Chimaeric rabbits from immunosurgically-prepared inner-cell-mass transplantation

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SUMMARY

Rabbit inner cell masses (ICM's) have been prepared by immunosurgery from 96-h-old blastocysts. Their properties are described. It is shown that ICM's prepared this way retain their developmental potentialities. In particular if they are injected into host blastocysts, they appear to be able to participate in the development of the composite blastocysts thus obtained: upon implantation into a foster-mother the latter give rise, in a high proportion of cases, to chimaeric rabbits. Immunoglobulin allotypic markers as well as progeny test were used to assess chimaerism.

INTRODUCTION

Chimaeric animals are of great interest for examination of various biological problems (Mintz, 1974; McLaren, 1976; Gardner, 1978a). In particular, they can be used to look at possible transfer of genetic information between different cells. Such transfer has been suggested, but not proved, during the process of antibody formation (see for instance Bell & Dray, 1973). Although chimaeric mice have been used to examine this problem (Munro, Day & Gardner, 1974), the domestic rabbit seems to be a better model, due to the well known polymorphism of the different allotypic markers on the polypeptide chains of its immunoglobulins (Oudin, 1956a, b; 1960a, b).

Such a study was undertaken by Gardner & Munro (1974). Two allophenic rabbits were obtained, but no conclusion was drawn, because no analysis of individual immunoglobulin was presented. We thus decided to reinvestigate this point and to look in chimaeric rabbits at the problem of the allelism or the pseudo-allelism of the structural genes coding for the allotypically different polypeptide chains of immunoglobulins, e.g. by the possible expression of unexpected allotypes. The first results of this study have already been published (Bordenave & Babinet, 1979).

In this paper we wish to present in detail the way by which tetraparental rabbits were obtained. Inner cell masses (ICM's) were prepared by immunosurgery...
surgery (Solter & Knowles, 1975) and subsequently injected by microsurgery into the cavity of suitably chosen blastocysts (embryo aggregation cannot be used in the rabbit because of inability of zona-pellucida-free embryos to implant into a foster-mother (Adams, 1965)). We show that, indeed, the rabbit ICM’s prepared this way retain their developmental properties and that the composite blastocysts give rise to chimaeric rabbits upon implantation into a foster-mother in a high proportion of cases.

**MATERIAL AND METHODS**

*Embryos*

Fauve de Bourgogne and Blanc de Bouscat rabbits (obtained from Ferme Expérimentale du Prieuré, Institut Pasteur, Rennemoulin 78450 Villepreux, France) were used as sources of host and ICM donor blastocysts respectively. Fauve de Bourgogne has brown hair and pigmented eyes whereas Blanc de Bouscat is white with unpigmented eyes. The blastocysts were obtained by flushing TC Tyrode solution (Paul, 1970; Supplied by Difco Laboratories Detroit U.S.A.) through the dissected genital tract of females which had been mated with fertile males 96 h before. Usually five to ten blastocysts were obtained from one female. Immediately after flushing the blastocysts were transferred into a serum-complemented medium (Menezo’s Medium (Menezo, 1976) Api-System La Balme-les-Grottes 38390 Montalieu-Vercieu, France). This medium was used throughout, except for the brief period of micro-injection when a phosphate-buffered medium, PB1, (Whittingham & Wales, 1969) supplemented with 10 % heat-inactivated (30’ at 56 °C) foetal calf serum was used.

*Preparation of ICM*

Blastocysts were treated with a 0.5 % Pronase solution (w/v in bovine serum albumin-free Whitten’s medium, 1970) preincubated 1 h at 37 °C and then filtered on Nalgene 0.45 μm filter unit (Nalge Company, Rochester, New York). This was done at 37 °C under a dissecting microscope so that the blastocysts could be transferred to normal medium as soon as the zona albumina and pellucida had disappeared. This happened usually after 3 to 5 min of incubation in pronase. The blastocysts were then washed three times before being transferred into an antibody preparation which consisted of a decocomplemented goat antiserum against normal rabbit serum diluted three times with Menezo’s medium. After 30 min of incubation at 37 °C, the embryos were again washed three times, and transferred to the complement solution (one to six dilution of a rabbit serum as described in Artzt et al. 1973). Around 10 min later, the outside trophoblastic cells began to lyse, the blastocyst collapsed and acquired a characteristic appearance of a bulk of lysed trophoblastic cells in which the rounded ICM was usually visible. The ICM was then separated
Fig. 1. Illustration of ICM fusion. (A) Two ICM's put side by side in culture at 37 °C. (B) The same, 1 h later. (C) 2-5 h later.
from the lysed cells by pipetting it a few times in fresh medium. ICM's prepared this way are shown in Fig. 1. 

Microinjections of ICM

Apparatus

The general equipment is mainly the same as the one used for micromanipulation of mouse blastocyst and has been described in detail by Gardner (1978b). Briefly, it includes left and right micromanipulator units with base plate (from E. Leitz, Wetzlar, West Germany) equipped with two instrument heads, one for each manipulator, and an Ergaval microscope which has an image-erected optics and a fixed stage (Carl Zeiss of Jena). The left micromanipulator holds the suction pipette (see below), which is controlled by an Agla micrometer syringe outfit. The right micromanipulator holds the injection pipette (see below) which is controlled by a de Fonbrune force pump (Beaudoin, France).

Microinstruments

Two microinstruments were used, one for holding the recipient blastocyst (holding pipette), and the other for transplanting the ICM of the donor blastocyst (injection pipette). Both instruments were made from the same thin wall capillary tubes (Drummond, 1 mm OD, 0.1 mm wall thickness).

Holding pipette

When collected at a given time (96 h post-coitum in our experiments), rabbit blastocysts show a great variation both in overall size and in the thickness of the zona albumina. The latter probably reflects, in part, the variation in internal pressure of the blastocoelic fluid. Therefore, in order to hold the blastocysts as firmly as possible, we used two kinds of holding pipettes, one for the 'big' blastocysts (> 500 μm) and one for the 'small' ones (< 500 μm). These were pulled by hand and cut with a de Fonbrune Microforge so as to get an outside diameter of 450 and 350 μm respectively. The thickness of the wall in both cases was around 50 μm. These pipettes were washed after each experiment and used repeatedly.

Injection pipette

The capillary was first pulled with an electric puller (Industrial Science Associates Inc, N.Y., U.S.A.) so that the taper was about 10 mm to the pointed tip. It was then cut with the Microforge at an outside diameter of 45 μm. The pipette was then bevelled with a glass pipette grinder (Industrial Science Associates, New York) at an angle of around 45°. It was then washed immediately by sucking in distilled water, in order to remove the glass fragments which otherwise could partially occlude the opening of the pipette.
Fig. 2. Illustration of ICM microinjection. (A) The blastocyst is held by the holding pipette, the ICM being oriented towards the latter. The injection pipette is positioned at the same level as the ICM (see text). (B) The injection pipette, containing the ICM to be injected, is introduced into the blastocyst. (C) Donor ICM has been deposited inside the cavity of the blastocyst. (D) Host blastocyst, after withdrawal of the injection pipette. Note the important enlargement of the zona albumina as a consequence of blastocyst collapse.
Microinjection

The microinjections were carried out in hanging drops of PBI medium in Leitz manipulation chambers as described by Gardner (1978), except that the volume of the drops was bigger, due to the size of the rabbit blastocyst. One blastocyst and one isolated ICM were placed in each drop. With the aid of the injection pipette, the blastocyst was oriented to be held firmly by the middle of its embryonic disc (Fig. 2a). The ICM to be injected was then gently forced into the injection pipette. The latter was positioned so that its tip was at the same level as the ICM (Fig. 2a) and could not go further towards the holding pipette. This was achieved by using the micromanipulator unit which controls the injection pipette as a stopper of the joystick. Using the joystick, the pipette was then positioned near the wall of the blastocyst opposite to the ICM. At this stage it was crucial to ensure that the maximum diameter of the blastocyst and the tip of the injection pipette were exactly in the same focal plane. The method of advancing the injection pipette (Fig. 2b) depended on the thickness of the zona albumina of the blastocyst to be injected: with thin zona, a slow motion was enough, while with thick zona, it was necessary to use its elasticity and force the blastocyst partly into the holding pipette, so as to maximize the useful distance for the injection pipette to find its way into the blastocyst. In this case, a firm and quick motion of the injection pipette was necessary. The ICM to be injected was then released at the desired place, namely on to the ICM of the host blastocyst (Fig. 2c). During this process, the blastocyst always retained its shape. It was only when the injection pipette was withdrawn that blastocoelic fluid was expelled and the blastocyst collapsed partly while the zona albumina enlarged dramatically (Fig. 2d). The operated blastocysts were then transferred to fresh medium and allowed to repair for 2 to 4 h. After this period they re-expanded almost completely and the injected ICM was no longer visible as a discrete mass of cells but had spread (Fig. 3). Occasionally, the trophoblast herniated out of the zona at the point of injection. This could be avoided by very slowly withdrawing the injection pipette which prevented sudden expulsion of blastocoelic fluid and consequent partial protrusion of some trophoblast.

Implantation

The operated blastocysts were transferred into Blanc de Bouscat foster-mothers which were rendered pseudo-pregnant, 12 h less advanced than the blastocyst donors, by mating with a Blanc de Bouscat vasectomized male. Anaesthesia was performed by intramuscular injection of 2 ml of hypnorm (UVA, Paris). The uteri of the recipient female were exposed via a ventral incision. About 1 cm below the junction of the uterus and the oviduct, the wall of the uterus was perforated with a needle. The operated blastocysts in Menezo’s medium were injected into the uterine horns (three to five blastocysts by uterine
Fig. 3. Blastocyst culture after ICM micro-injection. (A) Four blastocysts just after injection of ICM. In blastocysts 1, 2 and 3, the injected ICM is clearly visible (arrows). In blastocysts 1 and 3, the route of injection is also visible. (B) The same four blastocysts after 2-5 h culture. They have regained their blastocoelic fluid. As a consequence zona pellucida has a more regular shape and zona albumina has become thinner.
horn) by means of a flame-polished pipette through the passage created by the needle towards the uterine lumen. The ventral incision was then sutured.

This procedure is largely inspired by the one described by Dickmann (1971).

RESULTS

Properties of 96 h-isolated ICM

As described below, ICM’s isolated by immunosurgery were able to colonize the embryo and thus to give rise to chimaeric rabbits, when injected into blastocysts with differential genetic markers. Their behaviour in vitro was strikingly reminiscent of the one of the mouse ICM’s as was originally described by Gardner (1971).

Compaction and fusion

Isolated ICM’s appeared as a ball of cells. These cells had a great tendency to compact one with the other. Indeed, if the cells were partially dissociated simply by pipetting with a narrow-tip pipette, they readily recompacted after a short period in culture (15 or 30 min) at 37 °C. Furthermore, if pairs of ICM’s were put in culture, they would fuse and form a single mass after a few hours (Fig. 1b, c).

Behaviour in long-term culture

Single ICM’s were put in culture in 2-5 ml of Dulbecco’s modified Eagle’s medium supplemented with 15 % foetal calf serum. Fifteen h later, the ICM’s had attached and spread giving rise to a homogeneous colony of undifferentiated cells. After 48 h changes were already visible as some cells of the ICM’s would flatten out. Several days later, various types of cells appeared in culture which could be kept growing for several weeks.

Chimaeric rabbits

Whole ICM’s from blastocysts of (donor) white rabbits (Blanc de Bouscat) were injected by microsurgery into blastocysts (host) of brown rabbits (Fauve de Bourgogne). In addition to the coat colour marker, host and donor blastocysts differed at one or both of two unlinked loci which control the allotypic specificities carried by the variable region of the heavy chains (i.e. locus a) or by the constant region of the K light chains (i.e. locus b) of the rabbit immunoglobulins.

One hundred and ten operated blastocysts were implanted in a total of 12 foster-mothers. Eight became pregnant and gave rise to 33 newborns, 23 of which survived up to the adult age and could thus be analysed for the allotypic specificities of their immunoglobulins without the mother’s immunoglobulins perturbing the analysis. Among the latter, 16 exhibited allotypic specificities from both host and donor blastocyst origins and were thus chimaeric for their
Immunosurgically-prepared rabbit ICM for producing chimaeras

Table 1. Determination of chimaeric rabbits by means of immunoglobulin allotypic specificities

<table>
<thead>
<tr>
<th>Allotypic specificities*</th>
<th>Genotype of host blastocysts (brown rabbits)</th>
<th>Genotypes of ICM (white rabbits)</th>
<th>Phenotypes of the 7† resulting rabbits all homogeneously brown</th>
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<td>b4</td>
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* The immunoglobulin allotypic specificities coded by alleles of two unlinked loci were studied. The specificities of the a series (a1, a2, a3 . . .) are symbolized by a triangle which is black for the brown rabbits and white for the white rabbits and the specificities of the b series (b4, b5, b6 . . .) are symbolized by a circle which is black for the brown rabbits and white for the white rabbits. The host blastocysts came from an a1, b4 homozygous brown female which was mated with an a1, b4 homozygous brown male. The ICM's were prepared from the blastocysts of an a2, a3 white female which was mated with an a2, a3, b5, b6 white male. The foster-mother was an a3, b4 Blanc de Bouscat which was mated with a vasectomized Blanc de Bouscat male.

† Among the seven resulting rabbits, obviously six are chimaeric as they express immunoglobulin allotype specificities from the two parental strains while one is not a chimaera for the lymphoid system as it only expresses the specificities of the brown parental strain.

lymphoid system (an example is given in Table 1 for six of them and a detailed analysis of these rabbits will be presented elsewhere).

Surprisingly, none of these rabbits exhibited coat-colour chimaerism: all were of the brown pigmented type, including those which were chimaeric in their lymphoid system. This point will be discussed below. In the absence of other genetic markers, which would permit analysis of the extent of chimaerism in other organs than the lymphoid and the pigmentation systems, we investigated possible germ-line chimaerism. Some of the rabbits, which exhibited immunoglobulin allotype chimaerism, including males and females, were progeny tested by crossing them to each other. Three such intercrosses were performed. Cross number one gave rise to six rabbits all of the brown type.
Table 2. Progeny test made between two chimaera rabbits

<table>
<thead>
<tr>
<th>Allotypic specificities*</th>
<th>Phenotype of the male (brown)</th>
<th>Phenotype of the female (brown)</th>
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<tbody>
<tr>
<td>a1</td>
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<td>b5</td>
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</tr>
<tr>
<td>b6</td>
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Phenotypes of the 8 rabbits constituting the first Fl

| a1 | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| a2 | ▲ | △ |
| a3 |   |   |
| b4 | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| b5 |   |   |
| b6 |   |   |

Homogeneously brown

Grey

* For the meaning of the symbols, see Table 1. Two tetraparental rabbits were mated: an a1, a2, a3, b4, b5, b6 brown female with an a1, a3, b4, b5 brown male. The first offspring was constituted by seven a1, b4 brown rabbits and one a1, a2, b4, b5 grey rabbit. Obviously the female was a germ-line chimaera.

Cross number two and three gave rise respectively to eight and seven brown rabbits plus one grey rabbit, typical of an F1 (Fauve de Bourgogne × Blanc de Bouscat) individual. This was indicative of germ-line chimaerism of at least one of the parents in both crosses. The grey rabbit of cross number two died prematurely, but the other (born from cross number three) survived up to the adult age and its immunoglobulin allotype determinants could be analysed. From this analysis it was demonstrated that (1) it was indeed the product of gametes of both white and brown origin (2) the female progenitor was a germ-line chimaera (see Table 2).

DISCUSSION

Rabbit ICM’s were prepared by an immunosurgical method originally devised in the mouse (Solter & Knowles, 1975). Our results indicate that this method is appropriate in the rabbit as well. Rabbit ICM’s prepared this way seem to retain their potentialities as evidenced by their behaviour both in in vitro and in in vivo manipulations.

In vitro, it was shown that ICM’s prepared from 96 h-old blastocysts assumed a compact spherical form. This seemed to be a property of the component cells: indeed, if some cells are partially dissociated (e.g. by pipetting) they will recompact readily after a while in culture. Furthermore, whole ICM’s can fuse...
together: when put by pairs in culture, they will form a single mass after a few hours (see Fig. 1). When allowed to attach and spread in a tissue-culture dish they appear at first as a homogeneous colony of undifferentiated cells which will subsequently differentiate into various types of cells. When injected into a host blastocyst, they are able to participate in its development, as shown by the fact that implantation of the composite blastocysts into a foster-mother gives rise to chimaeric rabbits in a high proportion of cases. Taken together, all these properties are reminiscent of what was observed with the mouse ICM's (Gardner 1971).

In our experiments, the chimaerism of the rabbits born from the composite blastocysts was established by analysis of their immunoglobulin allotype constitution. However, no coat-colour chimaerism was observed. This is an interesting though intriguing observation. If it is assumed that both white and brown phenotypes of our strains have their origin in genes acting via melanocytes, one could hypothesize that absence of coat-colour chimaerism is due to an extreme case of clonal selection in our chimaeras. In other words the melanocytes of the brown type would have much higher proliferative properties than those of white origin, thus colonizing all the territories normally occupied by the melanocytes and giving rise to uniformly brown rabbits, in spite of the presence of both brown and white melanocyte ancestors in the chimaeric embryo. It should be noted that such clonal selection, although less extreme, has long been assessed in allophenic mice (see for instance Mintz, 1969). Results of another experiment (Babinet and Bordenave unpublished results) tend to the same conclusion. ICM's from Fauve de Bourgogne were injected into Blanc de Bouscat blastocysts. Three rabbits were obtained from such composite blastocysts. Two of them were white with no sign of chimaerism in their lymphoid system; the third one, which exhibited immunoglobulin allotypic determinants of both white and brown origin, was nevertheless uniformly brown. Here again, brown melanocytes could have overgrown the white ones. It should be noted that in another large scale experiment aimed at producing chimaeric rabbits, Moustafa (1974) got a low incidence of coat-colour chimaerism, but, in the absence of suitable markers, it was not possible to correlate it with chimaerism in other organs.

Finally injection of ICM into a blastocyst appears from our results to be a very efficient way of obtaining chimaeras. As was shown for the incidence of chimaerism in only one tissue, i.e. the lymphoid system, 16 out of 23 animals born from composite blastocyst were allophenic. A minimum of two of those were germ-line chimaeras. There is thus no reason why other organs should not be chimaeric, although it was not possible to demonstrate this point because of lack of suitable markers like isozymes.

Note added in proof

Since this paper was submitted for publication, a further brown rabbit, chimaeric for the lymphoid system, was progeny tested and found to possess germinal cells from the white parental strain only.
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