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Search for C-Type Particles in Human Neoplasia*

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ABSTRACT

The salient biologic and morphologic characteristics of RNA tumor (oncornavirus) viruses are reviewed. The ultrastructure of replicating oncornaviruses is illustrated in detail. C-type particles wide spread in at least three orders of animals were sighted in human sarcomas and leukemias. One case, an infantile fibrosarcoma, is presented from our cases surveyed for the presence of C-type particles. Tissue cultures derived from this tumor contained viral particles and had an elevated reverse transcriptase activity associated with the presence of 70 S RNA. The particles were larger (125 to 150 nm) than those of the murine or avian Type C particles.

An enormous effort is being exerted by hundreds of laboratories to detect and to identify possible causative viral agents of human neoplasia. The biologic and technological background information is quite extensive, well defined and successful in the identification of animal oncogenic viruses. A large number of these agents belong to a single group of RNA containing viruses, the oncornaviruses, the type species being the Rous Sarcoma virus. It may be puzzling why extension of the methods from animal tumors to human material is so frustrating. One technique, most successful in animal virus research, is electron microscopy. This technique is of limited sensitivity and low concentration of viruses in tissues or cultivated cells may be impossible to detect. Yet, oncornaviruses have been sighted in human tumors. Many reports, however, of human cancer viruses, even in current publications of prestigious journals, are not viruses at all. Rather, they are cellular organelles or artefacts confused with oncornaviruses. In this paper a review is presented of the most salient biologic and morphologic characteristics of the known oncornaviruses. One case is detailed to demonstrate the presence of C-type viral particles in a tissue culture derived from a human tumor.

General Considerations of Oncornavirus

The oncornavirus group includes the known avian, murine, feline and hamster leukosis complex, the monkey sarcoma, and monkey and murine mammary tumor viruses. Those of the group capable to infect and to transform human cells in tissue cultures are listed in table I. In tumors of the host of origin, both infectious virus and

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a virus induced tumor antigen, the transplantation antigen, are present.

Until recently, oncornaviruses were grouped with the myxoviruses which they resemble because both groups are of enveloped virions, their capsids are assembled in the cytoplasm and they bud from the cell surface of host cells where the nucleocapsid is enveloped. However, they form a group apart from the myxoviruses[16] that show helical capsid symmetry while the symmetry of oncornaviruses is unknown. Like all enveloped viruses they are sensitive to Tween 80-ether treatment. The diameter of oncornavirus virions is about 100 nm; they contain a high molecular weight RNA in the genome (MW 10 to 13 million) and also reverse transcriptase (RNA → DNA) and other enzymes that set this group further apart from orthomyxoviruses which have a simpler internal structure.

Oncornaviruses are characterized by a well defined structure and morphogenesis. Early in the search for viruses, it was learned that a critical evaluation of the electron micrographs and adherence to strict morphologic definitions were needed to separate virus particles from cell organelles and from artefacts. Bernhard[4,5] and then a group of electron microscopists[2] defined various RNA tumor virus particles on the basis of their ultrastructure and relationship to the host cell.

Intracellular particles were defined as *intracisternal A* and *intracytoplasmic A type*. In thin sections, these appear as double concentric rings cut from a double shelled sphere. The internal ring is usually more electron dense. The center is electron lucent. The intracisternal A is seen budding from rough endoplasmic reticulum into perinuclear or endoplasmic cisternae as shown in figure 1. These particles are often seen in spontaneous or experimental tumors, particularly plasma cell tumors of mouse. They have no known oncogenic activity.

TABLE I
Experimental Host Range

Oncornaviruses	Cell Transformation (in vitro)	Cell Transformation (in vitro) Tumor in vivo
Rous sarcoma	Human; bovine	Chicken, duck, rat hamster, monkey
Murine sarcoma	Human	Mouse, rat, hamster
Feline sarcoma	Human	Cat, dog, monkey

Intracytoplasmic A Particles

Intracytoplasmic A particles are encountered in mouse mammary carcinoma. They are usually present in large numbers and are located within the hyaloplasm. These particles are about the same size as the intracisternal ones. Their outer shell measures 65 to 70 nm and the inner shell about 50 nm.

Extracellular B-type Particles

The extracellular particles also fall in two types defined as B-type and C-type particles. *B-type particles* are formed when intracytoplasmic A particles bud through the cytoplasmic unit membrane where they acquire an envelope. The nucleoid condenses and is usually localized excentrically within the membraneous sac which measures 90 to 200 nm. The sac is covered on its external surface by characteristic spikes that make these particles recognizable by negative staining. The enveloped extracellular particle is referred to as B-type and is the mature or infectious virion of mouse mammary tumor. Similar particles were isolated also from a primary mammary tumor of a rhesus monkey and were observed in preparations of human milk. In figure 2 are shown negatively stained B-type particles.*

* Courtesy of Drs. Sarkar and Moore.[19]

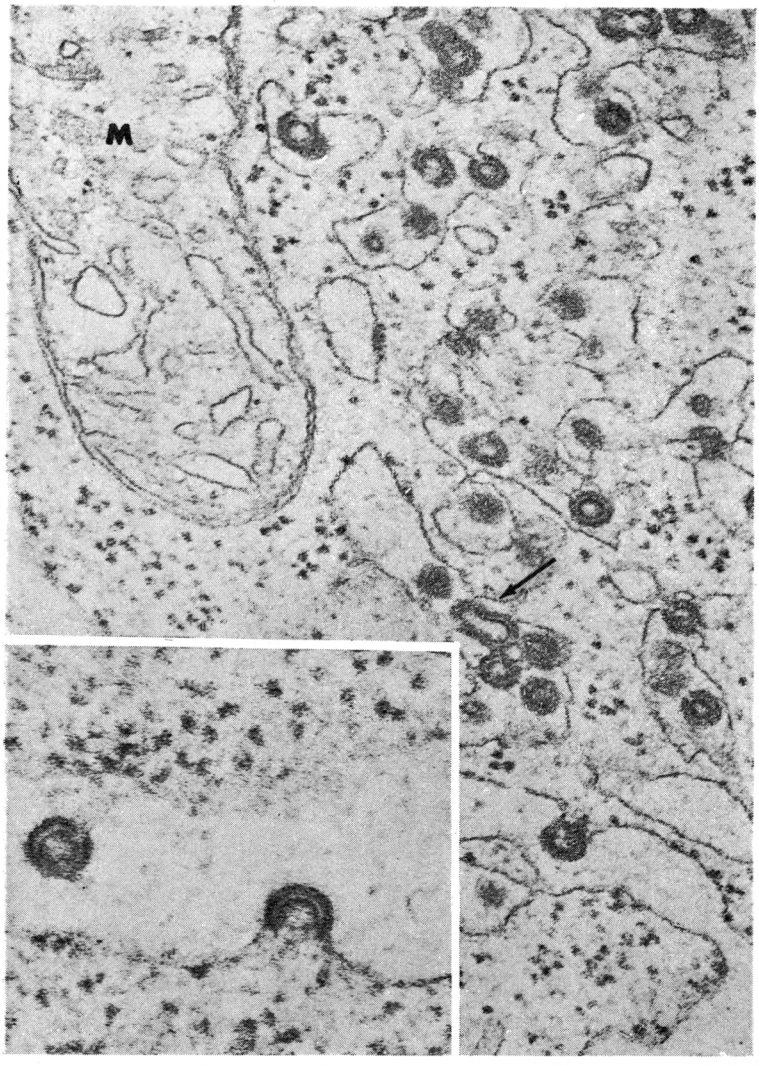

FIGURE 1. Intracisternal A-Type virus particles in plasma-cell tumor of mouse. All stages of development are evident. The particles bud into cisternae of the rough endoplasmic reticulum, forming double shelled doughnut-shaped structures. Some particles are double (arrow) the center is electron lucent. M-mitochondrion. Thin section × 68,000. Insert: Particle budding from the inner surface of the endoplasmic reticulum into cisternae. Thin section × 110,000.

EXTRACELLULAR C-TYPE PARTICLES

C-type particles are also formed by budding at the cell surface but no intracytoplasmic precursor is morphologically demonstrable. The nucleoid develops as a crescent at the site of budding. The periphery of the inner core is intensely osmiophilic. The intermediate layer is less dense and the cell's unit membrane contributes the viral envelope. In figure 3 are illustrated the successive phases of the developing C-type particle.† After the bud pinches off the cell membrane, the intermediate layer condenses in relatively short time and the particle matures by redistribution of the electron dense material in the nucleoid. The nucleoid is centrally located within the envelope which exhibits *no* substructures such as spikes. Identification of the mature virion therefore is not possible by negative staining. Identification is also unsatisfactory by thin sectioning as too many structures, such as secretory granules, pinocytotic vesicles, vesicular lacunae and artefacts, may mimic the simple target shape of the mature virion.[14] Identification

† Courtesy of Dr. Anderson[1] from laboratory of Dr. Dalton.

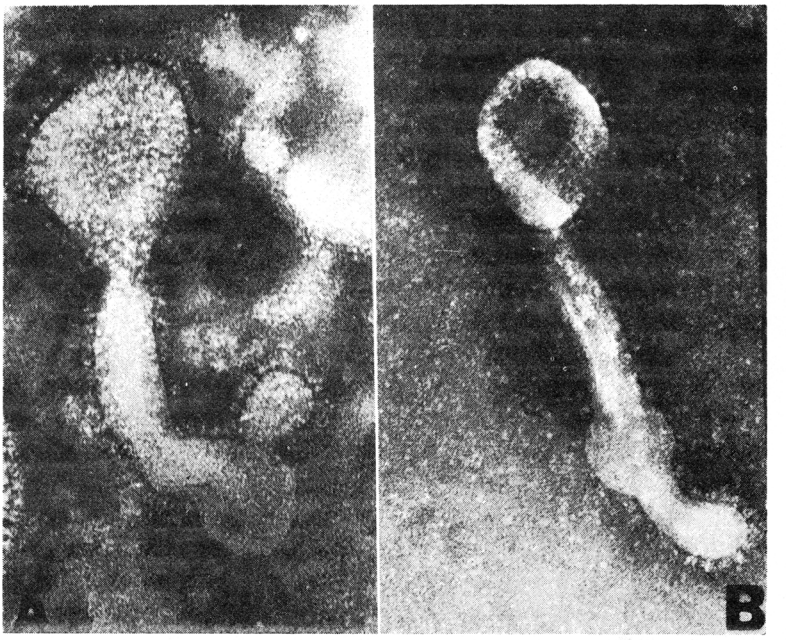

FIGURE 2. A. Extracellular B-type particle negatively stained from murine mammary tumor virus (MuMTV). B. Particle found in human milk isolates. Note the similarity of structure particularly the projections of the surface membrane. The regularity of the nobbed spikes and their constant size and distribution allows identification of B-type particles in negatively stained preparations. Figures 2 A and B × 240,000 through the courtesy of Drs. N. H. Sarkar and D. H. Moore.[19]

is also unreliable when only one or a few particles are present, particularly in plasma pellets or even in tissues in the intercellular space. Budding particles, particularly if fixed in Dalton's chrome osmium, are sufficiently specific to be identified as C-type particles.[9] All of the leukemia sarcoma complex viruses of avian, murine and hamster origin are of the C-type.

Frequency of virus particles in animal tumors was found to be variable. In general, high concentrations of virus particles are observed in inbred animals. Virus particles usually have a low concentration or are very scanty in animals in their natural habitat such as in feral mice and in cats. Experimental models are known where virus particles are not formed in the virally induced tumors. One example is the tumor induced in hamsters by the inoculation of murine sarcoma virus. The resulting tumor produces no infectious virions nor detectable viral antigens, but the sarcoma virions can be retrieved by co-cultivation of the tumor cells with murine fibroblasts in the presence of murine leukemia virus as a helper virus.[18]

With the independent discoveries in 1970 by Temin and by Baltimore of measurable virus-specific reverse transcriptase activity in oncornaviruses together with the presence of a high density (70S) RNA, these chemical methods have extended the ability of scientists to detect oncornaviruses. Until 1970, electron microscopy was the most reliable method of virus detection. However, biochemical studies eventually need to be validated by ultrastructural analysis.

C-Type Particles in Human Tissues

Searching for oncornaviruses in human tumors, in a natural rather than an inbred population, is difficult owing to the scarcity of identifiable particles. Oncornaviruses are widely spread in animals, including subhuman primates. Since their experimental host range extends to human cells, they are considered the prime candidate agent of human tumors. The step between sighting virions by the electron microscope and establishing a virus producing tissue culture presents difficult problems not yet solved for human tumors.

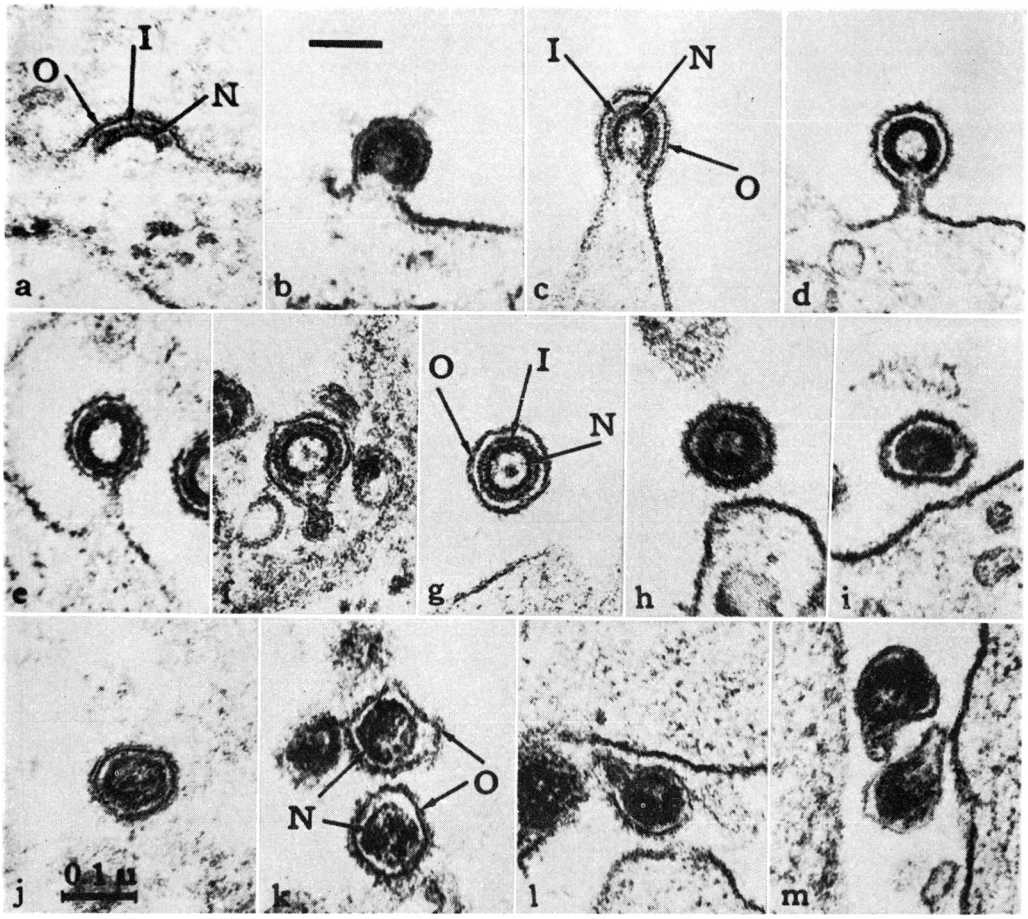

FIGURE 3. Formation of murine leukemia virus in tissue culture. Developmental stages advance as are shown: *a* through *d* early budding from the cell membrane, *e* through *i* the late bud and *j* through *m* the maturation of the free particle. N—nucleoid, I—inner membrane, O—outer membrane. Thin sections × 90,000, courtesy of Dr. D. R. Anderson.[1]

Sighting of virus or virus-like particles in human leukemias, lymph nodes, plasma pellets as well as in various soft tissue and bone tumors is the subject of many communications since 1958.[3,7,8,10,11,12,17,19,20,21] Hundreds of tumors were studied. Mature extracellular particles were observed in many tumors but budding particles were seen only in very few.

So far there are no human tumors where the causative agent is known to be an oncornavirus. There were false hopes. The cell lines ESP1 and RD114 are good examples. The C-type particles produced by these cultures were found to be of mouse origin in the ESP1 line[13] and a new endogeneous cat virus in the RD114 line.[15]

A new direction taken by electron microscopists leads to searching placentas and embryonal human tissues for C-type particles with some early successes noted.[6] Other candidate human viruses might come from overseas where at least two reports are of interest but not yet confirmed. Weiman and Ostertag[20] isolated a C-type virus from a cell line established from a patient with polycythemia vera. Zhdanov et al.[23] report the isolation of B-type and C-type particles from human cell lines.

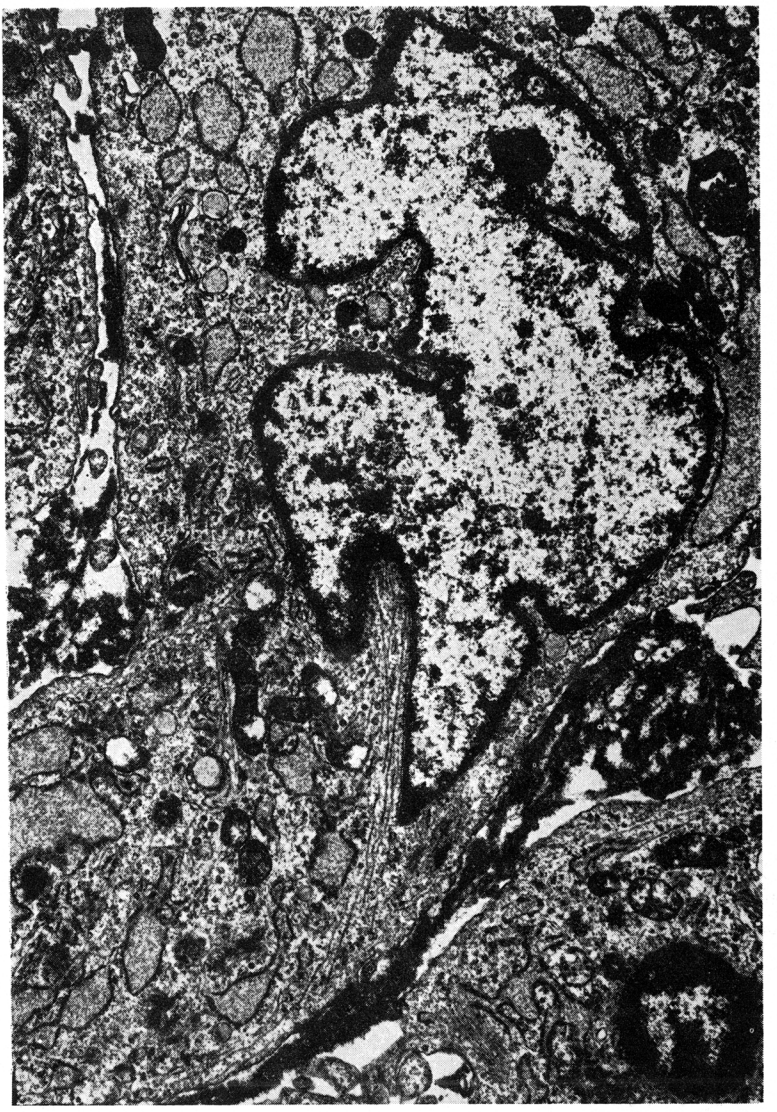

FIGURE 4. Tissue culture (R323) derived from an infantile fibrosarcoma. The cell illustrated is representative of the culture in general. Note the prominent nucleolus, the irregular nuclear shape and the cytoplasm rich in organelles. Large numbers of small vesicles in the Golgi area are to be differentiated from viral particles. Pinocytotic vesicles with dense deposits are also common. The endoplasmic reticulum is distended with moderately dense material and gives the cell its characteristic fibroblast appearance. Microtubules and polyribosomes are prominent. Mitochondria are sparse and rather small. Occasional cells contain aggregations of a few glycogen granules. The cells are surrounded by the dense fibrillary material interpreted as a precursor of collagen.

Virus Particles in an Infantile Fibrosarcoma

In our own laboratories a systematic search to detect viruses in human sarcomas has been underway since 1969. Herpes-type viruses, Epstein-Barr virus and cytomegalovirus were recovered from several of our case materials; C-type virus particles were found in one case, — a tissue culture derived from an infantile fibrosarcoma. The ultrastructural characteristics of the tumor with case history have been reported.[22] The infant, son of diabetic parents, was found at three weeks of age to have a tumor mass on his posterior upper thigh. The tumor, a 3.5 cm spherical mass, was excised at age 3.5 months and the child is well without recurrence during the three years that have followed. The tumor consisted of poorly differentiated fusiform cells without recognizable collagen on histologic sections. The tumor was thought to be compatible with fibrosarcoma, but a malignant Schwannoma could not be ex-

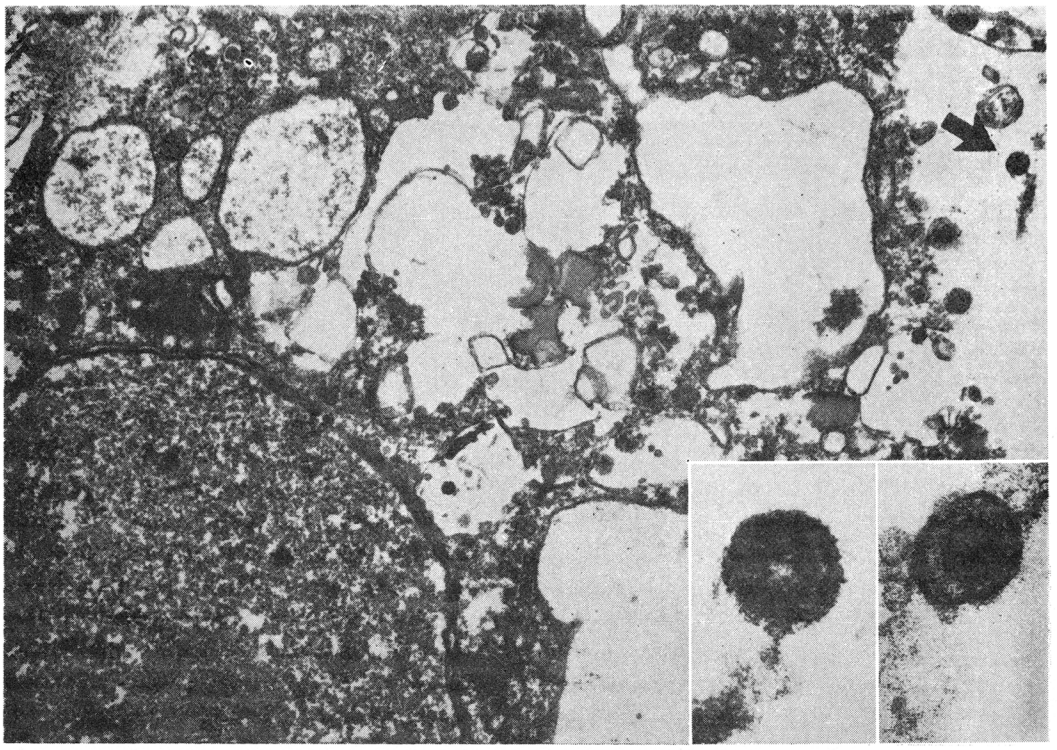

FIGURE 5. Tissue culture derived from infantile fibrosarcoma with C-type particle (arrow). Original magnification × 15,000. Insert: left, same particle, enlarged. This late bud is between the phases depicted as h. and i. in figure 3. Magnification × 100,000. Right, a free particle, corresponds in maturation to the phase depicted as i, in figure 3. Magnification × 100,000. Note: These particles are somewhat larger than murine C-type particles.

cluded. The ultrastructure revealed mesenchymal cells with some differentiation toward fibroblasts and occasional cells producing collagen fibers. An intercellular electron dense material (protocollagen?) was also present; it was prominent even in the tissue culture derived from the tumor.

Tissue Culture (R 323) and Virus Detection

Cultures were started in 250 ml Falcon plastic flasks. Sterile tumor tissue, minced to approximately one mm^3 fragments, was overlaid with McCoy's 5A medium containing 20 percent fetal bovine serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml. The cultures were incubated at 37° in an atmosphere containing 5 percent carbon dioxide. The primary cultures grew into confluent monolayers in 17 days. They were split 1:3 for the first passage. Confluent monolayer was obtained in 22 days. First passage cultures were split 1:2 for second passage. Growth stopped, and on the 19th day in the second passage the media was changed to Eagle's minimum essential medium (MEM) with 20 percent fetal bovine serum. In this medium, the cultures recovered in 16 days. The cultures from this point were carried in MEM with 20 percent fetal bovine serum. The following passages were split 1:4 and grew into confluent monolayers in eight days. Cultures were carried to the 14th passage and several early passages were saved frozen in liquid nitrogen.

In culture, the cells appear fibroblastic (figure 4). Cytoplasmic vacuoles and

dense granules are more prominent than in sections of the tumor. The cells scraped from the flask with a rubber policeman were fixed and prepared for electron microscopy and were screened for the presence of viral particles.

The cells harvested from minimum EM were fixed in 3 percent glutaraldehyde, adjusted to pH 7 and an osmolarity of 490 milliosmoles with Sorensen's phosphate buffer. The material was post fixed with osmic acid in Milloning's buffer and embedded in epon. The sections were stained with uranyl acetate and lead citrate. Many of the cells were ruptured. Those that were intact had unit membranes with few small microvilli. Few lipid droplets were present. Electron lucent vacuoles and dilated rough endoplasmic reticulum (cisternae) were numerous. The hyaloplasm was densely packed with microfilaments and occasional microtubules were seen. Polyribosomes free in the hyaloplasm were seen aggregated in some areas. Few glycogen granules aggregated in some of the cells into modest masses. Mitochondria, centriole and Golgi apparatus were inconspicuous. The nuclei were irregular with some infolding of the nuclear membrane. The nuclear chromatin was variously distributed.

First passage culture was extensively screened for the presence of virus particles. C-type particles were observed in the extracellular spaces and within vacuoles; particles were extremely rare. Some of the particles are illustrated in figure 5. The viral envelope and intermediate layer are well illustrated as is the central nucleoid. The central nucloid measures up to 80 nm and the envelope up to 150 nm. The average diameter of 16 particles measured is 144 nm. Morphologically, these particles are similar to those described in a liposarcoma culture by Morton et al[17] and resemble murine and avian type C particles.

Reverse transcriptase activity associated with the presence of high density (70S) RNA was detected in the 7th passage of this fibrosarcoma culture.* The culture exhibited activity of the same order of magnitude as avian MC 29 virus infected fibroblast cultures, which were used as controls. This finding provides added support that the viral particles belong to the oncornavirus group even though the particles found in the tumor and the tissue culture were few and therefore do not entirely satisfy the strict morphologic criteria. All our efforts to establish a virus producing cell line from this culture were unsuccessful.

Summary

In summary, attention is called to available standards and strict criteria for detection of oncornaviruses by electron microscopy. C-type particles are ubiquitous in three orders of animals. They were demonstrated to be most efficient carcinogens in a wide variety of species from birds to subhuman primates. They were sighted in numerous human specimens from leukemic patients and patients with various sarcomas. C-type particles were sighted in human fetal tissues and in placenta. Emphasis has been made by us that viruses are morphologic as well as biochemical entities and although electron microscopy is not the most sensitive method for detection, identification of the biochemist's "particles" will eventually have to be confirmed by morphologic observations.

Acknowledgments

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* By Dr. George S. Beaudreau of Oregon State University.

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