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Electron Microscopy of Chick Fibroblasts Infected by Defective Rous Sarcoma Virus and Its Helper

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Nonproducing Rous sarcoma cells of the chicken and their virus-producing as well as uninfected counterparts were studied with an electron microscope. The structural peculiarities of transformed cells included cytoplasmic annulate lamellae, aggregates of membrane-bound, glycogen-like granules, and empty, virus-like shells. Of 69 individual lines of nonproducing Rous sarcoma cells, 64 contained small numbers of viral particles. These particles were morphologically indistinguishable from mature avian tumor virus but lacked demonstrable infectivity. In sessile normal and leukosis virus-infected fibroblasts, microtubules and fibrils occurred in parallel arrays at the periphery of the cytoplasm. This cortical organization was absent from rounded Rous sarcoma cells. The characteristics of microtubular arrangement seemed to reflect differences in the locomotory activity of normal and transformed cells.

The Bryan strains of Rous sarcoma virus (RSV) are capable of transforming chicken fibroblasts without inducing the maturation of progeny virus (23, 47). Nonproducing Rous sarcoma cells are generally found after inoculation of tissue cultures with a high dilution of virus. Extensive analyses have shown that the nonproducing state always follows solitary infection with RSV. Under such conditions, maturation of infectious virus is regularly absent from Rous sarcoma cells, but may be initiated by superinfection of these cells with an avian leukosis virus. These findings indicate a defectiveness of RSV with regard to virus production and the need for a helper virus which can supply the missing link in virus maturation (23).

Nonproducing Rous sarcoma cells are free from viral envelope antigen, and RSV progeny released after superinfection with helper virus carries the envelope antigen of the helper (22, 24, 48). This suggests that the defect of Rous sarcoma virus lies in the production of the viral envelope, and that it is this viral component which must be provided by the helper.

Nonproducing Rous sarcoma cells are, however, not free from viral material per se. They contain the viral genome, as demonstrated by the activability of virus production after infection with helper virus in all such cells (23). In addition, nonproducing Rous sarcoma cells contain the group-specific antigen of the avian tumor viruses (12, 51). This antigen appears to belong to an internal component of the virion (1, 26, 28, 32).

The presence of such viral material in nonproducing Rous sarcoma cells suggested that morphologically identifiable precursors of RSV might be found in such cells, and prompted the electron microscopic investigations reported in this communication. Similar expectations led Dougherty and Di Stefano (10, 12) to study the ultrastructure of virus-producing and nonproducing Rous sarcoma cells. The principal observations coming from the two laboratories are in excellent agreement. In addition, we have found several ultrastructural characteristics which have so far gone unnoticed in Rous sarcoma cells.

MATERIALS AND METHODS

Virus. Two pseudotypes (41) of the Bryan high-titer strain of Rous sarcoma virus were used. They were genetically identical, but they differed with respect to their helper viruses, and thus also with respect to their envelope antigen. One of the pseudotypes was affiliated with Rous-associated virus type-1 (RAV-1) and was abbreviated RSV(RAV-1). The other had as helper virus Rous-associated virus type-2 (RAV-2) and was abbreviated RSV(RAV-2). Stocks of the Rous sarcoma viruses and of their helpers were prepared according to published techniques (23, 49). The Prague strain of Rous sarcoma virus (PR-RSV) was used only in interference tests. Stocks of this virus were obtained by harvesting the supernatant fluids of

heavily infected chicken fibroblast cultures from 6 to 9 days after inoculation.

Cell cultures and infectivity assays. Fibroblast cultures were prepared from individual chick embryos of Hyline Farms line 934D according to the procedures of Rubin (38). A small proportion of the cells from each embryo was tested for the presence of congenital avian leukosis virus infection and for genetic cellular resistance to avian leukosis viruses (7, 38). Only embryos which were free from avian leukosis virus and genetically susceptible to the viruses under study were used in the present experiments. RSV was assayed by focus formation on chick embryo fibroblasts according to standard procedures (39). The presence of avian leukosis virus in the supernatant fluid of cell cultures was tested by the interference technique (40). For this purpose, chick embryo fibroblasts were inoculated with the sample to be tested and were transferred three times at 2- to 3-day intervals. At the third transfer, portions of the cells were challenged with RSV(RAV-1), RSV(RAV-2), and PR-RSV. Resistance against any one of these viruses indicated the presence of an avian leukosis virus in the test sample.

Nonproducing Rous sarcoma cultures. Cultures were initiated by transferring a single Rous sarcoma focus on a layer of normal chicken fibroblasts as described previously (23, 48). The supernatant fluids of these cultures were tested for the presence of infectious Rous sarcoma virus after centrifugation at 4,000 rev/min for 20 min and sonic treatment for 2 min to eliminate intact Rous sarcoma cells.

Fluorescence microscopic techniques. The type-specific envelope antigen of avian tumor viruses was stained with fluorescein-conjugated chicken immune sera applied to nonfixed cells. Fluorescent antibodies were also prepared from sera of chickens which had been inoculated with sonically treated nonproducer cells in an attempt to immunize against a putative passenger virus, not belonging to the avian tumor virus group and possibly associated with nonproducer cells. The details of the conjugation and staining procedures have been published previously (48, 50). Group-specific antigen of the avian tumor viruses was demonstrated in acetone-fixed cells by use of hamster antisera against Rous sarcoma and fluorescent anti-hamster globulin in an indirect technique. These tests have also been described previously (28).

Electron microscopy. Cell cultures were fixed in situ by adding to the tissue culture fluid an equal volume of cold, 3% glutaraldehyde adjusted to *p*H 7.3 with 0.09 M phosphate buffer containing 1% calcium chloride (42). After 30 min at 10 C, the cells were scraped off the petri dish with a disposable rubber policeman, were pelleted at slow speeds, and were resuspended in a small volume of buffered 3% glutaraldehyde. They were then placed in vials and shipped by air to the electron microscopy laboratory. Upon receipt, the specimens were rinsed briefly in phosphate buffer containing 10% sucrose and were postfixed for a period of 30 to 90 min in 1% osmium tetroxide buffered to *p*H 7.4 with chromate buffer (8). To facilitate handling, some cultures were embedded in 4% agar and treated as tissue blocks (16). All cultures were dehydrated in a graded series of alcohols and propylene oxide and embedded in an Epon-Araldite mixture (31). Ultrathin sections were cut with glass or Dupont diamond knives on LKB ultramicrotomes (models 4800 or 8800). Sections were doubly or singly stained with uranyl acetate and lead citrate (35, 52). The specimens were examined with a Siemens Elmiskop I electron microscope with accelerating voltage of 60 kv and a 50-μ objective aperture. Occasionally, thick sections were stained for light microscopy to evaluate the proportions of the various cell types studied (36).

RESULTS

Biological characteristics of cell cultures used in electron microscopy. Cultures of nonproducing Rous sarcoma cells were initiated with single foci of RSV-transformed chicken fibroblasts, which had appeared in chick embryo cell cultures after a low multiplicity of infection with the virus. Under these conditions, the cells of a focus contained the progeny genome produced by a single virus particle, and so did any nonproducing culture derived from such a focus. The 69 nonproducing Rous sarcoma cultures entered in Table 1

TABLE 1. *Survey of cell cultures used in electron microscopy*[a]

Culture	Total no. of cultures	RSV in culture medium	RAV in culture medium[b]	Type-specific viral antigen at cell surface[c]	Group-specific viral antigen in cytoplasm[c]	Presence of transformed cells	Activability of RSV production by superinfection with leukosis virus
Nonproducing Rous sarcoma cells of the chicken	69	0/69	0/35	0/35	5/5	69/69	10/10
RSV-producing cultures	41	41/41	7/7	5/5	3/3	41/41	NA[d]
RAV-producing cultures	11	0/11	4/4	10/10	2/2	0/11	0/7
Uninfected chicken fibroblasts	48	0/48	0/17	0/20	0/3	0/48	0/8

[a] Number of cultures positive/number of cultures tested.
[b] Detected by interference test.
[c] Detected by fluorescent-antibody staining.
[d] Not applicable.

were all started with different foci. In addition to virus-transformed cells, nonproducing cultures contained the uninfected chicken fibroblasts onto which the original single focus of sarcoma cells had been planted, and which served as feeder cells. The cultures were transferred at intervals of 2 to 3 days. They grew with an approximate doubling time of 20 to 24 hr for the first 5 to 6 weeks after initiation. Later, their growth rate slowed down considerably, and by 8 weeks they had become practically stationary. During the first few transfers after the isolation of the single Rous sarcoma focus, only a few virus-transformed cells were visible. After 2 to 3 weeks, Rous sarcoma cells became more numerous, and they eventually gained predominance over the feeder cells. The average age of cultures at the time of harvest and fixation for electron microscopy was 35 days, although cells have been examined as early as 19 and as late as 59 days after initiation of the cultures. By the time of harvest for electron microscopy, normal fibroblasts were microscopically no longer detectable in many instances; in others, they constituted the minority of the cell population. Only a few cultures were used in which transformation was sparse. Occasionally, the isolation of a single Rous sarcoma focus was not successful. The plates then failed to show any transformed cells at all, and appeared to contain only normal feeder cells. Such cultures were included among the controls.

The absence of infectious RSV from nonproducing cultures was established by three to six tests of the supernatant media for focus-forming activity (Table 1). Such tests used one-tenth of the total undiluted supernatant fluid of a given culture. Their negative outcome meant, therefore, that at the time tested there were less than 10 focus-forming units of RSV per culture. For some of the infectivity assays, cells were detached from the culture dish with a rubber policeman and, after sonic treatment, were plated for infectious virus. No focus-forming activity was found. By the time the cells were fixed for electron microscopy, they had been tested for RSV production an average of four times, and had been invariably found negative. In addition, some of the cultures were also studied with other techniques (Table 1). The presence of viral envelope antigen was checked with fluorescent chicken antisera which were known to stain a wide variety of antigenically different avian tumor viruses, including many antigenic types of RSV. The failure to detect such viral envelope antigen in nonproducing Rous sarcoma cells was in keeping with previous observations (24, 48). The group-specific antigen of avian tumor viruses, which has been shown previously to occur in nonproducing cells (12, 28, 51), could be demonstrated in the cytoplasm of nonproducing Rous sarcoma cells with the indirect fluorescence technique. Nonproducing cultures were activable, and they released infectious RSV when superinfected with RAV. As long as they remained in the nonproducing state, no RAV was demonstrable in their culture fluids.

About 10% of the Rous sarcoma foci isolated for the purpose of initiating nonproducing cultures released virus upon the first transfer. Such foci had presumably been contaminated with helper virus, and RAV was indeed found in the fluid medium of these virus-producing cultures (Table 1). A few of the nonproducing Rous sarcoma cultures became virus-releasing at some time during their repeated transfers. This also appeared to be due to accidental contamination with RAV, which had occurred either during the original focus isolation, or during one of the transfer manipulations. RAV was again demonstrable in the supernatant fluids of such "spontaneous producers." RSV-releasing cultures were further obtained by superinfection of nonproducing cultures with RAV. By using parts of such cultures for electron microscopy before and after activation, descendants of the same focus could be studied in the nonproducing as well as in the producing state. The average age of RSV-releasing cultures at the time of harvest was 35 days, but some cultures had been virus producers for only the last week of their passage history. RSV-producing cultures showed type- as well as group-specific viral antigens (Table 1). Their content of Rous sarcoma virus ranged from 0.5 to 10 focus-forming units per cell, and they consisted predominantly of transformed cells.

RAV-producing cultures consisted of normal-appearing chicken fibroblasts that had been inoculated with RAV. Their passage history was the same as that of nonproducing cells. RAV was demonstrable by the ability of culture fluids to induce cellular resistance in vitro directed against RSV (Table 1). Type- and group-specific antigens were demonstrable with fluorescent antibodies in virtually all cells of such cultures.

Control cultures of normal chicken fibroblasts were derived from the same embryos which furnished the feeder cells in nonproducing Rous sarcoma lines. As mentioned above, feeder plates on which, for reasons unknown, the isolation of a Rous sarcoma focus had been unsuccessful, and which were free of virus-transformed cells as well as of RSV and RAV, were included among the controls. Control cultures were free of infectious RSV or RAV, did not stain when tested by immunofluorescence techniques for

viral antigen, and could not be activated to release RSV after infection with RAV (Table 1). They were harvested for electron microscopy at the same dates as nonproducing and virus-producing cultures.

Electron microscopy of uninfected control cultures. A total of 48 control cultures were examined; some of these were sampled repeatedly at different times during their culture history. From the number of sections and the average number of cells per section, it was estimated that the material inspected consisted of multiple profiles of at least 1,200 different normal chicken fibroblasts. These cells showed the general morphological characteristics observed in other fibroblast lines (18). Actively growing chicken fibroblasts were elongated and slender with a prominent centrally located nucleus (Fig. 1). The chromatin was clumped and marginated at the nuclear envelope and to a lesser extent around the nucleolus. The nucleolus varied widely in size. The cytoplasm contained few round mitochondria with distinct cristae. The rough endoplasmic reticulum was sparse, with short, narrow profiles. Free ribosomes occurred in moderate numbers and were arranged in clusters. β-Form glycogen particles and lipid droplets were seen infrequently. The Golgi apparatus was well developed. Present in the cytoplasms of cultured chick fibroblasts were also fibrils and microtubules similar to those found previously in various tissues (4, 9, 29, 43, 44, 46). An outstanding feature of these fibrils and tubules was their concentration adjacent to the plasma membrane, combined with a marked orientation parallel to the long axis of the cell (Fig. 2 and 2a).

With an increase in the number of culture passages, the electron microscopic appearance of normal fibroblasts changed. The nucleolus became more prominent, mitochondria tended to be elongated and branched, and the cisternae of the endoplasmic reticulum were dilated. Cytoplasmic fibrils and microtubules adjacent to the cell membrane showed a more rigorous orientation parallel to the long axis of the cell. Vacuolization as well as an increase in lysosomes and cytoplasmic fibrils was observed in stationary cultures. No structures resembling viral particles or precursors of viral particles were seen in any of the control cultures. Likewise, other structures related to RSV infection and described below were absent from uninfected controls.

Ultrastructural features of nonproducing Rous sarcoma cells. Sixty-nine nonproducing Rous sarcoma cultures were examined. The total number of transformed cells of which multiple sections were studied was approximately 1,400, giving an average of about 20 cells per culture. The general appearance of the cells was in agreement with previously described cytological and ultrastructural features of Rous sarcoma cells (5, 10, 12, 17, 19, 20). The structural changes to be described for Rous sarcoma cells were not due to environmental conditions such as the lowered *p*H of the medium in transformed cultures, because these structural alterations were not found in normal feeder cells growing together with nonproducing Rous sarcoma cells in the same culture vessel. The transformed cells were rounded and often larger than normal (Fig. 3–5). In a typical cell, the nucleus was located eccentrically, was hypertrophied, and often showed bizarre lobulation. The nuclear indentations faced the center of the cell. Multiple nuclei, common in the giant cells, were also present in some round cell forms. The distribution of the chromatin was not significantly altered. The nucleolus appeared enlarged. The Golgi apparatus was often hypertrophied and located within the cytoplasmic region extending into the indented area of the nucleus. The mitochondria were usually reduced in number, were smaller than in the control cells, and contained fewer cristae. The amount of endoplasmic reticulum in rounded cells was reduced, but appeared increased in giant cells where dilated cisternae apparently gave rise to some of the vacuoles. Free ribosomes were more numerous than in normal cells.

Cytoplasmic structures apparently related to RSV-induced transformation. The peripheral cytoplasm of nonproducing Rous sarcoma cells contained fewer tubules, but sometimes contained an increased number of fibrils as compared with control fibroblasts. However, in striking contrast to normal cells, fibrils and tubules were rarely concentrated close to the cell membrane in rounded, transformed cells, nor did they show the characteristic parallel arrangement described for normal fibroblasts (Table 2, Fig. 5). Only large, flattened polykaryocytes which are sedentary cells presumably arising from RSV-induced transformation occasionally contained peripherally concentrated fibrils (Fig. 6). But, even in these cells, the concentration or parallel orientation did not equal that seen in normal feeder cells of the same culture.

Also seen frequently in nonproducing Rous sarcoma cells were particles ranging from 300 to 400 A, associated with membranes of the endoplasmic reticulum. These membranes occurred in parallel arrays and were continuous with the rough portions of the endoplasmic reticulum (Fig. 7 and 8, Table 2). On the basis of morphological criteria and of the affinity of the particles for lead citrate stain, as well as the relative lack

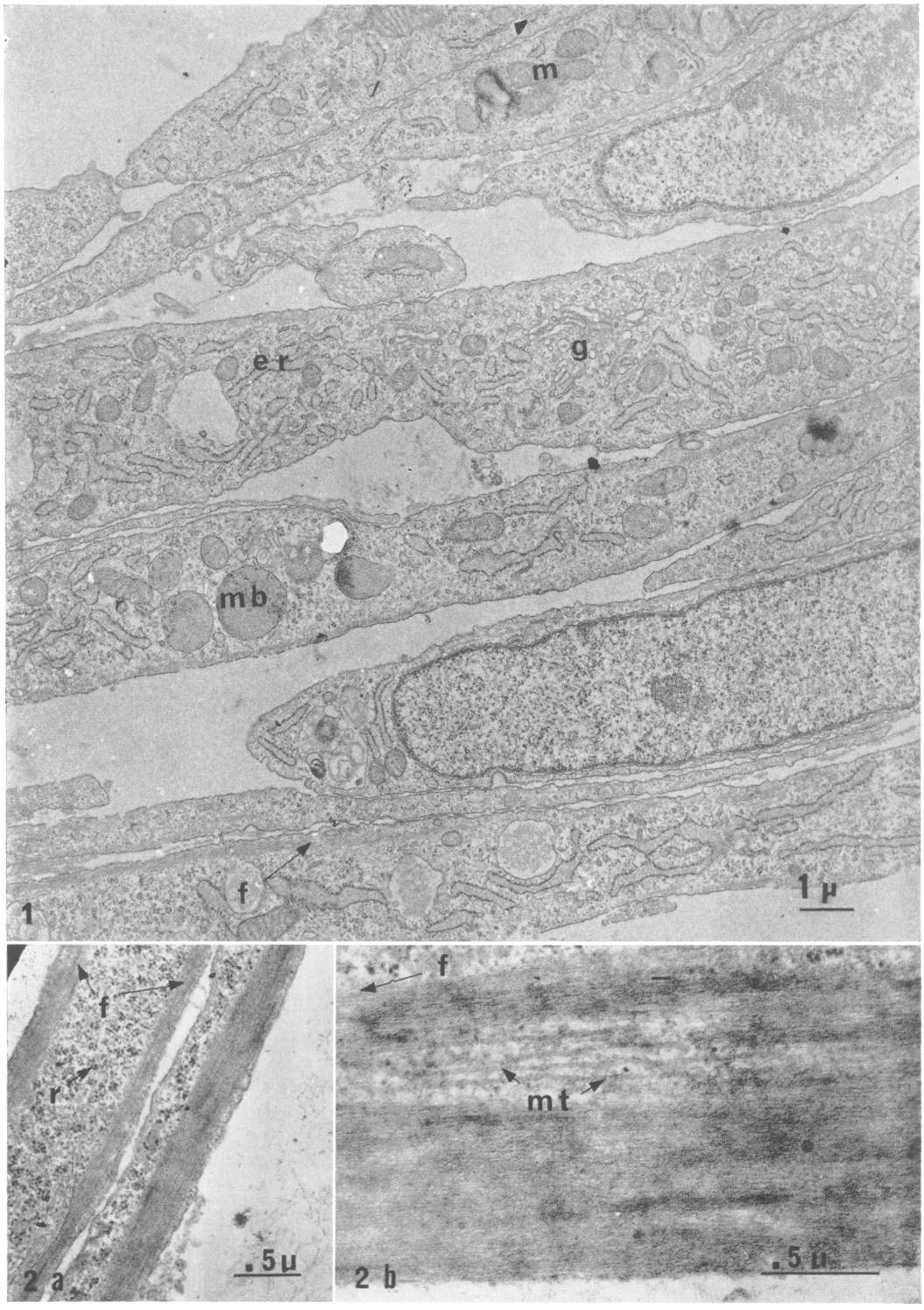

FIG. 1. *Survey micrograph illustrating the general characteristics of control chick fibroblasts. Note the beginning fibril condensation at the cell surface (f). Small round mitochondria (m) and short segments of endoplasmic reticulum (er) are characteristic. A golgi area (g) and occasional microbodies (mb) are identified.* × *8,250.*

FIG. 2a. *Detail of the surface of normal chicken fibroblasts showing cortical organization. Fibrils (f) occur at one or both surfaces. Clusters of free ribosomes (r) are visible.* × *20,000.*

FIG. 2b. *Higher magnification of cell cortex illustrating that microtubules (mt) appear to blend with fibrils (f) to form the surface complex.* × *35,000.*

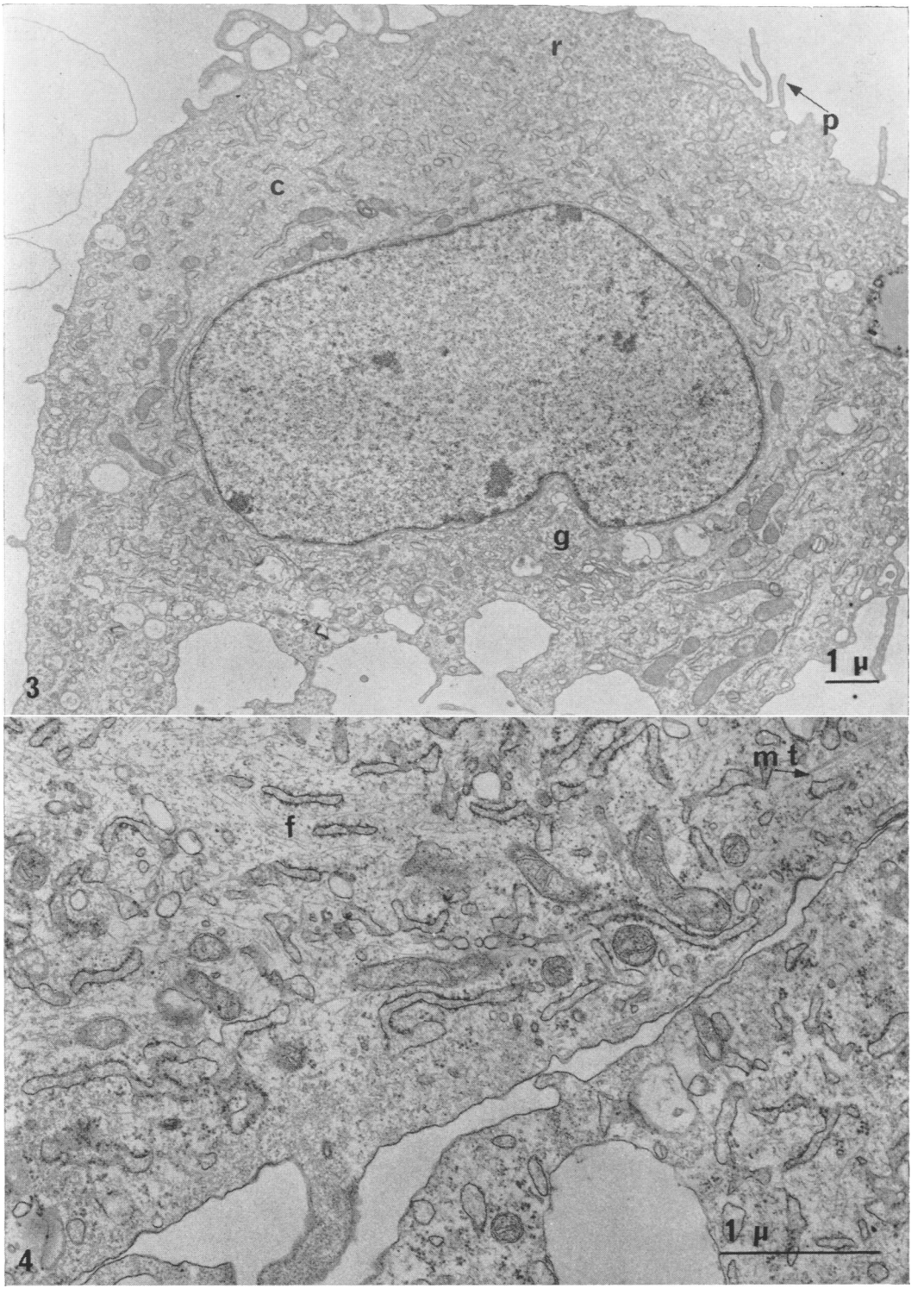

FIG. 3. *Survey micrograph of a nonproducing Rous sarcoma cell. The cell is rounded. A prominent golgi apparatus (g) lies in the area of the indented nucleus. The area of cytoplasm (c) above the nucleus contains a high concentration of fibrils. Cytoplasmic ribosomal clusters (r) are prominent in one area. Pseudopodia (p) mark the cell surface.* × *8,000.*

FIG. 4. *Cortex of a nonproducing Rous sarcoma cell shows lack of organized fibrils and microtubules. Fibrils (f) and occasional microtubules (mt) are distributed randomly through the cytoplasm.* × *24,000.*

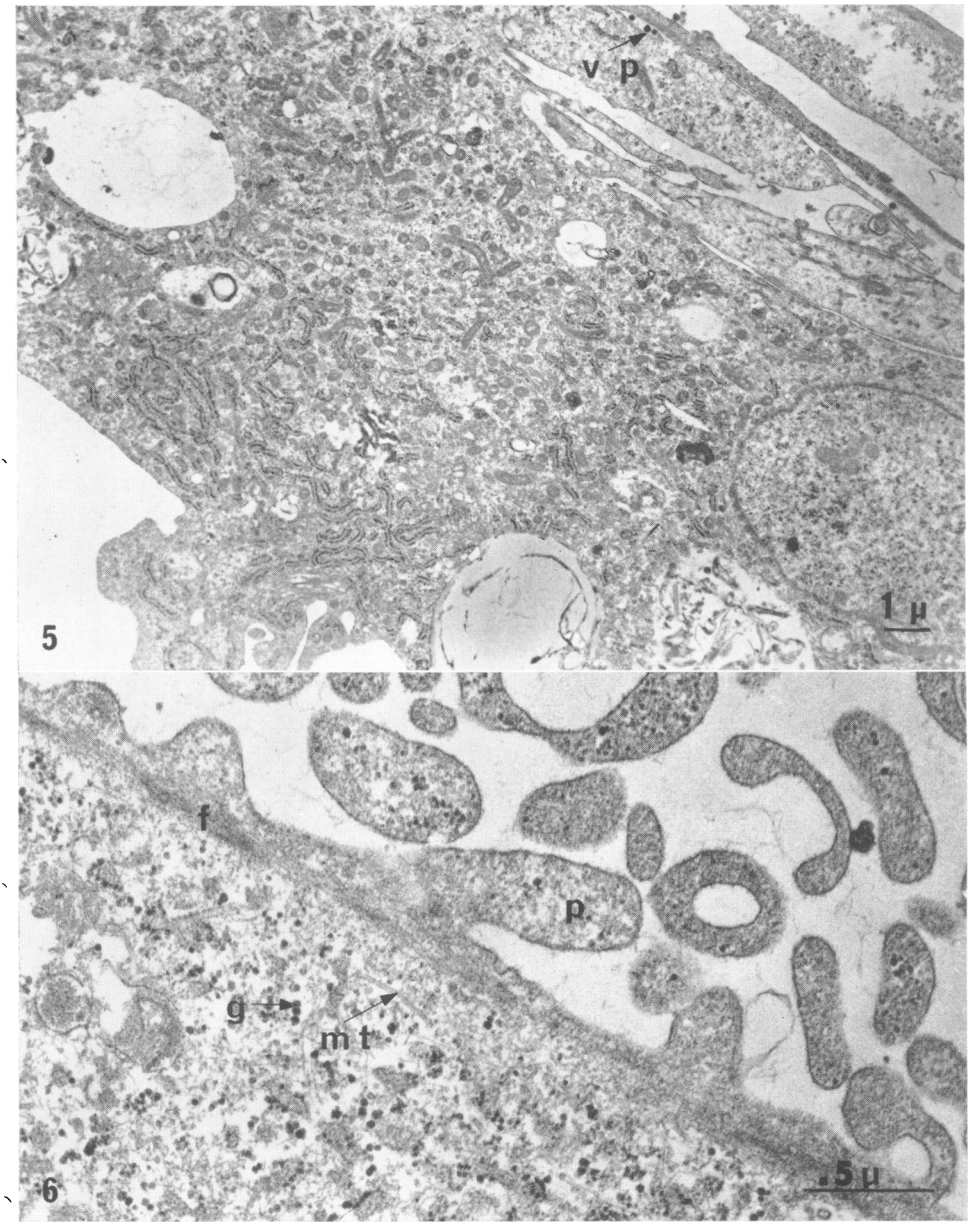

FIG. 5. *Portion of a polykaryocyte from a nonproducing Rous sarcoma culture. A few virus particles (vp) are noted at one edge.* × *6,000.*

FIG. 6. *Cortex of a polykaryocyte from a nonproducing Rous sarcoma culture contains some parallel arrays of microtubules and fibrils. Cytoplasmic protrusions (p) are also prominent. Scattered glycogen particles (g) are noted.* × *35,000.*

of staining with uranyl acetate (34, 45), these particles were tentatively identified as glycogen. However, further studies with chemical techniques will be required before definite conclusions on the nature of these conspicuous, membrane-bound particles in Rous sarcoma cells can be reached.

Still restricted to transformed cells but present only in about 10% of the inspected nonproducer cultures were cytoplasmic annulate lamellae

TABLE 2. *Incidence of various ultrastructural markers in cultures of nonproducing Rous sarcoma cells and corresponding controls*[a]

Marker	Uninfected chicken fibroblasts	Nonproducing Rous sarcoma cells	RSV-producing cells	RAV-producing cells
Peripheral organization of microtubules and fibrils	35/48	9/69[b]	1/41[b]	8/11
Parallel arrays of membrane-bound glycogen	0/48	29/69	9/41	0/11
Annulate lamellae	0/48	7/69	1/41	0/11
Intracytoplasmic viruslike shells	0/48	6/69	2/41	0/11
Avian tumor viruslike particles	0/48	64/69	41/41	11/11

[a] Number of positive cultures/total number of cultures
[b] Peripheral organization of microtubules and fibrils was detected in polykaryocytes only.

(Table 2, Fig. 9). Tangential sections of these lamellae gave the appearance of round vesicles, similar to those described by Di Stefano and Dougherty (10). At about the same frequency, cytoplasmic viruslike shells were detected near the nucleus (Table 2, Fig. 10). These structures showed no obvious resemblance to mature avian tumor virus particles. They had an average diameter of 850 A, with a double-layered external membrane and an electron-lucent interior, and were often associated with clusters of ribosomes. These shells appeared identical to similar structures observed previously in productive and nonproductive avian tumor virus infection (5, 10, 12, 19).

Presence of avian tumor viruslike particles in nonproducing Rous sarcoma cells. Viral particles morphologically indistinguishable from complete avian tumor virus were seen in 64 of 69 nonproducer cultures (Fig. 11). The negative cultures had been noted to contain only a small percentage of transformed cells, and it is likely that in these instances not enough transformed cells were examined to detect the presence of particles. The particles occurred at the cell surface and within cytoplasmic vacuoles, a distribution which was similar to that seen in virus-producing cells (Fig. 11). Budding of the particles from the cell membrane was suggested, as seen in Fig. 12.

The number of viral particles associated with nonproducing Rous sarcoma cells was determined in 10 cultures which consisted only of transformed cells, as judged from light microscopic inspection. In this material 0.2 particle was found, on the average, per cell section. Allowing 400 sections per cell, the number of particles associated with a nonproducing Rous sarcoma cell was estimated to be approximately 80. This value is not significantly different from the average of 50 particles per cell observed by Dougherty and Di Stefano (12).

Attempts to demonstrate infectivity and antigenicity of avian tumor viruslike particles occurring in nonproducing Rous sarcoma cells. The data compiled in the first line of Table 1 presented evidence that nonproducing Rous sarcoma cells were free of *infectious* avian tumor virus. The viral particles seen with the electron microscope were therefore either noninfectious avian tumor virus or resulted from infection with an unrelated passenger virus which was structurally indistinguishable from avian tumor viruses and showed a similar mode of formation at the cell surface. To test the presence of such infectious passenger virus, 0.5-ml portions of undiluted culture fluid from 22 nonproducing Rous sarcoma cultures were inoculated in separate dishes of normal chicken fibroblasts. These fibroblasts were transferred at 3-day intervals, and after the third transfer they were tested for the presence of virus. Different portions of each culture were stained with fluorescent antibody reacting with a wide variety of avian tumor viruses and with fluorescent chicken antibody prepared against disrupted nonproducing Rous sarcoma cells of the chicken. In no case was specific fluorescence detectable. The cells were also found to be sensitive to infection with RSV(RAV-1) and RSV-(RAV-2). Upon electron microscopic inspection, such chicken fibroblasts inoculated with medium from particle-containing, nonproducing Rous sarcoma cells failed to show particles themselves. Multiple sections of an estimated 500 cells were inspected and found negative for particles as well as for other structural features associated with nonproducing Rous sarcoma cells. A positive control infected with RAV showed an abundance of virus when examined with an electron microscope. These observations led to the conclusion that the avian tumor viruslike particles associated with nonproducing Rous sarcoma cells of the chicken were not infectious under the conditions of our tests and, therefore, did not represent an

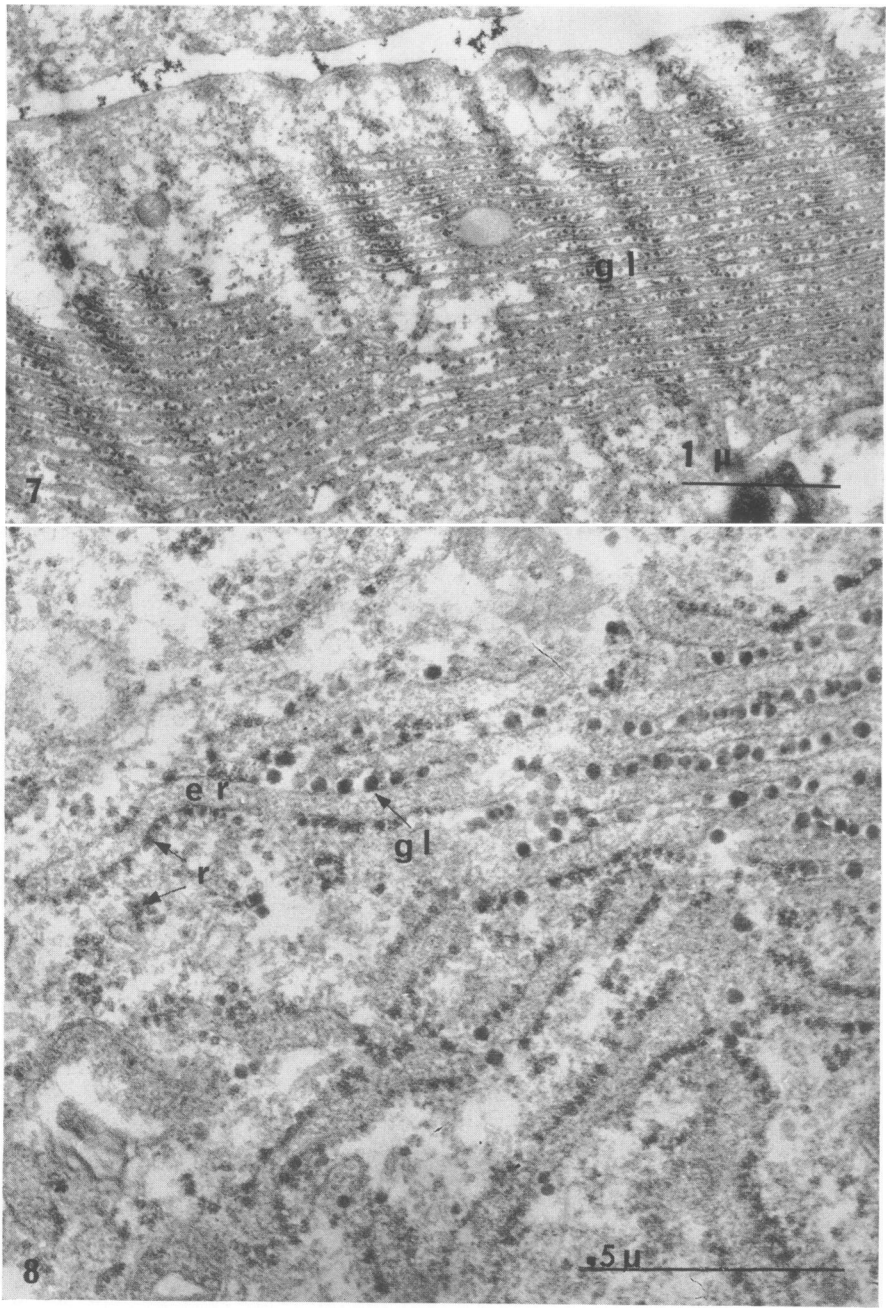

FIG. 7. *Low-power view of a nonproducing Rous sarcoma cell illustrating the membrane-bound, glycogen-like particles (gl).* × *20,000.*

FIG. 8. *Larger magnification of a similar structure as in Fig. 7. There is an apparent continuity of the rough endoplasmic reticulum (er) and membranes with glycogen-like particles. Free and bound ribosomes (r) are smaller and stain less densely than the glycogen-like particles (gl).* × *70,000.*

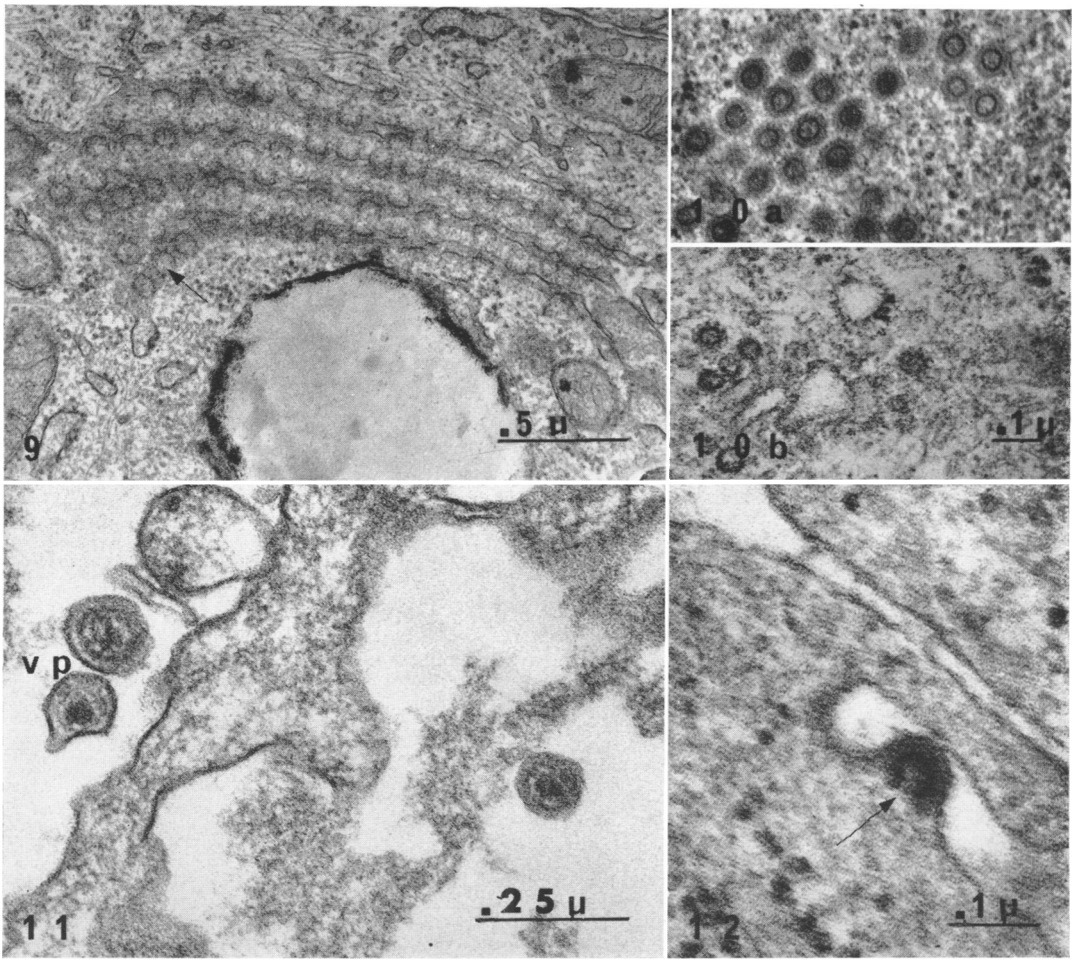

FIG. 9. *Annulate lamellae in the cytoplasm of a nonproducing Rous sarcoma cell. Tangential section gives the appearance of round vesicles (arrow).* × *35,000.*

FIG. 10. (*a*) *An example of cytoplasmic viruslike shells as seen in nonproducing Rous sarcoma cells.* (*b*) *Possible origin of these structures from cytoplasmic vesicles.* × *60,000.*

FIG. 11. *Viral particles (vp) at the periphery of a nonproducing Rous sarcoma cell. Dimensions and structure are that of avian tumor viruses. Outer and inner membranes as well as nucleoid are visible.* × *80,000.*

FIG. 12. *Budding of viral particle from the surface of nonproducing Rous sarcoma cell (arrow).* × *120,000.*

actively growing passenger virus unrelated to the avian leukosis and sarcoma complex.

Electron microscopic observations of virus-producing Rous sarcoma cells. Multiple profiles of an estimated 800 different cells from 41 separate RSV-producing cultures were examined. The findings were in agreement with previous studies on virus-producing Rous sarcoma cells (5, 13, 14, 17, 21, 25). The most outstanding and consistent difference between producing and nonproducing Rous sarcoma cells was the number of virus particles budding from cells and accumulating at the cell surface (Fig. 13). This was estimated to range from 500 to 5,000 per cell. The number of virus particles in producing cells was thus approximately 10 to 50 times that seen in nonproducing cells. Other morphological features of nonproducing Rous sarcoma cells, such as parallel arrays of membrane-bound glycogen, intracytoplasmic viruslike shells, and annulate lamellae, were also seen in producing cultures, as indicated in Table 2.

After prolonged staining with uranyl acetate, the internal structure of avian tumor virus particles associated with RSV-producing cells became more clearly visible (Fig. 14 and 15). The

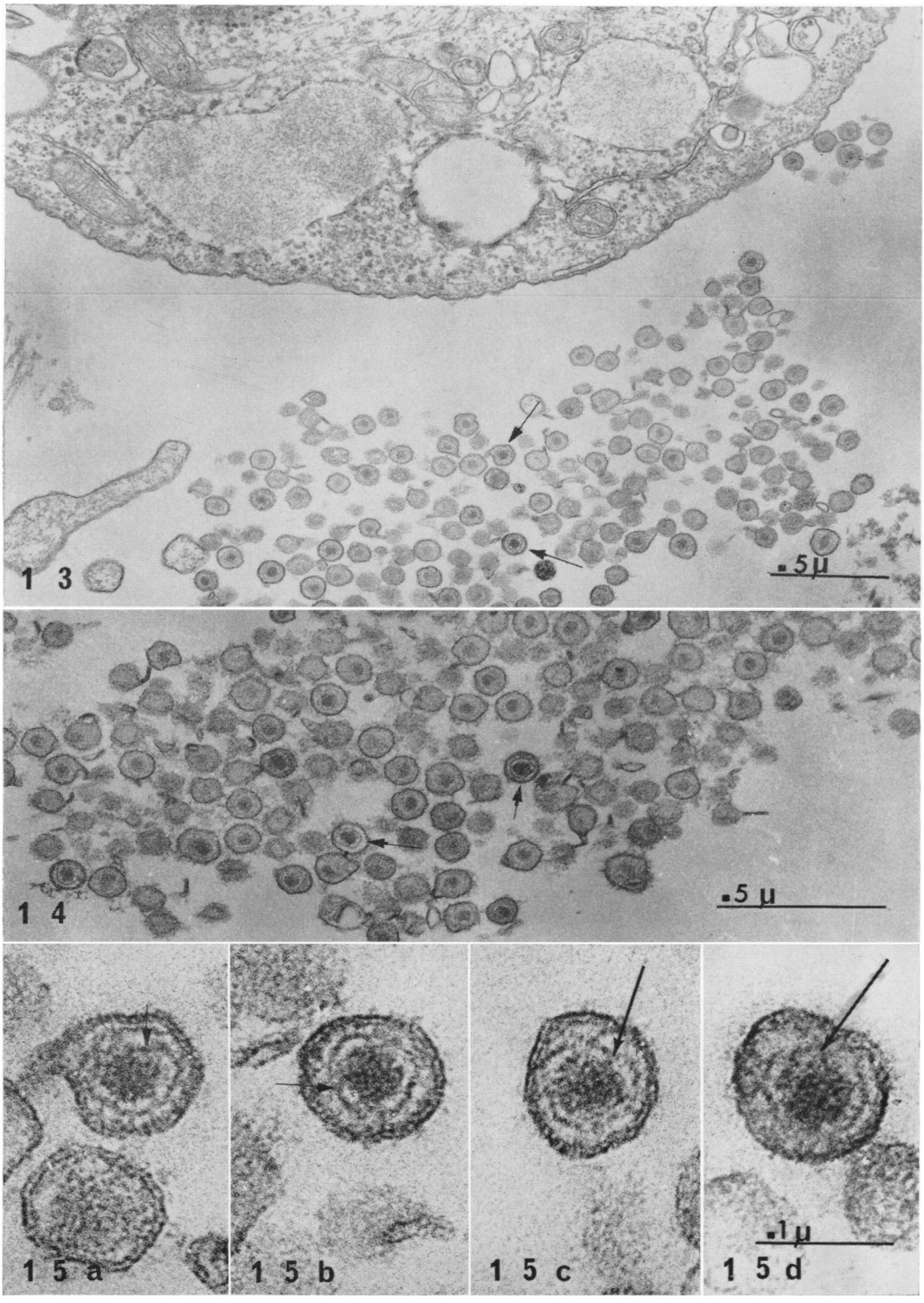

FIG. 13. *Group of virus particles on the surface of a producing Rous sarcoma cell. The cortical arrangement of fibrils seen in normal cells is absent.* × *35,000.*

FIG. 14. *Cluster of virus particles from a producing Rous sarcoma culture. Outer and inner membrane of the particles as well as nucleoids may be seen (arrows).* × *50,000.*

FIG. 15. *Selected virus particles from Fig. 13 and 14 magnified to 200,000* × *to demonstrate the relationship between nucleoid and inner membrane. The arrows point to a threadlike connection between nucleoid and inner membrane.*

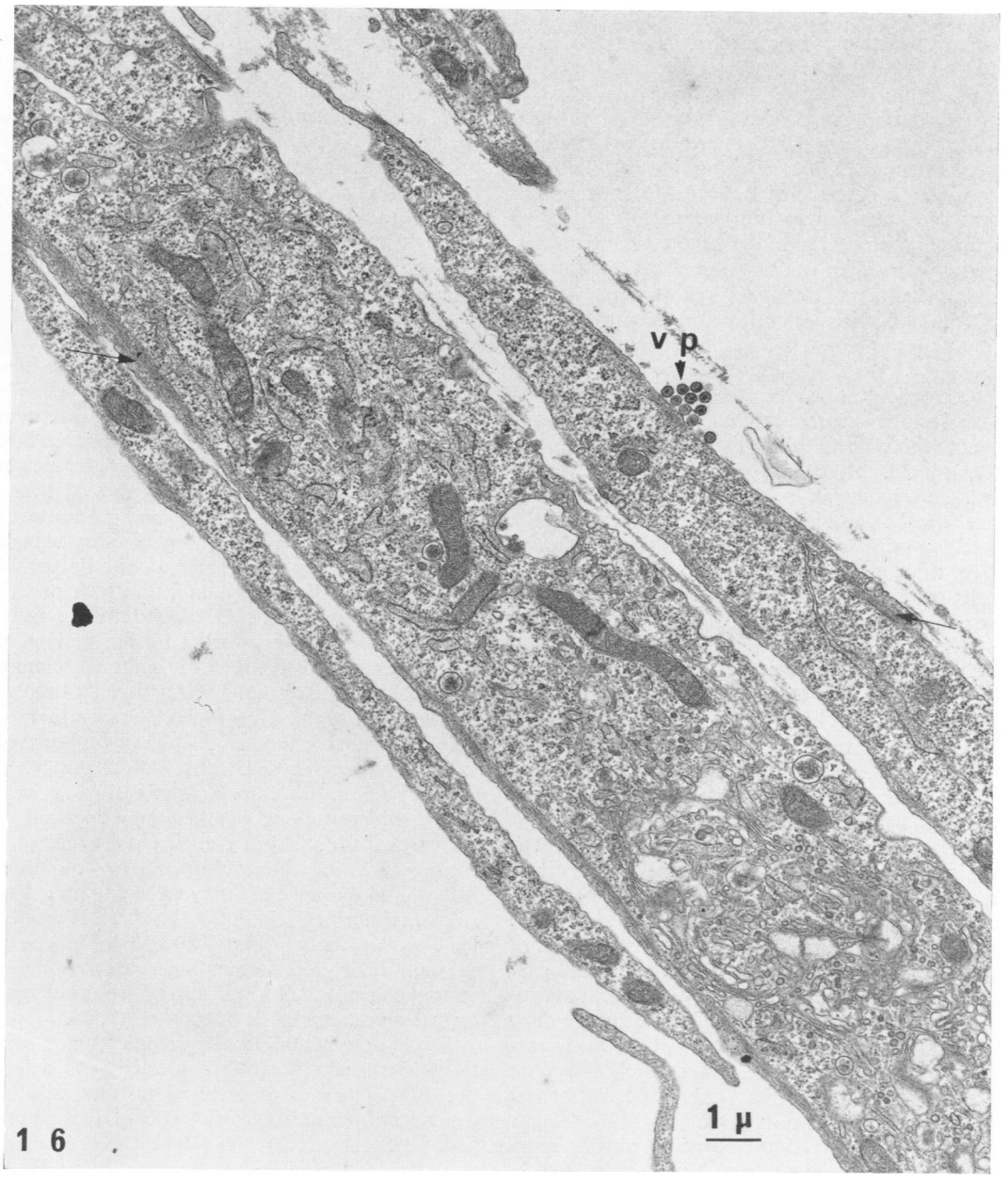

FIG. 16. *Part of RAV-infected chicken fibroblast. Virus particles are visible at the cell surface (vp). The cortical organization of the cell is the same as that of normal fibroblasts (arrows).* × 7,500.

electron micrographs indicate that the "internal membrane" of the particles is connected to the nucleoid. Figure 15 also casts some doubt on the membranous nature of the structures immediately inside the viral envelope. However, more information will be needed to define these structures and their relationships to viral nucleoid and envelope.

Ultrastructure of RAV-infected chicken fibroblasts. Several profiles of about 400 RAV-infected cells sampled from 11 different cultures were studied. Their general appearance in the electron

microscope was identical to normal fibroblasts except for the presence of avian tumor virus particles at the cell periphery (Fig. 16). No membrane-bound glycogen, annulate cytoplasmic lamellae, or cytoplasmic viral shells were seen, although presence of the latter two structures may have gone undetected because of infrequent occurrence. Particularly noteworthy were peripheral concentrations of fibrils and tubules which showed the same parallel orientation that had been observed in normal fibroblasts. Obviously, these structures remained undisturbed by the processes of virus assembly and release.

DISCUSSION

Our observations on the ultrastructural features of Rous sarcoma cells are in good agreement with reports from other laboratories (5, 10, 12, 14, 17, 21, 25). The changes observed in cellular organelles, such as the hypertrophy of the nucleolus and the Golgi apparatus, the swelling of the mitochondria, and the increase of free ribosomes, have been seen in malignant cells of different etiologies, and hence cannot be regarded as specific effects of RSV on the cell.

The parallel arrays of membrane-bound particles, tentatively identified as glycogen and restricted in our material to Rous sarcoma cells, have been found in other pathological conditions (2, 3, 45). Noteworthy occurrences of such glycogen-like structures are preneoplastic nodules in the livers of ethionine-treated rats and livers affected by viral hepatitis or yellow fever. Epstein and co-workers (15) have studied the ethionine-induced nodules in preneoplastic livers and found that the membrane-bound particles, contrary to glycogen in normal liver tissue, did not disappear on fasting or after the injection of glucagon. The relationship of glycogen-like particles to tumor cell metabolism in general and to RSV infection specifically is not clear. The chemical nature of these particles in Rous sarcoma material needs still to be established more convincingly, and until that time speculations as to their function appear futile.

A similar uncertainty regarding specific function exists with the annulate lamellae detected in a small proportion of nonproducing Rous sarcoma cells. Again, the occurrence of these structures in the Rous sarcoma is not unique; they have been described in diverse normal vertebrate and invertebrate cells, but they appear to be more prominent in malignant and other fast-growing tissues (6, 30, 33).

The viruslike shells occurring in the cytoplasms of Rous sarcoma cells (5, 10, 19) appear to be more specifically related to virus infection. They may also be found in avian leukosis (11) and in certain virus-induced murine tumors (Dalton, *personal communication*). Whether these shells represent viral precursors or aberrant forms of virus synthesis is not known.

In normal fibroblast cultures, the cytoplasmic cortex contained densely packed arrays of microtubules and fibrils which were oriented in parallel to the long axis of the cell. These structures appeared to become more prominent in crowded cultures in which movement and division had stopped. In contrast to normal chick fibroblasts, rounded Rous sarcoma cells showed no cortical organization of tubular and fibrillar elements, but these structures were randomly distributed in the cytoplasm. Since normal fibroblasts in a confluent monolayer are essentially sessile whereas the mobility of rounded Rous sarcoma cells is not greatly impaired by crowded culture conditons, it is tempting to relate the difference in the cortical structure of normal and of transformed cells to their mobility and response to contact inhibition. A highly organized system of tubules and fibrils at the cell periphery would indicate little cellular mobility, and lack of this arrangement would mark highly mobile cells. This speculation is supported by the finding of randomly distributed tubules in fetal cells whose adult counterparts often show a parallel tubular organization (4) and by the occurrence of randomly dispersed tubules in the cytoplasm of various malignant cells (9). Unfortunately, it cannot be decided on the basis of electron microscopic evidence alone whether the dispersion of the cortical tubule-fibril complex is a cause or a consequence of increased cellular mobility. Microtubules and fibrils may well play an active role in cellular locomotion, as has been suggested by Goldberg and Green (18) and by Kavanau (27), but their precise function as well as possible relationships to the mitotic apparatus or collagen formation remain to be established (9, 18, 37).

The finding of structurally complete virus particles in chicken fibroblasts infected with defective RSV alone is most puzzling. Our results, which confirm and extend those of Dougherty and Di Stefano (12) show that these particles may be seen regularly in nonproducing Rous sarcoma cells of chicken origin. They are absent from normal chicken fibroblasts, and, in structure as well as cellular localization, they are indistinguishable from complete avian tumor virus. Nevertheless, these particles are noninfectious under standard conditions and must therefore be functionally defective despite their apparent structural completeness. As suggested by Dougherty and Di Stefano (12), they may represent the internal, helper-independent viral components, i.e., RSV RNA and group-specific antigen,

wrapped in plain cell membrane. This virus would presumably be unable to perform the early functions of the infectious cycle which are controlled by the type-specific envelope antigen. Such an antigen is not detectable in nonproducing Rous sarcoma cells (24, 48) and, therefore, is probably lacking from the particles elaborated by such cells. However, the latter statement cannot be made with absolute certainty because of the small number of particles involved and the limited sensitivity of the immunological tests for type-specific antigen. It may be that small quantities of a type-specific envelope antigen are made in defective RSV infection, permitting some virus maturation, but that this antigen is ineffective in mediating the early steps of infection. A similar qualifying remark seems necessary with regard to the lack of infectivity in nonproducing cultures of Rous sarcoma cells. From our data, it may be estimated that less than 1 in 10^8 of the particles seen in nonproducing Rous sarcoma lines is infectious in the standard RSV focus test. However, one could imagine that the virus released from nonproducing cells is extremely unstable because of a defect in the viral envelope and that it can infect new cells only within a very short time after release. This short-lived infectivity would facilitate a cell-to-cell spread of defective RSV, but may escape detection in the focus test. The occurrence of such a cell-to-cell transfer of RSV was suggested by preliminary experiments (Vogt, *unpublished data*). Despite these hypothetical possibilites of vestigial infectivity and envelope antigenicity in the particles released by nonproducing Rous sarcoma cultures, it is obvious that this virus is greatly deficient if compared with that synthesized by producing Rous sarcoma cells. Much further work will be necessary before the nature and function of viral particles of nonproducing Rous sarcoma lines is understood.

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