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# Evidence for the Inactivation of an X Chromosome Early in the Development of the Human Female

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There is considerable evidence that as a result of some early embryonic event the genes of one X chromosome in each somatic cell of the mammalian female are not expressed [1]. A single active X is present by 16 weeks menstrual age in the somatic cells of the human female [2, 3]; however, at which developmental stage inactivation occurs remains to be determined. In the human embryos studied by Park [4], significant numbers of sex chromatin were seen first at the eighteenth day (32 days menstrual age); however, the appearance of sex chromatin or other cytological manifestations of X chromosome dimorphism may not be precisely correlated with the time of functional inactivation of the X. Furthermore, Park observed "unusual variation from place to place in the rate of appearance [of sex chromatin]", which might reflect a lack of uniformity in the time of X inactivation in different embryonic tissues.

Additional evidence that X inactivation may not occur at the same time in all cells of the embryo comes from observations of the distribution of pigmented melanocytes in mouse chimeras and natural heterozygotes for X-linked genes specifying pigment phenotypes. On this basis, Deol and Whitten [5, 6] have estimated that X inactivation occurs around the seventh day in cells of the retina and after the eleventh day in those of the inner ear. These estimates are consistent with the suggestion, based on the study of mouse chimeras "heterozygous" for coat colors specified by X-linked genes, that inactivation is not complete before the time of implantation [7], which occurs at 4½ days following ovulation in the mouse and at about 20 days from the first day of the last menstrual period in humans.

Therefore, because it is conceivable that not all cells of the human embryo have completed X inactivation, tissue samples as well as cultured cells were obtained from a variety of organs of early human embryos heterozygous for the common electrophoretic variant of glucose-6-phosphate dehydrogenase (G6PD;

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E.C. 1.1.1.49). We have examined these specimens for evidence of cells with two active X chromosomes.

## MATERIALS AND METHODS

### *Experimental Material*

The embryonic specimens heterozygous for G6PD A were from Negro females undergoing therapeutic abortion in the Fertility Control Unit of the Johns Hopkins Hospital, where every effort is made to assure the accuracy of historical data. Embryonic material from Caucasian females, and therefore presumably of the G6PD B genotype, was obtained from the same clinic as a control for the existence of an embryonic enzyme electrophoretically similar to the A variant.

Tissues were obtained from nonviable embryos which had been aborted between 42 and 98 days following the first day of the last menstrual period. Whenever possible, menstrual age was also estimated on the basis of mean foot length, shown to be related in spontaneous abortuses [8] and in embryos obtained after induced abortion (L. S. Burnett, unpublished data). Utilizing aseptic techniques and a dissecting microscope, the various tissues were identified and samples were obtained from as many distinct organs as possible. A fresh sample of each embryo was assayed directly for G6PD phenotype, while samples of the various embryonic tissues were established into culture. Maternal blood specimens were obtained for G6PD electrophoresis.

### *Cell Culture*

Small samples of the various embryonic tissues were minced into 60 mm plastic dishes and maintained in minimum essential medium enriched with nonessential amino acids (GIBCO) and 20% fetal calf serum. Cell proliferation was sufficiently rapid that cell transfers were made within 1 week following explantation. At that time the original petri dish containing explants and residual epithelioid cells as well as fibroblasts was reflooded with medium. Several days later, when the culture became confluent, it was typed for G6PD. Further typing was obtained from cells which had undergone at least one, and frequently several, subsequent subcultures to look for possible evolution of the enzyme phenotype in culture.

### *Cloning*

Cultures were established from the lung, umbilical cord, heart, intestine, and liver of an embryo aborted 50 days following the last menstrual period. Dilute suspensions of individual cells from aliquots of these cultures, which had been maintained for a short time in liquid nitrogen, were plated into 60 mm petri dishes; the clones thus obtained were transferred using cylinders.

### *G6PD Typing by Hypergel Cellulose Acetate Electrophoresis*

Blood specimens were prepared according to the method described by Sparkes et al. [9], and assays were carried out on red cell lysates within 18 hr following venipuncture. Solid tissues (10–20 mg) and fibroblasts ($10^5$–$10^6$ cells) in 0.05–0.1 ml of water containing NADP (1 mg/ml) were disrupted in an ultrasonic cleaner and frozen two times in an acetone–dry ice mixture. The lysate was used as the enzyme extract (1 $\mu$l lysate/slot). The G6PD electrophoresis was carried out on Titan III cellulose acetate plates (Helena Laboratories) according to the method of Ellis and Alperin [10] with the following modifications: the buffer was Tris EDTA borate, pH 8.7 (Gelman); NADP (5 mg/100 ml) was added to the buffer used to presoak the plates; and the gel ran at 350 V for 18 min. The plates were floated in 3–4 ml of the reaction mix [9] for approximately 20 sec, blotted, placed cellulose-acetate side down against a glass plate,

and sealed to the plate on all sides with tape to prevent drying. The gel was developed in the dark for 15–20 min and then fixed and stored in 1% formaldehyde in the dark where the bands remain stable for several weeks (fig. 1).

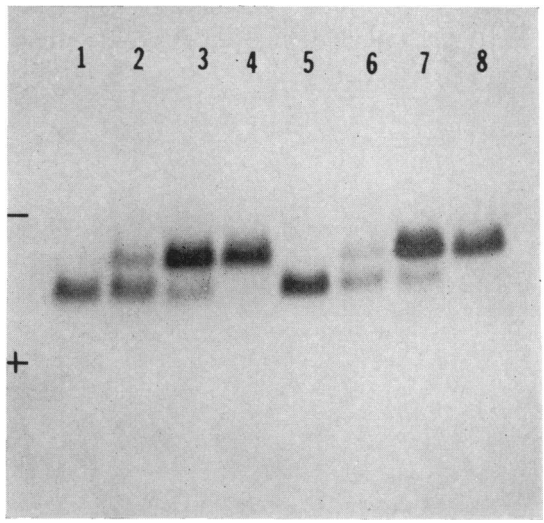


FIG. 1.—G6PD phenotype of nonclonal populations of embryonic fibroblasts and adult fibroblast controls. *Channels 1, 5,* Adult skin fibroblasts of A genotype; *channels 2, 6,* embryonic fibroblasts (K12); *channels 3, 7,* embryonic fibroblasts (K19); *channels 4, 8,* adult skin fibroblasts of B genotype.

## RESULTS

Twenty-two pairs of embryonic and maternal samples were obtained and analyzed for their G6PD phenotype. Seven of these embryos were from Caucasian mothers and ranged in age from 59–81 days following the last menstrual period. In each case maternal and embryonic specimens and the cells cultured from these embryonic specimens had only the B isozyme, as expected on the basis of maternal genotype. The remaining 15 specimens were from Negro mothers and embryos aged 42–98 days following the last menstrual period. Of the maternal samples analyzed, seven had the common B phenotype, while one had the A variant. The remaining seven were heterozygous for A and B. For the embryonic samples assayed directly, the G6PD types were as follows: six had only the B band; four had only the A band; and five had both A and B bands. Karyotypes were obtained from 13 of these, and five were male while eight were female. All of the embryonic specimens which had both A and B bands were female.

The five specimens with the heterozygous phenotype were of 42, 50, 61, 68, and 86 days menstrual age and all showed only two electrophoretic bands (fig. 1, table 1). The earliest specimen (K24) was derived from a mother also heterozygous for the G6PD variants, but the fetal origin of the specimen was clearly indicated by presence of the yolk sac, which was the tissue used for enzyme assay. An additional heterozygous embryo of uncertain menstrual age but of

TABLE 1

G6PD Phenotypes and Pertinent Characteristics of Embryonic and Maternal Specimens

| Maternal Race and Reference No. | Age of Specimen (Days) | | G6PD Phenotype | | | Embryonic Sex | Source of Embryonic Specimens | |
|---|---|---|---|---|---|---|---|---|
| | From Last Menstrual Period | Estimated Menstrual* | Maternal Red Cells | Embryonic Original | Embryonic Cultured Cells | | Original | Cultured Cells |
| Negro: | | | | | | | | |
| K23 ..... | 98 | 77 | B | B | B | ♀ | Thorax, spine | Cartilage, intestine, muscle, skin |
| K12† ..... | 86 | 79 | B | AB | AB | ♀ | Cord, intestine, heart | Adrenal, brain, cord, heart, intestine, kidney, liver, lung, skin |
| K18 ..... | 85 | ... | AB | B | B | ♂ | Cord | Cartilage, cord, muscle, skin |
| K2 ..... | 81 | ... | B | A | A | ... | Cord | Cartilage, eye, muscle, skin |
| K14 ..... | 74 | ... | B | B | B | ♂ | Thorax | Cartilage, muscle, skin |
| K13 ..... | 72 | 63 | AB | A | A | ♂ | Pool‡ | Brain, cartilage, eye, muscle, skin |
| K15 ..... | 70 | 56 | AB | A | A | ♂ | Spine | Cartilage, muscle, skin |
| K17† ..... | 68 | ... | AB | AB | AB | ♀ | Cord | Brain, cartilage, cord, eye, heart |
| K1 ..... | 68 | ... | A | A | A | ... | Pool | Cartilage, cord, muscle, skin |
| K22 ..... | 65 | ... | B | B | B | ♀ | Pool | Cartilage, cord, muscle, skin |
| K16† ..... | 61 | ... | AB | AB | AB | ♀ | Skull, brain | Cartilage, muscle, skin |
| K9 ..... | 61 | ... | B | B | B | ♀ | Fibroblast | Brain, cartilage, eye, muscle, skin |
| K8 ..... | 60 | ... | B | A | A | ♂ | Pool | Cartilage, eye, muscle, skin |
| K19† ..... | 50 | ... | AB | AB | AB | ♀ | Pool | Cartilage, cord, eye, heart, intestine, liver, lung, skin |
| K24† ..... | 42 | ... | AB | AB | AB | ♀ | Yolk sac | Eye |
| Caucasian: | | | | | | | | |
| K4 ..... | 81 | ... | B | B | B | ... | Pool | ... |
| K20 ..... | 80 | ... | B | B | B | ♀ | Limb | Cartilage, cord, heart, muscle, skin |
| K7 ..... | 76 | 77 | B | B | B | ♂ | Limb | Cord, lung |
| K10 ..... | 76 | ... | B | B | B | ♂ | Pool | Cord, eye |
| K11 ..... | 71 | ... | B | B | B | ♂ | Pool | Lung |
| K5 ..... | 63 | ... | B | B | B | ... | Pool | ... |
| K21 ..... | 59 | ... | B | B | B | ♂ | Limb | Cartilage, cord, heart, muscle, skin |

* Based on mean foot length [8].

† Heterozygous embryonic specimen.

‡ Composite of variety of embryonic tissues.

approximately the same developmental age as our earliest one was obtained from another hospital and also showed only two G6PD isozymes. The 50-day specimen (K19) was intact, with prominent tail and limb buds for lower extremities; it was possible to identify several distinct organs which were used to establish fibroblast cultures. Cultures derived from lung, umbilical cord, intestine, heart, and liver were the source of the fibroblast clones which were analyzed as a means to uncover tissues whose cells may not have completed X inactivation. The results of G6PD typing of these clones (table 2) indicate that X inactivation was complete in all cells examined, since in none of them was the heteropolymer expressed.

TABLE 2

EXPRESSION OF G6PD IN 145 CLONES FROM 50-DAY* EMBRYO HETEROZYGOUS FOR G6PD VARIANTS

| ORIGIN OF CLONES | CLONING EFFICIENCY† | PHENOTYPE | | |
|---|---|---|---|---|
| | | A | B | Heteropolymer |
| Lung | .27 | 8 | 15 | 0 |
| Umbilical cord | .42 | 5 | 15 | 0 |
| Intestine | .67 | 14 | 25 | 0 |
| Heart | .32 | 13 | 22 | 0 |
| Liver | .35 | 6 | 22 | 0 |

* From last menstrual period.
† Clones obtained per cells plated.

## DISCUSSION

The electrophoretic variants of G6PD are sensitive indicators of two active X chromosomes in a single cell. Heterozygotes for the A and B variants express only one allele per somatic cell, since the other is on the inactive X chromosome, and tissue samples have two bands (A and B) [11]. On the other hand, when both X chromosomes of a single cell are expressed, as in oocytes from these heterozygotes [3, 12] or in hybrids formed from fusion of male cells of the two G6PD types [13], both A and B bands again are present; but, in addition, there is the heteropolymer, an even more intensely staining band migrating in between. Therefore, while the simultaneous presence of both A and B bands indicates a mixture of the two parental cell types, the expression of the heteropolymer (under the assay conditions used) is specific for the presence of two active X chromosomes within the same cell.

All fetal specimens assayed had either an A or a B band, or both, but never the heteropolymer. Moreover, 145 fibroblast clones (progeny of single cells) obtained from multiple tissues of a 50-day embryo heterozygous for the G6PD variants had only a single G6PD allele per clone, thus confirming that, without exception, inactivation had already occurred (table 2). The efficiency with which clones were obtained (.27–.67) was similar to that for adult fibroblasts [14] and

indicates that the sample of cells assayed was probably representative of the fetal population of fibroblasts from which the clones were derived. Although the existence of rare cells with two functional X chromosomes cannot be excluded, it is clear that the majority of cells have already undergone inactivation.

Furthermore, the G6PD phenotype of these embryonic cells is stable. Without exception, the G6PD electrophoretic type observed initially on direct tissue typing was the one observed in fibroblasts derived from these specimens. Nor were any organ-specific differences apparent. On continued cultivation of cells homozygous or hemizygous for the variants, the G6PD phenotype did not change through the fifth subculture. However, in two of the *heterozygous* embryos a progressive decrease in intensity of one band was observed. One (K12), a female embryo of a B mother, was heterozygous on initial tissue typing and had both A and B bands in the first culture but ultimately lost the B band. The other (K19), an embryonic specimen from an AB mother, on initial tissue assay had A and B bands of equal intensity, but fibroblasts from a variety of tissues showed a progressive decrease in the intensity of the A band (fig. 1). Clones obtained from these fibroblasts were predominantly of the B type (table 2). We interpret this loss of the maternal, and more common, G6PD variant in the first embryo and of the A variant in the second to in vitro selection against one population of cells and not to the disappearance of an embryonic enzyme.

In fact, we found no evidence of an enzyme electrophoretically different from those commonly found in adult tissues, and the type of enzyme in the embryonic specimen was usually consistent with the maternal race and G6PD phenotype. However, in two instances the embryo was A while the mother was B, suggesting exclusion of the maternal G6PD allele. It is unlikely that these discrepancies occurred because of experimental error. More likely they are attributable either to selection against the maternal erythrocytes carrying the pertinent allele or to maternal heterozygosity for the relatively frequent $A^-$ variant, which is abnormally labile in red cells but is stable in nucleated cells such as leukocytes and liver [15].

Our inability to find any evidence of cells with two active X chromosomes in embryos as early as 6–8 weeks gestation (4–6 weeks from conception) indicates not only that X inactivation is initiated early, as has been suspected, but also that it is complete very early in human development. Therefore, the determination of the specific time and pattern of inactivation must necessarily involve analysis of earlier specimens, not easily available in human populations, and may have to await advances in the ability to maintain human blastocysts in vitro.

## SUMMARY

Studies of somatic tissues and cultured cells, including fibroblast clones, from human embryos heterozygous for the electrophoretic variants of glucose-6-phosphate dehydrogenase confirm that one X chromosome is inactivated very early in embryonic development and indicate that X inactivation has occurred in the majority of cells from a variety of tissues at least by 5 weeks from conception.

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