STUDIES ON THE MIGRATORY BEHAVIOR OF MELANOCYTES IN GUINEA PIG SKIN*

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In certain mammals, including guinea pigs, cattle, pigs, dogs, and even man, in which melanocytes are normally present in the superficial epidermis, spotted phenotypes display the phenomenon of "pigment spread" which has intrigued dermatologists and developmental biologists ever since its initial description in 1896 (1, 2). The phenomenon is well expressed in the guinea pig in which it was first described and repeatedly investigated (3, 4).

In newborn spotted guinea pigs, areas of pigmented skin bearing black hairs are sharply delineated from white spotted areas bearing white hairs. As the animals grow up, however, pigment slowly spreads into the initially unpigmented superficial epidermis of white skin areas. The white hairs are unaffected by this color transformation. Thus pigment spread, in conjunction with ordinary growth, gradually results in the development of "transitional" zones, up to 1 cm or more in width, of secondarily blackened white skin bearing white hairs separating completely nonpigmented areas and areas of black skin bearing black hairs. In a similar manner, red pigment encroaches upon white skin, and black pigment may even encroach upon red, although in the latter case the rate at which it occurs is exceedingly slow.

Pigment spread is easily initiated artificially by grafting black skin into white skin, or vice versa. Under these conditions the rate of encroachment is speeded up, facilitating experimental analysis.

Theories of Pigment Spread.—Although various hypotheses have been put forward to account for the phenomenon (5), only two have withstood critical evaluation. According to the first, spread results from a "phenotypic" transformation of amelanotic melanocytes into melanin-producing cells, or of phaeomelanin (red)-producing cells into eumelanin (black)-producing cells, by a serially transmissible "infective" agent derived from pigmented melanocytes, or possibly from some agent capable of derepressing certain genes. The second hypothesis attributes pigment spread to a physical

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migration of melanocytes, or more probably melanoblasts, from the pigmented into nonpigmented, or from black into red skin.

Numerous lines of evidence, necessarily of an indirect nature, appeared to favor the infection hypothesis. These included: (*a*) the demonstration that branched melanocytes, or what appeared to be melanocyte-like cells, form a complete reticular system of approximately even density throughout the superficial epidermis of the integument, irrespective of its color (6); and (*b*) the fact that melanocytes do secrete the pigment granules they synthesize directly into cells of the Malphighian system via terminal processes on their dendrites, hinting that they might also be able to "infect" other melanocytes in a similar manner (7).

The strongest case for the "infection" hypothesis derived from studies on the transformation of red skin into black, since in the epidermis of secondarily blackened red skin only black melanocytes were demonstrable, having approximately the same density as the original population of red melanocytes (8). So far as the transformation of white epidermis into pigmented epidermis is concerned, the hypothesis had two serious shortcomings: (*a*) The presence of amelanotic melanocytes in the basal layer of the epidermis of white skin has never been demonstrated. Their existence was inferred from the demonstrability of what appeared to be effete melanocytes in the more superficial and presumably nonviable strata of the epidermis (6, 9); and (*b*) although pigment spread can easily be initiated by "transplantation" of relatively few melanocytes in epidermal cell suspensions to small superficial lesions prepared in white skin, all attempts to initiate spread by means of cell-free extracts derived from pigmented epidermis have failed (8).

Strong supportive evidence for the premise that spread results from differential migration of pigment cells stems from the fact that, during ontogeny, melanoblasts *do* migrate from their presumptive position in the neural crest to their definitive position in the skin (10), and embryonic melanoblasts injected into the blood stream of early avian embryos are able to migrate into the skin (11). This hypothesis too seemed to have some weaknesses. For example, it failed to explain why white hairs continue to grow in initially white skin whose superficial epidermis had been secondarily blackened, or why melanocytes failed to migrate into putatively melanocyte-free lingual epithelium when black skin epidermis was experimentally juxtaposed to lingual epidermis (5).

Grafting experiments employing isoantigenically "labeled" melanocytes subsequently established, unequivocally, that pigment spread from grafts of black skin placed in white skin areas is due to migration of pigment cells or their precursors, but left open the question as to the mechanism underlying the encroachment of black into red pigmentation (4).

The purpose of the present communication is to present evidence that migration of pigment cells does indeed underlie the latter phenomenon (Part I). Experiments are also described (Part II) in which an attempt has been made to use melanocyte migration as the basis of an indicator system for the phenomena of "allogeneic inhibition" (12) and "contact-induced cytotoxicity" (13).

PART I

EXPERIMENTAL SUBJECTS AND PRINCIPLE OF METHOD USED TO DISCRIMINATE BETWEEN THE "INFECTION" AND MIGRATION HYPOTHESES OF PIGMENT SPREAD FROM BLACK INTO RED SKIN

Materials and Methods

Adult guinea pigs of domestically maintained sublines of the two inbred strains, 2 and 13, and their F_1 hybrid offspring were employed. Animals of these genotypes are phenotypically tricolored (e^p/e^p; s/s), usually being predominantly white with smaller patches of red and black skin. The uniformity of each of these strains with respect to its histocompatibility genes was attested by the permanent acceptance of intrastrain grafts and by the consistent genetic tolerance displayed by F_1 hybrid animals towards grafts from *either* of their parental strains. However, homografts of skin exchanged between strain 2 and strain 13 animals or transplanted from F_1 donors to hosts of either parental strain were consistently destroyed within 12 days as a consequence of a typical violent homograft reaction (14). Previous analyses have established that these two strains of guinea pigs differ with respect to alleles at 7–8 histocompatibility loci (15, 16). This means that grafts exchanged between them confront their hosts with 7–8 different foreign cellular isoantigens. The experiments to be described made use of these antigens as built-in melanocyte "markers".

A previous analysis had shown that encroachment of pigment from black into red skin occurs only at an experimentally useful rate if the red skin is of very pale color (8). It must be emphasized, however, that the density of distribution of melanocytes in pale red skin is certainly not inferior to that in darker red skin or in black skin (6, 17).

Careful screening of our animals yielded three with patches of appropriately pale red skin on their trunks, two belonging to strain 13 and one to strain 2.

A typical experiment is illustrated in Fig. 1. A relatively large oval graft, about 2.0 × 3.0 cm, was removed from the area of pale red skin on the strain 13 donor's trunk. After trimming away as much of its thick dermis as possible with fine curved scissors, the graft was transplanted to a highly vascular granulation tissue bed which had developed in a full-thickness cutaneous wound prepared 6 days beforehand in white skin on the right side of a F_1 hybrid "primary host's" chest. 80 days later, when the graft had become well established and bore a normal hair crop, two very shallow "split-thickness" beds 5–7 mm in diameter, were cut in its center into which a thin, black sole of foot skin graft and a black ear skin graft, respectively, from a strain 2 donor were carefully fitted as sources of black pigmentation. Grafts of sole or ear skin were used in preference to black trunk skin on account of their greater density of melanocytes and the fact that they differ qualitatively from trunk skin, so that their margins remain incisively distinct.

A very slow encroachment of black pigmentation from these grafts into the juxtaposed pale red skin occurred which required a period varying from 160 to 300 days to produce an annulus of spread 4–5 mm in width. At this time the entire grafted area was carefully shaved, a thin layer of vaseline applied, and seven exceedingly thin split-thickness rectangular grafts, comprising the superficial epidermis and as little of the dermis as possible, approximately 2–3 mm in length of side, were cut from the zone of secondarily blackened red skin with a straight-edged No. 11 scalpel blade. Particular care was taken to avoid including any of the central strain 2 grafts. One of the grafts of tinctorially transformed skin was transplanted to a split-thickness bed prepared in white skin on the F_1 donor itself as a test for viability of the melanocytes on a host known to be genetically compatible. Three grafts were transplanted to separate,

shallow white host skin beds prepared on the chests of (*a*) a strain 2 and (*b*) a strain 13 animal, respectively, designated as "secondary hosts". The grafting procedures have been described elsewhere (18).

The possible fates of these two latter groups of grafts were as follows: (*a*) On the strain 13 secondary host: Being of strain 13 origin, the stromal and keratinocyte components of the graft should be permanently accepted. If the black melanocytes present are also indigenous strain 13 (originally red) melanocytes whose tinctorial phenotype has been infectively trans-

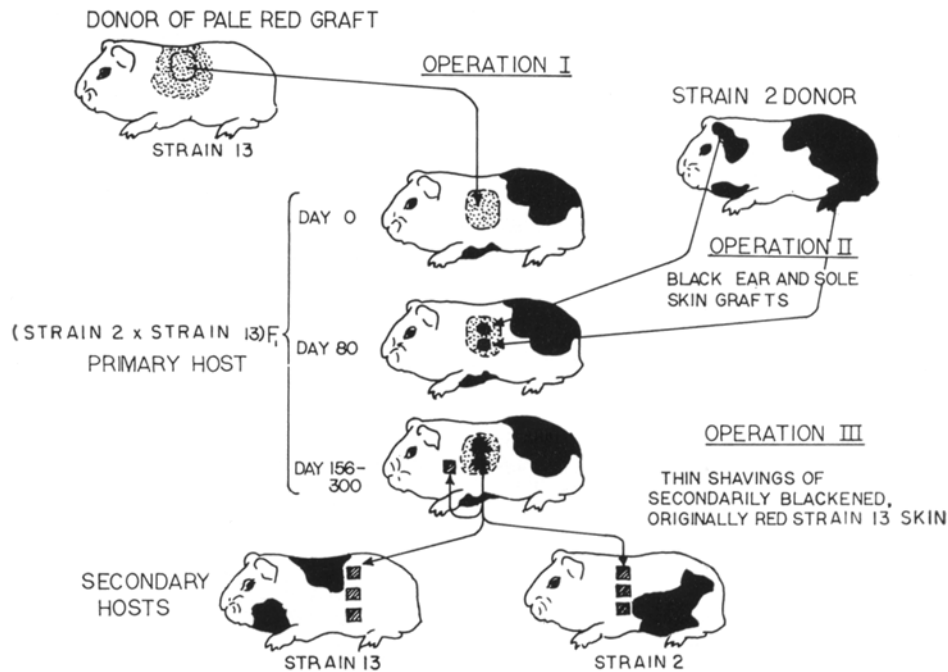

Fig. 1. This illustrates the principle of the experiments designed to determine whether migration of melanocytes is responsible for the spread of pigmentation from black into pale red skin grafts (see text).

formed, they too should survive and eventually initiate pigment spread in the white skin of their secondary hosts. If, however, these pigment cells are of migratory origin, they must necessarily be of strain 2 genotype and, consequently, antigenically foreign to the strain 13 secondary hosts, and so eventually undergo rejection.

(*b*) On the strain 2 secondary hosts: Since it is of strain 13 origin, the original native cell population of these grafts should enjoy only a transient survival. However, if the black melanocytes present are transformed (initially red) strain 13 cells, they too should succumb to the host's immune response. But, if these black melanocytes are of migratory origin, from the centrally inlaid strain 2 black sole or ear skin grafts, they should be exempt from immunologic attack by their hosts and become incorporated in the host's white epidermis, as this replaces that of the rejected graft. The development of foci of black pigment spread would then be expected at the graft sites.

Obviously, had some F_1 animals with appropriately pale red skin been available, operation I in the experimental plan could have been omitted, and the strain 2 black skin grafts could have been transplanted directly into intact native pale red skin.

Results

The results of six independent tests employing the above experimental design (see Fig. 1) are summarized in Table I.

TABLE I

Analysis of Origin of Black Melanocytes in Secondarily Blackened Pale Red Skin

Donor of red skin grafts	F_1 Host No.	Strain of donor of black skin inlaid into established red skin graft	Fate of black melanocytes when thin grafts of secondarily blackened red skin transplanted to white skin area on:			Fate of black melanocytes in thin grafts of secondarily blackened strain 13 red skin transplanted to sensitized strain 2 secondary hosts
			F_1 host	strain 2 host	strain 13 host	
Strain 13 Exp. 8	9	2	+ (20)	+++ (25)	+++ (25) --- (32)	++000 (30)
	10	2	+ (20)	+++ (35)	+++ (20) --- (55)	0000
	14	2	+ (20)	+++ (36)	+++ (26) --- (40)	000
	15	2	+ (20)	+++ (45)	+00 (20) -00 (36)	+++0 (44)
Strain 13 Exp. 11	12	2	+ (20)	+++ (22)	+++ (22) --- (60)	++00 (35)
	13	2	+ (20)	+++ (30)	+++ (20) --- (40)	+000 (40)
Strain 2 Exp. 40	5	13	+ (20)	000	+++ (23)	

+ indicates a graft site on which pigmentation developed, indicating melanocyte survival.

Figures in parentheses indicate day on which pigmentation was first observed on a graft site or on which it finally bleached out.

— indicates appearance of transient focus of pigmentation on one graft site.

0 indicates graft site on which pigment failed to develop during a 100 day observation period.

In every case, after a period of pigment dilution accompanying the healing-in phase, the control grafts on the F_1 animals all became intensely black within about 20 days of transplantation and initiated pigment spread, indicating the adequacy of the grafting technique.

When the secondarily blackened strain 13 pale red skin grafts were transplanted to the strain 2 secondary hosts, they all healed in and, after a chronic period of extreme pigment dilution lasting 22–45 days, the host sites became

superficially repigmented and developed a few black hairs. As a result of pigment spread, these initially small foci of black pigmentation underwent considerable expansion. 100 days after transfer of the grafts to these secondary hosts, by which time every graft site was represented by an area of intensely black pigmentation, each animal received two grafts of trunk skin, 1.5–2 cm in diameter, from a strain 13 donor, transplanted to separate full-thickness beds prepared on its left thoracic wall. In every case, rejection of these grafts was complete by the 10th postoperative day as a consequence of a typical violent homograft reaction; yet the strong level of sensitivity elicited against strain 13 isoantigens failed to bring about the bleaching-out of the pigment on any of the grafted sites on the right thoracic wall. This constitutes strong evidence that the melanocytes responsible for this pigmentation were of strain 2 genetic constitution, i.e., that migration of these cells into the original strain 13 pale red grafts was responsible for their color transformation.

In the case of the strain 13 secondary hosts, the grafts of secondarily blackened strain 13 pale red skin also healed-in well and, after a transient period of depigmentation, a few black hairs, accompanied by superficial pigmentation in four animals, developed on the graft sites within 20–26 days. Within 32–60 days, however, all trace of pigmentation had disappeared from these sites as the culmination of a progressive bleaching-out process. Although these animals were under observation for 200 days, pigmentation never reappeared after its bleach-out. These observations are interpreted as indicative of the slow development of immunity on the part of the hosts to which the black melanocytes in the grafts eventually succumbed—unequivocal evidence of their strain 2 origin. They afford independent confirmatory evidence that the transformation of the strain 13 pale red skin was due to migration of strain 2 melanocytes into it.

One final test was carried out. 20–28 days after the six strain 2 secondary hosts had received their strain 13 sensitizing skin grafts, each received an additional set of three to five very thin grafts of secondarily blackened pale red skin from its original F_1 primary host donor transplanted to its left thoracic wall. In this case it could be assumed that all strain 13 cells in the grafts would be promptly destroyed because of the high level of immunity directed against their isoantigens. If the melanocytes responsible for the black color of these grafts were, however, of strain 2 (migratory) origin, it seemed likely that some of them would survive and become incorporated in the host's white epidermis, eventually producing foci of pigment spread. This is indeed what happened in four of the six animals in which evidence of melanogenesis was obtained in at least one of the grafted sites after a delay of 30–44 days.

An additional experiment was carried out in which the donor of the pale red graft belonged to strain 2 and the donor of the black ear and sole skin grafts was a strain 13 guinea pig. The results (Table I) were similar to those described above, except that permanent foci of pigmentation developed on the strain 13 secondary host and not on the strain 2 secondary host.

Two points need emphasizing in connection with these findings. Firstly, because of the extreme thinness of the secondarily blackened pale red skin grafts employed, and the fact that the donor sites of these grafts only bore pale red hairs, the black hairs which arose on the grafted areas on the secondary hosts must be attributed to incorporation of black melanocytes from these grafts into the transected white hair follicle bases left behind in the shallow graft beds of the host's skin.

Secondly, in no case was pale red pigmentation evident after the black pigmentation had bleached-out on the sites to which secondarily blackened pale red skin had been transplanted on strain 13 hosts. This is not necessarily evidence that, when black melanocytes migrate into pale red skin, the native population of red melanocytes is in some way displaced. It is conceivable that red melanocytes were present in the bleached-out areas, but because of the slow rate of spread of red pigment into white skin, they failed to make their presence evident.

PART II

THE INFLUENCE OF HOST/DONOR HISTOINCOMPATIBILITY ON PIGMENT SPREAD

Over the past few years two groups of Swedish investigators (12, 13) have presented various lines of evidence based upon ingeniously contrived experiments conducted both in vivo and in vitro in mice, purporting to demonstrate that very intimate contact of cells of isoantigenically dissimilar types (normal or malignant) may result in growth inhibition (the phenomenon of "allogeneic inhibition") or actual death (the phenomenon of "contact-induced cytoxicity") of one or other components of the mixture, normally the one that is confronted by alien transplantation antigens. For example, when cells from a parental strain donor and an F_1 hybrid donor are mixed, it is the former whose growth or viability is affected.

Although lymphocytes are effective as the "attacking" cells, their capacity to react immunologically against the putative target cells is certainly not mandatory on the basis of various experimental findings. For example, genetically tolerant F_1 hybrid lymphoid cells will harm target tumor cells from a parental strain donor, or heavily irradiated node cells from a donor of one inbred strain will harm target tumor or normal cells from a different strain. The basic requirements for these interrelated phenomena to occur are: (*a*) the target cells *must* establish intimate contact with the attacking cells, and (*b*) the latter *must* confront the former with foreign antigenic specificities determined by histocompatibility genes. Indeed the phenomena may still express themselves even when homogenates or extracts of attacking cells are substituted for intact viable cells, or when attacking cells of nonlymphoid types are employed.

According to both the Hellströms and the Möllers (12, 13), where intact attacking cells are involved, the cause of death of the target cells is their contact with cells differing in surface structure and the arrangements of their histocompatibility antigens.

These workers offer no convincing explanation as to how such differences can harm cells. On the basis of their interesting and provocative observations, these workers have suggested that the phenomena may be manifestations of a natural "surveillance" or homeostatic mechanism capable of eliminating harmful (potentially malignant) cell variants displaying changed surface structures from a tissue with a given cell surface pattern, without the need for an antigen-induced stimulation of immunologically competent cells.

Since pigment spread has been shown to be due to migration of melanocytes and these cells are in very intimate contact with neighboring Malphighian cells of the keratinocyte system, it seemed reasonable to suppose that if the principles described above do apply to cells of the epidermal system, their operation might be demonstrable in terms of melanocyte activity. Specifically, spread from black F_1 hybrid guinea pig skin grafts placed in white skin of F_1 hosts should take place with greater facility, and be more extensive, than spread from black skin grafts derived from either strain 2 or strain 13 parental donors placed upon similar hosts, since in the latter case the migrating pigment cells would constantly be in contact with alien transplantation antigens on the surfaces of the contiguous host cells. The experiments to be described where designed to explore this possibility.

Materials and Methods

Adult strains 2 and 13 guinea pigs and their F_1 hybrids were used as before. In the first series of experiments, each F_1 host received black ear skin grafts of the same size from a parental strain and another F_1 hybrid donor carefully fitted into beds prepared in white skin on the side of its chest. Inspections were carried out at regularly spaced intervals over a 200 day observation period, and the progress and extent of spread recorded by anesthetizing the animals with ether, close clipping the operation field, and tracing the outlines of the grafts and the annuli of spread surrounding them as accurately as possible on superimposed sheets of cellophane. The outlines were then transferred directly to sheets of tracing paper and the areas of the graft and of the secondarily blackened host skin determined with the aid of a radial planimeter.

In the second series of experiments, suspensions of epidermal cells in isotonic saline solution, including melanocytes, prepared enzymically (8) from black ear skin of parental and F_1 animals were grafted to tiny, shallow beds, each approximately 1–2 mm in diameter, in white host skin (Fig. 2). Under these conditions the grafted melanocytes rapidly became incorporated in the epidermis, producing, virtually, point foci of pigmentation whose expansion, through spread, could also be followed. In these experiments it was felt that the grafted melanocytes would be exposed to the adverse effects of allogeneic inhibition immediately after grafting.

Black Ear Skin Grafts of constant size were prepared by punching out discs from the shaved and thoroughly cleaned pinnae with the aid of a sharp trephine 13 mm in diameter, and then stripping the skin from both sides of the median ear cartilage for use as grafts. The latter were then carefully fitted into full-thickness beds of appropriate size prepared in white skin on the side of the host's chest. The operative technique has been described in detail elsewhere (18). In each group of tests alternate F_1 hosts received their F_1 graft uppermost and their parental strain graft lowermost, and vice versa, to randomize any possible position effect biasing the results.

Epidermal Cell Suspensions were prepared from thin shavings of skin, of even thickness, cut from shaved and vaselined donor sites on black ears. The grafts were floated on a 0.1% solution of trypsin in Hanks' solution and incubated at 37°C for 20–30 min. The grafts were then rinsed in normal saline solution and spread out, cuticular surface lowermost, in a dry Petri dish. Starting at one corner of the graft, its dermal component was then peeled back and discarded. Light scraping of the exposed Malphighian layer of the resultant pure epidermal sheet detached both melanocytes and keratinocytes, which were taken up in citrated saline solution. Agitation of the initially coarse cell suspension with a pipette resulted in a monodisperse cell suspension. To attempt some degree of standardization, the cell suspensions employed contained the cells detached from 1 cm² of ear skin epidermis dispensed in 0.1 ml of saline solution. Epidermal cell grafts were transferred with the aid of a pipette to tiny, shallow (approximately 1.5–2 mm diameter) beds cut in the superficial epidermis of shaved and vaselined white skin areas. The presence of the vaseline helped to prevent the suspension from spreading over the skin surface. Each suspension was transferred to five different sites on each host's chest disposed on the perimeter of a circle (see Fig. 2). The cells were allowed to settle in their respective beds before applying a layer of very fine-meshed tulle gras. Despite every attempt to standardize this technique, it was obvious that there was tremendous variability in the amount of pigmented material left in each small bed after the tulle gras was in place. On this basis alone, highly variable results were anticipated.

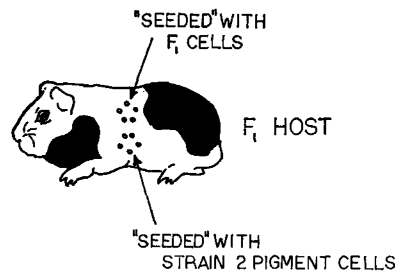

FIG. 2. This shows distribution of two quintettes of tiny graft beds prepared in the white skin of an F_1 hybrid host to receive melanocyte suspensions from F_1 and strain 2 donors, respectively.

Results

Spread from Strain 13 and F_1 Black Ear Skin Grafts in White Skin of F_1 Hybrid Hosts.—The results of 10 independent comparisons of the extents of the pigment spread into white skin on hybrid hosts from black ear skin grafts of 200 days' standing from genetically similar F_1 donors and genetically dissimilar strain 13 donors are set out in Table II.

Despite the pains taken to ensure that the grafts were of approximately uniform size, initially—trephined discs 13 mm in diameter—there was considerable variation in their final sizes. What is particularly striking is the tremendous variation in the extent to which pigment spread had occurred from the grafts of both series. Furthermore, extensive spread from one graft on a host was not necessarily accompanied by an equally impressive degree of spread from the other graft on the same animal, as indicated by the entries in the final column of Table II.

Statistical examination of the data, by applying the t test to a paired comparison of the differences between the areas of spread due to migration of melanocytes from the F_1 and strain 13 grafts on each of the hosts, indicated that confrontation of donor melanocytes with alien cellular antigens had no significant influence on their migratory or proliferative capacity ($P > 0.50$).

Spread from Strain 2 and F_1 Black Ear Skin Grafts in White Skin of F_1 Hybrid Hosts.—In this experiment, 11 independent comparative tests were made of

TABLE II

Areas of Pigment Spread Generated in White Skin of (Strain 13 × Strain 2) F_1 Hosts by Black Ear Skin Grafts from F_1 and Strain 13 Donors Transplanted 200 Days Previously

Animal	Areas in mm²				
	F_1 graft	F_1 graft spread	Strain 13 graft	Strain 13 graft spread	F_1 spread–strain 13 spread
7	122	52	110	51	+1
8	77	26	239	31	−5
9	161	84	142	91	−7
10	110	19	116	64	−45
11	122	7	142	43	−36
12	103	58	110	77	−19
35	161	239	110	210	+29
36	116	45	77	71	−26
37	168	237	200	139	+98
38	194	256	194	116	+140
Totals	1334	1023	1440	893	+130
Means*	133 ± 35	102 ± 100	144 ± 51	89 ± 53.6	+13

* ±Standard Deviation.

The differences between the areas of spread generated by the two paired series of grafts are not significant ($P = 0.5$).

the capacities of strain 2 and F_1 hybrid melanocytes to migrate from black skin grafts into white host F_1 skin (Table III). As in the previous experiment, there was wide variation in the final sizes assumed by individual grafts. Much more striking were the variations in the areas of pigment spread which developed from them. It will be noted that large grafts did not necessarily give rise to the largest amounts of spread.

Although the mean area of spread produced by each F_1 ear skin graft in the present experiment (98 mm^2) is closely similar to that obtained for the F_1 grafts in the previous experiment (102 mm^2), in the majority of individual tests the strain 2 grafts were much superior to the F_1 grafts as generators of pigment spread, the mean area of spread for 11 strain 2 grafts was 152 mm^2, and the difference between the two series is significant ($P < 0.01$).

Experiments With Melanocyte Suspensions.—In the experiments now to be described the capacities of melanocytes present in suspensions of epidermal cells from F_1 and parental strain donors to initiate and develop foci of pigment spread were compared. Each individual test entailed making two series of five very small shallow beds, distributed around the perimeter of a circle, one above the other in white skin on the lateral thoracic wall of an F_1 hybrid host. Samples containing F_1 melanocytes were placed in one series of microbeds and

TABLE III

Areas of Pigment Spread Generated in White Skin of (Strain 13 × Strain 2) F_1 Hosts by Black Ear Skin Grafts from F_1 and Strain 2 Donors Transplanted 200 Days Previously

Animal	Areas in mm^2				
	F_1 graft	F_1 graft spread	Strain 2 graft	Strain 2 graft spread	F_1 spread–Strain 2 spread
					mm^2
1	97	114	116	95	+19
2	137	124	174	116	+8
3	136	51	136	109	−58
4	116	84	137	140	−56
5	142	45	136	77	−32
6	155	58	155	74	−16
23	187	143	181	224	−81
24	181	116	200	167	−51
25	194	121	168	244	−123
26	220	70	206	168	−98
27	225	155	161	264	−109
Totals	1790	1081	1780	1678	−597
Means*	151 ± 41	98 ± 38	162 ± 23	152 ± 67	−54

* ±Standard Deviation.

The differences between the areas of spread generated by the two paired series of grafts are significant ($P < 0.01$).

melanocytes from a parental strain donor were placed in the other series of beds (see Fig. 2).

To randomize sources of error, in case the upper sites were more favorable than the lower ones, or vice versa, the group of sites that received F_1 cells were alternated from one host to the next in the series.

In each comparative test the actual number of micrograft sites on which melanogenesis was initiated in each quintette was scored together with the aggregate area of pigmentation deriving from each quintette 150 days after grafting. These tests were terminated at this time since many of the initially individual foci of spread had begun to coalesce.

Spread from F_1 Seedings Versus Spread from Strain 2 Seedings.—Table IV

TABLE IV

Comparison of Number of Foci of Pigment Spread and Aggregate Areas of Spread into White F_1 Host Skin from Seedings of F_1 and Strain 2 Melanocytes Performed 150 Days Previously

Animal	With F_1 melanocytes			With strain 2 melanocytes		
	Number of foci of melanogenesis	Total area of pigment	Mean area of pigment per focus*	Number of foci of melanogenesis	Total area of pigment	Mean area of pigment per focus‡
		mm^2	mm^2		mm^2	mm^2
13	1/5	8	8	5/5	123	25
14	3/5	27	9	4/5	117	29
15	0/5	—	—	2/5	72	36
16	0/5	—	—	2/5	90	45
39	3/5	76	25	3/5	56	19
40	3/5	93	31	0/5	—	—
41	2/5	55	28	4/5	103	26
42	4/5	210	53	5/5	97	19
43	3/5	90	30	2/5	72	36
Totals	19/45 (42%)	559		27/45 (60%)	730	

* Mean area of pigment per focus on all animals 26 ± 16 mm^2 (±SD).

‡ Mean area of pigment per focus on all animals 29 ± 9 mm^2 (±SD).

The difference between the areas of spread generated by the two types of melanocytes is not significant ($P > 0.5$).

TABLE V

Comparison of Number of Foci of Pigment Spread and Aggregate Areas of Spread into White F_1 Host Skin from Seedings of F_1 and Strain 13 Melanocytes Performed 150 Days Previously

Animal	With F_1 melanocytes			With strain 13 melanocytes		
	Number of foci of melanogenesis	Total area of pigment	Mean area of pigment per focus*	Number of foci of melanogenesis	Total area of pigment	Mean area of pigment per focus‡
		mm^2	mm^2		mm^2	mm^2
17	5/5	147	29	5/5	50	10
18	5/5	59	12	5/5	32	6
19	3/5	67	22	4/5	42	11
20	5/5	78	16	2/5	11	6
21	4/5	69	17	4/5	37	9
22	5/5	236	47	3/5	53	18
29	5/5	220	44	5/5	230	46
30	0/5	—	—	1/5	16	16
31	2/5	81	41	0/5	—	—
32	4/5	347	87	0/5	—	—
33	2/5	104	52	3/5	208	69
34	3/5	142	47	5/5	313	63
Totals	43/60 (72%)	1550		37/60 (62%)	992	

* Mean area of pigment per focus on all animals 38 ± 21 mm^2.

‡ Mean area of pigment per focus on all animals 25 ± 24 mm^2.

The difference between the areas of spread generated by the two types of melanocytes is not significant ($P > 0.2$).

summarizes the results of 11 independent comparisons of the fate of F_1 and strain 2 melanocytes in microbeds prepared in F_1 skin. Because of frequent fusion between areas of spread originating from neighboring seeding foci by 150 days, the mean area of spread generated per successfully established focus was made the basis of comparison. Melanogenesis was initiated in a higher proportion of sites with strain 2 cells than with F_1 cells (60% versus 42%), though the difference is not significant ($P > 0.05$). There was wide variation in the extent of the spread that developed from each established focus of melanogenesis. The mean areas of spread developing from each successful focus of spread were closely similar for each series (26.2 mm^2 for F_1 melanocytes and 29.3 mm^2 for strain 2 melanocytes), and examination of the data by the t test showed that the difference between the two means is not significant ($P > 0.5$). This confirms that the strain 2 melanocytes were at no particular disadvantage on F_1 hosts so far as their capacity to migrate or proliferate is concerned.

Spread from F_1 Seedings Versus Spread from Strain 13 Seedings.—In these tests, summarized in Table V, a higher proportion of the sites that received F_1 pigment cells generated foci of pigmentation than in the previous series, indicating the rather capricious nature of this grafting technique. Although the mean area of spread generated by foci originating from F_1 cells exceeded that of the spread originating from foci of strain 13 cells (36 mm^2 versus 27 mm^2), the difference is not significant ($P > 0.2$).

Taken as a whole, the present series of findings (*a*) emphasize the great variability in extent to which pigment spread may occur from a skin graft or from a microwound "seeded" with melanocytes in animals of similar genetic constitution; (*b*) lend no support to the thesis that parental strain melanocytes are at any demonstrable disadvantage as compared with F_1 melanocytes on F_1 hybrid hosts; and (*c*) show that the migratory activity of strain 2 melanocytes in F_1 skin is significantly superior to that of F_1 or strain 13 pigment cells in the same milieu, indicating that pigment spread must be under some kind of genetic control.

DISCUSSION

The findings that melanocytes from the annuli of spread arising from strain 2 black ear or sole of foot grafts placed in strain 13 pale red skin permanently survived their transplantation to strain 2 hosts, but only transiently survived when they were transplanted to strain 13 hosts, constitute unequivocal evidence that the pigment cells concerned must have been of strain 2 origin, i.e., that spread was due to migration. Confirmatory evidence in support of this interpretation was afforded by the observations that: (*a*) the foci of melanogenesis on the strain 2 secondary hosts did not bleach-out and disappear when these animals received, and subsequently rejected, relatively large "sensitizing" grafts of strain 13 skin; and (*b*) even when grafts of the secondarily blackened strain 13 skin were transplanted to strain 2 hosts highly presensitized against

strain 13 transplantation antigens, some of the melanocytes managed to survive and generate foci of spread.

Although no evidence was obtained concerning the fate of the keratinocytes or the fibroblasts present in these exceedingly thin grafts transplanted to hosts specifically immunized against them, knowledge of the vulnerability of even the thinnest skin grafts, or of pure epidermal grafts, to transplantation immunity makes it reasonable to assume that these were destroyed within about 1 wk, if not sooner. The survival, therefore, of melanocytes of host genetic origin which, through pigment spread had become incorporated among an immunologically susceptible population of Malpighian cells, suggests that there is a high degree of discriminatory specificity at the cellular level when homografts are destroyed. This makes it difficult to argue that the ultimate mediators of homograft rejection are nonspecific, diffusible, pharmacologically active substances liberated within the graft by the infiltrating mononuclear cells (19).

In view of the known high degree of histoincompatibility between guinea pigs of strains 2 and 13, and the intimacy of contact which normally exists between melanocytes and cells of the Malpighian system in the epidermis, failure to obtain evidence that pigment spread is less extensive from black parental strain grafts on white F_1 host skin, than from black F_1 skin on these same hosts is contrary to expectation if allogeneic inhibition and contact-induced cytotoxicity are phenomena that apply in in vivo situations. The present failure to obtain evidence in support of these concepts is in accord with an increasing variety of both in vivo and in vitro observations which are hard to reconcile with them. These include: (*a*) The fact that the naturally or experimentally procured states of cell chimerism in cattle, sheep, man, mice, rats, chickens, etc., are normally stable over long periods of time (20, 21). The most cogent example here is that afforded by allophenic mice resulting from fusion of early cleavage stage embryos of different genetic origins (22). (*b*) Observations that skin and other tissues transplanted from parental strain donors to F_1 hosts become permanently incorporated with no evidence of lesions at graft-host tissue interfaces. (*c*) Findings that, with the aid of heterologous anti-lymphocyte serum (ALS), skin heterografts transplanted orthotopically to mice (e.g., from human or rat donors), to hamsters (from rat or mouse donors), or to calves (from sheep or goats) are accepted and live in a state of complete normality for prolonged periods (23).[1] (*d*) The capacity of mixtures of embryonic cells from two different species, e.g., mice and chickens, to live juxtaposed in vitro forming interspecific renal tubules and other histochimeric tissues (24). In none of the homologous or heterologous combinations studied have any indications of cell or tissue incompatibility been observed.

[1] Shaffer, C. F. and W. J. Donowick. Personal communication.

Although it can now be stated that pigment spread, whether from black or red to white, or from black to pale red skin in the guinea pig, and probably in other species too, is the result of melanocyte migration, this must obviously be accompanied by mitotic activity on the part of the migrant cells; otherwise there would be a falling off in the intensity of the pigmentation and the density of the melanocytes, which is not the case. Among the still outstanding questions associated with this phenomenon are (*a*) why hair follicles are not affected, and (*b*) what is the fate of the initial native population of pale red melanocytes in epidermis that is secondarily blackened through pigment spread (25).

SUMMARY

Pigment spread is the natural or experimentally procured (through grafting) progressive encroachment of pigmentation from black or red skin areas into juxtaposed white skin areas, or from black skin areas into red skin areas in spotted guinea pigs and other mammals. So far as spread from black into white skin is concerned, it had previously been shown that migration of epidermal melanocytes into skin lacking homologues of these cells was responsible. However, since red skin already has its own complement of phenotypically "red" melanocytes, the intriguing possibility remained that when black pigment encroaches upon red, rather than melanocyte migration being responsible, phaeomelanin (red)-producing melanocytes are transformed into eumelanin (black)-producing cells by some kind of serially transmissible factor derived from contiguous eumelanotic melanocytes.

By utilizing two isogenic strains (Nos. 2 and 13) of spotted guinea pigs and their F_1 hybrids, the mechanism underlying the spread of pigment from black into red skin has been analyzed, employing cellular transplantation antigens as melanocyte "markers." The findings demonstrate unequivocally that a physical migration of pigment cells is responsible.

By comparing the extents of pigment spread from black ear skin grafts, or from epidermal cell suspensions prepared therefrom, from parental strain or from F_1 hybrid donors in white host skin areas of F_1 hybrid guinea pigs, it has been possible to evaluate the influence of the intimate contact of melanocytes with alien transplantation antigens on their survival and migratory behavior. No evidence was forthcoming that pigment spread takes place less readily when the cells responsible are confronted by epidermal cells bearing foreign antigens than when they are confronted by cells of their own antigenic constitution. These findings are contrary to expectation if the phenomena of allogeneic inhibition or contact-induced cytotoxicity apply to normal cells in in vivo situations.

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