Study on the participation of neoplastic cells in the development of mouse embryos

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SUMMARY

To test the ability of cloned committed erythroleukemic cells to participate in development we have injected Friend leukemia cells (FLC) into C57BI/6 mouse blastocysts together with Friend leukemia virus (FLV) and we have examined the newborn individuals derived from them. Five animals out of 32 born have FLC-derived neoplasia. The incidence of neoplasia is increased as compared with other similar experiments without the virus. In two of the animals with the FLC neoplasia the disease manifestation is an erythroid leukemia similar to the one obtained directly with the virus in normal DBA/2 mice.

INTRODUCTION

Blastocysts of a genetically marked mouse strain can be injected with embryo cells derived from different mouse strains with the result of building a chimaera (Gardner, 1968). Chimaeras are obtained also by injecting embryo cells which are in a more advanced stage of development than the recipient blastocysts (Moustafa & Brinster, 1972; Rossant, Gardner & Alexandre, 1978).

If injected into mouse blastocysts murine teratocarcinoma cells of some highly malignant strains assume normal duplicative capacity and participate in normal development (Brinster, 1974; Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975). So far no other attempts to colonize the embryo with other neoplastic cells have been published.

One large series of blastocyst injections with FLC (unpublished) was conducted by one of us (E. C.) in the laboratory of Dr B. Mintz. In this attempt more than 100 mice were born without signs of chimaerism as judged by the GPI assay in the blood and other organs. In the same series two mice were found at birth to contain a solid tumour derived from the injected FLC.

This latter type of experiments, aimed to test whether the normalization of

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duplicative capacity obtained for teratocarcinoma cells could be extended to other cancer cells, is based on a number of unproved assumptions. The main one assumes that in case of normalization the cell does not resume immediately its limited duplicative capacity: if this should be the case, then the descendants of the original injected cell would be too few to allow detection in the newborn animal.

In fact, this limitation might apply to any cell derived from normal adult tissue where a limited, relatively small number of cell duplications is already fixed for the particular cell (Hayflick, 1965). We might assume then, that in our case the birth of a normal non-chimaeric animal is due, at least some times, to the normalization of FLC followed by the disappearance of their detectability. If, together with FLC, one injects in the blastocysts an aliquot of FLV and if the virus transforms only erythroid cells of the same genetic type of the injected cell, then the normalized erythroblast cell inside the developing embryo might become ‘target’ to the virus and resume the neoplastic growth which would give among newborn individuals a higher incidence of tumours as compared to the experiment without the virus. The present paper reports an experiment where we registered two facts: (a) a significant increase in the number of tumours by comparing the newborns obtained with FLC alone versus those obtained with FLC plus FLV; and (b) the appearance of a type of leukemic disease which was never registered by grafting FLC.

MATERIALS AND METHODS

Virus

Friend leukemia virus (FLV). The virus we used was either the original sample obtained from Dr Ostertag or a subculture of it grown on SCI cells. The virus injected in young susceptible animals causes very rapid onset of leukemia with polycytaemia and splenomegaly. The CX titre (Rowe, Pugh & Hartley, 1970) of the preparation is $6.7 \times 10^7$ plaque forming units (pfu)/ml while the titre of foci on spleen of young DBA/2 mice is $10^7$ Focus Forming Units (FFU)/ml (Axelrad & Steeves, 1964). The same preparation counted in the electron microscope contained $5 \times 10^{10}$ virus particles/ml. The virus is suspended in Dulbecco's medium supplemented with 10% foetal calf serum. The amount injected equals to $4 \times 10^{-7}$ ml and contains approximately 24 pfu, 4 FFU and 20000 biologically non-titrable viral particles.

Cells

The Friend leukemia cells (FLC) used are a subclone of clone 745 of Dr Friend. The original line was obtained from a DBA/2 mouse treated with Friend virus. Our subclone line 5-86 produces very little or no XC titrable particles or particles capable of producing spleen foci. Our clone is typically erythroblastoid in appearance and has a doubling time of less than 12 h. Its karyogram gives a mean number of 38 chromosomes with 3 metacentrics.
Intravenous or intraperitoneal injection of these cells into DBA/2 mice results in growth of a solid tumour which metastasizes. The blood of the injected animals does not show signs of circulating erythroblasts.

Mice

Mice donors of blastocysts were inbred C57B1/6 CNEN (Casaccia, Rome), originally obtained from the Jackson Laboratories. Animals used as foster mothers were Swiss albino random bred mated with vasectomized males. All blastocysts were collected by flushing the uterus at day 3 of pregnancy, counting the day of the occurrence of vaginal plugs.

Blastocyst injection

The techniques employed were those described by Gardner (1968), and subsequently modified by Moustafa & Brinster (1972) and Mintz & Illmensee (1975). The type of needle used for injections is visible in Fig. 2. The tubing connecting the syringe to the needle has an inner diameter of 0.8 mm and contains air.

Prior to the injection the blastocysts are transferred into the virus suspension together with FLC. The cells are then injected into the blastocoele. The injection of the virus suspension is slowly continued for a few seconds in order to substitute the entire blastocyst fluid with the virus suspension. Before transfer into the uterus of foster mothers at day 2 of pseudopregnancy, injected blastocysts are allowed to recover from the injection procedure for approximately 3 h.

Detection of chimaerism

In order to detect chimaerism the electrophoretic differences which exist between the GPI (glucose-6-phosphate isomerase) of DBA/2 and C57B1/6 mouse strains (respectively GPI-1a and GPI-1b) were measured according to De Lorenzo & Ruddle (1969).

Histopathology

When required, animals were autopsied and samples of organs were fixed in acetone. Sections were coloured with haematoxyline and eosine.

RESULTS

The present experiment is planned to test the incidence of tumours in animals born from blastocysts injected with FLC and FLV. Since our final aim is to compare this incidence with the one registered in a previous unpublished experiment in which the virus was not used, we have adopted experimental conditions which were identical in all appreciable details. In both experiments the cell line used is the same and all blastocysts derived from C57B1/6 mice with common ancestors (Jackson Laboratories) and with the same strong resistance to the erythroleukemia induced by the FLV (FV-1b; FV-2r) (Lilly
& Pincus, 1973). The number of injected cells was 3 in both experiments with very few exceptions in the unpublished experiment in which 2-5 cells were injected.

One first series of 40 C57B1/6 blastocysts were injected with 3 FLC and incubated in alfa-medium supplemented with 10% FCS under an atmosphere containing approximately 40% air, 45% N₂ and 5% CO₂. Twenty-eight blastocysts which appeared after 2-4 h to have recovered from the injection procedure were kept under observation for approximately 200 h. All of them clearly showed, by the third day, multiplication of the injected cells. By the end of the observation period the multiplying injected cells had killed the embryo (Fig. 1), escaped the zona pellucida and were vigorously growing in the surrounding medium. In fact, under the in vitro conditions we used, their growth seems to overcome the growth of the embryo.

In a second series 14 C57B1/6 blastocysts were injected with virus alone and transferred into the uterus of pseudopregnant foster mothers which delivered eight normal animals.

In a third series 80 C57B1/6 blastocysts injected again with 3 FLC and FLV were transferred into ten foster mothers. Transfer was performed into the uterus except for one set which was transferred into the oviduct of a foster mother at day 0 of pseudopregnancy. Five animals out of a total of 32 delivered at term were found dead or died during the following 48 h. At autopsy samples were taken for both histological and enzyme analysis. Permanent cell lines were derived from fragments of two animals (no. 70.2.1 and 73.1.1) out of the five which were autopsied shortly after death. Cells from these cultures tested for GPI gave the pure GPI-1a pattern characteristic for FLC.

Table 1 summarizes the results of the experiment. The main outcome is the relative high number of animals born without neoplasia.

GPI electrophoresis of blood and tissue samples of all animals born showed that the five animals, who died soon after delivery, contained cells which originated from the injected FLC. In fact, beside the fast migrating GPI band (GPI-1b), we could observe the slow migrating band (GPI-1a) characterizing FLC from

Fig. 1. Embryo injected with one FLC after approximately 200 h of incubation in a thermostat. The FLC have filled the entire zona pellucida. The embryo is the dark mass of dead cells.

Fig. 2. Friend leukemia cells and virus being injected into the blastocoel. Outer diameter of the injecting pipette —13 μm. Nomanski Optics. (A) Injection pipette before penetration; magnification × 160. (B) Injection of cells and virus completed; magnification × 400.

Fig. 3. Histology of the solid tumour of mouse 73.1.1. (A) Magnification × 250. (B) Enlargement of the indicated field; magnification × 1000. The plane of focus in the two photographs is slightly different.

Fig. 4. (A) Panoramic view of the heart of mouse 37.2.1. The cavities are filled exclusively with erythroid cells. (B) Higher magnification (× 1000) showing the types of circulating cells. No mature erythroblasts are to be seen.
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Table 1. Blastocyst injections with Friend leukemia cells and Friend leukemia virus

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>No. of C57B1/6 blastocysts</th>
<th>No. of C57B1/6 mice born</th>
<th>No. of animals with neoplasia and code number</th>
<th>Histopathology</th>
<th>Culture†</th>
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<td>6</td>
<td>3</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>37-2</td>
<td>7</td>
<td>2</td>
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<td>Leukemia extreme</td>
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<tr>
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<td>5</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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<td>5</td>
<td>2</td>
<td>—</td>
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<td>6</td>
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<td>—</td>
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<tr>
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<td>2</td>
<td>—</td>
<td>—</td>
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<td>7</td>
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<tr>
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<td>2</td>
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<td>Tumour</td>
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</table>

* When the number of injected blastocysts transferred into the uterus of pseudopregnant foster mothers was less than 6 some albino blastocysts were also transferred.
† The two positive cultures resemble in all aspects the original 5-86 FLC clone. The GPI mobility tested after a few passages gives a single GPI-1a band.
‡ Data referring to mouse 37.2.1. were already published (Calef, Jucker, Fruscalzo & Ragaini, 1980).

the C57B1/6 embryos. The remaining animals had only the fast migrating GPI-1b band of the C57B1/6 strain.

Histology

Three of the five animals with enzymatic chimerism (animals no. 70.2.1, 73.1.1 and 86.1.1) had a visible solid hypogastric tumour which weighed approximately 200 mg (1/3 of the whole animal). Histology showed a highly undifferentiated reticulum sarcoma which appears indistinguishable from the one obtained by grafting FLC into an adult DBA/2 mouse (Fig. 3). No solid tumour was found in the other animals (37.2.1 and 70.2.2); both animals show infiltration of spleen and liver with cells which are erythroid in appearance; the blood vessels were also filled with the same type of cells. Animal 37.2.1 is an extreme case of leukemia, the blood vessels of all organs examined contain almost nothing else but a type of immature cells diagnosable as proerythroblasts (Fig. 4). The other animal (70.2.2) appears to be a somewhat less extreme case of leukemia and its vessels contain normal amounts of mature erythrocytes.
DISCUSSION

The introduction of committed neoplastic cells into an embryo results in the birth of a relatively small proportion of animals which bear descendants of the injected cell under the form of a tumour. If one accepts this low 'participation' as due to some significant mechanism occurring during development, then, among different explanations, we envisage one which allows a rapid progress of the committed cell towards a differentiated stage with loss of its proliferative capacity.

In our unpublished experiment mentioned above we have obtained birth of 142 animals by transferring 243 FLC-injected C57B1/6 blastocysts into the uterus of albino foster mothers. Among these 142 animals only 2 had FLC-derived tumours. This very low incidence of tumours or any other sign of participation of the injected cells might be due to failure of the injected cells to survive or to their disappearance, soon after implantation into the uterus. Here we have shown by incubating in vitro the injected blastocysts that most of the injected cells survive and eventually kill the host embryo. This observation allows us to dismiss the hypothesis of early death of the FLC by some trivial accident during the injection procedure.

In this experiment we have also tested the possibility that one might increase the signs of participation of the injected FLC by injecting them together with an aliquot of FLV.

The sample of 32 animals born from embryos injected with both FLC and FLV contains five individuals with FLC-derived neoplasia while others without enzymatic chimaerism present no signs of either tumour or leukemia. These results show that the virus suspension yields a small but significant increase of the presence of the descendants of the injected FLC. As a matter of fact, one important corollary of our present experiment relies on the assumption that embryonic cells of the developing C57B1/6 strain are just as resistant to the action of the virus as the fully developed animal. This assumption is met by our control in which we obtained eight normal animals born from FLV alone injected C56B1/6 blastocysts.

The question whether FLC resume normal duplication as teratocarcinoma cells do receives a positive, albeit indirect, answer. Indeed, we can state that our results are in agreement with our working hypothesis; however, they do not prove duplicative normalization of the neoplastic cell. Due to the long period of development, where we do not monitor the status of the injected FLC there might be other events to account for the increased number of tumours.

One can think, for instance, that the virus suspension increases the aggressiveness of the injected cells by mimicking the situation found in the in vitro cultivation of injected blastocysts. However, the interpretation that in early embryos FLC resume normal duplicative capacity is sustained by the finding among our five animals with FLC-derived neoplasia of two animals with circulating
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leukemic cells and absence of solid tumours (animals no. 37.2.1. and 70.2.2). These are typical manifestations of the primary disease induced by the virus, whereas the in vivo growth of cells from our 5-86 line always results in a solid tumour.

The authors wish to thank Dr V. Monaco (C.N.E.N. Casaccia-Rome) for generously providing animals used in this work, Dr C. Marchelli for counting the FLV preparation at the electron microscope and Mr N. Rizzo for skillful technical assistance.

This work was partially supported by Progetto Finalizzato Virus del C.N.R. (grant no. 96/81136) and by Progetto Finalizzato Crescita Neoplastica del C.N.R. (grant no. 96/91701).

REFERENCES


(Received 22 May 1981, revised 8 November 1981)