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Genetics

Manufacture of diploid/tetraploid chimeric mice

(cytochalasin B/genetic engineering)

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ABSTRACT **Tetraploid mouse embryos were produced by cytochalasin B treatment. These embryos usually die before completion of embryonic development and are abnormal morphologically and physiologically. The tetraploid embryos can be rescued to develop to maturity by aggregating them with normal diploid embryos to produce diploid/tetraploid chimeric mice. The diploid/tetraploid chimeric embryos are frequently abnormal: the larger the proportion of tetraploid cells, the greater the abnormality. By karyotype analysis and by the use of appropriate pigment cell markers, we have demonstrated that two of our surviving chimeras are in fact diploid/tetraploid chimeras. One surviving chimera is retarded in growth and displays neurological abnormalities. The coat color chimerism suggests that this chimera is about 50% tetraploid. Another chimera with about 10% tetraploid pigment cells in the coat is only slightly retarded in growth and is a fertile male. Tetraploid cells are distributed in many, if not all, tissues of embryos but evidently are physiologically inadequate to support completely normal development and function in the absence of substantial numbers of normal diploid cells.**

Most chimeric mice are made by aggregating eight-cell embryos of two different genotypes to make a single integrated embryo that develops into the typical chimeric mouse. However, it is possible to use three embryos to make triple chimeras (1) or four to make quadruple chimeras (2). Chimeras have also been made between lethal mutant embryos and normal embryos. However, none of these chimeric embryos has succeeded in carrying the mutant cells to term.

The strategy of aggregating semilethal embryonic cells and normal cells has been used in rescuing male jimpy mutants destined to die as juveniles (3). The jimpy/normal male chimeras developed into fertile adults that transmitted the jimpy allele to progeny. The cells of parthenotes have also been rescued by forming chimeras (4, 5). In this paper, we report the successful production of diploid/tetraploid chimeras.

Individuals of different degrees of ploidy from haploid up to hexaploid are relatively easy to produce in amphibia (6). However, mammalian embryos apparently require cells of diploid genotype to survive. No haploid, triploid, or higher degree of ploidy in adult mammals has been conclusively demonstrated. Nevertheless, a few humans have been discovered to be diploid/tetraploid chimeras with low numbers of tetraploid cells (7). Perhaps cells of other degrees of ploidy also exist in human embryos either alone (8) or in association with normal diploid cells.

Several different methods have been used to produce mouse embryos of different degrees of ploidy (9–14). Of these methods, cytochalasin B treatment as devised by Snow (11) has proved to be the most successful for producing tetraploids. Cytochalasin B treatment arrests cell division but permits nuclear division to continue. When the cells are binucleate, they are removed from the cytochalasin B; during the ensuing cell division, a tetraploid karyotype is established in both daughter cells. Such embryos at the blastocyst stage can be transferred to the uterus of a pseudo-pregnant mouse, and a few of them undergo extensive embryonic development. Snow reported that three tetraploid mice were born but did not survive (15). The vast majority of tetraploid and triploid mouse embryos are retarded in growth and exhibit abnormalities in nearly all tissues (15, 16).

In investigating the consequences of tetraploidy, Tarkowski *et al.* (17) used a somewhat different strategy that led to the production of diploid/tetraploid chimeras. He applied the same cytochalasin B treatment to early cleavage stage embryos in which the cells were not in synchrony for cell division. Consequently, at the termination of treatment some cells became tetraploid and others remained diploid. None of these diploid/tetraploid chimeric embryos was carried to term.

By the use of standard techniques for manufacturing chimeras (18), we have extended the work of other investigators to produce fully viable diploid/tetraploid chimeric mice.

MATERIALS AND METHODS

Embryos of albino genotype were from the ICR strain. Embryos of pigmented genotype were obtained by mating ICR females to $B6D2F_1$ males or by mating $B6D2F_1$ females with males of the same strain. A few pigmented embryos were also obtained from crosses of ICR females to SM/J males (a black strain). Eggs and cleavage-stage embryos were obtained by inducing superovulation in females by intraperitoneal injection of 5 international units of pregnant mare serum gonadotropin followed, 48 hr later, by 5 international units of human chorionic gonadotropin.

In these experiments we used the procedure for creating tetraploid embryos devised by Snow (11). Two-cell embryos were flushed from the oviducts of females 50 hr after injection of the chorionic gonadotropin. Embryos were then placed in cytochalasin B and cultured in the medium of Hoppe and Pitts (19). Embryos were cultured at 37°C under an atmosphere of 5% CO_2/95% air for 12–13 hr. A stock solution of cytochalasin B (1 mg/ml in dimethyl sulfoxide) was prepared. This was diluted to give a concentration of 10 μg/ml in the culture medium. After 12–13 hr in culture, the two-cell embryos were washed thoroughly in medium and placed in new medium without cytochalasin B. Some of the two-cell embryos were fixed and stained (20) to determine incipient tetraploidy. The presence of two nuclei in each blastomere (Fig. 1) was evidence that the treatment with cytochalasin B had been successful. On removal from cytochalasin B, a few embryos fragmented or cleaved within the next 4 hr. Cytological and karyotypic analysis revealed that such embryos were not tetraploids and they were discarded in subsequent experiments. The remaining

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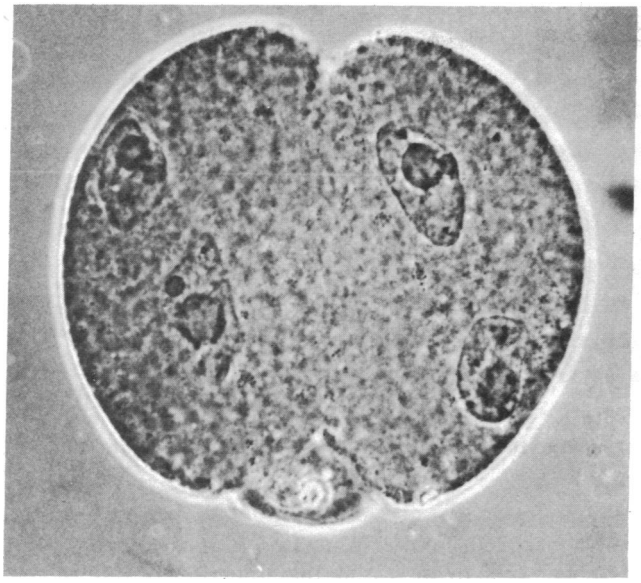

FIG. 1. Two-cell embryo after treatment with cytochalasin B for 12 hr. Note the binucleate condition of each blastomere.

two-cell embryos that divided within the next 5–12 hr were assumed to be tetraploid and were used in the production of chimeric embryos.

In preparing the embryos for aggregation into chimeras, the zona pellucida was removed by treating with acidified Tyrode's solution (pH 2.0) for a few seconds (21). The zona-free embryos were washed and placed in the aggregation medium (1% rehydrated Difco phytohemagglutinin P in culture medium). One tetraploid and one diploid embryo were gently pushed together. In some of the aggregations, one tetraploid embryo was sandwiched between two diploid embryos. These aggregates were placed in microdrops of the aggregation medium and incubated at 37°C under an atmosphere of 5% CO_2/95% air for 15–20 min. When the embryos adhered tightly as incipient chimeric embryos they were washed free of the aggregation medium and incubated in regular medium at 37°C under an atmosphere of 5% CO_2/95% air. Within 1–2 days of culture the aggregates developed into morulae or blastocysts. These chimeric embryos were then transferred to one horn of the uterus of pseudo-pregnant mice. Additional genetically marked diploid embryos were transferred to the other horn. These acted as an internal control and also helped to carry the pregnancy to term. A tetraploid four-cell embryo aggregated with a diploid eight-cell embryo, a diploid/tetraploid morula, and a diploid/tetraploid blastocyst are shown in Fig. 2.

Bone marrow cells were used in karyotype analysis (22). They were obtained from chimeric mice or normal control mice after injection of 0.02 ml of 0.025% colchicine (in isotonic saline) per g of body weight. Three and a half hours later the mice were killed by cervical dislocation, and the femur, tibia, and fibula of both hind legs, the humerus, ulna, and radius of both front legs, and the caudal vertebrae were removed, cut into 1-mm fragments, and centrifuged in 4 ml of 1% sodium citrate prewarmed in a 37°C water bath. The centrifuge tube was shaken vigorously for 6–8 sec and then incubated at 37°C for 10–15 min. The supernatant containing the cells was poured into a clean centrifuge tube and spun in a clinical centrifuge for 3–4 min at 400 rpm. Pellets of bone marrow cells at the bottom of the tube were removed and fixed in methanol/acetic acid, 3:1 (vol/vol). The cells were then resuspended and recentrifuged in the fixative two times. The cells were then dropped on microscope slides and dried with a flame. Control karyotypes from mice of the same sex and age as the experimental chimeras also were made by this procedure.

RESULTS AND DISCUSSION

The data on the development of diploid/tetraploid chimeric embryos are given in Table 1. Of 59 chimeras produced and transferred surgically to the uteri of pseudo-pregnant females, only 2 were born alive and continued to develop. In this experiment the tetraploid cells carried markers for normal pigmentation and the diploid cells were albino (see Table 1, Experiment 3). The chimeras showed mixtures of black and white fur and were putative diploid/tetraploid chimeras. Judged from

Table 1. Data on chimera production

Conditions			Results							
			Chimeric	Carrier	Foster	Offspring				
Diploid strain	Tetraploid strain	Aggregated embryos	embryos transferred	embryos transferred	mothers used	Chimeras, no.	Albino, no.	Pigmented, no.	Carriers, no.	Comment
					Experiment 1					
Albino ICR × ICR	Pigmented ICR × $B6D2F_1$	Diploid albino + tetraploid pigmented	30	29	3	0	5	0	8	No chimeras
					Experiment 2					
Pigmented $B6D2F_1$ × $B6D2F_1$	Albino ICR × ICR	Two diploid pigmented* + one tetraploid albino	11	10	1	2	0	1	6	Chimeras from pigmented diploids;* no white hair
					Experiment 3					
Albino ICR × ICR	Pigmented ICR × $B6D2F_1$	Diploid albino + tetraploid pigmented	18	0	1	2	2	1†	0	One chimera with 50% black hair; one chimera with 10% black hair
Total			59	39	5	4	7	2	14	7% of chimeric embryos born as chimeras

* These diploids were from a cross that segregated four distinct pigment phenotypes.

† This pigmented mouse represents a failure of tetraploidization of the pigmented embryo component of the chimera and also a failure of the chimera to develop to term as a chimera; the albino component must have been excluded during development of the embryo.

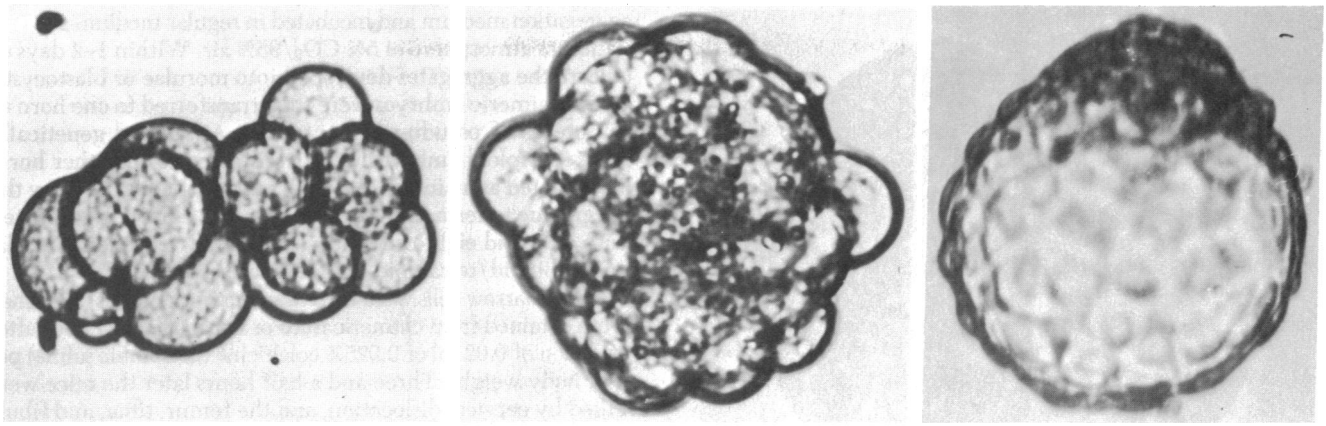

FIG. 2. (*Left*) Aggregated tetraploid (four cells) and diploid (eight cells) embryos to make a diploid/tetraploid chimera. (*Center*) Diploid/tetraploid morula. (*Right*) Diploid/tetraploid blastocyst.

the amount of pigment in the fur, one of the chimeras was about 50% tetraploid and the other about 10%. A few newborn mice were morphologically abnormal and did not survive. They presumably were chimeras of diploid and tetraploid cells because such abnormal newborn are very rare in the stocks of mice we use. Furthermore, several abnormal embryos were found in the uteri at examination on day 10 of the gestation period (about 21 days long). Eleven newborn mice derived from chimeric embryos showed no evidence of tetraploid chimerism and evidently were derived from diploid components of the original chimeric embryos. Two of these 11 were chimeras but were derived from the 2 diploid embryos that were aggregated with a tetraploid embryo in an effort to enhance rescue of the tetraploid cells by increasing the proportion of diploid cells. These embryos were thus triple chimeras. In sum, 22% of all transferred chimeric embryos survived the birth but only 7% were chimeras. The remaining 15% were not overt chimeras and probably developed entirely from only one component of the aggregated embryos. The results demonstrate that diploid/tetraploid chimeras do not develop as well as chimeras made between diploid embryos. Moreover, the larger the population of tetraploid cells in the chimera, the poorer is the development.

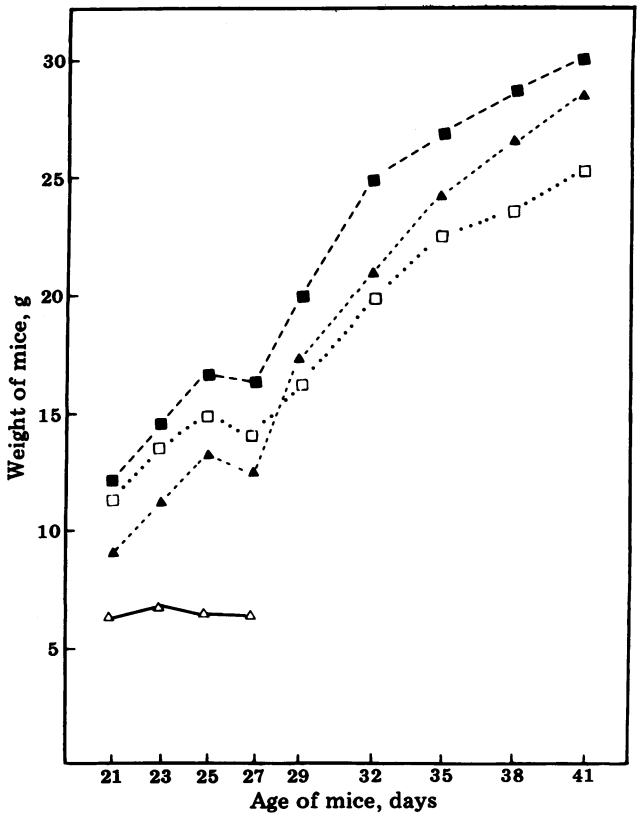

FIG. 3. Growth of diploid/tetraploid chimeras and their control littermates. ■, Diploid male mouse; □, diploid female mouse; ▲, chimera with 10% tetraploid pigmentation; △, chimera with 50% tetraploid pigmentation.

The data plotted in Fig. 3 show the effects of the tetraploid component on the rate of growth of the chimera. The chimera of approximately 50% tetraploid cells did not gain any weight after weaning, became moribund, and was sacrificed for karyotype analysis on day 27 post partum. The remaining surviving chimera grew at a reduced rate for a male but is apparently normal. Both are male and may be sex chimeras, derived from an XX ↔ XY aggregate. These generally develop as phenotypic males.

An extensive karyotype analysis was made on the sacrificed chimera and on diploid controls. At least one of our chimeras was, in fact, a diploid/tetraploid chimera, and the assessment of chimerism by an examination of metaphase figures (Fig. 4) is reliable (Table 2). Only about 3% of the metaphase figures from the chimera were tetraploid. On the basis of fur pigmentation we might have found a much larger fraction—as much as 50%. The low percentage found in the bone marrow cells may reflect variation in the distribution of tetraploid cells in different tissues of the body or it may reflect a disadvantage in cell division of tetraploid stem cells as compared to diploid cells.

The tetraploid cells may be physiologically deficient because of changed surface–volume relationships. They have twice the volume of diploid cells but not a correspondingly enlarged surface area. If they were spheres, the increased surface area would be only about 59% for the 100% increase in surface area, so the surface–volume relationship of normal cells is not maintained. Cell shapes other than a sphere would increase the ratio of surface to volume but not to normal.

Snow has made a detailed examination of morphological abnormalities in tetraploid embryos as they develop (15). He reported that most tetraploids develop little if at all; only about 60% implant, and only about 10% of these show substantial embryonic development. Developmental aberrations began to appear at 14 days of gestation. In the pure tetraploids, nearly all tissues and organs were abnormal to some degree but blood

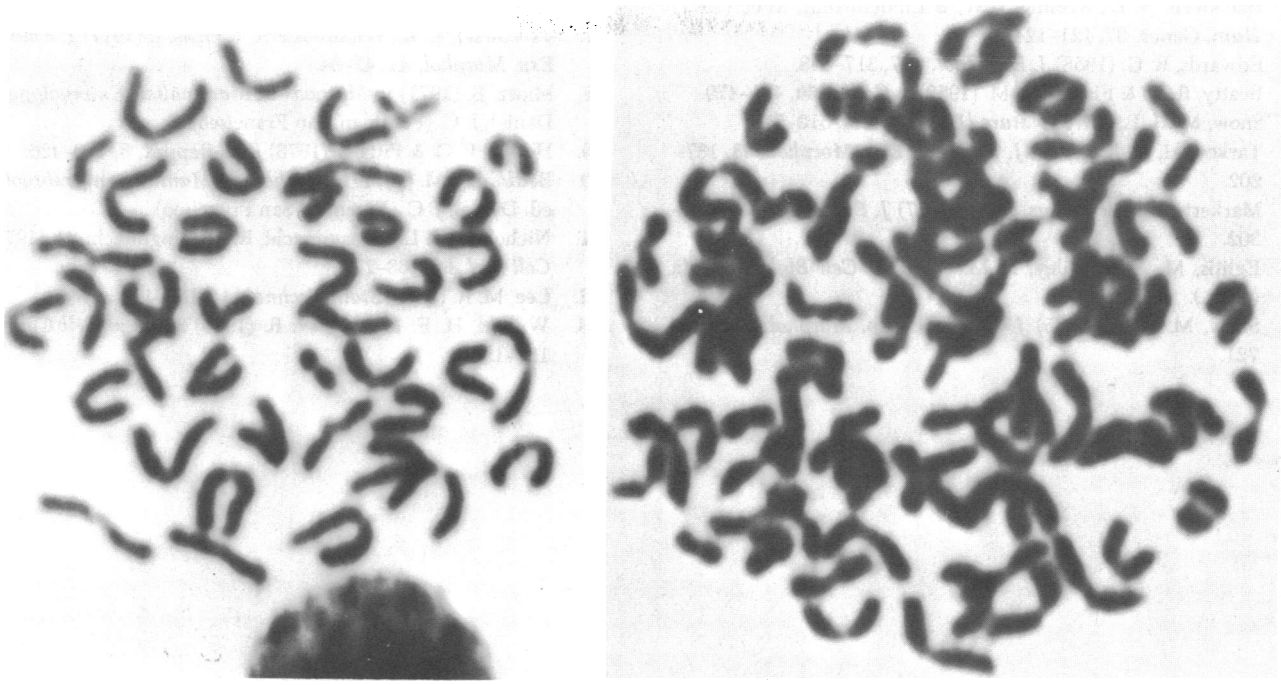

FIG. 4. Diploid (*Left*) and tetraploid (*Right*) karyotypes.

cells and brain were particularly abnormal. The tetraploid embryos were smaller than normal embryos and, because their cells were larger, they must have been composed of very many fewer cells, perhaps only about a fourth the normal number. This deviation in cell size and number may prevent normal morphogenesis (14) and normal physiological function in many tissues and organs.

Although replication of stem cells of the tetraploid may be less frequent than in their diploid counterparts, it does not seem likely that any significant number of the tetraploid cells regressed to diploid. Snow reported (15) that revertants were rare, probably less than 0.1%. In our surviving chimera we have seen no evidence of a changed proportion of pigment in the fur and in this case the pigment stem cells responsible for the pigmentation of the hair do not appear to be changing in relative abundance.

Our surviving chimera is a fertile male, now mature, and reproductively active. He has sired 24 albino progeny, presumably all from the diploid cell line.

A number of important problems should be accessible to investigation through the use of these chimeras. Is there any replicative competition among stem cells in the adult that would place the tetraploid cells at a disadvantage? In the case of pigment cells, for example, will the pigmented areas gradually become white through reduced multiplication or precocious death of the pigmented tetraploid cells? How important is cell size in the function of different specialized cells, particularly in the brain? Our chimera with the largest amount of tetraploid tissues exhibited neurological abnormalities.

Can tetraploid spermatogonia undergo meiosis to give rise to diploid sperm? If so, such sperm would prove useful in the production of normal adrogenetic diploid embryos when used to fertilize eggs in which the female pronucleus is later removed by microsurgery (13). Because half of these ploidy chimeras should be sex chimeras, when the male component is tetraploid, sperm could only be derived from tetraploid cells. It thus should be relatively easy to determine whether or not meiosis can occur successfully in tetraploid cells.

How many Barr bodies would be produced in tetraploid female and male cells? Does the presence of more than one X chromosome in a spermatogonium preclude successful gametogenesis? Or is the ratio between X chromosomes and autosomes the significant variable? Evidence from polyploid mammalian cells in tissue culture suggests that the sex chromosome-to-autosome ratio may determine the number of X chromosomes that can be active (23).

Table 2. Karyotype analysis

Slide	Diploid/tetraploid chimera, no. of cells		Diploid control, no. of cells	
	Tetraploid	Diploid	Tetraploid	Diploid
1	2	45	0	301
2	2	115	0	261
3	4	105	0	609
Total	8	265	0	1171

All metaphase figures on each slide were recorded. Bone marrow cells were cultured and arrested in metaphase as described in text. The proportion of tetraploid metaphase cells (about 3%) in the chimera is much smaller than the proportion of tetraploid pigment cells in the hair (about 50%). No tetraploid figures were observed in preparations from diploid control mice.

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