Tubular Structures in S49 Mouse Lymphoma Are Regulated through In Vivo Host-Cell Interaction and In Vitro Interferon Treatment

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ABSTRACT Malignant S49 mouse lymphoma cells that grow in suspension culture demonstrate in their cytoplasm characteristic tubular structures. These structures also appear in immunogenic, substrate-adherent variants of S49 cells that grow in culture. Upon transfer of both cell types into nude mice, the tubular structures of the adherent variants (and not the suspension-growing cells) undergo a profound alteration whereby their tubular components disappear and clusters of viruslike particles appear. These very closely resemble, on morphological grounds, precursors of B-type retroviruses. This specific in vivo interaction between the host and the S49 variant can be mimicked in culture by treatment of these cells for 24 h with 500 U/ml of mouse interferon. The suspension-growing S49 cells are unresponsive to interferon in this respect. Immunohistochemical analysis reveals that both tubular structures and the viruslike particles represent stages in the morphogenesis of mouse mammary tumor virus. A working hypothesis is advanced relating the regulation of the tubular system to the impaired tumorigenic potential of adherent S49 cells in syngeneic Balb/c hosts.

Substrate-adhering variants with impaired tumorigenic potential have been recently selected from highly malignant, suspension-growing S49 mouse lymphoma cells (7, 10). These adherent variants manifested increased immunogenic ability insofar as their inoculation into syngeneic mice protected the hosts against subsequent challenges with suspension-growing malignant S49 cells.

In a previous report, we have shown that S49 cells contain a prominent cytoplasmic marker system that resembles tubuloreticular structures at the electron microscopic level (6). These tubular structures are characteristic of suspension-growing S49 cells as well as tumors and suspension-growing sublines derived from the tumors. S49 adherent variants grown in culture demonstrate the same tubular system.

In the present paper, we report that in vivo passage of adherent S49 cells in nude mice results in a profound morphological change in the tubular system characterized by the disappearance of the tubular components and their replacement by clusters of viruslike particles. Similar morphological changes can be induced in vitro by incubation of adherent (and not suspension-growing) S49 cells with mouse interferon (IFN)[1], thereby representing a novel feature of IFN activity. Furthermore, immunoelectron microscopy demonstrates that both tubular components and the viruslike particles are mouse mammary tumor virus (MMTV)-related structures.

MATERIALS AND METHODS

Cells: Parental S49 cells (maintained in suspension culture) and the substrate-adhering variants derived from them (7) were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated horse serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C, in a humidified atmosphere containing 5% CO_2. Viability was measured using trypan blue exclusion.

Tumors: Cells ($1-2 \times 10^7$) were inoculated intraperitoneally into congenitally athymic (nude) mice as previously described (8).

Electron Microscopy: For electron microscopy, cells growing exponentially (2×10^7) were washed once in phosphate-buffered saline (PBS). The pellet was fixed for 10 min with a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed in 1% osmium tetroxide. Dehydration in graded ethanols was followed by embedding in araldite. Blocks were sectioned

[1] *Abbreviations used in this paper:* IFN, interferon; MMTV, mouse mammary tumor virus.

with glass knives on an LKB III ultramicrotome (LKB Instruments, Gaithersburg, MD), double stained with uranyl and lead salts, and examined using a Jeol 100 CX or a Philips 300 electron microscope. Tumorous tissue was cut into small pieces, and the same procedure was carried out. Structures were counted directly in the electron microscope at a magnification of 8,000.

Immunoelectron Microscopy: For direct immunocytochemical analysis of the tubular structures, we have used the protein A-gold technique as adapted for osmium-fixed tissues (3). Briefly, thin sections mounted on nickel grids were pretreated for 60 min with a saturated aqueous solution of sodium periodate before processing for immunoelectron microscopy. The grids were then washed in PBS and put on a drop of PBS containing 0.5% bovine serum albumin (BSA) for 10 min. This was followed by incubation for 60 min at room temperature on a drop of specific antiserum properly diluted in PBS containing 0.5% BSA. The grids were then washed with PBS and incubated for 60 min at room temperature with protein A-gold complex prepared as previously described (2). Colloidal gold (150 Å in diameter) was prepared as previously described (4), using 1% sodium citrate. The grids were then washed in PBS, rinsed in distilled water, dried, stained with uranyl acetate and lead citrate, and examined in a Jeol 100 CX or a Philips 300 electron microscope. Rabbit antisera against MMTV and intracysternal A particles were a gift from Dr. G. Smith, National Cancer Institute, Bethesda, MD, and goat antisera against MMTV and Rauscher murine leukemia virus were a gift from the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Institutes of Health, Bethesda, MD.

Extracellular Virus-associated Reverse Transcriptase: Growth medium (24 h) was harvested, and cell debris were removed by centrifugation at 5,000 *g* for 10 min. The virus was pelleted by centrifugation at 100,000 *g* for 1 h, and resuspended in TNE buffer (20 mM Tris HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA). Reverse-transcriptase activity was determined by the incorporation of ^{3}H-dTTP (2,000 cpm/pmol) into acid insoluble material using poly (A) oligo (dT) as template primer (12). $MgCl_2$ (6 mM) was used as the divalent cation. Enzyme activity is presented per 10^6 cells counted at harvest time.

IFN: Mouse IFN was produced in L-cells after infection with Newcastle disease virus, and purified to a specific activity of 5×10^7 U/mg protein according to Paucker et al. (13). IFN at concentrations indicated in the legends was added directly to the growth medium for 24 h.

RESULTS

Suspension-growing S49 cells (11) demonstrate in their cytoplasm tubular structures with a distinct morphology (6) that appear in ~20% of the cells in electron microscopic cross-sections. These structures are characteristic of S49 cells grown in suspension culture, of tumors derived from these cells (in syngeneic as well as in nude mice), and of suspension-growing sublines derived from these tumors.

Substrate-adhering variants derived from the suspension-growing cells (10) demonstrate, when grown in culture, the same tubular structures. These structures (Fig. 1*A*) demonstrate both tubules of various length, which, at times, can reach up to 3 μm (6), and round particles closely associated with them. These will be defined now as the tubular system. The round particles might merely represent cross-sections of the tubules at different planes. However, on morphological grounds alone it cannot be excluded that the tubular system is composed of two unrelated elements: (*a*) tubules of various lengths and cross-sections in them visualized as round viruslike particles, and (*b*) an independent system of round viruslike particles not related biochemically to the tubular structures except for their close spatial association in the cytoplasm. Sometimes tubules can be seen that seem to be composed of subunits (Fig. 1*E*). Although the substrate-adhering variants are immunogenic and cannot grow as tumors in syngeneic hosts, they give rise to progressive tumors in nude mice. These tumors demonstrate profound morphological changes in the tubular system (Fig. 1, *B* and *C*). The tubular components disappear and are replaced by clusters of round particles. These will be defined now as the viruslike system. This transition occurs in ~95% of the systems (Table I) and is found both in the solid as well as the ascitic components of the nude mice tumors derived from adherent cells. It indicates that the state of the tumor cells in vivo (whether in solid mass or free floating) makes no difference in the expression of the viruslike system. It also demonstrates that the round particles that appear in these tumors are not cross-sections of tubular structures aligned in parallel, but rather separate entities.

The adherent S49 sublines ZN1, DC1, T25 adh, and T40 adh (7, 9) have been independently selected from different suspension-growing S49 clones, and have all demonstrated an identical transition from the tubular to the viruslike system when inoculated into nude mice. Thus, in culture, 85–95% of the systems observed are tubular, while only 5–15% are viruslike. In vivo, however, between 95 and 100% of the systems in all the sublines examined have the viruslike nature. When various suspension-growing sublines ACT4, T8, T25, and T40 (7, 9) that were independently derived from different S49 clones were inoculated into nude or syngeneic Balb/c mice, no such transition occurred. In all these tumors, a preponderance of tubular systems (97–100%) was observed. These findings demonstrate the generality of our observations in all S49 sublines.

When tumors derived from adherent cells were transferred back to in vitro culture, we observed a reversion from the viruslike system to the pretumor tubular state within 15–20 d (Fig. 2). Reinoculation of these cells into nude mice again resulted in the disappearance of the tubular system and the appearance of the viruslike system. These findings indicate that the significant changes manifested upon alternating the adherent cells between the in vitro and in vivo states are not due to a specific cell selection, but rather to a change conferred upon the whole cell population. Moreover, these findings demonstrate that the changes taking place in the tubular system are the consequence of a specific in vivo cell-host interaction linked to the substrate-adhering ability of these variant cells. A support for this suggestion comes from a preliminary study in which suspension-growing revertants selected from the adherent cells and inoculated into nude mice demonstrated tubular systems characteristic of suspension-growing S49 cells (Hochman, J., and N. Mador, manuscript in preparation).

Effect of IFN

IFN is known to suppress assembly and budding of various retroviruses (5). Since the round viruslike particles very closely resemble cytoplasmic A particles, known to be intracellular precursors of B-type retroviruses (15), we examined the effect of IFN on the distribution of the tubular and viruslike systems in cultured adherent S49 cells (Table I). Whereas the majority

FIGURE 1 Electron micrographs of tubular and viruslike systems in S49 cells. (*A*) Tubular system in adherent cell culture (× 48,000); (*B*) adherent cell in tumor (× 9,250). Arrowheads mark viruslike system; (*C*) viruslike system in adherent cell tumor (× 61,000); (*D*) viruslike system in adherent cell culture treated with 500 U/ml IFN for 24 h (× 61,000); (*E*) section of a tubular system (× 70,000). Arrowheads mark subunits. Bars, 0.1 μm (*A*, *C–E*) and 1.0 μm (*B*).

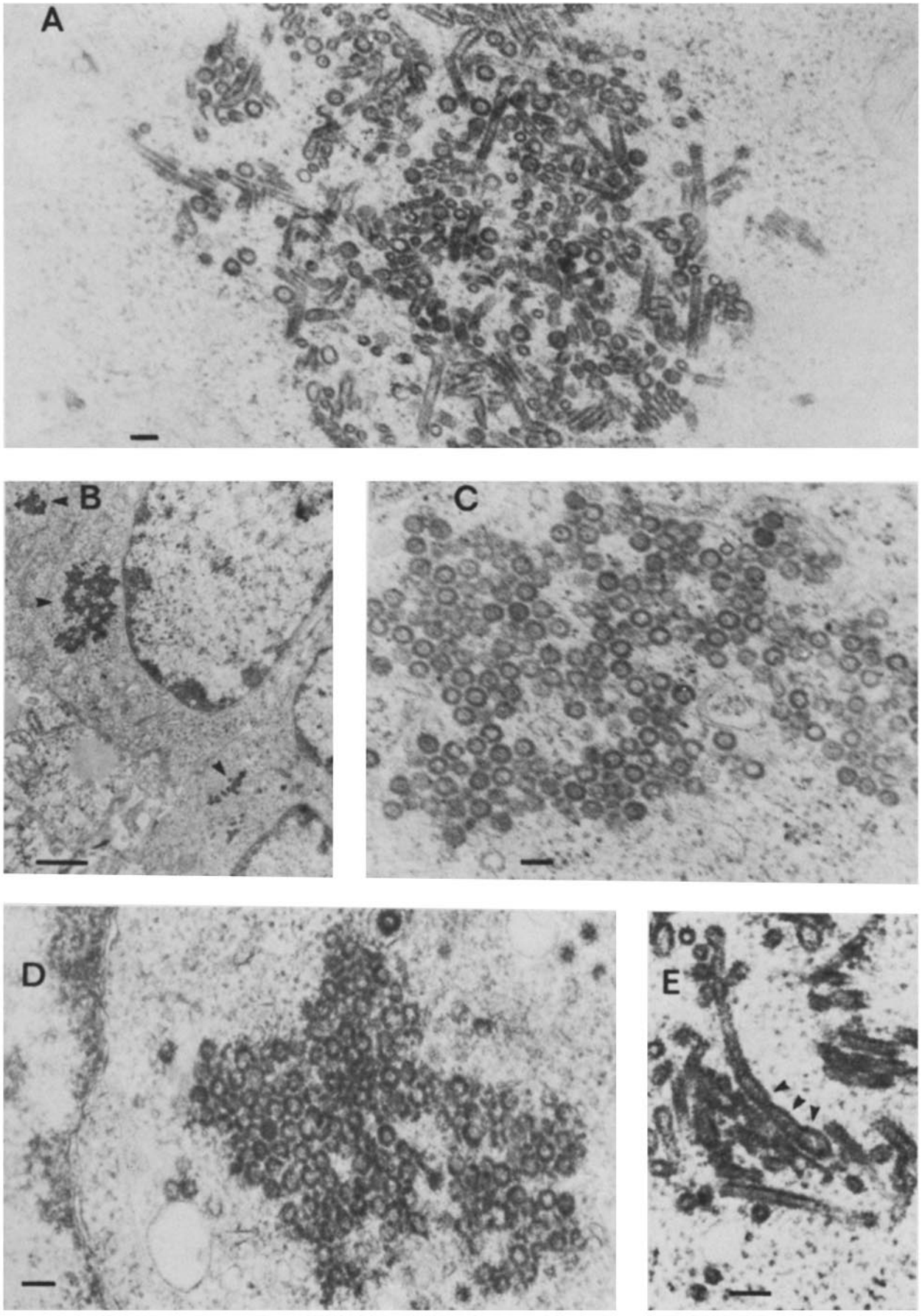

TABLE I

Effect of IFN on the Distribution of Tubular Structures and Viruslike Particles in Suspension Growing and Adherent S49 Cells

Cells	Treatment	Structure distribution		n
		Tubular system	Viruslike system	
		%		
Suspension cell tumor in Balb/c and nude mice	—	95 ± 1	5 ± 1	10
Adherent cell tumor in nude mice	—	5.1 ± 0.5	94.9 ± 0.5	10
Suspension cell culture*	—	98 ± 2	2 ± 2	5
Suspension cell culture	IFN§	90 ± 8	10 ± 8	4
Adherent cell culture‡	—	85 ± 10	15 ± 10	8
Adherent cell culture	IFN	24 ± 10	76 ± 10	6
Adherent cell culture	Serum-starved	89 ± 2	11 ± 2	2

The data represented are the mean ± S.D. of 2–10 different experiments (*n*). In the cell lines examined, 20–30% of cell sections demonstrated either one of the systems. That is referred to as 100% in this table.

* Subline T25 (7).

‡ Subline T25 adh (7).

§ 500 U/ml per 24 h.

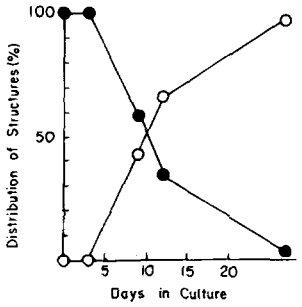

FIGURE 2 Kinetics of the distribution of tubular (○) and viruslike (●) systems in adherent S49 cells after transfer from a nude mouse tumor into culture. At each time point, 200 cellular cross-sections were counted. These represent ~50 systems (tubular and viruslike).

of systems (~ 90%) seen in the adherent cells grown in culture are of the tubular type, >70% of the structures observed after a 24-h treatment with 500 U/ml of IFN were composed of round viruslike particles.

This effect appears to be specific for the mouse IFN, since treatment with heat-inactivated mouse IFN or heterologous human IFN-α had no effect on the ratio of the tubular to viruslike system (not shown). Such IFN treatment inhibited retrovirus production in suspension growing and adherent cells, as indicated by the suppression of the reverse transcriptase activity released into the growth medium (Table II).

To investigate the possibility that the effect on the distribution of the two systems in adherent cells is induced by the antiproliferative activity of IFN, we inhibited cell growth by an alternative procedure, namely, serum depletion for 24 h (Table I). Since the latter procedure for growth arrest had no effect on the ratio of tubular and viruslike systems, IFN appeared to act through a different mechanism.

Electron micrographs of suspension-growing S49 cells, in tumors as well as in culture, reveal predominantly structures with tubular components similar to the adherent variants in culture (Fig. 1). In contrast to the adherent cells, however, treatment of suspension-growing cells with IFN has no significant effect on their tubular systems (Table I). Therefore, the effect of IFN on substrate-adherent variants in vitro mimics in this respect the interaction of these cells with their host in vivo. The percentage of cultured cells demonstrating tubular structures is somewhat decreased by IFN in both suspension growing and adherent S49 cells.

Immunocytochemial Analysis of Tubular Structures

To explore the molecular identity of the tubular as well as the viruslike systems, we applied the protein A-gold immunocytochemical technique (see Materials and Methods) using specific antisera against MMTV, murine leukemia virus, and intracysternal A particles. Fig. 3 demonstrates that antibodies to MMTV recognize the tubular structures (Fig. 3*A*). These antibodies recognize both the tubular components and the round viruslike (cytoplasmic A) particles in adherent (Fig. 3*A*) as well as in suspension-growing cells (not shown). Furthermore, these antibodies also clearly recognize the round viruslike particles in a tumor of adherent cells in a nude mouse (Fig. 3*B*) as well as in adherent cells treated with IFN (not shown). Antibodies to murine leukemia virus (Fig. 3*C*) and antibodies to intracysternal A particles (not shown) cannot recognize these structures in both adherent and suspension-growing cells. These findings demonstrate that the two components (tubules and round particles) share a close structural similarity with MMTV and might therefore represent different cytoplasmic stages in the morphogenesis of this type-B retrovirus.

TABLE II

Effect of IFN on Retrovirus Release in Suspension Growing and Adherent S49 Cells

Cells	Treatment	Retrovirus release
		Reverse transcriptase
		cpm/10⁶ cells
Suspension cell culture*	—	2,164
Suspension cell culture	IFN§	670
Adherent cell culture‡	—	1,990
Adherent cell culture	IFN	200

* Subline T25 (7).

‡ Subline T25 adh (7).

§ 500 U/ml per 24 h.

DISCUSSION

In this paper, we demonstrated that the tubular systems present in substrate-adherent variants derived from S49 lymphoma cells undergo a characteristic change upon passage in vivo (nude mice) or IFN treatment in vitro (culture). The loss of the tubular components occurs concomitantly with the appearance of compact clusters of round particles. These resemble cytoplasmic A particles known to be the cytoplasmic precursors of the type B retrovirus MMTV. Indeed, both components were found to share antigenic similarity with MMTV as shown by immunoelectron microscopy. It is therefore suggested that they represent two distinct stages in the morphogenesis of MMTV. In addition, the findings that the tubular components appear, at times, to demonstrate a sub-

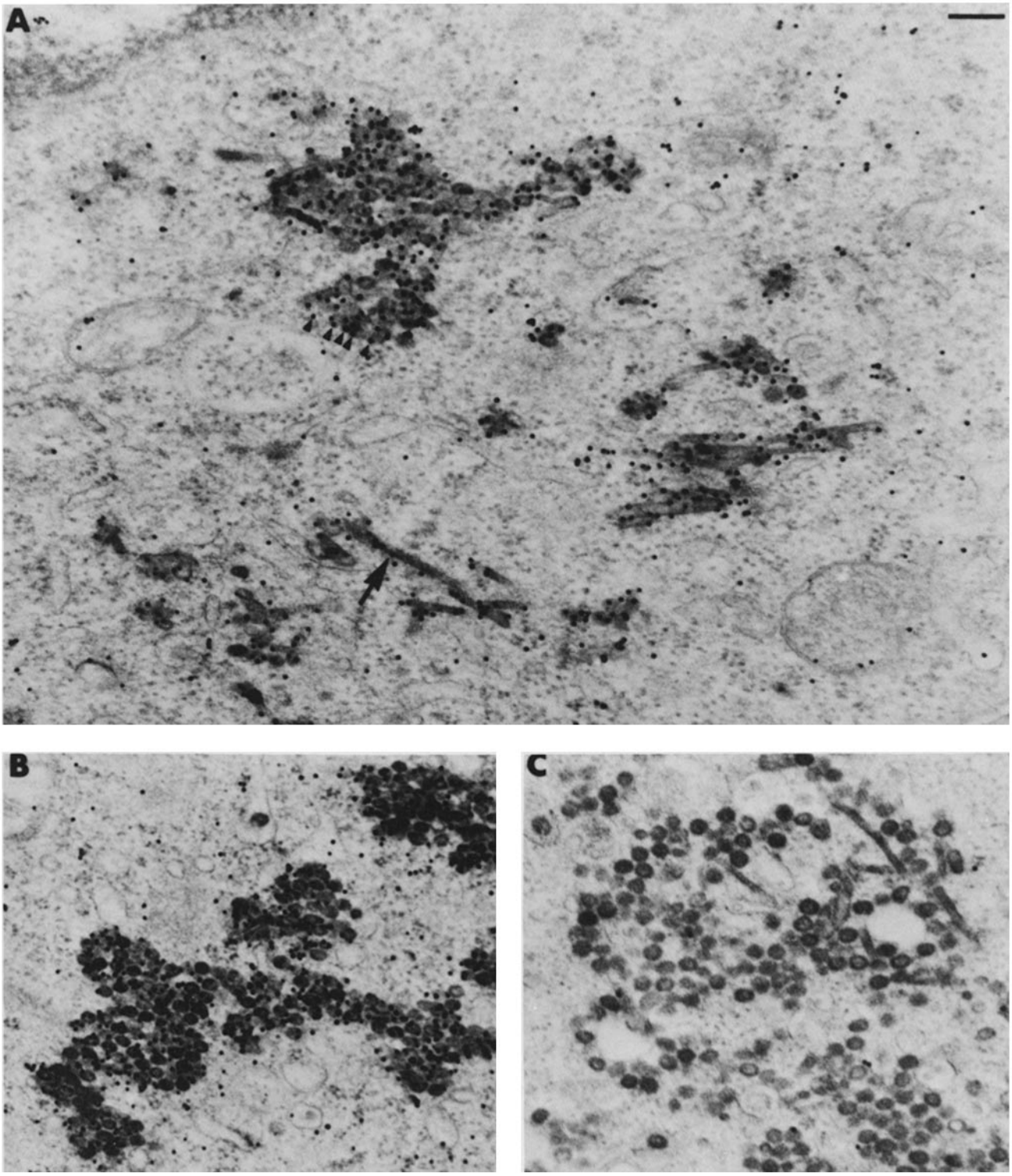

FIGURE 3 Immunoelectron microscopic analysis of tubular and viruslike systems in S49 cells. (*A*) Tubular system in adherent cell culture with anti-MMTV; (*B*) viruslike system in adherent cell tumor in nude mouse with anti-MMTV; (*C*) section of a tubular system in adherent cell culture with anti-murine leukemia virus. Bar, 0.2 μm; all magnifications, × 48,000.

unit structure (Fig. 1 *E*), might suggest a precursor-product relationship between the two viral components. Tubular intermediates in the morphogenesis of MMTV have not been reported before. However, it is noteworthy that in an earlier report (1), it was demonstrated that cytoplasmic A particles in a murine ependymoblastoma were closely associated, albeit infrequently, with tubular structures very similar to those in our model system. This supports our assumption that the tubular components are precursors in the normal morphogenesis of MMTV.

The above-mentioned morphological changes are the result of in vivo tumor-host interaction specific to the adherent cells only, and do not take place in tumors (either in nude or syngeneic mice) derived from suspension-growing S49 cells.

The findings that incubation of adherent cells with IFN in vitro can very closely mimic this tumor-host interaction, and that parental suspension-growing S49 cells are unaffected in this manner, offer a new aspect of IFN's mode of action. The effect of IFN on the morphogenesis of MMTV in adherent cells is independent from its inhibitory effect on the budding and release of mature viruses to the extracellular medium. That is because in both suspension growing and adherent cells, IFN significantly decreases reverse transcriptase activity (Table II).

Human IFN was recently found to induce "lupus inclusions" in cells of several human B and T lymphoblastoid cell lines (14). These inclusions are microtubular structures, somewhat resembling our model system, that are found in the glomerular endothelium and the peripheral blood lymphocytes of patients with systemic lupus erythematosus. Their nature and function are unknown, and they were referred to as "viruslike" (14). Taken together with our findings, it is tempting to speculate that these also represent events in the morphogenesis of endogenous viruses affected by IFN.

On the basis of the present data and our previous findings (7, 10) that adherent S49 cells demonstrate increased immunizing ability when compared with their parental suspension-growing cells, we propose the following working hypothesis. Upon inoculation of adherent S49 cells into nude (or syngeneic mice), a specific response to host factors occurs. These factors, of which IFN might be a representative, induce a chain of events whereby relatively stable intermediates (visualized as tubular structures) of endogenous MMTV are largely converted, within 24 h, into cytoplasmic A particles. This conversion results in a subsequent increase (or change of distribution) of MMTV-related antigens on the cell surface, with no budding or release of active viruses thereafter. These morphogenetic events render the adherent cells more immunogenic in the syngeneic host that ultimately rejects them. In the nude mice, a similar chain of events takes place but without immune rejection. Therefore, the profound changes in the tubular structures can be easily studied in these mice. Of particular interest in this respect are preliminary findings (Hochman, J., and N. Mador, manuscript in preparation) revealing that adherent cells rescued from syngeneic mice 3 d postinoculation demonstrate a shift from tubular structures to cytoplasmic A particles. When suspension-growing S49 cells are inoculated into both nude and syngeneic mice, their interaction with host factors does not result in altered MMTV morphogenesis, thereby allowing their progressive uninterrupted growth in nude as well as in syngeneic hosts.

In addition, our studies suggest that the various S49 sublines be used as a model system for the study of the morphogenesis of MMTV as well as the effect of IFN on this virus maturation.

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REFERENCES

1. Ames, R. P., and R. C. Rubin. 1970. Morphology of virus-like particles persisting in murine ependymoblastoma *in vitro*. *Cancer Res.* 30:1142–1148.
2. Bendayan, M., J. Roth, A. Perrelet, and L. Orci. 1980. Quantitative immunocytochemical localization of pancreatic proteins in subcellular compartments of the rat acinar cells. *J. Histochem. Cytochem.* 28:149–160.
3. Bendayan, M., and M. Zollinger. 1983. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. *J. Histochem. Cytochem.* 31:101–109.
4. Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold solutions. *Nature Phys. Sci.* 241:20–22.
5. Friedman, R. R. 1977. Antiviral activity of interferons. *Bacteriol. Rev.* 41:543–567.
6. Hochman, J., N. Ben-Ishay, and M. Castel. 1982. Tubuloreticular structures in S49 cells: relation to cAMP-dependent protein kinase. *Exp. Cell Res.* 142:191–195.
7. Hochman, J., A. Katz, E. Levy, and S. Eshel. 1981. Substrate adhering lymphoid cells show impaired tumorigenicity and increased immunogenicity. *Nature (Lond.).* 290:248–249.
8. Hochman, J., A. Katz, and Y. Weinstein. 1979. A transplantable mouse tumor deficient in cyclic-AMP-dependent protein kinase activity. *Eur. J. Cancer.* 15:11–16.
9. Hochman, J., and E. Levy. 1984. Growth regulation in S49 mouse lymphoma: involvement of cAMP and substrate adhesiveness. *In* Advances in Cell Growth Regulation. C. G. Bolis, L. Frati, and R. Verna, editors. Plenum Publishing Corp., London. 155–168.
10. Hochman, J., E. Levy, N. Mador, M. M. Gottesman, G. M. Shearer, and E. Okon. 1984. Cell adhesiveness is related to tumorigenicity in malignant lymphoid cells. *J. Cell Biol.* 99:1282–1288.
11. Horibata, K., and A. W. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell. Res.* 60:61–77.
12. Panet, A., and Z. Kra-Oz. 1978. A competition immunoassay for characterizing the reverse transcriptase of mammalian RNA tumor viruses. *Virology.* 89:95–101.
13. Paucker, K., B. J. Berman, R. R. Golgher, and D. Stancek. 1970. Purification, characterization and attempts at isotopic labeling of mouse interferon. *J. Virol.* 5:145–152.
14. Rich, S. A. 1981. Human lupus inclusions and interferon. *Science (Wash. DC).* 213:772–775.
15. Smith, G. H., and B. K. Lee. 1975. Mouse mammary tumor virus polypeptide precursors in intracytoplasmic A particles. *J. Natl. Cancer Inst.* 55:493–496.