EXPRESSION IN ORGAN CULTURE OF AGOUTI LOCUS GENES OF THE MOUSE¹

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ABSTRACT

CLEFFMANN (1963) reported that pigment cells of the lethal yellow (A^y/a) genotype change to black immediately upon cultivation, and continue to produce black pigment unless sulfhydryl reagents are added to the culture medium. We have attempted to repeat this observation and have not been able to do so. We also have been unable to induce the synthesis of yellow pigment by adding glutathione to cultures of agouti (A/A) skin. We therefore suggest that hypotheses which attempt to explain the action of agouti locus genes on the basis of effects of sulfhydryl compounds be considered with caution.

DESPITE the occurrence of numerous coat color phenotypes in mice, only two basic kinds of pigment granules are found—black or brown granules classified as eumelanin, and smaller yellow granules known as phaeomelanin (RUSSELL 1949). In the wild-type or agouti pattern determined by the A allele of the agouti locus, a yellow subterminal band appears in an otherwise eumelanin dorsal hair. This pattern results from a rapid shift from deposition of eumelanin to deposition of phaeomelanin and back again to eumelanin in the hair shaft. In nonagouti (a/a) mice, the dorsal hairs consist entirely of eumelanin, while dorsal hairs of lethal yellow ($A^y/-$) mice possess only phaeomelanin. Other mutations at this locus include extreme nonagouti (a^e), black-and-tan (a^t) and viable yellow (A^{vy}).

It has been shown that whether melanocytes produce eumelanin or phaeomelanin is not determined by the genotype of the melanocyte but by the genotype of the follicular environment (SILVERS and RUSSELL 1955; SILVERS 1958a, 1958b, 1961). It is clearly of some importance to understand the biochemical factors in the hair follicle which regulate the activity of the melanocyte. Not only does this have implications for constructing models of gene action (FOSTER 1965; PHILLIPS 1966), but an understanding of many pleiotropic effects is also involved. For example the A^y gene is associated with relatively high susceptibility to induced pulmonary tumors (HESTON 1942), spontaneous pulmonary tumors (HESTON and DERINGER 1947), induced skin tumors (VLAHAKIS and HESTON 1963), mammary gland tumors and hepatomas (HESTON and VLAHAKIS 1961a) and reticular neoplasms (DERINGER 1970). Lethal yellow mice tend to become

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obese quite rapidly because they have an increased food intake along with reduced energy requirements (DICKERSON and GOWEN 1947). HESTON and VLAHAKIS (1961b) have shown that the high tumor susceptibility of lethal yellow mice can be reduced to normal levels if these animals are maintained on a highly restricted diet.

It was reported by CLEFFMANN (1963) that the formation of yellow pigment can be induced by sulfhydryls. CLEFFMANN reported that under conditions of culture, yellow melanocytes change their color immediately to black, but that they could be induced to form yellow pigment again if glutathione or other sulfhydryls were added to the nutrient medium. He also reported that nonagouti melanocytes could be induced to produce phaeomelanin if given sufficiently high concentrations of sulfhydryls. It was the purpose of the present investigation to confirm and extend these findings.

MATERIALS AND METHODS

Mice of the C57BL/6J-*A^y* strain obtained from the Jackson Laboratory, Bar Harbor, Maine, were used to supply yellow and non-agouti (black) skin. Since *A^y/A^y* homozygotes are not viable, this strain is maintained by mating *A^y/a* with *a/a* segregants. Four of the *A^y/a* and four of the *a/a* donors were four days old when the cultures were initiated, while all other donors were less than 24 hours old. In order to identify the phenotype of the newborn mice, a skin graft of approximately 2.5 mm × 2.5 mm from each donor was transplanted to a C57BL/6J adult recipient (BILLINGHAM 1961). The color of the hairs which developed on these grafts permitted identification of the donor's phenotype.

Agouti (*A/A*) skin was obtained from CBA/Ss mice. In addition to obtaining samples from newborn and infant donors, skin was also obtained from 4-week-old animals which had been plucked 3 or 4 days previously. Plucking is known to initiate growth of the succeeding hair generation (CHASE, RAUCH and SMITH 1951) and has the advantage of producing follicles in a uniform stage of development.

Several explants, each approximately 2.5 mm × 2.5 mm, from the dorsum of each donor were grown in organ culture. The explants were cultured on stainless steel grids in media consisting of 70% Tyrode's solution, 20% chick embryo extract (GIBCO) and 10% human serum (CLEFFMANN 1963). Cultures were terminated at various intervals throughout a 10-day culture period. The cultured skin was fixed in phosphate-buffered formalin, sectioned, and lightly stained with hematoxylin and eosin. Histological preparations of cultured skin were compared with histological preparations of freshly excised dorsal skin from the same donor.

Sulfhydryl concentrations were determined by the method of ELLMAN (1959) using a Coleman-139 spectrophotometer. Solutions of known molarity of glutathione (GSH) were used to prepare a standard curve (concentration *vs.* absorbance) from which culture medium sulfhydryl concentrations were read. Normal culture medium was found to include approximately a $10^{-4.4}$ M concentration of sulfhydryls, which is in good agreement with the level which CLEFFMANN reported (10^{-5} M) for the sulfhydryl content of his medium (CLEFFMANN 1963).

GSH was added to the medium of some of the cultures of agouti skin at a level of 10^{-3} M.

RESULTS

A total of 30 explants of *A^y/a* skin, 18 of *a/a* skin (Table 1) and 29 explants of *A/A* skin (Table 2) were examined after various periods of culture. All of the black skin maintained its phenotype, in agreement with CLEFFMANN's observations (CLEFFMANN 1963). However, the yellow phenotype of the *A^y/a* skin was

TABLE 1

Numbers of A^y/a and a/a explants examined after various periods of culture

Days in culture	No. of explants from A ^y /a donors	No. of explants from a/a donors
1	2*	2*
2	2*	2*
3	4*	4*
4	5*	3*
5	3	1
6	4	2
7	3	1
8	1	0
9	4	2
10	2	1

* In each of these groups, one explant was taken from a 4-day-old donor. All other explants were taken from donors less than 24 hours old.

equally stable. There was no suggestion of a change from yellow to black in any of the explants examined.

Hair growth was significantly retarded in all cultures so that even after 10 days *in vitro* none of the follicles of A/A skin obtained from newborns had progressed to the stage of the hair growth cycle, the transition of the follicle from Anagen IV to Anagen V (CHASE, RAUCH and SMITH 1951), where they would have been expected to be initiating the yellow band (GALBRAITH 1964). Thus only black pigment was found in explants of newborn A/A skin even when they had been cultured in a 10⁻³M concentration of GSH—a concentration which, according to CLEFFMANN (1963), should have been sufficient to promote phaeomelanin synthesis by A/A melanocytes. Indeed, all our observations on A/A skin in culture indicated that sulfhydryl compounds did not play any role in determining the agouti pattern. For example, (1) skin explants from young adult A/A mice that had been plucked 3 or 4 days previously, and that were in the

TABLE 2

Numbers of explants of A/A skin examined after various periods of culture

Days in culture	With no added GSH		With 10 ⁻³ M GSH added	
	From plucked adults*	From newborns	From plucked adults*	From newborns
4	.	1	1	.
5	1	.	.	.
6	2	1	1	2†
7	2	1	.	1
8	3	2	.	1
9	1	2	1	.
10	2	2	.	2

* Explants taken 3 or 4 days after plucking.

† From 10 day old donors.

black phase of pigment formation, produced yellow pigment when cultured in the *absence* of supplemental GSH; and (2) 10-day-old *A/A* skin, which possessed follicles that had either completed or were just completing yellow band formation, continued to synthesize eumelanin when maintained *in vitro* for 6 days in a 10^{-3} M concentration of GSH.

DISCUSSION

According to CLEFFMANN's data (CLEFFMANN 1963), it was necessary to add 10^{-4} M GSH to the culture medium in order for *A^y/-* skin to produce yellow pigment. In our experience, *A^y/a* explants produced good yellow pigment without any added sulfhydryl compounds, and the basal levels of sulfhydryls in his and our experiments were comparable.

CLEFFMANN also reported that the addition of 10^{-3} M GSH induced yellow pigment synthesis by agouti explants. In our experience, however, agouti explants which were in the eumelanin phase of pigment production continued to synthesize black pigment when cultured in the presence of 10^{-3} M additional GSH.

Since our experiments utilized the same culture medium as CLEFFMANN used it is also difficult to reconcile the apparent difference in the rate of hair growth. While we found hair growth to be significantly delayed in the cultured explants, CLEFFMANN's report suggests that hair growth proceeds normally *in vitro*. It is hard to believe that differences in culture vessels (we used petri dishes and he used roller tubes) can account for this discrepancy, especially inasmuch as hair growth in similarly aged explants has likewise been reported to be retarded and abnormal under more favorable culture conditions (HARDY 1949).

While CLEFFMANN used a silver staining technique, in our experience the hematoxylin and eosin method resulted in excellent preparations, since with this method the yellow and black pigment granules retain their natural color.

The findings of CLEFFMANN were particularly puzzling because they implied that the agouti locus had two primary effects on pigment formation. In addition to its known role in altering the follicular environment (SILVERS 1961), CLEFFMANN's findings indicated that this locus also acted autonomously within the melanocyte determining its capacity to react to sulfhydryl compounds. Our observations are more consistent with the notion that all melanocytes are equivalent in their capacities to produce eumelanin and phaeomelanin, the pigment they do produce being determined solely by environmental conditions controlled by the agouti locus.

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