EXPLOITING RADIOSENSITIVITY IN THE MODERN MANAGEMENT OF NEUROBLASTOMA

Thesis submitted for the candidature for the degree of Doctor of Medicine in The University of Glasgow 1997.

The work included in this manuscript is entirely original and my own unless otherwise stated.

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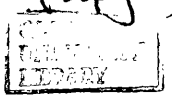

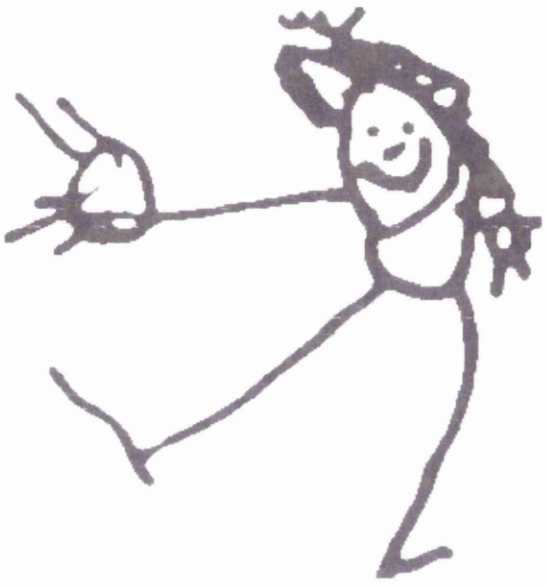

For Nicola Age 4

ABSTRACT

Neuroblastoma is a biologically diverse and clinically challenging tumour. At one end of the clinical spectrum, children with localised, low risk disease, can survive with little or no therapy. Spontaneous resolution of this tumour has been seen and children have survived despite residual macroscopic tumour remaining at the end of therapy.

In contrast, the majority of children present with widespread metastatic disease and are rarely curable. Paradoxically this is an aggressive form of disease despite appearing sensitive to both chemotherapy and radiotherapy. The majority of these patients relapse and only 20% survive 2 years.

This thesis is concerned only with the management of stage 4 patients and concentrates on 3 main areas.

Firstly, the role of control of the primary tumour was considered, in this essentially systemic disease. It was shown that complete surgical resection of the primary reduces local relapse and improves survival. Prognostic factors for stage 4 patients were examined during this analysis and the identification of prognostic subgroups was possible.

Secondly a retrospective analysis of all stage 4 patients treated within this centre was completed, to determine the usefulness of external beam radiotherapy in the palliation of advanced disease.

The final part of this thesis is experimentally based. Factors that may improve the clinical effectiveness of ^{131}I-meta-iodobenzylguanidine (^{131}I-mIBG) were investigated. This molecule is a targeting agent, which, when administered systemically, is selectively accumulated by tumours of neural crest origin. In clinical practice the individual tumour uptake can be variable and the optimum timing of administration is still undetermined.

Initially a new formulation of ^{131}I-mIBG was investigated. This 'no carrier added' (nca) formulation meant that smaller quantities of drug could be administered and tumour specific accumulation increased. Before clinical studies could be contemplated, laboratory investigations had to be completed, to determine if this new preparation behaved similarly to the traditional formulation. The work documented in this thesis confirms this is the case.

Another aim of this thesis was to examine biological factors that may modulate specific tumour accumulation of this agent. The effect of pre-dosing neuroblastoma cells in culture, with chemotherapy agents, resulted in a 2-5 fold increase in specific type 1 tumour accumulation. This may be a very significant finding for the future administration of ^{131}I-mIBG in combination therapy regimens. Unfortunately the combination of elevated temperature and ^{131}I-mIBG exposure resulted in decreased tumour accumulation of ^{131}I-mIBG.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α	alpha
APUD	amine precursor uptake and decarboxylation
ATP	adenosine tri-phosphate
β	beta
BOC	Beatson Oncology Center
CCSG	Children's Cancer Study Group
cGy	centigray
CI	confidence interval
CNS	central nervous system
CR	complete response
CRC	Cancer Research Campaign
CT	computerised tomography
CUSA	cavitron ultrasonic aspirator
cTNM	clinical TNM classification
D	mean inactivation dose
dfs	disease free survival
DA	dopamine
DM	double minutes
DNA	deoxyribonucleic acid
ed(s)	editor(s)
EM	electron microscopy
ENSG	European Neuroblastoma Study Group

EGF	epidermal growth factor
et al.,	et alia
GF	growth factor
Gy	Gray
Ha-ras	Harvey ras oncogene
HVA	homovanillic acid
i.v.	intravenous
in situ	in situation
I	iodine
INNS	International Neuroblastoma Staging System
INRC	International Neuroblastoma Response Criteria
K	potassium
LOH	loss of heterozygosity
M	metastases
MAO	monoamine oxidase
mdr	multiple drug resistance gene
mm	millimeter
mg	milligram
ml	milliliter
mIBG	meta-iodobenzylguanidine
MKI	mitoses karyorrhexsis index
mM	millimolar
MR	mixed response
MRP	multidrug resistance associated protein
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
N	nodes
ng	nanogram
Na	sodium
n.c.a.	no carrier added
NED	no evaluable disease
NGF	nerve growth factor

N-*myc*	nuclear N-myc oncogene
NSE	neurone specific enolase
NR	no response
OPEC	vincristine, cisplatin, etoposide, cyclophosphamide
PD	progressive disease
PCR	polymerase chain reaction
POG	Paediatric Oncology Group
PR	partial response
pTNM	pathological TNM classification
RB1	retinoblastoma gene
RHSC	Royal Hospital for Sick Children
RNA	ribonucleic acid
RT	radiotherapy
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SF_2	surviving fraction at 2 Gray
SJCRH	St. Jude Children's Research Hospital
T	tumour
TBI	total body irradiation
Tc	technetium
TNM	tumour, nodes, metastases
trk	tyrosine receptor kinase
UICC	Union Internationale Contre le Cancer
UK	United Kingdom
UKCCSG	United Kingdom Children's Cancer Study Group
VGPR	very good partial response
VMA	vanillyl mandelic acid

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CHAPTER 1 NEUROBLASTOMA

1.1. HISTORY

1.1.1. Earliest Reports

The first description of neuroblastoma was believed to be by Virchow (1846) and he suggested the tumour was of glial origin.

By 1879, Morgan documented a further case describing a baby with a hard lump over the left acromion. Within days, the child developed multiple bone and subcutaneous deposits. Thereafter, the child rapidly became unwell with increasing abdominal distention and died at the age of only seven weeks. At post mortem tumour affected multiple sites including the left adrenal, kidneys, liver and heart. Morgan believed the primary tumour to be the original subperiosteal deposit that he had observed, but the histopathological description of the small round cells, suggests that this was in fact a primary adrenal neuroblastoma with multiple metastases.

Six years later Dalton (1885) published a description of a baby with abdominal distention who was otherwise well at birth. The child's abdomen continued to enlarge until death. Post mortem examination revealed a small left adrenal tumour 'the size of a hen's egg' and a liver almost completely replaced by tumour. Dalton postulated that the adrenal was in fact the primary and the liver involvement was a secondary phenomenon.

Dr. William Pepper (1901), from The University of Pennsylvania, described a similar case of a female infant who died aged six and a half weeks, with an abdominal mass and a right adrenal tumour. On reviewing the literature and by identifying five similar cases, he established the constellation of disease now described in The International Neuroblastoma Staging System (INSS) as Stage 4S disease, although he believed he was dealing with a lymphosarcoma of the adrenal gland.

Hutchison (1907), from The London Hospital of Sick Children, reviewed a series of ten older children, seven his own cases. These children had multiple sites of disease, especially osseous metastases. In retrospect, this is the first collection of patients corresponding to INSS Stage 4 disease. This author however also misdiagnosed the disease as sarcomatous.

Finally Homer-Wright (1910) observed the characteristic histopathological rosette-like arrangement of neuroblastoma cells (now named the Homer-Wright rosette) and concluded that the tumour was neural in origin. He reviewed several cases, including those of Pepper and Hutchison, and observed that despite these widely differing clinical entities, they were in fact the same histopathological disease. He named it neuroblastoma.

1.1.2. Historical Review of Treatment

During the 1920's neuroblastoma was perceived as a hopeless condition and treatment of any kind was rarely attempted. Blacklock confirmed this,

having reviewed 136 published cases, all of whom died within a few months (Blacklock 1934).

More encouragingly, Cushing and Woolbach (1927) first observed the phenomenon of spontaneous differentiation of neuroblastoma. Less than ten years later, Ladd and Gross noted a favourable survival rate in patients with liver involvement and localised disease (Ladd and Gross 1941). Lehman, provided the first documented cure of neuroblastoma, by reporting the case of a child treated 15 years previously by surgical excision. This had been performed by Dr. Bartlett in St. Louis in 1916 (Lehman 1932).

Radiotherapy was introduced in 1928 but had made little impact on survival figures (Holmes and Dresser 1928). Wittenborg (1950) however, published results demonstrating an effective radiation response with doses as low as 400cGy, in a patient with Stage 4S disease. As a result, post operative radiotherapy became fashionable.

Around the same time, Gross (1953) noted the clinically favourable factor of young age. Soon afterward, chemical screening tests to detect neuroblastoma and monitor catecholamine levels became available (Kaiser and Von Studnitz 1961).

Single agent chemotherapy was introduced around 1960 (Tan, Dargeon and Burchenal 1959; Kontras and Newton 1961). Later, the combination of

multiple agents improved disease free survival but again this modality had little impact on overall survival (James *et al.*, 1965).

The first widely accepted clinical staging system was introduced in 1971 (Evans *et al.*,). This meant that, for the first time, different prognostic groups became apparent, in a disease previously universally perceived as fatal. Young children with localised disease did well and interest developed in minimising therapy to maintain good response rates and reduce treatment related morbidity.

By 1980, the prognosis of disseminated neuroblastoma remained poor. It was clear that the survival of children with widespread metastatic disease had changed little, despite the improvements in survival in other paediatric malignancies.

Combination chemotherapy, however, was becoming more sophisticated and drugs with different cell cycle specificities and non overlapping toxicities were used sequentially to improve response rates (Hayes, Green and Mayer 1977). The addition of cisplatin and epipodophyllotoxins (Hayes, Green and Casper 1981; Shafford, Rogers and Pritchard 1984) continued to improve overall initial response rates and it became possible to induce disease regression in up to 80% of patients. Cheung published a meta-analysis of 44 published clinical trials and indicated that the response rate, median survival and event free survival correlated strongly with the overall dose intensity of agents (Cheung and Heller 1991). Studies were piloted,

demonstrating the use of haemopoetic support enabling high dose therapy (Pritchard, McElwain and Graham-Poole 1982).

Besides using different agents in dose intensive regimens, new modalities became available during the late 1980's including mIBG. Antibodies, with radioactive nuclides attached, were also developed to specifically target neuroblastoma cells. Interest developed in agents such as retinoic acid, which induced differentiation in neuroblastoma cells in vitro and these were tried clinically.

The current management of poor prognosis neuroblastoma therefore is intensive, multimodality therapy including many of the above agents in an attempt to induce complete response rates in as many children as possible, since otherwise cure remains elusive.

1.2. EPIDEMIOLOGY

1.2.1. Introduction

Cancer is relatively rare in children, with an estimated incidence of 12 per 100,000 children per year in The United States (Miller *et al.*, 1995). This is nevertheless a significant cause of childhood death. Neuroblastoma is the fourth most common malignancy. As the table below shows, the annual incidence has remained relatively unchanged over the last twenty years at 0.9 per 100,000 (Miller *et al.*, 1995).

Table 1.1. Trends in age adjusted incidence rates per 100,000 for Children Aged 0-14 years, 1973-1987. (Miller *et al.*, 1995)

Tumour Type	1973-1977	1978-1982	1983-1987
All tumour types	12.0	12.2	13.1
Leukaemia	3.8	3.7	4.1
Lymphomas	1.6	1.5	1.6
CNS	2.1	2.1	2.4
Neuroblastoma	0.9	1.0	1.0
Renal tumours	0.7	0.8	0.8
Soft tissue sarcoma	0.8	0.9	0.9
Bone tumours	0.5	0.7	0.7
Epithelial tumours	0.5	0.5	0.5
Germ cell	0.4	0.4	0.4
Retinoblastoma	0.3	0.3	0.3
Hepatic	0.1	0.1	0.2

1.2.2. Age

Twenty five percent of patients are aged less than one year at diagnosis and ninety percent less than 5 years (Kinnier-Wilson and Draper 1974). Neuroblastoma is the commonest tumour to present in the first month and the first year of life. The median age at diagnosis is 2.5 years (Hayes and Green 1983). Occasional adult cases have been documented (Lopez, Karalousis and Roa 1980; Aleshire *et al.*, 1985; Kaye *et al.*, 1986).

1.2.3. Sex

Several studies report a slight male predominance of neuroblastoma cases (Fortner, Nicastri and Murphy 1967; deLorimier *et al.*, 1969; Grosfield and Baehner 1980; Halperin and Cox 1986; Huddart *et al.*, 1993).

1.2.4. Aetiology

The aetiology of neuroblastoma is largely unknown. The geographical variation, with the apparent lack of cases diagnosed in sub Saharan Africa, is probably due to inadequacy of diagnostic services (Lucas and Fischer 1990).

A genetic basis is likely since neuroblastoma has been associated with a number of congenital abnormalities: neurofibromatosis (Bowland and Towler 1970), Beckwith-Wiedemann syndrome (Emery *et al.*, 1983) nisidioblastosis (Grotting, Kassel and Demler 1979) and fetal hydantoin syndrome (Pendergrass and

Hanson 1976). It is occasionally associated with congenital heart disease, skull defects, soft tissue sarcomas and ependymomas. Osteosarcoma, fibrosarcoma, thyroid and renal cell carcinoma are occasionally seen in treated patients.

As with other childhood tumours, familial cases have been noted in monozygotic twins (Mancini *et al.*, 1982); siblings (Gerson, Chatten and Eisman 1974); cousins (Plochl, Kasser and Klien 1976); half siblings and parent child relationships (Khushner *et al.*, 1986). Both maternal and paternal affected parents are equally likely, although the poor prognostic factor of NMYC amplification in advanced neuroblastomas is more likely to be inherited from the father due to genomic imprinting of this allele (Cheung *et al.*, 1994). Knudson suggests that 25% of neuroblastomas could be inherited in an autosomal dominant manner, with two hits necessary for malignant transformation (Knudson and Strong 1972). This risk to siblings of affected children is 6% to 8%. Familial cases are more likely to have multiple primary tumours (23% compared to 5% unselected cases) and present at an early age. In a review of his own and published familial cases, Kuschner and colleagues (1986) noted a median age at diagnosis of nine months compared to 2.5 years in non familial cases, with sixty percent of cases presenting before the end of the first year of life compared to twenty five percent at less than one year in unselected cases. The oldest familial neuroblastoma was in a thirteen year old child (Gunby 1920). Familial cases do not differ from unselected cases in presentation and ultimate survival (Kuschner *et al.*, 1986).

In the literature there are anecdotal reports suggesting an association between maternal exposure to diuretics, alcohol and sex hormones and neuroblastoma (Kramer *et al.*, 1987; Mandel *et al.*, 1994).

1.3. PATHOLOGY

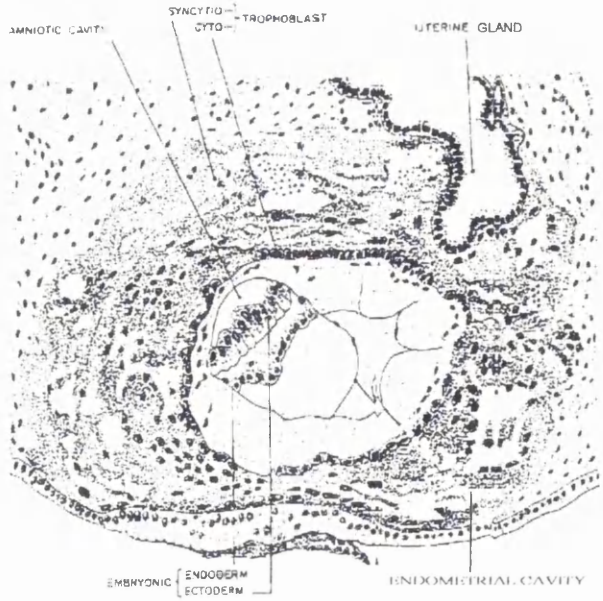
1.3.1. Introduction

Neuroblastoma originates from the neural crest. Different degrees of differentiation can exist within the same tumour giving a spectrum of pathological entities, neuroblastoma, ganglioneuroblastoma or ganglioneuroma.

1.3.2. Embryology of Neuroblastoma

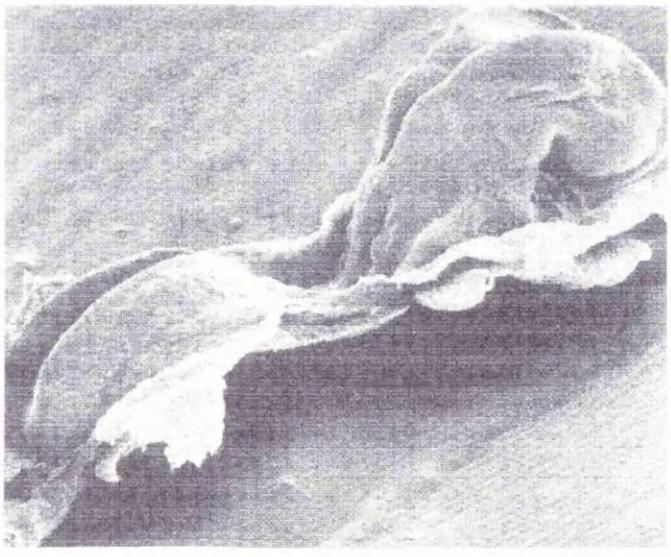
After fertilisation the ovum passes through the morula to the blastula stage when the conceptus is a ball of cells. During the third week the gastrula embeds within the endometrium and tissue differentiation begins.

Figure 1.1. The human conceptus at 12 days (*modified from*: A Companion to Medical Studies *eds.* R. Passmore and J.S. Robson. 2nd edition)



The development of the nervous system begins at the neurula stage when the neural plate, the forerunner of the nervous system, becomes established. This consists of a thickened plate ectoderm overlying the notochord process which acts as a primary inductor. A midline groove forms, creating a neural fold on each side. Each neural fold rises up and enfolds the neural plate forming the neural tube. The neural tube eventually forms the brain and spinal cord. As the neural folds fuse, some of the neural ectoderm at the lateral edges of the neural plate is not included within the neural tube. This is called the neural crest.

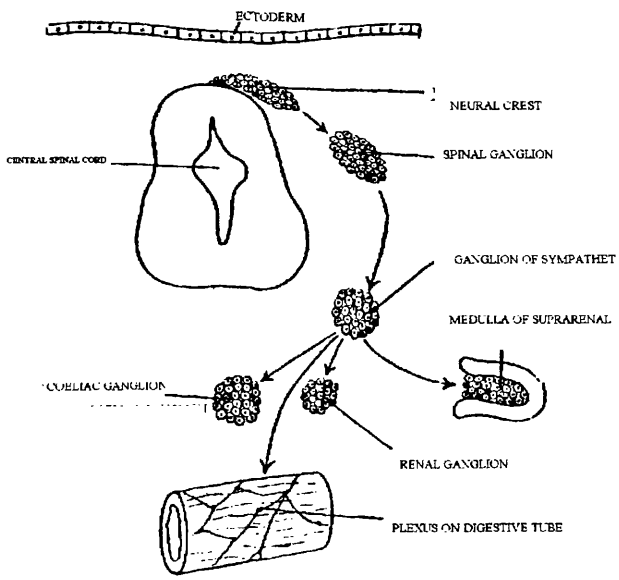
Figure 1.2. The development of the neural crest *(modified from:* Grays anatomy 38th edition).



The cells on the dorsal surface of the neural crest migrate and eventually form melanocytes. Those from the ventral surface develop into the spinal ganglia, the sympathetic chain, prevertebral ganglia, chromaffin bodies and cells of the amine precursor uptake and decarboxylation (APUD) system.

The cells migrating to the sympathetic nervous system also migrate to the adrenal medulla and the paraganglia of Zuckerkandl at the bifurcation of the aorta. Differentiation of these cells starts in the tenth week of gestation but continues into adulthood.

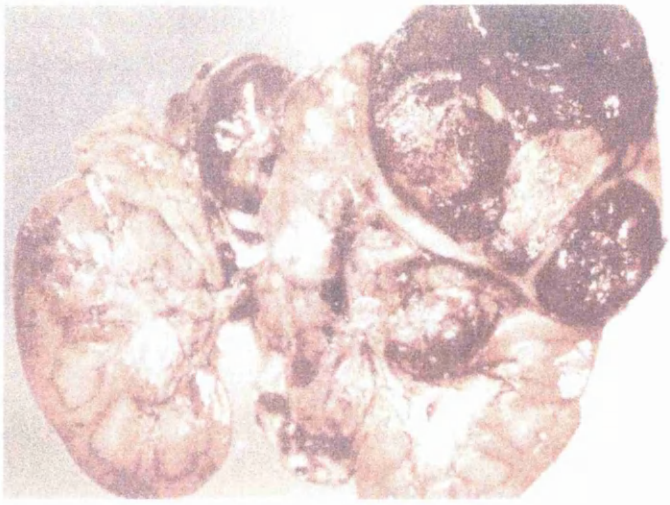
Figure 1.3. The migration of neural crest cells. *(modified from:* Grays anatomy 38th edition).



1.3.3. The Pathological Features of Neuroblastoma

The primary site of disease is commonly the adrenal medulla but any site of sympathetic tissue can be affected. Macroscopically the tumour appears grey or pink and friable. This may vary in size from a small deposit to a large cystic mass, which is often well delineated, rather than encapsulated. The cut surface of the primary tumour is characteristically grey with multiple areas of cystic degeneration, haemorrhage and calcification.

Figure 1.4. The macroscopic appearance of neuroblastoma (Reproduced with kind permission from Dr. Alan Howatson, RHSC, Glasgow).

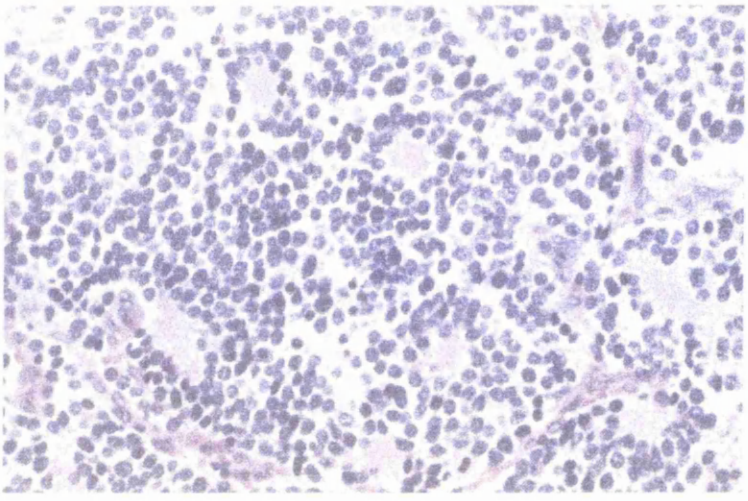


The tumour spreads locally within the retroperitoneum infiltrating around and encasing critical vessels and organs. In the mediastinum, the tumour may expand and permeate the vertebral bodies and ribs. Metastatic spread occurs rapidly with dissemination to the lymph nodes. Haematogenous spread is responsible for bone marrow infiltration, bone metastases and widespread visceral involvement.

Microscopically the tumour can vary depending on the degree of differentiation of the tissue. The undifferentiated tumour is composed of sheets of small round blue cells with scanty cytoplasm. The nucleolus is prominent and, in its most primitive form, the cells show minimal cohesion. In more differentiated tumours cells show clustering, large nuclei and cytoplasmic processes called neurofibrils. In a third of neuroblastomas, fibrils arranged at the centre of these aggregates

form a rosette type structure. These are named 'Homer-Wright' rosettes and are classical of neuroblastoma.

Figure 1.5. The microscopic appearance of neuroblastoma *(Reproduced with kind permission from Dr. Alan Howatson, RHSC, Glasgow)*



By E.M. studies cytoplasmic granules 100-500μm in diameter are seen. These are thought to contain catecholamines.

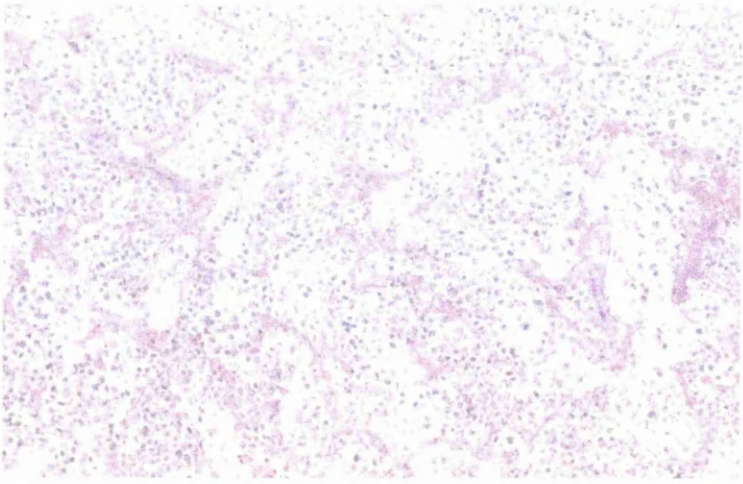
1.3.4. The Pathological Features of Ganglioneuroblastoma.

This tumour has an equal sex incidence and usually occurs before the age of ten years. Symptoms can be a result of local pressure effects or the release of vasoactive peptides. Macroscopically, the tumour may be well encapsulated and the cut surface demonstrates a glistening tan like appearance with focal areas of necrosis and haemorrhage.

Ganglioneuroblastoma is composed of mature sympathetic cells, neuroblasts, intermediate cells and schwann cells. Two prognostically distinct histological patterns are discernible, called 'complex' and 'composite' types.

A complex ganglioneuroblastoma has a lobular type appearance. Neuroblasts are mixed with intermediate and ganglion type cells within the same lobule. The composite arrangement is typified by the same mixture of cells but scattered throughout the whole sample. 65% of composite ganglioneuroblastomas remain localised compared to 18% of the complex type.

Figure 1.6. The microscopic appearance of ganglioneuroblastoma. *(Reproduced with kind permission from Dr. Alan Howitson, RHSC, Glasgow).*

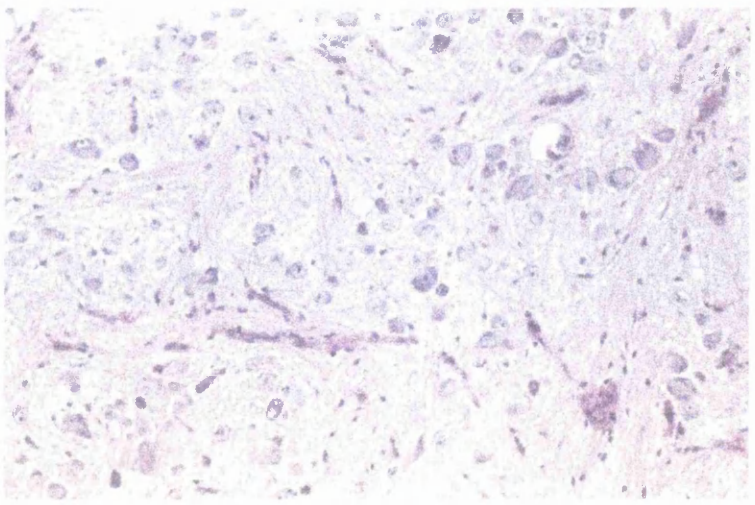


1.3.5. The Pathological Features of Ganglioneuroma

Ganglioneuroma consists of mature cells and represents the benign end of the clinical spectrum, occurring later in life, usually in adults aged 30 to 40 years

old. The primary can be found in similar sites to neuroblastomas but atypical sites such as the gut, uterus, ovary and skin can also be affected. Patients may be asymptomatic or present with nonmetastatic complications, for example, hypertension. Macroscopically, lesions vary in size ranging from small non-encapsulated lesions to large extra adrenal capsulated masses. The cut surface has a grey-white appearance and is composed of mature ganglion cells, sheathed neurites and schwann cells, encased in a compact oedematous stroma infiltrated by lymphocytes. The prognosis for ganglioneuroma is generally better than ganglioneuroblastoma and neuroblastoma. However, quite undifferentiated elements may exist within the same tumour and ultimately influence metastases and hence prognosis (Beckwith and Martin 1968).

Figure 1.7. The microscopic appearance of ganglioneuroma (Reproduced with kind permission from Dr. Alan Howatson, RHSC, Glasgow).



The Shimada classification is a prognostic system based on the pathological appearances of the tumour and is discussed later (Shimada *et al.*, 1981).

1.3.6. Neuroblastoma *in situ*

Small clusters of neuroblastoma cells have been frequently documented in the adrenal glands of infants aged less than 3 months old. The incidence of this phenomenon, called neuroblastoma *in situ*, varies. Turkel and Itabashi (1967) noted its presence in 100% of fetal adrenal glands. Beckwith and Perrin (1963) also noticed the frequent appearance of neuroblastoma nodules, in 3 month old infants, when post mortem examination was performed for other reasons, and estimated the frequency to be 40 times greater than that of neuroblastoma. The majority of these tumours must therefore spontaneously differentiate or degenerate.

1.3.7. Summary

Neuroblastoma and related tumours may be found anywhere along the craniospinal axis. Their propensity for extensive local infiltration and early widespread metastases make the management of this disease difficult. Different degrees of differentiation may be apparent within the same tumour. In general, the more undifferentiated the tumour, the greater the probability of spread and the poorer the prognosis. Neuroblastoma is composed of small round blue cells but signs of differentiation include cytoplasmic processes and neurofibril formation. The classical pathological feature of neuroblastoma is the 'Homer Wright' rosette.

1.4. CLINICAL PRESENTATION

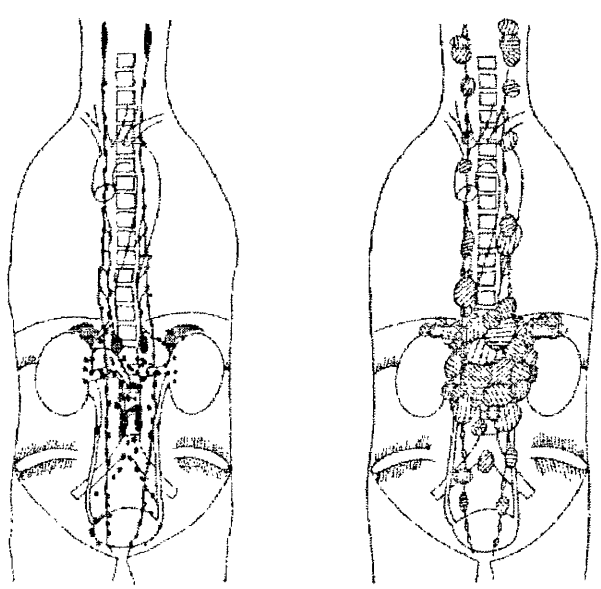
1.4.1. Introduction

Besides the non-specific symptoms of disseminated malignancy, presenting clinical symptoms may vary greatly. They depend on the site of the primary tumour, the presence of metastases and the production of metabolically active substances.

1.4.2. Primary Sites of Disease

As a result of their embryological origin, neuroblastomas are commonly found at sites of spinal and sympathetic ganglia (Figure 1.8).

Figure 1.8. The sympathetic nervous system and primary sites of neuroblastoma. (*With permission from* Cancer in Children Clinical Management. *Eds.* P.A. Voute, A Barrett, H.J.G. Bloom).



The most common primary site of tumour is in the abdomen, either the adrenal gland (40%) or the paraspinal ganglia (30%). Less commonly affected sites are the thorax (14%) pelvis (4%) and neck (2%). Occasionally the primary site of disease cannot be found and the child presents with widespread metastatic disease

Caution must be taken when interpreting data from older series of patients, as modern imaging techniques and diagnostic criteria did not apply. Table 1.2. below represents a summary of the incidence of the site of primary in previously published series of patients.

Table 1.2. Primary sites of disease in published series (% of cases)

adrenal	retroperitoneal	thorax	neck	pelvic	unknown	total	reference
50	19	8	3	4	14	98	Fortner 1967
	43	17	3	4	4	144	Stella 1970
39	33				4	180	Carslen 1985
58	17	25				36	Perez 1967
63	16	2	3	11		212	deLorimier 1968
40	5	11	2	6	16	217	Gross 1959
71		15	2	7	6	55	Halperin 1986
60	25	10	2	2	1	160	Grosfield 1980
42	29	14.4	2.2	4.18	7.9		mean values

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60	25	10	2	2	1	160	Grosfield 1980
42	29	14.4	2.2	4.18	7.9		mean values

Clinical signs vary, depending on the site of primary. For example, disease originating in the cervical region can be mistaken for lymphadenopathy, but in a very young child experiencing difficulty in feeding or with respiratory distress, neuroblastoma is a distinct possibility. In this area, the signs of Horners syndrome may be present (unilateral ptosis, narrowing of the palpebral fissure, meiosis, enopthalmus and anhidrosis). Since sympathetic innervation is associated with development and maintenance of normal eye colour, the subtle sign of heterochromia of the iris may indicate a cervical neuroblastoma.

Thoracic tumours generally arise in the posterior mediastinum and may be discovered on routine chest x-ray. When large, symptoms of dyspnoea or repeated respiratory infection can warn of mediastinal disease. Abdominal disease is the commonest presentation and the tumour can become large before giving symptoms of abdominal pain or renal complications from displacement and obstruction of the renal tract.

Pelvic disease usually presents with altered micturition or defecation or a palpable mass. This may invade through the sacro-sciatic notch and present as a lump in the gluteal region. Paravertebral tumours arise mainly in the cervical and thoracic region. They can extend through the intravertebral foramina in a classical dumb-bell configuration, to cause compression of the spinal cord. Symptoms of back pain, weakness, difficulty in walking and bowel and bladder dysfunction are suggestive of this complication requiring urgent treatment. With intraspinal disease 40% of cases may not be clinically apparent at presentation but the possibility of this unfortunate complication should always be considered since the prognosis for patients with dumb-bell tumours is otherwise good.

In some patients, a primary tumour cannot be found. This is less common in the days of modern imaging techniques but a favourable sub-group INSS stage 4S (Brodeur *et al.*, 1988) represent a group of young children with a small or undetectable primary site and metastatic disease confined to the liver, skin or bone marrow, without bone involvement.

1.4.3. Patterns of Metastatic Disease

50% of infants and 70% of older children, present with metastatic disease. Distinct patterns of metastatic disease are seen and these are of prognostic significance.

Due to foetal circulation, patients with INSS stage 4S neuroblastoma, have disease which is confined to the liver, skin and bone marrow. The liver, in this situation, often bears the brunt of metastatic deposition, resulting in massive hepatomegaly which can cause respiratory embarrassment and death. Despite this, the majority of patients have an extremely good outcome with minimal or no therapy; Spontaneous resolution is seen. However, occasionally, very young children, usually those aged less than six weeks, succumb to these mechanical complications. Skin nodules, when present, can demonstrate a bluish tone and blanch on pressure, due to the local release of vasoconstrictive noradrenaline. This is called 'the blueberry muffin' sign (Evans *et al.*, 1980).

This category of stage 4S disease is fascinating, in a tumour where metastatic disease is otherwise incurable. It is postulated that this condition arises from aberrant neural crest cells forming the entire peripheral autonomic nervous system. The resulting abnormal migration of these cells results in the widespread pattern of skin nodules and focal deposits of disease encountered above. It is thought that these abnormal cells remain at least partially under regulatory mechanisms, which induce spontaneous maturation of these deposits. This is known as 'the neurocristopathy hypothesis'.

In contrast, the older child presents with a wider pattern of metastatic disease. Lymphatic spread results in both local and regional lymphnode involvement. Haematogenous spread results in visceral involvement and bone marrow infiltration results in pancytopenia. Neuroblastoma has a predilection for the bones and soft tissues of the skull, particularly those of the orbital area. Infiltration results in periorbital swelling, ecchymosis and proptosis causing a 'racoon type' facies. The brain, heart and lungs are rarely sites of metastatic disease except in advanced cases, when involvement is a result of spread by lymphatics or direct extension through the meninges or diaphragm.

1.4.4. Non Metastatic Complications of Disease

The excretion of urinary catecholamines for example, homovanillic acid (HVA), vanillymandelic acid (VMA) and dopamine (DA) can suggest the diagnosis of neuroblastoma. Their production can result in episodic sweating, flushing, tachycardia, tachypnoea, pallor, headache, irritability, fatigue and hypertension (Mason *et al.*, 1957). These symptoms have been reported in pregnant women, in the last month of pregnancy, who later gave birth to children with neuroblastoma (Voute *et al.*, 1970).

In 2% of patients, a form of cerebellar encephalopathy, polymyoclonia-opsoclonus syndrome, often described as 'dancing eyes, dancing feet' is seen. It is characterised by progressive myoclonic jerking movements of the hands and feet, truncal ataxia, titubation of the head and conjugate jerking movements of

the eyes (Bray *et al.*, 1969; Moe and Nellhaus, 1970). This condition is often associated with localised disease of otherwise good prognosis. It is therefore unfortunate that 80% of the children, with this rare complication, have permanent symptoms of progressive dementia, extrapyramidal disease or recurring ataxia (Senelick *et al.*, 1973; Koh *et al.*, 1994). The cause of this syndrome is not known but it is unlikely this is associated with elevated levels of catecholamines as these are raised in 90% of children with neuroblastoma and this presentation is relatively rare. It is more likely to be due to an auto immune response to a common neurological antigen. This would explain its persistence in children apparently free of tumour.

Profuse diarrhoea is also occasionally seen as a non-metastatic complication, and is due to an excessive production of vasoactive intestinal peptide, a gut hormone and neurotransmitter.

1.5. CLINICAL EVALUATION

1.5.1. Introduction

In 1986, an international group of researchers representing every major oncology group in the world, agreed the minimum criteria necessary for the diagnosis of neuroblastoma (Brodeur et al., 1988).

1.5.2. Diagnostic Criteria

Ideally, a biopsy of tumour tissue, analysed by standard histopathological techniques, provides an 'unequivocal' diagnosis. This would also provide material necessary for the biological characterisation and hence prognosis of the individual tumour.

The majority of children have metastatic bone marrow involvement at diagnosis and patients often embark upon intensive chemotherapy before radical surgery. Therefore for practical purposes, an 'unequivocal' diagnosis of tumour cells in the bone marrow and increased urinary metabolite levels is sufficient for the diagnosis, if tumour tissue is not available. A radiological diagnosis of tumour, even in the presence of elevated catecholamines is however insufficient as ganglioneuroma (Hayes *et al.*, 1989), phaeochromocytoma (Samaan *et al.*, 1988) or peripheral neuroepithelioma can present this way.

Histologically, neuroblastoma is composed of small round blue cells and must be distinguished from rhabdomyosarcoma, Ewing's sarcoma, neuroepithelioma, acute megakaryoblastic leukaemia and non-Hodgkins lymphoma. Neuronal differentiation may be demonstrated by conventional light microscopy but in addition electron microscopy or immunohistological methods involving antibodies against neurone specific enolase (Dhillon *et al.*, 1982), synaptophysin (Gould *et al.*, 1987) and chromogranin A (Helman *et al.*, 1988) may be necessary.

Both the measurement of homovanillic acid (HMA) and vanillylmandelic acid (VMA) is necessary. In undifferentiated tumours, urinary or serum dopamine may be a more sensitive measure. Measurements must be corrected to per milligram creatinine and to be considered positive, be greater than 3 standard deviations above the mean value for that age group.

1.5.3. Assessment of Disease Extent

The greater the number of clinical investigations performed, the more accurate but extensive the initial extent of documented disease. In order to standardise staging and therefore ensure comparable groups of patients, a recommended minimum requirement for determining extent is recommended.

Site, volume and extent of the primary tumour or large metastases can be delineated by CT or MRI scanning. Ultrasound is considered inferior at providing the necessary three dimensional measurements, particularly with abdominal disease.

Liver involvement or lymphadenopathy may be demonstrated by standard CT or MRI imaging. The opportunity for histopathological confirmation of nodes arises at surgery when any node greater than 2cm within the operative field should be biopsied.

The standard means of detecting bone metastases is by ^{99m}Tc-diphosphonate scintigraphy in centres (for example United States and Japan) where mIBG

scanning is not available. It is however recommended that mIBG should be used as it appears to be more sensitive at distinguishing active, rather than inactive disease involving cortical bone (Voute *et al.*, 1985). It must be noted that any bone abnormality in a growing child should be confirmed by plain x-ray. In addition to bone deposits, any sites of occult tumour would simultaneously be demonstrated on mIBG scan. A disadvantage is that not all neuroblastomas accumulate mIBG, in which case ^{99m}Tc-diphosphonate should be used.

The likelihood of detecting bone marrow involvement increases when bone biopsy rather than aspirate is performed and with an increased number of samples taken. The recommended number of samples is therefore four, two bone marrow aspirates (at least 1cm of bone marrow) and two bone biopsies from each posterior iliac crest (Franklin and Pritchard 1983). One positive sample from four can confirm bone marrow disease but all samples should be negative to exclude involvement.

Both stage 4 and 4S disease may involve the bone marrow but have a strikingly different prognosis. Patients with stage 4S disease are commonly less than one year old at diagnosis and have a limited stage primary. They should also have limited bone marrow disease, less than 10% of the marrow infiltrated. For practical purposes, this should be less than 1% of the bone marrow containing infiltration. If a patient has a heavily contaminated marrow, even if the primary tumour otherwise fulfils the criteria of stage 1 or 2, the patient should be regarded as having stage 4 disease.

1.5.4. Definitions of Response

The overall response is determined by both the primary tumour and metastatic sites. When investigations are positive on initial assessment, they should be repeated. An interim assessment can be performed at two months after treatment but four months is recommended. If surgery to the primary is necessary, this should be completed before the final evaluation at the four month period but before any transplant procedure or maintenance therapy.

The response should be defined as one of the six categories proposed by the INRC (Brodeur *et al.*, 1993), as follows: Complete response (CR); Very good partial response (VGPR); Partial response (PR); Mixed response (MR); No response (NR) and Progressive disease (PD).

Table 1.3. Definitions of response to treatment.

CR	A complete response indicates a complete clearing of disease from both the primary sites and sites of metastatic disease. The catecholamine levels should have returned to normal if they were raised previously.
VGPR	A very good partial response indicates 90%to 99% volume reduction in the primary tumour with complete resolution of disease at other sites, other than bone. Bone lesions however should be healing, improved from previous scans and no new lesions evident. Catecholamines should be normal.
PR	There are no new lesions. The tumour volume and sites of metastatic disease have decreased in volume by 50%. Bone lesions should be healing with no new lesions. One residual positive marrow or biopsy is permitted if the extent is less than previously documented. Catecholamines should be reduced from 50-90%.
MR	There are no new lesions. A mixed response indicates a greater than 50% response at some sites but less than 50% response at others. For example, a large primary may respond less well than metastases and surgical excision could convert this category to one of the above.
NR	There are no new lesions. There is less than 50% reduction of all sites and any site of disease does not increase in size by more than 25%.
PD	There are new lesions. Any previously present lesion has increased in size by more than 25%.

1.6. STAGING

1.6.1. Introduction

For many years the lack of a commonly agreed staging system between countries prevented the reliable comparison of data. By 1988, there were three major classification systems, The Children's Cancer Study Group (CCSG) (Evans *et al.*, 1971); The St. Jude Children's Research Hospital (SJCRH) (Hayes *et al.*, 1983) used by the Paediatric Oncology Group (POG). This was later modified by both the Italian Co-operative Working Group (1987) and the Malignant Tumour Committee of the Japanese Society of Paediatric Surgeons (Nagahara *et al.*, 1990). Another major staging system, the TNM classification was implemented by the UICC in 1987.

1.6.2. The Children's Cancer Study Group Staging System

The first widely accepted staging system was that of Evans (Evans *et al.*, 1971) who defined the extent of disease clinically and radiologically. The surgeon's assessment of the disease extent at the time of surgery was also included. Previous staging systems relied heavily on the extent of surgical resection achieved but this is dependent on the particular surgeon's skill and experience (Pinkel 1968).

The CCSG system took account of the previously documented better survival in patients less than one year old. Because this applied to all stages, the group did not impose an age restriction to their categories. A special subgroup of patients,

was however defined. Children with a localised primary (stage I or II) and disease dissemination only to the liver, skin and or bone marrow were categorised as stage IVS (S denotes special). These patients have a good prognosis with 75% two year survival compared to 7% two year survival in the original series of 100 patients. This group of researchers also stressed the prognostic significance of the primary mass crossing the midline, which remains a powerful prognostic indicator (Evans *et al.*, 1990).

The Evans staging system, by allowing prognostic stratification, provided a major stimulus to research in the field. It soon became clear however that although it could separate low risk patients from those needing intensive treatment, more detailed definitions of localised disease were required.

Table 1.4 Children's Cancer Study Group staging classification (Evans *et al.*, 1971).

Stage 1	Tumours confined to the organ of origin.
Stage II	Tumours extending in continuity beyond the organ or structure of origin but not crossing the midline. Regional lymph nodes on the homolateral side may be involved
Stage III	Tumours extending in continuity beyond the midline. Regional lymph nodes may be involved bilaterally.
Stage IV	Remote disease involving the skeleton, organs, soft tissues, or distant lymph nodes groups
Stage IV-S	Patients who would otherwise be Stage I or II, but who have remote disease confined only to one or more of the following sites: liver, skin, or bone marrow (without radiographic evidence of bone metastases on complete skeletal survey).

1.6.3. The Paediatric Oncology Group Classification.

In an attempt to further define groups with localised disease, Hayes *et al.*, in 1983 developed a surgical pathological system stressing the importance of resection and lymph node involvement. Based on an analysis of 254 children treated at St. Jude Children's Research Hospital, Memphis, Tennessee, she found a difference in survival in patients with no regional node involvement (87%) compared to those with node dissemination (33%). The prognostic significance of involvement of lymph nodes has been confirmed (Evans *et al.*, 1990). This only holds if the tumour does not infiltrate beyond the midline. If it does, then the latter becomes the overwhelming factor. In addition, this system fails to distinguish between bone and bone marrow involvement. Both these factors may explain why, although useful, this system is a less powerful discriminator than that of the CCSG (Halperin and Cox 1986; Evans *et al.*,1990). In clinical practice this system was never very widely used.

1.6.4. The TNM Classification

This is a complicated system, based on clinical and radiological investigations but with a separate post surgical histopathological component. The rationale for this, is that the stage at presentation is often different from that at surgery, after intensive chemotherapy. It has the major disadvantage the tumour has both a clinical and separate pathological description. These bear no relationship to one another and give rise to sixteen different possible categories of disease. This system also fails to consider factors such as age and patterns of metastases. For these reasons, this system was not widely used in clinical practice.

Table 1.5. The UICC TNM staging system (UICC 1987).

Clinical Staging System		Pathological Staging System	
T_0:	not detected	pT_O:	no tumour
T_1:	tumour<5cm	PT_1:	complete excision
T_2:	tumour 5-10cm	pT_2:	incomplete removal
T_3:	tumour>10cm		A: microscopic residue
T_4:	multi-centric tumour		B: microscopic residue
			C: biopsy alone
		pT_4:	multi-centric
N_o:	normal lymph nodes	pN_O:	normal lymph nodes
N_1:	abnormal regional lymph nodes	pN_1:	abnormal regional lymph nodes
N_x:	no available information		A: complete removal
	on lymphnodes		B: incomplete removal
M_o:	no metastases		no information
M_1:	distant metastases present	pN_x:	no metastases
		pM_0:	distant metastases present.
		pM_1:	
CSI:	$T_1,N_0,N_x.M_0$	PSI:	$pT_1.\ pN_{0,x}.\ pM_0$
CSII:	T_2N_0,N_xM_0	PSII:	$pT_1.\ pN_{1a}.\ pM_0$
CSIII:	$T_{1,2,3},N_{0,x},M_0$	PSIIIA:	$pT_{3A}.pN_{0,1A,X}.pM_0$
CSIV:	$T_{1,2,3,}.\ N_{0,1,x}.M_1.$	PSIIIB:	$pT_{0,1,3A/B}.\ pN_{0,1A/B,x}.\ pM_0$
CSV:	$T_4.\ N_{0,1,,x.}\ M_{0,1.}$	PSIIIC:	$pT_{3C}.\ pN_{0,1A/B,x}.pM_0.$
		PSIV:	$pT_{1,3A/B/C.}\ pN_{0,1A/B,x.}\ pM_1.$
		PSV:	$pT_4.\ pN_{0,1A/B,x.}\ pM_{0,1.}$

1.6.5. The International Neuroblastoma Staging System.

Each system had the power to divide patients prognostically, but there were several areas of differences and one group within one system could not be directly compared with that of another. The major areas of contention were:

- The prognostic significance of tumour crossing the midline.
- The importance of the involvement of ipsilateral and contralateral lymph nodes.
- The importance of resectability of the tumour.

During November 1986, an international consensus was reached through a collaboration of delegates from every major oncology group in the world. The result was a universally agreed set of criteria for the diagnosis, assessment and staging of neuroblastoma. These were termed the International Neuroblastoma Response Criteria (INRC) and the International Neuroblastoma Staging System

(INSS) respectively (Brodeur *et al.*, 1988). These were subsequently modified slightly in 1993 (Brodeur *et al.*, 1993).

Stage 1 and 4 were similar to previous staging systems. Stage 1 was however later modified to include a resected specimen with adherent positive nodes if they were fully resected. Stages 2A and B in total account for only 10%-15% of neuroblastoma cases. The significance however of positive regional lymph nodes remains a contentious issue and by initially separating these categories it was hoped to determine if stage 2B more closely resembled that of stage 2A or stage 3 disease. Stage 3 was carefully defined as those tumours infiltrating 'by contiguous invasion to or beyond the opposite side of the vertebral bodies'. Finally the stage 4S disease category was maintained but limited to those patients less than one year old with bone marrow infiltration of less than 10%.

Table 1.6. International Neuroblastoma Staging System (Brodeur *et al.*, 1993).

Stage 1	Localised tumour confined to the area of origin; complete gross excision, with or without microscopic residual disease; Identifiable ipsilateral and contralateral lymph nodes negative microscopically (nodes attached to and removed with the primary tumour may be positive).
Stage 2A	Unilateral tumour with incomplete gross excision; Identifiable ipsilateral and contralateral lymph nodes negative microscopically.
Stage 2B	Unilateral tumour with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; Identifiable contralateral lymph nodes negative microscopically.
Stage 3	Tumour infiltrating across the midline with or without regional lymph node involvement; or unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral regional lymph node involvement.
Stage 4	Dissemination of tumour to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined in stage 4S).
Stage 4S	Localised primary tumour as defined for stage 1, 2A or B, with dissemination limited to liver, skin and/or bone marrow. (limited to infants less than one year of age).

1.6.6. Summary

The widely used staging system of Evans in 1971 provided the first clear identification of prognostic groups and as a result provided a major stimulus to research interest in this apparently fatal tumour. The large number of non directly comparable staging systems, however, were inhibiting the direct comparison of data between different centres. The need for consistent criteria for diagnosis, staging and assessment of cases led to the internationally developed and agreed staging system (INSS). This has been adopted widely and is the staging system used for this thesis unless otherwise stated.

Present systems concentrate on anatomical prognostic factors. Biological features are however assuming greater significance and may be incorporated into future classifications.

1.7. PROGNOSTIC FACTORS

1.7.1. Introduction

Homer-Wright when naming neuroblastoma noted its clinical diversity. Initially, prognosis was based on clinical features. Biological research has however identified better predictors of prognosis. These are crucial, in identifying those children who need minimal or no therapy, since the majority of neuroblastoma patients are very young children who are therefore even more vulnerable to the long term morbidity of treatment.

1.7.2. CLINICAL PROGNOSTIC FACTORS

1.7.2.1. Age

This remains the most powerful prognostic factor as children aged less than one year at diagnosis, regardless of stage, do well. Thurman (1967), and later Evans (1971), devised separate prognostic groups for children less than one year old. In the original CCSG retrospective analysis of 100 patients, 82% of infants survived compared to 10% of children aged two or more at diagnosis (Evans *et al.*, 1971) regardless of stage.

1.7.2.2. Stage

Children with localised disease have a better prognosis. Patients with stage 1 disease had a prognosis of 80% compared to 7% in the original review by (Evans *et al.*, 1971).

1.7.2.3. Site

Tumours in the mediastinum or cervical area are associated with a better outcome. This is not, however, entirely independent of stage. For example 62% of abdominal primaries have widespread metastatic disease at the time of diagnosis compared with 26% of thoracic cases (Evans *et al.*, 1971). Age, stage and site are however inter-related prognostic factors, since young children tend to present with localised disease and localised disease often presents earlier in non-abdominal sites.

1.7.3. BIOLOGICAL PROGNOSTIC FACTORS

1.7.3.1. Chromosomal Abnormalities

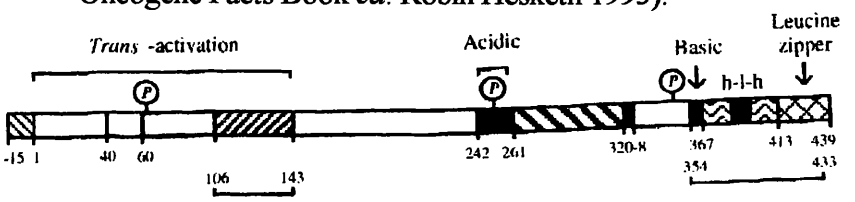
Chromosomal abnormalities are often found in malignant cells and often increase with each subsequent relapse (Feder and Gilbert 1983). Translocations, deletions, duplications or areas of gene amplification are seen. Homogeneously staining regions (HSR) and double minutes (DM) are chromosomal and extrachromosomal areas of gene amplification. In neuroblastoma, HSR and DM contain amplified copies of the N-myc oncogene (Shwab *et al.*, 1983). Abnormalities of the MDR1 gene are also frequently detected and, interestingly, vary inversely with N-*myc* expression (Nakagawara *et al.*, 1990). Loss of heterozygosity of 1p, 4p, 11q, 14q, and 17q occur in neuroblastoma (Srivistan *et al.*, 1991).

1.7.3.2. N-*MYC*

N-myc amplification is present in 30% of neuroblastomas and is associated with advanced stage (Brodeur *et al.*, 1984) and rapid progression. N-*myc* amplification of 1 copy, 1-3 copies and greater than 10 copies is associated with a 70%, 30% and 5% eighteen months disease free survival respectively (Seeger *et al.*, 1985). In 1983 Shwab *et al.*, and Kohl *et al.*, simultaneously but independently isolated an amplified nucleotide sequence that shared homology with the cellular and v-*myc* gene. N-*myc* is located on chromosome 2p23-24, and consists of three exons. Four nuclear peptides are generated by means of differential gene splicing. These range from 58 to 64 kilodaltons in size and

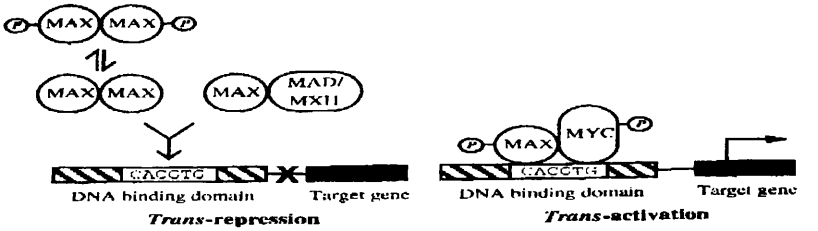
consist of three distinct regions: a DNA binding region, a helix loop helix structure and a leucine zipper motif. This suggests this short lived nuclear protein has a role in nuclear transcription. The C terminal certainly has structural hallmarks consistent with a binding site for a transcription factor. The helix-loop-helix and leucine zipper motif appear essential for the coupling of N-*myc* protein to other nuclear proteins such as the Max protein and RB1 nuclear protein (Wenzel *et al.,* 1991). The N-*myc* and Max proteins probably bind together and bind to DNA enhancing transcriptional activation.

Figure 1.10. The human *myc* protein structure (*Adapted from* The Oncogene Facts Book *ed.* Robin Hesketh 1995).



In normal cells, the level of *myc* protein is finely controlled. Therefore in the presence of gene amplification and deregulated expression, this fine balance is upset. The extra copies of *myc* protein possibly also bind to the RB1 suppresser gene product, lowering its critical nuclear concentration and hence inhibitory, antiproliferative effect on the cycling cells.

Figure 1.11. Tanscriptional regulation by myc and max proteins (*Adapted from* The Oncogene Facts Book. *ed.* Robin Hesketh 1995)



Recent work indicates that multidrug resistance associated protein (MRP) gene expression correlates with N-*myc* gene expression. The evidence for this is that when N-*myc* oncogene expression is downregulated, using antisense constructs, MRP expression, determined by both polymerase chain reaction (PCR) and Western blotting techniques, is also downregulated (Norris *et al.*, 1996).

The genes regulated are, at present, not fully elucidated but it has been shown for some time, that there is a correlation between the down regulation of N-*myc* RNA and induced differentiation of SMS-KCNR neuroblastoma cells (Thiele *et al.*, 1985).

1.7.3.3. 1-p Deletion

Deletion of the short arm of chromosome 1 is seen in 70% of neuroblastomas (Gilbert 1984). 1p deletion is often found alone in tumours. If present, in 1, 2, or 4S disease, it can identify high risk cases. A number of studies support the observation of N-*myc* amplification and 1p deletion occurring together in the same tumour sample. Both abnormalities are usually present in advanced cases, stage 3 and 4. Interestingly, in this situation, the 1p deletion is significantly larger, and the loss of the 1p paternal allele is usually due to genomic imprinting (Caron *et al.*, 1996).

When the 1p deletion is present without N-*myc* amplification the 1p alleles lost were of maternal origin and smaller (Caron *et al.*, 1994). This data suggests that of the two tumour suppresser genes present at this breakpoint area, one is susceptible to genomic imprinting, the other not. The area of the 1p deletion is at present being extensively mapped with gene probes. The deleted locus occurs

in the region of 1p32.12-1p32.6 (Fong *et al.*, 1992). There may be as many as 2-4 tumour suppressor genes in this area, but they have yet to be fully identified.

The site of the translocated material is often the 17q area but LOH of chromosome 11q and 14q have also been observed (Srivistan *et al.*, 1991). These are also the site of potential suppressor genes and the focus of current research (Van Roy *et al.*, 1996)

1.7.3.4. Ha-*ras* Gene

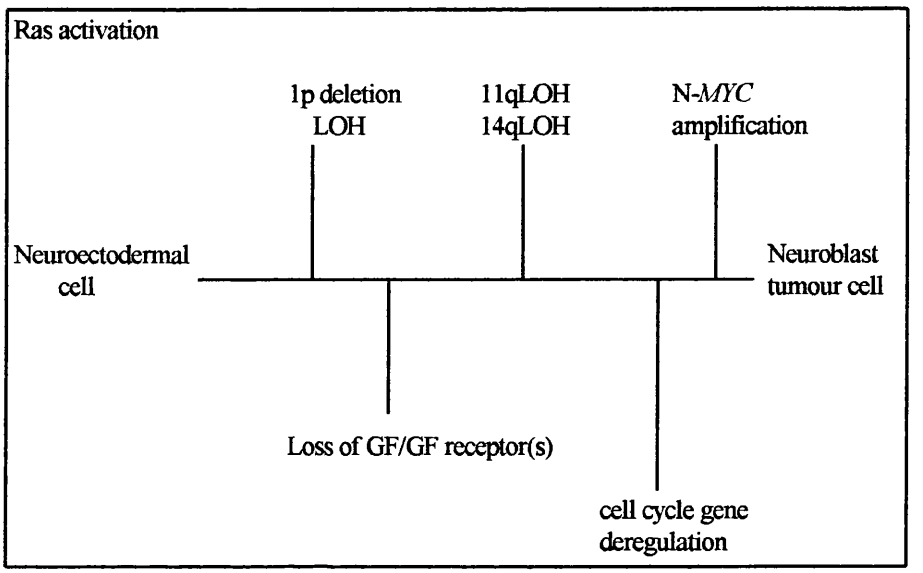
The Ha-ras protein is widely expressed in neural tissue. Expression of this gene can be determined by immunoperoxidase staining by means of an antibody directed towards the RNA C terminal region. Ras point mutations are rarely seen in neuroblastoma, but when present, a high Ha-*ras* expression appears to correlate with greater disease free survival (Taneka *et al.*, 1994) the converse of the relationship between N-*myc* and survival.

1.7.3.5. DNA Ploidy

In neuroblastoma, an abnormal DNA content of tumour cells is associated with a poor outcome. Ghering, in 1993, noticed stage 3 and 4 tumours were associated with near diploid or tetraploid DNA content. This was associated with 20% of samples having coexistent N-*myc* amplification and a higher proportion of 'S' phase cells. In clinical practice, these prognostic features were partly age dependent (Look *et al.*, 1991). Diploidy was a predictor of early treatment failure in all children less than two, whereas hyperdiploidy indicated poor prognosis in half those aged between one and two years. In children older than two, with metastatic disease, these factors did not predict outcome which was dismal. It is possible that these common molecular abnormalities indicate a

multistep transformation in carcinogenesis. The following is a model proposed by Tonini (1993).

Figure 1.12. Model of multistep transformation in neuroblastoma



1.7.3.6. Nerve Growth Factor

The trk gene encodes for high affinity nerve growth factor. It is expressed in good prognosis tumours. The lack of nerve growth factor expression is a sign of poor prognosis neuroblastoma (Suzuki *et al.*, 1993). Survival varies from as much as 74% to 0% in the presence or absence of the NGF receptor $gp140^{trk}$.

1.7.3.7. CD44

CD44 is a cell surface glycoprotein and an independent prognostic factor, associated with a favourable outcome. This correlates with histological differentiation but inversely with N-*myc* expression (Terpe *et al.*, 1994).

1.7.3.8. Neurone Specific Enolase

This is an enzyme which exists in dimeric form. The isoenzyme γ/γ is specific for tissue of neuroendocrine origin. It is not particularly specific as increased levels have been reported with Wilms tumour, Ewing's sarcoma, NHL, soft tissue

sarcoma and acute leukaemia (Cooper *et al.*, 1987). 96% of children with metastatic disease have elevated levels of the enzyme. A level greater than 100ng/ml is associated with a 2 year survival of 14% compared with 34% with low levels of NSE. The effect is particularly marked if children less than 1 year old are considered (25% compared with 100%) (Zelter 1983).

1.7.3.9. Ferritin

Ferritin may be present in the serum due to many mechanisms. It is unusual for children with low stage disease to have elevated levels. In addition the level will decrease to normal with successful treatment. In children aged less than one year, there is a 72% disease free survival with normal levels compared with no survival in infants with ferritin levels greater than 142ng/ml (Hann 1985).

1.7.3.10. GD2

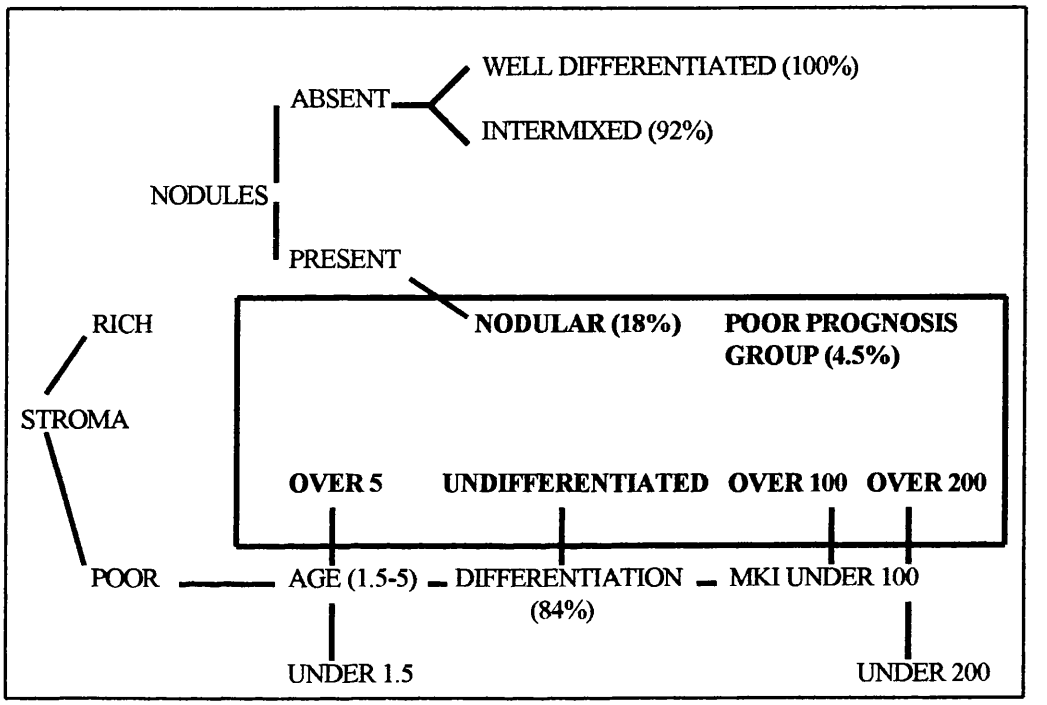
This is a cell membrane bound ganglioside. It can be measured in serum or from the tumour sample. The level will decrease with successful treatment and has been noted to increase on relapse of disease (Ladishc and Wu 1985).

1.7.3.11. Shimada Classification

Developed in 1984, (Shimada *et al.*, 1984) this prognostic staging system was based on histopathology from 295 surgically resected primary tumours. Patients underwent surgery before any chemotherapy or radiotherapy. Analysis was focused on primary tumour samples rather than biopsy of metastatic tissue. Therefore it is possible that this selection has concentrated on better prognosis patients. Despite this, it has remained a useful prognostic indication of survival

of neuroblastoma patients (Evans *et al.*, 1987). Shimada and colleagues published a flow diagram in the original paper to summarise their findings.

Figure 1.13. Flow Diagram of the Shimada prognostic staging system. (Overall survival)



Since both the neuroblastoma cells and its supporting tissues originate from the neural crest, the organisational pattern of the supporting stromal tissue is included in the evaluation. In the stromal poor group, neuroblasts at various stages of differentiation are separated by small amounts of fibrous tissue. Thereafter the cells themselves are examined for any signs of neuronal maturity. The mitosis-karyorrhexis index (MKI) is determined by counting the number of mitoses and karyhorrhexes in a field of 5,000 cells and a high index is usually associated with aggressive cell pathology. This, in addition to the patients age,

helps subclassify the pathology into prognostic groups. The survival of the groups is noted in brackets.

In the stromal rich group, the stroma is well developed consisting of Schwann cell sheaths, peri and endo neural components and even satellite cells. The well-differentiated group exhibited well-formed neuroblasts resembling mature ganglioneuromatous cells. The intermediate group showed variably differentiated neuroblasts. The nodular classification was ascribed if foci of stroma poor tissue were evident. This is significant since the survival of the nodular group is significantly poorer (18% compared to 100% well differentiated and 92% intermixed.)

1.7.3.12. Summary

Initially prognosis was determined by clinical factors, but biological features are becoming increasingly important. Patients can be stratified into prognostic groups on the basis of a combination of both clinical and biological characteristics. These are summarised below.

Table 1.7. Risk groups for neuroblastoma.

LOW RISK	INTERMEDIATE RISK	HIGH RISK
Hyperdiploid/Triploid	near diploid/tetraploid	diploid/ tetraploid
no 1p LOH	+/- 1p LOH, 14q LOH	1p LOH
no N-*myc* amplification	no N-*myc*	N-myc amplification
high TRK-A expression	low TRK-A	TRK-A low/ absent
< 1 year old	>1 year < 10 years	1-5 years
INSS stage 1,2 or 4S	stage 3 or 4	stage 3 or 4
NSE<20	NSE 20-100	NSE >100
90% cure	25-50% cure	<10% cure

1.8 TREATMENT

1.8.1. Localised Disease

The role of surgery in localised disease is well established. Surgery is known to be curative in INSS stage 1 and 2A disease. The addition of radiotherapy does not improve survival, even if residual gross or microscopic disease is present (Matthay *et al.*, 1989). The addition of chemotherapy similarly confers no survival advantage (Evans *et al.*, 1984). For more advanced disease, stage 2B and 3, complete surgical resection improves survival (Ninane *et al.*, 1982; Hayes *et al.*, 1983; Rosen *et al.*, 1984 and Haase *et al.*, 1989).

Post surgical treatment is reserved for special circumstances, such as spinal cord compression but may be considered if the tumour has homolateral lymph node involvement, that is, INSS stage 2B, or poor biological prognostic factors (Neider and Gauderer 1991). If the child is less than six months old, this point is less clear due to the otherwise better prognosis of children aged less than one year. The addition of cyclophosphamide and vincristine (Ninane *et al.*, 1982; Hayes *et al.*, 1981) leads to a survival of greater than 75% (Rosen *et al.*, 1984).

Chemotherapy is the principal modality of treatment for children with more advanced disease. In stage 3 disease, large primary tumours infiltrate across midline structures usually rendering the tumour inoperable at presentation. Curative surgery is delayed until either systemic chemotherapy or mIBG therapy has "down staged" the primary, making surgical resection easier. If possible, radical surgery is desirable

(Hasse 1989). The prognosis of this group is variable (40-70%) depending on the completeness of surgical resection (Le Tourneau 1985). Additional post operative radiotherapy is indicated for gross residual disease (Koop and Schnaufer 1975; Castleberry 1991).

1.8.2. Metastatic disease

Children with true stage 4S disease have an inherently good prognosis. By definition they should have good biological prognostic features for example: no N-MYC amplification or bone marrow contamination limited to less than 5% of cells obtained from aspirate and trephine. If poor biological risk factors are present, the patient would be more appropriately treated with intensive therapy. A number of these tumours regress spontaneously and therefore treatment is usually directed toward controlling symptoms. It should be noted, however, that a number of these patients subsequently relapse.

Multi-agent chemotherapy is indicated in children with stage 4 disease at presentation. Five chemotherapeutic agents are used, etoposide, cisplatin, cyclophosphamide, adriamycin and vincristine. Combinations of these drugs can be extremely effective, producing a partial or complete response in 80%-90% of children (Shafford *et al.*, 1984). The dose intensity of the first four agents appears important in the overall clinical response rates, but prolonged treatment is not necessary (Cheung and Heller 1991).

Large tumour bulk, high mitotic rates and frequency of multi-drug resistance (MDR) in neuroblastoma led to the rationale of dose intensive modern chemotherapeutic

regimens (using the highest tolerable dose delivered in the shortest possible time scale). Drugs are used with differing cell cycle specificity, in the optimum cell cycle combination, with non-overlapping toxicity.

Surgery at presentation is contraindicated in patients presenting with widespread metastatic disease unless a histological diagnosis cannot be obtained from tissue elsewhere, and biopsy is appropriate. The contribution of the extent of surgical resection to residual sites of disease, after induction chemotherapy, to overall survival is unclear. The need for aggressive surgery has been questioned until chemotherapy can definitely eliminate disease from all metastatic sites (Matsumura *et al.*, 1988).

In an attempt to clear any microscopic or small volume residual disease, and improve long term survival, consolidation chemotherapy, with or without TBI regimens, involving bone marrow transplant, has been tried. Large doses of chemotherapeutic agents are used, limited only by their non-haematological toxicity. Improved two year survival varies from 20% to 40% (Phillip *et al.*, 1991).

Despite this intensive therapy and apparent improvement in short term survival, longer term progression free survival drops from 40% to 25% to 13% survival at two, five and seven years respectively for patients undergoing high dose therapy in CR or VGPR. Unfortunately those with residual disease do less well. The two and seven year survival rates are 40% and 37% respectively in the same analysis (Phillip *et al.*, 1989). There appears to be little difference in success rates regardless of which drugs are used or if TBI is added.

1.8.3. mIBG

Neuroblastomas differ markedly in their selective uptake of mIBG (Moyes *et al.*, 1989) but encouraging responses have been seen. Voûte and colleagues noted a 58% total response rate in previously heavily treated patients with refractory disease (Voûte *et al.*, 1991). mIBG can also be used in combination with TBI, where the specific properties of ^{131}I-mIBG are used to target metastases 2-5mm in diameter (Gaze *et al.*, 1995).

1.8.4. Radiolabelled Antibody

Monoclonal antibodies can be manufactured specifically to target neuroblastoma cells and some types are also radiolabelled. UJ13A is a radiolabelled monoclonal antibody directed against neuroblastoma cells. There is however, heterogeneous expression of the antigen and the disadvantage that the reticuloendothelial cells accumulate the antibody.

1.8.5. Differentiating Agents

Retinoic acid is an analogue of vitamin A. 13-*cis* retinoic acid is the form of retinoic acid given clinically. This is converted to the biologically active *trans* isomer, which binds to nuclear retinoic acid receptors and cellular retinoic acid binding proteins. In vitro studies have shown that this agent induces differentiation and inhibits growth in neuroblastoma cells in culture (Hill 1986).

1.9. Screening

The majority of patients with neuroblastoma present with advanced disease. The recognition that prognosis is much improved in the minority of patients presenting with early stage disease, particularly those aged less than one year at diagnosis, lead to the anticipation that screening for neuroblastoma would be of benefit. 91% of neuroblastoma tumours excrete vanillylmandelic acid (VMA) and or homovanillic

acid (HMMA). Urine screening was simple and safe, resulting in a specificity of >99.99% and a predictive value of 50% (Woods *et al.*, 1994).

Mass screening was therefore implemented in some countries with enthusiasm, unfortunately without the benefit of detailed pre-screening epidemiological studies. This partly explains the continuing confusion as to whether screening is of value in this condition.

Screening the urine of six month old infants has been undertaken in Kyoto, Japan since 1974 (Sawada *et al.*, 1982). This was expanded to a nation-wide basis, in 1985. This resulted in a dramatic increase in incidence of the disease, partly due to 'the halo effect' of increased awareness and registration of neuroblastoma, but also due to the increased detection of early stage tumours. The screened population from 1985 resulted in 73% (438/598) of cases with prognostically favourable stage disease 1, 2 or 4S. As a result of this, an impressive overall survival rate of 97% has been quoted for neuroblastoma in Japan (Sawada *et al.*, 1994). Screening, however, detected patients with favourable prognostic criteria, aneuploidy, absence of 1p chromosome deletion and N-*myc* cellular oncogene non amplification (Hayashi *et al.*, 1988; Kaneko *et al.*, 1990; Nakagawara *et al.*, 1991). Some of these screen detected tumours regressed spontaneously when left untreated (Matsumura *et al.*, 1991).

In contrast, a significant number of cases, with poor prognostic criteria, developed after one year in infants previously screened as negative (Nishi *et al.*, 1989). Since it is speculated that the biological prognostic factors above remain constant throughout the life span of the tumour (Brodeur *et al.*, 1984) screening therefore appears unsuccessful in detecting poor prognosis neuroblastoma.

The failure to identify the poor prognosis group may lie in the timing of screening. Screening infants at six months means that only a small proportion of patients present before this age and those that do, are likely to have a good prognosis (Huddart *et al.*, 1993). However, it has been suggested that delaying screening for example until the eighth or twelfth month of life may increase the number of poor prognosis neuroblastoma cases detected (Kerbl *et al.*, 1993).

In summary, therefore, screening programmes have been less rewarding at detecting poor risk patients than hoped. They have however yielded additional prognostic information so that, if combined with recent advances in identifying poor risk groups by molecular analysis changes in timing or repeated testing may improve its effectiveness. Certainly high risk neuroblastoma is not reliably detected by screening infants aged less than one year.

1.10. Conclusion

Favourable neuroblastoma, with favourable biological characteristics, sometimes requires minimal therapy. Similarly children aged less than one year have a good prognosis regardless of their stage due to the same favourable biology. Unfortunately the majority present with widespread metastatic disease and unfavourable biological risk factors. This is precisely the group that remain incurable and which screening has failed to detect.

CHAPTER 2 mIBG

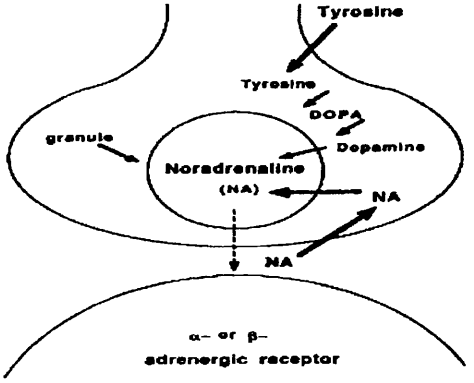
2.1. HISTORY

2.1.1. The Historical Development of mIBG

mIBG is the result of a systematic search for radiopharmaceuticals to image the human adrenal medulla which began in the 1960's by the prestigious Ann Arbour group in Michigan, USA. First attempts involved radiolabelling catecholamines and their precursors. Dopamine and noradrenaline appeared initially promising, but later proved inadequate (Morales *et al.*, 1976)

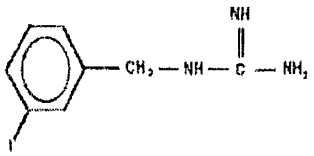
A complete change of approach, reasoning that adrenal medulla cells could be regarded as adrenergic neurons proved successful. These cells have the same property as APUD cells (amine precursor uptake and decarboxylation) where a specific reuptake of neurotransmitter from the synaptic cleft terminates the synaptic message.

Figure 2.1. The reuptake mechanism (*adapted from* Neuroblastoma: mIBG in its Diagnosis and Management. *eds.* J.Moyes, V.R. Mc Cready and Ann Fullbrook. Springer Verlag).

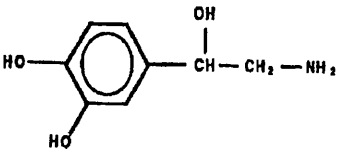


The adrenergic blocking drugs, bretylium and guanethidine, were therefore studied. Guanethidine itself cannot be readily iodinated but Korn was successful in the initial iodination of bretylium analogues (Korn *et al.*, 1977). The search for more effective anti-hypertensives resulted in bethanidine. This is formed as a result of fusing the o-bromobenzyl portion of bretylium with guanethidine (Boura *et al.*, 1961). Derivatives of this molecule were subsequently synthesized and investigated. The resulting aralkylguanidine compounds, in contrast, were easily radiolabelled with iodine. The next year, Weiland demonstrated good concentration of ortho-iodobenzyldimethyl-2-hydroxyethylguanidine in the canine adrenal medulla (Wieland *et al.*, 1979) and he continued his study of its three stereoisomers ortho-, meta- and para-iodobenzylguanidine (Weiland 1980).

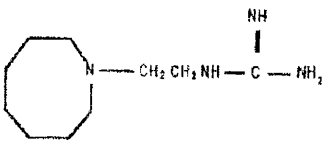
Figure 2.2. Chemical structure of catecholamines, adrenergic neurone blockers and meta-iodobenzylguanidine (*adapted from* Neuroblastoma: mIBG in its Diagnosis and Management. *eds.* J.Moyes, V.R. Mc Cready and Ann Fullbrook. Springer Verlag).



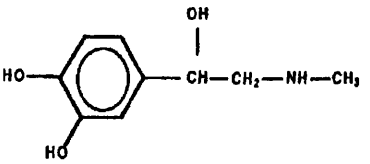
m-Iodobenzylguanidine



Noradrenaline



Guanethidine



Adrenaline

2.1.2. The Three Stereo-isomers of Iodobenzylguanidine

mIBG soon proved to be the superior agent. It had a five times greater affinity than o-IBG for the adrenal medulla. In addition the peak level of mIBG accumulation within the medulla was four times greater than the other isomers, leading to better images. mIBG also proved to be more resistant to the in vivo dehalogenation suffered by p-IBG and therefore had less thyroid accumulation. Finally, m-IBG is excreted by the urine, rather than the gall bladder, as o-IBG, avoiding a potentially confusing image.

2.2. THE CHEMICAL MANUFACTURE OF mIBG

2.2.1. The Traditional Method

The original synthetic method by Wieland (Wieland *et al.*, 1980) used the precursor metaiodobenzylamine hydrochloride which was reacted with cyanamide to produce mIBG. This product was then refluxed in a solution of ^{125}I-sodium iodide for 72 hours. During this period, the radioactive iodine atom exchanged for the stable isotope on the mIBG molecule. The unreacted radioiodine is then removed by means of ion exchange chromatography. This yielded mIBG with a specific activity of 30MBq and a radiochemical yield of 70%. Attempts to increase the specific activity by this method resulted in a decreased radiochemical yield. The long incubation period is also inadequate for the production of ^{123}I-mIBG, therefore alternative methods of synthesis were developed.

Manger was able to reduce the exchange period to 2 hours (Manger *et al.*, 1982). The thermal decomposition of an ammonium salt in air provided acidic conditions for this reaction which was completed at 150°C. A further modification enabled the reaction time to be shortened by one hour (Mock and Weiner 1988).

All of the above methods involve an iodide exchange step. Any exchange method will result in a lower specific activity preparation where 'cold' non-radiolabelled carrier molecules, are inextricably mixed with the active radiolabelled molecules. This leads to a poor therapeutic differential between target and non-target tissues. Even with the relatively high specific activity preparations used in clinical practice, the specific activity remains $>1.11 GBqmg^{-1}$ and in this preparation approximately one of every 2,000 molecules is radiolabelled.

2.2.2. No Carrier Added (n.c.a.) mIBG

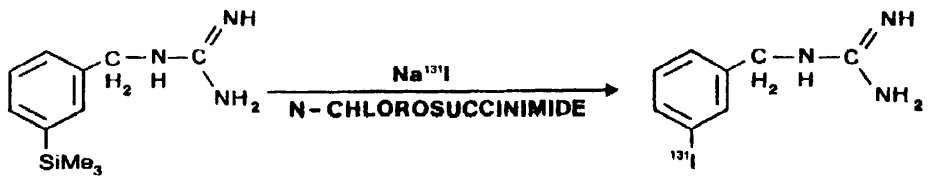
An exciting breakthrough in 1993 by Vaidyanathan and Zalutsky (Duke University, USA) resulted in a new chemical method of manufacture where almost all of the molecules of mIBG were labeled with the appropriate isotope (Vaidyanathan and Zalutsky 1993). This became known as no carrier added (n.c.a.) preparation.

Tumour uptake occurs by two processes: specific, high affinity, active transport (uptake 1) and passive diffusion (uptake 2). Most normal tissues,

unless sympathetically inervated, will concentrate the drug by uptake 2 mechanisms. The uptake 1 process will predominate at low concentrations of mIBG whereas nonspecific accumulation of ^{131}I-mIBG will occur at high concentrations. Therefore high specific activity preparations will exploit type one uptake and be more specifically accumulated by the tumour.

The iododesilylation method (Vaidyanathan and Zalutsky 1992) involves the addition of $Na^{131}I$ and N-chlorosuccinimide to m-trimethylsilybenzylguanidine (Figure 2.3). This reaction continues over 5 minutes at room temperature and gives excellent radiochemical yields of 98%.

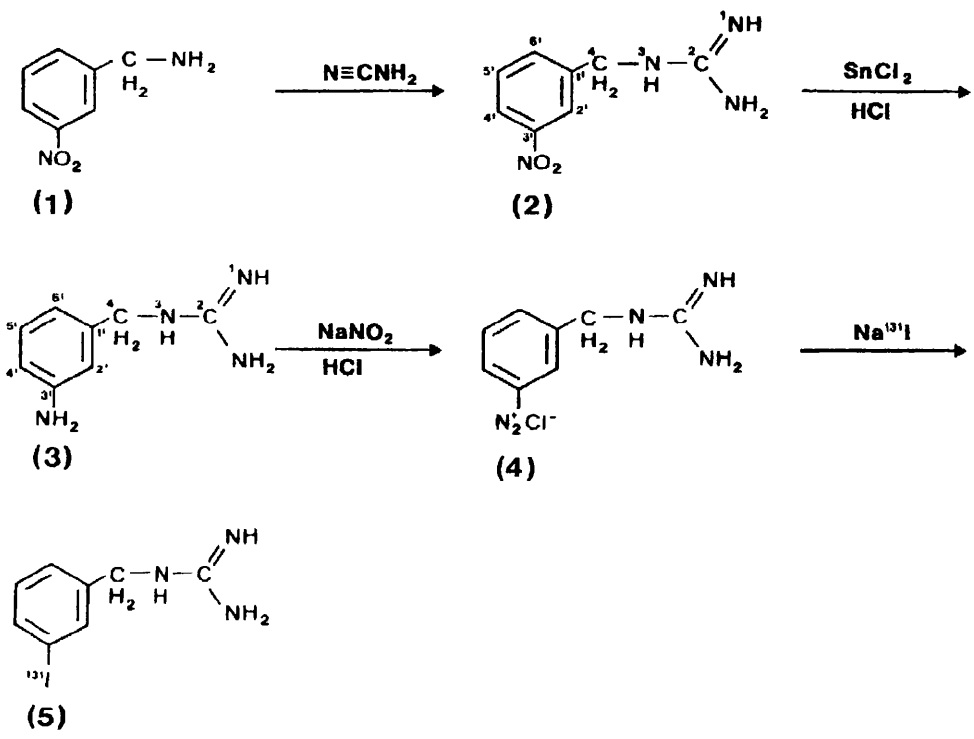
Figure 2.3. mIBG synthesis by the iododesilylation method of meta-trimethylsilylbenzylguanidine.



1 2

Figure 2.4 shows the synthesis of mIBG from a metadiazo derivative of benzylguandine. The reaction time necessary is similar to the iododesilylation method outlined above but the radiochemical yield is much lower, 13.4% compared to >98% by the iododesiylation method outlined above (Mairs *et al.*, 1994).

Figure 2.4. The synthesis of mIBG from a metadiazo derivative of benzylguandine



A typical therapy dose (7.4GBq) of commercially produced ^{131}I-mIBG contains at present 5mg of cold carrier mIBG molecules and plasma levels reach 100nM. The same radiation dose, if the n.c.a. preparation is used, would be 50μg in comparison with an equivalent plasma dose of 1nM. The n.c.a. preparation has at present reached the stage of clinical imaging in The Beatson Oncology Center, Glasgow.

2.3. IMAGING WITH mIBG

The first published report of mIBG use in neuroblastoma describes a case report of a two and a half year old child with strong localisation in an abdominal neuroblastoma (Kimmig *et al.*,1984). One year later, Geatti published ten cases where the uptake seemed so high that therapy appeared possible (Geatti 1985). Soon reports documenting localization in other

tumours of neural crest origin were commonplace (Endo *et al.*, 1984; Smit *et al.*, 1984; Von Moll *et al.*, 1987). These are listed below.

Table 2.1. Tumours imaged with I-mIBG (Von Moll *et al.*, 19870

phaeochromocytoma	chemodectomas
merkel cell skin cancer	small cell carcinoma
medullary thyroid cancer	schwannoma
carcinoids	choriocarcinoma

2.4. SENSITIVITY AND SPECIFICITY

Hoefnagel in 1991 reviewed over 2400 published cases where ^{131}I-mIBG had been used for diagnostic purposes. The high sensitivity (81%-91%) and specificity (95%-100%) of mIBG with phaeochromocytoma and neuroblastoma diagnostically led to its use as an imaging agent and later for therapy.

Table 2.2. The sensitivity of mIBG scintigraphy (Hoefnagel *et al.*, 1991).

DIAGNOSIS	PATIENTS	SENSITIVITY
Phaeochromocytoma	>1000	88.2%
Neuroblastoma	841	91.0%
Carcinoid	237	69.8%
Medullary thyroid carcinoma	178	34.5%
Neural crest tumours	144	39.6%

Table 2.3. The specificity of mIBG scintigraphy in neuroblastoma (Hoefnagel *et al.*, 1991).

	SCINTIGRAM	
NEUROBLASTOMA	POSITIVE	NEGATIVE
True	109	5
False	0	16
Total	109 (95.6%)	21 (4.4%)

2.5. PATIENT ADMINISTRATION

2.5.1. Storage and administration

The major contaminant of any I-mIBG solution is free iodine. This must constitute less than 5% of the injected dose. Decomposition occurs more rapidly in the light and with elevated temperature. Therefore clinical ^{131}I-mIBG is stored frozen and thawed by immersing it in a waterbath one hour beforehand. It is diluted in 0.9% saline and given as a slow iv. bolus injection since theoretically ^{131}I-mIBG can displace catecholamines and result in a hypertensive crisis.

2.5.2. Thyroid blockade

Any free iodine will be avidly accumulated by the thyroid gland. Lugols iodine should be administered 48 hours beforehand and continued for 5-7 days. Despite this precaution, even in fully compliant patients, thyroid accumulation is seen in 80% of therapy doses (Moyes *et al.*, 1985).

2.6. PHARMACOKINETICS

2.6.1. Pharmacokinetics

mIBG biodistribution can be described by an open three compartment model. Clearance follows a bi-exponential pattern, with a rapid initial phase and terminal mean biological half life of 35 to 37 hours (Ehninger *et al.*, 1987; Lashford 1988).

Clearance is predominantly renal (132 ml/min./m^2); However the total body clearance of 189ml/min/m^2 represents the renal excretion, plus other routes. This is probably the dissipation of the drug from the plasma to the tissues. To support this, the volume of distribution of the drug is large (307l/$min^{-1)}$ suggesting that much of the ^{131}I-mIBG is sequestered in tissues and then slowly released (Ehninger *et al.*, 1987).

2.6.2. Biodistribution

In the rapid initial phase, renal levels peak at 5 minutes but drop to a third of the value within five minutes. This is the major route of excretion over the next 24-48 hours. This means that urine passed by the patient will be contaminated, which has implications for false positive scanning, and, if urine is spilled, radiation protection.

Hepatic activity also rises over the first five minutes and thereafter remains constant due to the volume of the organ and blood flow. The liver has no specific uptake and the rapid increase in dose within the first five minutes is probably due to avid binding to non specific mucopolysaccharide molecules. Evidence to support this has been shown in the laboratory situation, where mIBG binds avidly to gels and interacts with small molecules.

Cardiac levels also peak rapidly but fall again within half an hour. The accumulation in heart tissue is inversely proportional to the level of circulating catecholamine in the plasma and blood. The peak levels, however, can be significant and this is the major barrier to the use of ^{211}As-mABG. This problem is the subject of intense research at present as blockers of cardiac uptake are being developed.

Table 2.4. The human biodistribution of mIBG represented as organ uptake with time after injection (Feine *et al.*, 1987).

ORGAN	30 minutes	6 hours	24 hours	48 hours
SALIVARY GLANDS	-	++	++	+
LUNGS	+	+	-	-
LIVER	+	++	++	+
SPLEEN	-	-	-	-
KIDNEY	++	-	-	-
ADRENALS	-	+	+	+
HEART	-	++	++	+
TUMOUR	+	+	++	++

Tumours are usually visible in scintigraphy between 24 and 72 hours post injection. Pulmonary uptake appears at 1-4 hours and is thought to be due

to passive type 2 uptake by endothelial cells. Interestingly splenic uptake is not seen in children but does occur, over time, in adults due to sympathetic innervation of this organ. It is not clear how mIBG reaches the lumen of the bowel but mIBG can be localised to the small bowel 18 hours post dose and later in the large bowel at 24 hours. This could be due to swallowed saliva or alternatively due to the autonomic innervation of the bowel. Incidentally, the salivary gland uptake mentioned is mediated by neuronal mechanisms since reduced uptake is seen in Horners syndrome (Nakajo *et al.*, 1984).

2.6.3. Metabolism

The biodistribution is unchanged at 72 hours but 60% is excreted after 24 hours, and only 10-25% remains after four days. The majority of product remains unmetabolised due to the guanidine side chain. Below is a summary of the urinary metabolites and their relative amounts (Manger *et al.*, 1986). Extremely small amounts (1-4%) are excreted in the faeces, saliva, sweat and exhaled breath.

Table 2.5. The urinary metabolites of mIBG

URINARY METABOLITE	RELATIVE AMOUNT	COMMENT
mIBG	84-89%	some in vivo deiodination does occur
^{131}I-Iodide	2-6%	can be high if catecholamine levels high
^{131}-I-metaiodohippuric acid	2-10%	
131I-metaiodobenzoic acid and ^{131}-I-4-hydroxy-3-iodobenzylguanidine	<2%	

2.6.4. Serum Concentrations

Peak serum concentrations during therapeutic infusions are one tenth the saturating concentration of 1μM. Animal experiments confirm that using doses of 0.003 to 30μM does not change the above biodistributions (Shapiro and Gross 1987).

In vitro studies suggest that short exposures to high concentrations of mIBG would optimize loading of mIBG. The rapid plasma to tissue translocation and early renal clearance results in short lived exposure but estimates of tumour accumulation by therapy doses of ^{131}I-mIBG suggest tumour concentrations of 3-30Gy are possible (Moyes *et al.*, 1989., Smets and Rutgers 1991).

It must be borne in mind that non target tissues are exposed despite this tumour specificity. In view of this, Lou Smets and Maria Rutgers suggest pre-dosing with unlabelled mIBG could block these non target tissues (Smets and Rutgers 1991). In contrast the n.c.a. preparation with small concentrations of I-mIBG exploit the specific uptake mechanism and reduce normal tissue exposure.

2.6.5. Toxicity

Transient elevation of blood pressure at 6hrs has been seen in some patients but this returned to normal 2 hours later (Hartmann *et al.*, 1987). There are no reported cases of hypotension although mIBG was originally developed from an adrenergic blocking agent. Short lived nausea is seen commonly and lasts for 2-3 days (Treuner *et al.*, 1987). Transient elevation of liver function enzymes occurs within one week of treatment (Hartmann *et al.*, 1987).

As expected, bone marrow depression resulting in thrombocytopenia and leucocytopenia is seen. Despite normal tissue accumulation of mIBG in the liver, adrenal gland, heart and salivary glands, no long term toxicity of these organs have been reported in the literature.

2.7. THE CELLULAR UPTAKE OF MIBG

2.7.1. Type 1 Uptake

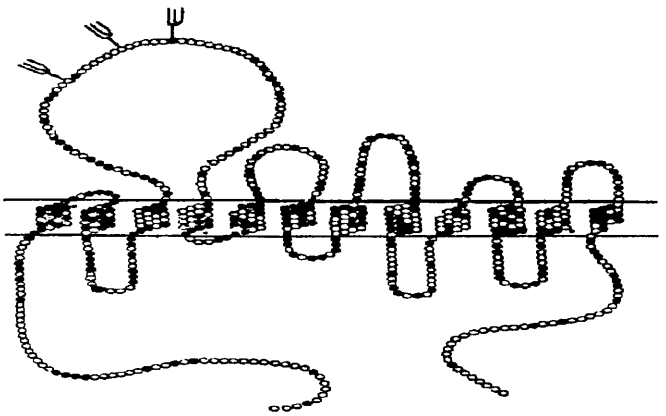
This specific type of uptake is used to terminate the synaptic signal and is characteristic of APUD cells and cells of the adrenal medulla. It is suggested that more differentiated cells express this type of uptake (Montaldo *et al.*, 1991). mIBG is accumulated via the noradrenaline transporter which permits effective accumulation of the drug, in fact thirty times that possible by passive diffusion. This type of uptake predominates at low concentrations of mIBG but becomes saturated at concentrations greater than 1μM (Smets *et al.*, 1989).

This process, however, is saturable, temperature, sodium and oxygen dependent and can be blocked by specific inhibitors of sodium-potassium dependent ATPase transport mechanisms, and monoamine reuptake inhibitors such as ouabin and desmethylimipramine. Noradrenaline is taken up by the same pathway and competitively inhibits uptake of mIBG if it is present in excess (Jaques *et al.*, 1984; Jaques *et al.*, 1987; Buck *et al.*, 1985; Gasnier *et al.*, 1985; Smets *et al.*, 1989; Ivaronne *et al.*, 1991; Montaldo *et al.*, 1991; Armour *et al.*, 1994).

2.7.2. The Noradrenaline Transporter

The human noradrenaline transporter has been isolated from SK-N-SH cells (Pachoiczyk, Blakely and Amamra 1991). The cDNA sequence predicts a protein 617 amino acids long, with a relative molecular mass of 69 kilodaltons. The N and C terminals are located on the cytoplasmic side of the membrane. The molecule contains 12-13 highly hydrophobic regions each 18-25 amino acids long which probably represent membrane spanning domains. The resulting structure also presents three possible glycosylation sites extracelluarly (Figure 2.5).

Figure 2.5. The noradrenaline transporter structure (*modified from* Pachoiczyk *et al.*, 1991).



The molecular sequence bears striking homology to the GABA transporter. 46% of DNA sequence is identical (68% allowing for conservative amino acid substitutions). These common areas are responsible for accumulating neurotransmitters against concentration gradients by means of coupling to the transcellular gradients of Na and Cl. The properties of this transporter are characterised by type 1, active uptake.

2.7.3. Type 2 Uptake

This form of uptake is, in contrast, unsaturable and sodium independent. It is however temperature dependent (Armour *et al.*, 1994). This is not non specific binding since the uptake increases linearly and is prominent at very high concentrations of ^{131}I-mIBG. This is probably due to the accumulation of small charged ions by electrochemical gradients (Lampidis *et al.*, 1989).

2.7.4. Type 3 Uptake

Experiments have indicated that, in the heart, non-neuronal mechanisms may account for 13-61% of cardiac uptake (Sisson *et al.*, 1987). Little is known at present about this type of uptake, but it appears only to occur when type 1 and type 2 uptake mechanisms have been blocked in pharmakokinetic studies of the rat heart. It may be due to passive diffusion mechanisms and intracellular binding (DeGrado, Zalutsky and Vaidyanathan 1995).

2.7.5. Neuroblastoma Cell Lines

Many neuroblastoma cell lines including SK-N-LO, IMR32, (Buck *et al.*, 1985), LNB-1, LA-N_1, CHP-212, N_1E115, NB4A3, Neuro-2A, (Smets *et al.*, 1989). SK-N-MC, N18 (Geurreau *et al.*, 1990), GI-LI-N AND GI-CA-N (Montaldo *et al.*, 1991) show no ability for active uptake of mIBG. SH-N-SH and its subclones, SH-SY-5Y and SH-EP (Buck *et al.*, 1985; Smets *et al.*, 1989; Geurreau *et al.*, 1990), NB-100 (Smets *et al.*, 1989) NB-G (Paffenholz *et al.*, 1989), LAN-5 (Montaldo *et al.*, 1991) and SKNBE(2c) (Montaldo *et al.*, 1991) demonstrate both active and passive uptake.

2.7.6. Storage and Retention

The radiobiological effect depends on the dose and time exposure of the therapy dose given. Therefore the intracellular storage location of the mIBG molecule is important. In phaeochromocytoma and the normal adrenal medulla mIBG is stored in the neurosecretory granules (Gasnier *et al.*, 1986; Smets *et al.*, 1989). In neuroblastoma cell lines however, the cytoplasmic location of mIBG suggests it may not be stored in this way (Geurreau *et al.*, 1990) but extragranularly, associated with the mitochondrion (Gaze *et al.*, 1991). This has obvious implications for the type of radioisotope used as the nucleus would be the obvious radiation target.

The other implication is that if ^{131}I-mIBG is stored differently within tumour and normal cells, there exists a possibility of exploiting this difference e.g. giving reserpine to deplete normal tissues or finding a pharmacological agent that will specifically block egression from tumour cells.

The intracellular level of mIBG is a result of a dynamic equilibrium of uptake and passive leakage from the cell. The level is not depleted by reserpine nor is exocytosis induced by acetylcholine or membrane depolarisation. Similarly this is oblivious to changes in calcium and potassium.

2.8. The METABOLIC EFFECTS OF mIBG

In clinical practice, the lethal effect of the radionuclide mIBG is due to the radioisotope attached. However, mIBG is itself cytotoxic when concentrations greater than 10mM are used (Bruchelt *et al.*, 1988). The cytotoxic effect is not limited to neural crest cells and appears mediated dependent upon the guanethidine side chain. The target is the cytoplasm (Smets, Bout and Wisse1988). The effects are primarily upon mitochondrial respiration. mIBG appears to interfere with glucose metabolism, by stimulating anaerobic glycolysis resulting in an increase in lactate production. The degree of cytotoxicity seems proportional to the degree of disruption of this glucose metabolism (Loesberg *et al.*, 1990).

Studies in isolated mitochondria show that mIBG selectively inhibits complex 1 of the mitochondrial respiratory chain, but that oxidative phosphorylation remains intact.

2.9. TREATMENT WITH mIBG

2.9.1. Refractory Neuroblastoma

mIBG was used for therapy for the first time in 1985 (Lumbroso *et al.*, 1985). In 1986 an international conference was held in Rome as clinical experience increased. From 76 heavily pretreated patients, 12 (16%) had responses of 90% or more. 14 (18%) had a response of over 50%; 50 patients (66%) had tumour shrinkage but less than 50% of the original size of tumour (Mastrangelo and D'Angio 1987). Groups have continued to treat patients with refractory disease. The main findings are that prolonged responses are seen, improving survival with little toxicity (Hutchinson *et al.*, 1991). In general patients with bone marrow involvement respond poorly and suffer toxicity (Matthay *et al.*, 1991)

The encouraging results in heavily pretreated patients with limited toxicity led clinicians to use mIBG in fitter patients, pre-chemotherapy. Mastrangelo (Mastrangelo *et al.*, 1993) still concerned about possible bone marrow suppression, used mIBG in 3 stage 3 patients. These patients have no bone marrow involvement by definition. He found that one patient had a

complete response with the two others showing a considerable reduction in tumour mass. There was no mIBG uptake in residual disease. Subsequent publications from this group indicate that these tumours, on relapse, still did not uptake mIBG suggesting that the mIBG effectively eradicated the mIBG sensitive clones. This group, encouraged by the lack of toxicity, went on to treat stage 4 patients.

2.9.2. Localised Disease

Strong radiobiological reasons exist for using mIBG when the patient first presents (See chapter 3). Hoefnagel and his colleagues therefore used mIBG when the patients first attended and found it rendered 80% of tumours surgically resectable (Hoefnagel *et al.*, 1991). There was some hesitation initially in using this approach for stage 4 patients, since widespread disease of varying size is present, and ^{131}I-MIBG is certainly suboptimal for very small tumour deposits, less than 1mm.

2.9.3. Combination Therapy

Meta-iodobenzylguanidine has also been used in combination with high dose chemotherapy and bone marrow rescue. (Corbett *et al.*, 1991). The rationale behind this is outlined in chapter three.

2.9.4 Palliative Therapy

When needed, mIBG can be effective in the acute palliation of symptoms, Treuner noted resolution of bone pain and fever as soon as 3 days (Treuner *et al.*, 1987).

2.10. Types of Radionuclide

Three types of radiolabelled isotopes of iodine exist for imaging and therapy. More recently astatine has been investigated.

Table 2.6. The properties of different isotopes

nuclide	decay	T1/2	energy (kV)	emission	comment
^{131}-I	γ,B	8.05 d	637, 723	B 69-190KeV	compromise
^{123}-I	γ,electron capture	13.2 hr	159	low energy electron	Near ideal problems expense and supply
^{125}-I	γ,electron capture	60 days	35	auger electron	
^{211} As	γ,electron capture	7.2 hr	6.87	5.9 MeV	high LET therapy

2.10.1 ^{125}I-mIBG

^{125}I-mIBG has been used primarily for biodistribution studies, where its long half life and low photon energy have practical advantages. The 14-21 low energy auger electrons emitted, have a path length of 1μm (Charlton *et al.*, 1978) and may be useful for therapy delivering selective radiation to

the cells accumulating the agent rather than irradiating neighbouring, possibly normal cells.

2.10.2 ^{123}I-mIBG

Both the half life and emission characteristics of ^{123}I-mIBG make it attractive for scanning and therapy. The short half life results in high activity being delivered to the tumour while the favourable gamma energy emitted is 159keV. This is sufficient for therapy and imaging, being free of β emissions and is similar to the gamma energy of ^{99m}Tc (140keV) so that ^{123}I-mIBG can be imaged easily with modern gamma cameras and the extremely sensitive SPECT (Single Photo Emission Computer Tomography) without additional shielding and radiation protection precautions. It has been confirmed in clinical studies that ^{123}I-mIBG is a superior imaging agent to ^{131}I-mIBG (Shapiro *et al.*, 1983). The major limitation of ^{123}I-mIBG at present is that, since it is manufactured in a cyclotron, it is expensive and not readily available. In addition, the short half life demands same day production and transportation of the agent for clinical use. A further disadvantage is that because of the short life prolonged scanning would not be possible.

2.10.3. ^{131}I-mIBG

^{131}I-mIBG represents a compromise between the three isotopes and is most widely used in clinical practice. The energy of the emissions is rather high,

364keV, requiring additional collimators to be fitted to the gamma cameras if imaging is desired. The same emission is efficient at cell kill but the range of the particle is such that neighboring cells are irradiated in a cross fire effect. The length of the half life allows for satisfactory tumour accumulation of radiation and repeated scanning to be performed if required.

2.10.4. ^{211}As-mABG

This isotope has a half life of 8 hours and is potentially the most lethal isotope, emitting high LET radiation, in the form of auger electrons, with a path length of 60μm in water. Impressive spheroid growth delay is seen with the above preparation, however preliminary animal studies demonstrate severe cardiac toxicity as well as tumour regression (Zalutsky *et al.*, 1994).

2.11 Summary

mIBG was deliberately synthesised in the search for an agent to image the adrenal medulla. Specific accumulation by neuro-endocrine tumours led to imaging then therapeutic use. The n.c.a. formulation should enable enhanced tumour specific uptake.

CHAPTER 3 THE RADIOTHERAPY OF NEUROBLASTOMA

3.1. INTRODUCTION

3.1.1. Radiotherapy in Neuroblastoma

The use of radiotherapy in neuroblastoma has traditionally been limited. In children with metastatic disease chemotherapy can induce effective remission. It is unnecessary in completely excised, node negative tumours but its role in incompletely surgically excised tumours is less clear.

Modern radiotherapy techniques include the use of total body irradiation and targeting agents. The regimens are based on scientific evidence and radiobiological modelling. They are used, in combination with other agents, in the setting of advanced metastatic disease.

3.1.2. The Radiobiology of Neuroblastoma

Radiobiological values are derived from *in vitro* work on human tumour cell lines and must therefore be interpreted carefully due the inherent limitations involved with doing so, but mean inactivation dose and SF_2 parameters, in particular, correlate well with clinical radioresponsiveness (Fertil and Malaise 1985). SF_2 is the fraction of cells surviving after a radiation dose of 2 Gy. This is clinically useful since it is more representative of a single radiotherapy treatment and more representative of the initial part of the slope of the cell survival curve (Deacon, Wilson and Peckham 1985; Steel and Wheldon 1991).

Table 3.1. Radiosensitivity parameters of cell lines (Fertil and Malaise 1985)

Group	Radio-sensitivity	α (Gy^{-1})	β (Gy^{-1})	D_0 (Gy)	mean inactivation dose	SF_2
1	high	0.54	7.6	1.35	1.52	0.27
2	moderate	0.38	4.2	1.47	2.1	0.43
3	low	0.28	4.6	1.16	2.49	0.51

Human neuroblastoma cell lines, *in vitro,* vary from one cell line to another but overall values of D_0, Dq, n and SF_2 indicate that they are highly radiosensitive and have little capacity for the accumulation of sublethal damage (Ohnuma *et al.*, 1977; Deacon *et al.*, 1985; Wheldon *et al.*, 1986; Plowman 1986; Holmes *et al.*, 1990). D_0, Dq, n are values defined in the multitarget model of radiobiology.

Do indicates the radiation dose required to reduce a cell colony to 0.37 of the previous value; Dq (the quasi-threshold dose) indicates the point at which the cell survival curve crosses the x axis. If this value is large, this indicates that the cell line can accumulate or repair SLD (sub-lethal damage) well; n is another mathematical parameter which indicates the number of critical DNA hits a cell can accumulate before cell damage is irreparable.

Holmes performed split dose recovery experiments on neuroblastoma cells, and found that this radiosensitivity was not due to poor repair mechanisms. Post radiation repair in some neuroblastoma cell lines can be greater than even radioresistant lines (Peacock *et al.*, 1988; Yang *et al.*, 1990; Holmes *et al.*, 1990). Radford, from his DNA neutral filter elution studies, suggested that the damage incurred by radiation may be greater (Radford *et al.*, 1986). Therefore, for neuroblastoma, this results in a higher incidence of both irreparable lethal as well as, reparable, potentially lethal damage (PLD).

This high susceptibility, of neuroblastoma, to single hit, lethal damage suggests that multiple small fractions, could induce significant tumour kill,

particularly that of critical late responding tissue. Theoretically therefore, on the basis of the above radiobiological evidence, it would seem that radiotherapy treatment of neuroblastoma should be by many small fractions. It is essential that the treatment time should not be excessively prolonged, to avoid repopulation of the tumour by clonogenically still active cells.

Extending this reasoning, it should be particularly suitable for targeting radiotherapy, as continuous low dose rate radiation could exploit the sensitivity of neuroblastoma cells to irreparable damage while minimising the effect to normal tissues. There is a critical dose rate effect however, since neuroblastoma cells, although radiosensitive, are not necessarily deficient at repair (Peacock 1988).

Traditional external beam treatment is given at dose rates of about 1Gy per minute. In targeted radiotherapy, the dose rate kinetics of dose delivered to the tumour deposit depend on the pharmacokinetics and pharmacodynamics of the carrier molecule as well as the properties of the radionuclide. In general, dose rates are low <20-30cGy per hour.

3.1.2.1. Repair and Repopulation

Repair of PLD has been observed in neuroblastoma cells in culture when dose rate falls below 2cGy per min (Holmes *et al.*, 1990). However repopulation only occurs when dose rates fall to much less.

3.1.2.2. Redistribution

During low dose rate therapy, cells blocked in the cell cycle redistribute into more radiosensitive parts of the cycle. This usually enhances the effect of the radiation but inevitably the effect will be influenced by repopulation. In practice this contributes little to outcome over the treatment period.

3.1.2.3. Reoxygenation

Hypoxic cells dominate the response of tumours to repeated large fractions of radiotherapy. In protracted courses of radiotherapy, these cells may gain access to more oxygen through redistribution of blood flow and because of lower oxygen utilisation. This makes this relatively hypoxic group of cells more sensitive. There is insufficient time for reoxygenation to occur during targeted therapy but the oxygen enhancement ratio for low dose rate therapy is small anyway. Therefore redistribution and reoxygenation, usually favourable factors, enhancing sensitivity to radiation, are of little benefit here.

3.2.1.4. Fractionation

The advantage of fractionation in sparing late responding tissues is limited in this situation since low dose rate radiation already does this. The exception would be if repeated dosing up-regulated the uptake of the mIBG. Clincal evidence suggests that repeated doses of mIBG do not this.

3.2. THE RADIOTHERAPY OF NEUROBLASTOMA

3.2.1. Radical Radiotherapy

The role of radiotherapy is best reserved for the post operative setting in those with INSS stage 2B, 3 or 4 disease (Jacobson *et al.*, 1983; Jacobson *et al.*, 1984; McGuire *et al.*, 1985; Castleberry *et al.*, 1991). Indications for treatment would be incomplete resection, spilled tumour at surgery and node positive disease.

Doses required for external control are however age related. Jacobson noted on review of 58 patients, treated with radiotherapy, that effective local control could be achieved with 15 Gy in children less than one year (Jacobson *et al.*, 1984). Earlier reports suggested local control could be established with smaller doses. Jacobson used 12 Gy, but in this particular series a four month old child treated died one month after treatment of sepsis with documented residual disease at post mortem (Jacobson *et al.*, 1983). Doses as low as this may therefore be inadequate to effectively control disease. For children aged one to two years, 15-25 Gy is required for tumour control. Older children appear to require larger doses: 40 Gy for children less than five years old and 50 Gy for older children (Jacobson *et al.*, 1984). Neuroblastoma in older patients probably represent a more biologically aggressive end of the spectrum, as older children are also more likely to harbour tumours of advanced stage disease with poorer biological risk factors.

3.2.2. Palliative Radiotherapy

Stage 4S patients are a heterogeneous group but a proportion of patients are destined to do well, with little or no treatment. However, gross hepatomegaly, if present, can induce respiratory compromise, inferior vena caval obstruction and compromise renal perfusion. Radiotherapy is reserved if organ function is threatened. Small doses, such as 4.5 Gy in three fractions, are adequate to induce tumour involution. This dose is too small to kill all the tumour cells but appears to provoke maturation.

Radiotherapy is given in small fractions of 1.5. to 1.8 Gy. This is an attempt to reduce late normal tissue damage. It should be noted that this strategy may not always be successful in very young children where growth and proliferation are continuing (Steel and Wheldon 1991).

In patients with incurable disease, treatment is less constrained by the long term potential morbidity of radiotherapy and the radiosensitivity of neuroblastoma can be exploited to control soft tissue and bone metastases effectively in one or two fractions of 6-8Gy (Halperin and Cox 1984).

3.2.3. Total Body Irradiation

Philip and Pinkerton (1989) reported a 20% 2 year survival, in a selected group of patients with recurrent neuroblastoma treated with intensive

therapy, compared to 0% in the control group. The use of megatherapy with or without TBI is based on this study. The European Bone Marrow Transplant Registry has reviewed 439 intensive therapy procedures (with bone marrow transplant) and concluded that TBI based procedures lead to a similar survival but that toxicity was greater, 16% in the TBI and chemotherapy group, compared to 8% in those ablated with chemotherapy alone (Landenstein and Philip 1992). Regimens using TBI are based on the following assumptions, $\alpha=0.85cGy^{-1}$; $\beta=0.065cGy^{-1}$ and finally, that total body irradiation, delivered in 7x2 Gy fractions results in a 6 log cell kill. Calculations suggest that this should be sufficient to sterilise small deposits of radiosensitive neuroblastoma cells of less than 1mm in diameter. The above regimens must therefore be unable to completely sterilise every potential neuroblastoma cell.

3.3. TARGETED RADIOTHERAPY OF NEUROBLASTOMA

3.3.1. Introduction

Radiation dose can be selectively delivered to tumours by carrier substances with radionuclides attached. This strategy depends on the target tissues being able to concentrate the carrier molecule to a high degree Ideally this would result in a high tumour dose, limited total body dose and therefore low normal tissue toxicity. The therapeutic efficiency of the dose given depends on the radiosensitivity of the neuroblastoma cells, the growth kinetics of the individual tumour and the dose rate profile of the

radiopharmaceutical used. The characteristics of the emitted radiation must also be considered.

3.3.2. Clinical Examples

Anti-neuroectodermal monoclonal antibodies have been developed for neuroblastoma targeted radiotherapy but unfortunately, cells other than those of the tumour also express the target epitopes resulting in a cross reaction with normal surface antigens. Differences between malignant and normal cells are therefore quantitative rather than qualitative. In addition, diffusion of macromolecular targeting agents into large tumour masses may be limited (Mairs *et al.*, 1992). Radiolabelled mIBG engineered for adrenal imaging, is dependent upon specific accumulation by a noradrenergic reuptake mechanism (Chapter 2).

In vitro studies of neuroblastoma cells expressing nerve growth factor (NGF) receptor have been shown uptake to preferentially ^{125}I-NGF (Mairs *et al.*, 1991). ^{131}Iodine labelled epidermal growth factor (EGF) although internalised and rapidly degraded, also shows enhanced killing of those cells that express EGF receptor (Capala and Carlsson 1991). Eventually it is hoped that targeting agents with specificity for cancer cell genes will be found.

3.3.3. Radionuclides

The effectiveness of any targeted therapy depends on the uptake of the agent, the site of its accumulation within the cell, and the range of the emitted radiation. Radioactive decay particles have a characteristic mean path length, over which distance potentially cytotoxic ionisations take place. There is an optimum size of metastases curable which is determined by the path length of each nuclide (Humm 1986.)

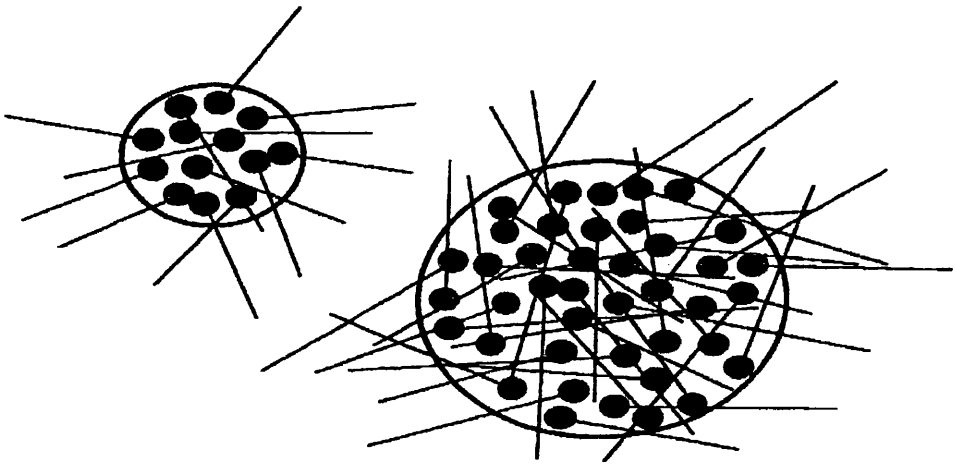
Table 3.2. Summary of characteristics of radioactive isotopes used in targeted radiotherapy.

RADIONUCIDE	HALF LIFE	EMITTED PARTICLE	RANGE	OPTIMUM SIZE
^{123}I	15 hrs	auger	1μm	1μm
^{125}I	60 days	auger	1μm	1μm
^{211}At	7 hrs	α	0.5mm	600μm
^{199}Au	3.1days	β	0.3mm	400μm
^{131}I	8 days	β	0.8mm	2mm
^{90}Y	2.7 days	β	5mm	4cm

Theoretically small tumour deposits have a small number of clonogenic cells and therefore require a small dose to sterilise them. Supposing the mean path range of the decay particle is greater than the size of the deposit, most of the disintegration radiant energy is deposited in surrounding tissue, outwith the tumour, contributing nothing to cure but causing toxicity. In a

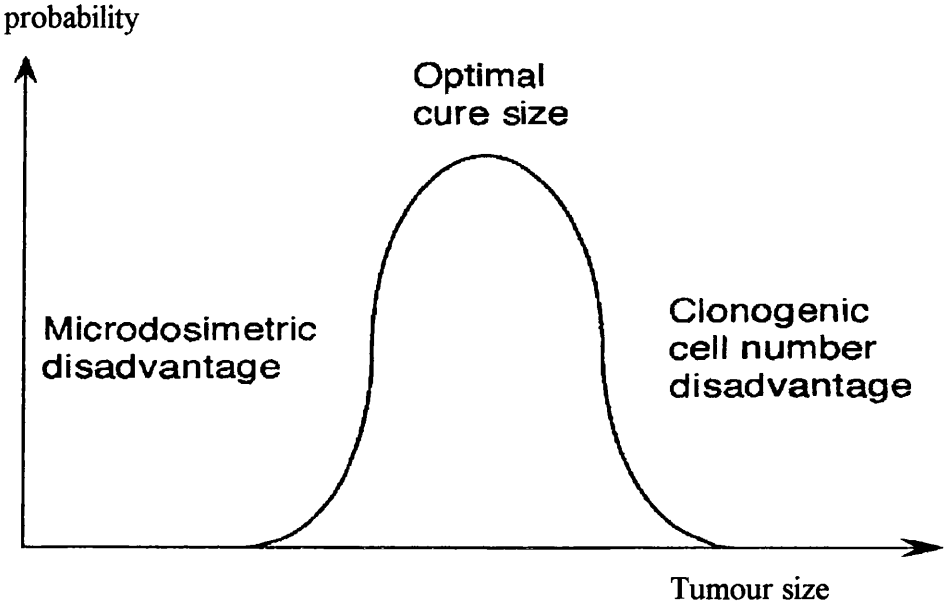
larger metastases the majority of the 'crossfire' radiation is absorbed within the tumour.

Figure 3.1. Model showing absorption of disintegration energy of isotopes in tumour deposits of various sizes.



There is an optimum size of deposit, where most of the radiation accumulated by the tumour cells is absorbed efficiently by the micrometastases and cure is likely. This is represented below (Figure 3.2.).

Figure 3.2. Diagram showing probability of tumour cure and tumour diameter (from Wheldon 1994)



Within any tumour, there is heterogeneity of uptake between cells and therefore between areas of tumour. If the path length of the emitter is sufficiently long areas that have not accumulated the targeting agent can still be irradiated. The path length of some β emitters can be several millimetres. With short range emitters, e.g. Auger emitters where the path length is short, the emitted disintegration energy of the isotope is absorbed within a single cell. To be of any benefit, an Auger emitter has to be delivered to the DNA of the cell.

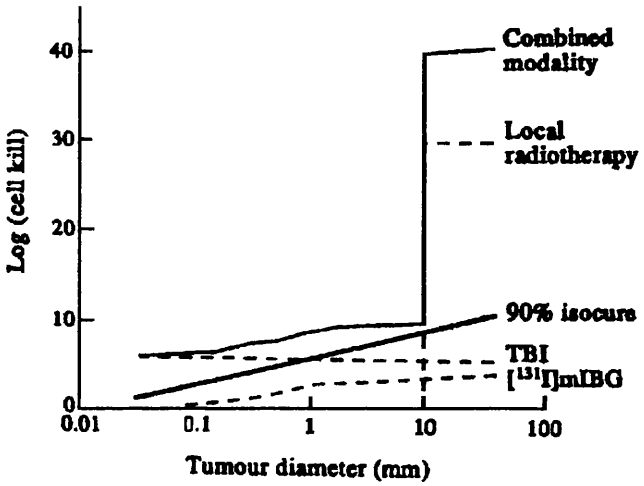
As tumour size becomes larger, the number of clonogenic cells increases, and the dose required for cure goes up. Since the maximum absorbed dose accumulated in large necrotic and hypoxic areas may be limited, the chance of cure decreases. In vitro work with ^{131} I-mIBG using spheroids of different sizes supports the above observations (Gaze *et al.*, 1992). Despite these unique characteristics and the specificity of targeted radiotherapy the use of mIBG as a single therapeutic agent would not be sufficient to eradicate all disease. (O'Donoghue *et al.*, 1991).

3.4. COMBINATION THERAPY

3.4.1. Introduction

Ablation of all sites of disease is a prerequisite for cure in neuroblastoma. Combination therapy for neuroblastoma exploits the complimentary characteristics of each modality.

Figure 3.3. Log kill versus tumour diameter for all components of combination therapy.



3.4.2. TBI Component

TBI is needed to ablate extremely small metastases, <1mm in diameter.

The dose of total body irradiation is determined by the intrinsic radiosensitivity of the tumour type. For neuroblastoma a minimum dose of 2 x 2Gy is sufficient (Amin *et al.*, 1993). The dose is also limited by whole body tolerance and in clinical practice 7 x 2 Gy has been tried (Gaze *et al.*, 1995). This probably results in overkill of very small single cell metastases. For neuroblastoma there may be an advantage for reducing the TBI component in order to increase the mIBG component. TBI should be fractionated to spare normal late tissues without compromising tumour cell kill.

3.4.3. mIBG

The optimum mIBG dose also depends on tumour radiosenstivity, uptake of targeting molecules, and the contributions from the external beam dose.

It is important that the critical tolerance of normal tissues is not exceeded. A total body dose of 2 Gy should not be exceeded. Typically 0.005 - 0.015 percentage uptake per unit mass of the injected radioactivity is absorbed by the tumour, over a period of two to three days. After this time, the dose rate will be too low for tumouricidal effect resulting in a proportion of the dose being wasted.

3.4.4. External Beam

External beam dose depends on the site and size of any residual local tumour and is limited by normal tissue tolerance. Doses in the range of 35-50Gy are used.

3.5. CONCLUSION

Local radiotherapy treatments are effective but used less commonly because of concern of long term morbidity from radiotherapy.

The prognosis for stage 4 neuroblastoma patients aged older than one year at diagnosis is poor. Relapse occurs within a predictable period (Collins, Loeffler and Tivey 1956). This implies the presence of occult disease in either the primary or metastatic sites. The combination strategy designed above covers both micro and macroscopic disease with encouraging results. Of the five poor prognosis patients treated, 2 were still alive at 17 and 18 months at the time of publication (Gaze *et al.*, 1995).

CHAPTER 4 CONTROL OF THE PRIMARY SITE IN METASTATIC NEUROBLASTOMA

4.1. INTRODUCTION

In 1982, a database of patients, treated at major paediatric oncology centres in Britain and Europe, was established by the United Kingdom's Children's Cancer Study Group (UKCCSG). For several years, relevant data, primarily concerning patient treatment, has been collected and the information obtained used to form the basis of research and clinical trials. The patients for the following analysis were obtained from this source enabling a substantial number of cases of this relatively rare tumour to be studied.

Only stage 4 patients were studied. This population of patients therefore represents a selected group of poor prognosis patients from specialist centres. This, however, is precisely the population of children with neuroblastoma in which cure rates remain poor and intensive research is still required.

The following chapter therefore performs a retrospective analysis of this sample population, with particular concentration on the effectiveness of local treatments in controlling the primary site in what is essentially a metastatic disease. During the analysis, it was also possible to examine prognostic factors influencing disease progression and survival.

The following results section is therefore divided into the following sections:

1. A description of this population of stage 4 patients.

2. An examination of the effectiveness of surgery and radiotherapy in controlling the primary site.
3. The sites and importance of residual disease after standard therapy.
4. Relapse and prognostic sub-groups.
5. Survival.

4.2. MATERIALS AND METHODS

4.2.1. Data Source

The original source of data was from details of patients registered within the United Kingdom Children's Cancer Study Group (UKCCSG) and European Neuroblastoma Study Group (ENSG) 'survey' data base. Additional information for complete analysis was obtained by surveying the participating centres directly.

The UKCCSG was formed in 1977 by a group of paediatric oncologists within the United Kingdom, with the aim of improving the management of children with cancer. The group is now much larger and includes interested pathologists, scientists and epidemiologists. Data managers have collected information on all patients with childhood cancer in Britain. Childhood tumours, being relatively rare, benefit from this clinical collaboration. The data collected is used to propose treatment protocols and to design future clinical trials.

The European Neuroblastoma Study Group is an independent European society with a specific interest in designing trials concerning the treatment of neuroblastoma. The survey database was initiated in 1982 and stored in the UKCCSG offices. It is, therefore, not a true population based registry but a database resulting from this international collaboration of clinicians from specialist centres. Basic patient details and proposed management were registered regardless of whether the patient was subsequently treated and followed up in a separate trial.

By May 1993, over 1,200 patients were registered but only those satisfying the criteria for Evans Stage IV (Evans *et al.*, 1971) or INNS stage 4 (Brodeur *et al.*, 1988) were selected for further study.

The database contained information on patient age, pattern of disease at presentation, disease extent and initial treatment. Details of surgical resection and pathological assessment of the surgically resected specimen were obtained by contacting the participating centres directly (Appendix 1). If it was indicated on this questionnaire that radiotherapy had been given, a separate questionnaire, asking for planning details was issued (Appendix 2). Non replying centres were subsequently re-contacted to increase the response rate. Follow up information was acquired from ENSG trial follow up forms along with the above sources.

This information, having been noted on specifically designed forms, was then entered on to a computer data spreadsheet (QPRO) and entry data checked

three times in total against the original patient data form. Details were converted to either categorical or continuous variables and a total of fifty seven variables listed for each patient. The types of variables used are summarised in Appendix 3.

4.2.2. Definitions

The extent of disease at presentation, after therapy and on relapse, was defined according to standard clinical diagnostic methods (Brodeur *et al.*, 1988).

The completeness of surgical resection for each patient was assessed by both the surgeon and pathologist, each independently examining the same resected specimen.

The extent of surgical resection was defined by the surgeon, according to three categories. Firstly, a complete surgical excision was defined by the surgeon as complete macroscopic removal of all visible tumour and involved nodes during the operative procedure. Pathological examination obviously later confirmed whether this was complete or incomplete. Partial excision was estimated by an assessment of the amount of residual tumour, for example 75%-99%. There was a separate category for less than 75% of tumour removed at the time of operation.

The pathologist was asked to examine the same resected tissue and comment on completeness of excision, firstly by macroscopic examination of the tumour

and nodes, then microscopically determining the extent of tumour at resection margins. In addition, the histopathology of the tumour was described. For statistical analysis, the most primitive tumour elements present were noted rather than the predominant component.

The documentation of residual disease at the primary site was based on the pathologist's assessment of surgical resection. Hence residual disease in the primary site could vary from microscopic residual disease through macroscopic residual disease to macroscopic non-resectable tumour.

The calculation of the external beam dose assumed α=0.85cGy, β=0.065cGy with a doubling time of 2 days.

Consolidation treatment was described as high dose chemotherapy, with or without radiotherapy, with bone marrow transplantation.

The end of treatment outcome defines the status of patient after initial chemotherapy and surgical excision, before additional high dose chemoradiotherapy, if appropriate.

Relapse was defined as a recurrence of disease, preferably histologically proven, in a patient previously free of neuroblastoma. The date of relapse provided an estimate of the time period from date of diagnosis to date of relapse and information on disease free survival. The length of time from date of diagnosis to death provided an estimate of the overall survival.

4.2.3. Statistical Analysis

Statistical analysis was performed by using a BMDP package (Dixon 1988) and graphical representation using Harvard Graphics. An initial examination of the data involved checking and examining the completeness of the data accumulated. 'Unknown' variables were, if possible, corrected. Otherwise missing data were checked for sources of error but were determined to occur in a randomly distributed manner.

Preliminary data analysis determined the basic characteristics of the study population. General information on age at diagnosis, sex and patterns of disease presentation and relapse were obtained in this way. These patterns were examined for sources of error, and checked. For example it was important to check if the patients in one subgroup had the same prognostic factors as the group(s) under direct comparison.

The next step involved a univariate analysis of variables e.g. relapse and death. Categorical variables were first cross tabulated and tested using the chi squared test. Ordinal variables, that is discrete variables connected in a specific order, for example patterns of metastases, were analysed using special chi-squared techniques. This type of information can be of limited use. For example, it is more informative to note not only whether or not, but how long it took to relapse. In this situation the disease free survival (dfs) indicates this variable more appropriately.

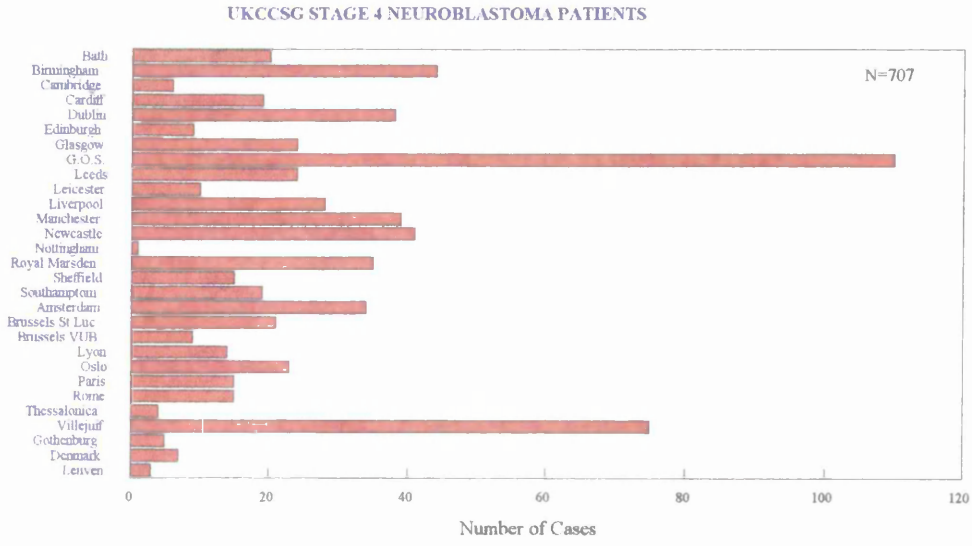
Continuous data were tested using Student's 't' test but an 'anova' test was necessary if more than two groups of variables were involved. Survival between different groups was compared using the 'Mantel Haenzel' test enabling two or more curves, rather than isolated points on a graph, to be compared. Prognostic factors were determined using 'Cox' multivariate analysis. These factors: age; sex; presence of bone marrow metastases at presentation and BMT were carefully considered throughout the analysis to ensure the variables were randomly distributed and did not result in bias.

4.3. RESULTS

4.3.1.1. Population of ENSG Stage 4 Patients

There were 727 patients registered, from 17 British and 12 European Centres, with the ENSG survey database from 1982 until May 1993. On average 60 cases were notified per year with no significant difference in the incidence of annual registered cases.

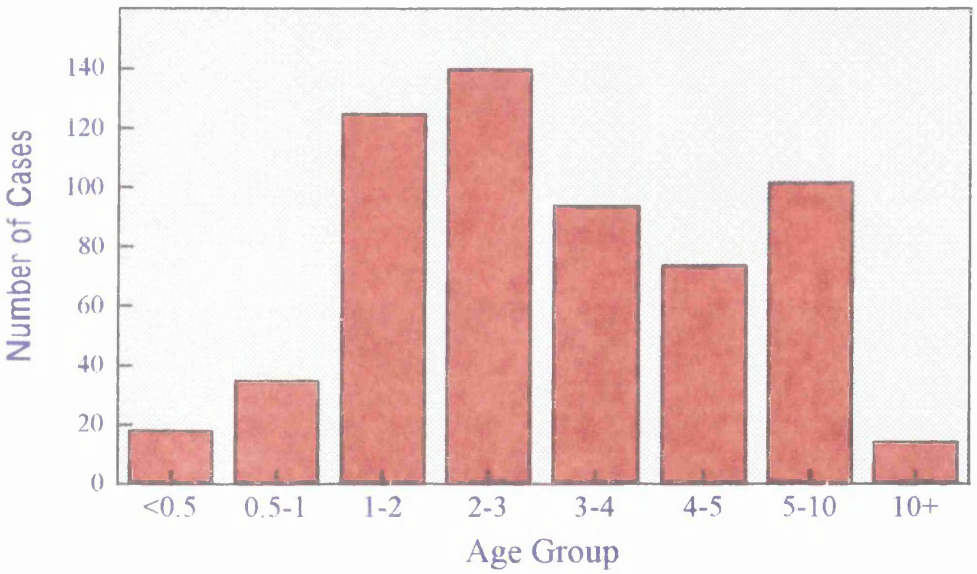
Figure 4.1. The number of ENSG Stage 4 patients recruited from each centre



4.3.1.2. Age

The mean age (SD) was 3.52 (2.76) years, with a range of 0-22.34 years. 73 (10.4%) children presented aged less than one year old at diagnosis. 80.6% of children presented before the age of five years and 20 children (2.8%) presented after the age of 10 years.

Figure 4.2. The age distribution of ENSG Stage 4 neuroblastoma patients.



Children aged less than one presented as follows: 25 patients less than six months; 12 aged six to eight months and 36 aged between eight months and one year.

4.3.1.3. Sex

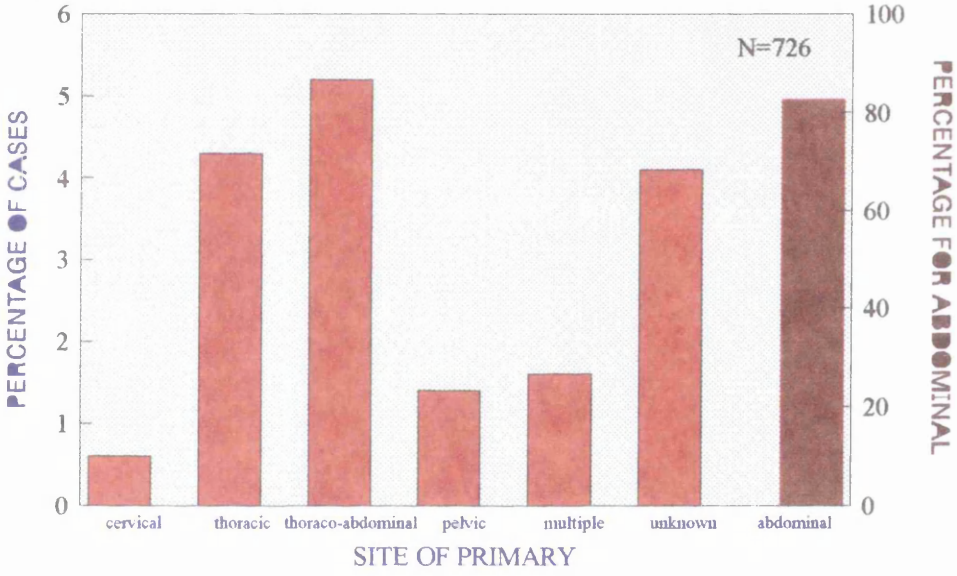
There were 402 boys compared to 304 girls in this series, indicating a slight male preponderance of 1.32:1.

4.3.1.4. Primary Sites of Disease

The abdomen was the site of primary disease in 601 patients (82.6%). This was followed by thoracoabdominal (38, 5.2%); thoracic (32, 4.3%); multiple (12, 1.6%); pelvic (10, 1.4%) and cervical (5, 0.6%) respectively.

In those aged less than one year at diagnosis the thoracic cavity was more commonly affected by the primary. In this series of stage 4 patients, the site of primary was not of prognostic significance.

Figure 4.3. Primary sites of disease.

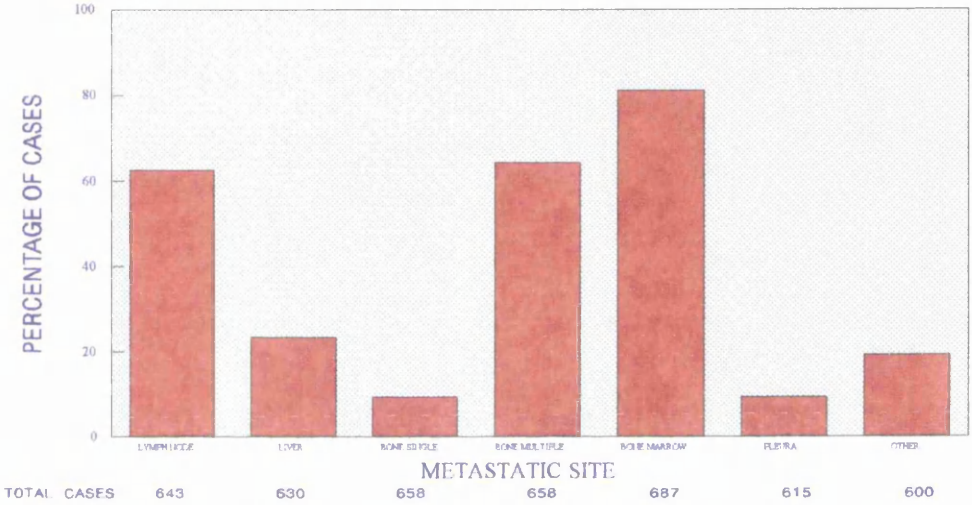


4.3.1.5. Metastatic Sites.

Bone (67.2%), bone marrow (76.6%) and lymphnodes (15%) were common sites of metastatic disease. The liver was involved in 20.4% of cases but never in the small number of cervical sites of primary. Similarly the pleura was an unusual site of spread, documented as 7.8% in this series and only in those cases of thoracic and thoracoabdominal sites of primary. A single bone

metastasis was seen in 8.4% of cases. The category defined as 'other' included various sites such as CNS, skin, testes and pancreas (15.8%);

Figure 4.4. Metastatic sites at presentation



There was no specific pattern of metastatic disease associated with the site of primary. The presence of bone or bone marrow metastases at presentation was associated with a poor outcome (p=0.002).

The number of metastatic sites involved at presentation was also found to be of prognostic significance (p=0.002).

Table 4.1. Correlation between the number of metastatic sites involved at presentation and outcome.

Number of met. sites	Alive NED	Alive with disease	Dead
0	2(50%)	1(25%)	1(25%)
1	27(36.5%)	3(4.1%)	44(59.5%)
2	26(13.6%)	25(13.1%)	140(73.3%)
3	19(12.9%)	11(7.5%)	117(79.6%)
4	16(20.3%)	4(5.1%)	59(74.7%)
5	3(13.0%)	2(8.7%)	18(78.3%)

4.3.1.6. Treatment

It was not a major aim of this study to analyse the systemic therapy given, nor was it necessary to treat the registered patient according to ENSG protocol. 495 patients received radical chemotherapy and 81% were treated by ENSG protocol. The remainder received similar regimens.

4.3.1.7. Population Summary

Children with neuroblastoma, present usually with an abdominal primary and widespread metastatic disease affecting the bone, bone marrow and lymphnode sites

Features of prognostic significance in this population are

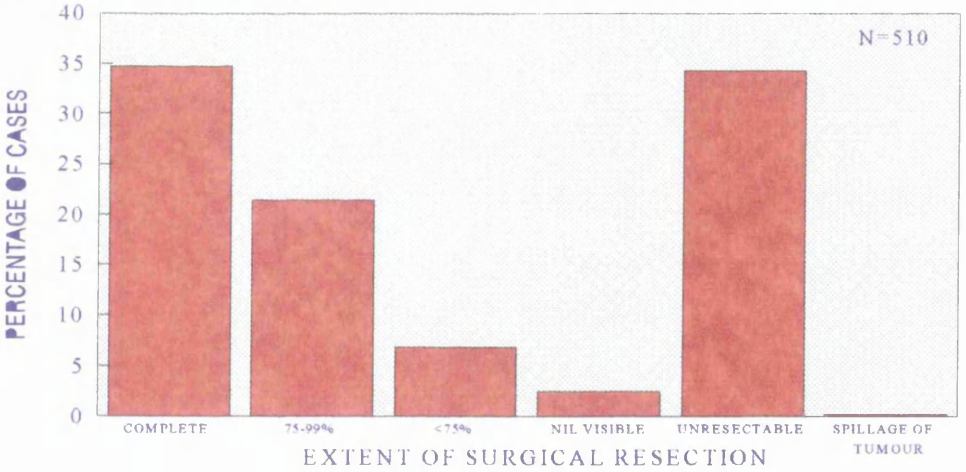
1. Aged less than one year at diagnosis ($p<0.0001$)
2. The presence of bone or bone marrow disease ($p=0.002$)
3. The number of metastatic sites involved at presentation ($p=0.002$)

4.3.2. Local Therapy For ENSG Stage 4 Patients

4.3.2.1. Surgery

In 510 cases, details of surgery are known. At the end of induction chemotherapy, surgery was still not possible in 175 (34.3%). The primary was considered technically unresectable or surgery contraindicated by the persistence of metastatic disease. Occasionally parental consent was refused or no visible tumour visualised by standard radiological means. The group receiving surgery is therefore comparatively selected, as it excludes those children who progressed, or died before completing chemotherapy or as a result of chemotherapy.

Figure 4.5. The surgeons estimate of the feasibility of surgical resection



311 (61.9%) patients had a surgical procedure performed to the primary site. 24 patients (4.7%) had surgery attempted at initial diagnosis. There was no difference in outcome if the surgery was performed 'upfront,' before any chemotherapy. Therefore for the analysis all patients undergoing surgery were considered as one group. Considering only the 335 cases who received surgery, the surgeon felt complete resection had been achieved in 53% of

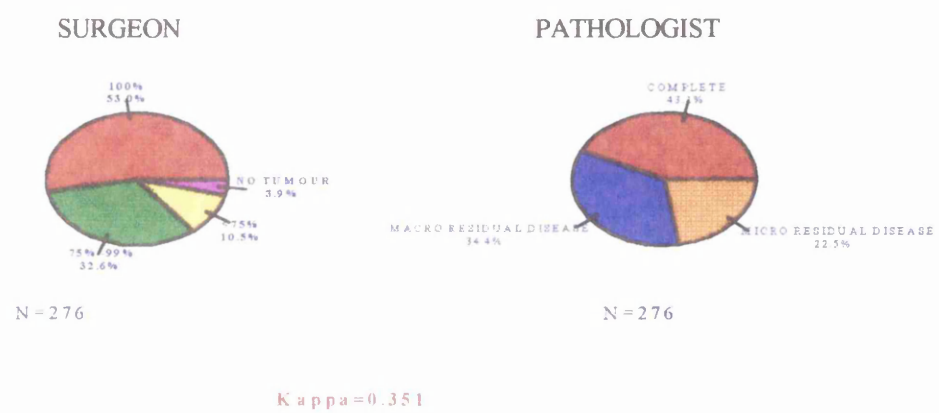
cases; 75-99% of the tumour had been removed in 33% and less than 75% of the tumour in 10%. No tumour deposits were visible at laparotomy, after chemotherapy, in 13 cases (4%). This indicates a good surgical selection of cases.

4.3.2.2. A Comparison of the Extent of Surgical Excision

A comparison was made between the extent of surgical resection and its effect on progression free and overall survival. It was found that the surgeon's estimate of completeness of resection, at the time of laparotomy, had prognostic significance concerning overall survival (p=0.047).

The pathologist's opinion on the same resected specimen was also noted and this showed, as expected, more extensive disease than the surgeon could estimate. This resulted in significant disagreement between the pathologist's and the surgeon's estimate of the extent of resection (kappa=0.351).

Figure 4.6. A comparison of estimates of complete resectio
In 276 cases, the extent of surgical resection, estimated by the surgeon at the time of surgery and the histological extent of surgical resection, determined independently by the pathologist were compared. A kappa value of < 0.4 indicates significant disagreement.

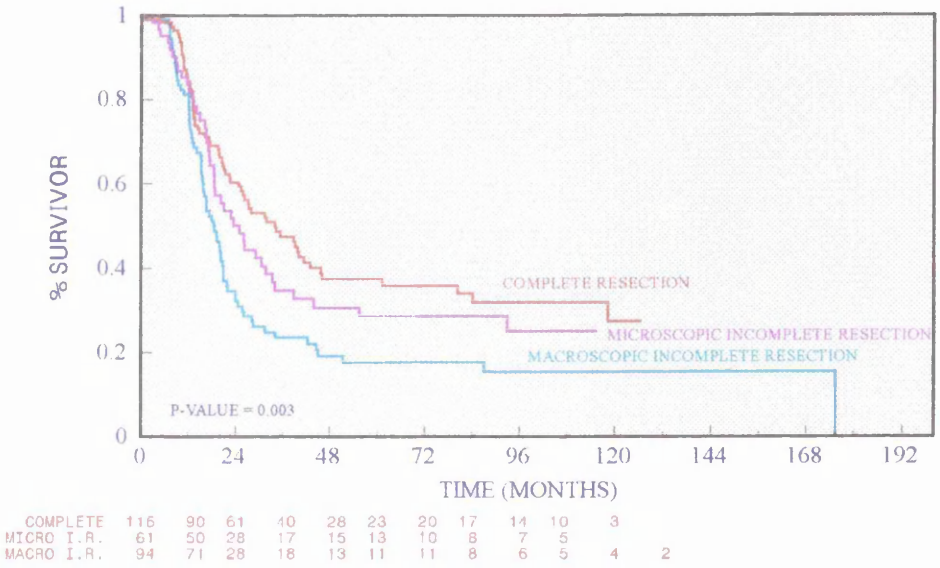


This point has been made since statistical analysis showed that the pathologist's estimate of the extent of surgical clearance was a more accurate predictor of outcome ($p<0.0001$ for progression free survival and $p=0.0032$ for survival). The pathologists estimate of the extent of resection is used to determine the extent of residual disease. The following categories therefore refer to complete resection, microscopic residual disease and macroscopic disease.

The graph below demonstrates the prognostic significance of the pathologists estimate of the extent of the surgical resection.

Figure 4.7. The effect of completeness of surgery on survival.

The following demonstrates a survival graph for 271 patients where the survival details and extent of surgical resection was determined independently by the pathologist.



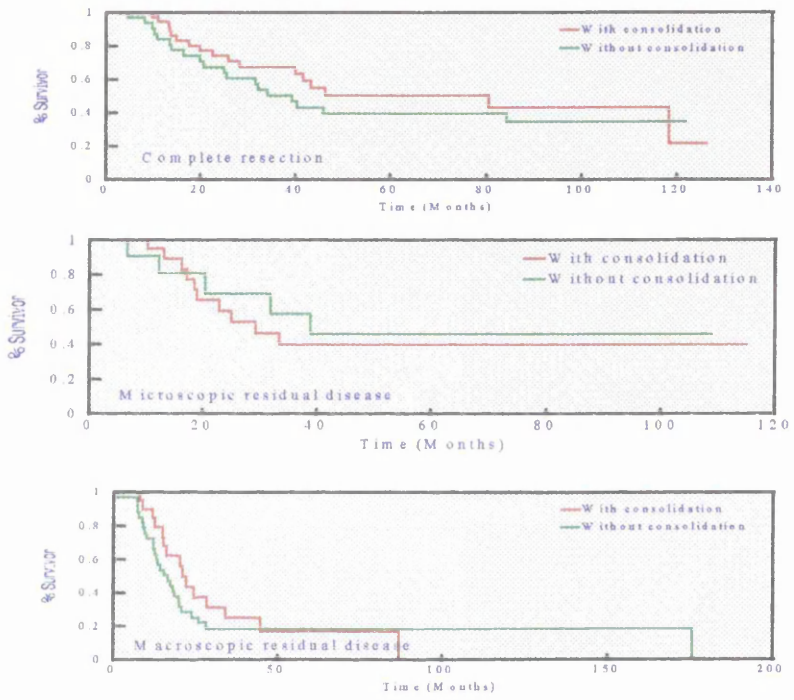
A multivariate analysis was used to determine the importance of each category of resection. Table 4.2. demonstrates that the categories of 'complete' and 'microscopic residual disease' groups have similar 'p' values. There is however, a larger difference if the surgeon leaves residual macroscopic disease, the outcome being similar to the non-resected group. This means therefore, that it is essential to obtain as complete a resection as possible.

Table 4.2. The significance of the extent of surgical excision

CATEGORY	APPROX. CHI SQ.	P-VALUE
complete resection	15.81	0.0001
microscopic residual	0.04	0.0625
macroscopic residual	0.5	0.477
non resectable	0.00	0.9647

This series of patients was large enough to determine the importance of complete excision in those patients undergoing a high dose consolidation procedure. The result was determined by comparing three groups of patients: Those with completely resected disease; Those with microscopic residual disease and finally those with macroscopic tumour remaining after surgery. For each of these three categories, patients were divided into those undergoing a transplant procedure and those not. The results showed that there was no significant additional advantage from the extent of surgery when comparing those in the transplant group ($p=0.12$).

Figure 4.8. The effect of the completeness of resection on patient outcome correlated with transplant.
For each of the above, histologically defined categories, 2 groups of patients are compared directly: those patients having a high dose consolidation procedure and those not.



4.3.2.3. Histology of Resected Specimens.

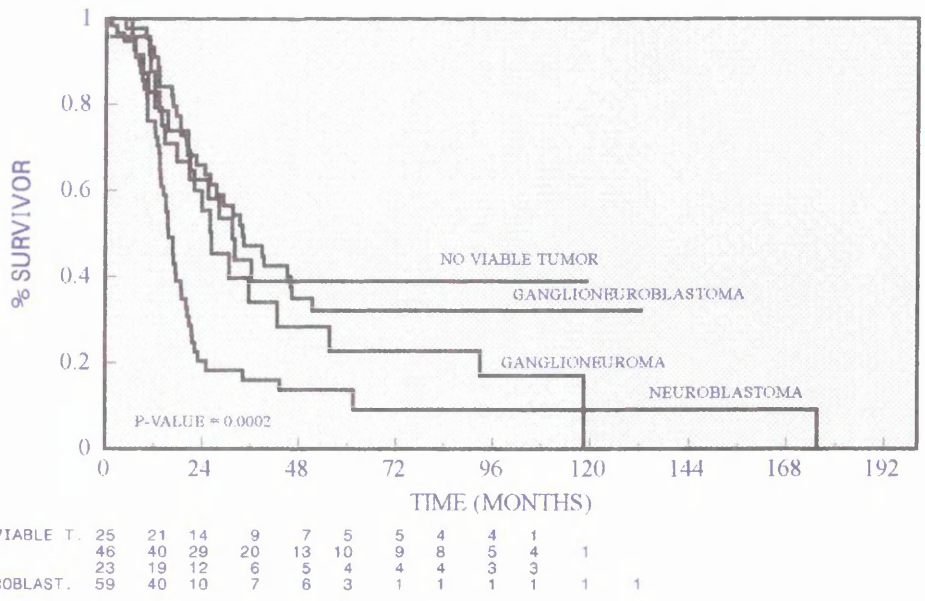
The pre-chemotherapy histology was known in 213 cases, with neuroblastoma predominating in 70% of cases. 25% of cases had ganglioneuroblastoma at presentation and only 5% ganglioneuroma. At the time of surgical resection, the majority of patients had been exposed to chemotherapy resulting in a different composition of histological types.

Table 4.3. The histology of resected specimens

TYPE	NUMBER (PERCENTAGE)
neuroblastoma	55 (34%)
ganglioneuroblastoma	41 (25%)
ganglioneuroma	25 (16%)
no viable tumour	23 (14%)
differentiated	14 (9%)

The histology of the resected specimen was an important prognostic variable with no viable tumour and ganglioneuroblastoma being associated with the best outcome (p=0.0002 for overall survival).

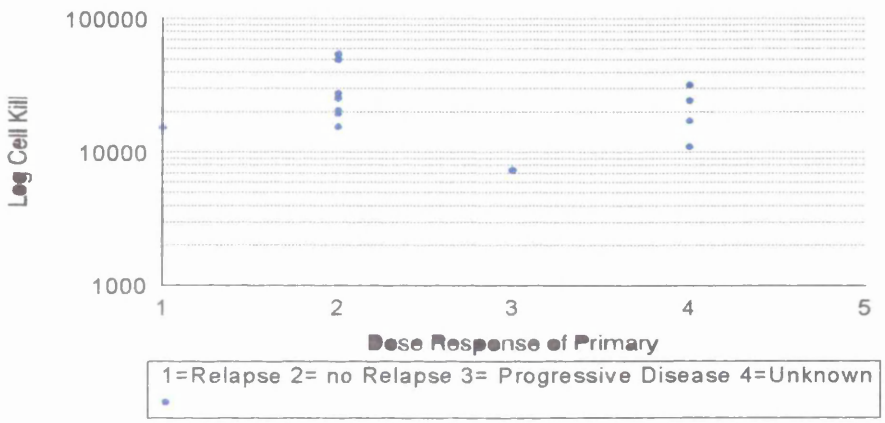
Figure 4.9. The relationship between histology of the resected tumour and survival.
In 153 cases the complete histopathological and survival details were known.



4.3.2.4. External beam radiotherapy

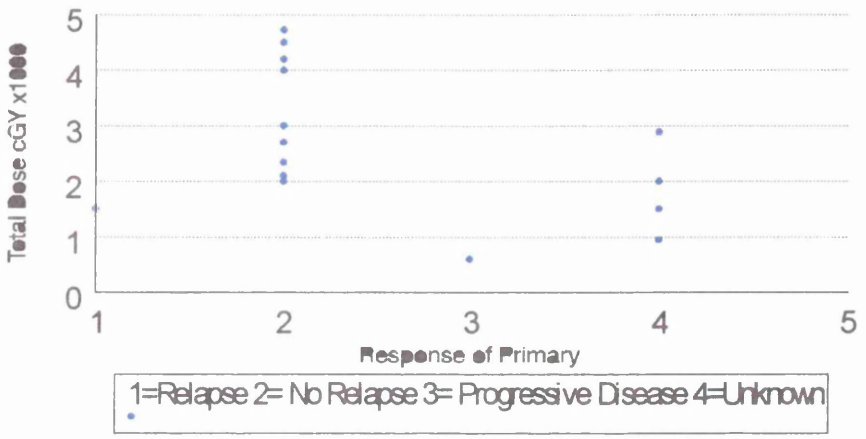
Data was available for only 17 patients. Therefore no firm conclusions could be made. A graphical representation suggested that the greater the degree of cell kill the more likely was control of the primary site.

Figure 4.10. Control of the primary correlated with cell kill



A greater degree of cell kill was achieved the larger the dose. Figure 4.11. below indicates clinical practice in that most children received doses greater that 2000cGy.

Figure 4.11. The relationship between dose and local control.



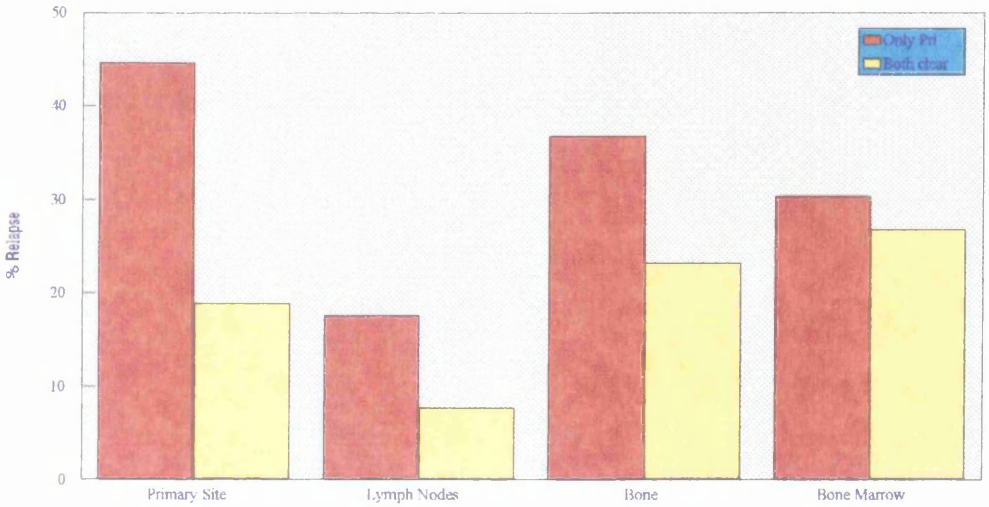
4.3.2.5. The Effect of Clearing the Primary Site

To observe the effect of clearing the primary site of tumour, an analysis was performed concerning two groups of patients. Firstly those children who are clinically and radiologically free of tumour and secondly, those children who have residual disease at the primary site but are free of disease elsewhere.

44.6% of those with residual disease in the primary site progressed at the primary site, compared to only 18.9% of those in whom the primary site was microscopically free of tumour ($p<0.0005$). Therefore clearing the primary site reduces the chance of local relapse.

Figure 4.12. The effect of clearing the primary site.

Two groups of patients are compared directly, those with a histologically confirmed clearance of the primary and those with residual disease at the primary site.



4.3.2.6. Summary of Local Treatments

- The surgical selection of cases is good.
- There was no difference in outcome if surgery was performed 'upfront.'
- The surgeon's estimate of the extent of resection has prognostic value ($p=0.047$).
- The pathologist's estimate of resection has much greater prognostic value ($p<0.003$).
- The surgeon and pathologist often disagree as to the extent of clearance ($k=0.351$).
- It is important to achieve as complete resection as possible as complete resection reduces local relapse and improves dfs and overall survival ($p=0.001$).
- The sample of patients having radiotherapy to the primary was small, therefore no firm conclusions could made.
- A surgical clearance of the primary site reduced local relapse from 44.6% to 18.9%.
- The histopathology of the resected specimen has prognostic significance ($p=0.0002$).
- Consolidation therapy and complete resection are independent prognostic factors.

4.3.3. Residual Disease

Patient disease status was assessed at this point after completion of induction chemotherapy and after surgical resection of the residual primary site, if this had been possible. Within this group of patients 145 underwent a high dose procedure involving bone marrow transplant. The status of metastatic sites was determined by standard radiological means. The status of the primary was determined by the pathologist's estimation.

Table 4.4. Sites of residual disease after induction therapy and surgery

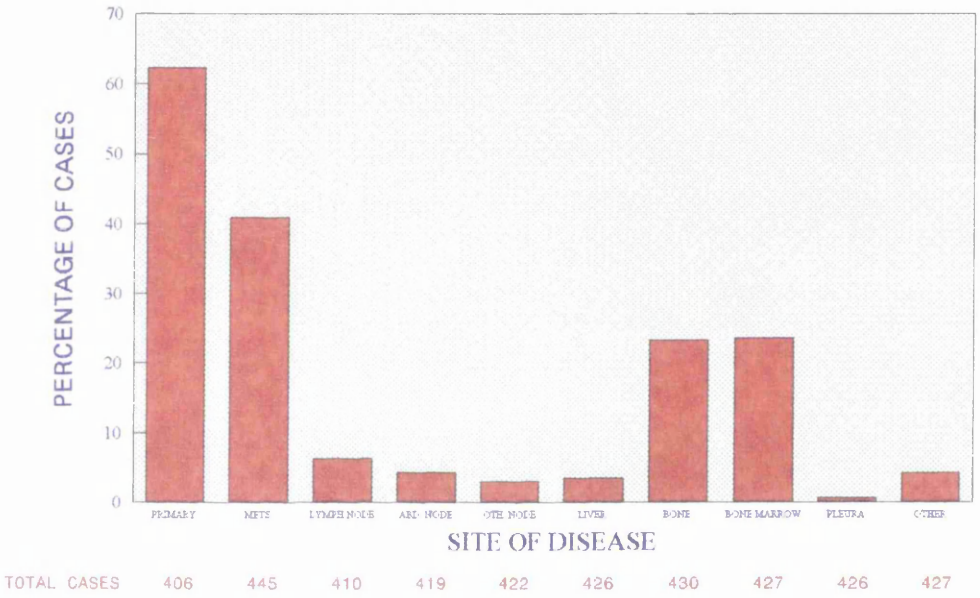
SITES OF DISEASE	NUMBER OF PATIENTS	PERCENTAGE OF CASES
DISEASE FREE	359	61%
BOTH PRIMARY AND METASTATIC DISEASE	133	22.6%
PRIMARY SITE ONLY	79	13.4%
METASTATIC SITE ONLY	17	2.9%

4.3.3.1. Sites of Residual Disease

The primary site therefore appeared to be the commonest site of residual disease (62.3%) but this included a range of patients from those with unresectable, gross disease to those with microscopic residual disease. Metastatic involvement remains in (40.9%) of cases. Bone (23.5%) and bone marrow (23.7%) are frequently involved. Children aged less than one year had the same incidence of

involvement and pattern of sites of residual disease despite having a better outcome.

Figure 4.13. Sites of residual disease.
Sites of residual disease in patients after chemotherapy and surgery. The numbers are expressed as the percentage of cases where these details were known for each category.

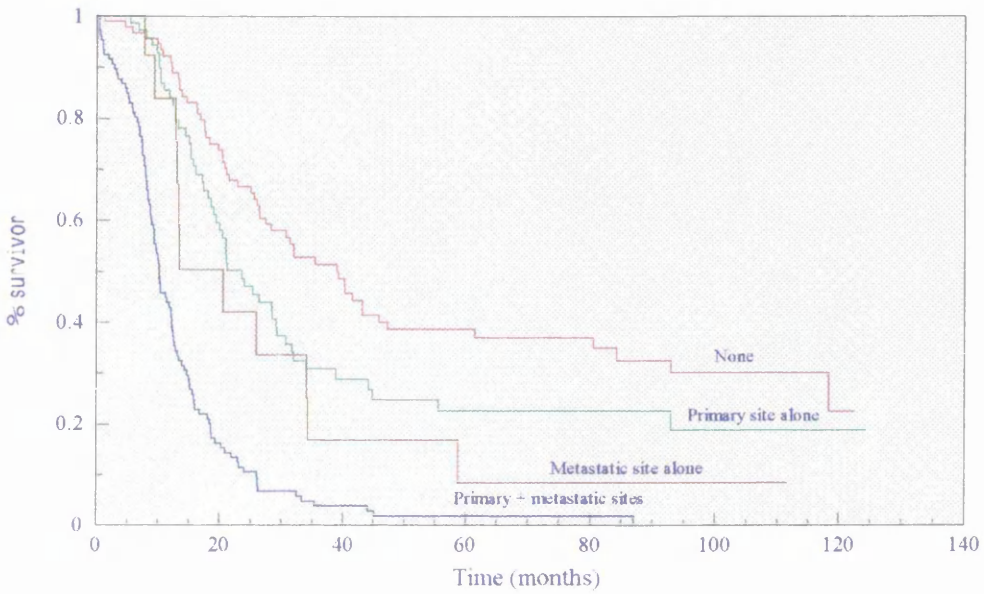


The 'other' category consisted of skin, orbital, CNS and pancreatic metastases and this accounts for 4.2%. Liver and pleura were sites of residual disease in 3.5% and less than 1% respectively.

4.3.3.2. Residual Disease and Survival

The presence of residual disease, at any site, adversely affected both the disease free survival ($p<0.0001$) and overall survival ($p<0.0001$).

Figure 4.14. The presence of residual disease and survival.
This graph demonstrates the survival outcome of those patients grouped in table 4.4.



The burden of residual disease was also important regarding the probability of relapse. If disease is present at any site, primary or metastatic, only 15.6% of patients survive compared with 34.2% ($p<0.0001$).

Table 4.5. Number of residual sites of disease and outcome.

Number of residual sites	Alive NED	Alive with disease	Dead	p value
0	38 (34.2%)	13(11.7%)	60(54.1%)	
1	14(15.6%)	14(15.6%)	62(68.9%)	
2	5(9.1%)	4(7.3%)	46(83.6%)	
3+	0(0%)	3(6%)	47(94%)	<0.0001

4.3.3.3. Summary of Residual Disease

- Common sites of residual disease are the primary, bone and bone marrow.
- The presence of residual disease adversely affects survival. ($p<0.0001$)
- The bulk of residual disease is important.($p<0.0001$)
- Residual disease in the bone or bone marrow particularly adversely affects survival.($p<0.002$)

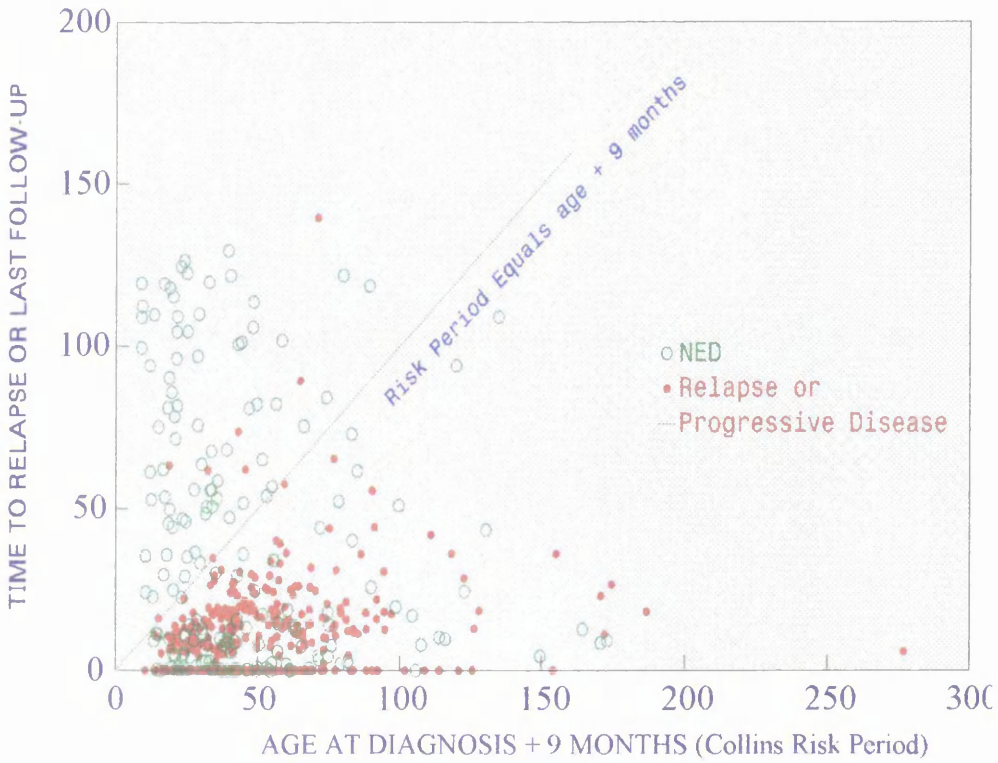
4.3.4. Relapse

246 patients (70%) of those who achieved complete remission with induction therapy or high dose chemoradiotherapy, relapsed. Only 114 (15.7%) of the total number of patients remain free of disease. 52 (7.1%) died during treatment either due to tumour progression or as a result of therapy. 133 (18.3%) suffered progressive disease or progression after a response. 11 cases were censored.

4.3.4.1 . Collins Risk Principle

The median follow up time was 1.31 years (range 0-14.62 years). Collins (1956) proposed a mathematical model for the risk period for relapse for paediatric malignancy. This was based on the observation of the growth rate of tumours. For neuroblastoma and other tumours this was the patients age at diagnosis plus 9 months. The application of the Collins risk period showed that only six patients developed a late recurrence or tumour progression beyond the Collins risk period of age at diagnosis plus nine months. There were no special characteristics of these patients.

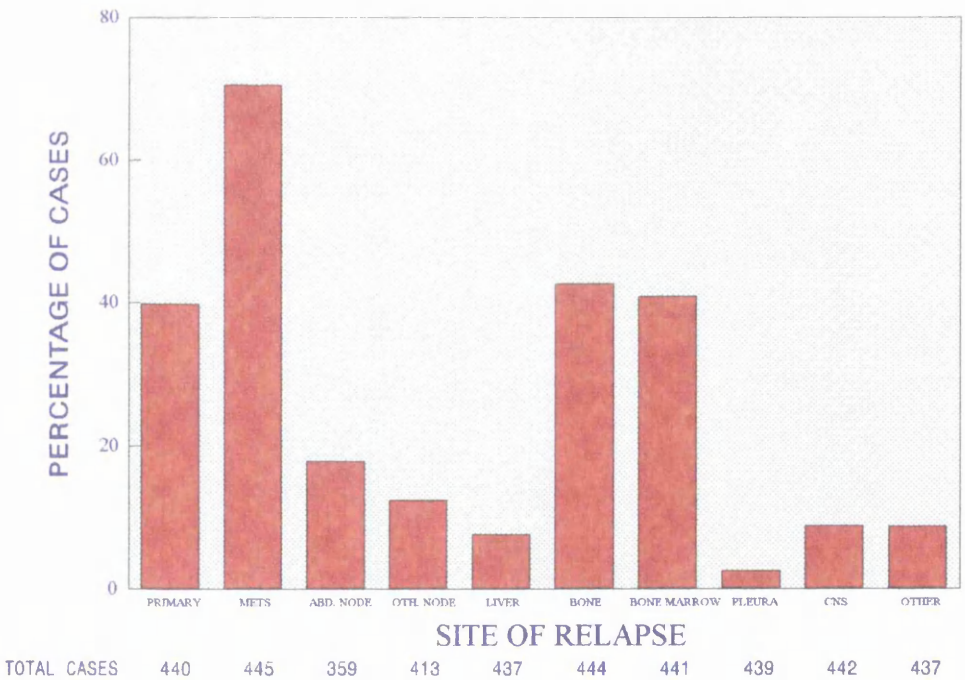
Figure 4.15. The Collins risk period of relapse for ENSG stage 4 patients. *(Please note both these axes are months)*



4.3.4.2. Sites of Relapse or Progressive Disease

Metastatic sites remained the most common sites for relapse or progressive disease, particularly bone (42.6%) and bone marrow (40.8%). Lymph nodes were a site of recurrence in 12.3% of cases. The liver, CNS and pleura were relatively uncommon sites, 7.5%, 5.6% and 2.5% respectively. The primary site was a common site for relapse and progressive disease 39.8%. These findings show a similarity to those sites of residual disease. This indicated that sites difficult to clear of disease e.g. bone or bone marrow were also common sites of relapse.

Figure 4. 16. Sites of relapse.

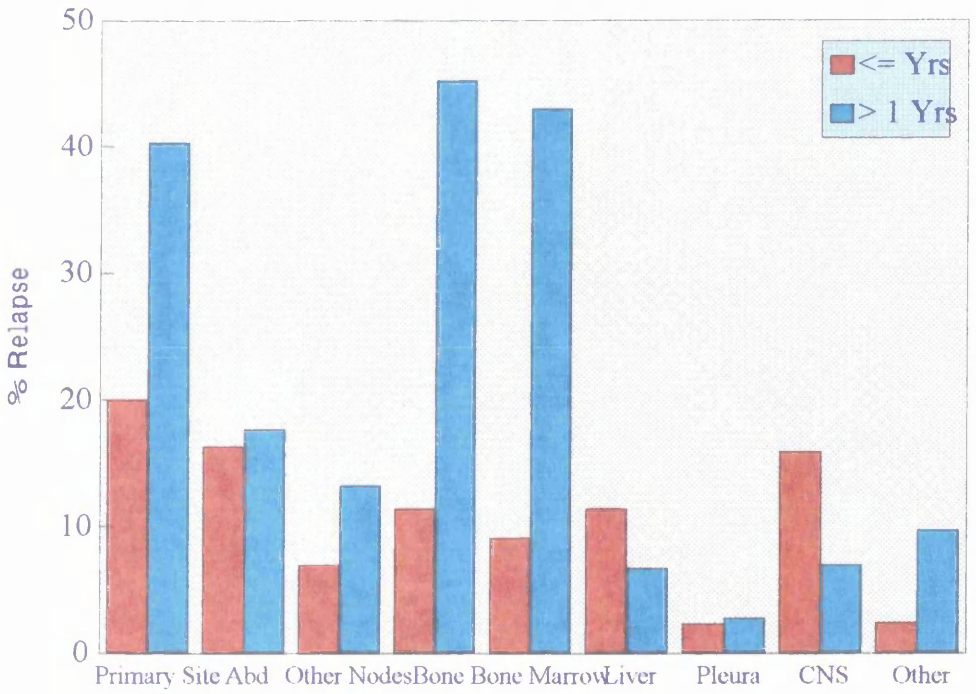


The factors of age ($p<0.0001$), residual disease ($p<0.0001$), bone marrow transplant ($p<0.001$) and extent of surgical resection ($p<0.0001$) were significant factors for progression free survival. There were sufficient cases in these groups to analyse their pattern of relapse separately.

4.3.4.3. Age

This group of patients were checked for bias but they were well distributed for all major variables. There was no difference in the pattern of metastatic disease and residual disease at the end of standard therapy in these children. One important difference was however that they were less likely to relapse. The difference was most marked in the bone and bone marrow sites. All sites of relapse were less frequently involved except CNS sites, in 16% of cases compared to 7% of older children. This probably reflects the different biology of neuroblastoma in children aged less than one.

Figure 4.17. A comparison of sites of relapse, in ENSG Stage 4 neuroblastoma patients of different age groups.



4.3.4.4. Consolidation therapy

This group of patients showed a reduction in relapse in the primary site. The bone and bone marrow are also less commonly affected, but the pattern of relapse did not differ from that of the general group. CNS relapse was more frequent in this group. This is important as CNS relapse becomes more important as control of systemic disease improves.

4.3.4.5. Residual Disease and Relapse

These factors have already been examined but briefly, the pattern of relapse for those children with disease remaining in the primary site was no different from that of the group as a whole. This implies that disease in the primary site eventually re-seeds to metastatic sites.

4.3.4.6. Summary

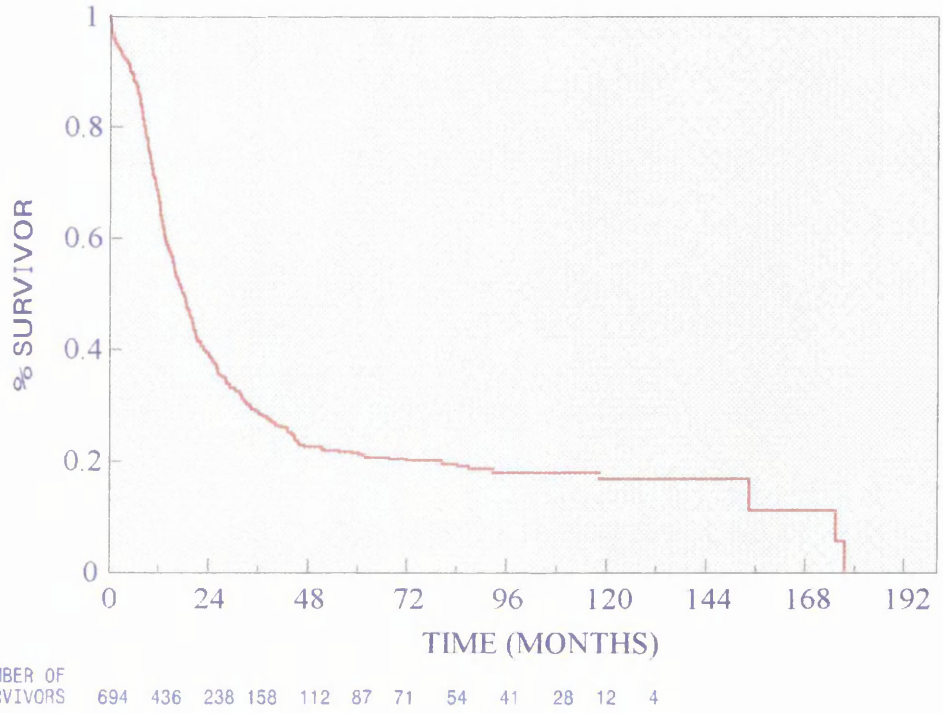
- The Collins Risk Principle holds for ENSG stage 4 patients.
- Sites difficult to clear of disease are often sites of relapse.
- Children aged less than one year and those having consolidation therapy are less likely to relapse but when they do so the pattern of relapse is the same.
- Achieving complete removal of the primary reduces local relapse and improves disease free and overall survival.

4.3.5. Survival

The median time of follow up was 1.31 years range (0-14.62yrs). At this time the number of patients alive with no evaluable disease was 117 (16.1%). 71 (9.8%) were alive but with disease. 509 children (69.9%) had succumbed to their disease. For 31 (4.3%) status is unknown.

The overall survival of this group of patients remains poor. Two, five and ten year survival was 38%, 20% and 18% respectively. In this series of patients there are no long term survivors beyond fifteen years.

Figure 4. 18. The survival of ENSG Stage 4 neuroblastoma patients



4.3.5.1. Summary

In this series the following factors affected overall survival.

- age $p<0.0001$
- high dose chemoradiotherapy $p<0.0001$
- presence of residual disease $p<0.0002$
- histopathology of resected specimen $p=0.0002$
- completeness of surgical resection $p=0.003$
- surgery attempted $p<0.0001$.

4.4 DISCUSSION

4.4.1. Population

This collaboration from ENSG members has the advantage of a significant number of cases of this rare tumour. The population characteristics of age, sex and distribution of primary site are similar to previously published, comparable series of patients (Rosen *et al.*, 1984; Shortner *et al.*, 1995).

On average 60 patients were entered to the ENSG survey each year. The number of referrals did not significantly vary over the ten year time period. The survey started in 1982 and patient details were collected in May 1993. The small number of patients from 1992 merely reflects the delay in notification of registration.

4.4.2. Age

As with other series, the majority of patients presented before the age of five. This emphasises the therapeutic challenge in this very young group within paediatric oncology.

Over this 11 year period 73 children presented, aged less than one year ,with Stage 4 disease. Of this group, only 25 presented aged less than six months, and 12 aged between six and eight months. The remainder, half of the group, presented after this period. This has implications for screening. The effectiveness of screening to detect neuroblastoma, remains a contentious issue. Its widespread implementation in Japan resulted in a large increase in incidence of

low stage, biologically favourable tumours. It is suggested that a significant number of these would have spontaneously resolved or been cured effectively with standard therapy. More seriously, a significant number of advanced, poor prognosis, tumours subsequently developed despite being screened as negative when children were tested at six months old (Nishi *et al.*, 1989; Nakagawara *et al.*, 1991). It is proposed that delaying the screening of infants from six to eight months may increase the number of clinically significant tumours detected (Kaneko *et al.*, 1990; Kerbl *et al.*, 1993).

Although the biological characteristics of *this* series of patients are not known, they are a group accrued from specialist clinical centres and the clinical characteristics of the sample are representative of other series of stage four patients. If screening was endeavouring to detect these patients and resources are limited, then it would appear that only 37 patients with stage four disease would have been detected, before the age of eight months over the 11 year period. These data therefore would support the delay of screening in the hope of detecting the patient with the poorer outlook.

Age remained a powerful prognostic indicator and as with other series, those aged less than one at diagnosis, even with stage 4 disease, had a significantly improved survival (Evans *et al.*, 1971; Grosfield 1980; Rosen *et al.*, 1984; Kretchmar *et al.*, 1984; Carslen *et al.*, 1985). This group was examined closely in an attempt to determine if there were any obvious explanations for this: The prognostic features are the same as for the general population. The thoracic site, prognostically a more favourable site, was more common, but insufficient to

explain this marked difference in survival. The response to therapy was identical to the group as a whole and sites of residual disease were identical to those older patients after therapy.

The difference in this group, however, was that they had a striking reduction in incidence of relapse. On relapse, the common sites of relapse (primary, bone and bone marrow) are affected but interestingly more unusual sites e.g. CNS are affected to a disproportionately larger degree. These unusual sites e.g. testes and CNS may be sanctuary sites for chemotherapy. These findings must be explained by the difference in biology of the disease in those aged less than one year.

It has been suggested that those children aged older than six years also have longer survival (Finklestein *et al.*, 1979) and this is attributed to favourable biological features (Blatt *et al.*, 1995). In this series there was no evidence to support this.

4.4.3. Pattern of Primary

In this series the site of primary was not of prognostic significance, since a favourable primary site is associated with young age and limited stage disease. The population characteristics were comparable to stage 4 patients contained within the following series.

Table 4.6. Population characteristics of previously published series of patients.

Series	adrenal %	abdomen %	thorax %	pelvis %	neck %	other %	total
Gross'59	41	13	12	6	2	27	217
Bodian'59	37	32	11	8	5	7	129
Stella '70	29	43	17	3	3	4	143
Wilson 1974	23	32	16	7	0	22	487
Rosen'84 < 1 year	40	25.5	22.5	2.5	2.5	7.5	40
Rosen > 1 year	37	28	19	4	4	4	78
Fortner' 67	50	19	8	4	2	17	133

Common sites affected by metastatic spread were the lymphnodes, bone and bone marrow. The pattern of metastatic spread in metastatic neuroblastoma is not random and is of prognostic significance. Specific sites of predilection have been described (Pepper 1901; Hutchison 1907 and De la Monte *et al.*, 1983). The patterns of metastases on presentation were analysed to determine whether or not a specific pattern of metastases is associated with an individual site. No correlation was found, highlighting the systemic nature of this disease.

The number of metastatic sites affected was of prognostic importance particularly if two or more sites were affected (p=0.0002). Table 4.6. indicated successively poorer survival with an increasing number of metastatic sites

affected by disease; This would indicate that the bulk of disease is also important in this illness.

4.4.4. Control of the Primary Site

The role of surgery in metastatic neuroblastoma has traditionally been limited to biopsy, determination of the extent of disease and provision of histological material for prognostic testing. The necessity for complete resection remains controversial (Ogita, Tokiwa and Majima 1985; Losty *et al.*, 1993;). Several authors demonstrated no survival advantage in comparing the extent of resection and survival (Sitarz *et al.*, 1983; Matsumura *et al.*, 1988; Shorter *et al.*, 1995).

Surgery can often be difficult, needing considerable expertise on the part of the surgeon. The length of a surgical procedure varied from one to ten hours, in one series from an experienced group (Kiely 1989). A complication rate of 18%-21% has been quoted (Haase *et al.*, 1991) and this appears greatest when resecting advanced tumours in small babies (Azizkhan *et al.*, 1984).

Neuroblastoma can infiltrate beyond the midline, encasing neurological, vascular and other vital organs. Operative and anaesthetic techniques involving haemodilution, hyperthermia and cardiopulmonary bypass may be required for the dissection of large central tumours. Special equipment, lasers or microwave knives, facilitate this hazardous dissection. The cavitron ultrasonic aspirator (CUSA; Cavitron, Stamford, CT) allows the fragmentation, irrigation and

aspiration of friable necrotic tumour tissue. This allows tissues, high in water content, to be selectively fragmented and aspirated while tissues high in collagen and elastin, blood vessels and pseudocapsular walls are spared (Loo *et al.*, 1988). Urological injury and haemorrhage are common complications (Fortner *et al.*, 1968; Azizkan *et al.*, 1985). Nephrectomy, if necessary, results in poorer survival (Tsuchida *et al.*, 1991). This may be because more advanced tumours are more difficult to resect but in addition potential post operative treatment may be compromised by inadequate renal function. Vascular accidents involving major vessels may result in haemorrhage or infarction of critical organs (Priebe and Clartworthy 1967; Azizkhan *et al.*, 1984). Thoracic duct damage and brachial plexus injury have also been described (Filler *et al.*, 1972). Wound infections or sepsis, urological injury and bowel obstruction secondary to adhesions have also been noted (Haase *et al.*, 1991).

Despite these difficulties, evidence is now accumulating that complete tumour resection is beneficial in prolonging disease free survival (Losty *et al.*, 1991) and overall survival (Tsuchida *et al.*, 1991; Haase *et al.*, 1991; Yokoyama *et al.*, 1994; La Quaglia *et al.*, 1994).

In this series, there was no difference in outcome whether surgery was performed as a primary or delayed procedure. This is consistent with previously published data (Tsuchida *et al.*, 1991; Haase *et al.*, 1991; Yokoyama *et al.*, 1994; Shorter *et al.*, 1995).

A delayed surgical procedure however has the advantage that it can downstage an inoperable tumour into one which is surgically resectable. The tumour itself may become less friable, less vascular and more fibrous, facilitating easier dissection (Azizkan and Haase 1993). Shamberger suggests that surgical complications can be reduced if the operation is delayed until after chemotherapy (Shamberger *et al.*, 1991).

The surgeons in this series achieved complete macroscopic resection in 52.8% of cases resected and in 85.3% more than 75% the tumour was resected. This indicates a good selection of cases. Overall the group undergoing surgery had better survival than those who did not. Any surgical group, in a retrospective analysis such as this, are of course selected. The only way to avoid this bias would be to prospectively randomise patients to surgery, or not, before any treatment is given.

The surgeon's estimate of the completeness of surgical resection at laparotomy predicted survival ($p=0.047$) but with less accuracy than the pathologist could achieve by study of the resected tumour ($p=0.0032$). Previous studies failing to demonstrate a survival advantage for complete resection have used the surgeons estimate for analysis (Sitarz *et al.*, 1983; Matsumura *et al.*, 1988; Shorter *et al.*, 1995).

This study indicates incomplete agreement between the surgeons and the pathologists (kappa=0.36) as to the extent of resection. Since previous studies are based on surgical estimates of resection, this may explain why these fail to

show value as a prognostic index. This does not seem unreasonable since we do not rely solely on the surgeon's estimate of clearance for other malignancies e.g. breast and colonic cancer.

Previously published analyses have compared only two categories-complete versus incomplete excision. From the ENSG series of patients, if the degree of surgical resection is analysed more closely, there is evidence for a large variation in outcome between the three groups, particularly between those with macroscopic and microscopic residual disease, while the outcome is much less different between complete and microscopic residual disease. This may be another explanation for the continued controversy.

This data confirms that effective control of the primary reduces local relapse. Tsuchida noted that complete resection reduced local relapse rates to 17% compared to 55% in the partially resected group (Tsuchida *et al.*, 1991). Ikeda also noted that effective local control reduced the local relapse rate to 17% (Ikeda *et al.*, 1992). The local relapse rate in this series was 18.5%.

This study confirms a difference in disease free and overall survival with complete resection. This is in keeping with other studies (Tsuchida *et al.*, 1991; Haase *et al.*, 1991; Yokoyama *et al.*, 1994; LaQuaglia *et al.*, 1994). Previous authors had speculated that surgical excision may be more important in certain subgroups, but had a insufficient number of patients to confirm this. This study indicates however there is no additional advantage in those patients proceeding to transplant. There, survival advantage is conferred by clearance of the primary

site and an additional separate survival advantage conferred for a transplant procedure.

Matsumara concluded that ablation of metastatic disease was much more important than the extent of surgical resection (Matsumura *et al.*, 1988; LeTourneau *et al.*, 1985). He based his conclusions on the observation that patients with complete macroscopic removal of the primary but with residual metastatic disease had a median survival of eleven months, while those with incomplete resection of the primary but free of metastases survived a median of 23 months. In this series, only 17 patients fulfilled the criteria of having metastatic disease only, therefore a direct comparison could not be done. It does not, however, contradict Matsumura's findings, who stated that residual disease in the primary site is also associated with a poorer outcome than when complete remission is achieved. Perhaps he did not demonstrate the above finding due to the broad grouping of surgical groups of less than 50% resected, more than 50% resected, and complete macroscopic excision and relying on the surgeons estimate of disease only.

Studies involving high dose intensity chemotherapy before surgery noted that not only was surgical excision easier but a clear survival advantage based on the extent of surgical excision was also evident in their patients, who achieved a higher incidence of complete remission pre-surgery (Tsuchida *et al.*, 1991; La Quaglia *et al.*, 1994).

The Philadelphia experience also failed to show a difference in survival with the extent of surgical resection and attributed this to the spread of favourable biological characteristics, within the incompletely resected groups (Shorter *et al.*, 1995). This study does not contradict this finding since no biological data were available for analysis. Indeed it supports the importance of the type of disease resected since the histopathology of the resected specimen also proved to be a powerful prognostic factor. The authors believe that the underlying biology of the tumour, will determine its response to therapy, e.g. favourable tumours will respond well to chemotherapy enabling ablation of metastases and easier dissection of the primary.

Insufficient information was available for multivariate analysis, to determine if the extent of resection or the pathology of the resected specimen was more important.

In summary, the extent of surgical excision does have a statistically significant importance in progression free and overall survival. It may be however that the histology of the resected specimen may be more important than the amount of tumour left behind.

Access to those children who received radiotherapy was limited, as despite re-surveying only a small number of patients were identified. Previous literature suggests that the response of neuroblastoma to radiation depends on the age and stage of the patient, in addition to normal radiotherapy considerations. Figures 4.10 and 4.11 merely demonstrate current clinical practice.

4.4.5. Residual Disease

The bone and bone marrow, commonly affected at presentation, remained involved after treatment in a substantial proportion of cases and these are also common sites of relapse. The outcome gets progressively worse with the increasing amount of residual metastatic disease. This is disappointing, more effective induction regimens are required to induce remission in metastatic sites and to downstage the primary as much as possible, to make surgery feasible.

4.4.6. The Collins Risk Period

Collins (1956) proposed a mathematical model for the risk period for relapse for paediatric malignancy. Rosen confirmed this finding in his published series of patients (Rosen *et al.*, 1984). This suggests that latent disease predictably recurs. There were no unusual features about the children regarding their presentation, response to therapy nor pattern of relapse. Recurrences have been seen after many years with neuroblastoma (Richards *et al.*, 1976). Jaffe (1973) reported a 7 year old, treated with chemotherapy, who relapsed in the bone three years later. This was cured but the patient again relapsed and died of disease 22 years later. Other reports in stage 4 patients document relapse after several years (Hinton *et al.*, 1968) but Hata (Hata *et al.*, 1991) reports one case and reviews a further 12. He suggests that those patients induced into remission with minimal treatment are especially susceptible to late recurrences. Other reports concern late relapse in children aged less than one year.

4.4.7. Relapse

The bulk of residual disease, regardless of site, is an important factor for relapse. Those patients with disease left only in the primary site have an identical pattern of relapse to the group as a whole. This implies that the primary eventually reseeds to metastatic sites.

A prognostically distinct group is composed of those patients aged less than one year at diagnosis. These patients had the same presentation characteristics and the same response to therapy. There was no difference between the bulk of disease or the sites of residual disease after induction therapy. They were, however, much less likely to relapse. This must be attributed to the favourable biology of the tumour in young patients.

The transplant group, on relapse, had the same relapse pattern as the group as a whole. They were also however less likely to do so. This finding was determined by comparing transplant patients with residual disease in the primary, with those clear of disease at the time of transplant. The additional therapy is likely to have increased control at all sites.

The overall survival of ENSG stage 4 patients was poor. The same prognostic factors were present for overall survival as disease free survival, since once the patient relapsed death was inevitable.

4.5. Conclusion

- This analysis did not have access to biological information. There was insufficient information to determine accurately the most important factor for survival in these patients.

- Patients aged less than one year at diagnosis have a better prognosis. They behave as other patients but they relapse less often and when they do so atypical sites are affected for example: CNS.

- For Stage 4 patients as a group: The presence of bone or bone marrow disease at presentation is a sinister prognostic factor. These sites were commonly affected, difficult to clear and frequent sites of relapse. More intensive chemotherapy regimens are necessary to induce complete remission in these sites.

- Effective control of the primary can be achieved by aggressive surgery, and reduce the incidence of local relapse. This improves overall survival. The histopathology of the tumour, after standard therapy, may be more important.

- The volume of residual disease is important. Residual disease in the primary will eventually reseed into metastatic sites and the outlook becomes poorer with increasing amounts of residual disease.

- This study therefore confirms the good prognosis of those aged less than one year and suggests that for older patients, intensive aggressive therapy of the primary and metastatic disease is necessary to improve outcome.

CHAPTER 5 PALLIATIVE RADIOTHERAPY IN METASTATIC NEUROBLASTOMA

5.1. INTRODUCTION

The majority of children with metastatic disease will die: less than 20% survive two years (Rosen *et al.*, 1984). It is common clinical practice among clinical oncologists to palliate symptoms from bone and soft tissue masses with external beam radiation in patients with end stage disease. There is, however, little in the literature to document the effectiveness of this in neuroblastoma.

A retrospective analysis of all patients with neuroblastoma treated palliatively with external beam radiation within The Beatson Oncology Centre, Glasgow was completed to examine the effectiveness of this therapy and, in addition, to determine whether a dose-response relationship could be established.

5.2. PATIENTS AND METHODS

The case notes of all children receiving palliative radiotherapy between 1980 and 1995 were reviewed. Staging was defined by INSS criteria (Brodeur *et al.*, 1988). Details of doses, fractionation schemes and sites of disease were obtained from radiotherapy records. Patient details were noted including information on analgesic requirements, response to treatment, and the simultaneous administration of low dose oral etoposide, which was commonly used in patients with relapsed disease.

Treatment was given for localised pain or loss of function from bone or soft tissue metastases by single field or parallel opposed pair of fields, depending on site. ^{60}Cobalt and 4MeV or 5MeV linear accelerators were usually used. 300kV was used for three treatments. The different dose fractionation schedules used were compared directly by an equivalent radiobiological total dose given as 2Gy fractions, assuming the α/β ratio for neuroblastoma tissue is 10Gy.

The principal pain measure was a regular clinical assessment of pain relief at the irradiated site by the clinician. In addition as a more quantitative assessment, the child's analgesic requirement (type and quantity) was noted and converted into a pain score. A complete response was defined as a total resolution of symptoms enabling all analgesia, in this advanced stage of illness, to be discontinued. Since radiotherapy is a local treatment, a reduction in analgesia was not always appropriate due to disease progression elsewhere. Often, when possible, this was not attempted in this 'terminal care' setting. Therefore a separate category was noted for those children who experienced relief in symptoms but in whom analgesia was not discontinued. A functional assessment of any painful or neurologically affected site was attempted. If functional impairment was due only to pain affecting motor function, improvement in clinical power (MRC scale) was graded. In more complicated cases of spinal cord compression where

additional sensory, bowel or bladder function were involved, the improvement of these respective functions had to be considered. The speed of onset of relief of symptoms was documented as the length of time to maximum response from the first day of treatment. A return of pain to any irradiated site before death is documented as a relapse.

Data was analysed by a non parametric test and significance values obtained by means of Kruskal-Wallis one way ANOVA analysis (Conover 1980). The distribution of survival time after treatment was estimated using the Kaplan-Meier actuarial method (Cox and Oakes 1984).

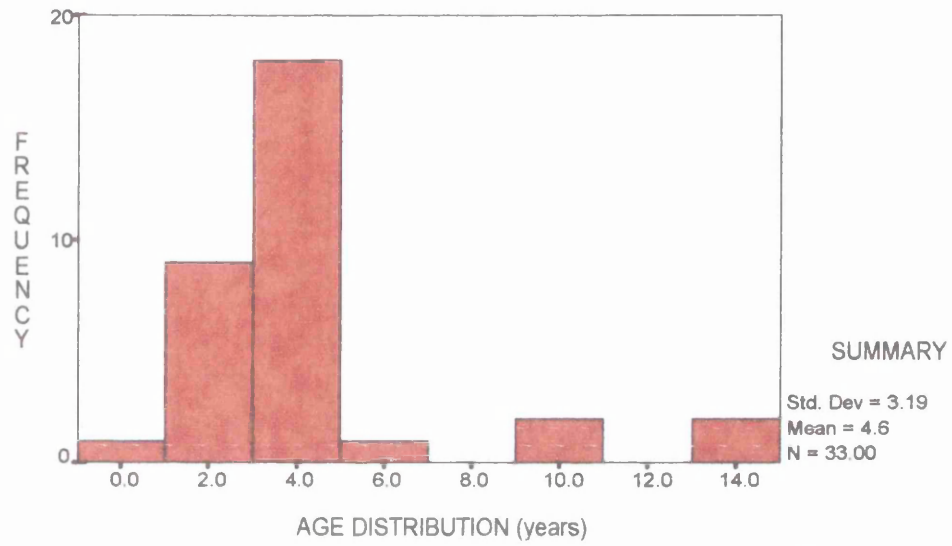
5.3. RESULTS

5.3.1. Patient Characteristics

From 1980 to August 1995, sixty-six children with histologically proven neuroblastoma were treated at The Royal Hospital for Sick Children, Glasgow and The Beatson Oncology Centre, Glasgow. Of these, fifteen patients received thirty-two palliative radiotherapy treatments.

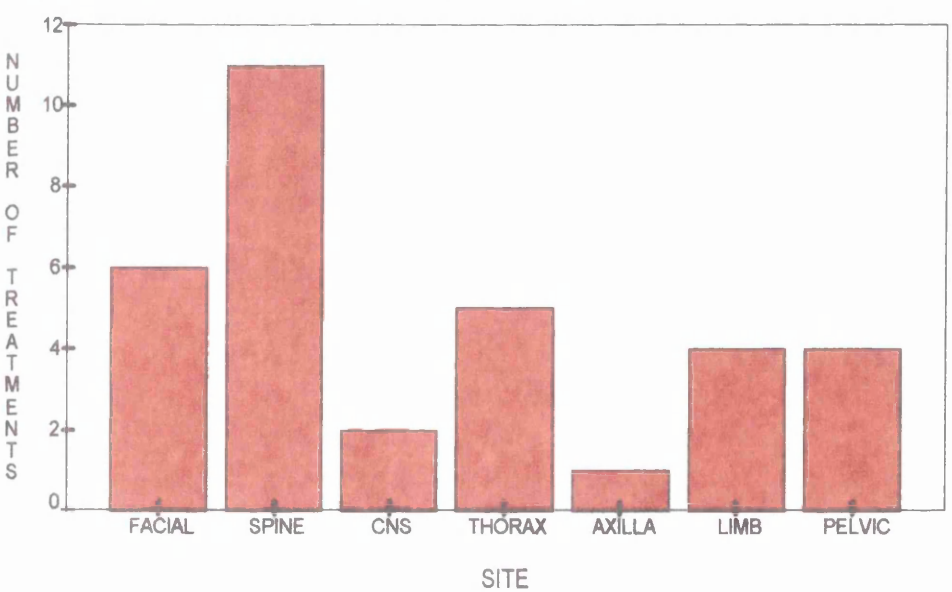
The mean age of the study population was 4.57 years, at diagnosis, with a range of six months to 14 years 9 months.

Figure 5.1. The age distribution of patients



Fourteen Stage 4, and one Stage 4S patient received radiation for the control of painful bone or soft tissue metastases accounting for 42.4% of treatments; spinal cord compression 33.3%; orthopnoea or recurrent respiratory tract infection 15.2%; headaches from intracranial metastases 6.1% and nerve involvement 3%.

Figure 5.2. Sites irradiated in palliative treatments.



5.3.2. Treatment Details

Patient details are summarised in table 5.1.

Table 5.1. Patient characteristics of all BOC neuroblastoma patients treated with palliative radiotherapy.

PATIENT	AGE (Yrs)	SITE	SIZE (cm)	FIELD	ENERGY (MeV)	DOSE (Gy)	FRACTIONS	DAYS	RESPONSE	RESPONSE TIME	ETOPOSIDE	DURATION OF RESPONSE
A.L	2.7	facial	5.5X7	single	4	30	10	12	CR	>1 month	ETOPOSIDE	
		spine	5X9	par. pair	1.33	8	1	1	PR	>1 month	NO	
		spine	7X12	par. pair	5	8	1	1	PR	>1 month	NO	< 1 month
		pelvis	5 X5	par. pair	4	8	1	1	CR	>1 month	ETOPOSIDE	< 1 week
		pelvis	10X12	par. pair	300 kV	20	5	5	PR	<1 month	ETOPOSIDE	< 1 month
A.M	0.6	axilla	NA		NA	24	13	17	PR	<1 month	NO	< 1 month
A.K.	4.4	facial	10X16	single	1.33	20	5	5	PR	>1 month	ETOPOSIDE	< 1 month
		thorax	11X11	par. pair	1.33	20	5	5	PR	>1 month	ETOPOSIDE	< 1 month
		limb	6X12	single	1.33	8	1	1	CR	>1 month	ETOPOSIDE	< 1 week
		limb	6X12	single	1.33	8	1	1	CR	>1 month	ETOPOSIDE	< 1 week
		limb	5X13	single	5	20	5	5	CR	>1 month	ETOPOSIDE	< 1 month
A.S	2.7	CNS	8X8	single	5	30	15	19	PR	<1 month	ETOPOSIDE	< 1 month
B.H	3.0	spine	8X10	par. pair	1.33	6	1	1	PR	<1 month	NO	< 1 month
		limb	6X10	par. pair	1.33	6	1	1	CR	>1 month	ETOPOSIDE	< 1 month
		pelvic	6X10	single	1.33	8	1	1	CR	>1 month	ETOPOSIDE	< 1 week
C.G	3.8	facial	5X5	single	300 kV	6	1	1	PR	>1 month	NO	< 1 week
		spine	6X19	par. pair	300 kV	6	1	1	PR	>1 month	NO	< 1 week
E.B	3.9	thorax	NA	single	4	30	10	14	CR	>1 month	NO	< 1 week
F.H	4.0	facial	4X4	par. pair	1.33	10	2	2	PR	>1 month	NO	< 1 week
		spine	7 X17	par. pair	1.33	20	5	5	CR	>1 month	NO	< 1 week
		thorax	8X10	single	1.33	25	5	5	NA	>1 month	ETOPOSIDE	NO RESPONSE
I.R	4.8	spine	7X12	par. pair	1.33	8	1	1	PR	< 1 month	NO	< 1 week
		spine	6X10	par. pair	1.33	6	1	1	NO	>1 month	NO	NA
J.T	2.2	CNS	18X13	single	4	20	5	5	PR	NA	NO	NO RESPONSE
K.E.	6.5	pelvic	8.5X9	par. pair	1.33	8	1	1	PR	>1 month	NO	NO RESPONSE
M.R	3.9	spine	7X10	par. pair	1.33	15	4	4	PR	>1 month	ETOPOSIDE	NA
S.T	2.7	facial	4X4	par. pair	1.33	12	2	2	NO	<1 month	NO	< 1 week
		facial	6X6	par. pair	1.33	20	5	7	PR	>1 month	NO	< 1 month
TMcN.	9.4	spine	5X7	par. pair	1.33	12	3	3	PR	>1 month	NO	NO RESPONSE
		spine	5X7	par. pair	1.33	12	3	3	PR	>1 month	NO	< 1 week
W.MN	14.9	spine	15X17	par. pair	1.33	23	7	9	CR	>1 month	NO	< 1 month
		spine	**8X18**	**par. pair**	**1.33**	**20**	**10**	**12**	**CR**	**>1 month**	**NO**	**< 1 month**

All treatments were short, simple and well tolerated. 50% of treatments consisted of two fractions or less. Therapy was given either with a single field or by a parallel opposed pair of fields, depending on site.

Total doses ranged from 6Gy or 8Gy given in one fraction to 30Gy administered in 15 fractions over 19 days for central nervous system treatments.

Patients often had multiple treatments. Infact two children had five palliative treatments, each to different sites of disease, throughout the advanced stages of their illness. Despite frequent multiple treatments, retreatment of a site of disease was only required in two instances.

Table 5.2. The number of treatments per patient

No. of Treatments	1	2	3	4	5
No. of Patients	7	4	2	0	2

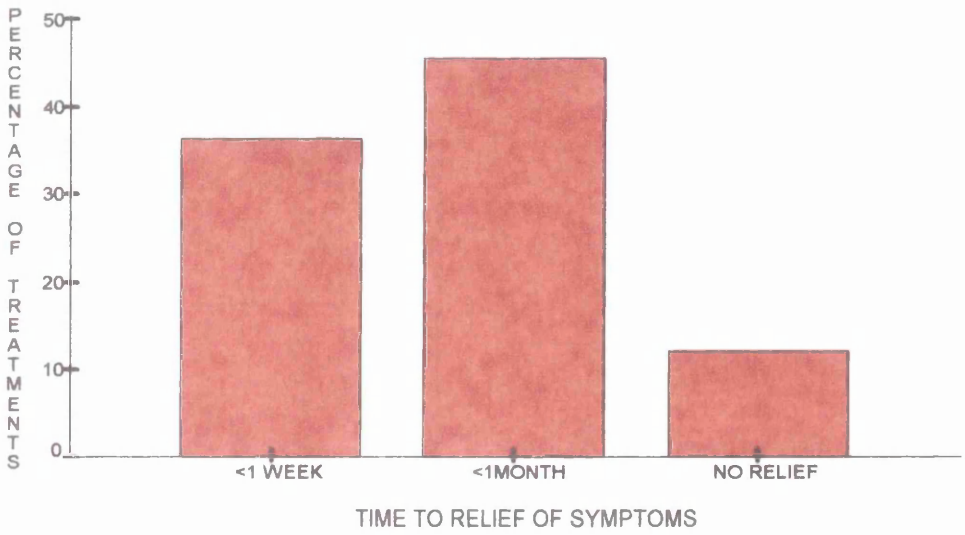
Of the two patients retreated, one had a further 8Gy treatment to a painful tibial metastasis eight months after an initial successful single 8Gy treatment. Thereafter the patient remained pain free for the remainder of his life.

The remaining re-treated patient had chemoresistant Stage 4 disease. He experienced no pain relief after a single 8Gy treatment to his lumbar spine and similarly no benefit from a single 6Gy retreatment twenty three days later.

One patient initially diagnosed with Stage 4S disease was included in this series. This seven month old child had incompletely resected local disease, which progressed during treatment with OPEC chemotherapy. Palliative treatment was given for brachial nerve plexus involvement. Pain relief occurred within twenty four hours and a measurable shrinkage of visible tumour mass was noted, after 2405cGy had been given, in thirteen fractions over seventeen days. Unfortunately recurrence of pain and regrowth of mass occurred within three weeks.

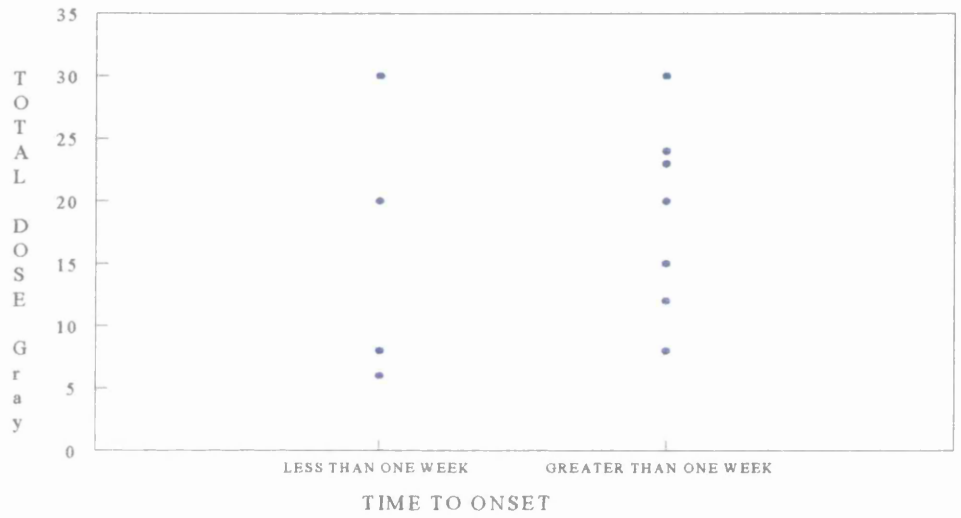
A complete response to symptoms, enabling all analgesia to be discontinued, occurred in 33.3% of cases. In 57.5% the local site treated was controlled clinically and the analgesic requirements decreased or left unaltered due to disease elsewhere.

Figure 5.3. Time after first day of radiotherapy until onset of effect.



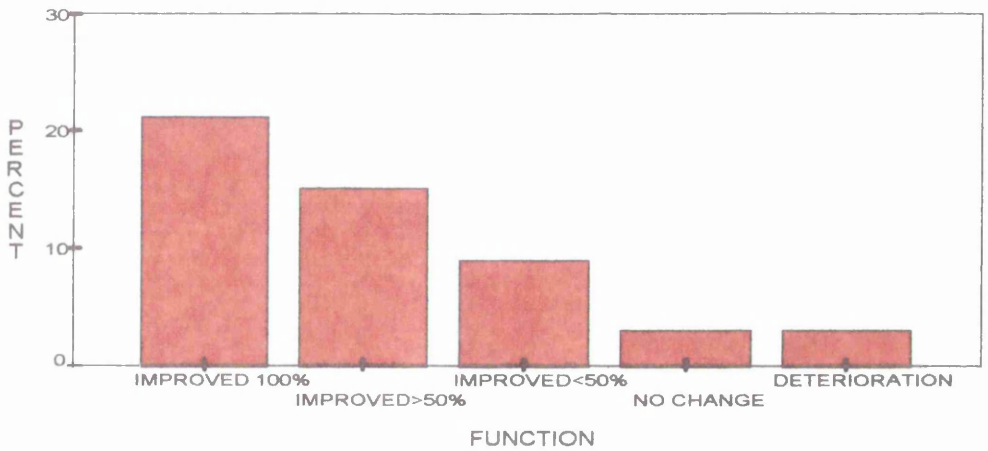
36% of cases experienced rapid relief of pain within one week, from the first day of treatment and the remainder within one month. No pain relief first occurred after this time.

Figure 5. 4. The relationship between dose per fraction and onset of analgesic effect.



Complete functional recovery in some instances, as for example treated spinal cord compression, took longer and for this reason the eventual response was noted.

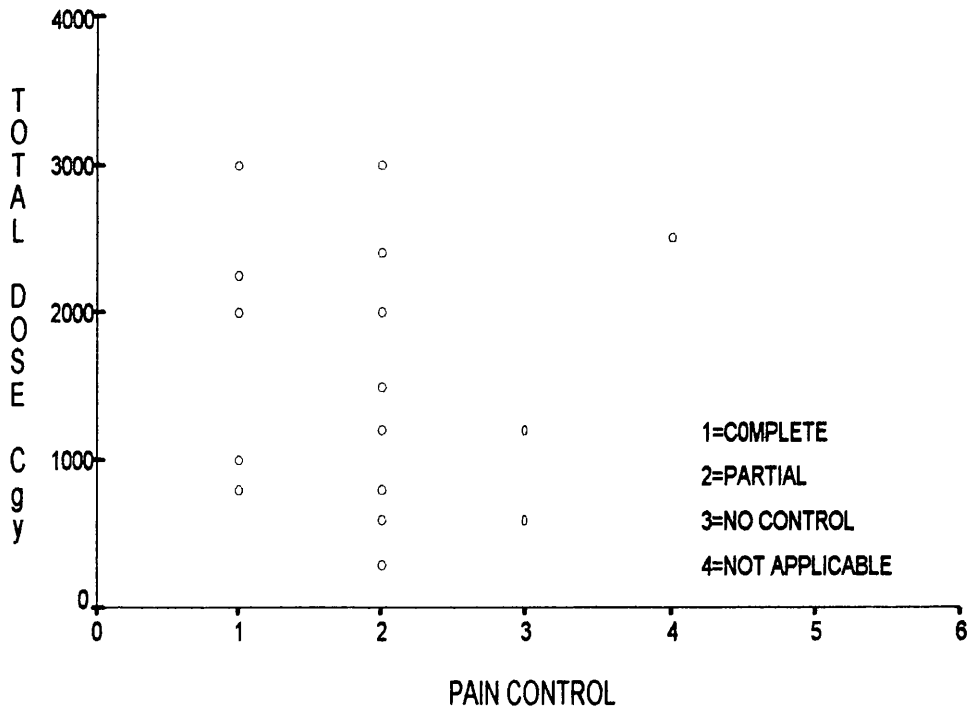
Figure 5.5. Functional improvement after palliative radiotherapy



A functional improvement of 50% or more occurred in 66.6% (12/18) of cases where there was functional impairment due to pain or neurological involvement.

There was no statistical difference in outcome, analgesic effect or speed of onset of relief, depending on site irradiated (p=0.47), total dose (p=0.08) or dose per fraction (p=0.74). Therefore no dose response could be determined.

Figure5.6. Total dose and symptom control.

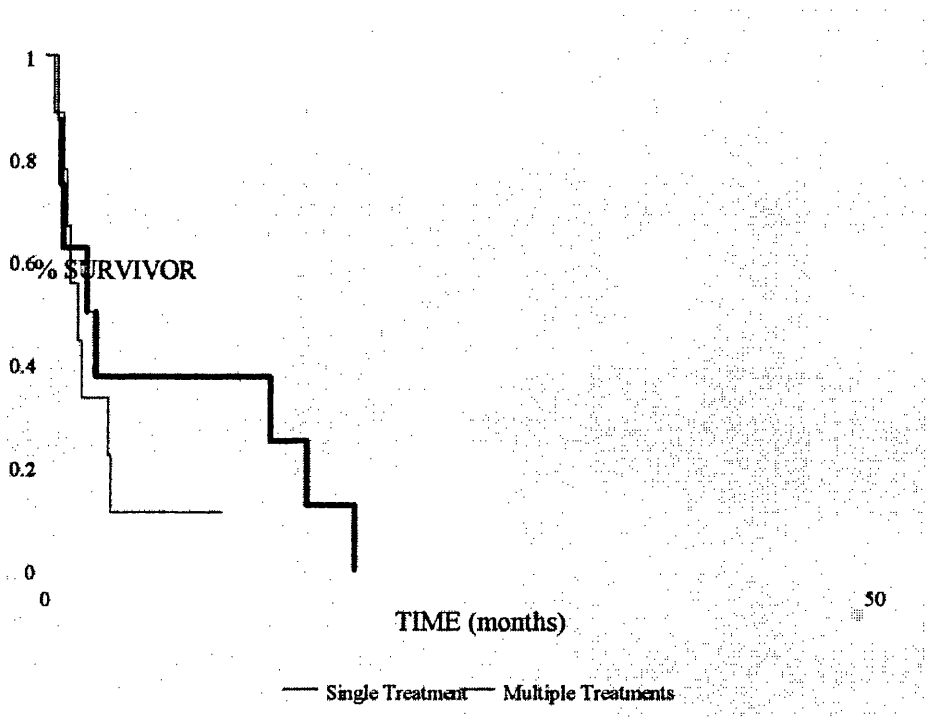


Half the children were receiving simultaneous palliative etoposide administration. There was no difference in outcome between the two groups nor was any additional toxicity experienced in the etoposide group.

The duration of response was usually life long. However there was a return of discomfort to six of the thirty two treated sites. This is strictly defined as any intensity of pain. The range of time to return of pain varied from 22 days to eight months.

Survival of each patient from the first treatment date is summarised in figure 5.7.. This demonstrates a median survival time of 2.16 months (95% confidence interval of 1.08, 3.74 months). The mean survival time was 5.21 months (S.E.=1.56). Obviously those patients living longer had multiple treatments.

Figure 5.7. Survival of all patients from first day of first treatment.



5.4. DISCUSSION

Neuroblastoma cell lines are radiosensitive *in vitro* (Deacon, Wilson and Peckham 1985; Wheldon *et al.,* 1986). Clinically, however, variable responses are seen. Historically there is documentation of tumour response *in vivo* to doses as low as 400 rads (4Gray) (Farber 1940; Wyatt and Farber 1941; Wittenbourg 1950) .

In the radical setting, a number of series indicate that local control of the primary site by local radiation is age, stage and dose dependent (Jacobson *et al.*, 1983; Jacobson, Sause and O'Brien 1984; Rosen *et al.,* 1984).

Clinically external beam radiotherapy provides highly effective palliation of bone and soft tissue metastases (Perez *et al.*, 1967; Halperin and Cox 1986; Dobbs, Barrett and Ash 1992). More recently, mIBG has been used to palliate pain (Hoefnagel 1991(b); Westlin 1995) . There is however little in the literature to highlight the effectiveness of the role of palliative radiotherapy in neuroblastoma and no dose response has been demonstrated.

A retrospective analysis of all patients, treated within this centre, from 1980 was performed to ascertain the effectiveness of our palliative regimens and see whether a dose response relationship was observed.

The age and stage composition of this population reflects the characteristics of children with poor prognosis neuroblastoma. Treatment regimens were simple, quick to complete and well tolerated. The concomitant administration of etoposide did not alter the effectiveness nor add side effects.

Short simple regimens, 1x8Gy or 2-5 fractions of 4-5Gy appear as effective as longer dose fractionation schedules. Extended fractionation was generally used for larger volumes to minimise normal tissue side effects which have not been specifically analysed here. Although different sites, doses and field sizes require different fractionation schedules, they could be compared directly by calculating the radiobiological equivalent dose. A critical dose response relationship could not be determined for this group of patients. This could be due to the small numbers involved or the inherent radiosensitivity of neuroblastoma that the fraction sizes used are too large to detect any critical threshold level for control. The small number of treatments whose effect was not life long did not differ significantly in any of the above criteria.

A major advantage is that the effect of the treatment was rapid. The subcutaneous dose of morphine was able to be reduced within 24 hours in one patient. 40% of patients had relief within one week of the first day of

treatment. All patients who responded did so within four weeks of the first day of treatment.

Complete pain relief was achieved in 33.3% of treatments and all analgesia, usually opiate, was able to be discontinued. In 57.5% of treatments, an improvement in symptoms or partial reduction in analgesic requirements was seen. These results are therefore similar to that of a large series of patients reported previously, in which the effectiveness of palliation of bone metastases in adults was assessed (Tong, Gillick and Henderson 1982). Halperin and Cox, performing a similar analysis, noted a total response rate of 65% with a relapse rate of 23% although doses used are not given. 6 (19%) of treatments failed to produce a complete disappearance of symptoms for the remainder of the child's life. In this series this relapse was defined as any return of pain to the treated site. This is more stringent than other studies where relapse was defined as a return of discomfort to the original pain intensity at the treated site (Halperin and Cox 1986). Half of these were successfully retreated. Two had a transient response of one month but both patients quickly deteriorated and died before retreatment was contemplated.

In all patients experiencing 50% or more improvement in function a significant increase in mobility was seen.

Any response obtained was usually sustained for the remainder of the patient's life. This was usually short. The median survival time after the treatment for pain relief was 2.16 months (95% C.I. 1.08, 3.74). This emphasises that the long term morbidity normally associated with radiotherapy in the young child can be largely ignored in this group of patients. The majority of patients having multiple treatments had different sites irradiated during the course of their illness and benefited from considerable pain relief, reduction in analgesic requirements and increase in mobility and improvement in quality of life.

5.5. Conclusions

The aim of palliative radiotherapy should be effective, simple treatments with a limited number of fractions causing minimal upset to the patient. Half of the regimens consisted of three fractions or less. There was no additional outcome benefit from protracted dose fractionation schedules and relief of pain to painful bony or soft tissue sites of disease was possible in 90.8% of treatments attempted. In a third of cases, complete pain relief enabling the discontinuation of opiate analgesia in the advanced stages of disease was seen. Treatments were well tolerated and the incidence of side effects low. No patient required increased analgesia as a direct result of the radiation therapy. Simultaneous oral etoposide therapy, used currently for palliation of systemic disease, could be continued without any additional toxicity.

The effect was usually sufficiently rapid in onset to be beneficial and sustained for the remainder of the patient's life. A dose response relationship could not be determined, possibly due to the small number of patients. However, it can be concluded that the palliation of painful metastases with small doses in one or two fractions appears to provide effective long lasting control, with the possibility of retreatment if necessary.

This mode of palliative treatment may not be considered where the radiotherapy department is separate from the children's hospital. However, any patient with localised persisting pain may well achieve long term effective pain control with one or two treatments.

CHAPTER 6 THE MODULATION OF ^{131}I-mIBG UPTAKE BY PRE-DOSING WITH CHEMOTHERAPY

6.1. INTRODUCTION

Laboratory studies confirm that neuroblastoma is responsive to both chemotherapy and radiotherapy *in vitro*. For children with advanced disease m-IBG is unlikely to be curative and is therefore often used in combination with other therapies (Voute *et al.*, 1995; DeKraker *et al.*, 1995; Mastrangelo *et al.*, 1995; Gaze *et al.*, 1995). In the United Kingdom, a multicentre study of ^{131}I-mIBG, used as the initial agent followed by multi-agent chemotherapy, has recently begun (Gaze and Wheldon 1996).

Several radiobiological arguments exist to consider the use of ^{131}I-mIBG before other therapies (Gaze and Wheldon 1996).

- Problems of heterogeneous tumour accumulation, due to poor tumour vascularity or variable cellular uptake, are minimised by the 'crossfire effect' of the attached isotope. This results in neighbouring cells within the path length range of the isotope being irradiated. For example, ^{131}I-mIBG would be most effective against deposits 2-5mm in diameter.
- Neuroblastoma is both chemosensistive and radiosensitive, but famed for acquired multiple drug resistance. ^{131}I-mIBG is not affected by this.
- Encouraging clinical response rates, with minimal toxicity, have been observed when this is used before conventional chemotherapy (Mastrangelo 1993; Hoefnagel *et al.*, 1994).

It is known that the administration of other therapeutic agents can alter the ability of neuroblastoma cell lines to accumulate ^{131}I-mIBG in culture. For example Smets *et al.*, (1991) demonstrated that 5Gy of external beam irradiation stimulated ^{131}I-mIBG uptake by neuroblastoma cell lines in culture conditions. In addition, neuroblastoma cells exposed to γ-interferon induced morphological differentiation and an increased transcription of the noradrenaline transporter gene (Montaldo *et al.*, 1992).

Mastrangelo *et al.*, (1995) reported a small group of patients treated with ^{131}I-mIBG in combination with cisplatin (CDDP). Although haematological toxicity was significant, treatment was reasonably well tolerated with no serious sequelae and encouraging results.

On a scientific level, the effect of previous chemotherapy exposure on mIBG uptake has not been investigated. The purpose of this study was to investigate the effect of exposure to cisplatin, etoposide, vincristine, cyclophosphamide drugs from the widely used OPEC regimen (Shafford, Rogers and Pritchard 1984) on ^{131}I-mIBG cellular accumulation.

6.2. MATERIALS AND METHODS

6.2.1. Cell Culture

SK-N-BE(2c), a neuroblastoma cell line with a high capacity for mIBG uptake (Mairs *et al.*, 1994) was used. This cell line was derived from the bone marrow

of a patient with progressive neuroblastoma following chemotherapy and radiotherapy (Biedler *et al.*, 1978). A2780, a variant of the cell line NIH:OVCAR-3, was used as a non neuronal control (Hamilton *et al.*, 1983). These cell lines were routinely screened for mycoplasma contamination and routinely refrozen and fresh batches taken, to prevent repeated selection on repeated subculture.

They were grown at 37^0C and maintained in a 5% carbon dioxide environment. Both cell lines were kept in a RPMI-1640 medium with 25mM HEPES buffer and supplemented with 10% foetal calf serum; 100 IU ml^{-1} penicillin and streptomycin; 2mM L-glutamine; 2mM amphoteracin and 2mM non essential amino acids. A2780 required 0.1%(v/v) insulin (Boehringer Mannheim). All media and supplements were purchased from Gibco (Paisley, UK) unless otherwise stated.

6.2.2. Reagents

^{131}I-meta-iodobenzylgunaidine (^{131}I-mIBG) (specific activity 37-185mBqmg^{-1}) was obtained from Amersham International (product code IBS 6711). Desmethylimipramine hydrochloride (DMI) was purchased from Sigma (Poole, Dorset, UK). 4-hydroperoxy-cyclophosphamide, the pro-drug of 4-hydroxy-cyclophosphamide was used. The active agent is quickly generated in aqueous solution. This was donated from Dr. Jorg Pohl, Asta Medica Pharmaceuticals, Germany, and was stored at -20^0C, in the dark.

The drug concentrations of chemotherapeutic agents employed were determined from repeated clonogenic assay experiments. A concentration x time exposure of drug was chosen to induce at least one log kill of cells, but which enabled the cell population to re-establish consistently in culture. Final concentrations of 2μM cis-dichlorodiammine-platinum II (cisplatin), 1μM etoposide, 10nM vincristine, 2.5μM 4-hydroperoxycyclophosphamide and 10μM non-radiolabelled m-IBG were used.

The values obtained were similar to previously published values where available. For example, 50% survival levels for LAN-1, CHP100, and CHP212 were 25-50ng per ml (8-16nM) for cisplatin and 5-20ng per ml (8-16μM) for VM26 (Pritchard, Whelan and Hill 1985). All drug solutions were freshly prepared before each assay, by dissolving them in normal saline aided by heating at 25^0C for 30 minutes and by filtering finally. Etoposide was dissolved initially in 200μL of dimethylsulphoxide (DMSO) and then diluted in medium, to the required concentration.

6.2.3. Treatment of Cells with Cytotoxic Drugs

When in exponential growth, cells were harvested by means of trypsinisation. Cells were then subcultured into 25cm^2 plastic tissue culture flasks. Once established and at least 50% confluent, these cells were then incubated with a single chemotherapeutic agent for a maximum of 24 hours.

Preliminary experiments indicated SK-N-BE(2c) had rapid growth in culture with a doubling time of 15.5 hours. In addition, the density of the cell population had a dramatic effect on the values obtained for the ^{131}I-mIBG uptake assay. Experimental conditions therefore had to be strictly defined to ensure that the cultures remained well nourished and in exponential growth at all points during the assay. If the cell number in the tissue flask became greater than one million, the experiment was terminated.

6.2.4 ^{131}I-mIBG Uptake

100 flasks per cohort were incubated with the appropriate chemotherapeutic agent. The monolayers were exposed to chemotherapy for this first 24 hours of the experiment. Cells were assayed for mIBG uptake at 0hrs, 2hrs and 24hrs after meticulous washing to remove the agent. The monolayers were then assayed thereafter every 24 hours for as long as the cells remained viable or the ambient conditions satisfactory for analysis as defined in section 6.3.1.

At the time of assay, the agent was removed by repeated washing with sterile phosphate buffered saline. Cells were then incubated with or without 1.5mM DMI for 30 minutes. This time period and concentration of DMI was previously shown to cause maximal inhibition of the active, type 1 uptake system (Mairs *et al.*, 1991). At the end of this period the medium was removed and replaced by medium containing 0.1mM ^{131}I-mIBG with or without DMI. The cell cultures were then incubated at 37^{0}C for two hours.

In order to measure the ^{131}I-mIBG uptake, the process was first terminated by washing with ice cold phosphate buffered saline. The radioactivity was then extracted from the cells by two washes of 0.5mls aliquots of 10% (w/v) trichloroacetic acid and measured in a sodium iodide crystal gamma counter (Canberra Packhard, Berkshire, UK). The mean number of cells per flask was calculated and ^{131}I-mIBG uptake quoted as picomoles of mIBG accumulated per million cells. This is expressed graphically as percentage uptake of the control.

6.2.5. Cell Viability Experiments

Clonogenic survival was assessed by colony forming assay. Trypan blue staining gave an immediate indication of the integrity of the cells.

For clonogenic assay, cells were plated at an initial concentration of 1,000 cells per flask. Once established and 70% confluent, they were incubated for 24 hours with the drug. This was then removed by repeated washing with phosphate buffered saline. The cells were sub-cultured immediately and then grown at 37^0C, in the above media, in 5% CO_2, until colonies were formed. Colonies were counted after 11 days growth. The surviving fraction was calculated as the number of colony forming cells in a treated group, relative to the control cells, that were not exposed to the drug, then corrected for cell number. A minimum of six replicates were taken for each concentration of drug and each time point, for both clonogenic assays and trypan blue assays.

6.2.6. Statistical Analysis

Each uptake experiment was repeated a minimum of three times and six replicates taken for each uptake point in the assay, or for each clonogenic flask. The points documented are the mean of uptake in picomoles of ^{131}I-mIBG per million cells. Values are expressed as a percentage of the control, un-exposed cells, +/- 2 standard deviations.

6.2.7. RT-PCR Analysis of Noradrenaline Transporter Gene Expression

6.2.7.1. First Strand cDNA Synthesis

Total RNA was extracted from 1x 10^{-6} control and cisplatin treated cells: prior to treatment, at the time of cisplatin removal and 24 hours later. The RNA was isolated according to the guanidine thiocyanate method and purified according to standard procedures (Maniatis 1990). The RNA concentration of the sample was measured by spectrophotometric analysis at 260nm. 1μg of the purified RNA was reverse transcribed using the commercial kit 'Gibco Superscript Preamplification System' (Paisley, UK) according to the manufacturer's protocol.

6.2.7.2. Amplification of cDNA

cDNA was PCR amplified using primers specific for the transporter sequence. The sense primer was 5'-CTGGTGGTGAAGGAGCGCAACGGC-3' and the antisense primer was 5'-ATGTCATGAATCCCGCTGCTCTCG-3' (Montaldo *et al.*, 1992). This amplification generated a 590 base pair PCR product. Semi-quantitation was achieved by comparison of the target signal with the signal generated by co-amplification of a reference sequence glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH primers were 5'-GCATTGCTGATGATCTTGAGGC-3' (sense) and 5'-TCGGAGTCAACGGATTTGG-3' (antisense). These generated a 300 base pair PCR product. Coamplification of the target cDNA sequences was performed in 10x synthesis buffer (pH 8.3) containing 100mM Tris-HCL; 1.5mM $MgCl_2$; 500mM KCl; 10nM of dNTPs; 20picomoles of each reference and GAPDH primer and 2 units of Taq polymerase. Thirty five amplification cycles, completed in a standard thermocycler in the following steps: one minute denaturation at 94^0C; one minute annealing at 65^0C and one minute extension at 72^0C. Products were electrophoresed through a 2% agarose gel in TBE buffer (Maniatis 1990). These were densitometrically scanned using Quality One Image Analysis software.

6.2.8. Observation of SK-N-BE(2c) with Chemotherapy

Cells were observed daily by light microscopy. In addition trypan blue staining was performed. The number of cells and surviving fraction was noted daily.

Morphological evidence of differentiation was noted by a change in cell shape and the development of neurite outgrowth. An increase in number and length of neurites was used as a marker of neuroblastic differentiation.

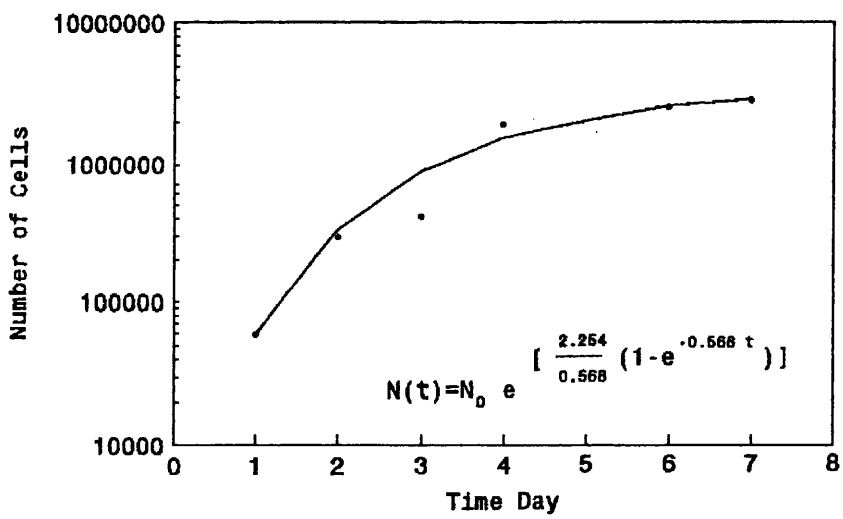
6.3. RESULTS

6.3.1. Preliminary Experiments Defining the Model System Conditions.

6.3.1.1. Cell Growth

Preliminary experiments showed that SK-N-BE(2c) had rapid growth in culture with a mean doubling time of 15.5 hours, in exponential growth.

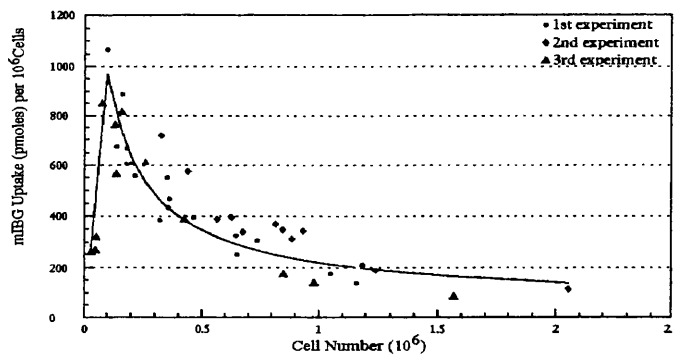
Figure 6.1. The cell doubling time of SK-N-BE(2c) in culture.



6.3.1.2. The effect of cell density on ^{131}I-mIBG uptake

Chemotherapy exposure resulted in large fluctuations in cell number. Initial experiments therefore explored the effect of cell density on ^{131}I-mIBG uptake and nor-adrenaline expression. The density of the cell culture dramatically affected the activity of both active and passive uptake of ^{131}I-mIBG. High culture densities resulted in a progressive decline in ^{131}I-mIBG accumulation. This finding has been noted previously (Montaldo *et al.*, 1992).

Figure 6.2. The effect of cell density on total ^{131}I-mIBG uptake.
The cell density is expressed as the number of cells in the culture dish. The mean ^{131}I-mIBG uptake is expressed as picomoles of ^{131}I-mIBG accumulated per million cells. Data points represent six replicates for each point of a series of three independent experiments on successive sub-cultured populations.



It shows two things, firstly, the large variability of uptake with very sparse cultures of low cell number, less than $1x10^{-5}$. For example total ^{131}I-mIBG uptake varies from 250 picomoles of ^{131}I-mIBG accumulated, per million cells,

when 2.5 x 10^4 cells are present to 1050 picomoles of ^{131}I-mIBG per million cells accumulated by $1x10^{-5}$, a 4.2 fold difference in cellular accumulation. In addition, low levels of ^{131}I-mIBG uptake occur in dense cell cultures, less than 200 picomoles per million cells, in cultures greater than 1 million in cell number. This is due to the large number of dying cells in a nutritionally depleted, acidic, hypoxic environment of overcrowded cultures as specific uptake is oxygen, energy and electrolyte dependent. This graph also illustrates the uptake characteristics of repeatedly sub-cultured populations of SK-N-BE(2c) and demonstrates little variability of ^{131}I-mIBG uptake in daughter populations.

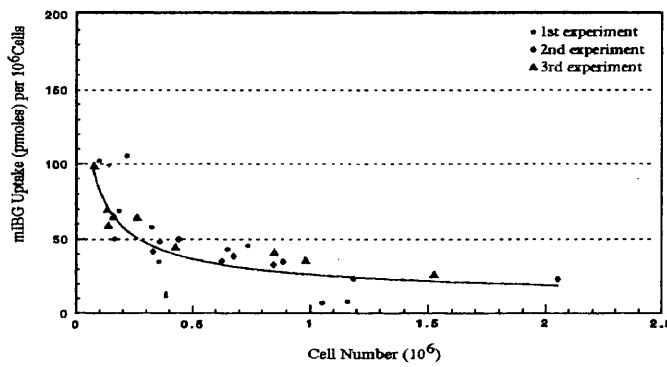
PCR analysis confirmed the reduction in ^{131}I-mIBG uptake, with increasing cell culture density, was due to a decreased expression of the noradrenaline transporter gene (Table 6.1).

Table 6.1. The effect of culture density on noradrenaline transporter expression in SK-N-BE(2c) Cells. (Final result obtained by Shona Cunningham).
Target to reference ratios were calculated from the intensity of PCR signals measured by scanning of ethidium bromide stained gels. R= reference sequence (GAPDH) and T= Target sequence (Noradrenaline transporter).

Culture Density (cell number X 10^{-5} per cm^2)	Target: Reference Ratio
0.09	0.96
0.16	1.01
0.19	0.82
0.37	0.45
0.65	0.32
0.86	0.25

Figure 6.3. The effect of cell density on passive ^{131}I-mIBG uptake

The cell density expressed as the number of cells in the culture dish. The mean ^{131}I-mIBG uptake is expressed as picomoles of ^{131}I-mIBG accumulated per million cells. Data points represent six replicates for each point of a series of three independent experiments on successive sub-cultured populations.



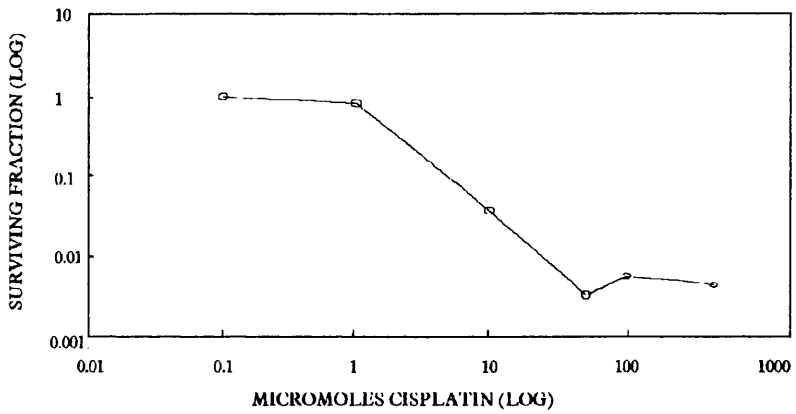
Passive uptake accounts for a small proportion of ^{131}I-mIBG uptake, less than 10%. This accumulation however also drops with increasing cell number again due to the large number of dying cells in an overcrowded culture environment.

6.3.1.3. Clonogenic Assays to Determine Chemotherapy Dose

Repeated colony assay experiments indicated the concentration of each chemotherapeutic agent for each cell line that would reproducibly cause at least one log cell kill.

Figure 6.4. Effect of 24 hours exposure of cisplatin on clonogenic survival of SK-N-BE(2c).

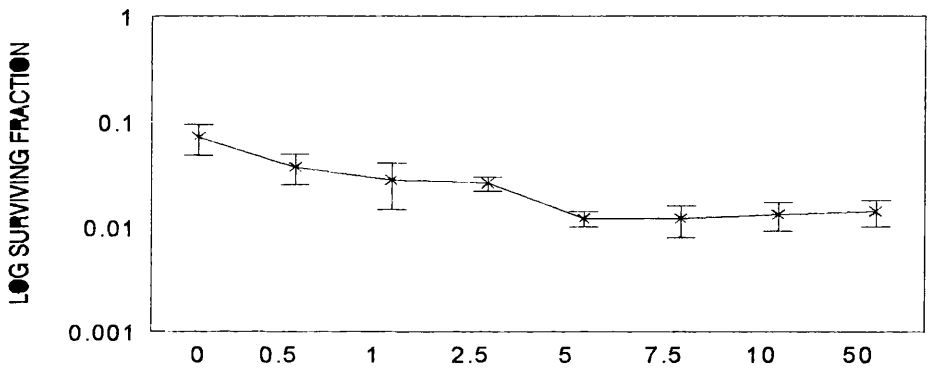
Results represent a mean value obtained from six replicates over a series of three independent experiments.



A 24 hour exposure of 4μM of cisplatin reduced the clonogenic potential of the population to 10% of the previously unexposed cells. The chemotherapy experiments were actually conducted within the range of 10nM to 5μM cisplatin.

Figure 6.5. Effect of 24 hours exposure of 4-hydroxyperoxycyclophosphamide on clonogenic survival of SK-N-BE(2c).

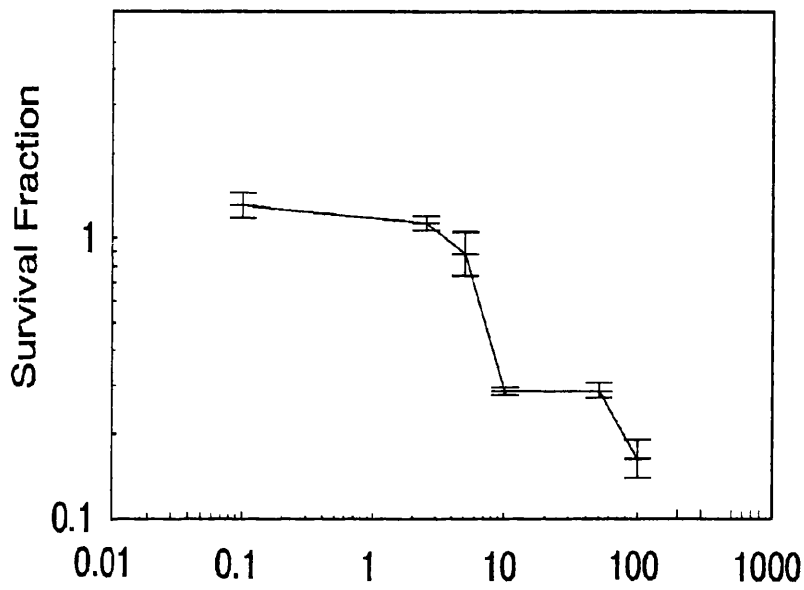
Results represent a mean value +/- 2 SD, obtained from six replicates over a series of three independent experiments.



10μM cyclophosphamide reduced the number of surviving cells to 10% of the control values. However, repeated colony assays predicted that a lower concentration, 2.5μM cyclophosphamide exposure would reliably induce cell kill but enable the remaining cell population to re-establish.

Figure 6.6. Effect of 24 hours exposure of vincristine on clonogenic survival of SK-N-BE(2c).

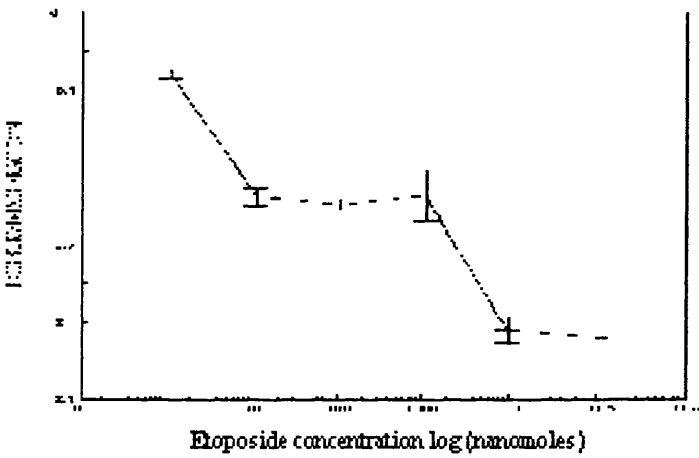
Results represent a mean value +/- 2 SD, obtained from six replicates over a series of three independent experiments.



10nM vincristine was the concentration used to reduce the cell number from $2.32x10^{-5}$ to $0.74x10^{-5}$.

Figure 6.7. Effect of 24 hours exposure of etoposide on clonogenic survival of SK-N-BE(2c).

Results represent a mean value +/- 2 SD, obtained from six replicates over a series of three independent experiments.

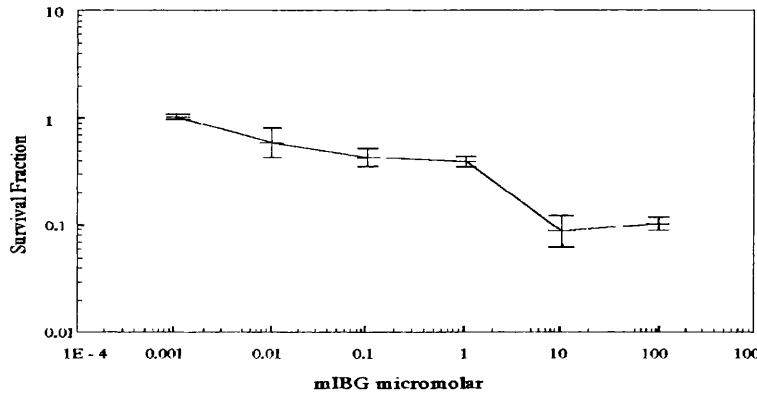


Etoposide concentration micromoles (log)

1μM etoposide proved sufficient to effectively reduce the cell concentration to 10% of the control values.

Figure 6.8. Effect of 24 hours exposure of m-IBG on clonogenic survival of SK-N-BE(2c).

Results represent a mean value +/- 2 SD, obtained from six replicates over a series of three independent experiments.



mIBG concentration micromoles (log)

10μM mIBG was noted to reduce the surviving fraction of cells to one tenth of the unexposed sample.

6.3.2. Cell Population Effects After Exposure to Cytotoxic Drugs

The chemotherapy agent was present in the medium for the first 24 hours. The cells continued to grow exponentially, regardless of the drug incubation. The cell culture consists of a heterogeneous population of dividing cells, distributed in different parts of the cell cycle, relatively quiescent cells and cells resistant to the drug used. For cell cycle specific agents, those cells in parts of the cell cycle past the block will proceed to division and increase the cell number. In addition there will be a number of cells unaffected by the drug.

The surviving fraction, a measure of the clonogenic ability of the population, drops noticeably at 2 hours with all agents then drops further at the end of the drug exposure period. For example, with cisplatin, the surviving fraction of SK-N-BE(2c) decreased from 1, in non-exposed cells, in these conditions, to 0.45 (SD 0.003) at 2 hours and 0.216 (0) at 24 hours. During the first day of the experiment, the cell number continues to increase despite these observations due to the unaffected cells continuing to divide rapidly. For 24 hours after the removal of the drug, there is a continued expression of cell damage with cell numbers continuing to fall, the surviving fraction remaining low and the proportion of trypan blue stained cells high. The cells after chemotherapy exposure became quite precarious and meticulous attention to their cell culture conditions was needed to ensure their survival. Medium was changed therefore twelve hourly during this period. By day 3, the monolayers show signs of recovery and quickly re-establish.

Trypan blue staining, indicating metabolically incompetent cells, increased to a maximum value of 46.7% at day 2, 24 hours after the removal of the drug from the medium. High levels of trypan blue staining persist but the cells are recovering by day 3, when for example the trypan blue values fall dramatically to 17% and 24% respectively. At this time the cell number stabilises and then continues to increase exponentially. After this critical 24 hour period, treated cells often showed ,with all OPEC agents, a stabilisation of cell number and constant surviving fraction. These observations often correlated with morphological evidence of differentiation.

Table 6.2. The cell population effects of OPEC drug exposure

Data points represent a mean value +/-2 SD obtained from six replicates from a representative experiment over a series of three independent experiments.

	0 hours	2 hours	24 hours	48 hours	72 hours	96 hours
CISPLATIN						
CELL NUMBER (SD)	0.116 (0.013)	0.11 (0.006)	0.499 (0.002)	0.254 (0.006)	0.438 (0.006)	0.633 (0.05)
SURVIVING FRACTION(SD)	1 (0.006)	0.454 (0.003)	0.216 (0.00)	0.17 (0.003)	0.216 (0.001)	
TRYPAN BLUE +	0%	1.5%	40%	46.7%	17%	24%
CYCLOPHOSPHAMIDE						
CELL NUMBER (SD)	0.285 (0.1)	0.285 (0.016)	0.534 (0.16)	0.31 (0.05)	0.965 (0.07)	1.2 (0.01)
SURVIVING FRACTION(SD)	1 (0.006)	0.21 (0.006)	0.21 (0.001)	0.188 (0.003)	0.2 (0.001)	
TRYPAN BLUE+	0%	5%	40%	55%	57%	
ETOPOSIDE						
CELL NUMBER(SD)	0.285 (1)	0.29 (0.07)	0.517 (0.019)	0.292 (0.134)	1.1 (0.108)	1.7 (0.016)
TRYPAN BLUE	0%	16.6%	32%	27%	13.4%	
VINCRISTINE						
CELL NUMBER(SD)	0.285 (1)	0.251 (0.037)	0.564 (0.160	0.261 (0.049)	0.583 (0.052)	0.9 (0.01)
SURVIVING FRACTION(SD)	1 (0.006)	0.663 (0.01)	0.323 (0.004)	0.216 (0.004)	1.24 (0.003)	
TRYPAN BLUE+	0%	5%	30%	24.6%	15%	26%

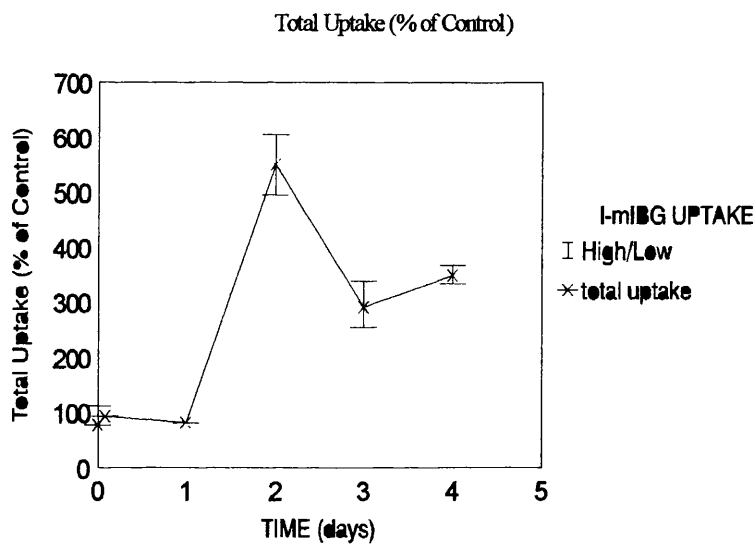
6.3.3. ^{131}I-mIBG Uptake

6.3.3.1. The Effect on ^{131}I-mIBG Uptake with 2μM Cisplatin

The cells were incubated with cisplatin for 24 hours. In the presence of the drug, type 1 uptake of ^{131}I-mIBG remains unaffected. On day 2 of the experiment, 24 hours after removal of the drug, the active type 1 uptake, increases dramatically from 104 to a mean of 574.08 picomoles of ^{131}I-mIBG per million cells (95% CI= 605-495 picomoles of ^{131}I-mIBG per million cells).

This represents a 6.8 fold increase in uptake. If a correction factor is applied to compensate for the difference in cell number between the two populations, this dramatic increase is still real.

Figure 6. 9. ^{131}I-mIBG uptake and cisplatin exposure.
Results represent a mean value +/- 2 SD, obtained from six replicates over a series of three independent experiments. Uptake is expressed as a percentage of the unexposed control value.



Additional experiments suggested a concentration dependent stimulation of active incorporation of ^{131}I-mIBG. These results are summarised below.

Table 6. 3. The effect of cisplatin concentration, type ^{131}I-mIBG uptake and noradrenaline transporter expression.

These data represent SK-N-BE(2c) cells 24 hours after cisplatin removal. ^{131}I-mIBG total uptake values represent mean value from three experiments in triplicate. Values are expressed as percentage of ^{131}I-mIBG accumulated and compared to the control 0 hours value.The noradrenaline transporter gene expression is expressed as the ratio of intensity of target sequence to intensity of the reference sequence. The values are expressed as the percent of control values of non-drugged cells(+/- 2SD).

Cisplatin (μM)	^{131}I-mIBG Uptake (% of control)	Noradrenaline Transporter Expression (% of control)	
		24 hours	48 hours
0.02	171% ($p<0.001$)	115 (+/-) 6.2	89(+/-) 11.2
0.2	162% ($p<0.001$)	120 (+/-) 7.5	94 (+/-) 8.4
2	355% ($p<0.001$)	129 (+/-) 7.9	134 (+/-) 9.3
20	431% ($p<0.001$)	125 (+/-) 8.2	165 (+/-) 10.6

Cisplatin induces a dose dependent stimulation of expression of the noradrenaline transporter molecule. This is assessed at the 24 hour time point, when the drug is removed and at the 48 hours time point, when the increase in ^{131}I-mIBG accumulation is observed. At 0.02μM and 0.2μM levels of cisplatin, the induced enhancement is not maintained after the removal of the drug. At 2μM and 20μM however the effect was prolonged. The findings above suggest

that the increased uptake of ^{131}I-mIBG was due to the increased synthesis of receptors rather than increased activity of existing receptors.

6.4. ADDITIONAL EXPERIMENTS

6.4.1. Inhibitor Studies

DMI, an inhibitor of type 1 uptake, enabled the contribution of passive uptake to be estimated. Results suggested that the increased accumulation was due to type 1 accumulation.

During the first 24 hours there was a rise in passive accumulation of ^{131}I-mIBG which peaked at 30% at the end of incubation. 24 hours after drug exposure, on day 2, when there was a dramatic increase in total ^{131}I-mIBG uptake, this passive uptake returned to normal values (<10%). SKF550 was used in monolayers, with or without DMI, in an attempt to define if type 3 uptake was present. The was no demonstrable type 3 uptake in SK-N-BE(2c) cells.

6.4.2. **Retention Studies** (completed by Shona Cunningham)

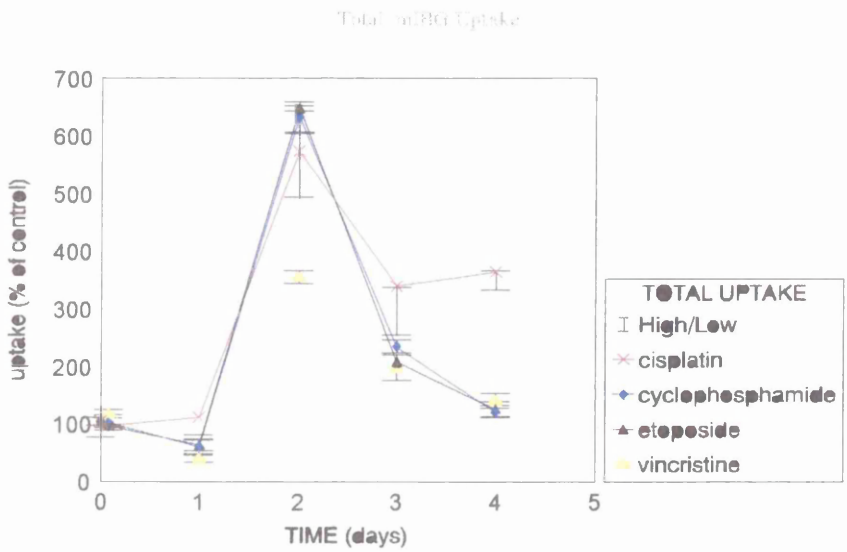
SK-N-BE(2c) cells have no storage mechanism for ^{131}I-mIBG. They retain a high intracellular level by a dynamic equilibrium of diffusion and re-uptake of the drug. To determine if this increased cellular accumulation was due to decreased egresion of the drug from the cells or storage in granules, reserpine

was used, which depletes these. No difference was found in these experiments between the cisplatin and control cells (Data not shown, Armour *et al.*, 1997).

6.4.3. The Effect of Pre-dosing SK-N-BE(2c) With OPEC Chemotherapy

Figure 6.10. The effect of pre-dosing SK-N-BE(2c) With OPEC chemotherapy.

The following values were taken from a representative experiment and correlate with the population data of table 6.1. The following agents were present for the first 24 hours of the experiment: 2μM cisplatin; 2.5μM cyclophosphamide; 1μM etoposide; 10nM vincristine.; ^{131}I-mIBG uptake was assayed at 0, 2, and 24 hours time point. The drugs were then removed and assays repeated on the surviving cell populations. Each point represents a mean value +/- 2SD, obtained from six replicates for every point of the assay and expressed as percentage of the control.



Cyclophosphamide, another alkylating agent gave similar results to cisplatin above with total uptake increasing from 104.4 picomoles per million cells to a mean of 633.6 picomoles per million cells, 95% CI=660.14-607.06 picomoles per million cells. This represents an increase of 608% in radionuclide accumulation.

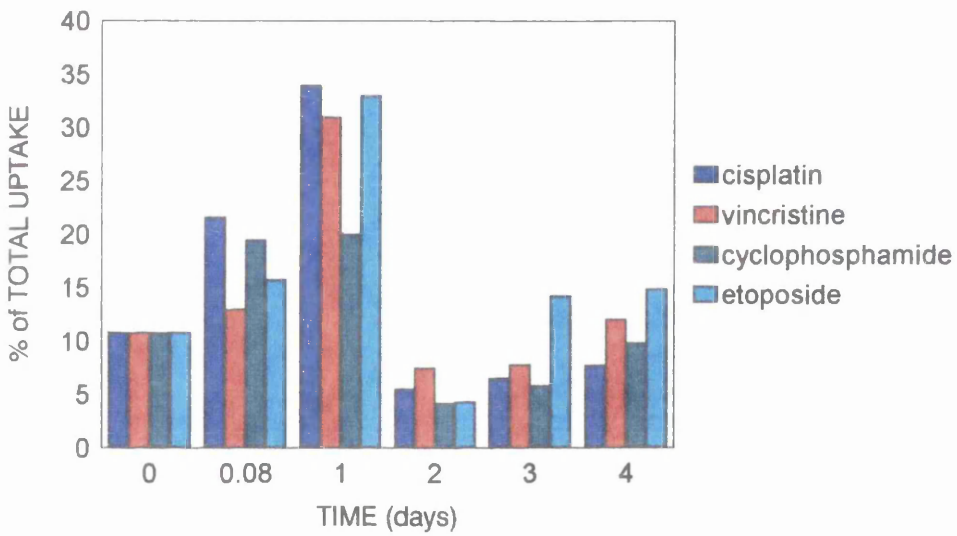
Etoposide, a cell cycle specific agent also provoked a 620% increase in type one uptake, from 104.4 to 648.3 (95%CI= 676.38-620.22) picomoles per million cells., interestingly at 24 hours when specific uptake accounts for only 60.7% of the control value. Similarly, 10nM vincristine elicited a specific type 1 increased accumulation of 340% (mean, 356.1 picomoles per million cells, 95% CI= 367.19-345.01 picomoles per million cells 24 hours after the drug is removed, but at 24 hours, type one accumulation accounts for only 37.8% of the control value.

6.4.4. The Passive Uptake of ^{131}I-mIBG in SK-N-BE(2c) with All OPEC Drugs

Type 2 passive uptake accounts for less than 10% of the total ^{131}I-mIBG accumulated by SK-N-BE(2c), under normal conditions. However, on exposing the cells to the individual OPEC agents, there was an increase in this non-specific uptake which reached a maximum value at the end of drug exposure when it accounts for a mean value of 29.5% (range 22%-33.98%) of total uptake. The simultaneous low cell number, surviving fraction and high degree of trypan blue staining suggested the chemotherapy induced damage was

expressed maximally at this point. The increased passive uptake returned to normal control values, 24 hours later, when the large increase in type 1 uptake was seen.

Figure 6.11. The passive uptake of SK-N-BE(2c) with all OPEC drugs .
The data is expressed as a mean of six replicates, taken from the same representative experiment outlined in table 6.1 and graph 6.10.

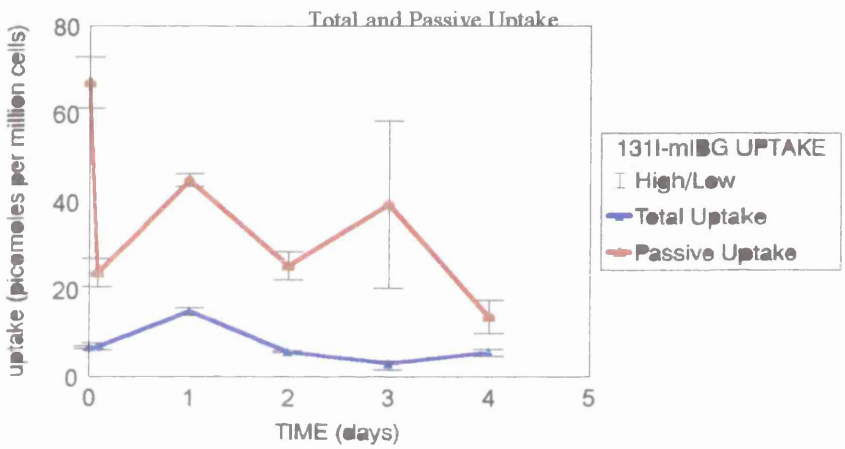


6.4.5. The Effect of ^{131}I-mIBG Uptake with mIBG Exposure

Predosing with mIBG showed an apparent reduction in specific type 1 uptake at 2 hours. This value remained low for the rest of the assay period. Passive uptake remained less than 10 picomoles per million and less than 10% of the total ^{131}I-mIBG accumulated. The amount of passive uptake peaks at 24 hours when it accounted for 15 picomoles per million cells (33% of total uptake). The total amount of uptake must be interpreted with caution since the presence of any

unlabelled m-IBG will ultimately compete for receptor molecules and interfere with the assay.

Figure 6.12. Total and passive uptake of ^{131}I-mIBG with 10μM m-IBG. *Each point represents the mean and 95% confidence intervals obtained from six replicates at each point in the assay.*



6.4.6. The Effect of Chemotherapy Exposure on Non-neuronal Control Cells.

Predosing non-neuronal control cells, A2780, with chemotherapy had no effect on the cellular accumulation of ^{131}I-mIBG. Notably there was no increase in passive accumulation of ^{131}I-mIBG at 24 hours as with the SK-N-BE(2c) cells.

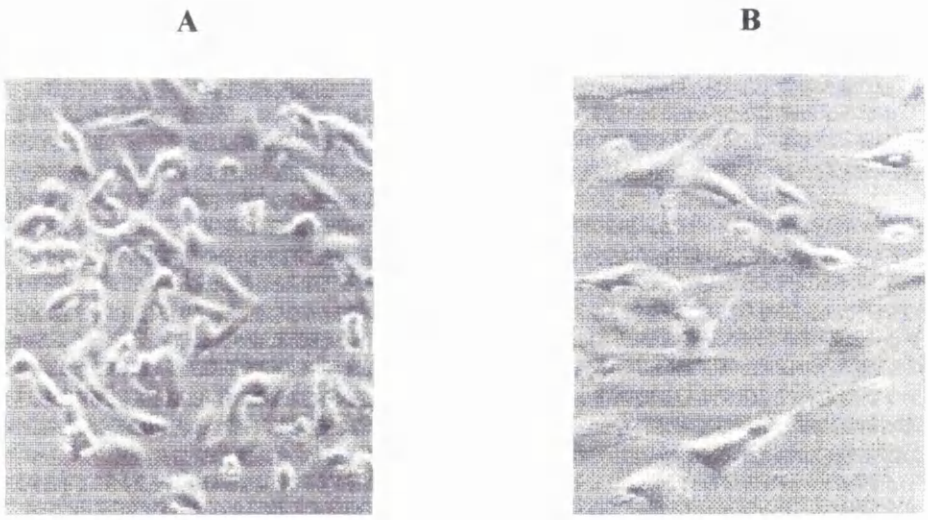
Table 6.4. Total and passive uptake of ^{131}I-mIBG of A2780 cell (picomoles per million cells) after exposure to 10nM vincristine.

	0 Hours	0.08 Hours	24 Hours	48 Hours
MEAN TOTAL UPTAKE (SD)	9.27 (0.87)	8.78 (0.43)	3.2 (0.4)	4.2 (0.015)
MEAN PASSIVE UPTAKE (SD)	6.77 (1.95)	8.2 (0.2)	1.75 (0.2)	1.22 (0.25)

6.4.7. Morphological Change in SK-N-BE(2c) After Drug Exposure.

Often after drug exposure, the cell population became fragile and the cell culture would fail to re-establish. Occasionally however, the cell number would remain stable, individual cells became less adherent and distinct neurite formation was seen. The following photographs were taken on day 4 of the experiment, 3 days after the removal of the drug. It must be stressed that these are preliminary observations and no firm semiquantitation e.g. serial neurite: cell body length measurements were applied.

Figure 6.13. The morphological change of SK-N-BE(2c) cells
A= control; B=with 10nM Vincristine



6.4.8. Summary of Results

- Preliminary experiments confirmed the rapid growth of SK-N-BE(2c) in culture. This resulted in a reduction of type 1 uptake and decreased expression of the noradrenaline transporter.
- Cisplatin exposure resulted in an increased specific accumulation of ^{131}I-mIBG. RT-PCR studies confirmed a concentration dependent enhancement of noradrenaline transporter gene expression.
- There was no increase in type 2 uptake nor increased retention of ^{131}I-mIBG, to explain this increased accumulation. Type 3 uptake was not demonstrated in SK-N-BE(2c) cells.
- This increased specific accumulation was observed when other OPEC agents were used.
- There was a transient increased passive accumulation of ^{131}I-mIBG at 24 hours but this has returned to normal 24 hours later.
- No effect was seen on non neuronal cells.
- The effect of predosing SK-N-BE(2c) with mIBG remains unclear.

6.5. DISCUSSION

6.5.1. Background

At present ^{131}I-mIBG is used in the transplant setting, against micrometastatic disease, but recent radiobiological modelling suggests that this may not be optimal. The exposure of neuroblastoma cells to the OPEC agents was investigated to determine if their prior exposure affected the specific type 1 accumulation of ^{131}I-mIBG.

6.5.2. The Experimental System.

A cell culture monolayer system was examined to determine the effect of chemotherapy exposure on the purely cellular uptake, isolated from the additional pharmacokinetic and pharmacodynamic factors involved in chemotherapy *in vivo*. The reported series of experiments were conducted over a period of several months. Preliminary experiments therefore were necessary to ensure the accuracy of the final results.

Firstly, theoretically, phenotypic variations are possible within a cell culture line within this period. Repeated freezing and changing of cell stock minimised the risk of this phenomenon. As well as these precautions, preliminary experiments confirmed that over the average length of each experiment, there was no difference in the ^{131}I-mIBG uptake ability of the cells (figure 6.1 and figure 6.2).

Secondly, specific ^{131}I-mIBG uptake is mediated through Na^+K^+ATPase pump. It has been demonstrated in retinal pigment epithelium and other neuroblastoma cell lines, that the expression of this type of pump is inversely correlated with cell density (Burke *et al.*, 1991; Montaldo *et al.*, 1992). Preliminary studies indicated that this was also true for the SK-N-BE (2c) cell line and the environmental conditions during the assay had to be strictly maintained and results discarded when the cultures became too dense.

The concentrations of agents used to predose the cells were, in some cases, clinically achievable concentrations but were primarily chosen to achieve at least one log cell kill in a population and to enable the resulting survivors to re-establish. This was an attempt to mimic the effects of repeated cycles of chemotherapy. In practice, slightly lower drug concentrations were necessary to allow the monolayers to re-establish but the cells exposed were reduced to a maximum of half their previous control values. The results summarised in table 6.1 indicate a representative degree of cell kill achieved in a typical experiment.

6.5.3. The Effects of Chemotherapy

During the 24 hour incubation period and for 24 hours afterwards, there is an increase in trypan blue staining, suggesting deficiency in the metabolic integrity of the cell. But more importantly, the falling cell number and reduced surviving fraction indicate the expression of chemotherapy induced damage.

6.5.4. Increased Active Uptake

The neuroblastoma cells pre-incubated with 2μM cisplatin, a clinically relevant concentration (Rosenberg 1985), more efficiently concentrated ^{131}I-mIBG than the control, un-drugged cells. This enhanced accumulation does not appear to be due to enhanced type 2 or 3 uptake, nor to a greater degree of storage of the molecule, since inhibitor studies with DMI, SKF550 and reserpine respectively, do not affect this enhanced uptake. These observations were also made when other OPEC agents, cyclophosphamide, etoposide and vincristine, were examined.

The OPEC agents differ in their modes of action and cell cycle specificity. The fact that all agents, regardless of their cellular target or cell cycle specificity up-regulated the accumulation of ^{131}I-mIBG suggested that this was a result of nuclear damage.

The next phase of experiments examined the expression of the noradrenaline receptor, which mediates type 1 uptake. Much is known about the cellular and nuclear effects of cisplatin and this drug was therefore chosen for further study. This was to determine if the increased accumulation of ^{131}I-mIBG was due to an increased expression of receptor or increased function of the remaining receptors. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis confirmed a concentration dependent enhancement of expression of this noradrenaline transporter gene.

It is known that chemotherapy, including cisplatin, disrupts signal transduction pathways (Tritton and Hickman 1990) and as a result, alters cell growth and differentiation. Cisplatin interestingly, initiates a cascade mechanism where DNA is degraded, in an attempt to protect the chromosomes from further DNA damage. The decoy DNA is probably sacrificed, in an attempt to neutralise the reactive cisplatin molecule in a similar manner to cellular glutathione and methionine (Scanlon 1990). This results in a transient change in gene expression associated with a change in expression of nuclear oncogenes and DNA repair enzymes.

There is evidence for increased DNA polymerase expression within 9 hours of cisplatin exposure (Scanlon *et al.*, 1991). The change in gene expression therefore occurs rapidly. DNA damage is usually expressed maximally at 48 hours post exposure (Pritchard *et al.*, 1985).

The mode of cell death induced by cisplatin is complex and dose dependent. At supra-lethal concentrations (100μM) rapid apoptotic death occurred in a murine leukaemic cell line, whereas lower concentrations (1-10μM) caused G_2 arrest followed by slow non-apoptotic death (Ormerod *et al.*, 1994). Others have shown that in human lymphoblastoid cells, DNA damage, by cisplatin, may result in p53 mediated apoptosis if cells are in G1/S phase or p53 independent apoptosis of p53 mutant cells in G_2/M phase of cell cycle (Allday *et al.*, 1995). It has been demonstrated previously that SK-N-BE(2c) cells respond to cisplatin treatment by undergoing G_2/M blockade and subsequent apoptosis (Piacentini *et al.*, 1993).

Ionising radiation, interferon γ and phorbol esters enhance the uptake of ^{131}I-mIBG in neuroblastoma cells (Montaldo *et al.*, 1992; 1996; Smets *et al.*, 1991). These agents, in common with cisplatin up-regulate p53 expression. Furthermore cisplatin can induce the expression of p53 dependent genes such as the CIP1 gene, encoding for the cell cycle kinase inhibitor p21 (El-Deiry *et al.*, 1994). It may be postulated, therefore, that the increased accumulation of ^{131}I-mIBG results from the transcriptional transactivation of the noradrenaline transporter gene via a putative p53 consensus sequence in the promoter.

In addition to the activation of apoptosis by the formation of DNA adducts (Dole *et al.*, 1995; Cece *et al.*, 1995) cisplatin may inhibit the growth of neuroblastoma cells by virtue of its capacity to promote differentiation.

Cytotoxic agents including epirubicin and gamma irradiation (Rocchi *et al.*, 1987; 1993) have been shown to induce biochemical as well as morphological evidence of differentiation *in vitro*. Importantly cisplatin has been shown to induce neurite outgrowth at concentrations of 0.4 to 13.2μM (Konigs *et al.*, 1994). This was found to be dose dependent and maximal at 3.3μM cisplatin. It is interesting that the cultures of neuroblastoma cells, exposed to 2μM cisplatin showed some features of differentiation, although these were not quantified.

SK-N-BE(2c) represents an intermediate (I) type of cell line. Different differentiating agents can cause it to differentiate towards either a 'N' neural, or 'S' substrate adherent type phenotype (Ross *et al.*, 1994; Spengler *et al.*, 1994).

N type cells are characterised by small round bodies and short bipolar neurites and adhere poorly to the culture vessel. An 'S' cell in contrast is flat, adherent and expresses markers of schwann cell, glial or melanocyte precursors. It has been suggested that a neuroblastic (N) type phenotype is associated with a higher type 1 accumulation of mIBG. (Iavarone *et al.*, 1991).

Not all differentiating agents causing maturation in neuroblastoma cultures, increase ^{131}I-mIBG accumulation. Montaldo performed a series of experiments noting the expression of the noradrenaline receptor using RT-PCR. He noted an increased expression of the noradrenaline receptor on treating LAN-5 neuroblastoma cells with a variety of differentiating agents (Montaldo *et al.*, 1996). LAN-5 shows a moderate constitutive level of mIBG incorporation. Accumulation doubled after a 48-72 hour exposure to interferon-γ (IFN) and there was a transient increase with TPA (a phorbol ester). Retinoic acid, a well-known differentiating agent, failed to show any increased expression and accumulation despite inducing differentiation in cells.

This may be explained by different cell signal transduction pathways being affected by the different agents. This is interesting since phorbol esters mediate gene induction (c-fos) via the transcription factor AP2 whereas interferon and cisplatin induce the metallothionine IIA gene via a different signal transduction pathway (Scanlon *et al.*, 1991).

In fact differentiation is commonly seen in neuroblastoma cells but not all neuroblastoma cells are capable of maturing. Ambrose using paraffin non-

isotopic in situ hybridisation (PNISH) found that cells with 1p deletion exhibited no evidence of differentiation and postulated that a gene in this area is responsible for maturation.

In the population of cells that survived cisplatin treatment, the relative proportions of cycling cells, quiescent cells, clonogens and doomed cells are not known. Therefore the sub-population that displayed the increased capacity for active uptake of ^{131}I-mIBG cannot be assigned with certainty. From a therapeutic perspective, this is less relevant since it is possible that even those cells which are destined to die as a result of cisplatin exposure, could still contribute to the radiation crossfire effect by virtue of the increased uptake of the radiopharmaceutical and hence 'cross fire' effect.

Recently cisplatin has been used in combination with ^{131}I-mIBG in patients with relapsed disease. Cisplatin, administered in two consecutive weekly treatments, followed 24 hours later by ^{131}I-mIBG, resulted in an impressive response rate of 67% (Mastrangelo et al., 1995). It is tempting to speculate that these *in vitro* findings may be relevant. Further spheroid and *in vivo* studies are necessary.

6.5.5. Passive Uptake

During drug exposure, the increase in passive, type 2 uptake by mIBG is significant, regardless of the agent used. With 2μM cisplatin, this increased from 11.3-37.82 picomoles per million cells (10% to 36%) and similar values were seen for the other drugs including the alkylating agent, cyclophosphamide (11.3

to 25.32 picomoles per million cells). This was a transient phenomenon and importantly had completely resolved 24 hours later as figure 6.14 clearly demonstrates. This has potentially significant implications, for systemic therapy, since this non-specific uptake is common to all cells, and this would lead to increased accumulation in non target tissues. The mechanism is probably due to a non specific membrane effect. Pioneering clinical work by Mastrangelo however at present administers ^{131}I-mIBG at this point and no significant sequelae have been noted. In addition on assessing the effect of cisplatin pre-dosing on a non-neuronal ovarian cell line no such increase was documented. The control line A2780 showed no such significant difference in ^{131}I-mIBG accumulation throughout the period of the experiments. This must be interpreted with caution however, since the uptake by these tissues is very low, so it is possible that any increase was too subtle for this assay to identify.

6.5.6. mIBG Pre-dosing

The effect of pre-dosing SK-N-BE(2C) cells with mIBG remains unclear. In the method of assay used, the mIBG exposed to the cells inevitably competed with the labelled ^{131}I-mIBG in the assay. Using different radiolabelled isotopes would be unlikely to solve this problem due to the inherent radiosensitivity of neuroblastoma cells.

6.6. CONCLUSIONS

The effect of acute exposure of chemotherapeutic agents on mIBG cellular accumulation has never before been investigated but appears to result in a five fold increased accumulation of specific, target tissue uptake of ^{131}I-mIBG.

Inhibitor experiments indicated that this was an increase in specific type 1 uptake. This was confirmed by RT-PCR analysis which indicated a concentration dependent stimulation of noradrenaline receptor expression. Preliminary observations suggested the cells had a more differentiated phenotype but the population of cells responsible for this increased accumulation are not readily identifiable.

Increased accumulation of ^{131}I-mIBG occurred with all OPEC agents. This means that eventually, if this effect is clinically relevant, the non-myelotoxic agents of cisplatin or vincristine may be preferred, in the sick, stage 4 patient, rather than the myleosuppressive agents of cyclophosphamide and etoposide. This is important since the major concerning toxicity of ^{131}I-mIBG, at present, appears to be that of the bone marrow.

^{131}I-mIBG therapy looks promising but its place in the multimodality therapy of neuroblastoma is not fixed. Theoretically the use of ^{131}I-mIBG 'upfront', before any chemotherapy would be optimal. Clinical studies suggest the administration of cisplatin and ^{131}I-mIBG 24 hours later is feasible. The above experiments, performed on neuroblastoma cell monolayers need further evaluation, with

spheroid and xenograft models. If relevant, ^{131}I-mIBG administration 24 hours after OPEC agent may increase the radionuclide uptake by the tumour *in vivo* several fold. This is an exciting breakthrough for targeted ^{131}I-mIBG therapy.

Children with neuroblastoma at present have a high chance of relapse. This suggests the presence of occult disease after intensive multimodality therapy. It would be reasonable therefore to use all therapies available to their maximum effectiveness in optimal combination to maximise cell kill.

CHAPTER 7 EVALUATION OF A NOVEL MIBG PREPARATION

7.1 INTRODUCTION

The cellular uptake mechanisms of mIBG have been described in detail in chapter 2. Briefly, specific uptake is mediated via a high affinity, energy, oxygen, Na^+K^+ATPase dependent mechanism, via the noradrenaline transporter. This accumulates mIBG in high concentrations and predominates over passive diffusion, at low plasma concentrations of less than 1μM.

No carrier added (n.c.a.) m-IBG is a new formulation where every mIBG molecule is radiolabelled with a radionuclide in contrast to the currently available commercial preparation where only 1 of 2,000 molecules is radiolabelled. Theoretically, there should be a greater differential of ^{131}I-mIBG accumulation between target and non-target tissues if ^{131}I-mIBG were given as a low concentration, carrier free preparation, than if the same total activity were administered as the standard preparation.

Before any clinical studies could be contemplated however, laboratory investigations had to be completed to determine if this new preparation behaved similarly to the traditional formulation on a cellular level.

Initial experiments examined the mIBG uptake characteristics of the well characterised cell line SK-N-BE(2c) with A2780 as the non neuronal control. The high potency accumulation of SK-N-BE(2c) has been well documented but the ovarian cell line completely lacks the noradrenaline receptor. Therefore any

drug accumulation will be by passive diffusion. Finally, uptake of n.c.a. mIBG was tested in a panel of neuroblastoma cell lines. Other members of the group determined the cytotoxicity and biodistribution of n.c.a. ^{131}I-mIBG to ensure its safety before clinical studies could be contemplated.

7.2. MATERIALS AND METHODS

7.2.1. Chemicals

Reagents were obtained from Aldrich Chemical Company (Dorset, UK). ^{131}I-sodium iodide and ^{131}I-mIBG (specific activity 37-185MBq mg^{-1} or >1110MBq mg^{-1}) were supplied by Amersham International (Buckinghamshire, UK) (product code IBS 6711). Non radiolabelled mIBG was synthesised from meta-iodobenzylguanidine hydrochloride according to the method of Wieland (Wieland *et al.*, 1980). No carrier added (n.c.a.) ^{131}I-mIBG was synthesised by iododesilylation of metatrimethylsilybenzylguanidine (Vaidyanathan and Zalutsky, 1993) then purified by solid phase extraction (Mairs *et al.*, 1994). These were supplied by Dr. R. Mairs and Miss Shona Cunningham. n.c.a. ^{131}I-mIBG was prepared as a dry powder following the evaporation of the methanolic solvent. The drug was reconstituted in phosphate buffered saline. Desmethylimipramine hydorchloride (DMI) was purchased from Sigma (Poole, Dorset, UK).

7.2.2. Cell Culture

A panel of neuroblastoma cell lines were used: SK-N-BE (2C) (Beidler *et al.*, 1978); SK-N-SH (Beidler *et al.*, 1973); NB1-G (Carachi *et al.*, 1987) and IMR-32 (Tumilowicz *et al.*, 1970). A2780, a variant of NIH:OVCAR-3 (Hamilton *et al.*, 1983) was used as a non neuronal control. All cell lines were screened for mycoplasma contamination and routinely refrozen where fresh batches of cells were used in experiments. This was done to ensure repeated selection did not occur with repeated cell culture. They were grown at 37^0 C and maintained in a 5% carbon dioxide environment.

NB-1 and SK-N-SH required MEN medium but all other cell lines enjoyed a RPMI-1640 medium with 25mM HEPES buffer. All media were supplemented with 10% fetal calf serum; 100 IU ml^{-1} penicillin and streptomycin; 2mM L-glutamine; 2mM amphotericin and 2mM non essential amino acids. A2780 required 0.1%(v/v) insulin (Boehringer Mannheim). All media and supplements were purchased from Gibco (Paisley, UK) unless otherwise stated.

When in exponential growth, cells were harvested, SK-N-BE(2c) and A2780 by means of trypsinisation, otherwise cells were readily dislodged from the culture vessels by shaking. Cells were then subcultured into six well plates. Once established, and at least 70% confluent, they were assayed for mIBG uptake.

7.2.3. ^{131}I-mIBG Uptake

All chemicals and media were first heated to the desired temperature. Half of the cells were then incubated with the inhibitor DMI for 30 minutes. This time period and concentration of inhibitor was previously shown to cause maximal inhibition of the active, type 1 uptake system (Mairs *et al.*, 1991). At the end of this period, the medium was removed and replaced by medium containing both the drug at the same final concentration and n.c.a. ^{131}I-mIBG.

In order to measure the ^{131}I-mIBG uptake, the process was first terminated after 2 hours, by washing with ice cold phosphate buffered saline. The radioactive lysate was then extracted from the cells by two washes of 0.5mls aliquots of 10% (w/v) trichloracetic acid and measured in a sodium iodide crystal gamma counter (Canberra Packhard, Berkshire, UK). The mean number of cells per well was calculated and ^{131}I-mIBG uptake quoted as picomoles of mIBG accumulated per 10^5 cells unless otherwise stated.

7.2.4. Uptake Inhibitors

In experiments comparing these two formulations, the uptake is expressed as the ratio of specific type 1 uptake to the total amount of mIBG accumulated by the cell. 1.5μM desmethylimpramine effectively inhibits specific uptake of mIBG (Tobes *et al.*, 1985. Lashford, Hancock and Kemshead 1991). The quoted value is therefore obtained by subtracting the DMI inhibited uptake from the total value accumulated.

Temperature dependence was determined by incubating cultures at 4^0C. The sodium dependency of uptake was investigated in two ways firstly by using medium containing 125mM lithium chloride instead of sodium chloride and secondly by using 1mM ouabin, an inhibitor of Na^+K^+ATPase (Jaques *et al.*, 1987). The effect of dissolved oxygen and energy dependence was determined by the addition of 1.5mM sodium dithionite to the medium (Buck *et al.*, 1985).

The effect of the competitive inhibitors, noradrenaline, dopamine and imipramine was observed by incubating with a range of concentrations (10^{-3} to 10^{-3}M) of these drugs. Finally the uptake of ^{131}I-mIBG was tested in a panel of neuroblastoma cell lines.

7.2.5. Statistical Analysis

Each experiment was repeated a minimum of three times and six replicates taken for each uptake point in the assay. The points documented are the mean of uptake in picomoles of ^{131}I-mIBG per million cells. Error bands represent two standard deviations from the mean. The data was analysed using Student's t test.

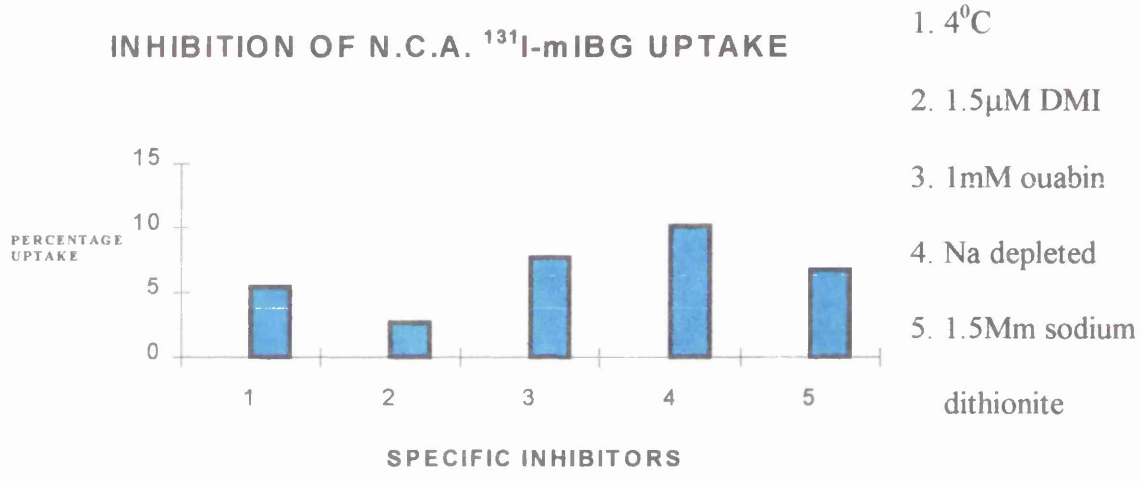
7.3. RESULTS

7.3.1. Characteristics of n.c.a. ^{131}I-mIBG Uptake

Figure 7.1 below demonstrates the uptake reduction of SK-N-BE(2c) cells with various inhibitors of specific type one uptake.

Figure 7.1. The inhibition of n.c.a. ^{131}ImIBG uptake.

SK-N-BE(2c) cells were incubated under the following conditions for 2 hours then assayed for ^{131}I-mIBG uptake. The following results are mean values, taken from six replicates for each point and expressed as a percentage of a control, non drugged sample.

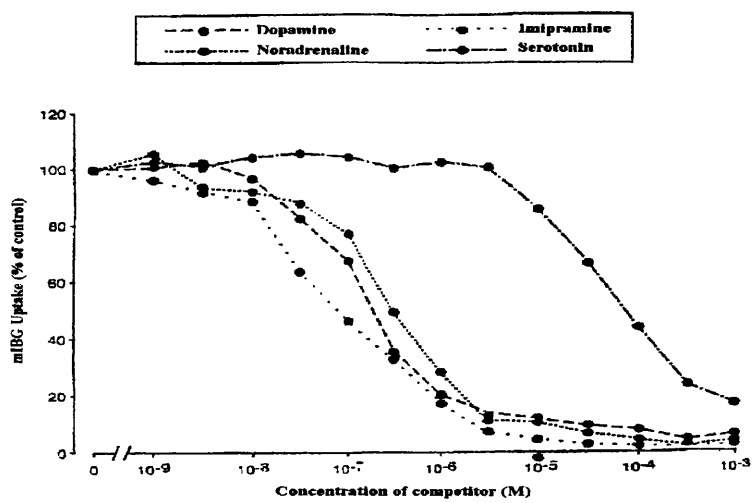


Sodium depletion of the medium resulted in the SK-N-BE(2c) cells accumulating only 10.2% of their previous values. Reducing the ambient temperature of the cells to 4°C also reduced the uptake to 5.5% of the control SK-N-BE(2c) cells. 1mM ouabain, a specific inhibitor of ATPase effectively reduced the uptake to 7.5% of the control value. Oxygen depletion similarly reduced ^{131}I-mIBG uptake to 6.8%. This indicates that for SK-N-BE(2c) the majority of drug amassed was due to specific type one uptake.

7.3.2. n.c.a. ^{131}I-mIBG Uptake in The Presence of Inhibitors

Increasing amounts of the biogenic amines dopamine, noradrenaline, imipramine and serotonin were incubated with n.c.a. mIBG. As the concentration of these compounds increased within the medium, the uptake of n.c.a.^{131}I mIBG reduced, suggesting that the new formulation was also competitively inhibited by these compounds. The degree of inhibition was 50% inhibition with 85nM imipramine; 190nM dopamine; 304nM noradrenaline and 80μM serotonin and is consistent with previously published evidence with low specific activity ^{131}I-mIBG (Lashford *et al.*, 1991).

Figure 7.2. The competitive inhibition of type 1 ^{131}I-mIBG uptake. *SK-N-BE(2c) cells were incubated with increasing concentration of the competitive inhibitors noted below. After 2 hours they were assayed for ^{131}I-mIBG uptake. The following results are mean values, taken from six replicates for each point and expressed as a percentage of a control, non drugged sample.*



7.3.3. n.c.a. ^{131}I-mIBG in Neuroblastoma Cell Lines.

The degree of specific uptake was determined in a panel of neuroblastoma cell lines and the rank order of accumulation was noted in table 7.1.

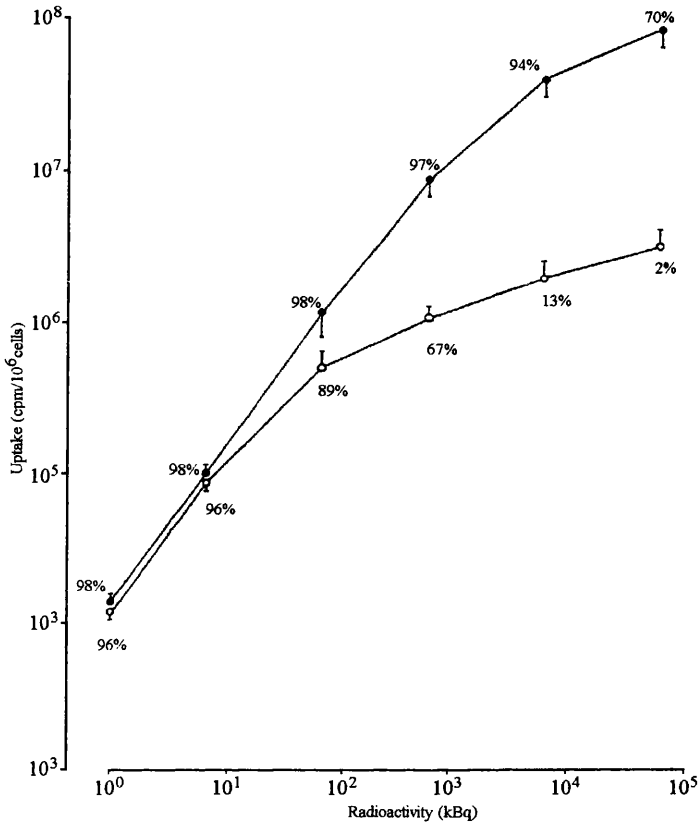
Table 7.1. The specific uptake of n.c.a.^{131}ImIBG
The following results are mean values (+/-SD), taken from six replicates for each point.

CELL LINE	DMI INHIBITABLE UPTAKE (cpm x10^{-3} per million cells)(SD)
SK-N-BE(2c)	114 (8)
SK-N-SH	107 (10)
NB1-G	26 (4)
IMR-32	12 (2)

7.3.4. Uptake of n.c.a. ^{131}I-mIBG and Low Specific Activity Preparation by SK-N-BE(2c) Cells

At 1kBq, both drug preparations are accumulated in similar amounts. As the radioactivity and hence amount of drug, added to the cells increases, the accumulation differs significantly. As the amount of radioactivity added increases, the uptake of n.c.a.^{131}I-mIBG by type one uptake accounts for 94% of total uptake while that of the commercial preparation is much less, only 13% at 9MBq. This is due to competitive inhibition by non-radiolabelled molecules in the commercial preparation.

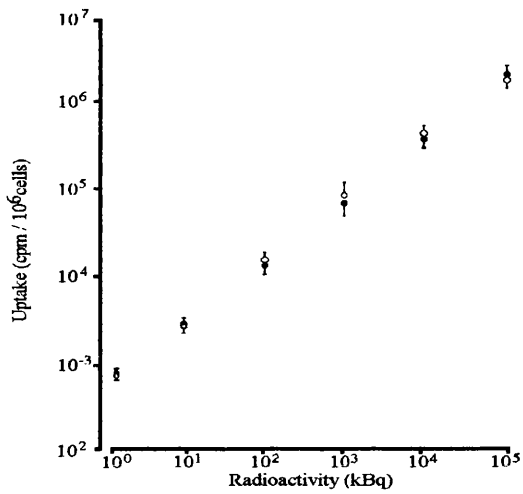
Figure 7.3. The uptake of n.c.a. ^{131}I-mIBG and low specific activity ^{131}I-mIBG. *Uptake in SK-N-BE(2c) Cells. (Open circles commercial ^{131}I-mIBG; closed circles n.c.a. ^{131}I-mIBG*



7.3.5. Uptake of n.c.a. ^{131}I-mIBG and Low SpecificActivity ^{131}I-mIBG by A2780 Cells.

There was no difference between the cellular accumulation of the two preparations. As the concentration of both ^{131}I-m-IBG preparations increased, the cellular accumulation of both increased linearly.

Figure 7.4. The uptake of n.c.a. ^{131}I-mIBG and low specific activity ^{131}I-mIBG in A2780 cells. *(Open circles commercial ^{131}I-mIBG; Closed circles n.c.a.^{131}I-mIBG)*



7.3.6. Summary of Results.

- Inhibitor studies confirmed that nca ^{131}I-mIBG is accumulated by type1uptake in SK-N-BE(2c) cells.
- nca ^{131}I-mIBG is competitively inhibited by biogenic amines suggesting accumulation by the noradrenaline receptor.
- nca ^{131}I-mIBG is accumulated in a predictable way by a panel neuroblastoma cell lines.
- For increasing amounts of specific activity the accumulation of nca ^{131}I-mIBG is greater than the present commercially used formulation.
- There was no increased accumulation of nca ^{131}I-mIBG in the non-neuronal control cell line.

7.4. DISCUSSION

Interference of the sodium dependent ATPase pump by ouabin or depletion of sodium from the medium resulted in a reduction in total uptake to 7.8% and 10.2% respectively. The method of high accumulation uptake, characteristic of SK-N-BE(2c) cells, also appears inhibited by a reduction in temperature to 4^0C (5.5%) and oxygen depletion of the medium by sodium dithionite (6.8%). This suggests that this mechanism of n.c.a. ^{131}I-mIBG uptake is via the type 1 uptake characterised in a number of neuroblastoma cell lines (Jaques *et al.*, 1984; Buck *et al.*, 1985; Gasnier *et al.*, 1986; Ivaronne *et al.*, 1991; Lashford *et al.*, 1991; Mairs, Gaze and Barrett 1991; Montaldo *et al.*, 1991).

Type 1 uptake of m-iodobenzylguanidine is thought to occur via the noradrenaline receptor. It is not unexpected therefore that the structurally similar compounds of noradrenaline and dopamine competitively inhibit the type 1 accumulation of m-IBG. Platelets accumulate ^{131}I-mIBG via the serotonin receptor. Serotonin appeared less effective at competitively inhibiting uptake in the neuroblastoma cell lines, probably due its differing structure. The order of effectiveness of inhibition, imipramine>dopamine> noradrenaline> serotonin corresponds to that of published studies in the same cell line, by low specific activity ^{131}I-mIBG (Lashford *et al.*, 1991) and by HeLa cells transfected with the noradrenaline receptor (Pacholczyk *et al.*, 1991). Therefore n.c.a. mIBG appears to be accumulated by type 1 uptake via the noradrenaline receptor.

The active, type 1 accumulation was tested in a panel of neuroblastoma cell lines with varying, but well documented ^{131}I-mIBG capacity. This rank order of uptake is compatible with known uptake ability and correlates with noradrenaline transporter gene expression (Mairs *et al.*, 1994).

The non specific uptake of mIBG, is in contrast, non saturable and sodium independent. It is however temperature dependent (Armour *et al.*, 1994) and is probably mediated via the facilitated diffusion of small ions (Lampidis *et al.*, 1989).

Passive uptake was studied in the ovarian cell line A2780, which has no noradrenaline receptor (Figure 7.3). There was no difference in passive accumulation between the neuroblastoma cell line and ovarian cell line accumulation.

On examining the specific type 1 uptake in detail, in the SK-N-BE(2c) cell line, increasing doses of radioactivity are applied to the cultures. At 1kBq, the accumulation of mIBG is similar for both preparations (98% v 96% of total uptake). Over a large range of doses up to 100MBq, the amount of nca mIBG accumulated by type 1 uptake remains high at 94%. In contrast however, when low specific activity ^{131}I-mIBG is used, the proportion of ^{131}I-mIBG accumulated by this specific uptake falls to 13%. This is as a result of saturation of the receptor by non-radiolabelled cold molecules contaminating the low specific activity preparation. Bruchelt (Bruchelt *et*

al., 1988) made similar observations comparing ^{131}I-mIBG of differing specific activities in SK-N-SH and SK-N-LO cells.

Bruchelt exposed both cell lines to ^{131}I-mIBG preparations of differing specific activity. SK-N-SH is a cell line with a high capacity for mIBG accumulation. In both cases 100μCi was used. The amount of radioactivity incorporated by SK-N-SH, on exposure to the low specific activity preparation was 5.2% of the amount of radioactivity accumulated using the high specific activity preparation.

7.5. CONCLUSIONS

The n.c.a.^{131}I-mIBG preparation represented an exciting breakthrough in mIBG targeting therapy. It enabled much smaller quantities of drug to be administered for each dose of radiation given. These laboratory studies indicate that, at a cellular level, the mechanism of ^{131}I-mIBG incorporation by neuroblastoma cell lines appears to be identical to the traditional low specific activity preparation. The new formulation, n.c.a. ^{131}ImIBG, is taken up via the noradrenaline transporter by an energy, temperature, ATPase dependent mechanism. The high specific activity of the n.c.a. preparation exploits the specific uptake mechanism relative to the traditional low specific activity preparation where the noradrenaline transporter becomes saturated with the non-radiolabelled molecules at higher doses.

CHAPTER 8 THE MODULATION OF ^{131}I-mIBG UPTAKE BY HYPERTHERMIA

8.1. INTRODUCTION

The critical requirement for any form of targeted radiotherapy is preferential uptake of the targeting agent by tumour cells relative to normal tissues. For mIBG therapy this would require high active uptake (type 1) in neuroblastoma cells relative to passive (type 2) uptake, as type 2 uptake occurs in normal as well as tumour cells. Clinically neuroblastomas differ widely in their capability for active uptake (Moyes *et al.*, 1989) making them of variable suitability for systemic ^{131} I-mIBG treatment.

The effect of combining ^{131} I-mIBG with elevated temperature had not been evaluated at the time of this study but there was a sound theoretical basis for exploring the potential effect of combination of both of these modalities.

8.1.1. Historical

At the beginning of this century several investigators using dilution assays, defined doses of heat that could inactivate tumour cells. By 1903 it was known that rat sarcoma cells could be inactivated by heating fragments of tumour at 45^{0}C for 30 minutes (Loeb 1903). In 1912 Lambert, by culturing tumour or normal connective tissue cells in plasma drops, demonstrated that normal tissue cells were more resistant to hyperthermia and that for both tissues, the degree of damage sustained depended on the height of temperature and duration of heating.

In 1921, Rodenburg and Prime demonstrated the synergistic effect of heat and radiation by dilution assays involving mouse sarcoma tumours. Later it

In 1921, Rodenburg and Prime demonstrated the synergistic effect of heat and radiation by dilution assays involving mouse sarcoma tumours. Later it was found that cycling cells appeared more sensitive to the effects of heat (Bucciante 1928) and that with repeated heating, thermotolerance would be observed (Crile 1961).

Systemic hyperthermia has been hampered for technical reasons but there are reports in the German literature dating back to 1888 of the regression of tumours with concomitant erysipelas infection (Busch 1888; Bruns 1928). A New York surgeon, Coley, chief of the Sloan Kettering Memorial Hospital Bone tumour service, reported in 1893, ten patients whose advanced tumours responded to an erysipelas induced fever. One patient in particular had a 'persistent small round cell sarcoma' still locally recurrent after five resections and this resolved completely during the long period of associated fever. The patient remained disease free at the time of reporting, seven years later. For hyperthermia to be effective, sustained high temperatures appeared necessary (De Courcey 1933; Nauts 1953). This could be achieved by many methods including the injection of bacterial toxins but had the major disadvantage that heating was inhomogeneous and uncontrolled.

8.1.2. Clinical Aspects

Localised tumours can be heated by a variety of methods including cautery, electromagnetic and ultrasound heating of tissues and these are reviewed well elsewhere (Meyer 1984). Methods used to induce whole body

hyperthermia include paraffin wax, hot air and radiofrequency techniques. Alternatively the patient can be heated by water perfused blankets or suits or by heated extracorporeal circuits. Whole body hyperthermia, treating to temperatures of 42^0C is now technically feasible but not without toxicity. Possible clinical toxicities are summarised below.

Table 8.1. Clinical toxicity associated with hyperthermia.

Cardiac arrythmias	Liver necrosis.
Agitation, seizures, coma.	Adult Respiratory Distress Syndrome (ARDS).
Low Mg Ca, K.	Second degree burns.
Vomiting and Diarrhoea.	Fatigue.
Coagulopathies, thrombocytopenia, anaemia.	

Observed cardiac arrythmias appear to be associated with acidosis. This can be reduced by ventilating the patient during treatment (Van der Zee *et al.*, 1983). Arrythmias and the neurological side effects of agitation and seizure are probably partly explained by the common electrolyte disturbances (These are not fully explained by renal or gastrointestinal losses). Cerebral oedema, ARDS and liver necrosis have been seen when tumour contaminates these sites.

Many early clinical studies appeared to support enthusiasm for systemic hyperthermia for deep seated tumours, but these are poorly defined by modern clinical investigation standards. In any case with the arrival of chemotherapy in the 1940's and megavoltage radiotherapy in the 1950's, interest inevitably waned in this field. There remains however, a small group of scientists and clinicians dedicated to this therapeutic modality as scientific

evidence and sound theoretical reasoning suggest that the combination of hyperthermia and radiotherapy appear to be synergistic.

8.1.3. Rationale for the Combination of mIBG and Hyperthermia

There are three main reasons to consider combining systemic mIBG therapy and whole body hyperthermia.

- Hyperthermia is effective in radioresistant cells.
- Hyperthermia potentiates the effect of radiotherapy.
- Heating can improve the delivery of mIBG to the tumour cell.

8.1.3.1. Hyperthermia Targets Different Cells.

It is debatable whether malignant cells are more sensitive to hyperthermia per se (Cavaliere *et al.*, 1967; Kim *et al.*, 1974; Hahn 1982) but there is evidence that those cells in a poorly nutrient (Hahn 1979; Li *et al.*, 1980), acidic environment (Overgaard and Bichel 1977) typical of tumours may be more susceptible to the effects of radiation.

The effectiveness of radiotherapy has traditionally been limited by hypoxic and 'S' phase cells (Gray *et al.*, 1953; Sinclair and Morton 1965) but these are equally sensitive to heat (Hahn 1974 ; Gerweck, Gillette and Dewey 1975).

Figure 8.1 Survival of hypoxic and aerobic cells with hyperthermia (from Hahn 1974).

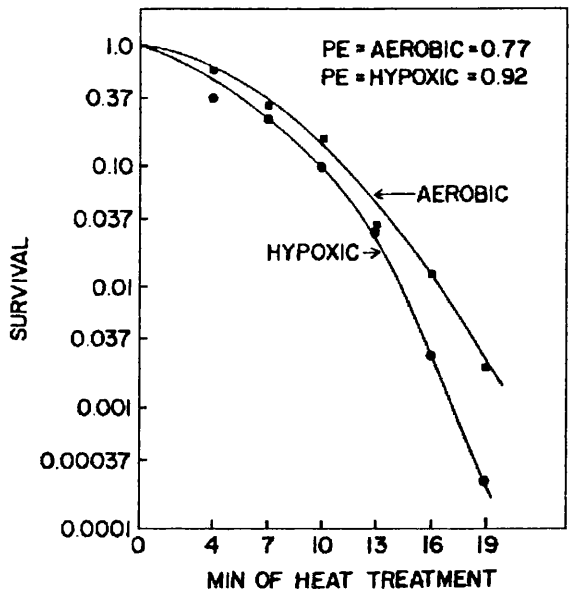
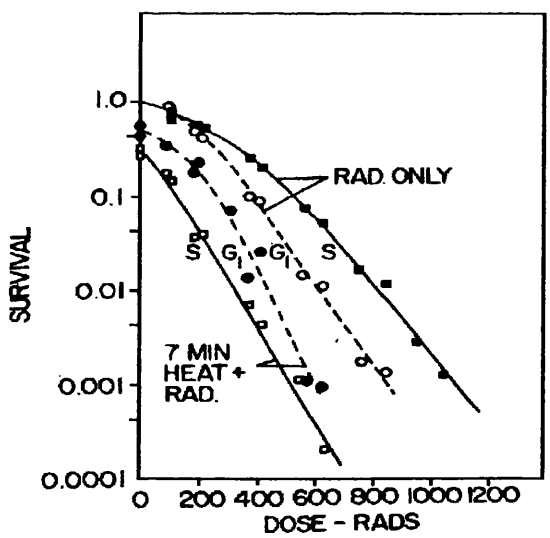


Figure 8.2. The effect of elevated temperature and cell cycle (from Gerweck *et al.*, 1975).

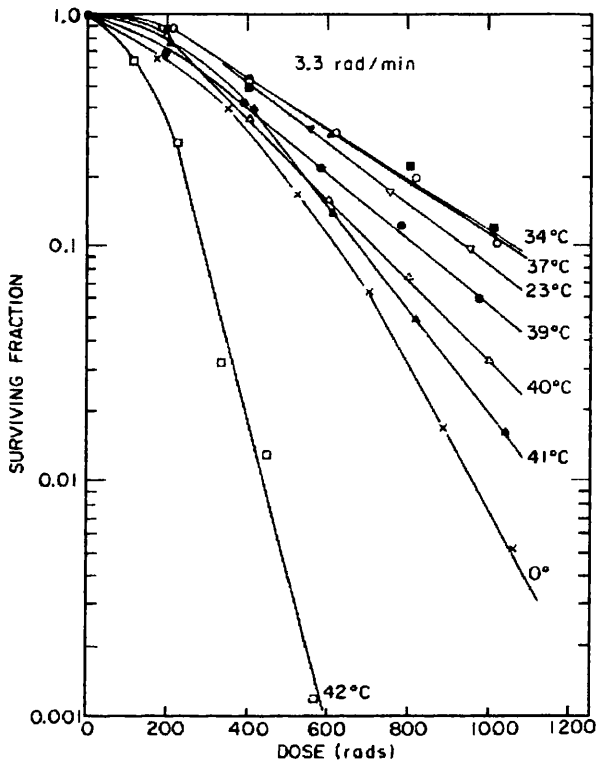


8.1.3.2. Hyperthermia Acts Synergistically with Radiation

Hyperthermia acts synergistically with radiation mainly through the inhibition of repair of potentially lethal damage and sublethal damage by heat (Ben Hur *et al.*, 1974; Hahn 1974; Gerweck *et al.*, 1975; Suit and Gerweck 1979). This is demonstrated in figure 8.3, where the survival curve of Chinese

hamster cells becomes straighter with increasing temperature, signifying the expression of more irreparable damage.

Figure 8.3. The effect of increasing temperature and repair (from Ben-Hur, Elkind and Bronk 1974).



Theoretically the above is particularly appealing as systemic mIBG can be compared with low dose rate radiotherapy. Recalling the radiobiological arguments outlined in chapter three, targeted therapy is effectively low dose rate radiotherapy. In this situation, the cells have the potential to repair and most of the cell kill is mediated through lethal non repairable damage. Neuroblastoma cells have a high sensitivity to this kind of single hit, lethal damage but can infact repair PLD and SLD extremely well.

Therefore the advantages are two fold. Hyperthermia should potentially enhance low dose rate radiotherapy by inhibiting repair, prominent in low

dose rate therapy in general but this is especially relevant with this particular tumour type where the capacity for this phenomenon is high.

8.1.3.3. The Delivery of the Radiopharmaceutical

The erratic disorganised blood flow of tumours, rather than resulting in a decreased perfusion of tumours can, due to poor homeostatic control mechanisms, create a 'heat sink' effect. This can lead to the tumour receiving prolonged effective heating regardless of blood flow.

Unlike external beam radiation, targeted radiotherapy is characterised by low dose rate irradiation which is delivered over a relatively long period of time. Whether the DNA damage induced is sufficient to sterilise tumour cells depends on a number of factors, including the cellular uptake and retention of the targeting agent. There is however, evidence that hyperthermia improves the effectiveness of chemotherapy by means of increased drug uptake, increased drug utilisation, and decreased repair of chemotherapy induced DNA damage (Field and Bleehan 1979). Therefore it is theoretically possible that heating could increase the delivery and uptake of m-IBG.

8.2. MATERIALS AND METHODS

8.2.1. Cell Culture Conditions

Two cell lines, SK-N-BE(2c) (Biedler *et al.*, 1978) and IMR-32 (Tumilowicz *et al.*, 1970) were used. ^{131}I-mIBG uptake is well characterised in both. These lines

were chosen as they represent extremes in mIBG uptake ability. A2780, a variant of the cell line NIH:OVCAR-3, was used as a non neuronal control (Hamilton *et al.*, 1983). Cell culture conditions were as those described in section 7.2.2. Once established, and at least 70% confluent, cell cultures were assayed for ^{131}I -mIBG uptake at 37^0C, 39^0C and 41^0C respectively.

8.2.2. Reagents

^{131}I-meta-iodobenzylgunaidine (^{131}I-mIBG) (specific activity 37-185mBqmg^{-1}) was obtained from Amersham International (product code IBS 6711). Desmethylimipramine hydorchloride (DMI) was purchased from Sigma (Poole, Dorset, UK).

8.2.3. ^{131}I-mIBG uptake

All chemicals and media were first heated to the desired temperature. The cell cultures were then incubated at 37^0C, 39^0C or 41^0C with or without DMI, for two hours. Cells were then assayed for ^{131}I-mIBG uptake under the conditions described in section 7.2.3. The temperature of the flasks was meticulously checked throughout the experiments.

8.2.4. Cell Viability Experiments

Cell survival was assessed by two methods, clonogenic assay and trypan blue staining in the manner described in section 6.2.5.

8.2.5. Statistical Analysis

Each experiment was repeated a minimum of three times and six replicates taken for each uptake point in the assay or for each clonogenic flask. The points documented represent the mean of uptake in picomoles of ^{131}I-mIBG per million cells. Error bands represent two standard deviations from this mean. The data was analysed using Student's t test.

8.3. RESULTS

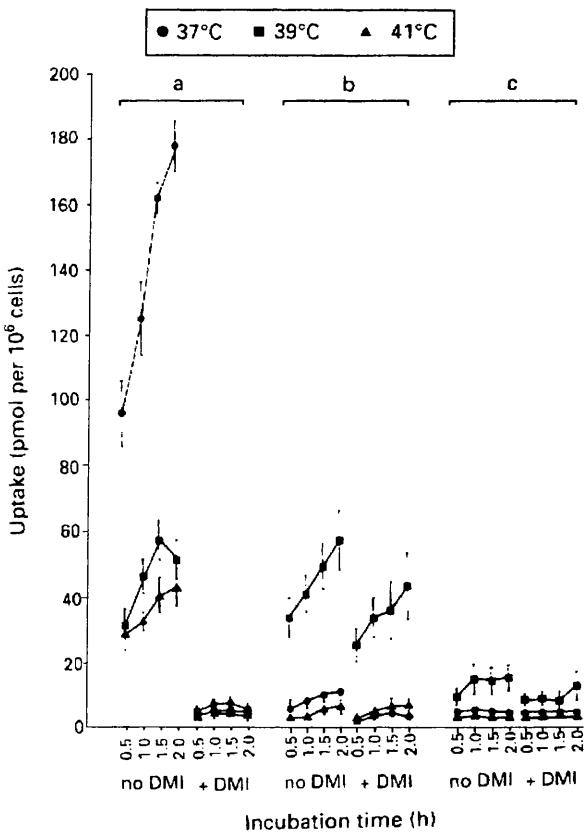
At 37^{0}C, comparison of the incorporation of ^{131} I-mIBG, at a concentration of 0.1mM, into SK-N-BE(2C) cells in the presence or absence of 1.5mM DMI indicated that 98% was due to active uptake (figure 8.4). At higher temperatures there was a dramatic, statistically significant reduction in type 1 intracellular drug accumulation ($p<0.001$). The inhibitory effect of the 41^{0}C incubation on type 1 uptake was slightly greater than that of 39^{0}C ($p<0.02$). DMI was added to the medium to obtain inhibition of specific transport. Elevated temperature had no significant effect on the non-specific uptake by SK-N-BE(2c). It appears the temperature mediated decrease in ^{131}I-mIBG uptake, by SK-N-BE (2c) was mediated by thermal denaturation of the mIBG transporter molecule.

The IMR-32 cell line has low level acquisition of ^{131}I-mIBG: approximately 10% of SK-N-BE(2c) levels throughout the two hour time course of the experiment. In this cell line, we observed a three fold increase in drug accumulation at 39^{0}C compared with that at 37^{0}C after 1.5 hours ($p<0.001$).

A2780 was used as a non-neuronal control and therefore has no type one mIBG uptake ability. A similar but less pronounced increase in ^{131}I-mIBG entry into these cells was seen. The increase in accumulation at 39^{0}C compared with that at either 37°C or 41^{0}C was nonetheless highly significant ($p<0.001$).

Clonogenic survival studies indicate that there was no temperature dependent survival difference for all three cell lines in the range of 37^{0}C-41^{0}C.

Figure 8.4. The modulation of ^{131}I-mIBG uptake and elevated temperature. A=SK-N-BE(2c); B=IMR 32; C=A2780.



8.4. DISCUSSION

The cell line SK-N-BE(2c) demonstrated active, specific type one uptake of ^{131}I-mIBG which has been well characterised (Lashford *et al.*, 1991). Neuroblastoma cells retain high intracellular levels of mIBG by a dynamic equilibrium of diffusion and re-uptake (Smets *et al.*, 1990). If mIBG therapy, given with hyperthermia, resulted in heat denaturation of the monoamine receptor ATPase, this might, if irreversible, diminish tumour uptake or inhibit re-uptake of the egressed drug. The experiments showed that the active uptake of mIBG was markedly reduced by elevated temperature. This suggests that the transport protein may have been structurally altered at 39^{0}C and 41^{0}C. This is plausible as it has been shown that some membrane proteins can undergo thermotropic change at temperatures as low as 39^{0}C (Verma *et al.*, 1977).

In addition, the functional ability of the Ca-ATPase of sarcoplasmic reticulum has been shown to be reduced at 40-45^{0}C (Cheung *et al.*, 1987). Na-K-ATPases (Smigielski and Janiak, 1978) as well as other membrane transport systems (Kwock *et al.*, 1978) have been shown to be inhibited at temperatures greater than 43^{0}C.

The effect of temperature alteration on the radiopharmaceutical uptake by the cell line IMR-32 was less clear. These cells have poor uptake 1 capacity. The elevated temperature dependent enhancement of mIBG uptake was observed both in the presence, and absence of the monoamine transporter inhibitor DMI.

Therefore the increased accumulation of mIBG at 39^{0}C must involve non-specific uptake mechanisms.

A similar effect of temperature elevation has been noted for passive molecular transport in Ehrlic ascitic tumour cells (Strom *et al.*, 1973). These exhibit an exponential increase in passive diffusion of radiolabelled uridine across the cell membrane with increasing temperature. Although the effects were marked at 44^{0}C, the data also support increased passive diffusion at 41^{0}C and below.

Mechanisms of mIBG uptake which do not involve the noradrenaline transporter are not yet well elucidated but may involve electrochemical gradients (Lampidis *et al.*, 1989). It has been shown that an abrupt reduction of membrane potential associated with increased alkali cation permeability occurs at temperatures greater than 38^{0}C in human erythrocytes (Mikkelson and Wallach 1977). As mIBG exists in cationic form at physiological pH, some of the entry into cells incubated at temperatures greater than 37^{0}C could be due to electrophoretic migration mediated by altered electrochemical gradient.

It is of concern that the observed increase in accumulation of the ^{131}I-mIBG at elevated temperatures occurred in IMR-32 and the control cell line. This suggests a non specific general effect affecting the integrity or function of the cell membrane. The membrane effects of hyperthermia have been extensively studied and are reviewed well (McLaren and Pontiggia, 1990; Marcocci and Mondovi 1990). In summary, these changes are mediated by a change in membrane proteins rather than by changes in lipid motion or order (Lepock 1982).

Although elevated non-specific accumulation of ^{131}I-mIBG may appear to be encouraging for clinical practice for those tumours that poorly accumulate mIBG, it is far outweighed by the disadvantage of increased accumulation of mIBG by normal cells. This phenomenon would result in a lower specificity of mIBG for the tumour cell. At 41^{0}C, uptake of mIBG is poor in all cell lines. This is probably due to the denaturation of critical membrane proteins.

8.5. CONCLUSIONS

The experimental evidence above indicates that hyperthermia should not be combined with targeted ^{131}I-mIBG in tumours where a good uptake of mIBG is anticipated as this will lead to an increased accumulation in non target tissues and hence a lower therapeutic differential.

CHAPTER 9 CONCLUSIONS AND FUTURE AIMS

SUMMARY AND CONCLUSIONS

Neuroblastoma is a rare illness, affecting young children. The majority present with metastatic disease, but different biological and prognostic patterns exist. For example, Stage 4S disease, affecting children less than one year, has no serious molecular abnormalities, responds to minimal therapy and has been seen to spontaneously differentiate. In contrast, older patients, with systemic disease, are at present incurable. Predictable relapse ensues, due to multiple drug resistance and the rapid repopulation of tumour from occult disease, remaining at the end of treatment. Screening protocols have failed to detect these older patients with multiple chromosomal abnormalities but have provided a wealth of biological information, enabling patients to be divided into low, intermediate and high risk categories.

The ENSG database, initiated by the UKCCSG in 1993 and analysed in chapter 4, represents the largest collection of neuroblastoma patients ever established.

The primary reason for the analysis was to determine if complete surgical resection of the primary site could affect survival, in this essentially systemic disease. This contentious issue was complicated by the fact that previously published studies had been small, relied

on the surgeons estimate of the completeness of resection and used broad categories to describe the extent of surgical resection. The data from this series determined that there was significant disagreement between the surgeon and pathologist when estimating the extent of residual disease and that the pathologist could more accurately determine the presence of residual disease. Secondly, if the description of the resection category was too large, important differences could be missed. The subsequent analysis, considering these factors, showed that complete surgical clearance of the primary site reduced local relapse and improved survival. It also suggested that if residual disease remained at the primary site, it would eventually re-seed to other metastatic sites.

Despite the large number in this database, it was not possible to tell if the histology of the resected specimen was more important than the extent of the resection. Shortner, (Shortner *et al.*, 1995) had access to biological information and concluded that this was more important. The findings do not contradict this, since biologically favorable disease will be more likely to respond to induction therapy and hence be more amenable to surgical resection. Indeed the biology of this disease is important and demonstrated by the increased survival of those aged less than one year.

The bulk of disease in this illness is, however, also important since the number of metastatic sites affected at presentation and the

presence of residual disease adversely affects survival. It is interesting that high dose consolidation therapy reduces the incidence of relapse, presumably due to the greater control of occult disease. Incidentally there are no published studies to examine the effect of surgical resection in those patients receiving a consolidation procedure. This series had sufficient numbers to do so and found that complete surgical resection and high dose consolidation are two independent prognostic variables.

The predictable relapse is the most crucial aspect of neuroblastoma. Current chemotherapy regimens are capable of inducing remission in the majority of patients, but the patient often relapses within a predictable time period. Patients, who are free of disease at the end of standard therapy, or who have a high dose consolidation, have a reduced incidence of relapse.

It is disappointing that at the end of standard induction regimens one third of patients are still unresectable. A series of Japanese papers currently advocate intensive chemotherapy regimens with aggressive surgery (Sawaguchi *et al.*, 1990; Tsuchida *et al.*, 1992 and Iwafuchi *et al.*, 1996). It would seem logical to conclude that more intensive regimens are necessary to ablate metastatic disease and enable complete surgical resection to be achieved.

The effectiveness of external beam radiotherapy was examined in two settings. Control of the primary site probably requires greater than 2000cGy but its usefulness in the palliative setting, as short simple, painless effective therapy was demonstrated.

The experimental agent mIBG was investigated. This is a targeting agent that can be administered systemically and accumulated by tumour cells specifically. The disadvantage is that the tumour uptake can be extremely variable. Since the tumour cell uptake had been well characterised, the aim of this experimental project was to examine factors that may enhance the tumour uptake. Two main areas were studied successfully:

1. A new nca preparation of mIBG
2. Biological factors that may influence the cellular environment of the cell.

In 1993, 13 years after the original manufacture of ^{131}I-mIBG, Zalutsky and his colleagues, discovered a method of preparation resulting in every m-IBG molecule being radiolabelled. This meant that for every amount of dose required, a much smaller quantity of drug could be given. Theoretically, tumour specific accumulation of the drug could be exploited. The experiments in chapter 6 confirmed that this new formulation had the same cellular accumulation characteristics as the low specific activity preparation and that on a cellular level, there was preferential accumulation of nca ^{131} I-mIBG. High specific activity nca ^{131}I-mIBG is now being used, on a trial

basis, for diagnostic scans at The Beatson Oncology Center, Glasgow.

The pre-administration of chemotherapy before the administration of ^{131}I-mIBG resulted in a dramatic, reproducible, increased accumulation of the targeting agent. Future work will concentrate on this interesting observation. Additional PCR studies confirmed the logical conclusion, that the increased uptake was by increased noradrenaline transporter molecules and not enhanced storage retention of the radiopharmaceutical or increased function of the existing receptor molecules.

Future studies should therefore examine if this effect, observed in an isolated cell monolayer system, is still observed in spheroids, where the cells are more crowded and more representative of a small metastasis. If still present and significant, then xenograft models would examine the effectiveness of this *in vivo*.

The observation that the cells differentiated was also exciting. cis-retinoic acid causes differentiation but no increased accumulation of ^{131}I-mIBG. It is known that retinoic acid causes differentiation along different signal transduction pathways. The further examination of the mechanisms behind the induction of differentiation and cell death induced by the OPEC agents, may unlock another critical

differentiation pathway as observed in more biologically favourable forms of this disease.

At present many agents are combined in the treatment of neuroblastoma. Either these must be combined in a more efficient manner, exploiting each agent to its maximum use or other methods of inducing differentiation in the remaining occult disease must be found.

On the basis of this thesis I would conclude that higher dose intensity chemotherapy is desirable, combined with aggressive management of all sites of disease. ^{131}I-mIBG, at present combined with TBI in the consolidation phase of the treatment should be considered before chemotherapy, or 48 hours after the first bolus. I feel intensive research should be directed to a genetic level to find the *crucial* differentiation pathway, responsible for the differentiation or apoptosis of the cell, since all of the above intensification of management may still fail to eradicate all occult disease.

APPENDICES

APPENDIX 1 Questionnaire Requesting Details of Surgery

PATIENT NAME	DATE OF BIRTH
WAS SURGICAL RESECTION COMPLETE IN THIS PATIENT?	YES/NO
SURGICAL RESECTION	1. 100% 2. 75 - 99% 3. LESS THAN 75%
PATHOLOGICAL EXAMINATION OF THE RESECTED SPECIMEN	1. MACROSCOPIC RESIDUAL DISEASE 2. MICROSCOPIC RESIDUAL DISEASE 3. CLEAR RESECTION MARGINS
TUMOUR HISTOLOGY AT RESECTION	1. NEUROBLASTOMA 2. GANGLIONEUROBLASTOMA 3. GANGLIONEUROMA 4. NO VIABLE TUMOUR

APPENDIX 2 Questionnaire Requesting Details of Radiotherapy

NAME	DATE OF BIRTH
DATE START OF TREATMENT	
DATE TREATMENT END	
DOSE	NUMBER OF FRACTIONS
SITE	FIELD SIZE
ENERGY	
DID THIS PATIENT HAVE LOCAL RELAPSE?	

APPENDIX 3 **Categories of Variables Listed About ENSG Stage 4 Patients**

ENSG NUMBER	DATE OF BIRTH	DATE OF DIAGNOSIS
ENSG CENTRE	SEX	STATUS
SITE OF PRIMARY	PREOPERATIVE CHEMOTHERAPY	SIZE OF PRIMARY (PREOP AND POST OP)
RESPONSE OF PRIMARY TO CHEMOTHERAPY TYPE	POST OPERATIVE CHEMOTHERAPY	RESECTION SIZE
RESIDUAL DISEASE (PRIMARY AND METASTATIC SITES)	SURGEONS ASSESMENT OF SURGICAL EXCISION	PATHOLOGICAL ASSESMENT OF EXCISION
PATH RESECTED SPECIMEN	RADIOTHERAPY GIVEN	RELAPSE
DATE OF RELAPSE	HISTOLOGICAL CONFIRMATION OF RELAPSE	DATE OF RELAPSE
SITES OF RELAPSE (PRIMARY AND METASTATIC)	DATE OF DEATH	CAUSE OF DEATH

APPENDIX 4 Papers published

1. Armour, A.A., Mairs, R.J., Gaze, M.N. and Wheldon, T.E. (1994). Modification of meta-iodobenzylguanidine uptake in neuroblastoma cells by elevated temperature. *British Journal of Cancer*, **70**, 445-448.

2. Armour, A.A., Cunningham, S.H., Gaze, M.N., Wheldon, T.E. and Mairs, R.J. (1997). The effect of cisplatin pretreatment on the accumulation of MIBG by neuroblastoma cells in vitro. *British Journal of Cancer*, **75**, 470-476.

3. Mairs, R.J., Cunningham, S.H., Russell, J., Armour, A., Owens, J., M^{c}Kellar, K. and Gaze, M.N. (1995). No carrier added iodine-131-mIBG: Evaluation of a therapeutic preparation. *The Journal of Nuclear Medicine*, **36**, 1088-1095.

Abstracts presented and published

Armour, A., Mao, J.H., Barrett, A. on behalf of members of the UKCCSG. The role of surgical resection of the primary tumour in stage 4 neuroblastoma. *Clinical Oncology*, **8**, 203.

Cunningham, S., Armour, A., Mairs, R.J., (1997). Cytotoxicity of 123I/125I/131I labeled mIBG to neuroblastoma cells in vitro. *Clinical Oncology*, **8**, 202.

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The role of surgical resection of the primary tumour in stage 4 neuroblastoma. Armour, A., Mao, J.H., Barret, A. on behalf of members of the UKCCSG **Advances in neuroblastoma research meeting, Philadelphia, USA. May 1996**

The role of surgical resection of the primary tumour in stage 4 neuroblastoma. Armour, A., Mao, J.H., Barret, A. on behalf of members of the UKCCSG
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