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Transformation of Mouse 3T3 Cells by Murine Sarcoma Virus: Release of Virus-Like Particles in the Absence of Replicating Murine Leukemia Helper Virus

(density gradient centrifugation/electron microscopy/[^{3}H]uridine)

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ABSTRACT **Small numbers of virus-like particles were observed by electron microscopy in each of two cloned lines of 3T3 cells transformed by murine sarcoma virus, even though these lines were free of detectable quantities of infectious leukemia and sarcoma virus. The morphology and occurrence of the particles were identical to those of the murine leukemia–sarcoma group. Moreover, the particles incorporated uridine and had a buoyant density of 1.16 g/ml in sucrose gradients. No evidence of sarcoma or leukemia virus infectivity was associated with the particles in cells of several susceptible species under various conditions, including both cosedimentation with leukemia virus and infection in the presence of inactivated Sendai virus. The particles may represent a form of murine sarcoma virus deficient in one or more of the viral components necessary for infectivity.**

Several continuous lines of mouse embryo cells are susceptible to transformation and focus formation by murine sarcoma virus (MSV) (1, 2). Titration patterns of MSV in various isolates of mouse 3T3 cells indicate that focus formation in monolayer cultures under the usual assay conditions is dependent on dual infection by MSV and a murine leukemia virus (MuLV), which acts as a "helper" (1, 2). Thus, the behavior of MSV in mouse 3T3 cells, under certain culture conditions, is identical to that originally reported for MSV focus formation in mouse embryo cells (3). However, when culture methods are used that permit the multiplication of single transformed cells, while restricting reinfection of cells with progeny virus, both cell transformation and focus formation by MSV occur in the absence of MuLV "helper" virus and the properties of 3T3 cells infected with MSV alone can be examined (2, 4).

Growth of cells in semisolid agar suspension cultures is one such selective procedure that favors the multiplication of transformed cells and, at the same time, restricts virus spread (5). When 3T3 cells are infected with the Moloney isolate of MSV and plated as suspension cultures in semisolid agar, colonies of transformed cells can be seen after a suitable incubation period (2). The quantitative aspects of colony formation in this system indicate that cell transformation depends only on infection with MSV and occurs independently of infection with MuLV "helper" virus. Indeed, certain lines of MSV-transformed cells isolated from individual semisolid agar colonies contain a rescuable MSV genome in the absence of leukemia virus replication and have been designated "sarcoma-positive, leukemia-negative (S+L−)" (2). S+L− cells do not contain detectable quantities of focus-forming MSV unless superinfected with MuLV, after which both MSV and progeny MuLV are readily recovered (2). Transformation of mouse cells by MSV, therefore, does not require the replication of MuLV, and the "helper" activity of MuLV must involve either a quantitative or a qualitative effect on MSV replication.

In addition to semisolid agar suspension cultures, monolayer cultures of 3T3 cells have also been used to study cell transformation by MSV in the absence of MuLV "helper" virus. When the BALB/3T3 cell line was infected with limiting dilutions of MSV, small foci of transformed cells, apparently resulting from solitary infection with MSV, could be seen after normal incubation periods (4). Isolation of individual foci under these conditions gave rise to "nonproducer" lines that contained MSV-transformed cells. Nonproducer cells do not release detectable quantities of MuLV or MSV, do not produce visible C-type particles, and do not seem to contain MuLV group-specific antigens (4). Addition of MuLV to such cells is followed by the release of focus-forming MSV and progeny MuLV.

In view of the similarities between the murine leukemia–sarcoma group of viruses and the analogous avian leukemia–sarcoma complex (6), we examined cloned S+L− mouse cell lines in order to determine whether their behavior is similar to that reported for the corresponding leukemia-negative Rous cells in which virus particles (7), group-specific antigens (8), and an altered form (9, 10) of sarcoma virus were found. This report describes the presence of apparently noninfectious, virus-like particles in two lines of S+L− mouse cells, analogous to similar particles previously reported in avian cells.

MATERIALS AND METHODS

Cell culture techniques

The isolation of S+L− cells from semisolid agar colonies of transformed 3T3 cells was described (2). For the experiments described below, two of the original S+L− cell isolates were

Abbreviations: MSV, murine sarcoma virus; MuLV, murine leukemia virus; S+L−, sarcoma-positive, leukemia-negative (3T3 cells).

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cloned by plating at limiting cell dilutions in microplates (Falcon Plastics, Microtest II). Individual colonies were isolated and the cloning procedure was repeated, giving rise to two different S+L− lines that were each twice cloned, each derived from a different semisolid agar colony.

MSV assay with 3T3FL cells, as well as the use of DEAE-dextran and optimal amounts of murine leukemia helper virus in MSV assays, have been described (2). Assays for infectious leukemia virus were based on the induction of lytic foci in S+L− cells after superinfection with MuLV (11).

Cat- and human-embryo cells were infected with a feline pseudotype of MSV under conditions that permit adequate focus visualization (12, 13).

Normal rat kidney cells were provided through the courtesy of Dr. R. C. Y. Ting, Bionetics Research Laboratories, Bethesda, Md. Assays with these cells were by the same techniques as those used for MSV assays in 3T3FL cells, except that kidney cells were plated at a concentration of 80,000 cells per 60-mm plastic Petri dish. Cultures were examined for up to 10 days for evidence of transformation and were often subcultured for several passages after infection in an effort to detect transforming activity in the test material.

The medium used for growth and maintenance of all cells, as well as for virus assays, was McCoy's 5a (Modified) supplemented with 10% fetal calf serum (Grand Island Biological Co.). For incorporation of radioactive uridine, cells were grown in enriched Eagle's Minimum Essential Medium (Hanks' salts) supplemented with 10% fetal calf serum.

Viruses

Moloney leukemia virus was obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Md., as crude tissue culture fluids from JLSV-9 (14) cells infected with this strain. This material contained 5×10^5 focus-inducing units (FIU) per ml when assayed in S+L− cells (11).

A second preparation of the Moloney leukemia virus, consisting of a 10% suspension of infected mouse spleens, was obtained from University Laboratories, Highland Park, N.J., and was used as a source of "helper" virus in MSV assays (3).

A feline leukemia virus pseudotype of MSV [MSV(FeLV)] (12) was obtained by filtration of tissue-culture fluids from infected cat cells. This preparation contained $10^{5.8}$ FFU/ml when assayed in feline cells pretreated with DEAE-dextran in the presence of optimal amounts of feline leukemia "helper" virus.

B-34 virus, a hamster-specific variant of MSV (Harvey) (15), was obtained from supernatants of B-34 cell cultures.

Electron microscopy

Cell monolayers were detached by gentle scraping with a rubber policeman, concentrated by centrifugation (1,000 rpm for 10 min), and washed in Dulbecco's phosphate-buffered saline (pH 7.2) before primary fixation with 5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 2 hr at 4°C. After glutaraldehyde fixation, the cells were washed three times in 0.1 M sodium cacodylate–0.2 M sucrose (pH 7.3). Cells were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate–0.2 M sucrose for 1 hr at 4°C, bathed in 0.5% aqueous uranyl acetate for 1 hr at room temperature, dehydrated in a graded ethanol series, passed through propylene oxide, and embedded in an Epon–Araldite mixture. Thin sections were poststained with uranyl acetate and lead citrate.

Radioactive labeling

Parallel cultures of normal 3T3FL cells, S+L− cells, and S+L− cells superinfected with a saturating amount of MuLV were labeled by the addition of 500 μCi of [5,6-^{3}H]uridine (New England Nuclear) to each 75-cm^2 flask. Culture fluids were harvested 24 hr after labeling and were immediately clarified by low-speed centrifugation ($10,000 \times g$, 20 min, 4°C).

Density gradient centrifugation

Clarified culture fluids (60 ml per sample) were treated with polyethylene glycol, at a final concentration of 5%, for 16 hr at 4°C (16). The resulting precipitate was sedimented, resuspended in one-tenth the original volume of buffer (0.1 M NaCl–0.01 M Tris-Cl (pH 7.4)–0.001 M EDTA (17), layered on a discontinuous sucrose gradient, and centrifuged (SW 41 rotor, 40,000 rpm, 75 min, 5°C) (17). Material banding at the density interface between the light (20%) and the heavy (65%) sucrose solutions was recovered and dialyzed against the same buffer to remove the sucrose. The dialyzed samples were layered on 12 ml of a linear sucrose gradient (10–65% sucrose in this buffer) and sedimented to equilibrium (SW 41 rotor, 40,000 rpm, 16 hr, 5°C). Fractions (500 μl) were collected dropwise from the bottom of the centrifuge tubes, and the density of every fifth fraction was determined refractometrically.

Trichloracetic acid-precipitable radioactivity in 0.1-ml aliquots of the fractions obtained after density gradient centrifugation of culture fluids was determined by the "Millipore" filtration technique (18), using 200 μg of yeast RNA as a carrier and 0.45-μm filters. Samples were counted in a Beckman LS-250 liquid scintillation counter.

Cosedimentation

Formation of interviral aggregates of S+L− particles and murine leukemia virus after cosedimentation was attempted by a modification of Fischinger and O'Connor's procedure (12). S+L− fluids (11 ml) were mixed with 1 ml of a suspension of MuLV containing 5×10^5 focus-inducing units per ml and centrifuged at $55,000 \times g$ for 60 min in a Spinco SW 41 rotor. The viral pellet was overlaid with tissue culture medium and held for 30 min at 4°C before resuspension. Focus-forming activity was determined in 3T3FL cells; optimal amounts of "helper" virus were added to alternate assay dishes. As a positive control, $10^{6.5}$ FFU of MSV(FeLV) was cosedimented with MuLV and the resulting pellet was assayed for focus-forming activity in mouse cells.

Cell fusion with inactivated Sendai virus

Virus preparations consisting of undiluted cell culture fluids to which DEAE-dextran had been added to give a final concentration of 20 μg/ml were mixed with 5×10^6 3T3 cells and allowed to incubate at 37°C for 15 min. The virus–cell mixtures were then chilled to 4°C and UV-inactivated Sendai virus (kindly supplied by Dr. H. Hanafusa, Public Health Research Institute of the City of New York), diluted in Eagle's Minimum Essential Medium buffered with Hepes (pH 7.4), was added. Preparations were then incubated at 4°C for 30 min and at 37°C for 30 min with periodic shaking. After treatment with Sendai virus, the cells were centrifuged and plated at twice the usual concentration. Murine leukemia "helper" virus was added to alternate dishes immediately

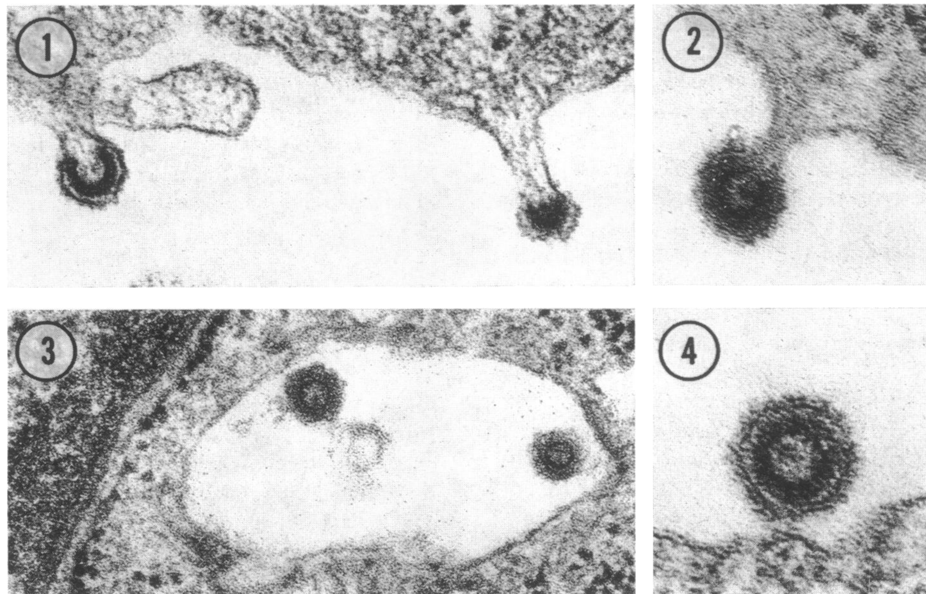

FIG. 1. Virus-like particles in early stages of budding from the cell membrane of an S+L− 3T3 cell. ×108,000.
FIG. 2. Particle in the final stages of budding. ×134,000.
FIG. 3. Particles observed in an intracellular membrane-bound vacuole. A portion of the S+L− cell nucleus can be seen. ×90,000.
FIG. 4. Virus-like particle, approximately 100 nm in diameter, after release from S+L− cell. The virion has an electron-lucent center surrounded by an electron-dense area. ×180,000.

prior to plating of cells. Plates were checked periodically for evidence of cell transformation, and supernatant fluids were tested for MSV-transforming activity.

RESULTS

Observation of virus-like particles in S+L− cultures

Examination of thin sections obtained from each of two cloned S+L− cell lines in the electron microscope demonstrated the presence of virus-like particles, conforming in size and morphology to viruses of the murine leukemia–sarcoma complex (Figs. 1–4). The particles measured 95–105 nm in diameter and contained a nucleoid consisting of an electron-lucent center surrounded by a dense ring-like structure and an outer limiting membrane. Particles were observed budding from the host cell membrane (Figs. 1 and 2), in intracellular membrane-bound vacuoles (Fig. 3), and extracellularly (Fig. 4). The morphology of the particles seen in S+L− cell lines was identical to that generally associated with C-type murine leukemia viruses.

Extremely small numbers of virus-like particles were seen in S+L− preparations, approaching the minimum number detectable by electron microscopy. In one quantitative study, only five particles were detected after examination of 200 cell sections. By contrast, S+L− cells superinfected with MuLV contained 50–100 times as many particles. No particles were observed in over 1000 cell sections of normal 3T3FL cells maintained in this laboratory under identical culture conditions.

Physicochemical properties of S+L− particles

The incorporation of radioactive uridine into fractions obtained after sucrose density gradient centrifugation of supernatant fluids from normal 3T3 cells, S+L− cells, and S+L− cells superinfected with MuLV is shown in Fig. 5. No peaks of radioactivity corresponding to particles with a density similar to that usually associated with murine leukemia virus were seen in fluids from normal 3T3 cells. Examination of fluids obtained from S+L− cells, however, showed a peak of [^{3}H]uridine incorporation corresponding to a density of 1.160 g/ml. A sample of this peak material, obtained from S+L− cells in a second experiment, was examined in the electron microscope by negative-staining procedures; typical C-type particles were observed, although no MSV or MuLV focus-forming activity could be detected. S+L− cells, therefore, release into the culture fluids particles that incorporate uridine and have a density similar to that regularly associated with viruses of the murine leukemia–sarcoma complex. Fluids obtained from S+L− cells superinfected with leukemia virus also showed incorporation of uridine into material with a density of 1.160 g/ml. Material recovered from this peak contained both infectious MSV and MuLV as determined by tissue-culture assay. The amount of [^{3}H]uridine incorporation in peak material from S+L− cells was only a fraction of that seen after superinfection with MuLV.

Typical microsomal peaks (ρ = 1.224) were also seen in all three samples. The unusually large microsomal peak found in material isolated from superinfected S+L− cells may be the result of extensive cell lysis after superinfection with leukemia virus (11).

Lack of infectivity of S+L− particles

All attempts to demonstrate cell-transforming activity of supernatants and cell lysates prepared from S+L− lines used in this study were negative, even though cloning experiments indicated that essentially all of the cells in one of the S+L− lines contained the MSV genome in a rescuable form. Infection of 3T3FL cells and mouse L cells did not result in any signs of cell transformation or focus formation. Addition of optimal amounts of MuLV helper virus (3), or pretreatment of cells with DEAE-dextran (19), did not result in observable transformation of cells by S+L− culture fluids. Similarly, infection of 3T3FL and L cells with 1000-fold concentrates of

S+L− culture fluids did not indicate any focus-forming activity. Also, 3T3FL cells chronically infected with Friend or Moloney leukemia virus were not transformed after infection with S+L− culture fluids. Neither normal nor feline-leukemia virus-infected cat cells were transformed by S+L− fluids, nor were human muscle-skin cells. Rat kidney cells, which have been shown to be susceptible to cell transformation and focus formation by a rat cell-derived variant of MSV in the absence of "helper" virus (20), also did not respond to infection with S+L− fluids, even when pretreated with DEAE-dextran.

In addition, two other techniques that are known to enhance the host range of RNA tumor viruses, cosedimentation and infection in the presence of inactivated Sendai virus, were each tried in an attempt to demonstrate infectivity of S+L− culture fluids.

Fischinger and O'Connor have shown that the host range of MSV pseudotypes can be altered after sedimentation in the ultracentrifuge in the presence of either murine or feline leukemia viruses (12). If the virus-like particles released by S+L− cells were a type of MSV with an altered host range, cosedimentation with the appropriate leukemia virus might be expected to result in the formation of an interviral aggregate possessing the transforming activity of MSV and the host range of the leukemia virus. Cosedimentation of an isolate of MuLV that readily infects 3T3FL cells with culture fluids obtained from S+L− cells was done according to the procedure of Fischinger and O'Connor (12). The resulting pellet did not contain any detectable focus-forming activity on 3T3FL cells, regardless of the addition of optimal amounts of MuLV "helper" virus and DEAE-dextran to the assay plates (Table 1). A preparation of MSV (FelLV) was included in the experiment as a positive control for the cosedimentation procedure. As previously reported (12), cosedimentation of MSV(FelLV) with MuLV resulted in the formation of virus aggregates able to transform mouse cells (Table 1). In a separate experiment, cosedimentation of feline leukemia virus (FelLV) and S+L− culture fluids did not result in any focus-forming activity when the resulting pellet was assayed in cat cells.

Infection of 3T3 cells with S+L− particles under conditions of cell fusion mediated by inactivated Sendai virus was also attempted. This technique has been used successfully to demonstrate infectivity of avian sarcoma virus preparations in otherwise resistant cells (21). No evidence for focus-forming activity or MSV replication was detected when S+L− culture fluids were used to infect 3T3 cells in the presence of inactivated Sendai virus, but a hamster-specific variant of MSV, B-34 virus (15), which does not ordinarily infect mouse cells, did induce a small number of foci in 3T3 cells in the presence of inactivated Sendai virus and optimal amounts of MuLV "helper" virus.

TABLE 1. *Cosedimentation of S+L− culture fluids and MuLV*

			FFU/ml*	
Group	Preparation	Centrifugation	(+)DEAE-dextran	(−)DEAE-dextran
I	S+L− fluids only	−	0	0
II	S+L− fluids only	+	0	0
III	S+L− fluids + MuLV	+	0	0
IV	MSV(FelLV) only	−	0	0
V	MSV(FelLV) only	+	0	0
VI	MSV(FelLV) + MuLV	+	115	220

* Optimal amounts of MuLV "helper" virus were added to all assay plates.

DISCUSSION

S+L− 3T3 cells are mouse cells that contain the MSV genome in the absence of detectable quantities of infective MSV or MuLV. We show that S+L− cells release relatively small numbers of virus-like particles that have several characteristics usually associated with members of the murine leukemia-sarcoma group of viruses. Electron microscopic observations of the particles present in S+L− cells show them to be identical in size and morphology to other murine leukemia and sarcoma viruses. In addition, S+L− particles mature by budding from cell surfaces.

Physicochemical properties of S+L− particles are also indistinguishable from those associated with murine leukemia and sarcoma viruses. The particles incorporate [^{3}H]uridine and have a buoyant density of 1.16 gm/ml in sucrose density gradients, properties regularly associated with murine leukemia-sarcoma viruses.

Infectivity studies with a variety of techniques and target cells have failed, thus far, to demonstrate any biological activity associated with S+L− particles. Infection of several different cell lines known to be susceptible to transformation by MSV, including the 3T3 mouse cell line and the normal rat

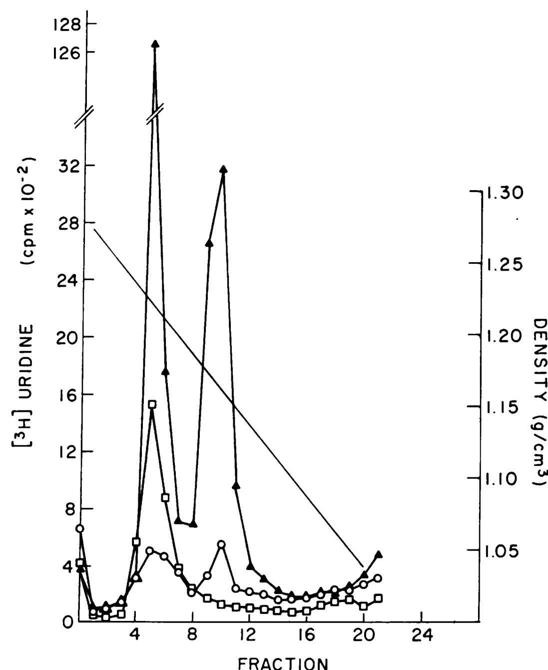

FIG. 5. Equilibrium density sedimentation patterns. Trichloracetic acid-precipitable radioactivity incorporated into 100-μl aliquots of fractionated culture fluids from 3T3FL cells, S+L− cells, and S+L− cells superinfected with MuLV after addition of [^{3}H]uridine. Absorbancy at 260 nm: 3T3FL, 0.249; S+L−, 0.176; superinfected S+L−, 2.140. See text for details. □——□ 3T3FL culture fluids; ○——○ S+L− culture fluids; △——△ superinfected S+L− culture fluids.

kidney cell line, did not result in any observable cell transformation. Techniques such as cosedimentation with "helper" viruses and infection of cells in the presence of inactivated Sendai virus, both of which have been shown to increase the host range of oncogenic RNA viruses, were also used in an effort to demonstrate transforming activity of S+L− particles. Cosedimentation of S+L− particles with MuLV did not result in focus-forming activity in 3T3FL cells, nor did infection under conditions of cell fusion mediated by UV-inactivated Sendai virus. In addition, no evidence of replicating MuLV could be found in either of two S+L− isolates as detected by the XC test (22). S+L− particles, therefore, differ from previously recognized forms of both MSV and MuLV in their inability to transform or to replicate in various cell-culture systems.

Some indication of the possible relationship of S+L− particles to MSV may be gained by comparison with the analogous avian system. Chick cells transformed with the Bryan high-titer strain of Rous sarcoma virus (RSV) in the absence of avian leukemia virus also produce virus-like particles, termed RSV(0) (10). One class of RSV(0) particles, RSVα(0), is not infectious for any known host cell, even under conditions of cell fusion, and may represent a "defective" form of RSV (21). A second class of RSV(0) particles, RSVβ(0), has been described that is infectious for certain types of avian cells, but has a host range different from that of RSV produced in the presence of avian leukemia virus. Furthermore, the host range of RSVβ(0) depends on the presence or absence of a factor in the host cell in which the sarcoma virus replicated. This factor has recently been identified and appears to be an avian leukosis virus (23, 24). RSVβ(0) particles obtained from cells lacking the factor are noninfectious under ordinary conditions, but are able to infect chick and quail cells under conditions of cell fusion mediated by UV-inactivated Sendai virus, a property that distinguishes them from RSVα(0) particles (25).

Particles released by S+L− mouse cells may thus represent a "defective" form of MSV, and may be analogous to RSVα(0) since they lack infectivity for mouse cells even in the presence of inactivated Sendai virus. However, the possibility that a susceptible host cell will be found after further investigation cannot be excluded.

Aaronson and Rowe have also reported the isolation of MSV-transformed mouse cells that do not produce detectable quantities of leukemia or sarcoma virus (4). In their case, however, there was no evidence for the presence of virus-like particles either by electron microscopy or by incorporation of [^{3}H]uridine. Several factors may account for this difference in the behavior of MSV-transformed cells including: (*a*) variations in the genetic properties of different stocks of MSV (cloned MSV originally rescued from the HT-1 hamster cell line was used in the studies of Aaronson and Rowe, while S+L− cells were obtained after infection of 3T3 cells with noncloned virus); (*b*) the presence in S+L− cells of a portion of the MuLV genome responsible for virus budding and particle formation. Such genetic information could have been present in 3T3FL cells before infection with MSV, similar to the case with the factor responsible for infectivity of RSVβ(0) present in certain chick cells, or may have been introduced into the cell at the time of infection with MSV, possibly in the form of a "defective" leukemia virus particle.

A detailed investigation of S+L− particles is being performed in order to determine their exact relationship to other viruses of the murine leukemia–sarcoma group. Preliminary results indicate that the particles do not contain appreciable amounts of RNA-dependent DNA polymerase (D. K. Haapala *et al.*, manuscript in preparation), and that they possess several of the antigens usually associated with MuLV. If S+L− particles represent a type of "defective" MSV, they may provide a unique opportunity to characterize MSV-specific RNA and proteins in the absence of MuLV.

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