THE CULTURE OF PREIMPLANTATION RAT EMBRYOS AND THE PRODUCTION OF ALLOPHENIC RATS

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Summary. Rat embryos at all cleavage stages of development were cultured *in vitro*. The various cleavage stages were successfully cultured through at least one mitotic cycle with the exception of the four-cell stage. No instance of development through the four-cell stage was observed. The eight-cell stage was cultured to the blastocyst stage with about 80% success. There was no difference in viability of eight-cell embryos if they were cultured without the zona pellucida.

Despite some difficulties with adhesion of embryos and cellular death, denuded embryo pairs aggregated into a single blastocyst during a 24-hr culture period. Of all such aggregation attempts, 45% proved successful. Twenty-five aggregate blastocysts were transferred to eight recipient females. Subsequently, seven transferred embryos were born to four of the host females.

Five of these transferred embryos were externally female while the remaining two were normal males. Two of these females were sibling aggregates (identical phenotypes) and no evidence of mosaicism should have been observed. Of the remaining five animals, three were of one parental phenotype or the other, while the remaining two (one male and one female) expressed some degree of parental phenotype mosaicism.

INTRODUCTION

Tarkowski (1961) and Mintz (1962a) independently devised methods for combining early cleavage stages of two mouse embryos into a single embryo. Embryos combined using these methods proved to be mosaics composed of cells derived from the separate sets of parents. Tarkowski (1961) referred to these quadriparental animals as chimaeras, but because of the diversity of meaning, Mintz (1967a) suggested that the resultant mouse embryos from her experiment be termed 'allophenic mice' since these animals could exhibit components of different phenotypes due to their multi-embryo origin. In this paper, the term allophenic will be applied to individuals of multi-embryo origin.

The mouse has been the only mammalian species in which allophenic

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individuals have been decisively demonstrated. The attempts by Nicholas & Hall (1942) with rats, and Pighills, Hancock & Hall (1968) with sheep both proved inconclusive. These investigators were handicapped in their work since they could not culture the embryos for sufficient lengths of time *in vitro* to observe the continued development and aggregation of the two embryos before transferring to a foster mother.

The intent of the investigations presented here was to study the development of the rat embryo and the feasibility of producing allophenic rats. The experiments were conducted in three phases. The first phase was an attempt to assure a culture period of sufficient length to confirm aggregation of any embryo pairs. The second phase consisted of an attempt to culture two eight-cell embryos into a single blastocyst. The final phase was concerned with the successful transfer and development of completely aggregated embryos and the subsequent birth of allophenic rats.

MATERIALS AND METHODS

In all experiments, dams were of the Sprague-Dawley strain of rat. Male rats were either of the same strain or of the Long-Evans 'hooded' strain. Although these two strains differ in the ability of their melanocytes to produce pigment (Sprague-Dawleys are complete albinos), both strains are homozygous for the 'hooded' gene which influences melanocyte distribution. All of the embryos used were the result of pairing dams with experienced males of the same strain or of the Long-Evans strain. Consequently, these embryos were either albino or pigmented with a 'hooded' pattern respectively. (Although the albino embryos also had a 'hooded' pattern of melanocyte distribution, this trait is epistatic to the albinism.) In this paper, the terms 'albino' and 'hooded' will be applied to these embryos and only signify their potential phenotypic expression. All dams were ovulating spontaneously and the morning the vaginal plug was found was taken as Day 1 of gestation. Embryos could be found in the oviduct at the one-cell stage on Day 1, two-cell stage on Day 2, and the four-cell stage on Day 3. Eight-cell and blastocyst stages were found at the anterior end of the uterine horns on Days 4 and 5, respectively.

Donor rats were killed by an overdose of ether on the appropriate day and the embryos were collected according to the method of Mintz (1971). Morphologically abnormal embryos were rejected after observation using a dissecting microscope. For this study, the standard mouse medium of Biggers, Whitten & Whittingham (1971) was modified by decreasing the sodium chloride concentration to 5.140 g/litre, increasing sodium pyruvate to 0.036 g/litre (W. K. Whitten, personal communication) and by substituting 25% fetal calf serum for the bovine serum albumin.

If the zona pellucida was to be removed, the embryos were transferred to a Petri dish of culture medium which contained 0.5% pronase (Cal. BioChem., Grade B) but no fetal calf serum. The membrane dissolved after 5 to 7 min at room temperature and denuded embryos were washed three times in culture solution and transferred to an incubator (37°C) with an atmosphere of 5% CO₂ in air. Embryos were cultured in microdrops of medium under a layer of

equilibrated mineral oil (Brinster, 1963). The culture period varied from 24 to 72 hr, depending on the developmental stage of the embryos.

Embryos were obtained at all preimplantation stages and were cultured until it became apparent that development had been arrested. The criteria for arrested development were a failure to meet the normal cleavage schedule (see New, 1967) and a degenerated appearance shown by a swelling of the blastomeres and a change in their colour.

All attempts at embryo aggregation were between eight-cell embryos. As often as possible 'albino' embryos were paired with 'hooded' embryos for aggregation. Where this was not possible, sibling aggregates (albino with albino, or hooded with hooded) were attempted. The eight-cell stages were obtained on Day 4 of pregnancy and donors were killed in the late morning or early afternoon. The zonae were removed from each group of embryos and one pair of eight-cell stage embryos was placed in each microdrop and cultured together. Contacts between embryos were induced by gentle pressure from a fine glass rod, continued as necessary. During the intervals between handling, embryos were kept in the incubator. The embryos were cultured to the blastocyst stage in 24 to 30 hr.

It was found that aggregation occurred more readily if the embryos were placed in contact with each other in a small V-shaped depression in the bottom of the plastic culture dish so that they would not separate when returned to the incubator. These depressions were made in the floor of the Petri dish with a blunt glass rod about 0.2 cm in diameter.

Embryo aggregates were transferred to recipient rats 24 or 48 hr behind the postovulatory cycle of the donor rats. The recipients were also of the Sprague-Dawley strain and were mated in the same manner as the donors. The recipient host was chosen so that, when possible, native neonates were phenotypically distinguishable from those transferred. The transfer procedures used followed those described by Dickmann (1971). A dorsolateral incision was made to expose the reproductive system and only one uterine horn was used. Females which did not deliver by the 20th day after transfer were killed and the uterine contents were examined.

RESULTS

Culture of preimplantation stages of the rat

Nineteen embryos were obtained at the early one-cell stage on the morning of Day 1 and all nineteen proceeded through the first cleavage within a 24-hr period. After an additional 24 hr, four of these embryos had undergone another mitotic cycle to the four-cell stage. Further culture resulted only in degenerative changes. If the two-cell stage was the initial culture stage, a larger percentage of embryos survived to the four-cell stage (22 to 33%). Again, there was no evidence of development beyond the four-cell stage. Even if the four-cell stage was the initial culture stage, no indication of development was observed after 48 hr.

The eight-cell embryos removed from the uterus on Day 4 developed into normal blastocysts with about 80% success (nineteen of twenty-four). Some of

the developmental stages are illustrated in Plate 1. Rat blastocysts cultured for an additional 24 hr failed to hatch from the zona pellucida, although one cultured blastocyst initiated hatching but did not completely escape.

Three of eight embryos obtained on Day 5 and cultured for a 24-hr period hatched completely. Table 1 summarizes the results of the culture experiments.

Aggregation of eight-cell embryos

All aggregation attempts were limited to eight-cell stages and no more than two embryos were joined together. Out of eighty-four attempts, thirty-eight pairings (45%) resulted in complete aggregation into single multi-embryo blastocysts. In some cases, a few cells died or for some reason were not incorporated into the aggregated structure. If the amount of excluded material

Table 1. Development of rat embryos in culture

<i>Day post coitum on which embryos removed from donor</i>	<i>State at start of culture</i>	<i>% of embryos showing no change in developmental stage during culture</i>	<i>% of embryos developing and final stage of development in culture</i>
Day 1	One-cell stage (19)	0	79 (15) to two-cell stage 21 (4) to four-cell stage 0 to eight-cell stage
Day 2	Two-cell stage (33)	33 (11)	67 (22) to four-cell stage 0 to eight-cell stage
Day 3	Four-cell stage (35)	100 (35)	0 to eight-cell stage
Day 4	Eight-cell stage (24)	21 (5)	79 (19) to blastocyst 0 to hatch from zona pellucida
Day 5	Blastocyst (8)	63 (5)	37 (3) to hatch from zona pellucida

Number of embryos given in parentheses.

was very minor (one or two small cells), the blastocyst was considered a multi-embryonic structure. If the portion of non-fused cells was greater than a few small cells, however, the aggregation was considered a failure.

Transplantation of aggregated blastocysts

Only completely aggregated blastocysts were removed from culture 24 to

EXPLANATION OF PLATE 1

Figs 1 and 2. Culture of rat zygotes.

FIG. 1: Zygotes at start of culture.

FIG. 2: Two-cell stage after 24 hr and four-cell stage after 48 hr in culture.

Figs 3 and 4. Culture of eight-cell stage rat embryos.

FIG. 3: Eight-cell stage at start of culture.

FIG. 4: Expanded blastocyst 30 hr after start of culture.

Figs 5 to 8. Aggregation of a pair of eight-cell rat embryos.

FIG. 5: At 21.00 hours on the first day of culture—adhesion of two denuded eight-cell embryos.

FIG. 6: At midnight on Day 1 of culture—single morula.

FIG. 7: At 11.30 hours on Day 2 of culture—blastocoele (arrowed) formation in an aggregated blastocyst.

FIG. 8: At 17.00 hours on Day 2 of culture 24 hr after start of culture—comparison of normal and fused (arrowed) blastocysts.

PLATE 1

(Facing p. 4)

PLATE 2

Eyes of phenotypically 'hooded', 'albino', and 'mosaic' neonatal rats. Mottled eye pattern—top; 'albino' eye—bottom right; 'hooded' eye—bottom left.

FIG. 9. Frontal view.

FIG. 10. Lateral view.

(Facing p. 5)

30 hr later (Day 5 *post coitum*) and these were transferred, when possible, to pregnant recipient rats. A total of twenty-five fused blastocysts were transferred. Three of these were the result of combining two 'hooded' embryos (hooded ↔ hooded), three were albino aggregations (albino ↔ albino), and the remaining nineteen were the products of hooded and albino unions (hooded ↔ albino). A total of eight transfers were made, four (of twelve aggregated blastocysts) to recipients 24 hr out of sequence with the postovulatory cycle of the donor and four to hosts 48 hr out of sequence. None of the recipients 48 hr out of sequence gave birth to offspring which could be identified as transferred embryos, while the four recipients 24 hr behind the donor's cycle each gave birth to one or more transferred embryos.

Table 2. Development of aggregated rat blastocysts transferred from culture to recipient hosts

Transfer no. in chronological order	Difference between donor and host post-ovulatory cycles (hr)	No. and type of transferred aggregated embryos	No. of neonates in litter	Phenotype of neonates	
				Same as native offspring	Positive transferred offspring
1	24	1 hooded ↔ hooded	13	12 albino	1 hooded
2	48	3 hooded ↔ albino	8	8 albino	
3	48	3 albino ↔ albino	6	6 hooded	
4	48	3 hooded ↔ albino	10	10 albino	
5	24	2 hooded ↔ hooded	8	7 albino	1 hooded
6	24	6 hooded ↔ albino	10	7 hooded	2 albino, 1 mixed*
7	48	4 hooded ↔ albino	8	8 albino	
8	24	3 hooded ↔ albino	14	12 albino	1 hooded, 1 mixed*

* Indicates mosaic individuals.

A total of three aggregated blastocysts (each a hooded ↔ hooded) were transferred to two hosts that had native albino embryos. A single hooded female embryo was present in both litters (transfers Nos. 1 and 5, Table 2) and was normal in every respect. There was no evidence of intersexuality at external and internal examination.

In the third successful transfer (No. 6, Table 2), six aggregated blastocysts (hooded ↔ albino) were transferred to a host female with native 'hooded' embryos. A litter of ten was removed from both uterine horns 20 days after transfer and each was fixed (10% formalin) and examined for intersexuality and hair and eye pigmentation. All ten embryos were sexually normal. Three of the animals removed from the right uterine horn were judged to be transferred animals, since two of the embryos (one female and one male) appeared to be completely normal albinos while the third expressed a normally 'hooded' coat pattern but a mottled pigmentation of the choroid layer of both eyes (Plate 2). (Such mottled eye pigmentation indicates a mosaic tissue of hooded (pigmented) and albino (non-pigmented) cell-types. Tarkowski (1964) found a similar situation in allophenic mice.)

The final successful transfer (No. 8, Table 2) involved three aggregated blastocysts (hooded ↔ albino). The host animal carried 'albino' embryos.

Two transferred animals (female) were found in the litter, one of which appeared to be a normal 'hooded' embryo. The second animal had a mottled pigment pattern. As coat development proceeded, the former animal developed the expected hooded pattern while the latter expressed a mosaic albino-hooded coat pattern (Plate 3).

These results are summarized in Table 2.

DISCUSSION

Culture of the rat embryo

Other workers have attempted to culture various stages of the rat embryo, but none of these has reported a study of all stages under the same conditions. Folstad, Bennett & Dorfman (1969) cultured two-, four- and eight-cell stages in various media. Bennett & Stewart (1969) subsequently cultured later embryonic stages and transferred cultured blastocysts to ascertain viability. Working independently, Suzuki & Ilizuka (1969) achieved limited success in their attempts to culture rat zygotes. They reported that 12% of their cultured embryos cleaved *in vitro*, but no development occurred beyond the four-cell stage.

In the present study, regardless of the initial stage of embryo culture (one-, two- or four-cell), development beyond the four-cell stage was never observed. This inhibition of cleavage at the four-cell stage is reminiscent of the blockage of development from the one- to two-cell stage observed in early mouse culture experiments (see Whitten & Biggers, 1968). There is no evidence for assuming that a similar cause is responsible for the difficulty in culture of these two species through specific cleavage stages, but an interesting parallel is suggested.

Another similarity to the mouse embryo culture was the ability to manipulate the embryo *in vitro* (Mintz, 1965). After exposure to 0.5% pronase for 5 to 7 min, the eight-cell rat embryos were cultured with about 80% success, the same rate of success as eight-cell stages cultured with the zona intact.

Aggregation of mammalian embryos in culture

All of the verified cases of aggregation of mammalian preimplantation embryos have been carried out in the mouse (Mintz, 1971; Tarkowski, 1971). Although some attempts have been published on the sheep (Pighills *et al.*, 1968) and the rat (Nicholas & Hall, 1942), complete aggregation of the embryos was not observed before transfer and hence the success of the attempts in these species is doubtful. The data in this study, therefore, represent the only

EXPLANATION OF PLATE 3

FIG. 11. Comparison of coat patterns of 'albino', 'hooded' and 'mosaic' 3-week-old rats. The animal on the left is a native albino. The middle animal has a mosaic coat pattern and the right hand animal is a normal hooded rat.

FIGS 12 to 15. Mosaic coat patterns of the allophenic rat.

FIG. 12. Note the white band on the left side.

FIG. 13. A similar white band seen from above.

FIG. 14. A ventral view showing the dark band ending mid-ventrally near the fore-leg.

FIG. 15. Frontal view showing the dark and light bands meeting on the upper lip.

PLATE 3

(Facing p. 6)

reported instances of complete embryo fusion *in vitro* in a species other than the mouse.

During the preliminary attempts, only 40% of the eight-cell rat embryo pairs aggregated into blastocysts, as compared to 95% aggregation in some mouse experiments (Mintz, 1971). This lower incidence of rat embryo aggregation may be in part attributed to a higher rate of cellular death *in vitro*. Since development was arrested in 20% of the eight-cell rat embryos before the blastocyst stage, a lower incidence of aggregation due to death of one or both embryos would be expected.

It also seems possible that rat blastomeres are not as adhesive at 37°C as are mouse blastomeres. A higher percentage of aggregation (50%) was achieved if the indentation technique described previously was used to ensure contact between the embryos during the initial culture period.

The ability of embryo pairs to reaggregate into a single embryo seems to be expressed in a similar pattern in both mice and rat embryos. The mouse aggregates (Tarkowski, 1964; Mintz, 1965) develop to the blastocyst stage after a set number of mitotic cycles. Aggregation of the rat embryo also does not noticeably alter the rate of development. The blastocyst is formed after the same length of culture period as that for non-fused embryos although it is about twice the size of the normal blastocyst due to the larger total number of cells (see Pl. 1, Fig. 8). Mintz (1962a, b, 1964a, b, 1965) has demonstrated the ability of the mouse embryo to effect regulation as late as the inception of the blastocyst by showing the random nature of blastocyst aggregation. A similar claim cannot be made at this time for the determination of the rat blastomere. Even though the data on aggregation of the rat embryo are not inconsistent with this comparison, additional studies are needed to verify a random nature for rat blastomere aggregation.

Transfer and development of allophenic embryos

It is generally believed that the embryo at the time of transfer should be about 24 hr older than, or the same postovulatory age as, the embryos of the recipient animal (Mintz, 1967b). The data in Table 2 support this concept. Eight females acted as hosts. Only the four individuals which mated 24 hr after the donor animal produced any identifiable transferred offspring. The other four females were 48 hr behind the postovulatory age of the donors, and no transferred embryos were recognizable in the neonates.

The offspring identifiable as allophenic rats showed many similarities to allophenic mice. All allophenic mice which were produced using both the techniques of Mintz (1971) and Gardner (1968) appeared to be physiologically normal. The allophenic blastocysts were twice the normal size at the time of transfer. It would appear that an adjustment is made after transfer which reduces the mouse embryo to a normal size. Furthermore, some investigators (McLaren & Bowman, 1969; Mintz, 1971) have reported instances of allophenic mice which express only one parental phenotype. The loss of one parental cell population is thought to occur during differentiation when embryonic and extra-embryonic determination occurs (Mintz, 1969). The random orientation of blastomeres in allophenic blastocysts (Mintz, 1965) may occasionally

result in complete exclusion of one parental cell type from the area which differentiates into the embryo proper. This situation has been postulated for tissues and organs as well as the whole organism (Mintz, 1969).

The allophenic rats in this study are consistent with these findings. All the neonatal rats which could be shown to have been transferred allophenic blastocysts were morphologically normal, despite the doubling of blastocyst size. The rat embryo also appears to be able to exclude cells of one genotype from the embryo after aggregation *in vitro*. In the sixth transfer (Table 2), six aggregated blastocysts (hooded $\leftrightarrow$ albino) were transferred to a host with native 'hooded' (pigmented) embryos. The litter produced contained only one animal with any evidence of mosaicism (mottled pigmentation in the choroid layer of the eye); while two embryos appeared to be completely of the 'albino' phenotype. If any of the other three transferred blastocysts were, in fact, 'hooded' animals, they were indistinguishable from the native offspring. Likewise, in transfer No. 8 (Table 2), three allophenic (hooded $\leftrightarrow$ albino) blastocysts were transferred to a host with native albino embryos. Two of the newborn offspring showed some degree of pigmentation and were therefore identified as transferred neonates. One animal expressed a mosaic coat pattern while the other appeared to be of a normal 'hooded' phenotype. Again, it seems that the hooded animal was composed of only one parental cell line.

While exclusion of one parental cell line from certain tissues in allophenic rats has been previously shown (McLaren & Bowman, 1969; Mintz, 1971), it is important to remember that the animals from this investigation were only examined for a few phenotypic characters. The presence of completely non-mosaic allophenic individuals cannot be conclusively demonstrated in the rat until a study is undertaken in which the two parental cell types differ by a number of identifiable characteristics.

The final comparison noted was the similarity between the coat mosaicism in allophenic rats and mice. Mintz (1967a, 1970) was able to construct an archetypal coat pattern from allophenic mice. It consisted of alternating transverse bands down each side of the animal. Each transverse band extended from the mid-dorsal to the mid-ventral region and was independent of its neighbouring area. A total of thirty-four areas or bands were found in these mice (seventeen on each side) with six in the head region, twelve in the body region and sixteen in the tail. Each band was interpreted as a clone of cells, each clone being derived from a single melanoblast precursor cell in the embryo. As these melanoblast clones increase in size, growing from the neural crest area (mid-dorsal), they invade laterally and ventrally to the mid-ventral area. This migration explains the semi-transverse nature of the bands. The hooded gene in rats results in a delay in melanoblast migration from the neural crest area (Searle, 1968). This delay results in the lack of melanocytes in most of the lateral and ventral regions in the posterior half of the rat. Within these limitations, one allophenic rat (Plate 3) shows a similar banding to that seen in allophenic mice. The predominant coloration in the anterior sections of the animal shows a definite mouse-like pattern with areas of pigmentation extending from the mid-dorsal to mid-ventral region. With the culture and aggregation techniques described here, it should now be possible to demonstrate

this conclusively, using a solid-coloured rat strain, and also to verify whether the results obtained with allophenic mice are species-specific or are more generalized mammalian patterns of development.

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