ISOLATED LIMB PERFUSION FOR MALIGNANT MELANOMA: CLINICAL AND LABORATORY STUDIES

Volume I of 2 volumes

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DECLARATION

I declare that the preparation and writing of this thesis has been carried out by myself.

The research described in this thesis was performed by myself except where the help of others has been specifically acknowledged.

No part of the subject matter of this thesis has been submitted in support of any application for another degree or qualification in this or any other University.

Roy N. Scott

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Mr. Ian Binnie, Medical Photographer, Gartnavel General Hospital took all the photographs which are reproduced as illustrations in this thesis. Ms. Sheila Pattison, Medical Artist, Western Infirmary drew the diagram in Figure 3.

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ABBREVIATIONS

A.U.	absorbance units
AUC	area under the curve (see III.5.6.)
B0008, B0010	two human melanoma cell lines
cm	centimetre
conf. int.	confidence interval
CR or PR	complete or partial response
degC, °C	degrees centigrade
DIL	Alkeran (melphalan) diluent
EDTA	ethylenediaminetetraacetate solution
ELND	elective lymph node dissection
E.O.R.T.C.	European Organisation for Research and Treatment of Cancer
Fig.	Figure
FZ	Fungizone (amphotericin-B)
g	gram
HPLC	high performance liquid chromatography
hr	hour
ILP	isolated limb perfusion
kg	kilogram
log	logarithm
µg, micg	microgram
mel, MEL	melphalan
MEL57	a human melanoma cell line
MEM	minimum essential medium
mg	milligram
min(s)	minute(s)
ml	millilitre
mm, µm	millimetre, micron
mM, M	millimolar, molar
mmHg	millimetres of mercury
M.R.C.	Medical Research Council
mv	millivolt
n, No.	number (of)
nm	nanometre
N.S.	not statistically significant ($p>0.05$)
p	probability
p.s.i.	pounds per square inch
pts.	patients
r.p.m.	revolutions per minute
S.D., Std.dev.	standard deviation
S.E.M.	standard error of the mean
SOL	Alkeran (melphalan) solvent
$t_{\frac{1}{2}}$	half-life
t_R	retention time
U.I.C.C.	Union Internationale Contra le Cancer
UV	ultraviolet
VRP	verapamil
WBC	white blood cell count
W.H.O.	World Health Organisation
5 or 10YS	five or ten year survival rate

ISOLATED LIMB PERFUSION FOR MALIGNANT MELANOMA:

CLINICAL AND LABORATORY STUDIES.

SUMMARY

Malignant melanoma is usually considered a rare cancer but data from countries with good records show the incidence doubling in recent decades. Only lung cancer in women is increasing at a higher rate. The current incidence rate of malignant melanoma in Scotland is 8-10 new cases per 100,000 annually. Public education may help to prevent the disease and increase the rate of diagnosis at an early curable stage, thereby improving the overall prognosis. There is, however, a need for an effective treatment of high risk primary lesions and advanced malignant melanoma.

In this thesis I assess the value of isolated limb perfusion as treatment for malignant melanoma. This technique presents unique clinical opportunities to control the physio-pharmacological environment of a tumour and thus to maximise the therapeutic effects of the cytotoxic drug. Although isolated limb perfusion has been used clinically for thirty years there has been surprisingly little study of the physio-pharmacology. Different perfusion techniques have evolved empirically around the world.

I was involved in making isolated limb perfusion available in Scotland. I have studied the physio-pharmacology of isolated limb perfusion in both the clinical setting and using in vitro models.

In Chapter I the cutaneous melanocyte system and benign

pigmented naevi are reviewed to introduce the pathology of malignant melanoma. Treatment is considered in the light of the clinical pathology. Important surgical controversies (including excision margins and elective lymph node dissection) are discussed in particular detail. The rationale, history and published results of isolated limb perfusion are given and this treatment is thus set in the context of a comprehensive review of malignant melanoma.

Chapter II describes the introduction and preliminary validation of isolated limb perfusion in Scotland. Patients were referred to us through the Scottish Melanoma Group. In a consecutive series of our first 61 isolated limb perfusions we have shown that we can safely perform the procedure in Glasgow. Our initial results for therapeutic perfusion are comparable with other published series. In pilot studies of the physiology of isolated limb perfusion, it was soon apparent that many aspects were incompletely understood, and that there was scope for basic research.

The clinical pharmacology of melphalan isolated limb perfusion is described in Chapter III. In a phase I study we showed that melphalan can be given safely in a bolus dose of 1.75mg/kg body weight (up to 165mg). Using a sensitive and specific HPLC (high performance liquid chromatography) assay we measured melphalan concentrations in the perfusate, in the systemic circulation, and in the tissues of the perfused limb. The pharmacokinetic data show that isolated limb perfusion consistently achieves

the aim of exposing the tumour-bearing limb to high concentrations of melphalan, while minimising systemic exposure. Estimates of tissue uptake of melphalan suggest that the drug is taken up by the tissues of the leg mainly during the first 30 minutes of isolated limb perfusion. However perfusion should last longer than 30 minutes to maintain the concentration gradients which drive the drug through diffusion barriers to the target cells.

There is no pharmacokinetic advantage in divided dose compared with bolus dose administration, and no increased regional toxicity as a consequence of the high peak levels generated by single bolus dose administration.

Isolated limb perfusion achieves levels of melphalan in large necrotic tumours which are significantly higher than the levels in fat, and similar to the levels in well vascularised healthy skin.

The physio-pharmacology of isolated limb perfusion is quite unlike that of systemic administration of cytotoxic drug and in Chapter IV I describe the use of in vitro models to study ways of controlling melphalan cytotoxicity at the cellular level.

Multicellular tumour spheroids were grown from three established human melanoma cell lines. Spheroids were used in studies of experimental chemotherapy with melphalan, in physio-pharmacological conditions based on those measured in our patients. The multicellular tumour spheroid model is particularly valuable in the study of factors which are difficult to isolate as variables in other experimental systems.

The important conclusions of my in vitro studies are that:

1) Hyperthermia (>37°C) enhances the cytotoxic effect of melphalan, though heat alone is not cytotoxic to spheroids at temperatures less than 43°C for one hour.

2) Hypothermia (<37°C) tends to reduce the cytotoxic effect of melphalan.

3) There is a simple relationship between duration of exposure and cytotoxic effect.

4) Verapamil does not enhance melphalan cytotoxicity.

5) The B0010 cell line forms spheroids which grow poorly in conditions which allow B0008 and MEL57 spheroids to grow well.

6) Melphalan cytotoxicity is enhanced at lower pH values, and this effect is not simply due to decreased hydrolysis of melphalan.

7) Similar dose-survival curves are derived from spheroid experiments by back-extrapolation from growth curves, from colony forming assay of disaggregated spheroids and from colony forming assay of exponential monolayers.

The implications of the in vitro studies for clinical isolated limb perfusion are that:

a) the limb should be warmed to at least 37°C during isolated limb perfusion because melphalan cytotoxicity is enhanced by hyperthermia and reduced by hypothermia.

b) the results of one hour exposure to the achieved tissue concentrations may be improved by longer exposures; and shorter periods of clinical perfusion are probably inappropriate.

c) the addition of verapamil during isolated limb perfusion with melphalan would not be expected to improve

results, unless the vasodilator effect is important.

d) the cytotoxic effect of melphalan may be enhanced by correcting the alkalosis which develops if the perfusate is oxygenated with 100% O_2.

Isolated perfusion with melphalan is now the treatment of choice for recurrent or advanced malignant melanoma confined to one limb, and patients with high risk limb primaries should be entered in trials of adjuvant perfusion. We are now involved in studies designed to optimise the treatment by manipulating physio-pharmacology during isolated limb perfusion with melphalan, and by exploring the use of monoclonal antibodies armed with radio-isotopes in this system.

CHAPTER I

A REVIEW OF CUTANEOUS MALIGNANT MELANOMA

I.1. SKIN, MELANOCYTES AND PIGMENTED NAEVI

I.1.1. Introduction

Malignant melanoma is the name given to cancer arising from specialised cells called melanocytes which produce the pigment melanin. Melanocytes are normally found in various tissues of the body. In the study of cutaneous malignant melanoma it is essential to have an understanding of the melanocyte system in healthy skin. In this chapter a brief description of the important benign melanocytic lesions is also included as a necessary introduction to cutaneous malignant melanoma.

The aims of this chapter are to consider the features of cutaneous malignant melanoma which are of clinical importance and to set isolated limb perfusion in the context of other available therapies.

I.1.2. The skin

The skin is the major site of interaction between the environment and the body. Skin provides protection against many potentially damaging environmental influences. Some of these, such as mechanical forces, thermal and chemical injuries, or invasion by micro-organisms are obvious; others, such as osmotic gradients and radiation are more subtle. The skin is the largest sensory organ, and provides friction for manipulation and locomotion. It is an organ of heat exchange and insulation. Vitamin D is activated in the skin by ultraviolet light.

The two main layers of the skin are epithelium, the epidermis and underlying connective tissue, the dermis. The strength and elasticity of the skin is provided by the dense, fibrous tissue of the dermis which is richly supplied with blood vessels, lymphatics and nerves. The epidermis protects the dermis from injury and particularly from drying out. It is a stratified squamous epithelium which is sub-divided into various layers (Fig.1). The majority of epidermal cells are keratinocytes derived from embryonic ectoderm. These cells are produced by cell division in the basal layer and at the end of their life cycle they are continuously shed as dead cells from the surface of the cornified outermost layer. There is no direct blood supply to the epidermis which is nourished by diffusion of nutrients through tissue fluids from the richly vascular underlying dermis. The epithelial melanocytes (melanoblasts, clear cells, dendritic cells) are found among the basal keratinocytes in the epidermis.

I.1.3. The melanocyte system

Normal melanocytes are not conspicuous on routine histological sections of skin but they may be detected by the presence of an artefactual clear space around a shrunken cell body, in the basal layer of the epidermis. Mammalian melanocytes are derived from cells of neural crest origin[1] which migrate to invade the skin of the foetus between three and six months in utero[2]. The concentration of melanocytes varies from site to site on the body surface[3].

Most melanocytes are found among the basal cells of the

epidermis, but some are found in hair bulbs and in the dermis. Melanocytes are also found in the pia arachnoid of the central nervous system and in the uveal tract and retinal epithelium of the eye. In all of these sites (except the retinal pigment epithelium) malignant melanoma may develop[4].

Skin pigmentation is controlled by several genes[5] which determine the "constitutive" skin colour. Various modifiers of pigmentation interact to induce reversible "facultative" changes in skin colour[4].

Melanocytes produce the brownish-black pigment melanin (from the Greek, melas meaning black). The melanin pigment is transferred from melanocytes to keratinocytes and this transfer is essential for the pigmentation to be clinically apparent[4]. Melanin is not the sole determinant of human skin colour which depends on the interplay of many factors[6] including the reflectivity and absorbance characteristics of the skin surface, the thickness of the skin and the oxygen saturation of the haemoglobin in the cutaneous blood vessels.

The study of melanin biochemistry has been complicated by the difficulty of dissolving the virtually insoluble pigment[4]. The amino acid tyrosine is converted via dihydroxy-phenylalanine (dopa) to melanin by a series of reactions (Fig.2) in which the enzyme tyrosinase is important. In 1928 Raper[7] was the first to describe the role of tyrosinase in melanogenesis. This enzyme is an aerobic oxidase which, for activity, requires copper to be present. Tyrosinase is found exclusively within the melanocyte, where melanin is formed on specific sub-

cellular organelles called melanosomes.

The cell body of the epidermal melanocyte is found in the basal layer. Long, slender processes called dendrites ramify from the cell body of the melanocyte among the surrounding keratinocytes. The melanocyte does not form keratin. The function of the melanocyte is to synthesise melanin and transfer melanosomes to keratinocytes.

Melanosomes are formed in the cell body as spherical vesicles containing tyrosinase without melanin. As the melanosome matures it becomes more elongated, and develops a regular fibrillar or lamellar inner structure. With increasing deposition of melanin the internal structure of the melanosome becomes obscured[8]. The melanosome matures, accumulating melanin, as it moves down the dendrite away from the cell body. Racial differences in skin colour do not depend on variations in melanocyte number. The melanocytes of darkly skinned races contain many mature melanosomes (1-1.3μm in transverse diameter) laden with melanin whereas the melanocytes in pale Caucasian skin contain fewer, smaller[9] melanosomes which are less mature[10].

The pigment is transferred from the dendrites of the melanocyte to the surrounding keratinocytes which appear to take up intact melanosomes[11]. Each melanocyte supplies approximately 30 keratinocytes with pigment, and this group of cells is described as the epidermal melanin unit[12]. As the keratinocytes mature they carry the pigment up through the epidermis.

Tyrosinase activity is regulated by various inhibitors and activators. The activity of tyrosinase is enhanced by

increased levels of cyclic AMP within cells (e.g. Melanocyte Stimulating Hormone or theophylline), but it is inhibited by phenols, catechols and quinones[4]. The tanning effect of ultraviolet radiation (solar and artificial) is well known, and it has become a recreational obsession for many people. Exposure to ultraviolet light tends to enhance the activity of tyrosinase[13] and increase melanin production. The actual number of melanocytes can also be increased by exposure to ultraviolet light[14]. Excessive exposure to ultraviolet radiation can cause sunburn, malignant melanoma and non-melanoma skin cancer. In normal skin the light absorbing property of melanin protects underlying tissues by reducing the transmission of damaging ultraviolet radiation[4]. The relatively low incidence of malignant melanoma in darker races[15,16] compared with Caucasians, who develop sunburn more easily[17], suggests that melanin has an important role in protecting the skin from the harmful effects of solar radiation.

I.1.4. Benign cutaneous melanocytic lesions

There is such confusion about the nature and terminology of benign and malignant pigmented lesions that a consideration of benign cutaneous melanocytic naevi is appropriate in this thesis. Undeniably there are some difficult aspects where experts cannot agree. Nonetheless clinicians must have a working knowledge of pigmented lesions, in order that appropriate clinical management decisions are made for the benign mole as well as the malignant melanoma.

The discrimination of benign from malignant lesions is especially important because the incidence of cutaneous malignant melanoma is increasing across the Western World[18]. More patients are likely to present with pigmented lesions as a result of public education campaigns and a greater proportion of the lesions presenting will be seen when their malignant potential may not be clinically obvious and when histological mis-diagnosis could be most damaging. In recent years there has been increasing interest in certain benign lesions which may be precursor lesions or indicators of melanoma risk.

An understanding of the terminology which is used to describe benign melanocytic naevi and their biology will clarify the subsequent discussion of cutaneous malignant melanoma.

I.1.5. Freckles and lentigines

In a freckle (ephelis) there is a focal increase in the quantity of pigment in and around the basal epidermal layer, and no other microscopic change. The appearance is distinct from that of a lentigo (from the Latin,lens meaning lentil) where there is an increase in basal pigmented melanocytes plus lengthening and widening of the rete ridges. Lentigines are usually 2mm or less in diameter[19], tend to occur on sun-exposed sites and they are often associated with changes of solar keratosis in the epidermis. Clinically a lentigo may be difficult, if not impossible, to distinguish from a small pigmented naevus.

I.1.6. Moles and pigmented melanocytic naevi

Melanocytes tend to be regularly spaced, singly, among the cells of the basal epidermis. The ratio of melanocytes to basal cells averages about 1:10 but more melanocytes are found on the face and fewer on the trunk[20]. Melanocytes may be found in the dermis where they are known as naevus (from the Latin, natus meaning born) cells.

The various lesions which we call moles (from the Old High German, meil meaning spot) consist of clusters of melanocytes and naevus cells in the skin. The characteristics of naevus cells are variable (e.g. site and extent of pigmentation) but they all share a common embryonic origin with melanocytes. The melanocytic lesions are termed "pigmented melanocytic naevi" to distinguish them from the angiomas and other congenital naevi.

This account is simplified to introduce widely accepted concepts, but the detailed natural histories of the various types of pigmented melanocytic naevi have not been fully determined. Various controversies have yet to be resolved, and the emphasis here is on those aspects which may be important in relation to malignant melanoma.

I.1.7. Congenital pigmented melanocytic naevi

The congenital pigmented naevi are divided on the basis of size into the categories giant, medium and small. All are present at or appear within a few weeks of birth. In careful studies, with assessment of neonates and biopsy of moles, the true incidence of congenital pigmented melanocytic naevi in Caucasian babies is about 1% [21]. The natural history of the small (diameter <1.5cm) and medium

(1.5-19.9cm) congenital naevi is that they tend to persist unchanged throughout life. Congenital pigmented naevi are usually larger than the common acquired pigmented naevi. The histological features of congenital pigmented naevi are variable but most of these moles have features typical of compound naevi (see I.1.8.).

Growing evidence[22,23,24] suggests that small and medium congenital naevi may be more likely to be associated with subsequent malignant melanoma than acquired naevi. Hence the management of smaller congenital pigmented naevi is controversial, with some recommending prophylactic excision[25].

Giant congenital naevi are rare and do not regress spontaneously. These lesions (diameter >20cm) may be very large e.g. "bathing trunk" naevus. They show variable pigmentation and may be hairy like the smaller congenital lesions. There is an increased risk of malignant melanoma in patients with giant congenital moles. The lifetime risk of malignant melanoma is about 3-6%[26,27] in this group. The giant congenital pigmented naevi present difficult clinical problems. The appearance of the lesion is often disfiguring and excisional surgery leaves an extensive area for skin grafting. Although excision and grafting may be justifiable on the basis of risk of malignancy, surgery is rarely a practical proposition. Multistage procedures involve repeated distress for the child and the cosmetic results may be poor. Management often consists simply of regular examinations and comparative photography, with biopsy of any suspicious change. Early shave excision for cosmetic improvement is advocated by some plastic surgeons

but this treatment may not prevent malignant change, especially since melanoma arising in giant congenital naevus may arise from cells deep in the dermis[27].

I.1.8. Acquired pigmented melanocytic naevi

There are several different types of acquired pigmented naevi. Common acquired naevi account for the greatest proportion, and they occur at any site on the skin. Common acquired pigmented naevi mainly appear in the second decade of life as small brown spots which grow to a diameter of about 5mm, and by the age of twenty a young adult usually has about thirty of these lesions. Such moles may remain unchanged or protrude a little, but they tend to fade and may disappear in later life[28,29].

The histological appearances at each stage are characteristic and give rise to a further classification of the naevi. In infancy and childhood the flat, impalpable acquired moles consist of local concentrations or clusters of pigmented naevus cells which are in contact with the dermo-epidermal junction (junctional naevus).

As the acquired naevus ages, an increasing proportion of the naevus cells are found in the superficial part of the reticular dermis. An intradermal pigmented naevus consists of numerous aggregates of small, polygonal naevus cells in the dermis. There is some associated fibrosis in the overlying papillary dermis and the naevus cells tend to become depigmented. Some of the deeper naevus cells concentrate around hair follicles and others are associated with peripheral nerve endings. The latter group of naevus cells may take on the characteristics of Schwann

cells in a process called "neurotization". Schwann cells arise from the embryonic neural crest like melanocytes, and they normally form the myelin sheath of peripheral nerve axons. At a final stage in the natural history of an acquired mole the intradermal naevus may regress completely leaving little or no histological evidence of the pre-existing mole.

A compound naevus has histological features of both a junctional and an intradermal naevus, and it represents an intermediate stage in the development of an acquired naevus. Moles on the palms, soles and genitalia do not follow the general pattern of ageing but they tend to remain compound into adult life.

The halo naevus is fairly common, occurring on the back in young people. In a patient aged between five to fifteen a symmetrical depigmented halo may appear around one or more pre-existing moles. After a few months the mole disappears leaving a depigmented area which may persist for years. Histologically the most characteristic feature is a dense lymphocytic infiltrate associated with the dermal component of the naevus. The halo naevus is entirely benign and thus there is no need for excision.

The Spitz naevus or "juvenile melanoma" is a rare lesion which can be much more difficult to discriminate from malignant melanoma. The Spitz naevus can appear at any age but classically the lesion appears on the cheek of a child as a pink, hairless nodule. For several months the lesion grows and it may persist as an indolent lesion about 5mm in diameter or it may disappear spontaneously. The blue naevus is a rare, but interesting mole which appears in

the teens and persists unchanged throughout life. It tends to occur on the face, extremities and buttocks. A blue naevus in the sacral region forms the "Mongolian blue spot", which is characteristic of oriental races. Histologically the cells of a blue naevus are elongated, heavily pigmented and associated with collagen bundles in the reticular dermis. These features account for the characteristic bluish coloration[29] but there is very little risk of malignant transformation.

I.1.9. Dysplastic naevi

The dysplastic naevus has excited particular interest and controversy because it has histological features and clinical associations which suggest that it is a potential precursor of malignant melanoma.

In 1977 Frichot & Lynch[30] described the association of familial melanoma with unusual benign pigmented lesions, which they termed the "familial atypical multiple mole syndrome". Clark and colleagues used the terms "B-K mole syndrome"[31] and later "dysplastic naevus syndrome"[32] for this familial condition. Elder and colleagues[33] were first to describe identical histological features in non-familial cases, and they provided criteria for the diagnosis of the sporadic dysplastic naevus which were later adopted and modified by the Consensus Development Conference of the National Institute of Health in the USA[34].

As stated in the report of the Consensus Conference "dysplastic naevi are acquired pigmented lesions whose clinical and histological definitions are evolving"[34]. A

typical dysplastic naevus is a maculo-papular lesion, 5-12mm in diameter (larger than common naevus) with an irregular, poorly defined edge. The colour of a dysplastic naevus may vary from tan to dark brown, and the lesion may occur anywhere on the body. Individuals with multiple dysplastic naevi may have more than 100 lesions, and there is typically considerable lesion to lesion variability. Dysplastic naevi begin to appear in adolescence and continue to appear into adult life. Single dysplastic naevi have been reported in 4% of the population in a study from California[35]. Multiple dysplastic naevi are much less common, although this is the variant which was first recognised.

The typical histological features of a dysplastic naevus are superimposed on those of a junctional or compound naevus. There is elongation of the rete ridges and melanocytic hyperplasia. Individual melanocytes show cellular atypia with enlarged hyperchromatic nuclei. The melanocytes are often aggregated in nests which tend to fuse with adjacent rete ridges producing bridging. Fibrosis of the papillary dermis occurs and there is a patchy or diffuse lymphocytic infiltrate of the superficial dermis. It is important to note that these features may only occur focally within a lesion. According to the Consensus Conference, these appearances can be distinguished from malignant melanoma in situ[34].

Familial dysplastic naevi may be inherited as an autosomal dominant trait. The term "dysplastic naevus syndrome" is applied when two or more members of a family have multiple dysplastic naevi. Sporadic dysplastic naevi occur in

patients with no family history of dysplastic naevi or malignant melanoma.

The risk of subsequent malignant melanoma is not uniform for patients with dysplastic naevi. The classification of dysplastic naevi according to Kraemer and colleagues is given in Table 1. It is well established that the risk is very high in patients with the dysplastic naevus syndrome who also have a positive family history of malignant melanoma (D-1, D-2)[36,37]. Conversely, any increased risk is probably very small in sporadic cases with few dysplastic naevi and no family history of malignant melanoma (A,B & C).

Although the dysplastic naevus appears to be an appropriate step in a hyperplasia-dysplasia-neoplasia sequence[32] and despite wide acceptance of the concept, there are still controversial aspects. Ackerman and Mihara have claimed that patients with familial melanoma do not always have the dysplastic naevus syndrome[38]. They cite families with many cases of malignant melanoma, in which none had dysplastic naevi. They found that malignant melanoma rarely developed in association with sporadic dysplastic naevus, and that it was only a little more common in the familial type. In spite of the commonly held view that the dysplastic naevus is a melanoma precursor, Ackerman and Mihara report that even in patients with the dysplastic naevus syndrome most of the melanomas arise de novo, and not in association with any dysplastic lesion[38]. In their view the dysplastic naevus is thus a marker for malignant melanoma risk rather than a precursor lesion.

I.1.10. Atypical naevi

Although the histological features of dysplastic naevi are now established, clinically atypical naevi do not always show these features[39]. Recent studies have indicated that there may be an increased risk of malignant melanoma in people with many benign or "banal" pigmented naevi of the junctional or compound type[22,40,41]. In a recent Scottish case-control study it was shown that patients with large numbers of benign pigmented naevi, and those with clinically atypical (not necessarily histologically dysplastic) naevi are at increased risk of malignant melanoma[19].

People with many pigmented naevi may be at increased risk of developing malignant melanoma because they have more pigment cells than do those with few moles. In those with many moles the individual pigment cells may be more susceptible to malignant transformation. Alternatively the naevus cells and melanocytes in moles may be initiated cells, awaiting a critical promoting stimulus which makes them transform and express malignant behaviour. The relative importance of heredity and environmental factors in the natural history of benign pigmented lesions has yet not been elucidated, and the relationship between benign pigmented melanocytic naevi and malignant melanoma is an area of active investigation.

I.2. CUTANEOUS MALIGNANT MELANOMA: THE DISEASE

I.2.1. History

Robert Carswell (1838)[42] is credited with naming "malignant melanoma" which had been discussed earlier as "la melanose" by Laennec in 1806 (cited by Pemberton[43]). It is likely that the first recorded mention of cutaneous malignant melanoma was much earlier, in the writings of Hippocrates which date from the fifth century B.C.[44] Malignant melanoma was a relatively rare tumour, becoming more common during this century for reasons which will be discussed. Only in the last three decades has our understanding of the biological behaviour of malignant melanoma become clear enough to allow it to begin to influence our clinical management of the disease.

The importance of malignant melanoma is not solely due to its rising incidence. The reputation of cutaneous malignant melanoma is of a sinister, capricious and frequently fatal malignancy. Although cutaneous malignant melanoma has the potential for metastasis and eventual fatality, early lesions have an excellent prognosis if managed properly.

I.2.2. Clinico-pathological classification

Four major types of cutaneous malignant melanoma are recognised:

1) superficial spreading melanoma
2) nodular melanoma
3) lentigo maligna melanoma
4) acral lentiginous melanoma.

The first three were described by Clark and colleagues in

1967[45], and the last by Reed[29,46] in 1976.

According to the hypothesis of Foulds[47], malignant tumours acquire differing capacities for invasion and metastasis by sequential changes which may proceed gradually or abruptly, in a process known as "tumour progression". The biological behaviour of melanoma can be described in terms of the pattern of tumour progression which characterises each type.

I.2.3. Superficial spreading melanoma

In studies of Caucasian populations this is the commonest type of cutaneous malignant melanoma and in the Scottish population it accounts for 48.5% of melanomas[48]. Typically the lesion is one centimetre or greater in diameter, irregularly pigmented brown or black, with a notched lateral border[49]. There may be a faint pink flare at the margin, representing neo-vascularisation and ectasia. Thicker lesions may have a palpable edge. Later lesions tend to have a more prominent nodular component which may be more or less pigmented and associated with ulceration, bleeding and crusting.

Superficial spreading melanoma may arise by malignant transformation of epidermal melanocytes or by malignant transformation of a pre-existing naevus[50]. This type of melanoma may exist for several years without metastasis occurring. It seems that the typical lesion may enlarge steadily at the periphery or grow radially (hence the term radial growth phase) over a period of about five years[51]. During the radial growth phase the tumour is invasive but metastases are not apparent. The work by

Holmes and colleagues[52,53] provides evidence that the malignant cells tend to remain locally rather than to spread early and lie dormant. In these studies there was a low incidence of lymph node metastasis in patients whose primary lesions were in the radial growth phase (compared with patients whose lesions were in the vertical growth phase) and they suggested that the cells in such lesions were not yet capable of full-blown metastasis.

In the radial growth phase histological examination shows tumour in the epidermis and in the papillary dermis, with a prominent local immune response by lymphocytes and macrophages. For the diagnosis of malignant melanoma there must be a break in the epithelial basement membrane and invasion of the dermis by melanoma cells[54].

At a later stage focal change in the superficial spreading melanoma may occur in a process named "intra-lesional transformation"[51]. When this occurs new populations of cells, distinct from those of the radial growth phase, invade the dermis deeply and the "vertical growth phase"[55] becomes established. The vertical growth phase co-exists with the radial growth phase but the capacity for metastasis may now be manifest. The host cellular response is less apparent than in pure radial growth phase. The step-wise development of superficial spreading melanoma exemplifies "indirect tumour progression" as described by Foulds[47].

I.2.4. Nodular malignant melanoma

Nodular malignant melanoma presents as a rapidly growing, more or less pigmented nodule. In the Scottish Melanoma

Group experience, 22.6% of malignant melanomas are of the nodular type[48]. They grow rapidly, becoming raised with loss of normal epidermal surface markings, and ulceration. Nodular malignant melanoma is an example of "direct tumour progression" because pure vertical growth phase, with full malignant potential, is apparent from the onset and there are no demonstrable intermediate appearances[51]. By the time of presentation this lesion has usually penetrated the dermis more deeply than a superficial spreading melanoma of comparable age.

I.2.5. Lentigo maligna melanoma

Lentigo maligna is the pathological term used to describe Hutchinson's melanotic freckle (1892)[56,57] and this is the pre-invasive form of lentigo maligna melanoma.

Lentigo maligna is seen as a characteristic slowly enlarging brown macule on sun-exposed skin, usually the face of an elderly person[54]. Over many years there is centrifugal enlargement of the lesion. It is characterised by variable pigmentation and depigmentation, and there are often islands of virtually normal skin surrounded by tan and black areas. Histologically there are signs of actinic damage with an atrophic epidermis in addition to abnormal melanocytes. The depigmented areas may show histological signs of the host response or regression. Subsequent invasion is not inevitable in lentigo maligna, and a recent study suggested that the lifetime risk of lentigo maligna melanoma is 2% at age 65[58].

Lentigo maligna melanoma is less common than the superficial spreading and nodular types, constituting

14.5% of cases in the Scottish population[48]. There is a very long radial growth phase (5-25 years) and a slow vertical growth phase[51]. Lentigo maligna melanoma is regarded by some authorities as a separate disease entity because of the different relationship to cumulative sun exposure and because it seems to have a better prognosis than other types of cutaneous malignant melanoma[59]. However recent evidence from the West of Scotland suggests that thickness for thickness the prognosis of lentigo maligna melanoma is similar to the other histological types of malignant melanoma[60].

I.2.6. Acral lentiginous melanoma

This type of malignant melanoma was first described by Reed[29,46], and it is a characteristic lesion which occurs on the extremities. Superficial spreading melanoma and nodular melanoma occur rarely on the palms, soles and subungual areas where acral (from the Greek, akros meaning limb) lentiginous melanoma usually develops. This lesion accounts for 6% of cutaneous malignant melanoma in Scotland[48]. Two thirds of acral lentiginous melanomas occur on volar sites and the remainder arise in subungual sites.

The volar tumours present clinically as an irregular brown or black "stain" on the palm or sole with normal skin markings preserved initially. During the radial growth phase this type of melanoma may come to occupy a large surface area, especially on the sole where it may not be noticed by the patient. Two thirds of the subungual lesions develop on the hallux or the thumb. A

characteristic feature of this "melanotic whitlow" (as subungual melanoma was first described by Hutchinson in 1886[61]) is pigmentation in the nail-bed matrix with a halo of pigmentation in the eponychium.

In acral lentiginous melanoma the radial growth phase is of intermediate duration between superficial spreading and lentigo maligna melanoma. There is usually an exuberant "lichenoid" host reaction and regression is common. Intra-lesional transformation may occur, and the onset of a vertical growth phase is often an abrupt, dramatic change. Clinically a blue or pink nodule develops in the long-standing lesion and metastases are common.

I.2.7. Epidemiology

The precise sequence of events which results in the development of cutaneous malignant melanoma has not been elucidated. The disease is probably caused by a combination of several factors. Epidemiological studies from around the world have begun to establish the relative importance of these factors.

There is clear evidence that the incidence of cutaneous malignant melanoma is rising in white-skinned people world-wide. The incidence is doubling each decade in Scandinavia[62], and quadrupling each decade in New Mexico[63] and Arizona[64]. Malignant melanoma is most common in the sub-tropical coastal area of Queensland, Australia[65] where the annual age-standardised incidence rate (near population-based) is 37.2 per 100,000[66] and the incidence is doubling there every fifteen years. The increase in Australia may be due to the high proportion of "Celtic"

immigrants from Scotland and Ireland, who may be particularly susceptible to malignant melanoma[67]. In New Mexico[63] and Arizona[64] the increasing incidence is confined to the "Anglo" population which is descended from north European immigrants.

Good epidemiological data shows that the incidence rate of malignant melanoma is rising rapidly[18] and there does not seem to have been a parallel increase in non-melanoma skin cancer[68] (though the epidemiological data for non-melanoma skin cancer is less satisfactory). It is important to note that the increasing incidence of cutaneous malignant melanoma is independent of improved diagnosis or certification[69,70].

When considering incidence data it is important to know whether the figures are based on the population at large and reflect the true incidence of the disease. If they are not population-based then clinical series will tend to reflect the interests of involved clinicians and local referral patterns. Comparisons between countries may be impossible if melanoma is included with other skin cancers in the national registry.

Data from British epidemiological studies show that the incidence of malignant melanoma is rising in England and Wales[71] and in Scotland[48,72,73]. The Scottish Melanoma Group has been gathering national data since 1979. In Scotland we are fortunate that this organisation provides accurate information on over 95% of the patients presenting with primary cutaneous malignant melanoma[48]. The incidence of malignant melanoma in Scotland has nearly doubled between 1979 and 1986.

The average age of those dying from malignant melanoma is falling[70] because the incidence of malignant melanoma is higher in people born in more recent years than in people born earlier this century[69,74]. Malignant melanoma is very rare in childhood but the average age at onset of malignant melanoma is lower than that of non-melanoma skin cancer.

The increasing incidence could be due to greater susceptibility of succeeding generations to the disease, an increasing exposure to or increasing power of causative factors.

The most recent figures published by the Scottish Melanoma Group[48] give annual age-standardised incidence rates of 2.77 per 100,000 for males and 4.75 per 100,000 for females. A similar female preponderance was reported in a previous large Scottish series[75] and in English studies[76,77]. In areas of high incidence, such as Australia, the incidence of melanoma in the sexes is approximately equal. It has been postulated[76] that a minor hormonal influence may be overridden by the critical effects of sun exposure in Australia.

Associations have been demonstrated between malignant melanoma and high socio-economic status, and with indoor rather than outdoor workers[78]. These findings support the theory which implicates intermittent recreational sun exposure in the aetiology of cutaneous malignant melanoma. Although malignant melanoma is less common overall in outdoor workers, in this group there is an excess of malignant melanoma of the face, head and neck[78] (where lentigo maligna melanoma tends to occur).

In occupational studies an excess incidence of cutaneous malignant melanoma has been reported among workers in a chemical plant[79] and specifically among workers involved in the production of polychlorinated biphenyls (PCBs)[80]. The incidence of cutaneous malignant melanoma is greatest in white-skinned populations living in sunny climates and the incidence generally increases with proximity to the equator[70]. In Europe however, there is a higher incidence in the Scandinavian countries than in the Mediterranean countries[81] and this may be due to differing susceptibility of the predominant skin types to malignant change in these populations. In Queensland the high incidence of malignant melanoma in the sub-tropical coastal area around Brisbane has been attributed to greater indulgence in coastal sun-bathing by the city-dwellers[66].

As previously stated melanin is an effective sun-screen[82] and malignant melanoma is widely considered to be less common in the darker skinned races than in Caucasians although there is a lack of good population-based incidence data. Several clinical studies show that a high proportion of melanomas which do occur in those of African descent tend to develop on the sole of the foot[83,84,85]. This tendency is not explained by the trauma of walking barefoot in the tropics because the excess risk is also apparent in North American Negroes[84], and instead it may be related to the greater proportion of acral lentiginous melanoma in those of African descent.

In white-skinned patients the distribution of malignant melanomas by anatomical site seems to reflect sex

differences of dress in relation to sun exposure. The site distribution of malignant melanoma has been compared with the distribution of melanocytes by site and there were apparent excesses of melanoma on the head and trunk of the male, and on the leg of the female[86].

Among white-skinned people, those at particular risk for malignant melanoma include those with blond or red hair and who burn easily in the sun[87], those who tan poorly and who freckle readily after sun exposure[88]. These features are quite common in some white populations and they are therefore weak indicators of an "at risk" group.

I.2.8. Aetiology

Solar radiation is heavily implicated in the aetiology of malignant melanoma. In addition to the general trend for rising incidence with decreasing latitude, a correlation between sun-spot number and melanoma incidence has been demonstrated[89]. There is a very low incidence of malignant melanoma at sites on the body which are rarely or never exposed to sun-light[70]. In the rare disease xeroderma pigmentosum, where there is defective repair of actinic damage to DNA with the development of multiple cutaneous malignancies, there is an increased incidence of malignant melanoma[90]. Swerdlow[91] reported a clear jump in the incidence of melanoma in the Oxford area two years after an especially warm summer. In a Scottish study a correlation was demonstrated between severe sunburn and the development of malignant melanoma in the subsequent five years[92]. The tendency is for intermittent intense exposure to be associated with the development of

malignant melanoma on the intermittently exposed sites[18]. This pattern holds for nodular and superficial spreading melanoma but does not apply to lentigo maligna melanoma. Lentigo maligna melanoma is associated with high cumulative exposure to sunlight and, like non-melanoma skin cancer[93], occurs in the elderly. Sunlight is the major source of ultraviolet (UV) radiation encountered by the skin. In recent years there has been increasing use of artificial sources of UV radiation e.g. sunbeds, lasers, arc welding equipment and ordinary white fluorescent tube lights.

Ultraviolet A (UVA) radiation has a wavelength of 315-400nm and it penetrates the skin more deeply than ultraviolet B (UVB). UVB has a wavelength of 280-315nm and is the more active radiation, causing more tissue damage, despite the poorer tissue penetration. UVB with wavelength less than 295nm does not reach the earth's surface because of filtering by the layer of ozone in the stratosphere. The ozone layer also significantly reduces the quantity of UVB with wavelength 295-310nm which reaches the earth. There is growing evidence that the layer of ozone (chemical symbol O_3) which absorbs UVB is being depleted by chlorine which is liberated from chlorofluorocarbons (CFCs)[94]. Chlorofluorocarbons are man-made compounds which are widely used as propellants in aerosol sprays and they are used in great quantities in industrial refrigeration plants. Chlorofluorocarbons have a half-life of many years in the stratosphere, and a single liberated chlorine atom may destroy thousands of ozone molecules[95].

Recent evidence suggests that there is already a

particular lack of ozone over Antarctica, a "hole" in the ozone layer which may act as a sink, draining the general level of ozone in the stratosphere[94]. Concern about the ecological consequences (including possible rise in skin cancer) led to the Montreal Convention which aimed to reduce the production of chlorofluorocarbons by half by the end of the 1990s[96].

The initial rise in incidence of malignant melanoma antedated the widespread use of sunbeds and solaria. In females there has been a particular increase in the proportion of melanomas occurring on the leg below the knee[70]. In males there has been an increase in the proportion of malignant melanomas occurring on the trunk . In both sexes there has been an increase in arm lesions[70]. It is tempting to blame these changes on obvious changes in the patterns of behaviour and dress in Caucasians around the world. Translucent stockings were first worn commonly by young women in the 1930s. Since World War II it has been more socially acceptable for men to work bare-backed in the sun. During this century it has become generally accepted that sunlight is beneficial and that a suntan is desirable because it indicates good health. Although the weight of scientific evidence does not support these misconceptions, the suntan cult shows little sign of abating.

Sunbeds emit UVA almost exclusively but recently UVA has been shown both to accelerate and potentiate the carcinogenic potential of UVB[97]. Exposure to UV from commercial solaria may also be associated with changes in the human immune system[98]. There is not yet enough

evidence to confirm that regular use of a sunbed is a risk factor for malignant melanoma[18,99] but circumstantial evidence is strong.

The role of sunlight in the pathogenesis of malignant melanoma is complex but an understanding of the interaction between individual phenotype (e.g. eye, hair colour), behaviour and sunlight is emerging.

In rare instances heredity may be directly involved in the aetiology of malignant melanoma[100]. Greene and Fraumeni[101] have reviewed the heritable variants of melanoma in depth.

Placental transmission to the foetus from a pregnant woman suffering from malignant melanoma has occurred several times[102]. The influence of heredity on the development of malignant melanoma is much more subtle and complex in most instances, and may concern the occurrence or distribution of benign pigmented naevi or some other unidentified factor. The relationship between familial malignant melanoma and dysplastic naevi is described in I.1.9.

An excess risk of malignant melanoma was reported in renal transplant recipients (who are routinely immuno-suppressed) and it has been postulated[103] that the immunosuppressed patient fails to mount an appropriate cellular response to malignant change in a precursor naevus. There is also an association between chronic lymphatic leukaemia and malignant melanoma[104]. Those with a previous primary melanoma are at increased risk of a second primary, and there may be an association between breast cancer and primary cutaneous malignant melanoma[105].

I.2.9. Prognosis: Staging

It is widely realised that the prognosis of a patient with cancer depends largely on the extent to which the disease has spread by the time of presentation. Generally, disseminated disease carries a poorer prognosis than locally or regionally confined disease. In order to describe groups of patients and compare treatments, systems have evolved to "stage" the disease.

One of the problems in the study of malignant melanoma is that different terminologies and various staging systems are used by different authors. In comparing published work on melanoma it is important to establish which particular staging systems have been used. Furthermore it cannot be ignored that the interpretation of the same staging system may vary from centre to centre and interfere with analysis[106].

For most clinicians a staging system simply offers a shorthand description which gives an estimate of tumour burden, and an indication of likely prognosis. In compiling a database for an epidemiological study or a clinical trial much more is required of a staging system. Here the system should convey comprehensive information concerning the stage at diagnosis and thereafter, enabling detailed analysis.

The two most widely quoted staging systems are the original three stage system[107] (Table 2) and the M.D. Anderson system[108] (Table 3), both based solely on clinical information. The simplicity of the former is both its main attraction and its major weakness. The M.D. Anderson system conveys more information about the pattern

of spread and is particularly useful in describing candidates for treatment by isolated limb perfusion. Neither system takes the characteristics of the primary lesion into account. In both of these systems, and in the more detailed T.N.M. (tumour, node, metastases) system recommended by the U.I.C.C. (Union Internationale Contra le Cancer), patients are not distributed evenly between the groups. Particularly in recent years there has been a great preponderance of patients in stage I at presentation[109]. An alternative system, which addresses these criticisms, has been proposed[110] and adopted by the American Joint Committee on Cancer, but it is much more complicated and is unlikely to be used widely, outside of American clinical trials.

In this thesis I will specify which staging system is used when appropriate.

Modern medical practice has available a multitude of investigations and tests which may be used by clinicians when assessing extent of disease. It is not ethical to subject a patient to any investigation which is unlikely to affect their clinical management, unless the investigation is part of a research protocol to which the patient has consented. In practice most patients with primary cutaneous malignant melanoma can be managed satisfactorily after a proper clinical assessment supplemented by a few simple and inexpensive investigations.

Even in the absence of specific symptoms or physical signs, routine postero-anterior chest X-ray and serum assays for alkaline phosphatase and lactate dehydrogenase

("liver function tests")[111] are appropriate.

When major surgery, such as isolated limb perfusion, is proposed for any patient then additional pre-operative tests are indicated. Before embarking on aggressive regional therapy of any sort it will usually be appropriate to exclude the presence of otherwise occult metastases by further investigation. The "gold standard" at present is imaging by computerised tomography[112], but when this is not available abdominal ultrasound or radio-isotope scans of liver, spleen and brain may be useful. Nuclear magnetic resonance imaging (N.M.R./M.R.I.) and the use of radio-labelled monoclonal antibodies[113] are new methods which are presently being developed and evaluated. In a recent study of 90 patients with newly diagnosed primary melanoma (Clark level III & IV, see Table 4) and no symptoms or signs of metastases an extensive screening protocol was evaluated[114]. The scheme included chest X-ray, full blood count, biochemical screening, C.T. brain scan and radio-isotope scans of liver, spleen and bones. This regime failed to detect any evidence of metastatic disease in any of the patients and the authors conclude that for patients in this category the scheme of investigation added nothing to clinical assessment.

If a patient has symptoms or signs suggestive of metastasis then investigations should be performed to exclude benign treatable conditions, to provide accurate staging for estimation of prognosis, and to plan appropriate therapy. Just as histological confirmation of the primary diagnosis is mandatory, so suspected metastasis should ideally be confirmed histologically.

Pigmented regional lymph nodes in a patient with malignant melanoma are not always metastases[115]. Modern biopsy techniques, such as fine needle aspiration biopsy are being used increasingly in surgical oncology, but depend ultimately on the skills of the cytologist. At present open biopsy is usually required. The incidence of occult residual melanoma is high after "node-picking" of clinically positive lymph nodes[116], and hence definitive regional lymph node dissection is preferred in these circumstances. The value of elective node dissection of clinically uninvolved nodes is discussed in I.3.5-7. In a world-wide review of five year survival in relation to stage (original three-stage system) of disease[117] the survival rates ranged from 65-85% for stage I and 27-42% for stage II. A previous literature review by Mastrangelo and colleagues[118] found reports of 55-80% five year survival rates for stage I, 14-39% for stage II and 0-18% for stage III disease.

Certain characteristics of the primary lesion are of particular prognostic significance (see I.2.10.), but it is not surprising that once overt metastasis occurs the characteristics of the primary lesion lose much of their prognostic significance[119].

Although loco-regional "recurrence" has been associated with a prognosis as bad as for distant metastases[120,121], in one recent large retrospective series (including 1722 extremity melanomas) patients with loco-regional metastases survived significantly longer than those who presented with systemic metastases[122].

In stage II disease it appears that the number of involved

regional lymph nodes[117,123,124] is a major prognostic determinant.

In a large (477 patients) retrospective series from the South-east of Scotland, the clinical stage (three-stage system) at diagnosis was considered[75]. 88% were in stage I, 10% stage II and 2% stage III. It is hoped that the recently initiated public education campaigns will influence the pattern of presentation so that an even greater proportion of melanomas will present in clinical stage I.

I.2.10 Prognosis: Primary lesion

Although malignant melanoma has a reputation for capricious behaviour, features of prognostic value have been recognised for many years. Allen and Spitz, in 1953[125], recognised that deeply invasive cutaneous melanomas had a poorer prognosis than superficial lesions. A prognostic classification based on level of invasion into the dermis was first defined by Mehnert and Heard in 1965[126]. The classification proposed by Clark and colleagues[127] has been accepted internationally[128], and is now commonly used (Table 4).

In the late 1960s Breslow was investigating the relationship between volume of primary tumour and prognosis, and he found that volume was itself unimportant but that tumour thickness was a good prognostic indicator[129]. He defined thickness as the greatest vertical distance in millimetres between the granular layer of the epidermis and the deepest invasive cell in the dermis [129]. The measurement is made on routine

paraffin sections using a simple ocular micrometer. In most situations the Clark level and Breslow thickness give similar information but in comparative studies the Breslow technique has been shown to be a more accurate guide to prognosis [130,131].

The prognostic value of Breslow thickness has been confirmed by many authors[132,130,133,134,135,136,137,138], and it has also been established in the Scottish population[48]. Day and colleagues have found that the inverse relationship between Breslow thickness and survival may be a step-wise relationship and they have proposed that the natural "break-points" for cutaneous melanoma are 0.85mm and 3.6mm[139]. Other workers have reported "break-points" which fit the data from their own study populations e.g. 0.75 mm & 3mm[140], 1mm & 4mm [138]. In the Scottish series the limits, 1.5mm & 3mm, were decided arbitrarily[18]. In all series thinner lesions are associated with fewer local recurrences and longer survival than thicker lesions.

The reasons why Breslow thickness is a better prognostic guide than Clark level are clearly discussed by Schmoeckel [140]. The Clark method relies more on the subjective judgement of the pathologist and its reproducibility is limited. The histological interface which separates level III and IV lesions (papillary and reticular dermis) is not distinct. Level I lesions are rarely reported, and are often omitted[18,48] because of the difficulty in distinguishing between level I lesions and dysplasia. The good prognosis level II group and poor prognosis level V group both contain few patients. For the majority in level

III and IV categories the survival rates are similar.

Breslow thickness takes account of the exophytic portion of a nodular tumour, which may be only superficially invasive according to the Clark level. Pedunculated tumours have a reputation for being particularly aggressive, but Schmoeckel and colleagues[135] have shown that exophytic and endophytic lesions have similar recurrence rates when Breslow thickness is similar.

Although in general the measurement of tumour thickness has proved to be relatively easy and reproducible, some problems may arise[141]. It can be difficult to differentiate the deepest tumour cells from other cell types; there may be intra-epidermal spread of tumour down hair follicles and sweat ducts or there may be accumulations of naevus cells at the base. The granular cell layer may be absent due to ulceration. Obliquely cut sections do not cause significant inaccuracy unless the angle is greater than 22 degrees. Thus, in general the problems with Breslow thickness are minor and guide-lines for special circumstances have been provided.

Studies have shown that overall there is no additional benefit from combining Clark level with Breslow thickness[130,134,140].

In recent series there is an increasing proportion of lesions less than 1mm thick[142,143,144] and there is some evidence that Breslow thickness may be a less important prognostic determinant in patients with such thin primary lesions. For primary melanomas of 0.75mm or less, Clark levels II and III may be associated with differing recurrence rates[135]. Furthermore in most series the

prognostic determination is made with reference to a database which is disproportionately loaded with thicker lesions[145]. In a regression analysis of 331 patients with lesions less than 1.5mm thick, it was found that tumour thickness had no influence on survival but females had a better chance of survival, and extremity tumours were associated with longer survival than axial lesions[145]. It will be important to know whether these results are reproduced in other large series of thin melanomas. As primary lesions are increasingly diagnosed and treated when less than 1mm thick, it will be important to define the likely prognosis for identifiable sub-groups, since high-risk patients are potential candidates for adjuvant therapies.

Histological evidence of ulceration in a primary malignant melanoma has been associated with a poor prognosis[125,146,147], especially when the ulcer is wider than 3mm[148]. The advantage of this histological feature as a prognostic determinant is that it is rather less subjective than some of the others.

In some studies the mitotic activity of a primary melanoma has shown some value as a prognostic indicator[127,134,148,149]. However, the detection of mitotic figures is subjective and other nuclear changes, or other cell types e.g. polymorphonuclear leukocytes may be confused with mitotic melanoma cells. The mitotic index is defined as the maximum number of mitoses seen in $1mm^2$ of the tumour. When the mitotic index is greater than $25/mm^2$ a 98% incidence of subsequent metastases has been reported[140].

Microscopic vascular invasion has been reported as an indicator of poor prognosis[140,146,147,150] but it is rarely seen convincingly, its detection is time consuming and in at least one report[138] it was of no clear prognostic value.

The host inflammatory response to malignant melanoma consists of a lymphocytic cellular infiltrate, associated with fibrosis and new vessels. The term "regression" is sometimes used to describe these histological features, which are common in superficial spreading melanoma. When these appearances extend more deeply than the measured Breslow thickness of a thin malignant melanoma, it may indicate that the original lesion was thicker before regression occurred. Hence the presence of regression in thin lesions may be associated with subsequent metastasis and a poor prognosis[151,152] though more recent reports have not confirmed these results[153,154].

In clinical practice a history suggestive of partial spontaneous regression is common. Complete clinical regression is very rare, though widely recognised in malignant melanoma and other cancers. Everson and Cole[155] collected 176 cases of spontaneous regression, including 20 instances in patients with metastatic malignant melanoma. Nine further examples in melanoma patients have been collected and the full series was reviewed by Bodurtha[156]. Regression of cutaneous and lymph node metastases was much more common than regression of visceral lesions. There was only one instance of histologically proven complete spontaneous regression of a visceral metastasis.

The syndrome of metastatic melanoma from an unknown primary is more commonly encountered, and it is generally considered to be due to spontaneous regression of the primary lesion prior to the patient presenting with metastatic melanoma[157]. Although an earlier report[158] indicated that the prognosis is almost hopeless for patients like this, in the series reported by Milton and colleagues[157] the prognosis of patients with metastases from an occult primary had a similar survival to patients with metastases from a known primary.

In various reports a poor prognosis has been associated with histological features e.g. severe cellular atypia[140,149], the small lymphocyte-like melanoma cell type[140] and poorly pigmented lesions[149]. The clinicopathological subtype is not an important independent prognostic factor[130,140,148].

I.2.11. Prognosis: Patient characteristics.

Female patients seem to have a better prognosis than males in many series[117,136,159,160,161,162] and this has also been observed in Scottish melanoma patients[163]. As mentioned above, sex may be the most important prognostic determinant for patients with thin primary malignant melanomas[145]. Females with clinical stage I melanoma have a better survival than males[136,160], but women with nodal metastases have a similar survival rate to men. In the Scottish series the better survival in females (especially in middle age) was related to thinner lesions in women and a preponderance of lower limb lesions[163].

A better prognosis has been reported for limb lesions as

compared with axial lesions[164,165] but the W.H.O. group findings did not support this[162]. Day and colleagues found that lesions on the back, posterior arm, posterior neck and posterior scalp (B.A.N.S. regions) seemed to have a particularly poor prognosis[166] but the significance of these findings is contested[167].

The age of patients at diagnosis has not been shown to be of prognostic value [117,168].

I.2.12. Prognosis: Interaction of factors

Various factors which are known to be of prognostic significance have been combined in attempts to enhance the quality of prognostic determination[146,169,170]. Ideally the factors used should each be of independent prognostic value. Many of the factors which have been shown to be of prognostic value in primary malignant melanoma are inter-related. For example, malignant melanoma tends to occur on the trunk in males and on the female lower limb, is the favourable prognosis in women due to sex, average thickness or site? Several histological features derive their apparent prognostic importance from a strong correlation with Breslow thickness. To determine which of many interacting factors are likely to be most important a multi-factorial analysis is required.

Applying multi-factorial analyses to data from eight major centres from around the world, the most significant prognostic determinants for a patient with stage I primary cutaneous malignant melanoma were[117]:

(1) tumour thickness,

(2) tumour ulceration,

(3) tumour site and

(4) patient's sex.

Overall the single most important prognostic determinant is Breslow thickness.

I.3. SURGERY FOR CUTANEOUS MALIGNANT MELANOMA

I.3.1. Introduction

The surgical management of cutaneous malignant melanoma is controversial. In the last four decades there has been a proliferation of information concerning the disease and its natural history. This knowledge should provide a rational basis for therapy. In assessing the place of isolated limb perfusion in the management of malignant melanoma it is essential to consider the other treatment options, surgical and non-surgical.

It is vital that critical appraisal of therapeutic studies is made because the quality of published data and analysis is variable, and the conclusions drawn can be misleading. The great value of a population-based database has already been discussed, and there is no doubt that well conducted randomised prospective controlled trials generate the most appropriate data to compare treatment options. Cohort effects have been reported to account for changes in the prognosis of melanoma in succeeding generations[172], and thus the use of historical controls is unsatisfactory.

A minority of studies are population-based, and there have been relatively few prospective randomised trials of available therapies. Although the advantages of prospective randomised trials in minimising bias are well recognised, even these can give rise to controversy.

In this section the appropriate surgical management for the various manifestations of cutaneous malignant melanoma will be discussed, based on critical assessment of the literature and our own experience.

I.3.2. Primary lesion: biopsy, excision margins

Any skin lesion which the clinician suspects, or which the patient reasonably fears may be a malignant melanoma should be completely excised for histological examination. Rarely one or more incisional biopsies may be appropriate where there is little spare skin e.g. face, hands, feet or if the lesion is large. There is no evidence that incision[173] or excision biopsy[171] induces subsequent local recurrence or metastases so there is no place for immediate wide excision on the basis of clinically suspected melanoma, without prior histology.

An adequate excision biopsy includes a comfortable margin (1-2mm) of adjacent normal tissue to minimise distortion of the specimen. Such a specimen gives the pathologist the best chance of complete histological staging[174,175], including the Breslow thickness, and hence a more accurate estimate of prognosis is possible.

There is no evidence that the time interval between initial diagnostic biopsy and definitive wide excision (when indicated) has any influence on the incidence of local recurrence or metastases[173,176,177].

The margin of normal skin which should be excised around a primary malignant melanoma has been the subject of both debate and dogma in the past. For much of this century "wide local excision" of the primary melanoma was supported and advocated on the basis of the Hunterian Lectures of Sampson Handley published in 1907[178]. Unfortunately this influential work was based on the study of patterns of spread in metastatic melanoma nodules, from a single post mortem study, and not on the behaviour of

primary lesions. Furthermore Handley advised margins of "about an inch" from the edge of the tumour, rather than the more radical excisions subsequently attributed to him[179,180].

The optimal excision margin for primary cutaneous malignant melanoma is still controversial, but the modern approach is based on an understanding of the natural history of the disease. As described in I.2.10., thick primary lesions are associated with a greater risk of loco-regional recurrence and metastasis. In general it is accepted that thin (good prognosis) lesions can be treated adequately by conservative excisional surgery[181,182,183,184] and that thicker lesions require wider margins[182,184,185,186].

Unfortunately authors have defined their terms differently. For some the term "local recurrence" includes disease in the vicinity of a scar from a previously excised melanoma as well as in transit metastases and invasion of the regional nodes[187]. Others apply the term more precisely to biopsy proven recurrent malignant melanoma within or contiguous to the scar of a previous local excision[184]. Ackerman has indicated that "true" local recurrence, due to the persistence of tumour because of incomplete excision of a primary, is now rare[188]. A third group[120,121] use terminology which implies that loco-regional recurrences (including "satellite" and in transit lesions- see below) are manifestations of systemic metastatic potential e.g. "regional non-nodal metastases" and "local metastases".

Whether "recurrent" melanoma in the vicinity of the scar

of primary excision is "true" recurrence or metastasis, the recognition of primary lesions likely to be complicated in this way prompts most authorities to recommend wide local excision for such primaries. The risk of "local recurrence" has been shown in several studies to correlate closely with Breslow thickness of the primary and not with excision margins[162,181,184,187,189,190]. In thin primary lesions (<0.75mm Breslow thickness) there is a minimal risk of "local recurrence"[130,140,162,181,182,184,186]. For primary cutaneous malignant melanomas of >0.76mm Breslow thickness there is a greater risk of local recurrence and satellitosis (metastases <5cm from the site of the primary). In patients with lesions >4mm Breslow thickness, the risk is 10-20%[182,184,186].

Historically the extent of "wide local excision" for melanoma has been the subject of dispute. Wide local excision may remove occult foci of malignant cells[191] and it may remove some of the "fertile soil" or "field change" which surrounds the site of a primary malignant melanoma. Wong showed that there were abnormal melanocytes in apparently normal skin up to five centimetres from the tumour[192] and Cochran demonstrated increased numbers of melanocytes in the skin around many melanomas[193].

There is however no evidence that wide local excision favourably influences survival and several recent studies have questioned the value of wide excision[194,181,184,188] by demonstrating that survival is independent of excision margin. This is despite the evidence of a high rate of local recurrence in thick primary lesions treated by

narrow excisions compared with similar lesions treated by wide local excision[195], and in spite of the poor prognosis associated with the appearance of local recurrence[120,121]. Logically, the modern approach is to tailor excision margins to the Breslow thickness of the primary. Hence an excisional biopsy with a margin of 5-10mm can be considered adequate treatment for malignant melanoma of <0.75mm Breslow thickness.

In modern practice there is general agreement that for primary lesions of >2mm Breslow thickness, 3cm of surrounding normal skin is a reasonable excision margin, although even this seems too radical for some[185]. The W.H.O. (World Health Organisation) recently reported a prospective randomised controlled clinical trial (Trial 10) comparing excision margins in lesions <2mm Breslow thickness, and there was no benefit from wide margins[196]. Wide, deep excisions were routinely performed for cutaneous malignant melanoma in the past[179], following the radical example of Hogarth Pringle[197]. However Olsen demonstrated an apparently increased incidence of metastases in patients treated by resections which included the deep fascia[159] and it was postulated that this might be due to the removal of an important barrier to metastases. More recently Kenady showed no difference in "local recurrence" or overall survival whether deep fascia was excised or not[198]. Anatomically the deep fascia proper is only found in the limbs and neck. Excision of this structure where it forms part of a muscular attachment, or where it is blended with the periosteum increases the morbidity of the surgery. It is therefore

considered adequate to excise down to deep fascia, without excising it, in most cases.

After conservative excisions direct primary closure of the wound can often be achieved. The development of modern plastic surgical techniques has resulted in the availability of a variety of possible methods of reconstruction after radical excisions. Although there is a theoretical risk, there is no evidence that primary closure conceals tumour to the detriment of patient survival[199]. Nonetheless most surgeons apply a thin split-skin graft to the site of a wide local excision in the belief that this gives the best chance of early detection of local recurrence[200]. There is no definitive study to commend either routine primary closure (with or without a flap) or routine skin grafting and the decision can safely be made according to technical considerations[180].

In the management of primary malignant melanoma arising on a digit, especially subungual melanoma, amputation of the digit is often the most appropriate procedure. Papachristou and Fortner reported a 22% incidence of local recurrence after conservative amputations for subungual melanoma, and no recurrence after amputation at the metacarpo-phalangeal joint[201]. It may be possible to select patients for conservative amputations (e.g. proximal to the inter-phalangeal joint of the thumb) which preserve function depending on tumour size, thickness and stage[202].

I.3.3. Loco-regional recurrence/ advanced disease

As discussed in I.3.2. above, there is a lack of

uniformity in the terminology used to describe loco-regional relapse after treatment of a primary cutaneous malignant melanoma.

In this thesis, unless otherwise stated, (cf. MD Anderson staging system, Table 3) "local recurrence" describes relapse in relation to the wound of primary excision, or skin graft; "satellitosis" refers to multiple cutaneous and subcutaneous metastases within 5cm of the primary excision; and "in transit lesions" are found in the skin or subcutaneous tissues between the primary site and the regional nodes. These definitions are somewhat arbitrary, and it is by no means certain that they are of prognostic value since these various manifestations of loco-regional relapse may occur together and thus the distinctions become blurred.

There have been no controlled trials of appropriate treatments for local recurrence[202]. The most widely considered options are surgical excision, isolated limb perfusion and radiotherapy. Although it is tempting to manage a solitary local recurrence by simple excision, the weight of recent evidence supports the view that "local metastases"[120] or "regional non-nodal metastases"[121] may be the harbingers of repeated recurrences, systemic metastases and death. As noted in I.2.9., the prognosis for loco-regional recurrence is better than for systemic metastases[122]. The median survival after local recurrence in clinical series is between 10[203] and 30[121] months, and the ten year survival rate is between 15[121] and 20%[202].

We have already considered how treatment of the primary lesion may influence the risk of subsequent local

recurrence (I.3.2.). The conventional explanation for satellitosis and in transit metastases is that they develop from lymphatic spread of melanoma[203]. It has been suggested that in transit disease and satellitosis are related to lymphoedema after lymphadenectomy, or to seeding of cells after cutting across lymphatics in discontinuous (as opposed to in continuity) procedures[204]. There is no convincing evidence to support these putative mechanisms, and the patients most at risk are those with thick, ulcerated primary lesions and patients with lymph node metastases[186,187,204,205,206].

The incidence of these various manifestations of loco-regional relapse varies from centre to centre, and it may seem particularly high in series reported from centres which attract referrals for specific treatments such as isolated limb perfusion.

Amputation of the limb has been advocated for recurrence confined to an extremity[207]. There are few reports in the literature describing the results of major amputations for malignant melanoma. McPeak[208] reported 34% 5-year survival in 46 patients treated by hip disarticulation for recurrent melanoma, but the amputation series of Bowers[209] and Cox[210] yielded 5-year survival rates of 18% and 15%. Major amputation is now rarely performed for advanced or recurrent malignant melanoma but this option may have to be considered when other treatments have failed and when the patient has distressing symptoms. The considerable morbidity and questionable quality of life after such extensive surgery encourages the application of limb-sparing procedures, such as isolated limb perfusion. Some

are convinced that the advent of isolated limb perfusion with cytotoxics has significantly reduced the numbers of melanoma patients coming to amputation[211,212,213,214,215]. Objective assessment of the value of major amputations is complicated by confounding variables which include the changing attitudes of patients and surgeons to radical surgery for malignant disease, and the changing prognosis of malignant melanoma. In general it seems inappropriate to inflict a major amputation, which requires prolonged rehabilitation, on a patient whose survival is probably limited because of advanced disease.

I.3.4. Involved regional lymph nodes

Management of lymph nodes is an important consideration in malignant melanoma because the regional nodal basin is the commonest site of first metastasis[121,187]. In malignant melanoma the importance of the regional nodes has long been established. Pemberton, in 1858, advocated wide deep excisions for primary lesions plus excision of the regional nodes if involved[43]. A few years after the influential publications of Sampson Handley[178], Hogarth Pringle[197] introduced his radical, in continuity excision of primary lesion, intervening skin ,lymphatics and regional lymph nodes. This kind of excisional surgery for cancer found favour with the generations of surgeons who practised Halsted's radical mastectomy for breast cancer. In this thesis, consideration will be restricted to the management of the regional nodes draining the limbs, since limb primaries are common, lymphatic drainage is predictable in the limbs and major studies have

concentrated on this aspect.

The commonest initial presentation of metastatic melanoma is involvement of the nodes draining the site of the primary lesion. Nodes containing metastatic melanoma are clinically firmer, rubbery, and less tender than in inflammatory lymphadenopathy. Fine needle aspiration cytology or open biopsy may be helpful when the clinical findings are equivocal[203], but if the nodes are clinically malignant then block dissection without biopsy is justifiable. There is no dissent about this indication for dissection of the regional nodes[203,216,217] but there is debate about the extent of surgery required.

For clinically involved inguinal nodes the surgical options are to perform an inguinal or ilio-inguinal dissection. An inguinal dissection involves clearing all the nodes in the femoral triangle including those superficial to the inguinal ligament, in relation to the femoral vessels down to the apex of the triangle, and the node of Cloquet (i.e. including the deep inguinal lymph nodes). In an ilio-inguinal dissection the inguinal ligament is divided and all the above lymph nodes are excised in continuity with the iliac and obturator nodes.

In patients with involved inguinal lymph nodes 25-50% will have involved iliac nodes as well, indicating to some that the ilio-inguinal operation is more appropriate and may offer better disease control[218]. Others have observed that the more radical operation may be associated with greater morbidity[203,217], that positive iliac glands indicate a poor prognosis[203], and that an inguinal dissection is sufficient when the disease appears to be confined to the

inguinal nodes[203].

I.3.5. FOR elective lymph node dissection

The point at issue is whether there is an identifiable sub-group of patients with primary malignant melanoma who may benefit from dissection of clinically uninvolved nodes ("prophylactic" or "elective lymph node dissection") or whether dissection of the lymph nodes should only be performed if and when the nodes are clinically involved (therapeutic or delayed dissection). Even the strongest supporters of elective node dissection agree that only a selected group of patients may benefit from the procedure[217,220].

The debate about routine elective lymph node dissection has been usefully summarised by Balch and Milton, who favour elective lymph node dissection, and by Cascinelli and Sim, who prefer delayed lymph node dissection[220]. In a recent review Balch has restated his position, but he presents no new evidence[217]. The debate is an important consideration for all surgeons involved in the management of cutaneous malignant melanoma, because it raises many important questions, ranging from interpretations of the natural history of the disease to motives for surgical intervention.

The rationale for elective node dissection is based on the hypothesis that cutaneous malignant melanoma spreads in a step-wise manner, from the primary to the regional nodes and thence to distant sites[217]. If this concept is true, then patients with occult micro-metastases in the regional nodes should benefit from elective node dissection,

because it is performed when the tumour burden is small and before systemic spread occurs. By the time clinically detectable nodal metastases are established most patients (70-85%) will already have distant micro-metastases[124] and the ten year survival is around 25%. According to the hypothesis the aim of elective lymph node dissection is therefore to provide definitive treatment at an early stage in the natural history of the disease.

Balch and Milton[220] contend that Breslow thickness of the primary melanoma is the major indicator of the group which might benefit most from elective lymph node dissection. They argue that primary melanomas of Breslow thickness 0.76-4mm have a high risk of occult regional metastases and a low risk of systemic metastases, and that this is the group which should benefit from elective lymph node dissection[130,168,182,221,222].

There is no evidence to suggest whether inguinal or ilio-inguinal dissection is more appropriate in the management of lower limb disease by elective node dissection. The major proponents perform an inguinal dissection[217,222] and the iliac glands are spared unless the inguinal nodes are found to contain metastases, when an iliac dissection is considered appropriate.

It has been argued that lymphatic stasis (common after elective node dissection) may provide a favourable environment for the survival and proliferation of melanoma cells[204]. Furthermore immediate elective node dissection may result in the seeding of intralymphatic cells in transit which might otherwise reach and be contained by the draining nodes. Such a mechanism might explain in

transit disease occurring in post-lymphadenectomy lymph stasis, and provides a rationale for "interval" node dissection. Petersen and colleagues[179] found that "interval" node dissection, delayed more than fourteen days after primary excision, was associated with a lower incidence of loco-regional recurrence. Wanebo and colleagues[132] and McCarthy and colleagues[204] found no benefit from similar approaches, and the only prospective study which addresses this question is too small to provide a definitive answer[223]. In the absence of good evidence to favour "interval" elective node dissection, protagonists thus tend to perform definitive excision of the primary and elective node dissection under the same general anaesthetic[220].

The main studies cited for and against elective lymph node dissection have been summarised in Tables 5-12 for ease of comparison.

I.3.6. AGAINST elective node dissection

Although the theory invoked in favour of elective node dissection is attractive, there are important objections to it.

Firstly, malignant melanoma does not always spread via the regional lymph nodes. Balch's contention that Breslow thickness allows prediction of site of metastases[147,182,217] has not been confirmed. In a W.H.O. study of 1164 Stage I patients treated without elective lymph node dissection, 51% of the 516 who suffered a relapse within ten years of treatment suffered first recurrence in the regional nodes, 22% presented with

distant metastases and 31% had simultaneous regional lymphatic and distant metastases[224]. The major prognostic determinants of primary melanomas (Breslow thickness etc.) can identify patients at risk of metastases and death, but these criteria do not accurately predict the site of recurrence[138,224]. In Milton's own study of the site and time of first recurrence after wide excision alone for stage I disease[187] he excluded a number who developed first recurrences at multiple sites, and a significant minority presented first with distant metastases.

Secondly, clinical stage I/pathological stage II (i.e. microscopic nodal metastases) is not directly comparable with clinical stage II (original three-stage system, Table 2). Protagonists[217,221] of elective node dissection often argue that the prognosis for patients who have occult regional nodal metastases, diagnosed after elective node dissection (referred to as clinical stage I/ pathological stage II), is better than the prognosis of those who have a therapeutic node dissection for clinically apparent nodal metastases (clinical stage II). It is not surprising that there is a survival advantage for patients with a microscopic tumour burden compared with patients who have clinically obvious malignant lymphadenopathy, since it has also been shown that the number of involved nodes is itself of prognostic value[117]. Although the overall survival figures for groups having elective lymph node dissection seem favourable (Tables 5-10) it does not follow logically that elective node dissection benefits <u>all</u> patients having the operation. This is because many patients treated by elective node dissection may be

subjected to an unnecessary operation, since they have no demonstrable metastases in their excised glands, and their prognosis would be excellent in any case. Conversely, patients having therapeutic dissection all have clinically apparent nodal metastases and their comparatively poor prognosis is due to the advanced stage of their disease.

The two major prospective randomised studies which have addressed the question of routine elective node dissection have both shown no benefit from the procedure[223,225,226,227] (Tables 11 & 12). Even the strongest advocates of elective node dissection agree with the principal finding, that not all patients with melanoma will benefit from elective node dissection[217,220].

In the W.H.O. study (Table 11) 19.7% of those having elective node dissection were discovered to have occult nodal metastases, and 24.2% of those having excision of the primary alone developed nodal metastases[225,226] in a five year follow-up period. There was no significant difference between these two sub-groups in terms of five year survival.

The data from both the W.H.O. study[226] and the Mayo Clinic study[227] support therapeutic node dissection when required, as opposed to routine elective node dissection.

Both of these major prospective studies of elective node dissection were mainly concerned with the general applicability of the operation and neither the W.H.O. study[225,226] nor the Mayo Clinic[223,227] study was designed to determine whether specific small sub-groups might benefit.

The W.H.O. Melanoma group study[225] was initiated before

the identification of some factors which are now recognised to be of considerable prognostic significance. Neither Breslow thickness nor ulceration was originally included in the stratification criteria. Balch has indicated that although tumour thickness splits evenly between the treatment arms[217] in the W.H.O. study, in the intermediate thickness group (1.5-4.99mm) there was a higher incidence of ulceration in those having elective node dissection (52%) than in those having wide excision alone (19%). However, less than 10% of the entire study population fell into this sub-group of patients having elective node dissection, and the "mal-distribution" occurred at random.

Balch states that in this small, highly selected group there may be a survival advantage from elective node dissection[217,220], after accounting for ulceration. Even if this advantage in the 20% with intermediate thickness lesions is real it represents only a 4% improvement in the whole group, of whom a majority would have been subjected to unnecessary surgery.

Furthermore the risks of mortality and morbidity associated with surgical intervention cannot be ignored. There were two deaths in the Sydney experience of elective node dissection[221], probably occurring in the early experience of the operation, but it would be difficult to justify any adjuvant treatment with a significant risk of mortality today. Elective lymph node dissection is also associated with morbidity. In a series of 58 patients from Birmingham, Alabama the incidence of lymphoedema after inguinal node dissection was 26%, 23% developed a seroma,

5% had a wound infection, 55 had persistent pain and 8% had a persistent "functional deficit"[203].

In his criticisms of the multi-national W.H.O. trial (Table 11) Balch points to differences between surgical treatment results and clinical staging according to country[217,220]. Variability in treatment results could be explained by the small numbers contributed by certain centres and randomisation within the participating centres would mitigate the effects of variable clinical staging.

Another possible criticism of the W.H.O. trial is that since only limb lesions were selected for study, 85% of the patients in this study were women. Thereby a group at particularly low risk of metastases may have been selected and the overall good prognosis might dilute any benefit from elective node dissection. Breslow[228] compared the mortality rates of patients in the W.H.O. study with those from patients in the study of Wanebo and colleagues[132] (Table 10), where there was twice the proportion of men. This comparison showed that thickness for thickness there was no significant difference in survival, and the W.H.O. group had a particularly high proportion of lesions thicker than 3mm.

In the Mayo Clinic trial[223,227] (Table 12) there were few patients in the categories which Balch and colleagues consider likely to benefit from elective node dissection.

In both prospective trials (Tables 11 & 12) there was careful follow-up of the group managed by wide local excision alone, to facilitate the early detection of any regional nodal metastases. In the W.H.O. trial the overall ten year survival was 15-20% for patients needing a

therapeutic node dissection[226]. Those patients with a single positive node at therapeutic dissection had a 44% ten year survival, and this is an incentive to the early detection and treatment of nodal metastases when elective node dissection is not practised. Clinical accuracy in the diagnosis of metastatic lymph nodes is poor. Gumport and Harris[229] noted clinical under-diagnosis of 29% (clinically negative/histologically positive) and over-diagnosis in 23%.

Balch argues that the incidence of nodal micro-metastases is as high as 60% in patients with intermediate thickness primary melanoma (1.5-4.99mm)[217]. From the W.H.O. data he argues that, because 56% of patients treated by excision alone for lesions in this category subsequently required therapeutic lymph node dissection, this figure gives an estimate of the true incidence of nodal metastases at the time of treatment of the primary. The fallacy in this argument is that there is no reason to suppose that all subsequent nodal disease is the result of micro-metastases dating from or before the time of treatment of the primary. At least a proportion of nodal metastases will develop as a result of delayed spread from loco-regional or distant sites. Balch's theory derives some support from the 42% incidence of occult nodal metastases in elective node dissection specimens, serially sectioned and reported in retrospective studies by Lane and colleagues in 1958[230], and Das Gupta in 1977[231]. However in the patients treated by elective node dissection in the major prospective studies we have seen that the incidence of nodal micro-metastases is 19.7% overall in the W.H.O.

trial[226] (21% in the intermediate thickness group) and 6% overall in the Mayo Clinic trial[227].

The studies performed by Balch and Milton, and cited by them in favour of elective node dissection[168,221,220,217] are described in the later publications as "prospective but non-randomised" trials. Unfortunately this is not an accurate description of their impressively large clinical series. In both the Alabama and the Sydney series a significant proportion of the data was retrospectively culled from case records, and in the "prospective" parts of these studies the criteria which were actually used to select patients for elective node dissection are not clearly defined. The University of Alabama Melanoma Registry began in 1975[168] but includes patients treated since 1955. The Sydney study comprises patients treated from 1950-1980 and its retrospective nature is made clear in an early publication[221] but it is described differently in later papers[168,203,217,220].

Breslow[228] has indicated that the literature concerning elective lymph node dissection prior to 1977, including some of his own work, is very difficult to evaluate because the studies were retrospective and liable to bias in patient selection. Breslow found, on reanalysing the data from his 1975 paper[232], that his surgical colleagues had been selecting patients for elective node dissection because they had limb lesions, rather than axial lesions which had a less predictable lymphatic drainage. In addition none of the Clark Level V lesions had elective node dissection. Inadvertently the surgeons had thus selected the patients with the best prognosis for elective

node dissection.

This kind of problem appears again in the retrospective study of Reintgen and colleagues (Table 8)[222], where patients were referred by thirty different surgeons for adjuvant therapy after initial surgery. A minority of patients actually had elective node dissection and there was a bias towards elective node dissection for limb lesions. As in the work of Balch and Milton the problem of selection bias is partly dealt with by retrospectively analysing the patient groups after stratification according to dominant prognostic factors revealed by multi-factorial analysis[221]. Statistically this is less satisfactory than prospective randomisation with stratification. None of these studies can deal with the improvement in prognosis which has occurred in recent succeeding decades.

Such criticisms do not render the studies supporting elective node dissection null and void, but they serve to emphasise that conscious and unconscious bias can corrupt retrospective studies. An alternative way for the vocal protagonists of elective lymph node dissection would be to present the truly prospective parts of their series as separate "trials", since presumably selection criteria were used to allocate patient to the different treatments; but to date this information is not available.

I.3.7. Elective lymph node dissection: Conclusions

The evidence of the two major prospective randomised studies (Tables 11 & 12) suggests that there is no overall survival advantage conferred by elective lymph node

dissection, but this conclusion is not accepted by the proponents of the procedure who cite large, mainly retrospective, series (Tables 5-10) to support their position. A proper answer to this important question should emerge from the trials of elective lymph node dissection in sub-groups of Stage I melanoma patients, which have been activated by the National Cancer Institute of the U.S.A. (primary melanomas 1-4mm Breslow thickness) and by the W.H.O. Melanoma Group (trunk lesions >2mm Breslow thickness). Until the results of these studies are forthcoming a clinician must manage patients on the basis of his interpretation of the available evidence.

Any advantage due to elective node dissection is likely to be small, even in selected sub-groups. For this reason, and because the risks of significant morbidity in the majority of patients who do not need the operation are considerable, I favour a policy of therapeutic rather than elective node dissection.

Evidence is emerging which may explain why, in both malignant melanoma and breast cancer[233], treatment of the regional lymph nodes fails to influence survival. It is now known that lymphatico-venous anastomoses often by-pass lymph nodes, and there are naturally occurring channels which allow intact cells to pass through lymph nodes[234,235]. Thus, cancer cells may disseminate simultaneously by the blood-stream and lymphatic pathways, and hence lymph node metastases can be considered "indicators, but not governors" of survival[234].

Furthermore studies have shown that the demonstration of circulating malignant cells[236], nodal malignant cells[233],

and even distant micro-metastases[237] are not invariably associated with clinical metastases or death. Therefore there is no inevitable advantage from excising nodes containing micro-metastases.

There is no evidence from the trials discussed that the removal of healthy nodes confers any advantage or disadvantage to the patient with malignancy.

If there was an effective adjuvant therapy for cutaneous malignant melanoma, then (as in breast cancer) elective node dissection might be justified as a sampling procedure for staging.

I.4. NON-SURGICAL TREATMENT OF MALIGNANT MELANOMA

I.4.1. Introduction

The only treatment which reliably cures patients with cutaneous malignant melanoma is surgical excision of thin, low-risk lesions. Once a patient with cutaneous malignant melanoma develops systemic metastases cure is unlikely. At this late stage the priorities of treatment should be to relieve symptoms, improve quality of life and prolong of survival.

For all stages of cutaneous malignant melanoma many different modes of therapy have been tried to improve prognosis. When a variety of treatments are advocated for a disease it usually means that none of the treatments are entirely satisfactory. The role of excisional surgery for early disease is beyond dispute, save for details of extent, and in many early cases no other treatment is necessary. Sadly for patients with advanced disease, there are many possible treatment options using a range of modalities, but few treatments are of proven efficacy, and several are associated with significant toxicity.

We have seen that the risk associated with a primary melanoma can be quantified according to Breslow thickness (I.2.9.). Both loco-regional recurrence and distant metastases are associated with a poor prognosis. The challenge for clinicians is to develop effective (and ideally non-toxic) treatment strategies which will improve the prognosis of patients who are likely to develop, or who already have developed advanced disease.

As in breast cancer therapy, some treatments which show benefit in advanced disease have been used as "adjuvants"

to surgical excision of a high-risk primary malignant melanoma, in the belief that such treatments are more likely to have a greater benefit when the tumour load is less (see also I.4.6). In animal models it is recognised that the failure of macroscopic disease to respond to chemotherapy does not automatically predict a similar failure of micro-metastases to respond to the same drug[240]. Macroscopic tumour nodules may be more resistant to treatment because of their smaller growth fraction and poorer blood supply.

The balance of evidence concerning adjuvant therapy for breast cancer has recently been pooled and scrutinised in a meta-analysis, with the result that survival benefit has been established for adjuvant chemotherapy in pre-menopausal women[241]. A major problem is that to establish such benefit requires the study of large numbers of patients in trials of many years duration, because of the long natural history of diseases like breast cancer and cutaneous malignant melanoma.

I.4.2. Advanced disease: Systemic chemotherapy

There are no universally accepted treatments for systemic melanoma metastases and clinical decisions have to be made on an individual basis. Obviously the patient and relatives should have an appropriate understanding of the situation, and they will require support no matter how the patient's disease is managed.

In the absence of a curative treatment it may be inappropriate to offer active therapy with available modalities to patients who are asymptomatic, elderly or

terminally ill. If the aims of treatment for a particular patient are realistic, and risks or toxicity are acceptably low, then active treatment may be appropriate.

Few effective chemotherapeutic agents have been discovered for malignant melanoma, and even the strategy of combining active agents has proved largely disappointing in this disease[242].

As in the consideration of surgical treatment options, the literature concerning chemotherapy for malignant melanoma is replete with studies which fall short of established ideals for clinical trials. Again the "gold standard" for assessing the value of any proposed treatment is the prospective randomised controlled clinical trial with survival, rather than "response rate" as the end point. All too often the early promise of new agents in uncontrolled studies has not been confirmed when the same treatment is subjected to the rigours of a prospective controlled trial in comparison with established regimens. Unfortunately, the prospective trials which have been performed are often subject to the criticism of small sample size, which may preclude the detection of small but significant differences between regimes[243]. Rarely is valid survival data available for assessment.

The agent which is widely regarded as most effective in the treatment of malignant melanoma is dacarbazine (5-[3,3-dimethyltriazeno]-imidazole-4-carboxamide; DTIC)[244]. The details of dose and schedule vary from study to study[245,246], but the overall (complete plus partial) response rates are fairly consistent in the range of 14-25%[243,247,248,249]. There are very few complete responses

[247,248] and the responses are often transient[250].

There are various toxic side effects associated with dacarbazine therapy[250]. Significant gastro-intestinal toxicity is common, and marrow toxicity is dose limiting in the short-term. Unlike the nitrosoureas (see below), which may cause cumulative marrow damage leading to irreversible marrow failure, dacarbazine marrow toxicity usually recovers rapidly.

It has been reported that women respond more frequently than men, and that cutaneous and nodal metastases are more likely to respond than visceral metastases[248,251,252,253]. It has been reported that patients who respond to chemotherapy live longer than non-responders[252,253]. It can be argued that response to chemotherapy is associated with, but not causally linked to the improved prognosis.

Vindesine (desacetyl vinblastine amide sulphate) was first reported as a promising single agent for patients with advanced malignant melanoma by Retsas and colleagues[254,255]. In these early studies the objective response rate was 24%, and similar results were obtained in a Scottish Melanoma Group study[256]. In this latter study a 26% response rate was achieved, with 17% complete remissions. Responses were longer lasting than in previous studies. Furthermore these results were achieved with less toxicity than is associated with DTIC. In particular gastro-intestinal upset was relatively infrequent and mild.

In conventional doses the alkylating agents (melphalan, cyclophosphamide, chlorambucil etc.) produce modest response rates[244]. From the early days of chemotherapy

with alkylating agents it was recognised that the major dose limiting toxicity was myelosuppression[257,258]. Various strategies have been developed in order to circumvent this problem (including isolated limb perfusion). The practice of high dose systemic chemotherapy and autologous bone marrow rescue was pioneered by Ariel and Pack[259]. McElwain and colleagues[260,261] have shown that the response rate can be boosted to 70% using very high doses of melphalan and intensive support, including autologous bone marrow transplant. Unfortunately these responses were of short duration, but the results indicate that regional delivery or effective targeting to tumour may be ways of exploiting the potential of melphalan.

Melphalan is discussed in much greater detail later in this thesis (Chapters III & IV) because it is currently the agent of first choice in the treatment of malignant melanoma by isolated limb perfusion.

Anti-metabolites, including high-dose methotrexate regimens[262,263], have not yielded encouraging response rates in malignant melanoma.

The nitrosoureas, including lomustine (CCNU), carmustine (BCNU) and semustine (methyl-CCNU), were once considered as suitable single agents[242], but they have been replaced by newer, less toxic drugs like vindesine and DTIC.

Cis-platinum is an active single agent[264] which has been incorporated into recent combination chemotherapy regimens.

Despite the large number of studies, there is no prospective controlled clinical trial of adequate size[242]

which conclusively demonstrates the superiority of any combination chemotherapy regime compared with the same drugs used singly for advanced malignant melanoma[252,253,243,265,266].

The lack of proven long-term benefit, and potential for toxicity, should preclude the use of systemic chemotherapy outwith clinical trial protocols.

I.4.3. Advanced disease: Hormonal therapy

There is an apparent hormonal influence over the human melanocyte system, both in health e.g. chloasma of pregnancy, and in disease e.g. female survival advantage in melanoma. In spite of early studies suggesting that non-toxic anti-oestrogen therapy with tamoxifen may be of benefit, many subsequent studies demonstrated a very poor response rate[242,244]. Pituitary ablation has been unsuccessful[267].

Corticosteroids, especially dexamethasone, are of proven value in relieving symptoms and signs due to raised intracranial pressure from brain metastases[268]. They can thus provide good palliation, but have no specific anti-melanoma activity.

I.4.4. Advanced disease: Radiotherapy

Malignant melanoma has long had a reputation as a radio-resistant cancer. In an early, oft-quoted study[269] there was a response rate of only 2.5% in patients with advanced disease, but precise details of the radiotherapy regimes used were not given. In recent years a greater understanding of the radio-biology of melanoma has led to

modified dose schedules which have resulted in more encouraging results.

Habermalz and Fischer[270] showed that large fraction sizes produced greater response rates in the radiotherapy of malignant melanoma. Similar results were reported by Hornsey[271] who explained this in relation to the large capacity of melanoma cells to repair sub-lethal damage. Considering the radiation dose/survival curve for melanoma cells, there is a broad "shoulder" during which sub-lethal radiation damage is repaired by the cells[272], and only at relatively large doses is there a linear relationship between increasing dose and cell kill. High doses per fraction overwhelm the cell's capacity to repair sub-lethal damage[273].

"Resistance" to radiotherapy may also be partly explained by the presence of high levels of superoxide dismutase associated with melanin granules[274]. This enzyme scavenges, and inactivates, the free radicals which are produced by ionising radiation and which cause cell damage[275]. Superoxide dismutase therefore removes the active agents, and larger doses may be required to overcome this. Furthermore there is evidence that in larger tumours there are cell compartments which are relatively hypoxic and resistant to radiation[274].

Retrospective clinical series have confirmed that high dose per fraction regimes may produce significantly greater response rates than can be achieved with conventional fractionated radiotherapy, without excess toxicity to normal tissues[276,277]. However other clinical studies suggest that large fraction size does not

help[278,279]. In a recent study the authors concluded that large fractions may be of value in the treatment of superficial metastatic melanoma[280], but the proximity of node masses and visceral metastases to sensitive structures could result in considerable morbidity when large fractions were used to treat these lesions.

Palliative external beam radio-therapy is often used for symptomatic bony metastases and may be used for inoperable soft tissue masses.

Radiotherapy has also been applied as part of some studies of multi-modality therapy for malignant melanoma. The role of hyperbaric oxygen, radio-sensitisers (e.g. misonidazole), hyperthermia, immunotherapy and chemotherapy in combination with radiotherapy has been reviewed by Brascho[274]. There is no convincing trial evidence to suggest that any particular radiotherapy combination should be applied routinely. Further studies are indicated, and some are in progress.

Diagnostic lymphangiography was developed by Kinmouth and Taylor in the 1950s[281], and the technique of intra-lymphatic injection was soon applied for the administration of diagnostic and therapeutic isotopes. The rationale for endolymphatic radiotherapy is based on the selective accumulation of isotope plus vehicle (e.g. lipiodol) in regional lymph nodes. Various isotopes have been used including ^{198}Au, ^{131}I and more recently ^{32}P. Theoretically this treatment should be most advantageous in those patients at high risk of harbouring nodal micro-metastases (cf. other adjuvants, I.4.6).

In a prospective randomised trial comparing standard

surgical treatment with surgery plus endolymphatic radiotherapy, in the management of Stage I melanoma, there was no significant ten year survival benefit from endolymphatic therapy[282]. This was in spite of an apparent reduction in the incidence of nodal metastases in the latter group. A major problem with this technique is that macroscopic tumour causes distortion of the lymph nodes with uneven distribution of isotope and inadequate irradiation where it is most needed. In the M.R.C. (Medical Research Council) trial there was a 35% rate of technical failure of injection. This elegant technique is not widely practised.

I.4.5. Advanced disease: Immunotherapy

This is not the place for a detailed critique of the immunology of malignant melanoma. Good reviews of the topic are provided by Peter[283] and Mastrangelo and colleagues[284].

The rationale for immunotherapy with agents like Bacille Calmette Guerin (BCG) or Corynebacterium parvum (C.Parvum) is that there are specific tumour associated antigens on melanoma cells. These antigens normally produce a weak immune response, and melanoma patients seem to have a reduced immune response. Vaccination with non-specific antigens, like BCG or C.Parvum can produce a demonstrable increase in the antibody response to melanoma antigens, and the ultimate aim of immunotherapy was to translate this into survival benefit[285].

Specific immunisation with irradiated melanoma cells, or with melanoma antigens, and combinations of

immunostimulation with chemotherapy and radiotherapy have also been used[283].

Despite early promise the later trials of non-specific and specific immunotherapy have been disappointing, and in some clinical studies the results have suggested that active immunotherapy may have enhanced tumour growth[286,287]. Systemic chemotherapy plus non-specific immunotherapy (chemo-immunotherapy) using C.parvum is not effective in the treatment of metastatic malignant melanoma[288]. Although intra-lesional injection of BCG may cause regression of injected dermal nodules there is no significant effect on subcutaneous nodules, lymph nodes or visceral metastases[284].

Overall the initial promise of immunotherapy has not been fulfilled.

Recent emphasis in the field of immunotherapy has been on the use of monoclonal antibodies and biological response modifiers. Monoclonal antibodies may be used for targeting drugs or radio-isotopes against melanoma but this approach is still largely experimental[244]. Initial trials with interferons (with and without adjuvants like cimetidine) have produced low response rates[289,290]. Early results with lymphokine-activated killer T-cells (LAK-cells), produced by stimulating the patient's T-cells in vitro using interleukin-2 (IL-2), seem to offer a new approach to immunotherapy in advanced malignant melanoma[291]. This regime is however relatively toxic and it is not as yet widely available. The same team of workers have recently reported that autologous tumour-infiltrating leukocytes (TILs) may be effective in producing regression of

advanced melanoma, though the response may be short-lived[292].

I.4.6. Adjuvant therapy: Principles

We have seen that surgical treatment is central to the management of cutaneous malignant melanoma, and that the treatment of advanced disease is unsatisfactory. The concept of multi-modality treatment is of proven value in animal models and in certain human cancers (e.g. seminoma, osteosarcoma). In the animal models the best results have been obtained by combining an effective local treatment, usually surgery, with systemic treatment e.g. chemotherapy, or occasionally immunotherapy.

Schabel reported improved survival in tumour-bearing animals treated by surgery plus adjuvant chemotherapy, compared to animals treated by surgery alone[240,293]. Similar results have been reported by Balch and Maddox[294]. General principles for adjuvant therapy which have emerged from this kind of animal work[285] are

a) that the efficacy of the regime is greatest when the tumour burden is least,

b) occult micro-metastases are more sensitive to chemotherapy than larger tumours,

c) treatments which are effective against advanced disease are more active against residual metastases after excision of apparent disease, and

d) lethal metastatic disease may arise from a solitary malignant cell, and therefore curative treatment usually requires eradication of all tumour cells.

Applying these principles to the clinical situation in

patients with malignant melanoma it is likely that the patients who should benefit from adjuvant therapy are those with occult micro-metastases at the time of primary treatment. We have seen that the patients at highest risk of bearing occult systemic micro-metastases are those with thick primary lesions [168,221,295], and patients with involved lymph nodes [124,226].

I.4.7. Adjuvant therapy: Clinical results

Based on the results of treatment of advanced disease, dacarbazine has usually been part of the adjuvant chemotherapy regimes. In spite of the evidence from animal models and encouraging results in other cancers, the results of adjuvant chemotherapy for malignant melanoma have been poor. In two representative prospective randomised studies[296,297], no significant benefit was demonstrated for surgery plus adjuvant chemotherapy compared with surgery alone. In the former study the group receiving dacarbazine seemed to have a lower survival, and in the latter (W.H.O.) study 18% of patients had to have chemotherapy stopped or the dose reduced because of toxic side effects. Other drugs and combinations have been studied but they are similarly disappointing[285].

In a prospective randomised study of Stage II (original three-stage system) patients, lymphadenectomy alone was compared with surgery plus adjuvant radiotherapy to the node-bed[298]. There was no significant difference between the groups in terms of disease control or survival. This was to be expected because the risk of recurrence in the node-bed after surgery alone is low. There have been no

other significant prospective trials of adjuvant radiotherapy.

The results of clinical trials of surgery plus adjuvant immunotherapy or chemo-immunotherapy have shown no convincing benefit from these forms of adjuvant treatment[297,285].

I.4.8. New drugs, alternative strategies

The rationale for amino-acid derivatives of the nitrogen mustards, like melphalan, was to exploit the unique biochemical pathway for melanin synthesis which usually persists in malignant melanocytes[299]. Tauromustine (TCNU) is an interesting new agent comprising a nitrosourea linked to the amino-acid taurine (cf. melphalan, III.1.). This drug has a remarkably short half-life, and can be given orally to out-patients. In a recent study of tauromustine therapy in advanced melanoma the overall response rate was 16%, no better than other nitrosoureas[300]. In a comparative trial oral tauromustine yielded results comparable to intravenous mitozolamide (overall response rates 9 and 12%)[301]. These new drugs seem to be no more effective than dacarbazine.

One approach to specific targeted melanoma therapy involves the use of agents like 4-hydroxyanisole which is a "false precursor" converted by tyrosinase to toxic metabolites[302]. Alternatively, the selective accumulation of radio-labelled thiouracil by melanoma cells, and incorporation of the agent into the melanin synthetic pathway suggest a possible role for thiouracil in targeted radiotherapy[303]. "Neutron capture therapy" is an exciting

concept which depends on the fact that a non-radioactive isotope of boron (^{10}B) absorbs thermal neutrons with the emission of short range α-particles. ^{10}B conjugates may be selectively accumulated by melanoma, and subsequent external irradiation may produce selective kill of melanoma cells without injury to surrounding tissues[304].

Occasionally there is a need for a simple, atraumatic method of dealing with a superficial tumour. In an elderly or unfit patient, for example, extensive or risky surgical procedures may be inappropriate and topical therapy is an option. Intra-lesional thio-tepa[305], cryotherapy[203] or topical azelaic acid[306] are alternative methods which have been reported to be simple and effective.

I.5. ISOLATED LIMB PERFUSION: REVIEW

I.5.1. Rationale

In general, modern cancer chemotherapy involves the use of the maximum dose of drug to achieve the greatest anticancer effect, without inducing intolerable toxicity. Such toxicity limits the dose which can be administered and thereby limits the clinical usefulness of the drug. The relationship between beneficial and detrimental effects of any treatment can be described as the therapeutic ratio.

In malignant melanoma few chemotherapeutic agents are effective, and even for effective treatments the therapeutic ratio is low. The melanoma cell seems to possess inherent resistance to drugs which are active against other malignancies. This resistance (cf. resistance to radiation, I.4.4.) may be partly due to the melanoma cell's ability to accumulate sub-lethal damage and to repair potentially lethal damage[244,307]. It has also been calculated that in melanoma a relatively small proportion of the malignant cells are actively dividing[308] (i.e. low growth fraction), favouring the repair mechanisms which are most effective in the non-dividing (G_0) cells.

A potentially effective drug may also fail because of restricted access of drug to the site of action, for various reasons[309]. Increasing the local concentration of drug in the vicinity of tumour should enhance the anticancer effect, by increasing tumour uptake (unless this is saturable), and overwhelming the capacity of the cell's repair mechanisms. It is clear that simply

increasing the systemic dose to achieve this will eventually result in dose-limiting systemic toxicity. In a Phase I clinical study of an anticancer agent the aim is to define the maximum safe dose by planned incremental dose escalation, until dose-limiting toxicity is observed. The dose-limiting toxicity for systemically administered melphalan in the treatment of malignant melanoma is myelosuppression, which may be partly circumvented by autologous bone marrow transplant[261,310].

When malignant melanoma is regionally confined, isolated limb perfusion is a rational strategy whereby a high dose of melphalan can be confined to the treated region, sparing the patient from systemic toxicity, and thus maximising the therapeutic ratio. Using an extra-corporeal pump oxygenator the circulation to a tumour bearing region is functionally isolated from the rest of the body and high concentrations of cytotoxic agent can be generated within and restricted to the limb (Fig.3).

Although high doses and concentrations are fundamental to the rationale, no Phase I studies have been previously performed in the isolated limb perfusion setting, and dose schedules have evolved empirically.

During isolated limb perfusion the physio-pharmacological conditions in the treated limb are uniquely well controlled, compared with systemic administration. It is clear that, in this setting, hepatic and renal handling of administered drugs is largely irrelevant, but the volume and constitution of perfusate, and the temperature, flows, pressures and pH in the circuit may influence the bio-availability and efficacy of the cytotoxic drug.

I.5.2. History

In 1946 Gilman and Philips[311] showed that nitrogen mustards cause regression of tumours in experimental animals. Clinical trials indicated that toxic effects on bone marrow limited the dose of nitrogen mustard which could be given to patients[312,313]. At doses which could be given safely the therapeutic effect on tumours was transient.

In 1950 Klopp[258] and Bierman[314], working independently, showed that by injecting nitrogen mustard into the artery supplying a tumour a greater effect on the tumour was seen with less marrow suppression than occurred after intravenous injection of the same dose. Klopp and colleagues[258] found that these beneficial effects were further enhanced by a proximal tourniquet which blocked venous return but permitted arterial inflow.

When phenylalanine mustard (melphalan) was developed it was hoped that the phenylalanine moiety would become involved in the melanin synthetic pathway in melanoma cells (III.1.1.), and that the drug might therefore have some selective effect on these cells[299]. In 1956 Luck[315] showed that melphalan was the most effective agent available to inhibit the growth of the Harding-Passey mouse melanoma in vivo. In spite of this early promise melphalan is no longer the agent of first choice for systemic chemotherapy of malignant melanoma (I.4.2.) but it is the drug used most widely for isolated limb perfusion.

Klopp and colleagues[258] had considered the use of an extra-corporeal circuit with a pump/oxygenator (such as

that used in open heart surgery) to maintain viability of the perfused region during prolonged administration of chemotherapy, but the technique was not applied until 1957 in the report of Ryan and colleagues[316]. They had successfully isolated and perfused the liver, mid-gut and limbs in experimental dogs. Ischaemic changes were seen after ninety minutes of isolation/perfusion, probably due to low flow rates and relatively low perfusion pressures.

In 1958 Creech, Krementz, Ryan and Winblad of Tulane University in New Orleans reported the first clinical case treated by isolated limb perfusion with melphalan[317]. The patient was a 76 year old man with satellitosis and in transit disease of the leg, two years after a wide excision and superficial groin dissection for a primary malignant melanoma of the skin at the ankle. At a time when systemic chemotherapy produced partial responses in less than 10% of patients with malignant melanoma[318] this man had a complete and permanent remission following isolated limb perfusion.

By 1961 the originators of the technique had treated 242 patients with regional perfusion of various sites in the body affected by different tumours[319]. Around the world other clinicians began to apply regional perfusion in the treatment of cancer.

Early U.K. experience was reported by Garai and colleagues[320] of the Royal Marsden, Westbury of the Westminster Hospital[321], and Irvine and Luck of St. Mary's Hospital[322]. In common with others[323,319], Westbury found that isolation of a limb was more satisfactory than visceral isolation, and that malignant melanoma responded

more convincingly than other tumours. Novel features of Westbury's technique at that time included "high flow rates" (150-200ml/min), high oxygen tension and hyperthermia of the perfusate. It was reasoned that with a "high" flow rate there would be "a more efficient distribution to the periphery". Pure oxygen fed the oxygenator because the "radiomimetic" drug melphalan might be enhanced by this, in the way that therapeutic radiation had been enhanced in the studies of Gray[324]. The temperature of the perfusate was raised to 41°C to increase the "speed of reaction" of melphalan and increase the "utilisation of the drug and consequently decrease the spill-over when isolation is incomplete". I will consider the importance of flow rates, oxygenation and temperatures in more detail in the subsequent chapters.

In 1961, Rochlin and colleagues[325] had suggested that at temperatures greater than 37°C the alkylating agent triethylene thiophosphoramide (thiotepa) was more rapidly bound by tissues, although their results hinged on an indirect assay and may have been explained by increased breakdown of the drug at higher temperatures.

Cavaliere and colleagues later reported that hyperthermic perfusion alone (without any cytotoxic drug) may selectively kill cancer cells[326]. They showed that in vitro Novikoff hepatoma cells and Ehrlich ascites cancer cells (both established tumour cell lines) consumed much less oxygen at 42°C than at 38°C. There was little difference in respiration comparing normal hepatocytes and regenerating liver at these temperatures. In the clinical part of their study 22 patients had 25 regional perfusions

at temperatures from 41.5-43.5°C for several hours without cytotoxic drugs. There were six deaths and three amputations due to complications. Three tumours failed to respond and four patients could not be evaluated. However, ten tumours "disappeared" and histological evidence of "complete massive necrosis" was seen in tissue from eight. The group which seemed most responsive comprised seven melanoma patients, of whom four were alive with a functional limb at 28 months after heated perfusion. These are indeed interesting observations, but the results do not suggest that hyperthermic perfusion is an appropriate palliative treatment, because of the unacceptable peri-operative mortality and morbidity.

Following this work, Stehlin adapted his perfusion method to administer heat with melphalan in an effort to exploit potential "synergy"[327]. His report also mentions an increase in duration of perfusion from 45 to 120 minutes, as well as the addition of hyperthermia to the regime. There was a conspicuous improvement in tumour response as compared with his previous standard regime[328] (Table 14), and this work is often cited as evidence that hyperthermic isolated limb perfusion is more effective than normothermic perfusion. However serious complications were also more common after hyperthermic perfusion and by the time of their 1975 publication, Stehlin's team were perfusing with a lower dose of melphalan and at lower temperatures in an effort to reduce the incidence and severity of toxic effects[329]. The duration and temperature were by now varied "according to the extent of the melanoma within the limb and the condition of the

peripheral vessels". It is probable that most of the changes ascribed by Stehlin to hyperthermia could have been due to the increased duration of perfusion. Similar improved results may have been obtained by simply increasing the dose of melphalan. Nonetheless hyperthermia has become an accepted part of isolated perfusion for melanoma in many centres around the world[211,212,330,331], including our own. We will consider the role of hyperthermia in Chapters III and IV of this thesis.

I.5.3. Results: Introduction

In reviewing the world literature in 1979, Au and Goldman[212] indicated how the true value of isolated limb perfusion was unclear, despite many favourable reports. They observed how the interpretation of existing studies was compromised by various limitations, including:

a) the tendency to include patients with early and advanced disease in the same group,

b) failure to define clearly the exact nature and extent of associated surgery and whether other treatments were used,

c) lack of information on established important prognostic factors e.g. Breslow thickness, and other factors of possible prognostic significance e.g. sex, age, arm or leg,

d) comparison of perfusion results with selected surgical series, which are subject to similar criticisms and which report a wide spectrum of results,

e) variations in perfusion technique.

The need for proper trials to assess the value of isolated limb perfusion has long been recognised[332] and it is perhaps surprising that the technique has been applied so widely and for so long without it's value having been firmly established.

Studies which have made some attempt to address the criticisms of Au and Goldman are discussed below, because these studies are our best guides, in the absence of adequate randomised prospective clinical trials.

I.5.4. Results: Therapeutic perfusion

There have been many studies , since the original case report, which attest the value of isolated limb perfusion in the management of advanced locoregional melanoma[211,329,333,334,335,336,337,338,339,340,341,342].

In the following discussion of results it is important to realise that, as in so many aspects of melanoma management, there is a lack of appropriate controlled prospective clinical trial data on which to base the arguments.

I will describe the reported results of isolated limb perfusion for loco-regional advanced disease in terms of response rates, incidence of subsequent recurrence and survival.

For any cancer treatment it is appropriate to consider first whether the treatment produces a measurable effect on obvious tumours. Two categories of tumour response are now generally recognised, as recommended by the U.I.C.C.[343]:

Complete response = "disappearance of all known disease"

Partial response = "≥50% decrease in measurable lesions and objective improvement in evaluable but non-measurable lesions. No new lesions. It is not necessary for every lesion to have regressed to qualify for partial response, but no lesion should have progressed"

In the management of cutaneous malignant melanoma recurrences may be excised before referral for limb perfusion, or at the time of perfusion. Thus there are relatively few studies of <u>response rates</u> following isolated limb perfusion. A summary of studies which do include data concerning response rates (using the U.I.C.C. criteria) is presented in Table 13. Response rates resulting from modern regimes of isolated limb perfusion are very encouraging, with overall response rates consistently greater than 70% , and superior to any other available treatment[339,340.341,344]. Disappointingly the duration of responses is rarely indicated though it varies between months to many years in the studies in Table 13.
A major criticism of response rate analyses is that they are founded on a series of subjective assessments, often made by the responsible physician or surgeon, and open to conscious and unconscious bias. In addition, unless the study is truly prospective, one cannot assume that uniform criteria of response were rigorously applied. These objections are not unique to studies of isolated limb perfusion, and apply to studies of other treatments. The use of response rates has been critically appraised by Watson[345].

If response rate data is not available because of prior surgical clearance of recurrent disease, an alternative assessment of disease control can be made by determining the incidence of further limb recurrence after isolated limb perfusion. Disappointingly this information is seldom clearly reported in studies of isolated limb perfusion and, for comparison, there are few studies which describe the prognosis for patients with loco-regional recurrence managed by excisional surgery [122].

Rosin and Westbury described a series of 116 patients who had surgical clearance of recurrent melanoma in addition to isolated limb perfusion[211], 58 of the patients (50%) remained free of further recurrences in the limb until death or follow-up (up to 15 years). Most of the further recurrences occurred in the early years after isolated limb perfusion. In another study, with minimum two-year follow-up, 22 of 39 (56%) patients treated by surgical excision of recurrent melanoma plus limb perfusion remained free of further loco-regional recurrences[346]. It is a consistent finding that if further recurrence develops after therapeutic isolated limb perfusion it tends to occur in the first year or so[340,347].

In a review of their experience of isolated limb perfusion for loco-regional recurrence Schraffordt Koops and colleagues[348] achieved 5-year local recurrence-free survival of 61% overall and 5-year disease-free survival of 35% overall (using actuarial methods). Clearly it would be valuable to consider this sort of analysis in larger

groups with better defined long-term follow-up, and including sub-group analyses.

There is preliminary evidence that the time interval to next recurrence may be prolonged by isolated limb perfusion[349], but it is doubtful whether this alone would be an appropriate argument for such an elaborate treatment.

Ghussen, from Cologne in West Germany, has reported the only prospective randomised trial of isolated limb perfusion compared with excisional surgery[350,351] there were 37 patients with stage II disease (M.D. Anderson) and 33 with stage III disease. In both categories disease-free intervals were significantly prolonged by isolated limb perfusion, but the trial was prematurely ended once this difference was evident and the sub-groups are too small for survival analyses.

There is considerable variability in reported survival rates following isolated limb perfusion for patients with loco-regional advanced and recurrent melanoma (Table 14). This is not surprising because this category is heterogeneous, including the patient with a single small indolent recurrence near the wound as well as the patient with rapidly advancing extensive in transit metastases and ulcerating lymph nodes. In Table 14 the M.D. Anderson staging system is used because it describes patterns of loco-regional disease.

From Table 14, it is apparent that patients with different patterns of loco-regional recurrent or advanced melanoma have often been studied as one group and hence reported

five-year survival rates after isolated limb perfusion vary from 25-74%. The influence of other factors, e.g. age, sex and race of patients; number, timing and type of recurrences; treatment before perfusion and variations in perfusion technique, has not yet been determined. Hence the groups of perfusion patients reported in these various studies are not directly comparable, and there is a lack of appropriate surgical controls.

As we have seen (I.2.9., I.3.3.), patients with loco-regional advanced or recurrent melanoma are at high risk of developing early systemic metastases[120,121,186,202,203], and therefore survival after conventional surgery is often limited. Survival rates have been reported as 50% at three years after first recurrence[202], 33% at eleven years from primary treatment[120], and 15% at ten years[121]. In the series of patients with regional non-nodal metastases reported by Cascinelli and colleagues 41% of their 291 cases had involved nodes at some time, 22% had distant metastases at diagnosis of regional non-nodal disease and 27% developed distant metastases within six months from diagnosis[121]. The long term survival is greater in the study by Griffiths and Briggs[120] because they record survival from time of primary treatment, and because they did not include patients with overt nodal and systemic metastases at the start.

The only study with comparable long term follow-up after isolated limb perfusion is that of Krementz[318], which indicates ten-year survival rates of 59% for patients in M.D. Anderson stage II, 23% in IIIA and 28% in IIIAB

(Table 14).

Unfortunately, there is insufficient evidence to decide whether isolated limb perfusion improves survival of patients with loco-regional advanced and recurrent disease or not. Apparent improvements in survival[318,347,352] could arise because of patient selection, or because of spurious comparisons with disease in less favourable anatomical sites.

In an attempt to identify important prognostic determinants of the efficacy of isolated limb perfusion, a retrospective study of patients with loco-regional recurrence of melanoma treated by therapeutic perfusion was made using a multi-factorial analysis[353]. In this study the number of involved lymph nodes was an important prognostic factor, and subcutaneous lesions seemed to confer a poorer prognosis. Vaglini[341] has indicated that patients with a solitary loco-regional recurrence may respond better to isolated limb perfusion than those with multiple lesions, but this has yet to be confirmed.

The study of variables related to different perfusion techniques is in its infancy. It is not proven that hyperthermic isolated limb perfusion with melphalan is more effective than normothermic perfusion but the weight of published opinion is broadly in support of this[318,329,340,351,354]. Kroon and colleagues, however, have shown that normothermic (tissues 37-38°C) perfusion can still produce an overall response rate of over 80%[344]. This debate is unresolved because of a lack of appropriately designed clinical trials.

I.5.5. Results: Adjuvant perfusion

In view of the encouraging results obtained by isolated limb perfusion for advanced disease, it seems logical to consider isolated limb perfusion as a possible adjuvant to surgical excision.

Clinical series have often included such adjuvant perfusions[318,329,336,337,350,351,355,356,357,358,359,360]. Although comparisons with historical controls suggest improved loco-regional control and prolonged survival from adjuvant perfusion as compared with surgery alone, there is no definitive proof.

Since the identification of features of the primary melanoma (especially Breslow thickness) which have been shown to be important prognostic determinants, it has been possible to identify patients at particularly high risk of recurrence, metastasis and death. No other form of therapy has consistently obtained response rates which are comparable with those achieved by isolated limb perfusion (see I.3, I.4; cf. I.5.4.), and no other form of therapy has proven to be of value as an adjuvant. Therefore, in recent years, there has been great interest in finally establishing whether or not isolated limb perfusion may be an effective adjuvant to excisional surgery for patients with high-risk primary malignant melanoma.

A summary of the important retrospective series which give results for isolated limb perfusion as an adjuvant to surgery for primary malignant melanoma is given in Table 15. This is not an exhaustive listing of such studies, but a selection of better designed studies, which helps make important points about the evaluation of isolated limb

perfusion in the adjuvant setting.

The studies of Janoff[361], Koops[362] and Krementz[318] are reports of clinical series without internal controls, relying on the results of surgery alone obtained in other centres for comparison. As in similar previous studies[329,336,363] there appears to be a survival advantage for the perfused patients.

Sugarbaker and McBride[357] used 71 historical controls from their own hospital, demonstrating an apparent long-term survival advantage of 26% from adjuvant isolated limb perfusion. Concurrent but non-randomised controls were used in the small study by Rege and coworkers[364] which detected a statistically significant survival advantage from isolated limb perfusion for clinical but not pathological stage I disease. The study by Tonak and colleagues[365] included a comparison with surgical controls, but no account was taken of known prognostic factors and the follow up was only three years.

Adjuvant isolated limb perfusion was first used long before Breslow thickness was recognised as a major prognostic determinant, and hence this important factor could not be accounted for prospectively. Hence in major series, which include patients from the pre-Breslow era, significant numbers of patients with (what are now known to be) low-risk, thin malignant melanomas were treated by adjuvant isolated limb perfusion. In the report by Krementz[318], retrospective microstaging of 161 of the 381 patients revealed that 37% had primary lesions less than 1.5mm Breslow thickness, and in other studies 44%[364] and 50%[361] of the patients were in this low-risk category. The

inclusion of significant numbers of low-risk patients may tend to inflate the apparent benefit of adjuvant isolated limb perfusion. Today the excellent prognosis of patients with such thin malignant melanomas would preclude their inclusion in trials of adjuvant therapy. It is interesting that when McBride and colleagues[366] re-evaluated the M.D. Anderson experience of stage I malignant melanoma, including the series reported in 1976[357], they found a significant survival advantage for surgery plus limb perfusion (compared with surgery alone = wide local excision +/- elective node dissection) only in patients with primaries of Clark level III and greater than 1mm Breslow thickness.

Perhaps the most interesting retrospective studies of adjuvant perfusion have been reported by Martijn, Schraffordt Koops and colleagues. In the first study[331] a retrospective comparison was made between patients with high risk primary lesions treated in Groningen, Holland (wide local excision plus isolated limb perfusion) and similar patients treated in Sydney, Australia (wide local excision alone without elective node dissection). There was a statistically significant benefit to the perfused group as assessed by rate of loco-regional recurrence, proportion disease free at ten years and ten-year survival. It is somewhat perplexing that a subsequent comparison of the same Dutch patients with surgical controls from the Westphalia region of Germany and Holland has failed to demonstrate benefit from isolated limb perfusion[367]. The reasons for these conflicting results have not been elucidated but may include differences of

skin type, sun exposure and immune function[368].

The need for a prospective study of adjuvant isolated limb perfusion has long been recognised[322]. Ghussen's prospective study of limb perfusion[350,351] included patients with stage I melanoma (M.D. Anderson) as well as patients with more advanced disease. Although this was a randomised study, it is important to look critically at this work, because it is commonly cited as evidence for adjuvant perfusion on the basis of the longer disease-free interval demonstrated. Patients with a primary lesion >1.5mm Breslow thickness in the distal two thirds of a limb were randomised between surgery alone (including "elective" node dissection) and surgery plus isolated limb perfusion. There was no stratification for thickness of primary or clinical stage. Eight patients were excluded after surgery because "final examination of the ... specimens revealed a tumour thickness of less than 1.5mm or invasion ... less than Clark's level IV". There were only 37 patients in stage I (M.D. Anderson). The trial was prematurely terminated after an interim analysis[350] revealed a significant difference in disease-free interval between the 54 patients treated by surgery alone (all stages) and the 53 treated by surgery plus isolated limb perfusion. This difference was also observed in the stage I sub-group when the average follow-up is extended to five years[351]. There are however only 18 controls and 19 patients in the perfusion group, and there is no information concerning distribution of prognostic factors within the sub-groups. This may be critical because the incidence of "recurrence" in the stage I control group

seems rather high (7/18 = 39%) and could be due to a preponderance of thick, poor prognosis primary lesions in this group compared with the perfused patients.

An international multicentre prospective randomised controlled trial of wide local excision +/- elective lymph node dissection compared with the same treatment plus isolated limb perfusion has been activated by the W.H.O and E.O.R.T.C. (European Organisation for Research and Treatment of Cancer). 600 patients must be randomised for stratification and subgroup analysis, and it is hoped that the large numbers accrued, and careful trial design (e.g. stratification by Breslow thickness, quality control of perfusion technique) will help to provide definitive results. Patients are now being entered from centres all over Europe, from America and Australia.

In the U.K. a trial of adjuvant isolated limb perfusion began under the M.R.C., but support was withdrawn because of poor patient accrual. It is hoped that this study will continue under the auspices of the British Association of Surgical Oncology (B.A.S.O.) and should have the advantages of uniform technique and single national population (cf. W.H.O./E.O.R.T.C. study).

Clearly patients with appropriate high-risk primary malignant melanomas of the limb should be offered entry into a prospective clinical trial of adjuvant isolated limb perfusion where possible.

I.5.6. Results of perfusion: Summary

On the basis of objective response rates and loco-regional disease control, isolated limb perfusion is the treatment

of choice for patients with advanced loco-regional malignant melanoma. Included in this category are patients with a single nodule near the primary or its excision scar, through to patients with intractable loco-regional symptoms (e.g. pain, fungating tumour) who may even have manifest systemic metastases. It is important to appreciate that the case for isolated limb perfusion in the management of loco-regional disease does not depend alone on whether it improves survival. We have seen that isolated limb perfusion produces consistently high response rates which are unattainable with other treatments. It seems that isolated limb perfusion can achieve control of recurrent or advanced disease in the limb, without recourse to other treatments which can be associated with serious morbidity or mutilation. The patient often derives encouragement from disappearance or regression of symptomatic or gross disease.

Adjuvant isolated limb perfusion is not yet proven to be of sufficient benefit to recommend routine use. It may be of value in the management of high-risk primary malignant melanoma, and appropriate patients should be considered for entry into one of the current prospective trials.

I.6. ALTERNATIVES TO ISOLATED LIMB PERFUSION

I.6.1. Intra-arterial chemotherapy

Intra-arterial administration of cytotoxic is not a new idea[258,314]. Apart from isolated regional perfusion, other interesting related regional infusion techniques were developed. In 1959 Sullivan and coworkers described the continuous intra-arterial infusion of methotrexate, combined with the systemic administration of citrovorum[369], in an attempt to reduce systemic toxicity. Intra-arterial therapy may be by bolus injection or by continuous infusion[321]. For this kind of regional therapy intra-arterial catheters may be placed surgically or by per-cutaneous radiological techniques[330].

The rationale for arterial infusion chemotherapy is similar to that for isolated limb perfusion, but the treated region is not isolated from the systemic circulation and the total administered dose may have access to the rest of the body. With continuous arterial infusion chemotherapy, which may last longer than twenty-four hours, it would seem likely that tumour cells are exposed to chemotherapeutic drugs for a greater proportion of the cell cycle than with isolated limb perfusion[330]. It is unlikely that the relatively sustained high concentrations of cytotoxic drug achieved with isolated limb perfusion are matched by arterial infusion techniques. Consequently, in theory, the therapeutic ratio with arterial infusion may be less than with isolated regional perfusion, and intra-arterial infusion chemotherapy should be reserved for anatomical sites where isolated regional perfusion is not technically possible

(e.g. head & neck) or where general anaesthesia is contra-indicated[330].

Although many drugs have been administered by intra-arterial infusion for malignant melanoma[330,370] most experience has been with dacarbazine (DTIC)[371,372,330]. In at least one clinical study systemic toxicity was greater than would be expected with isolated limb perfusion[371].

Karakousis has developed a novel method of intra-arterial administration which he calls "tourniquet infusion chemotherapy"[373]. An intra-arterial cannula is placed per-cutaneously and passed so that the tip lies distal to a pneumatic tourniquet round the proximal limb. The tourniquet is inflated to greater than systolic pressure for five minutes, while adriamycin is infused; then the tourniquet is deflated to the level of mean arterial pressure, and finally the tourniquet is completely deflated. Apparently this can be accomplished without general anaesthesia, and an initial small clinical series yielded encouraging results[373]. In a later publication by the same authors[374] the technique was compared with hyperthermic regional perfusion in a dog model. In this study the levels of cytotoxic drug were rather low in the perfused limbs and disappointingly high in the major organs, and the levels were at least as good with tourniquet infusion. This is simply explained by the omission of a proximal tourniquet in the perfusion animals, which were therefore not treated by <u>isolated</u> limb perfusion.

The development of interventional radiology has led to burgeoning interest in percutaneous vascular techniques.

This new technology has been applied in the field of intra-arterial chemotherapy. By inflating an intra-arterial balloon catheter to stop arterial inflow, cytotoxic drug can be injected intra-arterially through the catheter distal to the balloon (cf. first phase of tourniquet infusion). In a dog model this technique (intra-arterial infusion-occlusion chemotherapy or intra-arterial infusion with stop-flow) has been shown to result in higher tissue levels of 5-fluorouracil than either intravenous or intra-arterial infusion[375].

A per-cutaneous technique for isolated limb perfusion, using balloon catheters has been reported in a dog model and compared to the intra-arterial infusion-occlusion method[376]. Per-cutaneous isolated perfusion resulted in higher levels of cytotoxic drug in the plasma and tissues of the perfused limbs, and lower levels in the systemic circulation compared with the levels achieved by infusion-occlusion.

In an elegant study, using experimental rabbits, Kar and colleagues have directly compared the pharmacokinetics of 5-fluorouracil after (1) intravenous administration, (2) intra-arterial injection, (3) intra-arterial administration with stop-flow, (4) intra-arterial administration with out-flow occlusion and (5) isolated limb perfusion[377]. Systemic exposure to cytotoxic drug was greatly reduced by isolated limb perfusion, compared with all other methods. Tissue levels in tumour and normal tissues were significantly greater after both isolated limb perfusion and intra-arterial administration with venous outflow occlusion, compared with those achieved by

other methods.

On the basis of this evidence it can be seen that in the management of regionally confined malignancy there are clear theoretical advantages to isolated limb perfusion over alternative methods. In clinical practice isolated limb perfusion is still a major surgical undertaking and development of the experimental per-cutaneous techniques offers the prospect that in the future isolated regional perfusion may be accomplished without general anaesthesia.

I.6.2. Isolated limb perfusion: other drugs

Isolated limb perfusion for malignant melanoma has been performed with a variety of drugs apart from melphalan. These drugs include nitrogen mustard[318,321,378], dacarbazine (DTIC)[379,380,381,382,383,384], cis-platinum[381,385], and adriamycin[386].

Various combinations of drugs have also been used in isolated limb perfusion including melphalan + thiotepa[318,387], melphalan + thiotepa + nitrogen mustard[318], melphalan + thiotepa + actinomycin-D[335], melphalan + actinomycin-D[342,346], cis-platinum + actinomycin-D[381], vindesine + dacarbazine + cis-platinum[388] and cis-platinum + etoposide (VP-16)[389].

No single agent or combination has been demonstrated to be superior to melphalan alone in isolated limb perfusion. Many years of largely empirical development have led to the current regimes of melphalan administration in isolated limb perfusion. It is unlikely that optimal drug schedules have been applied in the few studies listed

above, which mostly deal with early experience of the alternative agents in small clinical series.

There is still much to learn about the use of melphalan in clinical isolated limb perfusion and appropriate models. Melphalan is currently the drug of first choice. In my opinion it is inappropriate to begin using another drug, which has not shown better objective response rates than melphalan, unless such a drug is shown to be less toxic than melphalan in clinical trials, or unless the drug is being studied in such a trial.

CHAPTER II

CLINICAL STUDIES OF ISOLATED LIMB PERFUSION

II.1. INTRODUCTION

II.1.1. The Scottish Melanoma Group (SMG)

In 1978 the Scottish Melanoma Group was formed[72] in response to an apparently rising incidence of malignant melanoma. At that time there was no good population-based epidemiological database for malignant melanoma. The major American and Australian series came from centres with a recognised interest in the disease, and the cases seen and reported by them were not necessarily representative of the disease in the whole population.

The original aims of the Scottish Melanoma Group[72] were:

1) to ensure registration of all patients with cutaneous malignant melanoma presenting in Scotland,

2) to promote the use of modern pathological techniques and to standardise terminology used in reporting malignant melanoma,

3) to follow all patients from registration to death,

4) to define accurately the type of surgery being performed on patients with primary cutaneous malignant melanoma.

These aims were selected because accurate information on Scottish melanoma patients was lacking, and this is an important prerequisite for the design of valid clinical trials.

The initial objectives of the Scottish Melanoma Group have been achieved, and the sound database which has been established[73] provides the basis for current efforts to

reduce the incidence of cutaneous malignant melanoma, and to improve the prognosis of patients with the disease.

II.1.2. The SMG and isolated limb perfusion

Because of favourable reports concerning the use of isolated limb perfusion in the management of malignant melanoma (see I.5.3-6) Mr. Alan J. McKay, Consultant Vascular Surgeon at Gartnavel General Hospital, was asked to examine the available evidence and determine whether the facility should be available in Scotland, and if so, how it could be made feasible.

After studying the techniques of Krementz and Schraffordt Koops at first hand, and having considered the evidence discussed in the previous chapter of this thesis, it was decided by Mr. McKay that isolated limb perfusion was a technique which could be performed safely in Glasgow, and which should be investigated.

Once convinced that the technique was safe, and following initial encouraging results, we made it known through the Scottish Melanoma Group that isolated limb perfusion was available to patients in Scotland.

II.2. AIMS OF CLINICAL STUDIES

This chapter reviews our experience with isolated limb perfusion for cutaneous malignant melanoma. The introduction and preliminary validation of this complex technique is described.

Many of our patients were referred with advanced disease when conventional therapy had reached its limits, and when the alternatives proposed were systemic chemotherapy or major amputation. Our major initial concerns have been to establish the safety of isolated limb perfusion with melphalan, and to examine whether the rationale for isolated limb perfusion is sound (see also Chapter III).

II.3. PATIENTS AND METHODS

II.3.1. Patients

Our provisional criteria for suitable candidates for isolated limb perfusion were:

1) Patients with recurrent or advanced melanoma confined to a single limb (or, rarely, causing severe local symptoms in the presence of distant metastases) - therapeutic perfusion.

2) Patients with primary melanoma ≥1.5mm Breslow thickness (high risk primary lesion) located on a limb. Such patients were suitable candidates for randomisation in the M.R.C. trial comparing surgery alone with surgery plus adjuvant isolated limb perfusion.

The contra-indications to isolated limb perfusion were severe cardio-respiratory disease precluding general anaesthesia, severe peripheral vascular disease, and disseminated melanoma.

Between August 1984 and May 1988 61 clinical isolated limb perfusion procedures have been performed in the treatment of cutaneous malignant melanoma by our team. This figure includes the eight occasions on which we have repeated the procedure, but does not include four other occasions when isolated limb perfusion was abandoned for clinical or technical reasons (see II.4.5.).

The technique has also been used in four patients to treat soft-tissue sarcomas, but these cases are excluded from the analysis.

12 men and 41 women were treated, and the lower limb (51)

was treated much more commonly than the arm (2).

II.3.2 Therapeutic isolated limb perfusion

Three patients referred for consideration of therapeutic isolated limb perfusion were excluded either because of serious cardio-respiratory disease, or very advanced malignant melanoma with no significant symptoms due to their limb tumours.

50 therapeutic perfusions for advanced or recurrent disease have been attempted in 42 patients. All visible disease was often excised by the referring surgeon, before referral for isolated limb perfusion.

Excluding repeat perfusions, the clinical details of candidates submitted to surgery for therapeutic perfusion are summarised in Table 16.

II.3.3. Adjuvant isolated limb perfusion

15 patients have been treated by adjuvant isolated limb perfusion for high-risk primary malignant melanoma. Their clinical details are given in Table 17.

Three of the patients were treated by isolated limb perfusion in the context of the Medical Research Council trial.

II.3.4. Method of isolated limb perfusion

Routine pre-operative assessment of each candidate for isolated limb perfusion was as for any major vascular procedure. A full clinical history was taken and clinical examination was performed. In all cases full blood count, serum biochemistry and chest X-ray were obtained.

Additional investigations were performed as indicated by clinical condition e.g. thoraco-abdominal computerised tomography (CT-scan) was performed in most patients who were candidates for therapeutic perfusion.

The nature of proposed treatment was explained to the patients pre-operatively, and consent for surgery was obtained.

Local Ethical Committee approval was obtained for all clinical experiments performed.

Separate records were prospectively maintained concerning the patients' clinical status, peri-operative data and post-operative follow-up.

Our surgical method of isolated limb perfusion is a modification of the techniques described by Krementz[390] and Schraffordt Koops[362,391]. Since the great majority of patients have melanoma of the leg, I will first describe isolated perfusion of the lower limb. Fig. 3 shows the isolated limb perfusion circuit in diagrammatic form.

With induction of general anaesthesia 1 gram of ceftizoxime (Wellcome) is given intravenously, as prophylaxis against wound infection. A radial arterial line is inserted for per-operative monitoring and for repeated systemic blood sampling. In our early experience a central venous line was also inserted for haemodynamic monitoring peri-operatively, but this is no longer routine.

The patient is placed supine on the operating table and rolled onto the side opposite the limb to be treated, for washing of the perineum and ipsilateral buttock (which come into contact with the tourniquet as it is applied).

Once the patient is returned to the supine position, thermistor skin probes (Yellow Springs) are applied to the skin of the calf and thigh of the affected limb, and the temperatures are continuously displayed per-operatively on a monitor screen (Siemens Sirecust). In eleven patients, early in the series, the temperatures were recorded simultaneously in the calf muscles using needle thermocouples (Ellab), and on the calf skin using thermistor probes. A stockingette (Tubigrip) on the leg, and gamgee round the foot protect the skin from direct contact with a heated water blanket (Hawksley Ripple-Heat system with custom blanket) which is then wrapped around the limb (Fig. 4), and enclosed in sterile drapes. The abdomen and inguinal region are washed with iodine antiseptic and draping is completed.

Under routine balanced general anaesthesia the external iliac vessels are exposed retro-peritoneally via an oblique incision in the iliac fossa. All minor branches of the external iliac artery and all tributaries of the external iliac vein, from the iliac bifurcation to the inguinal ligament, are ligated and divided. The external iliac lymph nodes are routinely excised during this dissection.

Heparin (150 i.u./kg) is given intravenously, prior to control of the vessels for cannulation. Polyvinyl chloride (P.V.C.) cannulae (Bard and Cimid) are placed through a longitudinal venotomy and arteriotomy and the cannulae are advanced so that the tips lie in the femoral triangle inferior to the inguinal ligament, and distal to the lower edge of the tourniquet. The cannulae are secured in place

by firmly applied cotton snares (Fig. 5). A Steinmann pin is driven into the iliac crest and is used to anchor a red rubber Esmarch bandage which tightly encircles the root of the limb proximal to the tips of the cannula (Fig.6), allowing perfusion high up into the femoral triangle (Fig.7).

The perfusion apparatus consists of a simple roller pump (American Optical) in series with a disposable hybrid oxygenator (Bard) which has an integral heat exchanger (Fig. 3). The pump/oxygenator (primed with 500ml Ringer's lactate solution and 700ml matched packed red cells plus 3000i.u. heparin, pre-warmed by the integral heat exchanger) circuit is then opened to the arterial and venous cannulae, and the limb is effectively "on by-pass", supplied solely by the isolated circuit. When the isolated circuit is stable (after 3-5min) 5ml of 20% fluorescein is injected into the perfusate and, using a portable ultra-violet lamp, the absence of a significant "leak" from the leg to the systemic circulation is confirmed by close inspection of the skin above and below the tourniquet (Figs. 6 & 7).

When the calf skin temperature is at least 37.5°C melphalan is usually administered as a bolus of 1.5-1.75mg/kg of body weight. Perfusion continues for one hour, during which time flow rate and limb temperature are monitored and recorded at ten minute intervals. The average flow rate during the hour of perfusion was calculated for each patient. In each of the eleven patients where temperatures were measured by intramuscular and skin surface probes the data were analysed by

calculating the area under the time-temperature curves (AUC values) for cutaneous and intramuscular temperatures from thirty minutes pre-perfusion until the end of perfusion. The paired AUC values for each patient were then compared (Wilcoxon signed rank test).

Unlike most other centres we oxygenate the perfusate with 100% oxygen rather than 95% oxygen/5% carbon dioxide. Venous and arterial blood gases have been obtained from a series of 22 patients during isolated limb perfusion in order to quantify the anticipated alkalosis.

After perfusion for one hour the limb circuit is "washed out" with two litres of Ringer's lactate, the tourniquet is removed, the cannulae are withdrawn and the vessels are repaired. Appropriate doses of protamine sulphate are given to reverse the heparin-induced anticoagulation.

The operation is completed about three hours after induction of anaesthesia.

Patients are nursed routinely in the high dependency area of our ward for 24-48 hours post-operatively. During this time, transfusion of two or three units of blood is often indicated on the basis of serial haematocrits. In the post-operative period the patient is cared for in the same way as for any major vascular operation, and any systemic or local toxicity is noted. Particular emphasis is laid on checking the full blood count 10-21 days after isolated limb perfusion, when any delayed myelo-suppression, might otherwise go undetected.

The patients are mobilised as soon as possible and they can usually be discharged about ten days post-operatively.

II.4. RESULTS

II.4.1. Therapeutic isolated limb perfusion

Of the patients treated by therapeutic isolated limb perfusion 21 (46%) had evaluable tumour which could be measured after treatment, for an analysis of response rates. Response was defined according to U.I.C.C. criteria[343] and the results are shown in Table 18.

Analysis of disease control is presented in Table 19, which shows the pattern and timing of subsequent recurrence in patients, from the time of first therapeutic perfusion.

Survival after therapeutic isolated limb perfusion was analysed from the time of first perfusion by the life-table method[392] and is presented in Fig. 8. In our patients the probability of survival to three years after therapeutic isolated limb perfusion was approximately 35%.

II.4.2. Repeat isolated limb perfusion

Eight patients had a second isolated limb perfusion. Six women with further limb recurrence, 4-18 months after first therapeutic perfusion were treated this way. A young man initially treated by adjuvant isolated limb perfusion suffered loco-regional recurrence and had repeat perfusion at 28 months after adjuvant perfusion. In the second patient requiring perfusion of an arm, satisfactory venous cannulation could not be achieved at the first operation, and the attempt was abandoned. Within a month a repeat perfusion was successfully performed in this patient.

II.4.3. Adjuvant isolated limb perfusion

The results of adjuvant isolated limb perfusion are summarised in Table 20.

Two of the patients who developed recurrence in the inguinal lymph nodes died of disseminated malignant melanoma at 9 and 17 months after adjuvant isolated limb perfusion. The other patient is alive and apparently disease-free at 34 months, six months after second (therapeutic) perfusion.

The patient who is alive with limb recurrence eleven months after adjuvant perfusion, had involved iliac lymph nodes at the time of perfusion.

II.4.4. Physiology of isolated limb perfusion

The data for flow rates, tissue temperatures and perfusate acid/base status are summarised in Tables 21 and 22.

II.4.5. Complications and toxicity

There was no mortality associated with isolated limb perfusion. None of our patients have required amputation for treatment of their disease or as a result of a complication of isolated limb perfusion. Our experience of problems associated with isolated limb perfusion for malignant melanoma is summarised in Table 23.

Attempted therapeutic isolated limb perfusion has been abandoned four times. In the third patient in our series a large "leak" from the leg to the systemic circulation occurred and could not be reduced; no melphalan was administered and perfusion was abandoned. Twice there was unexpected massive aorto-iliac lymphadenopathy which

precluded safe vascular cannulation and on these occasions isolated limb perfusion was not attempted. The other case was the second arm perfusion which was successfully performed a few weeks later, using a more extensive exposure to achieve satisfactory venous cannulation.

II.4.6. Hospital stay

In all 65 cases (including 8 repeat perfusions and 4 abandoned perfusions) both the hospital stay and post-operative stay have been recorded. The median hospital stay was 11 days (range 6-43 days), and the median post-operative stay was 10 days (5-42).

II.5. DISCUSSION

II.5.1. Introduction

The clinical series described here includes all the patients we have taken to theatre for isolated limb perfusion, without exception (to May,1988). It is therefore a heterogeneous group, and includes the heavily pre-treated patients who were our first cases along with later cases, usually seen at a much earlier stage. Nonetheless important general observations can be made.

II.5.2. Mode of referral

It is important to realise that no stable referral pattern has yet been established and that our rate of accrual of cases has been accelerating over the last two years. Since our unit is committed mainly to vascular and general surgery, and because there are established centres nearby with an interest in malignant melanoma (University Department of Dermatology, Western Infirmary and Regional Plastic Surgery Unit, Canniesburn Hospital) nearly all our cases come from units other than our own. This is a major factor determining the pattern of disease which we see and treat.

Our colleagues around Scotland are frequently willing to send patients with loco-regional advanced disease for therapeutic isolated limb perfusion, but they were understandably more cautious about referring patients across the country for randomisation in a trial of adjuvant perfusion. There is a natural aversion to sending an already anxious patient from some distance to Glasgow for randomisation, or to obtaining informed consent at the

referring centre for a trial procedure with which the referring clinician is unfamiliar. Hence, although about 100 new primary limb melanomas are diagnosed in Scotland each year (96 lower limb melanomas per annum from 1979-83, unpublished Scottish Melanoma Group data), relatively few patients have been referred for adjuvant perfusion.

II.5.3. Age and sex of patients

The female preponderance is in keeping with the previously reported sex incidence of malignant melanoma in the Scottish population[73]. The average age of candidates for therapeutic perfusion (59.5) is well within the range we associate with significant cardio-respiratory problems in the West of Scotland. Working in a vascular surgical unit we were surprised how rarely the melanoma patients had evidence of significant peripheral vascular disease. On one occasion it was clinically apparent that the patient had suffered an acute iliac occlusion eighteen months prior to presentation with severe advanced loco-regional melanoma. Digital subtraction angiography was performed and showed that although the common iliac artery was occluded, the external iliac and distal vessels were patent. It was thus possible to perform an iliac perfusion despite the absence of palpable pulses in the treated limb.

Our general impression was that in spite of their age and disease, the patients were in good physical condition. This is probably due to selection by the referring practitioners.

II.5.4. Stage of disease

28 of the 42 patients for therapeutic isolated limb perfusion had local recurrence and/or in transit disease, apparently confined to the limb (Table 16). Others had evidence of nodal involvement and one had systemic metastases. This group of patients is clearly at high risk (I.2.9., I.3.3).

In the group treated by adjuvant isolated limb perfusion, the median Breslow thickness of the primary lesion was 4mm (Table 17), though the exact Breslow thickness could not be measured for the two subungual primaries. Table 24 shows the five year survival for Scottish patients according to Breslow thickness. There is no doubt that with conventional therapy alone, patients with such thick primaries are also at significant risk.

II.5.5. Previous treatments

Most loco-regional recurrence of malignant melanoma occurs in the first three years after primary surgery[394,122]. Hence it is important to note that most of our therapeutic perfusions were performed on patients in this phase, and that they were not a selected group of long-term survivors with indolent recurrences (Table 16). Most of the patients had at least one operation since the primary surgery, sometimes a diagnostic biopsy, but often a series of wide excisions with repeated skin grafting. The patients who had previous chemotherapy were almost exclusively from the first ten patients we treated. Only one patient had been treated by radiotherapy, reflecting the widely held belief that malignant melanoma is a radio-resistant tumour

(I.4.4).

II.5.6. Pre-operative investigation/assessment

We included thoraco-abdominal CT-scans as part of the pre-operative assessment of candidates for therapeutic isolated limb perfusion because we would not usually perfuse patients with established systemic metastases. No patient with unexpected systemic metastases was identified in this way however, and CT-scans failed to demonstrate the extent of lymphadenopathy which precluded safe isolated limb perfusion on two occasions. On the basis of this evidence, the practical benefits of CT-scans in this setting are unproven. Again the patients with probable systemic metastases may not have been referred for perfusion, because of selection by the referring doctors, and it may be reasonable for us to obtain fewer CT-scans in future.

II.5.7. Anaesthesia and per-operative monitoring

We have only used general anaesthesia for isolated limb perfusion, and regional anaesthesia could only be considered where systemic heparinisation is not proposed. Although we do not routinely aim to achieve full "cardiac" heparinisation, we believe that systemic heparinisation helps to reduce the risks of thrombotic and embolic complications of the vascular manipulations involved in isolated limb perfusion. Furthermore the degree of sympathetic block associated with spinal and epidural anaesthesia may produce systemic hypotension during perfusion, risking leak from the limb to the rest of the

body[394] and compromise the haemodynamic response to relative hypovolaemia at the end of perfusion.

For the hour of perfusion the limb is maintained by a perfusate, which consists of a mixture of the priming fluid and the blood trapped in the leg vasculature at the time of cannulation. The perfusate has a low haematocrit. At the end of perfusion the perfusate is drained from the limb and is largely replaced by Ringer's lactate. Therefore the circulating volume within the limb is effectively lost, and only partially replaced with crystalloid solution, at the end of perfusion. The average blood loss was estimated at approximately 1.4 litres in the series of 15 patients reported by Goldberg and colleagues[394]. Furthermore there is evidence that systemic vascular resistance may be reduced by a vasodilatation response to exposure of the skin to sustained high temperatures[395]. If there is no attempt to pre-load the systemic circulation, or to replace immediately the circulating volume which is lost at the end of isolated limb perfusion then there is a serious risk of circulatory collapse, particularly in an older patient who may have a limited cardiac reserve. This is why central venous manometry has been described as routine and why it has been suggested that pulmonary artery wedge pressure monitoring should be considered in patients with poor cardiac reserve[396]. Central venous pressures were routinely monitored in our early experience but anticipation and correction of volume depletion, has resulted in a diminished need for invasive monitoring.

II.5.8. Technique of isolated limb perfusion

For lower limb perfusion we favour vascular access by the iliac vessels because they are usually of adequate calibre for cannulation, and because direct perfusion of both the deep and superficial femoral systems is achieved.

The retro-peritoneal dissection results in less gastro-intestinal upset than a laparotomy, and is usually straightforward unless there is extensive peri-vascular tumour or if there has been a previous ilio-inguinal dissection, perfusion, or appendicectomy.

Particular care is taken during dissection to identify and divide all (named and un-named) branches of the external iliac vessels, which might allow "leakage" during perfusion. This also results in a length of free artery and vein, for ease and safety of manipulation during cannulation.

In perfusion of the upper limb the anomalous and frequently multiple venous drainage can cause problems. Satisfactory isolation of the arm combined with perfusion of the regional nodes is less likely to be feasible than in the leg, because of the extensive periscapular anastomoses and the geometry of the shoulder. Using our standard technique of iliac perfusion, fluorescein staining of the skin of the inguinal region, right up to the tourniquet is usually seen. It is likely that this implies adequate perfusion of the inguinal lymph nodes but similar perfusion of the axilla is not possible, without a greater risk of systemic leak.

Schraffordt Koops and colleagues[348] describe the routine use of a second perfusion at six weeks in patients with

tumour in a sampled superficial inguinal node (femoral perfusion), and in patients with melanoma affecting the foot (popliteal perfusion). A significant incidence of foot drop in his early experience has led him to advocate the routine use of "prophylactic" fasciotomy, to decompress the anterior compartment of the leg[397]. It would appear that compartment syndromes were more commonly seen in the early days of perfusion (when low flow rates were used) and especially with the introduction of hyperthermic perfusion (producing increased oxygen demand). Wieberdink has suggested that the problem is due to tissue hypoxia and that it is circumvented by "high" flow rates[398]. In common with most centres[211,357,390] we do not consider that "prophylactic" fasciotomy is indicated after routine isolated limb perfusion.

There are numerous other subtle technical differences between the perfusion regimes described in various centres. The physio-pharmacological consequences of such differences may prove to be of critical importance (see Chapters III and IV).

It is clear, mostly from conversation with many clinicians who were practising in the early days of isolated limb perfusion, that major morbidity and occasional mortality due to complications of isolated limb perfusion were the simple reasons why the popularity of the treatment waned in the U.K. after a vogue in the early 1960s. There are relatively few unfavourable reports on isolated limb perfusion in the literature[356,399], but clinicians are perhaps reluctant to publish poor results. The resurgence of perfusion began with the failure of new cytotoxic drugs

to make a convincing impact on melanoma, combined with the publication of major series by careful investigators[211,329,330,333,357] which showed that the treatment could be performed safely and effectively. It should come as no surprise that (as with any surgical technique) the best results are obtained by those who have not merely flirted with the technique, but who have taken it, adapted it and developed their own routine over the years. Technical factors which may critically determine the quality of clinical results may not have been highlighted in the literature concerning isolated limb perfusion, but in this complex mode of treatment it is clear that there is ample scope for disaster as a consequence of sloppy technique.

II.5.9. Therapeutic isolated limb perfusion

In our series of patients with evaluable tumour the overall response rate was close to 100% (Table 18). The incidence of complete response was particularly encouraging when compared with the results for systemic chemotherapy (see I.4.2.). It should however be borne in mind that cutaneous and nodal metastases may be more likely to respond to systemic chemotherapy than visceral (e.g. lung, liver) metastases[248,251,252,253], and this might tend to bias results in favour of isolated limb perfusion.

Examples of the response to isolated limb perfusion are illustrated in the series of clinical photographs (see Figs. 9A, B and 10A-D).

"Disease-free" survival is not an appropriate parameter

for our series of therapeutic perfusions because a significant proportion of the patients do not have the tumour excised. Table 19 shows where and when recurrences developed in our patients after therapeutic isolated limb perfusion. In this context "recurrence" refers to those patients whose residual disease progresses, perhaps after initial response, as well as those who develop new lesions. Therefore the term "recurrence-free survival" may be applied to describe the time interval between therapeutic perfusion and subsequent progression of existing melanoma or de novo recurrence. Combined with the response data (Table 18), the figures indicate that temporary regional control of melanoma is achievable with isolated limb perfusion.

Some of our patients treated by therapeutic isolated limb perfusion have since died due to metastatic disease. An unknown number of the patients who developed systemic metastases would have occult systemic metastases at the time of isolated limb perfusion, and it is unlikely that the regional treatment would prevent these occult lesions from becoming manifest systemic metastases. It is interesting that the lymph nodes seem to be a relatively uncommon site of subsequent recurrence in this series. This could be due to patient selection, it may be an effect of treatment, or perhaps it is due to under-diagnosis of nodal disease in the face of more obvious regional or systemic tumour.

For completeness the survival data is illustrated in Fig. 8. The life-table estimate for probability of survival to three years after therapeutic isolated limb perfusion in

our series (M.D. Anderson Stages II-IIIAB) is about 35% (cf. Table 14).

Overall our results for therapeutic isolated limb perfusion, in terms of response rates, disease control and survival are certainly encouraging and in keeping with results from other centres (I.5.4., Tables 13 and 14).

In none of our patients has amputation been necessary, either for progression of disease or because of complications of isolated limb perfusion.

II.5.10. Repeat isolated limb perfusion

Our early experience led us to believe that repeat isolated perfusion of the leg was most satisfactory if the iliac vessels could be re-cannulated at the second operation. On three of seven occasions this was not technically possible, and the femoral vessels were cannulated instead. In two of these cases perfusion was terminated prematurely because of poor flow and suspected "leak" from the leg to systemic circulation, but both had been perfused for at least thirty minutes. As a result of experience gained with these cases and because of further responses at the second perfusion, we consider that repeat perfusion is a worthwhile procedure, but we have recently modified our technique.

In the more recent cases coming to repeat isolated limb perfusion we have tended to place the tourniquet around the root of the limb but cannulate distally at the femoral or popliteal vessels. Good perfusion of the whole limb can thus be achieved, circumventing the need for a potentially hazardous retroperitoneal dissection.

It has not been proven that there is any advantage to be gained from the use drugs other than melphalan, alone or in combinations, during initial or repeat therapeutic isolated limb perfusion[368].

II.5.11. Adjuvant isolated limb perfusion

Three of the patients treated by adjuvant perfusion later in our series were managed according to the M.R.C. Trial protocol. 5 others were randomised to surgery alone, and clearly no meaningful comparison can be made between such small groups. I have described how accrual to this trial was slow in Scotland (see II.5.2.), and in November 1987 the M.R.C. withdrew its support because the accrual rate was similarly poor across the U.K. Existing data has been transferred to the British Association of Surgical Oncology (B.A.S.O.), who plan to continue the trial in the U.K.

Although we only perfused three patients in the context of the M.R.C. protocol we were concerned that two of these patients developed severe regional toxicity after perfusion. A 48 year old lady developed marked swelling of the limb associated with cellulitis and interdigital sepsis 19 days after adjuvant isolated limb perfusion and she had to be re-admitted to our ward for treatment including antibiotics. In the second case a 49 year old man (C.M.) with a 2.3mm thick primary melanoma was referred from Inverness after randomisation, and he developed swelling of the limb (Fig. 11), associated with severe pain in the muscles of the calf. At seven days after perfusion there was a rise in serum transaminase

enzymes (AST and ALT), and the serum creatine kinase level was grossly elevated at >3000units, indicating significant muscle damage. Both of these patients made a full and satisfactory recovery, but the man developed obvious wasting of all muscle groups in the limb with a symptomatic foot-drop and he had to walk with a stick for two months after perfusion.

We were keen to determine why these problems arose. The major differences between the M.R.C. protocol and our standard method were that the protocol required that melphalan should be given in a total dose of 2mg/kg body weight, and administered as three aliquots during the hour of perfusion. Hence the total doses given to these patients were somewhat higher than our standard regime would have involved, and we would have given the smaller doses as single boluses. Subsequent pharmacokinetic studies were designed to determine whether the mode of administration influenced the exposure of tissues to cytotoxic drug, and hence whether the observed toxicity was likely to be due to total dose or mode of administration (see Chapter III).

Considering all the adjuvant perfusions, it is important to note that although most of these patients have lesions greater than 3.5mm thick, 11 of 15 have had no problem with melanoma at a median follow-up of 29 months (Table 20). Furthermore, only one patient has suffered local recurrence or in transit disease after adjuvant perfusion and she was the patient discovered to have involved iliac glands at the time of perfusion.

Although the W.H.O./E.O.R.T.C. trial (I.5.5.) should

answer whether adjuvant perfusion is effective, there are potential objections to such a large scale multi-centre study, particularly with a complex treatment like isolated limb perfusion, in that it will be difficult to ensure that uniform perfusion techniques are used in all participating centres. The treatment will be applied to many small, perhaps unrepresentative samples from many quite different populations. Such criticisms have already been made of previous W.H.O./E.O.R.TC. studies by those who contend that significant benefits to sub-groups may be hidden[217].

We believe that the significant toxicity we have observed using the M.R.C. protocol is not acceptable in the context of an adjuvant trial. There are certain advantages in studying a group of patients treated by a single team of investigators. Therefore it is proposed that in Scotland we should prospectively study adjuvant perfusion by comparing the clinical results of those referred for perfusion with those not referred for perfusion, using the established framework of the Scottish Melanoma Group. In such a study we would consider adjuvant isolated limb perfusion for any patient with a primary malignant melanoma thicker than 1.5mm.

II.5.12. Physiology of isolated limb perfusion

In the series of 57 patients for whom we have data on arterial flow rates during iliac perfusion it can be seen that there is considerable variability in the flow rates used (Table 21). This is because no specific attempt has been made to achieve a pre-set value for any case, apart

from the series in which we studied melphalan pharmacokinetics. In general the tendency has been to commence with a low flow rate of about 50-100ml/min and increase the rate to a maximum stable level, without inducing excessive pressures in the lines or rapid changes in the reservoir volumes. We have not measured arterial or venous pressures in the limb directly during perfusion. So far as possible we have tried to maintain constant conditions in the isolated circuit but occasionally some additional fluid (in divided amounts, up to 300ml) has to be added to the reservoir because of vasodilatation, with a fall in reservoir volume which is not associated with any leak of cytotoxic drug into the systemic circulation. Our mean flow rate is similar to those used in other centres[211,318,350,381,400], higher than those in some early series[322], but lower than the flow rates (up to 1.2l/min) achieved by the advocates of "pressure regulated perfusion"[354,401]. It is not clear that there is any definite benefit from very high flow rates, though it is obvious that ischaemia of normal tissue should be avoided by achieving at least a physiological flow rate during isolated limb perfusions[400].

In the 11 patients in whom we recorded the <u>temperature</u> in muscle and skin, we demonstrated that the use of deeply placed needle thermistors was unnecessary because there was no significant difference between the skin surface and intramuscular temperatures throughout the treatment (Table 21). We measured the temperature at two sites on the limb because of the patient who suffered a superficial burn due to mal-function of the single thermistor probe, leading to

under-estimate of tissue temperature (see II.5.13).

Our main reason for monitoring temperature continuously was to avoid damage to normal tissues.

The larger series (n=60) of cutaneous temperatures shows that our method of warming the limb achieves satisfactory moderate hyperthermia by the end of perfusion in most cases (the series includes a few instances where the heated water blanket was unavailable or punctured per-operatively!). The maximum cutaneous temperature achieved in this series was 41.2°C. It is perhaps surprising that there was no significant temperature difference between the thigh and calf, since one might have expected the vessels supplying the warm, insulated limb to act as a counter-current multiplier system and generate higher temperatures distally.

At the temperature levels achieved in this series one would not expect any cytotoxic effect due to heat alone, because the specific cytotoxic effect of hyperthermia is usually described at temperatures over 41.5°C[326,402]. It is often stated that hyperthermia is "synergistic" with certain cytotoxic drugs[352] but the use of hyperthermia with melphalan is paradoxical because the drug has a short half-life and because it is even more rapidly hydrolysed to inactive metabolites in warmer conditions (see also III.3.4., Figs. 19,21,25).

It is likely that the generation of at least physiological temperatures (normothermia, 37°C) will be required to prevent vasoconstriction and shunting, to allow adequate tissue perfusion. A recent clinical study indicated that isolated limb perfusion with "controlled normothermia"

(tissue temperatures 37-38°C) seemed to produce similar results to perfusion with "mild hyperthermia"[344].

No attempt was made to measure tumour temperature in our study. The accurate measurement of intra-tumoral temperature is difficult, as there is often uneven distribution of heat within larger tumour masses. Some have indicated that sluggish blood flow within the abnormal vessels of tumour masses causes poor heat dissipation, resulting in selective heating of tumour tissue[403,404]. The interaction of hyperthermia and melphalan cytotoxicity is examined in greater detail in Chapter IV.

When the vessels are clamped for cannulation prior to perfusion the limb is temporarily ischaemic and it becomes somewhat acidotic as anaerobic metabolism proceeds. Originally the oxygenator was used simply to maintain viability of the limb during treatment, though it was postulated even in the earliest report[317], that high arterial oxygen tensions might increase the efficacy of "radiomimetic" drugs like melphalan. Indeed it was later shown in a murine model that hyperbaric oxygen enhanced the anti-tumour activity of nitrogen mustard[405,406], and that an elevated tissue P_{O2} may be cytotoxic to hamster melanoma[407].

The measured gas tensions in our patients (Table 22) show that the initial ischaemic acidosis is soon corrected and reversed, and that a high P_{O2} is maintained on the arterial side of the circuit throughout the time of perfusion. We have made little attempt to control P_{CO2} in the circuit, and the carbon dioxide is rapidly extracted

from the circuit by diffusion across the oxygenator, with the result that the arterial P_{CO2} has fallen as low as 6mmHg in our patients. There is a resultant tendency towards respiratory alkalosis within the perfused limb, which progresses during the hour of perfusion (with pH rising as high as 7.8). In none of our patients has there been any problem with tetanic muscle spasms, described by Krementz and colleagues[408], and which led them to recommend the use of a 95%/5% mixture of oxygen and carbon dioxide[318] to supply the oxygenator.

It can be seen that although the conditions in the leg are controlled, they are not "physiological". Isolated limb perfusion affords a unique opportunity to manipulate the tumour micro-environment to influence the cytotoxic effect of anti-cancer agents. Since we have seen no significant morbidity on account of the alkalosis within the limb we have not made any attempt to correct it.

It is interesting to consider the possible effects of such a profound alkalosis in the tumour-bearing limb. A degree of vasoconstriction might be expected due to the relatively low P_{CO2} (a potent vasodilator) and the high P_{O2}, but it is postulated that tumour vessels, which are abnormal structurally and functionally[409], might not respond as do normal vessels. In this way there may be preferential shunting of perfusate into the tumour vascular bed, with an enhancement of the anti-tumour effect. Conversely melphalan is more stable at lower pH values[410], and it has been suggested that tumour uptake of the related drug nitrogen mustard may be greater when the perfusate is acidified[318]. These mechanisms might act

to reduce the cytotoxic effect of melphalan in alkalotic conditions. Melphalan has been shown to have no significant effect on pH, peripheral resistance or oxygen consumption in vivo[411]. The interaction of melphalan cytotoxicity and pH is considered later in this thesis (Chapter IV).

The advocates of "pressure regulated perfusion" (i.e. the use of continuous monitoring of venous and arterial pressures to maintain the limb arterial pressure 15mmHg less than systemic arterial pressure), who achieve remarkably high flow rates[354,401] may do so because they maintain <u>normal</u> blood gas tensions in the limb during perfusion, and in their system the vasoconstrictor effects of low P_{CO2} and high P_{O2} will be absent.

II.5.13. Complications and toxicity

Although we describe our early experience with a complex form of treatment, we are pleased to report no life or limb threatening complication of isolated limb perfusion in this series.

One of the patients (Table 23) suffering a deep venous thrombosis also developed pulmonary thrombo-embolism, and this was the most serious general complication, but does not seem to be a particular risk of isolated perfusion in this series. The patient who bled post-operatively quickly developed a haematoma in the wound, which was explored before the patient returned to the ward. A small vessel in the wound edge was the cause.

The regional toxicity due to melphalan in isolated limb perfusion can be described according to the index

described by Wieberdink and colleagues[400] (see Table 25). We would agree with their view that grade I reactions indicate undertreatment and that responses graded as II and III are probable indicators of optimal dosage.

I believe that the nerve problems in our three patients were due to local pressure from the tourniquet rather than due to the cytotoxic drug, because the doses given to these patients were not high. The patient who suffered a thermal burn was only our second patient, and the problem arose due to a mal-functioning thermocouple and the lack of a protective layer between the patient's skin and the heated water blanket. Fortunately the burn was superficial and did not necessitate skin grafting. In the remaining three patients the regional toxicity was directly due to melphalan. I have already indicated (II.5.11.) how two of the three patients we treated by adjuvant isolated limb perfusion as part of the Medical Research Council trial are in the group who suffered grade IV reactions. The other patient (Fig.22) was given a high total dose because of his body weight, and the dose was given in divided aliquots as in the M.R.C. protocol.

There was remarkably little in the way of systemic upset associated with isolated limb perfusion with melphalan, though our recovery room staff have indicated that they believe the perfusion patients are more nauseated and vomit more than routine surgical cases. This does not appear to be a problem after the first post-operative night, and may be due to the cytotoxic, anaesthetics or analgesics.

None of the haematological problems listed in Table 23

required any specific treatment, and all recovered spontaneously. The leucopenia usually began about seven to ten days post-operatively, and hence we ask the patient's general practitioner to check the full blood count for a few weeks after the patient is discharged home.

II.5.14. Hospital stay

The longest hospital stay (43 days) was in a patient who had wide excision, skin grafting, and adjuvant isolated limb perfusion under the one general anaesthetic. Unfortunately the graft failed to take because of sepsis, resulting in the protracted stay in hospital. Interestingly the same procedure has been performed on two other occasions without complication.

Most patients can be mobilised within 24-48 hours after isolated limb perfusion, and they are allowed home once we are happy for them to travel the (often considerable) distance home. The resulting medians of hospital stay and post-operative stay (11 & 10days) are appropriate for elective major surgery in the age group treated.

II.6. CONCLUSIONS

The major conclusion to be drawn from this report of our experience with isolated limb perfusion is that we can safely perform the technique in Glasgow.

Our clinical results show very encouraging response rates after therapeutic perfusion, at least as good as those reported by other perfusion groups (Tables 13 and 14).

The value of adjuvant isolated limb perfusion is as yet unproven, and we will be treating patients in this way only in the context of a clinical trial.

The pilot studies describing the physiology of isolated limb perfusion have indicated avenues for possible investigation, which we have pursued, and which I will describe in the following chapters.

CHAPTER III

PHARMACOKINETICS OF MELPHALAN IN ISOLATED LIMB PERFUSION

III.1. MELPHALAN

III.1.1. Drug development

Melphalan (L-phenylalanine mustard; L-PAM; L-sarcolysine; NSC-8806; CB3025) is currently the drug of first choice in isolated limb perfusion for melanoma (see I.6.2.). Its full chemical name is p-{di(2-chloroethyl)} amino-L-phenylalanine and the chemical structure is illustrated in Fig. 12.

The anti-tumour activity of mustard gas was recognised in the 1930s[412], and nitrogen mustards were developed for use in haematological malignancies in the 1940s[313,413].

Melphalan was one of many substances which were derived from the nitrogen mustards in the early 1950s. The synthesis and biological activity of the phenylalanine derivative were reported by Bergel and Stock[414,415] and by Larionov and co-workers[418].

It was originally hoped that the combination of the amino acid phenylalanine (which is involved in melanin synthesis) with the nitrogen mustard moiety might result in selective activity against malignant melanoma[299]. The L-isomer, melphalan, is more active than the D-isomer, medphalan[415]. Melphalan is the active component of the racemic (DL) form, merphalan or sarcolysine, and melphalan is therefore the isomer of choice.

Early in vivo work showed that the Harding-Passey murine melanoma could be controlled by melphalan to an extent not matched by other treatments[315]. In subsequent clinical

trials[299,417,418,243] however, the response rate of advanced melanoma to conventional systemic administration of melphalan has been no better than that obtained by most other single agents (I.4.2.). Using high dose systemic melphalan with or without autologous bone marrow rescue, patients require intensive support but response rates of up to 70% have been reported by some workers[260,261,310]. Similarly the use of high dose melphalan in isolated limb perfusion aims to exploit the apparent dose-response relationship, while avoiding the risks of major toxicity to bone marrow and gut.

Although melphalan has been used for thirty years in the treatment of a variety of malignancies[419], relatively little work has been done to determine the fate of administered drug[420]. This is due to the lability of the compound and the lack of appropriate assays[419] until recent years.

III.1.2. Mechanism of action of melphalan

In common with the other nitrogen mustards, melphalan is a bifunctional alkylating agent which can react and bind with cellular macromolecules such as DNA (deoxyribonucleic acid), RNA (ribonucleic acid) and proteins. It is currently thought that DNA is the major cytotoxic target for melphalan and cross-links between adjacent strands of DNA have been particularly implicated in the mechanism of action[421,422,423]. However, other effects on DNA e.g. base mis-coding[424] and DNA-protein cross-linking[425] may also have a role in the cytotoxic effect of melphalan. In a human melanoma cell line in vitro it has been shown that

maximum levels of DNA inter-strand cross-links are reached 6-12 hours after melphalan exposure (cf. nitrogen mustard, peak levels immediately after exposure) in spite of the labile nature of the molecule[423]. The hypothesis proposed by Kohn[426] is that rapid binding of the drug to a single strand of DNA as a mono-adduct is followed by a delayed interstrand cross-link when the second chloroethyl group binds to the complementary strand of DNA.

For the drug to be effective in the treatment of cancer it is therefore essential that enough active drug should penetrate to the nuclei of malignant cells. In vitro experiments have shown that the transport of melphalan into lymphoblasts[427] and leukaemic cells[428,429] occurs by active carrier mechanisms. In L1210 (murine leukaemia) cells in vitro the uptake of melphalan is a temperature-sensitive, partly sodium-dependent, active process with cell:medium accumulation of approximately 5:1 at 37°C[429]. This process is competitively inhibited by physiological concentrations of the L-isomers of the amino acids leucine and glutamine, with a corresponding reduction in melphalan cytotoxicity[428]. Interestingly, in vitro, the amino acid phenylalanine does not interfere with melphalan uptake[427]. Phenylalanine has little effect on the cytotoxicity of melphalan to leukaemic cells[430] and it does not inhibit melphalan cytotoxicity to cultured melanoma cells[431]. Melphalan rapidly effluxes from cultured cells into melphalan-free medium[432], by a separate mechanism, and at least 75% of radio-labelled intracellular melphalan is free to leave the cell[429].

The clinical implications of these experimental results

are not yet clear, but now that there is a satisfactory assay for melphalan the basic clinical pharmacokinetics can be described. The cytotoxic effect of melphalan is likely to be influenced by special features of the isolated limb perfusion circuit, and some of these influences may act by altering pharmacokinetics.

III.1.3. Measuring melphalan

The first reported attempts to describe the pharmacokinetics of sarcolysine were made in 1957 by Cohn[433] who used ^{14}C-labelled drug in a rat model. The distribution of radio-activity could be described but the proportions due to parent drug, hydrolysis products or metabolites were unknown. In the original report of clinical isolated limb perfusion Creech and colleagues[317] described the use of a qualitative in vivo bio-assay to determine the duration of anti-tumour activity in the perfusate. Klatt and colleagues[434] described a colorimetric method for the analysis of alkylating agents in blood and tissues. This required the use of nitrobenzylpyridine and was specific for alkylating activity, but could not distinguish between melphalan and its monohydroxy- hydrolysis product. The technique involved significant heating of samples (which we now know would increase hydrolysis of the parent compound) and lacked sensitivity, only being able to detect alkylating activity in excess of 5μg/ml of plasma[419]. To improve the sensitivity for clinical assay of melphalan, Chirigos and Mead reported a spectrofluorometric method, but they found that the specificity of this assay was poor[435].

Sensitive and specific gas-liquid chromatography was used by Goras and colleagues to measure melphalan for drug formulation[436], but the method is not applicable to biological systems. With the advent of high performance liquid chromatography (HPLC), sensitive and specific methods which were easy to apply to biological systems became practical. HPLC methods for melphalan assay were thus developed[436,437,438].

III.2. HPLC

Chromatographic systems permit the separation and analysis of the components in a mixture because of the differential association of constituents between a mobile and a stationary phase. Many chromatographic techniques have been developed, and each is suited to particular tasks. For example, gas-liquid chromatography (GLC) is suitable for analysing volatile substances, or volatile derivatives of non-volatile compounds but the technique often requires heat. High performance liquid chromatography (also known as high pressure liquid chromatography) allows the detection and measurement of heat-labile biological substances at ambient temperatures and without the need for expensive derivatisation. HPLC is now widely used for the detection and measurement of biological compounds.

In the 1960s, Giddings[439] demonstrated how GLC theory could be applied to liquid chromatography. Later that decade the first high performance liquid chromatographs were reported[440,441,442]. The use of high pressures overcame the effect of the higher viscosity of liquid compared with gas. In the early days the use of single or dual wave-length ultraviolet (UV) detectors meant that very small amounts (nanogram quantities) could only be measured for suitable UV-absorbing compounds. Variable wave-length UV detectors have enabled the detection of such small amounts of a wider range of compounds.

The efficiency and speed of HPLC were improved by the introduction of new support materials which allow the separation of compounds over a relatively short distance

of solute travel e.g. Horvath[440] described the use of glass beads coated with an ion-exchange resin.

In classical chromatography the column is gravity fed and separation takes hours or even days. HPLC makes accurate analysis possible in minutes (which is comparable with GLC). The use of HPLC has greatly facilitated the study of substances like melphalan.

III.3. PHARMACOKINETICS

III.3.1. Introduction

The study of pharmacokinetics is concerned with the absorption, distribution, biotransformation and excretion of drugs[443]. For a given dose these factors govern the concentration of drug at the site of action and they determine how the concentration varies with time.

In the special circumstances of isolated limb perfusion, absorption and excretion of melphalan are relatively unimportant. The dose administered, its distribution, biotransformation and the time of exposure are likely to be important because these factors may be varied and controlled in the isolated limb perfusion system.

III.3.2. Dose

No previous formal phase I (dose escalating) study of melphalan in isolated limb perfusion has been reported.

The dose of melphalan used in isolated limb varies from centre to centre. In most series the dose is calculated on the basis of body weight, and the most commonly recommended dose for an iliac perfusion is 1-1.5mg per kilogram of body weight[318,334,338,340,350,362]. In a few studies it has been suggested that doses as high as 2mg/kg can be given[211,215].

It is increasingly common for the dose of melphalan to be calculated on the basis of measured limb volume[354] as advocated by Wieberdink[400]. The rationale for this method of dosimetry is that limb volume varies considerably, and does not correlate closely with body weight[400]. Wieberdink's study[400] was a retrospective study in which

toxic reactions were graded and related to melphalan dose. Although Wieberdink is convinced that limb volumetry is superior to body weight as a basis for melphalan dosimetry, his was not a formal phase I study, 87% of the patients were treated in a narrow dose range (9-11mg/l limb volume) and all three patients treated with higher doses received more than 13mg melphalan per litre limb volume[400].

Apart from the total dose administered, it is clear that the volume of perfusate in which this dose is dispersed will determine the effective concentration of drug. In reports of isolated limb perfusion it is remarkable that this critical factor has rarely been considered. Formerly it was believed that the perfusate volume was mainly determined by the prime[400], but the total volume of perfusate also includes blood trapped in the limb vessels at the start of perfusion. The volume trapped in the limb vessels will vary depending on whether the artery or vein is clamped and cannulated first, and on how long cannulation takes. The volume trapped in limb vessels may be a litre or more[444,445]. Furthermore the volume of prime varies between 600ml[213,349] and two litres[211]. In fact, the volume of prime is often omitted in reports[340,354,357,359,364], even in studies of melphalan pharmacokinetics[446,447].

It is not known whether the dose of melphalan is better given as a single bolus, or in divided aliquots during isolated limb perfusion[448]. We were concerned by the toxicity observed in patients treated according to the M.R.C. protocol for adjuvant isolated limb perfusion,

which required that melphalan should be given in divided aliquots during perfusion (II.5.11.). The original rationale for dividing the dose was to minimise the consequences of "leakage" and it is not clear whether there is any pharmacokinetic advantage in bolus or divided dose administration.

III.3.3. Distribution

Clearly it is one of the major objectives of isolated limb perfusion to confine the distribution of administered cytotoxic drug to the tumour-bearing region. Ideally higher levels of cytotoxic would be generated in malignant tissue than in normal tissues.

There have been several studies of drug levels achieved in perfusate during isolated limb perfusion with melphalan[444,446,449,450,451]. In whole blood a constant fraction of about 37% of total melphalan is present within erythrocytes, and this fraction is independent of melphalan concentration[452]. The fraction binding to plasma proteins (mainly albumin) is constant at low concentrations of melphalan, but at high concentrations (>10μg/ml) small increases in total melphalan result in larger increases in pharmacologically active free melphalan[452].

Attempts have been made to estimate the proportion of administered melphalan which distributes to the tissues of the leg semi-quantitatively[450] and quantitatively[453]. One previous publication describes the measurement of tissue concentrations of melphalan achieved by isolated limb perfusion[448] in three patients.

Some data on systemic levels of melphalan during perfusion has been obtained[446,451]. However it would be expected that even if isolation of the limb were perfect during perfusion then some re-distribution of melphalan might occur after re-connection of the normal circulation. A proportion of the administered dose, not washed out by flushing at the end of isolated limb perfusion, may be free to distribute in the systemic circulation. There have been no previous reports on the relationship between melphalan levels in the limb during perfusion related to systemic levels during and after perfusion.

III.3.4. Biotransformation

In aqueous solution it has been shown that melphalan decomposes to give monohydroxy-melphalan and dihydroxy-melphalan[454]. Monohydroxy-melphalan is much less cytotoxic than the parent compound and dihydroxy-melphalan has no anti-tumour activity[455,456]. Using HPLC to measure melphalan incubated in various fluids of different compositions it has been established that melphalan is more stable at low temperatures[410,435,438], in acidic fluids[410], and in the presence of protein[452,457] and chloride ion[410]. Thus melphalan may be relatively protected from hydrolysis in protein-rich environments like the extracellular space or within the cell.

III.3.5. Duration of perfusion

The duration of isolated limb perfusion with melphalan is usually one hour[213,331,444] but it may be as short as 45 minutes[359] or as long as two and a half hours[327,446].

There is no clinical trial based evidence to suggest that the optimal duration for isolated limb perfusion has been found. In spite of the short half-life of melphalan in aqueous solution, and the relatively short time of exposure involved in isolated limb perfusion, it may be that the drug is somewhat protected from hydrolysis once it leaves the vascular compartment. Furthermore, as discussed in III.1.2., the drug in the tissues may continue to form cross-links for hours after exposure[423]. Hence there may be relatively little therapeutic gain achievable by prolonged clinical isolated limb perfusion, if maximal safe doses are already being given.

III.4. AIMS

The aims of my studies were:

1) to measure and compare melphalan concentrations in perfusate during isolated limb perfusion, with the levels in the systemic circulation during and after perfusion,

2) to study the pharmacokinetics of melphalan in isolated limb perfusion, as the dose was increased in a phase I study,

3) to compare the pharmacokinetics of bolus dose with divided dose administration, and

4) to measure the tissue levels of melphalan achieved by isolated limb perfusion.

III.5. PATIENTS, MATERIALS AND METHODS

III.5.1. Clinical perfusions

Local Ethical Committee approval was granted for all studies described here.

After a simple review of our experience with the first 26 patients treated by isolated limb perfusion, it was confirmed that, because of the absence of melphalan-induced toxicity, there was scope for a phase I clinical study. In the context of this study it was decided to study melphalan pharmacokinetics.

Candidates for inclusion in the study were all those patients coming to external iliac perfusion for melanoma. The starting dose of melphalan was 1.5mg/kg body weight, which is the maximum dose recommended in the protocols on which our technique is based[213,362]. In the absence of significant toxicity we planned that the dose would be escalated by increments of 0.25mg/kg body weight.

The perfusion technique has been described (II.3.3.).

Pharmacokinetic data was acquired on a total of 28 consecutive patients. The three main groups were:

A. 11 patients who had isolated limb perfusion by our standard method and melphalan dose of 1.5mg/kg body weight, given by a single bolus injected into the venous line at the start of perfusion,

B. 9 patients who had a standard isolated limb perfusion with a melphalan dose of 1.75mg/kg body weight, given by a single bolus (as Group A), and

C. 6 patients who had standard isolated limb perfusion except for the melphalan dose of 1.75mg/kg body weight,

which was injected into the venous line in three aliquots, at 0, 15 and 30 minutes during perfusion (as in the M.R.C. protocol).

Group B comprises patients in the second stage of our phase I study.
Group C patients were treated with the same dose as Group B, but it was given in divided amounts for comparison of the pharmacokinetic profile with that of group B. This was done because of our concern about the toxicity we observed with the M.R.C. protocol (II.5.11) for adjuvant perfusion. Pharmacokinetic data was also recorded for two of the three patients whom we treated according to the M.R.C. protocol.

III.5.2. "Mock" Perfusions

"Mock" perfusions were performed on 2 occasions to study how the levels of melphalan changed with time in a closed circuit consisting of identical perfusion apparatus (i.e. excluding the patient from the circuit). In the "mock" perfusions, melphalan (100mg) was added to the standard prime (one unit of packed red blood cells + 750ml Hartmann's solution). The perfusate was allowed to recirculate at 37°C and 39°C and samples were drawn, as in the clinical perfusions, at 5 minute intervals for melphalan assay. Melphalan was also incubated at 37°C and 42°C in human plasma and in Eagle's Minimum Essential Medium (MEM), at an initial concentration of 20μg/ml for 4 hours. Serial samples were taken for melphalan assay by HPLC.

III.5.3. Perfusate sampling protocols

In all cases a 5ml sample of perfusate was obtained from the arterial and venous ports of the oxygenator at 2,5,10,15,20,25,30,35,40,45,50 and 60 minutes. In clinical perfusions 5ml samples were also drawn from the patient's radial arterial line before perfusion, at 15,30 and 60 minutes (during perfusion), and at 75,90,120,150,180,240 and 300 minutes (after perfusion).

All perfusate and blood samples were collected in lithium heparin tubes, mixed, and immediately placed on ice. Samples were centrifuged (2,500r.p.m. for 10 minutes) within one hour, then the plasma was separated and stored at -20°C for the minimum possible time before melphalan analysis by HPLC.

III.5.4. Tissue samples

Samples of malignant melanoma tissue, as well as normal fat and skin, were excised from accessible sites during and after perfusion from 15 patients (i.e. all patients coming to perfusion with visible nodules during the course of the pharmacokinetic studies).

When tissue was obtained it was immediately placed in a universal container and immersed in liquid nitrogen. Thereafter the tissue was stored at -20°C for the minimum possible time until melphalan assay. At the time of assay, tissue samples were thawed, weighed, minced with scissors and scalpel, then homogenised using an Ultra Turrax homogeniser in a volume of ice cold 0.01M NaH_2PO_4/1mM EDTA at pH3.1. Care was taken to minimise heating of specimens during preparation. Samples were further homogenised using

a Potter 'S' homogeniser. The final volume is measured and duplicate 1ml samples of homogenate from each specimen were used for assay and subsequent analysis was as for plasma specimens, including the use of internal standard.

III.5.5. Melphalan assay by HPLC

The sensitive and specific HPLC assay which we used is based on the method described by Chang and colleagues[438]. 10μg of dansyl proline (Sigma) is added to a one millilitre or less portion of the thawed plasma sample. In the analysis dansyl proline acts as an internal standard, a substance which is chemically similar to melphalan (the losses of which parallel the losses of melphalan) but which generates a distinct peak on the chromatogram.

Four volumes of acetonitrile (BDH, HPLC Grade) with 1% hydrochloric acid (BDH, Analar Grade) are then added to the plasma in 15ml conical centrifuge tubes. The sample is vortex-mixed immediately for 15 seconds and then centrifuged at 2,000 r.p.m. for 10 minutes to precipitate protein. The clear supernatant is then transferred to 30ml vials, and the volume is reduced to 300μl or less under vacuum using a Buchler vortex evaporator (approximately 25minutes at 30°C). The volume of the sample is then made up to 500μl with 20% methanol (BDH, HPLC Grade). The samples are transferred to autosampler vials, sealed and loaded in the autosampler.

The samples are injected by an Altex autosampler (Model 500, Beckman RIIC) which has a 100μl loop (50p.s.i.). An Altex solvent programmer (Model 420, Beckman RIIC) and an Altex solvent pump (Model 100A, Beckman RIIC) deliver the

mobile phase at 1.5ml/minute to a 250x4mm stainless steel column (Waters), packed with μBondapak C18 (Waters). The elution buffer consists of 50ml 0.01M NaH_2PO_4 and 50ml methanol titrated to pH 3.0 with phosphoric acid (BDH, Analar Grade).

Eluted melphalan and dansyl proline were detected by a UV detector (Model LC-UV, Pye Unicam) set at 261nm wavelength. The UV spectrophotometer range was 0.01 or 0.02 A.U. The recorder was set at 1mv and the chart run at 10cm/hr.

The data was processed by a recorder-integrator (Model DP88, Pye Unicam) which measures the areas under peaks on the elution chromatogram. The t_R (retention time) value for melphalan was 6.68 minutes, and for dansyl proline 9.57 minutes.

Standard curves were generated which confirmed the accuracy of the method over the range of melphalan concentrations 0.2-200μg/ml (correlation coefficient, r = 0.9894). The coefficient of variation (standard deviation/mean x 100) for 20 identical samples at 2μg/ml was 4.2%.

III.5.6. Pharmacokinetic analysis

The concentration time curves for bolus administration (groups A & B) were described by bi-exponential curves (Fig.13), fitted by the method of non-linear least squares (fitting by an "in-house" programme based on the Marquhardt[458] algorithm - see Fig.14 and III.6.2.).

To estimate the tissue exposure to (or bio-availability of) melphalan during isolated limb perfusion, the area

under each concentration time curve was calculated for clinical and mock perfusions. The area under the curve (AUC) was calculated by the trapezoidal rule, from time zero to sixty minutes (AUC_{0-60}) for the arterial and venous concentrations in perfusate (AUC_a and AUC_v). The same method was used to calculate the total AUC ($AUC_{0-\infty}$) for systemic exposure to melphalan during and after perfusion (AUC_s).

Incomplete mixing in the immediate phase after injecting the bolus of melphalan resulted in relatively wide variation in the levels measured in perfusate at two minutes. These values (included in Tables 26, 27 & 28) were therefore omitted from graphical illustration and calculations.

Estimates of the tissue uptake of melphalan were calculated using two methods:

a) From the Fick principle[459], the amount of a substance taken up by an organ per unit of time is equal to the arterial level minus the venous level (A-V difference), multiplied by blood flow.

i.e. (1) $$E = (A-V) \times Q$$

when E = extraction rate, A = arterial level, V = venous level & Q = flow rate; assuming that arterial blood is the sole source of the substance.

Considering a controlled system like the isolated limb perfusion circuit, the amount of substance extracted by the limb can be estimated as being equal to the extraction rate multiplied by duration of perfusion. When a series of paired arterial and venous measurements are available, the AUC_a and AUC_v can be introduced into the

equation.

i.e. for melphalan in isolated limb perfusion,

$$(2) \qquad MEL_{ex} = E \times time = (AUC_a - AUC_v) \times Q$$

when MEL_{ex} = amount of melphalan extracted,

E = extraction rate, t = duration of perfusion,

AUC_a = arterial AUC, AUC_v = venous AUC,

and Q = flow rate.

Assuming that Q is constant, and that MEL_{ex} is accounted for by tissue uptake alone.

b) Briele described a semi-quantitative method for estimating the proportion of drug extracted by the perfused limb using the percentage change in AUC with time[450]. It is assumed that the entire AUC_{0-60} is an overestimate of melphalan exposure, because a proportion of administered drug is discarded when the perfusate is washed out at the end of perfusion. To account for this, the area bounded by lines having their origin at the 60 minute point concentration and projected back to the x and y axes is subtracted from the total AUC_{0-60} (cf. Fig. 13). The change in percentage of derived AUC_{0-30} and AUC_{30-60} is used to calculate the percentage of melphalan remaining in the perfusate which is then plotted against time, for comparison with the values obtained in a "mock" perfusion (Figs. 15,16,17).

III.6. RESULTS

III.6.1. Perfusate versus systemic melphalan levels

Tables 26, 27 and 28 show the concentrations of melphalan measured in perfusate and systemic circulation for patients in Groups A, B and C expressed as means with standard deviations and standard errors. Illustrating the data for Group A, Fig. 18 shows that the perfusate levels of melphalan are much higher than systemic levels during and after isolated limb perfusion. Error bars are omitted for clarity of presentation. Table 29 shows the arterial and venous AUC_{0-60}, and the systemic $AUC_{0-\infty}$ for each patient in Groups A,B and C. It is clear that within all three groups the median AUC_s is much lower than the AUC_a or AUC_v. No statistically significant difference was detected between the AUC_s values of Group A versus Group B, or of Group B versus group C (Mann-Whitney). The higher dose regimes are not associated with any increase in systemic exposure (AUC_s).

III.6.2. Pharmacokinetics and Phase I study

Fig. 13 shows the curves describing arterial and venous melphalan levels during isolated limb perfusion in Groups A and B. Again the small error bars are omitted for clarity. The mean arterial and venous melphalan concentration time curves for Group A and Group B can be fitted to lines described by bi-exponential equations of the form:

$$C_t = A.e^{-\alpha.t} + B.e^{-\beta.t}$$

where C_t is concentration at time "t" mins,

A and B are intercepts on the log concentration

axis at t=0mins, of the two linear components of the curve describing logC against time, and α and β are the rate constants of these two components[460].

The fitted lines are illustrated in Fig. 14. The values for the parameters A, B, α and β, are given in Table 30, along with the half-life ($t_{\frac{1}{2}}$) values derived from the fitted lines.

Figs. 15 and 16 show the curves describing melphalan concentrations in perfusate during "mock" perfusion at 37°C and 39°C. Lines were fitted (Fig. 19) to describe these curves according to the mono-exponential equations:

$C_t = 98.4 \times e^{-0.013.t}$ for the 37°C experiment,

where $t_{1/2} = 51.6$ minutes;

and $C_t = 104 \times e^{-0.02}$ for the 39°C experiment,

where $t_{\frac{1}{2}} = 36.6$ minutes.

It can be seen that the half-life of melphalan in the β phase $t_{\frac{1}{2}}\beta$ of clinical perfusions is longer than the $t_{\frac{1}{2}}\alpha$ and it approximates to the values for the half-life times in the "mock" perfusions.

Tables 26, 27, 29 and Fig 13. show that increasing the dose of melphalan from 1.5 to 1.75 mg/kg body weight resulted in higher levels of melphalan in perfusate. Comparing drug exposure in perfusate (Table 29) the values for AUC_a and AUC_v are both significantly greater in Group B than in Group A (p values <0.01, Mann-Whitney).

The regional toxicity for patients in the phase I study is described in Table 31. In group B we saw Wieberdink grade III reactions on two occasions. Since we had not seen such toxicity in either group A or in the pilot group of 26

patients, and because of the toxicity encountered with the 2mg/kg dose in the M.R.C. protocol (II.5.11) it was decided that 1.75mg/mg body weight was close to the maximal dose for our system.

III.6.3. Bolus or divided dose?

Fig. 20 shows the curves describing melphalan concentration after bolus (Group B) or divided dose (Group C) administration of melphalan 1.75mg/kg body weight. From data in Table 29, there is no significant difference between Group B and Group C (Mann-Whitney) in either the AUC_a, AUC_v or AUC_s. Table 31 shows that the incidence of regional toxicity is also similar in Groups B and C.

III.6.4. Calculations of tissue uptake of melphalan

The results for the calculation of MEL_{ex}, according to the Fick principle, in the three groups of patients are given in Table 32, along with the same calculation for a "mock" perfusion at 39°C.

The $t_{\frac{1}{2}}$ of melphalan in human plasma in vitro was 114 minutes at 37°C and 60 minutes at 42°C (see Fig. 21). In the "mock" perfusions the $t_{\frac{1}{2}}$ values (III.6.1.) were less than in vitro incubations (Figs. 21 & 25), suggesting that hydrolysis proceeds more rapidly or that a significant amount of melphalan may be "lost" to the constituents of the circuit and to the cellular components of the perfusate. Assuming that hydrolysis is similar in the three groups, the combined results of the calculations in table 32 suggest that approximately 25-40% of administered melphalan distributes to the tissues of the leg (after

correction for the proportion "lost" to the circuit) during isolated limb perfusion.

The slopes of the concentration time curves after bolus dose administration (Fig. 14, Table 30) indicate that melphalan disappears from perfusate more rapidly in the first half-hour of perfusion. Equation (2) can be applied separately to the AUC_{0-30} and the AUC_{30-60} for Groups A, B and C. Table 33 shows that after bolus administration (Groups A & B) more melphalan is apparently taken up during the first thirty minutes of perfusion than during the second thirty minute period. Melphalan extraction seems to continue during the second thirty minute period, especially after divided dose administration.

The results for calculations based on Group A and B data according to the method of Briele and colleagues[456] are illustrated in Fig. 17. Using this method, the results for Groups A and B were remarkably similar.

III.6.5. Tissue concentrations of melphalan

41 tissue specimens for melphalan assay were taken from 15 patients with recurrent melanoma at 55 minutes during isolated limb perfusion.

The measured tissue levels of melphalan are shown in Table 34. The levels in tumour were significantly higher than levels in surrounding fat ($p<0.01$, Wilcoxon signed rank test), but not significantly different from levels in adjacent normal skin.

III.7. DISCUSSION

III.7.1. Systemic exposure to melphalan

Although there has been increasing interest in the pharmacokinetics of isolated limb perfusion, there has been no previous work which quantifies the total systemic exposure to melphalan resulting from this treatment.

It is important to realise that good surgical technique, including awareness of anatomical variations and thorough dissection of the vessels, help to prevent sudden major "leaks" to the systemic circulation during perfusion. Even with perfect operative technique, however, there is a variable but inevitable escape from the "isolated" limb and this may be due to "leakage" through intra-osseous femoral vessels, vessels passing through the obturator foramen or calcified branches of the cruciate anastomosis which may not be occluded by the tourniquet. After isolated limb perfusion a proportion of the melphalan taken up by the tissues of the limb may diffuse back into bloodstream and be "washed out" into the systemic circulation.

Studies have shown, using radio-labelled albumin, that the measured "leak" from perfusate to the systemic circulation during isolated limb perfusion may be as much as 40%[445,446,450], despite very low systemic melphalan levels during perfusion[446,445]. On such evidence it is claimed that the radio-labelled albumin method simply over-estimates the "leak" of melphalan[445] but this line of argument neglects several important factors. Albumin is a relatively stable large molecule which will tend to remain within the vascular compartment. In contrast melphalan is

continually degraded by hydrolysis in aqueous environments, and the fraction of melphalan which "leaks" from the isolated limb to the systemic circulation will partition within a much larger volume of distribution (intravascular and interstitial fluid). Furthermore some of the melphalan taken up by the tissues (of the perfused leg and the rest of the body) may be protected from hydrolysis by associating with tissue proteins[457,461] and released later, when the melphalan concentration gradients are reversed after perfusion. Thus a significant fraction of the administered melphalan dose could "leak" and be "washed out" from the perfusate to systemic circulation, yet produce only low systemic plasma concentrations.

It is clearly important to establish whether the complex and expensive technique of isolated limb perfusion genuinely achieves its major aim i.e. maximum levels of melphalan in the tumour-bearing limb and minimum systemic exposure. For these reasons we measured systemic melphalan levels during and for several hours after perfusion to determine the total systemic exposure (Tables 26,27,28 & Fig. 18), described by the systemic $AUC_{0-\infty}$ (AUC_S).

The peak concentrations of melphalan were much higher in perfusate than in the systemic circulation in all three groups (ratios of peak perfusate:systemic levels in μg/ml - A 45:0.76, B 75.3:0.53, C 44:0.4).

Recent studies describing the pharmacokinetics of high-dose systemic intravenous melphalan (assay by HPLC) have shown mean AUC values of approximately 400μg.min/ml in adults[448,462,463]. From Table 29 it can be seen that in all our patients the AUC_a and AUC_v are both much higher

than this. In the great majority the AUC_s is much lower, being over 300μg.min/ml in only two patients. The mean ratio of AUC_a/AUC_s in our patients was 16.1/1.
I have therefore shown in this consecutive series, that isolated limb perfusion successfully achieves very high levels of melphalan in the limb perfusate, with little systemic exposure to melphalan during or after perfusion.

III.7.2. Pharmacokinetics of bolus administration

The concentration time curves describing melphalan in perfusate after bolus administration (Fig. 13) are biphasic, conforming to a two compartment model. In groups A and B the $t_{\frac{1}{2}}$ (half-life) for the first, or α phase, was shorter than in the β phase. The concentration time curves for "mock" perfusions were monophasic, conforming to a one compartment model. The $t_{\frac{1}{2}}$ in the β phase of clinical perfusion is similar to the half-life observed throughout "mock" perfusions, when the rate of decay depends on hydrolysis plus losses in the perfusion circuit.
It is inferred that association of melphalan with the perfusion apparatus and cellular components of perfusate account for the difference between the concentration time curves for in vitro incubations in plasma (Fig. 21) and "mock" perfusions (Figs. 15, 16 & 19). Similarly the rapid loss of melphalan from perfusate in the α-phase of clinical isolated limb perfusion is ascribed mainly to uptake by the tissues of the limb.

III.7.3. Phase I study and dosimetry

To take full advantage of the potential benefit of

isolated limb perfusion it is important that the tumour-bearing limb is subjected to the maximal safe melphalan exposure, which is a function of time and concentration and which can be quantified by the perfusate AUC.

There is a trend towards melphalan dosimetry for isolated limb perfusion according to limb volume[318]. The measurement of limb volume by water displacement[400] can be a cumbersome procedure, particularly in older patients, unless there is a suitable safe hoist. There have been no reports describing the accuracy and reproducibility of limb volumetry by water displacement. In a small prospective study, Van Os and colleagues (using 10mg/litre of limb volume) found that the equivalent of 1.77mg/kg body weight could be given safely in their patients (their previous mean dose 1.44mg/kg)[464]. Similar results would have been obtained in their study if the higher doses had simply been administered on the basis of body weight. For patients of "average build" body weight is an acceptable basis on which to calculate dose in isolated limb perfusion, but the use of limb volume dosimetry may be of specific value in the management of patients who have an abnormal habitus, or amputees.

It is generally accepted that a lower total dose of melphalan should be used when perfusing smaller regions of the body e.g. axillary perfusion. In this setting the limb volume method for dosimetry results in doses which are probably inadequate[464] since they are even lower than those calculated on the basis of body weight[390]. Although the rationale is plausible the practical benefits of routine dosimetry by limb volume are unclear.

It has been recommended that other factors to be considered when calculating the dose of melphalan to be administered include complexion and hair colour[465,333] - fair-skinned red-heads being supposedly more susceptible to toxicity than those of a dark complexion but these recommendations have not been validated.

Body weight is obtained easily and reproducibly on the ward, without special equipment. We believed that since no formal phase I study had been carried out using body weight for dosimetry such a study should be completed before adopting routine limb volume dosimetry.

In the phase I study it was found that the dose of melphalan could be increased from 1.5mg/kg body weight (Group A) to 1.75mg/kg (Group B) with an acceptable slight increase in regional toxicity (Table 31). The peak levels of melphalan were higher after 1.75mg/kg than after 1.5mg/kg, and the higher levels were maintained during perfusion. Comparison of the perfusate AUC data for Groups A and B (Table 29) confirms that the higher dose resulted in significantly greater bio-availability of melphalan within the limb, without any increased systemic exposure. It is interesting to note that of the five cases which had an AUC_S value greater than 200μg.min/ml none suffered significant systemic toxicity and, in particular, there was no detected bone marrow toxicity.

In reports of isolated limb perfusion it is remarkable that the volume of perfusate, which critically determines the effective concentration of melphalan, has been so neglected. The volume of prime is easily measured directly but the volume of blood trapped in the limb vasculature

is probably more variable. We have discussed how the volume of priming fluid varies in descriptions of isolated limb perfusion according to different authors (III.3.2). We used a fixed volume of prime and a standard cannulation sequence, but some of the variability in our pharmacokinetic results is likely to be due to the variable volume of trapped limb blood which contributes to the total perfusate volume.

Benckhuijsen and colleagues[444] and Lejeune[445] have recently described elegant methods for estimating perfusate volume (which equals the volume of prime plus the volume of blood trapped in the limb at cannulation). The validity of these methods has not been confirmed, but the attraction is that, based on pharmacokinetic parameters, accurate knowledge of the perfusate volume would allow the administration of a dose calculated to produce a predictable concentration (and AUC) in perfusate. Assuming that cytotoxic effect and toxicity are mainly dependent on melphalan concentration and AUC, this would represent an advance on dosimetry by body weight or limb volume. In an ideal hypothetical situation, this new approach would be combined with knowledge of the relative sensitivities of an individual patient's tumour (and normal tissue) to cytotoxic drugs, allowing "tailored" therapy.

III.7.4. Bolus or divided dose?

Our experience with the M.R.C. protocol for adjuvant isolated limb perfusion led us to question whether the mode of administration (bolus or divided dose) was as

important as total dose in determining regional toxicity, perhaps because of different pharmacokinetics.

While conducting our phase I study we treated two patients according to the M.R.C. protocol for adjuvant isolated limb perfusion. The significant regional toxicity suffered by one patient (Wieberdink grade IV, see II.5.11 and Fig. 11) was associated with high values for AUC_a (3117min.μg/ml) and AUC_v (2797min.μg/ml), after administration of melphalan (2mg/kg) in three divided doses to a total of 180mg (cf. Groups A & B in Table 29). The other patient was given 100mg according to the protocol, producing AUC_a of 1765min.μg/ml and AUC_v of 2359min.μg/ml without any toxicity.

Once we determined that melphalan could be safely given as a bolus dose of 1.75mg/kg body weight (Group B, Table 31), we set out to discover whether divided dose administration at this dose level (Group C) resulted in greater AUC_a and AUC_v values. There was no significant difference in these values between the two groups. However one patient in Group C (total dose 175mg ; AUC_a 2116 min.μg/ml) suffered a severe reaction (Wieberdink Grade IV, see Fig 22).

A possible explanation for these observations is that we routinely use a rather lower priming volume than some of those involved in preparing the M.R.C. protocol[332]. Our method may result in relatively high perfusate concentrations. Were this the case then a relationship between regional toxicity and AUC_a and/or AUC_v would be expected. From Table 29 it can be seen that serious regional toxicity bore no clear relationship to these values. The incidence of regional toxicity, however, is

low in our small series.

I have shown shown that up to 165mg of melphalan can be given safely as a bolus (calculated as 1.75mg/kg body weight) during isolated limb perfusion, and that there is no apparent pharmacokinetic advantage in divided dose administration.

III.7.5. Calculations of tissue uptake of melphalan

It would clearly be desirable to know what proportion of administered melphalan is taken up by the tissues of the leg, and also to discover how that proportion is distributed among the different tissues (especially any differences between benign and malignant). With this knowledge the effects of various manipulations (e.g. changing dose of drug, duration of perfusion or temperature) could be assessed.

It has been suggested that cellular uptake mechanisms for melphalan may be saturable in the perfused limb[444,450].

This is the first study where paired arterial and venous samples of perfusate were obtained for melphalan analysis throughout the hour of isolated limb perfusion. Hence we could use the perfusate AUC data in Formula (2) which is derived as described (III.5.6) from the Fick principle. Uncorrected, the results tend to overestimate the amount of melphalan taken up by the leg (MEL_{ex}) at 45-60% of administered dose. It is important to realise that the melphalan concentration measured is the plasma concentration, and that although hydrolysis is corrected for, melphalan is known to rapidly associate with the cellular components of blood[450,452] in a ratio of

cells:plasma equal to approximately 1:1[444]. During the hour of perfusion a fraction of the administered dose (approximating to the haematocrit as %) partitions to the red blood cells, and this contributes to the 22% correction factor as calculated by applying Formula (2) to the "mock" perfusion (see Table 32).

The uncorrected results for a similar calculation using the AUC_{0-30} and AUC_{30-60} values (Table 33) seem to support the suggestion that melphalan uptake mechanisms in the tissues of the leg are less effective in the second half-hour after bolus administration (Groups A & B), but dividing the dose (Group C) allows continued drug uptake during this period. It is not clear whether this is advantageous, or whether it is preferable to generate very high peaks by bolus administration. Yet another strategy would be to administer the cytotoxic by infusion into the arterial line of the circuit, while slowing the flow rate to maximise the effect of first-pass extraction. Homogeneous mixing of melphalan within the perfusate might be achieved by injecting the drug via the arterial line during the course of one circulation time[400].

Briele and colleagues[450] described a method for calculating the uptake of melphalan semi-quantitatively. Our results (Fig. 17) are similar to those obtained in the original paper[450] although it appears that in our patients (Group A & B) there is continuing uptake of melphalan by the leg in the second half-hour of perfusion. Briele has suggested that the duration of perfusion can be reduced, because no uptake of melphalan by the leg was calculated in the second half-hour, and this was

attributed to a saturable cellular uptake mechanism[450]. There are flaws in this argument, however. Firstly, an initial step in the method of calculating the percentage change in AUC is the subtraction of a portion of the total AUC (III.5.6.), which involves an assumption that the actual concentration of melphalan is unimportant in determining tissue uptake. In fact, in a tumour-bearing limb the effective plasma concentration will govern concentration gradients, which are particularly important determinants of drug diffusion in poorly vascularised tissues e.g. ischaemic tumour nodules. Secondly, it is probably important to maintain effective concentration gradients for at least a minimum time (which will vary depending on the cytotoxic drug used) because of the existence of barriers to penetration of cytotoxics[309], especially when it is known that even intracellular melphalan can egress rapidly down concentration gradients[432].

Estimates of tissue uptake based on changes in perfusate concentration of melphalan (e.g. our Fick based calculations, the work of Briele[450] and Benckhuijsen[453]) are merely indicators of the dynamic situation in the limb as a whole, but the critical question relates to the concentration of melphalan which is achieved at the site of action i.e. tumour cell DNA. The changes in perfusate concentration of melphalan will be, at best, crude indicators of critical events which govern the passage of melphalan from the capillary lumen to the melanoma cell nucleus.

Hence, and because we obtained different results using an

identical method to Briele and colleagues[450], I believe that it is premature to advocate shorter periods of perfusion.

III.7.6. Tissue levels of melphalan

In one previous report melphalan concentrations have been measured in the limb perfusate and tissues of three patients having isolated limb perfusion[448]. The main findings were that more melphalan was detectable in tumour than in subcutaneous fat, and that clinical response may be correlated with tumour concentration of melphalan[448].

Initially, in our studies, it was hoped to obtain sufficient tissue samples for analysis of the time course of tissue levels of melphalan. It has already been explained that many of our patients were referred after excision of recurrent melanoma and very few patients had multiple tumour nodules large enough for assay for melphalan. It was therefore decided to take as many samples as possible at the one time point, towards the end of perfusion. The tissue concentrations of melphalan which we measured seem to be lower than those recently calculated by Benckhuijsen and colleagues[453] but their estimates are derived from a selected sub-group of perfusate time-concentration curves and are expressed in terms of μg/ml of tissue water (approximately equivalent to 60% of tissue mass). Furthermore, as they admit, tumour uptake cannot be calculated from their estimates of total tissue uptake and, as others have shown, distribution within tumour masses may be uneven[309].

We were encouraged that the concentrations of melphalan in

large necrotic tumour nodules were similar to those in well perfused normal tissue like skin, and greater than the concentrations in fat. Furthermore, most of our tissue specimens were obtained during perfusion from the upper part of the thigh or inguinal region which may not be as well perfused as the lower leg and foot. The tissue levels in the distal part may well be higher than those we measured in the proximal part.

III.8. CONCLUSIONS

Isolated limb perfusion successfully and consistently achieves the aim of exposing the tumour-bearing limb to high concentrations of melphalan, while minimising systemic exposure.

Following bolus administration of melphalan the concentration time curve is biphasic, with a $t_{\frac{1}{2}}\alpha$ of 4-8 minutes and $t_{\frac{1}{2}}\beta$ of 33-180 minutes. Melphalan seems to be taken up by the tissues of the leg mainly during the first 30 minutes of isolated limb perfusion. However perfusion should last longer than 30 minutes to maintain the concentration gradients which drive the drug through diffusion barriers to the target cells.

In a phase I study we have shown that, using our standard technique of isolated limb perfusion, melphalan can be given safely in a bolus dose of 1.75mg/kg body weight (up to 165mg total dose).

There was no pharmacokinetic advantage in divided dose administration, and no increased regional toxicity as a consequence of bolus dose administration.

It is encouraging that isolated limb perfusion achieved levels of melphalan in large necrotic tumours which were higher than the levels in fat, and similar to the levels in well-vascularised healthy skin.

CHAPTER IV

IN VITRO STUDIES

IV.1. BACKGROUND

IV.1.1. Introduction

Clinical isolated limb perfusion presents a unique opportunity to alter the physio-pharmacological environment of a tumour in order to maximise the therapeutic effects of the cytotoxic drug used, while minimising systemic toxicity and maintaining the viability of the normal tissues of the limb. Differences in perfusion techniques have evolved around the world empirically (see I.5.2., II.5.8. & 12.) and the importance of resulting variables in the system (e.g. duration of perfusion, concentrations of drug, temperature, pH and P_{O2} in perfusate and tissues) is largely unknown. There have been no adequate clinical studies addressing such questions although isolated limb perfusion has been practised for thirty years[317,318]. Until such trials are reported, we must be guided by the results derived from appropriate experimental models.

It should be possible to exploit the special physio-pharmacological conditions within the limb which may allow us to enhance the anti-tumour effect of melphalan on malignant melanoma cells. In preceding chapters I have described our clinical method of isolated limb perfusion and the associated physio-pharmacology. Simple systemic administration of cytotoxic agents is clearly an inappropriate model for the special conditions associated with isolated limb perfusion.

In this chapter variables which may be of importance in determining the efficacy of isolated limb perfusion are examined in vitro.

IV.1.2. The multicellular tumour spheroid model

In recent years there has been pressure to reduce animal experimentation for ethical as well as financial reasons. Simultaneously there has been a dramatic increase in the application of in vitro tissue culture techniques, and recently there has been more success in the culture of human tumour cells.

For a combination of these reasons, in my efforts to optimise the clinical results of isolated limb perfusion, I have used in vitro models, particularly multicellular tumour spheroids.

Most routine cell cultures are maintained as a monolayer, a two-dimensional sheet of flattened cells, anchored to the base of a plastic or glass vessel and immersed in liquid culture medium. However, three-dimensional aggregates of animal cells have been used in embryology since the original work on morphogenesis by Holtfreter in 1944[466]. Using cellular aggregates formed from single cell suspensions, in 1957, Moscona[467] demonstrated the invasive potential of malignant melanoma cells in vitro. It was subsequently shown that tumour cells had a greater tendency than normal cells to form aggregates[468].

Multicellular tumour spheroids (Fig. 23) are three-dimensional aggregates of tumour cells which take more or less spherical forms. In suspension culture spheroids grow by division of cells in the outer viable rim which is the

part most accessible to nutrients in the liquid culture medium.

In this three-dimensional tumour model, even with small spheroids, there are metabolic gradients[469,470,471,472,473,474], resulting proliferation gradients[475,476,477,478,479,480], and drug penetration barriers[481,482,483]. These features are important characteristics of tumours in vivo which are not apparent in monolayers or single cell suspensions. The growth of multicellular tumour spheroids follows Gompertzian kinetics, like the growth of tumours in vivo[484]. The three-dimensional spheroid system is particularly attractive as a model for the solid tumours, which have generally proved more difficult to treat than other malignancies, and where "resistance" to therapy seems to be partly related to the three-dimensional nature of the primary tumour and its metastases.

Multicellular tumour spheroids can be regarded as a system of intermediate complexity between tumour cells in monolayer tissue culture and similar cells growing as xenografts in vivo. The size, morphology and growth pattern of the multicellular tumour spheroid makes it a particularly attractive model for the avascular micro-metastasis.

Sutherland and associates were first to report the use of "multicellular spheroid" aggregates in experimental radiation therapy[475,485,486,487]. They observed that the spheroids had a similar structure to murine[475,485] and human tumour nodules[488]. The cell survival curves after experimental radiation therapy in this in vitro model

resembled those of solid tumours[475,485]. In recent years the potential of this model for the experimental chemotherapy of human tumour cell lines has begun to be exploited (Table 35).

We have developed the use of the multicellular tumour spheroid model in our studies because this system allows us to simulate the uniquely controlled but abnormal physio-pharmacology which prevails in the limb during isolated perfusion.

IV.1.3. Drug levels and duration of perfusion

Studies of melphalan pharmacokinetics in isolated limb perfusion indicate that the plasma levels of the drug are much higher and more sustained than can be achieved even by high systemic doses with autologous bone marrow rescue (III.6.1.). It is important that the levels of drug used in an in vitro model should be similar to those achieved clinically. The relevant measurement is the level of melphalan in and near tumour nodules which will be related to, but not equivalent to, plasma levels of the drug. In accordance with the results of my own work on plasma and tissue pharmacokinetics of melphalan in isolated limb perfusion (Chapter III), the concentration range of 1-16μg/ml is used in most in vitro experiments described in this chapter.

Our routine for clinical isolated limb perfusion involves a one hour exposure to melphalan but the optimum time/concentration relationship has not been established. Tissue pharmacokinetics are dependent on plasma pharmacokinetics but distribution within the different

tissues is variable (III.6.4.). The efficacy of melphalan depends upon the formation of chemical cross-links, a process which reaches a maximum several hours after exposure to the drug[423]. Hence the relationship between duration of exposure to melphalan in isolated limb perfusion and the resulting cytotoxic effect is complex. There have been no previous studies describing the relationship between duration of exposure to melphalan and cytotoxic effect. As a base-line it is therefore appropriate to consider the effect of varying the duration of exposure in an in vitro model which is based on, but simpler than, clinical isolated limb perfusion.

IV.1.4. Melphalan and temperature

As demonstrated for our patients (II.4.4., Table 21), the temperature of the limb is usually less than 37°C before isolated limb perfusion. In 1960 Rochlin[325] showed that without active heating the limb temperature drops further during isolated limb perfusion. From elementary physiology it can be anticipated that low perfusate temperatures would be associated with vasoconstriction, increased perfusate viscosity, and poor perfusion of the tumour-bearing limb. The cellular uptake of melphalan by active carrier[427,428,429] would also be reduced in hypothermic conditions. To minimise these effects it seems appropriate to perfuse the limb at a temperature close to the physiological normal of 37°C.

As discussed in I.5.2., the value of heating the limb above the "normal" physiological temperature of 37°C, i.e. hyperthermia, in isolated limb perfusion with melphalan is

controversial and there is no definitive clinical study to indicate the optimal temperature conditions. Although widely quoted[213,350,364] as demonstrating the advantage of hyperthermic isolated limb perfusion, Stehlin's work[327,329] has already been criticised in this thesis (I.5.2.). Furthermore, normothermic perfusion has been reported to achieve response rates as high as hyperthermic isolated limb perfusion[344].

The rationale for hyperthermic isolated limb perfusion with melphalan is founded on the selective sensitivity of malignant cells to heat, and on the enhancement of melphalan cytotoxicity by heat. Heat has a long history in the treatment of cancer. Direct applications of red hot irons were used in the treatment of cutaneous cancers in the times of Ramajama (2000BC), Hippocrates (400BC) and Galen (200AD)[489]. The evolution of modern methods of hyperthermia for cancer treatment has been documented[326,489,490,]. Giovanella[491] has summarised the experimental evidence that malignant cells are more sensitive than normal cells to hyperthermia.

The combination of hyperthermia plus regional chemotherapy was first used to enhance the cytotoxic effect on tumours in 1960[492]. Shingleton and colleagues[493] used regional hyperthermia to enhance the local action of cytotoxic drugs (including melphalan) along with systemic hypothermia to reduce marrow toxicity. Suzuki[494] indicated that regional perfusion systems were particularly suitable for administering hyperthermia. He observed that tolerance to heat was likely to be variable depending on the relative sensitivity of tumour and normal tissues, and

that certain labile drugs may be inactivated by heat.

When used clinically in the treatment of cancer, regional perfusion with hyperthermia alone (up to 45.8°C, for up to 6 hours 50 minutes) produced some encouraging responses but was associated with unacceptable morbidity and mortality[326]. Damage to normal tissues was due to a combination of poor perfusion (low flow rates), direct hyperthermic damage and haemolysis. Some reports have shown no systematic difference in heat sensitivity comparing normal and malignant cells[495,496]. In other studies where malignant cells have been shown to be more sensitive than normal cells to heat[329], or heat plus melphalan[497,498,499] the normal cells were not those of the tissues which prove dose-limiting in isolated limb perfusion. In an elegant biochemical study of isolated perfusion Ghussen and Isselhard[500] have shown that damage to the muscle of the canine limb is dose-limiting at temperatures greater than 43°C for one hour.

It is paradoxical that melphalan should be used so widely in hyperthermic isolated limb perfusion because melphalan is a labile substance which is hydrolysed to inactive metabolites more rapidly at higher temperatures[419,438]. Indeed, Cavaliere[501] has stated that melphalan becomes ineffective at temperatures greater than 41.8°C. In spite of this, some studies using in vitro assays have indicated that hyperthermia may enhance melphalan cytotoxicity, especially at temperatures over 42°C[497]. Furthermore, in an in vivo study of thermochemotherapy (41°C) with various cytotoxic drugs, an absolute therapeutic gain was found only for melphalan[498] and this gain could not be explained

fully by changes in melphalan pharmacokinetics[499].

Since the relationship between temperature and melphalan cytotoxicity is controversial, I set out to explore the interaction using a controlled in vitro system (to circumvent any vascular effects of temperature or melphalan) and conditions which were closely modelled on those which occur in isolated limb perfusion for melanoma.

IV.1.5. Melphalan and verapamil

Verapamil is a phenylalkylamine which blocks the transmembrane flux of calcium ions (calcium channel blocker) and it is best known clinically as a vasodilator and anti-arrhythmic[502]. Verapamil is a potent vasodilator which increases tumour blood flow in tumour-bearing rats[503]. It is interesting that in mice bearing experimental fibrosarcomas[504,505] and in human melanoma xenografts[505] verapamil appeared to enhance melphalan cytotoxicity without influencing the fractional distribution of cardiac output. It was shown that verapamil altered the pharmacokinetics and increased tumour uptake of melphalan[505]. The vaso-active drug hydralazine has also been shown to selectively enhance the cytotoxic effect of melphalan in vivo, perhaps by reducing tumour blood flow and decreasing the rate at which melphalan is cleared from the tumour tissue[506].

Cancer cells may be inherently resistant to a cytotoxic drug or they may acquire resistance by exposure to the drug. Verapamil has been shown to partly overcome acquired resistance to chemotherapeutic agents, and to a lesser degree, to potentiate the efficacy of these drugs against

sensitive cells[507]. Verapamil reduces acquired adriamycin-resistance in human lung cancer cell lines[508] and in human glioma cell lines[509], probably by blocking the active efflux mechanisms which drive cytotoxic drug from the cell. Verapamil may also enhance the chemotherapeutic efficacy of adriamycin and thiotepa against human bladder cancer cell lines[510], and it may increase the efficacy of vincristine against primary human colorectal cancer tissue[511].

Unfortunately, for systemic chemotherapy, the concentration of verapamil required for the anti-resistance effect may be higher than the maximum prolonged plasma concentration which can be safely maintained clinically (0.45μM)[512]. In isolated limb perfusion it should be possible to use much higher levels of verapamil in the perfusate, albeit for shorter time periods.

I chose to study the possible interaction between verapamil and melphalan in vitro, and hence in a system where vascular effects are absent.

IV.1.6. Melphalan and pH

Melphalan is more stable in acidic conditions[410] and we found that the perfusate tends to alkalosis during our standard isolated limb perfusion (II.4.4.). pH has been shown to influence both the cellular uptake of cytotoxic drug[483] and the sensitivity of tumour cells to hyperthermia[513]. pH may also affect the affinity of melphalan for cellular and plasma proteins. Since pH can be adjusted easily during isolated limb perfusion, I studied the effect of pH on melphalan cytotoxicity in

vitro.

IV.1.7. Solvents and amphotericin-B

Solvents used to prepare anticancer drugs may have a cytotoxic effect on cells in culture e.g. ethanol, dimethyl sulphoxide[514]. Amphotericin-B (Fungizone, Gibco) which is routinely used in culture media has been reported to have a cytotoxic effect on mammalian cells at 43°C[515] and may enhance the cytotoxic effect of anti-cancer drugs[516]. Therefore specific in vitro experiments were designed to take account of these variables.

IV.2. AIMS

The aims of my in vitro studies were:

1) to grow multicellular tumour spheroids from established human malignant melanoma cell lines.

2) to minimise experimental artefact and compare the results of experimental chemotherapy with melphalan in conditions analogous to those in clinical isolated limb perfusion.

3) to study the influence of a) temperature, b) time of exposure, c) verapamil, and d) pH on melphalan cytotoxicity in the human melanoma spheroid model.

4) to compare the results of the spheroid assay, with the results of standard colony forming assays using disaggregated spheroids and monolayers of the same cell line.

IV.3. MATERIALS AND METHODS

IV.3.1. Cell lines

The cell lines used in these studies were B0008, B0010 and MEL57. All three are established human malignant melanoma cell lines. The characteristics of B0008 and B0010 (originally denoted Hs294T and Hs852T, respectively) were first described by Creasey and colleagues[517] of the University of California, Berkeley, U.S.A., from whom the cell lines were obtained. MEL57 was originally provided by Dr. C. Sorg of the Universitats Hautklinik, Munster, West Germany. All three cell lines are commonly used in our own and other laboratories, and the cells have been karyotyped (to confirm human origin), confirmed DOPA-positive (melanocytic) and regularly checked to be mycoplasma-free.

IV.3.2. Routine cell culture conditions

In these experiments all cell lines were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with foetal calf serum (10%), L-glutamine, penicillin, streptomycin and amphotericin-B (Table 36). The antibiotics and anti-fungal are added routinely because sepsis can be a major problem in experiments involving multiple manipulations and prolonged culture.

Strict aseptic technique was rigorously observed in preparing and performing each stage of all tissue culture experiments. Any infected or possibly infected experiments were abandoned, and the results are not reported.

IV.3.3. Formation of spheroids

An outline of the spheroid experiment protocol is

illustrated in Fig. 24. Cells were harvested from near confluent monolayers after initial washing in Earle's salts (Gibco). The cells were lifted off with 0.25% trypsin (Gibco) in Earle's salts, the trypsin was neutralised with supplemented MEM, and the cells were dispersed by repeated pipetting.

The resultant cell suspensions were used to initiate spheroids, by the "agar underlay" (also known as "liquid overlay") static method[525,526]. Cells are seeded at high density ($5x10^5$ cells per flask) in 5ml of supplemented MEM into $25cm^2$ flasks (Nunclon), which were previously base-coated with 5ml 1.25% Noble agar (Difco) in supplemented MEM. The mixed agar/MEM coating does not allow cells to adhere to the surface of the tissue culture vessels and should prevent leaching of nutrients from the liquid MEM. Flasks were thereafter incubated in an atmosphere of 7% CO_2 at 100% humidity. In cell lines which form spheroids, small spheroids (approximately 200-250μm) usually form within a few days, ready for experimental treatment.

IV.3.4. Experimental chemotherapy: drugs, pH

Melphalan (Alkeran Injection, Wellcome) was obtained from the manufacturer (in gram quantities from a single manufacturing run) as the sterile anhydrous base (>98% pure drug), as supplied for intravenous administration, and 50mg aliquots were prepared and stored. For each experiment the drug was prepared freshly as for clinical administration. The powdered melphalan base was rapidly dissolved in the manufacturer's solvent (hydrochloric acid and ethanol) to produce the hydrochloride salt

immediately prior to use. This solution was then diluted in the supplied diluent (propylene glycol-K_2HPO_4 buffer) to give 10mg/ml. Thereafter the solution was passed through a non-absorbent 0.2μm filter (Flowpore D, Flow) and serially diluted in MEM to appropriate final concentrations. Melphalan was always made up freshly before use, put on ice and added to cultures within ten minutes.

In separate control incubations at 37°C and 42°C the concentration of melphalan in supplemented MEM was measured by HPLC (Figs. 25 & 26). Thus the effects of temperature and pH on melphalan decompostition were studied in vitro, in conditions similar to those of spheroid treatment.

Verapamil was obtained as the pure anhydrous drug (Cordilox Injection, Abbott). The drug was freshly dissolved for each experiment in phosphate buffered saline (Dulbecco's, Gibco), passed through a 0.2μm filter, and then diluted serially to appropriate concentrations in MEM.

For studies of melphalan cytotoxicity in acidic and alkaline conditions, supplemented MEM was titrated to pH7 with 1N HCl, and to pH8 with molar NaOH. In experiments controlled for the presence of spheroids and melphalan a pH meter (Pye Unicam) was used to confirm that the desired pH was maintained throughout the period of drug exposure.

IV.3.5. Experimental chemotherapy: spheroids

Three or four days after initiation, the spheroids from several flasks were pooled, allowed to sediment in

Universal containers (Sterilin) and single cells were removed with the supernatant. The spheroids were resuspended in fresh MEM, sorted into equal amounts and transferred into thin-walled glass scintillation vials. The volume in each vial was made up to 5ml with fresh MEM. Freshly prepared solutions of melphalan (prepared as above) in MEM were added to each vial in a 5ml volume to give an appropriate range of final concentrations (based on levels achieved clinically in isolated limb perfusion). The vials were sealed with "Parafilm" (American Can Co.) and immersed in a water bath (Grant) at a pre-set temperature between 31-44.5°C (±0.1°C). The vials were then maintained in controlled temperature conditions in the insulated water-bath for varying time periods. During the period of treatment the vials were shaken at ten minute intervals by hand.

At the end of the period of treatment the spheroids were transferred to Universal containers, and allowed to sediment. The medium with drug was immediately removed and the spheroids were then washed, three times, in ice cold MEM. The spheroids were then resuspended in MEM at approximately 37°C and decanted into 5cm tissue culture Petri dishes (Flow).

Under laminar flow conditions, individual spheroids of approximately 250μm diameter were selected under low power microscopy (x40, Olympus inverted microscope, Model CK). Using a Pasteur pipette and Pi-pump (Shuco International), one spheroid was transferred from the Petri dish to each well of a 24-well plate (Corning 25820). The wells had been previously base-coated with 1% agar in MEM, and

each contained 0.5ml liquid MEM. The multi-well plates were incubated thereafter at 37°C in an atmosphere of 7% CO_2 at 100% humidity. Once a week 0.5ml of fresh supplemented MEM was added to each well.

IV.3.6. Measurement of spheroid growth

In spheroid experiments there were usually six treatment groups, with 24 spheroids in each group. Each individual spheroid in every well of the multi-well plates was measured two or three times weekly. The cross-sectional area of each spheroid was measured using a "Micromeasurements" image analysis system coupled via a television camera to an inverted optical microscope[527]. Area measurements were subsequently converted to volumes, assuming spherical geometry, using an "in-house" computer program.

IV.3.6. Spheroid disaggregation

After the transfer of selected spheroids to multi-well plates approximately two hundred spheroids remained in the Petri dishes. The remaining spheroids were transferred in MEM to fresh agar base-coated flasks and incubated for a 24-hour recovery period before disaggregation for colony forming assay. The spheroids were allowed to sediment in Universal containers and the supernatant MEM was removed. The spheroids were washed with Earle's salts, then after 5-10 minutes incubation with 0.125% trypsin in Earle's salts at 37°C, the trypsin was removed and replaced with fresh MEM. The spheroids were mechanically disaggregated to a single cell suspension by repeated

pipetting and colony forming assays were then performed as for the cells from monolayers (IV.3.8.).

IV.3.7. Treating monolayers

Cells were seeded at $5x10^4$/ml in 5ml supplemented MEM and placed in replicate 25cm² flasks (same concentration of cells in 15ml MEM in 80cm² flasks for higher drug doses and temperatures). The flasks were incubated at 37°C for three days. At this stage, with the cells in the exponential growth phase, the MEM was decanted and replaced with MEM containing freshly prepared melphalan at concentrations similar to those used in the spheroid experiments. The flasks were sealed with "Parafilm" and immersed in the same water bath as spheroids at controlled temperatures, usually for one hour. The flasks were shaken by hand at ten minute intervals during drug exposure.

Following exposure to melphalan the monolayers were washed three times with ice cold medium, then replenished with MEM at 37°C and returned to the incubator for a 24-hour recovery period. The MEM was then decanted, the monolayers rinsed with Earle's salts and, after 5 minutes exposure to 0.125% trypsin in Earle's salts at 37°C, the cells were harvested and the trypsin was neutralised with fresh MEM. From each treated monolayer a single cell suspension was obtained for colony forming assay by repeated pipetting.

IV.3.8. Colony forming assays

The single cell suspensions (from disaggregated spheroids or monolayers) were diluted and seeded at 1000 cells per 5ml MEM in 60x15 tissue culture Petri dishes (Nunclon

Delta) for controls and most treatment groups. For some of the higher drug concentrations and higher temperatures, more cells were seeded (at approximately the same cell density) in larger dishes. Four replicate plates were set up for each treatment group. Each plate was scrutinised at day 0 to ensure that single cell suspensions were plated. After incubation at 37°C for 10-14 days in an atmosphere of 7% CO_2 and 100% humidity, the MEM was decanted and colonies fixed and stained with a 1:10 solution of carbol fuchsin ('Ziehl Neelson', RA Lamb) in distilled water. Colonies of at least 40 cells were counted and the average plating efficiency for the untreated cells of the B0008 cell line was 12%. Following convention, the plating efficiency of the untreated cells was normalised to 100% and the results for treated cells were expressed as a percentage of control clonogenic survival.

IV.3.9. End-points and analysis

An advantage of the spheroid model is the fact that several end-points can be derived from the experimental data[528,529].

Serial measurement of spheroid volume allowed the construction of growth curves for the various experimental groups, taking median spheroid volume as the dependent variable for each group. From the spheroid growth curves the time for the median spheroid volume to reach 10x the original volume can be obtained and this is termed the growth delay (for control groups) or the regrowth delay (of treated groups).

The growth curve of each individual spheroid in a plate

was used to calculate the median regrowth delay data. The computer program calculated 95% confidence limits about the median volumes and regrowth delay data, with allowance for small sample size (n<30).

At higher doses of effective treatments an increasing proportion of spheroids fail to regrow during the period of observation. The proportion of spheroids which fail to regrow can be expressed as the percentage "cured"[529]. This proportion tends to increase with the more effective treatments, and "cured" spheroids cannot simply be excluded from an analysis of results. In our studies "cured" spheroids were assigned an arbitrary regrowth delay of 1000 days (to prevent inappropriate exclusion from calculations of regrowth delay).

Clonogenic cell survival was estimated directly by disaggregating spheroids and using conventional colony forming assays. An estimate of clonogenic cell survival in intact spheroids can also be derived from the regrowth curves of treated spheroids by the process of back-extrapolation[530,531]. This method requires that regrowth curves of treated spheroids should eventually return to the same growth pattern as control spheroids (Fig. 27). The surviving fraction (S) of clonogenic cells is taken to be the ratio of the volume obtained by back-extrapolation of the regrowth curve (V_E: the volume from which the spheroid "apparently " regrew) to the volume actually measured on day 0 (V_0).

i.e. $$S = V_E \div V_0$$

and $$\log S = \log V_E - \log V_0$$

This method does not entail disruption or explantation of the spheroid and should be free from artefacts due to such manipulations.

IV.4. RESULTS

IV.4.1. General

The results for each experiment are given as tables showing values for the growth curves (median log volumes with 95% confidence intervals), regrowth delay, and "% cured" (Tables 37A-62C); together with graphs of spheroid growth curves and regrowth delay (Figs. 28A-52B) For clarity of presentation the confidence intervals are omitted from figures showing growth curves.

IV.4.2. Control Experiments

Experiment 1 (See Tables 37A,B,C; Figs. 28A & B)

Pilot studies were conducted with B0008 melanoma spheroids and the results confirmed that, in keeping with measured tissue levels (see Chapter III), an appropriate melphalan concentration range for one hour exposure at 37°C would be 0-16μg/ml. Experiment 1 was also designed to show any cytotoxic effect of the Alkeran solvent (acid/alcohol) or the solvent/diluent mixture at concentrations equivalent to those in melphalan 32μg/ml.

Experiments 2 & 3 (See Tables 38A,B,C, 39A,B,C; Figs. 29A,B, 30A & B)

Experiment 2 was conducted to determine whether amphotericin-B (a routine additive in supplemented MEM) was cytotoxic to B0008 melanoma spheroids at 37°C or if there was any significant interaction between amphotericin-B and melphalan at 37°C.

Experiment 3 was designed to show whether amphotericin-B or the Alkeran solvent was cytotoxic at 43°C, either independently or in combination.

IV.4.3. B0010 cell line

Experiment 4 (see Tables 40A & C; Fig. 31)

B0010 cells formed typical "tight" multicellular tumour spheroids but all groups grew very slowly during the period of observation.

IV.4.4. MEL57 cell line

Experiment 5 & 6 (see Tables 41A,B,C, 42A,B,C; Figs. 32A,B 33A,B)

The MEL57 cell-line seemed to form less tightly packed spheroids than either B0008 or B0010. These spheroids grew more rapidly than the B0008 spheroids, and the regrowth delay of control MEL57 spheroids was significantly less.

Experiment 6 was a study of thermo-chemotherapy with melphalan in the MEL57 spheroid model, using 41°C incubations.

IV.4.5. Melphalan and temperature

Experiments 7, 8 & 9 (see Tables 43A,B,C, 44A,B,C 45A,B,C & Figs. 34A,B, 35A,B, 36A,B)

In these experiments B0008 spheroids were treated with melphalan, for one hour in hypothermic conditions at temperatures relevant to clinical isolated limb perfusion (31°C and 35°C).

Experiments 10, 11 & 12 (see Tables 46A,B,C, 47A,B,C, 48A,B,C; Figs. 37A,B, 38A,B, 39A,B)

B0008 spheroids were treated with melphalan for one hour in normothermic conditions (37°C).

Experiments 13-19 (see Tables 49A,B,C, 50A,B,C, 51A,B,C, 52A,B,C, 53A,B,C, 54A,B,C, 55; Figs. 40A,B, 41A,B, 42A,B,

43A,B, 44A,B, 45A,B)

B0008 spheroids were treated with melphalan for one hour over a range of clinically achievable hyperthermic temperatures from 39°C to 44.5°C.

IV.4.6. Melphalan and incubation time

Experiments 20 & 21 (see Tables 56A,B,C 57A,B,C; Figs. 46A,B, 47A,B)

B0008 spheroids were exposed to melphalan at 37°C for time intervals from 15 minutes to 2 hours.

Experiment 22 (see Tables 58A,B,C; Figs. 48A,B)

This experiment was performed as Experiments 7 and 8 except that controls and drug exposures were incubated for 30 minutes rather than one hour.

IV.4.7. Melphalan and verapamil

Experiments 23 & 24 (See Tables 59A,B,C, 60A,B,C; Figs. 49A,B, 50A,B

The influence of verapamil on melphalan cytotoxicity in the B0008 spheroid model was assessed in these duplicate experiments.

IV.4.8. Melphalan and pH

Experiments 25 & 26 (See Tables 61A,B,C, 62A,B,C; Figs. 51A,B, 52A,B

Melphalan cytotoxicity and the effect of pH during drug incubation was studied using large spheroids (Experiment 25) and small spheroids (Experiment 26).

IV.4.9. Comparison of assays

The "percentage cured" data from Experiments 7-19 are summarised in Fig. 53. In this graph each point represents the percentage of spheroids "cured" in a single plate. The lines join the values from separate plates in the same experiment.

The dose/survival curves for thermochemotherapy with melphalan in the B0008 cell line are illustrated, as derived:

a) by colony forming assay of disaggregated spheroids (Fig. 54),

b) by back-extrapolation from the spheroid growth curves (Fig. 55) and

c) by colony forming assay of cells treated as exponential phase monolayers (Fig. 56).

In Figs. 54 and 56 each point represents the mean of four replicate plates for each experiment. The lines link points derived from the same experiments.

In Fig. 55 each point is the result of a single estimate of surviving fraction, according to the method of back-extrapolation (IV.3.9. & Fig.27).

IV.5. DISCUSSION

IV.5.1. Model systems for isolated limb perfusion

In any model system the aim is to reproduce clinical conditions as closely as possible. In vivo, the ideal representation of clinical isolated limb perfusion would entail the use of isolated limb perfusion in an appropriate animal model because systemic administration of a drug is associated with quite different physio-pharmacological conditions. During isolated perfusion high doses of cytotoxic drug are given in order to achieve and maintain high concentrations within the perfused region, and the major organs of excretion play little role in handling melphalan.

In vivo studies of the physiology of isolated limb perfusion have been performed in the dog[411,532,533], and rat[534,535]. A rabbit model, originally described by Mori in 1962[536], has been used recently to study the pharmacokinetics of 5-fluorouracil in various forms of regional chemotherapy (including isolated limb perfusion) for intramuscular VX-2 carcinoma in the hind limb[377].

The larger mammal models are closer analogies to clinical perfusion than rat and murine systems, but there is a lack of a representative melanoma tumour and the financial costs of large animals are high. There are several murine melanoma cell lines but the technical demands of cannulating, perfusing and repairing small mammal vessels are obvious. In theory it should be possible to grow human melanoma xenografts on nude rats which might be treated by isolated perfusion. Survival experiments, with serial measurement of treatment efficacy are feasible but they

would require great technical expertise and would inevitably be time consuming and costly.

In fact, there have been no definitive studies of the therapeutic results of different methods of isolated limb perfusion for malignant melanoma in an animal model.

Implanted animal tumours (in syngeneic hosts) and human tumour xenografts (in immuno-deficient animal hosts) may be used to represent spontaneous tumours and metastases but they have their limitations[537]. There is increasing recognition of inter-species differences in metabolism and immunology, and the results of experiments in in-bred laboratory animals cannot be directly extrapolated to the clinical situation.

The particular advantages of using in vitro models include the fact that a greater number of standard experimental "tumours" can be studied than would be practical clinically or in animal experiments. The physio-pharmacological conditions of clinical isolated limb perfusion can be better reproduced and are easier to maintain in vitro than after systemic administration in vivo. Many variables, including those associated with host metabolism, immunology and physiology; the "tumour bed effect", host cell infiltration and oedema may be circumvented by using in vitro models[530].

The effects of therapeutic manipulations can be studied at the cellular level using various types of cell culture.

The primary culture from a human tumour biopsy is initially composed of a heterogeneous tumour cell population, of low growth fraction. Previously it was hoped that testing of primary cell cultures using the

Human Tumour Stem Cell Assay[538,539] would provide chemosensitivity information about an individual patient's tumour analogous to the information obtained about bacteria by antibiotic sensitivity testing. This system has not yet fulfilled its promise and there are important criticisms of the use of stem cell assays for predicting tumour sensitivities[540,541,542,543,544].

After passage or sub-culture the cell line expands and the growth fraction tends to increase, with the provision of large amounts of consistent tumour-derived material. An established or continuous cell line can be maintained in culture indefinitely but not all tumour cell lines become continuous, and some die out after a few subcultures (finite cell lines). Continuous cell lines are attractive sources of experimental material because they are easily maintained, but they are necessarily derived from a selected subpopulation and may not be entirely representative of the original parent tumour.

In common with most other workers in the field we grew spheroids from continuous cell-lines for use in this series of experiments. Cells from early passages of human tumours, including malignant melanoma[545], have been used to form and study spheroids. These early passage cells may prove to be better than continuous cell lines as indicators of how an individual patient's tumour will behave, but the prediction of chemosensitivity was not an aim of my work.

IV.5.2. Limitations of in vitro systems

When considering the results obtained from any in vitro

model there are important limitations of such systems, which should caution against direct extrapolation to the clinical situation. The major limitation, common to most vitro methods, is the lack of an assessment of normal tissue response to treatments. Furthermore certain physio-pharmacological conditions may tend to promote tumour sensitivity to a drug at the cellular level in vitro, but in the more complex clinical setting such effects may be enhanced or negated by the simultaneous effects of the same conditions on other systems e.g. tumour (or normal tissue) vasculature. With these provisos, the results of in vitro experiments are indicative, but not predictive of what may happen in vivo and clinically.

IV.5.3. Control experiments

The alcohol in the proprietary acid/alcohol melphalan solvent is diluted more than 3000-fold (when equivalent to melphalan 32μg/ml), and the acid is buffered when the diluent is added. Thus neither acid nor alcohol would be expected to have a significant cytotoxic effect on cells in these experiments or clinically.

In Experiment 1 the control growth curves were very similar suggesting that the solvent and solvent/diluent had little cytotoxic effect. Regrowth delay of spheroids treated with the solvent with or without diluent was similar to that of control spheroids. There were no differences in the "percentages cured" comparing these groups.

In the spheroids treated with 6μg/ml melphalan there is a period of little or no growth followed by regrowth of the

spheroids i.e. regrowth is delayed. At higher concentrations of melphalan there is little or no increase in the median spheroid volume during the month of the experiment. This is reflected in the long regrowth delay values and can be quantified by the proportions of spheroids "cured".

In Experiment 2 amphotericin-B (Fungizone, FZ) did not affect spheroid growth and had no significant effect on melphalan cytotoxicity.

The results of Experiment 3 showed no significant difference between the subsequent growth of spheroids incubated for one hour at 37 or 43°C in MEM with (+FZ) and without amphotericin-B (FZ-free). In this experiment exposure to 43°C and a high concentration of acid/alcohol solvent, with and without amphotericin-B, had no significant effect on subsequent spheroid growth.

The lack of a measurable effect due to amphotericin-B is probably because of the low concentration (2.5μg/ml) used in these spheroid experiments.

IV.5.4. B0010 spheroids

In Experiment 4 the B0010 cell line formed typical spheroids but they grew poorly as compared with the B0008 and MEL57 cell lines. A high percentage of the B0010 spheroids failed to increase volume significantly during the course of the experiment and this is illustrated by the flat profile of the growth curve (note the different y-axis on Fig. 31) and the high "percentage cured". Regrowth delays could not be calculated because most of these spheroids failed to increase their volume ten-fold.

Interestingly the B0010 cell line also gives rise to a much less aggressive tumour in vivo compared with the B0008 cell line[517].

IV.5.5. MEL57 spheroids

In Experiments 5 & 6 the regrowth curves of treated MEL57 spheroids seem to follow a different pattern as compared with the regrowth curves of treated B0008 spheroids. With increasing doses of melphalan or hyperthermia the spheroids grow at progressively slower rates. Hence the regrowth curves do not run parallel with the control curves and the method of back-extrapolation (IV.3.9. & Fig.27) cannot be applied to derive dose-survival curves from the spheroid growth data.

Large multinucleated "giant" cells were noted on microscopy of treated MEL57 spheroids, but they were seen only rarely in MEL57 controls and never in B0008 or B0010 spheroids. In the more heavily treated spheroids the giant cells comprised a greater fraction of the total cell population. Similar appearances have been described after experimental radiotherapy of lung cancer spheroids, and it has been postulated that such giant cells may be lethally damaged but metabolically active cells[546]. Such cells may distort regrowth curves in vitro but they might not be expected to survive in vivo.

In my experiments with MEL57 spheroids the cytotoxic effect of melphalan seemed to be enhanced by incubation at 41°C, but this was not statistically significant at all doses of melphalan.

IV.5.6. B0008 spheroids, hypothermia and melphalan

In Experiments 7-18 the B0008 human melanoma multicellular tumour spheroid model has been used to examine the influence of temperature on melphalan cytotoxicity at the cellular level, in a system which is independent of vascular effects. The growth curves and regrowth delay data are discussed first. In a separate section the "percentages cured", and dose/survival curves are considered (see IV.5.13.).

Experiments 7 and 8 were conducted in identical conditions (31°C incubations) and though the pattern of results is similar, the spheroids in Experiment 8 seemed less responsive to melphalan. In general the spheroids in Experiment 8 were significantly smaller than in those in Experiment 7. The smaller spheroids might be expected to be more sensitive to treatment (cf. experiments 25 & 26) but the regrowth delay of control spheroids was longer in Experiment 7 than in Experiment 8. Indeed the regrowth delay of control spheroids in Experiment 7 was longer than that of control spheroids in most of the other experiments involving B0008 spheroids.

The growth curves and regrowth delay data of Experiments 7-9 suggest, but fail to show statistically, that hypothermia significantly reduces melphalan cytotoxicity in vitro. There was certainly no evidence that hypothermia enhanced melphalan cytotoxicity by reducing the rate of its hydrolysis.

IV.5.7. B0008 spheroids, normothermia and melphalan

Most tissue culture and in vivo experiments are conducted

at 37°C, yet this is not the "normal" temperature of the skin of the extremities either before or during isolated limb perfusion (cf. Table 21, II.5.12.)

Experiment 10 was performed early in the series, several months before Experiments 11 and 12 which are duplicates. The median spheroid volume was less in Experiment 12, yet the smaller spheroids seemed to be more resistant to melphalan.

There was a linear relationship between regrowth delay and melphalan concentration at 37°C in the concentration range studied.

IV.5.8. B0008 spheroids, hyperthermia and melphalan

Experiments 13 and 14 (39°C) were duplicate experiments, although the spheroids in Experiment 13 were larger on Day 0. Again the smaller spheroids appeared less sensitive than the larger spheroids.

In the three experiments conducted at 42.5°C, (Experiments 16,17 & 18) the regrowth delays were significantly longer per unit dose of melphalan than those following incubations at 41°C (Experiment 15) or lower.

There was no evidence that the cytotoxic effect of melphalan was diminished at the higher temperatures.

In Experiment 18, two control plates were included, in one the spheroids were selected and "picked off" immediately after experimental treatment and in the other the process of "picking-off" was delayed for two hours. This was done to confirm that there was no bias due to the differing time interval between treatment and selection. In any experiment this delay was never more than two hours. This

delay had no significant effect on the growth curve or regrowth delay of spheroids in the relevant plates in Experiment 18.

Hyperthermia alone showed no significant cytotoxic effect in the spheroid system until 44.5°C was reached (Experiment 19), when growth curves could not be plotted because greater than 50% of control spheroids incubated at 44.5°C failed to regrow. In this particular experiment the results could only be described in terms of "percentage cured" (Table 55, Fig. 53).

In vivo hyperthermia is associated with vasodilatation and increased flow in the vessels of normal tissues. Tumour blood vessels are less responsive, may dissipate heat less well, and this may result in more damage to tumour tissue[547]. Furthermore, vascular damage and thrombosis may be caused by hyperthermia, and this may explain the greater cytotoxicity of heat in vivo compared with in vitro[548,549]. Murray and colleagues have recently emphasised the importance of the vascular response to chemotherapy by showing that a significant proportion of tumour shrinkage may be due to loss of vascular volume[550]. My spheroid experiments have shown that the cytotoxic effect of melphalan is significantly enhanced by hyperthermia, in a system which is not dependent on vascular effects.

IV.5.9. Melphalan and duration of exposure

The results of Experiments 20 & 21 show the near-linear relationship between duration of exposure to melphalan at 37°C and the regrowth delay of treated B0008 spheroids

(Figs. 46B & 47B).

The median initial spheroid volume was similar in Experiments 7 and 22. All half-hour exposures were associated with a significantly reduced cytotoxic effect, comparing the growth curves and regrowth delay with those of Experiments 7 and 8. These experimental data support my contention (III.7.5.) that it is premature to advocate such short periods of isolated limb perfusion (as suggested by Briele[450]).

IV.5.10. Melphalan and verapamil

There was no evidence from Experiments 23 and 24 that verapamil was itself cytotoxic in the B0008 melanoma spheroid model. Nor was there any evidence that verapamil 5 or 10μg/ml enhanced melphalan cytotoxicity. In fact, verapamil tended to decrease melphalan cytotoxicity (Experiment 23, plate 3 vs. plate 6 - Fig. 49B), and increasing the verapamil concentration seemed to reduce melphalan cytotoxicity further (plate 4 vs. plate 5 - Fig. 49B).

IV.5.11. Melphalan and pH

In Experiments 25 & 26 there was no significant difference between the regrowth of controls incubated at pH 7, 7.4 or 8 for one hour. There was a trend towards reduced regrowth delay i.e. reduced cytotoxic effect, at higher pH values in Experiment 25 (large spheroids - Fig. 51B) and this trend was confirmed by the results of Experiment 26 (small spheroids - Fig. 52B).

Although the drug is known to be stabilised in acidic

conditions, melphalan was incubated in supplemented MEM - titrated to pH values 7, 7.4 and 8 - and no difference in the rate of hydrolysis was detected (Fig. 26).

The increased cytotoxicity of melphalan in acidic conditions has not been described before. It may be due to an effect on the known active carrier mechanisms, to an effect on protein binding or a combination of these factors.

IV.5.12. Variability of spheroid results

Within each experiment the results are consistent, but there is some variability between experiments. Throughout care was taken to standardise technique from experiment to experiment. The observed variability between separate experiments (done on separate days) may reflect differences which are difficult or impossible to control.

From inspection of control spheroid growth curves the initial size of a spheroid would be expected to affect its growth pattern, smaller untreated spheroids tending to grow more rapidly. Smaller spheroids have a larger growth fraction than large spheroids[513] and may be more susceptible to therapy if the growth fraction is accessible by treatment. Spheroids of similar sizes were selected as far as possible.

An alternative hypothesis is that in any one experiment the predominant clone, sub-clone or blend of cells within the cell line may differ causing different growth patterns. Spheroids initiated in several different flasks were always pooled for experiments to minimise the potential for bias.

Experiments were conducted over a period of eighteen months, during which time the ambient temperatures in the laboratory varied. This may have influenced spheroid growth, although the spheroids were only removed from the incubator for measuring or replenishing culture medium. It was not possible to use a single batch of foetal calf serum, and the quality of supplemented MEM was therefore another potential source of variability. There was no particular pattern to support a seasonal or a MEM effect on spheroid growth. Genetic "drift" was minimised by using spheroids of established cell lines at similar passage numbers.

IV.5.13. Comparison of assays

In the studies of experimental chemotherapy, melphalan showed a clear dose-response relationship with all three cell lines. The melphalan dose-response relationship for the B0008 and Mel57 cell-lines can be quantified by the regrowth delay or "percentage cured".

In general there is a trend for increasing cytotoxicity of melphalan at higher temperatures. This is illustrated by the increasing regrowth delays obtained by Experiments 7-18 (Figs. 34B, 35B, 36B, ... 45B). Similarly Fig. 53 summarises the "percentage cured" data, and shows a gradation from very low values for incubations at 31°C, to high values for incubations at 44.5°C. Neither regrowth delay nor "percentage cured" data can be related directly to a conventional dose-survival curve.

To enable comparison of assays, and validate a method for deriving dose-survival curves from spheroid data, Figs.

54, 55 & 56 show the curves based on:

a) colony forming assay of disaggregated spheroids,

b) back-extrapolation from spheroid growth curves

and c) colony forming assay of exponential monolayers.

As illustrated in Fig. 27, derivation of surviving fraction by back-extrapolation from multicellular tumour spheroid growth curves depends on the treated spheroids regrowing parallel to the control spheroids. B0008 spheroids tend to regrow at the same rate, independent of melphalan dose (e.g. Fig 38A, 39A). Clonogenic cell survival cannot be derived from the spheroid growth curves of all cell lines[546]. For example, Fig. 32 shows how MEL57 spheroids regrow more slowly after higher doses of melphalan. The back-extrapolation method is therefore appropriate for B0008 spheroids but not for MEL57 spheroids. The particular advantage when this method can be applied is that direct comparison can be made with the dose-survival curve obtained after conventional colony forming assay of disaggregated B0008 spheroids and monolayers.

In these studies, using thermochemotherapy with melphalan and the B0008 cell line, we have found close concordance between the dose-survival curves derived from intact spheroid growth curves, from disaggregated spheroids and monolayers.

These dose-survival curves all tend to support the hypothesis that melphalan cytotoxicity is enhanced by hyperthermia.

IV.6. CONCLUSIONS

Initial studies have shown that we can grow multicellular tumour spheroids from established human melanoma cell lines. This three-dimensional system appears to be an appropriate model for avascular micro-metastasis, and we have used spheroids in studies of experimental chemotherapy. The multicellular tumour spheroid model has proved particularly valuable in the study of contributory factors which would be difficult to study in other systems. Three dimensional models are especially attractive to those of us researching treatments for the solid tumours, which have generally proved more difficult to treat than other malignancies.

The important conclusions of these studies are that:

1) Hyperthermia (>37°C) enhances the cytotoxic effect of melphalan, though heat alone is not cytotoxic to spheroids at temperatures less than 43°C for one hour.

2) Hypothermia (<37°C) tends to reduce the cytotoxic effect of melphalan.

3) There is a simple relationship between duration of exposure and cytotoxic effect.

4) Verapamil does not enhance melphalan cytotoxicity.

5) The B0010 cell line forms spheroids which grow poorly in conditions which allow B0008 and Mel57 spheroids to grow well.

6) Melphalan cytotoxicity is enhanced at lower pH values, and this effect is not simply due to decreased hydrolysis of melphalan.

7) Similar dose-survival curves are derived from spheroid experiments by back-extrapolation from growth

curves, from colony forming assay of disaggregated spheroids and from colony forming assay of exponential monolayers.

The implications for clinical isolated limb perfusion are that:

a) the limb should be warmed to greater than 37°C during isolated limb perfusion because melphalan cytotoxicity is enhanced by hyperthermia and reduced by hypothermia.

b) the results of one hour exposure to the achieved tissue concentrations may be improved by longer exposures, and shorter periods of clinical isolated limb perfusion are probably inappropriate.

c) the addition of verapamil during isolated limb perfusion with melphalan would not be expected to improve results, unless the vaso-active effect governs melphalan enhancement.

d) the cytotoxic effect of melphalan may be enhanced by correcting the alkalosis which develops if the perfusate is oxygenated with 100% O_2.