THE EFFECT OF CELLS TRANSFERRED INTO THE MOUSE BLASTOCYST ON SUBSEQUENT DEVELOPMENT*

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The interaction between cells in the early mammalian embryo is an important part of differentiation. This interaction has been the topic of numerous investigations which have increased our understanding of how differentiation proceeds. Studies where whole embryos are fused (12, 19) provide one method by which the type of embryo cells interacting can be partially regulated. Another method for controlling the cells that interact was first reported by Gardner (5, 6). In this technique a few cells from the inner cell mass of one embryo are placed into another blastocyst. Moustafa and Brinster (14, 15) used this technique to study the ability of older embryo cells to participate in the development of blastocysts into which the older cells were placed. These studies indicated that cells as much as 4 days older than the recipient blastocyst could participate in development of the embryo.

The ability of older and asynchronous cells to participate in embryo development suggested the possibility that cells taken from the adult animal might be capable of influencing the development of the embryo. To test this possibility, two types of nonembryonic cells were placed in mouse blastocysts and the blastocysts allowed to develop to term in a foster mother. The cells employed were (*a*) bone marrow cells from CBA T6T6 mice and (*b*) teratocarcinoma cells taken from ascites fluid of 129 SvSl mice in which the tumor was propagated as an intraperitoneal tumor. The results of these studies are reported here and indicate that the transferred cells did affect the offspring resulting from the blastocysts that received the donor cells.

Methods and Materials

Obtaining and Handling Blastocysts. The mouse blastocysts were obtained by superovulating random-bred Swiss albino females with gonadotrophins (3, 4). On the 4th day after mating, blastocysts were collected by flushing the uterus with culture medium. The medium was Eagle's

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basal medium supplemented with 5×10^{-4} M pyruvate and 10% fetal calf serum (EBMS).[1] The blastocysts from several females were pooled, washed three times, and stored in medium at 37°C under an atmosphere of 5% CO_2 in air (4). These blastocysts were the recipients for the transferred cells.

Obtaining and Handling Cells. The bone marrow cells were obtained by flushing the femur of adult CBA T6T6 animals with culture medium. The cell suspension was examined and individual cells selected for transfer. An attempt was made to select cells which had characteristics similar to stem cells. The characteristics used were those suggested by van Bekkum et al. (20) and Haskill and Moore (8). These cells were round, approximately 8–10 μ in diameter, and appeared to have a large nucleus with one or two nucleoli. However, a positive identification as stem cells was not possible.

Cells from mouse testicular teratocarcinoma OTT 6050 (17) were from ascitic fluid which develops in 129 SvSl mice after they have received an intraperitoneal injection of ascitic fluid containing teratoma cells (18). In the ascitic fluid are found tumor masses which morphologically resemble normal mouse embryos of the late morula and early blastocyst stage. These masses have ectoderm enveloped in a layer of endoderm (18). The embryoid masses were placed in phosphate-buffered saline containing 0.25% trypsin and separated into individual cells by pipetting them back and forth in a narrow pipette. The cells were placed in Eagle's basal medium with 10% fetal calf serum.

Blastocyst Manipulation. The apparatus used to make the cell transfers consists of right and left handed Leitz micromanipulators and a Leitz Laborlux II microscope (E. Leitz, Rockleigh, N. J.). The blastocysts were placed in the well of a cavity slide containing 100 μl of the culture medium (EBMS with the $NaHCO_3$ replaced by Hepes) and covered by liquid silicone. The left hand micromanipulator holds a blunt suction pipette (the inside diameter is 5 μ and the outside diameter is 100 μ) and the right hand micromanipulator holds the injection pipette. The diameter of the injection pipette is varied to just accommodate the type cell to be injected. The pipette should have a thin wall and a tapered tip. The blastocysts and cells are placed in the well of the cavity slide, and the blastocyst picked up on the end of the suction pipette. The cells are picked up with the injection pipette (previously filled with oil) and the pipette inserted through the zona pellucida and blastocoele wall into the cavity. 2–4 cells are placed near or against the inner cell mass. The pipette is slowly withdrawn to prevent the escape of the cells. The injected or recipient blastocyst is then removed from the slide and placed in a watch glass containing culture medium until transfer to a foster mother.

Transferring Blastocysts to Foster Mothers. The foster mothers are obtained by injecting random-bred Swiss albino females with 2 U of pregnant mares serum followed in 48 h with an injection of 2 U of human chorionic gonadotrophin. The females are placed with vasectomized males who have been proven sterile. The injections for the foster mothers are begun 24 h after those for the females producing the recipient blastocysts. The foster mothers are anesthetized with pentabarbitol and the hair clipped from a 3 cm square area over the area of the ovary. A 1-cm incision is made in the skin and abdominal wall over the ovary and the ovarian fat pad, ovary, and uterus withdrawn. The recipient blastocysts are picked up in a small volume of culture medium in a finely drawn pipette. The pipette is inserted through the uterine wall and the blastocysts and medium are gently forced into the uterine lumen by air pressure created by blowing on a tube connected to the pipette. 4–8 blastocysts are placed in the uterine lumen of each foster mother. The incision is closed and the female placed in a cage alone until the young are born.

Assessing Results. The results of the cell transfers were determined in four ways. First, the animals born to the foster mothers were observed for pigmentation of eye or coat color. The recipient blastocysts were always albino and both types of cells (CBA T6T6 and 129 SvSl) came from agouti animals. Second, lymphocyte cultures were prepared according to the technique described by Hayry et al. (9) from the blood of the animals resulting from blastocysts that received CBA T6T6 bone marrow cells. Chromosome preparations were made from these cultures and examined for the T6 chromosome. Third, the offspring resulting from blastocysts into which cells were transferred were placed with albino animals of opposite sex for mating. The young from these matings were examined to determine if any germ cells contained agouti genotype from the transferred cells. Fourth, skin grafts were placed according to the technique described by Billingham (1) on the young resulting from

[1] *Abbreviation used in this paper:* EBMS, Eagle's basal medium supplemented with 5×10^{-4} M pyruvate and 10% fetal calf serum.

the blastocysts which received bone marrow cells or teratocarcinoma cells. The survival time of these skin grafts was compared to the survival time of similar grafts placed on animals from the same strain as those supplying the recipient blastocysts (ICR Swiss albino males or females). Medium survival times, 95% confidence limits, and statistical significance were determined according to the methods described by Litchfield (10, 11).

Results

The first group of experiments involved transfer of CBA T6T6 bone marrow cells into blastocysts from Swiss albino mice. From these recipient blastocysts 77 offspring were reared to adults. 40 were males and 37 were females. None of the animals showed any pigmentation. Chromosome preparations were made from peripheral blood lymphocytes of 37 males and 18 females. None showed T6 chromosomes.

However, when CBA T6T6 skin was grafted to these 77 animals, there was a significant increase in survival time of the grafts on experimental animals compared to controls. These results are shown in Table I. The graft median survival time for male experimental mice was 10.8 days which was significantly greater than for male controls, and the median survival time for female experimental mice was 10.6 days which was also significantly greater than for female controls. The graft median survival time on male and female experimental animals was not significantly different.

The distribution of graft survival time for experimental animals was quite skewed. 40 animals rejected the grafts between day 7 and 10; 21 rejected grafts between day 11 and 20; and 14 rejected grafts between day 21 and 30. The remaining two grafts remained for considerably longer. One graft on a male lasted for 55 days and one graft on a female lasted for 65 days. On skin grafts that

TABLE I

Effect of Cells Transferred into Mouse Blastocysts on the Immune Response of Offspring Resulting from the Recipient Blastocysts

Cell type transferred	Skin graft median survival time	
	Males	Females
Bone marrow cells	10.8 [9.1–12.8] (40)	10.6 [8.6–13.0] (37)
None (control animals)	7.4 [6.8–8.1] (40)	8.0 [7.3–8.8] (37)
Teratocarcinoma cells	11.0 [9.6–12.6] (30)	8.9 [8.1–9.7] (30)
None (control animals)	6.8 [6.0–7.7] (30)	7.8 [7.3–8.3] (30)

Median survival time is given in days and is followed by the 95% confidence limits enclosed in brackets. The number of animals in each group is shown in parentheses. The significance of the difference between median survival times was calculated by the method of Litchfield (10, 11). The increase in median survival time was significant at the $P < 0.05$ level for all groups that had received cells. The difference between males and females was significant only for the animals that received teratocarcinoma cells.

remained in place for longer than 3 wk, a substantial growth of agouti hair occurred. Fig. 1 shows the appearance at 1 mo of one of these longer-lasting grafts that grew hair.

The second group of experiments involved transfer of teratocarcinoma cells from 129 SvSl (agouti) mice into blastocysts from Swiss albino mice. From these recipient blastocysts, 60 offspring were reared to adults. 30 were males and 30 were females. One of the males had several small patches or stripes of agouti hair. Fig. 2 shows the extent of these agouti areas. The areas resembled thin stripes of hair running from the dorsal midline laterally on the right thorax and flank. They did not extend across the dorsal midline or on to the belly hair. The pattern was similar to that seen in some animals resulting from whole embryo fusion (13). There was also a small area of agouti hair immediately anterior to the base of the left ear. This male was mated to numerous females but no offspring resulted. Skin from a 129 SvSl male was grafted on this male. The first graft was torn off by the animal shortly after the protective cast was removed. The second graft remained for 26 days before it was off.

Skin grafts were made on all 60 of the animals resulting from the blastocysts that received 129 SvSl cells, and there was a significant increase in survival time of these grafts when compared to those made on control animals. These results are shown in Table I. The graft median survival time for experimental males and females was 11.0 and 8.9 days respectively. Both these values are significantly greater than the corresponding controls. In addition, the graft median survival time on the males was 2.1 days longer than on the females. This difference was significant, but the difference in graft median survival time for the controls was not significant.

The distribution of graft survival time for the animals from the teratocarcinoma cell transfers was skewed in a manner similar to that found for animals from the CBA T6T6 cell transfers. 36 rejections occurred between 7 and 10 days; 21 between 11 and 20 days; and 3 after 20 days.

All of the animals resulting from the bone marrow cells transfers and those from the teratocarcinoma cell transfers were placed with albino mice to determine if there were any germ cells with agouti genotype. No agouti young resulted from these matings.

Discussion

The results of the experiments in which CBA T6T6 cells were transferred into blastocysts provide evidence that some cells taken from the adult body are capable of participating in development of the embryo. The absence of evidence for the presence of the transferred cells in the pigmented tissues of the adults, in the germ cells, and in the peripheral lymphocytes suggests strongly that the cells did not clone the embryo to a large extent in these experiments. However, in the studies with peripheral lymphocytes one cannot rule out the possibility that a very few T6T6 cells were present among the lymphocytes. All that can be said is that of the approximately 2,000 chromosome spreads examined from these

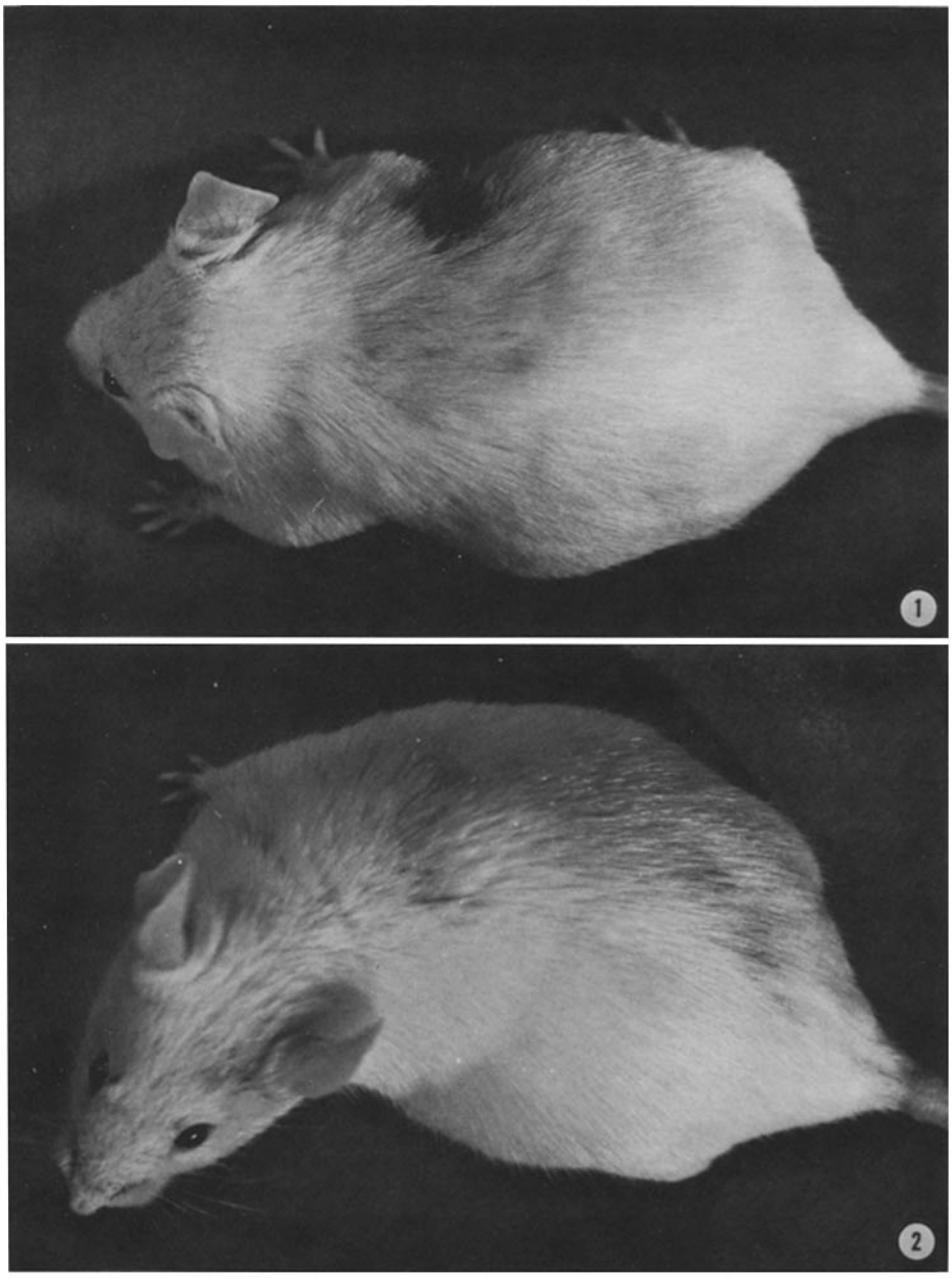

FIG. 1. A random-bred Swiss albino mouse with a CBA T6T6 skin graft. The graft is 24 days old and has grown agouti hair. The animal developed from a blastocyst of Swiss albino genotype that received CBA T6T6 bone marrow cells.

FIG. 2. A random-bred Swiss albino mouse with several stripes of agouti hair extending from the midline over the right flank and thorax. An additional group of agouti hairs is present at the anterior base of the left ear. The animal developed from a blastocyst of Swiss albino genotype that received 129 SvSl (agouti) teratocarcinoma cells.

animals, none contained T6 chromosomes. Perhaps the cloning efficiency is much lower than this under the conditions of these experiments.

The skin graft results from these animals, however, clearly indicate an effect of the transferred bone marrow cells. It seems very unlikely that such a strong immunological effect in the adult could result from the presence of the CBA antigens in the embryo from only a short time following the transfer of the cells. The highly significant prolongation of skin graft survival time in these animals seems more likely to result from the colonization of the embryo by a small number of cells for an extended period. A few CBA cells may still exist in those adults where the grafts were maintained for a week or more longer than controls.

The results of the experiments in which 129 SvSl teratocarcinoma cells were transferred into blastocysts provide evidence that this type of malignant cell is capable of participating in development of the embryo. In addition, the experiments indicate that the embryo environment can bring under control the autonomous proliferation of the teratocarcinoma cells. The interaction of the teratocarcinoma cells with embryo cells must be quite different than with adult cells, since in the adult the cells generally result in death of the animal in approximately 3 wk.

The appearance of the agouti hairs in one of the offspring resulting from the 129 SvSl cell transfers provides very strong evidence for the participation of the transferred cells in embryo development. Although the number of hairs is small, they are unequivocably present and could not arise from the albino recipient blastocyst genotype. The inability of the animal to maintain a 129 SvS1 skin graft indefinitely could have been due to the type of cell or small number of cells present. These may not have created sufficient tolerance to maintain a skin graft. A number of situations have been described in which chimerism exists without skin graft tolerance (2).

The skin graft data from the animals that received the teratocarcinoma cell transfers provide additional and convincing evidence for the participation in embryo development and for the presence in the adult of 129 SvSl cells. The reason for the 2.1 day difference in graft survival time between the males and females is not readily apparent. A possible explanation may be related to the sex genotype of the transferred cells. In the CBA cell transfers, the bone marrow cells came randomly from males and females and were placed into blastocysts of unknown sex, presumably random or half male and half female. However, the teratocarcinoma cells used in these studies came from a testicular teratoma (17, 18), and therefore all carried the Y chromosome. Perhaps it is more difficult for a cell with a Y chromosome to establish a colony in a female embryo because of antigens associated with Y chromosome expression. This could explain why the cloning of the females was less efficient than the cloning of the males by the male genotype teratocarcinoma cells. A similar effect in the CBA cell transfers might not be observed because the random combinations between transferred cells and blastocysts would decrease the differences. In addition the CBA strain may not react as strongly to the male antigen as does the 129 strain. Differences between strains in responsiveness to Y antigen have been demonstrated (16). However, in general the Y antigen has a weak effect (7), and the difference in median survival time between the male and female groups may result from other causes.

Summary

Bone marrow cells from CBA T6T6 mice and testicular teratocarcinoma cells from 129 SvSl mice were transferred into blastocysts from random-bred Swiss albino mice. The blastocysts were allowed to develop in foster mothers and the adults resulting from these blastocysts were studied for evidence of an effect of the transferred cells. A total of 137 adults resulted from the experiments, and one of the mice that had received teratocarcinoma cells in the blastocyst stage showed several thin stripes of agouti hair. All the adult animals received grafts of skin from animals identical to those supplying the cells. In all cases the animals that resulted from blastocysts into which cells had been transferred maintained skin grafts for a significantly longer period than controls. In a number of cases the graft developed agouti hair and in two cases the graft was maintained for approximately 2 mo. These experiments indicate that the transferred cells were able to establish small colonies in the embryos and that some of these cells persisted into the adult.

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References

1. Billingham, R. E. 1961. Free skin grafting in mammals. *In* Transplantation of Tissues and Cells R. E. Billingham and W. K. Silvers, editors. The Wistar Institute Press, Philadelphia, Pa. 1.
2. Billingham, Rupert and Willys Silvers. 1971. Immunological Tolerance. *In* The Immunobiology of Transplantation. Abraham Osler and Leon Weiss, editors. Prentice-Hall, Inc., Englewood Cliffs, N. J. 132.
3. Brinster, R. L. 1963. A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Exp. Cell Res.* **32**:205.
4. Brinster, R. L. 1972. Cultivation of the mammalian egg. *In* Growth, Nutrition and Metabolism of Cells in Culture, Vol. II. G. Rothblat and V. Cristofalo, editors. Academic Press, Inc., New York. 251.
5. Gardner, R. L. 1968. Mouse chimaeras obtained by the injection of cells into the blastocyst. *Nature (Lond.)*. **220**:596.
6. Gardner, R. L. 1972. An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J. Embryol. Exp. Morphol.* **28**:279.
7. Gasser, David L. and Willys K. Silvers. 1972. Genetics and immunology of sex-linked antigens. *Adv. Immunol.* **15**:215.
8. Haskill, J. S. and M. A. S. Moore. 1970. Two dimensional cell separation: comparison of embryonic and adult haemopoietic stem cells. *Nature (Lond.)*. **226**:853.
9. Hayry, P., M. Virolainen, and V. Defendi. 1970. Method for chromosome preparation from mouse peripheral lymphocytes. *Proc. Soc. Exp. Biol. Med.* **133**:637.
10. Litchfield, J. T., Jr. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96**:99.

11. Litchfield, J. T., Jr. 1949. A method for rapid graphic solution of time-per cent effect curves. *J. of Pharmacol. Exp. Ther.* **97**:399.
12. Mintz, B. 1962. Formation of genotypically mosaic mouse embryos. *Am. Zool.* **2**:432.
13. Mintz, B. 1970. Gene expression in allophenic mice. Symposia of the International Society for Cell Biology. Academic Press, Inc., New York. **9**:15.
14. Moustafa, L. A. and R. L. Brinster. 1972. The fate of transplanted cells in mouse blastocysts. *J. Exp. Zool.* **181**:181.
15. Moustafa, L. A. and R. L. Brinster. 1972. Induced chimaerism by transplanting embryonic cells into mouse blastocysts. *J. Exp. Zool.* **181**:193.
16. Silvers, Willys K. and R. E. Billingham. 1967. Genetic background and expressivity of histocompatibility genes. Science **158**:118.
17. Stevens, Leroy C., Jr. and C. C. Little. 1954. Spontaneous testicular teratomas in an inbred strain of mice. *Proc. Natl. Acad. Sci. U.S.A.* **40**:1080.
18. Stevens, Leroy C., Jr. 1970. The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. *Dev. Biol.* **21**:364.
19. Tarkowski, A. K. 1961. Mouse chimaeras developed from fused eggs. *Nature (Lond.)*. **190**:857.
20. Van Bekkum, D. W., M. J. van Noord, B. Maat, and K. A. Dicke. 1971. Attempts at identification of hemopoietic stem cell in mouse. *Blood J. Hematol.* **38**:547.