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C-type Virus Particles in Urethan-induced Pulmonary and Renal Carcinomas, in Cell-Graft-transmitted Carcinosarcomas, and in Filtrate-induced Lymphomas in Mice¹

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SUMMARY

Repeated injections of urethan into suckling BALB/c mice induced multiple papillary adenocarcinomas in the lungs and kidneys. When the pulmonary tumors were transplanted i.p. by cell graft into 6 suckling BALB/c mice, they induced disseminated carcinosarcomas within the peritoneal cavity in all inoculated animals. Tumors resulting from the transplantation of tumor cells were used for preparation of filtered extracts. The filtrates were inoculated into 6 suckling BALB/c mice and induced generalized malignant lymphomas in all animals.

The primary urethan-induced pulmonary and renal tumors, the carcinosarcomas that resulted from i.p. cell transfer, and also the generalized malignant lymphomas induced by inoculation of filtered extracts contained C-type virus particles.

Theoretically, it could be assumed that both the primary urethan-induced pulmonary and renal tumors, as well as the cell-graft-induced peritoneal carcinosarcomas, contained the C-type virus particles as passengers, not necessarily related etiologically to the tumors in which they were found. It is quite likely, however, that these virus particles were etiologically related to the filtrate-induced malignant lymphomas in which they were also found.

INTRODUCTION

In 1943, Nettleship and Henshaw discovered accidentally, and with surprise, that i.p. or p.o. administration of small doses of urethan (ethyl carbamate), which they used for anesthesia, resulted in the development of pulmonary adenomas in most mice of C3H and A inbred lines (17). This unexpected observation was confirmed and extended in other laboratories. Pulmonary adenomas were subsequently induced with urethan in mice of several inbred lines (10, 18) and also in rats (11, 13). All inbred strains of mice tested, such as C3H, A, C57 Black, Swiss, Bagg albino, Ak, etc., were found to be susceptible to the induction of pulmonary tumors with urethan. Curiously, however, the white-footed deer mice (Peromyscus leucopus noveboracensis), trapped at Brant Lake in the Adirondack Mountains, were found to be completely resistant to the carcinogenic action of urethan (10). Many studies have been made since these early experiments, and it is now realized that urethan is a powerful carcinogenic agent, capable of inducing not only pulmonary adenomas but also a variety of other tumors in several species of animals. Among the tumors induced with urethan, s.c. or i.p. fibrosarcomas have been observed in European hamsters (16); adenocarcinomas of the intestinal tract, such as cecum or colon, adenocarcinomas of the thyroid gland, or dermal melanocytomas in Syrian hamsters (25); leukemia and lymphomas (5, 26) in mice; or hepatomas in mice (12) and rats (13), etc. Pulmonary tumors in experimental animals and the carcinogenic action of urethan have been reviewed in several publications (1, 15, 21, 24).

The purpose of our study was to determine whether urethan-induced pulmonary tumors in mice are caused by an oncogenic virus and whether they could be transmitted by filtrates.

MATERIALS AND METHODS

Animals. BALB/c mice, bred in our laboratory by brother-to-sister mating since 1954, have been used in this study.

Urethan Injection. One g of urethan was dissolved in 100 ml of sterile water and a litter of four 2-day-old BALB/c mice was treated with i.p. injections (0.15 ml each) of the freshly prepared urethan solution. After an interval of 1 week, the mice were reinoculated (0.2 ml each) with a similar freshly prepared urethan solution; they received a 3rd inoculation (0.3 ml each) after an additional week.

Preparation of Tumor Cell Suspensions from the Urethan-induced Pulmonary Tumors. Several small pulmonary tumors induced with urethan were removed from one of the donor mice. The tumors were cut with small scissors and ground by hand in a sterile mortar with a sufficient volume of sterile 0.9% NaCl solution added to prepare a cell suspension of approximately 10% concentration. The cell suspension was then passed through a sterile voile cloth and used immediately for i.p. inoculation.

Preparation of Filtrates. Several peritoneal tumors induced by cell suspensions prepared from the urethan-induced pulmonary tumors were removed aseptically, pooled,
and used for the preparation of a cell suspension that was centrifuged at 3,000 rpm (1,400 \( \times g \)) for 20 min, followed by centrifugation at 10,000 rpm (7,000 \( \times g \)) for 5 min; the supernatant was then filtered through a Selas 02 filter candle. The resulting filtrate was used immediately for i.p. inoculation.

**Light Microscopy.** Tissues were fixed in Bouin’s fluid, dehydrated, and embedded in paraffin. Five-\( \mu m \) sections were stained with hematoxylin and eosin, periodic acid-Schiff or Mallory’s trichrome stains.

**Electron Microscopy.** Specimens were fixed in 4% phosphate-buffered glutaraldehyde followed by 1% phosphate-buffered osmic acid. They were then processed as previously described (4). Tissue blocks were sectioned with a Porter-Blum microtome using a diamond knife. Sections were coated with carbon, stained with uranyl acetate and lead citrate (27), and examined in a RCA-EMU 3G or a Phillips 300 electron microscope.

**RESULTS**

**Induction of Multiple Pulmonary Papillary Adenocarcinomas in the Lungs and Kidneys of the Inoculated Mice.** As a result of 3 consecutive injections of urethan within the 1st month of life, 3 of the 4 inoculated BALB/c mice developed multiple pulmonary tumors at ages varying from 11 to 15.5 months. One of them also developed multiple kidney tumors.

One of these mice, Mouse 405 (male), which served as a donor for the preparation of a pulmonary tumor cell suspension used for experimental transmission, developed multiple papillary adenocarcinomas of the lungs and similar tumors in the kidneys at age 15.5 months. The lungs showed distended enlarged bronchi with prominent hyperplastic lining cells. There were several circumscribed tumors, measuring between 0.3 and 0.6 cm, growing in close proximity to large bronchi (Fig. 1). The tumors were made up of coiled ribbon-like cords forming papillary projections and tubular structures, and occasionally growing in solid sheets (Fig. 2). The cells were large and polyhedral, with distinct outlines. Nuclei were large with prominent nucleoli; mitotic figures were infrequent.

Multiple tumors having the same histological patterns as the papillary adenocarcinomas of the lung were also present in the kidneys. These tumors, which measured up to 0.4 cm in diameter, were noted in the medulla and cortex and infiltrated the parenchyma, so that renal structures, such as glomeruli, were trapped within the tumor nodules (Figs. 3 and 3a).

Several pulmonary tumors were removed aseptically from this animal and used for the preparation of a tumor cell suspension.

**Electron Microscopic Examination of the Urethan-Induced Pulmonary and Renal Tumors.** Electron microscopic examination of sections of the pulmonary tumors (Fig. 2a) revealed the presence of large, irregularly shaped cells, frequently with cytoplasmic extensions. The cell surfaces contained microvilli (m); strands or rough-surfaced endoplasmic reticulum, a prominent Golgi apparatus, many ribosomes, and occasionally vacuoles and myelin figures (F) were observed in the cytoplasm. Characteristic budding, immature, and mature C-type virus particles (arrows) could be found. Fig. 2 represents a 1-\( \mu m \) light microscope section, embedded in Epon, consecutive to the ultrathin section illustrated in Fig. 2a.

When ultrathin sections of the urethan-induced renal tumors were examined in the electron microscope, a few immature C-type virus particles were also observed (Fig. 4, arrow).

Accordingly, both the urethan-induced pulmonary tumors and the renal tumors contained virus particles; however, it was easier to find the virus particles in the pulmonary tumors.

**Transmission of the Urethan-Induced Pulmonary Tumors into Newborn BALB/c Mice by Cell Graft.** A tumor cell suspension prepared from several pooled, small pulmonary tumors removed from Mouse 405, referred to above, was inoculated i.p. into a BALB/c litter, less than 6 days old, consisting of 2 females and 4 males.

After a latency of 5 to 7 months, all 6 mice developed multiple pulmonary tumors (Fig. 5, Arrow P) and a large number of small round tumors in their abdominal cavities (Fig. 5, Arrows C). The lung tumors were papillary and adenomatous carcinomas (Figs. 6 and 6a), grossly and histologically similar to the lung tumors of the donor.

**Description of the Multiple Abdominal Tumors Induced by i.p. Inoculation of Cell Suspensions Prepared from the Primary Pulmonary Tumors.** Large numbers of small, finely papillary, firm, white tumors were disseminated throughout the peritoneal cavity. They were implanted on the kidney and liver capsules without penetration of the parenchyma, as well as on the peritoneal surface of the diaphragm (Fig. 5). Histologically, some of the tumors had the structure of papillary adenocarcinomas. Other tumors in this group had many of the features of a carcinosarcoma or mesothelioma. The bulk of these tumors were made up of spindle cells with large, uneven nuclei forming a disorderly array of fibers. Irregular glands, lined by prominent hyperchromatic cells, were intermingled among the fibers and there were small papillary structures on the surface (Figs. 7 and 7a).

**Presence of C-type Virus Particles in the Peritoneal Tumors Induced by Cell Graft.** In sections of the peritoneal surface tumors examined in the electron microscope, budding, immature, and mature C-type virus particles were found in small numbers (Fig. 8, arrow).

**Transmission of the Urethan-Induced Renal Tumors by Cell Graft.** A cell suspension prepared from the kidney adenocarcinoma that developed in the original urethan-injection-treated donor was inoculated i.p. into a BALB/c litter of 8 mice, each less than 6 days old. At ages varying from 7.5 to 13 months, 6 of the inoculated mice developed malignant lymphomas (3 of them with concomitant generalized leukemia), 1 developed a sarcoma in the left groin, and the other developed a carcinosarcoma adhering to and infiltrating the diaphragm. Electron microscopic examination of the carcinosarcoma revealed the presence of a few budding and immature type-C virus particles.

**Induction of Malignant Lymphomas and Leukemia in Mice Inoculated with Filtrates Prepared from the Peritoneal...**
Carcinosarcomas. From 2 animals, a few of the multiple peritoneal tumors that had been induced by cell suspensions prepared from urethan-induced pulmonary tumors were removed aseptically, pooled, and used for the preparation of a filtrate. The filtrate was inoculated i.p. into a BALB/c litter (4 females and 2 males) less than 6 days old. All inoculated animals developed disseminated malignant lymphomas after a latency of 7.5 to 15 months. Among the 6 mice with malignant lymphomas, 2 also had generalized leukemia; one, the lymphatic form and the other, unclassified. None of the animals inoculated with the filtrate developed either pulmonary or i.p. tumors similar to those observed in the donor mice.

Microscopic examination revealed generalized infiltration with leukemic cells of the liver, spleen, thymus, lymph nodes, and kidneys. The tumors consisted of large pleomorphic, histiocytic cells. Some of the cells were very large with bizarre nuclei and prominent nucleoli. A small number of lymphocytes were present. The histology and pattern of infiltration of the tumors were those of a histiocytic lymphoma or reticulum-cell sarcoma (Fig. 9).

Electron Microscopic Examination of the Mediastinal Lymphoma Induced with Tumor Filtrate. A fragment of a mediastinal lymphoma was removed from one of the mice with filtrate-induced generalized leukemia. On electron microscopic examination of ultrathin sections prepared from this tumor (Figs. 10 and 11), large pleomorphic, histiocytic cells with indented nuclei, and occasionally small round lymphoid cells, were observed. C-type virus particles were found in the lymphoma; they were similar to those observed in the primary pulmonary tumors and in the peritoneal tumors induced by cell graft. A few were budding from plasma membrane; some were immature (Fig. 10, arrow), or mature, in the intercellular spaces. In addition, a cylindrical particle (Fig. 11, arrow), with an internal structure similar to an immature C-type particle, was also present.

DISCUSSION

Assuming, as a working hypothesis, that spontaneous or chemically induced malignant tumors are caused by oncogenic viruses (9), we have carried out an experiment dealing with the induction with urethan of pulmonary carcinomas in BALB/c mice, hoping to find virus particles in the induced tumors and expecting to transmit such tumors by filtrates inoculated into newborn mice. Viruses or virus-like particles have been previously described in spontaneous or urethan-induced pulmonary tumors in mice (2, 3, 14, 19, 20). One of these observations (14) described the presence of very small (about 20 nm in diameter) "elementary particles" in urethan-induced pulmonary tumors in strain A mice; according to Brooks (2), these small particles very likely represented glycogen. Svboda (22, 23), in a subsequent electron microscopic study of pulmonary adenomas occurring in either untreated or in urethan- or methylcholanthrene-treated Swiss mice, found no evidence of the presence of virus particles.

Rabotti (19) examined 6 spontaneous lung tumors from BALB/c mice and found virus particles measuring 120 to 140 nm in diameter, consisting of a nucleoid surrounded by a single membrane. The particles were seen in intercellular spaces and in cytoplasmic vacuoles. Brooks (2) observed type-C virus particles in pulmonary tumors, spleen, and other organs in a mouse of the A strain that had been treated with urethan and that had developed both pulmonary tumors and leukemia. Bucciarelli and Ribacchi (3) observed, in 6 out of 17 primary, hydrazine sulfate-induced lung tumors in BALB/c mice, cylindrical and spherical C-type virus particles, the latter measuring approximately 100 nm in diameter.

In our study, pulmonary and renal adenocarcinomas were induced by repeated injections of urethan into suckling BALB/c mice. In the original donor the carcinomas of the lungs and kidneys were multiple, relatively large, and histologically very similar. It is not possible to determine whether the kidney tumors developed as a result of metastatic transmission of primary lung tumors or vice versa, or whether the tumors developed in both organs independently and at about the same time. Multicentric development of foci of adenocarcinomas in salivary glands, following inoculation of a potent oncogenic virus, have been observed in our earlier studies dealing with the parotid tumor, i.e., polyoma virus in mice (7). It is conceivable, therefore, that a potent carcinogenic chemical could trigger a latent virus almost simultaneously in several sites of susceptible organs, such as lungs and kidneys in this case, causing the development of multiple tumor nodules in both organs.

The primary urethan-induced pulmonary and renal tumors contained C-type virus particles in the epithelial tumor cells. The carcinosarcomas disseminated in the peritoneal cavity, which resulted from i.p. cell transfer of the primary pulmonary tumors, as well as the lymphomas induced by inoculation of filtered extracts prepared from the cell graft-induced tumors, also contained C-type virus particles.

The fact that we observed C-type virus particles in the primary urethan-induced pulmonary and renal tumors is of particular interest and requires further study. It is not possible to determine whether these particles had an etiological relationship to the induction of tumors in which they were found. Normal, healthy BALB/c embryos contain latent, C-type virus particles in the thymus, liver, bone marrow, and spleen (4). More recently, we found C-type virus particles in the thymus of a normal, healthy adult BALB/c mouse. If left undisturbed, a few mice of this strain will develop leukemia spontaneously; the incidence varies from 5 to 20%. It is quite possible that repeated injections of urethan activated some of the latent leukemogenic viruses carried by these mice. On the basis of this assumption, we could theorize that both the primary, urethan-induced pulmonary and renal tumors, as well as the cell graft-induced peritoneal carcinosarcomas, contained the virus particles as passengers, not necessarily related etiologically to the tumors in which they were found. The malignant lymphomas induced with the filtrates also contained similar virus particles; in this case, however, one could assume, theoretically at least, that the virus particles were etiologically related to the lymphomas in which they were found, because similar C-type virus particles have been commonly found in, and have been known to be etiologically related to, leukemia and malignant lymphomas in mice.
The observation that filtered extracts prepared from the transplanted pulmonary tumors induced malignant lymphomas and leukemia following inoculation into newborn mice is reminiscent of previous experiments of Graffi et al. (8), who initially observed the development of leukemia and lymphomas in mice inoculated shortly after birth with filtered extracts prepared from transplanted mouse tumors. The early experiments of Graffi et al. have been repeated, confirmed, and extended by other investigators using a variety of transplanted tumors in mice (8).

Essentially, however, the attempted transmission of the pulmonary tumors by filtrates did not succeed, if pulmonary tumors similar to the initial, primary, urethan-induced lung tumors were expected to be reproduced. Instead, leukemia and malignant lymphomas were induced with filtrates prepared from the initially urethan-induced and then cell graft-transmitted tumors.

Assuming, as a working hypothesis, that pulmonary tumors like all other tumors are caused by oncogenic viruses, one could speculate that mice of several inbred strains tested, as well as rats and hamsters, carry latent oncogenic viruses that are activated by urethan. The fact that the white-footed deer mice are resistant to this mechanism of tumor induction or that they do not carry oncogenic viruses sensitive to activation by urethan. However, further studies are needed. The existence of an oncogenic virus responsible for the actual induction of pulmonary tumors, and presumably activated by urethan, still remains to be demonstrated.

REFERENCES

C-type Virus Particles in Urethan-induced Tumors

Fig. 1. Urethan-induced lung tumor (papillary adenocarcinoma) from the original donor. Coiled, ribbon-like structures made up of large polyhedral cells. H & E, x 400.

Fig. 2. One-μm light microscope section, embedded in Epon, of the preparation in Fig. 1. Large polyhedral cells forming ribbon-like strands, similar to those illustrated in Fig. 1. Periodic acid-Schiff, x 800.

Fig. 2a. Electron micrograph of an ultrathin section of a cell from a pulmonary tumor of the original donor consecutive to the section illustrated in Fig. 2. Immature and mature C-type virus particle (arrows) appear in vacuoles proximal to the cell surface. Also shown are microvilli and a myelin figure (F). Uranyl acetate and lead citrate, x 55,000.

Fig. 3. Urethan-induced papillary adenocarcinoma in the kidney of the original donor illustrating solid and papillary areas. Normal kidney tubules (left upper corner). H & E, x 80.

Fig. 3a. High power of papillary area of the same kidney tumor showing large polyhedral cells, somewhat irregular nuclei with enlarged nucleoli, and abundant cytoplasm. H & E, x 400.

Fig. 4. Electron micrograph of a fragment of the urethan-induced papillary adenocarcinoma that developed in the kidney of the original donor. An immature C-type virus particle (arrow). Uranyl acetate and lead citrate, x 50,000.

Fig. 5. At the age of 6 days this BALB/c male mouse was inoculated i.p. with a cell suspension prepared from a primary, urethan-induced, pulmonary tumor. Seven months later, this mouse, as well as its 5 siblings, developed papillary pulmonary adenocarcinomas (P, arrow) as well as multiple carcinomas disseminated throughout the peritoneal cavity (C, arrows). x 3.

Fig. 6. Microscopic section of a pulmonary tumor that developed following cell graft in a BALB/c male sibling of the mouse described in Fig. 5. Low-power view of a circumscribed papillary tumor showing close relationship to dilated, hyperchromatic bronchi. H & E, x 65.

Fig. 5a. High power of the same pulmonary tumor illustrating its papillary and adenomatous nature. H & E, x 400.

Fig. 7. Microscopic section of one of the peritoneal tumors that developed following cell graft in a BALB/c mouse, sibling of the male shown in Fig. 5. Solid, spindle-cellular tumor with irregular glands and papillary projections on the surface. H & E, x 65.

Fig. 7a. Detail of the peritoneal tumor shown in Fig. 7, showing characteristic sarcomatous morphology with glandular carcinomatous structure adjacent to sheets of spindle cells, suggesting a carcinosarcoma or a mesothelioma. H & E, x 400.

Fig. 8. Electron micrograph of the peritoneal carcinosarcoma described and illustrated in Figs. 7 and 7a. A mature C-type virus particle (arrow) with an electron-dense nucleoid is shown in an extracellular space. Uranyl acetate and lead citrate, x 89,000.

Fig. 9. Microscopic section of a histiocytic malignant lymphoma in liver that developed following inoculation into a suckling BALB/c mouse of a filtrate prepared from the peritoneal carcinomas. Pleomorphic histiocytic cells and lymphocytes; 2 mitotic figures are present (arrows). H & E, x 400.

Figs. 10 and 11. Electron micrographs of ultrathin sections of a mediastinal malignant lymphoma that developed in a BALB/c mouse following inoculation of filtrate prepared from peritoneal carcinomas described and illustrated in Fig. 9. An immature C-type particle (Fig. 10, arrow) and a cylindrical particle (Fig. 11, arrow) are present in intercellular spaces. The cylindrical particle has an internal structure similar to that of the immature type-C particle. Uranyl acetate and lead citrate. Fig. 10, x 85,000; Fig. 11, x 60,000.
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