CARCINOMA IN THE LEOPARD FROG: ITS PROBABLE CAUSATION BY A VIRUS*

By BALDUIN LUCKÉ, M.D.

(From the Laboratory of Pathology, School of Medicine, University of Pennsylvania, Philadelphia)

PLATE 15

(Received for publication, May 21, 1938)

For the experimental study of neoplastic diseases, warm blooded animals such as rodents and fowls have generally been used. The more primitive cold blooded vertebrates have been neglected because of the belief that among them tumors are rare and consequently not readily available for investigation. Recently, however, it has been shown that the leopard frog (*Rana pipiens*) is commonly affected with a carcinoma of the kidney. This is a particularly interesting tumor as its cell nuclei frequently contain large acidophilic inclusions such as suggest the presence of a virus (1). In the present paper an account is given of transmission experiments the results of which make it probable that this carcinoma is, in fact, induced by a virus.

The general characteristics of the spontaneous tumors, of which somewhat over 600 have been examined in this laboratory during the past 5 years, are briefly as follows:

The growths occur in one or both kidneys as solitary or multiple, white, solid or partially cystic growths varying in size from small nodules to large irregular masses several times the size of the kidney which they replace. The larger and presumably more rapidly growing tumors not uncommonly metastasize.[1] Histo-

* This study has been aided by grants from the International Cancer Foundation, and the National Research Council.

[1] This fact has developed from more recent studies and is contrary to our earlier report based on the finding of but three examples of metastasis among 276 cases of these tumors (2). But in a subsequent group of 146 tumors there were 17 with metastasis. The difference between the two groups lies in the much greater proportion of large tumors in the second group. A discussion of the incidence of metastasis of these tumors will be given elsewhere (3).

logically, the majority of the tumors have the appearance of adenocarcinoma. The component epithelial cells are quite atypical and much larger and more basophilic than normal kidney cells; usually they are crowded in disorderly multiple layers around irregularly shaped gland-like acini. Numerous mitotic figures denote active proliferation; the stroma is scanty and poorly vascular; a capsule is lacking; and marginal extensions of the tumor infiltrate and destroy the adjacent kidney.

In a smaller group the component cells are less atypical, the tumor tubules are single layered and more orderly, there are few mitotic figures, and, while no capsule is present, no extension of the periphery occurs. A frequent variation from this adenomatous growth is cystic dilatations with papillary projections into the cyst.

All gradations are found between the frankly malignant, invasive, and destructive adenocarcinoma and the structurally benign adenoma, cystadenoma, and papillary cystadenoma. In general the larger tumors nearly always have a malignant appearance, though many minute nodules are also evidently carcinomatous. The neoplastic disease, once established, appears to be progressive, for tumors with evidence of recession such as atrophy, extensive necrosis, and marked overgrowth of stroma are uncommon.

The outstanding characteristic of the frog tumor is the frequent presence of acidophilic intranuclear inclusion bodies which in their general appearance are like those found in herpes and certain other diseases known to be due to viruses. They invariably are confined to the cells of the tumor and have never been observed in normal renal epithelium of tumor-bearing kidneys, nor in the cells of other organs. In their typical form they are conspicuous and readily recognizable, and in such form they are observed within most of the tumors. It is obvious that there must be developmental stages, and the appearance of the early stages is still a matter of doubt. Moreover there appear to be seasonal variations; at least the inclusions are more frequent in winter and spring than in summer and autumn. Their common association with tumor cells, and their constant absence from related normal epithelium make it unlikely that they represent a virus which has only secondarily invaded an established neoplasm.

Material and Methods

Three series of transmission experiments are reported in this paper. In the first series the inoculation consisted of living tumor; in the second of desiccated or glycerinated tumor. The frogs used were adult leopard frogs (*Rana pipiens*) of both sexes and of average size, which came from New England. In a third series, alien species were used.

The frogs were kept in groups not exceeding 20, in large glass tanks. The tanks contained smooth rocks, and water of sufficient depth for frogs to submerge

themselves completely; change of water was provided by a constant inflow and outflow. The colony was housed in a cool basement room designed as an amphibian vivarium, in which the temperature ranged around 45–50°F. in the winter, and around 65–70° in the warmer months. Every group was inspected twice daily, and great care was taken to remove dead animals promptly. The frogs were fed, usually twice a week, with earthworms, mealworms, or insects; this food they learned to take readily. When living food was not available, bits of liver were fed by hand. During the coldest months no food was given. It proved possible to maintain the animals for from 6 months to more than a year in fairly good condition, excepting that despite isolation in small groups, recurrent epidemics (usually of red leg) destroyed considerable numbers.

The methods employed for inoculating living tumor were (*a*) to introduce small fragments, cut from healthy appearing areas with sharp scissors, by means of a hollow needle provided with a well fitting plunger (Bashford needle); or (*b*) to inject with a syringe a suspension of cells prepared by squeezing the tumor through a finely meshed sieve, or by grinding it in a rough bottomed mortar. Enough amphibian Ringer's solution was added to the cell mash to bring each dose of the suspension to 0.5 cc.

Desiccated tumor was prepared by the Flosdorf-Mudd lyophile process (4). The minced material was frozen at approximately −80°C. in a mixture of cellusolve and solid CO_2, and dried by high vacuum distillation from the frozen state; the containers were then sealed under vacuum and stored at refrigerator temperature for 2 or 3 weeks. For use the dried material was reduced to a fine powder by grinding and was suspended in sterile water; 0.5 cc. of the suspension was injected.

One group of frogs received an emulsion of a glycerinated tumor. The material, which has been stored for 20 days in 50 per cent glycerin at refrigerator temperature was washed repeatedly in amphibian Ringer's solution, and then ground to a fine emulsion of which 0.5 cc. was injected.

In the preparation of the tumors by these several methods aseptic precautions were observed. However, since it is virtually impossible to sterilize the skin of frogs without injuring it severely, the site of inoculation was merely moistened with 70 per cent alcohol both before and after inoculation.

The tumors used in the experiments varied greatly in size; many were small, early growths and furnished only sufficient material for inoculating relatively few animals, about 10 to 15; other tumors were large and represented an advanced stage of the neoplastic process. Now, since it seemed possible that in their various stages of growth, the tumors might vary in transmissibility, it became necessary to make a choice between using a variety of tumors with which to inoculate relatively small groups of frogs, or to rely on a few large tumors with which many frogs could be inoculated. Because frogs of uniform age and of pure breed were not easily available it seemed best, in these experiments at least, to choose the first of these procedures, and by using a variety of tumors, minimize the effect of variable factors in both tumors and animals. Thus, a total of 810 frogs, in groups of

from 10 to 40, approximately, received inoculations from 44 different tumors. (In this total are not included groups which because of infection failed to survive 6 months following inoculation.) As controls, 953 frogs were maintained under precisely the same conditions as prevailed in the experimental series.

For histological study, material was fixed either in formalin or in Susa fluid, and stained usually with hematoxylin and phloxin, or with a modified Giemsa solution.

Results with Living Tumor

Solid fragments or cell suspensions were inoculated into the dorsal or ventral lymph sacs (*i.e.*, subcutaneously), in the abdominal cavity, in the muscles of the thighs, or intracranially (by injection through an orbital plate). At none of these several sites did a progressive tumor develop. Usually, the injected material was rapidly destroyed and resorbed. Occasionally, fragments were found at autopsy for as long as 4 months, particularly those introduced in the lymph sacs. Such grafts had become attached and were vascularized. Some had undergone slight increase in size, but, histologically, all gave evidence of regression rather than of proliferation. While there were isolated well preserved areas of carcinomatous appearance, in which some of the cells showed mitotic figures, the dominating picture was that of atrophy and fibrosis. The process appeared to be one of long survival and of slow destruction of the grafts rather than one of successful transplantation.

From these experiments it may be concluded that local transplantation of this tumor cannot be accomplished by the methods used.[2]

However, from the examination of frogs which had been inoculated subcutaneously, intracranially, and intraabdominally, it became obvious that in a considerable number of them, tumors of the kidney had developed, and the proportion having kidney tumors became greater as the interval of time between inoculation and examination

[2] The fact that no local growth resulted at four different sites of inoculation may mean, of course, that none of these sites provided a suitable habitat for survival and multiplication of implanted tissue. Indeed it will be shown in a subsequent paper that successful local transplantation may be accomplished in the indifferent humors of the eye, and in the kidney itself, but not in the liver. In these experiments, even more strikingly than in the series here reported, there was evidence of selective affinity of the causal agent for a particular organ, for in over one-third of the frogs kidney tumors developed.

lengthened. Thus, in the animals examined within the first 3 months, the incidence of renal tumor corresponded with that observed in the control groups; but, in the next 3 months period, the incidence had become definitely greater among the experimental frogs, and decisively so in animals which survived inoculation more than 6 months. The results obtained with solid fragments and with cell suspensions were approximately the same. These findings are shown in Table I, in which are given, for each site of inoculation, the number of frogs examined at different intervals, and the number having kidney tumors.

TABLE I

Incidence of Kidney Tumors Developing in Frogs Inoculated with Living Tumor

Site of inoculation	Months after inoculation					
	0–3		4–6		Over 6	
Intramuscular	75	**3**	36	**1**	22	**1**
Subcutaneous	128	**1**	32	**2**	36	**5**
Intracranial	22	**0**	1	**1**	14	**4**
Intraabdominal	89	**0**	33	**5**	78	**17**
Controls	683	**16**	166	**10**	104	**7**

In each column is given the number of frogs inoculated and (in bold face) the number having tumors. It is seen that, excepting the intramuscular inoculations, there is a rise in incidence after the initial 3 months periods. Approximately 20 per cent of the animals which had survived over 6 months had developed kidney tumors. However, in frogs which had been inoculated in the muscles, the frequency of renal tumors did not rise appreciably. In the bottom line is given the incidence in the control series. It is seen that rise in incidence is slight; the possible significance of this is discussed in the text.

The table is based upon a total of 566 frogs examined. It will be noted that in the intramuscular group but few were found to have kidney tumor. In the other three groups the incidence rose from less than 2 per cent during the first 3 months period to approximately 20 per cent in frogs which survived for more than 6 months. The tumors found resembled in every detail those occurring under natural conditions (Fig. 1). From these experiments it may be concluded that inoculation of living tumor at various sites, either as fragments or cell suspension, results in the development of a tumor only in that

organ in which it occurs spontaneously. The time required for development of a kidney tumor appears to be, in most cases, at least 6 months.

Results with Desiccated and Glycerinated Tumor

234 frogs received intraabdominal injections of desiccates prepared from 10 tumors, and 10 frogs were injected with an emulsion of a glycerinated tumor. As shown below, the results obtained were similar.

TABLE II

Comparison of Percentage of Frogs Developing Kidney Tumor after Intraabdominal Inoculation of Desiccated or Glycerinated Tumor, and of Living Tumor. The Incidence in the Control Series Is Given in the Bottom Line

Material		Months after inoculation		
		0–3	4–6	Over 6
Desiccated or glycerinated tumor	Number of frogs examined	112	38	94
	Percentage having tumor	**6.3**	**10.5**	**21.3**
Living tumor	Number of frogs examined	89	33	78
	Percentage having tumor	**0**	**15.2**	**21.8**
Controls	Number of frogs examined	683	166	104
	Percentage having tumor	**2.3**	**6.0**	**6.7**

In inoculated animals surviving more than 6 months the results of the two series are approximately alike.

In none of the animals did any tumors develop at the site of injection, or in any of the viscera within the celomic cavity. But, as in the preceding series, there occurred a conspicuous increase in kidney tumors (Figs. 2 to 4). These findings are detailed in Table II where is given the number inoculated and the percentage having kidney tumor. For comparison, the results obtained after intraabdominal inoculation of living tumor are stated; in the bottom line, the incidence in the controls is recorded. It will be noted that the kidney tumor occurred in somewhat over 20 per cent of the frogs surviving injection with desiccated or glycerinated material for a period of more than 6 months.

This result corresponds closely with values obtained after inoculating living tumor.

Of the 10 frogs which received an emulsion of glycerinated tumor, only 4 survived the initial 3 months period; in 2 of these frogs the kidney tumor was present. The results are then, quite similar with both types of material. At least in the case of the desiccates, which were used in the great majority of the experiments, it may justifiably be assumed that the material used for inoculation did not contain living tumor cells.

From these experiments the conclusion is warranted that the tumor-inducing agent can resist conditions incompatible with the viability of animal cells. When one considers all the evidence of the present paper, and that previously given (1), it seems more than likely that the agent is a virus.

Retransmission

The question whether tumors which developed after inoculation would be transmissible with greater ease than the original tumors was investigated. Unfortunately the mortality among the 84 frogs used was, by chance, very great during the initial 3 months periods. Thus, but a single group remained in which it was possible to evaluate the results, which, however, are of sufficient interest to report here.

The original tumor (designated 126) was a typical adenocarcinoma with many inclusions. A suspension of its cells was injected into the abdominal cavity of 18 frogs. Of these 9 survived for more than 6 months and 4 of them were found to have developed renal tumors. One of these tumors was of sufficient size to be palpable during life, and this was removed and inoculated into the abdominal cavity of 13 frogs. Histologically it resembled the original tumor, excepting that inclusions were present to so great an extent as to occupy in some regions nearly every nucleus. 2 of the 5 frogs which were examined 4 to 6 months after inoculation, as well as 2 of the 4 which survived for more than 6 months had renal tumors. Other details are given in Table III.

This experiment does not answer the question for which it was designed, but the results indicate that a strain of tumor-inducing agent which had a high virulence initially retained this property after passage.

Controls. Incidence of Spontaneous Tumor

Examination of 953 control frogs gave very different results from those of the experimental series. During the first 3 months period from the inception of the corresponding experiments, slightly over 2 per cent had renal tumors. This incidence rose slightly, to 6 per cent in the second 3 months period, and to 6.7 per cent in frogs surviving for more than 6 months (see Table II, bottom line). While this rise is far below the striking increase in the experimental groups, it may have considerable significance. There exists a real possibility that the neoplastic disease is transmissible from frog to frog. In captivity, frogs are of necessity maintained under more crowded conditions than exist in their natural environment, and confinement in the laboratory

TABLE III

Kidney Tumor Developing after Intraabdominal Inoculation of Fragments from Tumor 126, and after Inoculation of One of the Tumors Which Developed (R 126)

Tumor	Months after inoculation					
	0–3		4–6		Over 6	
126	8	**0**	1	**0**	9	**4**
R 126	4	**0**	5	**2**	4	**2**
Controls	12	**1**	6	**0**	4	**0**

The figures in the first column of each group express the number of inoculated or control animals; the bold face figures, the number having tumors.

would favor not only direct contact by also indirect transference of various agents. Experiments are now under way to test the possibility that the tumor-inducing agent may be transferred by means other than inoculation. However, from the fact that relatively few tumors were found among the control animals while a very considerable number occurred among the experimental frogs, the inference may be drawn that transference by "natural" means does not readily come about.

The incidence of tumors observed in the control series, and in those of the experimental animals surviving inoculation for less than 3 months, is of the same order as the incidence of spontaneous tumors. Examination of 10,317 frogs, most of them from students' physio-

logical and pharmacological laboratories, revealed that kidney tumor occurred in 2.7 per cent. It is interesting to note that while there was some variation in frequency in different lots, the incidence on the whole was quite constant and varied but little from year to year. It should be pointed out that frogs purchased from dealers are not usually freshly captured animals, but during the winter at least, have been in captivity for months. No seasonal variation in frequency of tumors was observed.

Inoculation of Foreign Species

A group of 44 frogs consisting of approximately equal numbers of *R. clamitans*, and half grown bullfrogs, *R. catesbiana*, received intra-abdominal injection of mixed desiccates from 2 tumors.

Solid fragments and cell suspension of 4 other tumors were inoculated, also intraabdominally, into 65 frogs of a subspecies of *R. pipiens* occurring in New Jersey. This breed differs from the New England species in coloration, and possibly in certain other characters.

None of these frogs of foreign species or alien breed developed the renal tumors, indicating species specificity of the causal agent.

COMMENTS

The experiments here reported all support the indication, first given by the frequent presence of intranuclear inclusions within the tumor cells, that the kidney tumor of leopard frogs is caused by a virus. Living tumor inoculated at various sites did not lead to local growth, but tumors developed in the kidney of approximately 20 per cent of frogs which survived for more than 6 months. Inoculation of desiccated tumor, and in one group of glycerinated tumor, led to similar results; in somewhat over 20 per cent of frogs which survived for more than 6 months, renal tumors occurred. On the other hand, during the first 3 months, less than 2 per cent of the inoculated frogs were found to have this neoplasm, an incidence quite similar to that obtained in the controls and for spontaneous tumors from examination of a large number of frogs. Attempts to transmit the tumor to alien species proved unsuccessful. These results can best be interpreted as indicating the existence in the inoculated material of an organ-specific carcinogenic agent having the attributes of a virus.

That certain viruses induce neoplastic proliferation is a well established fact. The problem of the etiologic relation of viruses to tumors and the nature of virus-induced tumors have been so recently and fully reviewed by Andrewes (5) and by Rous (6), that no further discussion is necessary here. But, it is worthy of emphasis that such tumors have proven to be true neoplasms, that several varieties of them are known to exist, and that they are not confined to one particular class of animals. The kidney tumor of frogs, which usually is carcinomatous in character, would appear to be another example. This tumor is of interest not only because of its probable etiology, but because its ready availability makes possible the study of the general characteristics of tumor growth in another and more primitive class of vertebrates.

SUMMARY

An epithelial tumor with acidophilic intranuclear inclusions frequently occurs in the kidneys of leopard frogs. This tumor usually has the appearance of an infiltrating and destructive adenocarcinoma, which, when large, not uncommonly metastasizes; less often it is more orderly and adenomatous.

When inoculated as living fragments or cell suspensions into the lymph sacs, the cranial cavity, or the abdomen, no significant local growth results and the implanted material is resorbed. However, in approximately 20 per cent of the frogs surviving inoculation for more than 6 months, tumors develop in the kidney, which are like the "spontaneous" neoplasms. The incidence far exceeds that in the controls.

Desiccated and glycerinated tumor injected into the abdomen gives the same result as inoculation with living tumor; in somewhat over 20 per cent of animals surviving more than 6 months kidney tumors occur.

In alien species of frogs, no such tumors are produced by inoculation either with living or with desiccated tumor.

These experiments indicate the probability that the kidney tumor of the leopard frog is caused by an inclusion-forming, organ-specific virus.

BIBLIOGRAPHY

1. Lucké, B., *Am. J. Cancer*, 1934, **20**, 352.
2. Lucké, B., *Am. J. Cancer*, 1934, **22**, 326.
3. Lucké, B., *Am. J. Cancer*, 1938, **34**, 15.
4. Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.
5. Andrewes, C. H., The Oliver-Sharpley Lectures, *Lancet*, 1934, **2**, 63, 117.
6. Rous, P., Harvey Lecture, *Am. J. Cancer*, 1936, **28**, 233.

EXPLANATION OF PLATE 15

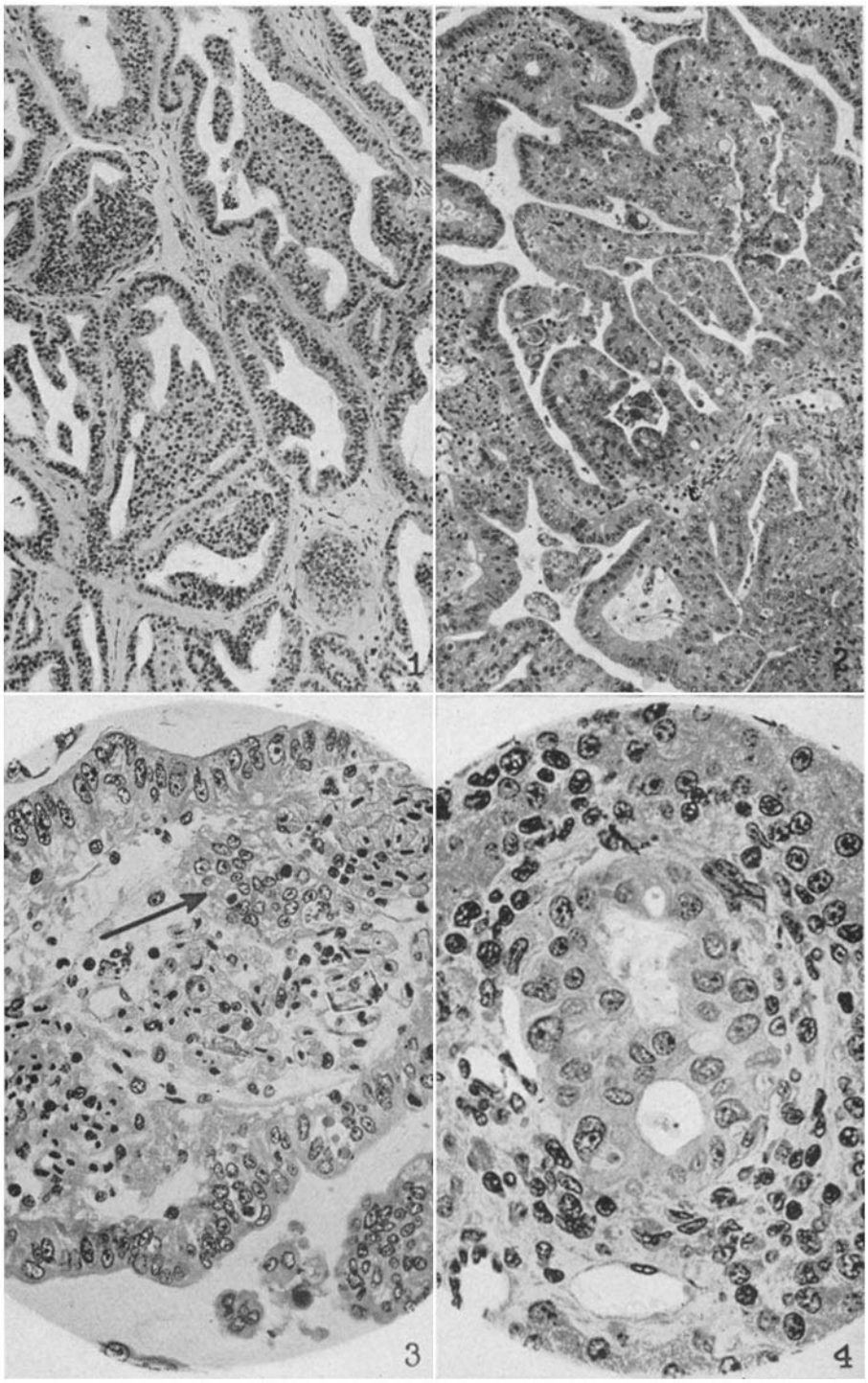
All sections were stained with hematoxylin and phloxin.

FIG. 1. Adenocarcinoma of the kidney found 176 days after intracranial inoculation with fragments of living tumor. The general character is like that of the spontaneous tumors. × 100.

FIG. 2. Adenocarcinoma of the kidney which developed after intraperitoneal injection of desiccated tumor. 355 days after inoculation, tumors were found in both kidneys; they had fused to form a large mass measuring 23 x 10 x 6 mm. Several metastatic nodules were located in the liver, and, on microscopic examination, tumor emboli were observed in some of the intrahepatic veins. × 100.

FIG. 3. Extension of tumor cells into the lumen of a thin walled vessel of the tumor shown in Fig. 2. The group of tumor cells is indicated by an arrow. × 300.

FIG. 4. A tumor cell embolus, also from the specimen shown in Fig. 2, is blocking a small intrahepatic vein. × 436.



(Lucké: Carcinoma in frog probably caused by virus)