[CANCER RESEARCH 27 Part 1, 293-296, February 1967]

Further Studies of a Transmissible Amphibian Lymphosarcoma[1]

LAURENS N. RUBEN AND MICHAEL BALLS[2]

The Reed Institute, Portland, Oregon

Summary

A variety of aspects of lymphosarcoma transmission were investigated in adult *Triturus viridescens* and *Rana pipiens*. Freshly biopsied and frozen cellular implants of lymphosarcoma from *Xenopus laevis* were used to induce the cancer in both the newts and the frogs. Cellular implants of these induced tumors were then used allogenically to transmit the cancer. Homogenates of Xenopus tumors and of tumors formed in the newts and frogs were successfully used to pass on the cancer. Filtered homogenate (filter size, 450 mμ) and supernatant fluids of centrifuged homogenate (20,000 × *g* for 30 min) were also infectious. Both frog and newt tumors could be back-transferred to Xenopus.

Horizontal transmission was demonstrated in newts, and urine collected from lymphosarcoma-bearing frogs was used to transmit the tumor to young adult Xenopus. Newts thymectomized prior to injection with tumor homogenate developed lymphosarcomas, which demonstrates that this lymphoid disease is not thymic dependent.

The implantation of methylcholanthrene crystals with and without the simultaneous injection of tumor homogenate indicated that the carcinogen will not induce the lymphosarcoma in either species tested in the absence of the lymphosarcoma agent. Previous reports of the induction of this tumor should be interpreted in terms of augmentation, rather than induction.

Introduction

Spontaneous lymphosarcomas in *Xenopus laevis laevis* (the South African clawed toad) were first described in 1962 (1). These cancers usually appear primarily in the liver, spleen, and kidneys, but true metastasis occurs to other visceral organs and, less frequently, to the head, trunk, and limb musculature. When cellular implants of the Xenopus lymphosarcoma were made into the abdominal cavity of *Triturus cristatus* (the European crested newt), lymphoid cancers developed in the visceral organs, particularly the liver, spleen, and kidneys. Transfer of cellular fragments of these newt tumors back to Xenopus led to the development of lymphosarcomas. Since newt lymphoid cells are morphologically distinguishable and considerably larger than Xenopus cells, it could be shown that the tumors were always composed of host and not of donor cells (3, 6). Further experiments demonstrated that lymphosarcoma from *X. laevis laevis* would induce the formation of similar tumors in other Xenopus species and subspecies and in other anuran species, e.g., *Bufo bufo bufo*, *Rana esculenta*, and *Rana pipiens* (5). These experiments, along with allogenic transfers into Xenopus forelimbs (12), suggested that the donor cancer tissue was destroyed by the host foreign tissue rejection mechanisms and that subcellular agent was thereby released to infect the host. The transmissible character of the lymphosarcoma in Xenopus has been dealt with elsewhere (7, 10), and a report characterizing the agent is being prepared. This present report will deal with aspects of the transmission of the lymphosarcoma in adult *Triturus viridescens* (the common American newt) and *R. pipiens* (the American leopard frog).

Materials and Methods

GENERAL. The adult *T. viridescens* used in these experiments were obtained from dealers in Massachusetts and Tennessee, while the *R. pipiens* had been purchased from a dealer in Vermont. The *X. laevis laevis* used were postmetamorphic populations bred in this laboratory from parents obtained from a California dealer. The newts and Xenopus were fed liver twice a week, while the frogs were fed meal worms. The general anesthetic used for all experiments was M.S. 222 (Sandoz.)

CELLULAR IMPLANT TRANSMISSIONS. Cellular implants made into *T. viridescens* were about 1 cu mm and were inserted intraabdominally through an incision made in the body wall near the spleen. In series where frozen implants were used, the fresh tissues had previously been removed, placed in sealed dishes, and stored at −22°C. Freshly biopsied tissues were used in all other series. Implants (3 cu mm) made to adult *R. pipiens* were generally inserted into the dorsal lymph sac through an incision made anterodorsal to the cloacal aperture and were moved with a glass probe to a position just anterodorsal to the pectoral girdle.

TRANSMISSION BY HOMOGENATES. Homogenates, whether prepared from normal or cancerous tissue, were all made in the same manner. The tissues were weighed in the frozen state and were then ground with sterile sand in a pestle and mortar which had been precooled with Dry Ice. The tissues most frequently used were liver, spleen, and kidney, and the calcium and magnesium-free amphibian Ringer's solution was added to make a 15% homogenate (w/v). After grinding, the crude homogenate was centrifuged at 8000 × *g* for 20 min at 4°C (Sorval RC2) to remove the sand and tissue debris. The pellet was discarded, and 1.5-ml aliquots of the homogenates were stored at −22°C in capped vials until thawed before use. Then, depending on the design of the experiment, the homogenate was injected directly (0.1 ml/

[1] Supported by USPHS Grant CA-08268 from the National Cancer Institute of the NIH and by the Jane Coffin Childs Memorial Fund for Medical Research.

[2] Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. Present address: School of Biological Sciences, University of East Anglia, Norwich, England.

Received June 30, 1966; accepted September 1, 1966.

newt or Xenopus or 1.0 ml/frog intraabdominally) or centrifuged at 20,000 × *g* for 30 min at 4°C (Sorval RC2) prior to injection. In 1 frog series the homogenate was filtered before injection through a 450-mμ ± 20-mμ filter (Size HA, Millipore Corp.) under negative pressure. In series where filtration was used, the integrity of the filter was checked following the procedure.

HORIZONTAL TRANSMISSION. In a test for contagion using *T. viridescens*, 10 males were injected with homogenate made from cancerous Xenopus tissues, then placed in the same 10-inch finger bowl with 10 females which were not injected. Twenty uninjected newts were kept in another bowl as controls. The different sexes were used merely for easy recognition of injected or uninjected test animals.

TRANSMISSION BY URINE. Urine was collected from *R. pipiens* which had earlier been given cellular cancer implants in the dorsal lymph sac. The urine, whether contaminated by fecal matter or not, was passed through a coarse filter (Whatman No. 1) and stored at −22°C. Urine from untreated frogs was collected and filtered in the same manner for use in control series. Urine was collected at five 2-week intervals, beginning 5 weeks postimplantation, until 2 of the implanted frogs died of the cancer. After that date urine was collected at essentially daily intervals, and this material was pooled. The frozen-stored urine was melted and injected into postmetamorphic Xenopus (0.1 ml/toad) as a test of infectivity. In 1 series the urine was passed through a 450-mμ filter under negative pressure prior to injection, while for a further series 80 ml of urine from cancerous frogs were concentrated to 10 ml in dialysis tubing using Aquacide No. 1 and then injected into toads.

THYMECTOMY. In order to test the effect of thymectomy on the initiation and development of this tumor, 14 newts were thymectomized. Ten of these were injected 1 week later with a homogenate made from Xenopus cancerous tissues, while the remaining 4 were kept as uninjected controls. Surgical thymectomy of newts is easily effected, since the 2 multilobed thymus glands lie subcutaneously immediately posterior to the articulation point of the quadrate and articular bones. If one follows the lower jaw to its posterior limit, pinches this region with watchmaker's forceps, and cuts deeply around the forceps points with iridectomy scissors, the thymus will usually be removed with the pinched skin. If the thymus is not removed by this procedure, it will nonetheless be exposed to view and can then be cut out. The newt heals well, even if the operation is a crude one. Inspection of the heads at the end of the experiment suggested that the thymus had not been regenerated.

CHEMICAL CARCINOGENESIS. Because it had been found in earlier work that 3-methylcholanthrene would initiate the appearance of lymphosarcoma in Xenopus stocks with a high incidence of "spontaneous" lymphosarcoma (2, 4), it was of some importance to know whether this carcinogen would initiate the development of lymphoid tumors in the absence of the lymphosarcoma agent and whether it could enhance the effect of an exogenously supplied agent in an injected homogenate. Eighty *T. viridescens* were divided into 4 groups of 20 animals. One group was used for untreated controls; another received an implant of packed methylcholanthrene crystals intraabdominally in the region of the spleen; a 3rd group received an injection of Xenopus tumor homogenate, while the 4th group received both an injection of the same homogenate and an implant of methylcholanthrene crystals near the spleen. The methylcholanthrene was packed into a 1-mm glass tube which was marked so that a similar amount (about 1 cu mm) of crystals would be given to each animal. The tube was inserted into the body cavity through an incision near the spleen, and a glass rod was used to push the crystals from the tube.

A similar experiment was established using *R. pipiens*, but the carcinogen volume was increased to 3 cu mm and the amount of homogenate to 1.0 ml/frog. Each of the 4 groups was originally composed of 15 frogs, but deaths unrelated to the treatment reduced the numbers to 13 in the untreated control group, 6 in the carcinogen group, 10 in the homogenate group, and 9 with both carcinogen and homogenate. The experiment was terminated after 188 days.

Results

CELLULAR IMPLANT TRANSMISSION. Several attempts to transfer the lymphosarcoma to *T. viridescens* using freshly biopsied Xenopus tumor as donor material led only to early death of the hosts (26 days on the average) with no indication of cancer development. It appeared that this early lethality was due primarily to immunologic considerations. We have previously reported (12) that fresh Xenopus lymphoid tumor implants initiate extreme inflammatory responses when allogenically grafted. This could be either because the lymphoid cancer provides an exceptional graft against host response or because it is much more antigenic than comparable implants of normal lymphoid and nonlymphoid tissues. At this time we cannot discern which of these alternatives apply.

In an effort to reduce the host immunologic response, the donor Xenopus cancer material was frozen prior to implantation. Of the 6 hosts receiving these cellular implants, 1 died at 24 days and the remainder were sacrificed at 25 days postimplantation. All 6 bore lymphosarcomas in the liver, spleen, kidney, mesenteries, and body wall. Cytologic examination indicated that the cancers were of host origin.

Fresh allogenic cellular implants made from tumor-bearing newts were inserted into 10 *T. viridescens* hosts, and while 1 died soon after the operation, the remaining 9 all developed lymphosarcomas.

All the adult *R. pipiens* in 2 groups (55 hosts) receiving fresh Xenopus tumor implants and another 3 groups (35 hosts) given frozen Xenopus tumor implants developed the lymphoid tumor if they survived the immunologic response period during the 1st weeks after implantation. Those receiving the frozen cancer material generally survived the initial responses. Tumors developed in the liver, spleen, kidneys, muscle, skin, stomach, intestine, and rectum. Ten weeks proved to be an adequate period for development of the tumor in the adult frogs.

Portions of these cancers of *R. pipiens* were frozen and subsequently implanted into 2 further groups of adult frogs (20 hosts). Five hosts died within a few days of the operation, but the remaining 15 frogs all developed the lymphosarcoma between 67 and 98 days after implantation.

TRANSMISSION BY HOMOGENATES. Injection of a homogenate of Xenopus cancer into 20 *T. viridescens* led to the development of clearly diagnosable lymphosarcomas in 16 of the hosts. All of the hosts died or were sacrificed between 30 and 60 days after injec-

TABLE 1

HOMOGENATE TRANSMISSION

Experimental treatment	Mean No. of days after injection when died or killed	Total No. of hosts	No. of hosts with cancer
Xenopus cancer homogenate	45	20 *Triturus viridescens*	16
Triturus cancer homogenate	32	10 *T. viridescens*	10
Supernatant fluid of 20,000 × *g* Triturus cancer homogenate	48	10 *T. viridescens*	10
Triturus cancer homogenate	51	20 *Xenopus laevis*	20
Xenopus cancer homogenate	188	10 *Rana pipiens*	6
Xenopus cancer filtrate, 450 mμ	102	9 *R. pipiens*	5
Rana cancer homogenate	95	10 *X. laevis*	10

TABLE 2

URINE TRANSMISSION

Collection No.	No. of Xenopus hosts	Days (mean) after injection when died or killed	No. of hosts with cancer
1	9	66	8
2	7	98	5
3	10	100	2
4	10	100	0
5	10	58	10
Pooled and concentrated	12	51	10
Diluted and filtered, 450 mμ	9	44	5

TABLE 3

CHEMICAL CARCINOGENESIS IN THE PRESENCE OR ABSENCE OF THE LYMPHOSARCOMA AGENT

Treatment	Days (termination)	Total No. of hosts	No. of hosts with cancer
1. None	41	20 *Triturus viridescens*	0
2. MC[a]	41	20 *T. viridescens*	0
3. Xenopus hom[b]	40	20 *T. viridescens*	20
4. MC + hom	34	20 *T. viridescens*	20
1. None	188	13 *Rana pipiens*	0
2. MC	188	6 *R. pipiens*	0
3. Xenopus hom	188	10 *R. pipiens*	6
4. MC + hom	188	9 *R. pipiens*	7

[a] MC, methylcholanthrene.
[b] hom, tumor homogenate.

tion, and the cancerous viscera of 5 of them were used to prepare a homogenate of newt cancer which was subsequently injected into 10 other newts, all of which became cancerous in a mean period of 32 days. The lymphoid cancer involved the usual viscera and, in addition, the ovaries, fat bodies, testes, and pancreas in a few instances.

Some of the newt cancer tissue homogenate was centrifuged at 20,000 × *g* for 30 min at 4°C, and the supernatant fluid was injected into 10 *T. viridescens*. All 10 hosts developed the lymphosarcomas in a mean period of 48 days, during which time they died or were sacrificed. None of the untreated control newts from the same stock developed tumors during this period.

Ten adult *R. pipiens* were injected with a homogenate prepared from Xenopus lymphosarcoma, and by 188 days 6 of them had developed the cancer.

Another aliquot of this same homogenate was passed through a 450-mμ filter and injected into 9 adult frogs, 5 of which formed the cancer by 102 days after injection.

Homogenates of both newt and frog lymphosarcomas induced tumors to form in all the 30 young adult Xenopus injected. Table 1 summarizes the homogenate transmission experiments.

HORIZONTAL TRANSMISSION. One of the 10 male newts given a tumor homogenate injection died soon after injection, but 8 of the remaining 9 developed lymphosarcomas in a mean period of 78 days, and all of the untreated females kept in the same bowl developed lymphosarcomas within 115 days. The same number of untreated males and females kept in a control bowl failed to form any cancers.

TRANSMISSION BY URINE. As indicated in the Materials and Methods section of this report, a number of urine collections were made from tumor-bearing and normal adult *R. pipiens*. For each bioassay group of cancerous frog urine a comparable control group of toads was injected with urine from untreated frogs collected on the same day. In no case did cancer appear in a Xenopus receiving urine collected from untreated frogs (61 hosts tested). The incidence of cancer in Xenopus injected with urine collected from cancerous frogs varied considerably (from 100% to zero). Table 2 summarizes the urine transmission experiments. The only correlation which can be made at this time bears on whether the urine contained much fecal matter. Collections were made by squeezing the abdomens of frogs while holding them over a beaker. Since the alimentary canal empties into the cloaca, on occasion fecal matter was caused to be released along with the urine. The urine containing the fecal matter induced more tumors than the "clear" urine. Collections numbered 3 and 4 (Table 2) were "clear."

Urine collected daily over a subsequent 3-week period and pooled was injected after concentration by dialysis using Aquacide No. 1. This concentrate induced lymphosarcomas in 10 out of 12 injected young Xenopus.

In 1 series, urine from cancerous frogs was diluted 10 times with calcium and magnesium-free amphibian Ringer's solution and passed through a 450-mμ filter before injection. Five of 9 injected immature Xenopus developed lymphosarcomas.

THYMECTOMY. The 10 thymectomized *T. viridescens* adults which received injections of a homogenate made from Xenopus lymphosarcoma all developed lymphosarcoma by 29 days postinjection (36 days postthymectomy). The thymectomized uninjected controls did not form the cancer.

CHEMICAL CARCINOGENESIS. All 20 *T. viridescens* implanted with methylcholanthrene and injected with a homogenate of

Xenopus lymphosarcoma, as well as the 20 newts simply injected with homogenate but not carrying the carcinogen, developed lymphosarcomas. None of the untreated 20 newts or those 20 given the carcinogen without homogenate formed the cancer. The 2 groups in which cancer developed were ended when 50% of the hosts had died of the disease. The other 2 groups were ended and prepared for histologic examination 1 day later.

The comparable experiment performed with the *R. pipiens* adults provided similar results in that no frog carrying only the carcinogen or untreated developed the cancer, while those 2 groups injected with the cancer homogenate with and without the methylcholanthrene developed a high incidence of the lymphoid disease (13 of 19 injected). These experimental groups were ended 188 days after injection or implantation. Table 3 summarizes the chemical carcinogenesis data.

Discussion

Although no spontaneous lymphosarcomas have been reported for *T. viridescens*, neoplastic lymphoid diseases have been reported in a number of amphibian species (1). Lymphosarcomas were induced in this newt species using freshly biopsied or frozen cellular grafts of Xenopus tumor. The newt tumors could be transferred to other newts or back to Xenopus by means of cell-free supernatant fluids or cellular grafts. Similarly, the tumor could be induced in frogs by means of fresh or frozen cellular grafts or cell-free filtrates of Xenopus tumor. The frog tumors could be transferred to other frogs or back to Xenopus.

That contagion might be involved in transmission of this lymphosarcoma in newts was suggested by the unexpected appearance of 16 cases out of 23 untreated stock animals which were being cared for by personnel handling cancerous Xenopus groups. The experiment reported here, where injected newts were kept in the same bowl as untreated animals, confirmed our suspicions. Our laboratory procedures were revised, and great care was taken regarding the feeding and handling of treated and untreated groups. With these procedures it has been possible to keep the control groups free from accidental contamination.

While a comparable group of experiments was performed to test for contagion using the adult *R. pipiens*, no clear-cut results were obtained. Lymphocytic foci could be identified in the frog viscera, but no lymphoblastic nodules appeared. This is most likely due to the large quantity of agent required to initiate the cancer in the adult frogs and to the relatively long developmental period of the cancer once initiated. That contagion is a likely possibility among *R. pipiens*, however, is suggested by our results using urine collected from cancerous frogs as a source of the agent. Since the urine containing fecal matter was more infectious than "clear" urine, it seems possible that most of the agent enters the cloaca from the alimentary canal rather than via the kidneys.

Removal of the thymus did not affect either the latency of cancer development or its eventual appearance. This lymphoid cancer does not therefore appear to be thymic dependent.

The experiments using methylcholanthrene in conjunction with cancer homogenates clearly demonstrated that the carcinogen will not initiate this tumor in either species within the experimental periods in the absence of the cancer agent. Thus, the previous reports of the initiation of this lymphosarcoma in Xenopus by methylcholanthrene (2, 4), benzpyrene (8), and urethan (9) should be interpreted in terms of augmentation and *not* induction. It seems likely, then, that the entire stock of Xenopus in the colony at the University of Geneva, where much of the previous work with this tumor was carried out, was infected with the lymphosarcoma agent. This seems especially likely in view of our earlier results, which suggested that even histologically normal allogenic grafts would initiate the development of lymphosarcoma in the implant site (11). Other experiments using "normal" heterogenic and xenogenic implants further indicated that increasing immunologic stress would enhance host cancer development (13). We must make clear, however, that, because frozen "normal" donor material and homogenates made from "normal" donor material fail to initiate cancer development, we are not suggesting transfer of the lymphosarcoma agent by the donor material but rather the activation of agent already present in the hosts.

Acknowledgments

We are grateful to Jean M. Stevens and Carolyn J. Balls for their competent technical assistance.

References

1. Balls, M. Spontaneous Neoplasms in Amphibia: A Review and Descriptions of Six New Cases. Cancer Res., *22:* 1142–54, 1962.
2. ———. Methylcholanthrene-induced Lymphosarcomas in *Xenopus laevis*. Nature, *196:* 1327–28, 1962.
3. ———. Xenoplastic Implantation of Amphibian Lymphoid Tumours. Rev. Suisse Zool., *70:* 237–44, 1963.
4. ———. Methylcholanthrene-induced Lymphosarcomas in the Anuran Amphibian, *Xenopus laevis*. *Ibid.*, *70:* 595–609, 1963.
5. ——— Lymphoid Tumor Transfers from *Xenopus laevis laevis* to Alien Subspecies and Species, including *Rana pipiens*. Cancer Res., *24:* 1261–68, 1964.
6. ———. Cell Transformation in Xenoplastic Transplantation of an Amphibian Lymphoid Tumour. Rev. Suisse Zool., *71:* 805–13, 1964.
7. ———. Transplantation of Spontaneously Occurring and Chemically Induced Lymphoid Tumors in *Xenopus laevis*. Cancer Res., *24:* 44–51, 1964.
8. ———. Benzpyrene-induced Tumours in the Clawed Toad, *Xenopus laevis*. Experientia, *20:* 143–45, 1964.
9. ———. Some Further Aspects of Lymphosarcoma in *Xenopus* (the South African clawed toad). Cancer Res., *25:* 7–11, 1965.
10. ———. Lymphosarcoma in the South African Clawed Toad, *Xenopus laevis:* A Virus Tumor. Ann. N. Y. Acad. Sci., *126:* 256–73, 1965.
11. Balls, M., and Ruben, L. N. Variation in the Response of *Xenopus laevis* to Normal Tissue Homografts. Develop. Biol., *10:* 92–104, 1964.
12. Ruben, L. N., and Balls, M. The Implantation of Lymphosarcoma of *Xenopus laevis* into Regenerating and Non-regenerating Forelimbs of that Species. J. Morphol., *115:* 239–54, 1964.
13. ———. Genetic Disparity and Cancer Induction by Normal Tissue Implants in Amphibia. Science, *146:* 1321–22, 1964.