Presence of Prostaglandin E_2 in Homozygous Lethal Yellow (A^y/A^y) Mouse Embryos

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ABSTRACT

Homozygous lethal yellow (A^y/A^y) mouse embryos develop with characteristic abnormalities and die before completing implantation. The heterozygous lethal yellow (A^y/a) condition is pleiotropic, affecting coat color and fat deposition, causing a diabetes-like syndrome, and shortening life expectancy. Lamoureux and Ong suggest prostaglandin E_2 (PGE_2) as a possible chemical messenger for the *agouti* locus gene action because prostaglandins are known to be endogenous to normal mouse embryos, involved in pigmentation and the implantation process, and could account for the pleiotropic effects of the lethal yellow gene. As a frist step in evaluating this hypothesis, the purpose of this research was to determine whether or not PGE_2 is present in the cells of homozygous lethal yellow murine embryos. Embryos were collected from heterozygous (A^y/a) matings and screened for PGE_2 by immunohistochemical assay. Whereas one-fourth of the embryos were expected to be homozygous for the lethal yellow allele, only 2 of the 15 embryos were positive for PGE_2. The results suggest that the presence of one or more A^y genes prevented the detection of PGE_2.

INTRODUCTION

"Lethal yellow" is a mutant allele at the *agouti* locus on mouse chromosome 2. The lethal yellow (A^y) allele is the dominant allele of the *agouti* locus. The A^y allele is pleiotropic; that is, it has multiple effects on the phenotype. In the heterozygous (A^y/a) mouse, the most obvious effect of the lethal yellow allele is the yellow coat of the mouse. The heterozygous condition is also associated with obesity, a diabetes-like syndrome, and an increased susceptibility to tumors. Homozygous (A^y/A^y) embryos develop with characteristic abnormalities and die before implantation on the sixth day of gestation (Silvers, 1984). We are interested in the A^y/A^y genotype because it is a lethal condition. Our research may help us understand why this gene is lethal.

The *Agouti* Locus Gene Action

The action of the *agouti* gene locus must involve some sort of chemical messenger to execute its function. This concept requires basic understanding of how coat pigmentation is determined. Each melanocyte, or pigment cell in the epidermal hair follicles, is capable of producing either eumelanin, a black pigment, or phaeomelanin, a yellow pigment. The *agouti* gene locus ultimately dictates which type of pigment the melanocyte will produce. However, the gene action and the resulting pigmentation are expressed in different tissue types, therefore requiring a chemical messenger. Experiments with dermal-epidermal recombination of mouse skin showed that the genotype of the underlying dermis (mesoderm), not the epidermis, determined the pigmentation in the melanocytes (Mayer and Fishbane, 1972).

A circulating messenger, such as a peptide hormone, cannot be the messenger between the dermis and the melanocytes. A circulating messenger would create a single pigmentation pattern throughout the entire mouse. Agouti hairs actually have alternating black and yellow bands in the hair shaft; the banding patterns are slightly different on individual hairs. Therefore, the *agouti* gene must act through a localized chemical messenger. A lipid messenger is suggested by the observation that the lipid content of the diet influences both the phenotype and breeding performance of mice (Lamoreux, personal communication). Lipid messengers include prostaglandins, thromboxanes, and interleukins.

Prostaglandins and the *Agouti* Locus

Two observations about prostaglandins could explain the previously mentioned pleiotropic effects of the lethal yellow allele. First, prostaglandins are found in virtually all cells; it is more uncommon to find a cell which does not contain prostaglandins. Also, just as there are many classes of prostaglandins, each type may have many different effects depending upon the target tissue. These two characteristic of prostaglandins could account for the diverse effects of the lethal yellow gene which influence a wide range of tissue types.

Prostaglandins and Pigmentation

Evidence suggests prostaglandin E_2, (PGE_2) may be involved with the *agouti* locus' gene action because of its influence on pigment cells. For example, in melanoma cells in culture PGE_2 increases both melanin synthesis (Abdel-Malek, *et al.*,1987; Nordlund, *et al.*, 1985) and the catalytic activity of tyrosinase (Abdel-Malek,*et al.*; Pawelek,1976), a key enzyme in the melanin pathway. The tyrosinase activity level is known to be lower in yellow mice than in nonyellow mice (Lamoreux, *et al.*, 1986), although no connection between lower activity and a

prostaglandin deficiency has been established. In addition, Nordlund, *et al.* found that PGE_2 greatly increases the number of pigment cells and accelerates melanogenesis in melanoma cells. However, other types of prostaglandins did not evoke a response by the melanocytes. These studies indicate PGE_2 may influence the phenotype of pigment cells.

Prostaglandins and Implantation

Although many substances can influence the implantation process. PGE_2 is not only required for implantation but is also produced by the embryo. However, this may or may not be the case for A^y/A^y embryos. Niimura and Ishida (1987) demonstrated that normal preimplantation mouse embryos contain PGE_2. This is true of embryo stages from single-cell through blastocyst. While it is known that prostaglandins are a major product of decidual tissue (Hyland, 1982; Harper, 1983), other researchers have shown that the embryos are also a source of the prostaglandin (Pakrasi & Dey, 1982; Dey et al., 1980; Racowsky & Biggers, 1983). Endogenous production of PGE_2 is significant because the cause of lethality for the homozygous lethal yellow embryos must lie within the embryos themselves and not the mother. To test the influence of the uterine environment, A^y/A^y embryos were transplanted to a nonyellow uterus (Robertson, 1942). While development proceeded further than expected in a heterozygous lethal yellow mother, the embryos ultimately failed to survive . It should also be noted that the mothers cannot be the primary source of the defect as they do give birth to lethal yellow heterozygotes as well as nonyellow offspring.

Finally, this study targeted PGE_2 because of its involvement in the implantation process in three ways: blastocyst hatching, changes in trophoblast morphology and adhesion properties, and vascular permeabilization at the site of implantation.

First, the blastocyst must hatch from its protective outer covering, the zona pellucida, before implantation can begin. Both prostaglandin antagonists (Baskar, *et al.*, 1981) and synthesis inhibitors (Holmes and Gordashko,1980), prevent hatching from the zona pellucida. In addition, prostaglandin inhibitors block the implantation process (Lau, *et al.*, 1973; Baskar, *et al.*). As with hatching, application of prostaglandins reverses the effects of prostaglandin antagonists, thereby allowing implantation to occur. Second, pre-implantation changes in both the adhesion properties of the trophoblast cells (Holmes and Dickson, 1973) and the morphology of the blastocyst are estrogen-induced processes; prostaglandins may work by activating estrogen. Holmes and Gordashko noted that embryos without prostaglandins seem to have been treated with an "impeded estrogen"; that is, morphological changes were initiated but underdeveloped and implantation did not occur. As further evidence for the role of prostaglandins in implantation, application of PGE_2 or $PGF_{2\alpha}$ was able to induce pregnancy in delayed-implantation (ovariectomized and progesterone-treated) mice. Although both succeeded in inducing implantation, PGE_2 was the more effective of the two compounds; $PGF_{2\alpha}$ was slow and weak to activate blastocysts. Third, PGE_2 increases vascular permeability of uterine tissue at site of implantation (Poyser, 1981). The resulting edema is considered a prerequisite for implantation (Psychoyos, 1967). Pakrasi and Dey found higher prostaglandin levels at implantation sites with blastocysts than either interimplantation sites or implantation sites without blastocysts. This suggests that the blastocyst, not the endometrium, is the major source of prostaglandins at the implantation site. Therefore, the endogenous prostaglandin level directly and indirectly affects the embryo's ability to implant in the uterus.

MATERIALS AND METHODS

A colony of inbred mice, strain C57BL/6J-A^y/a , was maintained under standard conditions at the Texas A&M Biology Department Vivarium.

Female, heterozygous lethal yellow *(Ay/a)* mice were superovulated to maximize the number of collected embryos. Each mouse was injected with 5 i.u. pregnant mare's serum gonadatropin (PMSG) (Sigma, St. Louis, MO). Forty-eight hours later, each mouse received an injection of 5 i.u. human chorionic gonadatropin (HCG) (Sigma). Fifty-four hours after injecting PMSG, each mouse was placed with a heterozygous lethal yellow male to mate. Copulation was verified by the presence of a vaginal plug.

Recovery of Embryos

Embryos were collected at 2.5 days *post coitum.* Each mouse was humanely sacrificed by CO_2 asphyxiation immediately before collecting embryos. Collection procedures followed those outlined by Hogan in Manipulating the Mouse Embryo.(1986).

1. The abdomen of the freshly killed mouse was wiped thoroughly with 70% ethanol. A small, transverse cut was made at the middle of the abdomen. The viscera were exposed, keeping the skin and fur out of the way. The two horns of the uterus, with the attached ovaries, were located by pushing the digestive tract to one side (see Figure 1).

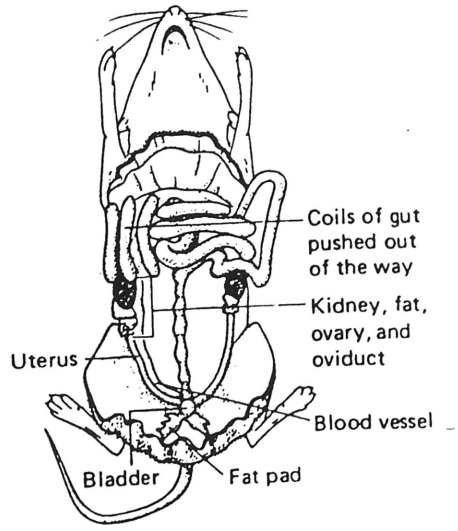

Figure 1: Dissection of Female Mouse Reproductive Organs (Hogan).

2. While grasping one uterine horn with forceps, the mesometrium was trimmed from the uterus, being careful not to cut the uterus itself. This procedure was repeated for the other horn. The uterus was pulled taut and cut between the oviducts and the ovaries. The cervix was also cut below the junction of the two horns, but above the bladder. (see Figure 2)

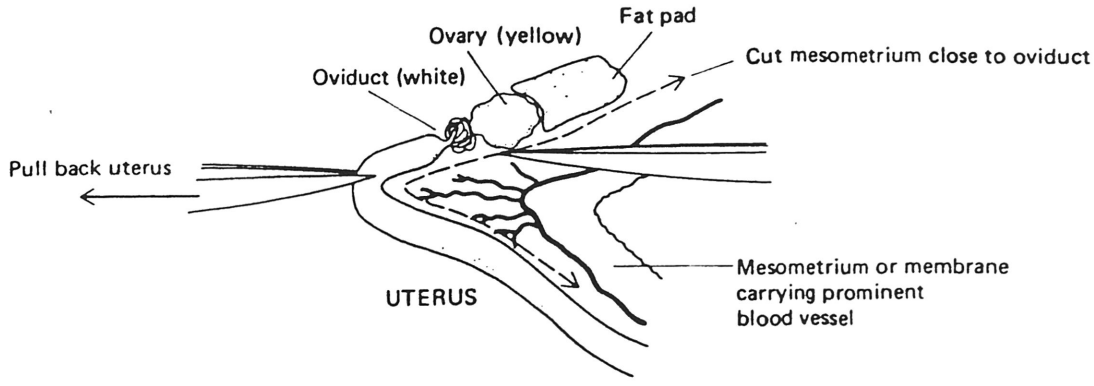

Figure 2: Removal of uterus and oviducts (Hogan).

3. The reproductive tract was placed in a petri dish with a small amount of phosphate buffered saline (PBS) (0.01M phosphate, 0.15M NaCl, pH 7.2-7.4). Using a scalpel, excess tissue was removed while viewing under a dissecting microscope. The reproductive tract was cut between the ovaries and the oviducts and again between the oviducts and the uterine horns.

4. The oviducts and the uterus were rinsed with PBS. Each was placed in a separate depression watch glass with a small amount of PBS. Using PBS and a syringe with a 27-gauge needle, one uterine horn was inflated from the cervical end; the cranial end was cut with a scalpel to release embryos and the fluid. The horn was reflushed from the cranial end toward the cervix. This procedure was repeated for the other horn. The uterus was lifted from the watch glass and rinsed with a small amount of PBS, letting the solution drain onto the watch glass.

5. The oviducts were flushed with PBS in a syringe fitted with a 30-gauge needle. The needle was ground to a dull tip to avoid punching holes in the oviducts. The coiled oviduct was grasped near the uterus-end with watchmakers' forceps; the needle was inserted into the lumen to flush the oviduct. If a white "puff" appeared in the surrounding medium, the flush was effective. The oviduct was rinsed with PBS as it was lifted from the medium.

6. Swirling the watch glass in a circular motion caused the embryos to accumulate at the center. Much of the adipose tissue floated to the surface while the embryos settled to the bottom of the watch glass. The embryos were collected with a micropipet and placed in PBS in another watch glass.

Manipulation of Embryos

Treatment of collected embryos followed the specifications of Niimura & Ishida. All incubations were carried out at room temperature in a humidified chamber.

1. The collected embryos were washed in PBS for 1 hour at room temperature.

2. The zonae pellucidae were removed by transferring the embryos to a drop of 0.2% pronase E (Sigma, St. Louis, MO) in PBS. The embryos were monitored until the zonae began to disappear. The embryos were then quickly removed from the enzyme solution.

3. The naked embryos were washed in PBS for 1 hour.

4. The embryos were fixed in 10% formalin-PBS for at least 1 hour. The embryos were held at this stage until convenient for assay.

5. The fixed, naked embryos were washed in PBS for 1 hour.

6. The embryos were incubated for 1 hour in rabbit anti-PGE2 serum (Polysciences, Warrington, PA) which was diluted 100 times with PBS.

7. The embryos were rewashed in PBS for 1 hour.

8. The embryos were incubated for 1 hour in goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Cappel, West Chester, PA) diluted 64 times with PBS. This was a light-sensitive compound. Therefore, it was kept shielded from light as much as possible.

9. Each embryo was rinsed quickly in PBS immediately before assay.

Fluorescent Microscopy Assay

The embryos were placed in a drop of PBS on a glass slide. They were observed under a light microscope with a mercury light source (Ziesse, West Germany) at 160x and/or 320x magnification.

Nonyellow mouse embryos were used for a positive control group. They were treated in the same manner as the experimental group. All embryos for positive control were expected to fluoresce. Negative control embryos were incubated in either rabbit serum or goat anti-rabbit IgG-FITC. This ruled out the possibility of nonspecific binding or autofluorescence. All negative control embryos were expected to lack fluorescence.

RESULTS AND DISCUSSION

The laws of genetics predict that if two heterozygous parents are crossed, then the genotypes of the offspring will appear in a 1:2:1 ratio (see Figure 3).

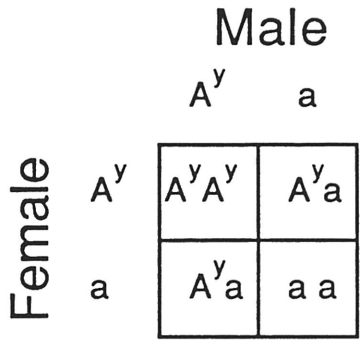

Figure 3: Genotypes from a heterozygous cross: 1 A^y/A^y : 2 A^y/a :1 a/a

A mating of two heterozygous lethal yellow (A^y/a) mice should produce one-fourth of the offspring with the homozygous lethal yellow (A^y/A^y) genotype, one-half with the heterozygous (A^y/a) genotype, and one-fourth with the nonyellow (a/a) genotype. According to our original hypothesis, if the A^y/A^y embryos lack PGE_2, then we expect 1/4 of the screened embryos to lack fluorescence.

Fifteen embryos were screened for the experimental group. Of these only two contained fluorescence and the remaining 13 failed to fluoresce. The hypothesis predicts that 75%, or 11.25 embryos, would have contained fluorescence and 25%, or 3.75 embryos, would have lacked fluorescence. When the data are applied to the Chi-squared test of statistical significance (see Table 1), we find that our results do not fit the expected ratio ($p > 0.01$).

Lethal yellow (A^y/A^y x A^y/A^y)

Fluorescence	Observed	Expected	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
yes	2	11.25	-9.25	85.56	7.61
no	13	3.75	9.25	85.56	22.82
				$\chi^2 =$	30.43

Table 1: Chi-squared results for the experimental group embryos.

Eight of the 10 embryos in the control group contained fluorescence. Although all 10 were expected to fluoresce, the Chi-squared test shows (see Table 2) that our data are statistically significant ($p < 0.01$)

Control Group (a/a x a/a)

Fluorescence	Observed	Expected	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
yes	8	10	- 2	4	0.4
no	2	0	2	4	0.0
				$\chi^2 =$	0.4

Table 2: Chi-squared results for the control group embryos.

Statistical analysis for the experimental and control groups fail to support the original hypothesis that homozygous lethal yellow (A^y/A^y) embryos lack PGE_2.

While the results of this investigation do not support the initial hypothesis that homozygous lethal yellow (A^y/A^y) embryos lack PGE_2, evidence does suggest a deviation from the normal condition. The hypothesis predicted that 75% of the embryos would test positive for PGE_2; this percentage accounts for the heterozygous (A^y/a) and nonyellow (a/a) embryos. Only 13.3% of the experimental group embryos contained PGE_2 while the control group statistically fit the expectation that all embryos would test positive. Because the control mice were genetically identical to the lethal yellow mice at all loci except *agouti*, this suggests that the lethal yellow (A^y) gene is responsible for the absence of PGE_2 in 86.7% of the embryos.

After noting an approximate reversal of ratios, the hypothesis was revised to account for all A^y alleles. The new hypothesis is that all embryos which are either heterozygous (A^y/a) or homozygous (A^y/A^y) for the lethal yellow gene test negative for PGE_2. This would predict that 75% of the embryos should test negative while the remaining 25% should test positive. When the observed results and this new ratio were applied to the Chi-squared test (see Table 3) the data are statistically significant ($p < 0.01$).

Experimental Group (A^y/a x A^y/a)

Fluorescence	Observed	Expected	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
yes	2	3.75	-1.75	3.06	0.82
no	13	11.25	1.75	3.06	0.03
				$\chi^2 =$	0.85

Table 3: Experiment data applied to the revised hypothesis.

The data are insufficient at this time to explain the observed effect of the lethal yellow allele on PGE_2 in embryos. The results may be due to a level of PGE_2 which cannot be detected with these methods. Niimura and Ishida, using the same immunohistochemical techniques, found that the amount of PGE_2 in the embryos depended upon their developmental stage. Because PGE_2 content was correlated with the strength of fluorescence, differences cannot be compared quantitatively. Another possible explanation for these results is an abnormal form of PGE_2 in embryos which carry one or more A^y alleles. Because prostaglandins are lipid-derived substances, no gene can code directly for PGE_2. However, the lethal yellow gene could affect PGE_2 through an enzyme or through a compound which affects prostaglandin sythesis. If an abnormal form of PGE_2 is produced, the compound may not be recognized by the antibody and ,thus, lack fluorescence.

The results for the control group embryos indicate effective methodology. Therefore, the differences between the embryos from yellow mice and the embryos

from nonyellow mice can be attributed to a genetic cause. The mice were genetically identical at all loci except *agouti*. The *agouti* locus, of course, is the location of the lethal yellow (A^y) allele. Although the results of this experiment cannot explain the mechanism for the A^y allele, the data does demonstrate that the lethal yellow gene does affect PGE_2 in embryos.

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