Transplantation of Human Melanocytes*

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Recent advances in the culturing of pigment cells from human beings have made it possible to begin the transplantation of autologous melanocytes into areas of skin that are hypopigmented. In a patient with piebaldism we were able to take pigment cells from a shave biopsy of the normally pigmented skin of the back, expand the cells in culture, and return them to an area devoid of pigment cells and get a perfect take. To grow the cells in culture we used 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as well as cholera toxin and isobutylmethyl xanthine. At this time, one can substitute basic fibroblast growth factor for TPA. The procedure of using autologous pigment cell cultures opens the door for further advances in the treatment of patients who do not have melanocytes in certain areas of the skin, as seen in patients with vitiligo or piebaldism, or as a consequence of severe mechanical or thermal trauma.
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There has always been a need to implant pigment cells into acquired or congenital white patches of skin so that the skin can be normal in color. Attempts along these lines have been reported periodically [1–9]. Only in the last few years, however, [10–12] have technical advances permitted the approach reported here; that is, implantation of cultured melanocytes, free of other cutaneous components such as blood vessels, keratinocytes, fibroblasts, lymphocytes, or Langerhans cells.

Pigment cells are lost from the skin of patients with vitiligo or following severe mechanical or thermal trauma. They are congenitally absent from the white patches in patients with piebaldism. In generalized albinism, the problem is different, in that the pigment cells are there, but the machinery that makes melanin is not functioning. The first group of patients would benefit greatly from pigment cell transplants. The second group, those with albinism, might benefit from transfection of the defective cells with the gene for tyrosinase if the lesion is a defective enzyme, or from the correction of a defect in the molecular controls for tyrosinase activity.

The problem is that epidermal melanocytes in vivo proliferate slowly. It is rare to see a mitotic pigment cell in a histologic section of normal skin. In patients with vitiligo, even under ideal conditions, when there is a good response over a year of treatment with psoralens and UV radiation, repigmentation will extend 3 mm at the most from the edge of a lesion or from a hair follicle. For many years, it has been possible to grow melanocytes in culture from metastatic melanomas from human beings and animals. Until recently, however, it has not been possible to grow large numbers of pigment cells from normal skin or from primary melanomas. In the early 1960s a technique was developed by Cruickshank and associates to grow melanocytes and keratinocytes together in culture [13]. Even though this method had some value, it was not useful for either keratinocytes or melanocytes because large numbers could not be grown. The situation was especially difficult with melanocytes because the melanocytes were overgrown by keratinocytes. The first real break came in 1982, when Eisinger and Marko reported the growth-promoting effects of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and cholera toxin in cultures of normal pigment cells derived from newborn foreskins and adult skin [10]. This culture technique was improved further by the addition of isobutylmethyl xanthine (IBMX) and human placental extract [14]. The cholera toxin and IBMX serve to keep the intracellular levels of cyclic 3′,5′-adenosine monophosphate (cAMP) high. The functions of TPA and placental extract are not known. It is possible that placental extract is a source of basic fibroblast growth factor (bFGF), which recently has been shown to promote the growth of human melanocytes synergistically with substances that increase intracellular levels of cAMP [12]. It is now possible to grow unlimited quantities of normal melanocytes in a defined medium supplemented with bFGF and IBMX or dibutyryl cyclic 3′,5′-adenosine monophosphate (dbcAMP). At the time of the experiment described here, TPA was an obligatory ingredient for obtaining rapid expansion of pure melanocyte cultures on the scale required for therapeutically effective melanocyte transplants.

The background for clinical studies was a pilot experiment carried out by one of us several years ago [15]. Mixed epidermal cells from black ears of recessively spotted guinea pigs were placed in culture for 1–3 weeks, harvested, and inoculated into epidermal blisters induced by ammonium hydroxide on the white flanks of autologous animals. Transplanted melanocytes assumed a nearly

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Abbreviations:

bFGF: basic fibroblast growth factor
cAMP: cyclic 3′, 5′-adenosine monophosphate
dbcAMP: dibutyryl cyclic 3′,5′-adenosine monophosphate
IBMX: isobutylmethyl xanthine
MEMS: minimal essential growth medium without calcium and magnesium
PBS: phosphate-buffered saline
TIC: melanocyte growth medium containing TPA, IBMX, and cholera toxin
TPA: 12-O-tetradecanoyl-phorbol-13-acetate

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basilar position among keratinocytes and produced and transferred pigment in a normal fashion. The pigmented spots expanded to more than 12 times their original size over a 4-month period. Some pigment cells migrated to hair bulbs and darkened regenerating hair as well.

In going on to the next step, the implantation into human beings of human melanocytes that have been expanded in culture in the presence of TPA, an important question was raised: Should cells grown with TPA be returned to human subjects? Are these cells normal? No one wants to implant transformed melanocytes into patients. In spite of the vast literature on TPA, it is striking that there has apparently not been a single incident reported in which TPA by itself produced malignant transformation. This point is important because when one considers the number of claims made in the literature based on poor experiments or poor judgment, one would expect simply by chance that someone would have stated that TPA was a transforming agent in some systems. In our hands, melanocytes from adult skin and from newborn foreskins always died after removal of TPA. Not one colony grew out from at least 5×10^8 cells that had been grown in culture with TPA and IBMX for a period of 3–6 months and were then transferred to medium without TPA. In contrast, cells from metastatic melanomas did not need TPA to proliferate in culture, and some were even inhibited by TPA. In addition, the karyotypes of melanocytes grown in TPA, IBMX, and cholera toxin were normal. Because the melanocytes in culture appeared normal by these tests, we decided to go ahead with the transplantation.

MATERIALS AND METHODS

The Patient The patient studied is a healthy 33-year-old white man with piebaldism (Fig 1), whose two siblings, his mother, aunt, grandmother, and newborn son are also afflicted (Fig 2). All of the patient's areas of hypopigmentation have been stationary, and he has never noticed any repigmentation. He has a white forelock. There are irregularly shaped areas of hypopigmentation over his arms, chest, abdomen, and legs. His back and buttocks are normal. Islands of hyperpigmentation measuring 10–20 mm in diameter are present within and at the margins of the hypopigmented areas. Hearing is normal. His son's piebaldism is almost an exact copy of his own.

Cell Culture Normal melanocytes were established in culture from a 3×3 cm^2 shave biopsy. The biopsy specimen was incubated in a 3 ml solution of 0.25% trypsin in minimal essential medium without calcium and magnesium (MEMS, GIBCO Laboratories, Grand Island, New York), supplemented with 200 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, Missouri), and 85 nM TPA (Consolidated Midland Corporation, Brewster, New York) at 2°C for 2 h. The epidermis was separated from the dermis, both tissues were shaken vigorously, and detached cells were plated in a 25-cm^2 flask in TIC medium. The latter consisted of 3 ml of Ham's F-10 medium (American Biorganics, Tonawanda, New York), 8% Nu-Serum (Collaborative Research, Lexington, Massachusetts), 8% newborn calf serum (GIBCO), penicillin and streptomycin, plus 85 nM TPA, 0.1 mM IBMX (Sigma), and 2.5 nM cholera toxin (TIC) (List Biological Laboratories, Campbell, California). After 1 week, the culture containing an estimated 2×10^5 melanocytes, mixed with fibroblasts, was incubated for 3 days with TIC medium that was supplemented with 100 μg/ml geneticin (G418 sulfate, GIBCO). This treatment eliminated the fibroblasts selectively [11], yielding a homogeneous culture of melanocytes (Fig 3). The cells were then grown in TIC medium and were passed 2 weeks later at a ratio of 1:1. Fresh medium was given twice a week. At the end of 1 month in culture, at the time of transplantation, there were approximately 2×10^6 melanocytes. Fifteen minutes prior to transplantation, the melanocytes were detached from the culture dish with the trypsin solution without TPA, centrifuged, and washed 2× with phosphate-buffered saline (PBS). A sample of cells was counted with the Coulter counter, and appropriate aliquots containing 2×10^5 and 5×10^5 cells were centrifuged. The melanocytes were resuspended in 200 μl PBS, and used for injection into suction blisters.

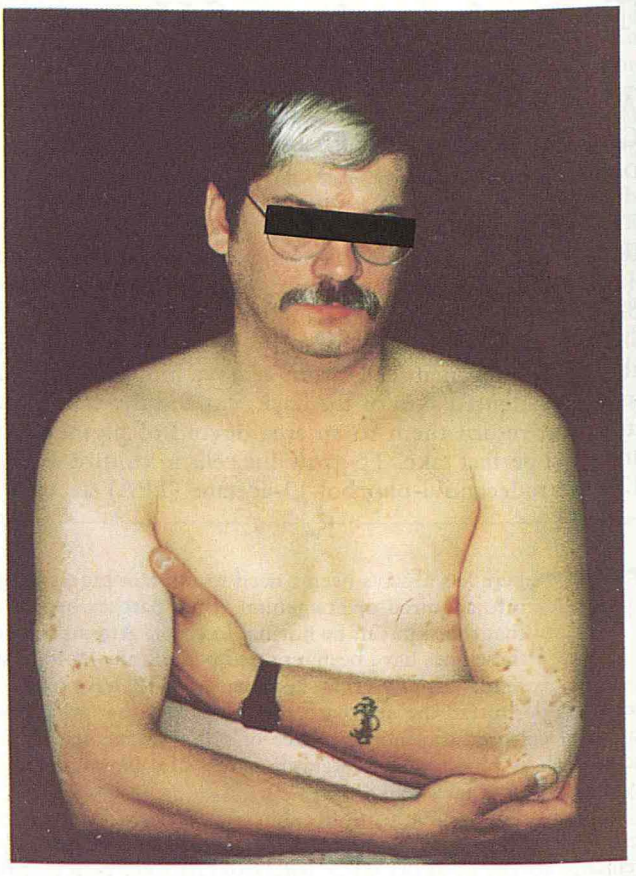

Figure 1. A patient with piebaldism, with a white forelock, large areas of hypopigmentation, and small macules of hyperpigmentation.

Transplantation Four suction blisters, approximately 1 cm in diameter each, were raised on the right side of the chest wall on areas without pigment. Vacuum pressures of 150 mm of mercury were applied to syringes placed upside down on the skin, each tube having a diameter of approximately 1 cm. After 1 h and 45 min, blisters began to form. The viscous blister fluid was aspirated

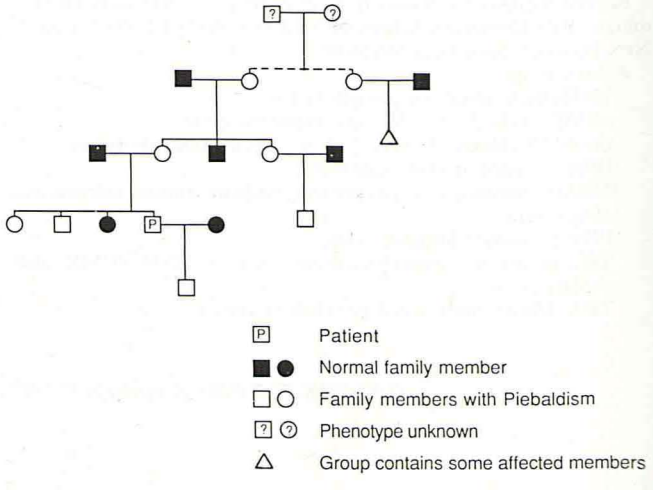

Figure 2. Pedigree of patient's family with piebaldism.

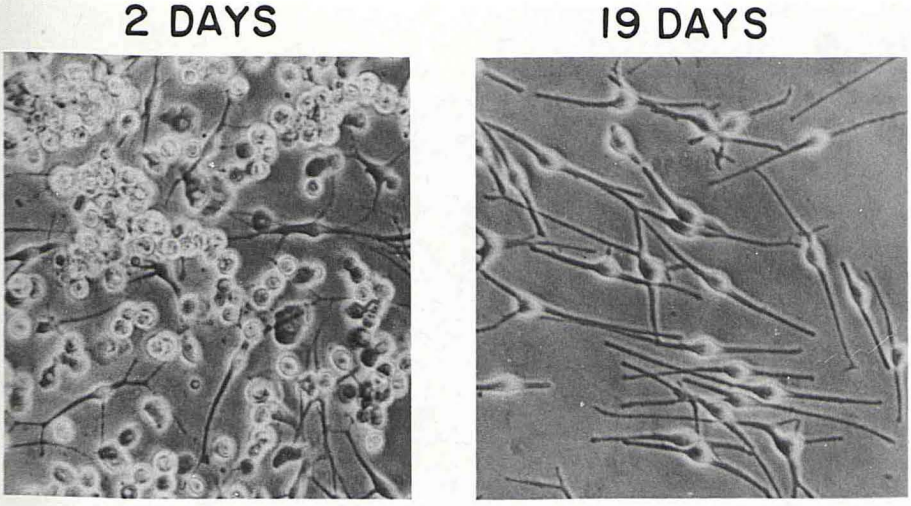

Figure 3. The patient's pigment cells in culture after they were dissociated from the shave biopsy specimen. At 2 days, there are melanocytes, debris, and rounded keratinocytes. After 19 days, one has a homogeneous culture of pigment cells. 224×.

with a 25-gauge needle and replaced with the pigment cell suspensions. Cells (2×10^5) went into each of two of the blisters, and 5×10^5 cells into the third. The fourth blister served as a control. The areas were bandaged and the tops of the blisters served to hold the transplanted cells in place. The patient returned in 6 days and again 3 weeks later for follow-up examinations. Healing was uneventful. The transplantation steps are summarized in Fig 4.

Electron Microscopy Biopsy specimens were fixed overnight in a cold solution of 2.5% glutaraldehyde plus 2% formaldehyde [16], buffered to pH 7.2 with 0.1 M sodium cacodylate buffer. They were refixed and stained 1 h at room temperature in a solution prepared of 1% osmium tetroxide plus 1.5% potassium ferrocyanide [17], buffered as above. The fixed specimens were dehydrated in ethanol and embedded in Spurr's epoxy mixture [18]. Ultrathin sections were cut on a diamond knife and counterstained with lead citrate [19]. Samples were thus prepared from piebald skin; from experimentally pigmented piebald skin 6 months after transplantation of autologous, cultured melanocytes; from a normally pigmented area similar to the one that had served as the donor site for pigment cells; and from one of the hyperpigmented islands.

RESULTS

Clinical We have shown in a patient with piebaldism that melanocytes can be isolated from a shave biopsy of normally pigmented skin, be expanded in culture, and returned to the patient into a hypopigmented area so as to get normal-appearing pigmentation. Four weeks after the injection of autologous melanocytes, the site that had received 5×10^5 cells had a fine stippling of pigmentation. There was less stippling in one of the other sites, but none in the third and fourth sites, the one injected with pigment cells and the control blister, respectively. Six months after the transplants we noticed excellent repigmentation at the site that had received 5×10^5 cells. All other sites had no pigmentation. There was no evidence of pigment spread from the pigmented transplant patch. Photographs were taken at that time but, because this patient is naturally light in complexion, only the original color slides and not the printed reproductions showed the perfect pigmentation.

Ultrastructural A biopsy specimen was removed from the middle of the repigmented spot for study with the electron microscope. The transplanted melanocytes had homed into the basal layer of keratinocytes and produced and transferred pigment in

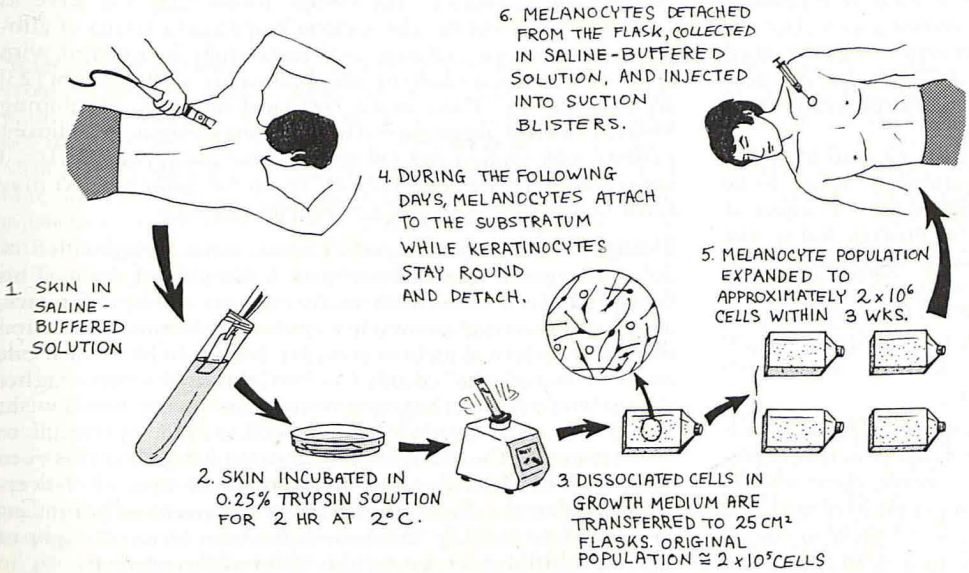

Figure 4. Summary of the technique for transplantation. The steps involve removal of pigment cells from the patient, expanding them in culture, and returning them to light areas of skin in the same individual.

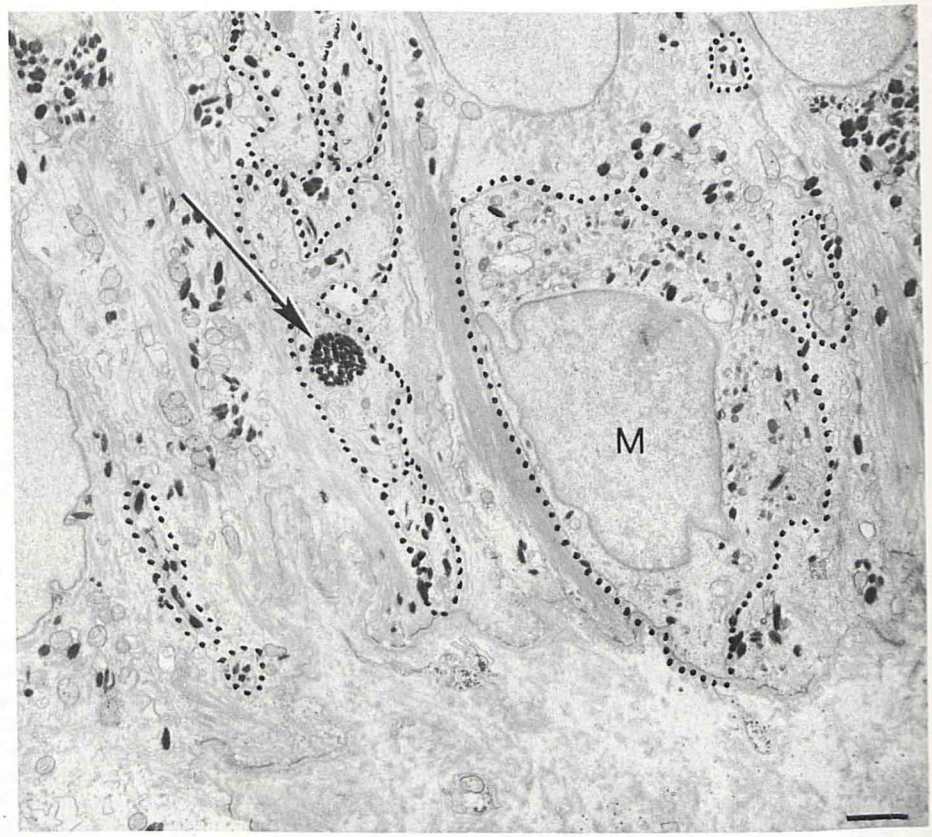

Figure 5. Electron micrograph from patient's normally pigmented epidermis. Under the dissecting microscope, the pigmentation of the specimen looked mottled, and the micrograph represents a relatively densely pigmented segment. In this view the epidermis is morphologically normal. A melanocytic nucleus is marked *M*, and all melanocyte plasma membranes are outlined with dots. The compound melanosome in a melanocytic dendrite (*arrow*) is typical of all pigmented epidermis of this patient, including that of the hyperpigmented macule and the experimentally pigmented piebald area. *Bar* = 1 μm

the same pattern as in the adjacent normal skin (Figs 5, 6). Like melanocytes in infants, solar lentigines, and repigmenting vitiliginous epidermis, the transplanted melanocytes were occupying a high basilar position rather than resting directly on the basement membrane [20]. We found no evidence of melanin or melanocytes in piebald skin that had not received melanocytes.

DISCUSSION

Procedure In any procedure involving the transplantation of skin, especially to exposed areas, there is concern about an appropriate match in color. In our patient, the match was perfect down to the level of ultrastructure. Since the time we started this experiment we have been able to modify the conditions for culture of melanocytes from newborn human foreskins and replace the medium that contained TPA and fetal calf serum with a defined medium containing bFGF and dbcAMP [12]. Whether this new medium will be as effective in supporting the proliferation of melanocytes from adult skin is not known.

The most difficult procedural problem is related to the preparation of the blisters into which the pigment cells are to be injected. Raising a suction blister takes almost 2 h. A variety of suggestions have been made to speed up the process, and several are being tested for feasibility in melanocyte transplantation in human subjects. It would be ideal if one could create small pockets between the dermis and epidermis in 10–20 min, without formation of viscous blister fluid. A combination of liquid nitrogen followed by simultaneous application of heat and friction appears to be promising.

Source of Pigment Cells An obvious source of pigment cells for transplantation is from an autologous patch of normally pigmented skin, which is what we used; however, there may be more efficient sources. For example, most people have nevi, and the pigment cells from nevi tend to be easy to grow in culture [14]. We could excise a nevus from the patient and use those melanocytes to expand in culture and to implant into white patches. There is, however, some reason for caution, since not all pigment cells in a nevus may have a normal karyotype [21]. Another potential source is hair bulbs. One would pluck anagen hair from the scalp or an eyebrow, grow the melanocytes in culture, and give them back to the patient.

The most practical source, however, may turn out to be allogeneic pooled populations of melanocytes grown from human foreskins, provided there is no family history of melanomas or dysplastic nevus syndrome in any of the infant donors. Pigment cells from newborn foreskins are easy to isolate and to grow in large numbers, and they are freely available in places where infant circumcision is routine. The vitiligo mouse [22] will serve as experimental model for the allogeneic approach. Grafts of allogeneic keratinocyte cultures, used successfully in a patient with recessive dystrophic epidermolysis bullosa in our department [23] are encouraging. These grafts contained normally functioning melanocytes that are presumed to be of donor origin. In addition, patients with vitiligo and vitiligo mice have suppressed delayed hypersensitivity responses [22,24]. This immunologic defect may favor the engraftment of allogeneic pigment cells.

Dosage In our patient, a perfect match was achieved with 5×10^5 cells injected into a blister with a diameter of 1 cm. This dosage translates into >6000 melanocytes per square millimeter, a high concentration when one considers the normal population density of epidermal melanocytes [25], but not so high when one considers that a "take" of only 1 in 3 will thrive. Whether a higher dosage would result in hyperpigmentation or, as one would wish, in pigment spread beyond the inoculated site [15,26] remains to be determined. The question of melanocyte dosage becomes even more critical when allogeneic pools are to be injected. Nevertheless, cultured collections of allogeneic pigment cells from infant foreskins could be grouped on the basis of racial origin of the individual donors and the skin color of their parents.

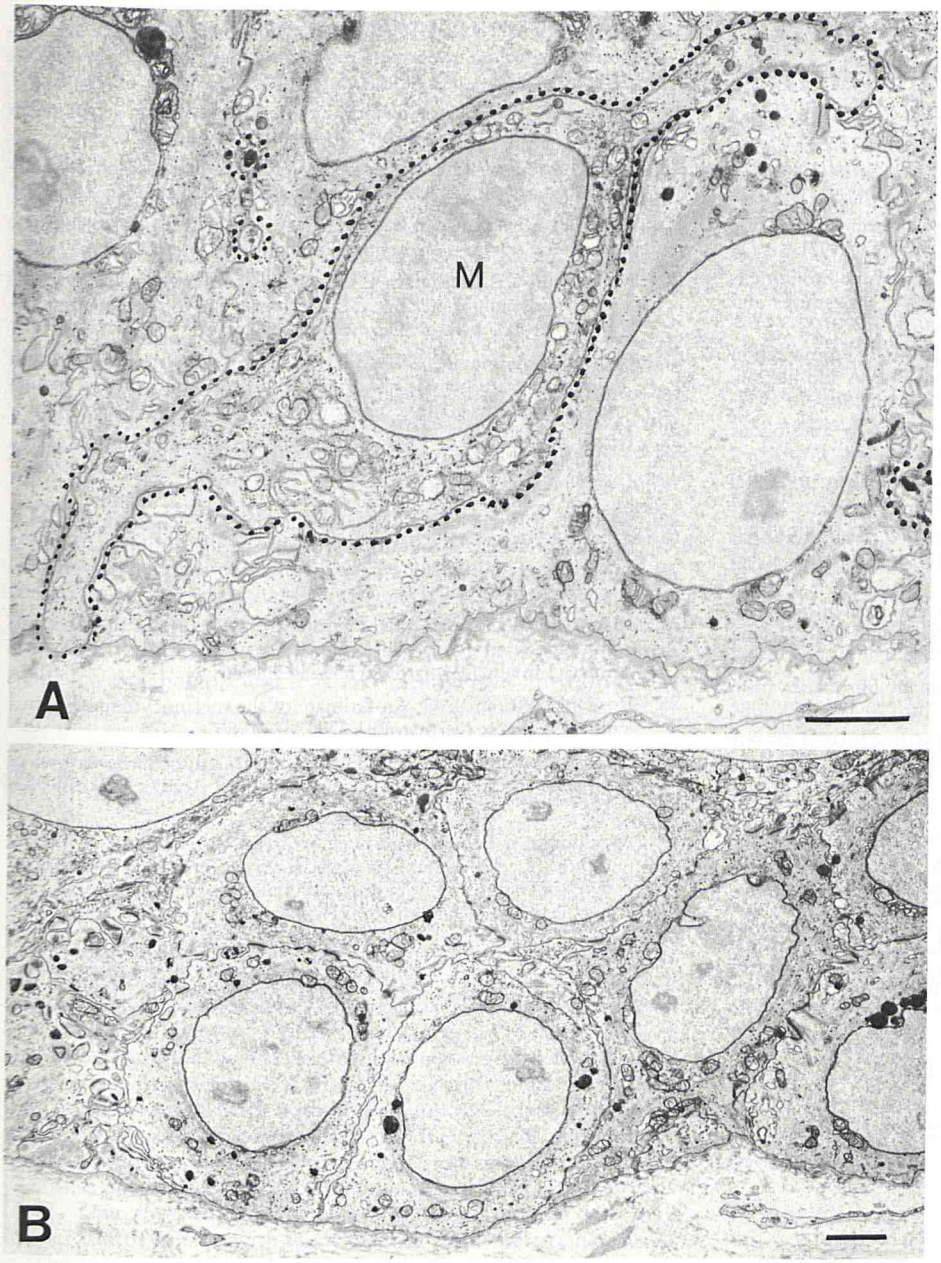

Figure 6. Electron micrographs from experimentally pigmented piebald epidermis at 6 months. As in Fig 5, morphologically normal segments of epidermis were selected for these illustrations. *A*, A melanocyte showing 2 dendrites is marked. The surrounding keratinocytes contain melanin granules dispersed singly or in packets. *B*, A lower-power overview of pigmented basal keratinocytes in the transplant area. *Bars* = 2 μm

Selection of Patients Although many patients with different causes of pigment-cell loss would benefit from a successful transplant of melanocytes, who should the first experimental patients be? It appeared too risky from the standpoint of success to begin with patients with vitiligo. Their pigment cells may not be normal, and if vitiligo is caused by an immunologic destruction of pigment cells, the cells might not take unless the patients were immunologically suppressed. We decided that the first case should be someone with piebaldism. Pigment cells would have been absent from birth, and yet, these patients were not known to have any immunologic defect, and their pigment cells as well as the skin as a whole were assumed to be normal. Our observations with the electron microscope have raised concern over the long-term effectiveness of melanocyte transplantation in piebaldism as well, however. The piebald epidermis of our patient, the experimentally pigmented piebald epidermis, and normally pigmented epidermis similar to that from which the melanocytes for culture and transplantation had been derived, all revealed a shocking surprise. In many respects they resembled the defective epidermis of patients with vitiligo [27] or Hermansky-Pudlak syndrome [28], and the melanocytes themselves possessed an inordinate number of compound melanosomes. Whether or not these defects apply only to our patient or also to other patients with piebaldism remains to be shown.

Our second subject will be one of our longstanding patients with vitiligo, who had a kidney transplant several years ago and is taking Imuran and steroids for immunologic suppression. If the second case is also successful, we will go ahead and do the procedure on patients with vitiligo who are not immunosuppressed. Continued intermittent treatment with psoralen plus UVA may be a helpful adjunct.

We thank our patient for his cooperation in these experiments. We also acknowledge Dr. Irwin Braverman for helping to obtain the initial shave biopsy from which the pigment cells were isolated; Dr. Robert Hartman for help with photography and the clinical management of the patient; and Elizabeth Kuklinska for expert assistance with the electron microscopic studies. We are indebted to Jack Schreiber for his secretarial skills.

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