[CANCER RESEARCH 55, 3781–3784, September 1, 1995]

Frequent Development of Pancreatic Carcinomas in the *Rana nigromaculata* Group[1]

Prince Masahito, Midori Nishioka, Hiroaki Ueda, Yo Kato, Ieharu Yamazaki, Kimie Nomura, Haruo Sugano, and Tomoyuki Kitagawa[2]

Department of Pathology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170 [P. M., Y. K., K. N., H. S., T. K.]; Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Higashihiroshima 724 [M. N., H. U.]; and Mitsui Memorial Hospital, Kandaizumi-cho, Chiyoda-ku, Tokyo 101 [I. Y.], Japan

ABSTRACT

In 1979, 2 species of pond frogs (*Rana nigromaculata* and *Rana plancyi plancyi*) were imported from China, and hybrids were made between these and Japanese, Korean, and Taiwanese pond frogs (*R. nigromaculata, Rana plancyi fukienensis* and *Rana brevipoda*) that had been kept for a number of years in the Laboratory for Amphibian Biology of Hiroshima University. From 1982, development of tumors, especially in the peritoneal cavity, was noticed frequently in the hybrids and also later, although rarely, in the Japanese pond frogs. Such tumors had never previously been observed among pond frogs in the laboratory.

Histological and immunohistochemical studies identified the i.p. tumors to be pancreatic carcinomas with occasional production of insulin and/or somatostatin. Ultrastructural investigation revealed both endocrine and exocrine secretion granules together with C-type retrovirus particles in the carcinoma cells. Other tumors included a retroperitoneal rhabdomyosarcoma, liver adenomas, and an unclassifiable mesenchymal tumor of the foot pad.

INTRODUCTION

In 1979, Nishioka and Ueda imported pond frogs (*Rana nigromaculata* and *Rana plancyi plancyi*) from China (caught in the suburbs of Beijing) and in 1980 produced hybrids between these and pond frogs of Japan (*R. nigromaculata* and *Rana brevipoda*), Korea (*R. nigromaculata*), Taiwan (*Rana plancyi fukienensis*) and their hybrids, which had been kept in the Laboratory for Amphibian Biology at Hiroshima University since 1974. From 1982 on, frequent occurrence of tumors, especially in the peritoneal cavity, was observed in the Chinese-related hybrid animals which died spontaneously, and by 1987 gross examination revealed a total of 101 tumors in 489 frogs (1).

Because at first tumors were observed exclusively in the Chinese-related hybrid frogs, Nishioka and Ueda (1) initially assumed that genetic factor(s) might be responsible for the tumorigenesis. Later, however, they found that i.p. tumors began to develop, although rarely, in nonhybrid Japanese and Japanese-Korean hybrids frogs, in which such tumors had never been observed previously. They then suspected viral participation in this matter but did not extend their observation in detail.

In the present study, precise histological, immunohistochemical, and ultrastructural observations were carried out on these tumors, revealing almost all of the i.p. tumors to be pancreatic carcinomas.

Spontaneous pancreas tumors in frogs and toads appear to be quite rare. To the best of our knowledge, there is only one reported case of a carcinoma involving both pancreas and stomach in a South African clawed toad (2). This paper documents the unprecedented observation of development of pancreas carcinoma in pond frogs together with their morphological and biochemical features.

Received 4/21/95; accepted 7/6/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by grants-in-aid from the Ministry of Education, Science and Culture, Japan.

[2] To whom requests for reprints should be addressed.

MATERIALS AND METHODS

In 1980, Nishioka and Ueda (1) of the Laboratory for Amphibian Biology (Hiroshima, Japan) made hybrids between Chinese and Japanese, Taiwanese, or Korean pond frogs. The species or subspecies and origin of the pond frogs used were as follows: (*a*) *R. plancyi plancyi* Lataste, from the suburbs of Beijing, China, collected by Dr. Chin-Ye Chang in 1979; (*b*) *R. nigromaculata* Hallowell, from the suburbs of Beijing, China, collected by Dr. Chin-Ye Chang in 1979; (*c*) *R. nigromaculata* Hallowell, from the suburbs of Hiroshima, Japan, collected in 1974; (*d*) *R. brevipoda brevipoda* Ito, from Okayama prefecture, Japan, collected in 1974; (*e*) *Rana plancyi chosenica* Okada, from Suwon, Korea, collected in 1974; (*f*) *Rana nigromaculata* Hallowell, from the suburbs of Suwon, Korea, collected in 1974; and (*g*) *Rana plancyi fukienensis* Pope, from the suburbs of Chiayi, Taiwan, collected in 1974.

Hybrids were made between female *R. plancyi* or *R. nigromaculata* from China and males of various species or subspecies from Japan, Korea, and Taiwan through artificial fertilization. Embryos and tadpoles were reared at room temperature, 18–22°C, in Petri dishes (18 x 6 cm) or in enameled pans (46 x 32 x 11 cm). The tadpoles were given boiled spinach or chard. The metamorphosed frogs were kept in plastic pancases (65 x 45 x 15 cm), initially 100/pan and later in reduced number (15–25) in one case as they grew in size. They were fed on tropical crickets (*Gryllus bimaculatus*), bred by the method of Nishioka and Matsuura (3), and kept in room temperature (25–28°C) from April through December and in hibernation temperature (8–10°C) from January through March.

Gross Inspection of Tumors. During 1982–1987, all frogs were examined after death for the presence of gross tumors. From 1988 on, abdominal palpation was carried out at the end of hibernation in March; when a tumor was detected, these frogs were sacrificed for autopsy, and materials for ultrastructural study were obtained from 2 of them. All the frogs having gross tumors were fixed in Navasin's fluid (4) for 24 h and then kept in 70% ethanol solution for future investigation.

Histological Study. A total of 29 frogs (3–10-years-old) with tumors, which were inspected within a relatively short period after death or sacrificed when an abdominal tumor was detected, were selected for this study. The tumors were cut out and processed for routine histological examination with hematoxylin and eosin staining.

Immunohistochemistry. Immunohistochemical study was carried out by avidin-biotin complex peroxidase method utilizing Guinea pig antiporcine insulin (Nichirei Corp., Tokyo) and rabbit antihuman somatostatin and glucagon antibodies (DAKO Corp., Copenhagen, Denmark).

Electron Microscopical Study. One Navashin-fixed and two fresh pancreatic carcinomas, together with normal pancreas tissues of frogs, were used for study. Pieces of Navashin-fixed tissue were kept in tap water overnight and then in 0.1 M phosphate buffer with 0.22 M sucrose for several days at 4°C. These and freshly obtained pieces of tissues were fixed in 2.5% glutaraldehyde in phosphate buffer for 1 h, then in 2.5% glutaraldehyde in phosphate buffer for 1 h, then in 1% osmium tetroxide in phosphate buffer for 1–2 h, dehydrated by ethanol, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and hydroxide and examined with a Japan Electron Optics Laboratory JEM 200CX electron microscope.

Table 1 *Incidence of abdominal tumors in Chinese-related and Chinese-unrelated pond frog hybrids inspected during 1982–1987*

Parent Status	1982	1983–1984	1985–1986	1987	Total
Chinese-related	7 /135 (5.2)[a]	32 /139 (23.0)	47 /180 (26.1)	8 /8 (100)	94 /462 (20.3)
Chinese-unrelated	0/13 (0)	0/1 (0)	0/13 (0)	0/0 (0)	0/27 (0)

[a] Numbers in parentheses, percentage.

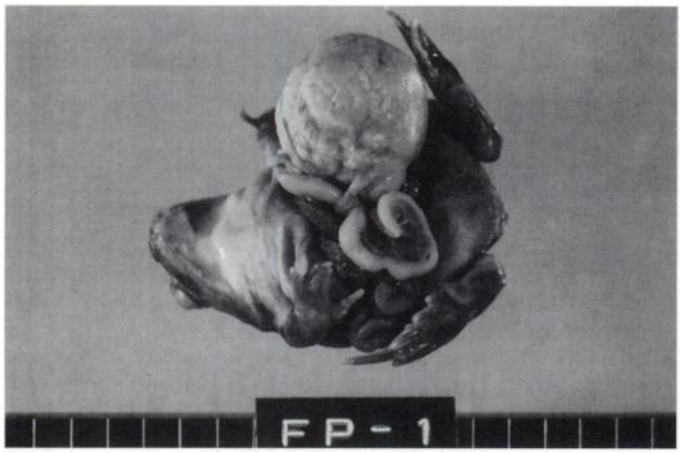

Fig. 1. Gross feature of a representative large pancreatic tumor. × 1.

RESULTS

The number of abdominal tumors recorded by gross observation and the number of frogs inspected during 1982–1987 are summarized by year and parent status in Table 1. Tumors began to appear exclusively in Chinese-related hybrids from the third year after birth, and the abdominal tumor incidence elevated with age. At the end of 1987 (8th year), all of the frogs (8 of 8) had a large abdominal tumor when inspected. A representative gross feature of the abdominal tumor is shown in Fig. 1. A total of 99 tumors was detected in 462 Chinese-related hybrids, 94 (95%) located in the abdominal cavity and the remaining 5 (1.1%) in the leg, the ovary, and the kidney. Until 1987, these abdominal tumors were observed exclusively in Chinese-related hybrids, regardless of the variation in the parent status. Later, however, an abdominal tumor (pancreas carcinoma) also developed in a Japanese and a Japanese-Korean hybrid frog. During 1982–1987, only 2 kidney tumors were noticed in 27 non-Chinese-related hybrid frogs (7.4%).

Gross Findings of Tumors. A total of 34 gross tumors, measuring 5–37 mm in largest diameter, were found in the 29 frogs used for the present morphological and immunohistochemical studies, with one having 2 tumors and another 3. Of these, 30 were located in the abdominal cavity, forming masses of various size between the liver and the duodenum. Their cut surface was yellowish-white, solid or cystic. The remaining 4 tumors presented as a large retroperitoneal node, 2 small hepatic nodules, and 1 small foot pad nodule.

Histological Findings. Histologically, all abdominal tumors were carcinomas of the pancreas. They were all similar in principle, being composed of uniform tumor cells with round nuclei, arranged in medullary patterns, occasionally forming rudimentary acinar or tubular structures often lobulated by thin connective tissue (Fig. 2*a*). In some tumors, such acini were widened by accumulation of protein-rich fluid negative for mucin staining (Fig. 2*b*). Occasionally, tumor cells demonstrated quasinormal acinar or tubular cell differentiation of pancreatic morphology (Fig. 2*c*). Microscopic metastases from pancreatic carcinomas to various tissues including the liver, kidney (Fig. 2*d*), heart, lung, stomach, and ovary were observed in 7 frogs.

The retroperitoneal tumor was a rhabdomyosarcoma, and the 2 hepatic lesions were a hepatocellular and a bile duct adenoma. The

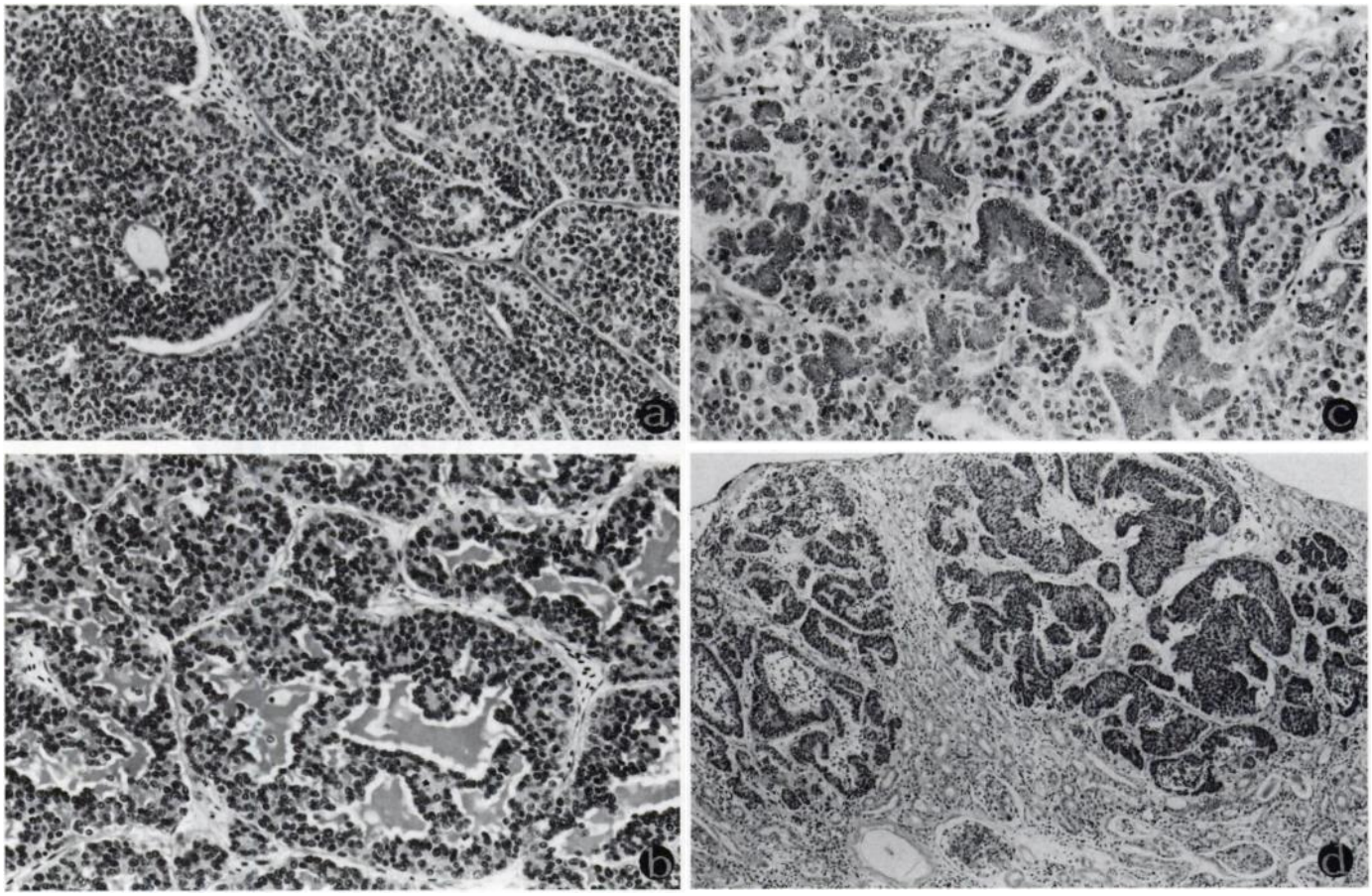

Fig. 2. Histology of pancreatic carcinomas demonstrating medullary growth of uniform tumor cells with round nuclei, lobulated by thin connective tissue (*a*), accumulation of protein-rich fluid in acini (*b*), differentiation to quasinormal acinar or tubular structure (*c*), and microscopic metastasis to kidney (*d*). H&E. *a* and *c*, × 150; *b*, × 300; *d*, × 75.

hind leg tumor was unclassifiable, being composed of round cells with clear cytoplasm.

Immunohistochemical Findings. The presence of insulin, somatostatin, and glucagon was clearly demonstrated in normal pancreas tissue of frogs. Among 11 pancreatic carcinomas studied, insulin and somatostatin were positive in 4 and 8, respectively, whereas glucagon was not detected. In 4 tumors, both insulin and somatostatin were demonstrated. Representative staining patterns of insulin and somatostatin are shown in Fig. 3, *a* and *b*.

Electron Microscopical Findings. Navashin-fixed material and two fresh pancreatic carcinomas were examined, together with normal pancreas tissue of hybrid frogs. The cells of the three tumors, as well as normal pancreatic cells, had endocrine secretion granules measuring up to 350 nm (Fig. 4*a*). In tumor sites where differentiation to exocrine cells was observed histologically, larger exocrine secretion granules (up to 1500 nm) were encountered.

Ultrastructural study also revealed mature C-type retrovirus particles measuring about 100 nm in maximum diameter in the extracellular space of all the tumor tissues (Fig. 4*b*) but not in the normal pancreas tissue. They were round or slightly irregular in shape with well developed envelopes. Some particles appeared to be budding from cytoplasmic membranes (Fig. 4*c*). There were also numerous immature C-type-like particles in the extracellular space.

DISCUSSION

Excluding the famous herpesvirus-induced renal adenocarcinoma among *Rana pipiens* in Minnesota and the Northeastern part of the United States (5, 6), spontaneous tumors in frogs and toads are rare. This is especially true of tumors of the pancreas. According to the reviews by Schlumberger and Lucké (5) and Balls and Clothier (6), only 22 and 66 spontaneous tumors had been registered in anuran amphibians from 1868 to the year of their publication, respectively, with no pancreatic tumors. In the registry of tumors in lower animals published by Harshberger *et al.* (7), collected from 25 or more countries from 1965 and 1981, no

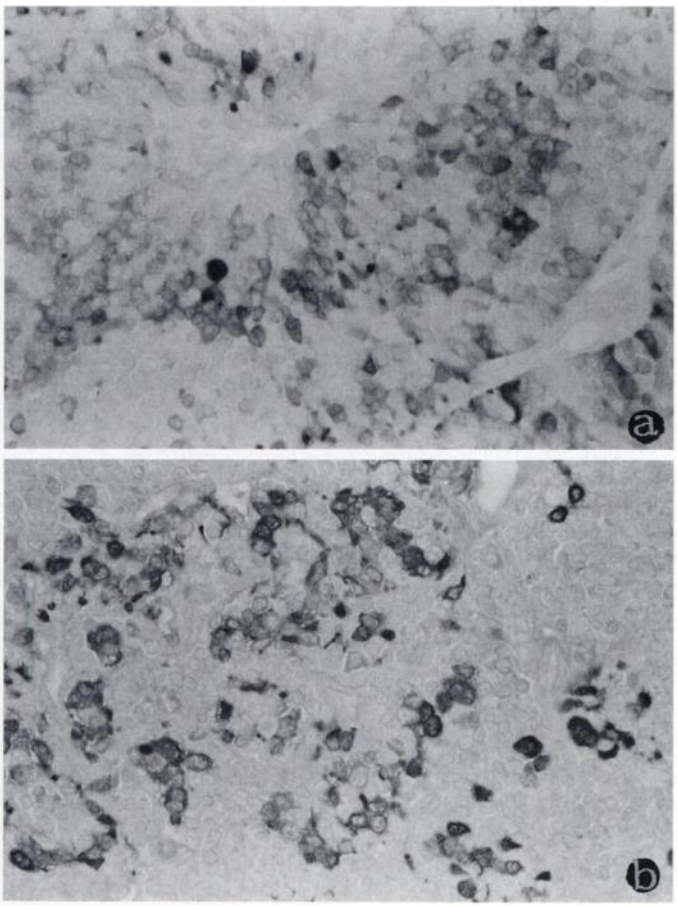

Fig. 3. Immunohistochemical demonstration of insulin (*a*) and somatostatin (*b*) in tumor tissues. × 150.

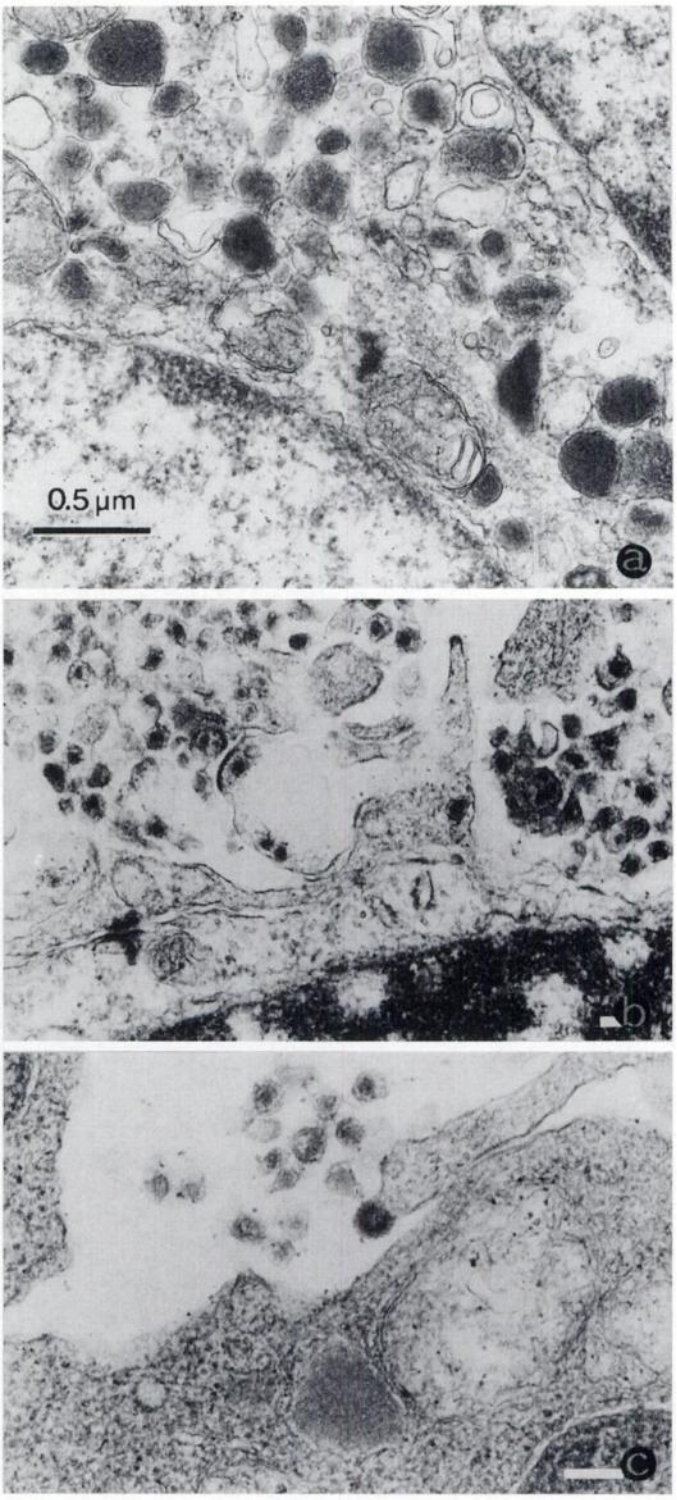

Fig. 4. Electromicroscopic demonstration of endocrine secretion granules measuring 200–300 nm (*a*), C-type retrovirus particles measuring about 100 nm in the extracellular space of tumor tissue (*b*), and budding of a particle from cytoplasmic membrane (*c*).

pancreatic neoplasm was seen among 36 frog tumors, except for a probable case reported by Elkan (2). The present observations are, therefore, unprecedented, and the contribution of histological, immunohistological, and ultrastructural investigations clearly identified that the lesions are indeed carcinomas of pancreatic origin. The tumor cells had a potential to differentiate into both endocrine and exocrine cells. Because in the pond frog the pancreas exists separately from the liver, the possibility of a hepatic-pancreatic origin of these tumors could easily be ruled out.

As to the underlying causal mechanisms, viral participation was immediately envisaged, because (*a*) spontaneous tumors are rare in frogs; (*b*) such tumors had never been observed in Japanese, Japanese-Taiwanese, or Japanese-Korean hybrid frogs until the introduction of the frogs from China and, later, tumors began to appear not only in Chinese-related hybrid frogs but also, although very rarely, in Japanese and Chinese-unrelated hybrid frogs; (*c*) C-type viruses were actually detected in tumor cells, although more studies are necessary to confirm that these really were causative agents and not merely passengers (8); and (*d*) there is a large body of evidence that DNA and RNA viruses are oncogenic in lower animals: in anuran amphibians, renal adenocarcinomas in *R. pipiens* (9) and melanomas in *Xenopus laevis* (10) were transmitted by herpesviruses; in fish, retroviral particles and reverse transcriptase activity were detected in lymphosarcoma of Northern pike (11) and in plasmacytoid leukemia of chinook salmon (12).

The nutrition of frogs has been very much improved by the introduction of a cricket feeding system since 1975 (3). Thanks to achievement with this system, frogs now grow more rapidly and live much longer (up to 10 years) than previously. Changes in nutritional condition and life span elongation often contribute to the appearance of spontaneous tumors that previously might not have been observed due to earlier death. In the present situation, however, such possibility can be ruled out because no abdominal tumor was observed in Japanese and Chinese-unrelated hybrid frogs during 1975–1987.

ACKNOWLEDGMENTS

We are grateful to Dr. Robert G. Mckinnell (University of Minnesota, MN) for his encouragement and critical reading of the manuscript. We also thank Takako Nanazoshi for help in preparation of the manuscript.

REFERENCES

1. Nishioka, M., and Ueda, H. Abundant tumors which recently occurred in the Laboratory for Amphibians. Zool. Sci. (Tokyo), *4:* 1069, 1987.
2. Elkan, E. A spontaneous anaplastic intestinal metastasizing carcinoma in a South African clawed toad (*Xenopus laevis* Daudin). J. Pathol. Bacteriol., *100:* 205–207, 1970.
3. Nishioka, M., and Matsuura, I. Two-spotted crickets, *Gryllus bimaculatus* De Geer, as an excellent diet for terrestrial anurans. Sci. Rep. Lab. Amphib. Biol. Hiroshima Univ. *2:* 165–185, 1977.
4. Nawaschin, M. Variabilität des Zellkerns bei Crepis-Arten in Bezug auf die Artbildung. Z. Zellforsch., *4:* 171–215, 1926.
5. Schlumberger, H. G., and Lucké, B. Tumors of fishes, amphibians, and reptiles. Cancer Res., *8:* 657–754, 1948.
6. Balls, M., and Clothier, R. H. Spontaneous tumors in amphibia. Oncology, *29:* 501–519, 1974.
7. Harshbarger, J. C., Charles, A. M., and Spero, P. M. Collection and analysis of neoplasms in sub-homeothermic animals from a phyletic point of view. *In:* C. J. Dawe, J. C. Harshbarger, S. Kondo, T. Sugimura, and S. Takayama (eds.), Phyletic Aproaches to Cancer, pp. 357–384. Tokyo: Japanese Sci. Soc. Press, 1981.
8. Levy, J. A. Endogenous C-type viruses in normal and "abnormal" cell development. Cancer Res., *37:* 2957–2968, 1977.
9. Naegele, R. F., Granoff, A., and Darlington, R. W. The presence of the Lucké herpesvirus genome in induced tadpole tumors and its oncogenicity: koch-Henle postulates fulfilled. Proc. Natl. Acad. Sci. USA, *71:* 830–834, 1974.
10. Asashima, M., Oinuma, T., and Komazaki, S. Electron microscopical and histochemical studies of the spontaneous tumors of *Xenopus laevis*. Zool. Sci., *6:* 899–905, 1989.
11. Papas, T. S., Dahlberg, J. E., and Sonstegard, R. A. Type C virus in lymphosarcoma in northern pike (*Esox lucius*). Nature (Lond.), *261:* 506–508, 1976.
12. Eaton. W. D., and Kent, M. C. A retrovirus in chinook salmon (*Oncorhynchus tshawytscha*) with plasmacytoid leukemia and evidence for the etiology of the disease. Cancer Res., *52:* 6496–6500, 1992.