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Original Paper

A New Allogeneic Model for Metastatic Melanoma

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Metastatic melanoma cells, clonally derived from an affected lymph node of an ultraviolet-irradiated laboratory opossum, were allografted subcutaneously into suckling young, juveniles and adults to determine their tumorigenicity and metastatic potential. All injected 1- and 3-week-old suckling young survived well beyond weaning at 8 weeks. One died 12 weeks after injection from the effects of rampant metastatic involvement, while the rest were killed 13 to 26 weeks after injection. At necropsy, most animals showed extensive primary tumour growth, many showed metastasis to nodes and/or lungs, and in some there was dissemination to distant sites including liver and spleen. Animals injected as juveniles or adults rejected the allografts. Injection of allogeneic malignant melanoma cells during early postnatal development facilitates successful, long-term allografting and metastasis without concomitant immunosuppressive agents. Developmental lack of self-recognition (immunological immaturity) or induced tolerance may be responsible. This unique model system will be useful for further metastasis studies and may be valuable for investigations of novel antineoplastic therapies.

Key words: melanoma, *Monodelphis*, allografts, metastasis, tolerance
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INTRODUCTION

THE LABORATORY opossum (*Monodelphis domestica*) is widely recognised in Europe and North America as a useful and unique mammalian laboratory model for studies on various diseases [1]. This marsupial is the only known mammalian model that develops cutaneous malignant melanoma as a direct result of chronic exposure of shaved adults to mid-wavelength ultraviolet (UVB) radiation [2, 3]. UVB-induced melanomas occasionally metastasise to regional lymph nodes in this species, but have not been reported to metastasise to visceral organs nor shown to be life threatening.

By commencing the UVB-exposure protocol soon after birth, we have been able to accelerate the progression of nevi to malignant melanoma with lymph node metastasis, and to increase the frequency of tumorigenesis [4]. Cell lines, derived from affected lymph nodes of animals exposed in this way, exhibit the pigmented, dendritic morphology reminiscent of other metastatic malignant melanoma cells *in vitro* and show remarkable karyotypic stability [4, 5]. Clonal derivatives of one of the established cell lines, tested *in vivo* for evidence of a tumorigenic phenotype by xenografting into athymic nude mice, were moderately tumorigenic and produced pigment in the immunoincompetent mice [4]. This result represented the first *in vivo* demonstration that accumulations of pigmented cells in lymph nodes of UVB-exposed opossums were metastatic melanoma cells and not benign cell aggregates or melanophages [6].

In an attempt to enhance growth, proliferation and spread of *Monodelphis* melanoma cells *in vivo*, we allografted melanoma cells into opossums at different stages of development from neonates to adults. The rationale for this experiment was based on evidence from earlier skin allografting studies on opossums. Allografting of normal full thickness skin on to the North American opossum (*Didelphis virginiana*) was successful in young from 3 to 17 days of age, whereas animals older than 3 months always rapidly rejected the skin allografts. Since *Didelphis* and *Monodelphis* are extremely immature at birth [8] and strikingly similar in their early postnatal growth and differentiation, we assumed that melanoma cell line allografts would be successful in *Monodelphis* if carried out early in postnatal life, and that the allogeneic cells would exhibit their tumorigenic potential and perhaps metastasise. These assumptions proved to be correct. In this report, we describe dramatic metastatic melanoma phenotypes with life-threatening capacity in genetically-diverse animals allografted as suckling young. In contrast, melanoma cells allografted into juveniles and adults were rejected.

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MATERIALS AND METHODS

Animals

Animals came from breeding stocks that were among the least inbred in the Southwest Foundation breeding colony. Details of the genetic diversity within this colony are available [9]. Inbreeding coefficients (F) of allografted animals ranged from 0.037 to 0.173 for suckling young and 0.165 to 0.220 for juveniles and adults.

Derivation of cell lines

The origins of the cell lines for this study are summarised in Figure 1. A lymph node biopsy was obtained at necropsy from a UVR-exposed *Monodelphis* to generate melanoma cell lines for growth *in vitro*, in conditions already described [4]. Cell lines TD15 L1 and TD15 L2 (hereafter referred to as L1 and L2, respectively), were established by ring cloning from "parental" TD15 L melanoma cells. A lung biopsy (from an animal injected at 3 weeks with L2 cells), containing pigmented metastatic melanoma cells, was cultured under similar conditions to establish an additional pigmented melanoma cell line, TD15 M1 (hereafter referred to as M1).

Allogeneic grafting

Different stages of development (neonates, 1-week-old and 3-week-old suckling young, juveniles and adults) were selected for allografting. The number of cells and the volume of the inocula were escalated for the different developmental stages to account for the increased body mass, except for M1 cell injections.

Suckling young. Cells were prepared for injection as a suspension of 1×10^7/ml in sterile phosphate buffered saline (PBS). Prior to injection of sucklings, the mother was lightly anaesthetised with the inhalant Metophane® [10]. Females of this species lack a pouch, so by placing the mother on her back, the suckling young were fully exposed, each attached to a teat (Figure 2a). Neonates (four litters, 1–3 days of age) were injected with a 27 gauge needle and an inoculum of approximately 25 μl (containing about 1×10^5 L2 cells in phosphate-buffered saline, PBS); half were injected subcutaneously (s.c.) and half intraperitoneally (i.p.). One-week-old young were s.c. injected with 2.5×10^5 L2 cells using a 27 gauge needle, at a site close to the dorsal midline of the trunk while the young were firmly attached to a teat. To reduce the possibility of accidental injection of melanoma cells into the peritoneal cavity, the cells were injected by insertion of the needle at a near-parallel angle to the dorsal skin, whilst manually restraining them. Three week old suckling young were readily removed from teats of their lightly anaesthetised mothers and were injected s.c. with 1.0×10^6 L2 cells (in 100 μl PBS), also under manual restraint. All of the young reattached soon after treatment. M1 cell (1.0×10^5 in 100 μl PBS) were injected s.c. into 14 animals at 3 weeks of age (two litters) at a lower cell number (1×10^5).

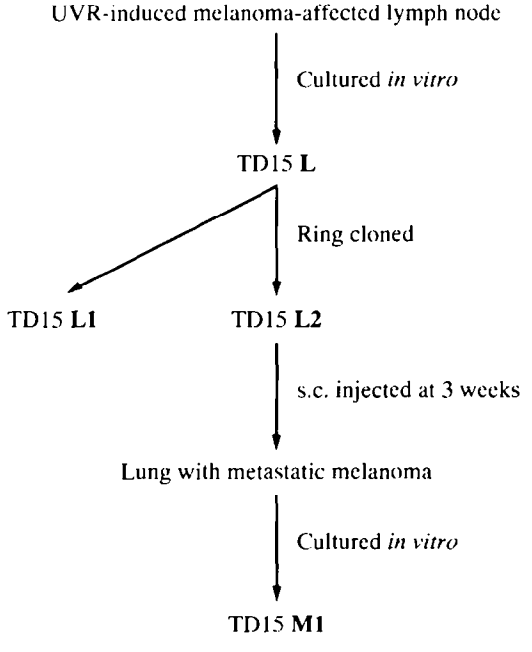

Figure 1. The derivation of melanoma cell lines L1, L2 and M1 from TD15 L.

Postweanling inspections and necropsy. Injected young were anaesthetised and shaved soon after weaning, then examined externally for subcutaneous and/or dermal lesions. Further shavings and external examinations were carried out occasionally until necropsy. Immediately before necropsy, animals were heavily sedated with Metophane® and 1–3 ml of blood were obtained by cardiac puncture prior to cervical dislocation. After weighing and shaving, sufficient skin was resected from the underlying fascia to expose the full extent of the primary tumour and any metastatic dermal and/or lymphoid involvement (particularly in lymph nodes which required dissecting away the surrounding fatty tissue). The pleural and peritoneal cavities were then opened and organs removed for detailed inspection. Other structures were then explored, including the cranium, brain, ribs, vertebral column, and any additional sites with indications of pigmented melanoma. Organs of interest were transferred individually to phosphate-buffered formalin (PBF) and the remains of the bodies were fixed in PBF. Photographs were taken when appropriate. Samples for histopathology were paraffin-embedded and cut at 7 μm. Sections of affected lung, liver and spleen were stained with H & E, or trichrome stain.

Juveniles and adults. Monodelphis reach sexual maturity at approximately 5 months. Animals from 3 to 7 months old were anaesthetised with Metophane® and were injected with 2.5×10^6 L2 melanoma cells (in 0.5 ml PBS) via a 27 gauge needle into the s.c. dorsal flank site. To determine the allografting potential of juvenile and adult animals, sibling pairs were selected from genetically diverse stocks within the colony at large, and presumably expressed different histocompatibility antigens. Four sib pairs were chosen for a total of eight animals. The s.c.-injected animals were periodically shaved and monitored for 3 months, and at the time of necropsy they were processed as described above.

RESULTS

Allografts of neonates and 1-week-old sucklings

The four litters of neonates (26 individuals, <0.2 g body weight) were all lost within 3 days. Two litters at 1 week of age (Figure 2a) had a crown-rump length of 13 mm and a total body weight of approximately 0.3 g. The epidermis of the skin is well formed but the follicular buds are small and few in number, particularly over the dorsal region. The subdermal layers are thin and delicate as is the subcutaneous connective tissue where the L2 inoculum was delivered (Figure 2b). After the injection, there was temporary blebbing around and beyond the needle tract. The highly melanotic nature of the cultured cells resulted

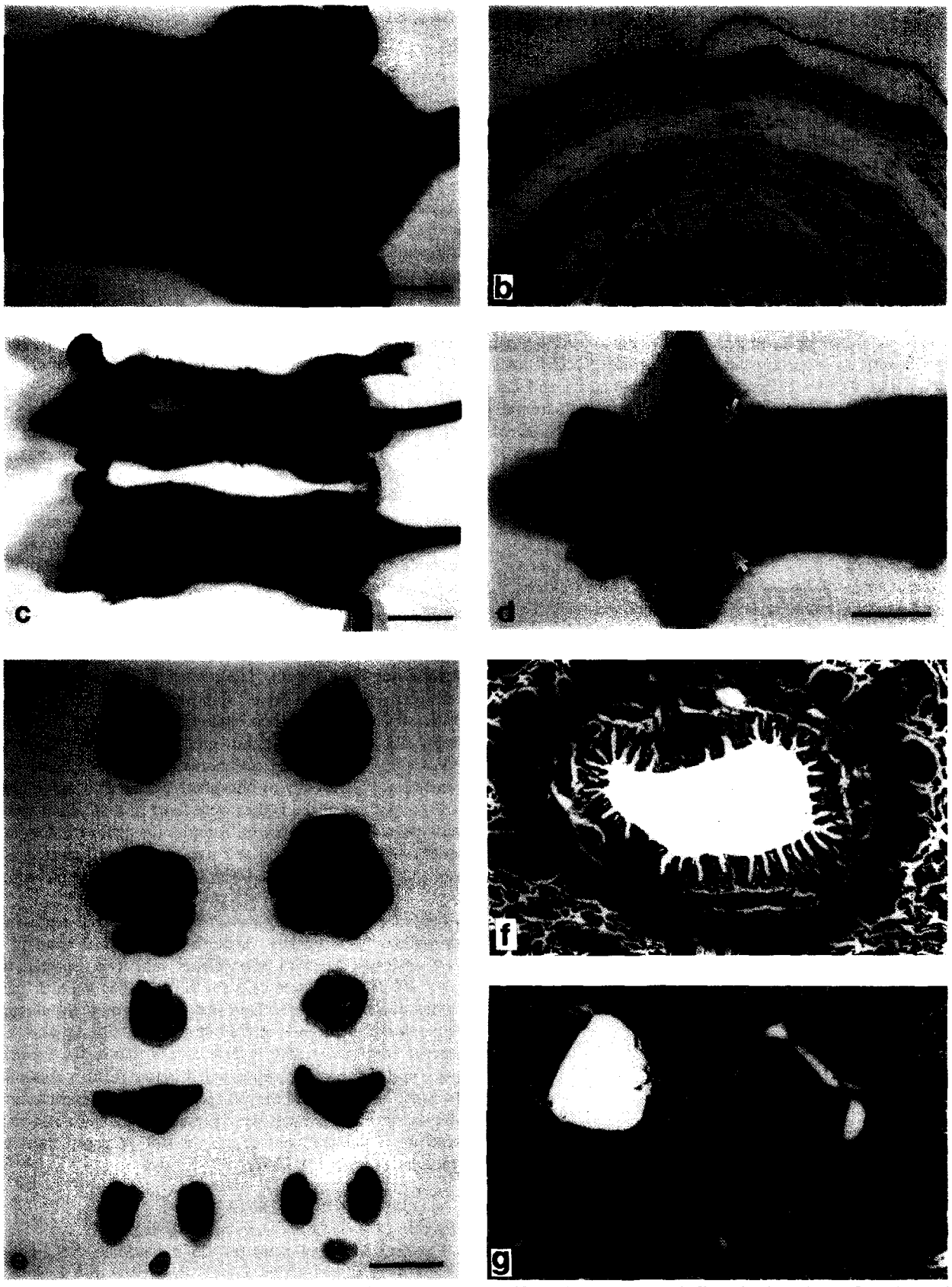

Figure 2. Morphology and histology of 1-week-old suckling young at time of L2 cell allografting and affected older animals. Scale bar = 1 cm (a), 2 cm (c,d,f) or 20 μm (b,g,h). (a) One-week-old litter of 10 suckling young; (b) transverse section of dorsal body region of 1-week-old suckling showing skin with developing hair follicle (hf), subcutaneous (sc) injection site (*), body wall (bw) and spinal cord (sp). H & E; (c) two shaved, anaesthetised weanlings from Litter 1 with numerous cutaneous metastatic melanomas and large, distinct primary tumours; (d) ventral view of adult (C6401) showing enlarged, affected axillary nodes (arrows) and blackened, affected internal organs *in situ*; (e) internal organs removed from C6405 (left) and C6397 (right) to show variation in tumour involvement of (from top) lungs, liver, stomach, spleen, kidneys and bladder; (f) section of lung of C6401 showing pigmented melanoma cells in alveolar tissue (around margin of field) with heavy concentration in connective tissue outside the smooth muscle layer of a bronchiole and invasion into the connective tissue between the muscle and mucosa. Trichrome stain; (g) section of liver of C6397 showing melanoma cells in connective tissue around ducts and vessels and between clusters of hepatic cells. Trichrome stain.

in the accumulation of black concentrates at the injection site which conveniently confirmed the successful delivery of the inoculum. All members of both litters survived to weaning (at 8 weeks of age) and beyond.

Clinical assessment. At the initial shaving and external examination of the 21 allografted animals, 7–8 weeks after injection, all showed evidence of a blue–black subcutaneous area on the dorsum indicative of the primary tumour at the injection site. All but two of the animals possessed a black lesion in the skin above or adjacent to the primary tumour. Most animals, in addition, displayed multiple, metastatic cutaneous lesions of varied size and shape over the head, limbs and body. Two such (anaesthetised) weanlings, shown in Figure 2(c), represent examples of extreme dermal dissemination to the pinnae of the ears, the jaws, buccal cavity, limbs, feet and base of the tail. Two animals showed no evidence of skin lesion formation at this time of assessment.

Routine daily checks of the 21 animals from the time of first inspection until necropsy revealed that, overall, they maintained a healthy appearance, feeding habits and behavioural patterns typical of this species. However, one animal (the lower specimen in Figure 2c; C6391) exhibited a rapid deterioration in health 11 weeks after injection, with weight loss, respiratory difficulties and impaired balance, and died within a week of the onset of symptoms. Another animal (C6397) showed early stages of similar symptoms together with abdominal bloating at 13 weeks after injection and was promptly killed. The remaining animals showed little increase in number or size of the skin lesions identified at the first inspection, but in several cases, the subcutaneous primary tumours expanded and became noticeably raised.

At necropsy. The two animals (one from each litter) without skin lesions at the initial examination failed to develop externally visible signs of melanoma by the time of necropsy. Assessments of the extent and severity of melanoma invasion and dissemination of the 21 animals are summarised in Table 1. The initial necropsies were performed on animals with advanced clinical conditions, but most animals were necropsied at intervals to determine progression at later time points. The graded scale used to record the extent and severity of lesions was heavy (+++), moderate (++) or light (+). There was no indication of a sex difference in either tumour susceptibility or progression. All animals in both litters showed marked proliferation and invasion of the primary tumour, with the most severely affected examples spreading anteriorly along the subcutaneous tissue, fascia and muscle of the body wall and ventrolateral extension particularly around the rib cage. In extreme cases, involvement of segmental nerves, vertebrae and ribs was clearly evident.

Metastasis reached global dissemination in seven animals injected at 1 week of age (including the individual that rapidly deteriorated and died), while only one of the 21 individuals was free of clinically evident metastatic involvement. One global example of dissemination is shown in an animal from Litter 2 (C6401) (Figure 2d). The exposed internal organs revealed blackened heart, lungs, liver and spleen. In two other heavily affected individuals (C6397 and C6405), the stomach, intestines, kidneys and bladder were significantly blackened by melanoma cell involvement, which sometimes extended to the long bones, cranial sagittal crest and meninges (Figure 2e). Pigmented ascites were not detected. Trends were noted in the patterns of organ and tissue involvement. For example, one or more (predominantly anterior) lymph nodes, together with the lungs, were affected in nine of the 10 animals possessing further dissemination (see Table 1).

Histopathology. Representative trichrome-stained sections of lung, liver (Figure 2f,g) and spleen from two heavily affected individuals contained pigmented metastatic melanoma cells and exhibited melanosis in the intercellular compartments of affected tissues, including association with soft tissue surrounding bronchioles and blood vessels in the lung. Melanoma cells were also abundant in the interlobular connective tissue around the hepatic ducts and blood vessels of the liver, and in the trabeculae and pulp of the spleen.

Allografts at 3 weeks

Results of s.c. injection of 3-week-old young are summarised in Table 2. Clinical assessment, necropsy procedures and histopathological procedures were the same as for 1 week allografted animals. Of the 10 animals injected with L2 cells (Table 2, experiment 2), eight possessed primary skin and/or s.c. lesions at necropsy, and five of these developed metastatic lesions. The anterior (suprascapular and/or axillary) nodes were involved, even though the cells were injected into the dorsal posterior s.c. compartment. Visceral metastases were occasionally observed, but only in animals that coincidentally exhibited positive lymph nodes. Affected lungs ranged in severity from a few pigmented metastases to nearly full involvement (Figure 3b, c). For comparison, an unaffected lung from this experiment is shown in Figure 3(a). The most affected animals exhibited no pronounced behavioural anomaly or respiratory distress prior to necropsy. In three of these four animals with lung metastases, other tissues also contained melanoma cells (e.g. vertebral column, thoracic muscle and liver). No animals demonstrated pigmented ascites.

The M1 variant cell line was established from an explant of an L2-induced metastatic lung melanoma (Figures 1 and 3c) and exhibited increased adherence to plastic cultureware, relative to the "parental" L2 line. All M1 allografted animals developed primary lesions (Table 2, experiment 3). Metastasis was more pronounced at 18 weeks after injection than at 10 weeks. At the later time point, three of seven animals had positive lymph nodes, and two of these had lung metastases, one of which also had melanoma in the liver.

Juvenile and adult allografting

Unlike the success obtained for suckling young, when juvenile and adult sibling pairs were s.c. injected with L2 cells, none developed metastatic lesions, and only one exhibited evidence of primary tumor establishment at necropsy. The single affected animal, injected as a juvenile, showed minimal primary tumour growth. Thus, rejection of the tumour cells was a feature of L2-injected older animals. Opossums that rejected the melanoma cells had lightly pigmented subcutaneous thin streaks, as evidence of the melanin present in the original injected cells.

DISCUSSION

The loss of all allografted neonates suggests that their extreme developmental immaturity and minute size require further refinements in the allografting procedures. Allogeneic grafts of melanoma cells were successful when carried out in older *Monodelphis* suckling young, with cell proliferation and dissemination being more pronounced in animals injected at 1 week rather than at 3 weeks. Dissemination to sites other than lymph node and lung in almost 50% of the 1-week-old animals might

Table 1. Individual assessments at necropsy of primary and metastatic tumour involvement for L2 melanoma cell allografts 1 week after birth

Animal ID	Sex	Weeks after injection	Dermis	Subcutis	Nodes	Lungs	Other
Litter 1							
C6391	F	12	+++	+++	+++	+++	+++
C6400	M	15	+++	+++	+++	+++	+++
C6397	M	13	+++	+++	+++	+++	+++
C6394	F	16	++	++	++	+++	—
C6393	F	16	++	++	++	++	—
C6395	F	16	++	++	+	++	—
C6399	M	16	++	+++	+	++	—
C6398	M	16	++	+++	++	+	—
C6392	F	16	++	++	+	—	—
C6396	M	16	—	+++	—	+	—
	Affecteds		9	10	9	9	3
Litter 2							
C6401	F	14	+++	+++	+++	+++	+++
C6405	F	13	+++	+++	++	+++	+++
C6410	M	14	+++	+++	+	+++	+++
C6408	M	22	+++	+++	—	+++	+++
C6403	F	22	+++	+++	+++	+++	++
C6406	F	26	++	+++	+++	+++	+
C6402	F	27	+++	+++	+	+	+
C6407	M	22	+++	+++	+	+	—
C6411	M	26	++	+++	++	—	—
C6409	M	22	+	+++	—	+	—
C6404	F	26	—	+++	—	—	—
	Affecteds		10	11	8	9	7
	Totals		19	21	17	18	10

The graded scale used to record the extent and severity of lesions was heavy +++, moderate ++, light + or no lesions —.

Table 2. Summary assessments at necropsy of primary and metastatic tumour involvement for all melanoma cell allografts

Experiment number	Cell line and (number)	Weeks after injection	Animals injected	Primary lesions	Lymph nodes	Lung metastases
1 week						
1	L2 (2.5×10^5)	11–26	21	21	17	18
3 week						
2	L2 (1.0×10^6)	11–22	10	8	5	4
3a	M1 (1.0×10^5)	10	7	7	1	0
3b	M1 (1.0×10^5)	18	7	7	3	2
Juveniles and adults						
4	L2 (2.5×10^6)	13	8	1	0	0

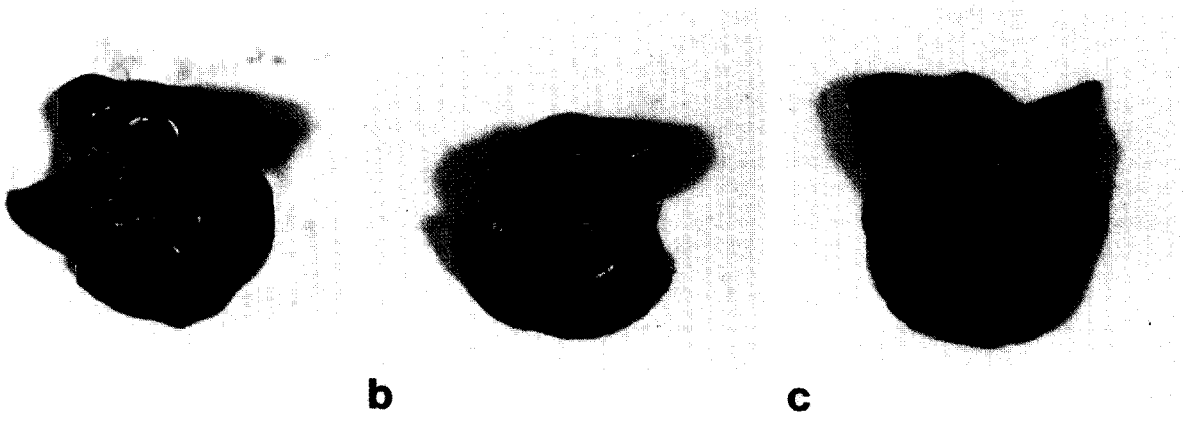

Figure 3. Lungs from representative adult *Monodelphis* injected s.c. at 3 weeks of age with allogeneic L2 cells: the lungs (and hearts) from three animals with various levels of melanoma progression obtained at necropsy are shown. (a) Normal lung from an animal with only a primary skin and s.c. lesion; (b,c) lungs from animals with primary and secondary tumours including lymph nodes and lungs. The M1 cell line was derived from a portion of the most severely affected lung (c).

have reached an even higher proportion if all the individuals in Litter 1 had been maintained longer. Profound dissemination occurred despite the lower cell number injected into the 1-week-old animals, necessitated by their small size and body weight. It is possible that during injection of the smaller young, the needle disrupted the musculature surrounding the vertebral column. This possibility could account for some animals being heavily affected at this site. Furthermore, accidental injection into the peritoneal or pleural cavities is most unlikely, since we never observed cases at necropsy of pigmented visceral lesions, without accompanying primary skin involvement.

Primary tumours in opossums were, in general, quite thin (less than 1 cm), irregular in outline and elongated, extending along the dorsal midline [5]. Thus, primary tumour volume is more difficult to quantify in allografted *Monodelphis* compared with mice injected with allo- or xenogeneic tumour cells, which typically result in spherical s.c. lesions [11].

A detailed description of the histopathology of this melanoma model is in preparation. The preliminary observations suggest possible preferential interactions with the extracellular compartment [12]. Dissemination patterns indicate that lymph nodes are involved prior to metastasis to other sites, such as lung. In humans, most cases of metastatic cutaneous melanoma are associated with lymph nodes (particularly the axillary and inguinal nodes), and melanoma staging criteria include assessment of nodal involvement as a marker of severity and poor prognosis [13–16]. Thus, our new model shares many similarities to the human condition. However, experimentally-induced metastasis in the opossum showed little evidence of tissue architecture remodelling, unlike that typically found in human melanoma metastases.

The consequences of injection of 1-week-old young clearly demonstrates the life-threatening potential of this metastatic model. The onset of symptoms and the speed of deterioration in condition were dramatic. One animal was permitted to die in order to establish this endpoint, and several more severely affected animals would probably have died of malignant melanoma if they had not been killed. It is remarkable, however, that several animals with rampant metastases in visceral organs showed no signs of abnormal behaviour.

Our results represent the first published demonstration of proficient, long-term survival and metastasis of allogeneic melanoma grafts in a marsupial, and to the best of our knowledge, in any immunocompetent mammal with genetic diversity. Melanoma cell line grafts have been successful in some rodent models, for example, syngeneic strains and immunodeficient athymic nude mice [11, 17]. Although no fully inbred or immunodeficient strains of the laboratory opossum exist for routine transplantation of syngeneic or xenogeneic tumour cells, allografting at an early age overcomes this deficiency. Earlier studies, involving injection of mouse melanoma cells into suckling young of *Monodelphis* on days 12 to 20, were not successful as long-term xenografts. In only one of 20 animals surviving to 7 weeks of age was there evidence of a remaining xenograft, and injections at later time points were also rejected [18]. Thus, successful long-term allogeneic, but not xenogeneic, tumour grafts are possible up to 3 weeks of age in genetically diverse *Monodelphis*.

Genetically diverse juvenile and adult sib pairs were chosen in an effort to survey the potential MHC I antigen diversity among the *Monodelphis* colony, since it is known in eutherian mammals that tumour cell recognition by T lymphocytes is dependent on MHC I expression. Our results indicate that juveniles and adults are proficient at recognising and rejecting the clonally-derived, transplanted, allogeneic tumour cells. Allografting of any tumour cell type into juvenile or adult *Monodelphis* is likely be compromised by the variability in the MHC I antigens between the host and the injected allogeneic tumour cells.

Neonatal eutherians have long been known to exhibit unusual immunological characteristics [19]. What, then, accounts for the ability of genetically diverse, non-immunosuppressed marsupial suckling young to accept and retain allografts? One plausible explanation is that this early stage in immunological development is not fully competent for T cell-mediated self-recognition (e.g. host MHC class I antigens are not yet fully expressed or recognised by T cell receptors). Another possibility is that injection of tumour cells at an early postnatal stage induces immunotolerance, similar to normal cell allograft results with fetal mice [20, 21]. Allotolerance is accomplished by copious and persistent expression of foreign tumour cell antigens [7, 21]. We are currently examining these and other aspects of melanoma allograft acceptance in suckling young of this species.

Our *Monodelphis* allografting model has a variety of advantages over other tumour grafting models. The melanoma cell lines reported here possess a diploid karyotype with a stable translocation, and produce substantial amounts of melanin, thereby permitting easy identification of primary and metastatic lesions

and cells derived from them [5]. Allografts are successful when injected into suckling young without requiring immunosuppressive agents, such as cyclosporin [20]. Moreover, it is likely that other allogeneic tumour cell lines (both melanoma and non-melanoma) will also be successful when injected at an early age. We also anticipate that this new melanoma model will be useful as an inexpensive *in vivo* system for the discovery of antineoplastic agents, including conventional chemotherapies and recently developed immunotherapies and gene therapies.

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