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Transplantation Studies of a Putative Lymphosarcoma of *Xenopus*[1]

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ABSTRACT

The fate of the putative transplantable *Xenopus* lymphosarcoma (M. Balls, Cancer Res., *22:* 1142–1154, 1962) was studied under three experimental conditions: (*a*) xenotransplantation, *i.e.*, transplantation of live "tumor" tissue between adults of *Xenopus borealis* and *X. laevis*; (*b*) inoculation of live "tumor" cells from *X. borealis* into the blastocoele of *X. laevis* embryos; and (*c*) transplantation of "tumor" tissue into recipient adults immunologically unresponsive to the donor tissue antigens. This condition is fulfilled by using *X. laevis-X. gilli* (LG) hybrids [H. R. Kobel and L. Du Pasquier. *In:* J. B. Solomon and J. D. Horton (eds.), Developmental Immunology, pp. 229–306. The Netherlands: Elsevier/North-Holland Biomedical Press, 1977] as donors, and triploid *X. laevis-X. gilli* and *X. borealis* (LGB) hybrids [C. H. Thiébaud, Dev. Biol., *98:* 245–249, 1983] as recipients. In all transplantation experiments, donor and recipient cells could be unambiguously distinguished upon quinacrine staining that yields typical nuclear patterns, for instance bright patchiness in *X. borealis*, visible also in LGB cells. The results of xenotransplantation between *X. borealis* and *X. laevis* indicated that all developing "tumors" were composed of the recipient cell phenotype. The inoculation of live "tumor" cells from *X. borealis* "tumor" into the blastocoele of *X. laevis* embryos resulted in "tumor" formation in the recipient tadpoles and in metamorphosed animals. The cell constituting these "tumors" all were of recipient, *X. laevis* cell phenotype. Finally, "tumor" tissues from LG clones transplanted into LGB hosts were replaced by "tumors" formed of cells with recipient, LGB phenotype. These experiments indicate that this *Xenopus* tumor-like growth is a transmissible and not a transplantable disorder.

INTRODUCTION

A spontaneous growth in the anuran amphibian *Xenopus* was first described by Balls (1) as a malignant lymphosarcoma ("tumor"). Later work on this disease revealed that the "tumor" could be successfully transmitted by the "tumor" tissue and cell free extracts into allogeneic and even xenogeneic recipients including animals belonging to different orders. The "tumor" foci were found primarily in the liver, spleen, and kidney and later metastasized to almost all other organs (2–4). A "tumor" inducing agent was later described to be filtrable through a 0.22-μm pore size Millipore filter, and that could not be eliminated from the "tumor"-bearing hepatic tissue homogenate (20–30% W/V) by centrifugation at 150,000 × *g* for 2 h (5, 6). A viral etiology for this "tumor" was proposed (7, 8), however no virus particle could be detected by electron microscopy studies (9). Based on its transmission to normal animals, its morphology, and the presence of acid-fast bacilli, C. J. Dawe expressed some doubts on the neoplastic nature of this *Xenopus* "tumor"-like growth (10–12). However since no cell marker for *Xenopus* species was known at that time, the issue of transplantability, (growth of transplanted tissue, *i.e.*, in tumor) *versus* transmissibility (induction of growth in the recipient, *i.e.*, infection) of this *Xenopus* "tumor", remained unresolved. In 1983 the presence of a stable nuclear marker in certain species of *Xenopus* was visualized by quinacrine staining (15). In the present study this marker was used to study the transplantation kinetics of this putative *Xenopus* lymphosarcoma.

MATERIALS AND METHODS

Animals. Embryos, tadpoles, postmetamorphic toadlets, and adults of outbred *X. laevis* and *X. borealis* of both sexes reared in our laboratory, were used. Tadpoles were fed on powdered stinging nettle, young toadlets on tubifex worms, and adult animals with chopped beef liver.

Isogeneic diploid clones of *Xenopus* hybrids of the two species of *X. laevis* and *X. gilli* (LG), as well as triploid hybrids of three species of *X. laevis*, *X. gilli*, and *X. borealis* (LGB), were prepared according to methods described before (13–15). Briefly, hybrid females of *X. laevis* and *X. gilli* (LG) produce diploid eggs that are genetically identical. When such eggs are inseminated by UV-irradiated sperm they develop into a genetically identical clone. If the eggs of the same female are fertilized by normal *X. borealis* sperm, vital triploid hybrid individuals (LGB) are formed that are immunologically tolerant to the tissue antigens of their LG sisters. At the same time LGB cells carry the *X. borealis* nuclear marker, and thus can be easily distinguished from LG cells.

Tissue Transplantation. Initial "tumor" material (provided by Dr. I. Hadji-Azimi) was a frozen "tumor"-bearing liver tissue that was originally derived from a spontaneous "tumor" of *X. laevis*. The "tumor" tissue had been maintained through serial *in vivo* passages and was stored in preservative (50% glycerol in APBS[3] (16), at −15° to −20°C. Depending on the experiment live (fresh) or frozen (dead) tissue of "tumor" or "tumor"-bearing liver or spleen were used for "tumor" induction as described before (17).

Implantation at Blastula Stage. *X. laevis* embryos were prepared and healthy embryos of stages 7 and 8 (18) were chosen and kept in Niu-Twitty complete buffered saline of pH 7.6 at 17°C. At this temperature the embryonic development was delayed and more embryos of the required stage could be accumulated. For each transplantation series, 20 embryos of stages 8 and 9 were placed in a 0.01 M solution of 2,4-dithiothreitol (Merck) for 4 min to remove the jelly layers. The embryos were then washed 4× in Niu-Twitty complete buffered saline of pH 7.6 and placed into small holes in hard agar for inoculation. The inoculation with "tumor" cell suspension (see below) was performed through the animal pole of the embryo with the aid of a micropipette (30–40 μm tip-size) attached to a micromanipulator (Singer Instruments Co. Treborough Lodge, Roadwater, Somerset, England). The inoculated blastulae were maintained at room temperature for 30 min and then transferred into the 10× diluted Niu-Twitty buffer and maintained at 26°C to develop.

Histological Techniques. Tissue fragments or whole embryos were fixed in Bouin's fixative for H&E staining or in Carnoy's fixative for differential staining with quinacrine to visualize the nuclear marker associated with *X. borealis* cells. These techniques are described elsewhere (15).

Cell Suspension. "Tumor"-bearing tissues were cut into small pieces and homogenized with a loose-fitting glass homogenizer in 0.1% EDTA containing APBS, and washed in Hanks' balanced salt solution of amphibian osmolarity. Cell viability was determined by trypan blue dye exclusion test.

Blood Collection. From post metamorphic and adult animals, blood was collected in a solution of APBS containing 0.1% EDTA by cardiac puncture. From tadpoles, the blood was collected through a cut at the very end of the tail in a Ringer solution containing 100 IU/ml heparin (Roche).

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[3] The abbreviation used is: APBS, amphibian phosphate-buffered saline.

RESULTS

Xenotransplantation. First, a "tumor" was induced in normal, adult *X. borealis* by placing a piece (about 2 mm^3) of a dead frozen tissue of "tumor"-bearing liver into the dorsal lymph sac through a small cut in the dorsal skin. After the development of "tumor," fresh "tumor"-bearing hepatic tissue of this animal was used for a dose-response study. The liver tissue was cut into pieces of different size, approximately 3 mm^3, 1 mm^3, or less than 1 mm^3. Three adult *X. laevis* each received a transplant of such defined size into the dorsal lymph sac. One of the animals which received the 3-mm^3 graft died within 16 days after transplantation. The animals that carried smaller grafts (1 mm^3 and less), survived longer and were examined at different periods after transplantation. The reciprocal "tumor" graft from *X. laevis* to *X. borealis* was also performed. The results of these experiments (Table 1) indicate that when the "tumor" or "tumor" foci were present in the implanted animals they were composed of the recipient cell phenotype as early as 16 days after transplantation. This was the first evidence that formation of "tumors" upon cross-species implantation is due to the effect of a "tumor" agent and not to the propagation of the transferred tissue itself.

Inoculation of "Tumor" Cells into the Blastocoele. In order to increase the chance of acceptance of the "tumor" graft, we performed cross-species implantation at early stages of development, when the recipient is immunologically incompetent. Each of the 240 blastulae received 0.2 μl of a suspension containing 5×10^5 *X. borealis* live cells/ml in Hanks' balanced salt solution (about 100 cells per blastula). Shortly after inoculation (150 min), 20 embryos were examined for the presence of *X. borealis* type cells. The inoculated *X. borealis* "tumor" cells were detected in 19 out of the 20 examined embryos. Out of the remaining 220 inoculated embryos, 90 survived up to stage 25. During further development, individuals with a sickish appearance and some apparently healthy ones were periodically fixed for histological examinations of "tumor" formation and determination of the phenotype of its constituent cells.

No "tumor" or cells of donor phenotype could be detected in 30 examined embryos of stages 30 through 49 (about 12 days after transplantation). No tadpole was examined between stages 49 and 52. The "tumor," however, could be readily detected in the viscera of tadpoles from stage 52 onward (Fig. 1). The histological examination of eight tadpoles of stage 52, four of stage 56, and five of stages 57–60, revealed the presence of "tumor" or "tumor" foci in almost all of the visceral organs and were composed only of the recipient, *X. laevis* cell phenotype. This was also the case in the 13 inoculated embryos who completed metamorphosis, of which six individuals survived even up to 2-month postmetamorphosis. Thymus and blood of these animals were also examined and turned out to be of pure recipient origin. No "tumor" was detected in the thymus.

Table 1 *Histological studies of "tumor" transplants between X. laevis and X. borealis*

One graft of 1–3 mm^3 was given to each of three animals per graft size. Histological examination was performed on tissue sections at various times after transplantation. L, *X. laevis*; B, *X. borealis*.

Donor to recipient	Days after transplant	Site of examination: Dorsal lymph sac "T"[a]	Dorsal lymph sac Phenotype[b]	Liver and spleen "T"	Liver and spleen Phenotype	Kidney "T"	Kidney Phenotype
B to L	16	+	L	−	L	−	L
B to L	16	+	L	−	L	−	L
B to L	30–40	+	L	+	L	−	L
B to L	60	+	L	+	L	+	L
L to B	16	+	B	−	B	−	B
L to B	80	+	B	+	B	−	B

[a] "Tumor" or "tumor" foci ("T"), determined by hematoxylin and eosin staining.
[b] Origin of cells determined by quinacrine staining.

Transplantation of the "Tumor" into Histocompatible Adult Host. In this experimental series, we tested the growth of the transplanted "tumor" in immunologically tolerant adult hosts. For this purpose "tumors" of LG hybrids were transplanted into the LGB individuals. The characteristics of these hybrids are described before (see "Materials and Methods").

First a group of LGB individuals that carry the *X. borealis* nuclear marker received a graft of normal skin from their LG siblings. Only those LGB individuals in which no sign of rejection of grafted tissue could be detected were used for the transplantation experiment. Then the "tumor" was induced in the dorsal lymph sac of LG hybrids by transfer of a dead-frozen "tumor"-bearing hepatic tissue of a *X. laevis*. After the development of the "tumor" in the LG individuals, a piece of their fresh (live) "tumor" tissue or "tumor"-bearing spleen (about 2 mm^3) was transplanted into the dorsal lymph sac of each LGB hosts (four animals for each tissue). After about a month the "tumors" formed in the dorsal lymph sac as well as in the liver and spleen of these LGB recipients, were subjected to histological examination. It was found that 0.41% of the cells in the induced "tumor" contained cells of LG donor phenotype (Table 2). Similarly, in liver and spleen of LGB hosts a very low proportion of donor cells were present in the "tumor" foci, (between 0.0 to a maximum of 4.72%, Table 2). The few LG cells were very dispersed and found mostly on the margin of "tumor" foci. A few dispersed LG cells could also be found in "normal" regions (no "tumor" foci present) of liver and spleen of these LGB hosts. The proportion of LG cells in "normal" region were found to be 0.75% in liver (out of 398 counted), and 0.77% in spleen (out of 385 counted). This experiment clearly shows that although the implanted donor (LG) cells could survive in LGB hosts long after transplantation, the "tumor" and "tumor foci" developed were mainly composed of the recipient cells.

DISCUSSION

The most prominent feature of cancer (malignant tumor) is a stable and heritable loss of controlled cell proliferation. This is the quality which leads to the growth and propagation of a cancer tissue upon implantation into a recipient animal. The results of the xenograft experiments in which we transplanted "tumor" tissue from *X. borealis* to *X. laevis* and *vice versa* showed that the "tumor" developed in transplanted animals was of recipient origin. This was expected since transplantation immunity has been demonstrated in these animals (19, 20). This experiment further indicated that a "tumor"-inducing agent is transmissible to other species.

The inoculation of "tumor" cells into the blastula was performed in order to overcome the problem of immunological reaction due to the histocompatibility antigens. The embryo is immunologically incompetent at this stage of development and grafts between different species of animals at the early embryonic stages are permanently accepted (21). The inoculation of the "tumor" cells into the blastocoele resulted in "tumor" formation in developing tadpoles from stage 52 and in metamorphosed animals. However, all the cells in "tumors" had the nuclear marker typical for the host species. This result demonstrated that the inoculated "tumor" cells did not proliferate

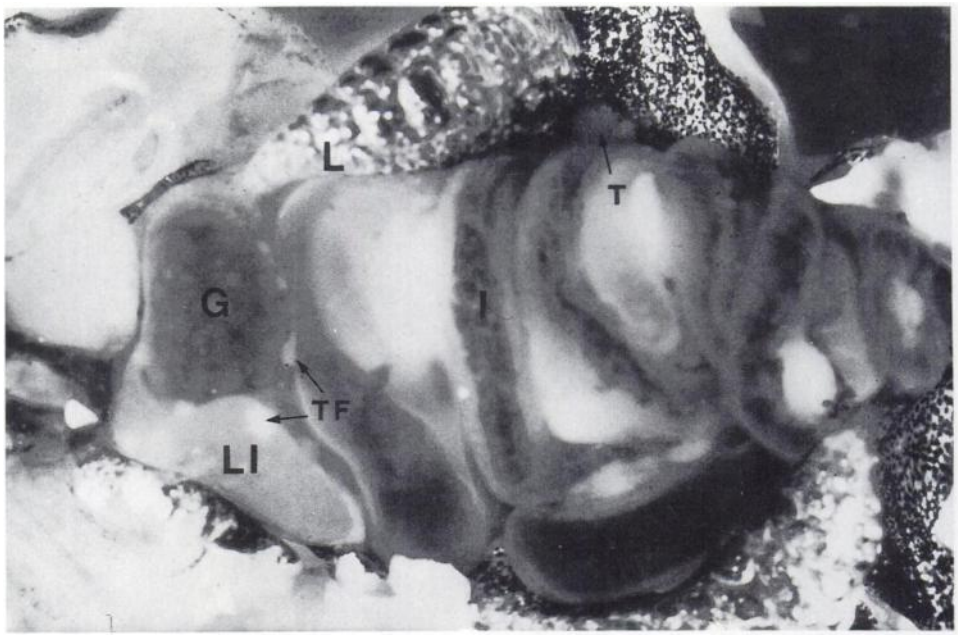

Fig. 1. A tadpole of stage 60 with "tumor" developed in the internal organs. The white patches or lumps are "tumor" foci or "tumor", respectively, × 19. *Li*, liver; *G*, gall bladder; *I*, intestine; *L*, lung; *TF*, "tumor" foci; *T*, "tumor."

Table 2 *Histological examination of LGB transplants into LGB hosts*

Fresh "tumor" tumor of LG was transferred into the dorsal lymph sac of LGB. Tissue sections of LGB were studied 30 days posttransplantation for the assessment of the "tumor" (or "tumor" foci) and of the phenotype of the cells as are described in Table 1.

LGB no.	Transplant	Tissue examined: "Tumor" B	"Tumor" C	Liver A	Liver B	Liver C	Spleen A	Spleen B	Spleen C[a]
1	"T"[b]	5038	0.41	2	423	0	2	476	0
2	"T"	4750	0.12	6	2081	0.09	4	2190	0.13
3	"T"-bearing spleen	2279	0.39	10	339	2.3	4	1202	0.66
4	"T"-bearing spleen			6	508	4.72			

[a] A, number of counted "tumor" foci; B, number of cells counted; C, percentage of donor cells.
[b] "T", "tumor."

in the immunologically unresponsive blastula. It could be argued that the elimination of the "tumor" cells was due to the microenvironment of the blastula which could not support the growth of the transplanted "tumor" cells. Nonetheless it was quite evident that the "tumor" agent could remain alive and active also in the embryo and could induce "tumor" formation. This experiment, however, did not provide new information on the kinetics of "tumor" development and the question of transmissibility and transplantability remained unresolved.

The most conclusive *in vivo* experiment for answering the question of transplantability of this "tumor" was the transplantation of the "tumor" tissue into the immunologically compatible adult host. As explained in detail, this approach involves transplantation from hybrid LG tissues to triploid LGB hosts, whereby donor and recipient cells can be clearly distinguished by the absence (LG) or presence (LGB) of the *X. borealis* nuclear marker upon staining with quinacrine. In this situation, a true tumor with the ability to divide indefinitely should have grown, as is the case for neoplasms of higher vertebrates. Yet, although implantation of the "tumor" tissue resulted in the formation of a cell mass at the site of transplanted tissue and in the development of "tumor" foci in internal organs (reminiscent of malignant metastasis), the participation of the cells from the recipient animal in the developed "tumor" and "tumor foci" by far exceeded those of the donor animal. The developed "tumor" masses and foci were thus not a result of uncontrolled cell proliferation of the "tumor" cells, but more likely were due to the accumulation of cells from the host at the site, where the "tumor" tissue or agent were placed.

The results of the transplantation experiments presented here, as well as intensive but vain attempts to establish an *in vitro* transformed cell line from this "tumor" (data not presented), imply that the cell constituting this "tumor" have an intrinsic short life-span. Moreover characterization of "tumor" cells by enzymatic (myeloperoxidase and α-naphthyl acetate esterase), phagocytic, and morphological criteria (data to be published elsewhere) indicate that the great majority of "tumor" cells are monocyte-macrophages at different stages of differentiation. These findings are incompatible with the concept of cancer but are suggestive of an intense inflammatory reaction resulting in proliferation and accumulation of cells of monocyte-macrophage lineage, and thus strongly support Dawe's remarks (10–12), on the granulomatous nature of this abnormal growth in *Xenopus*. Experiments which further investigate this possibility are presented in the accompanying paper (22).

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