the F(ab')₂ were more than 4 and 8 times higher than the respective values for the intact NY3D11 at 24 and 72 hours which would have made it even more favourable for imaging studies.

The reasons why intact NY3D11 did not localise to tumours in patients were discussed in the previous chapter and the same arguments apply to the F(ab')₂ in these studies. The most likely explanation for the observed distribution and failure to localise in tumours is that NY3D11, intact and F(ab')₂, bound to CD56 antigen on circulating NK cells and was taken up by the RES. The results of this study are similar to the only previous study where an anti-NCAM monoclonal antibody was administered to patients with SCLC (Michalides et al., 1994). Four patients were studied with 1mg of ¹³¹I- labelled MAb 123C3. The primary tumour was not imaged in any of the patients but a single liver metastasis was imaged in one subject. No details are given of the localisation pattern of the antibody although the authors commented that no high background due to binding of 123C to NK cells was observed. It has been reported that MAb123C can be internalised by SCLC cells and that this characteristic makes it particularly suitable for delivering radiation to tumour cells (Michalides et al., 1994; Kwa et al., 1996). This is the only anti-NCAM monoclonal that has been reported to internalise. Administration of
radiolabelled MAb123C to SCLC xenografts in mice produces remission of tumours but this has also been demonstrated in several studies with anti-NCAM monoclonals which are not thought to be internalised (see section 1.11.4). Anti-NCAM monoclonals, whether internalised or not, localise well in biodistribution and therapy studies in mouse xenograft models but in the two human studies conducted to date internalisation would not appear to confer any advantage.

Finally it should be considered whether further studies of anti-NCAM monoclonal antibodies for lung cancer imaging and therapy are worthwhile. If the hypothesis is correct that the reason for failure of NY3D11 to localise in tumours was due to binding to NK cells or possibly to circulating serum NCAM then it might be possible to saturate these binding sites with unlabelled NY3D11 prior to administration of the radiolabelled antibody. However the administration of larger amounts of 'cold' NY3D11 might also result in binding of this antibody to the tumour binding sites thereby blocking binding of subsequent radiolabelled antibody. Furthermore the effect of saturating NK cells with an antibody might result in harmful side effects to the patients through immunological mechanisms. Much larger doses of other antibodies up to 100 mg have been administered to patients without apparent ill-effects. No side effects were observed following administration of NY3D11 despite its expression by normal tissues and thus it might have been worthwhile to administer a larger dose of the antibody. However, even if some localisation was observed with a higher antibody dose, NY3D11 would still be unsuitable for therapy in patients due to its rapid distribution into the bone marrow and spleen which would result in unacceptable toxicity.

In conclusion, NY3D11 and its F(ab')2 fragments proved to be unsuitable for further patient studies despite promising results in mouse studies. Xenograft tumours grown in mice may differ from human tumours
in their architecture, blood supply, site of growth and through differing host factors. The need for caution in extrapolating results from animal studies to humans has once again been emphasised.
CHAPTER 7.
General Discussion

7.1 General conclusions and clinical relevance

NCAM is the major immunodominant antigen expressed by SCLC tumours and its potential as a target for antibody-directed therapy was soon recognised. However, despite extensive research into its function as an adhesion molecule in other tissues, the studies in this thesis were the first attempt to investigate the role of NCAM in the biology of SCLC. Monoclonal antibodies, peptides and antisense oligonucleotides were used to block NCAM-NCAM interactions but no effect was observed on formation of POC spheroids or the rate of aggregation of H69 cells in vitro. It was not possible to conclude whether the lack of observed effect was because the interventions were not NCAM function-blockers or that NCAM interactions were not involved. However studies with H69-derived clonal cell lines either rich or deficient in PSA found that the cells with high PSA aggregated less rapidly until PSA was removed with Endo N. This was evidence that PSA which is uniquely associated with NCAM, could influence H69 cell interactions.

It is unlikely that the polysialylated NCAM of SCLC contributes to its highly metastatic nature in vivo. Metastatic tumours tend to have down-regulation of adhesion molecules, but NCAM appears to be expressed strongly by both primary and secondary tumours in SCLC. It is more likely that NCAM expression in SCLC represents the oncophenotype of the tumour indicating a reversion to a more embryonal or primitive cell type in conjunction with the multiple other genetic abnormalities found in SCLC.

The NCAM gene has not previously been transfected into a NSCLC cell line. Although no biological differences were seen between the
parent cell line L23 and the NCAM-expressing L23T, an interesting finding was that the NCAM expressed by the transfectants was polysialylated. This implied that transfection with NCAM switched on the gene for polysialylation or that the RNA message for PSA was already present in the cell. The L23T cell line was sent to R. Gerardy Schahn in Germany to investigate these possibilities but unfortunately became degraded before it could be studied.

The basis for these transfection experiments were observations that NSCLC with NE features might have a different clinical behaviour from non-NE-NSCLC. Specifically it has been suggested that these tumours have 'SCLC-like' features which include chemosensitivity but poor prognosis. There has not been a conclusive clinical study to confirm these observations and at the present time it is not of clinical relevance in the treatment of NSCLC. However, there has recently been renewed interest in the use of chemotherapy to treat metastatic NSCLC and the continuing search for biological markers which may predict outcome or response to treatment is worthwhile.

The antibody NY3D11 appears to be unsuitable for further patient studies but the principle of targeting NCAM in patients with SCLC should not be discarded. The failure of this targeting study may have been partly due to properties of the monoclonal antibody and other anti-NCAM monoclonals might localise more effectively. Many tumour antigens on the surface of SCLC tumours have been identified to which monoclonal antibodies can be directed. Prior to this study, SCLC tumours were successfully imaged patients with radiolabelled antibodies to CEA (Macmillan et al., 1993) and virtually all known disease sites were imaged in SCLC patients after injection of a radiolabelled anti-GD2 ganglioside monoclonal antibody (Grant et al., 1996). The use of antibody directed
therapy in SCLC to destroy microdeposits of tumour cells remaining after chemotherapy remains a logical approach to this disease.

7.2 Future directions

In the future new biological approaches will be used to treat lung cancers. Although new chemotherapeutic agents may become available with improved effects on survival, it is unlikely that these drugs will ultimately produce a cure for lung cancer. The study of genetic changes in tumour cells such as mutation of p53, activation of ras oncogene and the correction of these abnormalities by inserting new genetic material or by antisense technology is likely to be part of future therapies (Woll, 1995). Growth of SCLC tumours is also sensitive to multiple autocrine and paracrine growth loops involving neuropeptides which can be blocked by specific antagonists (Sethi, 1995). Trials of such inhibitors in patients are likely to commence in the near future. Tumour angiogenesis in NSCLC tumours can predict metastatic disease and survival and the development of agents to target tumour vasculature is a new and promising area in cancer therapy (Fontanini, 1995). Other new approaches to cancer therapy include the development of anti-tumour vaccines, improved understanding of the mechanisms of drug resistance, enhancement of host resistance to cytotoxic insult, and genetic activation of prodrugs by tumour-specific promoters. With greater knowledge of the genetic changes, biology and prognostic markers of lung cancer, it should be possible in the future to tailor specific combinations of treatment to patients with lung cancer and improve the outcome for this disease.
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APPENDIX A

Preparation of Hites A

This formulation was kindly provided by Dr M. Seckl, ICRF, London. The following were added to RPMI 3.7%: hydrocortisone 10nM, transferrin 10μg/ml, estradiol 10nM, selenium 30nM and bovine serum albumin 1.25g per 500mls. The solution was filtered through 0.2μM Nalgene and insulin 5μg/ml was added (6mM HCL is required to dissolve insulin).
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H69</td>
<td>Classic SCLC</td>
</tr>
<tr>
<td>POC-1</td>
<td>Spheroid SCLC</td>
</tr>
<tr>
<td>H119-54.2</td>
<td>PSA+ve SCLC (derived from H69)</td>
</tr>
<tr>
<td>H119-45</td>
<td>PSA-ve SCLC (derived from H69)</td>
</tr>
<tr>
<td>L23</td>
<td>Large cell carcinoma</td>
</tr>
<tr>
<td>L23T</td>
<td>L23 transfected with pcDNA3/NCAM</td>
</tr>
<tr>
<td>L23C</td>
<td>L23 transfected with pcDNA3</td>
</tr>
<tr>
<td>MOR</td>
<td>Adenocarcinoma</td>
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</table>

<table>
<thead>
<tr>
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<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NCAM</td>
</tr>
<tr>
<td>Eric-1</td>
<td>NCAM</td>
</tr>
<tr>
<td>NKH-1</td>
<td>NCAM</td>
</tr>
<tr>
<td>735</td>
<td>PSA</td>
</tr>
<tr>
<td>SWA11</td>
<td>CD24</td>
</tr>
</tbody>
</table>

Table. 9 Summary of cell lines and antibodies
Additional discussion on methods of transfection

L23 cells have not previously been transfected and the initial choice of lipofection as the method for transfection was partly a fortuitous one but also based on its simplicity, relatively low cytotoxicity and high efficiency.

Several methods have been developed for introducing plasmid DNA vectors into cultured mammalian cells. The most widely used is transfection mediated by either calcium-phosphate or DEAE dextran. The calcium-phosphate method was first described by Graham and van der Eb (1973) who established the optimum conditions for the formation of calcium phosphate-DNA coprecipitates and reaction times for exposure of the cells to the precipitate. It is thought that transfected DNA enters the cell cytoplasm by endocytosis and is transferred to the nucleus. This method is highly efficient (up to 20% of cultured cells may be transfected at one time) and can be used to establish cell lines with both transient expression of foreign DNA and lines which carry integrated copies of foreign DNA.

The DEAE-dextran method was first described in 1965 by Vaheri and Pagano for the introduction of poliovirus into cells. It has subsequently been used for transfection of viral genomes and plasmids carrying viral sequences. Its mechanism of action may involve binding to DNA and inhibiting the action of nucleases and/or promoting endocytosis of the DNA. This method can only be used for transient expression of cloned genes and not for stable transformation. It works well with some cell lines e.g. COS, CV-1 but poorly with others possibly due to toxicity.

Electroporation is the use of high-voltage electric pulses to create micropores in the plasma membrane which allow DNA into the cells (Neuman et al., 1982). The procedure may be used for both transient expression and stable transformation. The advantage of electroporation is that it works well with cell lines refractive to other techniques. The
disadvantage is that transfection efficiencies vary widely and different conditions must be used for different cell lines. Transfection efficiency may be influenced by the strength of the electric field, the length of the electric pulse, temperature, conformation and concentration of DNA and the ionic composition of the medium. Considerable work may be needed to establish optimal conditions for a particular cell line and conditions established in one laboratory may not work well in another.

Transfection of cells using artificial membrane vesicles (liposomes) has become increasingly popular over the past decade. Liposomes interact spontaneously with DNA to form complexes which adhere to the cell surface, fuse with membrane and release DNA into the cytoplasm. The advantage of this method is that it is gentle and can be highly efficient. It is also relatively simple compared with the other methods and was chosen in this study for these reasons. Several lipofection reagents are commercially available and lipofectAMINE was chosen as the most effective on the basis of unpublished data from a BSc. project by R.Trivedi at UMDS comparing the efficiency of different formulations for transfection of primary muscle cultures.
Biodistribution of a radioiabelled monoclonal antibody NY3D11 recognizing the neural cell adhesion molecule in tumour xenografts and patients with small-cell lung cancer

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Summary The neural cell adhesion molecule (NCAM) is highly expressed on the surface of small-cell-lung cancer (SCLC) cells. We have produced a monoclonal antibody, NY3D11, that binds to NCAM to investigate whether this antigen could be used to develop antibody-directed therapy for SCLC. ¹²¹I-labelled IgG and F(ab')₂ fragments of NY3D11 localized selectively in human SCLC xenografts grown in nude mice. The human biodistribution of ¹²¹I-labelled NY3D11 after intravenous administration was investigated by gamma-camera imaging in six patients with SCLC. Three patients received IgG and three received F(ab')₂. No evidence of localization to primary tumours or metastases was seen and antibody accumulated rapidly in the liver and bone marrow. The probable explanation for this distribution is that NY3D11 reacted with soluble NCAM or natural killer cells that possess the CD56 (NCAM) antigen.

Keywords: small-cell lung cancer; neural cell adhesion molecule; monoclonal antibody; biodistribution

The mortality of patients with small-cell lung cancer (SCLC) remains greater than 90% at 2 years after diagnosis and new therapeutic approaches are urgently needed to improve the outcome of the disease (Souhami et al, 1990). The tumour is characterized by its initial responsiveness to chemotherapy, producing complete remission in about 50% of patients and early relapse. Eradication of persistent micrometastases at the end of chemotherapy would prevent the development of chemo-resistant tumour relapse. Antibody-directed therapy is one strategy used to target therapy specifically to the tumour site. Monoclonal antibodies recognizing tumour-associated surface antigens have been shown to localize specifically in many different tumour types. Therapy is most likely to be effective in small tumour-foci when the total tumour burden is low as antibody uptake is most efficient (Pedley et al, 1987; Olabiran et al, 1994). Adjuvant antibody therapy in patients with colorectal cancer has produced encouraging results (Reithmüller et al, 1994) and antibody-directed therapy of micrometastases of SCLC in the adjuvant setting could also be an effective new treatment.

The neural cell adhesion molecule (NCAM) is strongly expressed on the surface of SCLC cells (Souhami et al, 1991; Rygaard et al, 1992). Anti-NCAM monoclonal antibodies localize to SCLC xenografts in nude mice and produce regression of tumours when conjugated to a therapeutic dose of radioisotope (Boerman et al, 1991; Hosono et al, 1994). NCAM is also expressed by normal tissues including neural tissue, muscle, thyroid epithelium, testicular Leydig cells and natural killer (NK) cells in blood. It exists in membrane-associated forms and a soluble form that can be detected in the serum of patients with SCLC (Jaques et al, 1993). Serum NCAM levels are raised in patients with active disease and in relapse, suggesting that this antigen could be a useful target for therapy of micrometastases (Ledermann et al, 1994).

We have produced a murine monoclonal antibody, NY3D11, against the NCAM molecule on SCLC. The purpose of this study was to investigate the biodistribution of radioiabelled NY3D11 whole immunoglobulin and its F(ab')₂ fragments in nude mice bearing SCLC xenografts and to perform a localization study in patients with SCLC.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody NY3D11 was raised after immunization of RBS/DNJ mice (Robertsonian 8:12 translocation) with SCLC cell line UCH10. Spleen cells were fused with the Fox NY, NS1 variant myeloma line. For details see Olabiran et al. (1994). In common with most other anti-NCAM monoclonal antibodies, NY3D11 binds to immunodominant epitopes located in the 'stem region' C-terminal of the fifth Ig-like domain of the NCAM molecule (Gerardy-Schahn et al, 1994). The antibody for patient administration was purified by affinity chromatography on protein A-Sepharose from tissue culture supernatant (kindly produced by Celltech) and tested before clinical use according to guidelines in the Operation Manual for the Control of Production, Preclinical Toxicity and Phase I Trials of Anti-tumour Antibodies and Drug–Antibody Conjugates (1986).
For animal studies IgG and F(ab')2 fragments of NY3D11 were prepared from whole antibody by bromelain digestion (Mariani et al, 1991). In brief, freshly activated and desalted bromelain was added to the antibody in 0.1 M sodium acetate/3 mM EDTA pH 5.5 at a ratio of 1:50 (bromelain-antibody). The mixture was incubated for 1 h at 37°C, cooled to 4°C and a small amount of 0.1 M sodium hydroxide added to bring the pH to 6.0. The antibody/bromelain reaction mixture was immediately applied to a SP Sepharose column that was washed with 0.5 M sodium acetate/1.0 sodium chloride, pH 6.0 to separate the F(ab')2 fragments. F(ab')2 was then passed down a protein A column to remove residual whole IgG. The purity of the F(ab')2 product was assessed using sodium dodecyl sulphate polyacrylamide gel and high performance liquid chromatography before patient administration.

For animal studies IgG and F(ab')2, fragments of NY3D11 were labelled with 125I by the iodogen method to a specific activity of approximately 1.1 MBq mg⁻¹ protein. For patient studies, 0.5–1.0 mg NY3D11 were labelled with 74 MBq of 125I initially by the chloramine-T method (two patients) and subsequently by the iodogen method.

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Radiolabelling

For animal studies IgG and F(ab')2, fragments of NY3D11 were labelled with 125I by the iodogen method to a specific activity of approximately 1.1 MBq mg⁻¹ protein. For patient studies, 0.5–1.0 mg NY3D11 were labelled with 74 MBq of 125I initially by the chloramine-T method (two patients) and subsequently by the iodogen method.

In vitro studies of radiolabelled NY3D11

Immunoreactivity assay

Immunoreactivity of the labelled antibody with glutathione-S-transferase (GST) NCAM fusion protein (kindly provided by Professor Frank Walsh, UMDS, London, UK) was tested by enzyme immunoassay. The GST NCAM fusion protein contained a peptide sequence from full length human NCAM previously shown to bind anti-NCAM antibodies. A 96-well plate was coated with 100 μl of NCAM fusion protein 5 μg ml⁻¹ in carbonate buffer for 2 h at 37°C. The plate was washed three times with phosphate-buffered saline/Tween. Non-specific binding was blocked by adding 150 μl of 3% bovine serum albumin (BSA)/PBS to each well for 2 h at 37°C. The plate was washed again three times with PBS/Tween. An aliquot (200 μl) PBS was added to the first well of the row and 100 μl of PBS to the remaining wells. Antibody (1 μg) was added to the first well (to give a concentration of 5 μg ml⁻¹). A serial 1:2 dilution was made across the rows and the plate was incubated at 37°C for 1 h. After washing three times with PBS/Tween, 100 μl of alkaline phosphatase-conjugated anti-mouse IgG was added to each well. The plate was incubated at 37°C for 1 h and washed three times with PBS/Tween. Finally, 100 μl of fresh p-nitrophenyl phosphate substrate was added to each well and colour was allowed to develop for 1 h at 37°C. The absorbance of each well was read on a plate reader at 410 nm. Experiments were performed in duplicate on the same plate. The immunoreactivity of radiolabelled antibody was compared with that of unlabelled antibody.

Aggregate analysis by gel filtration on S300 column

The radiolabelled antibody was diluted 1:50 in PBS/azide and loaded onto a Sepharose 300 column. The activity of aliquots of each fraction collected was measured on a gamma-counter.

FACS analysis

Staining of NK cells with NY3D11 was investigated using double immunofluorescence on a FACSScan (Beckton Dickinson, UK) and three different markers of NK cells, CD8 (RFT8 Royal Free Hospital, Department of Immunology), CD16 (Leu 11B, kind gift from Professor J Thompson, Kentucky University, USA.) and CD57 (HNK1). Blood was obtained from two healthy donors. A commercial monoclonal antibody for NCAM, NKH1:CD56, (Coulter, UK) was used as a positive control.

Animal studies

Establishment of xenografts in nude mice

The human small-cell lung cancer cell line UCH10 or H69 was used to establish a xenograft model subcutaneously in the flanks of nude mice. The mice were female, 2-3 months old and weighed between 20 and 25 g. Subsequent passaging of tumours was carried out by subcutaneous implantation of small tumour pieces (approximately 1 mm3). The biodistribution study was commenced when the tumours reached a size of approximately 1.0 cm3. Immediately before the study, a xenograft tumour was examined for antigen expression by immunohistochemistry and binding of NY3D11 antibody and its F(ab')2 fragments was confirmed.

Immunohistochemistry

Immunohistochemical reactivity of NY3D11 with bone marrow and xenograft tissue was assessed using an avidin–biotin peroxidase technique. Xenograft tissue was snap frozen in isopentane, cooled in liquid nitrogen and 6 μm cryostat sections were cut. Bone marrow smears and cryostat sections were fixed before immunohistochemistry with acetone for 10 min.

Biodistribution study

Experiments to confirm specific localization of antibody in SCLC xenografts were performed with radiolabelled NY3D11 and the control antibody 4120, an IgG1 anti-human CD4 (a gift from...
Biodistribution of anti-NCAM MAb NY3D1

Professor Beverely, ICRF, London, UK). Two groups of 12 mice bearing UCH10 xenografts received 10 µg of radioiodinated NY3D11 or 4120 by injection into the tail vein. Animals were given food and water ad libitum. The water contained 0.1% potassium iodide to prevent thyroid uptake of iodide. Groups of four mice were bled and killed at 24, 48 and 72 h. Tissues were removed and placed into preweighed tubes filled with 7 M potassium hydroxide. When the tissues had dissolved, samples were counted on a gamma counter (Pharmacia, 1470 Wizard). The mean percentage uptake of the injected dose per gram of tissue (%ID g⁻¹) was determined and the tumour–non-tumour ratio was expressed as the radioimmunolocalization index (RI), calculated for each tissue according to the formula:

\[ RI = \frac{\%ID \text{ g}^{-1}\text{ of NY3D11 in tissue} \times \%ID \text{ g}^{-1}\text{ of NY3D11 in blood}}{\%ID \text{ g}^{-1}\text{ of 4120 in tissue} \times \%ID \text{ g}^{-1}\text{ of 4120 in blood}} \]

In experiments to compare the biodistribution of radioiodinated NY3D11 IgG and F(\(\text{ab}\)')₂, two groups of eight mice bearing H69 xenografts received IgG (5.3 µg) or F(\(\text{ab}\)')₂ (7.1 µg). At 24 h and 72 h, four mice from each group were bled and the following tissues removed for gamma radiation counting: liver, tumour, spleen, rectum, kidney, lung and muscle. Femur was taken from two mice in each group at both time points. Tissues were processed and analysed as described above.

**Patient studies**

**Patient eligibility**

Patients with SCLC, newly diagnosed or undergoing treatment, age 16–80 years, and WHO performance status 0–3 were eligible for the study. Patients with a history of allergy to iodine or immunoglobulins or a positive skin test to intradermal administration of antibody were excluded. Written informed consent was obtained from participants and the study was approved by the ethics committees of participating centres.

**Preliminary investigations**

Chest radiography, bone scanning and ultrasonography of the liver were used to assess tumour site(s). Full blood count, urea, electrolytes, creatinine, liver function tests and thyroid function tests were performed before antibody injection.

**Administration of antibody**

To prevent accumulation of radioactivity in the thyroid gland, patients received potassium iodate 85 mg t.d.s. for 7 days commencing 24 h before administration of radioiodinated antibody. Before the intravenous injection, patients received an intradermal injection in the forearm of 0.1 ml (1–2 µg) of radioiodinated antibody. At an adjacent site 0.1 ml of 0.9% saline was injected intradermally and the skin reaction at both injection sites was compared after 15 min. If erythema at the test site was greater than at the control site, intravenous administration of antibody did not proceed. The antibody (0.5–1 mg) was injected intravenously over 5 min.

**Study parameters**

**Gamma camera imaging**

Patients were scanned at 24, 48 and 72 h after injection of antibody. Scanning was performed using a large field of view gamma-camera with a high-energy parallel-hole collimator and an on-line computer.

**Venous blood sampling**

To determine the elimination of antibody from the circulation, 5 ml of venous blood were withdrawn immediately after injection (from the contralateral arm) and at 24, 48 and 72 h. Blood was stored in
preweighed EDTA bottles and activity was measured after several weeks when sufficient decay allowed accurate counting.

RESULTS

Biodistribution studies in mice

Tumour localization of NY3D11

Specific uptake of \[^{[125]}\]NY3D11 was seen in nude mice bearing UCH10 xenograft tumours compared with the uptake of the non-specific antibody 4120. Maximum tumour localization occurred at 48 h after intravenous injection. The mean uptake of radioactivity in tumour at this time was 15.9% ID g\(^{-1}\) (s.d. 2.46) for NY3D11 and 5.65% ID g\(^{-1}\) (s.d. 1.17) for 4120. Prolonged retention of radioactivity in the tumour was seen; 13.7% ID g\(^{-1}\) (s.d. 3.42) of NY3D11, five times the amount of non-specific antibody remained at 7 days. The radiolocalization index (Figure 1), which measures specific accumulation of NY3D11 in the tumour, increased from 1.8 at 24 h after injection to 3.7 at 7 days. For normal tissues this value ranged from 0.7 to 1.0 at 24 h and from 0.4 to 1.0 at 7 days.

Biodistribution of whole IgG vs F(ab\(^\prime\))\(_2\)

The biodistribution of radiolabelled NY3D11 IgG and F(ab\(^\prime\))\(_2\) at 24 and 72 h are shown in Figures 2 and 3. At 24 h, the % ID g\(^{-1}\) tumour of IgG vs F(ab\(^\prime\))\(_2\) was 19.7% vs 7.1% and at 72 h 26.7% vs 3.81% respectively. There was no evidence of localization to bone marrow or liver. These results show that a much greater proportion of injected IgG was taken up and retained by the tumour xenografts compared with the F(ab\(^\prime\))\(_2\) fragments. However, despite the smaller absolute uptake of F(ab\(^\prime\))\(_2\) fragments into tumour, the tumour-blood ratios of IgG vs F(ab\(^\prime\))\(_2\), at 24 and 72 h were 1.42 vs 6.18 and 2.54 vs 23.1.

Patient studies

The patient characteristics are summarized in Table 1. There were no positive skin reactions and no immediate or delayed adverse effects after injection of the antibody. No haematological or biochemical toxicity was observed. The first three subjects were studied with NY3D11 IgG after mouse studies indicated that uptake of IgG in tumour xenografts was higher than for F(ab\(^\prime\))\(_2\). No antibody localization was detected in primary tumours or metastases but accumulation in the bone marrow (spine and pelvis) and liver was observed in all subjects. The spleen was visualized in one patient (subject 3). The mouse studies had shown that F(ab\(^\prime\))\(_2\) might be more favourable for imaging (high tumour:blood ratio) and three further subjects were studied with NY3D11 F(ab\(^\prime\))\(_2\). The results were similar to those of IgG. No primary tumours or metastases were imaged but antibody again accumulated in bone marrow and liver. First-phase clearance of radiolabelled antibody from blood was rapid with less than 25% of initial activity remaining at 24 h (Figure 4). Whole antibody and F(ab\(^\prime\))\(_2\) were cleared at similar rates except in one patient (subject 4).

In vitro antibody studies

Aggregation

Antibody showed no evidence of aggregation in vitro either before or after radiolabelling. Incubation of radiolabelled antibody with a patient serum or previously stored serum with high soluble NCAM did not produce aggregation.

Immunoreactivity

After labelling using the chloramine-T method (subjects 1 and 2), comparison of the immunoreactivity of cold NY3D11 with
Biodistribution of anti-NCAM MAb NY3D11 in SCLC

Table 1 Patient characteristics and antibody received

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Primary</th>
<th>Metastases</th>
<th>Antibody</th>
<th>Dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>Right hilum</td>
<td>–</td>
<td>Whole Ig</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>Right lung (diffuse)</td>
<td>–</td>
<td>Whole Ig</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>Left hilum</td>
<td>Bone</td>
<td>Whole Ig</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>Mass left lung</td>
<td>–</td>
<td>F(ab')</td>
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</tr>
<tr>
<td>5</td>
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<td>Left hilar mass</td>
<td>Liver</td>
<td>F(ab')</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>Left hilar mass</td>
<td>Supraclavicular mass</td>
<td>F(ab')</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 4 Clearance of radiolabelled antibody from blood after injection at 0 hrs. ○, patient 1; □, patient 2; ●, patient 3; △, patient 4; ⬤, patient 5; ●, patient 6

Radiolabelled NY3D11 demonstrated approximately 50% loss of immunoreactivity. When the iodogen method was used for subjects 3–6, there was only a small reduction of immunoreactivity after radiolabelling.

Immunohistochemistry of SCLC xenografts and normal bone marrow
Immunohistochemistry of SCLC xenografts removed immediately before biodistribution studies confirmed that NY3D11 whole IgG and its F(ab')_2 fragments reacted with the tumour. After studies in humans demonstrated localization in bone marrow, immunohistochemistry on ten normal bone marrow samples was performed to investigate whether this distribution could be explained by reaction with a cellular element in bone marrow. These studies showed no evidence of antibody binding to normal bone marrow cells.

FACS analysis
The H69 SCLC cell line bound at least six to seven times as much NKH1 as NY3D11, despite the use of NY3D11 over a concentration range of 1–100 μg ml\(^{-1}\). Similarly, the percentage of CD8, CD16 or CD57 positive cells (NK cells) that stained with NY3D11 or NKH1 was always much less for NY3D11 than NKH1 (approximately 40% and 90% respectively) and the mean fluorescence intensity (MFI) of NKH1 on NK cells was six- to sevenfold greater than that of NY3D11. However, the MFI values suggested that NK cells could still bind large amounts of NY3D11.

DISCUSSION
These studies demonstrate that NY3D11 localized well to SCLC xenografts in mice but not to SCLC tumours in patients in whom it was cleared rapidly from blood and accumulated in bone marrow and liver. A previous study in patients with SCLC using the NCAM monoclonal MAb 123C also found no localization to primary tumours, although a single liver metastasis was detected (Michalides et al, 1994). This negative result occurred despite immunoscintigraphy studies in mice that suggest that MAb 123C has superior localization compared with other cluster-1 antibodies because of its internalization (Kwa et al, 1996). However, it is clear that other anti-NCAM antibodies that do not internalize can also localize well in mouse studies (Boerman et al, 1991; Waibel et al, 1993; Hosono et al, 1994) and the present study further highlights that successful localization of antibodies in mice is not necessarily reproduced in human studies. The reasons for this disparity between mice and man need to be understood for the successful future development of anti-NCAM antibodies.

The reticuloendothelial system (RES) can recognize and remove foreign particles from the circulation. Some antibody binding and uptake may occur through non-specific Fc receptor interactions. Blood clearance is also a function of size of the antibody. Murine F(ab')\(_2\) fragments are cleared more rapidly from blood than intact IgG in mice but clearance in man is similar (Lane et al, 1994). We observed a similar blood distribution of intact antibody and F(ab')\(_2\) fragments in patients implying that Fc interactions were not an important factor affecting blood clearance. Aggregation of antibody could also explain the observed distribution but there was no evidence from gel chromatography that aggregation had occurred during storage or after radiolabelling. The possibility that antibody combined with soluble NCAM in serum to form aggregates was also considered but no aggregation was seen after incubation with patient serum or serum containing high levels of soluble NCAM. Although Sepharose chromatography did not suggest binding to soluble NCAM, low-affinity antibody may occasionally not survive column chromatography. NY3D11 appears to have a relatively low affinity for NCAM and therefore aggregation cannot be completely excluded.

Gamma-camera scanning demonstrated accumulation in spine, pelvis and ribs that suggested that antibody was localizing in the bone marrow. However, no binding of antibody was seen in any of the normal bone marrow aspirates examined by immunohistochemistry. It is known that natural killer (NK) cells express...
NCAM (CD56) (Hida et al., 1991). When an epitope is present at low levels or affinity for the epitope is weak, immunohistochemistry may not be sufficiently sensitive to detect binding. NK cells comprise a very small percentage of bone marrow cells and this may explain why they were not detected by immunohistochemistry. FACS analysis subsequently showed that NY3D11 was six to seven times less immunoreactive than NKH1 on control H69 cells and NK cells. NY3D11 and NKH1 recognize similar epitopes on NCAM (Gerardy-Schahn et al., 1994). Despite this relatively low immunoreactivity, the mean fluorescence intensity values recorded in the FACS studies suggested that NK cells might still bind large amounts of NY3D11. Injected antibody may therefore have bound to circulating NK cells but excess localization to spleen would be expected and this was observed in only one patient in this study.

The importance of immunoreactivity of an antibody with its epitope for tumour localization remains uncertain. Some authors suggest that antibody uptake and penetration into tumours is enhanced by high affinity radioimmunoconjugates (Sclhom et al., 1992), whereas others propose that interaction of high-affinity antibodies at the surface of a tumour prevents penetration (Fujimori et al., 1989). Radioactive labelling of NY3D11 by the chloramine-T method led to a 50% loss of immunoreactivity compared with unlabelled antibody. After the labelling technique was changed to the iodogen method, the reduction in immunoreactivity was small. However, improvement in immunoreactivity failed to enhance tumour localization of antibody.

The accessibility of epitopes on tumour cells is also likely to be important for successful antibody localization (Perez et al., 1988). The limited success of radioimmunotherapy and wide variations in antibody localization amongst patients with cancers of the same histology has been attributed in part to heterogeneity of antigen distribution (Edwards, 1985). The distribution of NCAM expression in SCLC tumours in vivo has not been studied as it is difficult to obtain tumour samples, but accessibility of NCAM epitope to circulating antibodies in patients may be restricted. Antigens in vivo may be expressed at preferential sites (Perez et al., 1989) and the basa1 lamina may represent a physical barrier to extravasated antibodies (Dvorak et al., 1991). In colorectal adenocarcinomas, CEA epitopes expressed on the luminal surface of malignant acini or in the cytoplasm may be inaccessible to antibodies in vivo, whereas epitopes on the basal or basolateral aspects of glandular structures are more readily accessible (Boxer et al., 1994). However, it has been shown that CEA epitopes on lung cancer are accessible to circulating antibodies. When CEA was targeted in 21 patients with active SCLC, tumour was successfully imaged in 13 patients (62%) and 18 out of 38 known disease sites were imaged (Macmillan et al., 1993). This antigen is a promising target for further studies. The epithelial glycoprotein EGP-2 on SCLC has also been successfully targeted in patients (Kosterink et al., 1995). Six patients with SCLC were studied with indium-labelled monoclonal antibody MOC-31, which was identified as a cluster 2 antibody at the First International Workshop on SCLC antigens. Scintigraphy detected primary tumour or metastases in five patients and further studies are indicated.

In summary, reaction with soluble NCAM or circulating NK cells was the most likely reason for the failure of NY3D11 to localize to tumour in these studies. NCAM does not now appear to be a suitable target for antibody directed therapy in SCLC but the lung cancer antigen workshops have identified other surface antigens which should be investigated.

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The importance of complete excision in the prevention of local recurrence of ductal carcinoma in situ

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Summary Mastectomy probably represents over-treatment for the majority of women with screen detected ductal carcinoma in situ (DCIS) and breast-conserving surgery is now widely advocated. In this study, biopsy cavity shavings were used to ensure complete excision in 129 women undergoing breast-conserving surgery for screen detected DCIS. A margin was considered clear if DCIS was > 1 mm from any margin of excision and shavings were clear. Patients with involved margins (DCIS at resection margin) underwent re-excision, irrespective of shaving status. After re-excision, 101 women (78%) had clear margins and 28 (22%) close margins (DCIS ≤ 1 mm from resection margin). Cavity shavings were histologically clear of DCIS in all cases. Ipsilateral DCIS recurrence occurred in 12 (9.3%) patients. Two recurrences also contained invasive carcinoma. The median time to diagnosis was 14 months and all recurrences occurred at the site of the previous biopsy. Seven recurrences were detected at the first annual mammogram, four at the second and one at the third. Ipsilateral recurrence was related to margin status; only 2 out of 101 (2%) patients with clear margins recurred, compared with 10 out of 28 (36%) patients with close margins. Local recurrence and close margin status both correlated with a high modified Van Nuys prognostic index score. Our results indicate that local relapse represents residual DCIS rather than true recurrence in the majority of cases. Cavity shavings have proved ineffective in ensuring complete excision. We now ensure a minimum 10 mm margin of excision around all screen-detected DCIS lesions.

Keywords: Ductal carcinoma in situ; margin status; local recurrence

Ductal carcinoma in situ (DCIS) has become clinically important only since the advent of routine high-quality mammography, and now accounts for 20–25% of screen-detected breast malignancies (Verbeek et al, 1984). The majority of screen detected DCIS lesions, however, are asymptomatic and impalpable (Gump et al, 1987).

Despite the increase in diagnosis, the optimal surgical treatment for DCIS remains controversial. Until recently, DCIS was not differentiated from invasive breast carcinoma and was treated by mastectomy (Price et al, 1989). Since the widespread acceptance of breast-conserving surgery for early invasive breast cancer however, mastectomy is becoming more difficult to justify for localized screen-detected DCIS.

The main purpose of breast-conserving surgery for invasive or in situ disease is complete excision of the tumour (both macroscopically and microscopically) with a surrounding margin of normal tissue to prevent local recurrence, while maintaining a cosmetically acceptable breast. Unfortunately, there is no regular consensus regarding the definition of complete excision or of an adequate margin of excision. It is clear that the margin of clearance around an invasive tumour correlates with local control rates, with positive resection margins being associated with an increased risk of local recurrence (Veronesi et al, 1990). However, the volume of excised tissue is inversely proportional to the cosmetic outcome (Wazer et al, 1992).

Recurrence rates after local excision of DCIS vary widely among different studies and may reflect the type of surgery, adequacy of excision margins, DCIS pathology and patient selection criteria in each study. It is generally agreed that after local surgery up to 30% of women with DCIS will have recurrent lesions within 15 years. but, more importantly, up to 50% of patients will have invasive breast carcinoma on recurrence (Price et al, 1989).

Inadequate excision of the primary lesion appears to be one of the most important causes of local failure after breast-conserving surgery (Silverstein et al, 1994), and new prognostic index for DCIS has been proposed recently that includes resection margins as one of its predictive factors (Silverstein et al, 1996). The Van Nuys prognostic index (VNPI) also quantifies two other predictors of local recurrence, namely DCIS size and pathological classification. A numerical system is used to predict patients more likely to recur after breast conserving surgery.

The histological evaluation of excision margins is now known to be a critical part of the assessment of any patient with DCIS being considered for breast-conserving treatment, and various techniques have been used to improve the accuracy, including inking of specimen margins, two-dimensional radiography, cavity shavings and tumour bed biopsies. Biopsy cavity shavings are routinely used in our unit after wide local excision of invasive carcinomas to reduce the incidence of re-excision in patients with the tumour extending close to the main specimen margin.

The aim of this study was to determine the effectiveness of using biopsy cavity shavings to ensure complete excision of screen-detected DCIS lesions.

PATIENTS AND METHODS

Screening mammography is performed at the Nightingale Breast Screening Centre, University Hospital of South Manchester. Patients with mammographic evidence of malignant microcalcification or a