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Detection of Viruslike Particles in Germ-Free Mice

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ABSTRACT

KAJIMA, MASAHIRO (University of Notre Dame, Notre Dame, Ind.), AND MORRIS POLLARD. Detection of viruslike particles in germ-free mice. J. Bacteriol. **90**:1448–1454. 1965.—Viruslike particles similar in structure to the mouse leukemia agent were detected by electron microscopy in thymus tissues of germ-free AK mice which had developed spontaneous leukemia. In addition, unique viruslike particles (type B) were detected in tissues from spontaneous mammary adenocarcinoma of germ-free C3H mice. Leukemia virus-like particles were also observed in the thymuses of the control AK mice and of the C3H control mice as well as of those with mammary tumors. Germ-free mice are not virus-free. The routes of transmission of leukemia as well as of mammary tumor viruses may be "vertical," through the embryo or placenta.

The original germ-free strain of the Swiss-Webster mouse was caesarian-derived from conventional stock (Reyniers, 1959; Trexler, 1959), and each young animal was hand-fed a steam-sterilized diet up to weaning age (Pleasants, 1959). Thereafter, the mice were propagated by natural means through 21 successive germ-free generations. Mice of other strains were caesarian-derived and cross-suckled on germ-free Swiss-Webster mothers. When weaned, they were separated from the mothers and propagated by natural means. Thus far, seven strains of germ-free mice have been propagated and maintained in the Lobund Laboratory. In six of the strains, leukemia could be induced only by exposure to fractionated small doses of X rays (Pollard, 1964). Spontaneous leukemia has not been observed in them. On the other hand, mice of the germ-free AK strain developed spontaneous lymphatic leukemia (Pollard, Kajima, and Teah, Proc. Soc. Exptl. Biol. Med., *in press*). Germ-free mice are free from bacteria, protozoa, and fungi, as determined by standard bacteriological examinations (Wagner, 1959). They have been suspected of carrying some viruses which are probably transmitted vertically or by congenital routes (Pollard, 1964; Pollard and Matsuzawa, 1964). Leukemia viruslike particles have been detected by electron microscopy in tissues from normal germ-free mice (deHarven, 1964; Kajima, *unpublished data*) and from germ-free mice with radiation-induced leukemia (Kajima, *unpublished data*). The purpose of this communication is to describe viruslike particles in tissues from germ-free mice with spontaneous leukemia and in germ-free mice with spontaneous mammary cancer.

MATERIALS AND METHODS

Each of 14 germ-free AK mice was removed from the sterile isolator and examined by hematological and histological techniques. The mice were dyspneic and had rough fur. The AK mice showed gross, hematological, and histological evidence of lymphoid leukemia. The thymus glands were enlarged,

FIG. 1. *Thin sections from the thymuses of germ-free AK mice with spontaneous leukemia. (1a) An arrow indicates an early stage in the process of particle formation from the plasma membrane of a lymphocyte. A doughnutlike particle is seen in the intercellular space.* × *145,000. (1b) A later stage in particle formation. A doughnut like particle is connected to the cytoplasm by a narrow pedicle.* × *145,000. (1c) A portion of an epithelial cell. A cytoplasmic vacuole contains several viruslike particles. One particle shows a tail-like structure (Dalton, Hanguenau, and Moloney, 1962). An arrow indicates the formation of a particle in the cytoplasm.* × *30,000. (1d) An arrow locates a particle with an electron-dense, centrally located core.* × *70,000.*

FIG. 2. *Thin sections from the thymus of a germ-free C3H mouse with spontaneous mammary adenocarcinoma. (2a) Part of an epithelial cell. Characteristic vacuoles containing viruslike particles are indicated by arrows.* × *30,000. (2b) A higher magnification of the viruslike particle indicated by the arrow in the upper left-hand corner of Fig 2a. This particle may represent a transitional form between the typical doughnutlike particle and the particle with the dense nucleoid.* × *200,000. (2c) A higher magnification of particles shown by the arrow in the lower right-hand corner of Fig. 2a. These particles have large, centrally located, electron-dense cores (nucleoids).* × *200,000.*

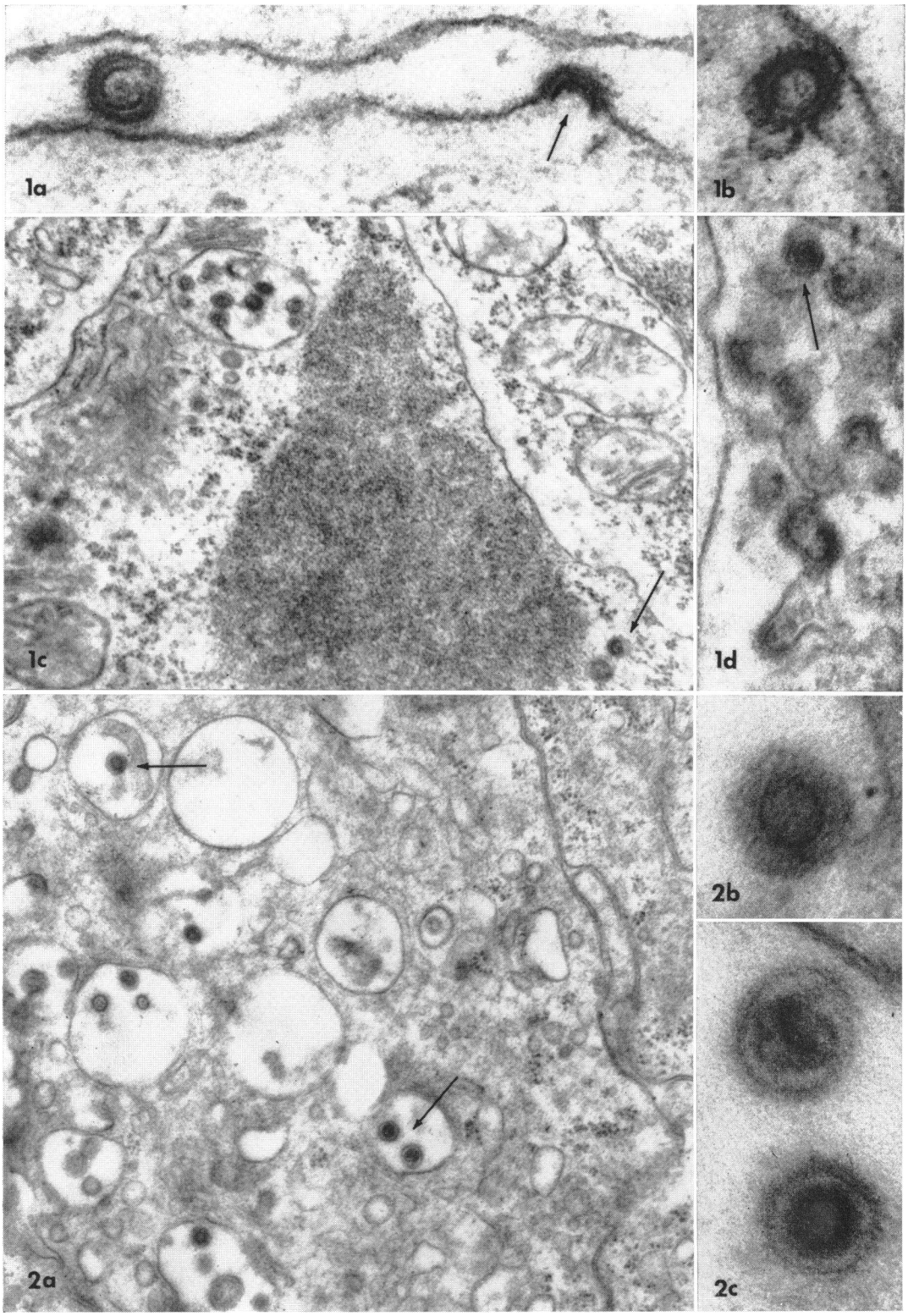

FIG. 1–2

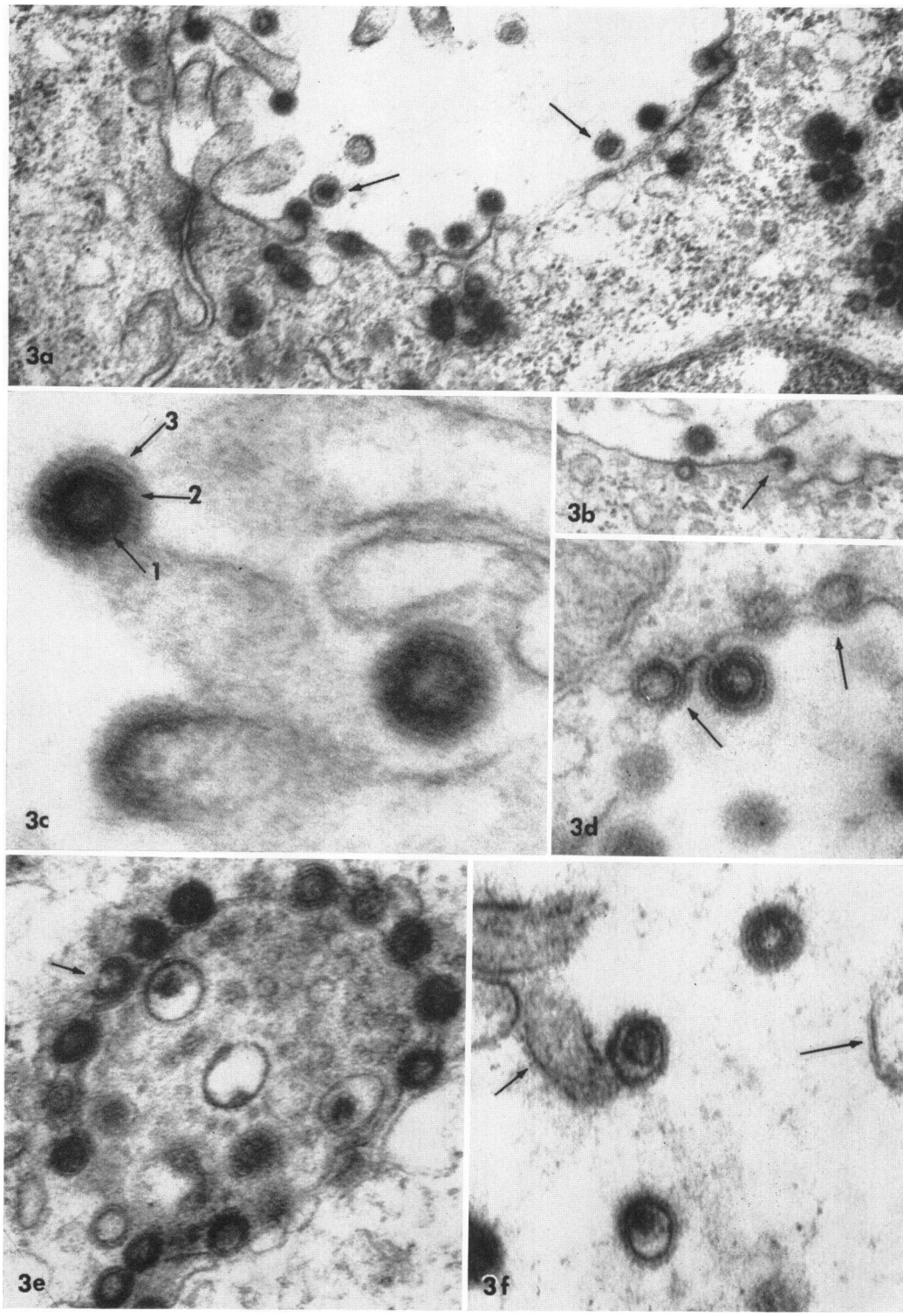

FIG. 3

and less frequently the lymph nodes, spleens, livers, and kidneys were also enlarged. The normal histology of organs was altered by heavy to moderate infiltrations by lymphoblastic cells, many of which were mitotic. The thymus glands of three such leukemic germ-free mice were examined by electron microscopy. The thymus glands were extracted in physiological saline, and cell-free filtrates thereof were inoculated into newborn mice.

Mammary tumor tissues from two germ-free C3H mice were identified histologically as mammary adenocarcinoma. Tumor and thymus tissues from such C3H mice were examined for viruslike particles by electron microscopy. The same organs from apparently healthy germ-free isologous mice served as controls.

Each tissue specimen was fixed for 1 hr in 2%, buffered osmic acid solution (*p*H 7.4; 4.5% sucrose; Caulfield, 1957), dehydrated in a graded series of ethyl alcohol solutions, and then embedded in Epon 812 (Luft, 1961). Tissue sections were made with a Porter-Blum microtome equipped with a glass knife, and they were then stained with uranyl acetate (Watson, 1958) or with lead hydroxide (Feldman, 1962), or both. An RCA EMU-3C electron microscope was used for observing and photographing the tissue specimens.

RESULTS

Examinations of thin sections from thymuses and from mammary tumor tissue cells revealed in them numerous viruslike particles similar to those reported previously in murine leukemia (Bernhard, 1960; deHarven and Friend, 1960; Dalton et al., 1961; Feldman and Gross, 1964) and in mouse mammary carcinoma cells (Bernhard, 1958; Dmochowski et al., 1959; Moore, 1962; Hairstone, Lyons, and Moore 1964*a*).

Two principal types of particles were observed in all of the enlarged thymuses from leukemic germ-free AK mice. One type was characterized by two concentric shells and an electron-lucid center (Fig. 1a). These doughnutlike particles were 70 to 100 mμ in diameter and resembled the type A1 particle (deHarven, 1962). The second type of particle had an electron-dense core and a single limiting membrane (arrow in Fig. 1d). The diameter of this particle was about 100 mμ. The latter type of particle has been described as a type C particle (Bernard, 1960; deHarven, 1962). Doughnutlike particles appeared to be formed by a budding process from the plasma membrane of the cell (Fig. 1a, b, and d). Although most of the viruslike particles were observed in intracellular vacuoles (Fig. 1c) and in intercellular spaces (Fig. 1a and d), a few particles were found in the cytoplasm (arrow in Fig. 1c). Epithelial cells and abnormal large lymphocytes of the thymus gland were mainly involved in the formation of virus particles. On the other hand, viruslike particles were also found in the thymus tissues of apparently healthy germ-free control mice. In the control mice, however, they were detected in smaller numbers and they were located either outside the cell or in cytoplasmic vacuoles of epithelial cells. The budding phenomenon was observed mainly in epithelium-type cells. The incubation period is not long enough to assess the presence of leukemia virus in the filtrates prepared from thymoma cells.

The thymus cells from the germ-free C3H mice with the mammary tumors contained leukemia viruslike particles similar to those observed in the control mice (Fig. 2a, b, and c). The mammary adenocarcinoma cells contained many smaller doughnutlike particles, 65 to 70 mμ in diameter, in the cytoplasm (Fig. 3a). These particles were similar to type A particles (Bernhard, 1960) or to type A2 particles (deHarven, 1962). Some appeared to have incomplete double concentric

FIG. 3. *Thin sections from mammary adenocarcinoma tissue of a germ-free C3H mouse with leukemia viruslike particles in the thymus. (3a) Acinar space and portion of a mammary tumor cell. Small doughnutlike particles are seen in the cytoplasm. Particles with a large, centrally located nucleoid are visible (arrows). The cytoplasm contains numerous ribosomes.* × *40,000. (3b) An arrow indicates the budding process in the plasma membrane. A doughnutlike particle is seen just within the plasma membrane. The fine fringe of the plasma membrane is also visible.* × *40,000. (3c) Formation of a particle at the tip of a microvillus. The particle shows two concentric shells and an outer fringe. Arrow 1 indicates the inner electron-dense shell; arrow 2, the outer limiting membrane formed from the plasma membrane; arrow 3, the additional outer fringe.* × *175,000. (3d) The plasma membrane, indicated by an arrow, shows budding which may result during the first stage in the passage of a doughnutlike particle to the outside of the cell from the cytoplasm. The particle showing three concentric shells, an additional outer fringe, and an attachment to the plasma membrane with a narrow pedicle, may represent the final stage in the release of the doughnutlike particle from the cell.* × *95,000. (3e) Doughnutlike particles are arranged near a cytoplasmic vacuole containing particles with an electron-dense, eccentrically located core. The arrow points out an incomplete doughnutlike particle.* × *95,000. (3f) Three different types of particles, characterized by their fine structure, are visible in acinar space. The particle at the upper right does not contain a nucleoidlike structure. The particle near the center contains a relatively large, electron-dense core (nucleoid). The particle at the bottom contains a smaller, eccentric, dense core surrounded by a faint membrane. The arrow indicates the fringe on the plasma membrane.* × *95,000.*

shells, which might represent a stage in the development of the smaller doughnutlike particle from the fine filamentous mass (arrow in Fig. 3e). Most small doughnutlike particles were aggregated as in an inclusion (Fig. 3a). Some were found singly in the cytoplasm (Fig. 3a), and sometimes near the plasma membrane (Fig. 3b and d). In addition, some of them seemed to be extruded into an intracytoplasmic vacuole or into intercellular spaces (Fig. 3d). In this process, particles appeared to acquire an additional coat consisting of plasma membrane and its fringes (Fig. 3d). This process might have produced the particle with three concentric shells and an outer fringe. On the other hand, budding of the plasma membrane resembling that in leukemia virus formation was also seen, though rarely (Fig. 3b and c). This process probably gave rise to particles having two limiting membranes and an outer fringe (Fig. 3c). Three main types of particles, measuring about 115 mμ in diameter, were observed outside the cell. The first was the particle without any electron-dense core (Fig. 3c and f). The limiting membrane of this type consisted of two or three concentric shells and an additional outer fringe. They were made either by the release of type A particle through the plasma membrane or by the budding process described above. The second type of particle contained a relatively large, centrally located, electron-dense core (about 50 mμ in diameter; Fig. 3a and f). This one resembled the type C particle (Bernhard, 1960; deHarven, 1962), except that its larger diameter might have been due to an additional fringe. The third type of particle was characterized by a smaller, eccentrically located, electron-dense core (about 30 mμ in diameter) which was surrounded by a faint membrane (Fig. 3e and f); it resembled the type B particle (Bernhard, 1960; deHarven, 1962). The first particle-type appeared to be the precursor of the latter two types. Although the type B particle was found in both of the mammary tumors, the particle which resembled the type C particle was present in one. Neither viruslike particles nor other suggestive structures have as yet been detected in mammary gland tissues taken from control, germ-free C3H mice.

DISCUSSION

Two types of virus particles have been detected in germ-free mice by electron microscopy: (i) those associated with leukemia, and (ii) those found in association with mammary carcinoma cells. Consideration of the procedures used to obtain germ-free mice (Reyniers, 1959; Trexler, 1959; Pleasants, 1959) indicates that both viruses may have been transmitted to progeny by vertical passage through the embryo. The first generation of germ-free Swiss-Webster mice were not nursed with their mother's milk (Pleasants, 1959); however, succeeding strains were foster-nursed by them. Vertical transmission of leukemia virus through the fetus (Gross, 1951) or through milk (Law and Moloney, 1961; Gross, 1962) has been demonstrated experimentally. Although the mammary tumor agent (MTA) is commonly transmitted in the mother's milk to newborn mice (Bittner, 1936), transmission through the embryo has also been described (Andervont, 1963). If MTA resides in germ-free C3H mice, the disease is elicited very rarely. A large colony involving generations of germ-free C3H mice has been observed for 4 years, and mammary tumors have been observed only in the two instances reported here.

That virus is a causative agent in mouse leukemia has been documented extensively (Gross, 1951; Graffi, 1957; Friend, 1957; Moloney, 1960). However, as observed by electron microscopy, the viruslike particles (presumably leukemia agent) persist in the tissues of disease-free mice.

In mammary tumor tissue, many viruslike particles were observed inside (type A particle) and outside (type B particle) of the cell. Type B particles were observed neither in the thymus tissues of leukemic mice (Fig. 1c and d), nor in the thymuses of the mice with mammary tumors (Fig. 2a, b and c). However, particles resembling leukemia virus (type C), except for their fringelike structure, were detected in mammary tumor tissue (Fig. 3a and f). It has been reported that "the leukemia particles are smaller and when negatively stained they do not have 100 A spines covering the external coat" (Hairstone et al., 1964*a*). Leukemia viruslike particles have been described only in mammary tumor tissue of strain A mice and in hybrid mice genetically related to strain A mice (Hairstone et al., 1964*a*; Hairfield, Sheffield, and Moore, 1964*b*). The different diameters of leukemia virus and the leukemia viruslike particle in mammary tumor tissue may depend on the quality of the membrane which they acquired in the budding process. The characteristic fringe of the virus particle in mammary tumor tissue is probably derived from the same material seen in the vacuole or on the surface of the plasma membrane of the cell (Fig. 3a, b, d, e, and f). Except for the fringe of the leukemia viruslike particle in mammary tumor tissue, its diameter and structure are similar to that of the leukemia virus (type C) in the thymus. The

thymus tissue from the C3H mice with the mammary tumors contained leukemia viruslike particles (Fig. 2a, b, and c). Leukemia virus, inoculated intraperitoneally, has been detected in cells of the mammary gland (Feldman, Gross, and Dreyfuss, 1963). The germ-free C3H mouse studied here has no genetic relationship to the strain A mouse. From these considerations, the type C-like particle observed in mammary tumor tissue is probably a variant of leukemia virus.

Virus-free mammary tumors have been described, and we have no way of relating the mammary tumors to leukemia virus. The latter may be ubiquitous in the host. However, the detection of type B virus particles in mammary tumor tissue would suggest that a second virus resides in the germ-free C3H mouse. This morphological description will have to be confirmed by biological test.

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