Further Aspects of Lymphosarcoma in Xenopus (The South African Clawed Toad)[1]

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SUMMARY

Evidence is presented which suggests that urethan (ethyl carbamate) increases the expected incidence of lymphosarcoma in *Xenopus laevis laevis* when this animal is repeatedly immersed in or given injections of a urethan solution. Similar results were observed when urethan was used as an anaesthetic in experiments involving forelimb autografts or homografts to the dorsal lymph sac. The implantation of hind-leg muscle without lymphosarcoma condensations led to the development of tumors in immature Xenopus as quickly as did the implantation of liver from the same animal bearing advanced tumors. Lymphosarcoma implants to adult Xenopus eyes did not grow well, but visceral lymphosarcomas resulted in four of five hosts. Eight young Xenopus, developed from tadpoles given lymphoid tumor grafts, all had lymphosarcomas when killed after metamorphosis. This indicates that metamorphosis does not prevent the growth and spread of the tumor.

The experiments of Leone (9), who induced lymphosarcomas in *Triturus cristatus*, and the experiments resulting in the induction of lymphosarcoma in *Xenopus laevis laevis* (2) have a number of similarities. Methylcholanthrene as carcinogen and urethan as anesthetic are common features of these experiments, but whereas spontaneous lymphoid tumors are known in Xenopus, none have yet been reported in Triturus (1). Thus, the role of urethan in Leone's experiments would seem to have been cocarcinogenic, whereas in the Xenopus experiments the tumor-augmenting property of urethan (14) may have played a part. The first part of this article is about two groups of experiments designed to test the role of urethan in carcinogenesis in Xenopus.

Both spontaneously- and chemically-induced lymphosarcomas have been found to be readily transplantable by homografting tumor fragments (3, 9); the implantation of histologically normal tissues has also led to lymphosarcoma formation in Xenopus (5). The second part of the article deals with experiments involving the transfer of histologically-normal tissues from tumor-bearing animals, and the transfer of tumors to Xenopus tadpoles or to the eyes of adult Xenopus.

MATERIALS AND METHODS

A. Treatment of Xenopus with Urethan

Ten immature *Xenopus laevis laevis* (mean nose-cloaca length 30 mm) were immersed and anesthetized daily in 5 per cent aqueous urethan for 40 days, and then kept under observation. One animal died 122 days after the first immersion, and another after 154 days. The remaining eight hosts and ten untreated hosts from the same mating were sacrificed after 165 days and studied histologically.

In a second experiment, two groups of 25 immature Xenopus (mean length 32 mm) from the same mating were treated thus:

Series 13A.—The hosts were given ten weekly injections of 0.1 ml of a 1% solution of urethan in distilled water.

Series 13B.—The hosts were given ten weekly injections of 0.1 ml of Niu and Twitty isotonic physiologic solution (7).

In each case the inoculi were injected into the dorsal lymph sac through the hind leg. Ten animals from each group were killed 160 days after the first injection, and the remaining fifteen after 170 days.

B. Tumor and Normal Tissue Transfers Following Urethan or MS222 Anesthetization

Group I.—Dorsal lymph sac lymphosarcoma was transferred to the dorsal lymph sac of two series of twelve immature Xenopus from the same mating. A 5% urethan solution was used to immobilize one series of hosts during the implantation procedure, and MS222 (Tricaine methanesulfonate) was used for the second series. Both series of hosts were killed between 27 and 30 days after implantation.

Group II.—Two series of twelve Xenopus, stage 66, derived from the same mating were anesthetized in urethan solution; the forearms were amputated and the right forelimbs dissected at elbow and wrist. In *Series*

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CS10 each forelimb was autografted to the dorsal lymph sac of the animal from which it had been removed, while in *Series CS11* the forearms were homografted to the dorsal lymph sac of other hosts within the series. Eight hosts died during the experiment; sixteen were killed 80 days after implantation.

Group III.—Two series of ten immature Xenopus from the same mating used in *Group B-I* were anesthetized in MS222 solution and the right forearms were autografted in one series (*Series CS27*) and homografted in the other (*Series CS28*). Four hosts died during the experimental period; sixteen were killed after 75 days.

Group IV.—Four series of twenty Xenopus, stage 66, from the same mating (*B-I*) were used in further forelimb grafting experiments. Two series were anesthetized in MS222 solution, the forelimbs amputated, and then autografted (*Series CS33*) or homografted (*Series CS34*). The other two series were anesthetized in urethan solution, the forelimbs amputated, and then autografted (*Series CS35*) or homografted (*Series CS36*) to the dorsal lymph sac. Thirty hosts died during the experimental period; the remaining 50 were killed between 80 and 100 days after implantation.

C. Transplantations

Group I.—Two animals bearing advanced lymphosarcomas were killed and lymphosarcoma of liver was transferred to five and five hosts respectively. Hind-leg muscle from the same donors was placed in five and eighteen hosts respectively. Hind-leg muscle was also removed and found to be histologically normal muscle tissue.

Group II.—Xenopus lymphosarcoma was implanted into tadpoles in two experiments. The first experiment was carried out by Dr. L. N. Ruben, who implanted lymphosarcomas into both hind limbs of two premetamorphic Xenopus (stage 58/9, Nieuwkoop and Faber [13]), which were later given to the author, who killed the resulting young toads 157 days after the operation. In a second series the author placed tumor fragments at the base of the tail of ten Xenopus tadpoles, stage 57–59. Four of the hosts did not recover from anesthetization (MS222), but the remaining six hosts began to metamorphose 6–10 days after the operation. The hosts were killed between 18 and 28 days after inoculation.

Group III.—Lymphosarcoma of liver was removed, placed in Niu and Twitty isotonic solution and cut into small pieces. Five adult Xenopus were then anesthetized in a 5% urethan solution and a small cut was made in the cornea with a fine blade, with care taken not to damage the iris. A single tumor fragment was then placed by inoculation in each eye with a micropipette and pushed between cornea and iris. The eyes were examined periodically but little change in the size of the implants was observed. The hosts were killed at 179 days and the visceral organs examined.

In each experiment hosts were kept in water at 20°–24°C. and fed with beef liver or *Tubifex*. When animals died or were killed at the end of the experiment, the visceral organs and any tissue at the implantation site were fixed in Zenker's fluid, and sections cut at 7–10 μ were stained with Mayer's acid hemalum and eosin.

RESULTS

A. Treatment of Xenopus with Urethan

The results of the frequent immersion of immature Xenopus in a 5% urethan solution are given in Table 1, which shows that three of ten developed lymphoid tumors as compared with none of ten Xenopus from the same mating who were not immersed in urethan. The lymphosarcoma of heart in one animal was unusual since, although Leone and Zavanella (10) report that such tumors often affect the heart in *Triturus cristatus*, very few such cases have been observed in Xenopus with spontaneous or transplant- or chemically-induced lymphoid tumors.

Histologic examination showed that 5 of 25 hosts given injections of physiologic solution bore tumors confined to the spleen, whereas 13 of 25 Xenopus from the same mating, but given urethan injections, developed lymphoid tumors which involved the liver, kidneys and/or lungs, as well as the spleen (Table 1).

B. Tumor and Normal Tissue Transfers Following Urethan or MS222 Anesthetization

The results of the histologic examination of the implantation sites and visceral organs are summarized in Table

TABLE 1

Incidence of Lymphosarcoma in Urethan-treated Xenopus

Group	Series	Treatment	Died or killed (Days after ttmt. began)		No. of hosts		No. of hosts with lymphosarcoma					
			Mean	Range	Total	With tumor	Liver	Spleen	Kidneys	Fat-bodies	Heart	Lungs
I	12A	40 immersions in urethan	160	122–165	10	3	3	2	1	1	1	—
	12B	Not immersed in urethan	165	—	10	0	—	—	—	—	—	—
II	13A	10 urethan injections	165	160–175	25	13	7	13	1	—	—	1
	13B	10 physiologic solution injections	165	160–175	25	5	—	5	—	—	—	—

TABLE 2

TISSUE TRANSPLANTATION FOLLOWING URETHAN AND MS222 ANESTHETIZATION OF XENOPUS

GROUP	SERIES	MATERIAL IMPLANTED	ANESTHETIC	MATING PROVIDING THE HOSTS	NO. OF HOSTS IMPLANTED	NO. OF HOSTS WITH LYMPHOID TUMORS	SITES AFFECTED BY LYMPHOSARCOMA (NO. OF ANIMALS)							
							Dorsal lymph sac	Skin	Muscle	Liver	Spleen	Kidneys	Lung	Pancreas
I	QQ	Tumor homograft	Urethan	A	12	12	12	12	12	12	12	12	—	1
	RR	Tumor homograft	MS222	A	12	12	12	12	12	12	12	12	1	—
II	CS10	Forelimb autograft	Urethan	B	12	7	6	6	4	6	3	2	—	—
	CS11	Forelimb homograft	Urethan	B	12	6	5	3	3	5	4	4	—	—
III	CS27	Forelimb autograft	MS222	A	10	0	—	—	—	—	—	—	—	—
	CS28	Forelimb homograft	MS222	A	10	0	—	—	—	—	—	—	—	—
IV	CS33	Forelimb autograft	MS222	C	20	0	—	—	—	—	—	—	—	—
	CS34	Forelimb homograft	MS222	C	20	0	—	—	—	—	—	—	—	—
	CS35	Forelimb autograft	Urethan	C	20	4	—	—	—	4	4	—	—	—
	CS36	Forelimb homograft	Urethan	C	20	5	—	—	—	3	4	—	—	—

TABLE 3

TRANSPLANTATION OF HISTOLOGICALLY NORMAL AND TUMOR-BEARING TISSUES FROM LYMPHOSARCOMA-BEARING XENOPUS

DONOR	DONOR TISSUE	SERIES	NUMBER OF HOSTS		DAYS AFTER IMPLANTATION WHEN DIED OR KILLED		SITES AFFECTED BY LYMPHOSARCOMA (NUMBER OF HOSTS)					
			Total	With tumor	Mean	Range	Dorsal lymph sac	Skin	Muscle	Liver	Spleen	Kidneys
A	Lymphosarcoma of liver	VI	5	5	25	—	5	2	5	5	5	5
	Hind-leg muscle[a]	V2	5	5	40	26–55	5	4	5	5	5	5
B	Lymphosarcoma of liver	ZZ1	5	5	32	30–34	5	5	5	5	5	5
	Hind-leg muscle[a]	ZZ2	18	18	33	30–34	17	13	11	16	18	9

[a] Histologically normal muscle taken from Xenopus bearing visceral-lymphoid tumors.

2. The anesthetic used clearly had no effect on the results of tumor transplantation, for all the hosts in *Series QQ* and *RR* bore tumors at the implantation site and in the viscera when killed only 30 days after the operation.

In *Group II* there was little difference between the results from forearm autografts or homografts following urethan anesthetization. About half the hosts in each series developed lymphosarcomas both in the dorsal lymph sac and in the viscera. Most of the hosts bore remnants of the implanted limbs in the dorsal lymph sac, but varied in that some contained limb tissue alone, whereas in others the limb tissue had been invaded by tumor. In one host, *Series CS11*, advanced tumors in the viscera had metastasized to the amputated, regenerating forelimb.

None of the hosts given forearm grafts following MS222 anesthetization bore tumors either in the dorsal lymph sac or viscera, though some contained remnants of the implanted forelimbs. Indeed, it was surprising to find that within *Groups II* and *III* there was little difference between the results of autografts and homografts, but that there was a difference between the total results of the two groups. However, since the hosts in *Group II* were not from the same mating as those in *Group III*, the results are not directly comparable.

The four series in *Group IV* involved hosts derived from the same mating and, although no tumors formed at the implantation site as in *Group II*, there is clearly a real difference between the results in the two series involving urethan anesthetization (9 of 40 positive for lymphosarcoma) and MS222 anesthetization (all 40 negative).

C. TRANSPLANTATION

I. *Transfer of histologically normal tissues from tumor-bearing animals.*—The results of the transplantation of histologically normal and tumorous tissues from lymphosarcoma-bearing Xenopus are summarized in Table 3, and show that the implantation of hind-leg muscle, not itself bearing lymphoid tumor condensations but taken from a Xenopus bearing visceral lymphosarcomas, led to the development of tumors in immature Xenopus as quickly as did the implantation of tumor-containing liver fragments.

II. *Tumor transfers to the anterior eye chamber.*—Al-

TABLE 4

TUMORS IN YOUNG TOADS DEVELOPED FROM TADPOLES GIVEN TUMOR IMPLANTS

SERIES	TRANSFER SITE	NO. OF HOST	SITES AFFECTED BY LYMPHOSARCOMA					
			Dorsal lymph sac	Dorsal muscle	Liver	Spleen	Kidneys	Fat-bodies
EA2	Hind limb	1	−	−	+	+	+	−
		2	−	−	+	+	+	+
XX	Base of tail	1	−	+	+	+	+	−
		2	−	+	+	+	+	−
		3	−	+	+	+	+	−
		4	+	+	+	+	+	−
		5	−	−	+	+	+	−
		6	−	−	+	+	+	−
Total		8	1	4	8	8	8	1

though the lymphosarcoma implants did not grow markedly in Xenopus eyes, four of five hosts which had been given inoculations developed lymphosarcomas of liver and spleen, whereas three of the four bore lymphosarcomas of kidney.

III. *Tumor transfers to premetamorphic Xenopus.*—All eight young toads, developed from tadpoles given lymphoid tumor grafts just before metamorphosis, contained visceral and/or muscular lymphosarcomas when they were killed after metamorphosis (Table 4).

DISCUSSION

Since the carcinogenic nature of urethan was established in 1943 (12), it has been shown that this chemical is a multipotential carcinogen (15), although the complexity of its role and the inadequacy of present knowledge is illustrated by reports that urethan induces thymic lymphoma in C57Bl mice (6), but inhibits virus induction of leukemia in C3H mice (8). Tannenbaum (14) suggested that urethan might not induce tumors *de novo*, but might augment the incidence of tumors normally occurring in a given population. The results obtained to date suggest that urethan immersion or injection, or the use of urethan as an anesthetic when histologically normal tissues are being transferred, does augment the incidence of lymphosarcoma in Xenopus from stocks liable to form spontaneous lymphoid tumors. However, the Xenopus lymphosarcoma transfers as readily after MS222 anesthetization as after urethan.

Urethan was also used as an anesthetic in experiments involving the implantation of methylcholanthrene or benzpyrene into Xenopus (2, 4) and of methylcholanthrene into Triturus (9). The successful induction of lymphosarcomas might have been a further reflection of the cocarcinogenic or augmenting properties of the urethan.

Following the success of Lucké and Schlumberger (11) in growing adenocarcinoma in the anterior eye chamber of *Rana pipiens* this site has been widely used with this renal tumor. Although the comparatively smaller size of the Xenopus eye makes the implantation procedure more difficult and damage to the eye more probable, four of five Xenopus given intraocular lymphosarcoma grafts developed visceral lymphosarcomas.

Since all eight toads, which developed from tadpoles given lymphosarcoma grafts at the base of the tail or under the skin of the hind leg, bore tumors when killed after metamorphosis, it may be said that the development of the tumor was not affected by metamorphosis.

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