CONTROL MECHANISMS INVOLVED IN PIGMENT CELL DIFFERENTIATION -- THE AXOLOTL AS A MODEL SYSTEM

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The focus of research in my laboratory centers around the study of factors that regulate pigment cell differentiation in animals--lower vertebrates in particular. For the past three years, these efforts have focused primarily on the axolotl (Ambystoma mexicanum), although a variety of other amphibians and reptiles have been used in these efforts including: the frogs, Bombina orientalis, Ceratophrys ornata, Dendrobates pumilio, and a variety of Hyla sp.; and the lizards, Bradypodion pumilion, Phrynosoma sp. and Crotaphytus collaris (to name just a few). Each of these species has contributed in various ways to our efforts to: (1) understand how normal pigment cells differentiate, and (2) understand how pigments and pigment cells interact structurally to produce an observed pigment pattern. This discussion will be restricted to a summary of our previous, current, and anticipated future efforts regarding the axolotl pigmentary system.

As an appropriate introduction to this problem, readers are referred to two recent reviews that summarize the status of pigment mutants in the axolotl prior to 1980 (Frost and Malacinski, 1980; Frost, Briggs and Malacinski, 1984). There are four recessive genes in axolotls that have profound direct effects on the normal pigment pattern of these animals: m (melanoid), a (albino), ax (axanthic), and d (white). Only the first three operate at the level of the neural crest pigment cell precursors, and it is these three genes that we have examined in some detail. Other recessive genes are also known to affect pigment phenotype, but only indirectly (see Frost and Malacinski, 1980, for a discussion).

Thus, as a prelude to experimental studies involving the pigment mutants in the axolotl, we began an in-depth analysis of the wild type pigment pattern and compared this to the mutant patterns observed in melanoid, albino and axanthic animals. During these studies, several points that had not previously been taken into consideration with regard to axolotls in particular became clear.

First, it was important to consider the age of animals when comparing pigment phenotypes. Although the axolotl does not normally metamorphose and thus does not undergo the characteristic changes in pigment pattern that accompany amphibian metamorphosis, it nevertheless undergoes developmental changes in the types of pigments and in the number and distribution of pigment cells present in the skin. For example, iridophores (reflecting pigment cells) appear relatively late in development in axolotl skin and are often not observed in large numbers until axolotls are approaching sexual maturity. The pteridine pattern observed in xanthophores also changes during development. Yellow coloration (due to pteridine pigments) is most intense in juvenile axolotls and fades somewhat in adults. This is paralleled by ultrastructural changes in the composition of pteridine-containing organelles (pterinosomes) such that organelles in juvenile skin have a more fibrous matrix than organelles in adult skin. Other changes in both the structure and composition (e.g., thickness) of the skin also occur during development which may also affect pigment pattern to some degree.

Second, there is considerable variability in what has been defined as the wild type pigment phenotype. Certain spawnings may contain individuals that are "darker" than normal or "more yellow" than normal, or as adults some individuals may have more iridophores than usual. This was an important consideration when we undertook an analysis of the bright-colored pigments in developing axolotls. After screening a large number of axolotls (>200 from many different spawnings), it became clear that the qualitative pattern of pigment extractable from wild type axolotl skin does not vary (except with developmental age). The variability is one of quantity (i.e., amount of pigment in a specified area of skin), and thus suggests variability in either the number of pigment cells or in the number of pigment organelles/pigment cell. At the present time our observations suggest that it is the number of pigment cells that gives rise to the variable phenotypes observed. Phenotypic variability has also been observed within the pigment mutants examined, and we believe that is within the expected range of variability that is observed in many amphibian pigment patterns.

Taking these factors into consideration, we recently completed analyses of the development of pigmentation in each of the three mutant phenotypes and in wild type axolotls. The wild type and melanoid analyses have been published (Frost, Epp and Robinson, 1984a & b), the albino description is nearing completion, and the axanthic manuscript will be submitted for publication later this year. Interested readers should refer to the above-mentioned publications for information on the wild type and melanoid phenotypes. The albino description will include the first clear documentation (i.e., transmission electron micrographs) of unpigmented melanophores containing well-defined premelanosomes from albino skin. Albino xanthophore and iridophore development is also described and compared to the wild type. DOPA-staining (i.e., histochemical localization of tyrosinase activity) of albino skin is also a part of this study. This seemed appropriate because previous reports claim that albinism in axolotls occurs because tyrosinase is somehow "inhibited."

The axanthic phenotype was relatively simple to define with respect to pigments and pigment cell development (it has only melanophores). However, every axanthic animal that we examined was infected with a virus. This virus was observed in pigment cells, in suspected pigment cell precursors and in other cells within the dermis of the skin. The significance (if any) of this observation is not clear at the present time.

More recently, I have returned to a project that involves the inhibition of the enzyme, xanthine dehydrogenase (XDH), and its effect on pigment pattern. I had previously shown that feeding salamanders allopurinol (AP), a drug that specifically inhibits XDH, causes animals to hypermelanize (Bagnara, Frost and Matsumoto, 1978; Frost and Bagnara, 1979). More specifically, if wild type axolotls were fed AP they gradually became phenocopies of the melanoid mutant.

Allopurinol is a drug used to treat gout in humans. In this regard its primary action is to prevent the formation of uric acid. AP acts by binding so tightly to XDH that the resulting enzyme--product complex is extremely slow to dissociate, effectively inactivating the enzyme.

XDH catalyzes the following reactions:

Purines: *hypoxanthine $\longrightarrow$ *xanthine $\longrightarrow$ uric acid

Pteridines: *pterin $\longrightarrow$ *isoxanthopterin
*pterin $\longrightarrow$ *xanthopterin $\longrightarrow$ leucopterin

Thus, it is easy to see why inhibiting XDH reduces uric acid, however, it is not so easy to imagine how inhibition of XDH promotes hypermelanization.

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*These compounds are present in axolotl skin.

Three points may be significant in this regard. First, in axolotls, purines function as iridophore pigments and pteridines as xanthophore pigments. Second, XDH requires several cofactors for normal activity, including NAD, molybdenum, a flavin, and a pteridine. Finally, it is known from in vitro studies of neural crest cell differentiation that melanophore differentiation appears to be the "permissive" state. For example, under normal culture conditions, neural crest cells develop into melanophores. However, if purines (like guanine) are added to culture medium, iridophores differentiate, and already differentiated melanophores can be "induced" to "transdifferentiate" to iridophores.

Inhibition of XDH activity promotes differentiation of melanophores over the other pigment cell types by mechanisms that are presently unknown. Our analyses of the melanoid phenotype suggest that xanthophores convert (in vivo) to melanophores thus accounting for the observed hypermelanization. Thus, the goals of my current research efforts are: (1) to determine what happens (both structurally and chemically) to pigments and pigment cells following administration of AP to axolotls, and (2) to compare AP-treated wild type animals with melanoid animals in an attempt to provide clues regarding how XDH activity and melanism are related.

The problem is a very basic one with regard to factors that control pathways of cell differentiation. Pigment cells and the pigment mutants in the axolotl provide an excellent model system for this type of study. At the present time we are producing melanoid phenocopies by feeding AP to wild type axolotls. When the phenotypic change is observed, we analyze tissues for XDH activity (using gel electrophoresis and enzyme assays), we extract skin and use TLC and HPLC to analyze purine and pteridine pigments, and we utilize transmission electron microscopy to observe ultrastructural changes associated with hypermelanization. In the future we hope to obtain purified axolotl XDH that can be used for the production of monoclonal antibodies, as an electrophoretic marker, and, if enough can be obtained, it may be possible to determine its effects on pigment cell differentiation in vitro.

Within the coming year we hope to finally have an answer to whether or not melanoid axolotls have normal XDH. Similarly, preliminary experiments are underway in which melanoid axolotls are being fed guanosine in an attempt to reverse the hypermelanization phenomenon. Much to our surprise, these animals exhibited a distinct reduction in melanin pigmentation that was especially noticeable in the appendages, the tail fin and along the dorsal midline. Concomitantly, yellow pigmentation was also increased in guanosine-treated melanoids and was most noticeable in the head and along the dorsal midline. Chemical and structural studies of this phenomenon are underway, and more experimentation is anticipated before the significance of any of these results can be determined.

One final note of interest regarding guanosine-treated melanoid animals involves a preliminary TEM examination of skin from two of these animals. In addition to containing many xanthophores (which in normal melanoids are lacking or very few in number), these animals were infected with a virus--a virus similar in location and appearance to that observed in axanthic animals!

Obviously there are many questions left to be answered regarding pigment mutants in the axolotl and pigment cell differentiation in general. In view of the regulatory roles that pteridines and purines (like GTP) are known to play in cellular functions, I anticipate that the next few years will be very busy and exciting as we pursue these particular lines of investigation.

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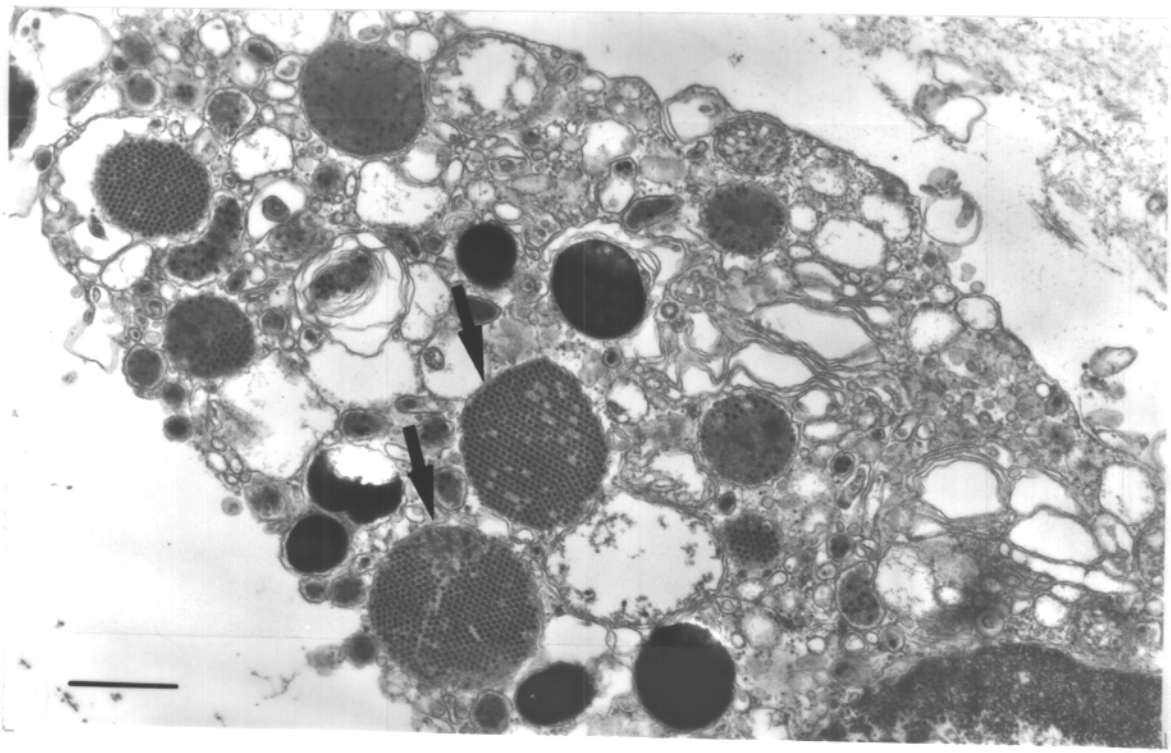

Example of virus particles (arrows) localized in dermal cells of axanthic skin. Bar = 0.5 μm