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TRANSPLANTATION OF MOUSE EMBRYOS, SEX RATIOS OF CHIMERIC MICE, AND MONOZYGOTIC TWINS

by

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A THESIS

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To Linda

and our children

Kevin, Kelly, & Kara

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ABSTRACT

TRANSPLANTATION OF MOUSE EMBRYOS, SEX RATIOS OF CHIMERIC MICE, AND MONOZYGOTIC TWINS

by

RICHARD J. MULLEN

Normal, zona-free, and chimeric mouse embryos were cultured overnight and 1,053 were transplanted to either one or both uterine horns of 106 pseudopregnant inbred or hybrid hosts. A higher percentage of transplanted embryos developed successfully when transplanted to hybrid hosts than to inbred hosts. Transplanting to both horns yielded a higher percent born and fewer resorptions than transplanting to only one horn. Removal of the zona pellucida with pronase reduced the percentage of mice born compared with normal embryos. Chimeric embryos were at least as viable as zona-free control embryos.

The sex ratios of 310 chimeric mice of various combinations of genotypes were studied. The male:female ratios varied from 1:1 to ratios approaching 3:1. Multicolored chimeras in which one genotype predominated ('unbalanced' genotypes) had normal sex ratios of 1:1 suggesting that sex was determined by the predominating component. Multicolored chimeras in which neither component

predominated ('balanced' genotypes) had sex ratios approaching 3:1 suggesting that most sex chimeras were developing as phenotypic males. Regardless of genotype, solid colored chimeras had normal sex ratios. Only two hermaphrodites (0.65%) were observed.

The possibility of producing monozygotic twin mice by separating blastomeres of the two-cell embryo was examined. Although one set of twins was produced the reduced viability of half-embryos made the technique quite impractical. Of 307 3.5-day *post coitum* half-embryos transplanted to 2.5-day hosts, 15 were born. After correcting for transplantation efficiency, it was estimated that 12-38% of half-embryos were totipotent. Varying the transplantation schedule to allow more time for the embryos to develop did not increase their viability. By culturing half-embryos for a period in Ca-free medium the number of cells was increased to normal before formation of morulae. This technique, though not injurious to control embryos, did not increase the viability of half-embryos. Of 241 chimeric embryos derived from the fusion of two half-embryos only 41 or 17.0% were born and only 30.8% were multicolored compared with normal chimeras of which 40-50% were born and 60-70% were multicolored. It was hypothesized that the reduced viability of half-embryos was due to the fact that some half-embryos received sufficient information to be totipotent but most did not.

MONOGRAPH

Culture of Preimplantation Mouse Embryos

In 1949 Hammond demonstrated that mouse embryos could be cultured *in vitro* from the eight-cell to blastocyst stage in a saline hen-egg extract medium. In 1956 Whitten reported that these stages of development could also be cultured in a chemically defined medium consisting of Krebs-Ringer bicarbonate with glucose and crystalline bovine albumin. The following year Whitten (1957) showed that by adding lactate to the medium mouse embryos could be cultured from the two-cell stage to the blastocyst stage. The gas phase of Whitten's medium was 5% carbon dioxide in air.

One-cell embryos were refractory to culture in a chemically defined medium until 1968 when Whitten and Biggers described a medium which allowed the embryos of certain hybrid mice to develop from the one-cell stage to the blastocyst. Shortly thereafter, Whitten (1969) found that if the gas phase was changed to 5% CO_2, 5% O_2, 90% N_2 then inbred as well as hybrid embryos could develop from one-cell zygote to blastocyst.

Although whole embryos are readily cultured in tubes, the microdrop technique of Brinster (1963) is more versatile and facilitates manipulation and observation of embryos particularly when working with zona-free embryos. The technique consists of simply putting small drops of medium under paraffin oil in plastic culture dishes. Details and reviews of culture media and techniques include those by Brinster (1968); Whitten (1970); and Biggers, Whitten, and Whittingham (1970).

Transplantation of Mouse Embryos

Transplantation of preimplantation mouse embryos is a means of demonstrating that some treatment or manipulation of the embryos, or the culturing *per se*, does not appear to reduce their viability. After culturing embryos for two days in Whitten's 1956 medium, McLaren and Biggers (1958) transplanted the embryos and obtained live young thus showing that cultured embryos were indeed capable of complete and normal development.

Although transplanting to a pregnant host is possible, it has many disadvantages. Native embryos and transplanted embryos must differ in pigmentation so they can be distinguished from each other. Also, more embryos might be lost because, as McLaren and Michie (1959) found, embryonic death increases when the number of implants in a single horn exceeds eight. These problems are eliminated when the embryos are transplanted to hosts which are pseudopregnant from matings with vasectomized males. This latter method is now used almost exclusively.

Whether transplanting embryos to test their viability or to obtain experimental animals the most efficient methods should be sought to maximize the percentage of embryos developing normally through birth. McLaren and Michie (1956) obtained better results with transfers of 3.5-day embryos to 2.5-day pregnant hosts than with synchronous transfers. Other reports (Noyes, et al, 1963; Gates, 1965) have shown that with pseudopregnant hosts synchronous transfers can be as successful as asynchronous transfers and that embryos can be transplanted at either earlier or later stages. Transplanting of 3.5-day embryos to 2.5-day hosts is generally recommended, however, for it insures that the blastocyst will be in the uterus for the entire period of time when the uterus is capable of accepting the implanting blastocyst. It also allows time for the embryo to catch up in its development as the processes of culturing, manipulating, and transplanting embryos may retard their development.

Whether there is any advantage of transplanting to both uterine horns rather than just one is not known. Recently, McLaren (1970) transplanted embryos to one horn and then removed the ovary from that side and found that the non-pregnant horn did not exert a luteolytic effect. However, of nine hosts, only five became pregnant and they had received a total of only 15 embryos (three per host). Although it does demonstrate that small numbers of embryos can be successfully transplanted to one horn, it does not rule out the possibility that, with normal numbers of embryos, having both horns pregnant could be advantageous.

Chimeric Mice

The progress in culturing mouse embryos *in vitro* and transplanting them made possible many new techniques in genetics and developmental biology. One such technique was the production of chimeric mice by the fusion of pre-implantation embryos. On Day 2.5 *post coitum*, the mouse embryo is at the eight-cell stage or beyond and is beginning to compact to form a morula. During this period, if the zona pellucida is removed, the embryos are found to be quite sticky and two embryos placed in contact will stick together. What is most interesting is that at this stage in development the morphogenetic movements are such that the two embryos will not only stick together but will actually fuse to form a single double-sized morula. These morulae continue developing into blastocysts and when transplanted give rise to 'normal' young. It should be emphasized that the embryos fuse but the cells do not. In this way, mice derived from these chimeric embryos are normal diploid but may have two completely different genotypes in different cells of the body.

This technique was first reported in 1961 by Tarkowski. Independently, Mintz (1962, 1965a) developed an improved technique using the enzyme 'pronase' to remove the zona rather than the mechanical method of Tarkowski. She also demonstrated that fusion occurred more readily at 37°C. Mintz (1967) termed these chimeric mice "allophenics".

At the time the embryos are fused their sex is not known. Thus, the association of embryos is random and it is expected that 25% will be male fused with male, 25% female with female, and 50% male with female. Genetically, the latter will be sex chimeras but the question of phenotypic sex (all males, all females, all hermaphrodites, or some of each) arises.

In Tarkowski's (1961) original work he observed 11 males, two females, and three hermaphrodites. This prompted him to speculate that some of the sex chimeras were developing as phenotypic males. Such an interpretation seems plausible in light of what is known about mammalian sexual differentiation.

The importance of the Y chromosome in sex determination is demonstrated by the fact that XO mice are female (Russell _et_ _al_., 1959; Welshons and Russell, 1959) and XXY mice are males (Russell and Chu, 1961). Also, fetal testicular hormones appear to have a dominating effect on sexual differentiation (Jost, 1965; Price and Ortiz, 1965). Since the classic studies on freemartins by Lillie (1916, 1917) most genetic, endocrine, and developmental studies have led to the generalization that the male determines and dominates mammalian sexual differentiation.

A preponderance of males among chimeras was also found by Mystkowska and Tarkowski (1968, 1970) who reported nine males, two females, and 23 males, 17 females, respectively. McLaren and Bowman (1969) reported 13 males, one female. However, Mintz (1968, 1969) produced more than 700 chimeras and reported their sex ratio to be more or less like that of normal mice with about 53% males. All workers found the incidence of hermaphrodites to be very low, about 1%.

There are several possible explanations for the sex ratios observed and the discrepancies between investigators. Although Mintz (1967) has obtained a relatively high percentage of young (up to 45%) from transplanted chimeric embryos it is not known whether chimeric embryos are as viable as normal embryos, since no comparisons have been made with normal embryos treated in a like manner but not fused. Thus, although almost all embryos fuse, it may be that all sex chimeras do not develop to term. This might explain the low incidence of hermaphrodites and the normal sex ratio observed by Mintz.

Another point to be considered is that not all fused and transplanted embryos with different pigmentation genotypes will develop as multicolred animals. In fact, Mintz (1967, 1969) found that 60% of the chimeras showed no overt evidence of chimerism; e.g., they were solid colored. This could result from chimeric blastocysts whose inner cell mass is composed entirely of cells of one genotype, the other genotype cells making up the trophoblast which does not contribute to the embryo proper. It might also result from selective or chance events occurring later which might eliminate one or the other genotype. Regardless of how it occurs it raises the question of whether sex ratios should include all chimeras or only those which are demonstrably chimeric.

In all of the earlier papers by Mintz, only the over-all sex ratio was given. Recently (Mintz, 1969), some of the data on multicolored and solid colored animals were reported. Although there was a preponderance of multicolored males, there was also a preponderance of solid colored females and the normality of the over-all sex ratio was stressed. Except for this latter report, Mintz also pooled all of the data on all chimeras regardless of the genotypes of the fused embryos.

Karyological studies by Mintz (1968) and Mystkowska and Tarkowski (1970) have demonstrated that sex chimeras can develop as phenotypic males, females, or hermaphrodites.

There is disagreement however whether or not the male component will usually predominate giving rise to an excess of males.

Totipotency of Half-Embryos

The studies with chimeric mice demonstrated that double-sized embryos are capable of normal and complete development. Similarly, the question of whether or not an isolated portion of a single embryo is capable of normal and complete development has also been asked. In addition to pioneering the work on chimeric mice, Tarkowski (1959a, b) also answered this question. One of the blastomeres of two-cell mouse embryos was destroyed by piercing it with a glass needle. These 'half-embryos' (Tarkowski also refers to them as "half-" or "isolated" blastomeres), still within their zonae, were transplanted to the oviduct where some of them underwent normal and complete development and several progeny were born, demonstrating conclusively their totipotency. The technique only allowed demonstration of the totipotency of single blastomeres. It is not known whether both blastomeres of a two-cell embryo might be totipotent. If they are, the production of monozygotic twins might be possible. In these studies Tarkowski was also concerned with the variations in the number of cells in the inner cell masses and trophoblasts of half-blastocysts, the ratio between them, and their volume. A few embryos appeared to be either entirely trophoblast or inner cell mass but most had both elements. On the average, only 30% of the total number of cells were in the inner cell mass compared with 60% for control whole embryos. Also, there was much more variation in the structure of half-embryos compared with controls.

The cytological work by Dalcq and co-workers (reviewed by Dalcq, 1957) led to the concept that the mammalian egg (one-cell) is characterized by polarity, bilateral symmetry, and two cytoplasmic zones, dorsal and ventral, whose cytoplasm will eventually come to lie in the inner cell mass and trophoblast, respectively. They further found that the first cleavage plane bore no fixed relation to the plane of symmetry so that the blastomeres of two-cell embryos could contain varying amounts of dorsal and ventral cytoplasm.

The great variation in the structure of blastocysts derived from a single blastomere of the two-cell embryo was interpreted by Tarkowski to be a consequence of this variable relation between the first cleavage plane and the plane of symmetry. Thus Tarkowski concluded that although the blastomeres of the two-cell embryo do possess regulating capacities and may be totipotent, they are limited to a large extent and determined by the composition of the blastomere's cytoplasm so that not all blastomeres would be totipotent.

Although the techniques used by Tarkowski were perhaps the only ones available at that time, they are subject to certain criticisms. Tarkowski found that when single zona-free blastomeres were transplanted to the oviduct they not only failed to develop but usually could not even be recovered at the time of autopsy. This necessitated leaving the zona intact and piercing through it to destroy one of the blastomeres. Thus, although there is only half an embryo, it is being bathed in the cytoplasm and debris of the destroyed blastomere. This might provide either a toxic or an enriched environment for the developing embryo. The former might explain the observed variations in the structure of half-blastocysts, the latter the fact that zona-free embryos did not develop whereas those with zonae (and debris) did.

A totipotent blastomere is labile but a labile blastomere may not necessarily be totipotent. However, the study of the lability of blastomeres can be used to determine whether the egg is prepatterned in the manner described by Dalcq and Tarkowski. Mintz (1964a, b; 1965b) fused two or more (up to 16) eight-cell embryos and followed the movements of cells through the blastocyst stage. She claimed that there was no selective sorting-out of cells as would be expected if the blastomeres were determined or prepatterned. She concluded that at least up to the eight-cell stage the blastomeres were morphogenetically labile, a concept that was at odds with the concepts of Dalcq and Tarkowski.

Likewise, cytochemical studies on half-embryos by Mulnard (1965) and additional studies by Tarkowski (1965) have also raised doubts about the concepts of Dalcq and the earlier interpretations by Tarkowski. More recently, Tarkowski and Wroblewska (1967) have studied the *in vitro* development of blastomeres isolated from 4- and 8-cell embryos. They concluded that for the inner cell mass to be formed certain blastomeres must become isolated from the exterior before the blastocoele begins to form. Thus, the abnormal or varied development of half-, quarter-, and eighth-embryos was attributed to the reduced number of cells rather than to variations in the segregation of dorsal and ventral cytoplasm.

The Uses of Mice Derived from Manipulated Embryos

In addition to studies on sexual differentiation discussed above, chimeric mice have been used in a variety of studies. These include studies on pigmentation (Tarkowski, 1964a; Mintz, 1967; McLaren and Bowman, 1969), muscle differentiation (Mintz and Baker, 1967), hematopoiesis (Mintz and Palm, 1969; Wegmann and Gilman, 1970), and liver development (Wegmann, 1970). Chimeric mice are useful in such studies because it is possible to combine two completely different genotypes within a single individual.

Chimeric mice are potentially useful in a variety of genetic and developmental inquiries. For example, does a mixture of mutant and normal cells result in a normal, abnormal, or some intermediate phenotype? The technique of immunofluorescence could be used to determine which cells are genotypically mutant and which are normal. If genotypically mutant cells appear normal in phenotype, this would suggest that they are deficient is some substance that can diffuse or be transported from the normal cells. Examples of this may be *short-ear* (*se*) and *vestigial-tail* (*vt*) described by McLaren and Bowman (1969) in chimeric mice. Conversely, if genotypically normal cells appear mutant in phenotype, it might indicate that the mutant cells are producing some detrimental material. This latter case has yet to be observed. Most of the genes studied to date have been related to pigmentation and all have expressed their normal or usual phenotype in chimeric mice.

Studies on a large number of mutants in mice have been hampered by the fact that many are lethal or do not reproduce when homozygous for the mutant allele. Consequently, litters with all members homozygous for the mutant allele cannot be produced. This means that homozygous mutant animals cannot be identified until the symptoms of the defect manifest themselves or until some closely linked marker (if there is one) expresses itself. It is, therefore, extremely difficult to study the very earliest stages and development of the defect. By fusing a normal embryo with a homozygous mutant it may be possible to obtain animals which, because of their chimeric constitution, are healthy but might produce only mutant bearing gametes. With such animals it would be possible to produce litters with all members homozygous for the mutant allele. Such animals would greatly facilitate the study of the genetic, biochemical, and developmental aspects of a mutant phenotype.

Mice derived from half-embryos are of little value as experimental animals because they do not differ from mice derived from normal whole embryos. However, if it were possible to obtain mice from both halves of an embryo they would be monozygotic twins and could be of considerable value as experimental animals. In spite of the fact that mice can be readily inbred, there are numerous genes and characters which show variable penetrance and expressivity even on an inbred background. Is this variability due to environmental influence and is it determined at the very earliest stages of development? Studies with monozygotic twins might help answer these questions. Also, the development of techniques for producing twins might be extended to other species where inbreeding is less practical than in mice.

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PAPERS

The following papers of which I am sole or senior author are submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. They are written in the form according to the specifications of the different journals.

Mullen, R. J., and S. C. Carter. Efficiency of transplanting normal, zona-free, and chimeric embryos to one and both uterine horns of inbred and hybrid mice. Submitted to and accepted pending revision by Biology of Reproduction.

Mullen, R. J., and W. K. Whitten. Relationship of genotype and degree of chimerism in coat color to sex ratios and gametogenesis in chimeric mice. Submitted to Journal of Experimental Zoology.

Mullen, R. J. Monozygotic twin mice and totipotency of half-embryos. To be submitted to Developmental Biology.

Efficiency of Transplanting Normal, Zona-Free, and Chimeric Embryos to One and Both Uterine Horns of Inbred and Hybrid Mice

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SUMMARY. Normal (zona intact), zona-free, and chimeric mouse embryos were cultured overnight and 1,053 were transplanted to either one or both uterine horns of 106 pseudopregnant inbred and hybrid hosts. A higher percentage of transplanted embryos developed successfully when transplanted to hybrid hosts that to inbred hosts. Transplanting to both horns yielded a higher percent born and fewer resorptions than transplanting to only one horn. Removal of the zona pellucida with pronase reduced the percentage of mice born compared with intact embryos. Zona-free chimeric embryos were at least as viable as zona-free regular embryos.

Transplantation of preimplantation mouse embryos is a means of demonstrating that some treatment or manipulation of the embryos did not appear to reduce their viability. Cholewa and Whitten (1970), for example, demonstrated that culturing embryos in the absence of fixed-nitrogen did not reduce their viability. It is also a means of obtaining animals that otherwise could not be produced such as the chimeric or allophenic mice produced by Tarkowski (1961) and Mintz (1965).

McLaren and Michie (1956) obtained better results with transfers of 3½-day embryos to 2½-day pregnant hosts than with synchronous transfers. After this study was begun, McLaren (1969) reported that removal of the zona by pronase reduced viability of transferred eggs. More recently, McLaren (1970) transferred small numbers of eggs and found neither a systemic nor a local luteolytic effect when one uterine horn was empty.

Experiments reported here were designed to compare the efficiency of inbred and hybrid hosts, the use of one and both uterine horns, and the transplanting of whole and zona-free embryos. Viability of chimeric embryos derived from the fusion of zona-free embryos is compared with non-chimeric zona-free embryos.

MATERIAL AND METHODS

Embryos were collected on Day 2 of pregnancy (plug = Day 0) from (C57BL/10GnDg x SJL/J)F_1, or reciprocal, females that had been mated with similar F_1 males. They were cultured overnight in drops of medium under paraffin oil and transplanted the following day to pseudopregnant hosts that had been mated to vasectomized males the day after the mating of donor females. Thus, 3½-day embryos were transplanted to 2½-day pseudopregnant hosts. Host females were either C57BL/10GnDg, (C57BL/10GnDg x SJL/J)F_1, or reciprocals. All females were judged to be in proestrus by the external appearance of the vagina (Champlin and Dorr, in preparation) and placed singly in pens with one stud male. The zona pellucida was removed with an 0.5% pronase (B grade, Calbiochem) solution. Details of culture medium and method of producing chimeric embryos will be presented elsewhere (Mullen and Whitten, in preparation).

Embryos to be transplanted were removed from culture dishes and pooled in a cavity slide. After anesthetizing with Avertin solution (tribromoethanol in amylene hydrate) and plucking the hair from the back of the host, a single one centimeter incision was made through the skin along the dorsal midline. A second incision was made through the abdominal wall directly over the ovary and the ovary and cranial end of the uterus were pulled through the incision by grasping the fat pad. A thread was passed through the mesometrium to aid in holding the uterus. The cranial end of the uterus was punctured with a 25-gauge needle and the embryos introduced with a small glass pipette attached to a micrometer control. When transplanting to both horns a similar procedure was done on the other side through a third incision. The pipette was drawn from 4 mm O.D. Pyrex glass tubing to a tube of about 0.3 mm O.D., 0.2 mm I.D., and 7 cm long. The fine end was fire polished before use. To assure that no air was injected, 5 to 6 cm of medium was drawn into the pipette before the embryos were picked up in an additional cm of medium. The medium was injected until the meniscus moved 3 to 4 cm. Whether transplanting to one or both horns, 8 to 14 embryos were transplanted per host. The operation from plucking of hair to final suturing lasted 4 to 5 minutes for one horn and 6 to 7 minutes for two horns.

Some hosts were sacrificed at either 16 or 17 days of gestation and the number of fetuses and resorptions ("moles") recorded. Others were allowed to deliver and the number of pups, resorptions, and placental scars recorded. Percentages of embryos developing were subjected to angular transformation and differences analyzed statistically.

RESULTS AND DISCUSSION

Of the 106 hosts used in this study, 100 or 94.3% became pregnant. Earlier results (Whitten, unpublished data) indicated that C57BL/10 females were better hosts that BALB/c or SJL females. C57BL/10 and B10SJLF$_1$ hybrids are resistant to pregnancy block whereas BALB/c and SJL are susceptible (Chapman and Whitten, 1969). Thus, the very high rate of pregnancy in this study may be partly attributable to the use of hosts resistant to pregnancy block.

The efficiency of transplanting whole and zona-free embryos into one and both horns of inbred and hybrid hosts is shown in Table 1. If the 2 C57BL/10 hosts that did not become pregnant were omitted the percentages would be 41.1% born and 23.3% resorbed.

Higher percents of embryos were born when transplanted to hybrid hosts than to inbred C57BL/10 hosts. The differences are not highly significant ($P<0.1$) but it might be expected that the hybrids provide a better environment for the development of the embryos.

Regardless of the host, a greater percent of embryos survived when transplanted to both horns than to only one horn ($P<0.01$). With F$_1$ hosts, part of this difference appears due to the fewer number of resorptions ($P<0.05$) when both horns are pregnant (6.9%) than when only one horn is pregnant (17.6%). McLaren and Michie (1959) found an abrupt increase in embryonic death when the number of implants in

TABLE 1

PERCENTS OF WHOLE AND ZONA-FREE EMBRYOS BORN AND RESORBED FOLLOWING TRANSPLANTATION TO ONE AND BOTH HORNS OF INBRED AND HYBRID HOSTS

			No. born or resorbed/No. transplanted %			
Hosts	Zona		Both horns		One horn	
C57BL/10	Intact	Born	55/112	49.1	37/112	33.0[a]
		Resorbed	18/112	16.1	21/112	18.8[a]
B10SJLF$_1$	Intact	Born	85/131	64.9	51/125	40.8
		Resorbed	9/131	6.9	22/125	17.6
B10SJLF$_1$	Removed	Born	51/129	39.5		
		Resorbed	7/129	5.4		

Total of 60 hosts, 12 per group.

[a] Includes 2 hosts that did not become pregnant.

a single horn exceeded eight. Since approximately the same number of eggs were transplanted into one horn as into both horns, the difference observed in this study could have been due to crowding. However, even though females receiving 8 eggs in one horn did show a slightly higher percent born than those receiving 10 or 14 eggs in one horn, the difference was not significant. There was no difference in number of resorptions, and so crowding could not explain the large difference between transplanting to one and both horns.

A non-pregnant horn could exert an effect, possibly luteolytic, that reduces the number of embryos that can be maintained throughout gestation. Recently, McLaren (1970) transplanted eggs to one horn and then removed the ovary from that side and found that the non-pregnant horn did not exert a luteolytic effect. However, only about 3 eggs were injected per horn and only 5 of 9 hosts became pregnant.

The observation that it is advantageous to have both horns pregnant is similar to the observations of Biggers _et_ _al_. (1962) that mice with only one ovary, though nearly as heavy and ovulating as many eggs as both ovaries in controls, had smaller litters.

When the zona pellucida was removed with pronase fewer ($P<0.01$) embryos survived until birth. Only 39.5% of the zona-free embryos were born compared with 64.9% of the whole embryos. A similar but more severe effect has been observed by McLaren (1969).

Results of transplanting chimeric embryos of various genotypes to hybrid hosts are shown in Table 2. The embryos were handled in the same fashion as the zona-free embryos referred to in Table 1 (i.e., collected on Day 2 and zona removed with pronase, except they were fused). It is quite apparent that chimeric embryos are at least as viable as regular embryos, if not more so. The somewhat poorer results with BALB/c↔C57BL/10 chimeras may be due to the slower development of BALB/c preimplantation embryos and the occasional difficulties in culturing them. Mintz (1967) has reported similar percentages born for chimeric mice, but did not compare survival with normal zona-free embryos.

TABLE 2

EFFICIENCY OF TRANSPLANTING CHIMERIC EMBRYOS TO HYBRID HOSTS

Component genotypes	No. of hosts†	No. born/No. transplanted	%
Both horns			
(C57BL/10 x CBA/J)F_1 ↔ SJL/J	8	58/113	51.3
BALB/cGnDgWt ↔ C57BL/10	8	30/ 81	37.0
One horn			
SJL/J ↔ C57BL/10	15	54/134	40.3
(BALB/c x SJL)F_1 ↔ C57BL/10	6	20/ 49	40.8
(SJL x 129/Rr)F_1 ↔ C3HeB/FeJ	5	14/ 32	43.8

†Does not include 4 hosts that did not become pregnant.

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Relationship of Genotype and Degree of Chimerism in Coat Color to Sex Ratios and Gametogenesis in Chimeric Mice

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The production of chimeric mice by the fusion of preimplantation embryos was first reported by Tarkowski ('61). Independently, Mintz ('62, '65) developed an improved technique and termed these chimeric mice "allophenics" (Mintz, '67). Chimeric mice have been used in a variety of inquiries into pigmentation (Tarkowski, '64a; Mintz, '67; McLaren and Bowman, '69), muscle differentiation (Mintz and Baker, '67), hermaphroditism (Tarkowski, '64b; Mintz, '68), hematopoiesis (Mintz and Palm, '69; Wegmann and Gilman, '70), and liver development (Wegmann, '70).

Since the classic studies on freemartins by Lillie ('16, '17) most genetic, endocrine, and developmental studies on mammalian sexual differentiation have led to the generalization that the male determines and dominates sexual differentiation. The finding of XO female mice (Russell *et* al., '59; Welshons and Russell, '59) and XXY male mice (Russell and Chu, '61) demonstrates the importance of the Y chromosome in determining sex regardless of whether it is a genic or chromosomal phenomenon (Mittwoch, '69). The dominating role of fetal testicular hormones in sexual differentiation has been reviewed by Jost ('65) and Price and Ortiz ('65).

Since it is expected that half of the chimeric mice produced by the fusion of two embryos will be sex chimeras (XX ↔ XY), but few appear as hermaphrodites, a basic question arises concerning the sex ratio of chimeric mice. A normal sex ratio would suggest that sex chimeras can develop as either males or females, an abnormal ratio would suggest that either the male of female component exerts a dominating effect on sexual differentiation. Although the question appears quite simple, the interpretations of the results have been controversial.

Tarkowski's ('61) original data of 11 males, two females, and three hermaphrodites prompted him to speculate that most sex chimeras appear as phenotypic males. A preponderance of males has also been noted in two subsequent reports by Mystkowska and Tarkowski ('68, '70), nine males, two females, and 23 males, 17 females, respectively, and by McLaren and Bowman ('69), 13 males, one female. Mintz ('68, '69), working with much larger numbers of animals (746), has reported a more or less normal sex ratio of 53% males. Mintz ('69) has shown the data on multicolored and solid colored animals for only two combinations of genotypes and although there is a preponderance of multicolored males (60%), the normality of the total sex ratio is stressed. All reports agree that the incidence of hermaphroditism is very low, around 1%.

In this report emphasis is placed on differences in sex ratios between chimeric mice of various combinations of genotypes and between mice that are overtly chimeric (i.e., multicolored) and those that may or may not be chimeric but most certainly have a very low degree of chimerism (i.e., solid colored). Progeny of chimeric mice were examined to see if there was any correlation between the genotypes of gametes produced with degree of chimerism in coat color.

MATERIALS AND METHODS

Animals

The mice were bred in the research laboratories or the Production Department of The Jackson Laboratory. The pigmented strains were either agouti (A/A) or nonagouti (a/a); the albino (c/c) strains were homozygous agouti except 129/Rr which was homozygous white-bellied agouti

($\underline{A}^{\underline{w}}/\underline{A}^{\underline{w}}$). The inbred strains and the crosses used to produce the embryos are given in table 1, together with the designations, relevant genotypes and colors. Females, whether they were killed for their embryos or used as recipients for transplanted embryos, were mated during proestrus stage as judged by the external appearance of the vagina (Champlin, Dorr and Gates, in preparation). Occasionally, donors were superovulated females. Hosts were usually B10SJLF_1 or reciprocal females that had been mated to vasectomized males the day after donor females had been mated.

Culture Techniques

The embryo culture medium was that of Whitten and Biggers ('68) with slight modifications. It contained NaCl, 5.140 gm; KCl, 0.356 gm; KH_2PO_4, 0.162 gm; $MgSO_4 \cdot 7H_2O$, 0.294 gm; $NaHCO_3$, 2.106 gm; Na pyruvate, 0.036 gm; glucose, 1.000 gm; penicillin G potassium salt, 0.075 gm (118,875 units); streptomycin sulphate, 0.050 gm (37,500 I.U.); Ca lactate$\cdot 5H_2O$, 0.527 gm; Na lactate (60%), 3.680 ml; phenol red (1% solution), 1 ml; and crystalline bovine serum albumin, 3.000 gm, per liter distilled water. The medium was filtered (0.20 µ Millipore filter) and gassed with 5% O_2, 5% CO_2, balance N_2 (Whitten, '69) or 5% CO_2, balance air. The 0.5% pronase solution containing 100 mg pronase (B grade, Calbiochem) and 20 mg polyvinylpyrrolidone

TABLE 1

Embryos used to produce chimeric mice

Strains and Crosses	Designations of embryos	Genotypes at agouti and albino loci	Color
Inbred strains			
C57BL/10GnDg	B10	a/a C/C	black
CBA/J	CBA	A/A C/C	agouti
C3HeB/FeJ	C3H	A/A C/C	agouti
SJL/J	SJL	A/A c/c	albino
BALB/cGnDgWt	BALB	A/A c/c	albino
SWR/J	SWR	A/A c/c	albino
129/Rr	129	A^w/A^w c/c	albino
Crosses			
B10♀ x SJL♂	$B10SJLF_1$	A/a C/c	agouti
SJL♀ x B10♂	$SJLB10F_1$	A/a C/c	agouti
B10♀ x CBA♂	$B10CBAF_1$	A/a C/C	agouti
BALB♀ x SJL♂	$BALBSJLF_1$	A/A c/c	albino
SJL♀ x 129♂	$SJL129F_1$	A/A^w c/c	albino
129♀ x SJL♂	$129SJLF_1$	A/A^w c/c	albino
$B10SJLF_1$♀ x B10♂	($B10SJLF_1$ x B10)	a/a C/C or or A/a C/c	black or agouti
$SJLB10F_1$♀ x B10♂	($SJLB10F_1$ x B10)	a/a C/C or or A/a C/c	black or agouti
$BALBSJLF_1$♀ x SJL♂	($BALBSJLF_1$ x SJL)	A/A c/c	albino

(PVP, Plastone C, General Aniline and Film Co.) per 20 ml of culture medium without albumin was filtered, frozen, then thawed and centrifuged before use. Embryos were cultured in small drops (4-10 µl) of medium under paraffin oil (Brinster, '63) in 60 x 15 mm plastic culture dishes (Falcon Plastics). There were 24-36 drops per dish. The paraffin oil was equilibrated with medium without albumin before use. Dishes were placed in an anaerobic culture jar (Torsion) and gassed prior to incubation at 37°C.

Collection, Fusion, and Transplantation of Embryos

All chimeras were derived from the fusion of a genotypically pigmented embryo with an albino. Day 2.5 (plug = Day 0) embryos were flushed with medium from the oviducts into glass cavity slides. Embryos from several females of the same genotype were pooled and then transferred to pronase solution and left until the zonae had almost disappeared. They were rinsed three times in medium before placing one embryo per drop in the culture dish which was then incubated. Embryos of the other component genotype were treated in a like manner and then each was 'dropped onto' an embryo already in the culture dish, by releasing it from the pipette directly above the other embryo and allowing it to drift down until contact was made. This procedure usually insured adhesion. Before incubation, all pairs of embryos that were not in contact were pushed together with a fine glass probe. They were checked two hours later to be sure they were still in contact. The following morning the chimeric embryos were transplanted to pseudopregnant hosts according to procedures described by Mullen and Carter ('71).

Subsequent Handling

If a pregnant host did not produce her litter by Day 19, the litter was delivered by Caesarean section and fostered. At birth the number of offspring and sex were recorded. At weaning the mice were earmarked and their sex and coat color recorded. Since all chimeras were derived from fusion of embryos with albino and pigmented genotypes, the approximate percentage of albino hair in the coat of multicolored chimeras provided some indication of the degree of chimerism in individual mice. Since the percentage of albino hair was estimated rather than measured, the values should only be considered as approximate relative percents as estimated by a single investigator. In designating chimeras, the pigmented genotype will always be given first and the symbol ' ↔ ' (Mintz, '64) will be used to indicate fusion.

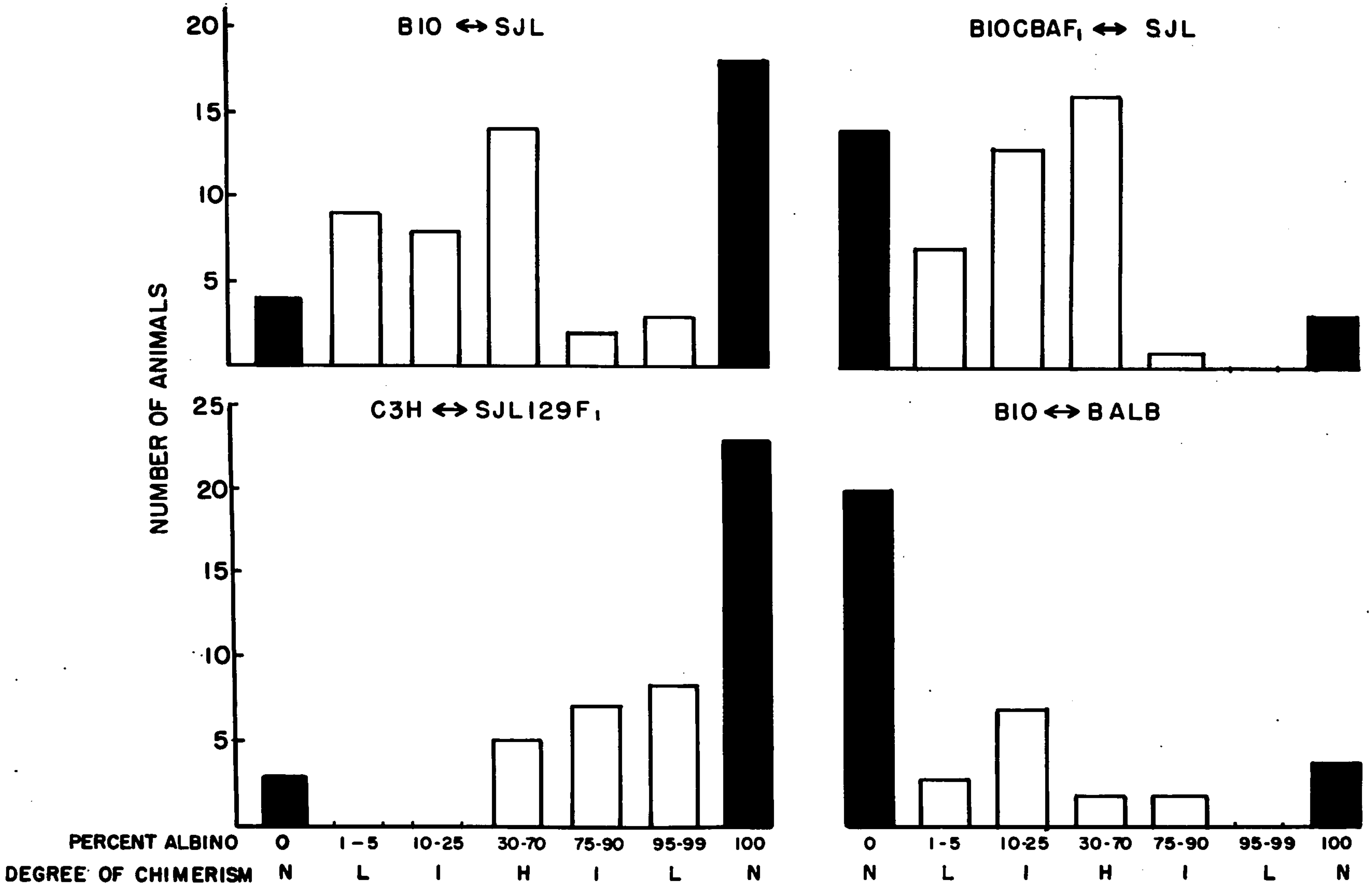
At seven or eight weeks each chimera was mated with B10 (a/a C/C) or SJL (A/A c/c) animals and sex and coat color of the progeny recorded. The coat color of the progeny indicated whether gametes of one or both genotypes were being produced; e.g., whether a B10 ↔ SJL chimera was producing B10, SJL, or both B10 and SJL gametes. Since the results obtained from matings with B10 and SJL were similar, most subsequent matings were with SJL so that progeny could be classified at birth by eye pigmentation.

RESULTS

Since pigmentation was the only overt indicator of chimerism, percent albino was used as a measure of the degree of chimerism in individual animals. Animals that were 1-5 or 95-99% albino were considered as having a low degree of chimerism; 10-25 or 75-90%, an intermediate degree; and 30-70%, a high degree. Combinations of genotypes which gave rise to large numbers of animals with a high degree of chimerism were considered to be developmentally 'balanced', that is, neither component appeared to predominate to any great extent. 'Unbalanced' genotypes were those in which one of the genotypes predominated in most of the animals. The histograms in figure 1 show the incidence of the various degrees of chimerism and of solid colored animals with obvious differences between chimeras of different genotypes.

The large number of B10 ↔ SJL chimeras showing high and intermediate degrees of chimerism indicates that these

Fig. 1. The histograms show the incidence of chimeras with varying degrees of chimerism and of solid colored animals which may or may not be chimeric. N = no overt chimerism (i.e., solid colored, shown in black); L = low degree of chimerism (1-5 and 95-99% albino); I = intermediate (10-25 and 75-90% albino); and H = high (30-70% albino). The upper two were considered balanced genotypes, the lower two unbalanced.


B10 ↔ SJL
B10CBAF₁ ↔ SJL
C3H ↔ SJL129F₁
B10 ↔ BALB
NUMBER OF ANIMALS
20
15
10
5
25
20
15
10
5
PERCENT ALBINO
0
1-5
10-25
30-70
75-90
95-99
100
0
1-5
10-25
30-70
75-90
95-99
100
DEGREE OF CHIMERISM
N
L
I
H
I
L
N
N
L
I
H
I
L
N

two strains tend to be developmentally balanced. It is interesting that, whereas the distribution of multicolored chimeras is skewed in favor of B10, the solid colored animals show a definite preponderance of SJL (i.e., animals that are 100% albino). B10CBAF$_1$ and SJL also appear to be balanced but in this case the preponderance of entirely pigmented animals (B10CBAF$_1$ component) is in agreement with the skewness of the distribution of multicolored chimeras. The variation in degree of chimerism between animals of the same balanced genotypes is shown in figure 2. Data on pigmentation were not available for all (B10SJLF$_1$ x B10) ↔ (BALBSJLF$_1$ x SJL) chimeras but these genotypes too appeared balanced.

C3H and SJL129F$_1$ are definitely unbalanced (fig. 1). Fifteen of the 20 multicolored chimeras were 85-99% albino, the SJL129F$_1$ component. Although the data are limited, B10 and BALB would also appear to be unbalanced with the B10 component predominating. For other combinations of genotypes, the data were insufficient to determine whether or not the genotypes were balanced.

The frequency of multicolored chimeras of the various genotypic combinations is shown in table 2. The table includes only data on chimeras derived from the fusion of two whole embryos. The frequency of multicolored chimeras

Fig. 2. These 14 $B10CBAF_1 \leftrightarrow$ SJL chimeras demonstrate the variation that can occur between animals of the same genotype. In some, the SJL component predominates (upper left), in others the $B10CBAF_1$ component (lower right), but most show a high degree of chimerism so the component genotypes were said to be balanced.

TABLE 2

Frequency of multicolored chimeras

	Multicolored/Total	%
Balanced genotypes		
B10 ↔ SJL	40/62	64.5
B10CBAF$_1$ ↔ SJL	38/55[1]	69.1
(B10SJLF$_1$ x B10) ↔ (BALBSJLF$_1$ x SJL)	15/22[1]	68.2
Balanced genotypes total	93/139	66.9
Unbalanced genotypes		
C3H ↔ SJL129F$_1$	22/65	33.8
B10 ↔ BALB	18/42	42.9
Unbalanced genotypes total	40/107	37.4
Others		
B10 ↔ BALBSJLF$_1$	13/23	56.5
B10 ↔ 129	2/ 2	83.3
B10 ↔ SWR	2/ 3	
(BALBSJLF$_1$ x BALB) ↔ B10SJLF$_1$	2/ 2	
(SJLB10F$_1$ x B10) ↔ 129SJLF$_1$	4/ 5	
Others total	23/35	65.7
Grand total	156/281	55.5

[1] Includes one hermaphrodite.

derived from two half-embryos, which was about 30%, will be discussed elsewhere (Mullen, in preparation). The frequency of multicolored chimeras appears dependent on the genotypes of the fused embryos. Those combinations of genotypes classified as balanced had a much higher incidence of overt chimerism (66.9% multicolored) than unbalanced genotypes (37.4% multicolored). In view of these differences, the over-all frequency of 55.5% multicolored is somewhat meaningless.

None of the differences in figure 1 and table 2 are due to changes in technique or increased skill of the investigators. B10 ↔ SJL (balanced, high frequency of multicolored animals) and C3H ↔ SJL129F_1 (unbalanced, low frequency) were made during the same period at the beginning of this study as were B10CBAF_1 ↔ SJL (balanced, high frequency) and B10 ↔ BALB (unbalanced, low frequency) during the latter part of this study.

Sex Ratios

The sex ratios of chimeras of the various genotypic combinations and of multicolored and solid colored animals are shown in table 3. The sex ratios vary considerably depending on whether the component genotypes, classified on the basis of pigmentation, were balanced or unbalanced, and on whether all mice or only the multicolored ones were

TABLE 3

Sex ratios of chimeric mice

	Total			Multicolored			Solid colored		
	♂♂	♀♀	%♂♂	♂♂	♀♀	%♂♂	♂♂	♀♀	%♂♂
Balanced genotypes									
B10 ↔ SJL	39	23	62.9*	29	11	72.5**	10	12	45.5
B10CBAF_1 ↔ SJL	35	19	64.8*	25	12	67.6*	10	7	58.8
(B10SJLF_1 x B10) ↔ (BALBSJLF_1 x SJL)	26	24	52.0	16	8	66.7	10	16	38.5
Total	100	66	60.2**	70	31	69.3**	30	35	46.2
Unbalanced genotypes									
C3H ↔ SJL129F_1	30	35	46.2	11	11	50.0	19	24	44.2
B10 ↔ BALB	21	21	50.0	8	10	44.4	13	11	54.2
Total	51	56	47.7	19	21	47.5	32	35	47.8
Others									
B10 ↔ BALBSJLF_1	9	14	39.1	5	8	38.5	4	6	40.0
B10 ↔ 129	1	1		1	1				
B10 ↔ SWR	2	1		2				1	
(BALBSJLF_1 x BALB) ↔ B10SJLF_1	2			2					
(SJLB10F_1 x B10) ↔ 129SJLF_1	3	2		3	1			1	
Grand total	168	140	54.5	102	62	62.2**	66	78	45.8

* $P < 0.05$

** $P < 0.01$

considered. All ratios were tested against an expected ratio of 1:1 (i.e., 50% males).

A significant excess in the total number of males over females occurred in the first two combinations of balanced genotypes, B10 ↔ SJL and B10CBAF$_1$ ↔ SJL (62.9 and 64.8% males, respectively). Examination of the data on multicolored and solid colored animals reveals that the over-all excess of males is, in both instances, due almost entirely to the significant excess of multicolored males (72.5 and 67.6%). The sex ratios of the solid colored animals are normal. The sex ratio of (B10SJLF$_1$ x B10) ↔ (BALBSJLF$_1$ x SJL) is normal but there is still an excess of multicolored males (66.7%). The pooled data on balanced genotypes show a significant ($P < 0.01$) excess of males (60.2%) that is entirely due to the significant ($P < 0.01$) value of 69.3% multicolored males.

The data on unbalanced genotypes are quite different. The over-all sex ratios are normal, as are the sex ratios of multicolored and solid colored animals.

When all of the data are pooled there is a slight, but not significant, excess of males (54.5%) but a significant ($P < 0.01$) excess of multicolored males (62.2%). The over-all ratios are shown only for comparison with other reports and to demonstrate that over-all sex ratios are not meaningful.

In addition to the mice referred to in table 3, two intersexes were observed. One was a $B10CBAF_1$ ↔ SJL, the other a ($B10SJLF_1$ x B10) ↔ ($BALBSJLF_1$ x SJL) and both were multicolored. The observed incidence of intersexuality was 0.65% (2/310) but may be somewhat higher since not all animals were thoroughly examined.

Gamete Production

Of 212 chimeras tested (125 multicolored, 87 solid) only seven failed to produce litters. These seven were multicolored, with balanced genotypes. They included three B10 ↔ SJL (one normal male, one cryptorchid male and one female), three $B10CBAF_1$ ↔ SJL (one male, one female, and one intersex), and one ($B10SJLF_1$ x B10) ↔ ($BALBSJLF_1$ x SJL) intersex. Two of the B10 ↔ SJL animals (the female and cryptorchid male) were litter-mates. Likewise, two of the $B10CBAF_1$ ↔ SJL (the female and the intersex) were littermates and all three were produced on the same day from the same batch of pooled eggs.

By mating many of the chimeras it was determined whether the gametes effecting fertilization were of one or both of the parental component genotypes. One hundred and eighty litters were obtained from 69 solid colored animals (i.e., possible chimeras) and all of the 1,344 progeny were of the genotype corresponding to the solid colored animal being tested. For example, all of the entirely albino B10 ↔ SJL chimeras, when mated with another albino, produced only albino progeny.

The mating of 110 multicolored chimeras produced 3,811 progeny in 486 litters. Examination of the genotypes and sex ratios of these progeny showed no evidence of functional sex reversal occurring in chimeras. There was no evidence of XX cells producing sperm or XY cells producing ova. Therefore, gonadal mosaic females, producing progeny with both parental component genotypes, must be XX ↔ XX and gonadal mosaic males must be XY ↔ XY. Those chimeras with non-mosaic gonads, producing progeny of only one genotype, may be XX ↔ XX or XY ↔ XY but are also possible sex chimeras, XX ↔ XY.

The incidences of chimeras with mosaic and non-mosaic gonads are shown in table 4. There are some obvious differences between chimeras of different component genotypes but what is most striking is the difference between males and females. Females show a high incidence of gonadal mosaics and relatively low incidence of possible sex chimeras as opposed to males which show a very low incidence of gonadal mosaics and an extremely high incidence of possible sex chimeras.

TABLE 4

Incidences of multicolored males and females with mosaic and non-mosaic gonads

	Males				Females			
	Mosaic testes[1]		Non-mosaic testes[2]		Mosaic ovaries[1]		Non-mosaic ovaries[2]	
	No.♂♂	%	No.♂♂	%	No.♀♀	%	No.♀♀	%
B10 ↔ SJL	3	13	20	87	6	67	3	33
B10CBAF_1 ↔ SJL	8	35	15	65	7	70	3	30
C3H ↔ SJL129F_1	0	0	10	100	2	20	8	80
B10 ↔ BALB	1	14	6	86	5	71	2	29
B10 ↔ BALBSJLF_1	0	0	4	100	4	57	3	43
Totals	12	18	55	82	24	56	19	44

[1] Progeny from both component genotypes, males must by XY ↔ XY, females XX ↔ XX.

[2] Progeny from only one component genotype, possible sex chimeras XX ↔ XY.

Additional differences between males and females are seen when gamete production is studied in relation to degree of chimerism (table 5). The values in the table are 'percent albino' as estimated for each animal at weaning.

Females producing progeny with only the pigmented or only the albino genotype tended to show a very low degree of chimerism. Those females producing only ova of the pigmented genotype were themselves predominantly pigmented, as indicated by their low percentage of albinism; those producing only ova of the albino genotype were predominantly albino in color. There are three possible exceptions to this generalization, the two B10 ↔ SJL chimeras which produced only albino (SJL) ova but showed very little albino in their coats (5*A* and 10*A*%), and the B10CBAF$_1$ ↔ SJL which was only 50% albino but produced only albino SJL ova. However, in spite of the fact that the two B10 ↔ SJL chimeras appeared to be predominantly B10 (resulting from the *C* locus of B10 acting in melanocytes) they did contain a considerable amount of SJL cells as evidenced by the very high percentage of agouti (resulting from the *A* locus of SJL acting in hair follicles). In table 5 this is indicated by *A*. The other exception, the B10CBAF$_1$ ↔ SJL referred to above, had only 13 progeny so it is not definite that it was producing only albino genotype

TABLE 5. Gamete production, as judged by progeny, in relation to the percent albino genotype in the coat

	Gametes produced	B10↔SJL	B10CBAF$_1$↔SJL	B10↔BALB	B10↔BALBSJLF$_1$	C3H↔SJL129F$_1$
		%SJL	%SJL	%BALB	%BALBSJLF$_1$	%SJL129F$_1$
Mosaic ovaries (must be XX↔XX)	Ova of both genotypes	5A, 10A, 25A, 50, 50, 60	15, 20, 50, 50, 50, 50, 85	20, 20, 25, 50, 75	50, 50, 50, 99	40, 85
Non-mosaic ovaries (possible XX↔XY)	Only pigmented genotype ova		1, 1	2, 15		
	Only albino genotype ova	5A, 10A, 95	50		97, 97, 99	85, 90, 95, 95, 97, 99, 99, 99
Mosaic testes (must be XY↔XY)	Sperm of both genotypes	2A, 25, 40	2, 5, 15, 20, 25, 30, 40, 50	50		
Non-mosaic testes (possible XY↔XX)	Only pigmented genotype sperm	1, 1A	2, 2, 5, 10, 10, 10, 10, 20, 40	1, 5, 15, 20, 20		
	Only albino genotype sperm	2A, 5, 5, 10, 20, 20, 40, 50, 50, 50, 50, 50, 50, 50, 60, 75, 95, 99	20, 30, 40, 50, 50, 60	90	40, 50, 60, 75	50, 50, 60, 60, 85, 90, 90, 90, 95, 99

Values in the table are percent albino as estimated for each animal at weaning. 'A' means that the pigmented parts of the animals were almost entirely agouti, which in B10↔SJL is part of the SJL genotype as is albino.

(SJL) ova. Females producing ova of both genotypes showed intermediate values, indicating intermediate and high degrees of chimerism. Thus, whether females were producing ova of one or both genotypes was correlated with degree of chimerism expressed in coat color.

Males, in addition to having much lower incidences of gonadal mosaicism, showed less correlation between gamete production and degree of chimerism. Whether they were producing sperm of only one or of both genotypes, they showed a wide range of degrees of chimerism.

The data in table 5 also demonstrate that for any combination of genotypes, one component will usually predominate in gamete production. SJL predominated in B10 ↔ SJL, B10 in B10 ↔ BALB, BALBSJLF$_1$ in B10 ↔ BALBSJLF$_1$, and SJL129F$_1$ in C3H ↔ SJL129F$_1$. Neither component appeared to predominate in B10CBAF$_1$ ↔ SJL chimeras which also had the highest incidence of gonadal mosaics (table 4).

DISCUSSION

Chimeric embryos are at least as viable as zona-free non-chimeric embryos (Mullen and Carter, '71). Although the viability of the chimeric embryos in this study (37-51% born) was similar to that reported by Mintz ('67, '69), the frequency of multicolored chimeras was much higher. The difference may be due to the different strains of mice used, the importance of which is clearly demonstrated in table 2, but may also be due to the differences in culture media and techniques. Multicolored animals are probably derived from blastocysts whose inner cell masses, and subsequently the embryonic ectoderms, are suitable mixtures of the two parental genotypes. Migration and intermingling of cells may be dependent on the culture medium and techniques.

If a greater number of different combinations of genotypes were available, there would probably be some combinations whose distribution of coat colors (as in figure 1) would be normal, some highly skewed, and many in between. In this study, the classification of different combinations of genotypes as either balanced or unbalanced is an oversimplification, but seemed warranted by similarities within each group. Those combinations of genotypes classified as balanced had higher incidences of multicolored chimeras (table 2), a preponderance of multicolored males (table 3), and higher incidences of gonadal mosaics (table 4) than did unbalanced genotypes. Melanocytes are not involved in sexual differentiation and chimerism in coat does not necessarily indicate internal chimerism. Nevertheless, the degree of chimerism in the coat appears to be a fair, and the only overt, indicator of the over-all degree of chimerism.

All of the strains used had approximately normal sex ratios except BALB/cGnDgWt. This strain has an abnormal ratio of only 37% males (Whitten and Carter, unpublished data) and although it might be argued that it should not be included in the sex ratio data, it has been because it is not known whether the abnormal ratio acts when in a chimeric constitution, and it is used only in combination with B10, and in B10 ↔ BALB the B10 component predominates so that the abnormal sex ratio of BALB probably cannot be detected.

Only 0.65% of the chimeras were hermaphrodites. The excess of multicolored males suggests that in sex chimeras the male component usually exerts a dominating effect on sexual differentiation and consequently most sex chimeras will develop as phenotypic males. This statement, however, requires qualification for sexual differentiation can be altered by genetic and developmental factors. Sex chimeras can develop as phenotypic females as verified by Mintz ('68) and Mystkowska and Tarkowski ('70), but are probably able to do so because of a preponderance of female cells in the embryo (more specifically, in the tissues involved in sexual differentiation). This concept has also been suggested recently by Mystkowska and

Tarkowski ('70). The principal genetic factor by which the female could predominate is the developmental superiority of one genotype over another as exhibited by combinations of unbalanced genotypes. With such combinations, the sex of an individual will usually be determined by the sex of the predominating component. In C3H ↔ $SJL129F_1$ chimeras the predominating component was $SJL129F_1$, and in B10 ↔ BALB, the B10 component. A developmental situation in which the female could predominate may occur with any combination of genotypes, regardless of balance. If the female component, by chance, predominated in an individual, then the sex of that individual would probably be female.

Multicolored chimeras of unbalanced genotypes would, therefore, be expected to have a more or less normal sex ratio as determined by the predominating component. Regardless of balance, all solid colored animals, the extreme case of one component predominating, would also be expected to have a normal sex ratio.

By definition, balanced genotypes are those that result in most animals having intermediate and high degrees of chimerism. Only in these multicolored animals would we expect to observe an excess of males as evidence of the male component exerting a dominating effect on sexual differentiation. Within this group there may be some sex chimeras which may have, by chance, a preponderance of XX cells, and these individuals will probably be females. Thus, the sex ratio of multicolored chimeras derived from the fusion of two embryos with balanced genotypes could approach, but theoretically never reach, a ratio of three males to one female. These expectations are met by the data in table 3. If more than two embryos are fused, a greater percent will be sex chimeras and the sex ratio could exceed 3:1 (Whitten, unpublished).

If sex chimeras had an equal chance of becoming either male or female, 50% of the multicolored females would be expected to be XX ↔ XY and would produce progeny of only one component genotype, because apparently XY cells in mice are not capable of differentiating as ova. However, if our theory is correct, multicolored females will usually be XX ↔ XX and have mosaic ovaries unless there is a preponderance of one component genotype. The latter was observed, as shown by the data in tables 4 and 5, where the majority of females produced both genotypes of progeny and those individuals that did not, were, in terms of coat color, preponderantly of one or the other genotype.

Gamete production by chimeric males is considerably less predictable and more complicated than in females. Mintz ('68) described genotype selection for germ cells and pointed out that it is much more extensive in males than females because of the continuous proliferation of spermatogonia. Our data fully confirm this, particularly the data on B10 ↔ SJL males. Most of the males produced only SJL (albino) sperm and even those producing both showed a marked preponderance of SJL (189 SJL and nine B10 progeny).

With a combination of balanced genotypes in which almost all sex chimeras develop as males one would expect one-third of the males to produce sperm of one genotype, one-third to produce the other genotype, and one-third both genotypes if there were no selection. This expectation is most nearly met by the B10CBAF_1 ↔ SJL males, suggesting that there is very little selection differential between B10CBAF_1 and SJL spermatogenesis. In view of the fact that the inbred SJL competes favorably with the vigor of a hybrid (B10CBAF_1) it is not surprising that it so completely predominates over another inbred as in B10 ↔ SJL chimeras discussed above.

B10CBAF_1 ↔ SJL males are also of interest because gamete production appears to be correlated with coat color. The animals producing only SJL sperm had the greatest proportion of albino in the coat (42% average); those producing only B10CBAF_1 sperm the lowest (12%); and the animals producing both sperm were intermediate with 23%.

Sterile animals (including hermaphrodites) appeared to be grouped in certain litters and it is tempting to interpret this as non-random distribution. However, because of the small numbers and the fact that the $B10CBAF_1$ ↔ SJL animals were from very large litters of 12 and 13 pups, such an interpretation at this time would be unwarranted.

Chimeric mice are another example of the phenomenon of male determining and dominating sexual differentiation. In view of the great variation between chimeras of different component genotypes and even between those with the same genotypes, as in figure 2, it is not too surprising that this dominating effect is not always observed. The sex ratios (male:female) of overtly chimeric mice derived from the fusion of two embryos will vary from 1:1 in those combinations where the components are developmentally unbalanced, to ratios approaching 3:1 when the components are developmentally balanced.

ABSTRACT Genotype and the degree of chimerism expressed in the coat color of chimeric (allophenic) mice were found to be related to the frequency of multicolored chimeras, the sex ratio, and the production of gametes. The sex ratios of 310 chimeric mice of various combinations of genotypes were studied. The male:female ratios varied from 1:1 to ratios approaching 3:1. Multicolored chimeras in which one genotype predominated ('unbalanced' genotypes) had normal sex ratios of 1:1 (19♂♂, 21♀♀) suggesting that sex was determined by the predominating component. Multicolored chimeras in which neither component predominated ('balanced' genotypes) had sex ratios approaching 3:1 (70♂♂, 31♀♀) suggesting that most sex chimeras (XX ↔ XY) were developing as phenotypic males. Regardless of genotype, solid colored chimeras had normal sex ratios. Only two hermaphrodites (0.65%) were observed.

A high incidence of gonadal mosaics which produced progeny with both parental genotypes and a relatively low incidence of possible sex chimeras which produced progeny with only one genotype occurred among multicolored females. A very low incidence of gonadal mosaics and a very high incidence of possible sex chimeras was found among males. These observations would be expected if most sex chimeras developed as phenotypic males.

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Monozygotic Twin Mice and Totipotency of Half-Embryos

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INTRODUCTION

Cytological studies by Dalcq, Mulnard and co-workers (reviewed by Dalcq, 1957) led to the concept that the mammalian egg is characterized by polarity, bilateral symmetry, and two cytoplasmic zones, dorsal and ventral, whose cytoplasm will eventually come to lie in the inner cell mass and trophoblast, respectively. The first cleavage plane bore no fixed relation to the plane of symmetry so blastomeres of two-cell embryos could contain varying amounts of dorsal and ventral cytoplasm. Tarkowski (1959a, b) destroyed one of the blastomeres of two-cell mouse embryos, then transplanted these isolated blastomeres or half-embryos back into the oviduct. Some underwent normal and complete development and several progeny were born, demonstrating conclusively their totipotency. However, there were many abnormal forms and great variation in the structure of half-embryos at the blastocyst stage which Tarkowski interpreted to be a consequence of the variable relation between the first cleavage plane and the plane of symmetry. He thus concluded that although blastomeres of the two-cell embryo may be totipotent, they are limited by the composition of the blastomere's cytoplasm (i.e., preponderance of dorsal or ventral) so that not all blastomeres would be totipotent.

The concept of cytoplasmic localization has been subjected to further experimentation and the results in general raise doubts about its importance in early development. Mintz (1964a, b; 1965b) fused two or more (up to 16) eight-cell embryos and followed the movement of cells through the blastocyst stage. She found there was no selective sorting-out of cells as might be expected if the blastomeres were determined or prepatterned to be either inner cell mass or trophoblast cells. She concluded that at least up to the eight-cell stage and possibly later, the blastomeres were morphogenetically labile. Similarly, Enders and Schlafke (1965) studied electron micrographs of early embryos and although the cytoplasm of blastomeres of late cleavage and early blastocyst stage did differ from the cytoplasm of the oocyte, no differences between inner cell mass and trophoblast could be discerned until late blastocyst. Mulnard (1965) confirmed and extended Tarkowski's work but suggested that the process of isolating the blastomeres might cause alteration of the structural pattern of the cytoplasm which might also explain the variable and abnormal structure of half-embryos at the blastocyst stage. More recently, Tarkowski and Wroblewska (1967) have studied blastomeres isolated from 4- and 8-cell embryos. Their conclusion was that for the inner cell mass to be formed certain blastomeres must become isolated from the exterior before the blastocoele begins to form. Thus, the abnormal and varied development of half-, quarter-, and eighth-embryos was attributed to the reduced number of cells rather than to variations in the segregation of dorsal and ventral cytoplasm.

The original objective of the present experiments was to determine if both blastomeres of the two-cell embryo were totipotent. Indeed, one set of monozygotic twins has been produced. I believe this is the first set of twins to be experimentally produced in mice or any mammal. However, the reduced viability of half-embryos makes the production of twins quite impractical. Therefore, additional studies were done to examine possible ways of increasing their viability and to examine further the role of cytoplasmic localization in early mammalian development.

MATERIALS AND METHODS

Embryos. The inbred strains of mice used were C57BL/10GnDg, BALB/cGnDgWt, SJL/J, and 129/Rr-$\underline{c}/\underline{c}^{ch}$. It was desirable to use hybrid embryos because of their vigor and also to have embryos differing in genotype at the agouti, albino, and pink-eye loci so that several pairs of twin embryos could be transplanted to a single host. Therefore, embryos were most often collected from a variety of female hybrids of the above strains which had been mated with either inbred or hybrid males. The embryos were collected from the oviducts 1.5-days *post coitum* (*p.c.*) at the two-cell stage.

Manipulation of embryos. The zona pellucida was removed with 0.5% pronase solution. Originally the blastomeres were separated in calcium- and magnesium-free medium with 0.01-0.02% Versene. Because of the possible toxic effect of Versene, this procedure was changed and the blastomeres were separated in simply Ca-free medium. After being in this latter medium for 10-15 minutes the blastomeres were readily separated by agitation or by passing a fine glass needle between them. These isolated blastomeres or half-embryos were cultured *in vitro* for two to three days before being transplanted to the uteri of pseudopregnant hosts. Details of the culture medium and techniques and the transplantation techniques are described elsewhere (Mullen and Whitten, 1971; Mullen and Carter, 1971). Chimeric embryos were made by placing two half-embryos with different genotypes in contact. At this stage the embryos are not very sticky and often had to be pushed together the following day which is the stage at which chimeric embryos are usually produced.

Culture in Ca-free medium. For reasons to be discussed, certain whole and half-embryos were cultured for periods of 14-24 hours in 'Ca-free' medium. Since the drops of Ca-free medium were under paraffin oil which had been equilibrated with medium containing Ca, it is not known whether any of this Ca diffused into the drops, thus the medium might more accurately be described as 'minimal Ca'. In Ca-free medium the embryos do not compact to form a morula, therefore, when attempts were made to transfer the embryos to standard medium the blastomeres (as many as sixteen) would often separate rendering it extremely difficult to reunite them. This problem was solved by leaving the embryos in the culture dish and withdrawing the Ca-free medium with a pipette, leaving the embryos in 'collapsed bubbles'. Standard medium was then introduced into the 'collapsed bubbles'. Carefully done, this procedure resulted in minimal disturbance to the embryo.

RESULTS

Development of half-embryos and production of twins. The usual schedule was to transplant 3.5-day embryos to 2.5-day hosts at about 10 AM. When whole embryos were transplanted by this schedule nearly 100% of the hosts became pregnant and, with zona-free embryos, 40% of the transplanted embryos developed normally through 17 days of gestation (Mullen and Carter, 1971). The results of transplanting half-embryos by this schedule are shown in the first column of Table 1. Since not all hosts became pregnant (only six out of 21) minimum percentage based on the total number of embryos transplanted and maximum percentage based on the number of embryos transplanted to hosts that subsequently became pregnant were calculated. Regardless of which value is considered it is readily apparent that under this schedule half-embryos were far less viable (5-15% born) than whole zona-free embryos (40%). Since a minimum of 5% and a maximum of 15% of the transplanted half-embryos were born but 40% was expected, it appeared that a minimum of 12% (5%/40%) and a maximum of 38% (15%/40%) of half-embryos were totipotent. It was thought that half-embryos might be retarded in their development so the schedule of transplanting was varied to allow more time for the embryo to develop. Extending the time of culture before transplantation to 3 PM had no effect, the percents being almost identical. Other schedules likewise yielded no improvement. It will be shown later that the technique of separating blastomeres did not reduce their viability (see Controls in

TABLE 1

DEVELOPMENT OF HALF-EMBRYOS ACCORDING TO VARIOUS TRANSPLANTATION SCHEDULES

Embryos (days *p.c.*)	3.5				4.5			
Hosts (days *p.c.*)	2.5		1.5		3.5	2.5		
Time of transplant	10am	3pm	10am	3pm	10am	10am	3pm	Totals
Number of hosts	21	11	2	2	4	4	2	46
Number of litters	6	4					1	11
Number of embryos trans.								
All hosts	204	103	17	18	19	24	19	404
Pregnant hosts	66	34					8	108
Number of sites[a]	13	9					1	23
Minimum %	6.4	8.7					5.3	5.7
Maximum %	19.7	26.5					14.8	21.3
Number born	10	5					1	16
Minimum %	4.9	4.8					5.3	4.0
Maximum %	15.2	14.7					14.8	14.8

[a] Includes number born plus number of resorptions. Minimum % based on all embryos transplanted; maximum % on embryos transplanted to hosts that became pregnant.

Table 3).

In the early stages of these experiments the procedure involved transplanting three or four pairs of monozygotic twin embryos of differing pigmentation genotypes to a single host. One set of B10SJLF$_1$ x 129-c/c (albino) monozygotic twins was born. The host had received four other half-embryos but they had pigmented genotypes. Since so few embryos were developing, the tedious procedure of transplanting twin embryos was abandoned. Thus, most of the embryos referred to in Table 1 and all of the half-embryos in the following experiments were derived from blastomeres separated at the two-cell stage but no further attempt was made to follow twin pairs of half-embryos.

Development in Ca-free medium. Whole embryos on Day 2.5 were at the eight-cell stage and began to compact to form morulae and subsequently blastocysts. Half-embryos on Day 2.5 had only four cells but still began to compact. Doubts about whether one should expect a normal blastocyst from a morula which began with only four cells prompted the development of a technique that would allow the number of cells to increase before compaction began. The technique which made this possible involved culturing the half-embryos for a period in Ca-free medium after being cultured in standard medium for one day. In Ca-free medium cleavage continued but, because of the lack of Ca, little or no compaction occurred. After a period the Ca-free medium was replaced with standard medium and the embryos went on to form morulae and blastocysts. The over-all result was to increase the number of cells from four up to the normal number of eight before compaction began. The results are illustrated in Figure 1.

In the original series of experiments the embryos were cultured for 24 hours in Ca-free medium. After returning the embryos to standard medium it was necessary to wait an additional 24 hours for the embryos to form adequate morulae and blastocysts. This necessitated transplanting 4.5-day embryos to 3.5-day hosts. Of the 73 half-embryos transplanted there were no births, resorptions, or any indication of their fate (Table 2). The period in Ca-free medium was therefore reduced to the minimum amount of time needed for the four-cell embryos to complete another cleavage and become eight cells (14 hours). Around midnight standard medium was introduced and by the following morning most embryos were morulae, some were becoming blastocysts. Of the 57 embryos transplanted there were no births but there were six resorptions. To determine the effect of the treatment, two controls were run. Whole embryos with zonae were cultured for the same period in Ca-free medium

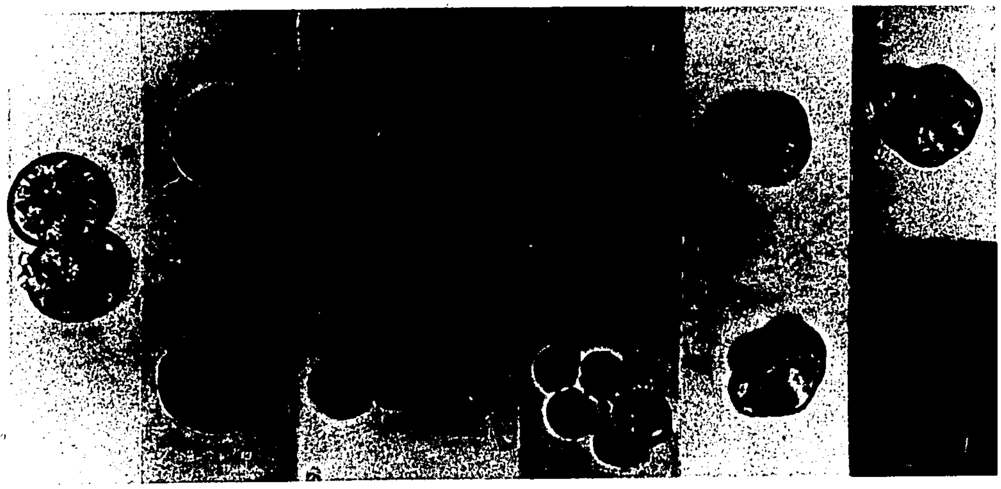

Fig. 1. Development of half-embryos before and after culturing for 14 hours in Ca-free medium. The blastomeres of the normal two-cell embryo on the left were separated to give two single blastomeres and after being cultured in standard medium for 24 hours each had four cells. If left in standard medium these would begin to compact to form morulae. However, by culturing these 4-cell embryos in Ca-free medium for 14 hours the number of cells was increased to eight which is the number of cells a whole embryo has when it begins to compact. These 8-cell half-embryos were then cultured in standard medium for 8-12 hours and formed morulae and blastocysts.

TABLE 2

DEVELOPMENT FOLLOWING CULTURE IN CA-FREE MEDIUM

Embryos	Period in Ca-free medium	Day of embryo & host	No. of hosts&emb.		No. born
Half-embryos	Day 2, 10am - Day 3, 10am (24hrs)	4.5-3.5	7	73	0
" "	Day 2, 10am - Midnight (14hrs)	3.5-2.5	4	57	0[a]
Controls					
Whole embryos	" " " " "	" "	1	12	9
Reunited half-embryos	" " " " "	" "	1	9	3

[a] Six resorptions (11%).

and after being transplanted nine out of 12 were born. To determine the effect of removing the zonae and separating the blastomeres, some two-cell embryos were treated in a manner identical to the half-embryos except after separating the blastomeres they were placed in culture and reunited. These were also cultured in Ca-free medium. Three out of nine of these embryos developed. Thus, the Ca-free medium treatment was not detrimental to the embryos but increasing the number of cells in the half-embryos before compaction began did not increase their viability.

Chimeric mice from fused half-embryos. It was estimated that as few as 12% of half-embryos were totipotent. If the dorsal cytoplasm, *d*, is considered (in terms of the concepts of Dalcq) as being the presumptive inner cell mass and the ventral cytoplasm, *v*, the presumptive trophoblast then it might be assumed that only half-embryos containing sufficient amounts of *d* and *v* would be totipotent and designated *dv*. The balance of the half-embryos would be divided equally between those which are predominantly *d* and predominantly *v*. The frequency of the various types of half-embryos might therefore be expressed as 0.44*d* + 0.12*dv* + 0.44*v*. If chimeric mice are made by fusing half-embryos, the only ones which will be viable will be those containing both *d* and *v* and the only ones which will be multicolored will be those containing d from both component genotypes. Thus, if half-embryos of strain A are fused with half-embryos of strain B, the results might be predicted by squaring 0.44d + 0.12dv + 0.44v. This yields a predicted value of 61% viable chimeric embryos. Since only 40% of transplanted zona-free normal embryos are born (Mullen and Carter, 1971), the 61% must be multiplied by 40% to equal a predicted 24% of half-embryo chimeras would be born. Of those born, it was predicted that 19% would be multicolored.

The above predictions were based on the minimum percent of half-embryos born (see above and Table 1). Similar calculations were made for maximum percents. Thus, combining the two, the original predictions were that 24-32% of half-embryo chimeras would be born compared with 40-50% for normal chimeras (Mullen and Carter, 1971) and that 19-47% of those would be multicolored compared with approximately 60-70% for normal chimeras of similar genotypes (Mullen and Whitten, 1971). To check the possibility that the manipulation of the embryos was reducing their viability, controls were run. The controls consisted of embryos which were treated in an identical manner except instead of placing the blastomeres with blastomeres of another strain, they were reunited with their sister blastomeres (i.e., blastomeres of two-cell embryos were separated and then reunited).

The results are shown in Table 3. The controls demonstrate conclusively that the procedure is not detrimental to the embryos for the control values (41-52% born) are almost identical with the values for normal chimeras (40-50%). The controls also suggest that the spatial arrangement of the blastomeres in relation to each other is not an important factor. After separating the blastomeres they were drawn into a pipette, introduced into the culture dish, and moved around until in contact, a procedure which undoubtedly resulted in the area of contact between blastomeres being different from the original area. In addition, these controls show that the technique of separating blastomeres, as done to the embryos referred to in Table 1, did not reduce their viability. The percentage of half-embryo chimeras born (17.0-21.6%) was significantly less ($P < 0.01$) than controls and, although close to the original predicted values (24-32%), the minimum value of 17.0% was significantly less ($P < 0.025$) than the original minimum predicted value of 24%.

The percent of half-embryo chimeras that was multicolored, 30.8%, was within the predicted range of 19-47% and was only about half of that observed with normal

TABLE 3

PREDICTED AND OBSERVED DEVELOPMENT OF HALF-EMBRYO CHIMERAS

	Born		Multicolored
Normal chimeras	40 - 50%		60 - 70%
Predicted for half-embryo chimeras:			
Original	24 - 32%		19 - 47%
Alternate	9 - 25%		
	Born/Trans. %	Born/Trans. %	Multi./Total %
Observed half-embryo chimeras	41/241 17.0 all 18 hosts	41/190 21.6 14 preg. hosts	12/39 30.8
Controls: reunited half-embryos	29/70 41.4 all 5 hosts	29/56 51.8 4 preg. hosts	

chimeras. Two of the chimeras died at birth so their pigmentation could nor be determined. The 39 chimeras included: 30 (B10SJLF$_1$ x B10) ↔ (BALBSJLF$_1$ x SJL); 5 B10 ↔ (BALBSJLF$_1$ x BALBSJLF$_1$); and 4 B10 ↔ SJL. As predicted, chimeras derived from the fusion of half-embryos had reduced viability and a reduced incidence of multicolored animals.

The above original predictions were based on concepts which are subject to various criticisms to be discussed. Therefore, alternate predictions were made based on a less restrictive concept. The concept was simply that a half-embryo which was totipotent had all the 'information' ($\underline{i}^+$) needed to initiate normal and complete development. A blastomere that did not have this information was designated $\underline{i}^-$ and consequently half-embryo chimeras would be totipotent only if they contained $\underline{i}^+$. A minimum of 12% and a maximum of 38% of half-embryos appeared totipotent, therefore, $(0.12\underline{i}^+ + 0.88\underline{i}^-)^2$ and $(0.38\underline{i}^+ + 0.62\underline{i}^-)^2$ yielded the alternate prediction that 9-25% of half-embryo chimeras would be viable. The observed values of 17.0-21.6% (Table 3) fell within this alternate predicted range. Prediction of the incidence of multicolored animals cannot be made by this method but, as will be discussed, such a prediction may not be valid because the incidence may depend on the size of the inner cell mass rather than cytoplasmic localization.

DISCUSSION

One set of monozygotic twins was produced. However, only 5-15% of the transplanted half-embryos were born compared with the expected 40% based on known efficiency of transplanting zona-free embryos (Mullen and Carter, 1971). The reduced viability did not appear to be due to the half-embryos being retarded in their development for varying the transplantation schedule to allow more time for the embryos to develop yielded no improvement.

The possibility existed that half-embryos had all the information needed to be totipotent but in most cases simply did not have enough cells. This idea is inherent to Tarkowski and Wroblewska's (1967) hypothesis. In this present study it was found that by culturing embryos in Ca-free medium formation of morulae could be delayed but cell division continued. Thus it was possible to increase the number of cells to normal before compaction began. None of the 57 half-embryos transplanted were born but there were six implantation sites (moles). The controls demonstrate that this technique is not detrimental to the embryos. Three out of nine reunited half-embryos and nine out of 12 whole embryos were born. The zonae of the whole embryos did not protect the embryos from the effect of the

Ca-free medium for they continued to cleave (up to 16 or more cells) but showed no evidence of compaction until they were returned to standard medium. Before using this data as evidence against the hypothesis of Tarkowski and Wroblewska it should be pointed out that the number of cells in these half-embryos at the time of blastocyst formation is not known and although the number of cells was increased, the total volume was not. The latter may be important if the formation of inner cell mass from cells isolated from the exterior is dependent on diffusion gradients. However, at the time when the embryo begins to compact, which may be an extremely important time during which the morphogenetic movements of cells results in some cells lying internally, these half-embryos had the normal number of cells but their viability was not increased.

Studies on chimeric embryos derived from the fusion of two or more whole embryos (Mintz, 1964a, b; 1965b) has been one source of evidence against the concept of cytoplasmic localization playing an important role in early development. However, it was thought that if the reduced viability of half-embryos was due to some embryos containing a preponderance of dorsal or ventral cytoplasm then it might be possible to predict the results of fusing two half-embryos of different genotypes. If the first cleavage plane is not fixed in relation to the plane of symmetry then there could be continuous variation in the ratio of dorsal to ventral cytoplasm in half-embryos. For simplicity, however, in the original predictions the half-embryos were thought of as either having approximately equal amounts of dorsal and ventral cytoplasm or a preponderance of one or the other. It was thought that this would lead to underestimating the results because it would not account for the case in which, for example, both half-embryos had a preponderance of ventral cytoplasm but together might have sufficient dorsal cytoplasm to be viable. On the contrary, the observed minimum percent born (17.0%) although close to the predicted 24%, was in fact significantly lower and less than half of that observed with normal chimeras (40-50% whole embryo chimeras born, Mullen and Carter, 1971).

The controls in this experiment are of extreme importance. They demonstrate that (1) the technique of separating blastomeres is not injurious as suggested by Mulnard (1965); (2) the spatial arrangement of the blastomeres at the two-cell stage is probably not important; and (3) the technique of fusing half-embryos is not detrimental to their viability. Unlike the half-embryos in Ca-free medium, these half-embryo chimeras must be considered as being completely normal with regards to the number of cells and volume yet they still have reduced viability.

Only 30.8% of the half-embryo chimeras were multicolored compared with 60-70% for normal chimeras. Although this is within the predicted range of 19-47% it is questionable whether the reduction is due to cytoplasmic localization or to the size of the inner cell mass. Undoubtedly, a half-embryo chimeric blastocyst has an inner cell mass which has fewer cells that that of a normal chimeric embryo. The reduced incidence of multicolored half-embryo chimeras could be simply a reflection of a reduced chance of both genotypes being incorporated into the embryo proper.

Another reason for making half-embryo chimeras was to try to determine why all normal chimeras (derived from two whole embryos) are not multicolored. The incidence of multicolored animals seems to vary between laboratories for Mintz (1969) has reported 40% multicolored whereas Mullen and Whitten (1971) found 56% multicolored but with a range from 34-69% depending on the genotypes of the fused embryos. Mintz (1967) has suggested that single colored animals "reflect the action of selection, chiefly during the period of reduction in embryo size from double to normal". It was reasoned that half-embryo chimeras would be normal in size and no reduction would occur so perhaps the incidence of multicolored animals would be increased.

As seen in Table 3 the results were to the contrary for only 30% were multicolored. Because of possible complications due to cytoplasmic localization, the results do not disprove Mintz's hypothesis. They do, however, suggest that the size of the inner cell mass and the number of cells incorporated into it could explain, at least in part, the incidence of multicolored animals.

The data presented here could be used as evidence in support of the concepts of Dalcq, Mulnard and co-workers and the early interpretations of them by Tarkowski (1959a, b). However, the percent of half-embryo chimeras born was less than predicted and the reduced incidence of multicolored animals could be explained without invoking the concept of cytoplasmic localization. Therefore, a less restrictive hypothesis was formulated.

The hypothesis is that some half-embryos receive all the 'information' needed to be totipotent whereas others do not. This hypothesis is based on the observations that (1) only 12-38% of half-embryos appeared to be totipotent; (2) increasing the number of cells by culturing in Ca-free medium, which was not detrimental to controls, did not increase the viability of half-embryos; and (3) chimeric embryos derived from the fusion of two half-embryos had reduced viability. The latter observation is probably the most significant. It was found that the blastomeres of two-cell embryos could be separated and then reunited without effecting their viability (41-52% born, Controls in Table 3). If only a certain percentage of single blastomeres possess the 'information' needed to be totipotent then it may follow that when single blastomeres from different embryos are joined, only a certain percentage of these chimeric embryos will be totipotent even though the number of cells and volume is normal. Thus, alternate predictions were made based on the estimate that only 12-38% of half-embryos are totipotent. The method of prediction did not allow for the situation in which the blastomeres might not have sufficient information to be totipotent by themselves but together they might be. This would lead to underestimating the predicted values. Nevertheless, the percent of half-embryo chimeras born, 17.0-21.6%, fell within the alternate predicted range of 9-25%. This concept implies cytoplasmic localization but it is much less restrictive in that it does not specify that the information is responsible or necessary for the formation of inner cell mass or trophoblast but only that it is responsible for the totipotency of half-embryos. It is not known whether there is any relation between this information and Dalcq's and co-workers' concepts of the structure and cleavage pattern of mammalian eggs.

Tarkowski and Wroblewska (1967) found that 30-40%

of single blastomeres from 4-cell embryos and only 15% of blastomeres from 8-cell embryos formed normal blastocysts. Thus, the decrease in number of cells was accompanied by a decrease in the percentage of embryos developing as blastocysts. They concluded that for the inner cell mass to be formed "certain blastomeres should become isolated from the exterior before the moment when blastocoelic fluid starts to accumulate between cells". Their hypothesis seems quite plausible and undoubtedly the number of cells is a factor in the viability of fragments of embryos. However, in light of the data presented here, particularly on half-embryo chimeras, it would not appear to be the primary factor.

The production of monozygotic twins by separating blastomeres of the two-cell embryo, though possible, seems quite impractical because of the reduced viability of half-embryos. It is hypothesized that the reduced viability is due to the fact that some half-embryos receive sufficient information to be totipotent but most do not.

SUMMARY

The potential of producing monozygotic twin mice by separating blastomeres of the two-cell embryo was examined. Although one set of twins was produced the reduced viability of half-embryos made the technique quite impractical. Of 307 3.5-day *p.c.* half-embryos transplanted to 2.5-day hosts, 15 were born. After correcting for transplantation efficiency, it was estimated that 12-38% of half-embryo were totipotent. Varying the transplantation schedule to allow more time for the embryos to develop did not increase their viability. By culturing half-embryos for a period in Ca-free medium the number of cells was increased to normal before formation of morulae. This technique, though not injurious to control embryos, did not increase the viability of half-embryos. Of 241 chimeric embryos derived from the fusion of two half-embryos only 41 or 17.0% were born and only 30.8% were multicolored compared with normal chimeras where 40-50% were born and 60-70% were multicolored. It was hypothsized that the reduced viability of the half-embryos and chimeras was due to the fact that some half-embryos received sufficient information to be totipotent but most did not.

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